DRUG DISCOVERY **UPDATE**

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NANOTECHNOLOGY AND IN SILICO TOOLS

Natural Remedies and Drug Discovery

Edited by

Mital Kaneria

Kalpna Rakholiya

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Nanotechnology and In Silico Tools

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Drug Discovery Update

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Natural Remedies and Drug Discovery

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Part I

Nanotechnology

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Chapter 1

Green route synthesis of silver nanoparticles (Ag-NPs) and their applications

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Introduction

As the most dynamic area of research in materials science, at present nanotechnology, is receiving massive interest regarding the synthesis and characterization of nanomaterials for their application in numerous fields, such as pharmaceuticals, catalysis, sensors, electrochemistry, biomedicines, cosmetics, food technology, etc (Bera, 2016; Frewer et al., 2014; Sarsar et al., 2013; Velez et al., 2017). Taking into account a particular size (<100 nm) and morphology-based characteristics, nanoparticles (NPs) are excellent molecular or atomic solid particles with novel or enhanced properties as compared to bulk molecules (Jahn, 1999; Stadler et al., 2019). NPs can be classified as inorganic or organic. For example, metallic NPs (Cu, Au, Ag, Al, etc.), semiconductor nanoparticles (CdS, ZnO, ZnS, etc.), and magnetic NPs (Co, Fe, Ni, etc.) are inorganic NPs while carbon NPs (like carbon nanotubes, fullerenes, quantum dots, etc.) are the organic NPs (Rafique et al., 2017). Presently, there is a wide and increasing industrial utilization of modified or fabricated NPs, such as in cosmetics, electronics, textiles, nano-based imaging, and drugs (Tortorella et al., 2014). At the start of the 20th century, the conventional chemical and physical approaches (Chemical reduction, milling, etc.) of the synthesis of NPs were costly and toxic to the environment (Ahmed et al., 2016; Vijayan et al., 2016). Therefore, aqueous plant extract and microbes-based bio-inspired technologies for the synthesis of metallic nanoparticles gradually became a major stream in the area of nanomaterial and nanotechnology research (Mohanpuria et al., 2008; Pathak et al., 2019). Till now, stable, cost-effective, environment-friendly, clinically adaptable, and bio-compatible NPs are being synthesized using diverse plant extracts and microbes (Ahmed and Ikram, 2016; Vanlalveni et al., 2021).

Among metal NPs, silver NPs (Ag-NPs) are receiving enormous interest among the scientific community due to their morphology-based wider application in cell biology, microbiology, food technology, pharmacology, chemistry, parasitology, etc (Bondarenko et al., 2013; Nour et al., 2019). NPs are synthesized by two common approaches: "top-down" and "bottom-up." Different techniques such as pulse laser ablation, ball-milling, evaporation−condensation (most commonly used), pulse wire discharge method, etc., are used in "top-down" approach by splitting proper bulk material into fine particles (by size reduction) (Rafique et al., 2017). In bottom-up approach, synthesis of NPs is achieved through a chemical method, i.e., mostly use general method and biological method by self-assembly phenomenon of atoms into new nuclei that grow into a nano-sized particle (Chitsazi et al., 2016). For the reduction of Ag ions, various inorganic and organic reducing agents are used in non-aqueous or non-aqueous solutions, such as sodium ascorbate, sodium borohydride ($NaBH_4$), N,N-dimethylformamide (DMF), citrate, poly-ethylene glycol block copolymers, Tollen's reagent, and essential hydrogen (Thombre et al., 2013).

Size stabilization of NPs is carried out by the additional use of capping agents which has one of the greatest advantages of synthesizing a considerable amount of NPs in a very short duration (Rafique et al., 2017). In this sort of

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chemical synthesis process, the used chemicals are generally toxic, impel non−eco-friendly byproducts which are the reasons that lead to the green-route biosynthesis of NPs without no longer use of toxic chemicals (Rafique et al., 2017). Therefore, the process development of green and eco-friendly synthesis of Ag-NPs is progressing as a main branch of nanotechnology where the application of biological entities such as plant extracts or plant biomass and microorganisms could be a substitute for chemical and physical methods (Rafique et al., 2017). The benefits of green synthesis, over physical and chemical methods, are that is environment friendly, cost-effective, and easy for large-scaled synthesis without high temperature, pressure, energy, and, harmful chemicals (Ahmed et al., 2016). Therefore, this chapter describes the green-route synthesis of Ag-NPs and their applications that have comparative advantages over conventional chemical and physical methods.

Conventional and green methods of nanoparticles synthesis

Physical methods

Mostly, evaporation-condensation and laser ablation are the important physical approaches (Table 1.1). The prepared thin films without solvent contamination and the uniform distribution of NPs are the advantages of these methods compared to the chemical approaches. Physical synthesis of Ag-NPs using a tube furnace at atmospheric pressure has some disadvantages, such as occupancy of large space, consumption of high energy, and required time to achieve thermal stability (Kruis et al., 2000; Magnusson et al., 1999). Synthesis of fine Ag-NPs from a small ceramic heater (with a local heating area) useful as inhalation toxicity studies and nanoparticles calibration device (Jung et al., 2006). Laser ablation method used for the synthesis of Ag-NPs where the ablation efficiency and characteristics of Ag-NPs depend upon the duration of the laser pulses, wavelength of the laser, laser fluence, the effective liquid medium with or without surfactants and ablation time duration (Tarasenko et al., 2006). The arc discharge method is another physical method to fabricate Ag-NPs suspension in deionized water without the addition of surfactants (Tien et al., 2008). In spray pyrolysis, a nanostructure is obtained by nanoporous nebulizer−based spraying or injecting of a precursor solution onto the hot substrate in the furnace that leads to the decomposition of the precursor to the final form of the desired material on the substrate (Tahir et al., 2020). Ball milling is a top-down type of physical method of synthesis of NPs, such as the processing of polymer nanocomposites (Gou et al., 2012). During vapor phase synthesis of nanoparticles, conditions are created in such a way that the vapor phase mixture is thermodynamically unstable relative to the formation of solid material to be prepared as nanoparticulate form (Swihart, 2003). Electrical explosion of wire or pulsed wire discharge required a high-density current from a capacitor discharge that is passed through a metal wire (resistive element) and this wire transforms and expands into heated vapor, boiling droplets, or plasma by Joule heating (Tanaka et al., 2021). As a combination of the advantages of both top-down and bottom-up approaches, nanosphere lithography (NSL) is a tool that promises inexpensive fabrication for the production of regular and homogenous arrays of NPs with different sizes (Colson et al., 2013). Further, quick synthesis of Ag-NPs has also been demonstrated by direct metal sputtering into the liquid medium (Iravani et al., 2014).

TABLE 1.1 Different approaches for synthesis of nanoparticles.

Conventional approach		Non-conventional approach
Physical	**Chemical**	**Green-route**
Evaporation-condensation	Chemical reduction	From bacteria
Laser ablation	Sonochemical	From fungi
Arc discharge	Microemulsion	From plant and plant-derived extract
Spray pyrolysis	Photochemical	
Ball milling	Electrochemical	From enzymes and biomolecules
Vapor and gas phase	Pyrolysis	
Pulse wire-discharge	Microwave	From yeast
Lithography	Solvothermal	From microorganisms
	Co-precipitation	

Chemical methods

The most common procedure for Ag-NPs synthesis is the chemical reduction approach with the help of organic and inorganic nature of reducing agents (Table 1.1) (Chitsazi et al., 2016). From an unusual route to known materials, high-intensity ultrasound can be used for novel nanomaterial production without long reaction time, bulk high temperature, and high pressure (Xu et al., 2013). Uniformly distributed and size-controlled Ag-NPs can be synthesized by microemulsion techniques that offer few advantages, like ultralow interfacial tension, being thermodynamically stable, large interfacial area, and afforded monodispersed nanoparticles (Chin et al., 2014). Photochemical processes of fabrication of metallic NPs offer enhanced spatial and temporal controls that have certain advantages as they avoid the use of hazardous compounds, do not rely on costly instrumentation and highly skilled manpower, and most importantly, it can be done at ambient conditions (Jara et al., 2021). Electrochemical deposition technique has been broadly used for metal NPs synthesis and deposition takes place at the interface of an electrolyte with the added metal to be deposited and a metal substrate with electrical conductivity (Singaravelan and Bangaru Sudarsan Alwar, 2015). Pyrolysis, in a broad spectrum, is commonly applied for the synthesis of carbon nanostructures by thermal treatment of organic waste. The functional distribution of NPs is determined by the mechanism of growth of the NPs based on the medium, growing size, and physio-chemical properties (Zahid et al., 2018). Microwave-based synthesis of NPs combines the advantage of the speed and uniform heating of the used precursor materials. The characteristic penetration of microwave irradiation makes it possible to homogeneously heat up the reaction solution (Onwudiwe, 2019). Solvothermal synthesis of NPs of different shapes and sizes involves the use of a particular solvent under moderate to high temperature and pressure to promote the interaction of precursors during NPs' synthesis (Li et al., 2016). As a simple and low-cost method, the co-precipitation method of synthesis of NPs engages simultaneous occurrence of coarsening, nucleation growth, and aggregation processes (Peternele et al., 2014).

Green-route methods

Using bacteria

The NPs, produced from conventional methods, are toxic, expensive, and non−environment friendly. To overcome these problems, precise green routes, i.e., the natural sources and their products are used for the synthesis of NPs (Table 1.1). Green route synthesis (Fig. 1.1) can be classified as (a) use of microorganisms like bacteria, fungi, yeasts (eukaryotes), and actinomycetes(prokaryotes), (b) utilization of plants and plant extracts, and (c) use of templates such as membranes, viruses DNA, and diatoms. Bacteria can aggregate Ag on their cell walls, hence recommending their application in the industrial recovery of Ag-NPs from ore materials (Pooley, 1982). Initially, Ag-resistant *Pseudomonas stutzeri* AG259 was reported to synthesize Ag-NPs (Klaus et al., 1999). Ag-NPs are synthesized using culture supernatants of *psychrophilic* bacteria (Shivaji et al., 2011) and *Bacillus licheniformis* present in the aqueous solution of $AgNO_3$ (Kulthong et al., 2012). Ag-NPs can be synthesized within 5 min by the reduction of aqueous Ag ions through various bacterial culture supernatants, i.e., *Enterobacter cloacae* (Enterbacteriaceae), *Escherichia coli*, and *Klebsiella pneumonia*(Shahverdi et al., 2007). Using $AgNO_3$ as precursor, intracellular/extracellular Ag-NPs of the different size range (2−500 nm) with diverse morphology (such as face-centered cubic, spherical, crystalline, cluster triangular, hexagonal, equilateral triangle) have been synthesized by different types of genera and species of bacteria (Rafique et al., 2017). Compared to the conventional methods; however, the main disadvantage of using bacterial nanofactories is the rate of slow synthesis of NPs and the limited number of their size and shapes (Kharissova et al., 2013). Therefore, fungi-based nanofactories and plants and plant extracts-based materials are being investigated for the Ag-NPs synthesis.

Using fungi

Compared to bacteria, fungi have the potential for the metallic NPs synthesis due to the capacity of metal bioaccumulation and their tolerance, the capacity of high binding, and bacterial-like intracellular uptake that causes easy to handle in research (Kharissova et al., 2013). Further, fungi are utilized through various methods for NPs synthesis, where fungi secrete enormous enzymes that reduce the $AgNO_3$ solution (Mandal et al., 2006). Fungi (more than 20) of different genera and species are reported to produce Ag-NPs using precursor solution of $AgNO_3$. Synthesized Ag-NPs are of intra/extracellular with varying sizes (1−109 nm) and different respective morphological attributes, such as random, spherical, ellipsoidal, polydispersed spherical, hexagonal, crystalline, etc (Rafique et al., 2017). During fungi-based synthesis, Ag-NPs are found on the surface of mycelia, not in solution. These extracellular Ag-NPs from eukaryotic systems confirmed that secreted enzymes are responsible for he reduction of Ag^+ particles. Compared with other classes of microorganisms, eco-friendliness (non-pathogenic), straightforwardness during taking care, stability, and reaction rate are the prominent advantages of fungi-based synthesis of Ag-NPs (Vigneshwaran et al., 2006)

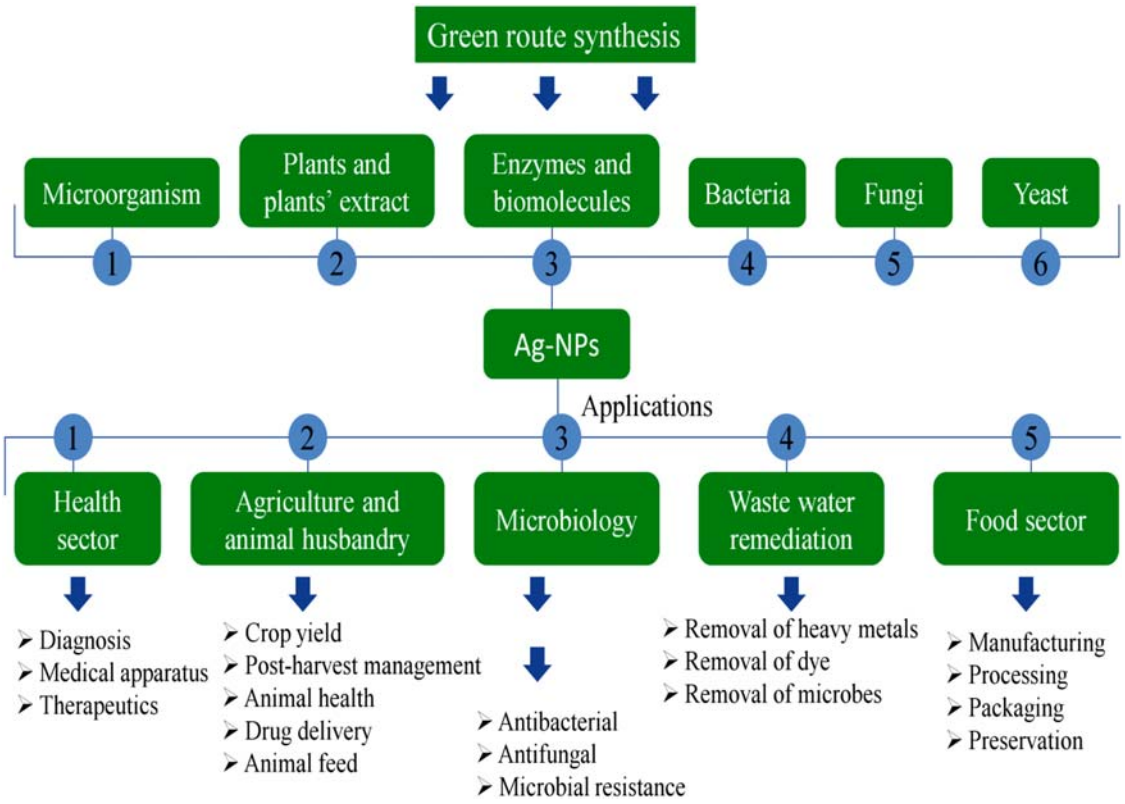

FIGURE 1.1 Green-route synthesis of Ag-NPs and their different applications.

Using plant and plants extracts

The first description of the synthesis of Ag-NPs (in shoots) using living plant system was demonstrated in *Alfalfa sprouts* (Gardea-Torresdey et al., 2003). The use of plant extracts in green Ag-NPs synthesis such as *Ananas comosus* (pineapple juice) (Ahmad and Sharma, 2012), extract of *Neem* and *Triphala* (Gavhane et al., 2012), *peanut* shell extract (Velmurugan et al., 2015), fruit extract of *Malus domestica* (Roy et al., 2014), etc., has stimulated numbers of investigations and studies till date. At room temperature, the formation of metal NPs using plant extracts is demonstrated to be finished in the metal salt solution within a short period of time depending upon the nature of the used plant extract. After plant extract selection, the central affecting parameters are the concentration of the metal salt, plant extract, pH, temperature, and contact time (Mittal et al., 2013). In addition to the formation parameters, the principal issue is the selection of the plant from which the extract could be used. The benefits of the synthesis of NPs from plants' origin are that they are easily available, safe to handle, and have active agents, at the large range, that can advance the required reduction of Ag^{+} ions (Kharissova et al., 2013). The plant parts such as roots, stem, latex, seeds, and leaves are mainly used for NPs synthesis. The exciting point is the active agents of these parts areinvolved in the stabilization and possible reduction and the plant extracts incorporated biomolecules acting both as reducing and stabilizing agents for the production of stable and shape-controlled NPs (Bindhu and Umadevi, 2013). Major compounds influencing the reduction and capping of NPs are biomolecules, like terpenoids, polysaccharides, phenolics, flavones, alkaloids, amino acids, enzymes, alcoholic compounds, and proteins (Bindhu and Umadevi, 2013). It is demonstrated that plant-based green synthesis appears to be faster compared to other microorganisms, such as bacteria and fungi. The utilization of plant and plant extracts in green synthesis has pinched attention because of their rapid growth, single-step technique, non-pathogenic nature, eco-friendly, and economical protocol for Ag-NPs synthesis (Rafique et al., 2017).

Characterizations of nanoparticles

Numerous techniques have been utilized to characterize the size, elemental composition, crystal structure, and other several physical properties of NPs (Mourdikoudis et al., 2018). Variation in strengths and limitations of each technique has complicated the choice of the most suitable method, and therefore often needed a combinatorial characterization approach

TABLE 1.2 Different techniques used for characterization of NPs (Mourdikoudis et al., 2018).

Sl No	Characterization techniques	Characterized entity
1	TEM, XRD, DLS, NTA, SAXS, HRTEM, SEM, AFM, EXAFS, FMR, DCS, ICP-MS, UV-Vis, MALDI, NMR, TRPS	Size (structural properties)
2	TEM, HRTEM, AFM, EPLS, FMR, 3D-tomography	Shape
3	XRD, XPS, ICP-MS, ICP-OES, SEM-EDX, NMR, MFM	Elemental-chemical composition
4	XRD, EXAFS, HRTEM, electron diffraction, STEM	Crystal structure
5	DCS, DLS, SAXS, NTA, ICP-MS, FMR, DTA, TRPS, SEM	Size distribution
6	XAS, EELS, XPS, Mossbauer	Chemical state—oxidation state
7	SAXS, NMR, TEM, Cryo-TEM, liquid-TEM	Growth Kinetics
8	XPS, FTIR, NMR, SIMS, FMR, TGA, SANS	Ligand binding/composition/density/arrangement/mass, surface composition
9	BET, liquid NMR	Surface area, specific surface area
10	Zeta potential, EPM	Surface charge
11	ICP-MS, UV-Vis, RMM-MEMS, PTA, DCS, TRPS	Concentration
12	Zeta potential, DLS, DCS, UV-Vis, SEM, Cryo-TEM, TEM	Agglomeration state
13	DCS, RMM-MEMS	Density
14	Sp-ICP-MS, MFM, HRTEM, liquid TEM	Single particle properties
15	3D-tomography, AFM, SEM	3D visualization
16	SEM, AFM, TEM	Dispersion of NP in matrices/supports
17	HRTEM, EBSD	Structural defects
18	TEM, SEM, STEM, EBSD, magnetic susceptibility	Detection of NPs
19	UV-Vis-NIR, PL, EELS-STEM	Optical properties
20	SQUID, VSM, Mossbauer, MFM, FMR, XMCD	Magnetic properties

(Mourdikoudis et al., 2018). Apart from two main parameters, i.e., size and shape, size distribution, surface charge, crystal structure, organic ligands present on the surface of NPs, surface area, degree of aggregation, and surface chemistry are measured in the overall characterization of NPs (Table 1.2) (Minelli, 2016). However, there are significant challenges in the analysis of NPs, such as interdisciplinary nature of the area, the absence of appropriate references for the calibration of analytical instruments, the difficulties in sample preparation, and interpretation of the generated data. Further, unmet challenges are there in the NPs' characterization, like the in situ and on-line measurement of their concentration, especially in mass production and their analysis in complex matrices (Mourdikoudis et al., 2018). During scale-up of NPs, waste, and effluent need to be monitored that require more reliable quantification techniques and therefore it is important to characterize the NPs, prepared in various ways, to the maximum extent.

Application of silver nanoparticles (Ag-NPs)

Health sector (diagnosis, medical apparatus, and therapeutics)

Out of the most vital and fascinating nanomaterials, Ag-NPs are the metallic nanoparticles involved in diverse plasmon sensor-based biomedical applications, such as cellular imaging, detection of squamous cell cancer of head and neck, detection of heavy metal and herbicide, detection the mercurial ions in solution, etc (Dawadi et al., 2021). The triangular shape of Ag-NPs has higher anisotropy and lightening rod effect which are widely used in the manufacturing of plasmon sensors or plasmon detectors (Dawadi et al., 2021). In case of electronic components and transportation, Ag-NPs have numerous applications in sensors, electrodes, integrated circuits, nanowires, and high-energy batteries (Rafique et al., 2017). The therapeutic applications of Ag-NPs are rapidly increasing, such as in dental cements, healing of burns and

wounds, skin therapy, reconstructive orthopedic surgery, bone cements, medical devices, and plastic catheters (Rafique et al., 2017). Further, Ag-NPs also has applications in targeted drug delivery, biolabeling, cancer therapy, coating of hospital textiles, gowns, coating of breath mask, coating of implants for joint replacement, orthodontic, and orthopedic fixations and implants (Rafique et al., 2017).

Agriculture and animal husbandry

By enhancing seed germination and plant growth, Ag-NPs have been widely used as a promising candidate to enhance crop yield (Kale et al., 2021). Regarding plant disease management and crop protection, Ag-NPs are proven to be active as anantimicrobial agent against plant pathogenic bacteria and fungus which are one of the reasons of significant loss in crop yield worldwide (Alloway, 2008). Though pest protection and nutritional enrichment of crops, Ag-NPs are expected to reduce the utilization of pesticides and frequent use of chemical fertilizers (Yokesh Babu et al., 2014). The green Ag-NPs, as an efficient pest management agents, are safe, non-toxic, and are used as enhanced pest control tool (Zahir et al., 2012). One of the important branches of agriculture is post-harvest management, including the preservation of agricultural products, which can be achieved by AgNPs-based antimicrobial packaging to increase the shelf life of vegetables and fresh fruits (An et al., 2018).

Nanotechnology devices such as nanosensors, microfluidics, nanomaterials, and bioanalytical are being utilized to improve various animal health conditions, reproduction, production, treatment, and prevention of their diseases (Kroubi et al., 2010). Due to the medicinal value of nanoparticles, they are being applied in animal husbandry as a source of drug delivery (Yang et al., 2013). Microbial infection in animal husbandry is a worldwide concern. The microbes secret many biochemicals that eventually contaminate animal feed which is causative of major diseases in animals and poultry (Frey-Klett et al., 2011). Nanotechnology bears the potential to explain many mysteries of animal health and therefore nanomaterials are being used as a food supplement in the diet of animals. Ag-NPs are widely utilized in animal feed for the treatment of diseases due to the physicochemical properties of Ag-NPs (Van Den Brûle et al., 2015). AgNPs are a promising tactic to inhibit fungal contamination that produces mycotoxins (Jogee and Rai, 2020). Few studies have observed that inclusion of nanomaterials could improve reproduction in livestock, poultry, and fisheries (Swain et al., 2015).

Antibacterial, antifungal, and microbial resistance

Novel broad-spectrum antibiotics ar a global high-interest concern due to increased microbial resistance and these anti-microbial agents can have several limitations, such as high cost, toxicity, low solubility, and severe side effects (Kakakhel et al., 2021). With the best example and lowest ecotoxicity to the environment, Ag-NPs have shown the best antimicrobial activity where gram-negative bacteria are more susceptible to Ag-NPs compared to gram-positive bacteria (Rajeshkumar and Bharath, 2017). Few green-routed Ag-NPs have exhibited excellent antibacterial activities on diverse microorganisms and have demonstrated cytotoxicity on HT115s and hSSCs (AlSalhi et al., 2016). Suggested mechanisms of antibacterial activity of Ag-NPs are 1: adhering to the cell membrane, Ag-NPs alter the membrane structure that causes leakage of cellular contents, 2: Penetration of Ag-NPs inside the cell causes DNA destabilization, 3: Destabilization of ribosome by the generated ROSs that leads to the oxidization of proteins and lipids, and 4: Genotoxicity that damages the DNA base causing duel inhibitions of replication and transcription (Behzad et al., 2021). Plant-derived Ag-NPs are reported to have promising antifungal and fungicidal properties against certain fungal isolates (Guerra et al., 2020; Jebril et al., 2020). *Rhizoctonia solani*, a wide host range plant pathogenic fungus, was reported to be inhibited by Ag-NPs (Elgorban et al., 2016). Along with antibiotics, antifungal activities of biogenic Ag-NPs have revealed promising results compared to the antibiotic alone (Padalia et al., 2015).

Wastewater management

Heavy metals toxicity and dyes such as methyl orange, rhodamin, congo red, etc., are common contaminants of water, and additionally, harmful bacteria and their toxins bear a considerable threat to the aquatic ecosystem and human health (Ganguly et al., 2021). Therefore, wastewater remediation, based on the application of Ag-NPs and its hybrids, has received massive interest in today's world (Ganguly et al., 2021). Preparations of hybrids of Ag-NPs with materials such as chitosan, cellulose, activated carbons, silicon dioxide, alginate, graphene oxides, titanium dioxides, etc., are utilized extensively in the treatment of wastewater (Ganguly et al., 2021). Ag-NPs have desirable antibacterial nature and capacity for enhanced adsorption that have prepared them valuable in the removal of contaminants from wastewater (Al-Qahtani, 2017). Enhanced surface reactivity of nanomaterials is based on the high surface free energy, high surface area, and great

density of active sites per unit mass which has resulted in making Ag-NPs good adsorbents (Zhang et al., 2016). Ag-NPs have favorable chemical and physical properties and therefore its adsorbents property has been investigated due to their satisfactory performance, high surface area and catalytic activity, exceptional biocompatibility, comparatively low cost, and high adsorption capacity (El-Tawil et al., 2019).

Food sector

As an important part of research and development, nanotechnology is being used for the large-scale manufacturing of processed foods, agricultural products and drinks, food packaging, and preservation across the globe (Moriarty, 2001). During nano-preservation of food, nanostructure-mediated encapsulation, water treatment, and humidity treatment are used in the nanostructures of "smart food" (Bajpai et al., 2018). Several studies have confirmed that these nanomaterials like Ag-NPs can successfully improve food safety, without altering taste and physical characteristics, by enhancing self-life and efficacy of the packaged food with added nutritional values (Das et al., 2018). In food packaging, Ag-NPs have been studied for the control and reduction of spoilage-related microflora in absorbent pads (Fernandez et al., 2010; Fernández et al., 2010), preservation of aseptic conditions in absorbent pads, etc (Fernández et al., 2009). In food processing applications, improved physical, microbiological, and chemical changes are observed in the stored green asparagus spears coated with Ag-NPs hybrids (An et al., 2008). In case of alternative non-thermal technology, antimicrobial nanocomposite packages, containing Ag and ZnO NPs, are observed to extend the shelf-life of fresh orange juice up to 28 days (Emamifar et al., 2010).

Shortcomings of nanoparticles and Ag-NPs

Plants and microorganisms are the raw materials for the synthesis of nanomaterials by green chemistry and biological synthesis. Interestingly, diverse biological molecules of plants and secondary metabolites, produced by microorganisms, play a major role in the bio-reduction of nanoparticles like Ag-NPs (Kakakhel et al., 2021). Further, there are some shortcomings and limitations in the synthesis of NPs using plants and microorganisms and therefore, it was important to find another biological method and direction for the synthesis of Ag-NPs (Kakakhel et al., 2021). Due to the seasonal dependency of plants, it is difficult to find specific plants for the standard and controlled synthesis of AgNPs. Monodispersed AgNPs are very critical for their chemical and physical properties and these NPs are simple to apply in the diverse fields of applied science with considerable results (Das et al., 2017). However, such monodispersed NPs, using plants, are very difficult to maintain that finally impacts their diverse applications (Das et al., 2017). Another limitation of plant-based synthesis of Ag-NPs is the reproducibility. If we are using the same plant extract, following another study, and even in case of same species, there could be an effect on their chemical makeup due to the environmental conditions in which the plants have grown (Kakakhel et al., 2021). Microbial-based syntheses of Ag-NPs also have some problems and limitations such as microorganisms especially fungi sometimes cause laboratory contamination that leads to adverse health effects in humans (Kakakhel et al., 2021). Further, there is still a break in the sustained stability of green-routed synthesis of Ag-NPs in various physical environments such as extreme temperature, pressure, and pH (Velgosová and Mražíková, 2017). Environmentally responsive, targeted, and controlled release of the materials by Ag-NPs, its bioaccumulation, and toxicity to the environment also needs dynamic research (Velgosová and Mražíková, 2017). In these consequences, green synthesis of Ag-NPs is still to gain popular in the industries compared to the chemical Ag-NPs synthesis (Velgosová and Mražíková, 2017).

Discussion and future prospect

The growing alertness for green chemistry and the use of biological synthesis of Ag-NPs have led to a need to develop techniques friendly to our environment. The plant-based synthesis of Ag-NPs needs further detailed standardization of the protocols where we can expect reproducing results in every trial. The biosynthesis of Ag-NPs from microorganism is an emerging and exciting field of nanotechnology and this process may significantly impact further advances in nanoscience. Different drawbacks of uses of microorganism like health contamination from fungus, pathogenic bacteria, etc., need to be refined and investigated in detail. A survey of scientific literature has revealed that these studies, on Ag-NPs applications, are carried out in in vitro condition, whereas no reports are there on the in vivo applications. Interestingly in in-vivo studies, the possible mechanisms of toxicity of Ag-NPs significantly have less available information compared with the in vitro studies. The diverse applications for Ag-NPs will continue to expand, however; in-depth understanding of the

accumulation of Ag-NPs in the environment and their potential long-term effects on humans and animal ecosystems need serious attention and research. Keeping view on the recent trends, we robustly believe that the biosynthesis of Ag-NPs will open novel directions toward various upcoming biomedical applications like nanodrugs, nanosensor in the future. There are also hopes for potential application of Ag-NPs in water disinfection, nano-weapon, against plant/animal diseases, and as surface plasma resonance enhancers. Also, these Ag-NPs could be potential tools to overcome the present energy crisis by an upcoming finding of their application in energy-driven devices. Further investigations are needed to identify the precise molecular mechanism of biosynthesis of Ag-NPs for better control over their size, shape, and stability.

Conclusion

During the last decade, number of efforts were carried out for the development of the green synthesis of Ag-NPs due to its certain advantages such as cost-effective, ecofriendly, large-scale synthesis, etc., over the chemical and physical methods. For the most competent miniaturized functional materials, exquisite and inventive methods are within nature itself. Recent awareness of green chemistry and green route synthesis of Ag-NPs has created a desire to develop and standardize eco-friendly methods. Simple prokaryotic to complex eukaryotic organisms are being utilized for the synthesis of Ag-NPs; however, the development of the microorganisms and dispersed formulation residue is very tricky compared with the other methods. The low rate of synthesis and a limited number of distributed size and shape has oriented the studies toward the uses of plants. Production of Ag-NPs from plants could have advantages over other biological elements, such as a slow rate of Ag-NPs synthesis using microorganisms, hygienic working environment, control over wastages, health and environment shielding, and final stable product formation. Through abundant applications for mankind such as medicine, cardiovascular implants, dentistry, therapeutics, biosensors, agriculture, etc., green-routed synthesis of Ag-NPs will be an important and major aspect of future nanotechnology. However, it will be very interesting to see how different shortcomings of the biological way of Ag-NPs synthesis would be solved for practical applications.

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Chapter 2

Biological synthesis of nanoparticles: a value of ethnomedicine

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Introduction

In recent, the potential of various plant biomasses and microbes for the synthesis of nanoparticles was explored. Various physical and chemical methods have been made for metallic nanoparticle synthesis, nanotechnology serves as a key method in the development of clean, less toxic, and less hazardous to save environmental methods for the synthesis and gathering of metallic nanoparticles. The biosynthesis of metallic nanoparticles by using plant extract is a new and emerging eco-friendly science of well-defined size, shapes, and controlled mono disparity (Ansilin et al., 2016). These particles are very tiny with their size ranging from 1 to 100 nm. Nanoparticles have brought considerable attraction due to their fabulous and interesting properties, with various applications (Daniel and Astruc, 2004). Green synthesis of metallic nanoparticles attracts an increasing interest because of their latest and distinct structures and characteristics that allow attractive applications in many fields such as antimicrobials (Priyadarshini et al., 2013), biotechnology, microelectronics, catalysis, medicine, optics, information storage, and energy conversion (Lee et al., 2003). Nowadays, nanoparticles are widely used as an effective antimicrobial agent against a broad spectrum of microorganisms, including antibiotic-resistant strains (Percival et al., 2007). Nanotechnology is referred to as the portrayal, manufacture, control, and application of structures controlling their shape and size at the different nanoscales (Sarsar et al., 2013). The area of nanoparticle study is most effective and interesting in terms of research in applied sciences, and the synthesis of nanoparticles is taking up significantly all over the world with their benefits. Nanoparticles show interesting effects taking into account particular characteristics, i.e., size (1−100 nm), shape, and structure (Slawson et al., 1999; Nalwa, 1999). These particles are categorized mainly as organic and inorganic nanoparticles. Inorganic nanoparticles include semiconductor nanoparticles such as zinc oxide, zinc sulfate, and xadmium sulfate, metallic nanoparticles like gold, silver, copper, aluminum, and magnetic nanoparticles like cobalt, iron, nikel, while organic nanoparticles incorporate carbon nanoparticles like quantum dots, fullerenes, and carbon nanotubes (Figs. 2.1−2.7).

Green synthesized nanoparticles from plants are a cost-effective, eco-friendly, and nontoxic approach to nanoparticle generation than the physical or chemical methods. Top-down and bottom-up methods are the two basic and old methods for the synthesis of Nanoparticles. In the top-down approach, suitable mass material breaks into fine particles by size reduction with different techniques, i.e., pulse laser ablation, evaporation−condensation, ball milling, pulse wire discharge method, etc. Through the Bottom-up approach, nanoparticles can be synthesized using biological (green synthesis) and chemical methods by the self-build phenomenon of atoms to new nuclei which stretch into a particle of nanoscale. In a top-down approach, evaporation−condensation is the most extensive method for metal nanoparticle synthesis (Swihart, 2003). Traditionally, researchers generally used the synthesis of nanoparticles in the bottom-up approach. The bottom-up approach is a nano-architectural phenomenon of self-assembly of materials from cluster-to-cluster, molecule-to-molecule, or atom-to-atom on top of a base substrate. The bottom-up approach is the adhesion of the surface layers to the base substrate. The most commonly used bottom-up methods are wielding and fixed.

The mechanism of biosynthesis of nanoparticles in plants can be associated with the phytoremediation, and bioremediation concepts metal oxide nanoparticles are viewed as potential next-generation or disinfecting agents, these are

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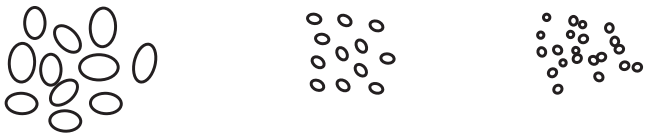


FIGURE 2.1 Bottom-up approach.

finding applications in the area of clinical concern, consumer products, and other industrial applications. Nanoparticles also have considerable attention to their unique antibacterial, antifungal, UV filtering properties, and high photochemical and catalytic activity (Mishra et al., 2011). The use of environment-friendly materials like plant leaf and root extract, and microorganisms such as bacteria, actinomycetes, fungi, and yeast offers extra benefits of compatibility for biomedical and pharmaceutical applications. These biological organisms can provide an environment for the synthesis of metal nanoparticles either intracellular or extracellular (Sadeghi & Gholamhoseinpoor, 2005). These organisms produce biominerals, which are composite materials and consist of an inorganic component and a special organic matrix like proteins, polysaccharides, or lipids, that control the morphology of the inorganic compound (Uosaki et al., 2014; Samberg et al., 2010). The formation of NPs has provided us with remarkable developments in the area of nanotechnology by demonstrating its potential over the last decade (Xu et al., 2006).

These particles can be categorized widely as inorganic and organic nanoparticles. Inorganic nanoparticles include semiconductor nanoparticles such as ZnO, ZnS, and CdS metallic NPs like gold, silver, copper, and aluminum and magnetic nanoparticles like Co, Fe, and Ni, while organic NPs incorporate carbon nanoparticles like fullerenes, quantum dots, carbon nanotubes. Many of these nanoparticles such as ZnO, SiO_2, FeO, and TiO_2, are frequently used due to their photocatalytic properties. Elemental metals such as Ag, Au, Fe, Cu, Pt, Pd, Ni, and Co are widely used for antimicrobial, optical, catalytic, electronic, etc. Wires made from Au, Cu, Si, and Co are used as conductors and semiconductors (Vadlapudi and Kaladhar, 2014). There is an extended interest in gold and silver nanoparticles as they enhance superior characteristics with useful flexibility (Bhainsa and D'souza, 2006). Silver nanoparticles have a required surface zone which results in catalytic activity, biochemical reactivity, and atomic behavior in contrast to large particles having the same chemical composition (Rajakumar et al., 2012). Noble metal nanoparticles such as silver, gold, copper, iron, and platinum are widely used in medicinal applications. There is a growing need to develop an environmentally—safe process for the synthesis of nanoparticles that do not employ toxic chemicals (Rajakumar et al., 2012; Shahverdi et al., 2007; Sharma et al., 2019).

Medicinal plants have been very useful in traditional medicine since old times and are a rich source of bioactive compounds having antiviral activities plants, and their parts are more effective and safer than synthetic drugs and chemicals in the treatment of certain diseases. Medicinal plants are a rich source of bioactive components including various secondary metabolites like alkaloids, flavonoids, phenolics, saponins, terpenoids, tannins, etc (Garg and Garg, 2018). The medicinal plants and their product use as medicines could be traced from the beginning of human civilization. The medicinal value of plants and almost all of their parts have been mentioned earlier in Hindu culture. It is found in one of the Vedas, Rigveda which have been written during 4500−1600 B.C., and it was a kind of library for human awareness that it is an ancient collection of Hindu culture in medicinal science (Huang et al., 2007). Plants are common and inexpensive sources of nanomaterials (Hutchison, 2008). Using plant parts that do not harm the environment, safe materials like leaf, stem, and root extract, bacteria, saccharomyces, actinomycetes, and fungi offer additional benefits of compatibility for biomedical and plant-made pharmaceutical applications. These biological organisms can provide an environment for the synthesis of metal nanoparticles either intracellular or extracellular (Romero et al., 2006). Plant extracts including secondary metabolites, such as phenolic acids, flavonoids, alkaloids, and terpenoids, play a major role in the formation of nanoparticles in an eco-friendly reaction (Hutchison, 2008). At present, various plants and herbal compounds are used to treat several diseases around the world. The use of plants has long been considered in the traditional medicinal system from the century (Amooaghaie et al., 2015).

In the field of nanotechnology research and studies have been improved rapidly throughout the world. Despite the potential of growing in the field of nanotechnology, there are still some problems with the possible risks and effects of nanoparticles on the environment and human health (Elangovan et al., 2015). Nanoparticles have become a significant subject of study in recent years due to their substantial applications in a variety of fields including diagnostics, bio-markers, cell labeling, antimicrobial agents, drug delivery, and cancer therapy (Goswami et al., 2017). The most practical and effective nanoparticles were found to be in the range of 1−100 nm. Some metal oxide nanoparticles are used as ingredients for rubber additives, catalytic converters, biomedical imaging, photovoltaic cells, sensors, and environmental remediation

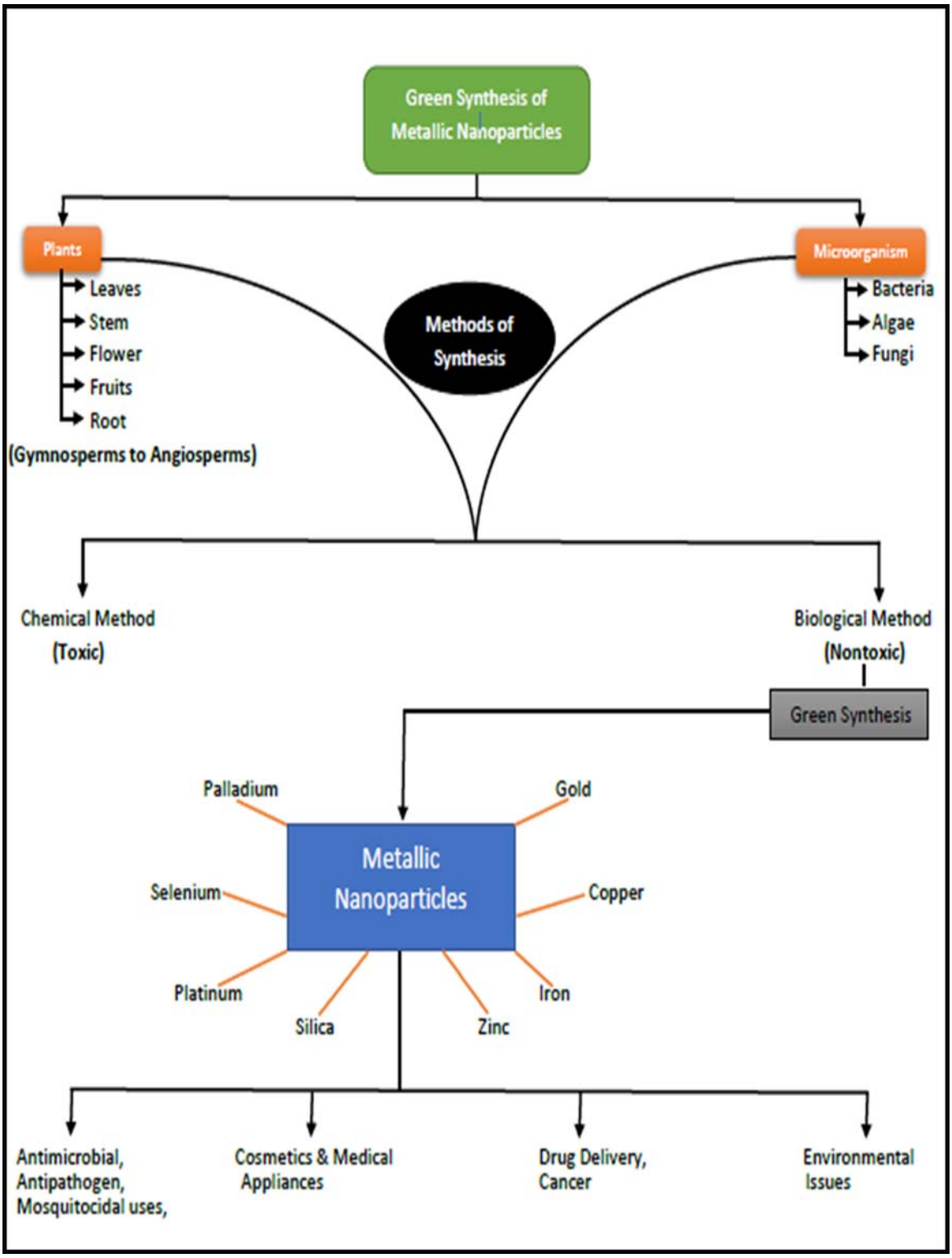


FIGURE 2.2 Showing different nanoparticles and their uses.

such as paints, cosmetics, and plastics products (Yates and Dionysiou, 2006). Some of the ongoing production methods for the fabrication of nanomaterials are discussed from a green point of view, and deficiencies are explained. This targeting is generally best accomplished by chemical modification of the nanoparticle's surface to increase its affinity for a specific cell (Krutyakov et al., 2008). Nanoparticles are unique because of their large surface area and this dominates the contributions

FIGURE 2.3 *Tinospora cordifolia.*

FIGURE 2.4 *Azadirachta indica.*

made by the small bulk of the material. Nanoparticles have different colors like yellow, gold, and gray. Especially silver nanoparticles have distinctive physicochemical properties, including high electrical and thermal conductivity, surface-enhanced Raman scattering, catalytic activity, chemical stability, and nonlinear optical behavior (Shinde et al., 2012). The absorption of solar radiation in photovoltaic cells is much higher in nanoparticles than it is in thin films of continuous sheets of the mass material since the particles are smaller, they absorb a greater amount of solar radiation. The superiority

FIGURE 2.5 *Ricinus communis.*

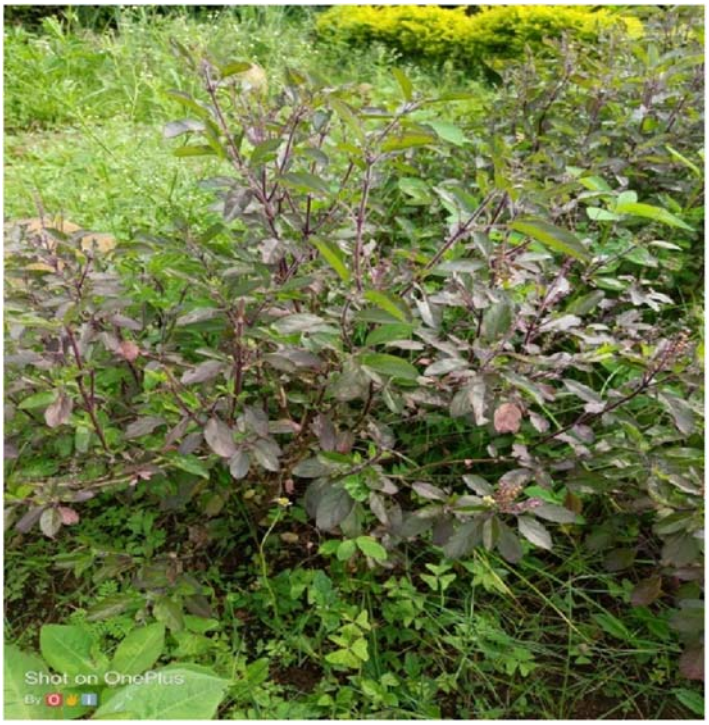


FIGURE 2.6 *Ocimum sanctum.*

FIGURE 2.7 *Moringa oleifera.*

of using nanoparticles for therapeutic drug delivery results shows two main basic properties first their nano size so these nanoparticles can penetrate through smaller capillaries and are occupied by cells, which allows efficient drug accumulation at the target sites. A second property is the use of biodegradable materials for nanoparticle preparation allows enduring drug release within the target site over days or even weeks (Yadav et al., 2013). While carrying drugs with nanoparticles have no biotoxicity for the carrier. Nanoparticles do not show any problem in large-scale production and sterilization but they only avoid organic solvents.

In this study plant parts of *Tinospora cordifolia, Azadirachta indica, Ocimum sanctum, Moringa oleifera, Ricinus communis* were used to evaluate their potential in the synthesis of various nanoparticles like gold, copper, silver, zinc, titanium, and iron.

Tinospora cordifolia

Kingdom—Plantae
Division—Magnoliophyta
Class—Magnoliopsida
Order—Ranunculales
Family—Menispermaceae
Genus—*Tinospora*

Species—*cordifolia*

Plant Description—*Tinospora cordifolia* commonly called gurjo, heart−leaved moonseed, Guduchi, or giloy. it is a herbaceous vine indigenous to tropical regions of the Indian subcontinent. It has been used as a traditional medicine to treat various disorders. It contains diverse phytochemicals, including alkaloids, phytosterol, glycosides, flavonoids, phenols, etc.

Azadirachta indica

Kingdom—Plantae
Division—Magnoliophyta
Class—Magnoliopsida
Order—Sapindales
Family—Meliaceae
Genus—*Azadirachta*
Species—*indica*

Also called neem, the fast-growing tree is valued as a medicinal plant, as a source of organic pesticides, and economically for its timber. This plant is native to the Indian subcontinent and to dry areas throughout south Asia. It has been also introduced to parts of Africa, the Caribbean, and numerous counties in South and Central America. The plant has long been used in folk medicine and also used in cosmetics and organic farming.

Ricinus communis

Kingdom—Plantae
Division—Tracheophyta
Class—Dicotyledonae
Order—Malpighiales
Family—Euphorbiaceae
Genus—*Ricinus*
Species—*communis*

Plant description—*R. communis* commonly known as the Castor plant is a fast-growing shrub. Castor oil and the roots and leaves are used in the Indian Indigenous Medicinal System for various diseases, and it has been investigated in a few limited studies for its potential as an antinociceptive and antiinflammatory herbal drug.

Ocimum sanctum

Kingdom—Plantae
Division—Magnoliophyta
Class—Magnoliopsida
Order—Lamiales
Family—Lamiaceae
Genus—*Ocimum*
Species—*sanctum*

It is commonly known as tulsi, holy basil which is an aromatic perennial plant. It is native to the Indian subcontinents. Tulsi is cultivated for religious and traditional medicine and essential oils purposes. It is widely used as a herbal tea commonly used in Ayurveda. Plant parts are used as antitubercular, insect-repellent, and nematocidal.

Moringa oleifera

Kingdom—Plantae
Division—Magnoliophyta
Class—Magnoliopsida
Order—Brasicales
Family—Moringaceae
Genus—*Moringa*
Species—*oleifera*

Plant Description—*M. oleifera* is a fast-growing, deciduous tree commonly called drumsticks. It has numerous applications in cooking throughout its regional distribution. Oil is used for hysteria, scurvy, prostate problems, and bladder troubles. The roots and bark are used for cardiac and circulatory problems, as a tonic, and for inflammation. The bark is an appetizer and digestive. The iron content of the leaves is high, and they are reportedly prescribed for anemia.

Methodology of nanoparticle synthesis

Chemical approach

In this method, the main components are the metallic precursors, stabilizing agents, and reducing agents are organic and inorganic both. Some of the reducing agents are sodium citrate, ascorbate, sodium borohydride, elemental hydrogen, polyol process, N, N-dimethylformamide (DMF), and polyethylene glycol is used (Zhang et al., 2016). chemical procedures start with reducing the metal ions to metal atoms which are followed by controlled bulk of atoms (Sotiropoulou and Chaniotakis, 2003).

Physical approach

Synthesizing nanoparticles is mainly a top-down approach in this method where the material is reduced in size by various physical approaches such as ultrasonication, microwave irradiation, electrochemical methods, and so on. In this method, various researchers used a tube heater which is utilized at barometrical weight by evaporation condensation for integrating nanoparticles. Evaporation condensation and laser removal are the essential physical methodologies. Different nanoparticles of silver, gold, lead, and cadmium have been synthesized and reported already (Mathur et al., 2018). The geometries can be also achieved by physical methods (Mandal et al., 2006) which can be utilized in varied applications. Ball milling, ion beam lithography, Photolithography, microcontact printing, evaporation−condensation, dip pen lithography, electrochemical synthesis, and nanoimprint lithography are reflected as novel techniques for nanoparticles (Chen and Pepin, 2001).

Green synthesis method

The biological method, which is represented as an alternative to chemical and physical methods, provides an environment-friendly way of synthesizing nanoparticles. This method does not require any expensive, harmful, or toxic chemicals. Metallic nanoparticles with various sizes and shapes, contents, and physicochemical properties can be synthesized. Synthesis can be done in one step using biological organisms such as bacteria, yeasts, molds, algae, and plants. Molecules in plants and microorganisms, such as proteins, enzymes, phenolic compounds, amines, alkaloids, and pigments perform nanoparticle synthesis by reduction (Shah et al., 2015; Nadaroglu et al., 2017; Nadaroglu et al., 2017; Cicek et al., 2015; Narayanan and Sakthivel, 2010; Mukhopadhyay and Yadav, 2011). Nowadays because of rapid development, affordable culturing costs, and easy control and manipulation of the growth environment, bacteria are targeted in the production of nanoparticles. At the same time, it is helpful to know some species of bacteria have special mechanisms to suppress the toxicity of metals or heavy metals. Bacteria preferred for these properties can perform nanoparticle synthesis in situ and ex situ. (Gao et al., 2014).

Methodology for preparation of plant sample

1. 20 g of the leaf was thoroughly washed and finely cut leaves were put in a 500 mL Erlenmeyer flask along with 100 mL of distilled water and then boiled for 5 min then the extract was filtered with Whatman No. 1 filter paper and stored at 4°C and used for further experiments (Mallikarjuna et al., 2011).
2. The plant extract was prepared by the Soxhlet extraction method (Okeke et al., 2001). Powder material (100 gm) was uniformly packed into a thimble and run in a Soxhlet extractor. It was extracted with solvent (methanol) till the solvent in the siphon tube of an extractor become colorless for about 48 h approximately. After that extract was filtered with the help of filter paper and then evaporated in a Rotary evaporator to get a syrupy consistency. Then the extract was kept in a refrigerator at 4°C for future experiments (Dubey et al., 2009).

Methodology for the synthesis of nanoparticles

1. Leaf extract (0.5 mL) was added to 10 mL of 1 mM $AgNO_3$ aqueous solution. UV−Vis spectrophotometer was used to measure the spectra of the solution. The suspension was diluted with distilled water to avoid errors due to the high optical density of the solution. (Mallikarjuna et al., 2011).
2. The dried leaf was added to double distilled water. After that, zinc acetate was dissolved in a water solution and the solution was obtained. The extract was added to the solution and evaporate for 12 h approximately to yield white zinc oxide nanoparticles and then desiccated at 100°C to obtain nanoparticles (Dhanemozhi et al., 2017).

3. Leaves (about 10 gm) were transferred into 100 mL of boiled double-purified water and kept for 10 min. After that, the hot leaf extract was filtered through filter paper and then kept for further process. In a conical flask containing 100 mL of 1 mM $AuCl_4$ solution with 10 mL leaf extract and stir well for about 30 min. After 30 min, the light yellow-colored mixture changed into a wine-red color, which represents the synthesis of gold nanoparticles (Balalakshmi et al., 2017).
4. 10 μg/mL of Methanol leaf extract was added to 1 mM silver nitrate solution at different ratios of 1:1, 1:2, and 1:3, and the volume was made up by the addition of distilled water. The solution was mixed by gentle shaking and kept at room temperature for 24 h.
5. An aqueous solution of salt $CuCl_2 \cdot 2H_2O$ (7.5×10^{-3} M) was heated to 85°C in an oil bath with the help of magnetic stirring; then neem leave's broth (20%) was added dropwise to this solution at different time intervals, the color changes were seen gradually from green, yellow, orange, radish brown, brown and finally dark brown with their stages.
6. For the synthesis of TiO_2 NPs, 100 mL of $TiO(OH)_2$ (0.1 mM) was stirred for 2 h 20 mL of the aqueous extract of plant extract was added to 80 mL of $TiO(OH)_2$ at room temperature under stirred conditions for 24 h. The pure $TiO(OH)_2$. After the reaction extract with $TiO(OH)_2$, the synthesized nanoparticles turned light green.

Metals synthesized from green synthesis:
Copper (Cu) and copper oxide (CuO):

Copper nanoparticles have recently created special attention because of their low-cost and novel optical, catalytic mechanical, thermal conduction, and electrical properties, which are different from that of their bulk metals (Brust and Kiely, 2002; Lee et al., 2009). It can be used in different formulations like nano−fungicides, nano−antimicrobials, and nano−fertilizers. UV−Visible spectrometer was used for the confirmation of copper nanoparticle formation 578-nm peak was obtained average size of copper nanoparticles was 40 nm (Karimi and Mohsenzadeh, 2015).

Silver nanoparticles

Silver NPs have been broadly considered for use in different fields like optoelectronics, catalysis, medicine, sensing, etc. Silver nanoparticles have a high surface area and unique chemical, and physical properties (Markowska et al., 2013). Among other noble metals, the synthesis of silver nanoparticles has established great significance as an antimicrobial agent (Panacek et al., 2006; Parashar et al., 2009). The unique properties of silver nanoparticles are mainly advantageous for cancer therapeutics. For the green synthesis of silver nanoparticles, the key requirements are a silver metal ion solution and a reducing biological agent. The easiest method for silver nanoparticle production is silver ion reduction and stabilization by a fusion of biomolecules such as polysaccharides, vitamins, amino acids, proteins, saponins, alkaloids, terpenes, and phenolics (Tolaymat et al., 2010).

Gold

Gold nanoparticles are paid attention to due to their high potential use in medicine (Jain et al., 2006). biocompatible nature (Sperling et al., 2008), less toxicity (Jeong et al., 2011), surface plasmon resonance, scattering and absorption properties (El-Sayed et al., 2005), facile synthesis, and easy surface functionalization (Ghosh et al., 2008). Gold nanoparticles have been investigated for potential applications in the field of biosensors (Kreibig and Vollmer, 1995; Chan and Nie, 2016), hyperthermia therapy (Huang et al., 2006), delivery platforms for therapeutic drugs (Paciotti et al., 2004), and antimicrobial drugs (Sondi and Salopek-Sondi, 2004; Hsiao et al., 2006) Employing plants as biological factories has the potential to deliver an environmentally friendly source of gold nanoparticles.

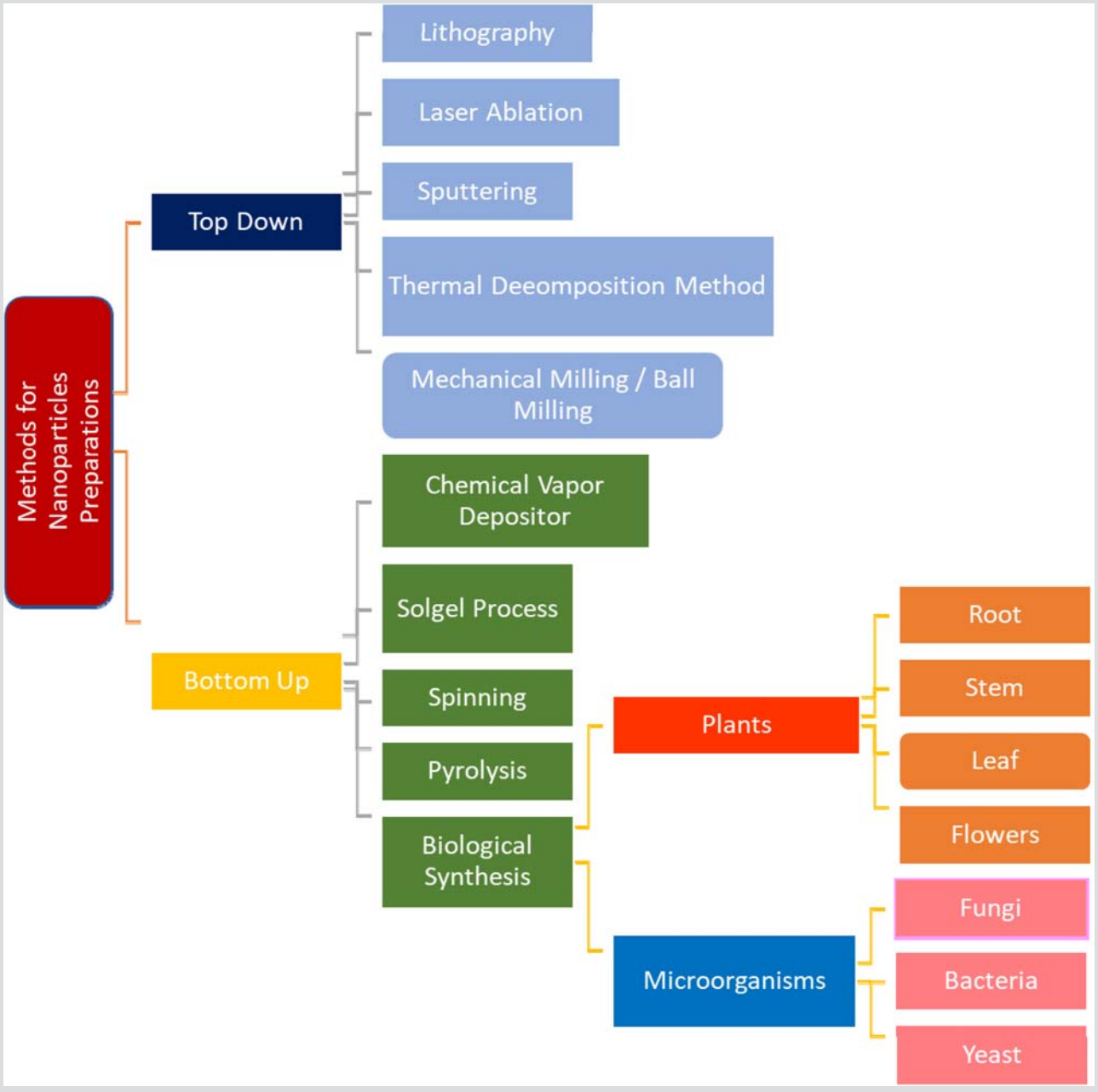
Zinc oxide nanoparticles (ZnO NPs)

Zinc oxide nanoparticles have created a lot of research interest because of their significant roles in biological fields and the energy sector. Zn oxide nanoparticles are less expensive, easy, and safe Zn oxide nanoparticles have been used in cancer therapy, and drug delivery and they possess antibacterial properties (Hameed et al., 2016). Zinc oxide NPs are one of the important particles to have received a lot of attention, due to their antimicrobial, and UV filtering characteristics Zinc NPs are successfully used as food additives and in cosmetics such as sunscreens, due to their ability to absorb ultraviolet rays (Kumar et al., 2011; Wang et al., 2004; Nohynek et al., 2007; Nohynek et al., 2008). The use of Zinc oxide nanoparticles is also used for agricultural purposes such as pesticides, without affecting soil fertility (Sharma et al., 2010). Numerous works have been reported now for this nanoparticle synthesis and utilization by plants, microorganisms, and others. Plant parts like stems, flowers, roots, seeds, and leaves can be used for the synthesis of Zn oxide nanoparticles, (Jadoun et al., 2021).

Titanium dioxide (Tio_2)

Titanium oxide nanoparticles are considered of great interest with their exclusive morphologies and surface chemistry. These nanoparticles are very useful in the preparation of paper textiles, cosmetics, plastics, tints, foodstuffs, etc. TiO_2 nanoparticles are vigorously used in the reduction of various toxic chemicals such as pollutants and dyes from water. Nowadays numerous plants have been used for their synthesis and applications. The synthesis starts with the reaction of a plant extract with TiO_2 salt. Initially, the preparation of nanoparticles can be confirmed by the changes in the color of the reaction mixture, after that the morphological and spectroscopic studies confirmed their shape and size formation. These nanoparticles are reported as light green changes into dark green color. TiO_2 nanoparticles were found mostly spherical shaped (Jadoun et al., 2021).

TABLE 2.1 Schematic representation of nanoparticles methods.



Antimicrobial studies of nanoparticles

Nanoparticles are using as antimicrobial agents for decades. It has a strong antibacterial activity which can decrease the microorganism concentration. Cu Nanoparticles are known for a wide range of antibacterial activity against different strains of gram-positive and gram-negative bacteria. Silver nanoparticles are good antimicrobial agents against many pathogenic microorganisms (Samadi et al., 2010). Recently, silver nanoparticles were demonstrated as having enhanced antimicrobial and biofilm inhibition activity as compared to that of silver metal salt (Naqvi et al., 2013; Singh et al., 2015) their tremendous antimicrobial activity inhibited pathogenic microorganisms like *Bacillus pumilus, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Aspergillus niger*, and *Penicillium chrysogenum*. Gold NPs have an antibacterial effect on a range of gram-positive and gram-negative bacteria (Shi et al., 2008). The synthesized Au NPs have shown enhanced antibacterial activity (Rajan et al., 2015). TiO_2 NPs where protein is used as a stabilizing agent, and its antimicrobial activity against *Bacillus subtilis* (Gram-positive), *Klebsiella planticola* (Gram-negative), and the fungal pathogen *Aspergillus niger* (Tables 2.1–2.3).

TABLE 2.2 Showing characterization techniques.

Characterization techniques	Function
UV–visible spectrometer	It detects the surface plasmon resonance which absorbs a specific wavelength of light
Fourier transformation infrared spectroscopy (FT-IR)	Detection of functional groups which are responsible for capping, stabilizing, and reducing agent/of metal NPs.
Transmission electron microscopy, high-resolution transmission electron microscopy, and scanning electron microscope (TEM, HR-TEM, and SEM)	It confirms the size, and morphological shapes of nanoparticles
Energy-dispersive spectroscopy (EDX)	It uses for the study of the elemental composition and purity of green synthesized nanoparticles.
Atomic force microscope (AFM)	Gives 2D and 3D structures of NPs.
Powder X-ray diffraction (XRD)	Detect the crystallinity of nanoparticles
Zeta potential	Detect surface charge of NPs which is responsible for stability.
Dynamic light scattering (DLS)	It detects the distribution and size of nanoparticles in colloidal solution, and detect clumps of nanoparticles.
X-ray photoelectron spectroscopy (XPS)	Used for the study of surface chemistry, elemental composition, electronic and chemical state of nanoparticles
Vibrating sample magnetometer (VAM)	Used for the study of magnetic properties of nanoparticles
Thermogravimetric analysis (TGA)	Thermal analysis.
Brunauer–Emmett–Teller (BET)	Surface area detection
Low-energy ion scattering (LEIS)	For the thickness of nanoparticles
Photoluminescence (PL) spectroscopy	Used for fluorescence, quantum dots, and metal nanoclusters study
Nanoparticle tracking analysis (NTA)	Used to detect nanoparticles size distribution in a liquid medium, capping efficiency refractive index between nanoparticles and lights.
Mass spectrometry (MS)	Study the elemental and molecular compositions and the chemical state of NPs, study the bioconjugation of NPs with target biomolecules inductively coupled plasma
Differential centrifugal sedimentation (DCS)	Detect size based on sedimentation rates
Ferromagnetic resonance (FMR)	Detect nanoparticle size, distribution, shape, surface composition, magnetic anisotropic constant, and demagnetization field.

TABLE 2.3 Depicts different medicinal plants with nanoparticles of different shape and sizes.

Plant name	Nanoparticle	Shape & Size(nm)	References
Azadirachta indica	Gold, silver	5–35 & 50–100 spherical, triangular, hexagonal	(Shankar et al., 2004)
Moringa oliefera	Silver	57 spherical	(Prasad and Elumalai, 2011)
Ocimum sanctum(root)	Silver	10 ± 2 & 5 ± 1.5 spherical	(Ahmad et al., 2010)
Ocimum sanctum (leaf)	Silver	10–20 spherical	(Philip and Unni, 2011)
Azadirachta indica	ZnO	40	(Sharma et al., 2018)
Tinospora cordifolia	Cu	50–130	(Elumalai and Velmurugan, 2015)
Azadirachta indica	CuO	49–324	(Ansilin et al., 2016)
Ocimum sanctum (leaves)	Cu	77	(Vasudev Kulkarni and Kulkarni, 2013)
Moringa oleifera	Cu	Spherical 6 & 61	(Galan et al., 2018)
Ocimum sanctum (leaf)	Gold	30 crystalline, hexagonal	(Philip and Unni, 2011)

Conclusion

In this chapter, we conclude that green synthesized nanoparticles of copper, silver, gold, titanium, and zinc are a great source of medicines as it was discussed by many researchers. These nanoparticles have been characterized by researchers and provide their shape and size. Some selected medicinal plants with their health benefits, ethnomedicinal properties, and antimicrobial studies of these particles are also included. Green synthesized nanoparticles will give great changes to our health and society also it will open various doors for researchers.

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Chapter 3

Recent trends of viral nanotechnology: an overview

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Introduction

Virus infections cause deadly human diseases with morbidity and sometimes mortality. Recently, viral infections have been used in the engineering of druggable candidates for the concomitant progression in pharmaceutics. Indeed, several viruses have similar patterns of composition and interacting patterns with specific receptors and co-receptors on host cell surfaces, which remain identical during the establishment of an infection (Tarafdar et al., 2013). Hence, the need to explore the "cell−virus interaction mechanism" remains as the thrust area for developing some effective antiviral agent in interdisciplinary nanosystems. Indubitably, viral nanoparticles (VNPs) and antiviral agents with vast properties and target-oriented applications to align them like a drug-cascade of nanosystems would be the challenging aspect of this endeavor. Mostly, VNPs of animal viruses are efficient with innovative applications for drug delivery in animal systems, due to the desirable biodegradability and biocompatibility, and specifically for the noninfectious nature with other hosts. Viruses also have individual characteristics such as high stability and well-defined three-dimensional (3D) structures with some precise interior and exterior surfaces that suitably lend to viral nanotechnology for use in health improvement (Koudelka et al., 2015; Rizvi and Saleh, 2018).

However, the unfortunate multidrug-resistance character in bacterial pathogens and the danger of further resistance to the newly added antibacterials are the commonplace occurrence causing clinical annoyance in the health sector today (Sahoo et al., 2021). The bacterial resistance to contemporary antibacterials/antibiotics and the added newer generations of antibiotics and/or modified versions of the obsolete antibacterials are a vicious cycle today (Sahoo et al., 2020b). As it is, the older drug formulations are ineffective against the present pathogenic bacterial flora, eventually requiring more/suitable drugs for the coveted control; those drugs are newly added or older ones are modified in turn. Thus, intuitively, it can be stated that the development of novel therapeutic stratagems by viral nanotechnology could solve the present problems, as viral nanotechnology has become a bold interdisciplinary research arena.

Moreover, viruses are predominantly small infective agents responsible for some serious morbidity or fatal diseases. Nowadays, viruses and virus-like particles (VLPs) are of considerable interest due to the inherent scope toward integration and facilitating the use of a broad spectrum for therapeutic purposes with viruses in nanotechnology (Singh et al., 2017). Moreover, viral nanotechnology has an interdisciplinary approach of the integration of virology, microbiology, chemistry, physics, immunology, and mathematical modeling (Schwarz et al., 2017). Viral nanotechnology includes the use of a viral supramolecular moiety with several recognizable coupling sites aligned on both outer and inner viral capsid surfaces having different shapes and dimensions unique to each virus. Those offer interesting opportunities for the use as nano-containers for the embodiment of different mixtures or as nano-fiber scaffolds to immobilize the functional units for manufacturing coveted agents. Viruses can be modified genetically to increase the selective binding site if necessary, ending up with varying properties of the whole template, as a path to create virus nanoparticles for newer therapeutic applications (Singh et al., 2006). Consequently, the diverse latent applications of viral nanotechnology with several opportunities in the fields of health, biomedicine, photonics, electronics, energy, and catalysis will be explored.

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This is a novel field demanding newer therapeutics that are already partially developed and, today, those being used in the development of vaccines, diagnostic kits, and drugs for prognosis as well as roles in gene therapy. As it is, viruses individually have some 3D nanoscale structures, composed of thousands of copies of one or more coat proteins (CPs) to protect the viral genetic material enclosed in capsids, required during newer infections (Ojasalo et al., 2021). Moreover, the understanding the live-cellular operations through the synthesized VLP-like nano-sized metal−organic frameworks, carbon nano-shells, and quantum dot (QD) nano-cores with veracious compatibility and the inherent interfaces could be increased; by the by, the precise updates for host cellular dysfunctions before their amplifications could be obtained. Additionally, VLPs of a kept nano-sized structure had been developed for the conveyance of infective nucleic acids to be inside capsid proteins with additionally introduced nucleic acids as a normal freight; but, a few investigations have described VLPs for mRNA and rDNA conveyances too. Since this century has received particularly much attention in the health system, both VNPs and VLPs have to do with some therapeutic exactitude, with possible use(s) as the building blocks of VNPs, adopting the technology that has some keen apparatus in a precise diagnostic and effective treatment, as exemplified (Bawa et al., 2016; Steinmetz and Manchester, 2011).

Viral nanoparticles

Principles of structure and characterization

Viruses are produced inside host cells as vehicles for inherent genomes during infection of newer hosts; but, the packaging of extraneous nanoparticles is planned in this field, which gave the idea of VNPs. In the early 1950s, bacteriophages were engineered as cloning vectors, which enabled the viral expression platform to be utilized in "bacteriophage therapy." The hollow protein shell is known as a capsid, which protects the genome from the exterior environment. Indeed, each virus particle has the potency to form a capsid, independently as the viral self-assembled structure (Buzón et al., 2020). Inclusions of viral self-assembled nanostructures into capsids as during viral encapsulation are possible. On the other hand, plant viruses (PVs) have a broad platform during viral synthesis for the molecular entrapment of nanoparticles, which could be utilized in the nano-technologic-based interdisciplinary fields (Wen and Steinmetz, 2016). Thus, there are an enormous platform in which viruses could be employed as a bandwagon of next-generation nanophoto/electromechanical systems, using nano-catalysis, nano-electronics, nano-optics, and biosensing, with the consequent plethora of biomedical applications (Steinmetz, 2010) (Table 3.1). A plant viral capsid gives bio-layouts for aligning of novel nanomaterials with

TABLE 3.1 Properties and applications of different types of viruses (Steinmetz and Manchester, 2011; Singh and Lillard, 2009).

S. No.	Virus	Properties	Application
1	Adenovirus	The average diameter is 70 nm with icosahedral symmetry	DNA and RNA delivery for cancer treatment
2	Potato virus X (PVX)	Approximate diameter of 13 nm with helical symmetry of average length in the range of 470−580 nm.	Genetic modifications, chemical conjugation, immune stimulation
3	Cowpea mosaic virus (CPMV) and cowpea chlorotic mottle virus (CCMV)	Approximate diameter is 28 nm along with T = 3 icosahedral symmetry	Chemical conjugation, bioimaging and encapsidation
4	Tobacco mosaic virus (TMV)	Shape is rod, length is 300 nm, and diameter is 18 nm with single structural protein and 2130 copies of helical symmetry	Modifications by genetics, conjugates by chemicals, and modification of noncovalent immune stimulation
5	M13 bacteriophage	7 nm in diameter with helical symmetry at the length of 900 nm.	DNA and RNA delivery, also platform for functionalization
6	Sulfolobus islandics rod-shaped virus 2 (SIRV2)	Shape is rod, length is 900 nm, and diameter is 23 nm with single structural protein with helical symmetry	Chemical conjugation

polar and nonpolar moieties, incorporated in an enhanced, rather an exact way for the assortment of sizes, the inside, outside, and extremities that are at the developmental process.

Indeed, capsid proteins self-assemble and orient into several nanoscale multivalent architectures, viz., icosahedrals, or any 3D structural symmetry of high mono-disparity. Particularly, PVs afford several protein interfaces between CP subunits to realign extraneous protein molecules with the inherent interacting properties resulting in a multivalent ligand connection of particles, which account for infusion, controlled self-assembly, encapsulation, and biomineralization, as well. This facilitates the utilization of viruses as an apparatus to be used for multidisciplinary applications (Zhang et al., 2018).

Viruses have distinct surface properties, which may be customized to accomplish the ideal capacity by hereditary building or concoction conjugation, or a mixture of both, permitting the exact nanoscale control of a VNP structure and sustaining capacity. If the prospect of material science is considered, VNPs can easily be produced in small numbers, interestingly being synthesized in the laboratory. The resulting particles are monodispersing, generally symmetrical, and polyvalent. Most viral capsids, utilized till now, were either rod-shaped or icosahedral. The average particle size of an icosahedral is 18−500 nm with the filamentous (rod-shaped) shape reaching up to 2 μm in length. VNPs are classically robust by nature. The essential capacity of the capsid is to encase and protect the nucleic acids, expanding their protection from temperature and pH boundaries. VNPs additionally retain the stability in the scope of dissolvable mixtures of buffers, which is fundamental for chemical alteration(s).

i. In the principle of construction, there are a plenty of paths in engineering a virus: viz., the entire genome can be manipulated, or changes can be introduced in the specific coding sequences of a nucleic acid meant for viral structural proteins for having changes to the structure and functionality of the virus per a specific "application requirement."
ii. The structural functionalization externally for the conjugation or immobilizations is an additional feature with VNPs.
iii. The encapsulation processes to utilize these viral capsids molecular cages to entrap small molecules are facilitated.

Nanomachines

These are mechanics that serve as the cascades of chemical reactions or electromechanical systems, in the range of micro/nano dimensions (10^{-9} m). Indeed bacteriophages, having been replicated in hosts and released in numerous exact copies, are of nano-sizes with abundant biologic entities, which enable those as efficient nanomachines. The bacteriophage M13 of the bacterium *Escherichia coli* is genetically, biochemically, and biophysically well studied (Hemminga et al., 2010). This phage is typical with a filamentous particle of the length of 900 nm, and the diameter is approximately 6.5 nm. Its hexagonal protein coat contains the single-stranded viral DNA genome of 6407 nucleotides; it lends itself to several interdisciplinary technologies through genetic modifications. Blithely, the fabrication of supramolecular nanostructures via self-assembly molecules enables the utilization of the phage as a tool for tissue engineering, with the generation of VNPs that have earned enormous importance in modern therapeutic development cascades. Thus, the M13 phage had been utilized for the development of optical, conducting, semiconducting, and magnetic materials.

Bionanomaterials from plant viruses

Several broad types of PVs have VNPs, which are not enveloped, but comprise only one or numerous kinds of subunits, organized either with icosahedral or helical symmetry around a genome; plant viral particles can be created in enormous amounts in plant cells. A PV capsid could be manipulated genetically and chemically or along with viral CPs (Zhang et al., 2018). PVs with the requisite properties are safely used for the intended modifications according to the therapeutic demands, since those are nonantigenic or nonpathogenic to animals and humans (Table 3.2).

X-ray analysis of viral nanoparticles

Indeed, through X-ray crystallography of a virus, 3D atomic structures are improved, and the viral life cycles are well understood; eventually, the development of antiviral drugs is pursued. The advancement of ultrafast X-ray free-electron lasers opens the vast possibilities for the atomic-resolution imaging of replicable objects, like biologic cells, viruses, nanoparticles, clusters, and single molecules perhaps, which enables achieving resolution(s) for a single-particle imaging better than a few tens of nanometers values. The in situ analysis of small-angle X-ray scattering of palladium (Pd) nanoparticles grown on tobacco mosaic virus (TMV) was reported; and the dissipated beams at small angles (under 2°) has been recorded on a 2D charge-coupled gadget detector (Meents and Wiedorn, 2019).

TABLE 3.2 Viral nanoparticle properties and their applications (Steinmetz and Manchester, 2011).

S. No.	Plant virus	Properties	Applications
1.	Tobacco mosaic virus (TMV)	Various properties of both exterior and surface, because of various amino acid composition, permit spatial and controlled statement of metals	As biotemplate for fabrication, range of nanotubular inorganic materials through metal deposition of PbS, CdS, Ni, Co, Cu, Pd, Pt, Ag, SiO_2, TiO_2, Al_2O, etc.
2.	Cowpea chlorotic mottle virus (CCMV)	**(1)** CCMV particles undergo reversible pH- and metal ion-dependent structural transitions. These structural transitions are described as a swelling mechanism which results in an approximately 10% increase in virus dimension **(2)** Altering of the interior surface of the CCMV cage from cationic to anionic, with the resulting particles favoring the encapsulation of cationic species	**(1)** The particular epitome of an anionic natural polymer, polyanetholesulfonic corrosive, into the CCMV confines **(2)** Particular capture followed by spatially compelled mineralization of iron oxide and polyoxometalate species, for example, vanadate, molybdate, and tungstate inside the CCMV particle
3.	Brome mosaic virus (BMV)	It has been discovered that CP monomers collect in vitro into unblemished VLPs and that BMV, like CCMV, has a pH and a particle subordinate expanding system. Polyethylene glycol (PEG)-covered particles were found to give best yields and result in the development of fundamentally unblemished and stable BMV VNPs	**(1)** Viral cages with incorporated AuNPs and QDs offer new instruments for biosensing applications, for example, following a viral infection process **(2)** The utility of VLPs containing NPs for single infection spectroscopy has been illustrated
4.	Red clover necrotic mosaic virus (RCNMV)	The self-assembly process of the RCNMV capsid is well understood. The assembly of the CP is stabilized by an internal protein/RNA cage and is initiated with the recognition of the origin of assembly site on the viral RNA by the CP and results in the formation of virions with encapsulated RNA	The hybrid metal-containing VNPs may be used as tools for biosensing purposes or as building blocks for the construction of new nanostructured materials
5.	Cowpea mosaic virus (CPMV)	**(1)** CPMV virions small particles evidently can diffuse through the capsid **(2)** CPMV wild and mutant particles show diverse responsive gatherings on the outside and inside surfaces, a wide scope of specific science is accessible permitting adjustment, notwithstanding standard (bio) conjugation procedures	Permitting efficient attachment of a range of molecules such as carbohydrates, peptides, polymers, and proteins as well as inorganic particles such as QDs, nanotubes, gold nanoparticles and a few more

Virus-like nanoparticles

Virus-like nanoparticles (VLNPs) are the noninfectious protein shells or capsids with the basic protein capsid units, and with the infectious, genetic material, they having been changed to be useful in nanomedicine. Some VLNPs from viral diseases can be dismantled and reassembled in vitro, permitting the segregation from the irresistible nucleic corrosive outer segment that would facilitate biosynthesis of VLNPs. The distinctive highlights of these biocompatible and biodegradable nanoparticles are evenness, polyvalence, and unmatching monodispersion. Furthermore, the recombinant VLNPs are self-collected from the heterologous articulation of basic proteins, with the unfilled inside cavity being manageable to both hereditary and substance adjustments. VLNPs can be created from beneficial infections of host material or by a recombinant expression of protein. Indeed, in biomimetic approaches, viruses serve as suitable systems because of their intrinsic

abilities to target host cells in the cytoplasmic delivery system. The surface designing of nanoparticles speaks to a promising method to confer infection highlights onto particles, allowing changes in topology that mirror the harshness of infections to encourage disguise to adjust the physicochemical highlights of cells to direct changes in size and charge to impersonate the contamination pattern of infections/transfections of nanomaterials with the virus. The vectors display chivalrous properties for consolidation of their capacities into a new age of developing bio-inspired engineered bearers, explicitly through surface adjustments to arrange natural obstructions that hinder free medications and the basic nanoparticle definitions (Parodi et al., 2017; Shoeb and Hefferon, 2019).

Moreover, wiping out of the utilization of viral parts is basic for instigating subsequent contamination, accommodating a more secure option in contrast to the utilization of infections as medication vehicles. This technique keeps on being filled with concerns of immunogenicity and wellbeing. Profoundly, infections to make novel biomimetic-medicated conveying stages can address a few confinements of the current nanoparticles and free medication plans, empowering the improvement of viable and straightforward answers for the expanded intracellular conveyance of genetic loads. Utilizing methodologies procured from infections, the developed agents have effectively shown the capability of impersonating inherent highlights, conferring nanoparticles with the expanded capacity to stay away from opsonization and depart from the endo-lysosomal pathways (Rohovie et al., 2017).

Self-assembling virus-like nanoparticles and virus-unlike nanoparticles

The utilization of self-assembling nanoparticles could adequately be joined by developing the control of a basic vaccine for the greatest effect in the structure of vaccine antigens. The VLPs are made from single or different viral antigens, now and again tied down in a lipid bilayer. Subsequently, utilizing the common or built-protein nanoparticle frameworks, biologists would now be able to include heterologous epitopes/antigens on the plain nanoparticle, a boundless wellspring of conceivable chimeric nanoparticle antigens. Chimeric nanoparticles would acquire through self-assembly or covalent connection of the substance of an antigen to a nanoparticle. Therefore, nanoparticles offer an aggregate quality of several restricting destinations and can give improved antigen constancy and immunogenicity against viruses. A few energizing advances have risen recently, including preclinical proof that the system might be relevant for the improvement of inventive new vaccines. Indeed, VLPs and nanoparticles are now ground-breaking entities for multivalent antigen introduction, corresponding to a few self-collecting proteins, which have been effectively utilized as frameworks to introduce complex surface glycoprotein (López-Sagaseta et al., 2016).

Amphiphilic systems

The chemical moiety or a cascade of processing with a hydrophilic and lipophilic system is considered an amphiphilic system, where the nature of molecules defines the way those are from the membranes, as those align themselves into bilayers via positioning the inherent polar groups toward the surrounding aqueous medium and the associated lipophilic chains toward the inside of the bilayer, sandwiching a nonpolar group between two polar ones (Xu et al., 2018). Hence, by having both hydrophilic and hydrophobic parts on molecules in proteins, the building of the reputation of manipulation in VNPs and the sophisticated engineering of VLPs through polymer vesicles, dendrimers, liposomes, and protein-based nanostructures is confirmed (Kundu et al., 2018). Each of these systems has individual merits and demerits in terms of biocompatibility, pharmacokinetics, toxicity, and immunogenicity, including QDs. Moreover, QDs can have multiple band energies, long-lasting fluorescence, broad bandwidth absorption, and narrow bandwidth emission. Hence, a QD is quite a promising attempt as imaging tools end up with the limitation of their cytotoxic nature. Moreover, dendrimers are simple and low-cost entities to be synthesized without any host in vivo toxicity. The only platform currently approved for clinical use is liposomes, which have shown promising advantages. The obvious approach for preparing VLPs via nanoparticles via condensation of nucleotide chains, liposomes, and cationic polymers has been useful. However, the high levels of transfection efficiency in a cell culture was displayed by polycation−DNA polyplexes, and simply polycation−DNA polyplexes further did not satisfy the practical application in vivo (Raup et al., 2016).

Synthetic virus-like nanoparticles in vaccine design

The integument of nanotechnology in vaccinology has helped to push the limits of nonlive subunit immunizations, bringing about several energizing, powerful, and invulnerable potentiations by nano-formulations. Thus, nanoparticle-based vaccines could improve humoral reactions against the target antigens as well as the advanced cell-based resistance with immunological memory. The nano-formulations are ready to give solutions toward several pressing and rising

human ailments; the VNP-based vaccine could be frequently discovered as some simple procedure of the collaboration elements between the invulnerable framework and infections in drug development. The nano-formulations are like a virus and nanoscale pathogenic creatures that have filled in as specific weights driving the advancement of immunity. Human insusceptibility and antibody systems keep on advancing, perceiving the central similarity between engineered nanoparticles and infections that may offer a clarification for the prevalence of nanoparticle immunizations over regular antibodies and may spike some new plan of methods of reasoning for vaccine development cascades (Fig. 3.1). Overall, vaccine nanotechnology is imagined to improve vaccine wellbeing, intensity, and accessibility, offering convincing stages toward tending to the numerous general well-being threats yet to have compelling prophylaxis and treatment alternatives (Chattopadhyay et al., 2017).

The episode X-beams, with energy of 12 keV, were illuminated as an example in a quartz capillary. The dissipated beams at small angles (under 2°) had been recorded on a 2D charge-coupled gadget detector. Furthermore, in vaccine design, artificial nanoparticles had shown a significant amplifying role along with improving their efficacy and safety over conventional formulations (Sun et al., 2018). Several chemical and physical formulations of nanostructures are like viruses, which are nanoscale materials carrying similar properties like antigens that provide selective stimulation and targeted pressure driving the evolution of the human immune system. A comprehensive strategy of development of B cell—based HCV (hepatitis C virus) through envelope glycoproteins E1 and E2 was responsible for cell entry of HCV optimization and nanoparticle display. Indeed, for the current pandemic of COVID-19, the world aimed at nanoparticle-oriented vaccines initially because nanoparticle-based vaccines do not require additional chemical dubbed moieties to help stimulate the immune framework. Since the adjuvant is an extra part that in a minority of cases can prompt insusceptible overcompensation, consequently, without it, the fabricating forms become simpler and simultaneously diminish the unfriendly impacts. The prepared AuNPs (gold nanoparticles) could serve for the preparation of antibodies as well as vaccines against infectious diseases.

The development of AuNP-based vaccines by experimentally examining the protective properties of vaccines for tick-borne encephalitis and other flaviviruses has been initiated. The vaccine was prepared by conjugating AuNPs (average diameter of 15 nm) to the soluble antigen. Mice were vaccinated intraperitoneally three times, each at 32 μg of antigen per injection. Several studies on AuNP-based vaccination and immunization have been utilized for such crucial objects as foot and mouth disease virus and influenza virus, with some precise recognition of antibodies that were obtained from the immunization of animals with 17 nm AuNPs coupled with pFMDV and pH5N1 antigens of these viruses.

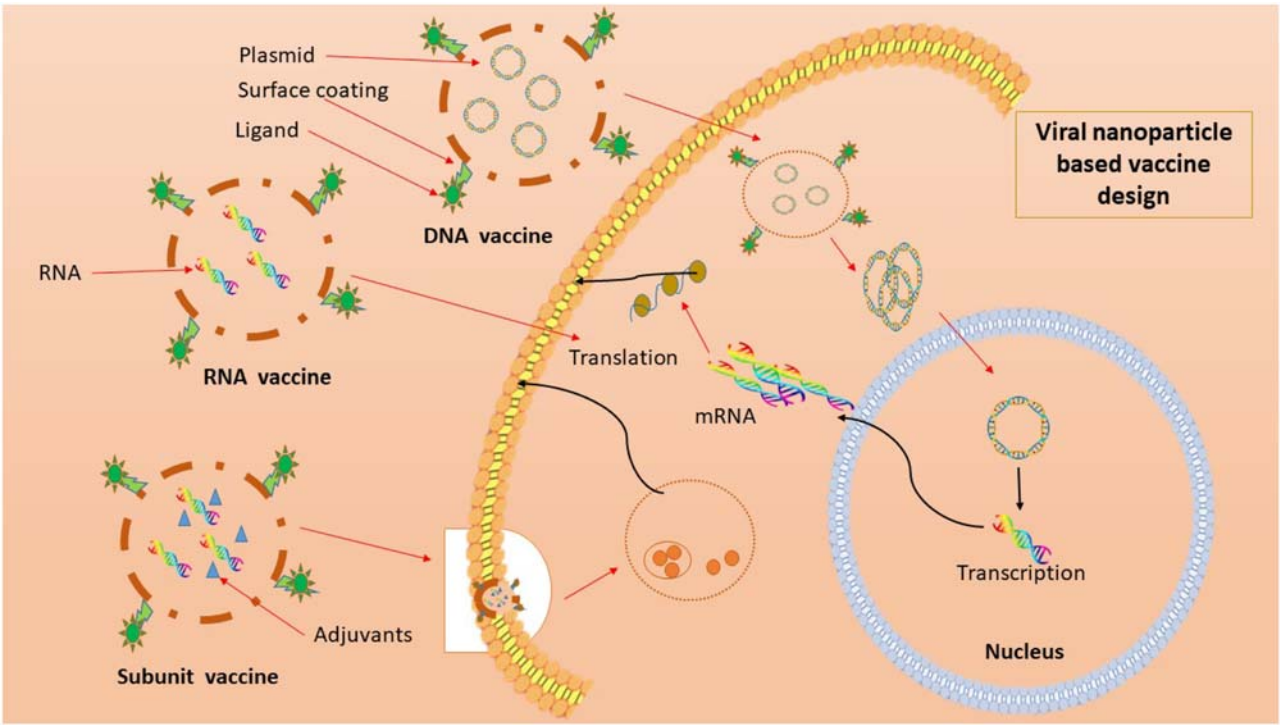


FIGURE 3.1 Structural illustration of viral nanoparticle-based vaccine design.

With AuNP/M2e + CpG conjugate vaccination, even after 15 months, the mice were able to produce M2e-specific antibodies, so these mice remained protected against the lethal H1N1 influenza virus. The most effective and direct strategy of aligning the nanomaterial as a vaccine is involved with optimizing band energy with biostability for recognition (Sun and Xia, 2016). Synthetic biology could likewise coronate an immunization with a basic property of the strength. A significant issue with the worldwide creation and delivery of immunizations is that those, by and large, should be refrigerated. In any case, manufactured nanoparticle−protein frameworks could make those unquestionably more steady than conventional vaccines, as the prospect of orientation would be involved.

Bacteriophage VLPs and vaccine development

Enormous platforms for vaccine development are available with the highly immunogenic VLPs, and the engineered bacteriophage VLPs have diverse surface peptides (Nahhas et al., 2021). The described multitude techniques for VLPs in vaccine discovery have opened alternative strategies similar to the filamentous phage display, while the identified bacteriophage VLP-based vaccines from a massive library ended up with an epitope discovery of immunization at the same platform (O'Rourke et al., 2015). In this connection, it could be concluded from earlier studies that HPV L2 epitope with VLP expression had induced an effective approach for inducing higher protective neutralizing antibodies; hence it could be protective for a large spectrum of diseases associated with HPV, namely, cervical and vaginal cancers, etc. It has been well discussed on RNA bacteriophage VLPs for vaccine development, due to the inherent unique viral structure with a single coat protein and the ability to encapsidate the RNA as well as other foreign nucleic acids. Even VLPs developed with bacteriophages have been known for their role to cure metabolic disorders, blithely. Similarly, a recent study demonstrated bacteriophage VLP-based vaccines targeting PCSK9 (proproteinconvertassubtilisin/kexin type 9) could help reduce morbidity-inducing levels of cholesterol and triglycerides. Thus, the vaccines can be used as an alternative to monoclonal antibody-based therapy. Previous work had recorded that the viral AP205-VLPs from the RNA bacteriophage were useful in the development of a novel and accurate epitope-based vaccine system with high immunogenicity suitable for protecting from influenza infection (Crossey et al., 2015; Frietze et al., 2016; O'Rourke et al., 2015; Tissot et al., 2010; Tumban et al., 2011).

Nanophytovirology

"Nanophytovirology" is an offshoot of nanotechnology dealing with distinctive strategies in the detection of viruses as well as the diagnosis and environmentally friendly management of plant viral diseases. Indeed, controlling PVs in commercial agriculture is a strenuous task. Numbers of techniques are used for virus detection, viz., electron microscopy, symptoms determination seed transmission, and mechanical transmission, but those being insufficient, several types of polymerase chain reactions (PCRs) such as the nested PCR, immuno-precipitation PCR, co-operational PCR, immunocapture PCR, multiplex PCR, reverse transcription PCR, and real-time PCR are used for viral nucleic acids, apart from standard serological techniques including enzyme-linked immunosorbent assay, restriction fragment length polymorphism, tissue blot immunoassay, transmission electron microscopy, phage display, and lateral flow devices (Pankaj, 2013). Additional methods for virus detection including hybridization techniques are microarray, in situ hybridization, next-generation sequencing, rDNA technique; and different amplification methods such as loop-mediated isothermal amplification, helicase-dependent amplification, rolling circle amplification, recombinase polymerase amplification, and nucleic acid sequence−based amplification are also known (Rastogi and Singh, 2019).

There are various methods for the detection of intact viral methods such as dynamic light scattering, fluorescence microarray, flow cytometry, interferometry, surface plasmon resonance, quartz crystal microbalance-based detection, and electrical conductance methods such as nanowire detection and conductometric sensor (Cheng et al., 2009). Indeed, the prospective uses and playbacks of traditional techniques such as microfabrication and nanotechnology are enormous. These include simplicity, enlightening the viral detection limit, which improves the ability to produce the desired type of viral analysis (Cheng et al., 2009). The development of nanophytovirology offers several unprecedented advantages, including carbon nanotubes, nanobiosensors, nanoherbicides, nanopesticides, nanofertilizers, and nano-based smart drug discovery systems. However, there are a few challenges seen in the path for drug development, such as the due development in the application of nanomaterials in plant viral disease identification, diagnosis, and management. As it is, nanoparticles are ultra-small in size and highly reactive, offering an advantage to the activity of viruses that might be affected (Khan and Rizvi, 2014). A rapid diagnostic tool would be necessary for the detection of viruses. Thus, the simple approach of nanotechnology would be used directly on seeds or foliage in the soil to defend plants from virus attacks (Khan and Rizvi, 2014).

CRISPR and RNAi are techniques to generate PV resistance. These are dsRNA-mediated mechanisms, which increase sequence-specific gene silencing at the posttranscriptional level. To overcome the challenge of addressing the uncertainty, the apparent dsRNA was sprayed on plants toward a practical application (Mitter et al., 2017), which demonstrated that dsRNA would be loaded on clay nano-sheets, which remain nontoxic and as degradable layered double hydroxide nano-sheets. Indeed, the CRISPR tool had come up with better advantages than dsRNA (Nalla and Shah, 2021). It induces permanent and irreversible genome modification, whereas dsRNA induces control of gene expression temporally. The challenges addressing CRISPR gene editing are the following: (i) technically it is time-consuming and labor-intensive, (ii) it could lead to the formation of the new Geminivirus variants, and (iii) the delivery of CRISPR tools into living plant cells is a tedious process (Kalinina et al., 2020). The use of QDs is an attractive alternative to fluorescent dye to detect viruses due to small emission spectra and more efficient luminescence (Duhan et al., 2017). A QD-FRET-based biosensor was developed to detect the plasmodiophorid *Polymyxabetae*. It is the single recognized vector of the beet necrotic yellow vain virus (Safarpour et al., 2012). Cadmium selenide quantum dots (CdSe-QDs) are used to detect the banana punchy top virus in a single amplification (Majumder et al., 2020).

Another set of major challenges is the trophic transfer of nanoparticles, toxicity, and biosafety of human health as well as the environment. Cellular toxicity of nanoparticles might lead to unwanted side effects, as membrane damage and an increased ROS generation concomitantly plant and human metabolism would be disturbed (Jazayeri et al., 2016). The main challenge associated with nanotechnology is to investigate the synthesis mechanism and cost analysis of the final product. A significant trend, in further development, is the production of nanomaterials on a commercial scale and their use in large-scale field applications for disease control. Nanophytovirology has immense potentiality to generate new approaches for "disease imaging and management." Nonetheless, it is in its infancy. Future studies are needed to focus on interactions between nanoparticles and plant cell walls. Moreover, further improvement and adaptation of all these technologies could enable scientific communities across the globe to manage better practices for the detection of PVs.

VNPs are recommended as a coveted option for cargo delivery of some pharmaceutics for the following reasons: (i) biocompatible, biodegradable, natural existence carriers, (ii) easy to transfer in drug delivery carrier, (iii) high stability and dynamism as carriers easily conjugating with proteins, and (iv) noninfectious to mammals (Czapar and Steinmetz, 2017). Viruses are effectively altered by genetic engineering principles to produce unique cancer-targeting imaging protein agents by integrating various agents such as fluorescent dyes and/or aiming moieties. VNPs with extraordinary discrimination specifically for the target of cancer-specific molecules could be formulated in utilizing in vivo screening for cancerous cells. VNPs seem like a key platform for engineering for the development of various drugs with imaging molecules and pointing ligands, the ancillary methods sought during drug locating. Thus, among the standard treatments of cancer and the targeted therapies, it could be the supreme way with fewer side effects as the delivery by the "cargo drug delivery system" (McCarthy and Weissleder, 2008).

Nowadays, the prodigious impact of nanotechnology on nanomedicine has been seen. Nanoparticles synthesized with plants are well known for the inherent effectivity in cancer treatment as plant VNPs. Several functional changes have been done so that those could be easily absorbed by cancer-affected tissues. Furthermore, computational modeling is the constructive approach to create the plant VNPs as anticancer druggable candidates. Plant VNPs offer an economical approach for pinpointing cancer-affected cells and transporting various drugs (Ohadi Rafsanjani et al., 2012). VNPs are created in various dimensions, and configurations involving viruses are remarkably symmetrical and uniformly arranged in VNPs. Furthermore, PVs (Fig. 3.2 and Table 3.3) would be functionalized for various nanomedicine applications in the future (Mukherjee et al., 2020).

Formulation of plant virus nanoparticles

Viral capsids are altered by several genetic modifications, addition/deletions of amino acid sequences with various functionalities, followed by modified functional groups added over virus capsids. The manipulation permits bioconjugation for the fluorescent-based dyes, targeting peptides in designing the coveted molecules such as drug molecules.

Additionally, azides ($-N_3$) and alkynes (C−C triple bond) are involved in incorporating requisite unusual amino acids by additional conjugation strategies. Moreover, some unnatural amino acids, namely, α-aminoisobutyric acid, had remarkable activity against bacterial strains (Nahhas et al., 2018). Micro-size cargo molecules are imparted into the integral virus, which could hold on to the capsid by electrostatic affinity interactions (Table 3.4). Moreover, manipulating the interlinkage of viruses and metal ions at the nucleated position by shape-repressed mineralization is reported. In the end, protein virus coatings are made to conduct to self-assemblage throughout cargo molecules (Wen et al., 2020).

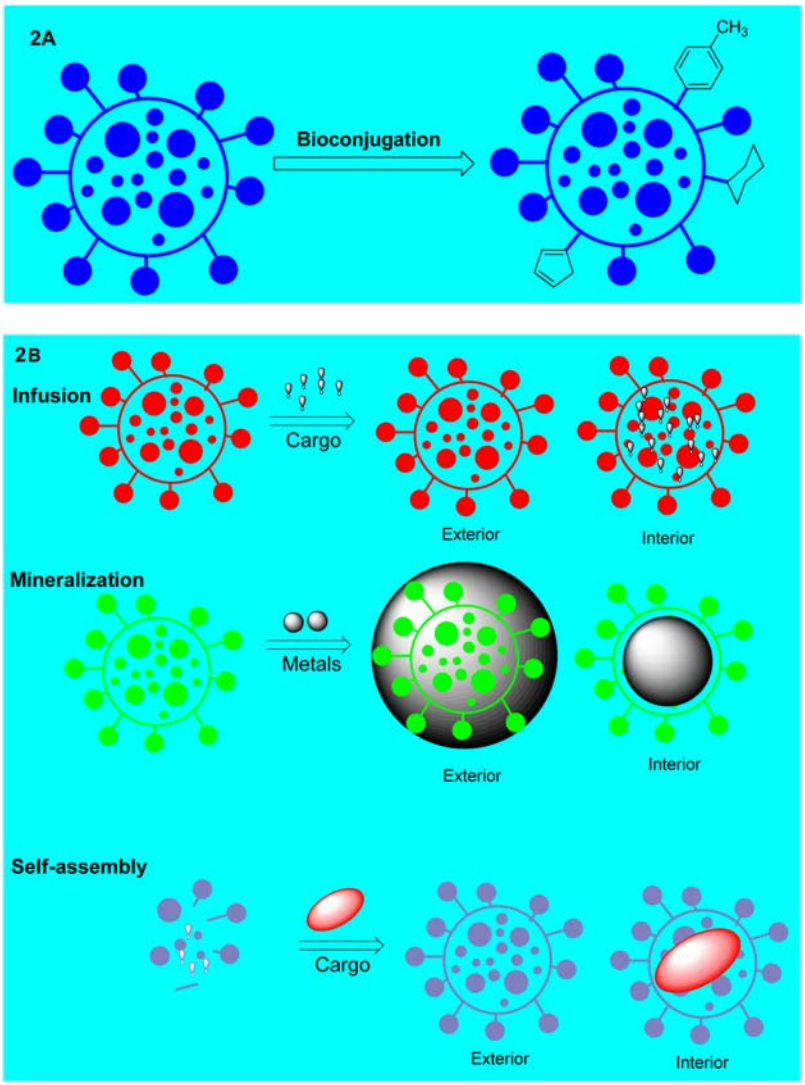


FIGURE 3.2 (A, B) Alteration techniques of virus nanoparticles.

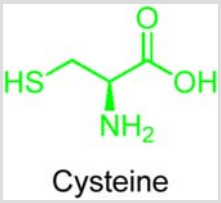
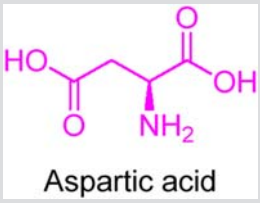
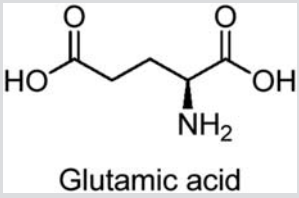
TABLE 3.3 Amphiphilic systems of viruses.

Virus	Mode of assembly	Types
CCMV	Template	DNA micelles
CCMV	Template	Lipid modified Gd^{+3} chelators
CCMV	Template	PDMS nanoemulsion

Entry and incorporation into cells

Now exteriors of plant VNPs have been functionalized, and those would favorably be absorbed by cancerous cells; innate PVs have been manifested for internalization into mammalian cells in the absence of any pointing moieties. Moreover, fluorophore-bioconjugated nanoparticles of *Sesbania* mosaic virus (SeMV) were used in a study to see the capability to enter into various types of mammalian cells. SeMV can enter these cancer cells, HeLa, MDA-MB-231,

TABLE 3.4 Some standard functionality for bioconjugation.

S. No.	The residue of amino acid	Functionality for bioconjugation	Structures
1.	Lysine residue	Amine ($-NH_2$) group	 Lysine
2.	Cysteine residue	Thiols (−SH) group	 Cysteine
3.	Tyrosine residue	Phenol rings (aromatic rings containing −OH group)	 Tyrosine
4.	Aspartic acid residue	Carboxylic acid (−COOH) group	 Aspartic acid
5.	Glutamic acid residue	Carboxylic acid (−COOH) group	 Glutamic acid

HepG2, and NIH/3T3 cells; it has an affinity toward the cell MDA-MB-231, particularly. Similarly, TMV-, CMV-, and SeMV-mediated nanoparticles were demonstrated to interact with vimentin protein on the outside of the mammalian cells. The vimentin protein present in the cytoskeleton responsible for cellular structure has been well known for exploitation as a host linkage protein for the control of various infectious agents. Furthermore, cancerous cells have shown the highest amounts of vimentin on cell surfaces. Cowpea mosaic virus (CPMV) (Beatty and Lewis, 2019) and

TABLE 3.5 Plant virus delivery system.

S. No.	Plant virus	Delivery system	Biologic delivered	References
Icosahedral plant viruses				
1.	Cowpea mosaic virus	Particles and empty virus	Not relevant	Shoeb and Hefferon (2019)
2.	Red clover necrotic mosaic virus	Protein cage coated by the virus has combined nearby anticancer drug cargo	Doxorubicin	
3.	Tomato bushy stunt virus	Protein cage coated by virus has compressed anti-cancer drug or drug covered through drug at outside of the cage	Not relevant	
4.	Hibiscus chlorotic ring-spot virus	Protein cage coated by virus has accumulated nearby cargo of anticancer drug	Doxorubicin	
5.	Johnson grass chlorotic stripe mosaic virus	During in vitro assembly nanocarrier loaded with anticancer drug	Doxorubicin	
6.	Sesbania mosaic virus	Fluorophores coupled with a surface of the virus particle	Fluorophores may be imaging cancer	
Rod-shaped plant viruses				
1.	Tobacco mosaic virus	Drug conjoins to outside of virus nanoparticles	Doxorubicin, phenanthriplatin	Shoeb and Hefferon (2019)
2.	Papaya mosaic virus	A particle that is like viruses are accumulating	No drug required	
3.	Potato virus X	The drug leads to binding with potato virus X in vitro or else transported all together with the uncovered nanoparticle of potato virus X	Doxorubicin	

SeMVs were demonstrated in getting entry into mammalian cells by the endocytosis pathway; this had proved that vimentin-like surface protein is essential in incorporation of the PV (Vishnu Vardhan et al., 2019). In recent years, the field of viral drug delivery has made great progress. Genetic insertion and chemical conjugation techniques employ some conventional methods (Chung et al., 2020). Currently, VNPs are accepted as the highly appropriate system for transporting drugs toward the cancerous tissue (Table 3.5). Indeed, the penetrability of PV nanoparticles was transformed through altering the pH and ionic strength as an incredible benefit to using as a drug delivery platform.

Bacteriophage-based nanoparticles

Several bacteriophages as icosahedral bacteriophages, namely, P22, HK97, T7, Qβ, and MS2, well-known head-tail pages, and without any tail P22, T7, as well as M13 phage, could be used in vitro for the development of VNPs. All VNPs had been used in cancer treatments, as those easily penetrate the human cell wall. Consequently, some animal viruses such as adenovirus and canine parvovirus-icosahedral animal virus and the icosahedral insect viruses and the flock house virus were employed as VNPs (Grasso and Santi, 2010). Moreover, by converting animal viruses and bacteriophages to VNPs, the generated recombinant DNA technologies plentifully have been used with types of vectors according to the respective host, and these VNPs were useful in treatments of various kinds of cancer cells.

Nowadays, viruses are being used for nanomaterial fabrications, and PVs are suitable moieties for nanomaterial synthesis. Nanoparticle-mediated PVs are considered as having distinctive features, and those lend themselves to novel opportunities for the use in industrial and biomedical sectors (Fig. 3.3). Several features of the biotemplate of nanomaterial fabrications are described in detail. Additionally, drug delivery is a new avenue in the ever-growing pharmaceutical sector. PV-mediated nanoparticles are usually stable for individual structural-physical characteristics. Moreover, PVs are less infectious in comparison to mammalian viruses. In the packing of the viral particles, the drug delivery system has been encapsulated with the viral genome for blocking viral propagation (Calzoni et al., 2019; Singh and Lillard, 2009). Consequently, a few more viral particles and associated proteins are being treated in this drug delivery cascade. Likewise, proteins covalently attached to drug molecules in viral capsid have actively been participating in the drug delivery process,

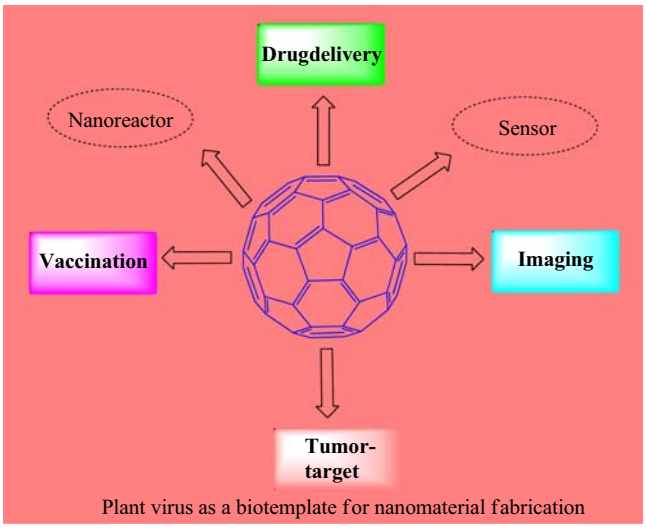


FIGURE 3.3 Schematic representation of nanomaterial fabrication.

duly ensuring chemical alteration and conjugation methodologies (Rohovie et al., 2017). Concomitantly, the encapsulation of the drug molecule is another feasible method of charge exchanges of negative to positive, viral capsid, and electrostatic interaction with viral RNA (Din et al., 2017; Perlmutter and Hagan, 2015).

The vaccination of cancer is the current prospective approach for the development of tumor-associated antigens (Guo et al., 2013). Indeed, PVs have repetitive protein structures, which trigger the development of vaccines (Hollingsworth and Jansen, 2019). These further activate immunotherapeutics of the body as the innate immunity, which suppresses the resistance of micro-environmental cancer as stimulating adaptive immunity. Moreover, episodes of cancer, namely, breast, colon, ovary, and a few more, have inhibitory properties, as reported. Furthermore, the viral carrier particles used as markers of surface tumors remain attractive to delivery of the drug with an accuracy of diagnosis and imaging tendencies (Hollingsworth and Jansen, 2019; Hosu et al., 2019). These have been further examined as neurodegenerative, infectious diseases, and a few more. Indeed, virus particles have the novelty as avenues to treating antiinflammation-related conditions.

Recently, viral particles have been recognized as upright techniques in growing biomedical sectors. These entities have high specificity and could reduce cost and time for validation of bioavailability and level of toxicity. Now, magnetic resonance imaging is an in vitro technique to illustrate several structures of the capsid with adopting macro-molar facilities to boost the image contrast and sensitivity. It has photodynamic properties with generating multimodal clarity of images (Hosu et al., 2019). Moreover, the detection of inorganic and biologic compounds by viral particles is a novel approach; particularly, the TMV (tobacco mosaic virus) is known as a carrier of the sensor in volatile organic compounds. Moreover, TMV is obliged as a convenient frame for harvesting light arrays. Likewise, its encapsulated enzymes mimic the proteins of virus particles in cell environments.

Future perspectives

Recently, a pandemic situation has been caused by the coronavirus (COVID-19), along with the consequent drastic economic recession (Sahoo et al., 2020c), even from its newer Omicron variant today. The development of newer drug candidates or vaccines has been the call of the day, for the last 2 years. Moreover, the anticipated advanced nanotechnology could have hope with several benefits, namely, wastewater treatment, textile industries, antimicrobial properties as well as anticancerous properties (Sahoo et al., 2020a; Turakhia et al., 2018, 2019, 2020), and antifungal, antiviral including anticoronaviral vaccines/drugs, and a few more. Indeed, the clinical establishment of prudent candidates with the elimination of side effects is vital in the improvement of the healthcare scheme, in general.

Conclusion

Purposefully, VNPs as exuberant nanocarriers could bring greater attention in the biomedical sector. The assemblage of a central protagonist as endeavors of "vaccination technology" and "cancer therapy" would be the prospects.

Additionally, VLNPs are noninfectious protein shells/capsids, with infection-causing basic proteins for interactions with host cells, which might be useful in the current nanotechnology sector as specific agents in the diagnostic and therapeutic treatments.

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Chapter 4

Biological synthesis of nanoparticles from selected medicinal plants

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Introduction

Nanotechnology is a rapidly growing and one of the promising tools for biology as it is concerned with the synthesis of particles ranging in size from 1 to 100 nm. Dry, wet, and computerized nanotechnology are subsets of nanotechnology. Dry nanotechnology work deals with physical, chemical, and surface science, such as the fabrication of structures. Wet nanotechnology is concerned with the study of biological systems, whereas computational nanotechnology is concerned with the modeling and simulation of biological systems (Sinha et al., 2009) (Fig. 4.1).

Nanotechnology broadens the scope of investigation by creating nanoscale particles, also known as nanoparticles. It can be used to investigate biological processes (Sondi and Branka, 2004) and biomedical sciences (Hütten et al., 2004) because they are analogous to the majority of biological molecules and structures. At the cell level, nanoparticles act as a bridge by regulating the interaction between synthetic materials and biological systems (Du et al., 2007). In addition, it has a key role in drug delivery, diagnostics, imaging, sensing, gene delivery, artificial implants, and tissue engineering (Morones et al., 2005). It has also been used in the development of environmentally friendly material synthesis technologies (Bhattacharya et al., 2005). Nanoparticles can be found naturally or created through nanotechnology. They can be synthesized via organic or inorganic compounds, whereas, in nature, it has been discovered in the earth's crust or through the weathering of Au (gold) deposits (Sinha et al., 2009). Nanoparticles are very complex molecules. It is made up of three layers, which are as follows (Shin et al., 2016) (Fig. 4.2) (Table 4.1).

1. Surface layer: This is made up of a variety of unique compounds like metal ions, emulsifiers, polymers, surfactants, etc.
2. Shell layer: This is chemically and physically distinct from the core.
3. Core layer: This is the central element of nanoparticles.

Nanoparticle size, shapes, and structures impact their reactivity, durability, etc. properties. Heavy metal nanoparticles, such as lead, mercury, and tin, have strong properties, which complicate decomposition and increase environmental hazards (Khan et al., 2019). As a result, it is necessary to develop nonheavy metal materials that are not hazardous and can be easily decomposed (Bhattacharya et al., 2005).

Nowadays, there is an increase in research on the synthesis of nanoscale metals using chemical, physical or green synthesis methods (Wang et al., 2007; Horwat et al., 2011). Each procedure has its own specific benefits and drawbacks. Alsammarraie et al. (2018) utilized green synthesis methods to gradually replace the physical and chemical methods due to their safety, cost-effectiveness, and environmental friendliness (Fig. 4.3).

Biological/green synthesis of nanoparticles

Green synthesis is an environmentally friendly, safe, and cost-effective method that has different ways to eliminate toxic waste, reduce energy consumption, and increase the use of ecological solvents. They can be synthesized from plants, bacteria, and viruses or their byproducts, such as proteins and lipids, using various biotechnology tools (Zhang et al.,

Nanotechnology and In Silico Tools. https://doi.org/10.1016/B978-0-443-15457-7.00021-6

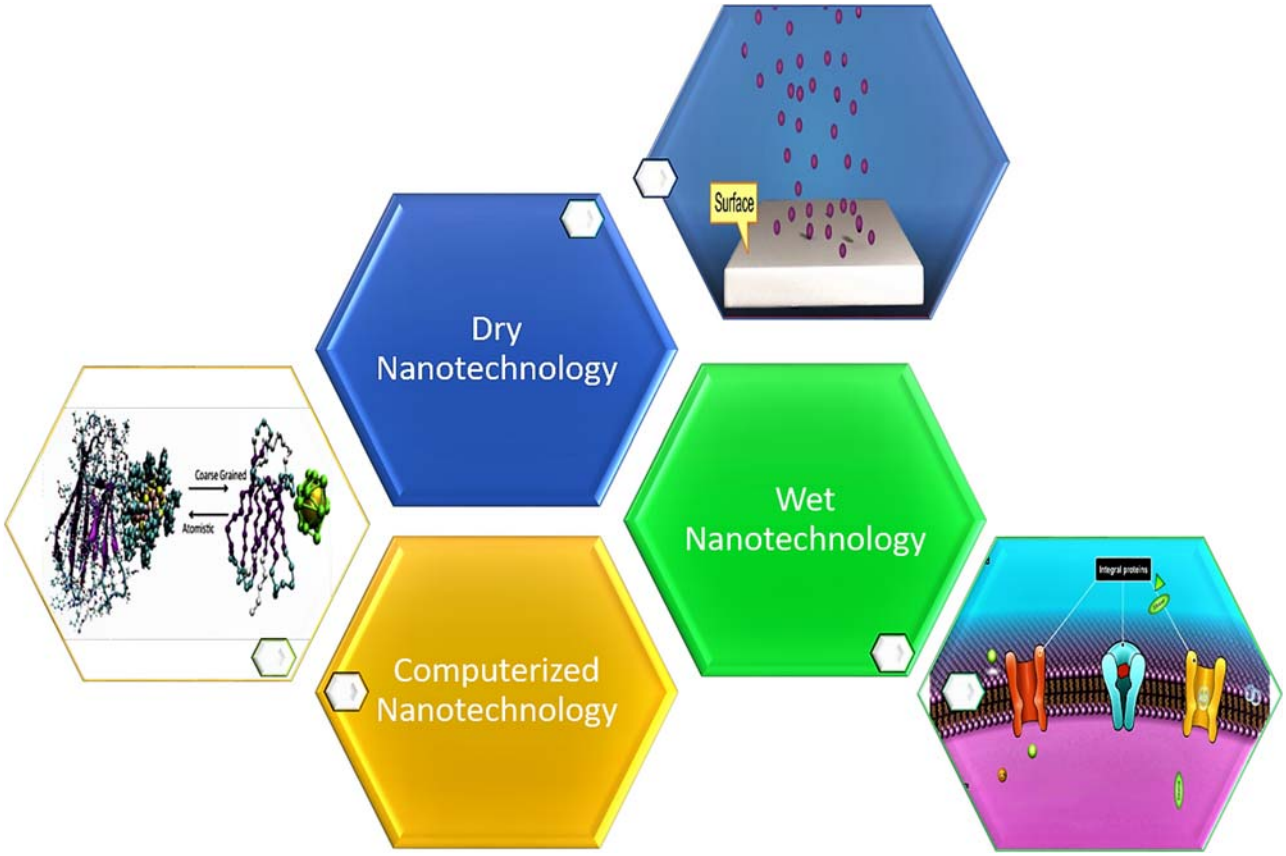

FIGURE 4.1 Depicts the three disciplines of nanotechnology disciplines.

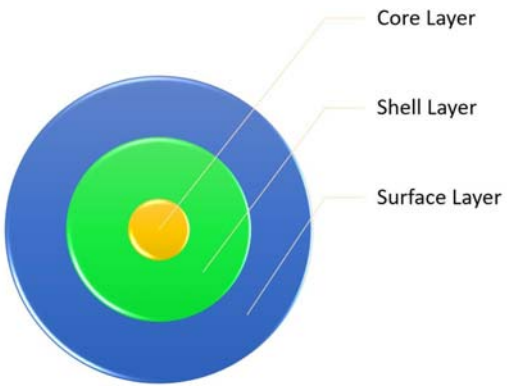

FIGURE 4.2 Schematic of nanoparticles layers.

2020). Plant components such as fruits, leaves, stems, seeds, flowers, bark, and so on are used in the production of nanoparticles because they contain a variety of beneficial elements, including bioactive compounds (Nathani et al., 2021). In an environmentally friendly standard biosynthesis pathway, bioactive compounds in plants could enhance the conversion of metal ions into biologically active nanoparticles (Munir et al., 2021). However, there are problems with the green synthesis of nanoparticles such as reaction time and quality of the final product due to the purity of extracted raw material. Nanoparticles can be synthesized using either a bottom-up or a top-down approach. The bottom-up approach will be primarily used in biologically based nanoparticle synthesis (Fig. 4.3). For the green synthesis of metallic nanoparticles (MNPs), three steps are followed (Fig. 4.4).

TABLE 4.1 Representative example of the biological synthesis of nanoparticles from medicinal plants.

Plant name	Part	Nanoparticles metal	Bioactivity	References
Tribulus terrestris L.	Fruit bodies	Ag	Antibacterial	Gopinath et al. (2012)
Tribulus terrestris	Leaf	Ag	Antibacterial	Gopinath et al. (2015)
Mentha piperita	Leaf	Ag, Au	Antibacterial	MubarakAli et al. (2011)
Sesbania grandiflora	Leaf	Ag	Anticancer	Jeyaraj et al. (2013)
Cinnamon	Leaf	Ag	Antibacterial	Premkumar et al. (2018)
Justicia wynaadensis	Leaf	ZnO	Antimiotic	Hemanth et al. (2019)
Caesalpinia pulcherrima	Leaf	Ag	Anticancer	Deepika et al. (2020)
Berberis aristata	Leaf	Zn	Antibacterial, Antioxidant	Chandra et al. (2019)
Mussaenda glabrata	Leaf	Ag, Au	Antimicrobial	Francis et al. (2017)
Cissus quadrangularis	Leaf	Cu	Antifungal	Devipriya and Roopan (2017)
Nyctanthes arbor-tristis	Flower	Zn	Antifungal	Jamdagni et al. (2018)
Rosmarinus officinalis L.	Leaf	Fe	Anticancer	Farshchi et al. (2018)
Boswellia serrata	Guns	Ag	Antibacterial	Kora et al. (2012)
Caria papaya	Fruit	Ag	Antimicrobial	Jain et al. (2009)
Cassia fistula	Steam Bark	Au	Antihypoglycemic	Daisy et al. (2012)
Cinnamon zeylanicum	Bark	Ag	Antibacterial	Sathishkumar et al. (2009)
Citrullus colocynthis	Callus	Ag	Antioxidant, Anticancer	Satyavani et al. (2011)
Citrus sinensis	Peel	Ag	Antibacterial	Kaviya et al. (2011)
Dillenia indica	Fruit	Ag	Antibacterial	Singh et al. (2013)
Dioscorea bulbifera	tubers	Ag	Antimicrobial	Ghosh et al. (2012)
Euphorbia prostrata	Leaf	Ag	Antiplasmodial	Zahir and Rahuman (2012)
Lippia citriodora	Leaf	Ag	Antimicrobial	Cruz et al. (2010)
Mentha piperita	Leaf	Au, Ag	Antibacterial	MubarakAli et al. (2011)
Mirabilis jalapa	Flower	Au	Antimicrobial	Vankar et al. (2010)
Hydrastis canadensis	Plant	Ag	Cytotoxicity	Das et al. (2013)
Gelsemium sempervirens	Plant	Ag	Cytotoxicity	Das et al. (2013)
Phytolacca decandra	Plant	Ag	Cytotoxicity	Das et al. (2013)
Thuja occidentalis	Plant	Ag	Cytotoxicity	Das et al. (2013)
Iresine herbstii	Leaf	Ag	Antibacterial, Antioxidant, Cytotoxic agent	Dipankar et al. (2012)
Melia azedarach	Leaf	Ag	Cytotoxicity	Sukirtha et al. (2012)
Tinospora cordifolia	Leaf	Ag	Antilarvicidal	Jayaseelan et al. (2011)
Trigonella-foenum graecum	Seed	Au	Catalytic	Aromal et al. (2012)
Withania somnifera	Leaf	Ag	Antimicrobial	Nagati et al. (2012)

Continued

TABLE 4.1 Representative example of the biological synthesis of nanoparticles from medicinal plants.—cont'd

Plant name	Part	Nanoparticles metal	Bioactivity	References
Acalypha indica	Leaf	Ag, Au	Antibacterial	Krishnaraj et al. (2010)
Aloe vera	Plant	In_2O_3	Optical properties	Maensiri et al. (2008)
Alternanthera sessilis	Leaf	Ag	Antioxidant, antimicrobial	Niraimathi et al. (2013)
Andrographis paniculata	Leaf	Ag	Hepatocurative activity	Suriyakalaa et al. (2013)
Argemone maxicana	Leaf	Ag	Antimicrobial	Singh et al. (2010)
Cyclopia intermedia	Shrub	Au	Anticancer	Aboyewa et al. (2021)
Boerhaavia diffusa	Whole plant	Ag	Antibacterial	Kumar et al. (2014)

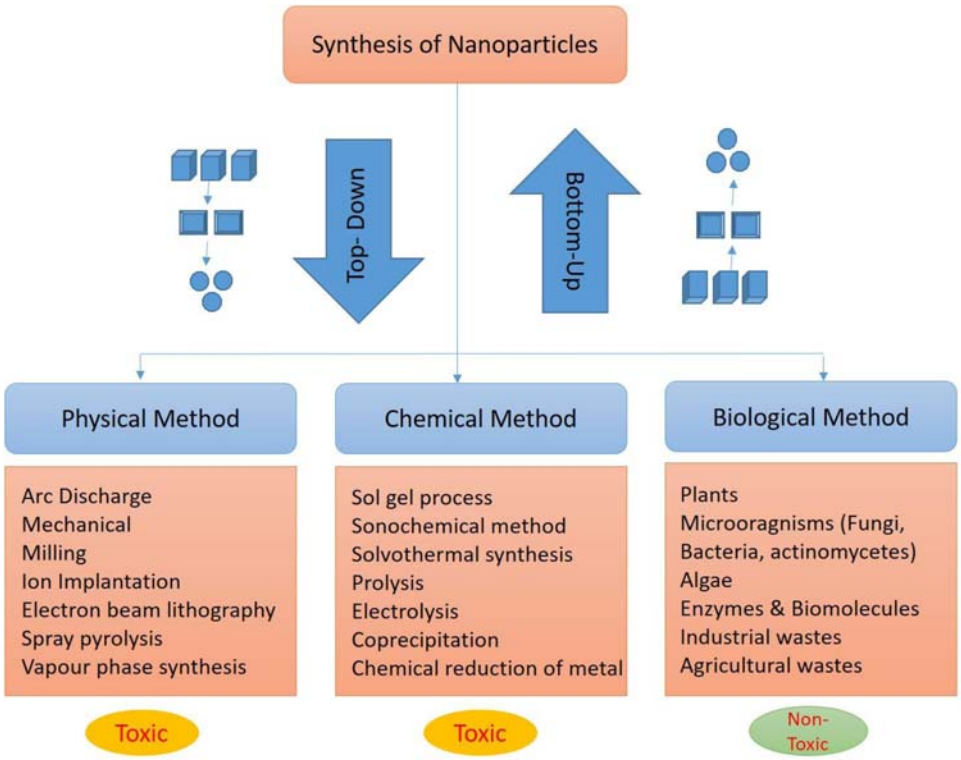

FIGURE 4.3 Various approaches and methods for the synthesis of nanoparticles.

1. Selection of a solvent medium
2. Selection of an eco-friendly and environmentally benign reducing agent
3. Selection of nontoxic material as a capping agent to stabilize the nanoparticles

In general, green synthesis of MNPs is summarized as follows: getting plant extracts followed by mixing with metal solution in specific conditions, reduction of metal particles followed by filtration and other steps to obtain nanoscale metal. This green nanoparticle synthesis method has an advantage over other conventional methods, but we need to take care of factors that are affecting the synthesis of nanoparticles (Singh et al., 2011; Mohanpuria et al., 2008).

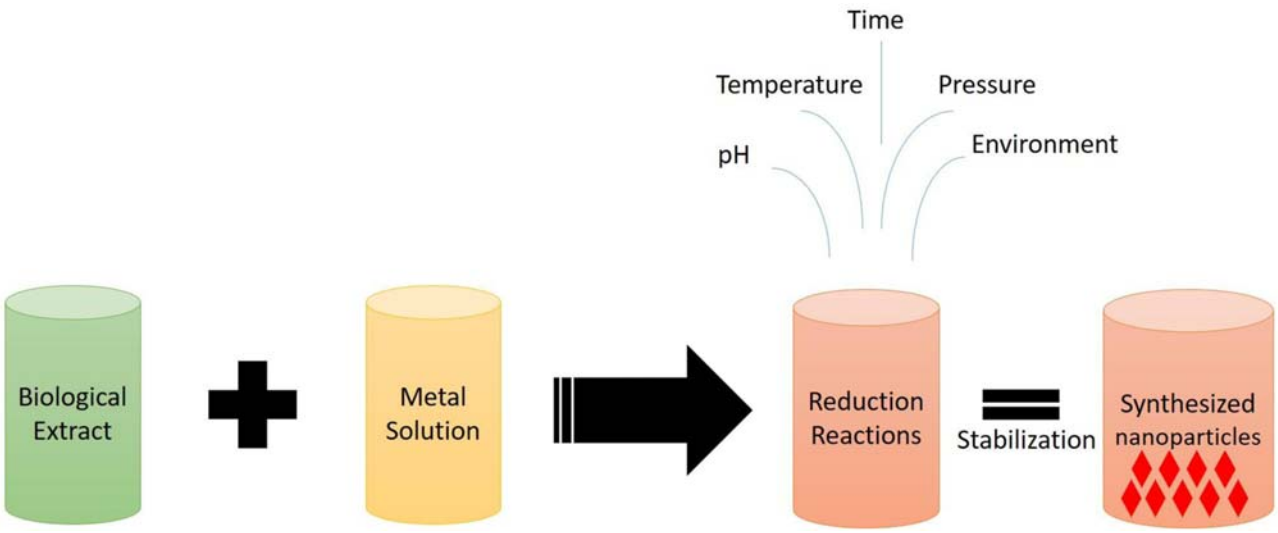

FIGURE 4.4 Synthesis of nanoparticles using green technology.

Key factors affecting the synthesis and stability of nanoparticles

There are several other important factors that affect the synthesis of nanoparticles, including pH of the solution, temperature, concentration of the extracts used, concentration of the raw materials used, and size (Baker et al., 2013). The following are some of the most important factors influencing nanoparticle biosynthesis.

pH: It is one of the important factors for the synthesis of nanoparticles using green technology because researchers have found that pH influences the size and texture of nanoparticles (Soni and Prakash, 2011).

Temperature: It is another important parameter for the synthesis of nanoparticles in all three methods. Each method requires a different temperature, such as physical methods requiring >350°C, chemical methods requiring <350°C, and green technology requiring 100°C or ambient temperature (Rai et al., 2006).

Pressure: It is also influencing the shape and size of synthesized nanoparticles (Pandey, 2012). The rate of reduction of metal ions depends on pressure, temperature as well as biological agents (Tran et al., 2013).

Time: In green nanotechnology, the quality of nanoparticles is influenced by the length of time. Variations in time have an effect on particle aggregation or shrinkage (Baer, 2011).

Particle shape and size: It's very important to determine the properties of nanoparticles. When the size of the nanoparticles reaches the nanometer scale, the melting point should be decreased, which makes the transformation of their shape easy (Akbari et al., 2011).

Environment: Nanoparticle quality is also affected by the surroundings of nature. This also affects the physical structure and chemistry of the synthesized nanoparticles. In many environmental conditions, nanoparticles get oxidized and become core-shell nanoparticles quickly by absorbing or reacting with other materials (Sarathy et al., 2008).

Proximity: When nanoparticles come into contact or near other nanoparticles, then alteration in the properties of nanoparticles is observed (Baer et al., 2008). This alteration in the properties leads to more tuned nanoparticles.

Other Factors: Secondary metabolites from the plants act as reducing and stabilizing agents in the synthesis of nanoparticles. This can shed light on the path that nanoparticle quality can vary depending on the type of plant, plant part, and extraction procedure (Park et al., 2011). It also depends on intracellular and extracellular enzyme quantities (Haverkamp et al., 2007). Not only that, every step during the synthesis of nanoparticles can influence the quality and quantity of nanoparticles.

Green synthesis of Au, Ag, and other nanoparticles

Green synthesis of Au (gold) nanoparticles

The typical green synthesis of Au NPs involves reducing gold ions with reducing agents derived from plant extracts or microorganisms. Soaking the plants in the solvent will yield an extract under suitable conditions. After that, the extract will

be mixed with the solution containing gold ions, which will turn the solution red. This red color of the solution indicated the production of Au nanoparticles (Smitha et al., 2009; Jafarizad et al., 2015; Vijaya Kumar et al., 2019). Spheres are the most common shape of green-synthesized Au nanoparticles followed by triangle and hexagon shapes. Paul et al. (2015) found Au nanoparticles in spheres and triangle shapes with varying diameters of 10−50 nm. However, when Jafarizad et al. (2015) used pelargonium, all of the Au nanoparticles were found in sphere shapes. Chloroauric acid concentration influences the Au nanoparticles in green synthesis (Ahmad et al., 2016). Researchers have explored Au nanoparticles as a catalyst. It is also documented that Au nanoparticles are used as diagnostic and therapeutic agents in the medical field (Lee et al., 2020). It is also used in antibacterial activity to deliver hydrophobic and hydrophilic drugs, peptides, and small molecules to the target site. It is also used in therapeutic moieties to exhibit enhanced anticancer effects (Sulaiman et al., 2020; Mugaka et al., 2019; Shikha et al., 2020; Singh et al., 2011; Thakkar et al., 2010).

$$HAu^{+}Cl_4\ 4H_2O + \text{plant extracts} \rightarrow \text{Au Nanoparticles} + \text{byproducts}$$

Green synthesis of Ag (silver) nanoparticles

The typical green synthesis of Ag nanoparticles procedure is the same as Au nanoparticles except for the mixing solution. After extracting, it will be mixed with silver nitrate and Ag nanoparticles are produced when the solution turns a brownish color (Khatami et al., 2018; Hemmati et al., 2019; Rautela et al., 2019; Rahimullah Shaikh and Bhend, 2019; Yu et al., 2019). Spherical, triangular, and hexagonal are the most common green-synthesized Ag nanoparticle shapes (Ping et al., 2018; Kumar et al., 2017b; Arokiyaraj et al., 2017). The stability of the green synthesized Ag nanoparticles depends on the plant extract, pH, and temperature. Increasing the stability of nanoparticles is a very crucial factor during the synthesis process. Researchers have explored various application processes of Ag nanoparticles such as catalysts and microbial growth inhibitors. Nayem et al. (2020) reported that Ag nanoparticles show good inhibitory effects on gram-positive as well as gram-negative bacteria. It has been used not only in the medical field but also in electronic devices as a photocatalyst, water purification, production of paints, disinfectants, kitchen appliances, and cosmetic industries (Kareem et al., 2020; Khatami et al., 2018; Rautela et al., 2019). Ag nanoparticles have a place in anticancer, antiviral, antifungal, and antibacterial therapeutic applications (Du et al., 2007; Markus et al., 2016; Lee et al., 2020; Tripathy et al., 2010).

$$AgNO_3 + \text{plant extract} \rightarrow \text{Ag nanoparticles} + \text{byproducts}$$

Green synthesis of other nanoparticles

The procedure for the synthesis of other metal nanoparticles will be the same as above. Cu (Copper) nanoparticles are not directly synthesized from simple copper salts but they require capping agents. The capping agents like surfactants control the size of the nanoparticles (Shende et al., 2015). It has a wide range of applications, including strong antimicrobial agents that can be used as disinfectants against infectious organisms (Anadozie et al., 2021). These nanoparticles are synthesized using citron juice. Citron juice synthesis of Cu nanoparticles is so simple and inexpensive that it is worthwhile to use on an industrial scale (Shende et al., 2015; Ramanathan et al., 2013).

$$CuSO_4\ 5H_2O + \text{Plant metabolites} \rightarrow \text{Cu nanoparticles} + \text{byproducts}$$

Pd (Palladium) nanoparticles are a precious high-density metal that is commonly used in medical diagnostics, catalysts, and biosensors. Various plant parts are used to synthesize the Pd nanoparticles. In 2017, Turunc and his team observed that the size of Pd nanoparticles was smaller than those prepared using synthetic agents.

$$Pd(CH_3COO)_2 + \text{Reducing extract} \rightarrow Pd + 2CH_3COOH$$

Zn (Zinc) nanoparticles have been discovered to be useful in food packaging and wastewater treatment. Zinc nitrate is required as a metal solution to produce Zn nanoparticles. It is very well known for antimicrobial and anticancer activities because Zn nanoparticles are found comparatively toxic against microorganisms, compared to all other metal nanoparticles (Sangeetha et al., 2011).

$$\text{Zinc nitrate} + \text{Plant extract} \rightarrow \text{ZnO nanoparticles} + \text{byproducts}$$

Medicinal plants used for nanoparticles green synthesis

In recent years, India has seen an increase in the trend of herbal-based products. This uprising is the consequence of public faith in Ayurveda and herbal products. This trust stems from an understanding of the adverse effects of chemicals derived or synthetic antimicrobial compounds on human health (Srivastava et al., 2014; Chandra et al., 2017).

As a result, in order to avoid toxic side effects, people are turning to natural medicinal plants that have already been mentioned in ancient literature. Researchers from various countries have shown metal nanoparticles to have therapeutic properties. Thus, a new era of nanomedicine is emerging, and the use of medicinal plants will benefit enormously from their use in the formatting of MNPs (Muhammad Mailafiya et al., 2019). Medicinal plants have an extra advantage over the other method for green synthesis. Because in the case of microbes it's required a multiple steps processes (isolation of potential microbe, specific culture preparation and subculturing, maintenance of the culture, etc.) to synthesize nanoparticles for applications. When this is compared with the plans various parts of it such as leaves, roots, stem, bark, flower, fruits, and seeds can act directly as reducing and stabilizing agents, which can be further mixed with the desired metal solution such as titanium oxide (TiO_2), silver nitrate ($AgNO_3$), and other metal solutions (Jha et al., 2009; Bar et al., 2009; Akhtar et al., 2013; Chandra et al., 2020). Various studies have reported the use of the green synthesis of nanoparticles from medicinal plants. Some of them are as follows.

Green synthesized nanoparticles have higher antimicrobial activity than the other nanoparticles. This activated antimicrobial activity could be the result of the synergistic action of a few proteins that function in capping and then stabilizing the biosynthesized nanoparticles (Roy et al., 2019).

Present knowledge/insights into the biological activities of green nanoparticles

Nanotechnology is highly urged for the future perspective because of the green synthesis process, cost-effectivity, and less toxicity. There has been much research work published and/or process about the MNPs in broad-spectrum propensity such as antioxidant, antidiabetic, biomedical devices, anticancer effect, antimicrobial agents, biomarker mapping, molecular mapping, tissue regeneration, regeneration materials, drug delivery, detection diagnosis, etc (Habeeb et al., 2022). With the advantage of green synthesis, there have been many challenges that need to be improved such as low yield, nonuniform particle size, stability of the nanoparticle dispersion, seasonal and regional availability of raw materials, etc (Guan et al., 2022). Although there are broad-spectrum of evidence in the literature published on green synthesized nanoparticles, they lack information regarding their fate in vivo. Literature studies reveal that various experiments have been done on biological models to check the toxicity of the nanoparticles but yet none has sufficiently established the exact mechanism involved in their toxicity. Hence, it's highly recommended to put efforts into assessing the efficacy of these nanoparticles in clinical research. Then only we can come up with a better approach that can make our nanoparticles into conventional medicine, with the benefit to overcome the many challenges associated with the present-day standard modes of treatment (Aboyewa et al., 2021).

Safety assessment for uptake, biocompatibility, and cytotoxicity of green nanoparticles

In the recent era, plant-mediated synthesis of nanoparticles has gained widespread attention, an emerging branch known as phytonanotechnology. Phytonanotechnology as an upcoming science has numerous advantages, in terms of scalability, biocompatibility, cost-effectiveness, and application in medicine, among others (Noruzi, 2015; Singh et al., 2016). The availability of plant tissues and comparatively safe profiles comprise the ideal properties for nanoparticle synthesis from plants. While several studies have documented the successful synthesis of plant-based nanoparticle synthesis, the factors involved and the mechanisms of synthesis still remain to be elucidated. In different plant species, different mechanism of nanoparticle synthesis has been reported (Baker S et al., 2013). The synthesis of MNPs via biological means exhibits safe profiles and improved biocompatibility, and defined new paradigms in the synthesis of nontoxic, scalability, biocompatibility, and well-defined structures, among others. Moreover, advances in synthetic biology and its application in the development of tailored MNPs have been extensively explored by researchers across the globe (Giessen and Silver 2016; Maddinedi et al., 2017).

The biological synthesis and wider application of nanoparticles have witnessed considerable success, however, safe parameters and challenges with uptake, biocompatibility, and uptake still imply the key concerns. In biological organisms, cell membranes are semipermeable and permit the passage of small ions/molecules while hindering others. The communication between the cells and nutrient uptake occurs through multiple mechanisms. The nanoscale biomolecules

enter the cell via endocytosis, the process in which the biomolecules in encapsulated into transport vesicles driven by a plasma membrane (Conner and Schmid, 2003). Studies have reported the uptake of engineered MNPs across plasma membranes of different cell types, including human mesenchymal stem cells (Greulich et al., 2011), human alveolar epithelial (Park et al., 2007), and human epidermal keratinocyte (HEK) cells (Monteiro-Riviere et al., 2013). However, different reported mechanisms of uptake contradict each other, necessitating an in-depth understanding of intracellular trafficking and uptake mechanism of NPs (Ahmad et al., 2018). The physicochemical properties, namely, surface chemistry, shape and size, and charges on the surface are key factors responsible for the cellular uptake of MNPs (Carnovale et al., 2019). Moreover, the endocytotic processes involve in the MNPs uptake comprise (1) pinocytosis (2) phagocytosis and (3) clathrin-mediated endocytosis (Nel et al., 2009; Verma and Stellacci, 2010; Ishimoto et al., 2008). The pinocytosis process (in all cells) functions in particle uptake of different sizes. A mammalian cell (neutrophils, monocytes, and macrophages), carries out the process of phagocytosis, which engulfs solid substances (diameter >750 nm) and forms phagosomes, and is employed in the uptake of nanoparticles in the cell (Ishimoto et al., 2008). The clathrin-mediated endocytosis is required for active uptake and nanoparticle internalization with a smaller diameter (<100 nm) (Harush-Frenkel et al., 2007; Li et al., 2008) and fuses with endosomes (Zhang et al., 2009). The different nanoparticles undertake specific mechanisms for entering the cell. When the gold nanoparticles are incubated with HeLa cells in the growth medium, the serum protein is adsorbed on the gold NP's surface toward cells via receptor-mediated endocytosis (RME) (Khan et al., 2007). Moreover, the mechanisms of cellular internalization are changed when the gold nanoparticles are encapsulated with different biological/organic molecules. The micropinocytosis pathway operates for the uptake of positively charged nanoparticles and uptake of negatively charged MNP's occurs by clathrin-mediated endocytosis (Dausend et al., 2008; Van Haute et al., 2018). In recent times, nanoparticle-induced autophagy, various MNP's like silver, gold, and neodymium oxide is known to induce autophagy in multiple cells (Chen et al., 2005; Li et al., 2010; Mishra et al., 2016).

Biologically synthesized MNPs and their biocompatibility

The information about the biocompatibility of synthesized MNP's is of primary importance and is attributed to the molecules which encapsulate the nanoparticle metallic core (Aditya et al., 2017). The different classes of biomolecules such as vitamins, flavonoids, proteins, terpenoids, carbohydrates, and tannins, including others render different properties of nanoparticles by regulating the net charge on the MNPs surface and it determines the MNPs mechanism of action (Sendra et al., 2017). Moreover, the different biomolecules functions as free radical scavengers, reducing agents and legends and binds to the MNPs metallic core (Sendra et al., 2017). The different types of cells exhibit varying levels of tolerance against biologically derived biomolecules. In a quest to understand the potential of biomolecules to nullify the toxicity of MNPs metallic core, studies into investigating the cellular models for the mammalian gene expression and its epigenetic modulation via capped biomolecules were undertaken. For example, cell viability assay (in a cervical cancer cell line (SiHa)) with tea polyphenols encapsulated platinum NPs and bimetallic Platinum-copper NPs showed the inhibition of SiHa cell lines, cell−cycle arrest in the G2/M phase, and increased number of cells in G0 death phase (Alshatwi et al., 2015; Athinarayanan et al., 2016). In another study on Parkinson's disease in zebrafish, the green platinum NPs reversed the neurotoxicity induced by MPTP via the regulation of mitochondrial enzymes, namely, dopamine, glutathione, superoxide dismutase, and glutathione peroxidase (Ganaie et al., 2017; Nellore et al., 2013). It is necessary to perform research toward gaining knowledge on whether biologically synthesized MNPs metallic core induces epigenetic modifications. While the studies on the evaluation of MNPs and cytotoxicity-related biomarkers, namely, mitotic index, micronucleus, ROS species generation, and cell death, showed that biological and chemical synthesized nanoparticles are less cytotoxic and potent genotoxic, compared to Ag^{+} ions alone (Panda et al., 2011).

MNP-mediated cytotoxicity and its mechanism

Multiple MNPs with different chemical and physical properties induce ROS-mediated cytotoxicity. The in vivo and in vitro induction of ROS-mediated cytotoxicity have been documented (Donaldson et al., 2001; Yang et al., 2009). Moreover, studies have shown that MNP-mediated cytotoxicity is due to the generation of ROS and oxidative stress processes. Nanoparticle-induced cytotoxicity is due to the following factors, NADPH-dependent.

Oxidation process activation, decrease in antioxidant enzymes, mitochondrial apoptosis, and perturbation in cell homeostasis. The cell-signaling pathways are activated by the MNP-mediated formation of ROS, and transcriptional activation, leading to the transcription of genes involved in fibrosis, cancer, inflammation, and genotoxicity (Ahmad et al.,

2018). For example, the overexpression of antioxidant enzymes leads to oxidative stress in biological systems, while mitochondrial apoptosis occurs due to severe oxidative stress. The estimation of ROS provides a tool to access the MNPs induced cytotoxicity (Ahmad et al., 2018).

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Chapter 5

Green synthesis of silver nanoparticles, characterization techniques and biological activities

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Introduction

The word "nano" refers to a billionth of a physical unit. Nanotechnology is a discipline of science that focuses on nanoscale (Satalkar et al., 2016). It is a rapidly developing field that has gained popularity in the last decade due to its wide range of applications (He et al., 2019). Nanotechnology is concerned with studying nanomaterials with sizes ranging from 1 to 100 nm and is defined as "the synthesis and application of materials to manipulate at the nanoscale using scientific knowledge" (Calipinar and Ulas, 2019). In several industrial fields, nanotechnology is among the most cutting-edge at the atomic and molecular levels. The materials produced have potentially unique properties related to their function and small size (Kakakhel et al., 2021). Nanostructured materials can form nanoparticles, nanopores, nanocubes, or nanotubes, but nanoparticles are among the first choice. They have grown in popularity in recent years. The primary reason for nanoparticle efficacy is their high surface-to-volume ratio and physicochemical and biological properties, making them an ideal agent for performing various functions at the cellular and subcellular levels (Viswanathan et al., 2019). Particle's chemical, physical, and biological properties change dramatically at the nanometer scale. Because of their small size, nanoparticles have enhanced properties that allow them to be used in many industries (Zhang et al., 2016).

Nanoparticles (NPs) are said to be raw materials employed in nanotechnology. Various raw materials (NPs) types include copper, nickel, iron, gold, and silver nanoparticles (AgNPs). Copper has been researched for several purposes. However, nickel nanoparticles may be crucial for biological functions (Magaye and Zhao, 2012). A category of nanomaterials called iron nanoparticles (FeNPs) is widely used in medical applications (Beheshtkhoo et al., 2018). Gold was once exclusively recognized as a metal. However, as nanotechnology developed, it became clear that its physio-chemical characteristics may make it a suitable material for forming gold nanoparticles. Gold nanoparticles attraction is due to their chemical and biological characteristics (Kalimuthu et al., 2020). AgNPs stand out among other metal nanoparticles' enhanced innate physical, chemical, and biological capabilities that vastly increase their value. Due to this versatility, AgNPs hold the pinnacle position in nanotechnology. Silver has a long history of being used for its medicinal and environmental benefits (Wongpreecha et al., 2018). AgNPs appear more biocompatible in comparison with traditional medications. A higher level of biocompatibility provides more site-specific distribution, boosting therapeutic effectiveness while reducing harmful side effects. As a result, AgNPs have enormous promise as medicines (Zhang et al., 2016).

With subjective evidence of the usage of colloidal silver in ancient Rome and Egypt, AgNPs are exceptional since they have been utilized for ages due to their excellent antibacterial action. AgNPs of various sizes and shapes are being developed. Many of them have a core-shell structure, which consists of a metallic silver core that can vary in size and shape and a coating that typically aids in controlling the size of AgNPs during synthesis and provides a surface charge to stabilize the AgNPs in solution (Reidy et al., 2013). Most wounds and infections were treated with colloidal silver (including AgNPs) during the 20th century. However, the discovery and development of modern antibiotics in the 1940s significantly decreased the usage of silver.

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Nevertheless, Ag has again gained popularity in the medical industry as an antibacterial due to the bacteria's resistance to antibiotics (Courtois et al., 2019). They demonstrate antibacterial activities and cover various characteristics, including antiviral, anticancer, thrombolytic, and anticoagulating activities. They are also helpful for diagnostics and imaging (Gomes et al., 2021).

AgNPs are considered benign and nontoxic to human and animal cells at low concentrations. They cause the least amount of environmental damage compared to other metal nanoparticles in the context of green chemistry, an emerging field essential for achieving the goal of sustainable development. However, their "greenness" is also determined by the synthesis technique used to synthesize them. Due to the numerous advantages AgNPs offer, it is essential to synthesize them.

Methods of synthesizing AgNPs

The bottoms-up and top-down approaches are the ones that are most frequently utilized for NPs synthesis. Top-down processes involve the physical milling, cutting, and shaping of materials with the aid of tools. Contrarily, bottom-up procedures, which use chemicals or biological processes (green synthesis) to self-assemble smaller particles into larger ones, are thought to be the best ways to synthesize nanoparticles (Alharbi et al., 2022). In this section, both approaches are discussed with more emphasis on the bottom-up approach specifically the green synthesis of AgNPs.

Physical methods

For the manufacture of AgNPs, evaporation-condensation, laser ablation, electrical irradiation, gamma irradiation, and lithography are the most crucial physical processes. Kimura and Bandow looked at the optical spectra of many metal colloid materials, solutions, and new inorganic solvent preparation techniques for metal colloids without using chemicals like electrolytes, glue, polymers, redox reagents, and other types of colloid stabilizers. The matrix isolation approach, the three different preparation techniques, the gas flow-cold trap method, the gas flow-solution trap method, and the silver nanoparticle synthesis (Keisaku and Shunji, 1983). Another approach for the production of AgNPs is the laser ablation technique, which has a variety of uses. The laser ablation approach is a novel, practical, and effective way to create and generate metal colloids without needing chemical reagents. In addition, by varying the number of laser pulses, this technique aids in controlling the size of the colloidal particle (Tsuji et al., 2002).

Pyatenko et al. created AgNPs by shining a 532 nm laser beam on an Ag target while submerging it in clean water. By utilizing a high-power laser and tiny laser beam spot sizes, this method successfully creates small nanoparticles with a narrow distribution in pure water without the addition of any chemical (Pyatenko et al., 2004). Using laser ablation and the thermal effusivity of a nanocomposite, Sadrolhesseini et al. developed a novel technique for producing AgNPs that are disseminated in graphene oxide. The ecologically friendly process releases the nanoparticles into the liquid solution without any chemicals, polymeric stabilizers, or surfactant stabilizers (Sadrolhesseini et al., 2013). Tsuji et al. reported the synthesis of colloidal solutions of AgNPs by laser irradiating and ablation of a silver plate in polyvinylpyrrolidone (PVP) aqueous solutions. Due to its procedural simplicity and extremely high rate of obtaining nanoparticles of different species and materials, such as metals, metal oxides, semiconductors, and organic materials, by irradiating those materials with intense laser light while they are suspended in solvents, this method is regarded as remarkable (Tsuji et al., 2008).

Another methodology for analyzing the synthesis of AgNPs in ethanol by laser ablation and determining the rate of laser pulse creation and concentration of created AgNPs is the pulsed photoacoustic (PA) technique (Valverde-Alva et al., 2015).

Researchers have investigated mechanisms and procedures such as plasma generation and cavitation dynamics bubble (Kudryashov et al., 2006; Liu et al., 2012), as well as the effects of solvents and laser settings on nanoparticles (Oseguera-Galindo et al., 2016). The straightforward and affordable nanofabrication technique known as nanosphere lithography (NLS) produces a wide range of well-ordered 2D NP arrays and nanoparticle shapes.

Jensen et al. studied periodic arrays of surface-confined molecules optical extinction spectra as a function of solvent NSL-produced AgNPs and four different NP array samples. They also examined four nanoparticle arrays; three samples yielded nanoparticles with truncated tetrahedral shapes but with different out-of-plane heights, and one sample included oblate ellipsoidal nanoparticles (Jensen et al., 1999). Additionally, they showed that the silver nanoparticle material system's localized surface plasmon resonance extinction maximum could be continuously modified across the visible, near-infrared, and mid-infrared spectrums (Jensen et al., 2000).

Chemical methods

Chemical reduction of AgNPs

Nanomaterials' chemical, physical, optical, and electrical characteristics are significantly influenced by their size, shape, and surface morphology. Therefore, chemical reduction is one of the most popular techniques for creating AgNPs using both inorganic and organic reducing agents. In general, various reducing agents are used to reduce the silver ions (Ag^{+}) in aqueous or nonaqueous solutions, including sodium citrate, ascorbate, sodium borohydride ($NaBH_4$), elemental hydrogen, polyol process, Tollens reagent, *N,N*-dimethylformamide (DMF), and poly(ethylene glycol)-block copolymers, hydrazine, and ammonium formate (Malassis et al., 2016).

Microemulsion techniques

A microemulsion is water, surfactant, oil, and cosurfactant mixture. Various surfactants are available to create the microemulsion while manufacturing the AgNPs. In general, a variety of surfactants can be used to create microemulsions, including nonionic surfactants like Triton X-100, cationic surfactants like cetyltrimethylammonium bromide and PVP, anionic surfactants like bis (2-ethylhexyl)sulfosuccinate, sodium dodecyl benzene sulfonate, and surfactant-coated water droplets serve as microreactors and provide a unique microenvironment for the creation of nanoparticles (Chhatre et al., 2012; Elmas et al., 2018; Nourafkan and Alamdari, 2014; Singha et al., 2014; Sun et al., 2001; Wani et al., 2013).

Microwave-assisted techniques

In contrast to the traditional heating approach, microwave synthesis methods allow the reduction of the AgNPs with a variable rate of microwave radiation. As a result, quick results are produced via microwave-assisted technology, which reduces the time it takes for chemical reactions from hours or days to minutes. Additionally, microwave irradiation facilitates the ripening of materials without aggregation and offers homogeneous heating for creating metallic nanoparticles (MNPs) (Guo et al., 2013; Pal et al., 2009; Pal et al., 2014; Wang et al., 2010).

Green synthesis

Due to the increased demand for ecologically friendly material synthesis technologies, the biosynthesis of nanoparticles has attracted much interest. Much work has been done on the environmentally friendly production of inorganic materials, mainly metal. "Green synthesis" is a bottom-up approach also referred to as "biological techniques" which are generally carried out using medicinal plants and have advantages over chemical and physical processes since they are less expensive, more environmentally friendly, and more widely accessible. They are using plant and microbial extracts to create nanoparticles (Samadi et al., 2009). At the same time, algae (El-Rafie et al., 2013), yeast (Jha et al., 2008), and fungi (Bhainsa and D'Souza, 2006) are continued now being investigated for the intracellular and extracellular creation of metal nanoparticles, as well as the similar usage of components from the entire plant. Using new methods for synthesizing nanoparticles, there is an exciting opportunity to be explored. In the literature, various bacterial strains such as *B. amyloliquefaciens* (Fouad et al., 2017), *A. calcoaceticus* (Singh et al., 2013), *P. aeruginosa* (Kumar and Mamidyala, 2011), *E. coli* (Divya et al., 2016), and *B. licheniformis* (Kalimuthu et al., 2008) were used effectively for the synthesis of AgNPs.

Due to the possible high yields and abundance of proteins, the synthesis of AgNPs utilizing microbes has garnered a lot of interest. However, there are challenges with this approach in terms of maintaining culture and growth (Guilger-Casagrande and de Lima, 2019). Plants are eco-friendly and simple to handle with therefore employing their extracts to synthesize NPs has various advantages over other environmentally friendly synthesis techniques. Additionally, it provides availability, cheap cost, great yield, minimal toxicity, and energy efficiency. Neo-clerodaneflavonol glycosides, ergosterol, iridoid glycosides, phytoecdysones, and other polyphenols are examples of phytochemicals in plants that act as reducing, capping, and stabilizing agents during the green production of NPs (Afreen et al., 2020; Pallela et al., 2018). AgNPs can be produced using a variety of plant parts, such as fruits, leaves, seeds, flowers, bark, roots, and stems. Upcoming era is an era to synthesize AgNPs using this green approach. Recently, few reported literature have utilized this green approach to synthesize AgNPs (Amaliyah et al., 2022; Chinni et al., 2021; Gomathi et al., 2020; Rather et al., 2022). Using new methods for synthesizing NPs, there is an exciting opportunity to be explored.

Mechanism of green synthesis of AgNPs

The process of synthesizing AgNPs using green synthesis from plants involves interacting silver nitrate (AgNO3) with the biomolecules found in plant extracts. There are three main stages in the formation of NPs: (a) an ion reduction reaction, (b) cluster formation that induces the growth of NPs and (c) stabilization of silver ions. Depending on the reducing agent, its concentration, $AgNO_3$, and pH, each stage has particular features. Amino acids, proteins, alkaloids, flavonoids, polyphenols, enzymes, tannins, carbohydrates, and saponins are examples of plant biomolecules that contain hydroxyl groups (OH), which stabilize and reduce silver ions (Ag^+) to Ag^0. It is further reduced to Ag^+, where silver nuclei are generated, leading to the production of AgNPs. Jasuja et al. reported that kaempferol, naringin, and glycosides are present in *Punicagranatum* peel extract. These compounds all have OH groups that can reduce Ag^+ ions and produce AgNPs (Jasuja et al., 2014). According to investigation carried out by Jain and Mehta on the putative mechanism of manufacturing AgNPs using Tulsi leaf extract, the quercetin in this plant combines with Ag^+ ions when the OH groups connect to the carbon atoms of aromatic rings, reducing Ag^+ to NPs and providing stability against agglomeration (Jain and Mehata, 2017). From one plant to another, this scenario could be different. In order to find biomolecular stabilizing and capping agents for the synthesis of AgNPs from plants, a thorough investigation is necessary.

Factors affecting the green synthesis of AgNPs

The key variables, namely pH, temperature, reactant and biomolecule concentrations, incubation time, and light intensity, can be used to control the structural and geomorphological physiognomies of NPs. These key variables can significantly contribute to optimizing the synthesis of metallic NPs using biological templates and are crucial for comprehending how environmental factors affect the synthesis of NPs.

pH

The pH of the substrate and medium, in general, plays a crucial part in regulating the shape and size of the NPs. The synthesis of AgNPs was best ensured at a pH of 7, where reduction of Ag^+ to Ag^0 takes place during AgNPs production, and the highest abundance of synthesized NPs was obtained at pH 7−9. When the substrate's pH is acidic, NPs of medium and large sizes are produced, with their shape and size altering accordingly. An alkaline pH resulted in smaller size distribution and shorter synthesis time for AgNPs (Binupriya et al., 2010; Du et al., 2015). Priyadarshini et al. also reported that at pH 8, AuNPs change from spherical to rod-shaped particles with an average size of 20−29 nm. AuNPs had an average size between 10 and 19 nm and a spherical form at pH 10. Small AuNPs are generated due to the reduction reaction intensifying as pH gradually rises (Priyadarshini et al., 2014).

Additionally, according to another study by Bergal et al., synthesizing AgNPs at an alkaline pH has numerous advantages, such as a stable and high yield of NPs, a quick growth rate, and an improved reduction process (Bergal et al., 2022). A basic pH enables more OH groups to participate in the reduction reaction, boosting the yield. Hydroxyl (OH) groups in plant extracts play an essential role in reducing and stabilizing functional groups during the production of AgNPs (Singh et al., 2009).

Temperature

AgNPs synthesis velocity, stability, and size are just a few examples of the factors that the temperature used during the synthesis of AgNPs that may have a considerable effect. A study by Birla and the group suggests that at temperatures between 60 and 80°C, *Fusariumoxysporum* produced more AgNPs at a faster rate. As the temperature rises, the fungal biomass produces more protein with faster synthesis (Birla et al., 2013). AgNPs are produced by *Aspergillus oryzae*, and high temperatures affect the production rate. Phanjom and Ahmed studied the effects of temperature on the production rate and synthesis time of AgNPs. They found that if the reaction starts at 30°C, temperatures can be completed at 6 h, but when 90°C is employed, the reaction can be completed in 10 min, while at 10°C, no synthesis took place (Phanjom and Ahmed, 2017).

Furthermore, even at a low temperature of 40°C, *Vitexagnus-castus* leaf extract reduced Ag^+ ions quickly. In contrast, another study found that the synthesis of AgNPs was most effective at temperatures between 60 and 80°C. As a consequence, it is widely known that high temperatures encourage nucleation, whereas low temperatures encourage growth in the synthesis of NPs (Kredy, 2018; Stavinskaya et al., 2019).

Effect of $AgNO_3$ and biomolecule concentration

$AgNO_3$ concentration influences the size of the produced AgNPs and their quantity. In experiments utilizing *Fusariumoxysporum*, it was found that between 0.1 and 1.5 mM, the concentration of AgNPs increased as the metal precursor quantity increased. However, no changes were detected at higher doses (Korbekandi et al., 2013). For the extracellular synthesis of AgNPs by fungal species in several different experiments, silver nitrate ($AgNO_3$) at a concentration of 1 mM was used. Smaller NPs were produced in some investigations utilizing a lower concentration of the metal precursor ($AgNO_3$), but smaller NPs were produced in other research using an intermediate concentration of $AgNO_3$ (Abdelrahim et al., 2017; Phanjom and Ahmed, 2017). $AgNO_3$ concentration increases absorption, and 1 mM is the ideal nanoparticle concentration. In addition, various biomolecules like plant and fungus extract to cover the surfaces of the NPs and act as reducing agents, preventing them from aggregating and enhancing their stability (Ahmed et al., 2016).

Incubation time

A crucial factor for improving the yield, stability, and size of synthesized AgNPs is incubation time. Within 2 min of incubation, a rapid color shift was seen when *Ananascomosus* (Pineapple) extract was utilized to synthesize AgNPs (Ahmad and Sharma, 2012). Another study by Jain and colleagues used *Ocimum Sanctum* (Tulsi) leaf extract to create stable AgNPs that were about 17 nm in size. After 15 min of incubation, the yield of biosynthesized AgNPs started to rise and kept rising over the next few hours (Jain and Mehata, 2017). Additionally, AgNPs were produced using an extract of *Origanumvulgare* L., and the yield of nanoparticles increased with reaction time up to 3 h. As AgNPs formed, the reaction mixture's color intensity slowly altered from yellow to brown (Shaik et al., 2018). Due to a rise in the amount of AgNPs generated, the absorption intensity also enhanced as the incubation period increased.

Light intensity

Light intensity is an essential factor and significantly influences the green synthesis of AgNPs. Therefore, sunlight-based light irradiation is a proper method to facilitate the environmentally friendly synthesis of AgNPs. It is anticipated that in sunlight, the reduction of Ag^+ ions can be finished in a short period, whereas under darkness, the reaction takes longer. This may be due to photons of a particular wavelength that stimulate the green synthesis process and promote the green synthesis of AgNPs under direct sunlight (Srikar et al., 2016). This approach was successfully applied to synthesize AgNPs from medicinal plants, such as *Piper longum* and *Carica papaya* fruit (Firdaus et al., 2017; Jayapriya et al., 2019). Additionally, the yield and concentration of AgNPs produced by the green synthesis of AgNPs utilizing sunlight are high and demonstrate good stability (Nguyen, 2020).

Characterization techniques

Before being used, appropriate techniques must first be employed to ensure the formation of AgNPs. The simplest way to track AgNPs synthesis is to visually examine the solution's color change from yellow to brown. Further, a spectrophotometer can detect nanoparticle peaks in the visible region of the UV-Vis spectrum at a wavelength between 400 and 450 nm. Other methods can be utilized to examine nanoparticle size, shape, dispersion, and composition, such as SEM, TEM, XRD, FTIR, DLS, and zeta potential. XRD analysis is a vital technique for assessing nanoparticle formation. Biomolecules that affect the production and stability of nanoparticles can be identified using FTIR spectroscopy.

UV-Vis Spectrophotometry

AgNPs that have been produced can be effectively characterized using UV-Vis spectroscopy. The absorbance spectra can confirm the synthesized AgNPs in a solution. Nanoparticles are typically detected at wavelengths between 400 and 800 nm. AgNPs with smaller average sizes and higher concentrations have a maximum wavelength (λmax) at lower and higher values, respectively. Additionally, broad and narrow peaks at longer and shorter wavelengths signify increased and decreased AgNPs size (Zhang et al., 2016). The intensity and location of the peak in the spectrum, which appears at wavelengths between 380 and 450 nm, can be used to demonstrate the quality of the produced nanoparticles. A broad peak at a high wavelength denotes aggregated AgNPs of enormous size, whereas a narrow peak at a low wavelength denotes nanoparticles of small size. Additionally, UV-Vis spectroscopy peaks at the same wavelength indicate that the stability of

green synthesized AgNPs can be preserved for several months. Sharma et al. used a spectroscopic approach to characterize AgNPs produced using *Ocimumgratissimum* aqueous leaf extract (Sharma et al., 2020).

Scanning Electron Microscopy (SEM)

SEM is a kind of electron microscopy that can be used to analyze the surface morphology of NPs, including their size, shape, and distribution. In SEM imaging, an electric current travels through electromagnetic coils and lenses to produce a focused beam of electrons. Secondary electrons are produced when this beam comes into contact with a sample surface. A very accurate reconstruction of the sample surface morphology is made using the information on the resultant electrons (Chandraker et al., 2021). Due to their high electrical conductivity, metal NPs like silver and gold are simple to scan with an SEM. This microscopy technique has a substantial benefit in that samples can be immediately placed on a black surface without worrying about unwanted incident beam scattering. The majority of particles range in size from 20 to 30 nm. Changes in the synthesis parameters discussed earlier in this chapter could be the reason for the shape variances. The AgNPs characterized by SEM often have various shapes: pebble-like, round, spherical, cubic, triangular, oval, and obovate (Arif and Uddin, 2021; Choi et al., 2021). SEM can be useful for learning about morphology, particle purity, and aggregation of AgNPs, but cannot provide information related to the internal structure of samples.

Transmission Electron Microscopy (TEM)

TEM can be used to characterize NPs as well. TEM can be used to get quantitative measurements of the produced NPs, such as their size, size distribution, and shape. A single drop of the sample solution must be coated with carbon copper grids for TEM imaging, which is conducted by passing an electron beam through a sample. Compared to SEM, TEM offers better spatial resolution, which permits a more thorough investigation of NPs. TEM examination showed that the produced NPs had a predominance of spherical form. In addition to spheres, AgNPs can also take the form of nanorods, nanowires, and triangles. Using TEM, rod-like, triangular, and quasi-spherical AgNPs have been found (Sreelekha et al., 2021). The requirement for a large sample section and high vacuum conditions is some of the drawbacks of TEM. Although tedious sample preparation for TEM analysis is necessary for getting accurate images. The TEM's studied region is relatively small and could not be an accurate representation of the entire sample. The sample could be harmed by the electron beam when considering biomaterials. Despite these limitations, TEM is a modern technology for gathering visual data about the shape, scale, structure, and dispersion of nanoparticles due to its atomic level accuracy (Chandraker et al., 2021; Zhang et al., 2016).

X-ray Diffraction (XRD)

XRD is a well-known characterization technique for qualitative and quantitative NPs evaluations. Bragg's law is the XRD's guiding theory. To establish the crystal structure of NPs and to verify their formation, XRD analyses are used. This method has also been applied to quantify the degree of crystallinity and determine the size of crystalline nanoparticles. Each material has a distinct diffraction beam; hence, the examination of materials using this method depends on the diffraction patterns. So, by comparing the diffraction beams to the reference database of the joint committee on powder diffraction standards library, a substance can be identified and characterized (Mehata, 2021). Impressive peaks can be seen in XRD patterns, and nano-sized AgNPs can be identified based on specific peaks (Alaqad and Saleh, 2016).

Fourier Transform Infrared Spectroscopy (FTIR)

Identification of the biomolecules that reduce Ag^{+} ions and stabilize the produced AgNPs can be identified by FTIR analysis. It is possible to identify the functional groups responsible for the production AgNPs using FTIR spectroscopy (Anandalakshmi et al., 2016). Functional groups like alkanes, ketones, and amines, among other functional groups, absorb infrared radiation at various wavelengths, allowing the identification of biomolecules. The form of the absorption spectrum profile in FTIR spectroscopy analysis exhibits different peaks representing the high concentration of specific types of chemical bonds. The functional groups involved in the surface coating and efficient stabilization of the generated NPs can be identified by comparing the FTIR spectra of a medicinal plant extract with that of biosynthesized AgNPs (Akintelu et al., 2020). This is why FTIR spectroscopy is a valuable and affordable method for figuring out how biological components contribute to creating and stabilizing green-produced AgNPs.

Dynamic Light Scattering (DLS)

The surface charge, size, and particle size distribution of NPs can all be examined using DLS. This method relies on the interaction of light traveling through a colloidal solution with spherical particles moving in a Brownian manner (Bamal et al., 2021). DLS can measure particles with a size between 1 and 500 nm, although it has trouble while measuring agglomerated particles. Only a minimum number of nanoparticles are needed to avoid numerous scattering effects in DLS. The scattered intensities from the time-dependent data can be used to estimate the hydrodynamic diameter. The electrical layers on nanoparticles' surfaces and the capping agents/stabilizers in solution typically impact their hydrodynamic diameter. Khader et al. used the DLS technique to report a hydrodynamic diameter of 203 nm while synthesizing AgNPs made from *Phoenix dactylifera* seeds (Vanin dos Santos Lima et al., 2022).

Zeta potential

Zeta potential is a crucial factor in controlling the stability, effective surface electric charge, and dispersion of NPs. High positive or negative charges in a particle cause them to repel one another and produce stable particles with a low inclination to coalesce, according to earlier studies by Baldassarre et al. (2015). Dispersions with a lower zeta potential may cause AgNPs to aggregate due to van der Waals attraction. Earlier studies said that AgNPs synthesized from *Annona squamosa* and *Bryophyllumpinnatum* leaf extract have zeta potentials of 37 and 26.7 mV, respectively. AgNPs having a value of 26.7 mV are more stable NPs (Anandalakshmi et al., 2016; Vivek et al., 2012).

Biological activities of AgNPs

Due to their distinctive physicochemical characteristics and biological activities, AgNPs have been one of the most attractive nanomaterials in the biomedical field. AgNPs have attracted the interest of numerous researchers and industries due to their wide range of biomedical uses (Burdusel et al., 2018).

AgNPs have long been used as antibacterial agents in several items, including cosmetics, textile coatings, and other products (Bruna et al., 2021). Studies have demonstrated that AgNPs are effective against many pathogens, including bacteria, fungi, and viruses (Algotiml et al., 2022). Additionally, AgNPs can efficiently harm or eliminate nematodes (Heflish et al., 2021) and worms (Li et al., 2015).

AgNPs are beneficial in cancer treatment because of their increased chemotherapeutic efficacy and low systemic toxicity. AgNPs anticancer activity is also influenced by several variables, such as size, shape, dose, and exposure duration (Gurunathan et al., 2015). AgNPs' anticancer mechanisms, however, are a lot more complex. It has been established that AgNPs can stop tumor cell growth by damaging cellular ultrastructures, causing ROS generation, and causing DNA damage. AgNPs can also activate proteins, control signaling pathways, and cause tumor cell apoptosis (Barcińska et al., 2018). They can also prevent tumor cell spread by preventing angiogenesis inside lesions.

AgNPs have other medical uses besides antibacterial and anticancer capabilities, including bone restoration and wound healing (Tian et al., 2007; Zhang et al., 2015). Additionally, AgNPs may be used as adjuvant in vaccine, an additive in dental materials, and medication carriers for precise and selective cell or tissue targeting, according to recent investigations (Asgary et al., 2016; Gherasim et al., 2020; Oei et al., 2012). The recent enhancements in AgNP biocompatibility and stability through surface modification and the excellent optical properties of AgNPs strongly suggest nanostructured systems based on silver as particular, selective, and adaptable candidates for drug-delivery applications (Qing et al., 2018; Xu et al., 2020).

This section will discuss AgNPs' potential mechanisms, antibacterial and anticancer characteristics, and other intriguing medical uses.

Antibacterial activity

Numerous pathogenic bacteria, fungi, and viruses, including *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, dermatophyte, HIV-1, etc., are successfully inhibited by AgNPs. AgNPs have diversified antibacterial effects on different bacterial strains (Yuan et al., 2017). It is interesting to note that AgNPs may behave differently against Gram-positive and Gram-negative bacteria. This might be because the two types of bacteria have cell walls that are different densities (Abbaszadegan et al., 2015). In addition to the bacteria strains, AgNPs may also have an antibacterial activity that is size-, shape-, concentration-, time-, and charge-dependent (Liao et al., 2019). In general, the antibacterial activity of

AgNPs increases noticeably when particle size is reduced. AgNPs exhibit improved antibacterial action, mainly when the size is less than 10 nm (Agnihotri et al., 2014). By extending the treatment duration of AgNPs, the antibacterial action can be markedly improved. The accumulation of AgNPs and silver ions throughout the exposure time may be responsible for the enhanced bacterial mortality (Dakal et al., 2016).

Additionally, AgNPs' structure may affect their antibacterial activity. Spherical-shaped AgNPs exhibit a stronger antibacterial activity than triangular, linear, or cubic AgNPs, according to a comparison study. This result suggests that AgNPs with a higher surface-to-volume ratio, associated with a higher effective contact and a larger reaction surface, may exhibit potent antibacterial activity (Raza et al., 2016). The surface charge of AgNPs also influences their antibacterial activity. Bacterial membranes are largely loaded with negative charges because they include lipopolysaccharide, peptidoglycan, and many groups, such as carboxyl, amino, and phosphate groups. On the other hand, AgNP adhesion to bacterial membranes can be aided by positive charges due to electrostatic attraction. As a result, modifying the surface charges of AgNPs may help to increase their antibacterial effect (Abbaszadegan et al., 2015; Dakal et al., 2016).

AgNPs antibacterial action may be affected by the stabilizers effects on AgNPs size, dispersion, and surface charge. It has been demonstrated that several stabilizers, including citrates, PVP, and polyvinyl alcohol, can change the properties of AgNPs to affect bacterial action (Anees Ahmad et al., 2020; Dey et al., 2015; Pencheva et al., 2012).

Numerous research has tested theories, even though the precise processes behind the antibacterial effects of AgNPs are still unknown. The antibacterial mechanisms of AgNPs have been explained by a variety of theories, including (i) causing bacterial membrane disintegration and allowing cellular contents to leak; (ii) producing reactive oxygen species (ROS) and shutting down the respiratory chains; (iii) destroying the DNA structure and preventing DNA replication; and (iv) inactivating enzymes and denaturing proteins (Qing et al., 2018). These mechanisms help AgNPs to have robust and broad-spectrum antibacterial activities.

Antifungal and antiviral activity

According to various research studies, AgNPs have antifungal effects in size- and dose-dependent manner against *Colletotrichumcoccodes*, *Monilinia* sp., *Candida* spp., and various plant pathogenic fungi (Lamsal et al., 2011; Malandrakis et al., 2020; Panácek et al., 2009). The type of culture media employed in the tests may affect the inhibitory activity, according to certain studies (Kim et al., 2012). Additionally, AgNPs exhibit potent antiviral action against influenza A (H1N1), hepatitis B virus (HBV), human parainfluenza virus, and herpes simplex virus (Gaikwad et al., 2013; Hu et al., 2014; Lu et al., 2008; Xiang et al., 2011). AgNPs with a size of less than 10 nm have good antiviral activity, possibly because of their sizable reaction area and potent adsorption on the surface of viruses (Kar et al., 2022). For instance, AgNPs can interact with the virus in size- and the dose-dependent way by binding to the glycoprotein knobs and inhibiting the reverse transcriptase (RT) of HIV-1 (Elechiguerra et al., 2005).

Antiprotozoal activity

Globally, *Giardia lamblia*, and *Cryptosporidium parvum*are responsible for gastrointestinal illnesses and are particularly resistant to chlorine treatments and other conventional methods of deactivation (Putignani and Menichella, 2010). Additionally, immune-compromised persons who contract *Cryptosporidium parvum* infection risk dying, making it crucial to find novel ways to destroy these organisms at deficient detection levels. Said *et al.* conducted an interesting *in vivo* study in which they examined the anti-G. lamblia activity of AgNPs, chitosan NPs, and curcumin NPs in rats. AgNPs, chitosan NPs, and curcumin NPs were combined to produce the most significant effect compared to when they were utilized independently (Said et al., 2012). The efficiency of AgNPs against *Cryptosporidium parvumis* being tested through various experiments (Cameron et al., 2016; Hassan et al., 2019).

Another major protozoal illness is leishmaniasis, one of the most commonly neglected tropical diseases in the world. The use of common anti-leishmanial drugs like antimonial and amphotericin B liposomes is limited due to the development of drug resistance. Many attempts were carried out to identify antileishmanial effect of silver nanoparticles as an alternative therapy (Allahverdiyev et al., 2011; Almayouf et al., 2020; Awad et al., 2021; Baiocco et al., 2011). Sharma et al. prepared AgNPs using *Fusariumoxysporum*and found that biosynthesized AgNPs had potential in vitro and in vivo activity against *Leishmaniaamazonensis* (Rossi-Bergmann et al., 2012). It was found that biologically

produced AgNPs were four times more potent than chemically created AgNPs in in vitro trials; additionally, they were much more successful in the in vivo model.

Malaria is one of the most widespread protozoal vector-borne infections (Chala and Hamde, 2021). The rapid rise in plasmodia resistance to antimalarial medications encourages ongoing research into novel strategies for combating malarial parasites and ways to limit the expansion of the mosquito vector (Shibeshi et al., 2020). AgNPs have also been tested against plasmodia in several studies and other treatments, and the findings were encouraging (Al-Quraishy et al., 2020; Avitabile et al., 2020; Mnkandhla et al., 2018). Yang et al., Panneerselvam et al. (2011) discovered anti-P. falciparum action in AgNPs with an average size of around 55 nm that were biologically produced using *Andrographispaniculata* Nees (Acanthaceae). Ponarulselvam*et al.*also reported anti-P. falciparum activity of AgNPs (average size of around 35−55 nm) made with an aqueous *Catharanthusroseus* leaf extract (Ponarulselvam et al., 2012). However, none of the reported research explains the antiplasmodial effects.

Anticancer activity

Currently, cancer is regarded as a significant contributor to morbidity and mortality on a global scale (Sung et al., 2021). Moreover, by 2035, there will likely be 14 million additional cancer cases, significantly affecting global society and the economy (Pilleron et al., 2019). In order to lessen the negative consequences of cancer occurrence, it is urgently necessary to develop effective and cutting-edge treatment approaches (Schirrmacher, 2019). Cancer and tumors are frequently treated with surgery, chemotherapy, and radiotherapy. However, the results are affected by the drawbacks and side effects of conventional therapy. For instance, standard chemotherapy can have harmful side effects, including myelosuppression, liver and renal failure, immunosuppression, and local reactions such as thrombophlebitis and tissue necrosis (Sedighi et al., 2019).

Additionally, cancerous tumors may become multidrug resistant (MDR), which could make chemotherapy ineffective (Bukowski et al., 2020). Therefore, it is crucial to create new medications to enhance their therapeutic benefits. A new discipline of anticancer therapy called cancer nanomedicine has emerged in recent years due to the increased interest that nanoparticles have received in cancer therapies due to their unique physical and chemical features (Bae et al., 2011; Bor et al., 2019). MNPs can be exploited as innovative therapeutic agents or drug carriers in combination with treatment candidates, compared to conventional anticancer medicines, and undesired side effects can be avoided using a tailored approach (Gurunathan et al., 2018; Khursheed et al., 2022).

In the hunt for anticancer drugs, AgNPs stand out among these nanoparticles. In cases of breast cancer, cervical cancer, colon cancer, ovarian cancer, pancreatic ductal adenocarcinoma, lung cancer, hepatocellular carcinoma, melanoma, osteosarcoma, and other cancers, AgNPs have been seen to exhibit good anticancer effects (Jain et al., 2021). Numerous studies show that different cancer cells respond differently to AgNPs with varying sizes, shapes, and doses/concentrations for their anticancer effects. The anticancer effect of AgNPs is further influenced by several variables, including tumor microenvironment, cell lines, exposure period, and pH of lesions. AgNPs generally show a broad spectrum of anticancer activity that is size, dose/concentration, and time dependant. AgNPs of smaller diameters can cause more pronounced cytotoxicity and genotoxicity, as well as accelerated endocytosis. Because they have a larger surface-to-volume ratio than other shapes, spherical AgNPs are more cytotoxic. Additionally, a larger AgNP dose typically results in more apoptosis than a lesser one (Avalos et al., 2014; Wu et al., 2019; Yeasmin et al., 2017; Zielinska et al., 2018).

Conclusion

Silver has been used as an antibacterial agent to eradicate various kinds of germs, including fungi, bacteria, and viruses. Nevertheless, due to the rising concern over antibiotic resistance brought on by drug overuse, this aspect has recently received more and more attention. AgNPs synthesized from medicinal plants exhibit significant antitumor activity, which is of utmost importance. Researchers are taking a closer look at the green synthesis of AgNPs for a variety of reasons, including cost-effectiveness, environmental friendliness, and preservation of human health. The synthesis of AgNPs by plant biomolecules, which has the potential to affect the advancement of nanotechnology, is a new and exciting topic of nanotechnology. The current advances in the green synthesis of AgNPs particularly using medicinal plant biomolecules, mechanism of synthesis, optimization conditions, characterization techniques, and biological activities of AgNPs have been highlighted in this chapter.

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Chapter 6

Synergistic effects of plant extracts and nanoparticles for therapy

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Introduction

Plant-based products and phytocompounds are well-known natural therapeutic agents that used for the treatment of various infections and diseases for ages. At present, almost 25% of industrial pharmaceutical products are derived from natural resources and these natural products exhibit significant chemical diversity, biological activity, macromolecular specificity, and reduced toxicity (Patra et al., 2018). These natural compounds are primarily plant-based products that are extensively explored for the treatment of several fatal diseases such as cancer, diabetes, cardiovascular, inflammatory, and microbial infections (Shende et al., 2017; Ghosh et al., 2015a). However, it has been shown recently that approximately 70% of these herbal drugs that are available on the market exhibit poor water solubility which makes them difficult to absorb after oral administration (Rahman et al., 2020). Moreover, these natural products have poor gastric and intestinal stability which makes them difficult to act as effective drugs. Thus, a novel and efficacious delivery system for these phytochemicals are required wherein nano-based carrier has recently been demonstrated as one of the potential therapeutic approaches for targeted delivery of these plant-based extracts (Bhagwat et al., 2018; Ghosh et al., 2016a,b). Hence, this chapter emphasizes the importance and synergism of plant extract with nanomaterials as a novel and efficient treatment option.

The high ratio of surface area to volume of nanomaterials makes them unique carriers of drugs and other pharmacologically important molecules (Majeed et al., 2022; Shende et al., 2018). Polymeric nanomaterials made up of chitin, alginate, lipids, polyethylene glycol (PEG), poly(capro) lactone (PCL), and poly (D, L-lactic-co-glycolic) acid (PLGA) are used for the encapsulation of plant extracts obtained from *Mentha piperita, Punica granatum* L., *Lycium barbarum*, *Zizyphus jujuba, Centella asiatica* L., *Campomanesia xanthocarpa* O. Berg., *Nasturtium officinale, and Passiflora serratodigitata* L. These nanoencapsulated complexes demonstrated improved antioxidant, antimicrobial, anticancer, and other biological activities. Silver, gold, zinc oxide, and other metallic nanoparticles are also conjugated and surface functionalized with extracts of *Leucas Aspera*, *Geranium wallichianum*, *Pyrenacantha grandiflora* Baill., *Aloe barbadensis* Miller, *Sterculia setigera, Withania somnifera*, and several other plants to enhance their therapeutic properties that are summarized in Table 6.1.

Therefore, further exploration of the biocompatibility, stability, in vivo cytotoxicity as well as economic viability would provide useful data on the feasibility of the potential biomedical application of these nanomaterials and phytoextract conjugates (Ahmad et al., 2021).

Polymeric nanomaterials based conjugates

Several polymeric nanomaterials are used for the encapsulation of phytochemicals. Rahnemoon et al. (2021) recently demonstrated the nano-encapsulation of *Punica granatum* L. peel extract (PPE) and evaluated its antimicrobial activity. Alginate nanospheres were synthesized using water in oil emulsification technique following which the obtained nanospheres were loaded with PPE. External gelation of the PPE-encapsulated alginate nanospheres was carried out using calcium chloride nanoparticles. A ratio of 4:1 and 9:1 for the alginate: PPE and alginate: calcium chloride were optimal for

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TABLE 6.1 Applications of plant extract nanoconjugates.

Nanomaterial	Plant component	Size of nano-conjugate (nm)	Application	References
Polymeric nanoparticles				
Alginate nanospheres	*Punica granatum* L. peel extract	205.1 ± 0.1	Potential preservative for inhibiting the growth of psychrophiles	(Rahnemoon et al., 2021)
Chitosan nanoparticles	*Zizyphus jujuba* pulp and seed extract	70–300	Enhanced stability of antioxidant activity and total phenolic content	(Han et al., 2015)
Chitosan, *Alyssum homolocarpum* gum (AHG), and 1:1 complex of chitosan and AHG (CCA) nanoemulsion	*Mentha piperita* phenolic extract	108.66, 65.18, and 70.81	Enhanced antioxidant activity	(Roshanpour et al., 2021)
CSNPs	*Punica granatum* L. peel extract	127.3	Enhanced antioxidant activity and antimicrobial activity against *S. aureus*	(Soltanzadeh et al., 2021)
Gelatin nanoparticles	*Centella asiatica* L. extract	115	Enhanced skin-protective activities	(Kwon et al., 2012)
Aloe vera gel template	Curcumin	Base size- 50–100 and depth- 60	Improved antioxidant activity and in vitro drug release	(Kitture et al., 2015a)
Liposomes	*Lycium barbarum* leaf extract	141.6 ± 2.360	Cytoprotective activity against oxidative stress	(Păvăloiu et al., 2021)
PCL nanoparticles	*Passiflora serratodigitata* L. stem and leaf extracts	379.2 ± 16.4 and 383.8 ± 18.2	Improvement in antiulcerogenic activity	(Strasser et al., 2014)
PLGA nanoparticles	*Campomanesia xanthocarpa* O. Berg. fruit extract	202.5 ± 50.8	Enhanced antioxidant and antimicrobial activity	(Pereira et al., 2018)
PLGA/PEG nanoparticles	*Nasturtium officinale* extract	205	Enhanced anticancer activity	(Adlravan et al., 2021)
Solid lipid nanoparticles	Triptolide	179.8 ± 5.7	Reduction of gastric mucosal irritation	(Zhang et al., 2013)
Metal-based nanomaterials				
AgNPs	*Pyrenacantha grandiflora* Baill extracts	13 and 3–25	Enhanced antibacterial activity	(Murei et al., 2021)
AuNPs	*Sterculia setigera* bark extract	–	Antitrypanosomal activity	(Abdulrazaq et al., 2021)
AuNPs	*Withania somnifera* extract	20	Enhanced anticancer activity	(Tabassam et al., 2020)
AuNPs conjugated with PLA-PEG-PLA copolymer	*Leucas aspera* extract	25	Antiinflammatory activity	(Reena et al., 2016)
ZnONPs	*Geranium wallichianum* leaf extract	18	Anticancer, antileishmanial, antibacterial, antifungal, and antioxidant activity	(Abbasi et al., 2020)
ZnONPs	*Aloe barbadensis* Miller leaf extract	14	Enhanced antibacterial activity and intracellular ROS production	(Ali et al., 2016)
ZnONPs	*Pterocarpus santalinus* extract	–	Enhanced antidiabetic activity	(Kitture et al., 2015b)

TABLE 6.1 Applications of plant extract nanoconjugates.—cont'd

Nanomaterial	Plant component	Size of nano-conjugate (nm)	Application	References
Fe_3O_4NPs	Curcumin	15–20	Enhanced antioxidant activity and tumor suppression	(Kitture et al., 2012)
Fe_3O_4NPs	Diosgenin	19–21	Enhanced anticancer activity	(Ghosh et al., 2015a,b)

the maximum encapsulation efficiency and effective particle size of 83.90 ± 0.53% and 205.1 ± 0.1 nm, respectively. Thereafter, the antimicrobial activity of the nanospheres encapsulating 200 mg/L of PPE was evaluated which showed enhanced inhibition against *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* sp. The antimicrobial activity of the nanoencapsulated plant extract was investigated by coating the nanoconjugate on chicken meat samples. The total microbial count was significantly less after 14 days of storage at 4°C as compared to the control sample. Therefore, this study highlighted the potential application of plant extract nanoconjugates as preservatives that can inhibit the growth of psychrophilic microbes.

Han et al. (2015) reported the nanoencapsulation of *Zizyphus jujuba* pulp and seed extract for enhanced antioxidant activity. The plant extracts were encapsulated in chitosan nanoparticles wherein sodium tripolyphosphate (STPP) was added to facilitate the nanoencapsulation. The size of the extract containing nanoparticles ranged from 130 to 270 nm which increased with subsequent increase in the chitosan/tripolyphosphate (TPP) ratio. Likewise, the zeta potential of nanoconjugate also increased with an increase in the ratio of chitosan and TPP. Moreover, a chitosan/TPP ratio of 3.0 and 8.5 mg/mL of pulp and seed extract resulted in more than 90% entrapment efficiency. The spherical morphology of the plant extract nanoconjugates was revealed by transmission electron microscope (TEM) images with a size range of 70–300 nm. Thereafter, the antioxidant activity of the encapsulated plant extracts was investigated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ABTS) radical-scavenging activity which highlighted the enhanced stability of the antioxidant activity of nanoconjugate as compared to the free plant extract. In addition, the total phenolic content of the plant extract was also enhanced after nanoencapsulation.

Similarly, Roshanpour et al. (2021) reported the enhanced antioxidant activity of *Mentha piperita* extract after nanoencapsulation. A double nanoemulsion of water-oil-water was prepared wherein an initial water-in-oil microemulsion was prepared using *M. piperita* phenolic extract and soybean oil followed by coating the microemulsion with biopolymers composed of chitosan, *Alyssum homolocarpum* gum (AHG), and a 1:1 complex of chitosan and AHG (CCA), respectively. The size distribution of the nanoemulsion prepared was dependent upon the pressure of the homogenizer wherein higher pressure resulted in the formation of larger droplets because of droplet re-coagulation. The lowest average harmonic diameter of the nanoemulsions prepared using chitosan, CCA, and AHG biopolymers were 108.66, 65.18, and 70.81 nm, respectively. The polydispersity index (PDI) of the nanoemulsions prepared using AHG and CCA biopolymers was less than 0.5 which highlighted the uniform size distribution of the particles. Additionally, the zeta potential of the chitosan, CCA, and AHG-coated nanoemulsions was 28.69, 20.16, and −37.4 mV, respectively. The initial encapsulation efficiency of chitosan, CCA, and AHG-coated nanoemulsions was 80.5%, 90.8%, and 88.7%, respectively which eventually reduced to 24.3%, 19.9%, and 23%, respectively after 24 days of storage at 30°C. The longest half-life period of 63 days was obtained for the CCA nanoencapsulated powder. Thereafter, the peroxide value of nanoencapsulated phenolic extracts of *M. piperita* was evaluated which showed improvement in the oxidative stability of soybean oil after nanoencapsulation with maximum antioxidant activity observed in CCA coatings. Likewise, the *p*-anisidine release value was maximum in CCA-coated nanoemulsions which were secondary oxidation indicator. The release of phenolic compounds from the nanoencapsulated extracts into soybean oil was maximum in CCA-coated extracts when stored at 60°C for 24 days.

In another recent study, Soltanzadeh et al. (2021) encapsulated *Punica granatum* L. peel extract (PPE) in chitosan nanoparticles (CSNPs) and evaluated its antioxidant as well as antimicrobial properties. An ionic gelation technique was carried out for the synthesis of CSNPs using STPP as the cross-linking agent. The zeta potential of CSNPs was reduced from 26.5 to 2.95 mV after PPE encapsulation at chitosan:PPE ratio of 1:0.5 which indicated a decrease in the physical stability of the nanomaterial. Moreover, the highest encapsulation efficiency of 69.7 ± 1.05% was achieved at chitosan:PPE ratio of 1:0.25 along with a PDI and loading capacity of 0.260 ± 0.015 and 13.8 ± 0.15%, respectively. Additionally, the loading capacity of CSNPs increased with a subsequent increase in PPE concentration. Scanning electron

microscope (SEM) images then revealed the spherical shape of the CSNPs that were homogenously distributed without aggregation as evident from Fig. 6.1. The average size of the CSNPs increased from 90.6 to 127.3 nm after PPE encapsulation. The total phenolic content of the PPE-loaded CSNPs was lower as compared to the pure PPE. Thereafter, the ROS scavenging potential of the PPE-encapsulated CSNPs was evaluated by a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay which showed a 56% inhibition in the antioxidant activity as compared to 85% inhibition of pure PPE that was attributed to the protective effect of CSNPs. Later, the antimicrobial activity of the PPE-loaded CSNPs was investigated wherein no activity was observed against *E. coli* whereas the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the encapsulated nanomaterial for *S. aureus* were 1.09 and 2.19 mg/mL, respectively. Thus, this study exhibited the protective function of CSNPs on PPE after encapsulation which could aid in maintaining important activities of the plant extract.

Aqueous extracts of *Centella asiatica* L. were also nanoencapsulated by Kwon et al. (2012) for the improvement of its skin-protective activities. Gelatin was used as the edible polymer for the nanoencapsulation process wherein TEM images revealed the successful formation of liposomes that were spherical in shape with an average diameter of 115 nm. The PDI of the nanoparticles ranged from 0.15 to 0.19 along with the highest asiaticoside content of 48.9 ppm which was quite similar to the free aqueous extract of *C. asiatica* L. The cytotoxicity of the nanoformulation was evaluated on human skin fibroblasts which showed lower cytotoxicity (10%) of the nanocomplex as compared to 15% toxicity of crude plant extracts. The treatment of UV-irradiated cells with 0.5 mg/mL of the plant extract containing gelatin nanoparticles resulted in a significant reduction in the expression of matrix metalloproteinase (MMP)-1 and hyaluronidase. Further in vivo studies of the plant extract-loaded nanocomplex was carried out on hairless mouse models which showed a twofold increase in the flux of the plant extract on mice skin cells as compared to crude extract. The confocal laser-scanning microscopy results showed a homogenous distribution of the nano-encapsulated extract throughout the dermis which further facilitated the sustained release of the active compounds.

In another study, Kitture et al. (2015a) reported the encapsulation of *curcumin* extracted from *Curcuma longa* in an *Aloe vera* gel template. The weight loss curve of the *curcumin*-loaded- *Aloe vera* (CLA) structures showed 15% entrapment of the bioactive compound. The UV-Vis spectroscopic results demonstrated two absorbance peaks at 256 and 423 nm corresponding to the *Aloin* present in the *Aloe vera* gel and *curcumin*, respectively. Atomic force microscopy (AFM) results then demonstrated the porous nature of CLA structures with a base size and depth of around 50−100 and 60 nm, respectively. The antioxidant assays of the CLA structures were then performed wherein 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and hydroxyl radical scavenging activity of the nanocomplex was investigated. Almost 86.30% DPPH radical scavenging activity and 91.38% hydroxyl radical scavenging activity was obtained which was higher than the radical scavenging ability of *curcumin* and *Aloe vera* extracts. The nitric oxide scavenging activity was also enhanced up to 61.17%, whereas superoxide radical scavenging activity of CLA structures was 35.05% which was lower as compared to 57.91% activity of *Aloe vera* extract alone. Thereafter, the in vitro trans-membrane release study of CLA structure revealed a steady increase in the release of *curcumin* with a subsequent increase in the incubation time. Almost 10.24 ± 0.12 μg/mL of *curcumin* was released from CLA structures within 5 h. Hence, the efficient drug delivery ability of the nanoencapsulated structures was evident in this study.

Similarly, Păvăloiu et al. (2021) recently reported the liposome encapsulation of polyphenols from *Lycium barbarum* leaf extract. A hydration film method in combination with sonication and extrusion was performed for the encapsulation of *L. barbarum* extract into liposomes. The highest entrapment efficiency of $84.60 \pm 2.230\%$ was achieved along with PDI and particle size of 0.187 ± 0.001 and 141.6 ± 2.360 nm, respectively. The entrapment efficiency was reduced to $79.85 \pm 1.030\%$ after 3 months of storage at 4°C under dark conditions which suppressed photooxidation and hydrolysis of lipids and maintained the stability of the liposomes. A pH of 7.4 demonstrated a slower polyphenol release as compared to the attenuated burst effect of the free plant extract. The cytoprotective effect of *L. barbarum* leaf extract-loaded liposomes was also confirmed after 24 h of nanoconjugate treatment against peroxide-mediated cytotoxicity on mouse fibroblast cells as observed in Fig. 6.2.

The antiulcerogenic activity of *Passiflora serratodigitata* L. extracts was evaluated by Strasser et al. (2014) after nanoencapsulation. The ethyl acetate fraction (EAF) and dry crude (DC) extracts of *Passiflora serratodigitata* L. leaves and stems were nanoencapsulated by a solvent displacement technique. The nanoparticles prepared in this study were biodegradable and composed of poly(epsilon-caprolactone) (PCL) wherein the diameters of nanoencapsulated DC (NDC) and nanoencapsulated EAF (NEAF) were 379.2 ± 16.4 and 383.8 ± 18.2 nm, respectively. In addition, the zeta potential of NDC and NEAF was -20.2 ± 1.8 and -27.3 ± 1.1 mV, respectively. Furthermore, the entrapment efficiency of NDC and NEAF was $90.6 \pm 2.5\%$ and $79.9 \pm 2.7\%$ (w/v), respectively. Thereafter, the antiulcerogenic activity of the nanoencapsulated plant extracts was studied in ethanol-induced ulcer rodent models wherein the NDC treatment showed a fourfold reduction in lesion area as compared to DCE administration. Likewise, NEAF showed a 10-fold increase in

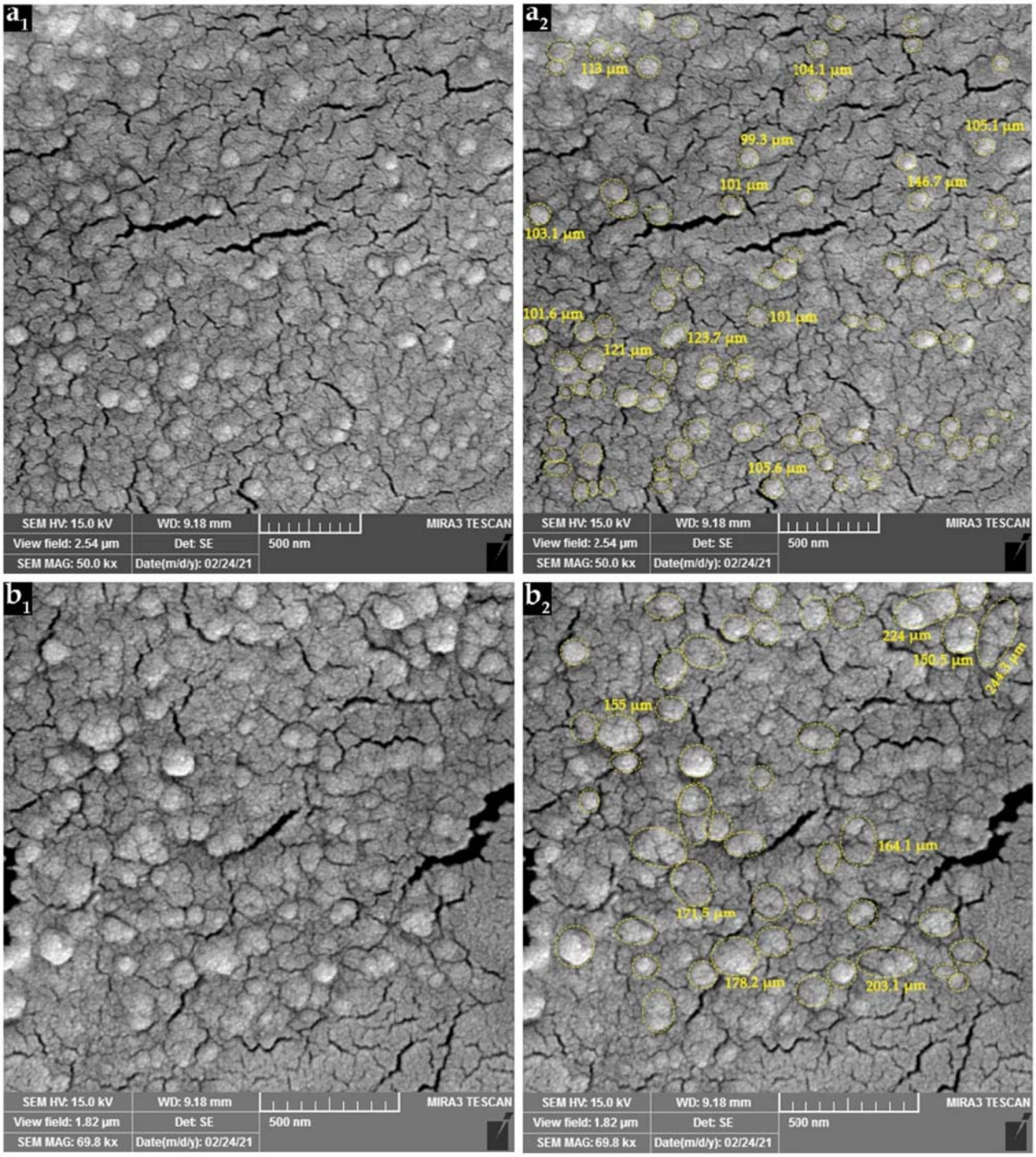

FIGURE 6.1 SEM image of (a_1) empty CSNPs, and (b_1) PPE-loaded CSNPs at chitosan:PPE ratio of 1:0.50 (w/w). Images (a_2, b_2) represent processes figures using ImageJ. *Reprinted from Soltanzadeh M, Peighambardoust SH, Ghanbarzadeh B, Mohammadi M, Lorenzo JM: Chitosan nanoparticles as a promising nanomaterial for encapsulation of pomegranate (Punica granatum L.) peel extract as a natural source of antioxidants,* Nanomaterials *11(6):1439, 2021.*

antiulcerogenic activity as compared to similar concentrations of EAF. Thus, it was proposed that nanoencapsulation of the plant extract must have increased its solubility and dispersive properties in the intestinal area of the animal models.

Pereira et al. (2018) also reported the nanoencapsulation of *Campomanesia xanthocarpa* O. Berg. (guabiroba) fruit extract for enhanced antioxidant and antimicrobial activities. Poly (D, L-lactic-co-glycolic) acid (PLGA) nanoparticles

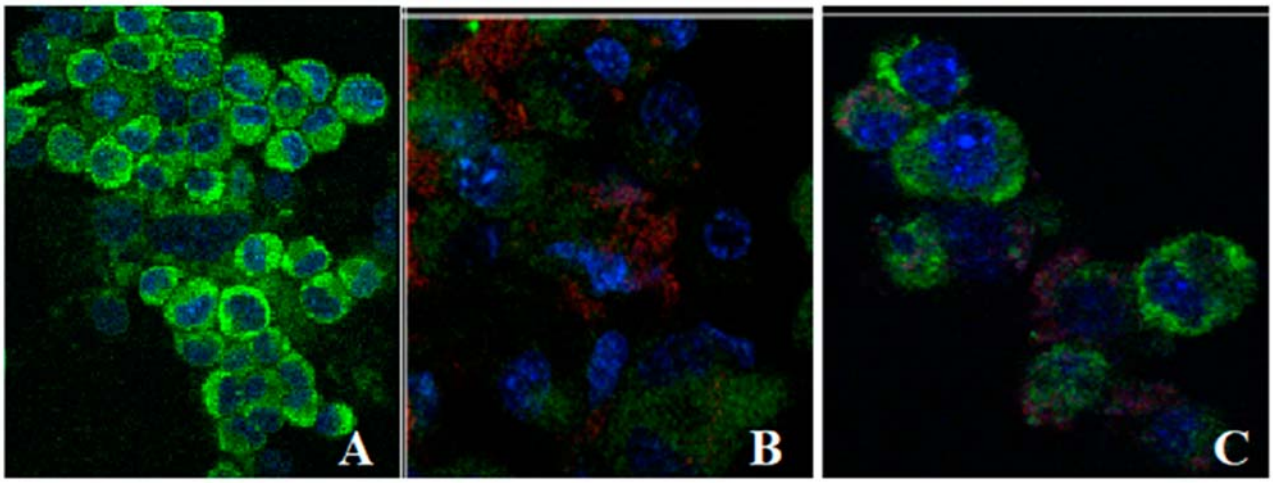

FIGURE 6.2 Confocal microscopy images of the uptake of liposomes loaded with *L. barbarum* by L-929 cells. (A) Control without liposomes; (B) cells treated with L1_LB; (C) cells treated with L2_LB; the liposomes are labeled with rhodamine B (red); cell membrane is stained with WGA-AlexaFluor 488 conjugated (green); cell nuclei are stained with hoechst (blue). For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article. *Reprinted from Păvăloiu RD, Sha'at F, Neagu G, Deaconu M, Bubueanu C, Albulescu A, Sha'at M, Hlevca C: Encapsulation of polyphenols from* Lycium barbarum *leaves into liposomes as a strategy to improve their delivery,* Nanomaterials *11(8):1938, 2021.*

were prepared using an adapted emulsion-evaporation technique. The obtained nanoparticles were then used for the encapsulation of phenolic guabiroba fruit extracts wherein the highest entrapment efficiency of 39.2 ± 1.0% was obtained with a 50:50 ratio of lactic to glycolic acid. The size and PDI of the nano-encapsulated extract were 202.5 ± 50.8 nm and 0.37 ± 0.04, respectively. TEM images of the nanoparticles further demonstrated their spherical morphology. An initial burst effect was observed for the plant extract-loaded PLGA nanoparticles with a 57% release of phenolic compounds in the first 15 min which was then gradually reduced to 32% after 12 h. A higher antioxidant activity of the nanocomplex was observed as compared to the free plant extract wherein the oxygen radical absorbance capacities of the nanocomplex and free plant extract were 174.7 ± 20.0 and 229.0 ± 15.6 μmol/L Trolox equivalent/g, respectively. A 10-fold lower concentration of the plant extract-loaded PLGA nanoparticles as compared to the free plant extract was required for a significant reduction in the ROS generation in noncancer human colon cells (CCD-18Co) which highlighted higher ROS inhibition of the guabiroba fruit extract after nanoencapsulation. The antimicrobial activity of the guabiroba fruit extract-loaded PLGA nanoparticles was investigated which showed a lower MIC of 2670 μg/mL for *Listeria innocua* as compared to a MIC value of 8107 μg/mL by the free plant extract.

Nasturtium officinale extract (NOE) was also nanoencapsulated in PLGA/PEG nanoparticles by Adlravan et al. (2021). A ring-opening technique was followed for the synthesis of a triblock copolymer composed of PLGA/PEG which was then used for an oil-in-water single emulsion solvent evaporation process. The prepared PLGA/PEG nanoparticles were then used for the encapsulation of NOE. Encapsulation efficiency of 83.5% was achieved along with a loading capacity of 12.6 ± 2.5 wherein the shape of the particles was spherical having a core-shell structure as observed in field emission scanning electron microscopy (FESEM) images. The average diameter of the particles was 205 nm while the zeta potential was −7.35 ± 2.4 mV. SEM images confirmed the homogenous distribution of the particles in the medium. The in vitro release kinetics of NOE from the nanocomposite were then evaluated wherein 74% of the plant extract was released at a pH value of 7.4 within 120 h of incubation whereas more than 90% of the extract was released at a 5.5 pH value. MTT assay then demonstrated the in vitro cytotoxicity of the nanoencapsulated complex against A549 lung cancer cells in which the IC_{50} value was 35.0 μg/mL after 72 h of exposure. Moreover, the viability of A549 cells was investigated using 4′,6-diamidino-2-phenylindole (DAPI) staining wherein the cells treated with NOE-loaded PLGA/PEG nanoparticles showed brighter and smaller nuclei with discrete globular structures that resembled apoptotic bodies as compared to cells observed after treatment with free plant extract. Additionally, the deoxyribonucleic acid (DNA) content of the cells was measured after treatment with 55 μM of NOE-loaded PLGA/PEG nanoparticles for 48 h. All the treated cells were arrested at the synthesis phase of the cell cycle which suggested that the nanoencapsulated plant extract might behave as a DNA synthase inhibitor. The expression of apoptotic markers such as p53, Bax, and Caspase-3 was also evaluated by quantitative polymerase chain reaction (q-PCR) assay which showed significant overexpression of these protein markers by 1.3-,

1.1-, and 1.2-folds, respectively. Moreover, the reduction in expression of bcl-2 and CyclinD1 was also observed after the treatment with the nanoparticle-plant extract complex.

Zhang et al. (2013) reported the preparation of solid lipid nanoparticles loaded with triptolide which could be delivered orally for the reduction of gastric irritation. A microemulsion process was carried out for the preparation of the nanoconjugate wherein an oil-in-water microemulsion was prepared which was then added to cold water for the precipitating lipid phase forming fine particles. A five-level central composite design (CCD) was employed for the evaluation of the optimized particle size, encapsulation efficiency, and triptolide loading capacity of the nanocomposite. Hence, the optimized nanoconjugate prepared had a lipid fraction along with a surfactant to the co-surfactant ratio of 49.73% and 3.25, respectively. The optimal lipid-to-triptolide ratio was 55.27 which resulted in a particle size of 179.8 ± 5.7 nm. Also, the optimal encapsulation efficiency and triptolide loading capacity of the prepared nanocomposite was $56.5 \pm 0.18\%$ and $1.02 \pm 0.003\%$, respectively. The in vitro release of triptolide was also investigated wherein the nanoconjugate revealed a sustained triptolide release which was 1.68-fold slower as compared to the control suspension of triptolide that completely released the phytoactive compound within 2 h. Thereafter, the ability of the triptolide-loaded nanoparticle to reduce gastric mucosa irritation was evaluated on Sprague Dawley rat models. Myeloperoxidase levels were quantified in rats after treatment since it is a known facilitator of neutrophil infiltration in the gastric mucosal tissues. The levels of myeloperoxidase were considerably decreased in rat models that were orally treated with the triptolide-loaded nanoconjugate as compared to rats treated with triptolide suspension through intraperitoneal injection. No inflammation was observed after oral administration of the nanoconjugate as compared to triptolide suspension. Therefore, this study highlighted the reduction of triptolide toxicity after nano-encapsulation.

Metal-based nanomaterials

Several metal nanoparticles are used for functionalizing bioactive principles and/or crude phytochemicals from medicinal plants for multiple applications. Silver nanoparticles (AgNPs) were conjugated with *Pyrenacantha grandiflora* Baill extracts by Murei et al. (2021) for enhanced antibacterial activity. A chemical reduction method was followed for the preparation of AgNPs using 1 mM of $AgNO_3$ and 1% tri-sodium citrate. TEM images of the obtained AgNPs showed their quasi-spherical morphology with an average size of 13 nm. In addition, biogenic AgNPs were also synthesized using *Magnetospirillum magnetotacticum* bacterial strain which had spherical morphology and a size range of 3−25 nm as seen in Fig. 6.3. Thereafter, *P. grandiflora* tuber extracts prepared in methanol, water, and acetone were conjugated with the AgNPs where the successful conjugation was confirmed by FTIR analysis that revealed the presence of −OH, C=C, C−H, and C=O groups of the plant extract. Next, the antimicrobial activity of biogenic AgNPs conjugated with plant extract was evaluated wherein acetone plant extract nanoconjugate exhibited considerable growth inhibition against methicillin-resistant *Staphylococcus aureus* (MRSA) with an inhibition zone of 24 mm. Likewise, chemically synthesized AgNPs also showed effective antibacterial activity against *E. coli* and *S. aureus* after conjugation with aqueous extracts of *P. grandiflora* tubers. Moreover, the MBC of 0.05 mg/mL was obtained when AgNPs were conjugated with acetone plant extracts.

In another study, Abdulrazaq et al. (2021) reported the conjugation of *Sterculia setigera* bark extracts with AuNPs and investigated its biological activity. AuNPs were synthesized using the bark extracts as a reducing agent wherein the AuNPs demonstrated a strong SPR absorption signal at 531 nm. The high-resolution TEM (HRTEM) images demonstrated the spherical morphology of the biogenic AuNPs. Thereafter, the prepared AuNPs were conjugated with powdered *S. setigera* bark extract to obtain a nanoconjugate which was further functionalized by PEG and diminazene aceturate. The antitrypanosomal activity of the nanoconjugates was then evaluated in infected mice wherein a considerable decrease in parasitemia was observed after 6 days of treatment with the plant extract-AuNPs conjugate.

In another similar study, AuNPs were conjugated with withanolide-A obtained from *Withania somnifera* (Tabassam et al., 2020). A chemical synthesis approach was carried out for the preparation of AuNPs. The obtained AuNPs were spherical in shape with a characteristic SPR peak at 523.5 nm. The conjugation of AuNPs with 10 μg/mL of withanolide-A resulted in a slight change in the SPR peak to 525.5 nm. TEM images showed an average particle size of 20 nm with uniform distribution of the particles wherein the size of AuNPs was not altered after phytoconjugation. Later, the cytotoxicity of the obtained phyto-nanoconjugate was determined against breast cancer cell lines (SKBR3) using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A dose-dependent cytotoxic activity was observed wherein high antiproliferative activity was facilitated by the phytochemicals conjugated with the AuNPs. The growth of SKBR3 cancer cells was inhibited at a half-maximal concentration of phyto-nanoconjugate as compared to free withanolide-A.

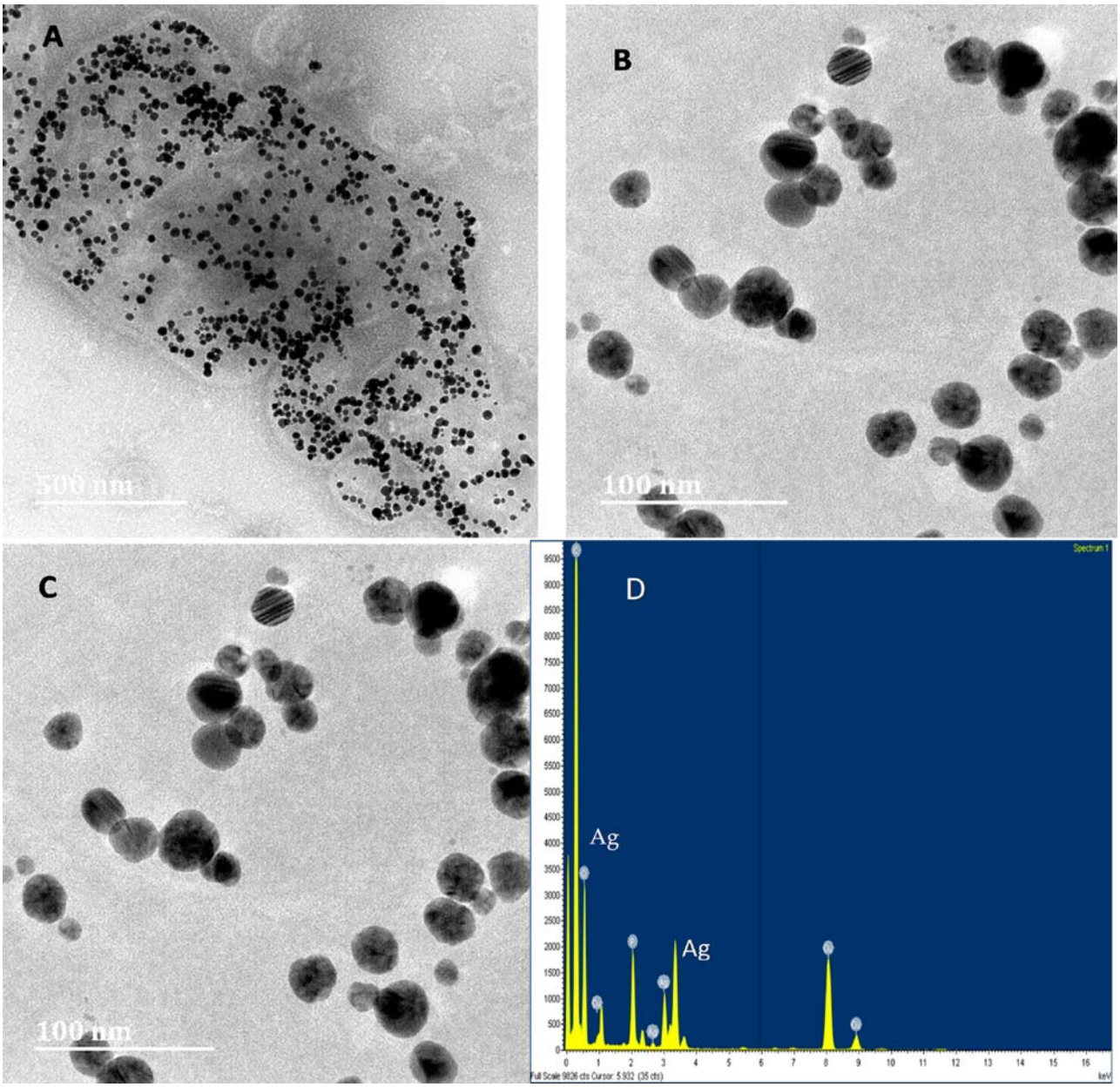

FIGURE 6.3 Analysis of biological synthesis of silver nanoparticles. TEM images of *Magnetospirillum magnetotacticum* with silver nanoparticles (A), silver nanoparticles (B), lattice fringes (C), and EDX pattern (D). *Reprinted from Murei A, Pillay K, Govender P, Thovhogi N, Gitari WM, Samie A: Synthesis, characterization, and in vitro antibacterial evaluation of* Pyrenacantha grandiflora *conjugated silver nanoparticles,* Nanomaterials *11:1568, 2021.*

In another study, Reena et al. (2016) prepared biogenic gold nanoparticles (AuNPs) that were loaded with *Leucas aspera* extract. A water-in-oil emulsion method was carried out for the preparation of AuNPs using the *Leucas aspera* plant extract along with a poly lactic acid-co-poly ethylene glycol-co-poly lactic acid (PLA-PEG-PLA) copolymer. The PLA-PEG-PLA copolymer was prepared by the ring opening polymerization of D, L-lactide through supplementation of PEG-1500 (PEG1500) which had an inherent viscosity of 0.24 dL/g. The plant extract loaded AuNPs nanoconjugate was synthesized using an ultrasonication-induced water-in-oil emulsion technique wherein the copolymer and plant extract were dissolved in chloroform and ethanol, respectively while the auric chloride was dispersed in water. The ultraviolet-visible (UV-vis) absorption spectra of the plant extract-loaded gold nanoconjugate demonstrated surface plasmon resonance (SPR) at 540 nm along with π-π^* transition of the plant extract at 260 nm. The X-ray diffraction (XRD) pattern of the nanoconjugate highlighted its crystallinity while TEM images showed its spherical morphology with an average

particle size of 25 nm. In vitro cytotoxicity of the nanoconjugate was analyzed against the Vero cell line of South African green monkey's kidney cells wherein more than 97% cell viability was observed in the presence of 25 μg/mL of plant extract loaded gold nanoconjugate. The antiinflammatory activity of the nanoconjugate was evaluated in which the antiproteinase activity of the plant extract-loaded gold nanoconjugate increased with a concomitant increase in the copolymer concentration. The antiinflammatory activity of the AuNPs was increased only after the encapsulation of *Leucas aspera* extract.

Geranium wallichianum leaf extracts were also conjugated with zinc oxide nanoparticles (ZnONPs) by Abbasi et al. (2020) for improvement in its bioactivity. ZnONPs were prepared by the reduction of $Zn(NO_3)_2 \cdot 6H_2O$ using *G. wallichianum* leaf extract which showed SPR at 398 nm. TEM images, as seen in Fig. 6.4, further revealed the hexagonal morphology of the ZnONPs along with an average size of 18 nm while the zeta potential and PDI were −8.53 mV and 0.232, respectively. Moreover, Raman and Fourier transform infrared (FTIR) spectroscopy analyses indicated the involvement of *G. wallichianum* leaf extract in preventing agglomeration of the ZnONPs that were present on the surface of the particles. The inhibition concentration-50 (IC_{50}) of the *G. wallichianum* leaf extract-ZnONP conjugate was 39.26 μg/mL against liver cancer (HepG2) cells. Likewise, the antileishmanial property of the ZnONPs against *Leishmania tropica* showed an IC_{50} of 15.60 μg/mL. Furthermore, the antibacterial activity of the biogenic ZnONPs-plant extract conjugate was investigated wherein considerable activity was observed against a wide range of Gram-positive and negative bacterial strains such as *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*. Antifungal activity of the biogenic nanoconjugate was also evident against *Aspergillus flavus*, *Aspergillus niger*, *Fusarium solani*, and *Mucor racemosus*. The antioxidant ability of ZnONPs was also evaluated, wherein the total antioxidant capacity was 52.43% at a concentration of 200 μg/mL of the biogenic nanoparticles.

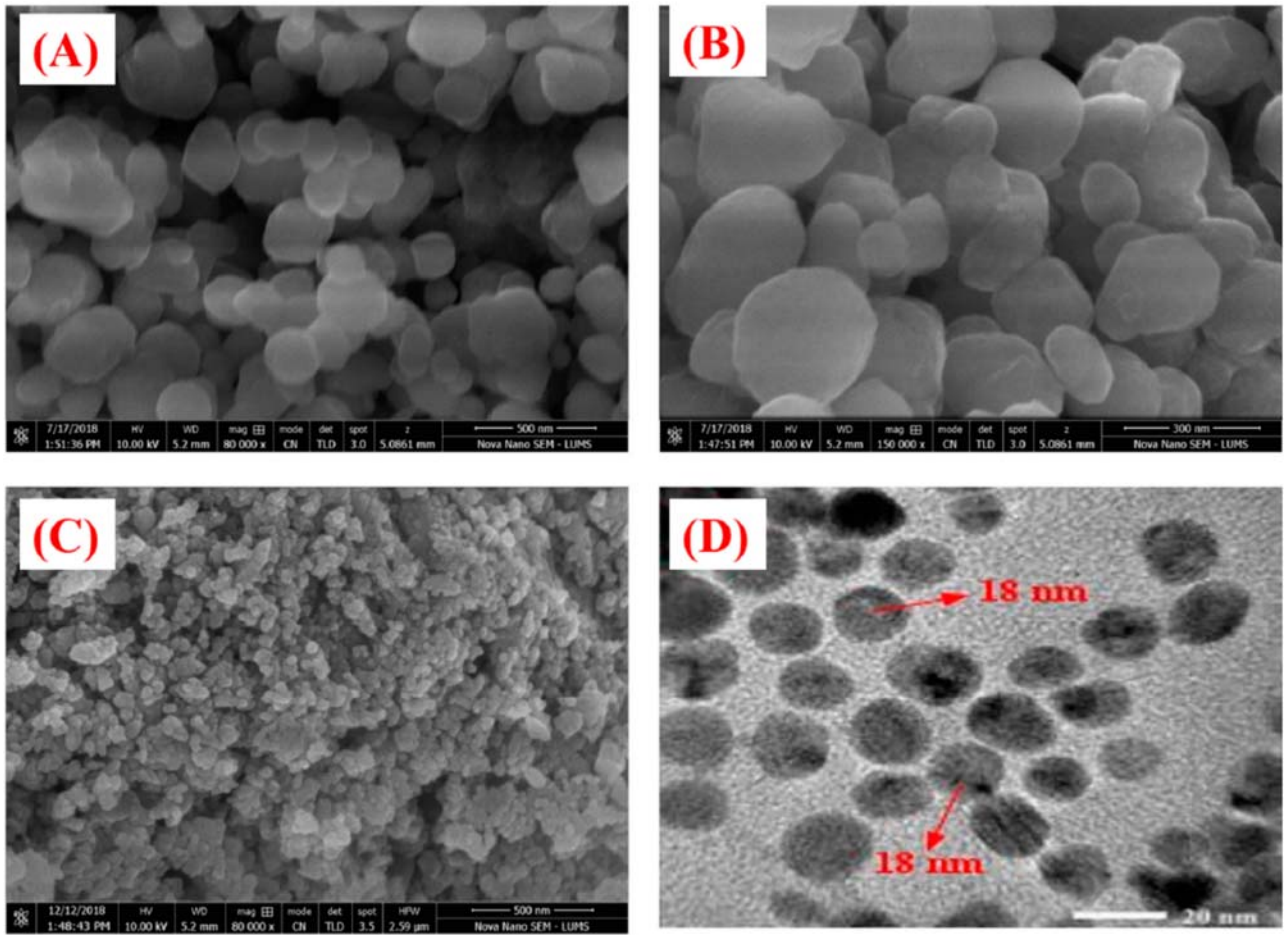

FIGURE 6.4 SEM and TEM images of *Geranium wallichianum* mediated ZnONPs using zinc nitrate as a precursor (A−C) HR-SEM images (D) TEM image. *Reprinted from Abbasi BA, Iqbal J, Ahmad R, Zia L, Kanwal S, Mahmood T, Wang C, Chen JT: Bioactivities of* Geranium wallichianum *leaf extracts conjugated with zinc oxide nanoparticles,* Biomolecules *10:38, 2020.*

Ali et al. (2016) also prepared surface functionalized ZnONPs for targeting multi-drug resistant bacteria. ZnONPs were biologically synthesized and capped by *Aloe barbadensis* Miller leaf extract (ALE) which showed a characteristic UV-vis absorption peak at 375 nm. XRD pattern of the obtained ALE-capped ZnONPs showed its crystalline nature whereas SEM and TEM images demonstrated its pleomorphic shapes some of which were spherical, oval, and hexagonal with an average size of 14 nm. Later, the antibacterial and antibiofilm activity of the capped ZnONPs was investigated against extended-spectrum beta-lactamase (ESBL) producing as well as methicillin-sensitive and resistant bacteria. The MIC and MBC values of ALE-capped ZnONPs against ESBL-producing *E. coli* and *P. aeruginosa* were 2200 and 2400 μg/mL and 2300 and 2700 μg/mL, respectively. Similarly, the MIC and MBC values for ALE-capped ZnONPs against methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) were 2000 and 2200 μg/mL, and 2200 2400 μg/mL, respectively. The SEM images of the bacterial strains after treatment with plant extract capped-ZnONPs revealed cellular damage. The biofilm-forming ability of ESBL-producing *E. coli*, *P. aeruginosa* and MRSA-1 were inhibited by $21.9 \pm 3.0\%$, $60.5 \pm 5.0\%$, and $5.3 \pm 3.0\%$, respectively. In addition, the production of extracellular polysaccharides by ESBL-producing bacteria was also reduced after nanoconjugate treatment. Flow cytometry results further confirmed the internalization of ALE-capped ZnONPs in both Gram-negative and Gram-positive bacteria with a 9.7- and 1.9-fold increase in the granularity of *E. coli* and MRSA cells, respectively. Moreover, a reactive oxygen species (ROS) fluorescent dye indicator dichlorodihydrofluorescein diacetate (DCFH-DA) was used for the detection of ROS generation in the bacterial cells after treatment with ALE-capped ZnONPs. The presence of 250−2000 μg/mL of ALE-capped ZnONPs resulted in the production of 102 ± 4−$358 \pm 8\%$, 118 ± 8−$256 \pm 6\%$, and 100.8 ± 6−$137 \pm 6\%$ of ROS in *E. coli*, *P. aeruginosa*, and *S. aureus*, respectively.

In another interesting study, Kitture et al. (2015b) prepared zinc oxide nanoparticles (ZnONPs) *Pterocarpus santalinus* (red sandalwood) conjugate and evaluated its antidiabetic properties. Zinc acetate was used for the preparation of ZnONPs which were then conjugated with the plant extract. Field-emission scanning electron microscopy (FE-SEM) images of ZnONPs revealed their monodispersed nature with an average size of 20 nm whereas X-ray diffraction (XRD) studies confirmed the wurtzite phase of the ZnONPs. Ultraviolet-visible (UV-Vis) spectral analysis then revealed the presence of characteristic absorbance peaks at 380 and 197 nm in the nanoconjugate corresponding to ZnONPs and the plant extract, respectively. Around 65% loading efficiency was observed for the plant extract onto the surface of ZnONPs. The antidiabetic activity of the nanoconjugate was investigated wherein the nanoconjugate showed 20% inhibition of α-amylase as compared to 83.7% inhibition by acarbose which was used as the positive control. The ZnONPs-red sandalwood conjugate exhibited 61.93% inhibition of murine pancreatic glucosidase activity as compared to 21.48% and 5.90% inhibition by ZnONPs and crude plant extract, respectively.

In a similar study, Kitture et al. (2012) conjugated *curcumin* with iron oxide nanoparticles (Fe_3O_4NPs) and explored its radical scavenging activity, tumor suppression ability, and cancer hyperthermia. A reverse coprecipitation method was employed for the synthesis of Fe_3O_4NPs that were then conjugated with *curcumin* using a citrate linker. TEM images of the Fe_3O_4NPs conjugate revealed monodispersed nanoparticle with size ranging from 15 to 20 nm. The XRD analysis confirmed their crystalline behavior. The magnetic properties of the nanoconjugate were studied using a vibrating-sample magnetometer which showed a saturation magnetization value of 60 emu/g that was higher than Fe_3O_4NPs. Thereafter, the tumor suppression activity of the surface functionalized nanoparticles was investigated wherein complete tumor suppression was obtained in the presence of 10 ppm of the nanoconjugate. Additionally, superior superoxide anion and superoxide radical scavenging highlighted the enhanced antioxidant activity of the nanoconjugate.

Diosgenin obtained from *Dioscorea bulbifera* was also used for the functionalization of iron oxide nanoparticles (Fe_3O_4NPs) by Ghosh et al. (2015b) that were then used as anticancer agent. The synthesized Fe_3O_4NPs capped with citrate were functionalized with diosgenin. The functionalized Fe_3O_4NPs were monodispersed with a size range of 19−21 nm. Diosgenin functionalized Fe_3O_4NPs demonstrated $51.08 \pm 0.37\%$ antiproliferative activity against human breast cancer cells (MCF-7) which was higher as compared to citrate-capped Fe_3O_4NPs and diosgenin alone. The migration of MCF-7 cells was also inhibited by $40.83 \pm 2.91\%$ after treatment with Fe_3O_4NPs-diosgenin conjugate, whereas untreated control cells demonstrated $91.53 \pm 2.44\%$ motility. The nanoconjugates showed apoptosis induction wherein phosphatidyl serine from the inner leaflet of the cell membrane was translocated to the outer membrane. Hence, it was proposed that the decrease in cell viability after nanoconjugate treatment was a result of apoptotic induction. Further analysis revealed the specific cleavage of poly-ADP ribose polymerase (PADP) after nanoconjugate treatment which highlighted the activity of Fe_3O_4NPs-diosgenin conjugate as an effective apoptotic inducer.

Concluding remarks and future prospects

Medicinal plants are a reservoir of diverse phytochemicals such as alkaloids, coumarins, fatty acids, flavonoids, glycosides, naphthoquinones, saponins, steroids, and terpenoids with promising therapeutic activities (Bloch et al., 2022). However, often these phytochemicals are nonpolar and sparsely soluble in an aqueous medium. Hence, bioavailability is a major drawback for obtaining desired therapeutic activity. Hence, it is important to develop and design an efficient delivery vehicle for the effective transport of the bioactive principles to the target site followed by sustained release (Ghosh, 2019). Biogenic nanoparticles are more advantageous as carriers due to their biocompatibility and large surface area (Ghosh, 2018). Gold, silver, copper, platinum, palladium, and other nanoparticles synthesized by bacteria, fungi, algae, and plants can be functionalized with the plant extracts for anticancer and antimicrobial activities (Bloch et al., 2021; Nitnavare et al., 2022; Ghosh et al., 2018). Numerous plant extracts have shown promising potential as prebiotics that can promote the growth and activity of probiotics. The probiotic microflora has tremendous nanobiotechnological potential (Ghosh et al., 2022). Hence, nanoparticles synthesized by the probiotic microbes can be used as effective functionalization surfaces for the plant extracts. Certain plant compounds and nanoparticles can effectively adsorb hazardous dyes and/or heavy metals and convert them to nontoxic forms. Hence such plant extracts and nanomaterials can be combined together as conjugates for the enhancement of their activity (Luikham et al., 2018; Ghosh, 2020). Likewise, numerous bioactive principles from medicinal plants exhibit potent apoptotic induction in cancer cells. Conjugating these phytocompounds with magnetic nanoparticles may result in the magnetic field-mediated effective targeting of the composite to the cancer site (Ghosh et al., 2019). Biofilms in bacteria are resistant to conventional antibiotics and play a significant role in multidrug resistance. Plant extracts and nanoparticles with biofilm-inhibiting and eradicating effects should be conjugated for enhanced antibiofilm activity (Ranpariya et al., 2021). Further, antibiotics can be conjugated with these composite systems to synergistically increase the antimicrobial activity and spectrum of affectivity. Such strategies may prove to be extremely powerful for controlling nosocomial infections.

In view of the background, it can be concluded that plant extract-conjugated nanoparticles can help in synergistic augmentation of the therapeutic activity. This can result in the development of promising nanomedicine with enhanced penetrability, targeted delivery, sustained release, and simultaneous monitoring and/or bioimaging. However, thorough pharmacokinetic and pharmacodynamic studies should be carried out along with toxicity tests before they can be approved for entering mainstream therapeutics.

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Chapter 7

Biogenically efficient production and characterization of silver nanoparticles using the marine fungus *Hamigera terricola* along with their antimicrobial and antioxidative efficacy

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Introduction

Nanoparticles (NPs) are define as a material with a measurement lengthwise 1−1000 nm in at least one dimension; although, they are most usually characterized as possessing a diameter of 1−100 nm (Jeevanandam et al., 2018). There are three methods and two approaches for the synthesis of NPs; methods are chemical, physical, and biological, whereas approaches involve top to bottom and bottom to top (Ijaz et al., 2020; Baig et al., 2021). Chemical and physical methods are conventionally utilized methods for the synthesis of NPs; however, they are not reliable due to the use of toxic chemicals for the optimal size and shape of the NPs, being expensive, lots of energy and time consumption, and mostly not being environment friendly. As a substitute of aforementioned traditional methods, researchers are moving toward the use of the biological methods due to the higher bioactivity, stability, and eco-friendliness of NPs and the cost effectiveness of the method (Ahmad et al., 2017; Mohd Yusof et al., 2019; Shafey, 2020; Zahoor et al., 2021). Microorganisms (such as bacteria, fungi, algae, and archaea) and plants are often used as a source of synthesis in biological processes. Amid all the biological sources, fungi are the most suitable microorganism for the fabrication of NPs due to their sustainable properties toward the harsh fermentation conditions (e.g., aeration and agitation), ease to produce in bulk, and tolerate wide range of pH. Fungi specially isolated from extreme environmental niches (such as marine environment) tends to secrete biomolecules with higher bioactivities and stability at room temperature. Silver is the extensively used metal by mankind from the ancient time in various form due to its antimicrobial properties Likewise, silver NPs are also known and being the interest of topic due to their efficient antimicrobial, antioxidative, larvicidal, and anticancer potency. Earlier research studies reported that the genus like *Aspergillus*, *Trichoderma, Hamigera,* and *Fusarium* are known for the effective synthesis of AgNPs (Mistry et al., 2021; Thakor et al., 2022a; Mistry and Bariya, 2021).

In this study, biogenesis of AgNPs was carried out utilizing cell-free filtrate (CFF) of marine procured fungi *Hamigera terricola*. Mycosynthesized AgNPs were characterized using various spectroscopic and microscopic techniques such as UV-visible spectroscopy, Fourier transform infrared (FTIR), X-ray diffraction (XRD), and transmission electron microscopy (TEM). Efficacy of mycosynthesized AgNPs was also tested for their antioxidant capacity and antimicrobial activity (against three Gram-positive, three Gram-negative, and one plant pathogenic fungi).

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Experimental

Biogenic synthesis of AgNPs

Hamigera terricola (GenBank accession number: MT647133) was cultured in the potato dextrose broth under shaking condition (120 rpm) for 96 h at 28°C. The fungal biomass (6 g) was separated using Whatman filter paper no. 42, cleansed in sterilized double-distilled water, then again suspended in 100 mL of sterilized double-distilled water, and incubated at 60°C for 24 h in water bath. The biomass was again separated by Whatman filter paper no. 42, and the CFF was collected. The acquired CFF was subjected for the biogenesis of AgNPs. The reaction mixture utilized for the biosynthesis of AgNPs consisted of 10 mL CFF (pH-9) and 90 mL silver nitrate solution (10 mM $AgNO_3$, MW- 169.87, HiMedia). The reaction mixture was incubated at 60°C in the water bath under dark conditions to prevent any photochemical reaction (Mistry et al., 2021).

Characteristics analysis of AgNPs

The UV-visible spectrophotometer (Shimadzu, 1800 series) was employed to analyze the unique absorption spectrum of mycosynthesized AgNPs in the range between 300 and 700 nm. Band gap energy (Eg) of AgNPs was estimated by Tauc's plot. FTIR analysis was conducted to study the surface chemistry of the AgNPs in the range of 4000 cm^{-1} to 650 cm^{-1}. The XRD pattern of AgNPs was obtained using an XRD operated at 30 kV and 100 mA. The CuKα radiation with the wavelength of 1.5406 nm was used for obtaining the spectral scan in the 2θ range from 0 to 90 degrees. The surface morphology (e.g., size and shape) of the biosynthesized AgNPs was studied through TEM imaging (Thakor et al., 2022a).

Antioxidant activity of AgNPs

With a few minor alterations, the procedure described by Keshari et al. (2020) was used to evaluate the antioxidative potential of the synthesized AgNPs. The capability of AgNPs to scavenge the free radicals was determined by performing 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. 1 mL of freshly made DPPH (1 mM) solution was vortexed with varied concentrations of 1 mL AgNPs (10, 20, 30, 40, 50, 75, and 100 μg/mL). The solution was incubated at room temperature under dark conditions (for 30 min). At 517 nm, readings of the solution were recorded. Methanol served as a blank, and all the reagents together with DPPH were used as a control. The free radical scavenging activity was denoted as inhibition percentage, which was calculated by the following equation as determined below.

$$\text{Free radical scavenging activity } (\%) = \frac{P_c - P_s}{P_c} \times 100$$

Where, Pc = absorbance of control and Ps = absorbance of sample.

Antimicrobial activity of AgNPs

The representative microorganisms utilized for the evaluation of antimicrobial activity consisted of six bacterial strains viz. three Gram-positive bacteria specifically, *S. aureus*, *B. megaterium,* and *B. subtilis*, three Gram-negative bacteria explicitly *E. coli*, *P. aeruginosa,* and *S. typhi,* as well as *Fusarium oxysporum* (plant pathogenic fungi). The well diffusion method was employed to study the antimicrobial activity.

Antibacterial activity

For performing antibacterial activity, the bacterial strains were cultured in nutrient broth and incubated at 37°C for 24 h. The bacterial growth was adjusted to 0.5 according to McFarland standards. In laminar air flow, 100 μL bacterial suspension was spread on a nutrient agar plate. Each well (12 mm in diameter) was inoculated with 100 μL of synthesized AgNPs, 10 mM $AgNO_3$, and standard streptomycin (1 mg/mL). Distilled water and streptomycin were used as negative and positive controls, respectively. The zone of inhibition was measured using zone scale (HiMedia) after overnight incubation at 37°C.

Antifungal activity

The antifungal activity of the synthesized AgNPs was assessed against plant pathogenic fungi *Fusarium oxysporum*. 1×10^6 spores/mL spore suspension of *F. oxysporum* was adjusted in hemocytometer and spread on potato dextrose agar

plate (Thakor et al., 2022a). The sterilized cup borer was employed to punch the well of 12 mm diameter under aseptic conditions. Each well was filled with 100 μL of the synthesized AgNPs, $AgNO_3$ (10 mM), and cycloheximide (1 mg/mL) as an antifungal agent. Cycloheximide acts as a positive control. All the inoculated plates were incubated at 28°C in an upright position for 72 h. The zone of inhibition was measured utilizing zone scale (HiMedia) (Pawar and Patil, 2020).

Statistical analysis

Obtained raw data were processed and analyzed with IBM SPSS Statistics 24. All the experiments were performed in triplicates to acquire mean and standard error (SE) values. One-way analysis of variance (ANOVA) analysis was also performed to assess the significance level at $P < .05$ value. Graphical illustrations were produced in Origin 2018b (9.55) (Thakor et al., 2022b).

Experimental outcomes

Fig. 7.1 represents the morphologic and microscopic images of the fungi *Hamigera terricola,* which was obtained from the marine water sample (Diu, India). The reaction of the CFF of *H. terricola* and $AgNO_3$ solution culminated in an expeditious color change of the solution from colorless to yellow followed by brown at 24 h as the reaction continued.

UV–visible spectroscopic analysis

On the reduction of silver ions, AgNPs produce brown color that indicates the formation of AgNPs. This color change is due to the unique surface plasmon resonance (SPR). Stable and constant synthesis of AgNPs was monitored though UV-visible spectroscopy (Shimadzu, UV-1800 series). The peak for the synthesized AgNPs was found at 425 nm, which indicates the spherical and smaller size of NPs (Alsharif et al., 2020; Patel et al., 2020). Fig. 7.2A indicates that the concentration/synthesis of the AgNPs was increased with the reaction time. No blue or red shift has been observed even after 6 months of storage at room temperature proves that the AgNPs are highly stable. This stability was observed might due to the capping of a proteinaceous agents present into the CFF of the *H. terricola,* which prevents NPs to get aggregate (El-Naggar et al., 2018).

Band gap energy (eV) analysis

Band gap energy of AgNPs is depended on the size of the particles. Several studies reported that the band gap energy of NPs is inversely proportional to the size of the particles, which means smaller size of the particles lead to the higher band gap energy. Band gap energy of AgNPs was estimated from the Tauc's plot by extrapolating the linear portion of the UV-visible curve. Fig. 7.2B indicates that the mycosynthesized AgNPs possess the value of band gap energy of about 2.18 eV. Large value of band gap energy pursuing AgNPs can be additionally used in optoelectronic devices, sensors, and batteries as a semi-conductive material. The value of band gap is much similar to earlier reported literature, and this value could be due to quantum confinement effect (Mistry et al., 2021; Thakor et al., 2022a; Das et al., 2016).

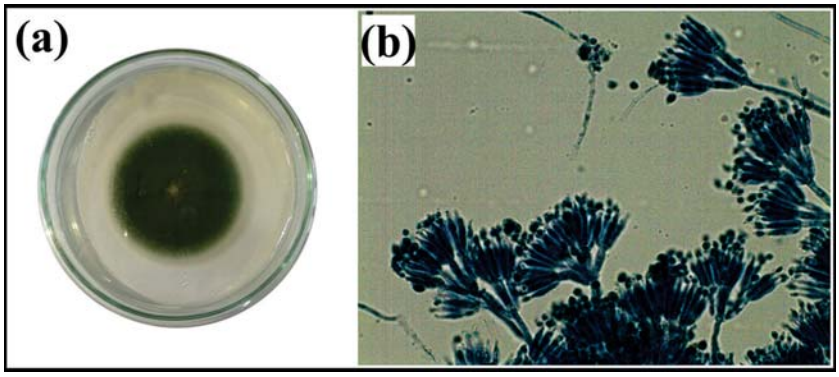

FIGURE 7.1 Hamigera terricola grown in petri plate with growth medium (A) along with its specific spore structure (B) observed under microscope (40X).

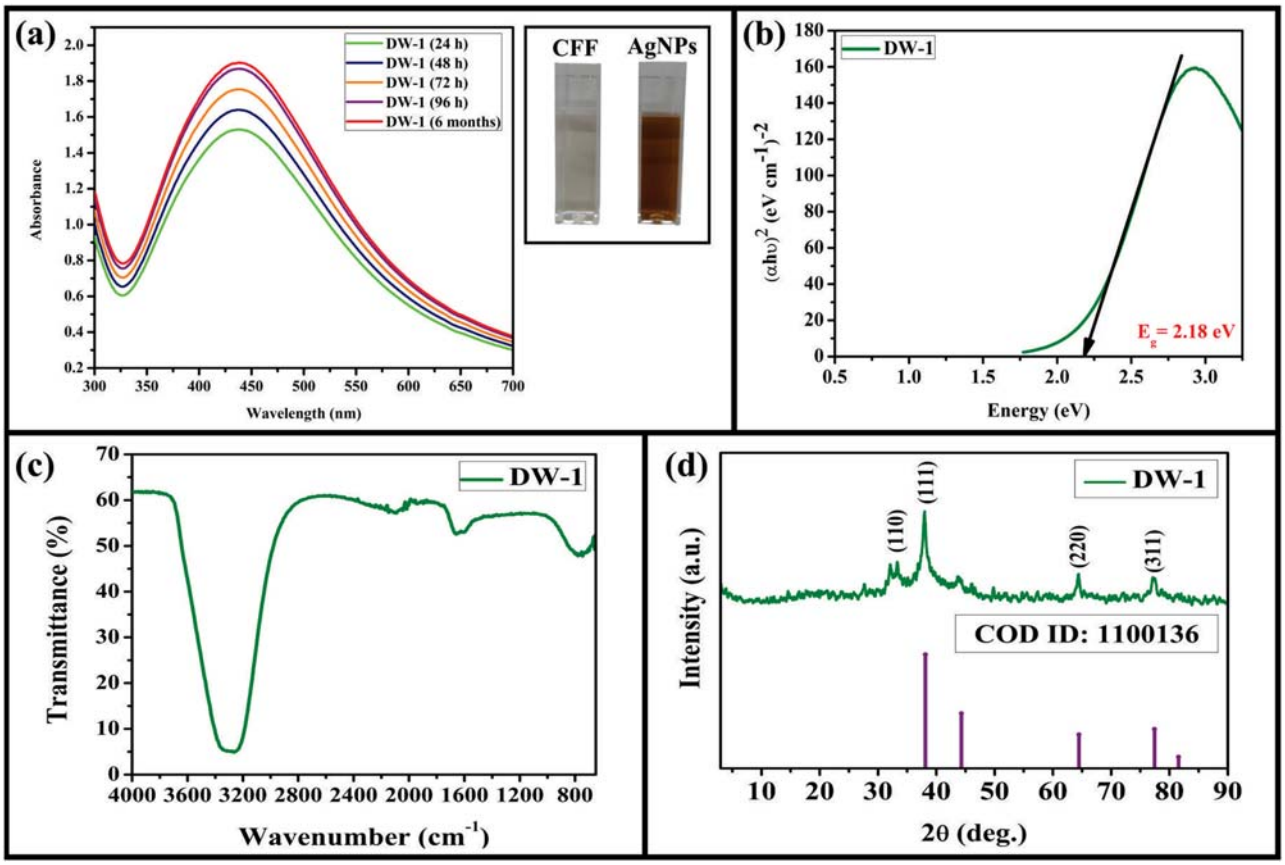

FIGURE 7.2 UV-visible spectroscopy (A), band gap energy graph (Tauc's plot) (B), Fourier transform infrared (FTIR) (C), and X-ray diffraction (XRD) spectra of AgNPs synthesized from cell-free filtrate (CFF) of *Hamigera terricola* (D).

Fourier-transform infrared spectroscopy analysis

In the optimum reaction condition, biomolecules secreted by fungi get immediate attachment on the surface of reduced silver ions, which provide stability and bioactivity to the AgNPs (Rheder et al., 2018). Surface chemistry of mycosynthesized AgNPs by DW-1 was studied though the ATR-FTIR spectroscopy in the range of 4000 cm^{-1} to 650 cm^{-1}. FTIR spectral analysis is represented in Fig. 7.2C. The peak obtained at 3266 cm^{-1} was due to the presence of O—H stretching of alcohol or phenol (Thirunavoukkarasu et al., 2013). Peaks at 2093 cm^{-1} and 1660 cm^{-1} were attributed to the C═S stretching of sulfur containing compound and C—C or C—N stretching from alkene or amines, respectively (Kumar et al., 2014; Koyyati et al., 2014). The peak acquired at 744 cm^{-1} represents the attachment of C—H bending from alkanes (Jyoti et al., 2016).

XRD analysis

X-ray diffraction peaks exhibited the nanocrystalline structure of the *H. terricola*—assisted AgNPs. The crystalline nature of materials at atomic level is studied by XRD analysis (Zhang et al., 2016). Fig. 7.2D indicates the unique XRD patterns of the *H. terricola* synthesized AgNPs, which indicates the face centered cubic (fcc) crystallographic planes of Ag. In the XRD pattern, the diffraction peaks were corresponded to [110], [111], [220], and [311] at 2θ and represented with the respective values of 32.30, 37.91, 64.38, and 77.2 degrees. Obtained XRD spectrum was compared with the COD ID no. 1100136 and the mean particle (grain) size was determined by the formula as given below (Lotfy et al., 2021).

$$D = \frac{0.9\,\lambda}{\beta \cos\theta}$$

Where, D = crystalline size in nm
λ = X-ray wavelength (0.1541 nm)

TABLE 7.1 X-ray diffraction interpretation.

Sr. no.	2θ	θ	FWHM	Crystallite size D (nm)	D nm (average)	(hkl)
1	32.3	16.15	1.87	4.42	5.94 ± 0.6	110
2	37.91	18.955	1.47	5.71		111
3	64.38	32.19	1.48	6.34		220
4	77.2	38.6	1.39	7.31		311

The mean particle size (n = 4) of grain size ± standard error.

β = full width at half maximum (FWHM) for angular line of the peak (in radians)
θ = Bragg's diffraction angle (Lotfy et al., 2021)

The estimated average size of the AgNPs synthesized by *Hamigera terricola* was 5.94 ± 0.60 nm ranging from 4.42 to 7.3 nm as indicated in Table 7.1.

TEM analysis

The TEM imaging exhibited the surface chemistry of the biosynthesized AgNPs mediated by the CFF of *H. terricola,* which includes size and shape, under the optimal working conditions. The AgNPs expressed polydispersity and spherical shape, with the mean particle size of 4.7 ± 1.26 nm in the range of 0.56−11.5 nm, as per the results obtained by TEM analysis. In addition, the selected area (electron) diffraction (SAED) pattern was also acquired by TEM imaging. Fig. 7.3A−C depicts the TEM images at the 50 nm scale, SAED pattern, and the size distribution curve of AgNPs. The SAED diffraction pattern is one that uses reverse space in the lattice planes. The SAED pattern and the XRD analysis are in correspondence with each other indicating the crystallinity of the biosynthesized AgNPs. The ring-like electron diffraction patterns indexed as [110], [111], [220], and [311] lattice planes relate with the face-centered cubic (FCC) structure of silver (Ag), which corresponds with the XRD analysis, and were determined by the SAED pattern acquired from the synthesized AgNPs (Wang et al., 2021).

Antioxidant activity

Assessment of antioxidant activity by the biosynthesized AgNPs was executed by the DPPH radical scavenging assay. Antioxidants are crucial for the functioning inside the living systems and act against reactive oxygen species (ROS) such as H_2O_2, hydroxyl radicals, and singlet oxygen. The potency of antioxidants for scavenging free radicals is beneficial for preventing many fatal diseases (Taha et al., 2019). The results depicted the concentration-dependent scavenging activity of the AgNPs synthesized by *H. terricola*. The scavenging activity of the DPPH was also elevated with the increase in the concentration of AgNPs (10−100 μg/mL), indicating the effective antioxidative potency of the synthesized AgNPs as shown in Fig. 7.3D. The AgNPs exhibited a dose-dependent pattern for their ability to scavenge free radicals, which increases with the increased dosage. The biosynthesized AgNPs exhibited an antioxidant activity of 80.83 ± 0.45% at a maximum concentration of 100 μg/mL. The biosynthesized AgNPs revealed the IC_{50} value of about 48.23 μg/mL against DPPH, which displays the potentials of AgNPs as an efficient antioxidant in different fields (Keshri et al., 2020; Mistry et al., 2022; Thakor et al., 2022a; Verma et al., 2022).

Antimicrobial activity of AgNPs

Antimicrobial activity of biogenic AgNPs was assessed against three Gram-positive, three Gram-negative, and one plant pathogenic fungi. Table 7.2 represent the antimicrobial potential of AgNPs in the form of zone of inhibition (ZOI) in mm. Experiment was carried out in triplicates to obtain the mean and standard error values. ANOVA analysis indicates that the obtained data are highly significant at $P < .001$. The highest ZOI was observed against *S. typhi,* which was about 29 ± 1.15 mm, and the lowest ZOI was observed against *E. coli,* which was about 24 ± 0.57 mm. AgNPs have also shown effective antifungal activity against plant pathogenic fungi *F. oxysporum,* which was about 22.33 ± 0.33 mm of ZOI. Obtained data revealed that the AgNPs have exhibited higher antimicrobial potency compare to the standard antibiotic used for bacteria and fungi which were streptomycin and cycloheximide. Where no inhibition

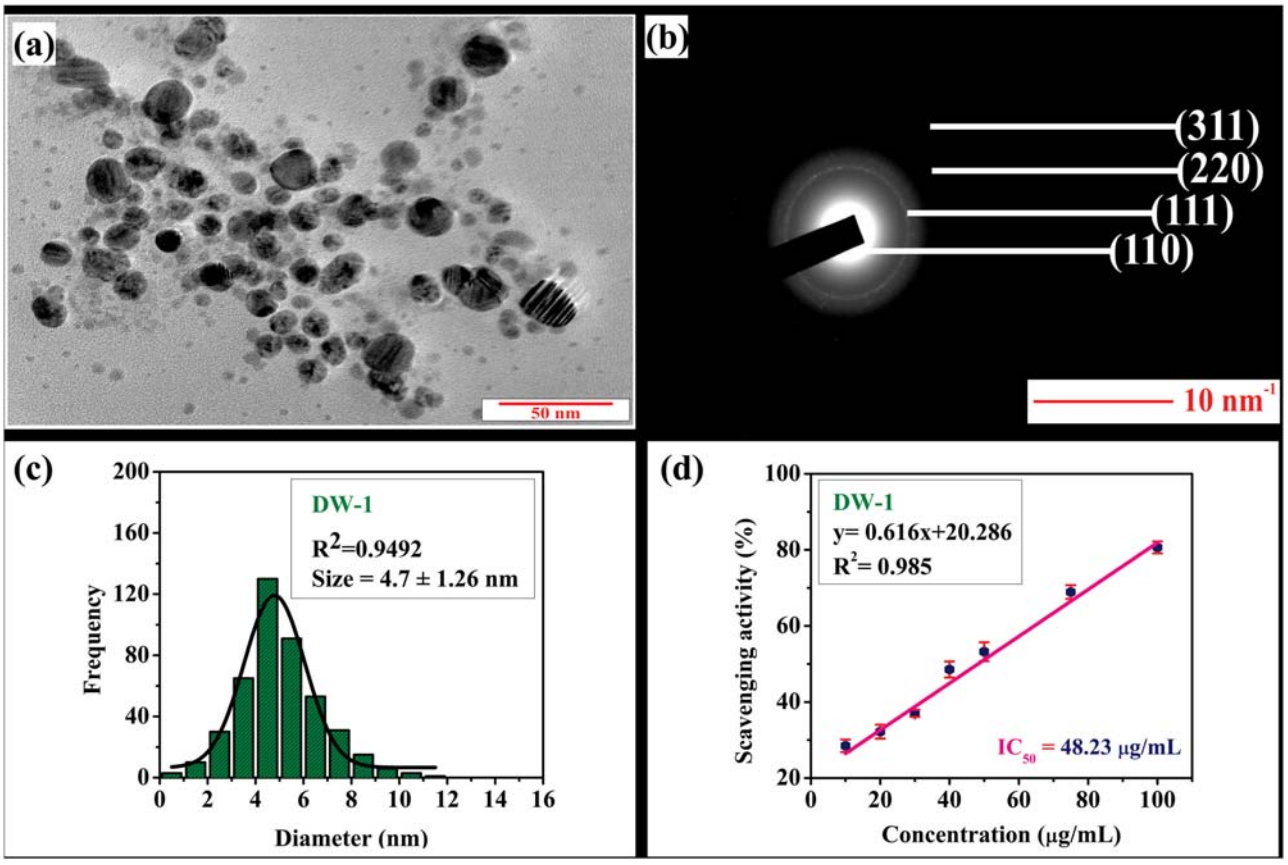

FIGURE 7.3 TEM images (at 50 nm) (A), selected area (electron) diffraction (SAED) pattern (B), size distribution curve (C), and antioxidant activity of AgNPs (D).

TABLE 7.2 Antimicrobial activity of mycosynthesized AgNPs.

Antibacterial activity				
Sr. no.	**Bacterial strain**	**Zone of inhibition (mm)**		
		AgNPs (100 μL, 1 mg/mL)	**Streptomycin (100 μL, 1 mg/mL)**	**10 mM AgNO3 (100 μL)**
1	*E. coli*	24 ± 0.57	16.66 ± 0.33	12.33 ± 1.2
2	*S. typhi*	22.66 ± 1.2	17.33 ± 0.33	12 ± 0.57
3	*P. aeruginosa*	23 ± 1	18.66 ± 1.33	12.33 ± 1.45
4	*B. subtilis*	27.33 ± 0.88	19.33 ± 1.76	11.66 ± 0.33
5	*B. megaterium*	25.33 ± 0.66	22.66 ± 1.76	11.66 ± 0.33
6	*S. aureus*	27.66 ± 0.88	24.66 ± 1.45	12.33 ± 0.88
P Value		<0.0001	**F Value**	52.47
Antifungal activity				
Sr. no.	**Fungal strain**	**Zone of inhibition (mm)**		
		AgNPs (100 μL, 1 mg/mL)	**Cycloheximide (100 μL, 1 mg/mL)**	**10 mM AgNO3 (100 μL)**
1.	*F. oxysporum*	22.33 ± 0.33	12.33 ± 0.66	11 ± 1.52
***P* Value**		0.0003	**F Value**	39.85

The mean particle size (n = 4) of grain size ± standard error.

was observed for microorganisms treated with distilled water. Mechanisms which may be responsible for the antimicrobial activity of the AgNPs synthesized by *H. terricola* are: (1) AgNPs form free radicals which consequent in oxidative stress (Mistry et al., 2022); (2) cause damage to the cell membranes of the microbes, ensuing into the lysis of the cell (Ismail et al., 2018); and (3) disrupts the replication of DNA and ATP synthesis via microbial absorption (Ansar et al., 2020). Earlier reports have been denoted that the AgNPs are less effective against Gram-positive bacteria due to deposition of thick peptidoglycan layer on the surface of cell wall. But in this experiment, AgNPs also had shown their efficacy against Gram-positive bacteria (Mistry et al., 2022). Due to effective antimicrobial activity against human pathogenic bacterial strains and plant pathogenic fungal strains, these AgNPs can be employed for the treatment of multidrug resistance pursuing microbes as a broad-spectrum antibiotic.

Conclusion

Synthesis of the AgNPs from the CFF of the marine procured fungi *H. terricola* was first time reported in this study. Synthesized AgNPs were characterized and had proven to pursue spherical shape with small size. Biomolecules present in the CFF provide reducing as well as capping agents to the AgNPs. These attached biomolecules contribute to the enhancement of bioactivity of AgNPs. Higher antioxidative and antimicrobial efficacy suggests that the mycosynthesized AgNPs can further be used as a broad-spectrum antimicrobial, antidiabetic, larvicidal. and anticancer agents.

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Chapter 8

Green synthesis of silver nanoparticles and their potenial biological applications

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Introduction

There are many challenges in front of humanity that need to be taken care of before they threaten our existence. Recent technological advancements have tried to solve or reduce their environmental effects. The chapter will discuss one such advancement for dealing with biomedical-related issues which is nanotechnology. Currently, many pieces of research in nanotechnology are taking place in and around the world to help in dealing with various problems at our hands. Physicist Richard Feynman was the first to present the idea of nanotechnology in "There's Plenty of Room at the Bottom" at the American Physical Society in 1959 (Feynman, 1992) whereas the term was first coined in 1974 by Taniguchi, a Professor at Tokyo Science University. Nanotechnology, which has strong linkages to nanoscience, is the molecular or atomic-level manipulation of matter to create novel materials and devices with newly remarkable qualities (Bhagyaraj and Oluwafemi, 2018). According to the National Nanotechnology Initiative (NNI, 2010), nanotechnology consists of "The understanding and control of matter at dimensions between approximately 1 & 100 nm, where unique phenomena enable novel applications" (McNeil, 2005). It deals with particles in the 10^{-9} range which is unimaginably small, but it doesn't just mean making particles less in size, it is meant to increase the surface area of the particles without altering their volume. There is quite a relationship between surface area and volume, but the question is "How the ratio of surface area to volume inevitably influences a nanoparticle's qualities?" Nanosized particles have more surface area susceptible to interacting with others at the same volume as the bulk matter, which reduces the amount of the required particles for treatment, resulting in reduced toxicity.

Nanoparticles are unimaginably small particles whose construction is quite challenging but fairly completed using two methods namely the top-down approach and the bottom-up approach (Whitesides and Love, 2001). The top-down approach is a destructive-based technique in which the small particles are created from bulk material and transformed into appropriate nanoparticles (Mijatovic et al., 2005). Whereas in the bottom-up approach, nanostructures are formed by stacking the building block, i.e., atoms on one another. In this approach, the use of single-molecule components chemical properties causes them to self-organize or self-assemble into a suitable shape or rely on positional assembly (Iravani, 2011). The generation methods for nanoparticles are mainly classified into three types, viz., physical, chemical, and iological methods. These three methods of nanoparticle generation include various techniques for them having their ups and downs for the particle and the surroundings as well. Various nanoparticles obtained via different techniques possess different characteristics so an appropriate technique should be chosen for the production. The downsides of these techniques motivate researchers to continue the work of finding better production techniques. The drawbacks of the physical methods mainly include time consumption, thermal stability, increase in environmental temperature, and occupying large spaces (Kawasaki and Nishimura, 2006). Whereas chemical methods include the use of toxic elements and harsh reducing agents (Pal et al., 2007). The biological method should be used because physical and chemical methods continue to have issues. In the biological method, plants and microbes are utilized for nanoparticle production. There are various drawbacks associated with biological methods such as maintenance of large-scale culture, slow production, and pathogenicity issues

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(Korbekandi et al., 2009). There is a new way of synthesizing nanoparticles to try and eliminate these drawbacks by using plants for the same. This method is generally known as the green synthesis method in which plants or plant parts are used for production purposes. This use of plants or plant parts for synthesis is an efficient and affordable process (Iravani et al., 2014). Additionally, we are not required to maintain large-scale cultures and this is a nonpathogenic process. Various nanoparticles produced via the green synthesis method allow us to try and resolve various issues at our hand. Due to its antibacterial capabilities, the silver nanoparticle is of vital importance for a variety of biomedical issues. The production of the same, i.e., silver nanoparticles, and their biomedical applications are discussed further.

Nanoparticle

Nanoparticles are larger than atoms or basic molecules that are subject to quantum mechanics but considerably smaller than common things that are governed by Newton's rule of motion (Horikoshi and Serpone, 2013). The transition when occurs from microparticle to nanoparticle several changes take place which make them act differently than bulk material, like a rise in surface-to-volume ratio and the size that is affected by the quantum realm. As the particles gradually get smaller, the surface area to volume ratio rises, which causes the atoms on the particles surface to have a stronger magnetic field than the atoms inside the particle. The increased surface area of nanoparticles results in a lot of interaction between the particles, increasing strength and chemical resistance, among other unique features. Due to their adjustable physicochemical properties, such as melting point, wettability, electrical and thermal conductivity, catalytic activity, light absorption, and scattering, which produce better performance compared to their bulk counterparts, nanoparticles have become more significant in technological advancements (Jeevanandam et al., 2018). Out of the many properties of nanoparticles, one of the fundamental draws and features of the nanoparticle is its optical property. Even before the fourth century AD, artists and sculptors utilized nanoparticles for their optical properties in their works. The Lycurgus cup is the most notable illustration, a remarkable cup fashioned of dichroic glass, a highly unusual sort of glass that changes color when exposed to light. Analysis revealed that it has these peculiar optical qualities because it contains a very small amount of a mixture of the incredibly tiny (70 nm) metal crystals of Ag and Au in about a 14:1 M ratio.

Types of nanoparticles

Nanoparticles made from different bulk materials possess significant properties favoring their usefulness for different purposes. These nanoparticles are of different types, each having unique properties from the other which can be more helpful in some particular manner than others. The nanoparticles are present in many forms like core-shell NPs, magnetic nanoparticles coated with a polymer, inorganic nanoparticles, and metallic nanoparticles (Ahmad et al., 2019). There are various types of nanoparticles mainly classified based on the bulk material they are made from like organic, inorganic, semiconductor, metallic, polymeric, and carbon-based. Nanoparticles included in organic-based are liposomes, micelles, dendrimers, and lipid nanoparticles made from biomolecules that naturally occur in our body which makes them less toxic, whereas inorganic nanoparticles mainly include nanoparticle's small size, high surface area to volume ratio, and unique structure all support the use of NPs for biological applications. Various instances of metallic nanoparticles are CuNPs, AuNPs, AgNPs, PdNPs, and PtNPs, out of them there is an expanding need in research for gold and silver nanoparticles because of their superior characteristics and flexible applicability (Vadlapudi and Kaladhar, 2014). Silver nanoparticles have a significant reactive surface area which helps in noteworthy biomedical reactivity, catalytic activity, and atomic behavior (Xu et al., 2006). The formation of silver nanoparticles is significantly increased for biomedical applications due to their antibacterial efficacy against microorganisms that are resistant to many drugs. Fullerene and carbon nanotubes (CNT) are the major classes of carbon-based nanoparticles. Fullerene is a hallowed structured particle and draws attention to itself because of its adaptability, electron affinity, high-strength structure, and electrical conductivity. CNTs are long tubular structures with 1−2 nm in diameter possessing either metallic or semiconducting based on their diameter telicity property (Khan et al., 2019).

Construction

The size of nanoparticles is in the nanometer range, which is unimaginably small and cannot be visualized by the naked eye, a conventional instrument for visualizing nanoparticles is an electron microscope. To construct such a small particle is quite challenging with the required characteristics. Nanoparticle construction is generally done by breaking the bulk material into the required size or by respectively stacking the building blocks, the former is known as the top-down approach, and the latter is called the bottom-up strategy. The top-down technique frequently employs classic workshop

or microfabrication procedures, in which materials are cut, milled, and shaped into the required shape and order using externally controlled equipment. Bottom-up methods should be able to build devices in parallel and at a lower cost than top-down methods, but they risk becoming overwhelmed as the size and complexity of the desired assembly grow. Nanoparticles are generated mainly by three methods, viz., physical, chemical, and biological methods. Every method has various techniques included in them and has pros and cons which are discussed further.

Physical methods

Laser pyrolysis arc discharge technique, high-energy ball milling, electrospraying, evaporation-condensation, and metal sputtering are only a few examples of the physical processes used to create nanoparticles. Various noble metal nanoparticles are formed by evaporation-condensation methods. Silver nanoparticles can be created using a small ceramic heater and a nearby heating source, as has been demonstrated (Mafuné et al., 2001). Due to the steep temperature gradient in the area surrounding the furnace, the vapor generated by heating is condensed quickly, allowing for the generation of silver nanoparticles with a high concentration. Silver nanoparticles are generally formed by laser ablation technique from bulk material in solution. The features of the generated silver nanoparticles and the effectiveness of the ablation are influenced by the laser's wavelength, the length of the laser pulses, the duration of the ablation, and the effective liquid medium (Ankamwar et al., 2005). The absence of chemical pollutants is the primary benefit of the physical technique over the chemical method which doesn't intoxicate the produced nanoparticle.

Chemical methods

The most popular chemical procedures for creating nanoparticles include the sol-gel method, the microemulsion technique, hydrothermal synthesis, polyol synthesis, and chemical vapor synthesis. The chemicals used for the production of nanoparticles accts as reducing agents, the most common reducing agent for silver nanoparticle generation is $NaBH_4$. Sodium citrate, hydroquinone, gallic acid, and elemental hydrogen are some other examples of reducing agents. The nanoparticle generation generally takes place in solution in this type of method, so they possess colloidal properties. The reducing agents reduce Ag silver ions to metallic silver (Ag^0), and they get agglomerated into an oligomeric cluster. In the presence of surfactants, the generated clusters are stabilized and protected from sedimentation, agglomeration, or loss of their surface qualities, which ultimately results in the production of a colloidal metallic silver particle (Mohanpuria et al., 2008).

Biological methods

Metallic nanoparticles are produced biologically, mostly using plants and microorganisms like bacteria, algae, fungi, and yeast. For the production of nanoparticles, reducing and capping agents are the primary prerequisite in the biological process, but most of the time, the components of the organisms cells serve in this capacity. Four out of the five kingdoms of living things monera (prokaryotic creatures without a true nucleus), protozoa (unicellular organisms with a true nucleus), fungus (eukaryotic, saprophyte/parasite), and plants have all been used in the AgNPs synthesis (eukaryotic, autotrophs).

Green synthesis

Nanoparticles and the environment both suffer from the traditional physical and chemical methods of nanoparticle formation. The production process releases so many toxic components and pollutants into the environment and much energy dissipation takes place increasing the temperature. So, there has been a need of finding an alternative to these methods for nanoparticle synthesis which is simple, cost-effective, efficient, and increased product concentration. It can be achieved by the use of biological agents like plants and bacteria found naturally in the environment. Green nanoparticle synthesis refers to the production of nanoparticles using biological agents. Various biological agents used for the production of nanoparticles are bacteria, algae, fungi, yeast, and plants. Some of the techniques using the biological agents are described below.

Bacteria mediated

Bacteria are single cellular organisms producing various inorganic materials intracellularly or extracellularly. In the intracellular form of synthesis, silver accumulated in the cell initiates the process and progresses due to microbial

expansion. After optimum bacterial development, the cells and the nanoparticle are extracted. The cell then goes through a unique process to release the produced nanoparticle. Contrarily, in the extracellular form of synthesis, bacteria's extracellular secretion is used for the synthesis and doesn't need to be treated in any unique way. They are a compelling source for producing nanoparticles like gold and silver however, some of them are resistant to silver and can build up a dry mass of silver on their cell wall (Paulkumar et al., 2013). The earliest evidence of silver nanoparticle synthesizing bacteria was established by isolating the *Pseudomonas stutzeri* AG259 strain from a silver mine (Prabhu and Poulose, 2012). In the presence of alpha-nicotinamide adenine dinucleotide phosphate reduced from NADPH-dependent nitrate reductase, AgNPs were synthesized in vitro, which converts nitrate to nitrite. There have been numerous hypothesized mechanisms for silver nanoparticles from bacteria, but the most plausible one was the synthesis of AgNPs in the presence of the enzyme nitrate reductase (Chen and Schluesener, 2008). Rathod et al. studied the synthesis of AgNPs from alkaliphilic actinobacterium *Nocardiopsis valliformis* and reported that they possess antimicrobial and cytotoxic properties (Rathod et al., 2016). Lateef et al. suggested that crude extracellular keratinase can be used for the synthesis of AgNPs obtained from *Bacillus safensis*, a keratin-degrading bacterial strain (Lateef et al., 2015). Patrycja et al. reported the increased activity of the antibiotics when conjugated with AgNPs synthesized by *Pilimelia columellifers* subsp. *Pallida*, acidophilic actinomycetes (Golińska et al., 2016). The primary drawback of utilizing bacteria to produce nanoparticles is their slow rate of synthesis and constrained size range when compared to other methods (Rafique et al., 2017). Some other examples of bacteria-mediated synthesis of AgNPs are given in Table 8.1.

Fungi mediated

Fungi are advantageous for the creation of metallic nanoparticles due to their high binding capacity, ability to bioaccumulate metals, and high intracellular intake (Ahmad et al., 2003). The benefit of synthesizing nanoparticles using fungus is that it grows faster and is simpler to handle and can easily be fabricated in a laboratory and can withstand severe environmental conditions. The mechanism of the metallic nanoparticle is by reducing their ions by enzymes secreted by them (Mandal et al., 2006), and reduction is said to be facilitated by extracellular enzymes such as naphthoquinones and

TABLE 8.1 Bacteria-mediated synthesis of AgNP.

Organisms	Size	Remarks	References
Pseudomonas mandelii	1.9–10 nm diameter	Studied larvicidal activity against *Anopheles subpictus* and *Culex tritaeniorhynchus*.	Mageswari et al. (2014)
Corynebacterium glutamicum	15 nm	Showed enhanced antimicrobial activity against pathogenic strains	Gowramma et al. (2015)
Bacillus thuringiensis	Under 100 nm	Amorphous, spherical shapes	Pourali et al. (2016)
Bacillus licheniformis	77–92 nm	In vitro antimicrobial activity against human pathogens and antiviral activity against bean yellow mosaic virus	Elbeshehy et al. (2015)
Acidophilic *P. columellifera* subp. *pallida*	–	Antifungal activity against *Malassezia furfur, Trichophyton rubrum, C. Albicans* and *Candida tropicalis*	Anasane et al. (2016)
Pseudomonas fluorescens	–	Sensitive against *K. pneumoniae* and *Xanthomonas campestris*	Syed et al. (2016)
Bacillus Strain CS11	42–92 nm	Nanoparticle exhibited absorption peak at 450 nm	Das et al. (2013)
Pseudomonas stutzeri AG259	Upto 200 nm	Characterization of nanoparticles established that crystals are embedded in organic matrix of bacteria	Klaus et al. (1999)
Bacillus licheniformis	50 nm	Color change from whitish yellow to brown indicates the formation of silver nanoparticles	Kalimuthu et al. (2008)
Klebisella pneumonia	1–6 nm	Culture supernatant utilized for synthesis and by the use of visible light it was observed that mixing process affects the silver nanoparticles formation	–
Nocardiopsis volliformis	5–50 nm	Bacteria is alkaliphilic actinobacterium and exhibited antibacterial and cytotoxicity activity	Rathod et al. (2016)

TABLE 8.2 Fungi-mediated synthesis of AgNP.

Organisms	Size	Remarks	References
Candida albicans	20–80 nm	Maximum antimicrobial activity against *S. aureus*	Rahimi et al. (2016)
Fusarium sp.	12–20 nm	Antibacterial activity against *E. coli, S. typhi,* and *S. aureus*	Singh et al. (2015)
Fusarium graminearum	45.5 nm diameter	Antimicrobial activity against *Pseudomonas aeruginosa, Salmonella* sp., *C. albicans,* and *E. coli*	Shafiq et al. (2016)
Trichoderma viride	1–50 nm	Inhibited growth of all tested pathogenic bacteria	Elgorban et al. (2016)
Aspergillus versicolor ENT7	15.5 nm	Antioxidant and antimicrobial activity	Netala et al. (2016)
Sclerotinia sclerotiorum MTCC 8785	25–30 nm	Antimicrobial activity is due to the lysis of AgNPs to the deoxyribonucleic acid	Saxena et al. (2016); Durán et al. (2016)
Colletotrichum sp.	20–50 nm	DNA of treated *E. coli* showed deformed and damaged deoxyribonucleic acid	Azmath et al. (2016)
F. oxysporum	–	Antibacterial effect on textile fabrics	Durán et al. (2007)
Aspergillus flavus	8.92 ± 1.61 nm	Nanoparticles are monodispersed	Vigneshwaran et al. (2007)
Hypocrealixii	123–195 nm	Used deadmass for the process. It is new, effective, and environmental friendly bioprocess for silver nanoparticles	Salvadori et al. (2015)

anthraquinones. Early in the 20th century, the fungus Verticillium was used to create AgNPs in the first experiment for the fungi-mediated production of metallic nanoparticles (Mukherjee et al., 2001). In fungus-mediated nanoparticle manufacturing, the extract of the saprophytic straw mushroom fungus *Volvariella volvacea* produced gold, silver, and silver-gold nanoparticles (Philip, 2009). Kathiresan et al. reported in vitro production of silver nanoparticles utilizing *Penicillium fellutanum* isolated from coastal mangrove silt and $AgNO_3$ as a substrate (Kathiresan et al., 2009). In fungi, the process of producing nanoparticles involves the formation of NPs on the mycelia's surface rather than in solution. First, the electrostatic interaction between positively charged Ag ions and negatively charged carboxylate groups in enzymes causes Ag^+ particles to be deposited on the surface of the fungal cells. The fungus's cell wall's enzymes then break down the Ag particles, resulting in the creation of Ag nuclei. Some other examples of fungi-mediated synthesis of AgNPs are given in Table 8.2.

Algae mediated

Algae are aquatic photosynthetic bacteria with sizes ranging from microscopic (Dinoflagellates) to macroscopic (Rhodophyta). According to Sinha et al. (2015), when silver ions come into contact with the algal extract of *Pithophora oedogonia*, AgNPs are produced within minutes. In vitro, Ehrlich Ascites Carcinoma was used by Khalifa et al. (2016) to examine the antitumor activity of AgNPs produced by the blue-green algae *Anabaena oryzae, Nostoc muscorum*, and *Calothrix marchic*. Aqueous extract of the green alga *Caulerpa racemose* was used to make AgNPs, which showed high catalytic activity for the breakdown of methylene blue and antibacterial activity (Edison et al., 2016). *Spirogyra varians* is used to reduce silver ions, creating AgNPs with a size of 17.6 nm and significant antibacterial capabilities against different harmful microorganisms (Salari et al., 2016). Nafe et al. proclaimed that AgNPs synthesize by *Chlorella pyrenoidosa* offered a high degree of consistent morphology, and confirmed antibacterial and photocatalytic properties of these AgNPs (Aziz et al., 2015). AgNPs were made using an aqueous extract of *Sargassum polycystum* C. Agardh, and their potential for cytotoxicity and antibacterial activity was assessed (Asha et al., 2015). Some other examples of algae-mediated synthesis of AgNPs are given in Table 8.3.

Yeast mediated

Yeasts are eukaryotic, single-celled organisms that are utilized to synthesize various nanoparticles. The yeast strain MKY3 aids in the extracellular creation of silver nanoparticles because it is silver-tolerant, When it is exposed to a 1 mM silver

TABLE 8.3 Algae-mediated synthesis of AgNP.

Organisms	Size	Remarks	References
Chlamydomonas reinhardtii	–	Internalization was evidenced for the first time	Navarro et al. (2015)
Spirulina Platensis and *Nostoc* sp.	11.5 and 20.3 nm respectively	Potential antibacterial activity against human pathogens like *S. aureus, S. epidermidis, K. pneumoniae,* and *E. coli*	Ahmed et al. (2015)
S. plantensis	3.2–7 nm	Nanoparticle synthesized as powder algal biomass and showed antimicrobial and antioxidant activity	Kaliamurthi et al. (2016)
Chaetomorpha linum	30 nm	Amines, peptides, flavonoids, and terpenoids present in *C.* linum acts as capping and efficient stabilization	Kannan et al. (2013)
Gelidium amansii	27–54n	Potential anti-micro-fouling coatings for various biomedical and environmental applications	Pugazhendhi et al. (2018)
Chlorella vulgaris	15–47 nm	Aqueous extract was used as reducing agent	Annamalai and Nallamuthu (2015)
Caulerpa racemose	25 nm	Showed antibacterial activity and catalytic activity for silver nanoparticles toward degradation of methylene blue	Kathiraven et al. (2014); Edison et al. (2016)
Sargassum wightii	–	Tested for their antibacterial potential	Govindaraju et al. (2009)
Pterocladia capillacae	100 ppm conc/7 nm	Cotton fabric treated with silver nanoparticles showed antimicrobial activity	El-Rafie et al. (2013)
Isochrysis galbana	53.1–73.9 nm	Showed zone of inhibition against *klebsiella* sp., *P. vulgaricus, P. aeruginosa, E. coli*	Merin et al. (2010)

solution, the strain produced AgNPs, and based on differential sample thawing, the pure silver nanoparticles were isolated (Kowshik et al., 2002). Commercial yeast extract (0.5 gm) incubated overnight after treating with $AgNO_3$ produced AgCl NPs indicated by a color change to darkish brown (Sivaraj et al., 2020). Jha et al. synthesized AgNPs in suspension culture containing yeast cells in presence of 0.025 M $AgNO_3$ and sooty gray NPs were filtered which were formed extracellularly (Jha and Prasad, 2008). Using yeast extract as a reducing and capping agent, Shu et al. (2020) generated well-dispersed, evenly shaped Ag-NPs. The mixture of Ag^+ ions and yeast extracts forms yeast micelles. In the reduction of Ag^+ as well as in the supply of high stability, monodispersity, and regulated size distribution for the created Ag-NPs, biomolecules such carbohydrates, reductive amino acids, aminobutyric acid, and -linolenic acid play a significant role. The amino acids present on the surface of AgNPs have a negative charge and exhibit electrostatic repulsive nature in alkaline solution providing stability without causing precipitation (Roychoudhury, 2020). Some other examples of yeast-mediated synthesis of AgNPs are given in Table 8.4.

Plant mediated

In recent years, plant-mediated nanoparticle synthesis has gained popularity due to its quickness, environmental friendliness, simplicity, and nonpathogenic, plant possess a large variety of metabolites that are helpful in AgNP production (Sondi and Salopek-Sondi, 2004). The nanoparticle produced using plant extracts is having higher kinetics than other biological or chemical agents. Alfalfa sprouts were used by Gardea-Torresdey et al. (2003) to provide the first proof of plant-mediated production of metallic nanoparticles. Alfalfa roots can take silver from an agar medium and transmit it to the plant's shoots in the same oxidation state, where the silver atoms are then rearranged to produce AgNPs. Various crops such as *Oryza sativa*, *Helianthus annus*, *Saccharum officinarum*, *Sorghum bicolor*, *Aloe vera*, *Zea mays*, *Basella alba*, and *Capsicum annuum* have been used for AgNPs synthesis which possess pharmaceutical as well as biological application (Kasthuri et al., 2009). AgNPs synthesized by the leaf extracts of *Justicia glauca* were reported that they possess antibacterial and antifungal properties (Emmanuel et al., 2015). *Hemidesmus indicus* leaf extract was used by Latha et al. (2015) to study the synthesis of AgNPs, they showed rapid synthesis and better antibacterial activity against *Shigella sonnei* bacteria at 40 μg/mL. According to dynamic light scattering, banana peel extract works best when used as a

TABLE 8.4 Yeast-mediated synthesis of AgNP.

Organisms	Size	Remarks	References
S. cerevisiae	–	Antifungal activity against some fluconazole-susceptible and fluconazole-resistant strains of *Candida albicans*	Niknejad et al. (2015)
Extremophilic yeast strain from acid mine drainage	<20 nm	Grow in presence of $AgNO_3$ up to 1.5 mM	Mourato et al. (2011)
S. cerevisiae, Rhodotorula glutinis, and *Geotrichum candidum*	2.5–20 nm	AgNPs are synthesized extracellularly by direct exposure to $AgNO_3$ solution	Zahran et al. (2013)
Yeast strain, BDU-XR1	–	Detected by absorption wavelength of 410–420 nm in UV–visible spectrophotometer	Jafarov et al. (2017)
Saccharomyces cerevisiae	Intracellularly—4 nm Extracellularly—9 nm	Glucans on the cell wall functioned as reductive reagents in Tollens' reaction for generating NPs	Li et al. (2018)
Commercial extract of yeast	–	Luciferase reporter phage shows antimycobacterial activity of silver chloride nanoparticles	Sivaraj et al. (2020)
P. capsulate	5–25 nm	NADh-dependent protein similar to nitrate reductase was suggested to mediate the reduction process	Subramanian et al. (2010)
Candida sp. VITDKGB	87 nm	Antibacterial activity against the MDR pathogens *S.aureus* and *Klebsiella pneumoniae*	Kumar et al. (2011)
Yorrowia lipolytica NCIA 3590	7 nm	Displayed effective antifungal properties against wall-disfigurement causing fungus	Apte et al. (2013)

reducing agent to create AgNPs from silver nitrate solution. When combined with levofloxacin antibiotics, AgNPs demonstrated good antibacterial action against representative yeast and bacterial pathogens (Ibrahim, 2015). From the root extract of *Erythrina indica*, spherical AgNPs with diameters ranging from 20 to 118 nm were created. They exhibited a cytotoxic impact on breast and lung cancer cell lines and had potent antibacterial activity against both gram-positive and gram-negative bacteria (Sre et al., 2015). Palaniyandi et al. synthesized silver nanoparticles from gum extract of *Azadirachta indica* when mixed with silver nitrate solution showed antibacterial activity against *Salmonella enteritidis* and *Bacillus cereus* (Velusamy et al., 2015). Table 8.5 lists several further instances of AgNP production by plants. Table 8.5 lists several further instances of AgNP production by plants.

Advantages of green synthesis

The production of nanoparticles by physical or chemical methods has many disadvantageous effects and affects the environment severely. In physical methods, a lot of room is taken up, a lot of heat is produced, and it takes a long time. In contrast, the use of toxic substances in chemical processes may render the generated nanoparticle poisonous and cause the release of toxins into the environment. Due to this urgent requirement for an environmentally acceptable synthesis process, the employment of biological agents was initiated. The biological agents used are bacteria, algae, fungi, yeast, and plants, and nanoparticles produced using them are nontoxic, efficient, cost-effective, and more environmentally friendly. The mechanism of nanoparticle generation is by reducing metal ions to their metallic particles. This process requires stabilizing and capping agents for preventing them from agglomerating. In most cases, stabilizing and capping agents are secreted by the biological agents themselves, so not required to add externally. These stabilizing and capping agents have a reputation for reducing toxicity, preventing the agglomeration of produced nanoparticles, and enhancing antimicrobial activity. If they also possess antimicrobial activity, then the synergistic effect of nanoparticle and capping agent may be found.

Application

Since the development of the first antibiotic penicillin from Penicillium mold, there have been various progress achieved in this field. But gradually microorganisms also developed resistance against the produced antibiotics, requiring new research for forbidding the threat of microorganisms. There have many advancements for the same but microorganisms develop

TABLE 8.5 Plant-mediated synthesis of AgNP.

Species	Part	Remarks	References
Eugenia jambolana	Leaf extract	Presence of alkaloids, flavonoids, saponins, and sugar compounds	Gomathi et al. (2017)
Saraca asoca	Bark extract	Presence of hydroxylamine and carboxyl groups	Banerjee and Nath (2015)
Rhynchotechum ellipticum	Leaf extract	Presence of polyphenols, flavonoids, alkaloids, terpenoids, carbohydrates, and steroids	Hazarika et al. (2014)
Abelmoschus esculentus	Pulp extract	IC_{50} dose leads to an increase in intracellular ROS and exhibits good antimicrobial activity.	Mollick et al. (2019)
Alpinia calcarata	Root extract	Good antimicrobial activity was confirmed by the resazurin dye reduction assay method.	Pugazhendhi et al. (2015)
Rosa indica	Ethanolic extract	Evaluated antibacterial activity against human pathogenic microbes and anticancer activity using human colon adenocarcinoma cancer cell line	Manikandan et al. (2015)
S. trilobatum	Unripe fruit extract	Antibacterial activity against human pathogenic bacteria and anticancer activity in vitro against human breast cancer cell line	Ramar et al. (2015)
Aloe vera	Leaf extract	Antiglycating activity, useful in the treatment of diabetes-related diseases	Ashraf et al. (2016)
Ananas comosus	Pineapple juice	Stabilizing as well as reducing agent and synthesized silver nanoparticle	Ahmad and Sharma (2012)
Argemone Mexicana	Leaf extract	Capping as well as reducing agent by adding to $AgNO_3$	Singh et al. (2010)

multidrug resistance. So, the use of nanoparticles was undertaken to overcome this problem. Silver is well-known for possessing antimicrobial properties against pathogenic microorganisms. So, AgNP-antibiotic conjugates were utilized for multidrug-resistant bacteria. Different applications of silver nanoparticles are discussed below.

Cell wall and membrane damage

Silver nanoparticles show antibacterial activity against both gram-positive and gram-negative bacteria because the cell walls of gram-positive bacteria contain peptidoglycan and those of gram-negative bacteria contain lipopolysaccharide. The bacterial cell wall is difficult for AgNPs to adhere to. The negative charge of the cell wall and the positive or less negative charge of the silver nanoparticles generate an electrostatic attraction that makes it easier for the particles to connect to the cell wall. After the AgNP is attached to the cell wall, the cell experiences morphological changes brought on by the nanoparticles, which cause the membrane's permeability to be disrupted, the respiratory system to become depolarized, and ultimately the integrity of the cell to be disrupted, resulting in cell death. The major mechanism of antibacterial action is thought to be the cell breakdown caused by nanoparticle attachment (Roy et al., 2019). Following brief contact, peripheral damage and dense pit formation may be seen with the help of modern imaging techniques. Silver nanoparticle attachment results in the production of irregular-shaped pits that enable them to enter the periplasmic space and eventually within the cell.

Intracellular penetration and damage

AgNPs may also penetrate the cell wall and interact with DNA and proteins to impact the cell's essential processes. Silver ions released from AgNP are known to make bacterial DNA condense from a normal relaxed state and DNA molecules lose their replication ability. DNA can potentially be damaged or denaturized by silver ions. Silver ions possess a high affinity for sulfur and amine groups of proteins leading to the accumulation of AgNP on the outer membrane and then they are taken up. DNA breaks down when AgNP enters a cell because silver ions are attracted to DNA physically and interact with nucleosides, which dissolves hydrogen bonds between complementary strands of DNA (Pramanik et al., 2016). The DNA damage can be dose-dependent, i.e., more concentration of AgNPs leads to cut DNA into small fragments which can

be demonstrated by agarose gel electrophoresis. According to research by Dutta et al., overexpression of the genes for Cu and Mg transporters, which code for proteins involved in sulfur transport, metal ion influx, antibiotic resistance, and antioxidants, upsets the intracellular antioxidant balance and caused cell damage (Nagy et al., 2011).

Oxidative stress

Reactive oxygen species (ROS) have a high redox potential, and under normal circumstances, cellular antioxidant defenses and ROS production are in balance. But certain changes can either cause an increase in ROS production or a decrease in the antioxidant capacity of cells or even both, which leads to oxidative stress. Biomolecules found in cells are harmed by ROS and free radicals such as hypochlorous acid, hydrogen peroxide, superoxide anions, etc., as oxidative stress grows. Both nanoparticles and antibiotics can harm DNA and proteins because they bind to the bacterial cell wall and enter through lipid peroxidation caused by nanoparticles. Das et al. reported that ROS generation significantly contributes to the antibacterial activity of silver nanoparticles when tested against multidrug-resistant *E. coli* and *S. aureus* (Das et al., 2017). AgNP-antibiotics don't show the synergistic effect with ROS but also enhanced their generation. In the presence of ROS, the antibacterial activity is increasingly demonstrating the importance of AgNP-induced ROS production for antimicrobial activity.

Antifungal activity

Silver has numerous pharmaceutical uses in industries like agriculture, textiles, and most notably medicine. It is highly toxic to microorganisms (Ahmad et al., 2019). Since microorganisms have developed multidrug resistance and there has been an increase in the number of invasive fungal infections. Therefore, there is a need to find more potent antifungal agents, and silver nanoparticles are a viable option for this purpose.

Antiviral activity

Silver nanoparticles possess antiviral activity which was evaluated using Hut/CCR5 cells. Silver nanoparticles also inhibited human immunodeficiency virus 17%−187% more efficiently than azidothymidine triphosphate, a reverse-transcriptase inhibitor (Sun et al., 2005). A sufficient release of silver ions from the membrane, functioning as an antiviral agent, was made possible by the antibacterial and antiviral qualities of silver nanoparticles in combination with polysulfone.

Wound healing

Wound healing is a major factor affecting antiinflammatory and antimicrobial activity, for this reason, there have been many improvements for minimizing the same, and treating cotton with nanoparticles exhibited potent healing power (Hebeish et al., 2014). Bacterial cellulose that was efficient against both gram-positive and gram-negative bacteria was created using *Acetobacter xylinum* strain TISTR 975 and a silver nitrate solution (Maneerung et al., 2008). Collagen was added to a polyurethane solution containing silver ions to increase its hydrophilicity, and then it was reduced by dimethylformamide by electrospinning. In an animal model, these silver nanoparticles with collagen incorporation have improved wound healing (Chen and Chiang, 2010). To regulate health-related infection, Jacob et al. biosynthesized tissue saturated with AgNPs, which has the property of inhibiting borne bacterial growth on the surface of the tissue (Jacob et al., 2019).

Cardiovascular implants

Silver's antibacterial property has made it advantageous to use to make cardiovascular implants. A prosthetic silicone heart valve with a silver coating was the first cardiovascular device to reduce endocarditic. The silver coat over the implant prevented bacterial contamination and reduced the inflammation reaction of the heart (Grunkemeier et al., 2006). Heart valves and stents coated with silver nanoparticles along with diamond-like carbon exhibited antithrombogenic and antibacterial properties (Andara et al., 2006). The biocompatibility, calcification resistance, and toughness of polymeric heart valves were improved by the incorporation of nanostructure material into the polymer's backbone (Ghanbari et al., 2009).

Catheters

Artificial catheters implanted in patients are highly inclined to contamination which leads to complications. Catheters made up of polyurethane are coated with silver nanoparticles for preventing biofilm formation. The silver nanoparticle-coated catheter is nontoxic and reduces bacterial growth and helps avoid Catheter-Associated Ventriculitis.

Orthopedic implants

The greatest challenge in orthopedic surgery has always been bacterial contamination, so to reduce bacterial resistance, silver nanoparticles (AgNPs) were used to make the prosthesis. Silver nanoparticles also began to be used in orthodontic adhesive for increasing the shear bond strength and expanding resistance to bacteria.

Dentistry

Antibacterial applications of silver nanoparticles are also seen in dentistry. They can be incorporated in the adhesives to increase the bond strength or in composites to prevent bacterial contamination and even increase the antifungal efficiency. Silver nanoparticle-incorporated endodontic fillings proved to be an efficient antibacterial agent against *Streptococcus milleri*, *S. aureus*, and *Enterococcus faecalis* (Lee et al., 2006).

Anticancer

In cancer, the affected cells grow uncontrollably and spread to other parts of the body. The human body is made of trillions of cells, and all the cells are replaced when they got old by cell division. This process occurs in an orderly manner but in cancerous sites the growth is unlimited and they are undifferentiated. There are many anticancer drugs on the market to control the cancers present in the body, but this anticancer drug has significant side effects and systemic toxicity. So to avoid these toxicities, nanotechnology is significantly used as they have high specificities and are less invasive (Fig. 8.1) (Conde et al., 2012). Metallic nanoparticles possess efficient cancer diagnostics and therapeutic properties because of their high penetration and target specificity for diagnostic and therapeutic, respectively (Ovais et al., 2016). It is possible to combine metallic nanoparticles with other biological particles to target cancer cells cell surface proteins or receptors in a precise manner (Sperling and Parak, 2010). ROS are produced in greater amounts as a result of communication between silver nanoparticles and mitochondria, which interferes with the function of the cell's electron transfer mechanism. Thus, the primary mechanism underlying anticancer activity is believed to be oxidative stress brought on by ROS. Due to their enormous surface area, silver nanoparticles can easily enter cells and interact with their constituents, disrupting the cellular signaling cascade. By producing ROS, which trigger apoptosis, Gurunatham et al. produced silver nanoparticles from *Bacillus funiculus* culture supernatant and showed antiproliferative activity in human breast cancer cells (MDA-MB231)

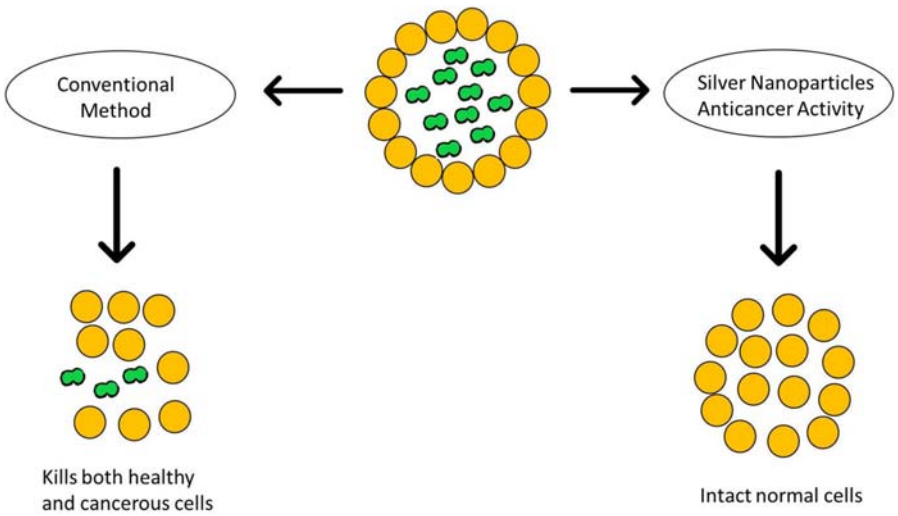

FIGURE 8.1 Anticancer activity of silver nanoparticles.

(Gurunathan et al., 2013). Using the *Penicillium shearii* AJPO5 fungus, Fageria et al. created silver nanoparticles with capped proteins. They demonstrated that these particles have an anticancer effect on mesenchymal (osteosarcoma) and epithelial (hepetoma) cells by producing ROS particles. They also claimed that these particles sensitize cancer cells, making them cisplatin-resistant (Fageria et al., 2017). Firdhouse et al. Utilizing *Alternanthera sessilis* plant extract to create silver nanoparticles, which showed a cytotoxic effect against prostate cancer cells (PC-3) (Firdhouse and Lalitha, 2013). Both antibacterial and anticancer properties are present in biologically produced silver nanoparticles. For example, Sankar et al. produced silver nanoparticles from the aqueous extract of *Briganum vulgare* (oregena), which exhibits dose-dependent efficacy against pathogens and human lung cancer cells (A549) (Sankar et al., 2013). Silver nanoparticles were created by Rajasekhurreddy et al. from the seed extract of Sterculia footed L. These nanoparticles had effective anticancer action against human cervical cancer cell lines (HeLa) and antiangiogenic activity (Rajasekharreddy and Rani, 2014). Kotecherlakota et al. synthesized silver nanoparticles using *Olax scandrns* plant extracts which possess biocompatible, imaging agent, anticancer, and antibacterial agent (Kotcherlakota et al., 2019). It was discovered that its anticancer mechanism involved the production and activation of ROS in p53. *Rosa indica* (rose petals), ethanol extract was used to biosynthesize silver nanoparticles that have antitumor action against human colon cancer cells (HCT-L5) (Kalimuthu et al., 2008). The HCT15 line of human colon cancer cells is resistant to the anticancer effects of silver nanoparticles that have been biosynthesized using *Vitex negunda* leaf extracts. Investigating apoptotic shifts and nuclear condensation with propidium iodide staining and DNA fragmentation by gel electrophoresis revealed that biosynthesized silver nanoparticles at an IC50 of 220 g/mL inhibited colon cell line HCTL5 proliferation, halted the Go/G1 process, observed decreased DNA synthesis, and induced apoptosis (Prabhu et al., 2013). When *Silanum trilobatum*'s unripe fruit extract was tested for cytotoxicity against the breast cancer MCF7 cell line, it was discovered that silver nanoparticles caused the cell line and mitochondrial pathway to die (Green and Reed, 1998). Leaf extract of *Punica granarum* biosynthesize silver nanoparticles possess 50% cytotoxicity at 100 μg/mL (Sarkar and Kotteeswaran, 2018). Silver nanoparticles produced by A. Calamus via the rhizome have anticancer action against A431 carcinoma cells with an IC50 value of 78.5822.7 g/mL. *Taraxacum officinde* leaf extract created silver nanoparticles that are effective against human liver cancer cells (HepG2) (Saratale et al., 2018). Silver nanoparticles affect the structure of cells, reduce cell stability, impair physiological function, and raise oxidative stress, which leads to mitochondrial malfunction. These are just a few of the early effects of silver nanoparticles (Gurunathan et al., 2015). Silver nanoparticles ability to absorb cytosolic proteins affects the function of cellular components and controls the expression of genes and pro-inflammatory cytokines (AshaRani et al., 2012). ROS generation is boosted by increased intracellular ion concentration. Endocytosed silver nanoparticles are destroyed by lysosomes which leads to the leakage of Ag^{+} ions into the cytoplasm causing cellular injury (De Matteis et al., 2015). Phycocyanin extract of *Nostoc linckia* biosynthesize silver nanoparticles possessing cytotoxicity activity against MCF-7 cells with IC50 of 27.79 ± 2.3 μg/mL (El-Naggar et al., 2017). Kuppusamy et al. (2016) Synthesize silver nanoparticles and gold nanoparticles using *Commelina nudiflora*, which reduces cell viability and exercises improved cytotoxicity against HCT-116 colon cancer cells with IC50 of 100 and 200 μg/mL. Methotrexate (MTX) is an anticancer drug possessing toxicity toward renal and hepatic sites at high dosages. PE-capped Methotrexate silver nanoparticles were produced via chemical methods and they showed anticancer activity against MCF-7 cell line and hemolytic activity was reduced (Muhammad et al., 2016).

Drug delivery

The dispersion of the drug in designated locations and its delayed and sustained release, which can be achieved by active and passive delivery techniques, are the main requirements for efficient drug delivery systems (Torchilin, 2010). To distribute pharmaceutical elements with certain therapeutic efficacy it is very important to design efficient drug delivery techniques. Combining pharmaceutical components and silver nanoparticles for effective drug delivery that is thermally adjustable, and pH-modified to target inflammation, and infectious (KJ, 2017). The drug delivery systems must be provided with flexible drug concentration and discharging characteristics (Fig. 8.2). The therapeutic efficacy should be as same at a lower concentration than the main compound to minimize the side effects (Kumar et al., 2017). Silver nanoparticles made from *Butea monosperma* plant extract were used by Kotecherlakota et al. to create a cancer drug delivery system that was then loaded with the FDA-approved chemotherapy drug doxorubicin (Patra et al., 2015). Silver nanoparticle-delivered doxorubicin showed more cytotoxicity than pristine drugs. Kumar et al. used Delftia sp. Strain KCM-006 for synthesizing silver nanoparticles and showed delivery of the antifungal drug miconazole, which prevented the growth of fungus biofilms and the manufacture of ergosterol (Kumar and Poornachandra, 2015).

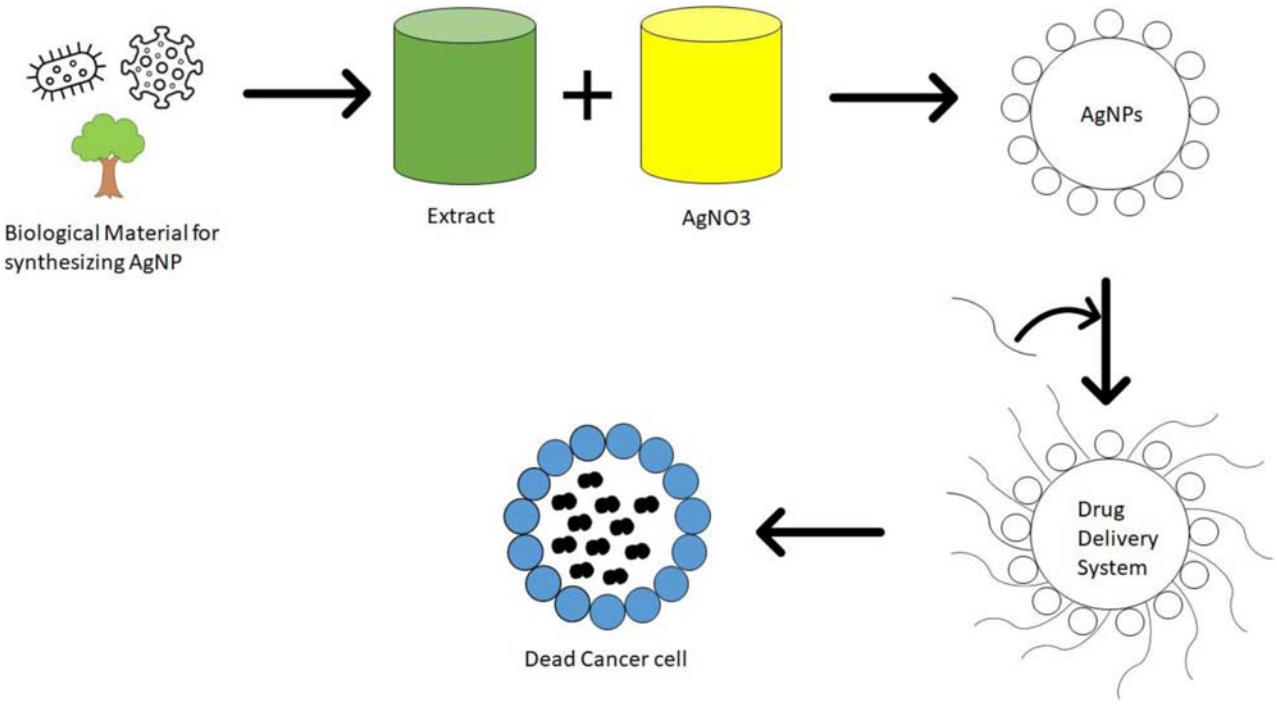

FIGURE 8.2 Drug delivery system.

Antimicrobial

Microorganism pathogenicity has grown to be a serious hazard to human health. Numerous antibiotics are available on the market to treat these infections, but it is quite dangerous because the microorganisms are becoming resistant to them. Nanotechnology offers a different approach to treating microbial illnesses. Due to their efficiency, which is greater than that of the antibiotics used in clinics, silver nanoparticles are powerful antimicrobial agents. Singh et al. synthesized silver nanoparticles from *Raphanus sativus* (entophytic fungus) using culture supernatant which showed an antibacterial effect on Gram-positive (methicillin-resistant *Bacillus subtilis*, MTCC441) and Gram-negative (*E. coli,* MTCC443) bacterial pathogen (Singh et al., 2017). Silver nanoparticles showed antibacterial activity by disrupting cell membrane and DNA (Fig. 8.3). Karunagaran et al. (2017) used a culture supernatant of *Bacillus thuringiensis* SSU1 for synthesizing silver oxide nanoparticles, which showed antibacterial effects. Kotcherlokota et al. used plant extract *Olax scandews* for synthesizing silver nanoparticles and they displayed a potent antibacterial effect and inhibited colony formation. By causing cell death, they weaken the bacterial cell walls and disrupt the cellular catalase enzyme levels (Mukherjee et al., 2014). Bombyx mori silk fibroin was used to biosynthesize silver nanoparticles by Fei et al. (2013) which showed an antibacterial impact in bacterial biofilm. Nanda et al. created silver nanoparticles from the culture supernatant of Staphylococcus aureus and tested their effectiveness against methicillin-resistant strains of S. aureus, S. epidermidis, and Streptococcus pyogenes, as well as *Salmonella typhi* and *Klebsiella pneumoniae* (Nanda and Saravanan, 2009). Singhal et al. Biosynthesized silver nanoparticles of size 4−30 nm using leaf extracts of *Ocimum sanctum* and evaluated on both gram-negative and gram-positive bacteria, and their antibacterial effects (Singhal et al., 2011). Krishnaraj et al. (2010) used leaf extracts of *Acalypha indica* for synthesizing silver nanoparticles, resulting antibacterial effect against waterborne pathogens. Shaik et al. (2016) used root extract of *Salvadora persica* L. For producing silver nanoparticles exhibiting antibacterial effect against both Gram-positive and Gram-negative bacteria. Kaviya et al. (2011) biosynthesized silver nanoparticles using peel extract of *Citrus sinensis* and examined their antibacterial both Gram-positive and Gram-negative bacteria are affected.

Diagnosis

Diagnosis of any medicinal problem is very important to find a cure. Without a proper diagnosis, we may never be able to find a cure or find a wrong cure. In today's scientifically driven world, nanotechnology is a promising approach to a proper diagnosis (Parveen et al., 2012). Nanotechnology has significantly improved the sensitivity, specificity, and accuracy of the process (Parveen et al., 2012). It is most commonly used in the diagnosis of pathogens in communicable diseases and

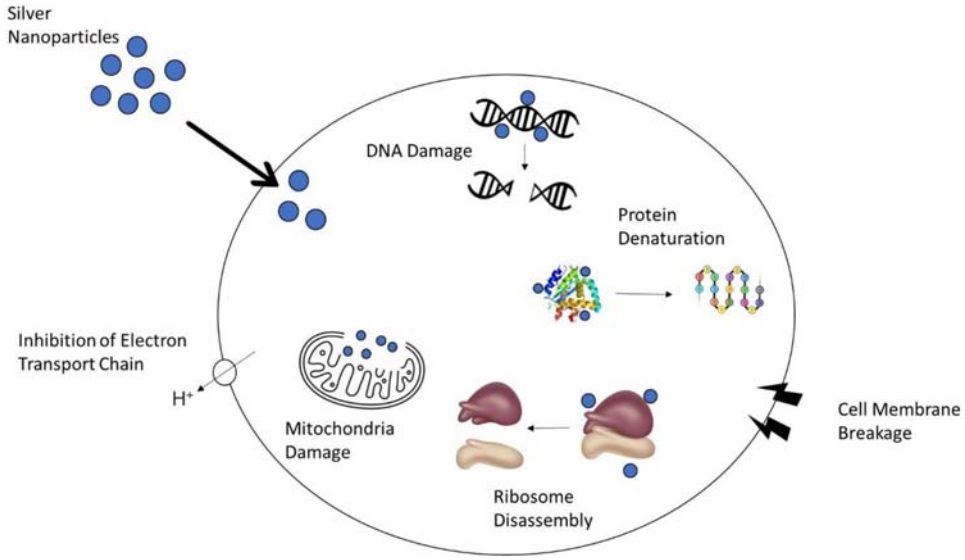

FIGURE 8.3 Antimicrobial activity.

cancer biomarkers present in cancerous cells. Nanoparticles possessing magnetic, fluorescent, and metallic properties are utilized for diagnosis (Graham et al., 2006). The diagnostic process for pathogens and cancer biomarkers has evolved and been optimized by nanotechnology, making the process more practical but also much more sensitive as the majority of the complex procedures are integrated into a simple device with the ability to be used for on-the-spot diagnosis (Tay et al., 2016). This also reduces the amount of sample required, materials used in the procedure, and time for analysis (Patil et al., 2016). Kotcherlakota et al. used biosynthesized silver nanoparticles from *Olax scandens* leaf extract for imaging B16F10 cancer cells (Tay et al., 2016). This nanoparticle internalized in cancer cells exhibits red inflorescence while normal cells do not exhibit fluorescence (Fig. 8.4). Biosensing is also an important part of diagnostic applications, in which a device incorporated with a biologically active agent is used to identify the presence of particular chemicals. Silver nanoparticles are widely used in biosensors mainly because of the extreme sensitivity toward the surrounding medium due to Surface Plasmon Resonance (Ahmed et al., 2016). Pandey et al. used a polysaccharide solution of *Cyamopsis tetragonoloba*, which acted as a reducing agent and the produced nanoparticle showed biosensing properties toward ammonia with a very less response time (2−3 s) and detection limit (1 ppm) at room temperature (Pandey et al., 2012).

Challenges

We have seen the beneficial application of the green synthesized silver nanoparticle but everything which has advantageous effects also possesses harmful effects which should not be neglected. So silver nanoparticles should also be assessed for their long-time toxicity, immunological interaction, efficacy, and biosafety in *in vivo* studies before proceeding to

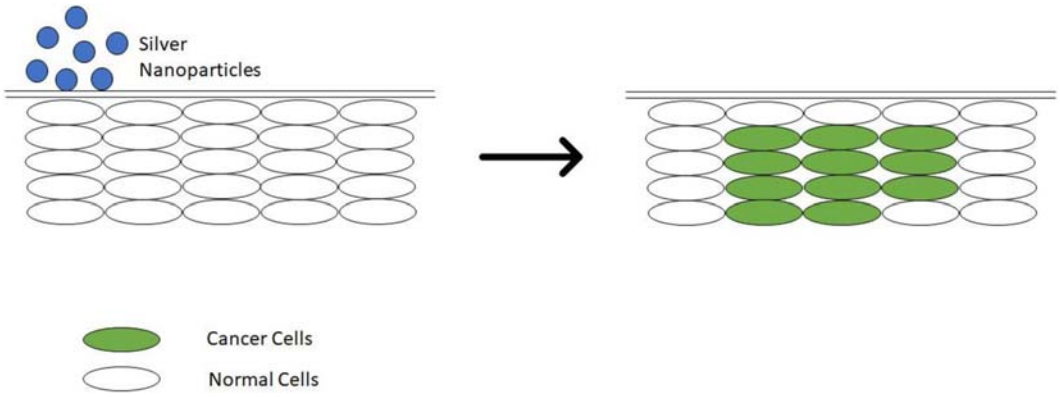

FIGURE 8.4 Diagnostic application of silver nanoparticle.

clinical trials is essential. Researchers should evaluate biocompatibility, dosage, route for administration, effective uptake, retention, and combinatorial approach for a conjugating drug with biosynthesized nanoparticles before proceeding to clinical trials. For the active uptake of nanoparticles, proper diffusion and penetration through the cell and tissue barriers are essential. Intestinal tissue invasion, immunological rejection, drug release by dispersion into the cytoplasm, a crossing of the endothelium to reach the target locations, and many other problems might arise with intravascular uptake administration. Before they may be used in clinical practice, all the previously described difficulties that depend on efficacy, regulation, and safety need to be satisfactorily overcome.

Conclusion

Since the beginning mankind had faced many issues and have tried to overcome them, in this chapter biomedical-related problem was discussed. We have seen the solution for multidrug-resistant bacteria by treating them with silver nanoparticles. Nanotechnology, which has strong linkages to nanoscience, is the molecular or atomic-level manipulation of matter to create novel materials and devices with newly remarkable qualities. The production method for synthesis is mainly classified into physical, chemical, and biological methods. These methods used for nanoparticle generation have various harmful effects on nanoparticles and the environment. So, researchers have found a new way of synthesis which is more efficient and simpler for nanoparticles which are known as green synthesis. In "green synthesis", biological agents such as bacteria, algae, fungi, yeast, and plants are used to create nanoparticles. This produced nanoparticle requires stabilizing and capping agents for preventing them from agglomerating. The silver particle possesses antimicrobial activity which is beneficial against multidrug-resistant bacteria. The mechanism of action of silver nanoparticles against multidrug resistance and various applications in different fields is discussed.

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Chapter 9

Green synthesis of silver nanoparticles, characterization and their biological efficacy

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Introduction to nanotechnology

Nanotechnology is defined as "a science, engineering, and technology conducted at the nanoscale (1−100 nm), where unique phenomena enable novel applications in a wide range of fields, from chemistry, physics, and biology to medicine, engineering, and electronics" by the National Nanotechnology Initiative (NNI) in the United States (Bayda et al., 2020). The word "nano" is a Greek prefix that denotes one millionth of a meter and implies "dwarf" or "very small" (109 m). Making the distinction between nanotechnology and nanoscience is crucial. Nanotechnology is the study of how to observe, quantify, control, assemble, and build materials at the nanoscale. When it comes to manipulating materials at the atomic and molecular levels, physics, materials science, and biology come together to form nanoscience.

Nanotechnology is one of the most important objectives in the domains of chemical engineering, physics, biology, and medicine (Poole and Owens, 2003). Area of nanotechnology, which is the science and engineering mainly concerned with the design, synthesis, characterization, and usage of materials and devices whose smallest functional organization in at least one dimension is on the nanoscale scale, or one billionth of a meter (Silva, 2004).

Pharmaceutical nanotechnology, which is based on life sciences, allows for the creation of nanostructures that can support novel drug delivery systems as treatment alternatives for a variety of diseases, as well as nanomaterial-based biosensors for cutting-edge diagnostics (Dourado, 2020). Many different kinds of nanosystems have been developed, including carbon nanotubes, paramagnetic nanoparticles, dendrimers, nanoemulsions, etc. It has been discovered that the success of the majority of drug delivery methods is significantly influenced by particle size. Drug nanoparticles have a larger surface area and smaller particle size, which increases their bioavailability and improves their solubility (Beye et al., 2018; Mazayen et al., 2022).

History of nanotechnology

Research into nanoparticles is not new. The term "nanometer" was first used by Nobel Prize winner in chemistry Richard Zsigmondy in 1925 (Hulla et al., 2015). The concept of nanotechnology was first proposed by Nobel Prize−winning American physicist Richard Feynman in 1959. The lecture Feynman delivered at the California Institute of Technology for the American Physical Society's annual meeting was titled there's Plenty of Room at the Bottom (Caltech) (Feynman, 1961). K. Eric Drexler's "Engines of Creation: The Coming Era of Nanotechnology," the first book on the subject, was released in 1986 and contributed to the increased popularity of the concept of "molecular engineering" (Drexler, 1986). Researchers used a transmission electron microscope to investigate the cup in 1990 to better understand the dichroism phenomena (TEM) (Bayda et al., 2020). By coincidence, a new class carbon nanomaterials is known as carbon dots (Xu et al., 2004).

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Nanomedicine 2014 will be held in conjunction with SELECTBIO and the British Society for Nanomedicine (BSNM) (Poole and Owens, 2003). BSNM is a registered charity that was founded to give industry, academia, medical professionals, and the general public free access to news and information on ongoing research in nanomedicine. Participants in the Nanomedicine 2014 program learned about a wide range of cutting-edge nanotechnologies, from therapy to diagnostics (Salata, 2004).

Branches of nanotechnology

Although there are many formulations using nanotechnology available today, research is still underway. Nanotechnology is developing branches that could destabilize the global market for agricultural, nonfuel, and mineral goods. Nanotechnology is currently recognized as a revolutionary field in terms of its impact on industrial applications. Nanoengineering, green nanotechnology, and wet nanotechnology are a few subfields of nanotechnology.

Nanoparticle

Nanoparticles are objects with overall dimensions in the nanoscale, or less than 100 nm. With therapeutic applications ranging from contrast agents in imaging to carriers for the delivery of medications and genes into cancers, these materials have recently emerged as key players in modern medicine (Murthy, 2007). In recent years, substantial scientific research has been done on the different uses of nanoparticles in manufacturing, electronics, building, cosmetics, and medicine (Mohajerani et al., 2019). This creates the opportunity for the development of materials, especially those for medical uses when traditional approaches could be constrained (Chong-Cerda et al., 2020). Due to their enhanced permeability and retention effect, which passively targets tumors, these drug carriers are ideal for the delivery of chemotherapeutics in cancer treatment (Wang et al., 2012).

Types of nanoparticles

Different pharmaceutical nanotechnology-based systems, often known as nano pharmaceuticals, have revolutionized drug delivery. Examples include polymeric nanoparticles, magnetic nanoparticles, liposomes, carbon nanotubes, quantum dots, dendrimers, metallic nanoparticles (MNPs), and polymeric nanoparticles (Fig. 9.1).

Carbon based nanoparticles

Fullerenes and carbon nanotubes are the most well-known kinds of carbon-based nanoparticles. Fullerenes are a prominent type of nanomaterial. Additionally, because of their high potential for reactivity, these particles are used in a range of applications (i.e., biomedicine, cosmetic products, solar cells, and catalysts) (Astefanei et al., 2015). The main characteristics of carbon nanotubes are their lightweight, small size, high aspect ratio, good tensile strength, and exceptional conducting properties, which make them perfect as fillers in a variety of materials, including ceramics, polymers, and metallic surfaces (Xu et al., 2004).

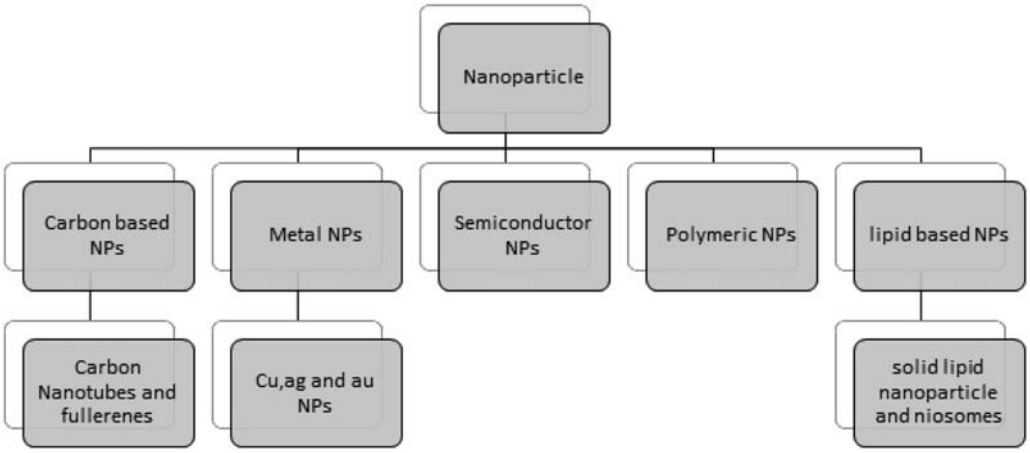

FIGURE 9.1 Overview of types of nanoparticles.

Metal nanoparticles

MNPs often have an organic, inorganic, or metal oxide shell surrounding an inorganic metal or metal oxide core. Numerous applications of metal nanoparticles can be found in daily life (Nasrollahzadeh et al., 2019).

Gold nanoparticles (GNPs)

GNPs are a wine-red material that is high in antioxidants. GNPs are available in a range of dimensions, from 1 nm to 8 m. Gold nanoparticles have been produced using a variety of green techniques, including seed-mediated growth, conducting the synthesis in the presence of ionic liquids, and various reduction techniques, such as the hydrazine reduction method and the sodium borohydride reduction method (Chaturvedi and Dave, 2021).

Iron oxide nanoparticles (IONPs)

Because of its unique qualities, such as superparamagnetism, a high surface-to-volume ratio, a huge surface area, and straightforward separation processes, iron oxide nanoparticles have attracted a lot of attention. IONPs are being employed increasingly often in biomedical research, which leads to the rapid creation of new IONPs types and increased exposure of cultured cells to a wider variety of IONPs (Ali et al., 2016; Murthy et al., 2020).

Silver nanoparticles (AgNPs)

Among the many MNPs used in biomedical applications, silver nanoparticles (AgNPs) are one of the most important and fascinating nanomaterials. In particular, AgNPs have a significant impact on nanomedicine and nanoscience (Zhang et al., 2016). Silver nanoparticles work well against a variety of fungus and algae (Kusat and Akgöl, 2021). More and more silver nanoparticles (NPs) are being used in contemporary products, which guarantees their entry into environmental systems (Sweet and Singleton, 2011).

Ceramic nanoparticle (CENPs)

Ceramic nanoparticles (CeNPs), which are inorganic metalloid solids made of oxides, carbides, carbonates, and phosphates, are made by heating materials to high temperatures and then rapidly cooling them. Biomedicine is the field in which ceramic nanoparticles are used most frequently In the biomedical field, ceramic nanoparticles are regarded as the best carriers of drugs, DNA, proteins, imaging agents, etc. (Thomas et al., 2015; Bhardwaj et al., 2021).

Semiconductor nanoparticle

Nanoparticles in semiconductors are fluorescent compounds. They have a layer of silica added to reduce photo-bleaching. The semiconductor nanoparticles coated with an extra layer of semiconductor substantially enhanced the luminescence of these core-shell assemblies (Karmakar et al., 2016). Due to their high surface area or quantum size effect, semiconductor materials undergo a significant change in their chemical and physical properties when reduced to the nanoscale (Sahu, 2019).

Polymeric nanoparticle

Polymeric nanoparticles are particles between 1 and 1000 nm in size that may include active ingredients that have been internalized or surface-adsorbed onto the polymeric core (Zielińska et al., 2020). Polymeric nanoparticles have great promise for targeted drug delivery (Masood, 2016). Polymeric nanoparticles are made from biocompatible and biodegradable polymers (Madkour, 2019). Polymeric nanoparticle-based therapies have tremendous promise for the treatment of a variety of illnesses because of the adaptability of their structures and the intricate definition of their compositions, architectures, and abilities (Elsabahy and Wooley, 2012).

Lipid based nanoparticles

Lipid-based nanoparticles (LBNPs) have generated a lot of attention in the fields of drug discovery and cancer therapy. These LBNPs include solid lipid nanoparticles, liposomes, and nanostructured lipid carriers (García-Pinel et al., 2019). The duration of pharmacological activity is extended by these nanoparticles' long half-lives and controlled drug release. They are capable of transporting both hydrophobic and hydrophilic molecules (De Jong and Borm, 2008).

Brief about silver nanoparticles (AGNPS)

For more than 2000 years, silver has been utilized as a preservative and for medical purposes. Ancient Greek and Roman societies preserved drinking water in silver pots. Since the nineteenth century, silver-based compounds have been widely used in antibacterial applications, burn and wound therapy, etc (Klasen, 2000).

Since those early days, silver has been used in a wide range of medical devices, such as bone prostheses, sutures, needles, cardiac implants, catheters, dental treatment, wound care, and surgical textiles (Lansdown, 2006).

Over the past few decades, silver has been transformed into nanoparticles with diameters ranging from 1 to 100 nm. Nanoparticles have the highest activity to weight ratios due to their small size, which maximizes their surface area (Khaydarov et al., 2009).

The healing properties of silver have been known for more than 2000 years. Since the nineteenth century, silver-based compounds have been used in numerous antibacterial applications. There have been several reported applications for nanoparticles in the physical, biological, and pharmaceutical fields. It is stated that silver nanoparticles have an effective antimicrobial impact and are used as antibacterial agents in China in a variety of public environments, such as train stations and elevators (Prabhu and Poulose, 2012). The substantial threat that microorganisms pose to silver ions and silver-based compounds, including more than 15 bacterial species, is well established (Slawson et al., 1992; Zhao and Stevens, 1998). Silver is an excellent choice for many medical applications because of its feature.

Properties of silver

Since silver is an inert metal, human tissues are unaffected by it in its "pure," nonionized form. Water, wound fluids, and exudates all help silver easily ionize and release Ag^{+} or other biologically active ions, which attach to proteins on the surfaces of cells, including those of bacteria and fungi. Silver may form a variety of compounds, such as Ag^{2+} or Ag^{3+}, despite being uncommon and unstable (Lansdown, 2004).

Silver is an electron-positive element, much like all other metals, and its cation, Ag^{+}, interacts and binds with proteins and anions in a surprising way. Additionally, Ag^{+} binds to receptor groups on the surfaces of neighboring cells, bacteria, and fungi/yeasts. The majority of silver compounds, including silver metal, partially ionize in the presence of water, physiological fluids, and tissue exudates, releasing Ag^{+} or other "biologically active silver ions" for antibiotic action or absorption into surrounding human tissues (Zhao and Stevens, 1998).

Metallic silver appears to pose little risk to health in comparison with soluble silver compounds, which are more readily absorbed than metallic or insoluble silver and may have adverse effects on human health (Rosenman et al., 1979; Rosenman et al., 1987; Drake and Hazelwood, 2005).

When silver is shrunk to a nanosize, its surface area increases, and there is a substantial amount of silver at the site of action.

Properties of silver nanoparticles

Surface effects and quantum effects are two characteristics that set nanomaterials apart from bulk materials (Roduner, 2006). These procedures have had a substantial impact on the chemical, mechanical, optical, electrical, and magnetic properties of materials.

The size, morphological substructure of the substrate, and form of nanoparticles are their primary properties (including proper aspect ratios). Silver nanoparticles have a wide range of properties, including catalysis, magnetic and optical polarizability, electrical conductivity, microbiological activity (antibacterial, antifungal, antiviral, antiinflammatory, etc.), and enhanced Raman scattering (Pandiarajan and Krishnan, 2017).

Mechanism of action of silver nanoparticles on microbial cells

AgNPs have been shown to be effective against more than 650 pathogens, including viruses, fungi, and Gram-positive and Gram-negative bacteria, although the precise mechanism behind this antibacterial mode of action is still not fully known (Fig. 9.2) (Malarkodi et al., 2013; Dakal et al., 2016).

Mainly AgNPs key mechanisms are as below:

1. Structural changes
2. Formation of free radicals
3. DNA damage
4. Modulating the signal transduction in bacteria

Structural changes

Silver nanoparticles' ability to enter and adhere to the bacterial cell wall enables structural alterations in the cell membrane, including enhanced membrane permeability and cell death. Nanoparticle accumulation and the development of "pits" on the cell surface are both taking place (Sondi and Salopek-Sondi, 2004).

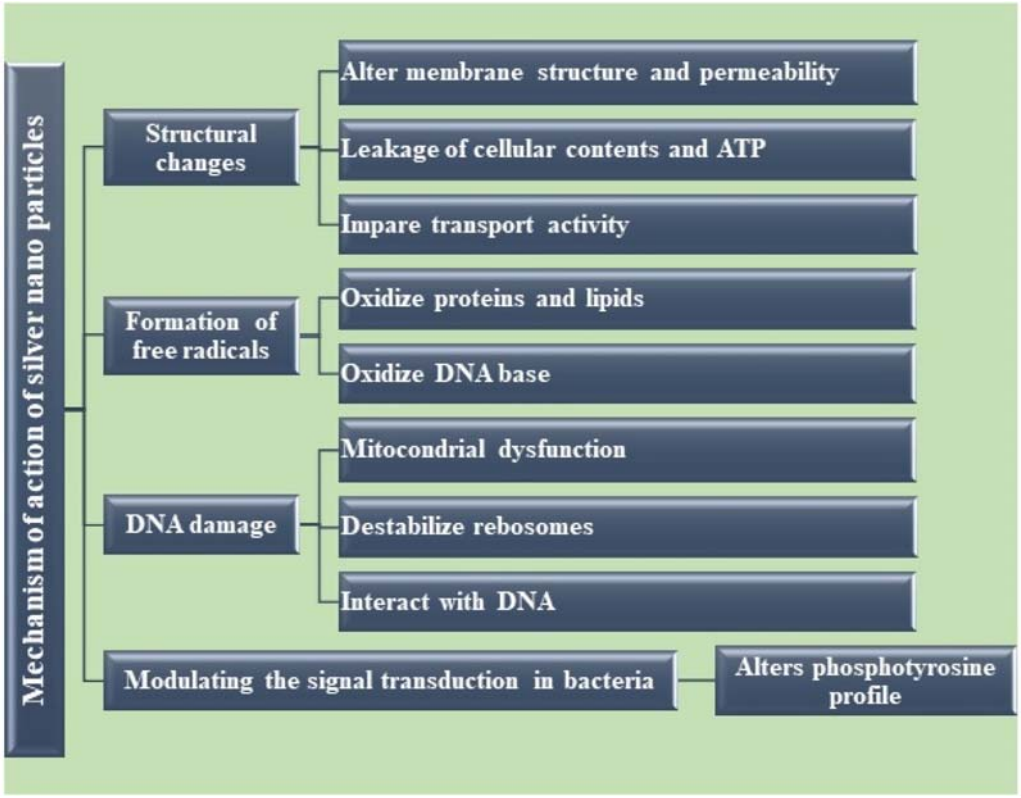

FIGURE 9.2 Various mechanisms of silver nanoparticles.

Formation of free radicals

Although the exact mechanism underlying these actions is unknown, it has been demonstrated that Ag nanoparticles have growth-inhibitory effects on microorganisms. One explanation for the growth inhibition is the generation of free radicals on the surface of Ag. Unchecked free radical generation can harm membrane lipids, which will therefore inhibit the membrane from working (Mendis et al., 2005).

The hypothesis of free radical involvement close to the Ag nanoparticle surface in the antibacterial activity of Ag nanoparticles was taken into consideration based on electron spin resonance investigations (Kim et al., 2007).

DNA damage

The fact that silver is a soft acid illustrates the natural tendency of an acid to react with a basic, in this case, a soft acid to react with a soft base (Morones et al., 2005). Most of the cells are composed of the soft bases phosphorous and sulfur. These nanoparticles' impact on the cell may trigger a reaction that leads to cell death. Another reality is that the bulk of DNA is composed of the two elements phosphorus and sulfur; nanoparticles can act on these soft bases and degrade DNA, which unquestionably results in cell death (Alcamo, 1997; Feng et al., 2000). The interaction of the silver nanoparticles with the sulfate and phosphorus in the DNA can cause issues with DNA replication.

Modulating the signal transduction in bacteria

Furthermore, it has been found that nanoparticles can affect the signaling processes of bacteria. It is widely known that phosphorylation of protein substrates affects bacterial signal transmission. Tyrosine residues have only been observed to dephosphorylate in gram-negative bacteria. The phosphotyrosine profile of bacterial peptides is altered by the nanoparticles. It was shown that the nanoparticles dephosphorylate the tyrosine residues in the peptide substrates, which prevents signal transduction and halts proliferation. To completely substantiate the claims of growth slowing, it is necessary to understand that further research on the topic is required. However, it's critical to understand that additional research on the topic is required to completely substantiate the assertions (Shrivastava et al., 2007).

Green synthesis

Nanoparticles have outstanding application in biomedical, drug delivery, chemical industries, electronics, foods, opto-electronic devices, nonlinear optical devices, space industries, energy science, and photoelectrochemicals (Nath and Banerjee, 2013). There are three most common approaches for synthesis of nanoparticles, physical, chemical, and biosynthetic. Generally chemical methods are expensive and nanoparticles synthesize by chemical synthesis are toxic, carcinogenic, and hazardous due to the use of toxic chemicals and toxic solvents in their preparation (Husen and Siddiqi, 2014). Therefore, eco-friendly method or green synthesis is required for the synthesis of nanoparticles. Biological method by the bottom-up approach is the best alternative to overcome this problem. Metallic/silver nanoparticles can be synthesized in a more sustainable and "green" process through utilizing biological materials such as bacteria, fungi, algae, plant extracts, enzymes, and biomolecules. This greener approach can be a single-step process that requires a lower energy level, lower temperature, and lower pressure than conventional processes. Another benefit of green-inspired synthesis of AgNPs is more cost-efficient than the physical and chemical methods (Srikar et al., 2016).

Methodology of formulation of silver nanoparticles by green synthesis

Biological methods are more useful for the manufacturing of exceptionally stable, properly characterised and safer AgNPs than the chemical techniques and physical methods.

Bacteria-mediated nanoparticle generation

Bacteria are capable to reduce metal ions and precipitate metals at nanometer scale. Bacteria are effective bio-factories for the synthesis silver nanoparticles because they produce various inorganic materials either intra- or extracellular. Silver is known for its bactericidal action but some bacteria are resistant to silver, and they store silver in their cellular wall (Singh et al., 2019). Most common mechanism is enzyme nitrate reductase converts nitrate (NO_3^-) to nitrite (NO_2^-) and silver ion reduced to metallic silver leads to synthesize silver nanoparticles. Some functional groups present in the bacterial cell wall are also responsible for the reduction of Ag^+ to Ag^0.

Bacteria are producing AgNPs either by intracellular or extracellular processes. Intracellular process involves the trapping, bioreduction, and capping of various nanoparticles. Extracellular process involves enzyme secretion, bio-reduction, and particle capping (Venil and Devi, 2021). Extracellular synthesis has been found to be more efficient and easier for the extraction of AgNPs. Bacteria are favorable tools due to their diversity and high adaptability to extreme conditions.

Fungi, actinomycetes, and yeast-mediated nanoparticle generation

Same as the bacteria; fungi, actinomycetes, and yeast also produce AgNPs either by intracellular or extracellular processes. Fungi contain higher amount of enzyme, proteins, and reducing components at their cell surface so it produce more amount of AgNPs than bacteria. Fungi accumulate Ag^+ into their cell surfaces and reduce by using naphthoquinones and anthraquinones present in fungal cell systems (Siddiqi and Husen, 2016). Very few researches are available on yeast synthesizing silver nanoparticles. Silver nanoparticles have been synthesized by various actinomycetes such as *Streptomyces* sp. *Thermoactinomyces* sp., and *Rhodococcus* sp. (Alani et al., 2012).

Algae-mediated nanoparticle generation

Many researchers have done work on the synthesis of algae AgNPs. AgNPs are produced either by intracellular or extracellular process. Algae are rich in protein, enzyme, pigments, lipids, DNA, RNA, and secondary metabolites; these may act as reducing agents at the surface or as a capping agent (Vijayan et al., 2014). Algae grow very fast, are easy to handle, and their biomass growth is 10 times faster than plants.

Plant-mediated nanoparticle generation

The specific quantity of the plant sample is taken for the preparation of the extract in solvents, and the extract filtrate is diluted with sterile distilled water. The diluted filtrate is used for the synthesis of silver nanoparticles. Reduction of silver ion by the mixture of molecules like polysaccharides, proteins, enzymes, secondary metabolites, and vitamins. Many plants are reported for the successful synthesis of AgNPs (Ahmed et al., 2016). There is a variation in chemical compositions of

plant extracts of the same species when it is collected from different regions and may lead to different results in different laboratories.

Green synthesis of AgNPs

AgNPs produced and synthesized with the addition of plant extracts or biomass in the silver salt, 1 mM of silver nitrate (95 mL) is mixed with 5 ml of (diluted) plant extract/biomass and stirred in aconical flask. The formation of the nanoparticle is confirmed by the development of brown color in the solution (Fig. 9.3).

Factors affecting formulation of silver nanoparticles by green synthesis

Different factors, including pH, temperature, and reaction time control the synthesis and stabilization of nanoparticles synthesized by way of biological entities. A number of the factors that influence nanoparticle system synthesis are as follows.

pH of the response medium

The pH of the response medium plays an important function in the production of nanoparticles. Different hydrogen ion concentrations result in variations in the size and shape of the nanoparticles. Many researchers have stated that a basic medium is favorable for AgNPs synthesis due to better stability, rapid growth rate; enhance reduction, small, and uniform size formation (Singh et al., 2009). The aqueous solution of AgNPs with different pH values showed different surface plasmon resonance (SPR) behavior. This is explained in terms of size and size distribution of AgNPs. Very high pH (pH $>$ 11) was associated with the drawback of the formation of agglomerated and unstable AgNPs (Tagad et al., 2013).

Response temperature

Temperature plays a very vital position in the synthesis of silver nanoparticles with the aid of specially affecting the sizes and styles of the ensuing nanoparticles. The synthesis of AgNPs lower as the reaction temperature increases but stability is

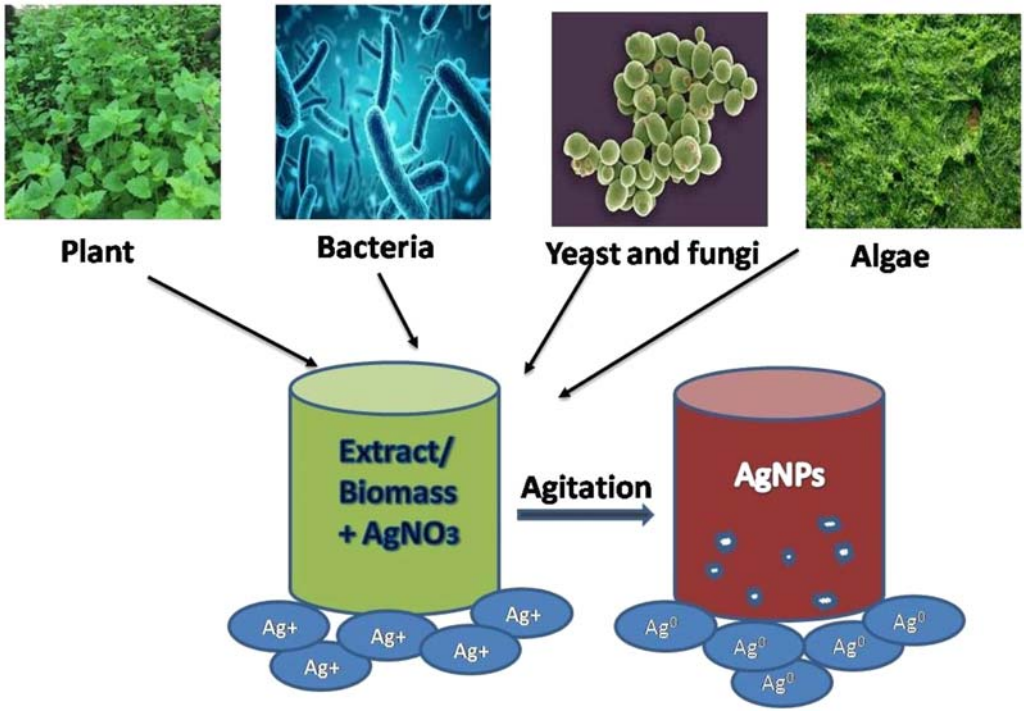

FIGURE 9.3 Green synthesis of silver nanoparticles.

increased, ensuing in a change in their morphology. It was demonstrated that lower response temperatures ended in large nanoparticles, whereas excessive temperatures produce small nanoparticles (Lade and Shanware, 2020). It was determined that *Vitexagnus-castus* leaf extract could lead rapid reduction of silver ions even at a relatively low temperature of 40°C while the effective synthesis of AgNPs was registered only at 60−80°C (Stavinskaya et al., 2019). Reaction temperate is also depending on the biomaterial, up to 100°C used for plant extract and biopolymers while for microorganisms it's restricted to 40°C.

Incubation time

The time period of incubation for nanoparticle response media substantially influences the first rate and morphology of the nanoparticle, long incubation time may result in agglomeration, that have probabilities to purpose a reduction within the nanoparticles capability (Pal et al., 2019). In the synthesis of A. fumigatus DSM819 silver nanoparticles, it was observed as small peaks at 10−20 min. After that, an increase in peak intensity was recorded after longer time periods up to 90 min due to the increase in the numbers of AgNPs, and then, the peak intensity starts to go down at 120 min (Othman et al., 2019).

Light intensity

Light intensity is considered a critical element for influencing the synthesis of AgNPs. The light irradiation approach that makes use of daylight is the best approach to support the green synthesis of AgNPs. *Piper longum* catkin extract and *Carica papaya* fruit mentioned that the absorbance multiplied with mild intensity improved. For this reason, it's expected that under sunlight, the reduction of silver ions can be completed within a couple of minutes, whereas the response requires an extended period in the dark. This arises because of because of photons of a selected wavelength in direct sunlight, which catalyze the green synthesis process (Kumar et al., 2017). It was observed by many researchers that the AgNPs with spherical shapes were formed and a higher number of particles was produced after continuous exposure to higher light intensity (Mokhtari et al., 2009). The rate of silver reduction could be efficiently accelerated through visible light exposure.

$AgNO_3$ concentrations and biomass concentration

For the formation of very minute particles, a very small quantity of the reactant is required. If the concentration of the reactant is increased, the reduction of silver ion will not be successful and the accumulation could be observed. One study reported the effect of silver nitrate ($AgNO_3$) concentrations (0.5, 0.7, and 0.9 mM) on the production of AgNPs. Surface electrical conductivity results showed that $AgNO_3$ concentration 0.5 mM produced higher electrical conductivity compared to those of 0.7 and 0.9 mM concentrations (Htwe et al., 2019). Higher concentration of silver nitrate leads to the formation of larger-sized nanoparticles. Biomass concentration should be optimized, as higher amount of biomass leads to agglomeration of AgNPs and formation of larger-sized nanoparticles (Singh et al., 2014).

Characterization of silver nanoparticles by green synthesis

The physicochemical characteristics of nanoparticles determine their behavior, biodistribution, safety, and effectiveness. AgNP characterization is therefore essential for evaluating the functional attributes of the produced particles. Table 9.1 lists many analytical methods and assay procedures that are employed for characterization.

Particle size and size distribution

For nanoparticles, particle size and size dispersion are the most important variables. They determine the distribution of nanoparticle systems in vivo, their biological fate, their toxicity, and their targeting ability. Numerous studies (Panyam and Labhasetwar, 2003) have demonstrated that submicron nanoparticles offer a number of advantages over microparticles as a medication delivery strategy. According to another study, 100 nm nanoparticles were absorbed in a Caco-2 cell line 2.5 times as much as 1 m microparticles and 6 times as much as 10 m microparticles (Desai et al., 1997).

Nanoparticles have a far higher intracellular uptake than microparticles due to their small size and relative mobility, which makes them available to a wider range of biological targets. Studies on anticancer drugs also show that particle size influences the release of the drug (Win et al., 2005).

In other cell types, microparticles do not absorb as well as nanoparticles (Zauner et al., 2001). If the particle size is larger than the drug, the release of the drug is significantly impacted (Redhead et al., 2001).

It is challenging for researchers to make nanoparticles with the smallest size while maintaining the best stability. Smaller particles have a larger risk of aggregating during storage and transportation of nanoparticle dispersion (Dunne et al., 2000).

The best methods available today for determining particle size are photon-correlation spectroscopy or dynamic light scattering, and the outcomes of these approaches are examined using scanning or transmission electron microscopy (SEM or TEM) (Boylan and Swarbrick, 2002).

Photon-correlation spectroscopy (PCS)

The average particle diameter of each fraction's intensity is calculated using PCS. This technique creates a correlation function from the time-dependent light-scattering fluctuations brought on by particles moving with Brownian agitation using an autocorrelator. The usual translation diffusion coefficient that results directly from this measurement is inversely proportional to the particle size, as stated by the Stokes–Einstein law (Huve et al., 1994).

Scanning electron microscopy (SEM)

Because of advancements made in a number of high-resolution microscopy techniques, SEM is a surface imaging technique used in electron microscopy that is fully capable of resolving a range of particle sizes, size distributions, forms of

TABLE 9.1 Characterization of nanoparticles and their techniques.

Parameters	Analytical techniques/methods
Particle size and size distribution	Scanning electron microscopy (SEM)
	Transmission electron microscopy (TEM)
	Photon-correlation spectroscopy (PCS)
	Dynamic light scattering (DLS)
	Fluorescence correlation spectroscopy (FCS)
Optical properties	UV-spectroscopy
Surface properties	Atomic force microscopy (AFM)
	Zeta potential
Crystallinity	X-ray powder diffraction (XRPD)
Thermal property	Differential scanning calorimetry (DSC)
Structure and composition	Nuclear magnetic resonance (NMR)
	Fourier transform infrared spectroscopy analysis (FTIR)
Drug loading	–
% Encapsulation efficiency	–
Drug release	Sample and separate (SS)
	Continuous flow (CF)
	Dialysis membrane (DM)
Antibacterial assays	Zone of inhibition plates (ZOI)
	Minimal inhibitory concentration test (MIC)
	Minimal bactericidal concentration test (MBC)
Cell viability	LDH test
	MTT assay
	MTS assay
Stability	–

nanomaterials, and surface morphology of manufactured particles at the micro- and nanoscales. A very energetic electron beam is used to explore objects on a very microscopic scale in order to learn more about nanomaterials. One can investigate the morphology of the particles and produce a histogram from the photographs by manually counting and counting the particles or by using specialized software (Patil and Chougale, 2021).

Transmission electron microscopy (TEM)

TEM is a helpful technique to obtain accurate measurements of particle and size distribution, morphology for nanomaterials. It is a major and widely utilized technology. TEM images allow one to see the form of synthesized NPs. The magnification is primarily determined by the ratio of the distances between the objective lens and the specimen and the objective lens's image plane.

In a conventional TEM, a thin specimen is subjected to an electron beam with a constant current density. The electron intensity distribution behind the item is magnified using a three- or four-stage lens system and is then shown on a fluorescent screen. A photographic emulsion or an image plate may be directly exposed in order to capture the image, or a CCD (Charge-Coupled Device) camera may capture the image digitally.

Higher spatial resolution and the ability to perform more analytical experiments are two benefits of TEM over SEM. Two limitations are a small sample section and high vacuum needs (Williams and Carter, 1996; Hall et al., 2007; Lin et al., 2014).

Dynamic light scattering (DLS)

Dynamic light scattering is a method that depends on the interaction of light and particles. This method is effective for determining narrow particle size distributions, especially in the range of 2−500 nm (Tomaszewska et al., 2013). DLS is the technique used the most frequently to characterize nanoparticles (Jans et al., 2009; Zanetti-Ramos; 2009).

To quantify the light scattered from a laser as it passes through a colloid, DLS largely relies on Rayleigh scattering from the suspended nanoparticles (Fissan et al., 2014). The fluctuation of scattered light intensity as a function of time can then be analyzed to determine the hydrodynamic size of the AgNPs (Koppel, 1972; Berne and Pecora, 2000; Dieckmann et al., 2009).

Fluorescence correlation spectroscopy (FCS)

FCS can generate quantitative data such as diffusion coefficients, hydrodynamic radii, average concentrations, and kinetic chemical reaction rates by fitting an appropriate autocorrelation analysis to the temporal fluorescent variation.

One significant advantage that FCS has over DLS or NMR is that it only needs a small amount of fluorescent probe particles at sub- to nanomolar concentrations. This allows it to monitor the probe particles, avoid medium interference, and probe AgNPs' sizes in the nanometer to hundreds of nanometer range (Magde et al., 1972; Krichevsky and Bonnet, 2002; Boukari and Sackett, 2008; Sapsford et al., 2011).

Optical analysis

By observing the transformation of the autolysat with AgNO3 from colorless to brown, UV-visible (UV) spectroscopy in the wavelength range of 200−800 nm, with a resolution of 1 nm, was employed to visually and spectroscopically monitor the synthesis of AgNPs (Wypij et al., 2021).

This is accomplished by counting the SPR frequencies of the solution's particle constituents. Mukherjee et al. claim that the strength of the peak obtained is connected to the quantity of nanoparticles present and that the breadth and height of the peak are directly proportional to the particle concentration. The quantity of particles in the test solution determines its height, and the range of the UV spectrum peak represents its dispersion (Mukherjee et al., 2008).

Surface properties

Among the different surface properties that are commonly viewed as significant aspects are surface composition, surface energy, wettability, surface charge, and species absorption or adhesion. Numerous characteristics of nanomaterial interfaces are caused by the atomic or molecular composition of the surfaces and the physical surface structures, which respond to interactions between the nanomaterial and the surrounding species (Sapsford et al., 2011).

Atomic force microscopy (AFM)

The size, shape, sorption, and structure of nanomaterials, as well as their dispersion and aggregation, are frequently examined using atomic force microscopy (AFM). Unlike electron or light microscopy, AFM enables high-resolution direct inspection of the object without the requirement for an incident beam. This surface imaging technique measures the force acting on a sample of living organisms at subnanometer resolution under physiological conditions by scanning a sharp tip over the sample's surface. The force acting on the tip as it is pushed toward and then pulled away from the sample is measured using the pico newton (10−12 N) sensitivity. There are three distinct scanning modes: contact mode, noncontact mode, and intermittent sample contact mode (Patri et al., 2006; Picas et al., 2012; Lin et al., 2014).

Zeta potential

The zeta potential of the nanoparticle is commonly used to explain the surface charge characteristics of nanoparticles. Nanoparticles with a zeta potential larger than (+/−) 30 mV have been shown to be stable in suspension due to the surface charge. The zeta potential can be used to detect the presence of an encapsulated charged active material or an adsorbate on the surface of nanoparticles (Zhang et al., 2016).

Crystallinity

X-ray powder diffraction (XRPD)

XRD is the fundamental technique for ascertaining the crystalline nature at the atomic level (Das et al., 2014). X-ray powder diffraction is a non-destructive technique with a lot of potential for characterizing both organic and inorganic crystalline materials. The guiding concept of X-ray diffraction is Bragg's law. For X-ray diffractography, wide-angle elastic scattering of X-rays serves as the usual basis (Waseda et al., 2011). Typically, diffracted beams come from the sample and show the physical, chemical, and structural properties of a powder specimen. Thus, XRD can be used to analyze the structural properties of a variety of materials, including inorganic catalysts, superconductors, biomolecules, glasses, polymers, etc. (Gill et al., 2010).

Thermal property

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) is a technique for thermal analysis that tracks the temporal variations in a sample's physical properties. Based on the difference in temperature between the sample and the reference material, DSC measures a heat quantity that is excessively radiated or absorbed by the sample during a temperature shift (Bothun, 2008).

The analysis of DSC measurements thereafter can disclose the structure and stability of the subject material (Pan et al., 2006). DSC can access material transitions such as melting, crystallization, glass transition, and decomposition of nanomaterial-bioconjugates.

Structure and composition

Nuclear magnetic resonance (NMR)

Dendrimers, polymers, and derivatives of fullerenes, as well as the conformational changes that occur during interactions between ligands and nanomaterials, have all been studied using NMR spectroscopy (Wang et al., 1999; Tomalia et al., 2003; Lundqvist et al., 2005).

Using specifically created radio frequency and/or gradient pulse sequences, NMR spectroscopy provides methods to analyze dynamic interactions of the species under a variety of situations, such as relaxation, molecule conformation, and molecular mobility. The structures and chemical compositions of the species are also assessed using NMR spectroscopy (Jiang et al., 2005).

Fourier transform infrared spectroscopy analysis (FTIR)

Fourier transform infrared (FTIR) spectroscopy is frequently utilized for nanomaterial applications in order to reveal nanomaterial-biomolecule conjugation, such as proteins connected to NP surfaces, and to display the conformational states of the bound (Shang et al., 2007; Tom et al., 2007; Baudot et al., 2010; Perevedentseva et al., 2011).

The study of nanoscaled objects has become a new application area for FTIR spectroscopy analysis, a method based on infrared spectroscopy, in the last 10 years. The various chemical bonds that are present in a substance can be seen using infrared FTIR spectroscopy (Patel et al., 2013).

Drug loading

High drug loading capacity is one of the most desired properties of a successful nanoparticle. The large loading capacity of NPs reduces the quantity of polymer carriers required for the administration of a vaccination or treatment inside the body (Liu et al., 2020). 45 Drug loading is an important consideration while making drug-loaded nanoparticles. Drug loading is governed by the following equations (Manjunath et al., 2005).

$$\text{Drug Loading} = \frac{\text{mass of the drug}}{\text{total mass of the drug loaded nanoparticles}} = \frac{(\text{w})\text{drug}}{(\text{w})\text{drug} + (\text{w})\text{nanocarrier}}$$

% Encapsulation efficiency

The amount of free drug that is present in the dispersion medium can be utilized to determine how well the system entraps drug. The dispersion media can be separated using centrifugation. Encapsulation efficiency is governed by the following equation (Savaser et al., 2018).

$$\text{Entrapment Efficiency} = \frac{(\text{wt.of the drug in system} - \text{wt.of drug in aqueas phase})}{\text{wt.of drug in system}}$$

Drug release

The drug release mechanisms play a key role in the creation of drug polymers. They affect the effectiveness of the advised application and keep drug delivery successful.

Based on the physical or chemical characteristics of a polymer, drug release mechanisms from polymer matrixes can be categorized into three main groups: (1) drug diffusion from a nondegraded polymer (diffusion-controlled system); (2) enhanced drug diffusion caused by polymer swelling (swelling controlled system); and (3) drug release caused by polymer degradation and erosion (erosion-controlled system) (D'Souza et al., 2014).

The behavior of dose forms as well as the safety and effectiveness of a product can both be determined by looking at the drug release kinetics. To assess drug release from nano-sized dosage forms, one of the three categories of techniques listed below, namely sample and separate (SS), continuous flow (CF), and dialysis membrane (DM), can be utilized. Recent reports also mention devices that integrate the SS and DM or CF and DM concepts (Chitra et al., 2014).

Antibacterial assays/quantification of antibacterial activity

Antibacterial activity is discovered using zone of inhibition plates, the minimal inhibitory concentration test, and the minimal bactericidal concentration test.

Zone of inhibition (ZOI) plates

It is a test to determine how sensitive a microorganism is to antibiotics. Antibiotic-loaded agar plates are then filled and left to incubate. The growth inhibition or bacterial destruction is identified by looking at the empty zone surrounding the antibiotic after the incubation period (Wiegand et al., 2008).

Minimal inhibitory concentration (MIC)

The substance that prevents bacterial development is present in the smallest amount. In order to estimate MIC, solutions of various concentrations are incubated with bacteria. The outcomes are then evaluated using an agar dilution or a broth microdilution. Some advantages include small-scale experiment applicability, low preparation required, relatively straightforward preparation and execution, and possibly short test turnaround times (Crisan et al., 2021).

Minimal bactericidal concentration (MBC)

Bacteria can be killed by substances in even the tiniest quantities. A procedure similar to that used to evaluate MIC is utilized to determine MBC, resulting in a methodology that has several advantages. It makes it possible to determine the minimum agent concentration needed to kill bacteria and can be used to rank antimicrobial agents based on their effectiveness (Mahmoudi et al., 2012).

Cell viability

Cytotoxicity assays come in two different varieties: in vivo and in vitro research. While in vivo toxicity tests (cell-based assays) are time-consuming, expensive, and fraught with moral ambiguity, in vitro toxicity tests (cell culture-based assays) are more expedient, useful, affordable, and devoid of such complications. Due to these advantages, in vitro experiments are the method of choice for assessing the toxicity of the majority of nanomaterials (Korzeniewski and Callewaert, 1983).

The three in vitro methods that are most frequently used to assess the cytotoxicity of nanoparticles are LDH, MTT, and MTS tests.

LDH test

The LDH test, which measures the quantity of LDH, a marker of cell membrane integrity released into the culture media by wounded cells, is frequently a colorimetric assay. Cellular toxicity can be evaluated using this assay in a quick, simple, and accurate manner (Meerloo et al., 2011).

MTT assay

The MTT assay is an additional possible technique to detect the cytotoxicity of NPs. MTT, also known as 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is a yellow substance that is converted into purple, insoluble formazan crystals by mitochondrial succinate dehydrogenases in live cells. This process is directly impacted by the number of viable cells (Malich et al., 1997).

MTS assay

In the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) experiment, tetrazolium salt is converted into a vibrant soluble formazan product by live cells using mitochondrial dehydrogenase enzymes. In reality, the MTS assay generates a colorimetric product, just like the MTT assay. The amount of living cells in the culture has a direct relationship with the amount of formazan produced (Reidy et al., 2013).

Stability

Given that one of the main contributors to silver toxicity is silver ions, the stability of silver nanoparticles significantly affects how poisonous they are. Additionally, it was claimed that the aggregation of silver nanoparticles affected their toxicity. Numerous factors, such as ionic strength and composition, pH, dissolved organic matter, ambient humidity, dissolved oxygen concentration, temperature, size, shape, and coating as well as their concentration, all have an impact on the durability of nanoparticles (Tarannum and Gautam, 2019). Stability study should be performed as per ICH Guideline.

Application of silver nanoparticles

The development of nanoparticles is quick, cheap, and eco-friendly. To create the stable nanostructures required for medical applications, the nanoparticles are coated with capping agents derived from natural substances, obviating the need for the postsynthetic coating step necessary in the case of chemical synthesis. AgNP capping agents, which have a wide range of medical applications, enable the surface modifications and attachments of other molecules (Mohanpuria et al., 2008; Gurunathan et al., 2014; Salomoni et al., 2015; Wypij et al., 2021).

Antibacterial

AgNPs have effectively suppressed *Staphylococcus aureus* (Rónavári et al., 2018), *Pseudomonas aeruginosa* (Abbaszadegan et al., 2015), and other hazardous bacteria, fungi, and viruses. The antibacterial effect of AgNPs affects microorganisms in many ways. Gram-negative bacteria are more sensitive to AgNPs than Gram-positive bacteria (Agnihotri et al.,

2014). AgNPs' antibacterial activity increases significantly as their particle size decreases. AgNPs have more antibacterial activity, especially when they are smaller than 10 nm (Jiraroj et al., 2014). Increasing the AgNPs treatment time can significantly boost the antibacterial effect (Hong et al., 2016). The most effective antibacterial action is seen for AgNPs with a spherical form. This phenomenon may lead to increased antibacterial activity in AgNPs with higher surface to volume ratios, which are associated with more effective contact and larger reaction surfaces (Mandal et al., 2016). AgNPs may function more effectively against bacteria if their surface charges are altered. AgNps stabilized by various polymeric systems have demonstrated enhanced antibacterial activity against the gram-negative pathogens *P. aeruginosa* and *E. coli* when compared to unstabilized AgNps. The costabilization of the bioactive copolymer pluronicTM F68 has significantly enhanced the antibacterial activity against both microbes (dos Santos et al., 2012; Mallmann et al., 2015).

Antifungal and antiviral

AgNPs been proven to have antibacterial effects on both bacteria and fungi. AgNPs that have been reduced with ribose and stabilized with sodium dodecyl sulfate (SDS). The effectiveness of these particles against *Candida tropicalis* and Candida albicans was also tested (Mohammed et al., 2020). In many different uses, especially as an antifungal, antiviral, and antibacterial action, it is thought to be cost-effective. The fungus was successfully used to create silver nanoparticles, which then showed antifungal activity when beginning with $AgNO_3$. It has been discovered that the use of NPs in treating bacterial, viral, and fungal infections in humans is effective and promising (Barbosa et al., 2019).

Anthelamic

Most anthelmintic drugs used to treat parasitic worm infections either target certain proteins or regulate muscle and neuronal electrical activity. Some D. flagrans extract has the ability to generate AgNP, which has a nematicidal impact (Ghareeb et al., 2022). Silver nanoparticles produced from the brown alga Colpomenia sinuosa had a greater nematicide activity (Gomes et al., 2021).

Anticancer application

Silver nanoparticles are being investigated more and more as a result of their distinctive physical, chemical, and optical characteristics, which allow them to be employed in a range of applications, such as the delivery of drugs to a particular target in the body (Ma et al., 2017). A system that integrates cancer detection and treatment modalities has been developed using nanoparticles (Patra et al., 2018). The initial generation of nanoparticle-based therapy utilized lipid systems that have currently received FDA approval, such as liposomes and micelles (Park et al., 2006). These liposomes and micelles might also include gold or magnetic nanoparticles (da Silva et al., 2019). In contrast to standard anticancer medications, MNPs can be used as novel therapeutic agents or drug carriers in combination with therapy candidates, preventing adverse side effects.

AgNPs have been reported to have effective anticancer action in cases of breast cancer (Gurunathan et al., 2015), colon cancer (Gurunathan et al., 2018), ovarian cancer (Yuan et al., 2017), pancreatic ductal adenocarcinoma (Zielinska et al., 2018), lung cancer (Fard et al., 2018), and other cancers. For ex, according to research, the globular B. tequilensis and *C. indica* AgNPs (multishaped, especially rod). The apoptotic potential of these two types of AgNPs was studied in human breast cancer cells. The findings of various biochemical and cellular tests showed that both B-AgNPs and F-AgNPs exhibited significant cytotoxic effects on breast cancer cells (Gurunathan et al., 2015). Some recent scientific research have focused on the use of AgNPs in combination with anticancer pharmaceutical medications in an effort to boost antineoplastic efficacy, particularly when utilized synergistically with natural anticancer agents used in their manufacture (Green-Chemistry approaches). This is despite the fact that AgNPs are extensively used in in vitro studies using various cancer cell models (due to their intrinsic anticancer effect) (Mihai et al., 2019).

Other medical application

Wound healing and infection control

The traditional way of treating wounds uses topical medicines like antibacterial or colloidal chemicals to lower the risk of infection and promote the healing process (Mihai et al., 2019). Because they heal quickly, burns, other serious wounds, and surgically created wounds are all regarded as acute wounds (Li et al., 2007). Antimicrobial wound dressings have developed into useful substitutes in recent years for reducing bacterial colonization and infection in wounds (Negut et al.,

2018). While *Pseudomonas aeruginosa* and *Escherichia coli* are typical of chronic wounds and infect deeper skin layers, *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) are the most commonly detected, affecting the early stages of wound healing (Mihai et al., 2014).

Bone healing

Bone repair is a growing field of knowledge for orthopedic surgery. Technologies for bone healing are required, and they must resemble bone in terms of biochemistry and structure. Nanotechnology might offer a solution as hydroxyapatite and nanoscale collagen fibers make up bone (Brannigan and Griffin, 2016). In orthopedic traumatology and bone healing, numerous nanometer-sized entities, structures, surfaces, and devices with typical lengths ranging from a few nanometers to a few micrometers are used (Harvey et al., 2010). An ideal scaffold for bone tissue engineering applications should be biocompatible, produce breakdown products that are nontoxic, and be simple to be excreted by the body in addition to serving as a 3D template for both in vitro and in vivo bone production (Stylios et al., 2007).

Dental applications

Dental innovations and diagnosis make use of nanostructures. Some nanoparticles are utilized in prosthetics, dental implants, and drugs that fight oral disorders. Furthermore, nanoparticles deliver oral fluids or drugs, preventing and treating various oral disorders and substantially preserving oral health (Priyadarsini et al., 2018). Nanomaterials show significant promise for improving the health of dental patients, and nanotechnology advancements are paving the way for the future of dentistry (Khurshid et al., 2015).

Vaccine adjuvant

The term "nano-adjuvant" is used to characterize nanoparticles (NPs), which can act as adjuvants for vaccinations (NA). NPs can either encapsulate or adsorb the vaccination antigen or DNA in a suitable formulation, improving stability, cellular absorption, and immunogenicity (Garg and Dewangan, 2020). When compared to unconjugated antigens, vaccine components can be delivered via nanocarrier-based delivery systems, which also increase cellular absorption and trigger potent innate, humoral, cellular, and mucosal immune responses (Pati et al., 2018).

Biosensing and imaging

Genome analysis, the food and beverage industry, environmental protection, and healthcare are just a few of the industries that use enzyme, tissue, immuno-sensors, DNA, thermal, piezoelectric, and radiofrequency biosensors in a range of applications (Shrestha, 2022).

Antibacterial mechanism of silver nanoparticles

Conventional broad-spectrum antibiotics have been widely used to inhibit bacterial infections. Although, these antibiotics are ineffective to inhibit multidrug-resistant bacterial strains. This is because such bacteria are getting more resistant to bactericidal action of antibiotic molecules. On that account, it's a need to develop some new formulation that work against multidrug-resistant strains (Nikolelis and Nikoleli, 2018). Due to the large surface area, AgNPs exhibit wonderful antibacterial activity. However, AgNPs are more effective against gram-negative bacteria than gram-positive because gram-positive bacteria have one cytoplasmic membrane and a relatively thick cell wall of 20−80 nm with several peptidoglycan layers which act as a natural barrier and prevent penetration of nanoparticles, while gram-negative bacteria have an external layer of lipopolysaccharide, middle thin layer of peptidoglycan and an innermost plasma membrane as well its negative charge accelerate the adhesion of AgNPs (Liao et al., 2019). The antibacterial activity of AgNPs also depends on the shape, size, charge, and dose of AgNPs.

Antibacterial mechanism of nanoscale silver is not exactly clear but it involved the following one or more mechanisms.

The first one is AgNPs having more surface area and continually release silver ions, nano silvers are able to penetrate the outer membrane of cell wall, accumulating in the inner membrane can cause cell membrane denaturation and afterward change the structure of the cell membrane. The denaturation of cytoplasmic membrane can rupture organelles, and even result in cell lyses (Perni et al., 2014; Slavin et al., 2017). AgNPs also interact with sulfur-containing proteins in the cell wall of bacteria; an interaction with protein may cause structural damage leading to cell wall rupture. Outer surface

damages and dense cavities on the cell surface can be visualized by advanced imaging techniques such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), and AFM (Gopinath et al., 2017).

The second proposed mechanism for antimicrobial activity of AgNPs is based on silver ion release from the nanoparticles, which has an adverse effect on both DNA and proteins.AgNPs can also enter the cell and interact with sulfur or phosphorus groups, present in intracellular content such as DNA and proteins altering their structure and functions. Intracellular effect of AgNPs is not limited to DNA, it also interacting with thiol groups of protein and respiratory enzymes and enzymes will deactivate interrupt intracellular O_2 reduction, inducing reactive oxygen species and free radicals generating that lead to damage to intracellular cells, activating the apoptosis pathway and terminate the cell multiplication.

Another mechanism that is proposed to occur in parallel with the earlier is the released of silver ions can interact with cellular components altering metabolic pathways, membranes, and even problems in DNA replication (Satishkumar et al., 2012). Silver ions can also inhibit the synthesis of proteins by denaturing ribosomes in the cytoplasm. In addition, silver nanoparticles can be involved in bacterial signal transduction. Bacterial signal transduction is affected by phosphorylation of protein substrates, and nanoparticles can dephosphorylate tyrosine residues on the peptide substrates (Duran et al., 2016; Ramkumar et al., 2017).

Toxicity of silver nanoparticle

Silver nanoparticles are very effective antimicrobial agents while being nontoxic to mammals. However, numerous studies stated that AGNPs causes destruction or imbalance in ecosystem release posing toxic impacts on plants, algae, and microorganisms. There is a need to understand the toxicity of AgNPs at the cellular level of these organisms and their further impacts. Study results do not conclude on the effective/lethal/sublethal/optimum concentrations of AgNPs organism-wise, in this context some regulatory measures can be made. As AgNPs are highly used in household, industry, and biomedical products, they enter into the body via ingestion, inhalation, dermal contact and, directly in systemic circulation via parental route (De Matteis, 2017). Due to exposure to AgNPs, they accumulate in the body and causes chronic toxicity, leads to trigger the immune system and inflammatory action.

In vitro studies have demonstrated the toxic effects of Ag NPs on rat liver (BRL3A) and neuronal cells, human lung epithelial cells, and murine stem cells (Stensberg et al., 2011). In vitro cytotoxicity characterized by oxidative stress, DNA damage and modulation of cytokine production, stimulate the production of radical oxygen species (ROS), resulting in oxidative stress and genotoxicity leading to cell necrosis and cell death (Hussain et al., 2005; Asharani et al., 2009).

AgNPs causes phytotoxicity in plants to a great extent which can be observed variably by analyzing different physical, physiological, biochemical, and structural traits (Tripathi et al., 2017). They damage the cell membranes; interrupt ATP production as well as DNA replication. The increase in the production of ROS and subsequent generation of oxidative stress lead to various toxic impacts and may also affect the gene expressions and damage to DNA (Yin et al., 2012). Silver nanoparticle increases the production of hydrogen peroxide (H2O2) in the plant cells and it affects the growth and development of the plants and causes cell lysis. The toxicity of AgNPs can be seen from the seedling growth stage up to full developed stage of the plants.

Compared with in vitro studies, significantly less information is available on the potential mechanisms of toxicity of Ag NPs from in vivo studies. Exposure of laboratory rodents to Ag NPs has resulted in a myriad of toxicological responses, including effects on circulatory, respiratory, central nervous, and hepatic systems. Effects on dermal tissues have also been reported after topical administration of Ag NPs (Hadrup et al., 2018).

Neil et al. concluded in their review that, a case of mortality was observed at intrauterine exposure to ionic silver at 64 mg/kg bw. Localized argyria has been reported with exposure to silver ions, metallic surfaces, and nanocrystalline silver. Generalized argyria was observed with ionic and nanocrystalline silver in humans at cumulative doses in the range of 70−1500 mg silver/kg body weight. Silver is observed to have a low potential for skin irritation. Eye irritation and some cases of allergic contact dermatitis have been reported. Silver may cause genotoxicity. Other reported toxicities include hepatic, renal, neurological, and hematological effects (Hadrup et al., 2018).

Majority information about the mechanisms of Ag NPs toxicity is found in in vitro studies, very rare in vivo studies reported three main mechanisms of toxicity of Ag NPS have been proposed: oxidative stress, DNA damage, and cytokine induction. There is a need to explore the toxicity effect of leached Ag NPs from commercial products.

Conclusion

Green chemistry has emerged as a cutting-edge idea for the creation and application of chemical processes to reduce or eliminate the use of hazardous materials. The potential of silver nanoparticles as antibacterial agents is enormous. Although

silver's antibacterial capabilities have long been recognized, the development of nanotechnology has made it possible to fully utilize this feature. Due to their powerful antibacterial activity, silver ions and silver compounds have been used as antimicrobial agents for decades in a variety of industries. Keeping all the potential benefits of silver nanoparticles in mind, there is an active need to encourage pharmaceutical industries and other related laboratories to explore the avenue of biological synthesis of silver nanoparticles.

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Chapter 10

Evaluation of antimicrobial, antioxidant, and photocatalytic activity of zinc oxide nanoparticles synthesized from *Parmotrema perlatum*

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Introduction

Nanoparticles provide great benefits for mankind and society, giving rise to "nanomedicine." Nanomedicine is blend research area of biology, chemistry, and medicine, and it possibly alters the ways to treat diseases through novel and highly developed therapeutic and diagnostic methods. It deals with design and development of various types of novel products (Seleci et al., 2016; Zhu et al., 2019). Nanoparticles have small size, which increase surface to volume ratio; these properties enhance their functional capability as compared to their bulk materials. Over a last decade, nanoparticles have emerged as a novel drug molecule, which have various application because of their unique structural, physical, electronic, chemical, optical, and catalytic properties in various fields such as pharmaceutical, medical, biotechnology, catalysis, drug delivery, bioengineering, and photo-electrochemical (Chaudhury et al., 2014). Synthesis of nanoparticles using various metal like gold, silver, copper, zinc, iron, platinum etc. is an active area of analysis in materials science.

Metal nanoparticles are synthesized by different technique like physical, chemical, and biological. But, biosynthesis of nanoparticles by using various biological systems like plant, bacteria, and algae has been increased in last few years. These biological systems have significant interest mainly because of being nontoxic, cheap, an easy process parameter, and eco-friendly compared to traditional methods. Various organic phytochemical and metabolites from plants are considered as significant sources for the synthesis of the nanoparticles, which act as both the reducing and stabilizing agent (Behzad et al., 2021; Jeevanandam et al., 2022).

Zinc oxide nanoparticles have great interest because of its wide range of application as in ultraviolet lasers, gas sensors, semiconductors, electric and piezoelectric devices, catalysts, solar cells, and pigments. Zinc oxide nanoparticles are believed to be nontoxic, biosafe, biocompatible, and possess potential applications and are used in the form of powders, antiseptic creams, surgical tapes and shampoos, to relieve skin irritation, diaper rash, dry skin, and blisters (Mirzaei and Darroudi, 2017; Bandeira et al., 2020; Barman et al., 2020).

Lichens are mutualistic relations between fungi and algae. Several exclusive extracellular secondary metabolites are formed as a result of this association in these microorganisms, which comprise amino acid derivatives, sugar alcohols, aliphatic acids, macrocyclic lactones, mono-cyclic aromatic compounds, carotenoids, xanthhones, dibenzofuranes, depsides, chromones, depsones, terpenoids, quinones, steroids, depsidones, and diphenyl ethers (Díaz-Reinoso et al., 2021). Some phytochemical produced by lichens are physically and chemically similar to broad-spectrum antibiotics. Lichens possess many kind of important biological exploit like antibacterial, antifungal, antioxidant, antiviral, and antigenotoxic (Ranković and Kosanić, 2021; Anar et al., 2016).

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The aim of present work was to evaluate antimicrobial, synergistic antimicrobial, antioxidant, and photocatalytic activity of zinc oxide nanoparticles from *P. perlatum* lichen.

Materials and methods

Chemicals

All the chemicals, solvents, and reagents used in the research were of scientific grade and were used without any further purification. All chemicals were prepared freshly using distilled water and stored in the dark to avoid any photochemical reactions. All glassware used in the research were thoroughly washed with distilled water. Zinc nitrate hexahydrate ($Zn(NO_3)_2$) were purchased from Hi media India, respectively. Distilled water was used throughout in all the experiments.

Preparation of lichen extract

For the synthesis of ZnONPs, 5 g of dry *P. perlatum* powder was mixed with 100 mL distilled water and boiled it on a magnetic stirrer heater at a temperature of 80°C for 60 min. The extract was cooled to room temperature and filtered by Whatman filter paper. The filtrate was used for the synthesis of ZnONPs.

Synthesis of ZnONPs

50 mL of *P. perlatum* extract boiled for 60 min was taken in a beaker and heated at 80°C on a magnetic stirrer heater. Five grams of zinc nitrate hexahydrate ($Zn(NO_3)_2$) (0.8 mM) was added to the *P. perlatum* extract when the temperature reached 80°C. The mixture was heated until it was reduced to a deep yellow colored paste. The paste was collected in a ceramic crucible and heated in a Muffle Furnace at 400°C for 2 h. A light yellow color powder of ZnONPs was obtained, which was carefully collected and stored at 4°C for further analysis (Padalia and Chanda, 2017).

Characterization of the synthesized ZnONPs

UV-vis spectroscopy

Synthesis of nanoparticles is initially observed by ultraviolet-visible (UV-vis) spectroscopy. The reduction of the metal ions in solution was observed by UV-vis spectra of the solution. UV-vis spectra was monitored on a spectrophotometer (Analytik Jena, Specord 200 Plus) in 300−700 nm range operated at an interval of 10 nm.

Fourier transforms infrared spectroscopy

Possible functional groups involved in the synthesis and stabilization of nanoparticles were studied by FITR spectroscopy. Nanoparticles were mixed with potassium bromide (KBr) and pressed into a pellet. The pellet was placed into the sample holder and spectra were observed in wavelength region 400−4000 cm^{-1} Nicolet IS10 (Thermo Scientific, USA). The different modes of vibrations were recognized and assigned to determine the various functional groups in the ZnONPs.

Transmission electron microscopy

TEM analysis was done to determine the shape as well as size of the bio-synthesized zinc nanoparticles. The sample was dispersed in double distilled water. A drop of thin dispersion nanoparticles was positioned on a "staining mat." Carbon-coated copper grid was introduced into the drop with the coated side upward. After about 10 min, the grid was removed, air dried, and then analyzed in transmission electron microscope (JEOL JEM 2100).

Biological activity ZnONPs

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of ZnONPs was determined by Resazurin microtiter plate method (Palomino et al., 2002) against *Bacillus cereus* (BC) (MCC 2240), *Staphylococcus aureus* (SA) (MCC 2043), *Staphylococcus epidermidis* (SE) (MCC 2044), *Bacillus subtilis* (BS) (MCC 2244), *Escherichia coli* (EC) (MCC 2246), *Klebsiella*

pneumoniae (KP) (MCC 2716), *Serratia marcescens* (SM) (MCC 2689), *Klebsiella Sp* (KS) (MCC2103), *Candida albicans* (CA) (ATCC2091), *Candida glabrata* (CG) (NCIM3448), and *Cryptococcus neoformans* (CN) (ATCC34664). MIC test was performed in 96 well microtiter plates. 20 μL of nanoparticles and added into the well along with 150 μL broth and 30 μL of microbial culture at a density of 6×10^5 CFU/mL was added to the wells. Antibiotics were used as positive controls. For bacteria plates were incubated 37°C for 24 h and for fungi 28°C for 48 h. Add 50 μL of 0.01% w/v of Resazurin in each well to observe the microbial activity. Plates were further incubated for 2 h, and the color change was observed from blue to pink, which indicates microbial growth and determines MIC values.

Synergistic antimicrobial activity

Agar disc diffusion method was used to evaluation of synergistic effect of synthesized ZnONPs alone, antibiotics alone, and ZnONPs plus antibiotics against selected microorganism. Antibiotics used in the study were penicillin (P), streptomycin (S), tetracycline (TE), chloramphenicol (CH), and gentamicin (GEN).

Synergistic effect of ZnONPs with all antibiotics was evaluated by using agar disc diffusion method (Rakholiya and Chanda, 2012). 20 mL of molten brain heart infusion agar inoculated with 200 μL test culture containing 1×10^8 cfu/mL in Petri plates. Plates were allowed to solidify. 20 μL of ZnONPs (5 mg/mL) dissolved in DMSO was added to the standard antibiotics paper discs and sterile paper discs (for control) and allowed to saturate for 30 min. The saturated discs were placed on the surface of the Muller-Hinton agar plates, which had earlier been inoculated with test microorganisms. All plates were incubated for 24 at 37°C for bacteria Results were observed by determining the zone of inhibition appearing around the discs.

Increase in fold area

Increase in fold area (IFA) was calculated by using the formula:

$$(B^2 - A^2) / A^2$$

Where, B, zone of inhibition ZnONPs + antibiotics and A, zone of inhibition of antibiotics.

Determination of 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity

The free radical scavenging activity of ZnONPs was measured by using DPPH by the modified method of (McCune and Johns, 2002). The reaction mixture consists of a 1.0 mL methanol, 1.0 mL of different concentrations of ZnONPs, and 1.0 mL DPPH (0.3 mM). Reaction mixture was incubated in dark condition for 10 min, and the absorbance was measured at 517 nm. Ascorbic acid was used as standard. Percentage of inhibition was determined using the formula: % Inhibition = $[1-(A/B)] \times 100$.

Where, A is OD of the sample, B is the OD of the blank.

Photocatalytic activity

The photocatalytic activity of ZnONPs was detected under UV-irradiation and sun light in terms of degradation of dye. Mercury lamp with 300 W high-pressure (wavelength 365 nm) was used as source of UV-irradiation (Desai et al., 2021) and sun light also. Photoreactor cell was filled with methylene blue, Malachite green, methyl red (100 mM) solution, and subsequently ZnONPs (10 mg) were then dispersed in the solution. The dispersion was kept in dark conditions for the establishing adsorption−desorption equilibrium. UV-vis spectrophotometer (UV-1601 pc Shimadzu) was used to monitor the dye concentration after an irradiation for fixed time intervals (Tripathi et al., 2014). In order to check the photocatalytic activity, ZnONPs were collected by centrifugation and supernatant were used for determination of photocatalytic activity.

Results and discussion

Synthesis of ZnONPs

Synthesis of ZnONPs was carried out by addition of zinc nitrate to *P. perlatum* extract, reaction mixture turned to deep yellow color paste. The paste was further heated in a Muffle Furnace at 400°C for 2 h. A light yellow color powder of ZnONPs obtained indicated formation of ZnONPs (Fig. 10.1).

FIGURE 10.1 Synthesis of ZnONPs using *P. perlatum* extract.

Characterization of ZnONPs

UV-vis spectroscopy

The UV-Vis spectrum of synthesized ZnONPs is given in Fig. 10.2. The ZnONPs showed their characteristic absorption maxima between 350 and 370 nm. The maximum and narrow absorption peaks were observed at 368 nm. Lu et al. (2019) reported green synthesis of ZnONPs by *Codonopsis lanceolate* root extract, and synthesized ZnONPs shows maximum absorption peak at 356 nm which is due to surface plasmon resonance.

Fourier transform infrared spectroscopy

FTIR spectrum of synthesized ZnONPs was determined in the region of 500−4000 cm^{-1} is given in Fig. 10.3. FTIR spectra of synthesized ZnONPs showed intense peak at 3541.42, 2345.52, 1427.37, 1365.65, 1180.47, 979.87, 879.57, 802.41, 732.97, 686.68, 532.37, 493.79, 447.50, and 408.92 cm^{-1}.The absorption band at 3541.42 cm^{-1}relates to O−H stretching of alcohol or phenol group. The absorption band at 1427.37 cm^{-1} and 1365.65 cm^{-1}relates to vibrations of O−H stretching ofcarboxylic acid of proteins and C−H stretching of alkynes, respectively. The absorption band at 979.87 and 879.57 cm^{-1}relates to C=C stretching of alkene. The peaks at 532.37 and 447.50 cm^{-1}relate to ZnONPs formation. Vasantharaj et al. (2021) reported that different functional groups of *Ruellia tuberosa* extract was responsible for the capping, reduction and formation of ZnONPs. Qualitative phytochemical analysis of *P. perlatum* revealed presence of different phytochemicals like flavonoids, saponins, cardiac glycoside, and triterpenes, tannins, etc (Table 10.1). FTIR spectrum suggested that ZnONPs synthesized using *P. perlatum* extract are capped by various phytochemicals, which act as stabilizing and reducing agents for nanoparticles.

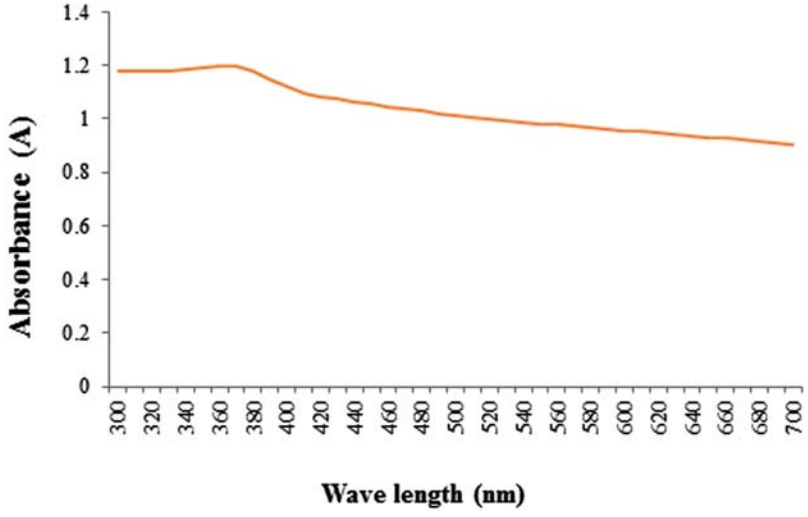

FIGURE 10.2 UV-visible spectroscopy of ZnONPs.

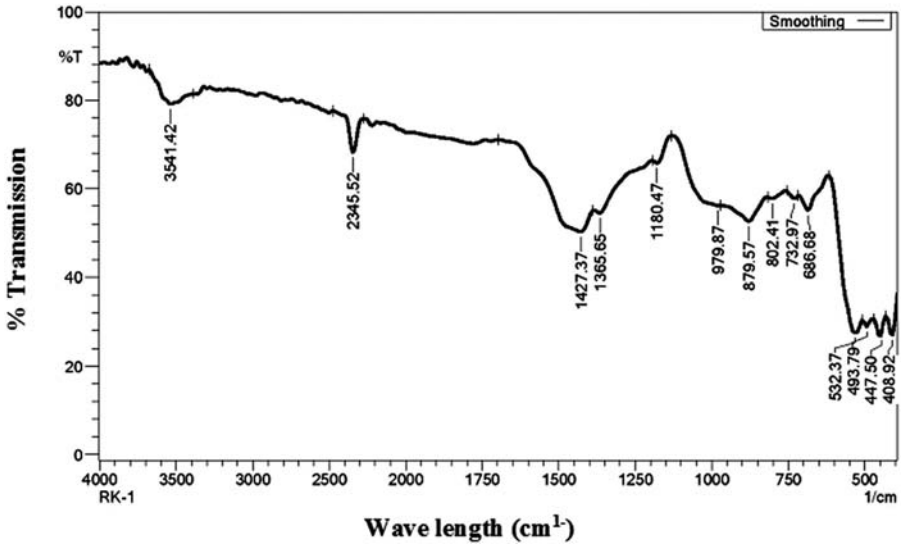

FIGURE 10.3 Fourier transform infrared (FTIR) spectrum of ZnONPs.

TABLE 10.1 Qualitative phytochemical analysis of dried powder of *P. perlatum*.

No.	Test	*P. perlatum*
1.	Flavonoids	++
2.	Triterpenes	+++
3.	Tannins	++
4.	Saponins	+++
5.	Cardiac glycoside	+++
6.	Phlobatanins	–

Transmission electron microscopy analysis

The size and shape of the ZnONPs characterized by TEM analysis are given in Fig. 10.4. The TEM image showed the size of ZnONPs between the 40–70 nm, and average size of nanoparticle was 51.21 nm. Shape of synthesized ZnONPs was spherical and irregular. Fowsiya et al. (2016) reported ZnONPs synthesized using *Carissa edulis* extracts, which revealed the size of ZnONPs ranging from 50 to 55 nm.

Biological activity zinc oxide nanoparticles

Determination of minimum inhibitory concentration

The synthesized ZnONPs were tested for antimicrobial activity at various concentrations (10–0.019 mg/mL) against selected microorganisms. The MIC values are given in Fig. 10.5. All four Gram-positive bacteria were inhibited by ZnONPs but higher MIC values. *S. aureus* was the most susceptible bacterial pathogen (MIC: 5 mg/mL). ZnONPs inhibit all four Gram-negative bacteria at lower MIC values as compared to Gram-positive bacteria. ZnONPs inhibit *E. coli* at lower MIC values 0.156 mg/mL. ZnONPs showed good antimicrobial activity against fungi at lower MIC vales as compared bacteria.

The MIC values of ZnONPs for *C. albicans, C. glabrata,* and *C. neoformans* were 0.156 mg/mL. The results show that ZnONPs showed strong antimicrobial effect against Gram-negative bacteria and fungi. In this study, ZnONPs showed strong antimicrobial effect against fungi when compared with bacteria. Among the bacteria, a higher antimicrobial activity was observed in Gram-negative bacteria when compared with Gram-positive bacteria.

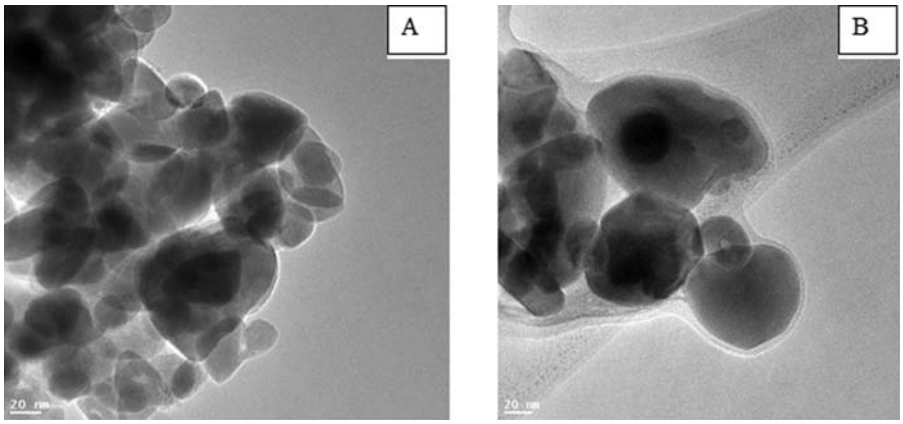

FIGURE 10.4 Transmission electron microscopy (TEM) image of ZnONPs.

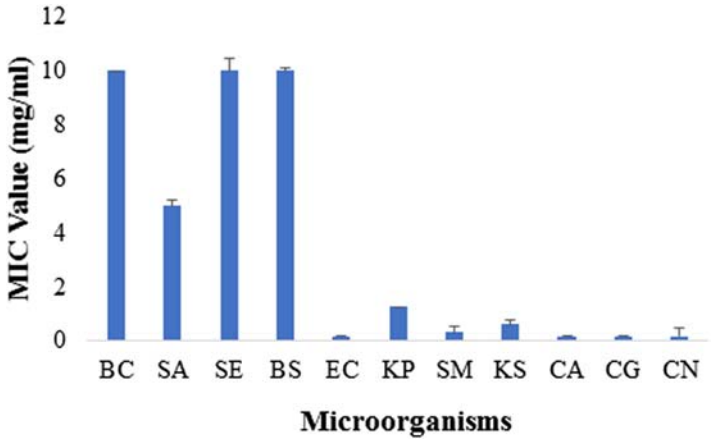

FIGURE 10.5 Minimum inhibitory concentration (MIC) values (mg/mL) of ZnONPs.

Similarly, zinc oxide nanoparticles synthesized using *Cassia fistula* and *Melia azedarach* showed significant antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* (Naseer et al., 2020). Vijayakumar et al. (2017) also reported green synthesis of ZnONPs using aqueous leaf extracts of *Plectranthus barbatus,* and antibacterial results shows that ZnONPs exhibited good antibacterial activity against *B. subtilis*, *V. parahaemolyticus,* and *P. vulgaris.* Azizi et al. (2016) reported green synthesis of ZnONPs using *Citrullus colocynthis* extracts and evaluated antimicrobial activity, results show that ZnONPs inhibited the growth of pathogenic bacteria like *Peseudomonas aeruginosa, Bacillus subtilis*, *Staphylococcus aureus,* and *Escherichia coli.* So, results suggested that use of *P. perlatum* synthesized ZnONPs can be more economic against pathogenic microorganisms. This is might be due to ZnONPs high surface to volume ratio and small size to interact with cell wall of microorganisms and exhibited inhibitory effect.

Synergistic antimicrobial activity of ZnONPs and antibiotics

Synergistic antimicrobial activity of ZnONPs and antibiotics (penicillin (P), streptomycin (S), tetracycline (TE), chloramphenicol (CH), and gentamicin (GEN)) is shown in Tables 10.2 and 10.3. Synergistic antibacterial activity of ZnONPs and antibiotics alone and their mixture evaluated against *B. cereus, S. epidermidis, B. subtilis, S. aureus, E. coil, K. pneumonia, S. marcescens, and Klebsiella sp.*, and increase in fold area was calculated.

Synergistic activity, i.e., increase in fold area of synthesized ZnONPs with antibiotics against Gram-positive bacteria, is given in Table 10.2. It was observed that S had the highest increase in fold area 1.25 against *B. subtilis.* P, TE, CH, and GEN had the highest increase in fold area 0.89, 0.56, 0.32, and 1.04 against *S. epidermidis, S. aureus, S. epidermidis,* and *S. epidermidis* respectively. Among all the five antibiotics tested, ZnONPs plus GEN antibiotics showed more synergistic activity than antibiotics alone against all for Gram-positive bacteria.

TABLE 10.2 Synergistic activity of ZnONPs with different commercial antibiotics against gram positive bacteria.

	B. subtilis			*B. cereus*			*S. epidermidis*			*S. aureus*		
Antibiotic	AB (a)	AB + ZnONPs(b)	IFA	AB (a)	AB + ZnONPs(b)	IFA	AB (a)	AB + ZnONPs(b)	IFA	AB (a)	AB + ZnONPs(b)	IFA
P	15	18	**0.44**	10	10	**0**	8	11	**0.89**	9	10	**0.23**
S	10	15	**1.25**	10	11	**0.21**	9	11	**0.49**	15	15	**0**
TE	22	25	**0.29**	18	21	**0.36**	18	20	**0.23**	20	25	**0.56**
CH	25	26	**0.08**	20	22	**0.21**	20	23	**0.32**	10	10	**0**
GEN	22	26	**0.39**	18	21	**0.36**	14	20	**1.04**	20	25	**0.56**

A, antibiotic; ***IFA***, increase in fold area. Increase in fold area was calculated as $(b^2 - a^2)/a^2$, where, **a**, the inhibition zones for antibiotics, **b**, the inhibition zones for antibiotics + ZnONPs respectively. All values are express in mm.
Antibiotics: Penicillin (P), Streptomycin (S), Tetracycline (TE), Chloramphenicol (CH), Gentamicin (GEN).

TABLE 10.3 Synergistic activity of ZnONPs with different commercial antibiotics against gram-negative bacteria.

	E. coli			*K. pneumoniae*			*S. marcescens*			*Klebsiella sp.*		
Antibiotic	AB (a)	AB + ZnONPs(b)	IFA	AB (a)	AB + ZnONPs(b)	IFA	AB (a)	AB + ZnONPs(b)	IFA	AB (a)	AB + ZnONPs(b)	IFA
P	12	13	**0.17**	24	26	**0.17**	–	–	–	17	17	**0**
S	11	15	**0.8**	7	7	**0**	8	11	**0.89**	16	16	**0**
TE	16	19	**0.41**	10	12	**0.44**	8	9	**0.26**	23	25	**0.18**
CH	21	26	**0.53**	16	17	**0.12**	10	15	**1.25**	30	30	**0**
GEN	11	8	**0.89**	10	10	**0**	9	12	**0.77**	26	26	**0**

AB, antibiotic; ***IFA***, increase in fold area. Increase in fold area was calculated as $(b^2 - a^2)/a^2$, where, **a**, the inhibition zones for antibiotics; **b**, the inhibition zones for antibiotics + ZnONPs respectively. All values are express in mm.
Antibiotics: Penicillin (P), streptomycin (S), tetracycline (TE), chloramphenicol (CH), and gentamicin (GEN).

Synergistic activity increase in fold area of synthesized ZnONPs with antibiotics against Gram-negative bacteria is given in Table 10.3. It was observed that Sand GEN had the highest increase in fold area 0.89 against *S. marcescens and E. coil,* respectively. P, TE, and CH had the highest increase in fold area 0.17, 0.44, and 0.53 against *E. coil, K. pneumonia, and E. coil,* respectively. Among all the five antibiotics tested, ZnONPs plus TE antibiotics showed more synergistic activity than antibiotics alone against all for Gram-negative bacteria. So our results show that ZnONPs combination with antibiotics exhibited enhance antimicrobial activity as compared to alone.

Reyes-Torres et al., (2019) reported synergistic effect of ZnONPs with antibiotics ampicillin against *E. coli, E. faecalis, P. aeruginosa,* and *S. aureus* and results shows that combination of the antibiotic with ZnONPs showed six-fold decrease for the strains of *E. coli, E. faecalis* and *S. aureus*, whereas that for *P. aeruginosa* a three-fold antimicrobial reduction activity was observed. Abo-Shama et al. (2020) reported synergistic effect of ZnONPs with antibiotics (azithromycin, oxytetracycline, cefuroxime, cefotaxime, fosfomycin, and oxacillin) against *E. coli* was significantly increased compared to antibiotic only. Ghosh et al. (2012) reported green synthesis of nanoparticles by using *Dioscorea bulbifera* tuber extract and also evaluated synergistic antimicrobial activity of nanoparticles combination with various antibiotics, results suggested that nanoparticles combination with antibiotic streptomycin showed highest 11.8-fold higher in zone diameter against *E. coli*. Several mechanisms proposed by different researcher for antimicrobial activity of ZnONPs like, hydrogen peroxide formation from ZnO surface, ROS and free radical generation, and electrostatic attraction of ZnONPs to bacteria damage to cell membrane leading to leakage of intracellular content (Agarwal et al., 2018).

DPPH radical scavenging activity

DPPH radical activity of ZnONPs is given in Fig. 10.6. DPPH antioxidant activity was measured by calculating IC_{50} value. The IC_{50} value is defined is the amount of sample required for 50% scavenging of the free radicals. So, lower IC_{50} value indicates good antioxidant activity. Synthesized ZnONPs also show good antioxidant activity with IC_{50} value 666 µg/mL. Antioxidant activity of ZnONPs may be due to presences of phytochemical like phenol that have tendency to donate the H ion (Prabu et al., 2019; Bharathi and Bhuvaneshwari, 2019). Similarly, Ravichandran et al. (2020) also reported synthesis of ZnONPs from *Durian waste,* and results show that ZnONPs exhibited good DPPH radical scavenging activity (IC_{50} value-6.39 mg/mL). Murali et al. (2017) also reported good dose dependent DPPH free radical scavenging activity of biosynthesized zinc oxide nanoparticles from *Ceropegia candelabrum* L.

Photocatalytic activity

Photocatalytic activity of ZnONPs was observed under irradiation of sun light and UV light at different time intervals 30, 60, and 90 min (Figs. 10.7−10.12). Dye degradation was identified by decolorization, and the corresponding absorbance was recorded using a UV-vis spectrophotometer. The results from the absorbance of dye degradation reveal that there is no shift of absorption peak of dye. Obviously, the major absorption peak of methylene blue, Malachite green, and Congo red is located at 668, 620, and 490 nm, which clearly decreases in intensity with increasing irradiation time. All the three dyes showed significant reduction in absorbance at 90 min time interval. Figs. 10.13−10.15 showed the plot of contact time of ZnONPs with dyes verses the absorbance of methylene blue, Malachite green, and Congo red, respectively. It has been observed the ZnONps were shown highest degradation of methylene blue under the influence of sun light/UV light with

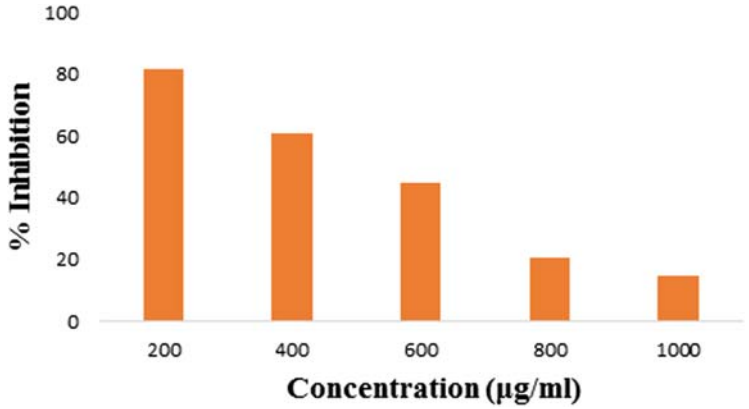

FIGURE 10.6 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of ZnONPs.

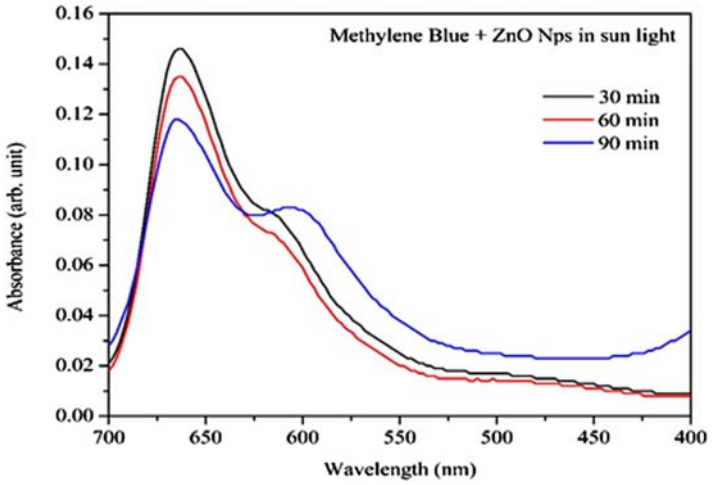

FIGURE 10.7 Photocatalytic degradation of methylene blue dye under sun light.

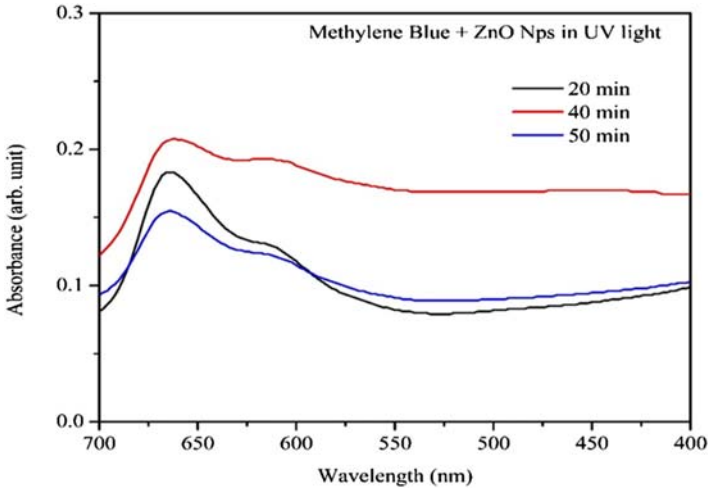

FIGURE 10.8 Photocatalytic degradation of methylene blue dye under UV light.

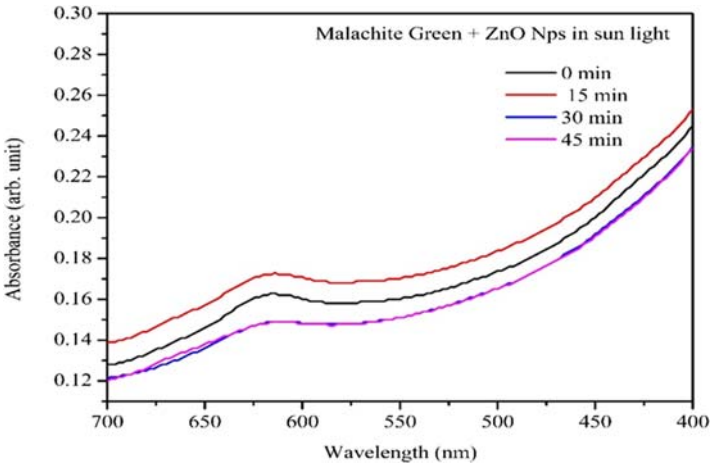

FIGURE 10.9 Photocatalytic degradation of malachite green dye under sun light.

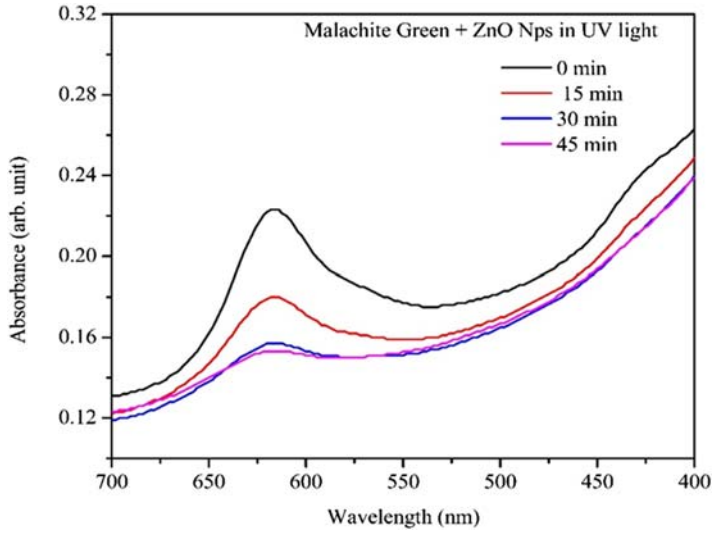

FIGURE 10.10 Photocatalytic degradation of Malachite green dye under UV light.

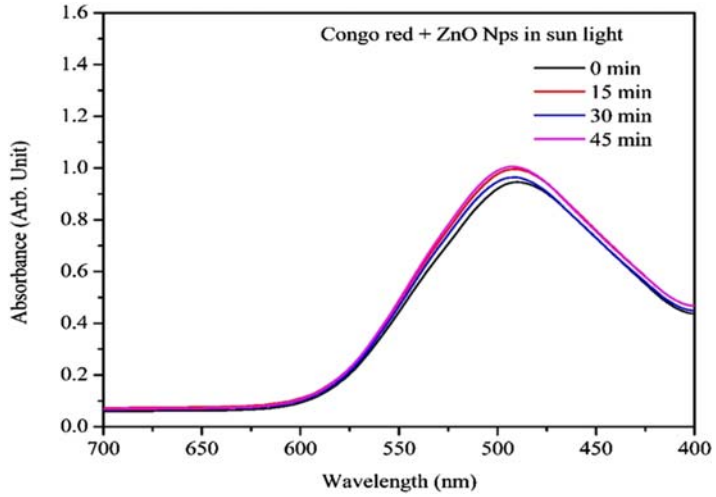

FIGURE 10.11 Photocatalytic degradation of congo red dye under sun light.

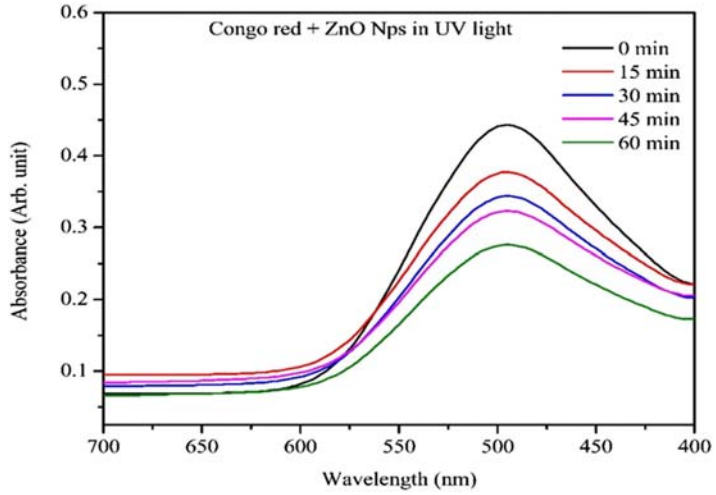

FIGURE 10.12 Photocatalytic degradation of congo red dye under UV light.

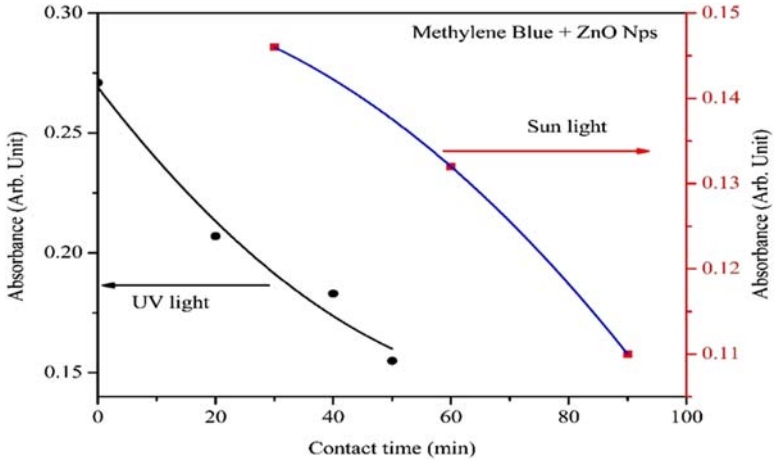

FIGURE 10.13 Photocatalytic degradation of methylene blue dye under sun/UV light with contact time ZnONPs.

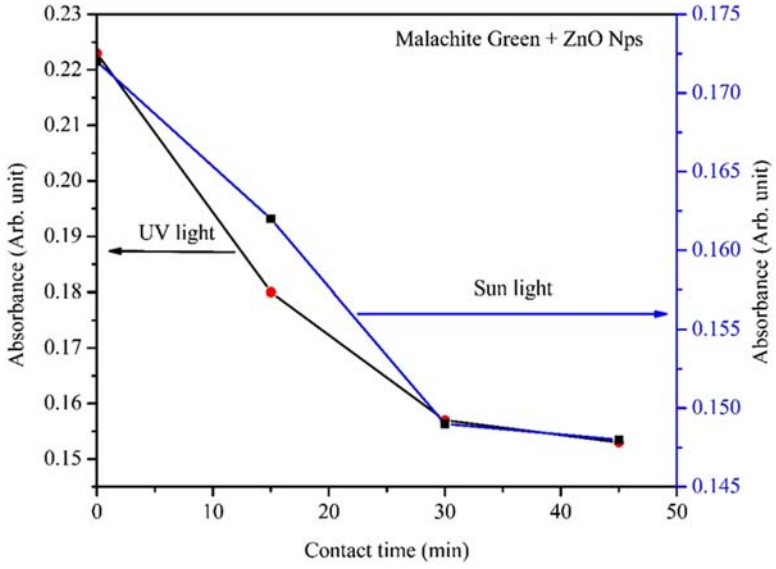

FIGURE 10.14 Photocatalytic degradation of malachite green dye under sun/UV light with contact time ZnO nanoparticles.

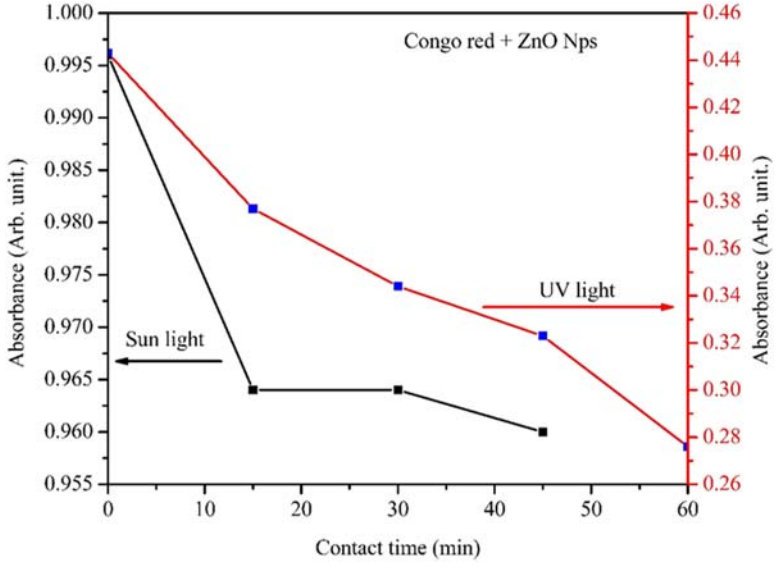

FIGURE 10.15 Photocatalytic degradation of congo red dye under sun/UV light with contact time ZnONPs.

respect to other two dyes. The photocatalytic activity of ZnONPs usually due to a zinc interstitials and high concentration of oxygen vacancies (Tripathi et al., 2014). These results indicate that ZnONPs have exclusive properties of photocatalytic activity for degradation of the methylene blue.

Conclusions

The present study involved the determination of antimicrobial, antioxidant, and photocatalytic activity of ZnONPs synthesized from *Parmotrema perlatum.* Aqueous extract was used for synthesis of ZnONPs; size of nanoparticles was in range of 40−70 nm. ZnONPs shows good antimicrobial activity against pathogenic microorganism with lower MIC values and good DPPH-free radical antioxidant activity. ZnONPs exhibited better synergistic antimicrobial activity combination with antibiotics as compared to ZnONPs alone. ZnONPs showed good the photocatalytic degradation of methylene blue dye under UV light. *P. perlatum* synthesized ZnONPs can be use as potential antimicrobial, antioxidant and photocatalytic agent.

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Chapter 11

Versatile strategies for multifaceted nanoparticle synthesis—An overview

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Introduction

In this chapter, we provide a brief overview of the current research worldwide on the use of microorganisms such as bacteria and actinomycetes (both prokaryotes), as well as algae, yeast, and fungi (eukaryotes) in the biosynthesis of metal nanoparticles and their applications. The present review focuses furthermore specifically on the bacteria-mediated synthesis of AgNPs, their mechanism, and their characterizations. Also, studies have shown that the size, morphology, stability, and properties (chemical and physical) of the metal nanoparticles are influenced strongly by the experimental conditions, the kinetics of interaction of metal ions with reducing agents, and the adsorption processes of stabilizing agents with metal nanoparticles. Hence, the design of a synthesis method in which the size, morphology, stability, and properties are controlled has become a major field of interest. Subsequently, this chapter focuses on the fundamentals, advantages, and disadvantages of each synthesis method are discussed with much consideration toward silver nanoparticles (SNPs).

In the recent scenario, soil microorganisms have adapted to the environment in such a way that other organisms have partially clogged showing antagonism against them, especially bacteria. Thus, an artificial approach is needed for the synthesis of metabolites/particles to inhibit this Multi-Drug Resistant (MDR) bacteria and fungi. Nanotechnology is one such multidisciplinary branch of science that has the prospect to create an impact on this clinical disease-causing organism. This field plays an increasingly crucial role in many key technologies of the new millennium (Mandal et al., 2006) Nanomaterials may provide solutions to technological and environmental challenges in the areas of solar energy conversion, catalysis, medicine, and water treatment (Sharma et al., 2009). These materials often show unique and considerably changed physical, chemical, and biological properties compared to their macro-scaled counterparts. The noble metals, especially silver, and gold, have attracted great attention due to their innumerable applications in various branches of science, namely catalysis, photonics, photography, chemical sensing, Surface Enhanced Raman Scattering (SERS), and most importantly, in the medicinal field as antimicrobial agents (Sarkar et al., 2007). Colloidal silver is of particular interest because of its distinctive properties, such as good conductivity, chemical stability, and catalytic and antibacterial activity.

Nevertheless, over the past few years, it has been demonstrated that the utilization of biological organisms has been emerging as a novel method for the synthesis of metal nanoparticles, which can be preferred over the existing chemical and physical methods. It is well known that many organisms can provide inorganic materials either intra or extracellularly (Brasier et al., 1990; Mann, 1990). For example, unicellular organisms such as magnetotactic bacteria produce magnetite nanoparticles (Lovely et al., 1987; Spring et al., 1995; Dickson et al., 1999) and diatoms synthesize siliceous materials (Oliver et al., 1995; Kroger et al., 1999). Multicellular organisms produce hard inorganic−organic composite materials such as bones, shells, and spicules using inorganic materials to build a complex structure (Lowenstam, 1981). Thus, nanomaterials are being actively synthesized and researched for specific functions such as microbial growth inhibition, as carriers of antibiotics, and as killing agents by utilizing culture filtrates/supernatant or biomass of the organisms.

Nanotechnology and In Silico Tools. https://doi.org/10.1016/B978-0-443-15457-7.00023-X

Bacteria in nanoparticle synthesis

Among the microorganisms, bacteria have received the most attention in the area of biosynthesis of nanoparticles. The first synthesis of Ag nanoparticles by bacteria was reported by Joerger et al. (2000) used *Pseudomonos stutzeri* AG259 to synthesize Ag nanoparticles with a size of less than 200 nm. Bacteria were grown on Lennox L (LB) agar substrate, containing 50 mmol/L $AgNO_3$, at 30°C for 48 h in the dark. In 2008, the biosynthesis of silver nanocrystals by *Bacillus licheniformis* was studied (Kalimuthu et al., 2008). Aqueous silver ions were reduced to silver nanoparticles when added to the biomass of *B. licheniformis*. This was indicated by the change in color from whitish-yellow to brown. The use of diverse organisms involved in the formation of various metal nanoparticles has been depicted in Fig. 11.1 and Table 11.1.

Actinomycetes in nanoparticle synthesis

Actinomycetes, which are gram-positive filamentous bacteria, produce a lot of other secondary metabolites or compounds which are exploited for nanoparticle fabrication processes. The monodispersity of the silver/gold nanoparticles produced either intra or extracellularly by the different methodologies was not very high and far inferior to that obtained through conventional methods. Moreover, by chance then by design, it was observed that alkalothermophilic (extremophilic) actinomycete, *Thermomonospora* sp., when exposed to gold ions, reduces the metal ions extracellularly, yielding gold nanoparticles with much polydispersity (Ahmad et al., 2003a,b). A complete reduction of the 10^{-3} M aqueous HAuCl solution at pH 9.0 and 50°C resulted in spherical and reasonably monodisperse nanoparticles. In contrast, intracellular synthesis of gold nanoparticles occurred in alkalotolerant actinomycete *Rhodococcus* sp., where particles are more concentrated on the cytoplasmic membrane than on the cell wall (Ahmad et al., 2003a,b). Also, the cell-free supernatant of *Streptomyces cyaneus*strainAlex-SK121 isolated from sediment samples collected in Egypt was found to reduce Ag^{+3} ions to AgNPs by gamma irradiation process (El-Batal et al., 2014) (Figs. 11.2 and 11.3).

Fungi as a mediator in nanoparticle synthesis

The use of fungi in the synthesis of nanoparticles is a relatively recent addition to the list of microorganisms. The use of fungi is potentially exciting since they secrete large amounts of enzymes and are simpler to deal with in the laboratory (He et al., 2008; Hong et al., 2009; Mukherjee et al., 2001). Using the fungus *Verticilliam*, a novel biological method for the synthesis of silver nanoparticles was reported in 2001. Contact of Ag^{+} ions to this fungal biomass resulted in the intracellular fabrication of metal ions and the formation of silver nanoparticles in the range of 25 ± 12 nm (Mukherjee et al., 2001). In another study, quite surprisingly, the plant pathogenic fungal strain *Fusarium oxysporum* behaved considerably differently indicating the reduction of the metal ions which occurred extracellularly, resulting in the rapid formation of highly stable gold and silver nanoparticles of 2−50 nm dimensions (Mukherjee et al., 2002; Ahmad et al., 2003a,b). Moreover, the aqueous extract of the fungal biomass can reduce gold and silver ions to their corresponding nanoparticles. Most probably, the probable mechanism could be due to the reduction of the $AuCl^{4-}$ and Ag^{+} ions due to the reductases released by the fungus into the solution, thus opening up a novel fungal/enzyme-based in vitro approach to nanomaterials. However, Sanghi and Verma reported efficient, simple, and environment-friendly biosynthesis of gold nanoparticles (GNPs) mediated by pH-dependent fungal proteins of *Coriolus versicolor* intracellularly (Sanghi et al., 2010).

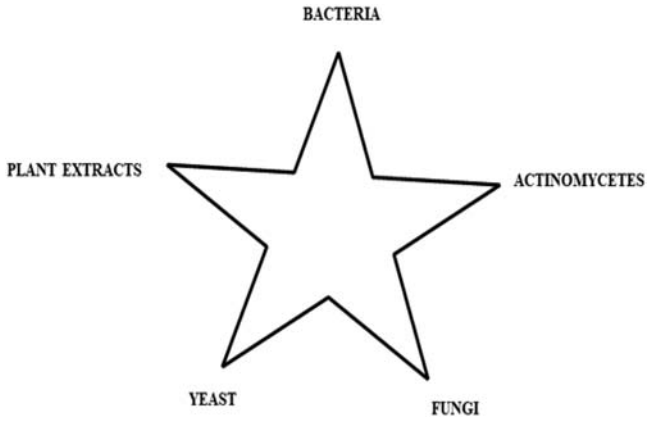

FIGURE 11.1 Different organisms involved in nanoparticle synthesis/fabrication process.

TABLE 11.1 Synthesis of nanoparticles by different biological entities.

Biological entity	Nature of nanoparticle	Intracellular/ extracellular	Size range	Name and date
Bacteria				
Bacillus licheniformis	Ag	Extracellular	50 nm	Kalimuthu et al. (2008)
Klebsiella pneumonia	*	*	28.2–122 nm (52.5 nm)	Shahverdi et al. (2007)
Escherichia coli	*	*	28.2–122 nm (52.5 nm)	Shahverdi et al. (2007)
Enterobacter cloacae	*	*	28.2–122 nm (52.5 nm)	Shahverdi et al. (2007)
Pseudomonas stutzeri AG259	Ag	Periplasmic	<200 nm	Joerger et al. (2000)
Klebsiella aerogenes	Cadmium sulfide	Cell surface	20–200 nm	Sweeney et al. (2004)
Staphylococcus aureus	*	Extracellular	120–180 nm	Nanda and Saravanan (2009)
Actinomycetes				
Thermomonospora sp.	Au	Extracellular	*	Ahmad et al. (2003a)
Rhodococcus	Au	Intracellular	*	Ahmad et al. (2003c)
Streptomyces cyaneus	Ag	Extracellular	*	El-Batal et al. (2014)
Fungi				
Fusarium oxysporum	Au Ag	Extracellular Extracellular Extracellular	20–50 nm 2–5 nm 5–15 nm	Gardea-Torresdey et al. (2003) Ahmad et al. (2003a)
Aspergillus fumigatus	*	Extracellular	5–25 nm	Bhainsa and D'souza (2006)
Verticillium	Ag	Intracellular	25 ± 12 nm	Mukherjee et al. (2001)
Aspergillus flavus	*	*	8.92 ± 1.61 nm	Vigneshwaran et al. (2007)
Cladosporium cladosporioides	*	Extracellular	10–100 nm	Balaji et al. (2009)
Penicillium fellutanum	*		5–25 nm	Kathiresan et al. (2009)
Coriolus versicolor	Au	Intracellular	*	Sanghi and Verma (2010)
Yeast				
Candida glabrata	Cadmium sulfide	Intracellular	*	Reese and Winge (1988) Dameron et al. (1989)
Torulopsis sp.	Lead sulfide	Intracellular	2–5 nm	Reese and Winge (1988)
Schizosaccharomyces pombe	Cadmium sulfide	Intracellular	1–1.5 nm	Mukherjee et al. (2001)
MKY3	Ag	Extracellular	2–5 nm	Kowshik et al. (2002)
Plant extracts				
Alfalfa Sprouts	Au	*	2–20 nm	Gardea-Torresdey et al. (2002)
Pelargonium graveolens	Ag	Extracellular	16–40 nm	Shankar et al. (2003)
Azadirachta indica (Neem)	Ag	Extracellular	5–35 nm	Ahmad et al. (2002)
Jatropha curcas (latex)	Ag	*	20–40 nm	Bar et al. (2009)
Aloe vera	*	Extracellular	15.2 ± 4.2 nm	Chandran et al. (2006)

Continued

TABLE 11.1 Synthesis of nanoparticles by different biological entities.—cont'd

Biological entity	Nature of nanoparticle	Intracellular/ extracellular	Size range	Name and date
Cinnamomum camphora	*	Extracellular	55–80 nm	Huang et al. (2007)
Cassia auriculata	*	Extracellular	20–40 nm	Sundaravadivelan and Padmanabhan (2014)
Capsicum annuum	Ag	*	10–12	Li et al. (2007)
Diospyros kaki	Au/Ag	*	2–12 nm	Song et al. (2008)

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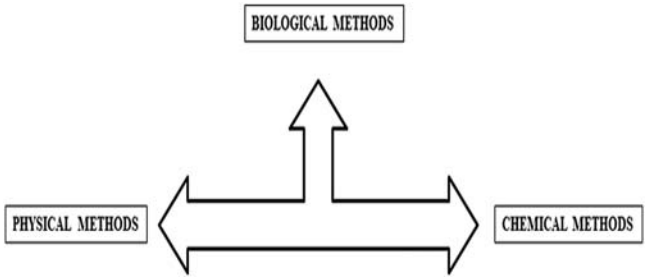

FIGURE 11.2 Schematic representation of different methodologies to synthesis nanoparticles.

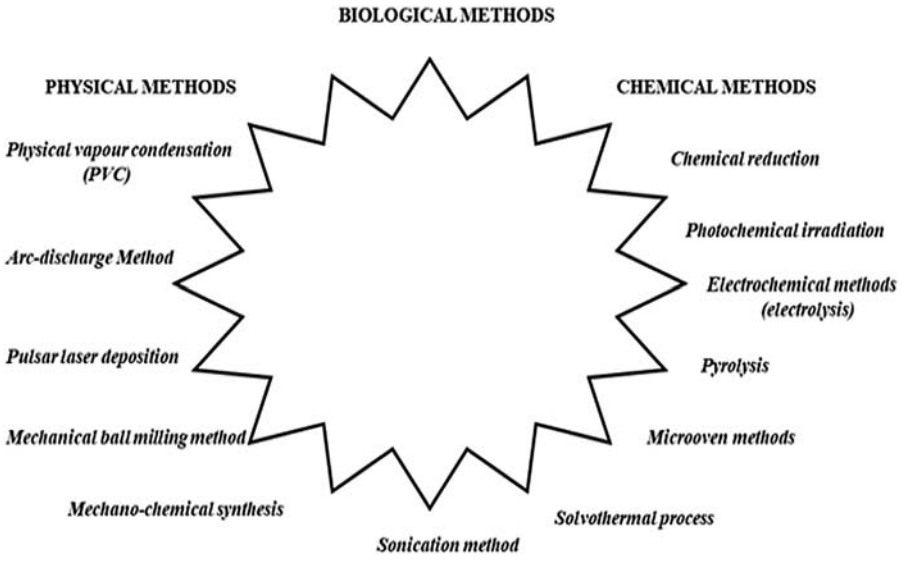

FIGURE 11.3 Divergent strategies used in the synthesis of nanoparticles.

Yeast in nanoparticle synthesis

It has long been recognized that among eukaryotes, yeasts are explored mostly in the biosynthesis of semiconductor nanoparticles. However, very few if any, reports exist about the yeast-mediated synthesis of metallic nanoparticles. In an individual report, silver nanoparticles in the size range of 2–5 nm were synthesized extracellularly by a silver-tolerant yeast strain MKY3, when challenged with 1 mmol/L soluble silver in the log phase of growth was demonstrated (Kowshik et al., 2002). However, in another investigation, contact of *Candida glabrata* to Cd^{2+} ions leads to the intracellular

formation of CdS quantum dots. The synthesis of particles is activated in the presence of cadmium ions (Reese et al., 1988; Dameron et al., 1989).

Plant extracts in nanoparticle synthesis

A number of plant extracts are being currently investigated for their role in the synthesis of nanoparticles. These plant resources have been successfully applied for silver nanoparticle synthesis, due to their potential medicinal property, huge availability, and the possibility of a faster rate of synthesis and may also reduce the steps in downstream processing, thereby making the process cost-efficient. Gold nanoparticles with a size range of 2−20 nm have been synthesized using live alfa plants (Gardea-Torresdey et al., 2002). Shankar et al. reported on the use of Geranium (*Pelargonium graveolens*) leaf extract in the extracellular synthesis of silver nanoparticles (Shankar et al., 2003). On treating aqueous silver nitrate solution with geranium leaf extract, rapid reduction of the silver ions was observed leading to the formation of highly stable, crystalline silver nanoparticles in the solution. In 2009, silver nanoparticles were successfully synthesized from $AgNO_3$ through a simple green route using the latex of *Jatropha curcas* as a reducing and capping agent (Bar et al., 2009). Crude latex was obtained by cutting the green stems of *Jatropha curcas* plants. The mixture was heated at 85°C with constant stirring for 4 h in an oil bath and silver nanoparticles were obtained gradually. The plant leaf extracts of *Capsicum annuum* (Li et al., 2007), *Diopyros kaki* (Song et al., 2008), *Chenopodium album* (Dwivedi et al., 2010), *Acalypha indica* (Krishnaraj et al., 2010). *Garcinia mangostana* (Krishnaraj et al., 2010), *Myrica esculent* (Phanjom et al., 2012), *Geranium* sp (Shankar et al., 2004), *Magnolia Kobus* (Shankar et al., 2004), *Coriandrum* sp.

Narayanan et al. (2008) have been effectively used for silver nanoparticle synthesis and analyzed its antimicrobial activity against various pathogenic organisms. Thus, subsequently, the advantage of using plants for the synthesis of nanoparticles is that they are easily available, safe to handle, and possess a broad variability of metabolites that may aid in the reduction process.

Synthesis methods of silver nanoparticles

Metallic nanoparticles are of great interest due to their excellent physical and chemical properties, such as high surface-to-volume ratio and high heat transfer (thermal conductivity).

Traditionally, nanoparticles are prepared and stabilized by physical and chemical methods. The most widely used chemical approaches include chemical reduction, physical methods, and biological methods.

Nanoparticles can be produced using many divergent techniques, typically classified as bottom-up or chemical methods and top-down or physical methods. In the bottom-up approach, the structure of nanoparticles is constructed by atoms, molecules, or clusters. The bottom-up approach is a process that builds toward larger and more complex systems by starting at the molecular level and maintaining precise control of the molecular structure. In top-down approaches, a bulk piece of required material is reduced to nano-sized dimensions using cutting, grinding and etching techniques, i.e., nanomaterials are prepared from larger entities without atomic-level control (Rodgers et al., 2006). Biological or biosynthesis techniques are also considered as bottom-up or chemical processes (Liu et al., 2003).

Chemical reduction

The most common approach for the synthesis of silver nanoparticles is a chemical reduction by organic and inorganic reducing agents. In general, different reducing agents such as sodium citrate, ascorbate, sodium borohydride ($NaBH_4$), elemental hydrogen, polyol process, Tollens reagent, N, N-dimethylformamide (DMF), and poly (ethylene glycol)-block copolymers are used for reduction of silver ions (Ag^+) in aqueous or nonaqueous solutions. Some of the chemical-reducing reactions can be carried out at room temperature. However, most of them need elevated temperatures for a higher reaction rate. In 2009, Janardhanan and colleagues synthesized silver nanoparticles by an aqueous chemical method with an organic base and with no external capping agents (Janardhanan et al., 2009).

Photochemical irradiation

Silver nanoparticles (AgNPs) can be successfully synthesized by using a variety of irradiation methods. For example, laser irradiation of an aqueous solution of Ag salt and surfactant can fabricate Ag NPs of well-defined shape and size distribution. No reducing agent was required in this method. Silver nanoparticles having narrow size distribution were synthesized in ethylene glycol−water mixtures without the use of a stabilizer. They used the pulse radiolysis method to produce nanoparticles by silver perchlorate (Jacob et al., 2007).

Electrochemical method

The electrolysis process has long been used for the reduction of metal ions. However, there are a few reports about using this method in the synthesis of metal nanoparticles, especially silver, however, this could be classified in the synthesis of Ag NPs. It is possible to control particle size by adjusting the electrolysis parameters and to improve the homogeneity of silver nanoparticles by changing the composition of the electrolytic solutions. In one of the studies, monodisperse silver nanospheroids 1−18 nm were synthesized by electrochemical reduction inside or outside zeolite crystals according to the silver exchange degree of the compact zeolite film-modified electrodes (Zhang et al., 2002). Furthermore, spherical silver nanoparticles 10−20 nm with narrow size distributions were conveniently synthesized in an aqueous solution by an electrochemical method (Ma et al., 2004).

Pyrolysis

Another fascinating method of synthesizing Ag nanoparticles is spray pyrolysis. In 2009, nano silver powder with about 100 nm average grain size had been fabricated by spray pyrolysis, using $AgNO_3$ solution, 336 mL/h flux of $AgNO_3$ solution, 0.32 MPa flux of carrier gas, and at 720°C furnace set temperature. In another work by Sawai and his coworkers (2008) metal silver nanoparticles were deposited on the surface and in the pores of activated carbon by supercritical water impregnation (SCWI). The aqueous feed solution was prepared by dissolving silver acetateAg (CH_3COO) in distilled water. All experiments were performed using batch-type reactor.

Micro-oven method

Microwave-assisted synthesis is a promising method for the synthesis of silver nanoparticles. It was reported that silver nanoparticles could be synthesized by a microwave-assisted synthesis method employing carboxymethyl cellulose sodium as a reducing and stabilizing agent previously. This heating method is especially important as its use provides increased reaction kinetics, rapid initial heating, and, hence, enhanced reaction rates culminating in clean reaction products with rapid consumption of starting materials and higher yields (Nadagouda et al., 2011). Additionally, starch has been employed as a template and reducing agent for the synthesis of silver nanoparticles with an average size of 12 nm, using a microwave-assisted synthetic method. Starch functions as a template, preventing the aggregation of the produced silver nanoparticles (Sreeram et al., 2008).

Solvothermal process

Solvothermal synthesis, is a versatile low-temperature route in which polar solvents under pressure and at temperatures above their boiling points are used. Under solvothermal conditions, the solubility of reactants increases significantly, enabling reactions to take place at a lower temperature.

Sonication

The sonication method involves powerful ultrasound radiations (20 kHz−10 MHz) applied to molecules to enhance the chemical reaction. Acoustic cavitation is a physical phenomenon that is responsible for the sonochemical reaction. This method was initially proposed for the synthesis of iron nanoparticles and is nowadays used to synthesize different metals and metal oxides (Khalil et al., 2004). The sonolysis technique involves passing sound waves of fixed frequency through a slurry or solution of carefully selected metal complex precursors. Ultrasound power affects the occurring chemical changes due to the cavitation phenomena involving the formation, growth, and collapse of bubbles in liquid (Pol et al., 2003). The main advantages of the sonochemical method are its simplicity, operating conditions (ambient conditions), and easy control of the size of nanoparticles by using precursors with different concentrations in the solution.

Mechano-chemical synthesis

In this process, a chemical reaction is induced by mechanical energy. The chemical forerunners are mostly a mixture of chlorides, oxides, and/or metals that react during milling or subsequent heat treatment to produce a composite powder in which ultrafine particles in stable salt matrixes are dispersed. These ultrafine particles are recovered by washing with a suitable solvent from selective removal of the matrix. In one of the studies, copper nanoparticles were prepared using SPEX 8000; the only contamination recorded was (2%) iron while for planetary ball mill under tempered steel as the milling medium, with a milling time of about 240 h.

Mechanical/ball milling method

Milling is a solid-state processing technique for the synthesis of nanoparticles. This technique was first used by Benjamin et al. (1976) for the production of superalloys. In the milling process, the raw material of micron size is fed to undergo several changes. Different types of mechanical mills are available which are commonly used for the synthesis of nanoparticles.

Pulsar laser ablation/deposition method

The laser ablation method is a commonly used technique for the preparation of copper nanoparticles in the colloidal form in a variety of solvents (Marine et al., 2000). Laser ablation is the process of removing material from a solid surface by irradiating it with a laser beam. At low laser flux, the material is heated by absorbed laser energy and evaporates or sublimates. At higher flux, the material is converted to plasma. The depth over which laser energy is absorbed and the amount of material removed by a single laser pulse depends on the material's optical properties and the laser wavelength. Carbon nanotubes can be produced by this method.

Arc-discharge method

In 2008, a novel technique for preparing a nanosilver water suspension without surfactants and stabilizers was studied using the arc-discharge method. Silver wires (99.99%) 1 mm in diameter submerged in deionized water (pH = 5.8, conductivity = 0.8−0.9 μS) were used as electrodes. During the arc-discharge, the surface layer of the Ag wires evaporates and condenses in the water. The transparent solution converts to a characteristic pale yellow color and then a silver suspension is created (Tien et al., 2008).

Physical vapor condensation (PVC)

In order to fabricate nanoparticles, the vapourization method has been frequently used, in which the target materials are vaporized by a heat source and then rapidly condensed. The vaporization process can be subdivided into physical and chemical methods depending on whether the reaction is present. If the resultant nanoparticles have the same composition as the target materials, they are prepared by physical vapor condensation (PVC). However, nanoparticles having a different composition from the target are usually obtained by chemical vapor condensation (CVC), because the chemical reaction occurs between the vapor and other system components during the vapourization and condensation (Tavakoli et al., 2007).

Mechanism of silver nanoparticle synthesis

Microbes encounter metals and metalloids of various kinds in the environment and attain certain genetic and biochemical metal resistance mechanisms to survive (Deshpande et al., 1993). Some of these mechanisms are extracellular precipitation, extracellular binding, and complexation, segregation into complex compounds by thiol-containing molecules, intracellular deposition, alteration in solubility and toxicity by varying the redox state of the metal ion, cellular efflux pumping system, and lack of a specific metal transport system (Das et al., 2010; Durán et al., 2011). For most metals, establishing this resistance and homeostasis involves combinations of the abovementioned mechanisms. Interestingly, *P. Stutzeri* AG259 exhibits intracellular accumulation of silver ions as AgNPs (Joerger et al., 2000). This can involve the reduction of metal ions to elemental metal through cellular machinery. Though the mechanistic aspect of nanoparticle synthesis is still poorly understood, various hypotheses have been proposed to elucidate the role of bacterial genes and proteins in the synthesis of AgNPs.

Genetics of AgNPs can be premeditated by the accumulation of silver ions, which may occur in two stages: (i) nonspecific and energy-independent attachment to the cell surface and (ii) intracellular accumulation *A. baumannii* exhibits plasmid-mediated silver resistance rendering bacteria capable of accumulating silver and retaining it by tight binding to a cysteine-rich metalloprotein (Shakibaei et al., 2003; Deshpande et al., 1993). Three major gene homologs, namely *silE, silP*, and *silS*, of silver resistance machinery have been suggested to play a significant role in AgNPs production (Parikh et al., 2008). Silver-binding gene homolog (*silE*) encodes a periplasmic silver-binding protein (*silE*) responsible for silver uptake by presenting histidine sites for silver ion binding. On exposure to silver ions, *silE*-based silver-binding machinery of bacteria gets activated leading to cellular uptake of silver ions. The ions are presented to bacterial silver reduction machinery where biomolecules, generated by silver reduction machinery, bind to the ions and reduce them to metallic silver nuclei or seed nanoparticles. These particles undergo growth and assembly to form AgNPs of

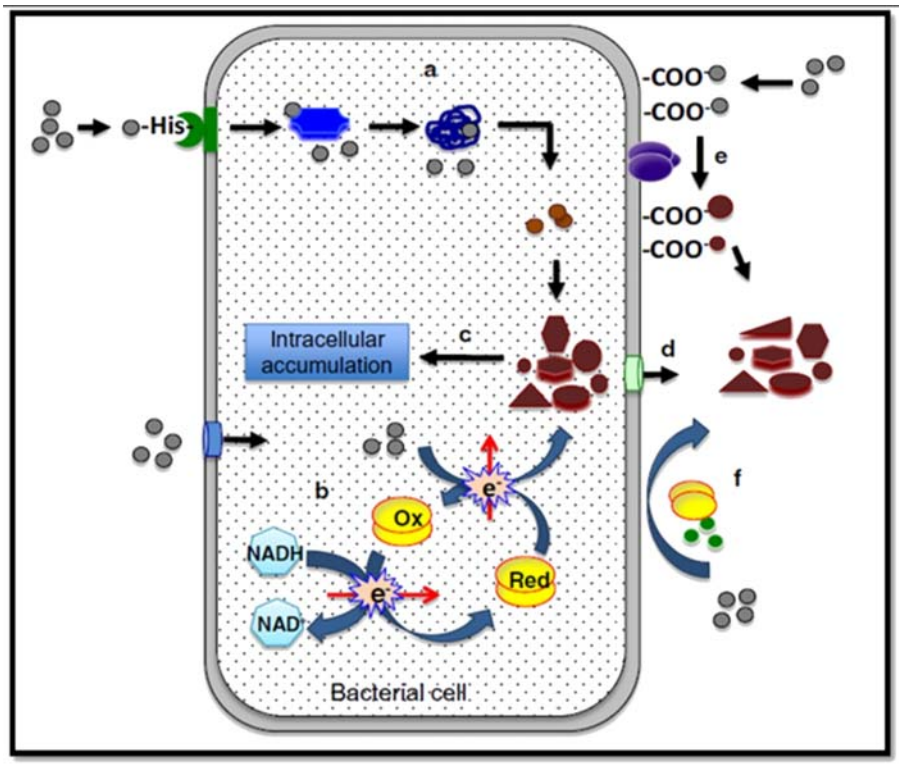

FIGURE 11.4 Proposed mechanism of bacteria-mediated synthesis of AgNPs. (A) Cellular uptake of silver ions and activation of silver reduction machinery; (B) electron shuttle system involving various cofactors and enzymes; (C, D) intra or extracellular localization of AgNPs; (E) electrostatic interaction between silver ions and cell wall components; and (F) reduction through extracellular enzymes and other organic molecules released in solution (Ramanathan et al., 2011).

different shapes (spherical or plate-like) followed by their release from the cells via the cellular efflux system as shown in Fig. 11.4 (Ramanathan et al., 2011). Alternatively, bacteria create an extracellular microenvironment during growth in which silver-specific proteins from silver resistance machinery are released outside the cells. These proteins might reduce silver ions subsequently forming stable extracellular AgNPs (Parikh et al., 2008). Further studies are required to decipher the exact role of *silP* and *silS* genes in AgNPs production. The involvement of periplasmic c-type cytochrome (MacA) and outer membrane c-type cytochrome (OmcF) in the surface reduction of Ag (I) to Ag (0) has been revealed through knockout studies (Law et al., 2008). MacA was involved in electron transfer from the inner membrane to the periplasm, while OmcF reduced extracellular electron acceptors. However, complete blockage of silver ion reduction in mutant strains was not obtained, indicating the possibility of multiple electron transfer pathways to Ag (I), which should be investigated further (Law et al., 2008).

Role of cell wall

The cell wall of bacteria also plays a major role in the biogenesis of nanoparticles owing to the involvement of cell wall components and enzymes. The nucleation of clusters of silver ions during the initial phase of AgNP synthesis causes an electrostatic interaction between the ions and negatively charged carboxylate groups of the cell wall (Li et al., 2012). Trapped silver ions on the bacterial surface are thereby reduced by cellular reductases and other redox proteins released by the cells to nanosilver form (Mahdieh et al., 2012; Debabov et al., 2013) as shown in Fig. 11.4. These nanoclusters can remain diffused through the cell wall (Nair et al., 2002). The Slayer of bacteria physically masks the negatively charged peptidoglycan sheet of the cell wall and, thus, can also be involved in bacteria-metal surface interaction (Namasivayam et al., 2011). Another potential mechanism across the cell wall is conferred in bacteria with electrokinetic potential through the generation of the transmembrane proton gradient. This gradient can indirectly drive the active symport of sodium along with silver ions from the surroundings. ATP binding employs special silver-binding proteins attached to membrane lipids on the external surface of bacteria, which attract the silver ions readily initiating AgNP synthesis (Namasivayam et al.,

2011). Since AgNPs are produced both extra and intracellularly, the role of intracellular electron donor and membrane transport system should be investigated for detailed elucidation of the mechanism of synthesis.

Enzymes and reducing agents

Silver reduction machinery involves an electron shuttle enzymatic silver reduction process. NADH generated during bacterial glycolysis and electron transport chain via energy-generating reactions creates a cellular reducing environment, due to hydrogen atoms, conducive for the synthesis of AgNPs (Jha et al., 2010). NADH-dependent enzymes, especially nitrate reductase, have been implicated to play a significant role in AgNP synthesis (Kalimuthu et al., 2008). Nitrate ions of $AgNO_3$ salt, induce nitrate reductase. The enzyme gains an electron from NADH and oxidizes it to NAD^+ and then undergoes oxidation to reduce the silver ions to nanosilver (Fig. 11.4). Further, nitrate ions (NO_3^-) get converted to nitrogen dioxide (NO_2), followed by nitrogen oxide (NO) and nitrous oxide (N_2O), and ultimately to gaseous nitrogen (N_2) (Karthik et al., 2012). However, Gaidhani and her coworkers (Singh et al., 2013), reported the nitrate reductase-independent synthesis of AgNP in *Acinetobacter*. Nitrogenase and hydrogenase classes of reducing enzymes, present in cyanobacteria, can reduce silver ions to form nanosilver (Brayner et al., 2007). The concentration of the cellular nitrogenase dictates the size of AgNPs, where a higher concentration in heterocysts leads to the rapid formation of larger shaped AgNPs near the cell wall and the intermediate content forms small and unaggregated nanoparticle colloids (Brayner et al., 2007). NfsA, an oxygen-in-sensitive nitroreductase present in Enterobacteriacae, has also been suggested to reduce $AgNO_3$ to AgNPs (Shahverdi et al., 2007). High pH and partial pressure of gaseous H_2 are important factors in bacteria-mediated AgNP production (Karthik et al., 2012). High pH catalyzes the opening of monosaccharide rings to open chain aldehyde forms that, in the presence of silver ions, undergo oxidation to corresponding carboxylic acid simultaneously reducing silver ions to AgNPs (Sintubin et al., 2009). High pH also activates reductases of oxidoreductase enzymes (Jha et al., 2010). Besides this, glutathione and thioredoxin systems are significant to maintain the reducing conditions indirectly and regulate the activity of enzymes (Jha et al., 2010). The reducing cofactors generated by the activity of various spore-associated enzymes, like glucoseoxidase, alkaline phosphatase, laccase, and catalase, can stimulate the biogenesis of AgNPs (Hosseini-Abari et al., 2013), also, Liu et al. speculated that to overcome metal stress, *Gluconacetobacter xylinum* secretes chloride ions from the cytoplasm and generates reductases to bioreduce the silver ions to form Ag/AgCl nanoparticles as a byproduct (Liu et al., 2012).

Peptides

Peptides received major attention for the synthesis and stabilization of AgNPs after Naik and his associates demonstrated the biogenic formation of AgNPs employing silver-binding peptides (Naik et al., 2002). Peptides interact with preformed silver nanoclusters in solution and generate a reducing environment around them leading to the reduction of silver ions and the formation of polydispersed AgNPs. Peptides containing amino acids like arginine, cysteine, lysine, methionine, glutamic acid, and aspartic acid are involved in the recognition and reduction of silver ions to silver crystals (Nam et al., 2008). Physiological conditions also play a significant role in dictating the metal-peptide interfacial interactions. For example, tyrosine undergoes conversion to a semiquinone structure under alkaline conditions through ionization at the phenol group, which reduces silver ions (Selvakannan et al., 2004). Tryptophan is converted to transient tryptophyl radical at high pH, which donates electron to reduce silver ions (Si et al., 2007). Peptides with disulfide linkage can also be used for peptide-coated AgNP synthesis (Graf et al., 2009).

Optimization of physicochemical parameters

Although biological methods are regarded as safe and biocompatible, it is difficult to control the morphology of nanoparticles (Shedbalkar et al., 2014). In chemical methods, only a single reducing agent is responsible for the reduction of metal ions to nanoparticles, and hence, well-defined monodispersed nanoparticles are easy to obtain (Li et al., 2012). On the other hand, several factors like enzymes, amino acids, media components, cofactors, etc. interact with metal ions differently, thereby forming polydispersed nanoparticles in bacteria genic synthesis. However, in other studies, it has been shown that the morphology of bacterial AgNPs can be controlled by regulating various other physicochemical parameters, such as culture age, silver ion concentration, temperature, and incubation time, and it is possible to produce size-controlled AgNPs through an appropriate combination of these factors (Singh et al., 2013). Various combinations of these parameters result in the formation of large aggregated particles into smaller monodispersed spherical AgNPs. These factors also affect the yield of AgNPs. Other factors that affect the morphology and productivity and synthesis of nanoparticles are inoculum size,

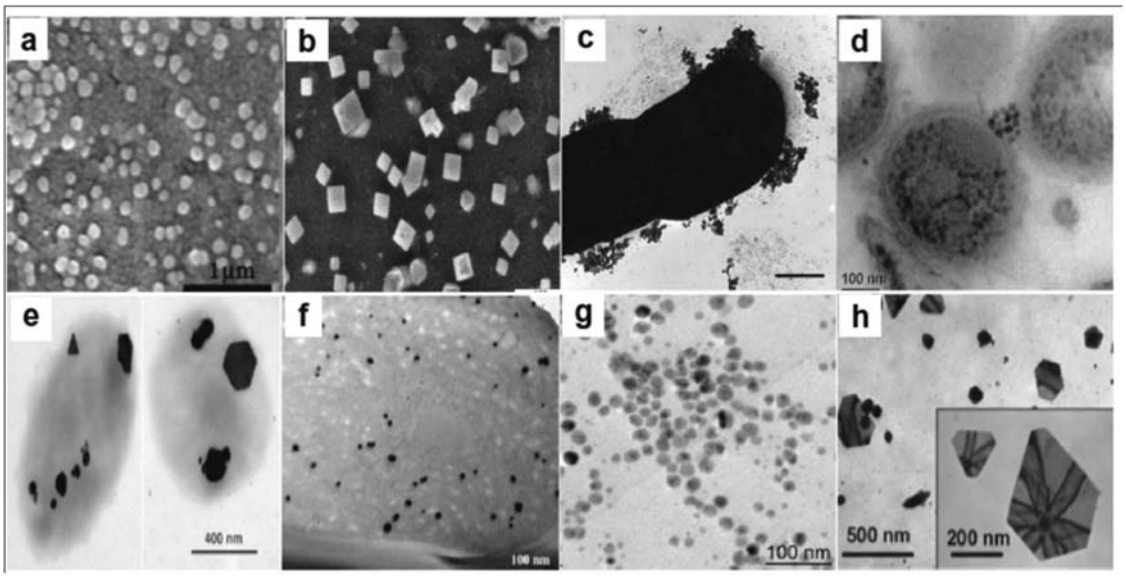

FIGURE 11.5 Electron micrographs of bacterial AgNPs. (A) SEM image of extracellular AgNPs by *Streptomyces hygroscopicus* (Sadhasivam et al., 2010), (B) FESEM micrograph of cuboid AgNPs produced by *Stenotrophomonas maltophilia* OS4 extracellularly (Oves et al., 2013), (C) TEM images of AgNPs precipitated on the surface of *Plectonemaboryanum* UTEX 485, (D) intra and extra cellular synthesis of AgNPs by *Bacillus* sp., (E) intracellular synthesis of triangular and hexagonal AgNPs by *Pseudomonas stutzeri* AG259 (Klaus et al., 1999), (F) spherical AgNPs in Anabaena cells (Brayner et al., 2007), (G) extracellular spherical AgNPs synthesized by *Acinetobacter calcoaceticus* (Singh et al., 2013), and (H) silver nanoplates by *Morganella psychrotolerans* (Ramanathan et al., 2011).

TABLE 11.2 A summary of characterization techniques for nanoparticles.

S. No.	Technique	Measures	Sample	Sensitivity
1	TEM	Particle size and characterization	Required <1 μg sample. Solid on substrate	Down to 1 nm
2	SEM	Particle size and characterization	Conductive or sputter Coated	Down to 1 nm
3	AFM	Particle size and characterization	Air or liquid	1 nm–8 μm
4	PCS	Average particle size and size distribution	Very dilute suspension	1 nm–10 μm
5	XPS	Solid	Elements, functionalization ratio	3–92 nm
6	XRD	Average particle size for a bulk sample	Arger crystalline samples (>1 mg) required	Down to 1 nm
7	FTIR	Substituent groups	Solid for ATR-IR or liquid	20 Å–1 μm
8	RAMAN	SP3 indicated by D mode	Solid	0.2–10 μm
9	UV-Vis	Side wall functionalization	Solution	Scanned and visible regions are 200–400 and 400–800 nm, respectively

Abbreviations: *AFM*, atomic force microscopy; *FTIR*, Fourier transform infrared spectroscopy; *PCS*, photon correlation spectroscopy; *SEM*, scanning electron microscopy; *TEM*, transmission electron microscopy; *UV*, ultra-violet visible spectroscopy; *XRD*, X-ray diffraction; *XPS*, X-ray photoelectron microscopy.

nutrient medium, pH, enhancers, etc. Both single-factor optimization and statistical response-surface methodology have been adopted to obtain size-controlled AgNPs and maximize synthesis yield (Deepak et al., 2011; El-Naggar et al., 2014).

Characterization techniques for nanoparticles

Preliminary detection of nanoparticle synthesis in a laboratory is usually done by visual observation for color change. The reaction medium turns reddish-brown or brown during the synthesis of AgNPs (Shahverdi et al., 2007). Change of color from purple to deep brown was observed for Au core-Ag shell nanoparticles at different molar ratios of $AuCl_4$ and $AgNO_3$ (Govindaraju et al., 2008). However, various analytical techniques such as UV-Vis spectroscopy (Shahverdi et al., 2007) FTIR, energy-dispersive X-ray analysis (Singh et al., 2013), X-ray diffraction (Dhoondia et al., 2012), electron microscopy (Klaus et al., 1999; Oves et al., 2013) atomic force microscopy (Sadhasivam et al., 2010), dynamic light scattering (Singh et al., 2013) and zeta potential (Krishnaraj et al., 2013) are often used to determine the nature, surface properties, composition, purity, stability, and morphology of any nanoparticles. Other techniques to characterize nanoparticles are atomic absorption spectroscopy (Zhang et al., 2005), X-ray photoelectron spectroscopy (Paulkumar et al., 2013), neutron activation analysis (Kiran et al., 2010), and thermogravimetric analysis (Shanmugasundaram et al., 2013). Some of the electron micrographs of bacteria genic AgNPs are represented in Fig. 11.5. Similarly, the commonly used techniques for characterizing nanoparticles are summarized in Table 11.2.

Concluding remarks and future prospects

Nanotechnology has a wide range of applications in the fields of biology, medicine, optical, electrical, mechanical, optoelectronics, etc. Silver nanoparticles have also been used for a number of applications such as nonlinear optics, spectrally selective coating for solar energy absorption, bio labeling, and antibacterial activities. The antibacterial property of silver nanoparticles against *S. aureus, P. aeruginosa,* and *E. coli* have been investigated (Rai et al., 2009). Silver nanoparticles were found to be cytotoxic to *E. coli.* Thus, biological agents in the form of yeast, plants, and microbes have emerged as an efficient candidates for the synthesis of nanoparticles. These biogenic nanoparticles are cost-efficient, simpler to synthesize and focus on a greener approach. But the exact mechanism of synthesis of biogenic nanoparticles needs to be worked out. Silver nanomaterials exhibit broad-spectrum biocide activity toward bacteria, fungi, viruses, and algae. This motivates its use in several agricultural applications. However, if the amount of nano-scaled silver entering sewage becomes higher than the tolerable levels for microbial communities in wastewater treatment plants, critical environmental infrastructure might be having a great impact. Further, there is mounting evidence that silver nanoparticles exhibit an array of cytotoxic and genotoxic effects in higher organisms. This raises concern about possible impacts on higher organisms including humans. Although significant progress has been made to elucidate the mechanisms of silver nanomaterial toxicity, further research is required to fully understand the processes involved and to safely exploit the tremendous antimicrobial properties of silver without jeopardizing human health, critical infrastructure, and the environment. Future, in vivo and environmental studies, should consider more systematically the various effects of aquatic chemistry on nano-scaled silver fate, transport, and toxicity.

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Chapter 12

A comprehensive review on eco-friendly synthesized gold nanoparticles and its advantages

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Introduction

Nanotechnology encompasses the intersection of disciplines between the 1 and 100 nm size ranges. Numerous disciplines, including chemistry, biology, physics, materials science, engineering, and medicine, have made use of this technique. There are essentially two types of nanomaterials, those occur in nature and those are created in a laboratory. Synthetic nanomaterials may be further split into four families: metal-based, carbon-based, dendrimers, and nanocomposites, whereas natural nanomaterials include viruses, compounds in bone matrices (such as calcium phosphate crystals), and corals. Nanosized metals including copper (Cu), iron (Fe), palladium (Pt), gold (Au), aluminum (Al), zinc (Zn), and silver make up metal-based synthetic nanomaterials (Ag). Catalysts, sensor components, optical devices, and biological applications are just some of the many uses that may be found for metallic NPs because of their useful features. Carbon nanomaterials include hollow spherical nanospheres like fullerenes, oblate nanorods like graphite, and cylindrical nanotubes. Batteries and very sensitive sensors have both been made possible because of the development of techniques for manipulating nanomaterials (Abou El-Nour et al., 2010; Lou et al., 2017).

Bottom-up and top-down methods may be used to broadly classify the many routes to nanomaterial synthesis. The bottom-up strategy involves putting together various components using chemical or biological processes. Bottom-up synthesis is crucial in the production of nanostructures because it yields materials with a more consistent chemical make-up. On the downside, this approach often introduces internal tension, which in turn raises the likelihood of surface flaws and contaminations. The opposite process, known as top-down synthesis, involves dismantling larger objects into more manageable components. This method facilitates large-scale manufacturing of nanomaterials, but it comes with some serious drawbacks, including poor surface structures and potential significant crystallographic damage. If not carefully managed, these constraints may have a significant impact on the physical characteristics and surface chemistry of nanomaterials, which in turn affects their intended uses (Thakkar et al., 2010).

Inorganic NPs may be synthesized using tried-and-true techniques including chemical and physical synthesis. To create NPs, physical techniques use temperature and pressure, whereas chemical synthesis relies on chemical reagents (like NaBH4) that function as reducing and stabilizing agents. Unfortunately, conventional synthesis techniques result in the creation of hazardous by-products that pose a threat to both human health and the environment. The organic solvents utilized in synthesis complicate subsequent nanoparticle extraction, which is a major roadblock for medicinal applications. Such efforts are necessary for the production of CdS and noble metal nanoparticles (NMNPs) in the form of amphiphilic colloids. However, when the nanoparticles are utilized as drug delivery vehicles, the inclusion of precursor components (such as PVA) may consequence the damage to healthy cells. The effects of nanoparticles on cardiac and vascular functions have been studied and need cautious evaluation.

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Mother Nature, as is often said, has all the answers to all the issues that arise. In several scientific domains, sustainability initiatives that use green chemistry to enhance and/or safeguard our planet's ecosystem are currently taking center stage. The usage of diverse biological entities has drawn a lot of attention in the field of nanobiotechnology instead of utilizing hazardous chemicals to reduce and stabilize metallic nanoparticles. Among all noble metals, gold and gold nanoparticles are precious, Scientists and engineers in the medical and pharmaceutical industries have shown a great deal of interest in the green synthesis of metal nanoparticles, particularly gold nanoparticles (AuNPs). As a result, numerous eco-friendly, energy- and money-saving procedures have been created. One billionth part, or 109, is denoted by the prefix "nano," which is used in science. The size range of metallic nanoparticles is 1−100 nm (Alanazi et al., 2010; Khan et al., 2014). Metallic nanoparticles have been used in a variety of sectors, including synthetic biology, health care, cellular transportation, food, and optical devices, because of their unique physical and chemical properties (Mohanpuria et al., 2008). Gold nanoparticles (Au NPs) stand out from other nanoparticles due to their distinctive surface morphologies, stable nature, and regulated geometry. A number of disorders can be identified, diagnosed, and treated using au NPs (Álvarez et al., 2015).

Recently, a variety of techniques, including physical (sonication, laser ablation, and radiation), chemical (condensation, sol-gel method, and reduction), and biological procedures, have been employed to create NPs. In (Fig. 12.1) Biosynthesis of nanoparticles is a secure, dynamic, and energy-effective process (Hurtado et al., 2016; Sathishkumar et al., 2009). This method employs a variety of biological resources ranging from prokaryotes to eukaryotes to produce NPs in vivo (Hurtado et al., 2016). The proposed mechanism of Au_{+3} to metallic Au0 NP conversion by these bio reductants is emphasized in (Fig. 12.2) (Aromal and Philip, 2012; Thakkar et al., 2010). These stem from the fact that conventional synthetic approaches frequently require the administration of toxic chemical entities during the production process, which may cause harmful reactions in the environment and possibly in animal and human health; additionally, such unpleasant chemicals may severely limit the application possibilities and biocompatibility of the generated particles (Wiley et al., 2005).

Green synthesis approach of AuNPs

Green synthesis is an easy and straightforward method for producing AuNPs. Producing the NPs does not need extreme conditions like high temperatures or pressures. Creating nanoparticles extracellularly is the norm. The HAuCl4 (tetrachloroaurate) salt is the catalyst for manufacturing. After the ingredients are combined, they are agitated on a stirrer to create the NPs. If the solution becomes a bright crimson or deep purple, then means the AuNPs were successfully created. After this, the NPs are centrifuged and dried before being characterized and put to use (Abbasi et al., 2015).

Because of their various surface functions and distinctive surface plasmon resonance (SPR), AuNPs have been researched more than any other noble metal NPs. Current civilization makes extensive use of AuNPs in a wide variety of

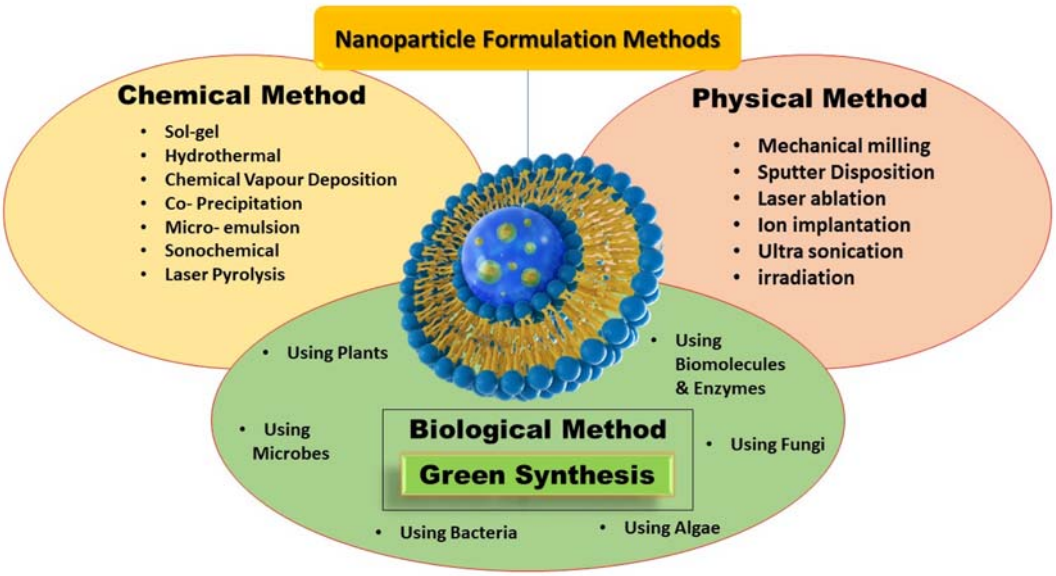

FIGURE 12.1 Methods to synthesize nanoparticles (gold nanoparticles).

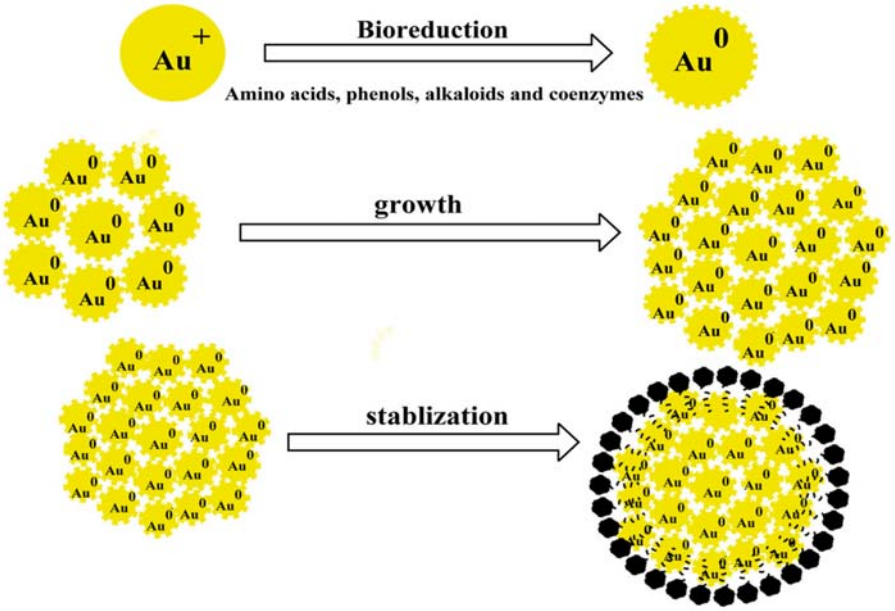

FIGURE 12.2 Process of synthesize gold nanoparticles. *Reproduced from Nadeem M, Abbasi BH, Younas M, Ahmad W, Khan T: A review of the green syntheses and anti-microbial applications of gold nanoparticles,* Green Chem Lett Rev *10(4):216–227, 2017. https://doi.org/10.1080/17518253.2017.1349192. 2017*

contexts (such as heavy metal determination, optical sensing, and imaging catalysis). Furthermore, AuNPs may be mixed with a wide variety of nano-biological assemblies to improve the performance of oligonucleotides, antibodies, and proteins. The adaptability of AuNPs for modification is enhanced by the fact that their association with biomolecules changes their SPR, conductivity, and redox activity. Here, we'll talk about green synthesis, the process of making AuNPs by biological means. As can be shown in Fig. 12.3, plant, fungal, bacterial, enzymes, and biopolymers are all green materials that may operate as reducing agents.

The optimal control of the physicochemical features of AuNPs is essential for their biomedical applications, and these properties are strongly influenced by variations in parameters and reaction environment. To achieve the desired properties of AuNPs, several parameters (such as HAuCl4 concentration, green extract quantity, reaction duration, temperature, and pH) must be properly analyzed. For instance, agglomeration causes the NPs to grow in size and become more irregular in form when the concentrations of HAuCl4 and green extract are increased (Ghosh et al., 2008). To provide just one example, increasing the Lantana camara linn leaf extract concentration from 100 mg/mL to 500 mg/mL caused the size of the AuNPs to expand from 6 to 100 nm. The consequences of lengthening the response time or raising the temperature are essentially the same (Yeh et al., 2012).

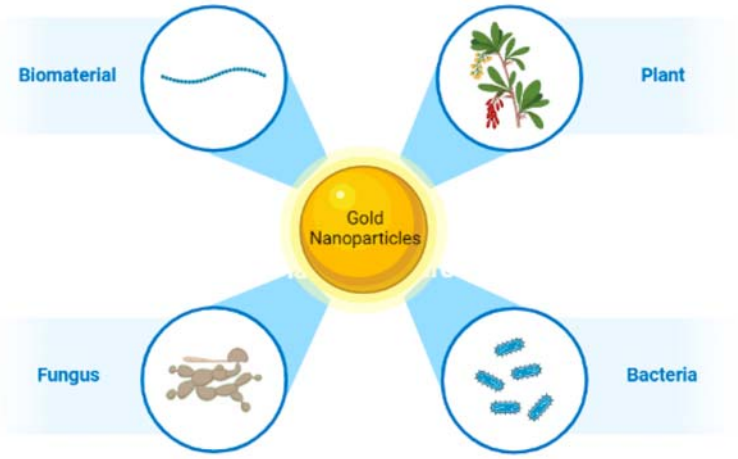

FIGURE 12.3 Biomaterial used for green synthesis of gold nanoparticles AuNPs.

Notably, the lowering ability of the green materials has a significant impact on the reaction time. The synthesis of AuNPs by Garcinia mangostana peel extracts took just a few minutes, whereas the synthesis of AuNPs by Lantana camara berry extract took 72 h. Increasing the temperature may shorten the response time, but it also causes NPs to aggregate (Kumar et al., 2017; Lee et al., 2017).

Production of gold nanoparticles from green plants

Nanoparticles are created through a variety of physiochemical processes, all of which have a negative impact on the environment. Plant-based nanoparticle synthesis is a simple process in which a metal salt is mixed with plant extract and the reaction takes minutes to a few hours at normal room temperature. The metallic salt solution is reduced to nanoparticles (Figs. 12.4 and 12.5) (Mittal et al., 2013). This trend of simplification has received a lot of attention in the last decade, especially with gold nanoparticles (Au NPs), which are safer than other metallic NPs (Rai et al., 2008).

Moreover, their synthesis is quick, cost-effective, environmentally friendly, and easily scaled up. Tea's antioxidant and anticancer properties are widely thought to provide significant health benefits. Recently, tea leaf extracts were used in the green synthesis of AuNPs. This review focuses on the use of phytochemicals found in plant extracts, as pure compounds, and in various consumable foods to synthesize nanoparticles.

Nanomaterials in various fields ranging from oil and gas to cosmetics and nanomedicine have propelled the world into a new era known as the nanotechnology era (Mokhatab et al., 2006; Mu and Sprando, 2010). Carbon nanotubes (CNTs), gold nanoparticles, liposomes, and paramagnetic nanostructures are among the most studied nanostructures (Daniel and Astruc, 2004; Jurgons et al., 2006; Liu et al., 2020; Park et al., 2004). Gold colloids are increasingly being used in fields such as chemistry, biology, engineering, and medicine. They have numerous applications in biomedicine, including diagnostics, therapy, and immunology (Dykman and Khlebtsov, 2011).

Gold nanoparticles are an excellent material for research because they are one of the most stable, nontoxic, and simple to synthesize nanoparticles and exhibit a variety of fascinating properties such as assembly of various types and quantum

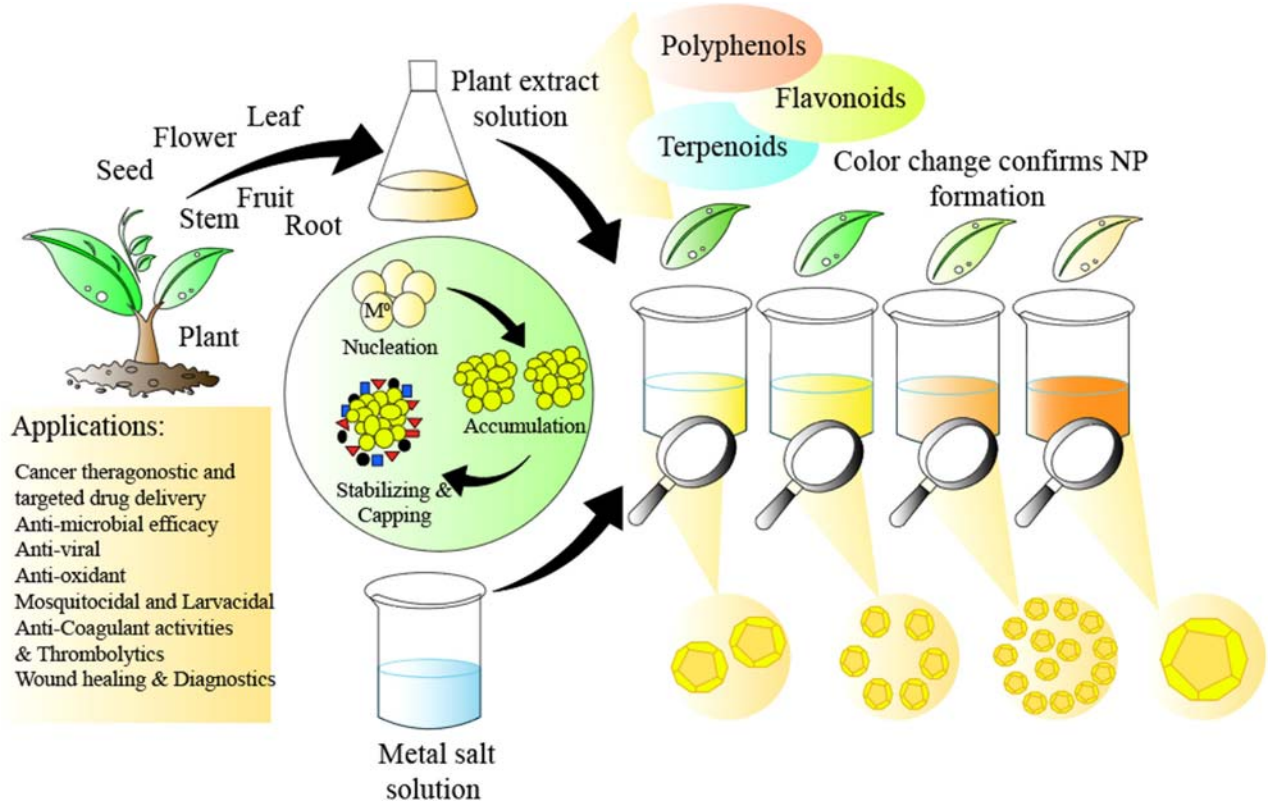

FIGURE 12.4 Process of formulation of green gold nanoparticle. *Reproduced from Shreyash N, Bajpai S, Khan MA, Vijay Y, Tiwary SK, Sonker M: Green synthesis of nanoparticles and their biomedical applications: a review,* ACS Appl Nano Mater *4(11):11428–11457, 2021. https://doi.org/10.1021/acsanm.1c02946. 2021*

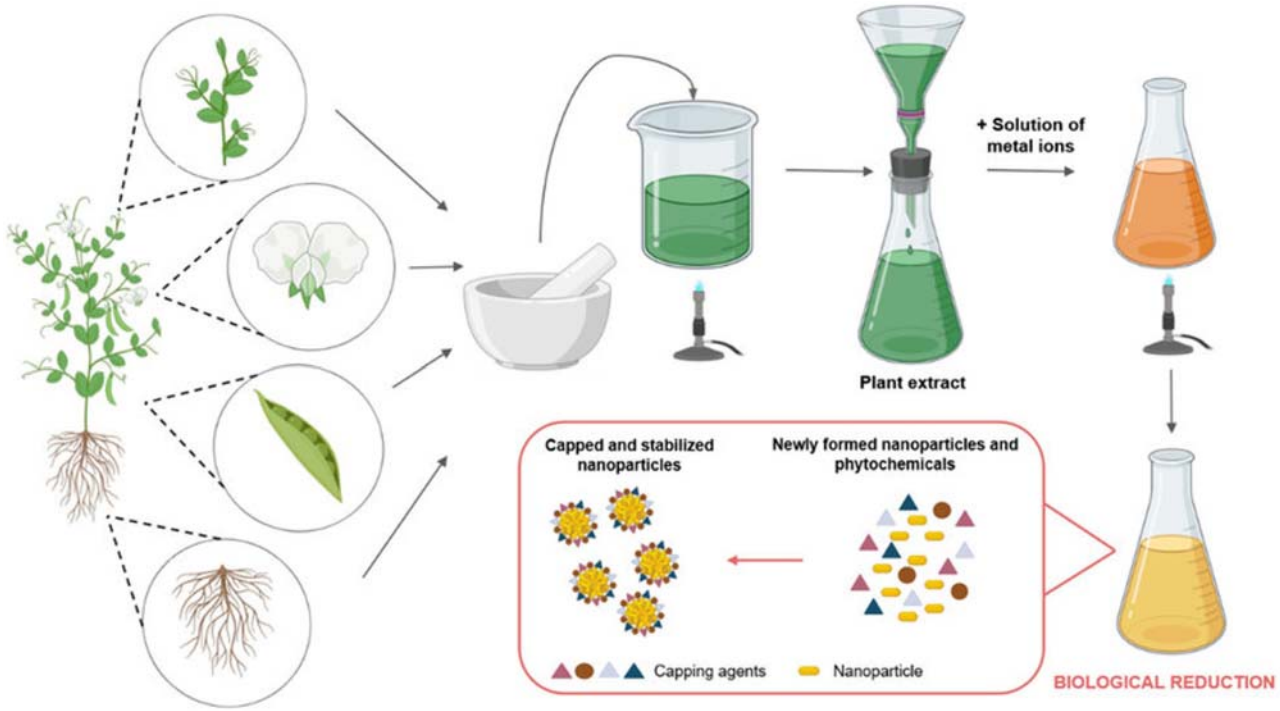

FIGURE 12.5 Process of formulation of green gold nanoparticle. *Reproduced from Rónavári A, Igaz N, Adamecz DI, Szerencsés B, Molnar C, Kónya Z, Pfeiffer I, Kiricsi M: Green silver and gold nanoparticles: biological synthesis approaches and potentials for biomedical applications,* Molecules *26(4), 2021. https://doi.org/10.3390/molecules26040844. 2021*

size effect (Daniel and Astruc, 2004). Gold nanoparticles have emerged as an excellent candidate for use in delivering various payloads to their destination (Paciotti et al., 2006; Paciotti et al., 2008). These payloads range from small drug molecules to large biomolecules such as DNA, RNA, and proteins. Table 12.1 shows that a variety of plant extracts were employed in the production of AuNPs.

TABLE 12.1 Gold nanoparticles synthesized sustainably from plant extracts.

Plant species	Part used	Shape	Size (nm)	References
Areca catechu	Nuts	Spherical	13.7	(Rajan et al., 2015)
Acorus calamus	Leaves	Spherical	<100	(Ganesan and Gurumallesh Prabu, 2019)
Aloe vera	Leaves	Spherical	15.2	(Chandran et al., 2006)
Anacardium occidentale	Leaves	Spherical	6–17	(Sheny et al., 2011)
Anacardium occidentale	Oils	Hexagonal	36	(Sheny et al., 2012)
Ananas comosus	Fruit	Tetrahedral	16	(Bindhu and Umadevi, 2014)
Azadirachta indica	Leaves	Planar	3–45	(Shankar et al., 2004)
Camellia sinensis	Leaves	Irregular	40	(Vilchis-Nestor et al., 2008)
Cinnamomum camphora	Leaves	Triangular and spherical	55–80	(Huang et al., 2007)
Dioscorea batatas	Rhizomes	Diverse	18.48–6.18	(Sreekanth et al., 2015)
Emblica Officinalis	Fruit	Spherical	16.8	(Ankamwar et al., 2005)
Punica granatum	Fruit	Triangular and spherical	5–17	(Lokina et al., 2014)
Zingiber officinale	Rhizome	Diverse	5–15	(Ganesh Kumar et al., 2011)

Production of gold nanoparticles from microorganisms

The use of harsh chemical conditions and organic solvents, as well as the production of toxic residues during the synthesis and functionalization of NPs, are the main drawbacks of this synthetic approach. As a result, over the last 2 decades, alternative (green/biological) methods for producing various types of NPs have been introduced and widely used (Kumari et al., 2016; Salvadori et al., 2014).

Due to the obvious limitations and disadvantages of traditional physical or chemical methods of metal nanoparticle synthesis, green chemical processes emerged as a new direction in the chemical industry about 2 decades ago (Rónavári et al., 2018). Since then, these biologically inspired green syntheses have garnered a lot of attention as a promising way to keep the economy going while also protecting the environment. Biological synthesis protocols provide a clean, highly tenable, and environmentally friendly method for producing nanoparticles with diverse sizes, shapes, physical, chemical, and biological properties and compositions. The formed nanoparticles have a significant advantage over conventionally produced materials: they are more environmentally friendly than the materials covering their surface and are also naturally formed, making them biocompatible. Several microorganisms, primarily bacteria and fungi, have recently been used to produce various metal nanoparticles, including silver, gold, silver-gold alloy, iron, copper, zinc, palladium, and titanium nanomaterials (Gade et al., 2014; Molnár et al., 2018). Although certain metals, such as silver, are well known for their toxicity, some silver-resistant bacteria can accumulate metals on/in their cell wall. This phenomenon inspired the first pioneering silver and gold nanoparticle synthesis using the silver-resistant bacterium Pseudomonas stutzeri. Several studies have suggested that metal nanoparticles (NPs) such as silver and gold can be easily and quickly biosynthesized intracellularly. Small, monodispersed gold nanoparticles, for example, were produced using Pseudomonas and Bacillus strains (Gericke and Pinches, 2006a). This synthesis method was successfully extended by Nair et al. to produce silver, gold, and silver-gold alloy nanoparticles (Hurtado et al., 2016). It was also proposed that the formation of nanoparticles using specific yeast strains could have the greatest potential for nanoparticle manipulation, particularly in terms of controlling culture parameters such as growth and other cellular activities (Gericke and Pinches, 2006b). In addition to the strains mentioned above, Pseudomonas fluorescens, Geobacillus stearothermophilus, and Staphylococcus epidermidis have been successfully used for the bioproduction of spherical gold nanoparticles ranging in size from 5 to 90 nm (Shukla and Iravani, 2018). Filamentous fungi can produce a wide range of metal NPs, including gold (Anshup et al., 2005; Liu et al., 2005; Nune et al., 2009), Silver, iron oxide (Ghosh et al., 2008), and even bimetallic nanoparticles (Giljohann et al., 2007) have been discovered. In general, fungi-mediated NP synthesis is divided into two categories: in vivo and in vitro methods. We were also interested in metal sorption by nucleic acids because the carbonyl groups of the nucleobases guanine and thymine serve as binding sites for metal DNA complexes (Aich et al., 1999; Kunoh et al., 2018; Mandal and Sankar Nandi, 1978; Pneumatikkais et al., 1978). Most previous methods for producing AuNPs from biomolecules required electron beam irradiation or the use of reducing agents to reduce Au cations (Vo et al., 2014).

Stabilization and functionality of AuNPs

Metal nanoparticles are an extremely intriguing class of materials due to their distinct characteristics that differ from their bulk state. Silver and gold nanoparticles (AgNPs and AuNPs) are the most common and widely used metals as nanoparticles, while aluminum, copper, palladium, and platinum can also be used to make metal plasmonic nanoparticles. Copper should have strong plasmonic performance based on its dielectric properties, but the application of plasmonic CuNPs is constrained by copper's inclination to oxidize (Wang et al., 2020).

SPR are observed in metal nanoparticles as a result of their interaction with light, which shows itself in the diverse hues of the related colloidal solutions depending on particle size and shape (Sangwan and Seth, 2022). To comprehend and utilize the plasmonic reactions of such metal nanoparticles when coupled with electromagnetic radiation, research fields such as detection, photocatalytic degradation, electronics, and biomedicine have been extensively studied (Ustarroz et al., 2017). Changes in particle size, shape, composition, and arrangement can have a considerable impact on the plasmonic characteristics of metal nanoparticles.

The three stages of preparation, storage, and application make up the lifespan of nanoparticles, including plasmonic nanomaterials. Particularly for plasmonic nanoparticles, whose size, shape, and chemical stability influence the total level of plasmonic and application performance, conservation of the particles' physical and chemical properties is crucial and must be carefully regulated (Grillet et al., 2013). Therefore, maintaining the appropriate plasmonic performance depends on stabilizing the developed nanoparticles. Due to the wide range of fields in which plasmonic nanoparticles are used, numerous stabilizing techniques have been devised (Dai et al., 2014). Stabilizers frequently have the added benefit of enhancing or improving the plasmonic characteristics of the nanoparticles. To endow the nanoparticles with colloidal stability, stabilizing agents must be present during and after nucleation and growth.

AuNPs cannot maintain their structures without the appropriate stabilizers; instead, they will aggregate or disintegrate and lose their plasmonic properties (Ustarroz et al., 2017). Other, stronger stabilizers tailored to the requirements of a certain application can take the place of first stabilizing chemicals. For in vivo applications, where the nanoparticles must keep their plasmonic capabilities until they reach their site of action and carry out the necessary function within a complex biological matrix, picking the right protective materials is key part of the development. AuNPs, on the other hand, need to be kept as a solution at low temperature because they are not a stable substance and rapidly combine to lose SPR absorption. However, the solution aggregates when frozen and cannot be redispersed. Additionally, a number of variables, including pH, temperature, and salt concentration, all of which eventually cause particle dissolution or aggregation, have an impact on the dispersion stability in solution (Kang et al., 2019; Králik, 2014). Therefore, to increase the stability of their dispersion, metal nanoparticles are frequently either stored in a citrate solution or react with thiolated molecules.

One of the most significant developments in AuNPs synthesis has come from the innovative work of Turkevitch and Frens, who invented and improved the citrate reduction of HAuCl4 (Frens, 1973). Citrate serves as a reducing and stabilizing ingredient in this process, which is frequently employed to create colloidal gold nanomaterials. Mulvaney and Giersing reported stabilizing AuNPs with alkenethiols with different chain lengths in 1993. Schiffrin and fellow scientists in 1994 provided a clearer illustration of this two-phase, thiolate-stabilized technique, which has allowed scientists to easily adjust AuNPs size at lower temperatures while maintaining relatively high stability (Brust et al., 1994).

There have been various studies throughout the years on PEGylation, a method for stabilizing gold nanoparticles (AuNPs) in their dispersion using poly(ethyleneglycol) (PEG). PEG is a well-known neutral, water-soluble polymer having a flexible backbone that may form hydrogen bonds with water (Wang et al., 2020). Moreover, PEG is biocompatible and can shield gold surfaces from immune system recognition as well as from aggregation in vitro and in vivo. The capacity to resist salts under physiological conditions is a crucial quality when biological applications are taken into account. Aggregation is thought to result from the addition of salt or an electrolyte, which increases the ionic strength. In order to prevent aggregation, adequate surface modification is necessary. The advancement of nanoparticle sciences requires the establishment of a new PEGylation technology because PEGylation and other functionalizations of AuNPs have mostly been restricted to chemisorption by the gold-sulfur processes (Nam et al., 2011).

Properties and characteristics of gold nanoparticles

Understanding the properties of NMNPs and exploring their application potential are two major driving forces behind the synthesis of a large variety of nanomaterials. Many properties of nanoparticles arise from their large surface-area-to-volume ratio and the spatial confinement of electrons, phonons, and electric fields in and around these particles (Sau et al., 2010).

Similarly, AuNPs exhibit deviation from the usual bulk arrangements due to their high surface area to volume ratio (Herron and Thorn, 1998) In other words, these properties of AuNPs depend on their size and shape (Eustis and El-Sayed, 2006; Sujitha and Kannan, 2013). The surface of a nanoparticle may be unstable due to the high surface energy and large surface curvature.

Important physical properties of AuNPs include SPR and the ability to quench fluorescence.

Surface plasmon resonance

Gold nanoparticles are shown to display a range of colors from red to dark purple as the core size of the particles increases from 1 to 100 nm. The red color indicates smaller particles with mostly spherical shape, whereas purple color exhibits larger particle size with mixed morphology (Siddiqi and Husen, 2017).

Plasmon is a surface-charge oscillation quantum; oscillation is begun as a result of an external field of electric forcing the surface particle charges to amass at one end (Hedkvist, 2013). Metals that best display such free-electron plasma performance including alkali metals, Al, Mg, and noble metals such as Ag, Au, and Cu. Surface plasmons (SPs) are plasmon types correlated with the metal's surfaces (Rhodes et al., 2006; Xia and Halas, 2005).

When a gold particle is exposed to light, the oscillating electromagnetic field of the light induces a collective coherent oscillation of the free electrons (Huang and El-Sayad, 2010). Furthermore, these spherical nanoparticles show a size-relative absorption peak from 500 to 550 nm which arises due to the collective oscillation of the conduction electrons due to the resonant excitation by the incident photons. This is known as a surface plasmon band (Amendola et al., 2017).

Various factors affecting the surface plasmon band are size, shape, solvent, surface ligand, core charge, and temperature. Proximity of the neighboring nanoparticles also affects the SPR of the gold nanoparticles (Hu et al., 2008; Mustafa et al., 2010; Yeh et al., 2012). As the aggregation of particles takes place, the SPR frequency moves toward the red end and

broadens the surface plasmon band. Thus, the color of the nanoparticles also changes from red to blue which is due to interparticle plasmon coupling (Su et al., 2003).

The color changing ability of the gold nanoparticles due to aggregation can be effectively used in various biomedical diagnostic tests (Elahi et al., 2018). One such example of such a test is the specific binding between negative charges within (Human Chorionic Gonadotropin Hormone (HCG)) and the positive charges within particles in pregnancy-positive samples of urine might be anticipated, whereas such a reaction will not happen in samples of negative urine (Kuppusamy et al., 2014; Rojanathanes et al., 2008).

Quenching fluorescence

Fluorescence refers to the emission of electromagnetic radiation, usually visible light, caused by the excitation of atoms in a material, which then reemit almost immediately. This is made possible due to the presence of a chemical compound that is called fluorophore (Hötzer et al., 2012).

AuNPs also exhibit the property of quenching fluorescence of proximate fluorophore by inducing the deactivation pathway. This is possible due to a significant overlap between the between the surface plasmon band of the AuNPs and emission spectrum of excited fluorophores (Lichtman and Conchello, 2005). The aforementioned phenomenon is commonly referred to as fluorescent resonance energy transfer (FRET). It is commonly observed in gold nanoparticles that are less than 1 nm in size. This is due to the fact that radiative and nonradiative decay rates of fluorescent molecules are both distinctly affected by the nanoparticles (Bigioni et al., 2000; Oh et al., 2005; Shi et al., 2015).

Another such mechanism by which the gold nanoparticles can cause quenching is the photo-induced electron transfer. In this process, the nanoparticles act as electron acceptors and cause quenching (Ipe et al., 2002; Thomas and Kamat, 2003).

Melting point

Melting point of gold nanoparticles varies in accordance with the size of the particles (Koga et al., 2004; Qiao et al., 2014). In general, the melting points of various forms of gold nanoparticles vary between 615 and 1115 K which is significantly more than 1336 K which is the melting point of bulk gold (Qiao et al., 2014).

The reduction of the core's attractive forces of interaction as a consequence of a decrease in the number of nearby atoms is what causes the decrease in melting point. This reduces the interaction between inner and surface atoms and increases the surface energy of surface atoms. Similarly, the electrical properties of the gold nanoparticles also differ from the bulk material. This arises from the fact that the electrical conductivity is inversely proportional to the surface area (Sambles, 1971; Zawrah et al., 2016).

Drug release pattern and kinetics of AuNPs

Drug release kinetics is of utmost importance in the pharmaceutical studies because the drug release from pharmaceutical nanoparticles is a key determinant in its biological effect. It is frequently advantageous to employ kinetic models to clarify release mechanisms, which can then be used to manage and understand drug release. The ability to express multiple release data with one or two parameters is another benefit of the kinetics. A general model can be used to derive parameters that can be used to compare various drug delivery methods and to correlate with bioavailability data. It is evident that parameters acquired from data fitting to different kinetic models cannot be used for comparison or correlation. Herein we discussed all such models used to determine the kinetic behavior and drug release pattern of AuNPs.

Mathematical kinetic models

Drug release kinetic studies are frequently helpful in getting one or two physically significant parameters that are used for comparison and linking the release parameter with important aspects like bioavailability. Additionally, a kinetic parameter can be utilized to investigate how formulation parameters affect drug release in order to optimize and regulate release. Models are deemed acceptable general models if they had both a single-figure overall error (OE) and a number of single-figure OEs that was higher than 75% of the total number of sets (England et al., 2015). The kinetics and mechanism of drug release from gold nanoparticles may be assessed using mathematical kinetic models. Such various models and their equations are given in Table 12.2.

TABLE 12.2 Various mathematical kinetic models used to analyze the data obtained from release of drug from gold nanoparticles (Barzegar-Jalali et al., 2008; England et al., 2015; Paarakh et al., 2018; Sibanda et al., 2004).

Model name	Model	Significance
Zero-order kinetic model	$f = k_0 t$	The zero-order model is associated with drug dissolution that is independent of drug concentration.
First-order kinetic model	$\ln(1-f) = -Kft$	The first-order model is associated with drug dissolution that is dependent of drug concentration.
Simplified Higuchi model	$F = k_H\sqrt{t}$	The simplified Higuchi model utilizes the equation to describe drug release from matrix and polymeric systems.
Hixson–Crowell model	$1-\sqrt[3]{1-F} = k_{1/3}t$	It is applicable to powder drug delivery describing cube root of drug % remaining in matrix versus time.
Korsmeyer–Peppas model	$\frac{M_t}{M_\infty} = k't^n$	It is applicable to swelling hydrogels describing and it is applicable to log cumulative % drug release versus log time.
Weibull model	$m = 1-\exp\left[-\frac{\{(t-Ti)b\}}{a}\right]$	Here b represents the shape of dissolution curve progression, it basically shows site-specific biphasic release kinetics.
Hopfenberg model	$\frac{Mt}{M\infty} = 1-\left[1-\frac{k0t}{CL}a\right]n$	It is used to identify the mechanism of release from the optimized oil sphere having good solubility and intermediate release rate.

Typically, diffusion and/or dissolution control the rate of drug release from nanoparticles. Regardless of the mechanisms involved in the release, its rate under sink conditions can be stated by a single generic equation, as shown below.

$$\frac{dw}{dt} = \frac{D}{h}SC_s$$

where w is the total amount of drug release from nanoparticles at time t; dw/dt is the release rate; D is diffusion coefficient; S is effective surface area; Cs is the solubility of drug in the medium; and h is the length of diffusion medium. This equation embodies both the Fick's first law of diffusion, which is used for diffusion rate limited release processes, and the Noyes-Whitney law of dissolution, which is applied to dissolution rate limited release (Barzegar-Jalali et al., 2008).

England et al. had developed layered gold nanoparticles of chemotherapeutic agents, cisplatin and paclitaxel and evaluated for release kinetics of these two drugs through zero-order kinetic model, first-order kinetic model, simplified Higuchi model, and Korsmeyer-Peppas model. Among all, Korsmeyer-Peppas model showed significant and adequate drug release of each formulation type and simplified Higuchi model best-described paclitaxel release from two-layered gold nanoparticles, they concluded (England et al., 2015).

Applications of AuNPs

Antimicrobial activity of green synthesized AuNPs

Gold nanoparticles (AuNPs) have recently received a lot of attention due to their unique properties and applications in biomedicine (Ahmed et al., 2015). The multifunctionality of gold nanoparticles, in particular, has facilitated their nano-biological adherence with oligonucleotides, drugs, protein, and antibodies (Ahmed et al., 2015; Giljohann et al., 2007). Furthermore, the optical nature of AuNPs enables them to play an important role as a marking agent in the science of biological imaging (Yong et al., 2009). On the other hand, the presence of bacteria in river and pond water is harmful to both humans and living organisms, causing long-term diseases. Because of their excellent antibacterial activity, several inorganic materials and nanomaterials have already been reported to help solve the problems caused by these bacteria. Metal nanoparticles, such as gold and silver nanoparticles, have piqued the interest of researchers due to their antibacterial and biocompatible properties with high surface-to-volume ratios.

Despite the use of physical and chemical methods for nanoparticle synthesis, there is still a critical need to develop environmentally friendly procedures that do not involve the use of highly toxic chemicals, particularly for medical purposes (Kuppusamy et al., 2015). Synthetic techniques including the utilization of natural items as reducing agents, on the other hand, must be prioritized in order to reduce the vulnerable impacts on nature and mankind.

Algae, fungi, and enzymes were discovered to be effective in the manufacture of AuNPs (Naveena and Prakash, 2013; Dhanasekar et al., 2015). Furthermore, as a result of the difficulties faced during microbial-aided synthesis (Zhang et al., 2011), plant-mediated synthesis is gaining popularity due to its ease of use and increased control over the form and size of nanoparticles. Plant-mediated synthesis has numerous advantages because it is reasonably safe, quick, and successful even at room temperature and does not require any additional physical equipment. Surprisingly, practically every part of the plant has been reported to be beneficial, notably the leaves, with some studies focused on fruit as well (Dash et al., 2015; Kumar et al., 2007; Yang et al., 2014).

Toxicity and anticancer activity of green synthesized AuNPs

Cancer, as we know, is the uncontrolled proliferation of a healthy cell that results in genetic abnormalities and mutations that spread across cells and tissues, resulting in carcinogenesis (the development of cancerous cells) (Iqbal et al., 2017). Nanotechnology is a fast-expanding field that employs nanoscale materials for diagnostic and therapeutic purposes. Cancer therapy is one medical field where nanomaterials (NMs) have a wide range of applications (Shi, 2021). The merger of nanoscience with pharmaceutical science can pave the way for important changes in medical research, such as the development of drug delivery systems for cancer therapy. Numerous nanostructures, including gold nanoparticles (AuNPs), CNTs, dendrimers, liposomes, and micelles, are frequently utilized as drug delivery vehicles (Chakraborty et al., 2014; Perissutti et al., 2017). Gold nanoparticles (AuNPs) have been used in targeted therapy to deliver drugs (Thambiraj et al., 2018). Without a doubt, adopting a regulated drug delivery system is a vital technique for increasing drug therapeutic effects and reducing drug-molecule adverse effects (Patel et al., 2017). AuNPs have also been used as delivery vehicles in combination with photothermal therapy and to deliver medications to cancer cells effectively (Sansone et al., 2018). As a result, the potential risks, such as carcinogenicity and undesired toxic consequences, limit the use of NPs in a variety of applications, particularly biomedicine. Traditional cancer treatments are harmful to the body, generating side effects or unintended effects on healthy cells, the development of drug-resistant cells, rapid drug metabolism, and the shortening of effective treatment time (Chugh et al., 2018). Plant-mediated green synthesis has the ability to generate NPs solutions containing biologically active compounds derived from natural extracts, which can have an effective anticancer activity on human cancer cells. A substantial number of biosynthesized AuNPs have been shown to have anticancer activity; nevertheless, their efficacy and cellular effects are dependent on the biological extract employed during the synthesis procedure (Li et al., 2017). Plants, which contain a wide diversity of phytochemicals and medicinal characteristics, play an important role in the treatment of various diseases around the world, including cancer. Because plant extracts are the widely available, cultivable, and cost-effective, green synthesis of MNPs utilizing plant extracts has numerous advantages over other biological resources (M. Khan et al., 2018). MNPs' optical, magnetic, and thermal properties make them promising candidates for use in a variety of medical applications, such as medication administration, medical diagnostics, therapeutic aims, and so on (Alalaiwe, 2019).

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Chapter 13

Green synthesis of nanoparticles from *Catharantus roseus* and study of its therapeutical applications

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Introduction

Nanotechnology has brought research breakthroughs in a variety of industrial applications. Among these, it has reached prominent levels in medicine such as bio-diagnosis, drug delivery, and cancer treatment (Shittu et al., 2017). Nanomedicine is an interaction between the field of nanotechnology and medicine, involving the development of new therapeutic and diagnostic modality using precisely designed nanoscale materials (Lucian et al., 2013).

Nanoparticles are nanoscale-sized particles that exhibit new properties such as high surface-area-to-volume ratio and excellent optical, physical, and chemical properties. These particles find advantages in the drug delivery area and overcome the limitations of most traditional particles. And important is the metal nanoparticles that are being brought to the fore as a new platform for targeted delivery of therapeutic agents. The green synthetic approach is basically aimed at building reliable and environmentally friendly processes (Velayutham et al., 2012). These processes aim to reduce/minimize waste generation and focus on the use of environmentally friendly solvents and renewable resources. Studies have reported the green synthesis of metal nanoparticles using a variety of biological materials (bacteria, fungi, algae, and plant extracts) (Kholoud et al., 2010).

The synthesis of green nanoparticles via plant extracts is considered to be a simple and easy approach (Maliszewska et al., 2011). Furthermore, the varying concentrations of phytochemicals in plant extract can act as a source of reduction and stabilization in nanoparticle synthesis procedure (Narayanan et al., 2010; Malik et al., 2014). The biological synthesis of nanomaterial can solve environmental challenges like solar energy conservation, agricultural production, catalysis, electronic, optics, and biotechnological area. Green synthesis of the nanoparticle is cost-effective, easily available, eco-friendly, nontoxic, largescale production, and act as a reducing and capping agent compared to the chemical method which is very costly as well as it emits hazardous by-product which can have some deleterious effect on the environment (Goodsell, 2004).

Biological synthesis utilizes naturally occupying reducing agents such as plant extract microorganism, enzyme, and-polysaccharide which are simple and viable which is the alternative to complex and toxic chemical processes (Subbaiya and Masilamani, 2015). *Catharantus roseus* (Periwinkle or Sadabahar) is a traditionally used medicinal plant belonging to the family Apocynaceae. It is also known as *Lochnera rosea*, *Ammocallis rosea*, and *Vinca rosea* (Maria et al., 2020). It occurs in every tropical or subtropical region of the world. Various phytochemical studies of *C. roseus* have been reported in the literature and its extract is known to exhibit antiviral, antibacterial, antioxidant, and antifungal activities (Jaleel et al., 2009). Alkaloids are considered the major chemical constituents of this plant.

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FIGURE 13.1 *Catharanthus roseus* plant.

It is considered a chemical factory of alkaloids producing 130 different kinds of terpenoidindol alkaloids like vinblastine, vincristine (anticancer agent), ajmalicine (antihypertensive agent), serpentine (antineuro inflammatory agent), and vindolicine (antidiabetic agent) (Jayanthi et al., 2010) exhibit the percentage of alkaloids that exist in different parts of *C. roseus* (Fig. 13.1). Besides alkaloids, *C. roseus* contain tannins, flavonoids, polyphenols, and carbohydrates (Lorena et al., 2015; Kanika et al., 2016).

Silver nanoparticles

Silver nanoparticles find applications in biomarking, sensors, antimicrobials, catalysts, electronics, and other medical applications such as drug delivery and disease diagnosis (De Jong et al., 2008). The main goal was to develop and characterize silver nanoparticles through biotransformation of *Catharanthus roseus*. It is a readily available plant known for its antibacterial, antifungal, antioxidant, antibiotic, and anticancer properties (Venkata et al., 2016). Pink periwinkle (*Catharanthus roseus*) is a perennial herbaceous plant grown commercially for medicinal purposes in India, Australia, Africa, and southern Europe. Contains more than 70 alkaloids, most of which are indole types. Alkaloids such as azamalicin, serpentine, and reserpine are well known for their hypotensive and antispasmodic properties (Siddharthan et al., 2019).

Sample collection and preparation of leaf extracts

Green synthesis, silver nanoparticles, Madagascar periwinkle, antiangiogenesis, Zebrafish. Various parts of Madagascar periwinkle were collected, air-dried, and isolated. An extraction medium was prepared for synthesizing silver nanoparticles. In addition, extracts and nanoparticles characterized by multiple characterization methods, so it was investigated for antiangiogenesis or inhibition of angiogenesis Property.

Crushed dried plant stems, leaves, and roots into a blender for fine powders and stored in a dry atmosphere to avoid moisture and microbial contamination. Add fine powder of stems, leaves, and roots (25 g) separately in the cup. Then the Powdered plant sample dissolved in various solvents such as acetone and ethanol, Methanol, and petroleum ether in a ratio of 1:10 (w/v). Evaporate the filtered solvent extract (air dry) and treated with silver nitrate aqueous solution (100 mL 2 mM) Possibility of Ag Nps biosynthesis (Kathiravan et al., 2020).

Synthesis of silver nanoparticles

Silver nitrate solution (2 mM) was prepared, then reactions with various solvent extracts (from C. roseus), was reddish brown. Maximum value at different times 8-h intervals were taken using UV Visible spectroscopy. The mixture was centrifuged to separate nanoparticles. Then discard the supernatant and the pellet was removed by washing it with distilled water impurities and reactants (Kathiravan et al., 2020).

The obtained pellets were alcohol-precipitated, dried in a watch glass, and stored at room temperature for further characterization and investigation of biological activity.

And then the extracted plant sample obtained from a variety of solvents was tested for their bio-reducing ability in the production of Ag Np using UVV in spectrum analysis.

Characterization of silver nanoparticles

UV-spectra analysis

Silver nanoparticles synthesized from Catharanthus roseus leaf extract were identified by measuring the wavelength of the reaction mixture in the UV-visible spectrum of a Perkin Elmer spectrophotometer with a resolution of 1 nm (300−700 nm) in 5 mL quartz (Siddharthan et al., 2019). A cuvette with an optical path length of 1 cm. After 24 h of incubation, the extract solution was collected with a sterile pipette and centrifuged at 4000 rpm for 10 min. Centrifuged samples were analyzed spectrophotometrically as described above (Nabeel et al., 2014).

Fourier transmission infrared spectroscopy

FTIR is a chemical analysis method that measures the intensity of infrared light, the wavelength or wavenumber of light. It is used to analyze binding or interaction with possible biomolecules. IR spectroscopy reveals the vibrational properties of chemical functional groups in a sample. When infrared radiation interacts with matter, chemical bonds take the form of stretching, shrinking, and bending. Chemical functional groups tend to absorb infrared radiation within a certain range of wavenumbers with respect to their structure and position in the rest of the molecule. In the synthesis of silver nanoparticles, FT-IR data accurately reflect the reduction of silver ions and stabilization of the formed silver NPS by measuring the interaction between the silver salt and protein molecules. The properties of functional groups on the surface of Ag nanoparticles of plant extracts were investigated by FTIR analysis, and the spectrum was scanned in the range of 4000−400 cm^{-1} at a resolution of 4 cm^{-1}. Samples were prepared by homogeneous dispersion of Ag NPs in a dry KBr matrix compressed into an almost transparent disk. K Br was used as a standard for sample analysis (Venkata Subba et al., 2013).

Antimicrobial activity of silver nanoparticles

The antibacterial activity of silver nanoparticles against various pathogenic bacteria (*Klebsiella* sp., *E. coli*, *Pseudomonas* sp.) was evaluated. This strain was resistant to all broad spectrum antibiotics. This bacterial culture was inoculated into a nutrient solution and cultured overnight. This bacterial suspension was applied to Mueller-Hinton agar (MHA) plates using a sterile swab and a well cutter was used to create five wells in each MHA plate. One well in the center was kept as a control using commercial silver nitrate for comparison with varying concentrations of plant synthetic silver nanoparticles in four adjacent wells.

The maximum hole diameter is 5−7 mm. The antibacterial activity of Ag nanoparticles against bacterial pathogens was evaluated using the hole diffusion method.

Different concentrations of silver nanoparticles (100, 150, 200 and 250 μL at a concentration of 1 μg/μL) were added to the wells of the MHA plate of each strain. Plates were incubated at 37°C. for 24 h. After the incubation period, the area of inhibition measured in millimeters can be observed using the scale supplied with the antibiotic, and the results are entered into a table (Siddharthan et al., 2019) (Table 13.1).

TABLE 13.1 Synthesis and characterization of nanoparticle's from *Catharantus roseus*.

Nanoparticles	Synthesis	Incubation period	UV spectrophotometer	FTIR
Silver	10 mL leaf extract +90 mL silver nitrate solution	Incubate at RT/24 h, dark Brown appears	Maxima B/W 300−700 nm At 400−480 nm	Alkynes, ketones, alcohols, carboxylic
Cobalt	15 mL leaf extract +50 mL of 0.03 m calcium chloride	Heat for 30−60 min at 70°C Change of color observed	4000−5000 nm	Hydroxyl Group of Flavio
Titanium dioxide	200 mL of leaf extract +80 mL of 5 mM of titanium oxide	Incubate for 4 h Color changes to light green	200−400 nm	Hydroxyl, Ti−O
Gold	0.5 mL leaf extract +9.5 mL of 1 mM $AuCl_2$ (dichlorogold(1-)	Color changes from yellow to ruby red	Between 200 and 800 nm Peak 545.5 nm	Hydroxyl and carboxyl group

Characterization of TiO_2 nanoparticles

The synthesized nanoparticles were identified by X-ray diffraction (XRD) spectroscopy (PerkinElmer Spectrum One instrument, PW1830 instrument operating at a voltage of 40 kV and a current of 30 mA, CuKα radiation). The Fourier transform infrared (FTIR) spectrum of the sample was measured in diffuse reflection modes with a resolution of 4 cm^{-1} on KBr pellets using a PerkinElmer Spectrum One instrument. The powder sample for FTIR was prepared in the same manner as the powder diffraction measurement. We analyzed the FTIR spectrum of the synthesized TiO_2 nanoparticles and discussed the possible functional groups for forming nanoparticles. In the scanning electron microscope study, a 25 μL sample was sputter coated on a copper die and an image of nanoparticles was examined using a scanning electron microscope (SEM; JEOL, model JFC1600). Topography was examined using an atomic force microscope (AFM; PARKS scanning probe microscope) operating in noncontact mode. AFM images were processed using the XEI software provided by the PARKS system (Horcas et al., 2007).

Cobalt nanoparticles

The synthesis of cobalt nanoparticles using a methanol extract of *C. roseus* has been reported for the first time (Rajmohan et al., 2015; Patel et al., 2015).

After synthesis, these nanoparticles were characterized and evaluated for their antioxidant, antibacterial, hemolytic, and catalytic activities. Biosynthesized nanoparticles are considered an effective approach to wastewater treatment containing the dye alizarin red S. The catalytic activity of CoNP was studied by modifying the surface of the synthesized nanoparticles with cetyltrimethylammonium bromide (CTAB), a cationic surfactant, and conducting comparative studies on decomposition. The anionic dye alizarin red S was performed under various experimental conditions.

Synthesis of cobalt nanoparticles

C. roseus was a collected were collected plant (including roots, stems, leaves, etc.). The flowers were thoroughly washed with water to remove dust. The particles and shadows were dried at room temperature for 25−30 days. Then, it was pulverized into a fine powder.

The amount of plant powder (50 g) was added to 300 mL of 30% methanol solution into a 500 mL flask and mix thoroughly. The mixture was then heated to 70°C for 30 min with continuous stirring. After heating, the mixture was cooled and then filter with pleated filter paper. For green synthesis of cobalt nanoparticles (CoNP) by using *C. roseus* methanol extract, 15 mL plant extract was added dropwise to 50 mL of a 0.03 M aqueous solution with constant stirring at 80C. Cobalt ion (CoII) was reduced to Co within 30°C for about 60 min after the color of the reaction mixture changed there will be the formation of CoNP was shown. The solution was centrifuged at 5000 rpm for 40 min. After that separated nanoparticles were completely washed, dried, and stored in the oven and then used for further research.

Characterization of cobalt nanoparticles

FTIR spectra of *C. roseus* plant extracts were recorded using a FTIR spectrometer ranging from 4000 to 500 cm^{-1}. The synthesized CoNPs have been characterized by various methods. The structural properties of cobalt nanoparticles were determined using X-ray diffraction. The morphology of green synthetic nanoparticles was performed using field emission scanning electron microscopy. The resulting CoNPs were scanned for elemental composition using energy-dispersive X-ray spectroscopy (Maria et al., 2020).

Antioxidant activity

The antioxidant activity of nanoparticles can be assessed using 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging assay. That is, nanoparticles of different concentrations were added to a fixed volume of a DPPH solution of a certain concentration. The reaction was then accelerated by placing these solutions in the dark at room temperature for 30 min. After using a conjugate such as methanol, the absorbance was recorded using a UV spectrophotometer. Experiments can be performed in the same manner using ascorbic acid as a standard. Absorption rate is evaluated based on absorption rate, the lower the absorption rate, the higher the cleaning power (Maria et al., 2020).

Antibacterial activity

Irradiated and nonirradiated samples were tested separately for Gram-positive bacteria (Bacillus subtilis JS 2004) and Gram-negative bacteria (*Escherichia coli* ATCC 25922). To test the antibacterial activity of nanoparticles, we used the disk

diffusion method. A suspension of test microorganisms (100 mL) containing 107 CFU/mL (colony-forming units) of bacterial cells was inoculated into nutrient agar medium. A filter disk with a diameter of 9 mm was separately impregnated with the test solution and placed on an agar plate already inoculated with the test microorganism. Discs without samples were used as negative controls. Plates were incubated for 18 h at 37°C. for bacterial strains.

Then, the antimicrobial activity was evaluated as the diameter of the growth inhibition zone (Maria et al., 2020).

Titanium nanoparticles

Nanoparticles have received a lot of attention due to their diverse uses is *Lactobacillus* sp. Reported TiO_2 Nanoparticles synthesized using *Saccharomyces cerevisiae* had antibacterial and antifungal properties. Using plant extracts for the synthesis of nanoparticle s can be an advantage over other environmentally friendly biological methods because it eliminates the tedious process of maintaining cell culture. Recently, green TiO_2 nanoparticles have been synthesized using natural products such as Nyctanthesarbortristis extract (Hanna et al., 1998).

Synthesis of titanium dioxide nanoparticles

C. roseus broth solution fresh leaves are washed well and chop 10 g of leaves take in 250 mL Erlenmeyer flask and add 100 mL sterile redistilled water and then bring the mixture to a boil it for 10 min before the final decantation. The extract was filtered by using Whatman filter paper #1 and stored at 15°C, and it can be used within a week. The filtrate was treated with 20 mL of leaf extract and was added to 80 mL of 5 mM (39.94 mgTiO_2 powder in 100 mL MilliQ water) dissolve in an Erlenmeyer flask with stirring at 50°C. After 4 h of continuous stirring, the formation of light green color take place that change indicated the formation of TiO_2 NPs (Fig. 13.2) (Velayutham et al., 2012).

Gold nanoparticles

Gold nanoparticles have been synthesized by traditional chemical and physical methods. However, these methods are toxic and can be costly because they rely on harsh reaction conditions. The use of plant extracts has been proposed as a possible environmentally friendly alternative for the synthesis of nanoparticles for medical and technical applications due to their common abundance and nontoxic properties. It has been reported that nanoparticles can be synthesized using plant extracts at rates comparable to chemical methods (Umesh et al., 2011).

Synthesis of gold nanoparticles

Fresh leaves of *Catharanthus roseus* were washed with clean water to make sure they were dust-free and sand-free. The plants were air-dried at room temperature for 3 weeks to prevent the destruction of the heat-unstable components of the plants by direct sunlight. The leaves were mixed with the powder and kept in a safe condition. 5 g of weighed plant sample

FIGURE 13.2 Particles are extracted from Whatman filter paper.

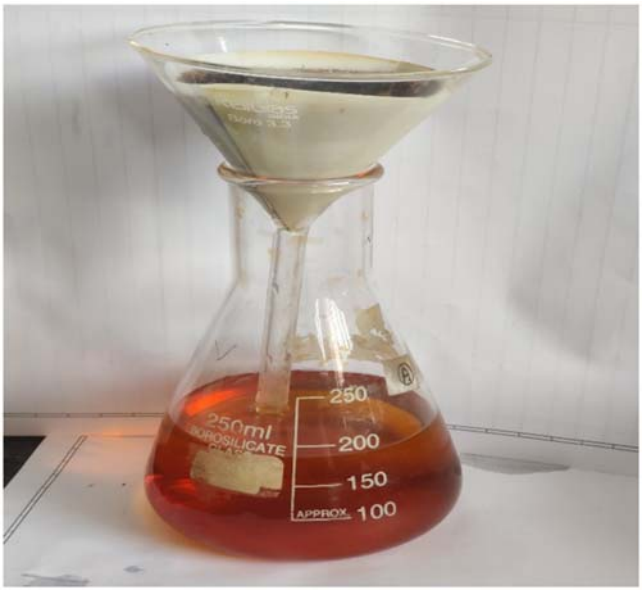

FIGURE 13.3 Change of color observed in Erlenmeyer flask after boiling for 5 min.

was added to 100 mL of sterile distilled water in an Erlenmeyer flask, and boiled for 5 min and filtered. Approximately 0.5 mL of filtrate was pipetted into a test tube, 9.5 mL of 1 mM $HAuCl_4$ was added, and Au^{3+} ions were reduced according to the method.

Color changes were observed and the wavelength was scanned using a UV spectrophotometer (Fig. 13.3).

Functionalization of gold nanoparticle

The functionalization of the gold nanoparticle turned into finished the usage of a trendy drug (Lincomycin) and polyethylene glycol (Molecular Weight = 3000). Three formulations of the three composites have been made.

In the First formulation trendy drug-lined nanoparticle and polyethylene glycol turned into organized with the aid of using the addition of 0.5 mL of biosynthesized *C. roseus* gold nanoparticle (AuNPs) with 5 g polyethylene glycol and 0.58 g of the usual drug with right stirring of 1 h. In the Second formulation, similarly 0.5 mL of biosynthesized *C. roseus* gold nanoparticle turned into introduced to 5 g polyethylene glycol and stirred for 1 h. In the Third formulation, 1 mL sterile deionized water was turned into combined with 0.58 g of trendy drug and 5 g of polyethylene glycol with right stirring of 1 h. All the formulations have been made into pill shape and left for 24 h in an incubator at 370 c to allow digestion of the mixture, after which will air dried (Joanne et al., 2011).

Antimicrobial activity

Antimicrobial activity of gold nanoparticles was tested in triplicate. *C. roseus* tested and recorded the results for gram-positive, gram-negative, and fungi bacteria. Gram-positive bacteria were more sensitive than gram-negative bacteria. Selected microorganisms demonstrated high sensitivity to biosynthesized nanoparticles. The antimicrobial activity of the test sample was investigated using the (measure the inhibition zone) Disc diffusion test against various pathogenic microorganisms. In the current study, higher (30 L/disc) sample concentrations had higher sensitivity than lower (15 L/disc) concentrations in all tested microorganisms (Joanne et al., 2011).

All pathogens were fairly affected in this study, and no effect was observed in the test samples. The gold nanoparticles not only interact at the cell membrane's surface but also enter the bacteria and cause cell damage by interacting with phosphorus/sulfur-containing DNA and its replication. In bacteria, the test sample was most effective against B5, with a lesser effect on B4. This was effective against F4 fungi but had a smaller effect on F2. All the microbial strains are more sensitive to higher concentrations (30 L), and he concludes that gold materials are an effective alternative to antibiotics for treatment. In bacterial cells, the nanoparticles release gold ions, which improves their performance.

Drug release

For the drug and each formulation, test tubes containing 3 mL sterile deionized water were prepared. Each tablet's height and diameter were measured, and the tablets were immersed in 3 mL of sterile deionized water for 3, 6, 9, 12, 15, and 18 min.

The absorbance and standard drug concentration of various dissolved formulated tablets were measured spectrophotometrically, and antibacterial activity was recorded (Jaleel et al., 2009).

Zinc oxide nanoparticles

ZnO-NPs have found tremendous use in biomolecular, diagnostic, and microelectronic applications (Singhal et al., 1997). Even though bulk zinc oxide cannot absorb arsenic, ZnO-NPs have been used to remove sulfur from water. This is because nanoparticles have much larger surface areas than bulk particles (Dhermendre et al., 2008). ZnO-NPs are always in the spotlight due to their fascinating properties and wide range of applications. The bio-inspired synthesis of ZnO-NPs was accomplished using environmentally and environmentally friendly accepted systems. Zinc oxide nanomaterials are used in the preparation of substances with medicinal and cosmetic properties. Zinc oxide is used on the skin to treat skin irritation, diaper rash, dry skin, and blisters due to its antibacterial properties. It is available in powders, antiseptic creams, surgical tapes, and shampoos. Zinc oxide is combined with iron oxide to make calamine lotion, and it is combined with eugenol to make zinc oxide eugenol, which is used in dentistry (Ferracane et al., 2001; Van Noort Richard et al., 2002).

Synthesis of ZnO nanoparticles

The pH of 0.025 M aqueous Zinc acetate was adjusted with *C. roseus* aqueous leaf extract. The result was a pale white solution. To remove impurities, the precipitate was washed with distilled water followed by ethanol after stirring. The solution was vacuum dried and used for ZnO Nanoparticles characterization.

Conclusion

Vinca rosea (*Catharanthus roseus*) is a low cost, easily available perennial herb grown commercially for its medicinal uses in India, Australia, Africa, and Southern Europe. It contains more than 70 alkaloids, mostly of indole types; Alkaloids like Ajamalicine, Serpentine, and Reserpine are well known for their antibacterial, antiinflammatory, antifungal activity, antimalarial activity, and antiviral effects.

The root base contains the alkaloid Alstonine, which has an ability to reduce blood pressure. Vinblastine and Vincrispine are the alkaloids which act as anticancer drugs in the treatment of different types of cancers like Lymphomas, Hodgkin disease, Breast cancer, Acute Lymphocytic leukemia, soft tissue sarcomas, Multiple myeloma, and Neuroblastoma.

The surface plasmon absorption bands were observed in the range between 400 and 480 nm. In the present study, the heating at 60°C was done prior to "silver nitrate addition" in order to overcome any physical alteration caused by heat application (in the presence of plant extract) on silver nitrate which is the source for nanoparticles formation.

The UV-vis spectrophotometer shows the maxima value around 300−700 nm at 400−480 nm wavelength range which corresponds to the production of silver nanoparticles. The silver nanoparticles absorb radiation intensely at a wavelength of 400 nm due to the transition of electrons. It is known that the silver nanoparticles exhibit a characterized absorption peak in the UV−visible spectrum around specified nm due to the Surface plasmon excitation. The silver nanoparticles extract exhibits maximum inhibition against *Pseudomonas aeruginosa* with zone diameter of 22 mm, followed by *Klebsiella* sp. which showed a maximum zone diameter of 18 mm, *Escherichia coli* showed a maximum inhibition zone size of 15 mm. It was seen that all the gram-negative organisms tested [*Escherichia coli, Pseudomonas aeruginosa, and Klebsiella*] showed greater zone of inhibition when compared to control zone size (using silver nitrate). In the present study, it was noted that when the nanoparticles concentration increased the zone size also increased for all the tested organisms. Hence, for the treatment regimen, the organism load is needed to be identified before fixing the concentration when silver nanoparticles are the therapeutic agent.

Nanoparticles have been used to alter and improve the pharmacokinetic and pharmacy dynamic properties of various types of drug molecules. The high bacterial activity might have occurred due to the silver cations released from the silver nanoparticles that act as bacterial agents.

So, these pathogenic bacterial organisms causing infections can be controlled using silver nanoparticles. The now-recommended antibiotics for the bacterial infections are Penicillin, Ampicillin, Tetracycline, chloramphenicol, and so on. Though antibiotics play a role in controlling bacterial infections most of the pathogens are developing resistance to them. Hence there is an urgent need for the discovery of alternatives to control pathogens. The antibacterial activity of silver nanoparticles may differ according to their structural symmetry which can vary according to the source of production and other physiological conditions.

The present study attempted to synthesize silver nanoparticles from *Vinca rosea* (*Catharanthus roseus*) using the modified procedure, and the inhibition activity against antibiotic-resistant pathogenic strains was tested. Basically, these chemical methods produce more toxic substances, so the most important feature of this study is that it is free from the chemical process. Hence the biological method is the alternative approach for the synthesis of silver nanoparticles. The nanoparticles and nanotechnology being one of the fast-growing fields in the life sciences, the use of plant extracts is also an environmentally friendly alternative. *Vinca rosea* is an easily available plant known to possess antimicrobial, antifungal, antioxidant, antibiotic, and cancer remedies properties.

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Chapter 14

Supercritical solvent-assisted green isolation of naturally occurring therapeutically active nanomaterial—a review

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Sustainable green synthesis

A sustainable green synthesis is an approach to the synthesis or manufacturing of chemical compounds with a higher magnitude of environmental friendliness. In conventional manufacturing processes, the primary approach was aimed at higher economical outcomes only, but in the sustainable synthesis approach, the focus is aligned with a viewpoint of complementarily with ecological components also. Broadly if the synthesis is done with a balanced focus on ecological as well as economical facets, then the adverse effects of industrialization may be lowered. Sustainability is to be taken care of at mainly four stages as described in Fig. 14.1.

Anastas and Zimmerman have pioneered in coining the concept of green chemistry and give 12 principles of green chemistry. Principle 1 focuses on prevention; Principle 2 focuses on atom economy in production processes; Principle 3 focuses on less hazardous synthesis; Principle 4 focuses on safer chemicals; Principle 5 focuses on safer solvents; Principle 6 focuses on energy efficiency. Principle 7 Targets durability; Principle 8 focuses on the reduction of derivatives; Principle 9 focuses on catalytic processes; Principle 10 focuses on design for degradation; Principle 11 focuses on real-time analysis for pollution prevention; and Principle 12 focuses on inherently safer chemistry for accident prevention (Anastas and Zimmerman, 2003; Paul et al., 2020).

If in nanoparticle synthesis, Principle 1 of green chemistry is adopted, it suggests that it is always better to prevent hazardous waste than to have to clean it up once it has already been created. It is also better to design a process to be safe instead of having to figure out ways to protect people from toxic chemicals being used or dangerous processes. So the practices of nanoparticle manufacturing must be rerouted to more sustainable ways like the use of supercritical fluid-assisted synthesis.

Supercritical fluid especially $ScCO_2$ and ScH_2O has below noted benign characteristics

1. Very low human toxicity potential by ingestion.
2. Very low human toxicity potential by exposure to both dermal and inhalation.
3. Very low terrestrial toxicity potential.
4. Very low aquatic toxicity potential.
5. Very low ozone depletion potential.
6. Very low photochemical oxidation potential.
7. Very low acidification potential.

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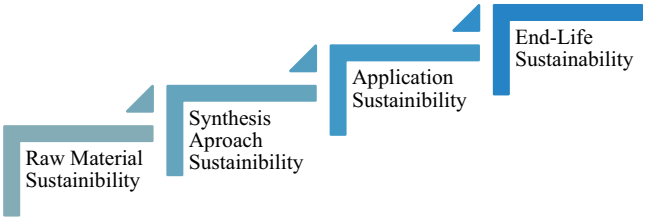

FIGURE 14.1 Nanomaterial sustainability.

Nanoparticles

Natural nanomaterials are can be isolated from plants and a single unit of them possess a size range of 1−100 nm. Nanoparticles possess significant potential as therapeutically active compounds. Their remarkable activities are because of the higher surface area to volume ratio. As this ratio goes higher, the interaction between the nanoparticles and substrate becomes much swifter and more effective (Kurajica et al., 2016).

Natural or synthetic nanomaterials found their effectiveness in the sectors like biomedical, bionic organ implantations, nanorobotics, energy sectors, electromechanical, agriculture, and pharmaceuticals. These eclectic activities boost the emergence of various interdisciplinary fields like nano-chemistry, neuroprotection, nano-biotechnology, biosensing, stem cell growth, nano-mechanical technologies, immunoassays, nano-pharmacology, etc. (Aiba et al., 2011).

There are mainly three classifications of nanoparticles based on sources. The natural sources have been found to be the most ancient but least explored, more focus must be exerted on making them more widespread for the sake of the global diverse demography. In semisynthetic sources of nanoparticle procurement, the precursors can be found from plant sources, and then functional group modifications can be deployed to make them more effective toward selective substrates. In synthetic sources, the nanoparticles are made exclusively with laboratory procedures by either mimicking the natural processes or by using advanced technological assistance. The sources are depicted in Fig. 14.2. In ancient times, practices of applications of natural materials as preventive and curative agents had been found (Balfourier et al., 2020). The main sources for procurement of these therapeutically active nano-compounds were plants, animals, microorganisms, and minerals which are shown in Fig. 14.3.

There are many advantages if nanomaterials are derived from natural sources. Some advantages are, that they are more environment friendly compared with synthetic nanomaterials, and they are more economical because of the obsolesce of multistep synthesis. They are more sustainable because of lower e-factor, they possess more selectivity and diversity, and they can be isolated without state-of-the-art infrastructural facilities (Askenase, 2021). The solvent sustainability is shown in Fig. 14.4.

The isolation of natural nanomaterial has also been distributed in two separate classes, i.e., unsustainable isolation and sustainable isolation. The techniques which are associated with the utilization of toxic solvents, higher energy, and low yield production techniques are classified as unsustainable and the techniques which utilize nontoxic, biodegradable solvents and utilities, have less energy consumption in production stages, and higher yields are classified as sustainable isolation techniques. For these reasons, more focus should be made to isolate natural nanomaterial with environmentally benign sustainable solvents. The method of isolation is shown in Fig. 14.5. Supercritical solvents are found to be the most nontoxic and environmentally benign for the isolation of natural nanomaterials. There are several supercritical solvents that can be utilized for isolation, like Supercritical CO_2, H_2O, CH_4, C_2H_6, C_3H_8, C_2H_4, C_3H_6, CH_3OH, C_2H_5OH, C_3H_6O, N_2O (Maio et al., 2021; Yousefi et al., 2019; Manjare and Dhingra, 2019).

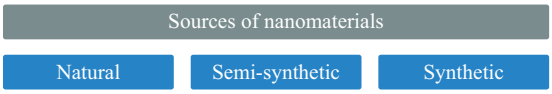

FIGURE 14.2 Nanomaterial sources.

FIGURE 14.3 Natural nanomaterial sources.

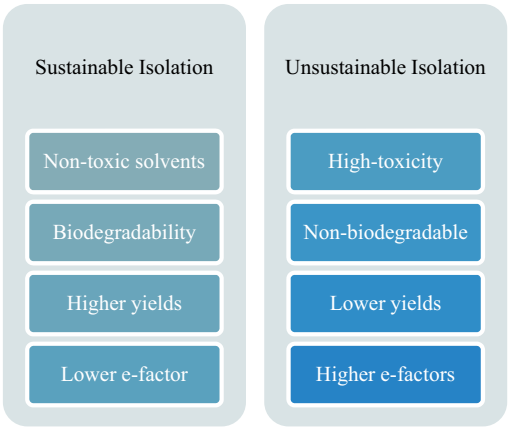

FIGURE 14.4 Solvent sustainability.

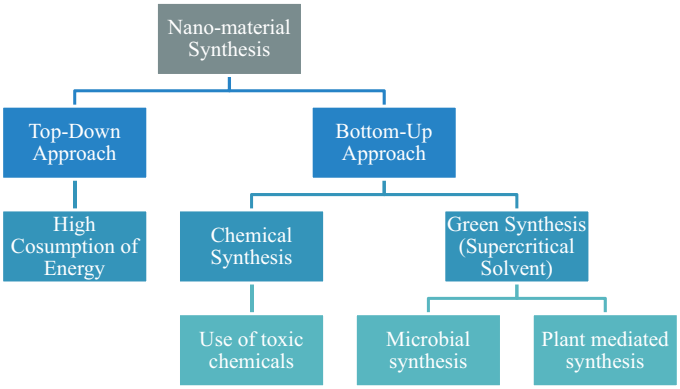

FIGURE 14.5 Nanomaterial synthesis approach.

Therapeutically active natural nanomaterial

Plants, animals, microorganisms, and geological activities produce nanomaterials by eclectic means as described in Fig. 14.6. There have been prominent studies undergone in the nanomaterial synthesis, natural enzymes, artificial enzymes and nanoenzymes timeline as shown in Fig. 14.7 (Wang et al., 2016).

Prominent natural nanomaterials

There are abundant amount of therapeutically active compound present in various plants, which can be extracted in nanoform, as the classification is given in Fig. 14.8. Paul et al. (2020) estimated *Moringa oleifera* leaf extract for the synthesis of silver nanoparticle-mediated drug delivery system. Sharma et al. (2020) synthesized Tungsten Nanoparticles from *Moringa oleifera* and reported antimicrobial and antiplatelet activities compared with known standard drugs.

Another prominent application of NENPs (Naturally extracted nanoparticles) is mimicking the natural enzymes for therapeutic and preventive causes (Wei and Wang, 2013). Iron oxide NPs (Nano Particles) and NENPs as peroxidase mimics were reported by Gao et al. (2017). Peroxidase, containing a huge family of enzymes, boosts the oxidation of its substrate with peroxide. Peroxidases NPs play a vital role in detoxifying reactive oxygen species and act as pathogen defender (Epp et al., 1983).

$$2GSH + H_2O_2 \rightarrow GS{-}SG + 2H_2O$$

FIGURE 14.6 Natural nano-material synthesis.

FIGURE 14.7 Natural enzymes, artificial enzymes and nanoenzymes timeline.

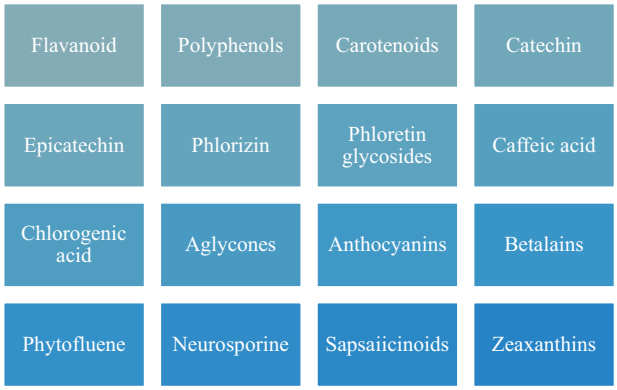

FIGURE 14.8 Prominent nanomaterials.

Natural Nanoparticles have reportedly been isolated from *Scutellaria barbata* exert potential anticancer activity in the pancreatic cancer cell (PANC-1) (Wang et al., 2019), breast cancer (Marconett et al., 2010), colorectal cancer (Yang et al., 2017), hepatocarcinoma (Kan et al., 2017), skin cancer (Suh et al., 2007), lung cancer (Chen et al., 2017), and ovarian cancer (Lee et al., 2017).

Sustainable synthesis of nanoparticles of copper oxide from fruit extract of *Syzygium alternifolium* (Wt.) Walp., has been studied by Yugandhar et al. (2018) reported antiviral activity. The synthesized nanoparticles have great properties specifically, spherical shape, size (ranging from 2 to 69 nm), nonagglomerated, and polydispersed nature. Calculation of EID_{50} was carried out by the following equation for viruses (Yugandhar et al., 2018).

$$\text{EID50} = \frac{(\%\text{ of infected eggs at dilution above 50\%}) - 50\%}{(\%\text{ of infected eggs at dilution above 50\%}) - (\%\text{ of infected eggs at dilution below 50\%})}$$

Nonmetallic nanoparticles from *Cyperus rotundus* root extract were extracted and studied (Sasidharan and Pottail, 2020). Ponarulselvam et al. (2012) have synthesized silver nanoparticles using leaves of *Catharanthus roseus* Linn. G. Don, the NPs exhibited potential antiplasmodial activities. Gold-Nano particles (AuNPs) were extracted from *Morus alba* (white mulberry) and reported as potential inhibitor against human pathogens (Adavallan and Krishnakumar, 2014). *Hibiscus rosa-sinensis* mediated eco-friendly synthesized AuNPs was studied by Yasmin et al. (2014), and the mechanism of AuNPs is shown in Fig. 14.9.

Experimental: sustainable greener isolation of natural nanomaterials

In the recent era due to various challenges like greenhouse gas emissions, land-water-air pollution, and toxicological effects, the whole focus of the natural nanomaterial synthesis approach is redirected toward sustainable ways. One among the various such ways is synthesis assisted by supercritical solvent. Traditionally the solvents like hydrocarbons, aliphatic/aromatic ketones, aliphatic/aromatic ethers, oximes, amides, etc. were utilized for the same. But due to their adverse chemical, physiological, and toxicological effects; nowadays these methods were oblivious in the field of synthesis.

Supercritical solvent-assisted synthesis can improve:

Waste minimization can protect the environment and often turns out to have positive economic benefits. Waste minimization can improve:

Efficient production practices. Waste minimization can achieve more output of product per unit of input of raw materials.

Economic returns. More efficient use of products means reduced costs of purchasing new materials improving the financial performance of a company.

Public image. The environmental profile of a company is an important part of its overall reputation and waste minimization reflects a proactive movement toward environmental protection.

Quality of products produced. Innovation and technological practices can reduce waste generation and improve the quality of the inputs in the production phase.

Environmental responsibility. Minimizing or eliminating waste generation makes it easier to meet targets of environmental regulations, policies, and standards. The environmental impact of waste will be reduced.

Green principle-5 given by Anastas and Warner (2000) focuses on safer solvents and auxiliaries using solvents (liquids used to dissolve other materials), separating agents (chemicals that help two other things separate completely), or other

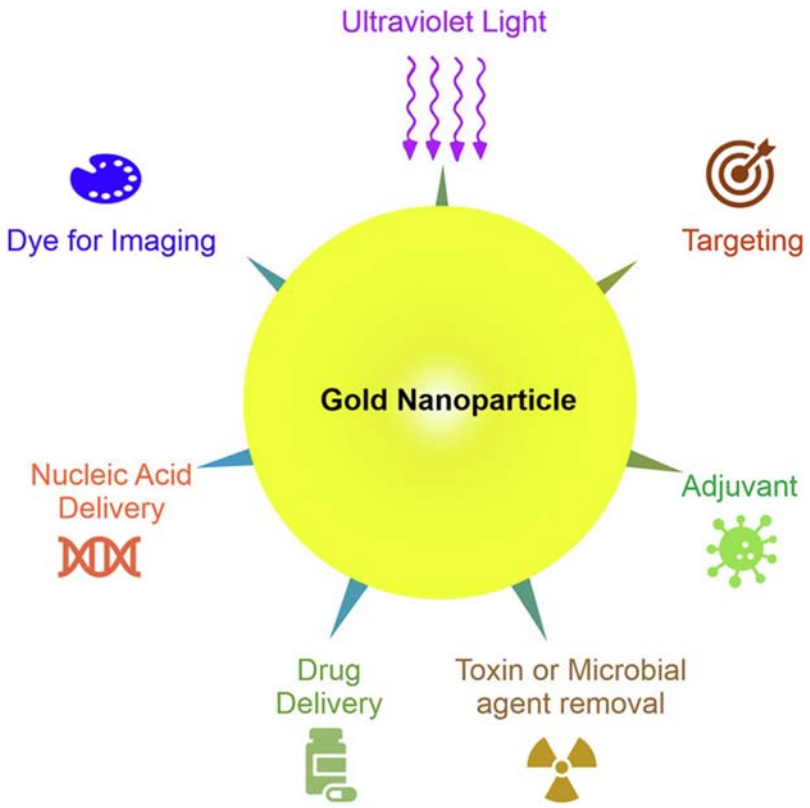

FIGURE 14.9 Mechanism of gold nanoparticle.

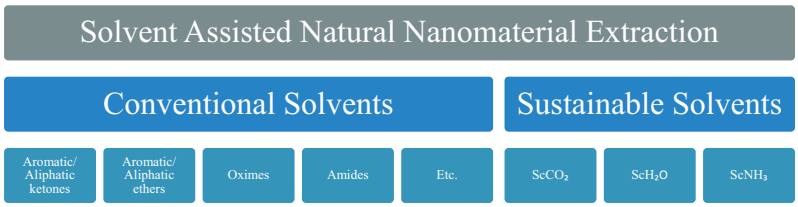

FIGURE 14.10 Solvent-assisted natural nano-materials (NNMs) extraction.

auxiliaries should be cut out of a procedure as much as possible or utilized if it is environmentally benign. Solvent-assisted natural nano-materials (NNMs) extraction is shown in Fig. 14.10 and Factors to be considered while selecting solvents for extraction of NMs is shown in Table 14.1.

For the extraction of natural nanomaterials, as shown in Fig. 14.11 (Kaiser et al., 2001), the subject is charged in the extractor in the pulverized palletized or intact form in the series of extractors, in a separate compression chamber CO_2 is pressurized and temperature is reduced to the point where it gets transformed in the supercritical phase. Simultaneously in another chamber co-solvent if/when required can be charged. These solvents and gases pass through the animal, plant, or mineral-based natural raw materials in the countercurrent system. The nanomaterial with some other impurities gets extracted by $ScCO_2$ or a selected supercritical solvent. In the separator, the solvent and CO_2 can be separated from the outlet stream and recycled in the super cold state to the inlet stream via a high-pressure pump. The nanoparticles then pass through a multistage crystallization process for ultrapurification. The phase diagram of supercritical fluid is shown in Fig. 14.12 for consideration of temperature and pressure optimization while extraction of NPs.

Future scopes and challenges

The current global pathway is heading toward achieving higher standards of sustainability. Global Sustainable Development Goals-2030 is aiming at responsible production and consumption as one of the 17 prominent goals. The supercritical solvent-assisted extraction and synthesis processes must be optimized for the same as rapidly as possible for ensuring energy and material efficiency in manufacturing industries. These nanomaterials must also be assayed for nanotoxicological aspects.

TABLE 14.1 Factors to be consider while selecting solvents for extraction of NMs.

Attributes of concern for conventional solvents for natural nano-particle synthesis	Carcinogenicity Neurotoxicity Acute mammalian toxicity Reproductive and developmental toxicity Repeated-dose toxicity Environmental fate and toxicity

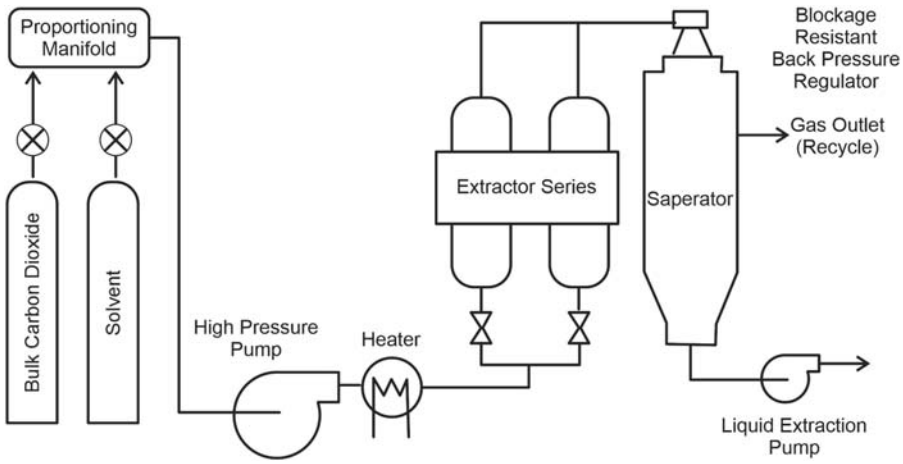

FIGURE 14.11 ScCarbon dioxide-assisted natural nanomaterial extraction unit process.

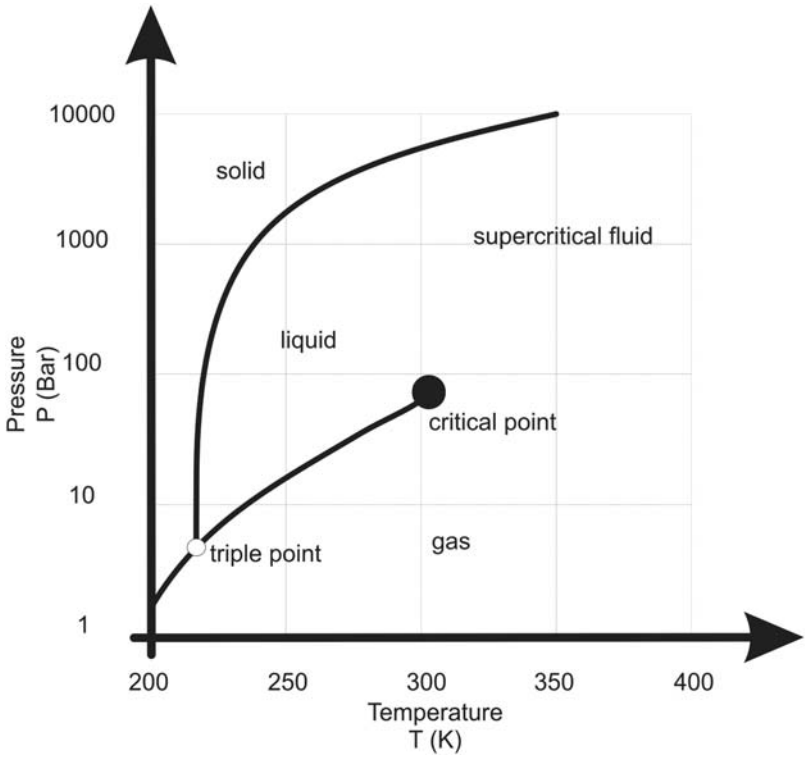

FIGURE 14.12 Phase diagram-supercritical fluid.

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Part II

In silico tools and techniques

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Chapter 15

In silico studies of phytochemicals of antiviral plants against human papillomavirus-16 E6 Oncoprotein: potential therapeutic drugs for cervical cancer

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Introduction

Human papillomaviruses (HPVs) are the cause of almost all cervical cancers, and it also contributes to a substantial fraction of anogenital and oropharyngeal cancers (Langsfeld and Laimins, 2016; de Martel et al., 2017). About 4.5% of all cancers worldwide are caused by HPV, affecting more women than men (de Martel et al., 2017). Cervical cancer is the third most common cancer in women worldwide (Carcinogenic HPV Infection, 2016; zur Hausen, 2009; Ma et al., 2019). A study reports that among all the women worldwide who are carriers of HPV, 32% are infected with HPV16 or HPV18, or both, which are considered as high-risk HPV (de Sanjosé et al., 2007). These two HPV types account for 70% of cervical cancer and precancerous cervical lesions. HPV infections are primarily transmitted through direct sexual contact (Carcinogenic HPV Infection, 2016; Schiffman et al., 2016) and have much higher prevalence in HIV-infected patients (Shiojiri et al., 2021). HIV-infected men in a same-sex relationship display the highest prevalence of anal infection and incidence of anal carcinoma (Donà et al., 2022).

Due to the existence of effective HPV vaccines, Cancers caused by HPVs, especially cervical cancers are one of the most preventable cancers if detected early and treated effectively (Goldie et al., 2003; Roden and Stern, 2018). When detected and diagnosed early they are treated with surgery, radiotherapy, or a combination of both. Due to a large misconception against the use of vaccines and the lack of proper medical facilities for early screening of HPV many still fall victim to this preventable virus. A study shows that in developing countries nearly over 500,000 new cases of HPV-related cervical cancers are diagnosed globally and every 2 min a woman dies from cervical cancer (Beddoe, 2019). To this current day, there is no effective treatment for HPV virus, and most of the infections due to HPVs are effectively managed by the host immune system. Most low-risk HPVs do not progress beyond warts formation. The problem begins only when high-risk HPVs, like types 16/18 becomes cancerous. The carcinogenicity of these HPVs results predominantly from the activity of the oncoprotein E6 and E7, which are viral proteins that impair growth regulatory pathways in the host cell (Carcinogenic HPV Infection, 2016). The present study is an effort to discover an effective antiviral agent against HPV 16 virus using in silico methods. Twelve medicinal plants in north-east India, exhibiting antiviral properties were screened for potential inhibitors against HPV 16 by analyzing its interaction with the target E6 Oncoprotein (PDB ID: 4GIZ) (Zanier et al., 2013).

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Materials and methods

Selection of plants and phytoconstituents

Twelve medicinal plants from north-east India, a region of the biodiversity hotspot that shares both the Himalayas and the Indo-Burma region (Tripathi, 2016) with antiviral properties were chosen for this study. The details of all the 12 medicinal plants, all the 138 phytoconstituents, and the reference journals are listed in Table 15.1.

Target protein description and active-site selection

E6 is a potent oncogene of HR-HPVs, and its role in the progression to malignancy has been and continues to be explored. E6 is known to interact with and subsequently inactivate numerous cellular proteins pivotal in the mediation of apoptosis, transcription of tumor suppressor genes, maintenance of epithelial organization, and control of cell proliferation (Tungteakkhun and Duerksen-Hughes, 2008). E6 interacts with a number of host cellular proteins like p53, E6AP, MAML1, retinoblastoma family proteins are proteins containing PDZ domains. E6 inactivates many of these proteins and affects many cellular pathways such cell proliferation and apoptosis (Garnett et al., 2006). The prevention of the binding of E6 to FADD and procaspase 8 will eventually restore the natural apoptosis pathways to cells infected by HPV (Tungteakkhun et al., 2008).

Hence, the interaction of E6 with FADD and procaspase 8 becomes a promising target in the treatment of HPV-associated cancer (Manzo-Merino et al., 2013; Tan et al., 2012). The HPV 16 E6 protein consists of 151 amino acids with high numbers of α-helical and β-sheet secondary structure (Tungteakkhun and Duerksen-Hughes, 2008). A number of flavonols were identified to be having th modest ability to inhibit the binding of E6 to FADD and Procaspase 8 (Yuan et al., 2012). To this current day, the exact knowledge of specific binding site on the E6 oncoprotein is not available. Recent study reveals that E6 forms a complex with the cellular E6AP ubiquitin ligase, ultimately leading to p53 degradation. The elucidated X-ray structure of a HPV16 E6/E6AP complex showed that HPV16 E6 forms a distinct binding pocket for E6AP. This gives evidence that the E6AP binding pocket is druggable, opening new possibilities for rational, structure-based drug design (Zanier et al., 2014). The HPV 16 E6 binding sites identified in a previous study with reference to six know inhibitors (Kolluru et al., 2019) were taken as the binding site for the current study, the binding site consists of amino acid residues viz., Leu50, Cys51, Val62, Leu67, Arg102, Gln107, and Arg131 (Fig. 15.1).

Target protein and ligand preparation

The sdf structure of most ligands (phytoconstituents) was downloaded from PubChem. Few of the ligands whose structures were not available were built using Gaussian 09 (Frisch et al., 2009) and were optimized with a decent methods and levels of theory [DFT B3lyp, 6-311G(d,p)]. All the ligands were prepared with Amber12:EHT force field using MOE 09 (Molecular Operating Environment, 2022). Hydrogen and charges were added and all the ligands were compiled into a single database together with the reference ligand (Myricetin). The Protein was also prepared with the same force field using MOE 09. Structural preparation was performed with corrections of the structure, addition of hydrogens and charges, and energy minimization with Amber12:EHT force field.

Molecular docking

The ligand database created with 138 phytoconstituents together with the reference compound were docked into the selected site of the target protein using triangle matcher method with induce fit model. The first 30 poses were generated with London dG scoring methodology and the best five poses were selected using GBVI/WSA dG methodology. The results of the docking interactions were visualized with Biova discovery studio, and both 2D and 3D images of the docking interactions were obtained.

Molecular dynamics simulation

MD simulations were performed using VMD (Humphrey et al., 1996) and NAMD (Phillips et al., 2020). CHARMM-GUI was used to prepare topology files for the protein and the ligands (Jo et al., 2008). Solvation of the system and analysis of MD trajectories were performed using VMD plugins. NAMD was used to perform MD-simulation using CHARMM36m force field (Huang et al., 2017). RMSD, RMSF, and Hydrogen bonding plots were prepared using QtGrace.

TABLE 15.1 List of 12 antiviral medicinal plants with their phytoconstituents, docking score, and the reference journals.

No.	Medicinal plants (botanical name)	S. No.	Phytoconstituents	Docking score (kcal\mol)	Antimicrobial properties and references
1	*Allium sativum* Linn	1	Ajoene	−5.1236	Antimicrobial, anti-viral (Sanjay et al., 2018)
		2	Allicin	−4.5374	
2	*Bergenia ciliata*	3	1-pentanol	−3.7610	Anti-microbial, anti-viral, anti-oxidant (Ahmad, 2018)
		4	Afzelechin	−5.2519	
		5	Bergenin	−5.7449	
		6	Camphor	−4.1590	
		7	Cianidanol	−5.3012	
		8	Decanoic acid	−4.8546	
		9	Gallic acid	−4.2790	
		10	Methyl cinnamate	−4.2775	
		11	Methyl nonanoate	−5.1371	
		12	Quercetin	−5.2893	
		13	Quercetin-3-O-D-xylopyranoside	−6.0698	
3	*Bidens pilosa* Linn	14	3,4-dicaffeoylquinic acid	−7.1965	Antimicrobial, antiviral (Bairwa, 2010)
		15	4,5-di-o-caffeoylquinic acid	−6.7875	
		16	Centaurein	−7.6227	
		17	Chlorogenic acid	−5.6891	
		18	Hyperoside	−6.2524	
		19	Isochlorogenic acid	−7.1055	
		20	Jacein	−7.1006	
		21	Phenylheptatriyne	−4.8953	
		22	Rutin	−7.6356	
		23	6,7,3′,4′-tetrahydroxyaurone	−7.2649	
		24	6-O-β-D-glucopyranosyl-6,7,3′,4′-tetrahydroxyaurone	−6.7239	
		25	6-O-(6″-acetyl-β-D-glucopyranosyl)-6,7,3′,4′-tetrahydroxyaurone	−6.6352	
		26	6-O-(4″,6″-diacetyl-β-D-glucopyranosyl)-6,7,3′,4′-tetrahydroxyaurone	−7.2042	
		27	6-O-(2″,4″,6″-triacetyl-β-D-glucopyranosyl)-6,7,3′,4′-tetrahydroxyaurone	−7.2834	
		28	7-O-(4″,6″-diacetyl-β-D-glucopyranosyl)-6,7,3′,4′-tetrahydroxyaurone	−7.2503	

Continued

TABLE 15.1 List of 12 antiviral medicinal plants with their phytoconstituents, docking score, and the reference journals.—cont'd

No.	Medicinal plants (botanical name)	S. No.	Phytoconstituents	Docking score (kcal\mol)	Antimicrobial properties and references
		29	Okanin-4-methyl ether-3′-O-β-D-glucoside	−6.6535	
		30	Okanin-4′-O-β-D-(3″,4″,6″-triacetyl)-glucopyranoside	−7.3996	
		31	Okanin 4′-O-β-D-(4″,6″-diacetyl)-glucopyranoside	−7.7990	
		32	Iso-okanin 7-β-D-(2″,4″,6″-triacetyl)-glucopyranoside	−6.8993	
		33	5-O-methylhoslundin	−6.5522	
		34	Centaurein	−7.4796	
		35	Luteolin	−5.2313	
		36	Isoquercitrin	−6.2661	
		37	Quercetin 3-O-rabinobioside	−7.0073	
		38	3,4-*Di*-O-caffeoylquinic acid	−7.1162	
		39	4,5-*Di*-O-caffeoylquinic acid	−6.9403	
		40	3,5-*Di*-O-caffeoylquinic acid	−7.3891	
		41	4-O-(2-O-acetyl-6-O-*p*-coumaroyl-β-D-glucopyranosyl)-*p*-coumaric acid	−6.8311	
		42	Caffeic acid ester	−6.1104	
		43	3-O-Caffeoyl-2-C-methyl-D-erythrono-1,4-lactone	−5.5707	
		44	3-Propyl-3-(2,4,5-trimethoxy) benzyloxy-pentan-2,4-dione	−6.3895	
		45	2-O-Caffeoyl-2-C-methyl-D-erythronic acid	−5.5845	
		46	Stigmastero-3-O-β-D-glucoside	−7.6502	
		47	Germacrene D	−4.8878	
		48	β-Amyrin	−5.9621	
4	*Centella asiatica* Linn	49	(−)-(1S,5S)-beta-pinene	−4.0939	Antimicrobial, anticancer, antiviral, antioxidant, antiinflammatory (Shaik Md., 2013)
		50	(+)-beta-caryophyllene	−4.5940	
		51	Apigenin	−5.4360	
		52	Ascorbic-acid	−4.4352	
		53	Betulinic-acid	−6.1532	
		54	Nicotinic-acid	−3.8948	

		55	Quercitrin	−6.3916	
		56	Stigmasterol	−6.5182	
		57	Naringin	−6.6766	
		58	Betulic acid	−6.1842	
		59	Alpha-pinene	−4.1645	
		60	Beta-pinene	−4.0355	
		61	B-caryophyllene	−4.6838	
		62	Linolenic acid	−6.1808	
		63	Oleic acid	−6.2175	
5	*Cucurma angustifolia* roxb	64	10-epi-eudesmol	−5.0481	Antiviral (Shailja et al., 2019)
		65	Alpha-fenchene	−5.5128	
		66	cis-Thujone	−4.9761	
		67	Curzerene	−5.0986	
		68	Curzerenone	−4.8606	
		69	Eucalyptol	−4.1060	
		70	Germacrone	−4.9171	
		71	Limonene	−4.4125	
		72	Linalool	−4.7210	
		73	Myrtenol	−4.2251	
6	*Cynodon dactylon* Linn	74	3-(Chloromethyl)-4-methoxybenzaldehyde	−4.4953	Antimicrobial, anti-oxidant, antiviral (Al-Snafi, 2016)
		75	3-hydroxy-1-methylpyridinium-hydroxide	−3.8546	
		76	5-Methylfurfural	−4.0150	
		77	Acetovanillone	−4.5200	
		78	Ethyl-D-glucopyranoside	−4.7721	
		79	Ferulic-acid	−4.5466	
		80	Levoglucosan	−4.4592	
		81	Levoglucosenone	−3.9194	
		82	Levulinic-acid	−4.1093	
		83	Pantolactone	−4.0504	
		84	Phenylmalonic-acid	−4.2016	

Continued

TABLE 15.1 List of 12 antiviral medicinal plants with their phytoconstituents, docking score, and the reference journals.—cont'd

No.	Medicinal plants (botanical name)	S. No.	Phytoconstituents	Docking score (kcal\mol)	Antimicrobial properties and references
		85	Syringic-acid	−4.8815	
		86	Vanillic-acid	−4.6370	
7	*Elshotzia blanda* Benth	87	Cynaroside	−6.7049	Antiviral, antiinflammatory, antioxidant, antimicrobial (Guo et al., 2012)
		88	Elemicin	−5.4188	
		89	Apiin	−8.1138	
		90	Galuteolin	−6.8189	
		91	Luteolin 3′-glucuronyl, methyl ester	−6.0868	
8	*Equisetum arvense* Linn	92	Genkwanin-5-glucoside	−6.5759	Antiviral (Asgarpanah et al., 2012)
		93	Oleanolic-acid	−4.5632	
		94	Onitin	−5.1304	
		95	Palustrine	−5.5471	
		96	Ursolic-acid	−5.6678	
9	*Houttuynia cordata* Thunb	97	3,4-Dihydroxy-N-(4-hydroxyphenethyl)benzamide	−5.4994	Antiviral, antimicrobial, antiinflammatory, antioxidative (Jiangang et al., 2013)
		98	5-Methoxy-1-methylpyrrolidin-2-one	−4.2327	
		99	7-Chloro-6-demethylcepharadione-B	−5.7881	
		100	Afzelin	−6.3976	
		101	Aristolactam-AII	−5.0917	
		102	Aristolactam-BII	−5.5117	
		103	Avicularin	−6.0611	
		104	Caldensine	−5.4979	
		105	Cepharadione-B	−5.8333	
		106	Isorhamnetin	−5.4867	
		107	Lysicamine	−5.5845	
		108	Methyl-chlorogenate	−5.8857	
		109	Methyl-vanillate	−4.6244	
		110	N-4-hydroxystyrylbenzamid	−5.1167	
		111	Noraristolodione	−5.4651	
		112	Norcepharadione-B	−5.7859	

		113	Phlorizin	−6.5040	
		114	Piperolactam-A	−5.0739	
		115	Protocatechuic-acid-4-glucoside	−5.5096	
		116	Splendidine	−6.1440	
10	*Mentha spicata*	117	1,8-Diazacyclotetradecane-2,7-dione	−4.7525	Antiviral, antimicrobial, antioxidant (Mahboubi, 2021)
		118	4-Hydroxycoumarin	−4.0647	
		119	Alpha-tocopherol	−8.1583	
		120	Carvone	−4.5273	
		121	Chrysosplenetin	−6.4380	
		122	cis-carveol	−4.5034	
		123	Demethylsulochrin	−5.5538	
		124	Eriodictyol	−5.3289	
		125	Eugenol	−4.3813	
		126	Ferreirin	−5.6461	
		127	Meprednisone	−5.8198	
		128	Phthalide	−4.0777	
		129	Pulegone	−4.5986	
		130	Retusin	−5.9828	
		131	Rhamnocitrin	−5.3571	
		132	Trans-5-O-(4-coumaroyl)-D-quinate	−5.6548	
11	*Mirabilis jalapa*	133	Avicularin	−6.1621	Antiviral (Kataoka et al., 1991)
		134	Ononin	−6.3944	
12	*Nicotiana plumbaginifolia*	135	3,5,3′-trimethoxy-6,7,4′,5′-bis(methylenedioxy) flavone	−6.7015	Antimicrobial, antiviral (Singh et al., 2010)
		136	3,5,5′,6,7,8-hexamethoxy-3′,4′-methylenedioxyflavone	−6.5850	
		137	4,7,9-Trimethoxy-6-(3,4,5-trimethoxyphenyl)-[1,3] dioxolo[4,5-g]chromen-8-one	−7.0624	
		138	Exoticin	−7.4195	

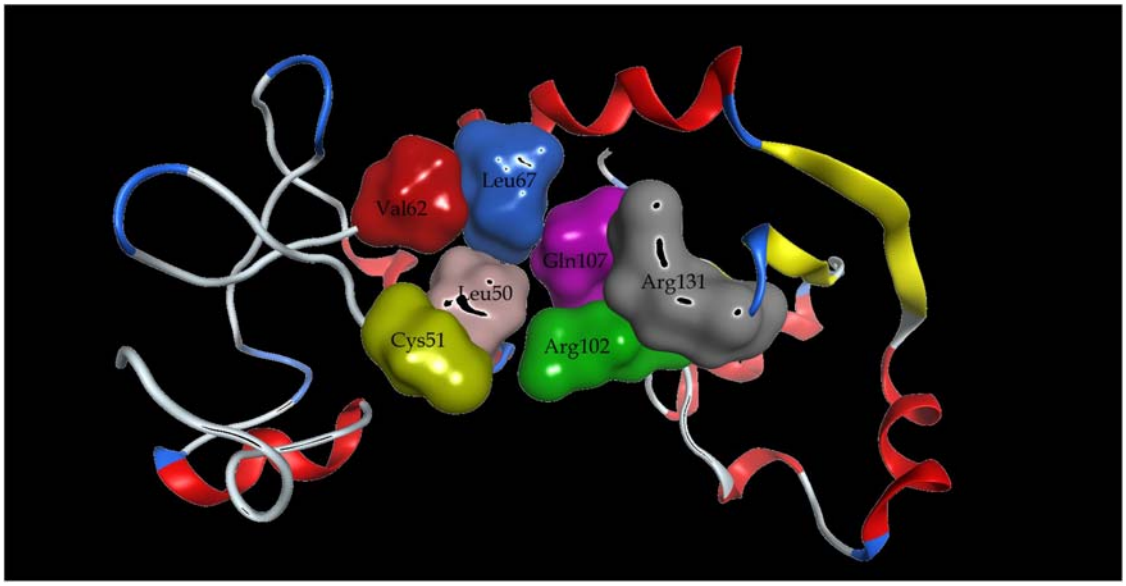

FIGURE 15.1 Binding site residues of the target protein (pdb: 4GIZ).

Results and discussion

The docking scores reveal that many of the phytoconstituents display greater binding affinity than the reference compound (Table 15.1). The top eight compounds with the best binding affinity are listed in Table 15.2. Compared to Myricetin with a binding score of −5.4435 kcal/mol, the top listed compound shows a much higher binding affinity for the target protein. Alpha-tocopherol and Apiin with very good binding scores of −8.1583 and −8.1138 kcal/mol, respectively (Table 15.2).

As flavonols are considered to be good inhibitors of HPV16 E6 (Yuan et al., 2012). The docking results compliment this activity of flavonols, as four of the top eight compounds, viz., Apiin, Rutin, Centaurein, Exoticin, consist of a flavonol unit in the molecular structure (Figs. 15.2 and 15.3). Myciretin, the reference compound, is also a flavonol (Fig. 15.4). The uniqueness of flavonols in these interactions is its ability to form strong hydrogen bonding interactions with Cys51 residual amino acid in the active site. Cys51 is the most important active site residue which appears in all the top binding interactions including the reference compound. The carbonyl oxygen of the flavonol ring and an adjacent methoxy oxygen atom forms strong hydrogen interactions with Cys51. Cys51, Lys11, Leu100, and Arg102 are the common amino acids that are involved in crucial interactions with the flavonol moiety. Okanin 4′-O-β-D-(4″,6″-diacetyl)-glucopyranoside and Okanin-4′-O-β-D-(3″,4″,6″-triacetyl)-glucopyranoside are among the top binding ligands which consist of a chalcone moiety in the molecular structure (Figs. 15.2 and 15.3). The carbonyl oxygen of the chalcone group contributes effectively to the binding score by forming hydrogen bonding interaction with Cys51. The residual amino acid Leu100 also seems to be an important active site residue where it is involved in interaction with all the top binding ligands except Rutin.

The RMSD plot of the protein backbone over a simulation of 20,000 frames reveals that except for Okanin 4′-O-β-D-(4″,6″-diacetyl)-glucopyranoside, all the other complexes are within acceptable deviation after 500 frames (Fig. 15.5). Protein-exocitin shows a considerable low value of RMSD compared to all other complexes and its average RMSD is about 1 Å beyond 500 frames. Rutin, Okanin-4′-O-β-D-(3″,4″,6″-triacetyl)-glucopyranoside, Alpha-tocopherol, Centaurein, and Myricetin in complex with the target protein showed very acceptable RMSD values. The RMSF plot for the alpha-C background of the protein complements the RMSD results, where Protein-Exoticin complex showed very low RMSF value compared to all the other complexes. Similar to the RMSD plot, the RMSF values of Okanin 4′-O-β-D-(4″,6″-diacetyl)-glucopyranoside shows very large fluctuation at certain regions (Fig. 15.6). All the complexes show minimum fluctuations around the residues Cys51, Lys11, Leu100, and Arg102 which indicates that the hydrogen bonding interactions between the protein and the ligands are maintained over the course of the simulation.

The plot on hydrogen bonding interactions (Fig. 15.7) reveals that protein-Apiin complex maintains the three hydrogen bonding interactions over the course of the simulation. Okanin-4′-O-β-D-(3″,4″,6″-triacetyl)-glucopyranoside-protein

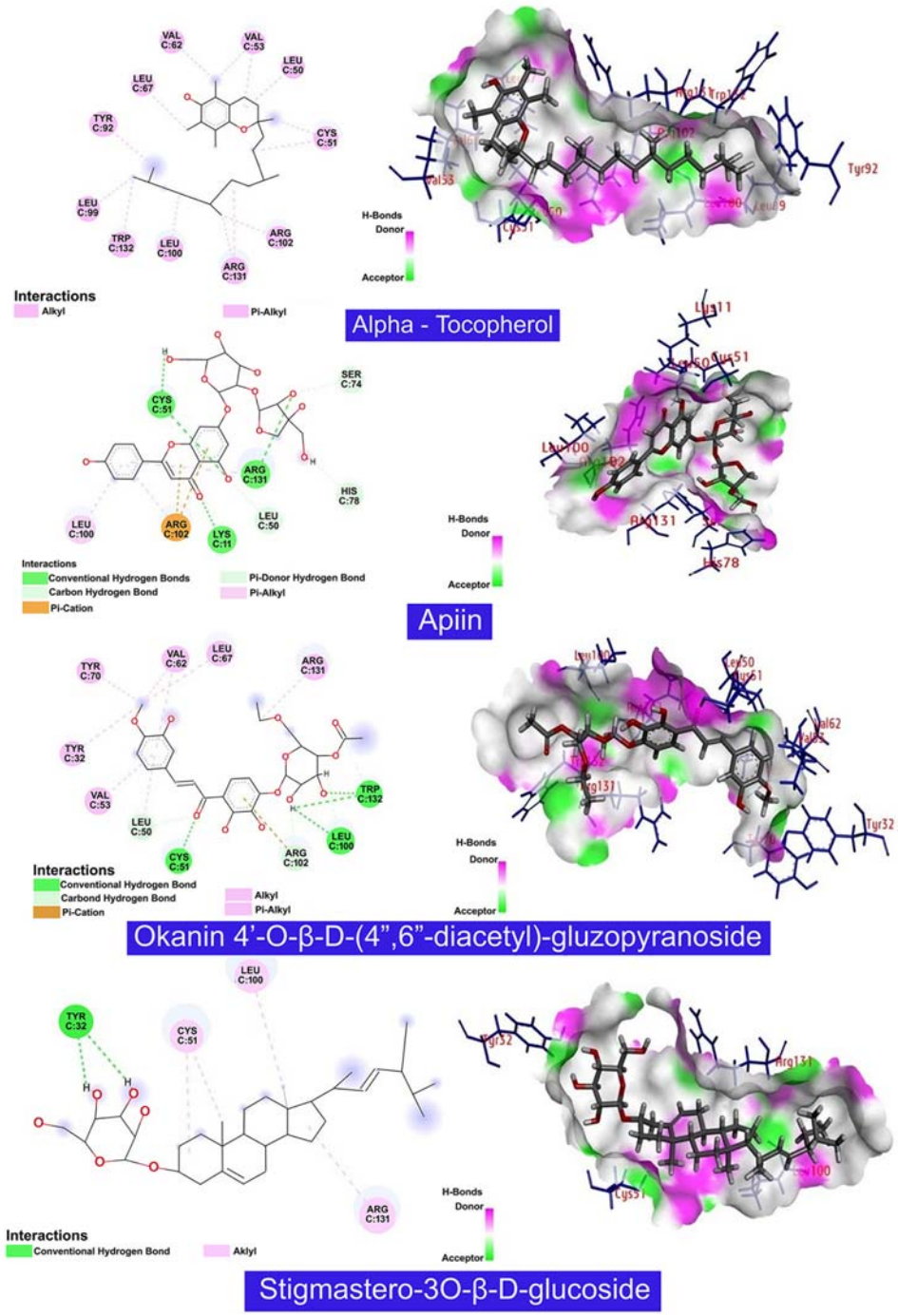

FIGURE 15.2 2D and 3D interaction diagram of the top 1-4 scoring ligands with the target HPV16 E6 oncoprotein (pdb ID: 4GIZ).

complex maintains three hydrogen interactions over the course of the simulation and the fourth hydrogen bonding interaction appears occasionally. As already seen in the study of docking interactions, alpha-tocopherol-protein complex doesn't exhibit any hydrogen bonding interactions (Fig. 15.2). The hydrogen bonding interactions in Exocitin-protein complex and Stigmastero-3-O-β-D-glucoside complex are completely lost over the course of the simulation.

TABLE 15.2 List of top 8 phytoconstituents docked with the target, HPV16 E6 Oncoprotein, together with the docking scores and the interacting amino acid residues at the binding site.

S. No.	Ligand	Docking score (kcal/mol)	Interacting amino acids
1	Alpha-tocopherol	−8.1583	Val53, Leu50, Cys51, Val62, Leu67, Tyr92, Leu99, Leu100, Trp132, Arg102, Arg131
2	Apiin	−8.1138	Cys51, Ser74, His78, Arg131, Leu50, Lys11, Arg102, Leu100
3	Okanin 4′-O-β-D-(4″,6″-diacetyl)-glucopyranoside	−7.7990	Leu67, Val62, Tyr70, Tyr32, Val53, Leu50, Cys51, Arg102, Leu100, Trp132, Arg131
4	Stigmastero-3-O-β-D-glucoside	−7.6502	Tyr32, Cys51, Leu100, Arg131
5	Rutin	−7.6356	Tyr54, Arg55, Ile52, Cys51, Val53, Arg131
6	Centaurein	−7.6227	Lys11, Leu50, Cys51, Leu67, Tyr32, Val62, Tyr70, Arg102, Trp132, Leu100
7	Exoticin	−7.4195	Tyr32, Leu67, Tyr70, Ser71, Ser74, Gln107, Arg131, Trp132, Leu100, Lys11, Asp49, Leu50, Cys51, Val53, Val62, Arg102
8	Okanin-4′-O-β-D-(3″,4″,6″-triacetyl)-glucopyranoside	−7.3996	Tyr32, Leu67, Tyr70, Leu50, Cys51, Lys11, Arg102, Leu100
9	Myricetin	−5.4435	Cys51, Tyr32, Leu50, Arg131, Arg102

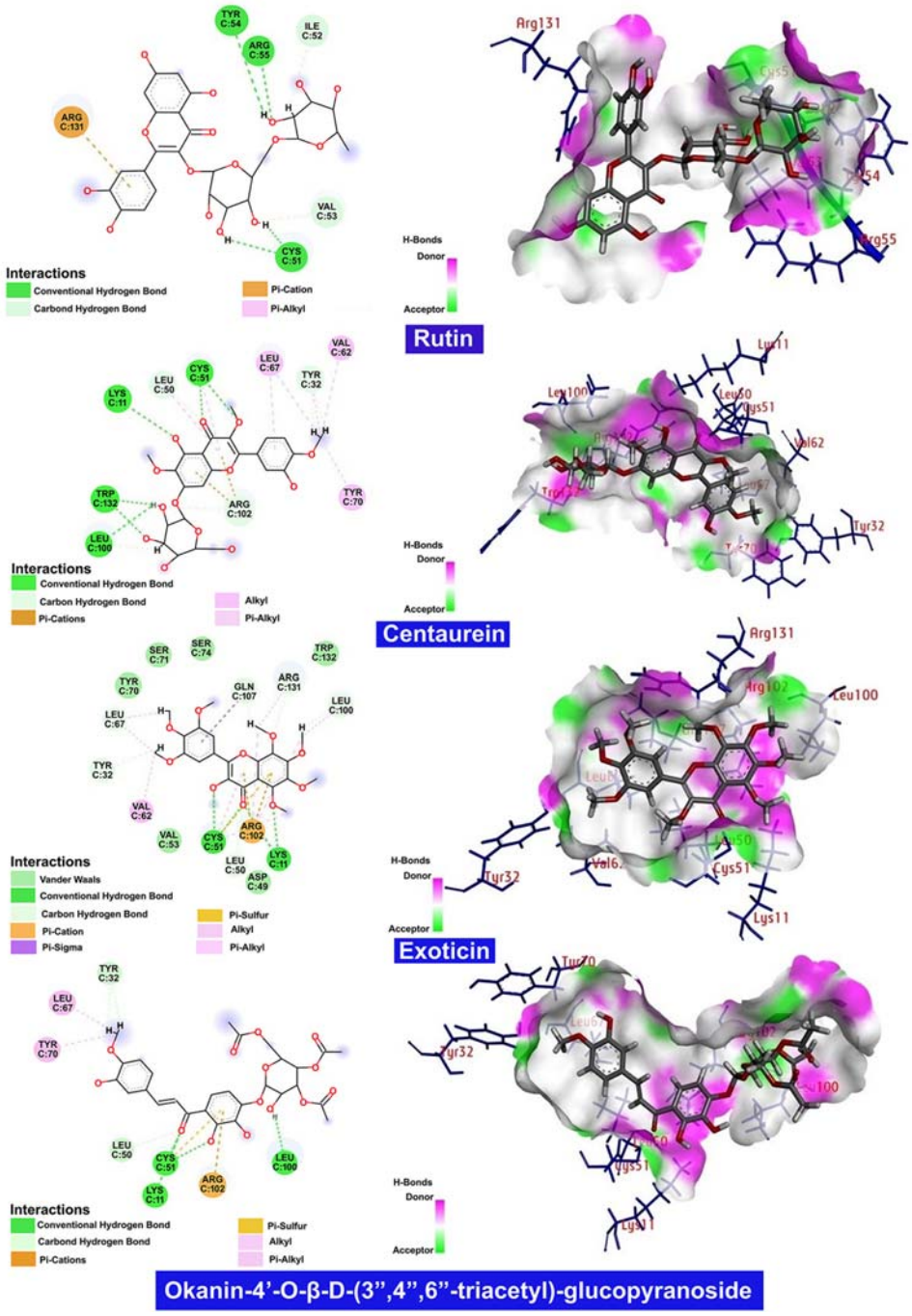

FIGURE 15.3 2D and 3D interaction diagram of the top 5-8 scoring ligands with the target HPV16 E6 oncoprotein (pdb ID: 4GIZ).

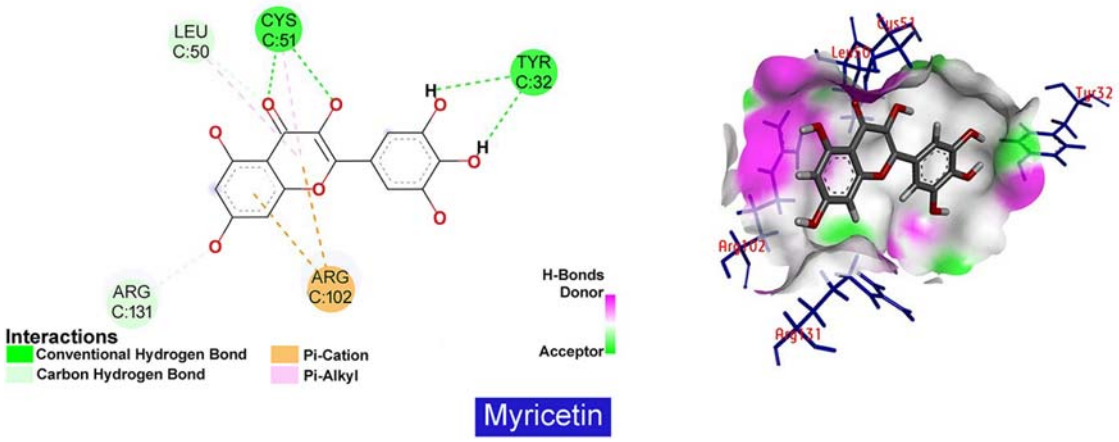

FIGURE 15.4 2D and 3D interaction diagram of the reference compound with the target HPV16 E6 Oncoprotein.

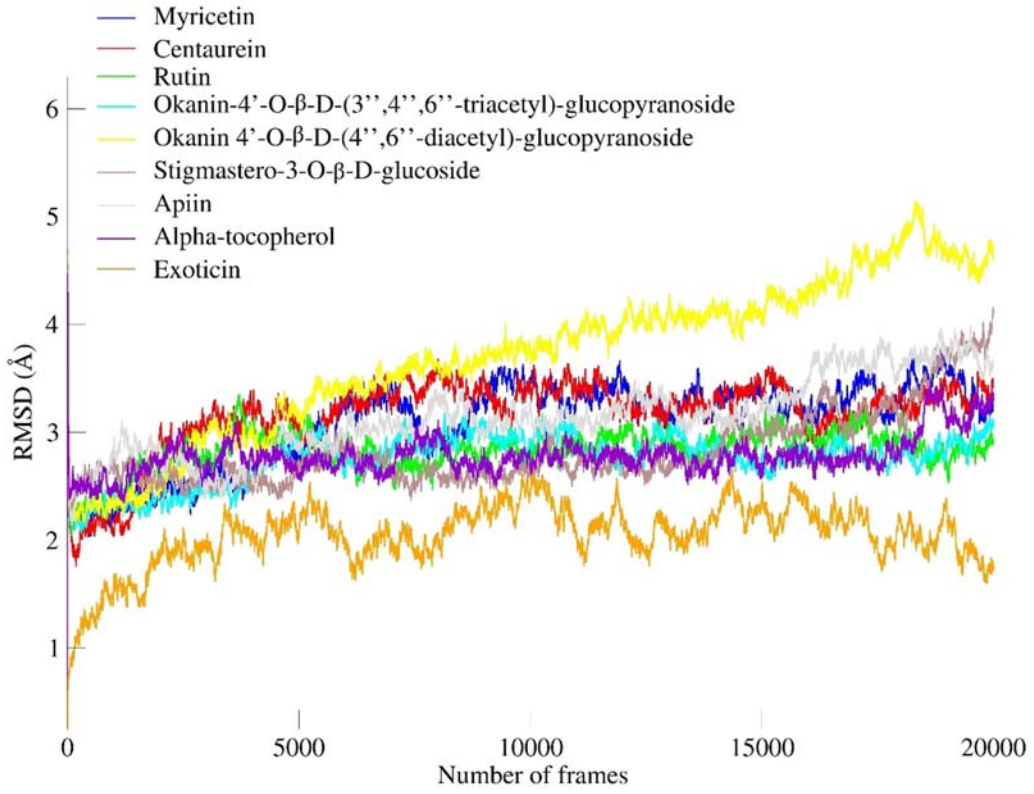

FIGURE 15.5 RMSD plot of Protein backbone for top scoring phytoconstituent -protein complex with the reference-protein complex, over 20000 frames.

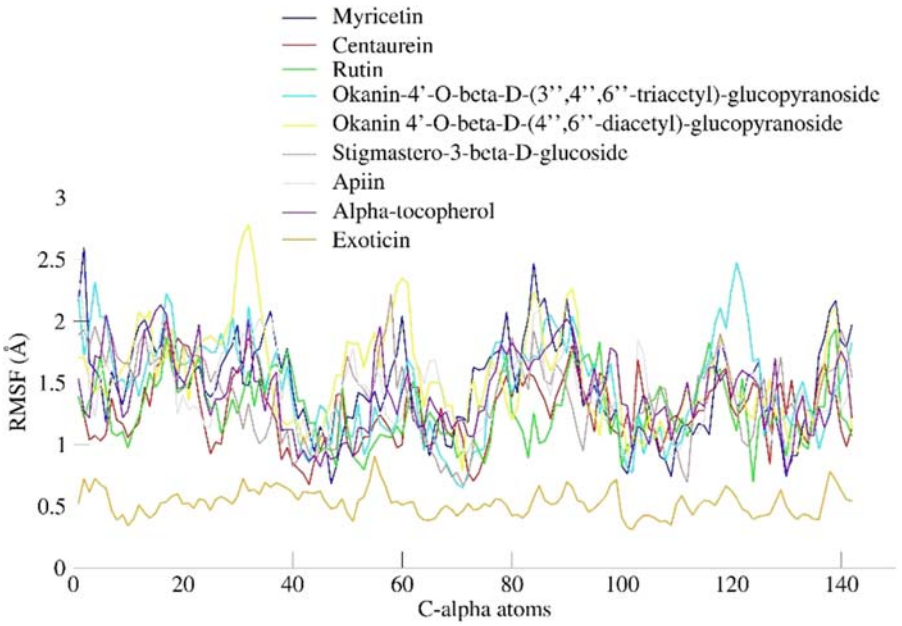

FIGURE 15.6 RMSF plot of C-alpha carbon atoms from the top phytoconstituent-protein complexes.

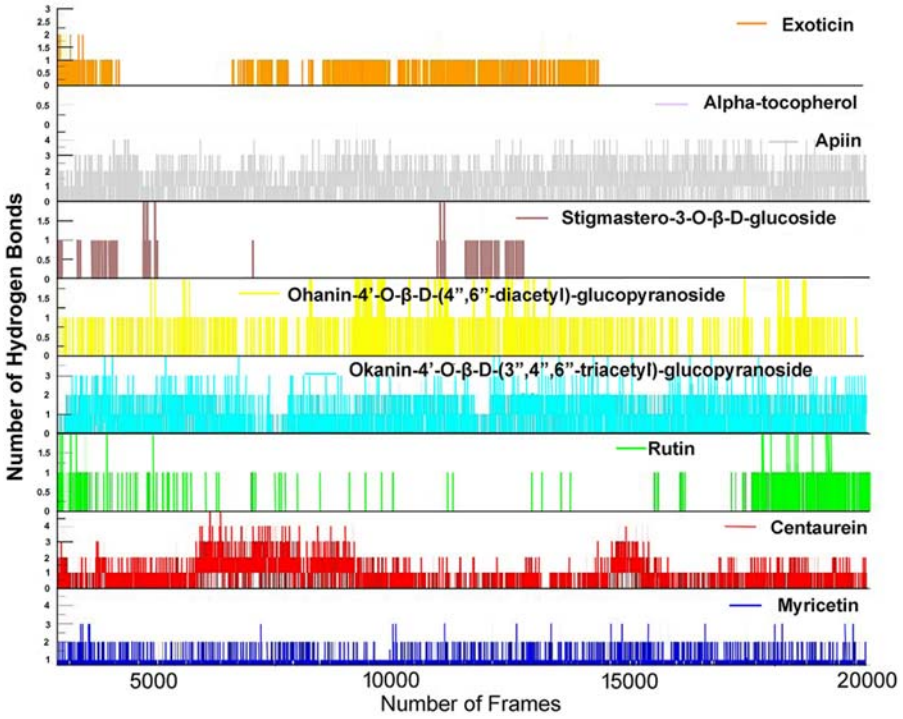

FIGURE 15.7 Hydrogen bonding interaction between Protein and phytoconstituents over 20000 frames.

Conclusion

In conclusion, the study investigated 12 medicinal plants in northeast India, with antiviral properties. 138 phytoconstituents from the plants were selected for in silico docking study against HPV16 E6 Ocoprotein, in an effort to find potential therapeutic plant-based drugs against cervical cancer. The study identified eight possible drug candidates with much higher binding affinity for the target protein as compared to the reference drugs, Myricetin. Among the top eight phytoconstituents identified, four consist of a flavonol moiety and two consist of a chalcone unit. Among the top phytoconstituents Apiin, Centaurein, and Okanin-4′-O-β-D-(3″,4″,6″-triacetyl)-glucopyranoside showed good results in molecular dynamics simulation analysis and can be possible inhibitors against HPV16 E6 Oncoprotein. Centaurein and Okanin-4′-O-β-D-(3″,4″,6″-triacetyl)-glucopyranoside are phytoconstituents in the plant Bidens Pilosa Linn, and Apiin is a phytoconstituent of Elschotzia blanda benth. The results of this study have to be substantiated by in vivo and in vitro analysis of the plants. More phytochemical profiling has to be carried out on many antiviral plants as they consist of a multitude of potent phytochemicals which can be explored for their antimicrobial and antiviral properties.

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Chapter 16

A computational approach to finding novel drug targets and their natural product inhibitors for *Aspergillus flavus*

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Introduction

Aspergillus flavus has a worldwide distribution and occurs typically as saprophytes in soil and on many kinds of decaying organic matter. These fungi widely infected essential crops such as corn, cottonseed, peanut, and tree nuts. *A. flavus* can grow at a temperature of 12 to 48°C; for optimum growth, 25−42°C temperature was required. This fungus is semi-thermophilic and semi-xerophytic. A. flavus can produce polypeptide-derived secondary metabolites called aflatoxins, which are highly toxic, mutagenic, and carcinogenic in plants and humans as well. A. flavus grows by producing thread-like branching filaments known as hyphae (Fig. 16.1). The mycelium, a network of hyphae, secretes the enzymes to digest complex food. Individual hyphae cannot be seen with the naked eye; thick mycelium mats with conidia are frequently visible (Kluczkovski et al., 2020). It is a taxonomic group found in various climates worldwide and includes organisms whose characteristics have high pathological, agricultural, industrial, pharmaceutical, and biotechnological importance. *A. fumigatus*, *A. nidulans*, *A. oryzae,* and *A. niger* are currently the four predominant Aspergillus strains studied because of their considerable importance (Seekles et al., 2022).

The cell membrane of *Aspergillus* comprises a phospholipid bilayer interspersed with globular proteins and ergosterol as the major sterol instead of the cholesterol, which is found in the membrane of animals and phytosterols in plants (Zhang et al., 2022a). Further, in the composition of the cell wall, mainly different polysaccharides (α-1,3-glucan, β-1,3-glucan, β-(1,3)-(1,4)-glucan, Chitin, galactomannan and a polymer of galactosaminogalactan) with proteins and glycoproteins are present (Latgé et al., 2022). This composition changes during cell growth, differentiation, and stress exposure (Qin et al., 2022). Previous studies have implicated the role of sphingolipids, particularly sphingosine and ceramide, in fungal cell signaling and cytoskeletal organization (Sugawara, 2022). The major sterol in filamentous fungi is ergosterol instead of cholesterol in mammalian cells and phytosterols in plants (Zhang et al., 2022b). Depending on the age of the culture, the developmental stage, and the growing environment, different Aspergillus species produce different amounts of ergosterol (dos Santos Nascimento et al., 2022). Both the membrane and fungal cell wall components are potential drug targets; they harbor most of the fungal antigens (Latgé et al., 2022). Understanding the mechanisms to maintain cell wall integrity in *Aspergillus* is crucial for developing antifungal drugs. It will also help to understand the pharmacology of existing anti-fungal drugs.

Besides, *A. flavus* makes aflatoxins, a class of furanocoumarins generated from polypeptides. Out of 16 aflatoxins structurally related, only four main aflatoxins, such as AFB1, AFB2, AFG1, and AFG2, can contaminate agricultural products and pose a risk to livestock and people's health. It causes Aspergillosis, a life-threatening human disease, particularly in a patient who is immunosuppressed or has chronic lung disease. Also, humans have reported aflatoxicosis in numerous countries in Southeast Asia and Africa (Hatipoglu et al., 2022). Aflatoxins have been implicated in

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FIGURE 16.1 Microscopic view of *Aspergillus flavus*.

hepatocellular carcinoma, acute hepatitis, and cirrhosis in malnourished children and kwashiorkor (Spearman et al., 2022; Cao et al., 2022). According to WHO and FAO regulations, the content of aflatoxin B should be less than 0.5 μg/kg in milk. The maximum acceptable limit for aflatoxins is 20 μg/kg in edible food and 20−300 ppb in animal feed in the United States (Álvarez-Días et al., 2022).

Currently, for identifying potential drug targets and developing antifungal drugs, in silico subtractive genome analysis is the most powerful bioinformatics approach by identifying genus or species-specific genes or groups of genes responsible for a unique phenotype. The accessibility of pathogen genome sequences has generated a vast amount of information that can help identify therapeutic targets. One of the most current approaches is based on a subtractive genomics strategy. A group of genes believed to be crucial for the pathogen but missing from the host is revealed by the subtraction dataset between the host and pathogen genomes. These will subsequently be classified into (i) drug targets in pathogen-specific unique metabolic pathways and (ii) drug targets in host-pathogen common metabolic pathways. Based on the above fact and the unavailability of new targets for *A. flavus*, the present study has been conducted to identify potential drug targets using subtractive genome analysis.

Materials and methods

The schematic diagram of the process from retrieval of the sequence to molecular docking has been illustrated in Fig. 16.2.

Retrieval of the proteome of host and pathogen

The complete proteome sequences of *A. flavus*, plant, and human were taken from NCBI in FASTA format for further analysis. In the case of plants, four plant proteome sequences, i.e., flowering plant (Taxonomic Id-3398), higher plant (Taxonomic Id-3193), vascular plant (Taxonomic Id-58023), seed plant (Taxonomic Id-58024) against *A. flavus* were taken (Don et al., 2011).

Identification of essential proteins in *A. flavus*

All reference proteome sequences of *A. flavus* were subjected to BLASTp with an E value of 10^{-4} to eliminate homologous protein in the host and pathogen. Further, these sequences were run with plants and human proteome sequences. During the BLASTp of plant and *A. flavus* database was selected nonredundant for repeated sequence elimination. In the BLASTp of humans and *A. flavus,* the database was selected as a reference protein, and the threshold value was set at 10^{-4}. The protein sequences of *A. flavus* showing no significant similarity were retrieved manually.

Metabolic pathway analysis using KASS

After BLAST, the metabolic pathway analysis of the essential proteins of *A. flavus* was done using KAAS (KEGG Automatic Annotation Server) server (www.genome.jp/tools/kaas/). The KAAS gives functional annotations of genes by comparing BLAST results to the manually curated KEGG Genes database (Georrge et al., 2012).

Subcellular localization prediction and active site identification

Subcellular localization analysis of the essential proteins has been done by Proteome Analyst Specialized Sub Cellular Localization Server v2.5 (PA-SUB) to identify the surface membrane proteins, which could be probable drug targets

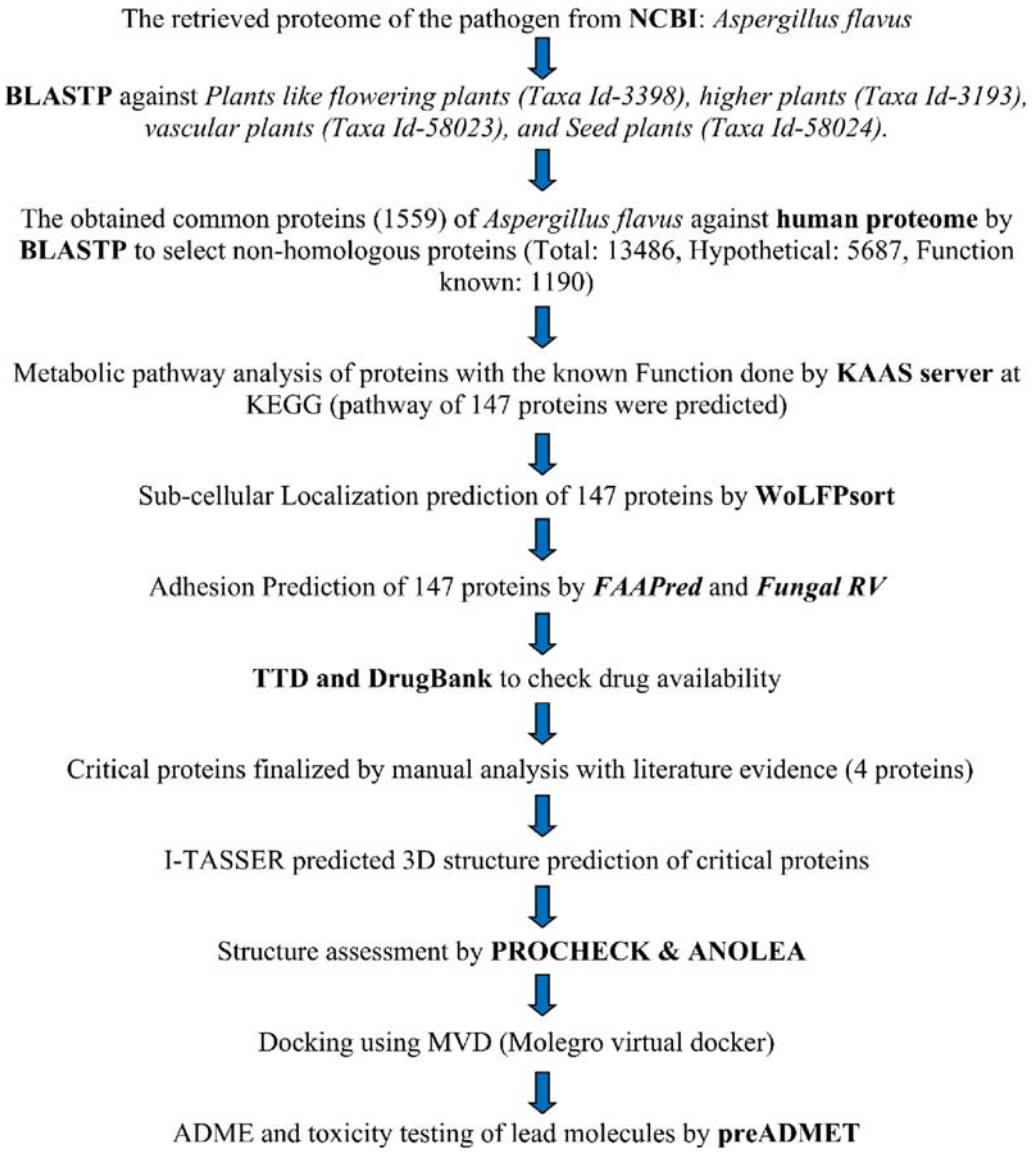

FIGURE 16.2 Steps for materials and methods.

(Saravanan et al., 2019). The critical protein present in which WoLF PSORT predicted part of the cell for protein localization. After that, this critical protein checks out in DrugBank and the target therapeutic database (TTD) for novelty. DrugBank is a comprehensive, reliable, freely available internet resource that provides data on drugs and therapeutic targets (Wishart et al., 2018). Outer membrane proteins, by Transmembrane prediction, were also predicted to identify the surface membrane proteins. For 3D structure prediction and stereochemical quality, I-TASSER, and PROCHECK finder have been used, respectively.

Ligand Library construction and molecular docking

To construct the Ligand Library, antiviral, anticancer, antifungal molecules, and chitinase inhibitor molecules were retrieved from the PubChem bioassay database [http://pubchem.ncbi.nlm.nih.gov/]. Drug-like compounds of NCI database [http://ligand.info]. Herbal compound molecules were obtained from the natural product database, which also contains Dr. Duke's Phytochemical library [www.ars-grin.gov/duke]. The approved drugs were selected due to their FDA approval and thus will not require preclinical trials. As they are already targeting fungal proteins, they are more likely to complete the inhibition of an organism (Joseph et al., 2015).

Molegro Virtual Docker has been used for molecular docking; it is a comprehensive framework for predicting interactions between proteins and ligands. It handles every step of the docking procedure, including identifying the target protein's probable binding sites and predicting the ligands' binding modes.

Results and discussions

A. flavus is one of the fungi that produce a mass amount of aflatoxin, the most worldwide toxin and carcinogenic compound for humans, plants, animals, birds, etc. The main source of these fungi is edible nuts, cereals, oilseeds, etc., and the target organ in humans for infection and damage to the kidney, liver, and brain. Gambian children: continuous low-level exposure to dietary aflatoxins may enhance susceptibility to infection (Syamilah et al., 2022). Hepatitis B Virus infections/carriers potency of aflatoxins in HBsAg+ is significantly higher than in HBsAg individuals' impact on the prevalence of liver cancer impairs children's growth. According to statistics, infectious diseases are the six biggest causes of death worldwide (Ali & Hussain, 1998). Despite the increasing demand for new antifungal drugs, the new drugs identified are a few reasons like less market and competition with newly developed agents (Donlin et al., 2022). Many new algorithms, tools, and databases have been developed as a result of the advancement in bioinformatics which has facilitated the automation of microbial genome sequencing, comparison of genomes, identification of gene product function, and simplified the process of development of antifungal agents, and rational drug design (Jampilek, 2022).

In silico subtractive genomics, a tactic is a powerful method to recognize the specific genes present in the pathogen but absent in the host, thus helping in the identification of novel genus-specific genes which can be used as drug targets. The primary concept of in silico drug target discovery is that "a good drug target is a gene essential for fungal survival yet cannot be found in the host" (Georrge et al., 2011). Earlier, novel drug targets have been identified successfully for various pathogens with the help of a subtractive genomics approach.

Identification of essential genes

In the study of essential gene identification, nonhomologous essential genes of *A. flavus*, and their protein product not similar to the plant and with human, has been identified using the subtractive genomic approach, which led to the development of drugs that strongly bind with the pathogen. The summary of several targets identified is shown in Fig. 16.3. *A. flavus* comprises 13,486 reference proteins that were retrieved from NCBI.

The 5687 hypothetical protein sequences whose function is unknown. No scientific proof exists that the hypothetical protein is expressed in living things. So, it was eliminated, and after running the BLASTp with four different plants, i.e., flowering plant, higher plant, vascular plant, and seed plant, nonhomologous 1559 reference sequences were essential for

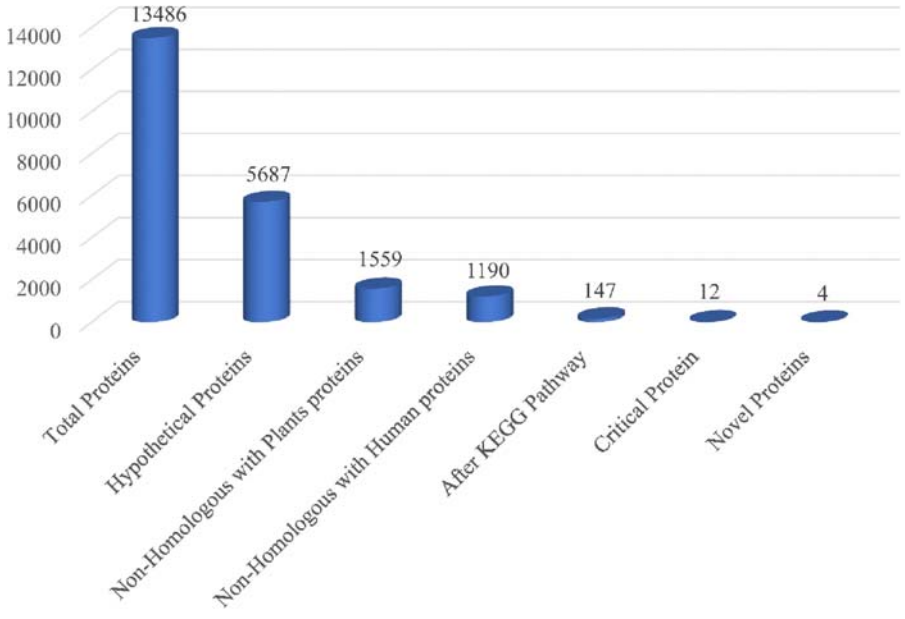

FIGURE 16.3 Summary of target identification.

A. flavus, not for the host (plant). The further step proceeds with the nonhomologous reference sequence of *A. flavus*. Further, 1559 nonhomologous sequences were BLASTp with human proteome. The database was selected as the reference database. Among the 1559 reference sequences, only 1190 nonhomologous sequences were found, which were not present in humans and plants.

Metabolic pathway analysis

These 1190 nonhomologous sequences were deployed in the KAAS server for Metabolic pathway analysis of the essential proteins. Out of 1190 reference proteins, only 147 essential proteins were predicted (Table 16.1). Studies were done on pharmacological targets' interactions with metabolic pathways. A comparative investigation of the metabolic pathways of

TABLE 16.1 The 147 proteins identified from the pathway analysis using the KAAS server.

SN	Gi no. and accession no.	KO id	Pathways
1.	gi|238507049|ref|XP_002384726.1|	K10254	Myosin-cross reactive antigen
2.	gi|238505184|ref|XP_002383821.1|	K00329	ko00190 oxidative phosphorylation
3.	gi|238504978|ref|XP_002383718.1|	K11309	Regulator of Ty1 transposition protein 109
4.	gi|238504090|ref|XP_002383277.1|	K10424	ko04962 vasopressin-regulated water reabsorption, Ko05016 Huntington's disease
5.	gi|238503520|ref|XP_002382993.1|	K15378	Solute carrier family 45, member 1/2/4
6.	gi|238502613|ref|XP_002382540.1|	K15204	Transcription factor C subunit 3
7.	gi|238501956|ref|XP_002382212.1|	K11362	Transcriptional coactivator HFI1/ADA1
8.	gi|238500914|ref|XP_002381691.1|	K01607	ko00362 benzoate degradation
9.	gi|238500694|ref|XP_002381581.1|	K01266	D-aminopeptidase
10.	gi|238500660|ref|XP_002381564.1|	K07078	Unclassified; poorly characterised; general function prediction only
11.	gi|238498896|ref|XP_002380683.1|	K03004	ko00230 purine metabolism, ko00240 pyrimidine metabolism, ko03020 RNA polymerase
12.	gi|238498480|ref|XP_002380475.1|	K15160	Mediator of RNA polymerase II transcription subunit 16, fungi type
13.	gi|238497213|ref|XP_002379842.1|	K10254	Myosin-crossreactive antigen
14.	gi|238497157|ref|XP_002379814.1|	K08329	ko04140 regulation of autophagy
15.	gi|238496729|ref|XP_002379600.1|	K11676	Ino 80 subunit 2
16.	gi|238496579|ref|XP_002379525.1|	K11697	Kinetochore protein Fta7
17.	gi|238496547|ref|XP_002379509.1|	K14770	U3 small nucleolar RNA-associated protein 16
18.	gi|238494392|ref|XP_002378432.1|	K03024	ko00230 purine metabolism, ko00240 pyrimidine metabolism, ko03020 RNA polymerase, ko04623 cytosolic DNA-sensing pathway
19.	gi|238493227|ref|XP_002377850.1|	K07734	Transcriptional regulator
20.	gi|238492949|ref|XP_002377711.1|	K09123	Hypothetical protein
21.	gi|238492337|ref|XP_002377405.1|	K13690	Alpha-1,3-mannosyltransferase
22.	gi|238492267|ref|XP_002377370.1|	K07443	Methylated-DNA-protein-cysteine methyltransferase related protein
23.	gi|238491938|ref|XP_002377206.1|	K15147	Mediator of RNA polymerase II transcription subunit 5
24.	gi|238491814|ref|XP_002377144.1|	K14315	ko03013 RNA transport
25.	gi|238491804|ref|XP_002377139.1|	K02272	ko00190 oxidative phosphorylation, ko04260 cardiac muscle contraction, ko05010 Alzheimer's disease, ko05012 Parkinson's disease, ko05016 Huntington's disease
26.	gi|238491546|ref|XP_002377010.1|	K07112	Unclassified; poorly characterized; general function prediction only

Continued

TABLE 16.1 The 147 proteins identified from the pathway analysis using the KAAS server.—cont'd

SN	Gi no. and accession no.	KO id	Pathways
27.	gi|238490480|ref|XP_002376477.1|	K11835	Ubiquitin carboxyl-terminal hydrolase 4/11/15
28.	gi|238489541|ref|XP_002376008.1|	K11560	Kinetochore protein Mis13/DSN1
29.	gi|238487946|ref|XP_002375211.1|	K10735	M00286 GINS complex
30.	gi|238487404|ref|XP_002374940.1|	K02307	ko04111 cell cycle—yeast
31.	gi|238487162|ref|XP_002374819.1|	K11561	kinetochor protein Mis14/NSL1
32.	gi|238486958|ref|XP_002374717.1|	K02141	ko00190 oxidative phosphorylation
33.	gi|238484155|ref|XP_002373316.1|	K07160	Unclassified; poorly characterized; general function prediction only
34.	gi|238483955|ref|XP_002373216.1|	K15529	ADP-ribose 1″-phosphate phosphatase
35.	gi|238483615|ref|XP_002373046.1|	K11595	Chromo domain-containing protein 1
36.	gi|238500355|ref|XP_002381412.1|	K11553	DASH complex subunit DAD1
37.	gi|238498280|ref|XP_002380375.1|	K00457	ko00130 ubiquinone and other terpenoid-quinone biosynthesis, ko00350 tyrosine metabolism, ko00360 phenylalanine metabolism
38.	gi|238494206|ref|XP_002378339.1|	K11376	Elongator complex protein 5
39.	gi|238493119|ref|XP_002377796.1|	K11572	DASH complex subunit SPC19
40.	gi|238490934|ref|XP_002376704.1|	K14334	ko00362 benzoate degradation
41.	gi|238488022|ref|XP_002375249.1|	K15136	Mediator of RNA polymerase II transcription subunit 18, fungi type
42.	gi|238485878|ref|XP_002374177.1|	K11270	Chromosome transmission fidelity protein 8
43.	gi|238508868|ref|XP_002385615.1|	K14300	ko03013 RNA transport
44.	gi|238508822|ref|XP_002385594.1|	K14654	ko00740 riboflavin metabolism
45.	gi|238508778|ref|XP_002385573.1|	K07101	Unclassified; poorly characterised; general function prediction only
46.	gi|238508701|ref|XP_002385536.1|	K05994	Bacterial leucyl aminopeptidase
47.	gi|238508686|ref|XP_002385529.1|	K00698	ko00520 amino sugar and nucleotide sugar metabolism
48.	gi|238508629|ref|XP_002385502.1|	K04486	ko00340 histidine metabolism
49.	gi|238508627|ref|XP_002385501.1|	K00997	ko00770 pantothenate and CoA biosynthesis
50.	gi|238508469|ref|XP_002385427.1|	K12273	Translocation protein SEC66
51.	gi|238508427|ref|XP_002385407.1|	K06100	ko03015 mRNA surveillance pathway, ko04530 tight junction
52.	gi|238507531|ref|XP_002384967.1|	K14822	rRNA-processing protein CGR1
53.	gi|238507413|ref|XP_002384908.1|	K04835	ko00660 C5-Branched dibasic acid metabolism, ko00910 nitrogen metabolism
54.	gi|238507357|ref|XP_002384880.1|	K07407	ko00052 galactose metabolism, ko00561 glycerolipid metabolism, ko00600 sphingolipid metabolism, ko00603 glycosphingolipid biosynthesis—globo series
55.	gi|238507063|ref|XP_002384733.1|	K07078	Unclassified; poorly characterised; general function prediction only
56.	gi|238506941|ref|XP_002384672.1|	K02263	ko00190 oxidative phosphorylation, ko04260 cardiac muscle contraction, ko05010 Alzheimer's disease, ko05012 Parkinson's disease, ko05016 Huntington's disease
57.	gi|238506911|ref|XP_002384657.1|	K11599	ko03050 proteasome
58.	gi|238506907|ref|XP_002384655.1|	K10967	ko00514 other types of O-glycan biosynthesis
59.	gi|238504206|ref|XP_002383335.1|	K12597	ko03018 RNA degradation
60.	gi|238503259|ref|XP_002382863.1|	K01113	ko00627 aminobenzoate degradation, ko00790 folate biosynthesis, ko02020 two-component system
61.	gi|238502923|ref|XP_002382695.1|	K03854	Mannosyltransferase

TABLE 16.1 The 147 proteins identified from the pathway analysis using the KAAS server.—cont'd

SN	Gi no. and accession no.	KO id	Pathways
62.	gi|238502635|ref|XP_002382551.1|	K07107	Acyl-CoA thioester hydrolase
63.	gi|238502493|ref|XP_002382480.1|	K15053	ko04144 endocytosis
64.	gi|238501984|ref|XP_002382226.1|	K15156	Mediator of RNA polymerase II transcription subunit 14
65.	gi|238501982|ref|XP_002382225.1|	K06902	MFS transporter, UMF1 family
66.	gi|238501850|ref|XP_002382159.1|	K02139	ko00190 oxidative phosphorylation
67.	gi|238501408|ref|XP_002381938.1|	K11761	Chromatin structure-remodeling complex protein RSC7
68.	gi|238499811|ref|XP_002381140.1|	K03854	Mannosyltransferase
69.	gi|238499555|ref|XP_002381012.1|	K05528	ko00513 various types of N-glycan biosynthesis
70.	gi|238498856|ref|XP_002380663.1|	K00763	ko00760 nicotinate and nicotinamide metabolism
71.	gi|238498270|ref|XP_002380370.1|	K03786	ko00400 phenylalanine, tyrosine, and tryptophan biosynthesis
72.	gi|238498220|ref|XP_002380345.1|	K00505	ko00350 tyrosine metabolism, ko00740 riboflavin metabolism, ko00950 isoquinoline alkaloid biosynthesis, ko00965 betalain biosynthesis, ko04916 melanogenesis
73.	gi|238498172|ref|XP_002380321.1|	K07008	Glutamine amidotransferase
74.	gi|238497171|ref|XP_002379821.1|	K01235	Alpha-glucuronidase
75.	gi|238496811|ref|XP_002379641.1|	K15152	Mediator of RNA polymerase II transcription subunit 21
76.	gi|238496645|ref|XP_002379558.1|	K12249	ko00909 sesquiterpenoid biosynthesis
77.	gi|238496187|ref|XP_002379329.1|	K03381	ko00361 chlorocyclohexane and chlorobenzene degradation, ko00362 benzoate degradation, ko00364 fluorobenzoate degradation, ko00623 toluene degradation
78.	gi|238495783|ref|XP_002379127.1|	K11135	Pin2-interacting protein X1
79.	gi|238495596|ref|XP_002379034.1|	K13507	ko00561 glycerolipid metabolism, ko00564 glycerophospholipid metabolism
80.	gi|238495422|ref|XP_002378947.1|	K01673	ko00910 nitrogen metabolism
81.	gi|238495372|ref|XP_002378922.1|	K14663	Protein N-terminal amidase
82.	gi|238495340|ref|XP_002378906.1|	K04627	ko04011 MAPK signaling pathway—yeast
83.	gi|238495164|ref|XP_002378818.1|	K11213	ko04011 MAPK signaling pathway—yeast
84.	gi|238495134|ref|XP_002378803.1|	K00698	ko00520 amino sugar and nucleotide sugar metabolism
85.	gi|238495066|ref|XP_002378769.1|	K10845	ko03022 basal transcription factors, ko03420 nucleotide excision repair
86.	gi|238494776|ref|XP_002378624.1|	K08968	ko00270 cysteine and methionine metabolism
87.	gi|238494736|ref|XP_002378604.1|	K01193	ko00052 galactose metabolism, ko00500 starch and sucrose metabolism
88.	gi|238494392|ref|XP_002378432.1|	K03024	ko00230 purine metabolism, ko00240 pyrimidine metabolism, ko03020 RNA polymerase, ko04623 cytosolic DNA-sensing pathway
89.	gi|238493227|ref|XP_002377850.1|	K07734	Transcriptional regulator
90.	gi|238492949|ref|XP_002377711.1|	K09123	Unclassified; poorly characterized; function unknown
91.	gi|238492337|ref|XP_002377405.1|	K13690	Alpha-1,3-mannosyltransferase
92.	gi|238492267|ref|XP_002377370.1|	K07443	Methylated-DNA-protein-cysteine methyltransferase related protein
93.	gi|238491938|ref|XP_002377206.1|	K15147	Mediator of RNA polymerase II transcription subunit 5
94.	gi|238491814|ref|XP_002377144.1|	K14315	ko03013 RNA transport

Continued

TABLE 16.1 The 147 proteins identified from the pathway analysis using the KAAS server.—cont'd

SN	Gi no. and accession no.	KO id	Pathways
95.	gi|238491804|ref|XP_002377139.1|	K02272	ko00190 oxidative phosphorylation, ko04260 cardiac muscle contraction, ko05010 Alzheimer's disease, ko05012 Parkinson's disease, ko05016 Huntington's disease
96.	gi|238491546|ref|XP_002377010.1|	K07112	Unclassified; poorly characterised; general function prediction only
97.	gi|238490480|ref|XP_002376477.1|	K11835	Ubiquitin carboxyl-terminal hydrolase
98.	gi|238489969|ref|XP_002376222.1|	K15564	CTD kinase subunit beta
99.	gi|238489541|ref|XP_002376008.1|	K11560	Kinetochore protein Mis13/DSN1
100.	gi|238494286|ref|XP_002378379.1|	K01720	ko00640 propanoate metabolism
101.	gi|238494128|ref|XP_002378300.1|	K10696	E3 ubiquitin-protein ligase BRE1
102.	gi|238493889|ref|XP_002378181.1|	K10531	L-ornithine N5-oxygenase
103.	gi|238493849|ref|XP_002378161.1|	K12763	ko04113 meiosis—yeast
104.	gi|238493689|ref|XP_002378081.1|	K07241	High-affinity nickel-transport protein
105.	gi|238493425|ref|XP_002377949.1|	K08095	Cutinase
106.	gi|238493007|ref|XP_002377740.1|	K00698	ko00520 amino sugar and nucleotide sugar metabolism
107.	gi|238492034|ref|XP_002377254.1|	K11770	Chromatin structure-remodeling complex subunit SFH1
108.	gi|238491956|ref|XP_002377215.1|	K11551	Central kinetochore subunit Mis15/CHL4
109.	gi|238491800|ref|XP_002377137.1|	K11888	Protein Cut8
110.	gi|238491698|ref|XP_002377086.1|	K11675	Ino 80 subunit 1
111.	gi|238491524|ref|XP_002376999.1|	K15130	Mediator of RNA polymerase II transcription subunit 8, fungi type
112.	gi|238491146|ref|XP_002376810.1|	K13281	UV DNA damage endonuclease
113.	gi|238490942|ref|XP_002376708.1|	K00505	ko00350 tyrosine metabolism, Ko00740 riboflavin metabolism, ko00950 isoquinoline alkaloid biosynthesis, ko00965 betalain biosynthesis, ko04916 melanogenesis
114.	gi|238490037|ref|XP_002376256.1|	K08504	ko04130 SNARE interactions in vesicular transport
115.	gi|238489809|ref|XP_002376142.1|	K14679	tRNA ligase
116.	gi|238489155|ref|XP_002375815.1|	K00418	ko00190 oxidative phosphorylation, ko04260 cardiac muscle contraction, ko05010 Alzheimer's disease, ko05012 Parkinson's disease, ko05016 Huntington's disease
117.	gi|238488641|ref|XP_002375558.1|	K04070	Putative pyruvate formate lyase activating enzyme
118.	gi|238488535|ref|XP_002375505.1|	K00551	ko00564 glycerophospholipid metabolism
119.	gi|238488303|ref|XP_002375389.1|	K00698	ko00520 amino sugar and nucleotide sugar metabolism
120.	gi|238487916|ref|XP_002375196.1|	K10771	ko03410 base excision repair
121.	gi|238487840|ref|XP_002375158.1|	K11763	Chromatin structure-remodeling complex subunit RSC9
122.	gi|238487534|ref|XP_002375005.1|	K01178	ko00500 starch and sucrose metabolism
123.	gi|238487008|ref|XP_002374742.1|	K15205	Transcription factor C subunit 6
124.	gi|238486924|ref|XP_002374700.1|	K01636	Unclassified; metabolism;
125.	gi|238486836|ref|XP_002374656.1|	K01417	Unclassified; Metabolism;
126.	gi|238486564|ref|XP_002374520.1|	K12609	ko03018 RNA degradation
127.	gi|238486548|ref|XP_002374512.1|	K07973	ko04011 MAPK signaling pathway—yeast
128.	gi|238486022|ref|XP_002374249.1|	K00416	ko00190 oxidative phosphorylation, ko04260 cardiac muscle contraction, ko05010 Alzheimer's disease, ko05012 Parkinson's disease, ko05016 Huntington's disease

TABLE 16.1 The 147 proteins identified from the pathway analysis using the KAAS server.—cont'd

SN	Gi no. and accession no.	KO id	Pathways
129.	gi|238486018|ref|XP_002374247.1|	K13507	ko0056 glycerolipid metabolism, ko00564 glycerophospholipid metabolism
130.	gi|238485440|ref|XP_002373958.1|	K15134	Mediator of RNA polymerase II transcription subunit 17, fungi type
131.	gi|238485402|ref|XP_002373939.1|	K08257	Mannan endo-1,6-alpha-mannosidase
132.	gi|238485352|ref|XP_002373914.1|	K01560	ko00361 chlorocyclohexane and chlorobenzene degradation, ko00625 chloroalkane and chloroalkene degradation
133.	gi|238484903|ref|XP_002373690.1|	K11563	Kinetochore protein Spc7/SPC105
134.	gi|238484335|ref|XP_002373406.1|	K03860	ko00563 glycosylphosphatidy linositol (GPI)-anchor biosynthesis
135.	gi|238484091|ref|XP_002373284.1|	K15206	Transcription factor C subunit 7
136.	gi|238484003|ref|XP_002373240.1|	K14130	Tryptophan 4-dimethylallyltransferase
137.	gi|238483999|ref|XP_002373238.1|	K02140	ko00190 oxidative phosphorylation
138.	gi|238483951|ref|XP_002373214.1|	K11559	Kinetochore protein Mis12/MTW1
139.	gi|238483247|ref|XP_002372862.1|	K01483	ko00230 purine metabolism
140.	gi|238483061|ref|XP_002372769.1|	K02127	ko00190 oxidative phosphorylation, ko05010 Alzheimer's disease, ko05012 Parkinson's disease, ko05016 Huntington's disease
141.	gi|238483033|ref|XP_002372755.1|	K01067	ko00620 pyruvate metabolism
142.	gi|238483247|ref|XP_002372862.1|	K01483	ko00230 purine metabolism
143.	gi|238483061|ref|XP_002372769.1|	K02127	ko00190 oxidative phosphorylation, ko05010 Alzheimer's disease, ko05012 Parkinson's disease, ko05016 Huntington's disease
144.	gi|238483033|ref|XP_002372755.1|	K01067	ko00620 pyruvate metabolism
145.	gi|238482681|ref|XP_002372579.1|	K02304	ko00860 porphyrin and chlorophyll metabolism
146.	gi|238496903|ref|XP_002379687.1|	K01720	ko00640 propanoate metabolism
147.	gi|238482419|ref|XP_002372448.1|	K00450	ko00350 tyrosine metabolism

the host and the pathogen was carried out to identify pharmacological targets engaged in metabolic processes particular to pathogens. The percentage distribution of novel drug targets involved in different metabolic pathways/biological processes is shown in Fig. 16.4. After manual analysis, only 12 critical protein pathways crucial for the organism's survival were identified (Table 16.2). They are Amino acid and nucleotide sugar metabolism, porphyrin and chlorophyll metabolisms, cutinase, snare interactions in Vascular transport, pin2-interacting protein x, ubiquitin carboxyl-terminal hydrolase, tryptophan 4-dimethylallyltranferase, t-RNA ligation, transcription factor c subunit 7. Critical proteins mean alternative pathways were not present for these proteins.

Subcellular localization prediction and identification of novel drug target

Adhesion constitutes one of the initial stages of infection in fungal diseases and is mediated by adhesins. To comprehend adhesin-mediated pathophysiology and how to take advantage of its therapeutic potential, adhesins, and adhesin-like proteins must be identified and well understood (Vaishnav et al., 2015). However, the understanding of fungal adhesins is poor compared. Fungal adhesins are important for xenotypic aggregation and homotypic, foraging, and bio-film development. Adhesins are cell surface proteins that give fungi the capacity to adhere to various substrates, such as cells and tissues. Adhesins are crucial components of a pathogen's pathogenicity because they serve as the first line of defense against host cell invasion.

Subcellular localization of proteins has been performed using WoLF PSORT, and adhesion properties have been identified using Faapered and FungalRV in metabolic pathway analysis. After performing WoLF PSORT and Faapered,

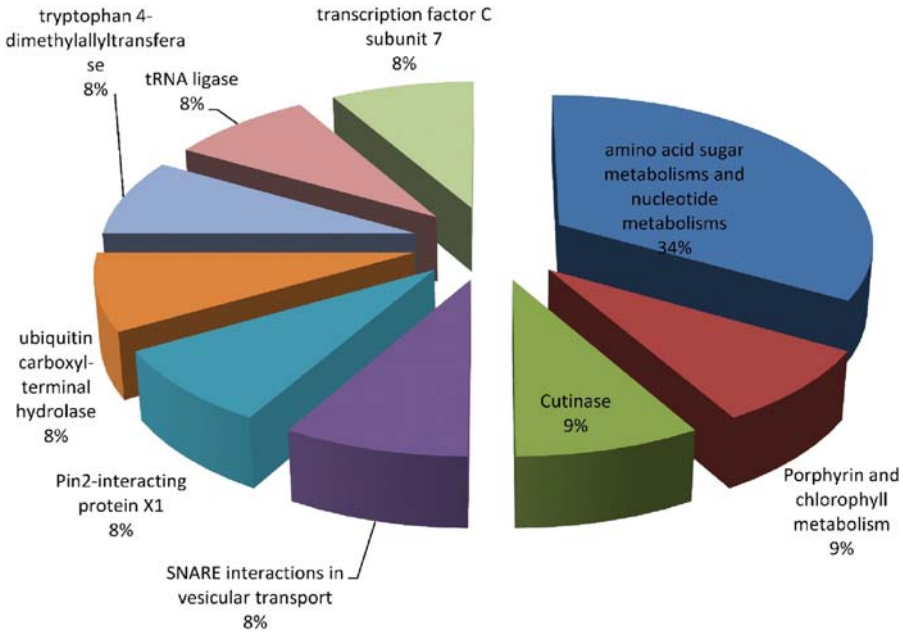

FIGURE 16.4 Percentage distribution of novel drug targets involved in different metabolic pathways/biological processes.

TABLE 16.2 List of 12 critical proteins identified using the KAAS server.

KO id	Enzyme name	Pathways
K00698	Chitin synthase B	ko00520 amino sugar and nucleotide sugar metabolism
K00698	Chitin synthase chs G	ko00520 amino sugar and nucleotide sugar metabolism
K00698	Chitin synthase A	ko00520 amino sugar and nucleotide sugar metabolism
K00698	Chitin synthase C	ko00520 amino sugar and nucleotide sugar metabolism
K02304		ko00860 porphyrin and chlorophyll metabolism
K08095		Cutinase [3.1.1.74]
K08504		ko04130 SNARE interactions in vesicular transport
K11135		Pin2-interacting protein X1
K11835		Ubiquitin carboxyl-terminal hydrolase
K14130		Tryptophan 4-dimethylallyltransferase
K14679		tRNA ligase
K15206		Transcription factor C subunit 7

and FungalRV, we got mainly five proteins (Table 16.3). Among the five proteins, only one is adhesin and critical, i.e., Endo 1,4 Beta xylanase, and the other four were only critical chitin synthase. In fungi, these five proteins are essential for survival. All five proteins were identified as novel drug targets after protein localization, and adhesion mechanisms were analyzed. Screening for drug targets was carried out using DrugBank and TTD to identify novel drug targets (Okella et al., 2020). Four proteins such as chitin synthase A, B, C, and G were not found in DrugBank except Endo 1,4 -beta Xylanase. The Endo 1,4 -beta Xylanase is present at the experimental level but not targeted for approved drugs. But when these five proteins were analyzed in TTD, we found drug target values like Chitin synthase B got the value of e−120, Chitin synthase A got value of 0. Chitin synthase G got the value of e−148, and Chitin synthase C got the value of e−145. After the TTD and DrugBank results, these five proteins run for 3D structure prediction by I-TASSER.

3D structure prediction and analysis

The 3D structure of the chitin synthase B, Chitin synthase C, Chitin synthase A, chitin synthase G, and endo 1,4 Bta-Xylanase was modeled by ab initio protein modeling tool I-TASSER as no template was available in PDB. The top

TABLE 16.3 List of predicted five plasma proteins and extracellular adhesion proteins.

KO id	Name of enzymes	Location	Adhesive
Plasma membrane proteins			
K00698	Chitin synthase A	Plasma membrane	No
K00698	Chitin synthase B	Plasma membrane	No
K00698	Chitin synthase C	Plasma membrane	No
K00698	Chitin synthase G	Plasma membrane	No
Extracellular adhesive protein			
K01181	Endo 1,4 beta-xylanase [EC:3.2.1.8]	Extracellular	Yes

model predicted by I-TASSER was considered for further work based on the C-score. A confidence score, or C-score, is used by I-TASSER to gauge the caliber of predicted models. It is determined using the importance of thread pattern matches and the optimum variables from simulations of the structure construction. The C-score normally falls between [−5, 2]. The best I-TASSER models had a negative c-score (Asmani et al., 2022).

The quality of the predicted structure was analyzed by PROCHECK and ANOLEA servers. ANOLEA assesses the packing quality of the model. This will immediately reveal if there are regions with steric clashes (atoms coming too close) since such regions will have high energy. The PROCHECK program was assessed using the Ramachandran plot. In the Ramachandran plot, the residue in the most favorable region and its allowed region should be 90%. But Chitin synthase-A showed a poor 3D structure in I-TASSER and was not considered for further work. Only four proteins, namely Chitin synthase B, Chitin synthase C, Chitin synthase G, and Endo 1,4−beta Xylanase, were used for further analysis. The PROCHECK program was assessed using the Ramachandran plot. It is evident from the Ramchandran plot that the predicted model has 96.7%, 95.1%, 96.4%, and 95.1 of ChsB, ChsC, ChsG, Endo 1, 4, beta Xylanase residues in the most favorable regions and the allowed regions, respectively.

Ligand Library construction and molecular docking

For the construction of the Ligand Library, A total of 97,782 antiviral, 41,640 anticancer, 8394 antifungal molecules, and 26 chitinase molecules were retrieved from the PubChem bioassay database [http://pubchem.ncbi.nlm.nih.gov/.Drug-like] compounds of NCI database [http://ligand.info]. Herbal compound molecules were obtained from the natural product database, and 1569 molecules were retrieved from the natural product, which also contains Dr. Duke's Phytochemical library [www.ars-grin.gov/duke]. The approved drugs were selected due to their FDA approval and thus will not require preclinical trials. As they are already targeting fungal proteins, they are more likely to complete the inhibition of an organism (Bonturi et al., 2022).

As a case study, the Chitin synthase B is used as a target, as its predicted structure quality is good. Molecular docking has been performed using Molegro Virtual Docker. The top three natural compounds have been identified via molecular docking (Table 16.4). Further, out of three ligands, the PubChem CID: 2,879,872; 1,840,499 were known for antifungal.

TABLE 16.4 Docking energy of the top three natural product molecules.

Sr.	IUPAC name. of the molecules	PubChem	MVD docking energy	MVD Rerank
1	2-[1-(3-ethoxycarbonyl-4,5,6,7-tetrahydro-1-benzothiophen-2-yl)-2,5-dioxopyrrolidin-3-yl]sulfanylbenzoic acid	2879872	−156.043	−103.036
2	(E)-3-[5-(4-acetylphenyl)furan-2-yl]-2-[(5-methyl-1H-1,2,4-Triazol-3-yl)sulfanyl]prop-2-enoic acid	1840499	−150.899	−98.0905
3	5-benzyl-N-[2-(4-methoxyphenyl)ethyl]-3-(4-nitrophenyl)-4,6-dihydro-3aH-pyrrolo[3,4-d][1,2]oxazole-6a-carboxamide	11958634	−198.869	−122.734

Conclusion and future prospectus

The targets found are inevitable for the organisms' growth; these proteins neither have a substitute nor an alternative pathway to accomplish the process. The current study designed a drug that can block Chitin synthase. It explores the possibilities of making new drugs from available natural products. The fungi are fast acquiring resistance to the existing drugs, so designing better and more effective drugs should be made faster. Thus, the current study can be the best replacement for the current therapies available. This fungus *A. flavus* is infected by all living things like plants, humans, animals, insects, birds, etc. So we can remove those infected diseases from the environment. Further, to confirm the result from in silico work, we need to do biological confirmations such as MIC, MBC, Antibiotic assay, Enzyme assay, and Crystallography.

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Chapter 17

Virtual screening of natural product as TAM family of RTK inhibitor: A potential tool for anticancer drug design

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Introduction

Cancer is a group of diseases characterized by the development of abnormal cells with uncontrolled growth and the ability to infiltrate and destroy normal cells (Aktipis et al., 2015; Dholakia et al., 2019). It is responsible for a million deaths annually, is generally dreaded, and around 50% of newly diagnosed cases may be cured. Cancer research mainly falls into two major categories—diagnosis and treatment and etiology and prevention. Diagnosis and treatment pertain to the techniques leading to the detection of cancer that already exists so that suitable remedial therapies such as surgery, irradiation, or chemotherapy can be performed (Arruebo et al., 2011). The fields of etiology and prevention aim to understand the mechanisms behind the development of neoplastic disorders and their underlying nature (Shah et al., 2018b). Traditional cancer therapies, including surgery, chemotherapy, radiation, or a combination, are rapidly losing efficacy. Even though the exact causes of cancer are not fully understood, it is recognized that several factors—many of which may be changeable, such as cigarette and alcohol use, excess body weight, and inherited genetic mutations—can raise the chance of developing cancer (De Pergola and Silvestris, 2013; Pelucchi et al., 2006; Pomerantz and Freedman, 2011). These risk factors may operate concurrently or sequentially to begin or encourage cancer development. Smoking cigarettes continues to be the leading cause of lung cancer mortality in the United States, accounting for around 80% of all lung cancer cases (Warren and Cummings, 2013). Smoking cigarettes also raises the risk of various cancers, including those of the liver, uterine cervix, kidney, bladder, stomach, colorectum, oral cavity, and throat. It also increases the chance of acute myeloid leukemia and lung, esophageal, larynx, and pancreatic cancers. Excess body weight, alcohol use, poor nutrition, and a sedentary lifestyle cause about one-fifth of all malignancies (Gallaway et al., 2018).

In this modern era, natural product research is a powerful method for identifying novel physiologically active substances with distinct modes of action (Sureja et al., 2022). Given the diversity of nature, it seems sensible to suggest that chemical leads that can interact with the majority of therapeutic targets can be developed. As a result, innovative and effective medications can be created to treat and cure cancer safely. The primary source for developing new drugs has always been natural items and will continue to be so. Virtual screening has a positive effect on finding leads (Bhakhar et al., 2021; Dhameliya et al., 2022; Gajjar et al., 2021). In the postgenomic age, drug development will increasingly rely on the chemical information of natural products combined with virtual screening as functional genomic investigations reveal more and more novel potential targets (Shah et al., 2018a). This chapter has instances of how virtual screening has affected the identification of active compounds and the technique used to find leads from currently understood natural products.

Background and epidemiology

Worldwide, an estimated 19.3 million new cancer cases (18.1 million excluding nonmelanoma skin cancer) and almost 10.0 million cancer deaths (9.9 million excluding nonmelanoma skin cancer) occurred in 2020. Female breast cancer has surpassed lung cancer as the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%), followed

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by lung (11.4%), colorectal (10%), prostate (7.3%), and stomach (5.6%) cancers. Lung cancer remained the leading cause of cancer death, with an estimated 1.8 million deaths (18%), followed by colorectal (9.4%), liver (8.3%), stomach (7.7%), and female breast (6.9%) cancers (Sung et al., 2021).

The American Cancer Society gathers the latest information on population-based cancer incidence each year. It predicts the number of new cancer cases and cancer-related deaths in the United States. The National Center for Health Statistics gathered mortality statistics from 2010 to 2018 in its report. In the United States, it is anticipated that there will be 1,898,160 new cancer cases and 608,570 cancer deaths in 2021. Because of decreases in smoking and advancements in early identification and treatment, the mortality rate from cancer has been steadily declining from its high in 1991 through 2018, or a total drop of 31%, after growing for most of the 20th century. Accordingly, there were 3.2 million fewer cancer fatalities than there would have been if peak rates had remained. Long-term death decreases for the four most common cancers have slowed for breast and colorectal cancers and stopped for prostate cancer, while lung cancer has accelerated. Lung cancer accounted for over half of the overall mortality drop from 2014 to 2018. The rate of the yearly decline in lung cancer mortality increased from 3.1% to 5.5% from 2009 to 2013 to 2014 to 2018 for males, from 1.8% to 4.4% for women, and from 2.4% to 5% overall. Consistent decreases accompany this pattern in incidence (2.2%−2.3%) but quick improvements in survival, particularly for nonsmall cell lung cancer (NSCLC). For instance, the 2-year relative survival rate for NSCLC went from 34% for patients diagnosed in 2009−10 to 42% in 2015−16, with definite improvements of 5%−6% for each stage of diagnosis; the survival rate for small cell lung cancer stayed at 14%−15%. Despite slowing momentum for other significant diseases, improved therapy has accelerated progress against lung cancer and contributed to a historic decline in total cancer mortality (Siegel et al., 2021).

In India, there are predicted to be 26.7 million disability-adjusted life years ($DALYs_{AMI}$) affected by cancer in 2021, and that number will rise to 29.8 million by 2025. The burden was most significant among men and in the northern and north-eastern areas of the nation (2408 $DALYs_{AMI}$ per 100,000 and 2177 $DALYs_{AMI}$ per 100,000, respectively). The seven most common cancer sites—lung (10.6%), breast (10.5%), esophagus (5.8%), mouth (5.7%), stomach (5.2%), liver (4.6%), and cervix uteri—contributed to more than 40% of the overall cancer burden (4.3%) (Kulothungan et al., 2022).

Across all areas of India, ovarian cancer ranked third in incidence among females, trailing only breast and cervical cancer in order of occurrence. Regarding incidence, esophageal cancer fell between the fifth and twelfth positions, whereas mouth cancer fell between the fourth and eighth. Lung cancer had the most significant crude incidence rates among males in the country's southern, northern, and eastern areas in 2016, while the western, central, and north-eastern regions had the highest incidence rates of mouth and esophageal cancers. Esophageal cancer incidence in the other areas ranged from fifth to tenth (Mathur et al., 2020).

RTKs (receptor tyrosine kinases)

RTKs were discovered early on as proto-oncogenes, with abnormal expression associated with tumor development in many malignancies. As a result, RTKs have emerged as essential targets for small-molecule inhibitor-based selective treatment (Bennasroune et al., 2004). There are now 58 RTKs in the human genome. Based on structural similarity in their extracellular areas and amino acid sequence identity within the kinase domain, these are divided into 20 subfamilies (Robinson et al., 2000). Some of them are EGFR/ERBB receptor kinase, Insulin receptor kinase, PDGF receptor kinase, FGF receptor kinase, VEGF receptor kinase, HGF receptor kinase, TRK receptor kinase, EPH receptor kinase, TAM receptor kinase, LTK receptor kinase, TIE receptor kinase, ROR receptor kinase, DDR receptor kinase, RET receptor kinase, KLG/CCK receptor kinase, MuSK receptor kinase, etc (Lemmon and Schlessinger, 2010; Yamaoka et al., 2018).

Cellular signaling is often mediated by receptor tyrosine kinases (RTKs). Numerous human disorders, including cancer, are caused by the dysregulation of RTK signaling. Multi-domain transmembrane proteins called RTKs serve as sensors for external ligands. They are responsible for relaying environmental signals to the cytoplasm and nucleus. RTKs function in this way to regulate essential cellular processes, including adhesion, motility, differentiation, growth, and cell survival (Lemmon and Schlessinger, 2010; Mele and Johnson, 2019).

When RTK's expression or activity is altered, they may become transformative in cellular and animal models. Since increased RTK expression or activation has also been fundamentally linked to the pathogenesis of various human malignancies, tyrosine kinase inhibitors have generated a lot of attention as prospective cancer therapeutics (Du and Lovly, 2018).

TAM receptors

In 1991, the family of RTKs known as the TAM receptors—TYRO3, AXL, and MERTK—was identified 3 decades ago. The name of the TAM family is derived from the first letter of its three constituents—TYRO3, AXL, and MERTK

(Prasad et al., 2006). TAM receptors are extensively dispersed in various organs, including the nervous system, and they play a role in immunological responses and cell survival, migration, invasion, metastasis, and chemosensitivity. The heart, liver, hippocampus, and cerebellum of the brain, as well as platelets, monocytes, and endothelial cells, all have high levels of TAM expression (Pierce and Keating, 2014).

The TAM family differs from other RTKs due to the KW (I/L) A(I/L) ES sequence, which is conserved in the extracellular region's adhesion molecule-like domains and a kinase domain. Specifically, each family member has an ectodomain nearly wholly made up of two immunoglobulin-like (Ig) domains and two fibronectin type III (FN III) domains. These motifs mimic the structure of the neural cell adhesion molecule (NCAM), which is thought to be important in cell-cell interactions and includes five Ig domains and two FNIII domains. The TAM receptor genes encode transcripts varying in size from 3 to 5 kb and have a similar genomic architecture (Linger et al., 2008).

The TAM receptors are activated upon binding with their extracellular ligands. Growth arrest-specific protein 6 (Gas6) and protein S (Pros1) were the first discovered and are the most studied ligands for the TAM receptors. The vitamin K-dependent protein Gas6, a 678-amino acid protein, was first identified as a ligand for AXL in 1995. The related vitamin K-dependent anticoagulation factor, protein S, was described as a ligand for Tyro-3 (Recarte-Pelz et al., 2013). Although numerous subsequent studies confirmed that Gas6 binds to and activates all three members of the TAM receptor family, the validity of Protein S as a ligand for any TAM receptors became subject to extensive debate. The ligands Gas6 and ProS have similar domain structures, including a C-terminal globulin that binds sex hormones, four repetitions identical to epidermal growth factor (EGF), and an N-terminal-carboxyglutamic acid (GLA)-rich domain. Gas6 and ProS show Ca^{+2}-dependent binding to negatively charged phosphatidylserine (PtdSer)-presenting cell membranes, and these protein ligands share 43% amino acid sequence similarity. The formation of a tetrameric complex by binding TAM receptor dimers to paired Gas6 or ProS molecules connected to the contacting cell membrane by PtdSer occurs in apoptotic cells (Lemke, 2013; Lemke and Burstyn-Cohen, 2010). Additional but less studied ligands include Tubby (interacts with all TAM members), Galectin-3 (LGALS3, interacts with MERTK and TYRO3), and tubby-like protein 1 (TULP-1, interacts selectively with MERTK). These three proteins interact with MERTK, stimulate MERTK phosphorylation, and promote efferocytosis. Gas6 is a ligand for all three TAMs but has a higher affinity for AXL than Mer and TYRO3, while Pros1 is a ligand for TYRO3 and MERTK. Pros1 and Gas6 must function as bridging ligands for phosphatidylserine (PtdSer), generally produced on the outer membrane of dying cells, generating the best signaling via the receptor (Caberoy et al., 2010, 2012; van der Meer et al., 2014).

MERTK

The RTK MERTK is expressed in numerous human malignancies and is a novel target in cancer therapeutics. This family of proteins has been implicated in reversible cell growth arrest, survival, proliferation, and cell adhesion (Linger et al., 2010). MERTK is physiologically expressed in a wide range of cell types, including hematopoietic lineage cells, such as monocytes, macrophages, microglial and dendritic cells, natural killer (NK) cells, natural killer T (NKT) cells, platelets, as well as epithelial cells in the retina, lung, testes, ovary, prostate, and kidney. MERTK is also expressed in the heart, brain, and skeletal muscle at low levels (Paolino and Penninger, 2016).

AXL and MERTK have been associated with cancer progression, metastasis, and drug resistance and are overexpressed in many human cancers, including various leukemias and solid tumors. Abnormal expression and activation of MERTK have been implicated in the oncogenesis of many human cancers, including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), NSCLC, melanoma, and glioblastoma, where MERTK functions to increase cancer cell survival, thereby promoting tumorigenesis and chemo-resistance (Linger et al., 2010). The currently available literature provides a rationale for developing MERTK kinase inhibitors as cancer therapeutics targeting both cell autologous and immune-modulatory antitumor effects. A better understanding of how MERTK and AXL are overexpressed in cancer cells may aid in determining the best strategy for targeting these RTKs. MERTK activation and subsequent signaling regulate numerous oncogenic pathways. Depending on cancer type, context, and the specific ligand (GAS6, PROS1, Tubby, TULP-1, or Galectin-3), MERTK signaling results in increased proliferation, antiapoptosis, and survival, migration and metastasis, anchorage-independent growth, cancer stem cell maintenance, and expression of the immune checkpoint protein PD-L1. In specific contexts, MERTK signaling may be transforming (Cummings et al., 2013).

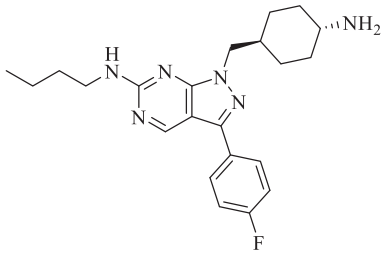
Current status of various MERTK inhibitors

TAM receptor inhibitors are being developed primarily because of their oncogenic significance in cancer progression. The proliferation, migration, invasion, and tumor development of cancer cells may all be inhibited by blocking TAM receptors.

Additionally, these compounds may suppress TME as well as tumor development. TAM receptor inhibition may lessen the immune system's ability to suppress macrophages, NK cells, and Treg cells (Aehnlich et al., 2021; Wium et al., 2018). The status of various MERTK inhibitors is mentioned below.

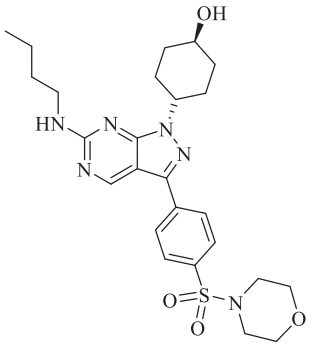
Agents in the preclinical development stage

UNC569 is a reversible, ATP-competitive, orally active MERTK inhibitor ($IC_{50} = 2.9$ nM, Morrison Ki = 4.3 nM). It also inhibits the other TAM family kinases (TYRO3 $IC_{50} = 48$ nM, AXL $IC_{50} = 37$ nM) (Christoph et al., 2013; Liu et al., 2012).



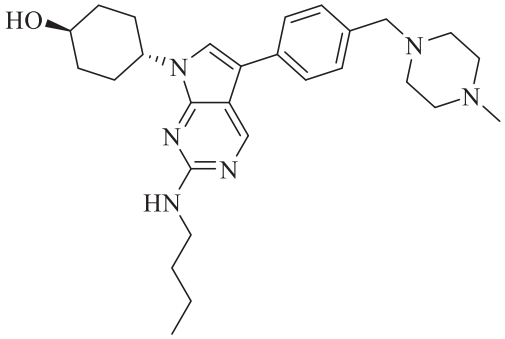
UNC569

UNC1062 is a potent MERTK inhibitor with an IC_{50} of 1.1 nM and a Ki of 0.33 nM. It also exhibits specificity against AXL and TYRO3 with IC_{50s} of 85 and 60 nM, respectively. It reduces activation of MERTK-mediated downstream signaling, induces apoptosis in culture, reduces colony formation in soft agar, and inhibits invasion of melanoma cells. It potently inhibits MERTK kinase activity (Schlegel et al., 2013).



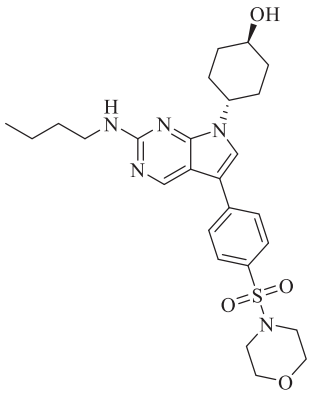
UNC1062

UNC2025 is an orally active ATP-competitive potent inhibitor of MERTK/FLT3 with IC_{50} values of 0.74 and 0.8 nM, respectively. It has greater than 45-fold more selectivity toward MERTK compared to AXL ($IC_{50} = 122$ nM; $K_i = 13.3$ nM) (DeRyckere et al., 2017; Zhang et al., 2014).



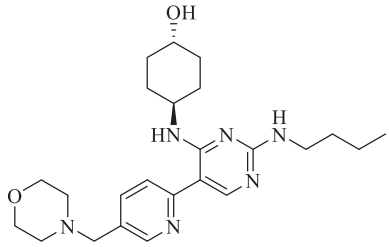
UNC2025

UNC1666 is a potent, specific, dual-acting ATP-competitive drug that diminishes MERTK and FLT3 phosphorylation. It ultimately leads to downstream signaling in acute myeloid leukemia (AML) with IC_{50} of 0.55 and 0.69 nM, respectively (Lee-Sherick et al., 2015).



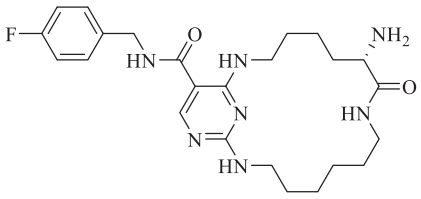
UNC1666

UNC2250 is a potent and selective inhibitor of MERTK with an IC_{50} of 1.7 nM. It is about 160 and 60-fold more selective for MERTK with respect to other closely related AXL and TYRO3 kinases, respectively (Zhang et al., 2013).



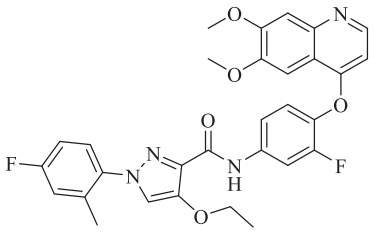
UNC2250

UNC2541 is a potent and specific MERTK inhibitor with an IC_{50} of 4.4 nM that binds in the MERTK ATP pocket and inhibits phosphorylated MERTK (pMERTK; EC_{50}, 510 nM). It has more selectivity against MERTK over AXL, TYRO3, and FLT3 (McIver et al., 2017).



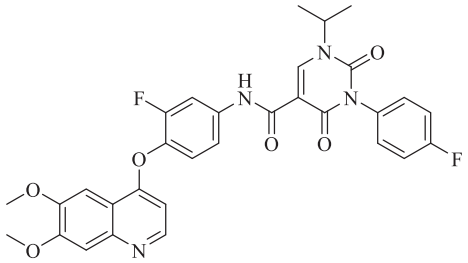
UNC2541

LDC1267 is a highly selective TAM kinase inhibitor having IC_{50s} of <5, 8, and 29 nM for TYRO3, AXL, and MERTK, respectively (Paolino et al., 2014).



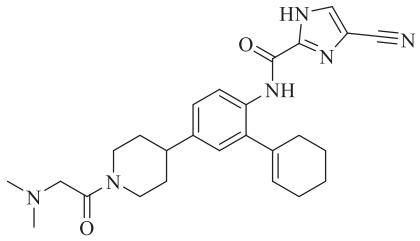
LDC1267

RXDX-106, also known as CEP-40783, is a potent, selective inhibitor of AXL and c-MET with IC_{50} values of 7 and 12 nM, respectively (Friedman et al., 2013; Miknyoczki et al., 2013).



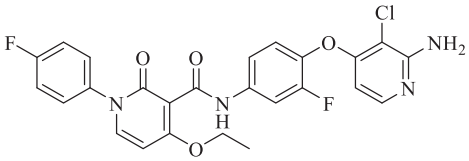
RXDX-106

JNJ-28312141 is an orally active CSF1R (Colony-stimulating factor-1 receptor) inhibitor that also inhibits FLT3 (Manthey et al., 2009).



JNJ28312414

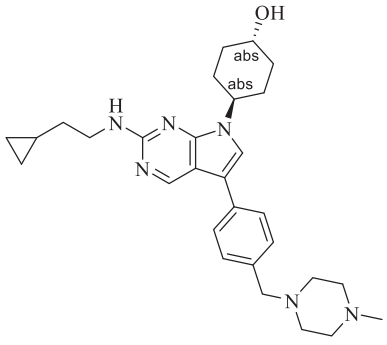
BMS 777607 (BMS 817378) is a Met-related inhibitor for c-MET, AXL, RON, and TYRO3 with IC_{50} values of 3.9, 1.1, 1.8, and 4.3 nM, respectively. It shows 40-fold more selectivity against Met-related targets concerning LCK, VEGFR-2, and TRKA/B. It also shows >500-fold greater selectivity against all other kinases (Dai and Siemann, 2010; Schroeder et al., 2009).



BMS 777607

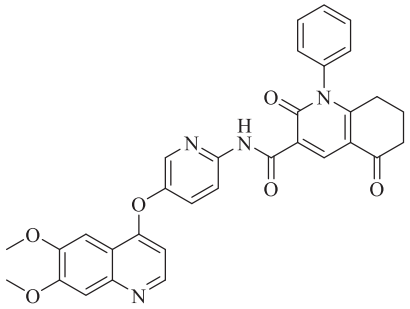
Agents in Phase-1 clinical development

MRX-2843, also known as UNC2371, is an orally active, dual-acting ATP-competitive inhibitor of MERTK and FLT3 with enzymatic IC_{50} values of 1.3 and 0.64 nM, respectively (Minson et al., 2016).



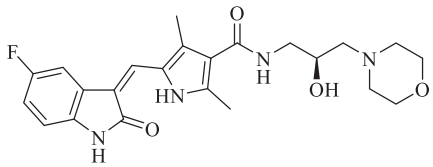
MRX-2843

ONO-7475 is a potent, selective, and orally active AXL/MERTK inhibitor with IC_{50} values of 0.7 and 1.0 nM, respectively (Ruvolo et al., 2017).



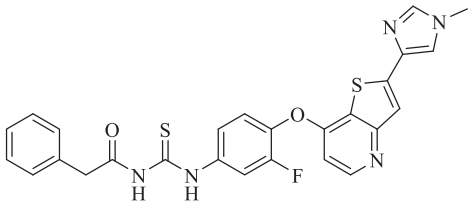
ONO-7475

SU14813 is a multi-targeted tyrosine kinases inhibitor active against VEGFR2, VEGFR1, PDGFRβ, and KIT receptors with IC_{50} values of 50, 2, 4, and 15 nM, respectively (Patyna et al., 2006).



SU-14813

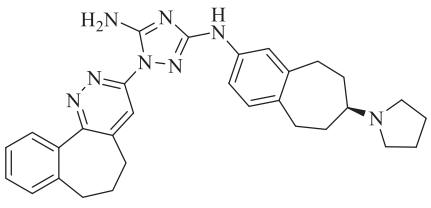
MGCD265, also known as Glesatinib, is potent, orally active, a dual-acting inhibitor of MET/SMO. It antagonizes P-glycoprotein (P-GP) mediated multidrug resistance (MDR) in NSCLC (Cui et al., 2019; Morgillo et al., 2017).



MGCD265

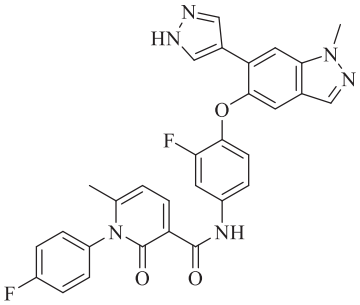
Agents in Phase-2 clinical development

Bemcentinib (R428; BGB324) is a potent and selective inhibitor of AXL with an IC_{50} of 14 nM (Holland et al., 2010).



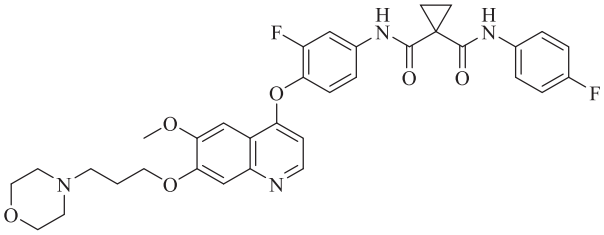
Bemcentinib

Merestinib (LY2801653) is a potent, orally active inhibitor of c-MET ($K_i = 2$ nM). It also shows potent activity against MST1R, FLT3, AXL, MERTK, Tek, ROS1/DDR1/2, and MKNK1/2 with IC_{50} values of 11, 7, 2, 10, 63, 0.1/7, and 7 nM respectively (Barat et al., 2016; Yan et al., 2013).



Merestinib

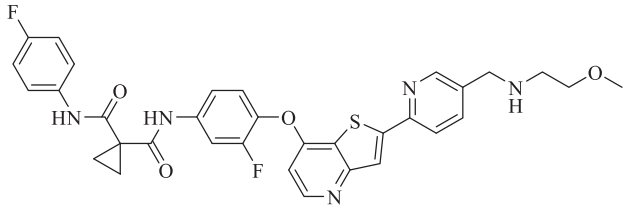
Foretinib is a multi-targeted tyrosine kinase inhibitor of Met and KDR with IC_{50} values of 0.4 and 0.9 nM, respectively (Eder et al., 2010; Qian et al., 2009).



Foretinib

Agents in Phase-3 clinical development

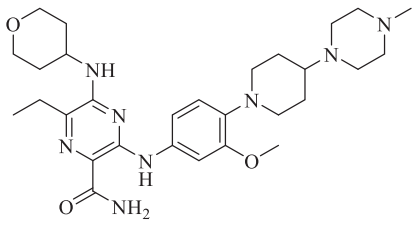
Sitravatinib (MGCD516) is an orally active RTK inhibitor with IC_{50} values of 1.5, 2, 2, 5, 6, 6, 8, 0.5, 29, 5, and 9 nM for AXL, MERTK, VEGFR3, VEGFR2, VEGFR1, KIT, FLT3, DDR2, DDR1, TRKA, and TRKB, respectively (Patwardhan et al., 2016).



Sitravatinib

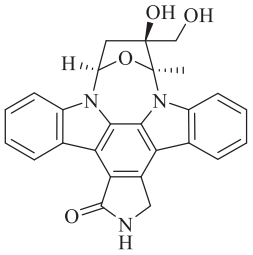
Approved drugs

Gilteritinib (ASP2215) is a potent and ATP-competitive inhibitor of FLT3 and AXL with IC_{50} values of 0.29 nM/0.73 nM, respectively (Mori et al., 2017).



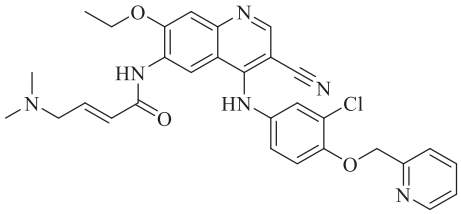
Gilteritinib

Lestaurtinib (CEP-701; KT-5555) is an ATP-competitive multi-targeted tyrosine kinase inhibitor having potent activity against the Trk family. It inhibits JAK2, FLT3, and TRKA with IC_{50} values of 0.9, 3, and <25 nM, respectively (Jatiani et al., 2010; Miknyoczki et al., 1999).



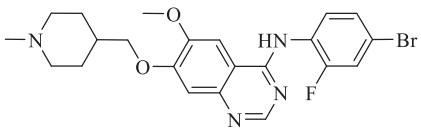
Lestaurtinib

Neratinib (HKI-272) is an orally active, irreversible inhibitor of HER2 and EGFR with IC_{50} values of 59 and 92 nM, respectively (Rabindran et al., 2004).



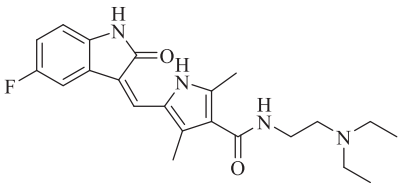
Neratinib

Vandetanib (D6474) is a potent, orally active inhibitor of VEGFR2/KDR with an IC_{50} value of 40 nM. It also has an activity for VEGFR3/FLT4 and EGFR/HER1 showing IC_{50} values of 110 and 500 nM, respectively (Inoue et al., 2012; Wedge et al., 2002).



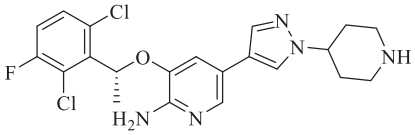
Vandetinib

Sunitinib (SU 11248) is an ATP-competitive, multi-targeted RTK inhibitor with IC_{50} values of 80 and 2 nM for VEGFR2 and PDGFRβ, respectively. It effectively inhibits autophosphorylation of Ire1α (O'Farrell et al., 2003; Sun et al., 2003).



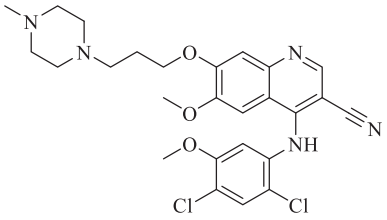
Sunitinib

Crizotinib (PF-02341066) is an ATP-competitive, orally active ALK and c-MET inhibitor with IC_{50} values of 20 and 8 nM, respectively. It is also a ROS1 inhibitor and has effective tumor growth inhibition (Cui et al., 2011; Zou et al., 2007).



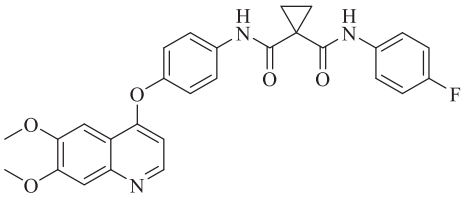
Crizotinib

Bosutinib is a dual inhibitor of SRC and ABL with IC_{50} values of 1.2 and 1 nM, respectively (Golas et al., 2003, 2005).



Bosutinib

Cabozantinib is a potent, orally bioavailable VEGFR2 and MET inhibitor, with IC_{50} values of 0.035 and 1.3 nM, respectively. It also displays potent inhibition of KIT, RET, AXL, TIE2, and FLT3 with IC_{50} values of 4.6, 5.2, 7, 14.3, and 11.3 nM, respectively. It disrupts tumor vasculature and promotes tumor and endothelial cell apoptosis (Yakes et al., 2011; You et al., 2011).



Cabozantinib

Natural products as anticancer agents

Since ancient times, the use of natural products as a primary source for the treatment and curing of diseases has been acknowledged (Cragg et al., 1997; Farnsworth et al., 1985). Despite significant advancements in science and technology, drugs of natural origin continue to play an essential role in drug discovery and development today (Bahar et al., 2007; Itokawa et al., 2008; Potterat and Hamburger, 2008). Due to the enormous and distinguished chemical diversity in the millions of species of plants, animals, marine creatures, and microbes, nature is a desirable source of novel therapeutic agents to treat various diseases (Trivedi et al., 2016). The influence of evolution on the selection and conservation of self-defense mechanisms, which represent the tactics used to fend off or eliminate predators, is shown in the different chemical moieties of many living creatures (Farnsworth, 1990).

Many effective chemotherapeutic agents developed and approved in the past had a natural origin. In the 1950s, when vinca alkaloids (vinblastine and vincristine) were identified as anticancer drugs, the search began for anticancer drugs from natural sources (Cragg and Newman, 2005). After that, natural products have been a critical component of cancer chemotherapy and chemoprevention, providing established anticancer drugs, new lead compounds for synthetic modifications, and understanding cellular and molecular mechanisms of action for their anticancer potentials (Kinghorn et al., 2016; Mann, 2002; Newman and Cragg, 2007; Nielsen, 2002).

These natural product derivatives are used as anticancer agents in either unmodified (naturally occurring) or synthetically modified forms (Kinghorn et al., 2009; Williams et al., 1989). For instance, anthracyclines (like doxorubicin), bleomycin, dactinomycin (actinomycin), and mitomycin C are anticancer antibiotics derived from microorganisms. In turn, chemicals from four plant-derived substances—bisindole (vinca) alkaloids, camptothecins, epipodophyllotoxins, and taxanes—are often employed as antitumor medications (Clardy and Walsh, 2004; Kinghorn et al., 2009). Additionally, there are numerous examples of promising natural product-derived anticancer agents that are currently in advanced clinical development or have just received approval, including those derived not only from plants and microbes (such as combretastatin and homoharringtonine analogs) but also from marine sources (e.g., the bryostatins, ecteinascidin 743, kahalalide F) (Kinghorn et al., 2009).

Since the 1940s, a total of 155 anticancer drugs have been licensed for use in both Western and Japanese medicine, and 47% of them were categorized as natural items per se (14%) or semisynthetic derivatives of natural goods (28%), or otherwise derived from natural products (5%). Many drugs produced from marine sources are currently being evaluated in

preclinical and early clinical trials as anticancer agents (Schwartsmann, 2000; Schwartsmann et al., 2001). Current natural anticancer drugs in clinical use, as well as those of this class now undergoing advanced clinical trials, are known to display significant structural diversification (Butler, 2008; Cragg and Newman, 2004; Cragg et al., 1997; Newman and Cragg, 2004, 2007; Rayan et al., 2017).

To reduce cancer occurrence, chemoprevention using natural or synthetic medicines is a promising strategy (De Melo et al., 2018; Mohan Shankar et al., 2022). The idea of cancer chemotherapy has significantly changed over the last several years. According to experimental research in animal models, it is possible to reverse or reduce premalignant lesions with chemopreventive drugs. In animal models, naturally occurring substances such as dietary phytochemicals, tea polyphenols, and resveratrol have chemopreventive action. Additionally, there have been continuing clinical trials to evaluate the efficacy and safety of a variety of natural substances in preventing or treating human cancer (Epstein et al., 2010; Jurenka, 2009; Wilken et al., 2011).

However, several difficulties and challenges must be overcome when creating new agents from natural sources instead of synthetic ones. For instance, it could be challenging to access the sample's origin, get adequate volumes of the sample to identify and isolate the sample's active constituents, and synthesize the required concentrations of the target chemical.

Objectives

Since cancer is the leading cause of mortality, finding active medications to treat the condition is essential. Studies have demonstrated that compounds obtained from natural sources hold promise in this attempt. Natural resources have been the primary sources of bioactive agents. They will continue to serve as the leading players in the search for novel medications since they naturally have a more excellent range of structural diversity than synthetic molecules. Searching active chemical constituents of natural origin assisted by virtual screening has shown considerable potential in drug discovery. One may correlate these studies to computational chemistry, which is gaining popularity nowadays owing to the prospect of creating alternative tactics that might help select the right group of compounds while avoiding wasting money on resources that produce undesired stuff. This study aimed to implement a strategy for multiple investigations that use virtual screening techniques to choose natural compounds with potential anticancer activity.

Virtual screening and AutoDock Vina

Computer-aided drug design (CADD) explores molecular features to create new drug discovery approaches using computational methods and data sources. When used broadly, it refers to computational methods for creating or choosing compounds as potential candidates before they are synthesized and evaluated for their biological activity (Song et al., 2009).

A CADD approach called virtual screening (VS), a popular technique, includes an in silico screening of a library of chemical compounds or an extensive database of ligands to determine which molecules or ligands are most likely to bind to a specific target (Voet et al., 2013). It is the computational equivalent of biological or pharmacological screening for scoring, rating, and filtering molecules most likely to attach to a target. The estimated affinities and probable binding are calculated in virtual screening (Seidel et al., 2019). It also helps scientists to pick a considerably smaller and manageable selection of compounds by allowing them to search through sections of the vast possible compounds in the chemical space. As a result, many cheminformatics issues in the design, selection, and analysis phases of the drug development process now revolve around virtual screening techniques (Seidel et al., 2019; Wermuth et al., 1998).

In virtual screening, two elements of the search are essential since hundreds of thousands of chemicals may be examined on a virtual screen. The docking approach must first be trusted to locate a relevant conformation, which is the first requirement. To confirm that the docking technique can accurately duplicate the observed binding mode, docking methods are often tested using "redocking" studies (Stahura and Bajorath, 2005).

There are now over 50 software programs available for protein-ligand docking, including Auto Dock Vina, Glide, FlexX, GOLD, and DOCK, to mention a few (Pagadala et al., 2017). We have used Auto Dock Vina as an example of a common and probably most popular freely available molecular docking program for virtual screening. Being free and the quality of the findings, particularly for ligands with eight or more rotatable bonds, explains their popularity. It incorporates an effective optimization approach based on a novel scoring function for calculating protein-ligand affinity and a new search algorithm for foretelling the likely binding modes (Morris et al., 2009).

Vina attempts to predict where and how a putative ligand may best bind to a specific protein in one execution. Vina may repeat numerous times with different randomizations (the configuration parameter exhaustiveness regulates how often the calculations are repeated). The coordinates of a cuboid, which we refer to as the docking box, specify the region of the

protein surface where the tool seeks to bind. The Vina documentation refers to this as the "search space." Due to the randomized computations seeding, repeating the same execution on the same ligand-protein combination by default might result in different binding modes (Jaghoori et al., 2016).

To ensure that the docking results may be duplicated, Vina does, however, let the user explicitly set an initial randomization seed. Vina may do the repeated computations in a single iteration in parallel on a multicore system since they are independent. It creates several threads, which operate concurrently whenever an application has open cores. When launching the docking experiment, the maximum number of concurrent threads may be managed (using the command-line option CPU). Vina defaults to trying to generate as many threads as there are cores available (Morris et al., 2009).

Virtual screening has the following benefits over traditional processing for drug testing on natural products (Good, 2007).

1. A substantially greater hit rate is achieved using virtual screening than conventional screening techniques, such as HTS. Additionally, for virtual screening, gathering and analyzing a large number of samples of purely natural products is unnecessary, which saves money and time on medication research.
2. The history of pharmaceuticals suggests that natural compounds supplied the market with a significant number of medications, but less than 10% of the natural product has been documented.
3. The pipeline of virtual screening can include the prediction of ADMET and other drug-like features, which may improve the hit compound quality and reduce failures in the drug development stage.

Docking studies

For the virtual screening of potential hits as MERTK inhibitor, a library of 492 natural compounds, especially polyphenols derived from food, were retrieved from the http://phenol-explorer.eu/ database (version 3.6 updated on 10-12-2016) (Neveu et al., 2010). The molecular docking was performed using the latest version of Autodock Vina v.1.2.0 software. The X-ray crystal structure of the MERTK kinase domain in complex with Merestinib was downloaded from the Protein Data Bank (PDB ID: 7AAY, Resolution: 1.87 Å, R-Value Free: 0.224, R-Value Work: 0.195, and R-Value Observed: 0.197). The binding site grid box was constructed near the protein's active site by employing the AutoDock tools grid setting feature to include essential amino acids for ligand binding. The grid box of size $37 \times 37 \times 37$ was constructed at grid center coordinates of (x, y, z) 24.7701, −12.5014, 13.0927 of the pocket to provide enough space for free movements of the molecules. The ligand and protein structures were optimized by adding missing hydrogen atoms, which is a step necessary for the correct calculation of partial atomic charges. Docking calculation was generated with the software free energy binding own scoring function. The binding affinity of the ligand was expressed in kcal/mol. Ten different poses were calculated for each protein with num_modes = 10 and exhaustiveness = 8. Molecular interactions between ligand and protein were generated and analyzed by BIOVIA Discovery Studio Visualizer.

Discussion

Virtual screening may be done using various naturally occurring ligand libraries to evaluate the anticancer activity (Shah et al., 2018a). Several sizable databases are accessible, most of which include commercially available chemicals, including ZINC, eMolecules, NCBI PubChem, ChemBridge, Otava, and Asinex for a number of these distinct libraries. Other libraries, such as lead-like compounds, nutraceuticals, natural products, and metabolome libraries, are tailored to particular purposes. Furthermore, the FDA-approved drug library may help repurpose compounds already showing biological activity and acceptable safety/toxicity profiles (Cosconati et al., 2010). We employed a natural product library that consists of polyphenols isolated from food that is well known and widely used in biomedical research.

Natural polyphenols were evaluated for their antitumor activity against MER Tyrosine kinase receptors using Autodock Vina software. The docking score values and no interactions were obtained using Autodock Vina. With the results obtained from the study, it was found that all polyphenols have significant binding energies with MERTK ranging from −11.3 to −4.6 kcal/mol. The results of the virtual screening experiment were ranked according to the binding energy of the best scoring conformation. The Top 10 compounds with the highest binding energies identified by virtual screening and their binding energies are represented in Table 17.1.

The analysis of the results revealed that all the 10 compounds tabulated in Table 17.1 were found to bind inside the kinase domain, surrounded by interacting residues with noticeably high binding energies that ranged from −11.3 to −9.8 kcal/mol. The 2D and 3D interactions of amino acid residues of MERTK Kinase (PDB ID: 7AAY) with Procyanidin

TABLE 17.1 Virtual screening identifies the top 10 compounds with their binding energies in kcal/mol.

Sr. No.	Molecule	Binding energy (kcal/mol)
1.	Procyanidin trimer T2	−11.3
2.	Naringin 6′-malonate	−10.6
3.	(+)-Gallocatechin 3-O-gallate	−10.4
4.	Rhoifolin	−10.2
5.	Cyanidin 3-O-(6″-succinyl-glucoside)	−10.2
6.	Cinnamtannin A2	−10
7.	Chrysin	−10
8.	Pallidol	−10
9.	Sesamolinol	−9.9
10.	Kaempferol 3-O-galactoside 7-O-rhamnoside	−9.8

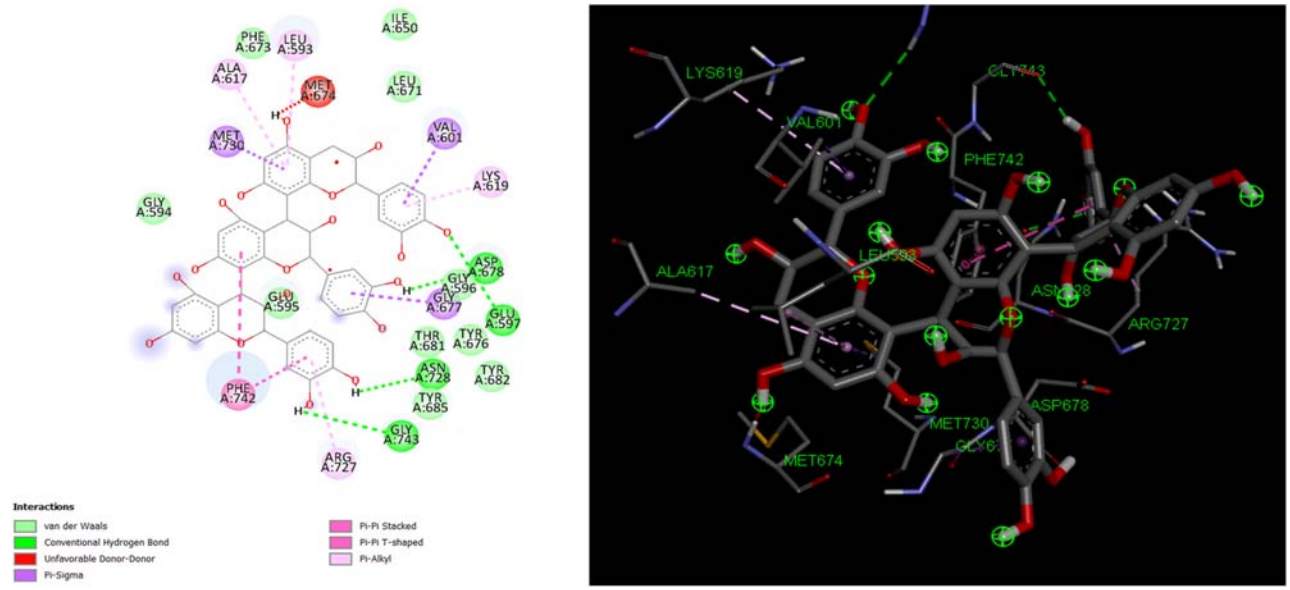


FIGURE 17.1 The 2D and 3D interactions of amino acid residues of MERTK kinase (PDB ID: 7AAY) with Procyanidin trimer T2.

trimer T2 (Fig. 17.1), Naringin 6′-malonate (Fig. 17.2), (+)-Gallocatechin 3-O-gallate (Fig. 17.3) and Chrysin (Fig. 17.4) are shown.

The docked poses of Procyanidin trimer T2 predicted that it interacts with residues of Leu593, Ala617, Lys619, and Arg727 for π-alkyl interaction. The π-π stacking with Phe742 residues was observed, as was the π-sigma interaction with Val601, Gly677, and Met730 residues. The H-bond formation was also observed with Glu597, Asp678, Asn728, and Gly743, whereas VdW with Gly594, Glu595, Gly596, Ile650, Leu671, Phe673, Tyr676, Thr681, Tyr682, and Tyr685 residues were found.

Naringin 6′-malonate established H-bonds (Met674, Asp741, and Phe742), π-sigma (Val601), π-alkyl (Leu593), π-π stacking (Phe673 and Phe742), and VdW (Gly596, Glu597, Ala617, Lys619, Ile650, Leu671, Lys675, Gly677, Met730, and Ala740).

Similarly, H-bond for (+)-Gallocatechin 3-O-gallate was found to form with Leu593, Glu603, Met674, Asp678, His680, Thr681, and Asp741, Carbon H-bond with Arg727, π-alkyl with Leu593, and Arg727, π-π stacking with Phe673,

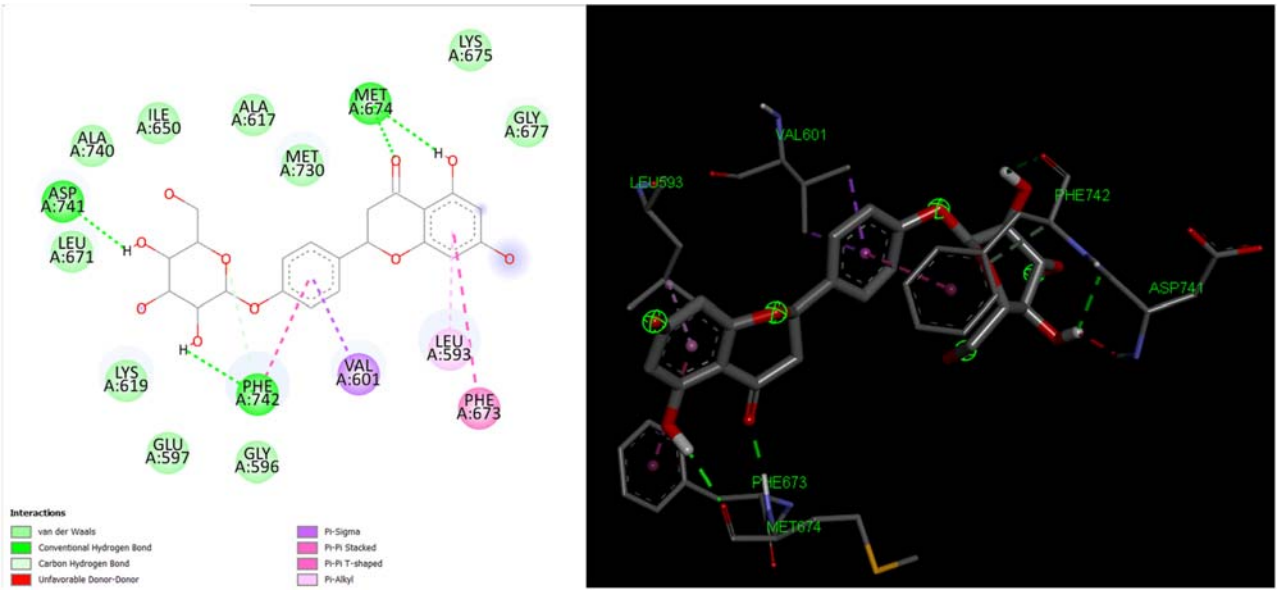


FIGURE 17.2 The 2D and 3D interactions of amino acid residues of MERTK kinase (PDB ID: 7AAY) with Naringin 6′-malonate.

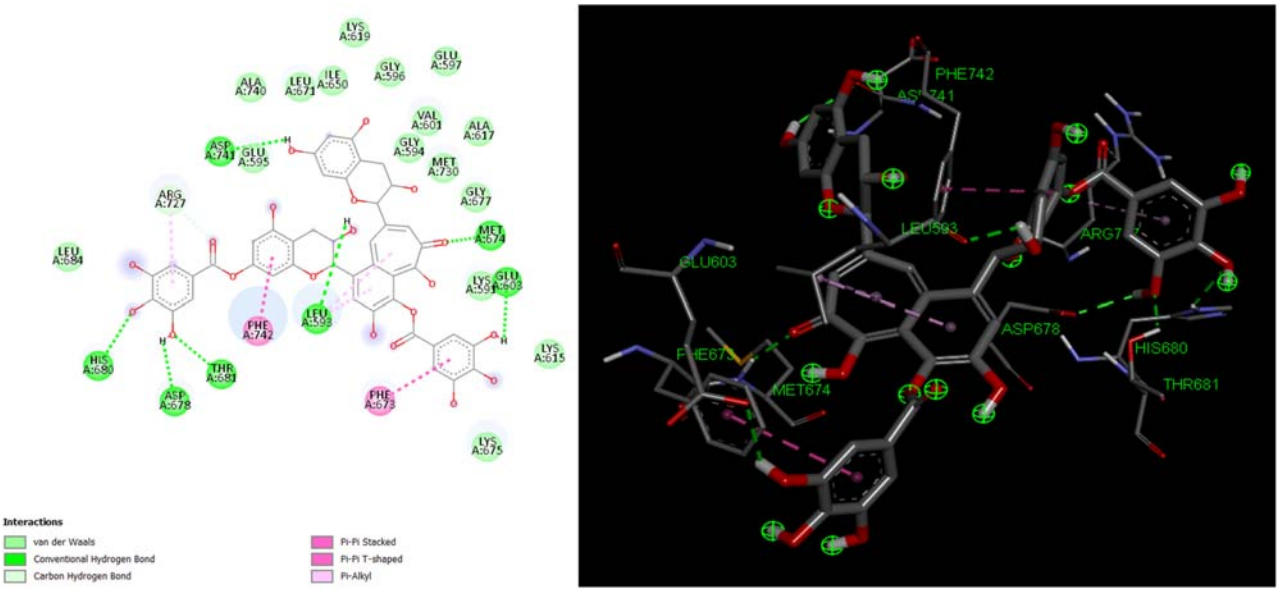


FIGURE 17.3 The 2D and 3D interactions of amino acid residues of MERTK kinase (PDB ID: 7AAY) with (+)-Gallocatechin 3-O-gallate.

and Phe742, and VdW with Lys591, Gly594, Glu595, Gly596, Glu597, Val601, Lys615, Ala617, Lys619, Ile650, Leu671, Lys675, Gly677, Leu684, Met730, and Ala740.

The interacting residues involved in the binding site of MERTK with Chrysin were found to form H-bonds with Leu593, Met674, Lys675, Asp678, Asp741, and Phe742, Carbon H-bond with Gly677, Thr681; π-alkyl with Leu593, Val601, and Ala617; π-sigma with Met730, and VdW with Gly594, Lys619, Ile650, Leu671, Pro672, Phe673, Tyr676, Tyr682, Tyr685, Pro692, Ala740, and Gly743 residues, respectively. It also shows π-sulfur and π-π stacking with Met730 and Phe742 residues, respectively.

Conclusion

In the years to come, natural products will still be the primary source of anticancer medications. It is anticipated that the natural product molecules involved in clinical trials and/or the drug development process will find a place in current anticancer therapy and contribute to the ongoing fight against cancer. Identifying natural product leads and optimizing

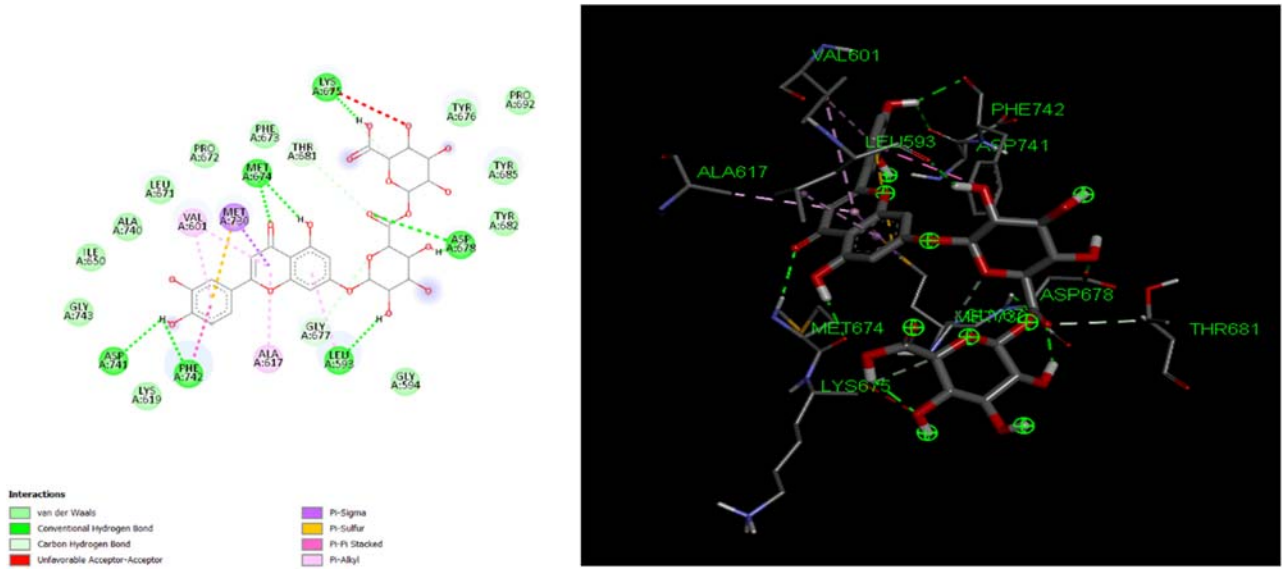


FIGURE 17.4 The 2D and 3D interactions of amino acid residues of MERTK kinase (PDB ID: 7AAY) with Chrysin.

through virtual screening, combinatorial and medicinal chemistry, total synthesis, and combinatorial biochemistry are all essential components of any successful drug discovery and development endeavor. Given the distinctive chemical diversity found in natural source materials, it is reasonable to assume that new anticancer drug leads capable of interacting with the majority—possibly even all—therapeutic targets will be found in naturally occurring or semi- or synthetic substances derived from natural substances.

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Chapter 18

In silico tools to comprehend legumes-derived bioactive peptides in diabetes and hypertension therapeutics

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Introduction

The coexistence of diabetes and hypertension

Globally the silent killers (type 2 diabetes mellitus and hypertension) are drastically dwindling the population at a fleet. The development of diabetes mellitus and hypertension commences a vicious cycle and the two together continue to precede each other in the chase (Tsimihodimos et al., 2018). The primordial mechanisms like upregulation of rennin angiotensin aldosterone system, oxidative stress, and activation of the immune system subsequently contribute to risk factors like dyslipidemia, obesity, endothelial dysfunction, vascular inflammation, arterial remodeling, and atherosclerosis. Further, stated risk factors aid in the progression of type 2 diabetes and hypertension in an individual (Petrie et al., 2018). Both the conditions commonly coexist and are complex over and above they exhibit heterogeneous phenotypes, which further elevates the risk for cardiovascular disease (Petrie et al., 2018; De Boer et al., 2017; Zhou et al., 2017; Ferrannini and Cushman, 2012). Genetic and pathophysiological factors are shared by type 2 diabetes mellitus and hypertension. One of the genetic factors includes Nuclear factor erythroid 2-related factor 2 (Nrf2). It is a nuclear transcription factor and is known to be an expert regulator of cytoprotective response. It exerts antioxidant effects by activating the transcription of target antioxidant genes such as superoxide dismutase, catalase (CAT), glutathione peroxidase (GPx), Glutathione-S-transferase (GST), heme oxygenase 1 (HMOX-1), NAD(P)H dehydrogenase [quinone] 1 (NQO1), metallothionein-1A (MT1A), and γ-glutamylcysteine synthase (γGCS) (Sarutipaiboon et al., 2020; Yagishita et al., 2020). Studies by Lopes et al. on male Wistar Kyoto rats and stroke-prone spontaneous hypertensive rats (SHRSP) reported down-regulation of Nrf2 especially in SHRSP wherein there was increased oxidative stress contributing to vascular dysfunction (Lopes et al., 2015). Furthermore, the pathophysiological features shared by type 2 diabetes and hypertension include vascular dysfunction and injury in endothelial tissues. On one side of the vicious circle, diabetes-associated factors augment the progression of hypertension. The following diabetes factors like raised oxidative stress, inflammation, and formation of advanced glycation end products along with their respective receptor promotes chronic hyperglycemia and insulin resistance. All the above factors combine and develop a vascular complication in diabetes and therefore heighten the risk of hypertension (Petrie et al., 2018; Brownlee, 2005). While on the other side, hypertension pathophysiology contributes to the risk of diabetes. The prime pathway crucial in the regulation of blood pressure is the renin angiotensin aldosterone pathway (RAAS), moreover exaggerated activity of RAAS directs the development of diabetes mellitus. Insulin resistance in skeletal muscle and hindrance in the intracellular insulin signaling pathway due to profound Angiotensin II from the RAAS pathway can diminish insulin secretion from pancreatic β-cells (Goossens, 2012). Thus hypertension-associated risk factors contribute to the development of diabetes. To summarize, similar risk factors and complex and highly interwoven pathways facilitate the coexistence of hypertension and type 2 diabetes.

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Treatment involved in diabetes and hypertension

Treatment aspects for both diseases must include lifestyle changes wherein physical activities and the inclusion of healthy food can help in moderating and reversing the risk factors associated with the disease condition. The impact of lifestyle modification undoubtedly results in reversing the risk factors however may require a long period. While, another approach i.e., pharmacological intervention, works immediately on an individual but can exhibit after effects due to routine and prolonged intake. In this regard, both approaches should be considered in the treatment and management of diabetes, hypertension, and their shared risk factors. Pharmacologic treatments for T2DM allow better control of glycemic conditions and hypertension and reduce blood lipid concentrations (Chiasson et al., 2003; Yadav et al., 2014). In the case of diabetes treatment medicines presently used include metformin, sulfonylurea, a thiazolidinedione, and sodium-glucose co-transporter-2 (SGLT2) inhibitor. Among all, the outstanding drug refers to metformin for type 2 diabetes. It helps in the reduction of blood glucose levels and additionally it displays an insulin-sensitizing effect on tissues involved in glucose metabolism (liver, skeletal muscle, adipose tissue, endothelium, etc.). Nevertheless, it is reported to have adherence issues and due to this, it is inappropriate for antidiabetic therapy via the oral route (McGovern et al., 2018). Overall, routine consumption of drugs in diabetes treatment is known to have a series of adverse effects such as insulin resistance, hypoglycemia, hypothyroidism, weight gain, obesity, atherosclerosis, and abdominal pain (Bodmer et al., 2008; Forst and Bramlage, 2014; Heine et al., 2005). Similarly, in the treatment of hypertension, a large number of antihypertensive drugs like captopril, enalapril, ramipril, etc act as angiotensin-converting enzyme inhibitors (ACE inhibitors), angiotensin receptor blockers, mineralocorticoid-receptor blockers, and calcium-channel blockers are known to exhibit direct vaso-protective effects. Additionally, the use of the above drugs to some extent reported to reduce vascular complications in patients with diabetes and concomitant hypertension (Cameron et al., 2016). However, these synthetic formulations inevitably possess adverse side effects, such as coughing, taste disturbances, and skin rashes (Atkinson and Robertson, 1979; Timmermans et al., 1992). Currently, an array of improved drugs with myriad targets and classes help in the management of diabetes and hypertension both. Blood glucose values lowering drugs including SGLT2 inhibitors and glucagon-like peptide-1 (GLP-1) receptor agonists are described to have a beneficial impact on diabetic patients possessing risk for hypertension and cardiovascular disease (Libianto et al., 2018; Geva et al., 2019; Nauck et al., 2017). To emphasize more GLP-1, it has a short life span as it is degraded by the dipeptidyl peptidase-IV enzyme (DPP-IV) present in the blood. Thus by exploring a peculiar DPP-IV inhibitor there is the possibility to increase the half-life of GLP-1. Thereby, stimulating insulin secretion from β cells of the islets of Langerhans and finally modulating blood glucose homeostasis (Singh et al., 2021). One of the DPP-IV inhibitor drugs linagliptin is associated with the reduction of obesity-related insulin resistance and reduces inflammation by governing M1/M2 macrophage polarization status (Zhuge et al., 2016). Also, incretin (gut hormone) is reported to reduce postprandial and fasting glycemia in type 2 diabetes mellitus (Müller et al., 2019; Gribble and Reimann, 2021). Novel drugs such as bardoxolone methyl target to increase Nrf-2 activity and thus enhances the inhibitory pyrin domain containing 3 (NLRP3) inflammasome. As a result, the drug exerts a remedy for oxidative stress, inflammation, and fibrosis (Zhuge et al., 2016). Moreover, statin and metformin both are known to enhance Nrf-2 activity (Kvandová et al., 2016). Lastly, statin and clopidogrel exhibit vaso protective nature as they possess antioxidant and antiinflammatory properties (Petrie et al., 2018). In conjunction, inhibition of specific digestive enzymes also contributes to combating type 2 diabetes mellitus, as they are involved in the breakdown and absorption of dietary carbohydrates and lipids. In the present state, inhibitors of enzymes such as α-amylase, α-glucosidase, and pancreatic lipase are searched and explored as therapeutics for diabetes mellitus (Patil et al., 2015; Irondi et al., 2018). In addition, some of the digestive enzyme inhibitory drugs available on market including acarbose and orlistat are effective but possess a wide range of side effects like diarrhea to liver toxicity hence their use is limited in the human population (Lunagariya et al., 2014). A similar response is also exhibited by ACE inhibitors, effective but have side effects. Therefore, bio-functional compounds from natural sources are investigated for the treatment of diabetes and hypertension.

Introduction to legumes sources and their health attributes

Dry beans are categorized as legumes they are considered to be a part of the diet for a long time and now they are being endorsed for their nutritional benefits (Geil and Anderson, 1994). The third largest family of angiosperm is Fabaceae/Leguminosae. It includes edible podded and nutritious dicotyledonous seeds such as beans, peas, lentils, peanuts, and others (Gepts et al., 2005; Messina, 1999; Çakir et al., 2019). Legumes can be classified into two: oilseeds (soybeans and peanuts) which are grown for both their protein and oil content; and grain legumes (common beans, lentils, lima beans, cowpeas, fava beans, chickpeas, and common peas) which are grown primarily as a protein source (Geil and Anderson, 1994). Legumes are the main source of protein and calories in Afro-Asian diets for both cultural and economic reasons

(Iqbal et al., 2006). Legumes are considered poor man's meat, as they are the cheapest sources of supplementary proteins in Indian diets. Legumes are also known to possess a significant amount of biomolecules like 20%−45% proteins, 60% complex carbohydrates with a low glycemic index, and 5%−37% dietary fibers and have a relatively low-fat content, minerals, and vitamins (Çakir et al., 2019; Maphosa and Jideani, 2017). The symbiotic association between legumes and nitrogen−fixing bacteria in the root nodules helps to raise significant level of protein content in the legumes. Henceforth legumes in terms of protein are preferred over cereals for human consumption (Kouris-Blazos and Belski, 2016; Keskin et al., 2022). High fiber and high resistant starch content present in the legumes can be a contributing factor in the prevention or treatment of diabetes as well as colon cancer (Mitchell et al., 2009). Additionally, they have higher calcium content than grains and are rich in iron, zinc, magnesium, and phosphorus. They also possess higher potassium levels, which are crucial for controlling hypertension. Trace amount of copper and manganese is also detected in the legumes (Singh and Pratap, 2016). It has been reported that germinated legumes are rich in vitamin C and there is an increase in the riboflavin as well as niacin contents upon germination (Tharanathan and Mahadevamma, 2003). Protein, vitamins, and minerals significantly increase throughout the sprouting process. Thus sprouting a simple bioprocessing technique can enhance legumes nutritional value and digestibility (Singh and Pratap, 2016). Legumes have several nonnutritive phytochemicals such as phytates, saponins, and oligosaccharides which may also have a role in cancer prevention. Furthermore, a number of bioactive compounds present in the legumes such as antioxidants, soluble, and insoluble dietary fiber are reported to lower the incidences of cardiovascular diseases (Bouchenak and Lamri-Senhadji, 2013), reduce blood cholesterol in people with hypercholesterolemia (Becerra-Tomás et al., 2019). It has been demonstrated that eating pulses reduce blood LDL cholesterol and triglycerides, which are the major risk factors for congenital heart disease, hypertension, diabetes, and obesity. Intake of legumes in diet is known to exert beneficial effect in weight management (Singh and Pratap, 2016). Therefore, globally the web of life style related diseases is getting complex and traditional approach wherein, legumes are included in daily diet is greatly acknowledged by human population to abscond the trap (Gharsallaoui et al., 2009; Boukid, 2021). Consumption of legumes provides certain essential amino acids and bioactive peptides. Functional food legume protein-derived bioactive components are reported to treat various disease conditions by interacting with enzymes associated with respective disease. Thus legumes can be involved in the development of food products with acceptable quality as they offer source for essential amino acids and bioactive peptides (López-Barrios et al., 2014; Ortiz-Martinez et al., 2014; Boye et al., 2010; Dhull et al., 2020).

Bioactive components in legumes

Legumes contain a huge depot of bioactive compounds. Some of the bioactive compounds include mucilage, essential oils, sterols, triterpenoids, saponins, carotenoids, alkaloids, flavonoids, phenolic acids, tannins, bitter principles, coumarins, micronutrients, and amino acids. All the bioactive compounds are known to exhibit varied beneficial effects to treat different metabolic conditions (Teoh and Das, 2018). Phytochemicals like saponins are known to exhibit anticancer activity, and also possess benefits in incidences of hyperlipidemia (Chang et al., 2006; Ellington et al., 2006). Kalogeropoulos et al. reported protective effect of legumes on type 2 diabetes mellitus due to the presence of high fiber content, low glycemic index, and a range of potentially bioactive nutrients including isoflavones and lignans (Kalogeropoulos et al., 2010). Decrease hunger and acute food intake aids in the management of obesity and obesity-related mortality and thus consumption of legumes can be benefitted as they are rich in low glycemic index carbohydrates, resistant starch, oligosaccharides, and fiber (Muzquiz et al., 2012; Rebello et al., 2014; Wanders et al., 2011). Furthermore, bean extracts containing digestive enzyme α-amylase inhibitors can also reduce starch digestion leading to a significant reduction in body weight, body mass index, and fat mass (Celleno et al., 2007). Therefore, legumes' bioactive compounds support and help in reducing the risk and progression of the metabolic disease condition.

Bioactive peptides from legumes

Bioactive peptides promote human health by reducing the risk of chronic diseases or boosting natural immune protection (Korhonen and Pihlanto, 2006). The protein/s are usually concentrated in the seeds (Kehinde and Sharma, 2020) and enzymatic hydrolysis during gastrointestinal digestion and microbial fermentation during food production can yield the bioactive peptides (Korhonen and Pihlanto, 2006; Acquah et al., 2022; Möller et al., 2008). Bioactive peptides are considered to promote diverse activities, including antimicrobial/antibacterial, antioxidant, antihypertensive, inhibitor of dipeptidyl peptidase IV, immunomodulatory, hypocholesterolemic, opiate-like, mineral binding, and antithrombotic (Singh and Vij, 2018; Sun et al., 2016; Singh and Vij, 2017; Singh et al., 2015; Sarbon et al., 2018; Singh et al., 2014; Paiva et al., 2016; Nongonierma and Fitzgerald, 2013; Malaguti et al., 2014). Fig. 18.1 depicts schematic representation

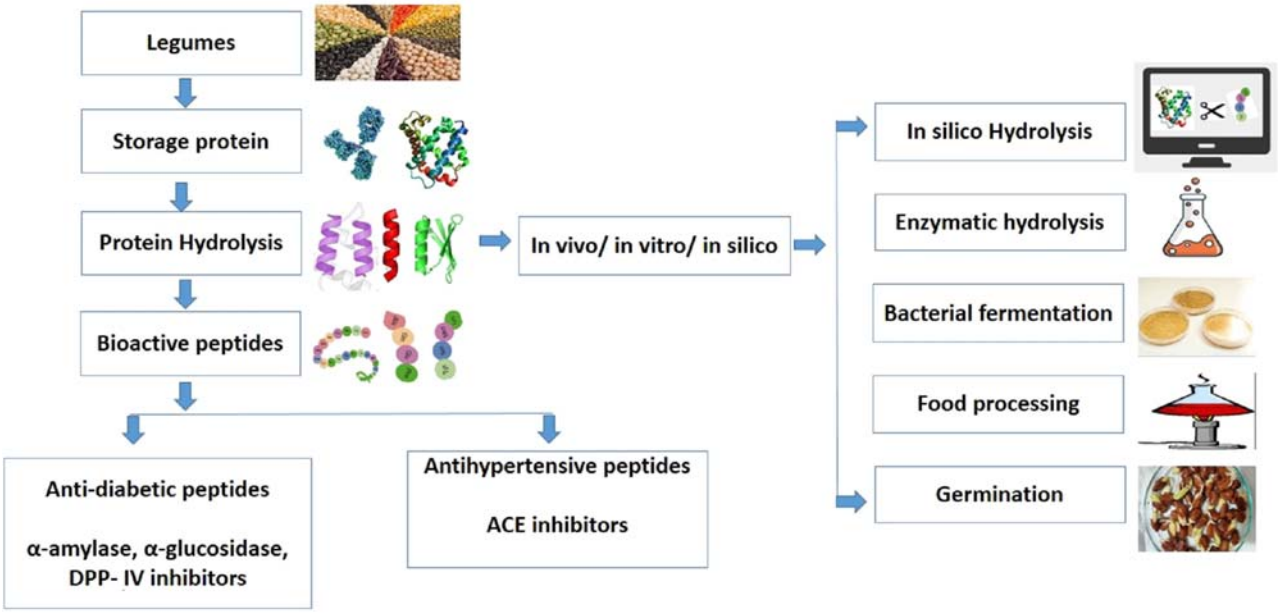

FIGURE 18.1 Schematic representation of different approaches for generation of bioactive peptides from legumes and their antihypertensive and antidiabetic property.

of different approach for generation of bioactive peptides from legumes and targeted enzymes for diabetes and hypertension. Studies on some legumes such as kidney beans (*Phaseolus vulgaris* L.) and peanuts (*Arachis hypogea* L.) showed significant cholesterol-lowering effects in vitro studies (Trinidad et al., 2010). Additionally, peptides extracted from hyacinth bean (*Lablab purpureus* L.), mungbean (*Vigna radiata*) and cowpea seed (*Vigna unguiculata*) exhibited remarkable antihypertensive activity (Castillo et al., 2017; De Leon et al., 2013; Distor et al., 2015; Viernes et al., 2012). Thus, bioactive peptides isolated from various legumes could be one of the approaches to treating diverse metabolic conditions.

Characteristics and properties of bioactive peptides

In order to deliver the benefits in regulating disease condition, bioactive peptides should exhibit certain traits. Following are some of the traits required by the bioactive peptides. During digestion, the bioactive peptides can be absorbed through the digestive system to enter the blood circulation and exert systemic effects (Saadi et al., 2015). Essentially, to reach their target organs bioactive peptides must be absorbed; small peptides (di- and tri-peptides) are more efficiently absorbed than larger ones, which are prone to hydrolysis by enterocyte peptidases (Bouglé and Bouhallab, 2017). These small bioactive peptides possess low molecular weight, high bioavailability, and flexible molecular structure that allow them to interact easily with different receptors in vitro and within the human body (Martínez-Sánchez et al., 2020).

Legumes-derived bioactive peptides in the treatment of hypertension and diabetes

The legume-derived bioactive peptide specifically are known to target ACE in case of hypertension and α-amylase, α-glucosidase, DPP- IV in diabetes treatment. The fundamental pathway playing a key role in hypertension is rennin angiotensin aldosterone pathway (RAAS), renin (secreted by the kidneys) converts angiotensinogen to the angiotensin I. Furthermore, with help of angiotensin-converting enzyme (ACE), angiotensin I is converted to angiotensin II, and an active form of the hormone. This hormone raises blood pressure by a direct effect on the blood vessels (Martin and Deussen, 2017; Bhatnagar et al., 2018). ACE is also known to degrade bradykinin allowing the release of aldosterone in the adrenal cortex (Puchalska et al., 2015; Teneva-Angelova et al., 2018). As a result, heighten activity of ACE contributes to the progression of hypertension (Teneva-Angelova et al., 2018; Jayathilake et al., 2018) and therefore, inhibiting ACE can be one of the targets in the treatment of hypertension (Bhatnagar et al., 2018; Piovesana et al., 2018). As stated earlier pharmacological interventions such as the consumption of benazepril, captopril, and enalapril is known to control ACE activity (Piovesana et al., 2018; Hanafi et al., 2018). However, the above

drugs are costly and often cause side effects such as dry cough, angioedema, allergic reactions, taste disturbances, and skin rashes (Hanafi et al., 2018; Orona-Tamayo et al., 2019). Thus a natural and safe way in treating hypertension could be introducing legumes in diet due to their immense nutritional and health benefits. To support this Table 18.1 represents different legumes based studies in controlling and treating hypertension. Considering diabetic conditions, α-amylase, α-glucosidase, DPP- IV inhibition activity from legume are studied for Anti-diabetic activity. α-amylase is involved in the dietary starch digestion and allows the release of ooligosaccharides. Further breakdown of oligosaccharides yields glucose which is rapidly absorbed by the body. Therefore, α-amylase inhibition is first-line strategy for managing diabetes (Gropper and Smith, 2012). The second enzyme targeted for the treatment of diabetes is α-glucosidase. It is a membrane-bound enzyme located in the epithelium of the small intestine. α-glucosidase helps in the breakdown of starch into oligo- and di-saccharides to yield glucose. The glucose so generated is then easily absorbed by the body. Therefore, inhibition of α-glucosidase is considered to be another effective strategy for lowering serum glucose levels (Shobana et al., 2009; Gropper and Smith, 2012). Besides, glucagon-like peptide-1 (GLP-I), glucose-dependent insulinotropic-polypeptide (GIP), and gastric inhibitory polypeptide all three gut hormones play a significant role in reduction of postprandial and fasting glucose levels in type 2 diabetes mellitus patients. For β-cell survival, increasing β-cell mass and insulin production all above-mentioned hormones are significant. Besides the hormones also regulate glucose homeostasis by enhancing insulin levels in the blood (Ng and Kong, 2007; Paliwal et al., 2015; Lammi et al., 2016). Another enzyme DPP-IV present in the blood plasma regularly breakdown GLP-1 and GIP and thus this leads to inactivation and reduction of GLP-1 and GIP half-life (i.e., from 5 to 2 min). Thus, finally inhibiting the insulin secretion from the β-cells. In this regard, DPP-IV enzyme inhibitors are known to exert a significant effect by improving the half-life of incretin hormones. This further elevates the half-life of incretin hormones. Therefore, inhibiting the cleavage of gut incretins GLP-1 and GIP can be a mainstay in managing type 2 diabetes mellitus. Studies supports legumes-derived bioactive peptides in inhibiting α-amylase, α-glucosidase, and DPP- IV and thus represented in Table 18.2. Thus varied legume based research study claims beneficial activity exhibited by legumes in the treatment of hypertension and diabetes. The mode of study can be in vivo, in vitro or in silico, where in the first two system required huge finance and could be timeconsuming. Therefore, the present work instigates the role and progress of in silico tools in similar line of work.

In silico approach involved characterizing bioactive peptides using virtual screening methods

In silico or bioinformatics is an emerging area using computer technology and helps to carry out simulation studies. Specifically, to screen the promising candidates and their interaction with the particular enzyme in the disease. Studies on food-derived bioactive peptides and their protein sources are well recognized due to the innovative and impressive databases. The present database on peptides gives a glimpse and better understanding of potential bioactive peptide sequences (Keska and Stadnik, 2017; Nongonierma et al., 2014; Agyei et al., 2019; Dziuba and Iwaniak, 2006; Kalmykova et al., 2018). One of the widely used databases is BIOPEP-UWM previously known as BIOPEP, which is extensively used in food and nutrition science as a source of information about peptides being the focus of interest as putative components of functional foods involved in the prevention of chronic diseases (Agyei et al., 2018; Tu et al., 2018; Piovesana et al., 2018). A researcher can simply search for the occurrence of bioactive peptide fragments in a protein sequence, and to predict the bioactive profile of the peptides generated from the in silico simulated protein digestion with proteases (Minkiewicz et al., 2019). Another database specific to antihypertensive peptides is AHTPDB. It is a manually curated database of experimentally validated Antihypertensive peptides and at present contains 6000 entries of about 1700 unique peptides. Each entry provides comprehensive information about a peptide that includes its sequence, inhibitory concentration (IC_{50}), log value of inhibitory concentration (pIC_{50}), and toxicity (Kumar et al., 2015). The data of the peptide sequence so obtained from LC-MS/MS can be further evaluated using any of the above-mentioned databases. Peptides exhibiting similarity in function as per the database can be further synthesized and validated using in vivo approach. This favors in development of novel foods and ingredients (Korhonen and Pihlanto, 2006). Furthermore, computer-based programs can predict the peptides released on proteolysis by using different enzymes and also help in a quantitative structure−activity relationship (QSAR) using software programs (Dziuba et al., 2003; Dziuba et al., 2004; Vermeirssen et al., 2004; Pripp, 2005; Pripp et al., 2005). Structure and function prediction between the peptide and substrate can also be explored using multivariate techniques (Pripp, 2007). On comparing in silico with in vitro and in vivo techniques, in silico approach has to add on benefits. It is known to reduce time, the cost of purification is scaled down and identification of the potent compounds is swift. Furthermore, in silico approach also reflects on the mechanism of action of the compounds, this in turn can be a major advancement as only precise molecules (exhibiting virtual activity) can be further considered for validation studies using in vitro/in vivo systems (Arcanjo et al., 2017).

TABLE 18.1 Legume derived bioactive peptides as ACE inhibitors.

Sources	Bioactive peptides generation methods	Bioactive peptide sequences	IC 50 value mL^{-1}	Study	References
Phaseolus vulgaris L. (Kidney bean)	Fermented and enzymatic hydrolysis (α-amylase, pepsin)	FVVAEQAGNEEGFE	0.28 ± 0.03 mg	In vitro	Jakubczyk et al. (2017)
Vigna radiate (Mung Bean)	Enzymatic hydration (alcalase)	KDYRL, VTPALR, KLPAGTLF	0.64 mg	In vitro	Li et al. (2006)
Glycine max (Soybean)	Enzymatic hydrolysis (trypsin, chymotrypsin, pepsin, pancreatin)	VLIVP, LAIPVNKP, LPHF, NVVGPLV, YLAGNQ, IPPGVPYWT, DQTPRVF, ASYDTKF, DTKF, PNNKPFQ, RPSYT	180 μgm	In vitro	Mallikarjun Gouda et al. (2006), Kodera and Nio (2006)
Glycine max (Soybean)	Enzymatic hydrolysis (thermolysin first, followed by pepsin and trypsin)	IVF, LLF, LNF, LSW, LEF	less than 10 μM	In vitro	Gu and Wu (2013)
Glycine max (Soybean)	Germinated followed by enzymatic hydrolysis (pepsin and pancreatin)	NALKPDNRIESEGG, SSPDIYNPQAGSVT, NALKPDNRIESEGG, RQNIGQNSSPDIYNPQAG, NALKPDNRIESEGG, VVAEQAGEQGFE, HKNKNPF	1.49 ± 0.14 mg	In vitro and in silico	González-Montoya et al. (2018)
Glycine max (soybean)	Fermented	AW, GW, AY,AI, VG	10 μM, 30 μM, 48 μM, 690 μM, 1100 μM respectively	In vivo	Nakahara et al. (2011)
Glycine max (Soybean)	Enzymatic hydrolysis (pepsin)	IA, YLAlGNG, FFL, IYLL	**153** μM, **14** μM, **37** μM, **42** μM respectively	In vivo and in vitro	Chen et al. (2002)
Glycine max (Soybean)	Synthetic peptide	AF, IF	165 μM, 65.8 μM respectively	In vitro	Zhu et al. (2008)
Glycine max (Soybean)	Steamed at 120 8C followed by fermentation	LVQGS	43.7 μM	In vitro	Rho et al. (2009)
Glycine max (Soybean)	Fermented	LAIPVNKP, LPHF, SPYP, WL	70 μM, 670 μM, 850 μM, 65 μM respectively	In vitro	Kuba et al. (2005)
Pisum sativum (Pea Bean)	Enzymatic hydrolysis (alcalase)	IR, KF, EF	2.25 ± 0.31 mM, 7.23 ± 0.69 mM, 2.98 ± 1.24 mM respectively	In vitro	Li and Aluko (2010)
Cajanus cajan L. (Pigeon pea)	Fermented	VVSLSIPR	–	In silico	Nawaz et al. (2017)

TABLE 18.2 Legume-derived bioactive peptides as α-amylase, α-glucosidase, and DPP- IV inhibitors.

Sources	Bioactive peptides generation methods	Bioactive peptide sequences	IC 50 value mL^{-1}	Study	Activity	References
Phaseolus vulgaris L. (Pinto bean)	Enzymatic hydrolysis (protamax)	PBP1: PPHMLP PBP3: PLPWGAGF PBP6: PPHMGGP PBP7: PLPLHMLP PBP9: LSSLEMGSLGALFVCM	6.08 mM 6.64 mM 6.14 mM 5.92 mM 0.31 mM respectively	In vitro	α-amylase inhibition	Ngoh and Gan (2016); Ngoh et al. (2017), Patil et al. (2020)
Vigna Unguiculata (Cowpea)	**(a)** Enzymatic hydrolysis (alcalase) **(b)** Germinated Followed by enzymatic digestion with pepsin and pancreatin	Less than 10 kDa	0.58 mg SP –	In vitro	DPP-IV inhibition	de Souza Rocha et al., (2014)
Phaseolus vulgaris L. (Kidney bean)	Fermented	INEGSLLLPH, FVVAEQAGNEEGFE	1.94 ± 0.16 mg	In vitro	α-amylase inhibition	Jakubczyk et al. (2017)
Phaseolus vulgaris (Black bean)	Enzymatic hydrolysis (alcalase)	AKSPLF, ATNPLF, FEELN, LSVSVL	–	In vitro, In vivo, In silico	blocking glucose transporters GLUT2 and SGLT1	Mojica et al. (2017a)
Phaseolus vulgaris L.(Common Bean)	Enzymatic hydrolysis (porcine and pancreatin)	KKSSG, KTYGL, GGGLHK and CPGNK	0.09–0.99 mg 36.3 to 50.1 mg^{-1}	In vitro and in silico	DPP-IV and α - glucosidase inhibition	Mojica et al. (2017b)
Soy and Lupin	Synthetic peptide	IAVPTGVA, LTFPGSAED	106 μM 228 μM respectively	In vitro and in silico	DPP-IV inhibition	Lammi et al. (2016)
Glycine max (Soybean)	Synthetic Soymorphin-5 peptide	YPFVV	–	In vivo	Hypoglycemic	Yamada et al. (2012)
Glycine max (Soybean)	Synthetic peptide	IAVPTGVA	223.2 μM	in vitro	DPP-IV inhibition	Lammi et al. (2018)
Glycine max (Soybean)	Germinated followed by enzymatic hydrolysis(pepsin and pancreatin)	NALKPDNRIESEGG, SSPDIYNP-QAGSVT, NALKPDNRIESEGG, RQNIGQNSSPDIYNPQAG, NALKPDNRIESEGG, VVAEQA-GEQGFE, HKNKNPF	~0.7 mg	In vitro and in silico	DPP-IV inhibition	González-Montoya et al. (2018)
Soy protein powder	Enzymatic hydrolysis by protease (papain and trypsin)	LLPLPVLK, SWLRL, WLRL	237.43 ± 0.52, 182.05 ± 0.74, and 162.29 ± 0.74 μmol/L, respectively	In vitro	α-glucosidase	Wang et al. (2019)

In silico tools for proteolysis, characterization, and bioactivity of peptide/s sequence

In silico tools can simulate the GI tract by cleaving the food protein into peptide sequences as done by the specific digestive enzymes. The peptide sequences so generated could have health benefits. To analyze bioactive peptides generated from the legume protein source, the best adopted in silico tool is the ExPASy Peptide cutter. Using tools is simple and helps to determine potential cleavage sites in a given protein sequence by using proteases or specific cleaving chemicals (Gasteiger et al., 2005). These tools allow for the theoretical prediction of the potential of various substrates with known protein sequences to generate bioactive peptides, using enzymes with known cleavage specificities. Using a Peptide cutter, the cleavage sites within the Bambara proteins using thermolysin were predicted (Mune et al., 2018). The thermolysin-derived peptides were searched for their ACE and DPP-IV inhibitory activity. Pea protein underwent proteolysis by pepsin, trypsin, and a-chymotrypsin using the Peptide Cutter program resulting in the generation of bioactive peptides (Pripp et al., 2005). Thus peptide cutter program is easy-to-use software and helps to generate promising results which can be further evaluated using other in silico tools. ProtParam is another tool and it is known to characterize the physical and chemical parameters for the stored protein reflected in Swiss-Prot, TrEMBL, or for a protein sequence entered by the researcher. A good deal of information such as molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index, and grand average hydropathicity (GRAVY) about the protein or peptide sequence can be achieved using the tool ProtParam. In this program, if the peptide instability index is smaller than 40, which is predicted as stable; when the instability index value is above 40, it is predicted as may be unstable. As per the study on pigeon pea seeds, data obtained from the 40 pigeon pea proteins/domains reported physical and chemical features of the protein exhibited similarity with the SDS profiling of the pigeon pea seeds (Boachie et al., 2019). Thus results obtained from the ProtParam hold a good homology with the data obtained by the in vitro analysis. Additionally, if the data exhibits a higher value, it is interpreted to possess higher hydrophobicity also the factors leading to higher hydrophobicity could be the presence of amino acids such as Met and Trp. The presence of hydrophobic amino acids could act as a hydrogen donor or acceptor and thus participate in the antioxidant property of the peptide (Ji et al., 2019; Jakubczyk et al., 2020; Zhang et al., 2020). Another software routinely used to understand the bioactivity of the unknown peptide sequence or protein is PeptideRanker, the predictions done by the software are based purely on structural chemical features (Mooney et al., 2012). The interpretation for the PeptideRanker score above 0.5 is the peptide can show the bioactive property in the in vitro and in vivo systems (Ding et al., 2020; Shang et al., 2018). Ngoh and Gan (2018) used the database PeptideRanker to select the following five peptide fragments from the common bean with scores above 0.8: PPHMLP, LSSLEMGSL-GALFVCM, PPHMGGP, PLPLHMLP, and PLPWGAGF. The authors concluded that these fragments demonstrated ACE inhibitory activity in in vitro assays with IC50 values of 1.52lM, 1.84lM, 11.04 lM, 27.32lM, and 31.88lM, respectively (Ngoh and Gan, 2018). Studies on a pool of pinto beans peptides, subjected to Peptide Ranker software helped to identify a few novel peptides exhibiting DPP-IV-inhibitory activity (Martini et al., 2021). Therefore, for preliminary screening of the peptides to understand their potency, above-mentioned tools could be preferred to filter out the peptide of our interest.

Evaluation of bioactive peptides using toxicity and solubility prediction programs

The bioactive peptides with potential traits need to undergo further analysis to address some genuine concerns for the development of peptide-based nutraceuticals. The major issue is the bioactive peptide should be devoid of any toxicity and should be soluble too. The searched bioactive peptide if shows toxicity cannot be considered for human consumption. Similarly, solubility is also an important characteristic of the peptides, which will influence the absorption, distribution, and elimination of the peptides in the human body (Balakin et al., 2006). Thus a program named ToxinPred simple, and efficient tool that helps in the prediction of toxicity of the given peptide sequence (Gupta et al., 2013). ToxinPred tool allows the identification of highly toxic or nontoxic peptides from a large number of peptides submitted. Toxicity is one of the fundamental factors that should be taken into consideration for the various products of foods and drugs. The solubility prediction of a given peptide sequence can be done using a computational tool PepCalc-Peptide property calculator by Innovagen. Simply entering the single letter code peptide sequence in the sequence bar from the N−C terminus and on clicking enter key calculates the physiochemical properties of the peptide including several residues, molecular weight, extinction coefficient, Iso-electric point, net charge at pH7, and estimated solubility. The tool additionally provides notes on solubility for the given peptide sequence. In silico study on soybean protein on proteolysis resulted around 12 tripeptide sequences HDW, RPR, EGW, DMG, LPR, CIR, MDL, DTW, MDY, DVF, AGR, and RRR being nontoxic and had good solubility. Solubility is a key parameter for tri-peptide to perform physiological functions (Zhao et al., 2019).

ADME prediction of bioactive peptides

Absorption, distribution, metabolism, and excretion (ADME) properties are critical factors to identify the bioactivity of peptides. These predictions can be made using admetSAR server (Cheng et al., 2012). The admetSAR operates with codes of peptides that should be translated into simplified molecular input line entry specifications (SMILES) by novoprolabs. ADMET properties prediction plays a vital role in identifying novel peptides with enhanced pharmacokinetic profiles (Kauthale et al., 2017). In silico ADMET properties includes human intestinal absorption (HIA), and blood−brain barrier (BBB) penetration. These are the two major factors of well-absorbed peptides. Human intestinal absorption (HIA) is an important absorption factor for the identification of tri-peptides with bioactivities. Movement across the intestinal epithelial barrier affects the exertion of bioavailability (The et al., 2011). If the HIA% of the tripeptide is higher than 30%, it represents the degree of food absorption done by the human intestinal cells above the cut-off value and hence can be labeled as HIA+. Those peptide sequences exhibiting lower than 30% values are poorly absorbed by the human intestinal cells and hence can be labeled as HIA- (Shen et al., 2010). Thus the prediction of the HIA parameter of peptides was well-absorbed compounds of importance for the identification of potential peptides candidate (Kumar et al., 2017). The BBB penetration is to avoid central nervous system (CNS) side effects. It is used to understand the compounds possibility to get through CNS and therefore is considered to be an important pharmacokinetic parameter (Murugavel et al., 2017). Additionally, other parameters include cytochrome P450 (CYP 450) enzymes CYP2D6 and CYP3A4 inhibition prediction is important in the metabolism, The CYP inhibitors would increase the concentration of the drug due to loss of enzyme function can be computed by admetSAR. Additionally, the Caco-2 permeability is the key part of the permeability of a compound. If a tri-peptide with the Caco-2 permeability value is more than or equal to 8×10^{-6} cm/s, it is labeled as $Caco^{2+}$, otherwise, it is labeled as $Caco^{2-}$ (The et al., 2011). In silico assay with ADMET was performed with soybean tripeptides, highlighting that seven peptides had a good probability of absorption in HIA and four in BBB indicating the searched peptides with good potency (Cheng et al., 2012).

Molecular docking screening of bioactive peptides

In biological processes, peptide−protein interactions participate to exhibit signal transduction, immune responses, and cellular regulation. Around 40% of the protein-protein interactions are due to the short peptides (Petsalaki and Russell., 2008; London et al., 2013). The promising peptides need to be investigated for their interaction with the target molecule using a molecular docking system. The existing database with an algorithm is used to predict how likely the peptide is to be bioactive (Mooney et al., 2012; Trabuco et al., 2012). Molecular docking is a computational approach involving the docking of small molecules of interest into the structures of macromolecular targets or enzymes. Using a docking algorithm, the identification of the optimal binding model can be visualized between the ligand or lead candidate and the active site of the target enzyme. The docking score so obtained reflects the potential complementary interaction between the ligand-enzyme and further helps in hit identification and lead optimization (Kitchen et al., 2004). Furthermore, ligand-protein docking can then be explored for virtual screening. The libraries of the small molecules are screened to identify the most relevant molecular structure likely to bind the enzyme. Thus, the docking score plays a significant role in the identification of structures with prominent binding and physiological activity. The molecular docking studies however need to be further experimentally validated. For computational studies, binding sites or cavities exhibiting a variety of shape and size present on the protein surface plays a crucial role. As the protein-ligand interactions are mainly dependent on the cavity present in the protein. Currently, two proteins−peptide docking algorithms i.e., local and global peptide docking are available. If information about the binding site is known or the binding sites are predicted by certain algorithms like PepSite or PEP-SiteFinder are also well known as protein-peptide binding modes are obtained through a high-resolution docking refinement within the binding site (Trabuco et al., 2012; Saladin et al., 2014; Yan and Zou, 2015). Therefore, algorithms for local peptide-docking are only performed for docking at the known binding site. While if the binding site is unknown, global peptide docking is required to blindly search for putative peptide binding conformations around the whole protein. Due to the relatively larger search space, global peptide docking is much more challenging than local peptide docking. Recently, several global peptide docking algorithms such as AnchorDock (Ben-Shimon and Niv, 2015), CABSdock (Kurcinski et al., 2015), pepATTRACT (Schindler et al., 2015), and MDockPep (Yan et al., 2016) have been developed for the blind prediction of protein-peptide complexes, among which CABS-dock is available as a web server and pepATRRACT has a web version for its rigid docking protocol (de Vries et al., 2017). CABS-dock is one of the few methods that does not require knowledge of the binding site or information about the peptide conformation (Blaszczyk

et al., 2016). This method allows simultaneous search for interaction "hot spots" or "hot segments" or binding areas that dominate the interaction to identify potential peptide-protein interface binding sites. However, an experimental assessment is required to validate the specific bioactivity potential of the peptides. In the case of diabetes studies, sitagliptin can be used as a control in Diabetes studies. The principal target proteins in diabetes pathogenesis need to be identified, followed by the 3D structure of the corresponding protein can be obtained from the PDB database and saved in PDB format. AutoDock tool helps to set the parameters and further calculations Docking Server and for designing BIOVIA Discovery Studio can be explored. Studies on chickpea simulating digestive enzymes and bromelain resulted in the generation of bioactive peptides. The two peptide sequences PHPATSGGGL and YVDGSGTPLT on molecular docking showed a good affinity for the DPP IV catalytic site compared to the bromelain hydrolysates (Bikadi and Hazai, 2009; Chandrasekaran et al., 2020). In the case of hypertension studies, docking can be carried out between ACE and the ligand/peptide of interest. The 3D structure of ACE can be obtained from protein data bank and can be selected as a receptor (Lafarga et al., 2014). The energy can be minimized by Discovery Studio. Water molecules can be removed and the zinc ion needs to be retained in the ACE model (Zhao et al., 2019). The CHARMM-based program (CDOCKER) of Discovery studio 2017 R2 Client software can be used for docking. The binding site with a specific radius and coordinates should be considered for the study. Discovery Studio helps to distinguish salt bridge, conventional hydrogen bond, carbon-hydrogen bond, pi-alkyl, van de Waals, alkyl, and metal-acceptor bond at the ACE active sites (Guo et al., 2017). As per the study by Zhao et al. Docking simulation suggested that one of the tripeptides DMG established two sites with an S1 pocket (Ala354 and Glu384) and all the five sites with an S2 pocket (Gln281, His353, Lys511, His513, and Tyr520) of ACE. Therefore, tripeptide DMG from soybean, as an ACE inhibitor, could be included in functional food (Zhao et al., 2019). Another study of major storage protein globulin from *Arachis hypogea* leads to a conclusion that a tripeptide IKP acts similar to Lisinopril in inhibiting ACE (Jimsheena and Gowda, 2010).

Conclusion

All over the world legume consumption is routine as well as they represent a cheap source of protein. As stated by several studies, legumes can be treated processed and can generate bioactive peptides. Globally diabetes and hypertension incidences are also raising and the safest way to overcome the symptoms associated with the disease condition is to have safe and natural therapies to treat the condition. Adopting varied in vitro or in vivo bioprocessing techniques to generate bioactive peptides can be laborious. Thus, in silico approach can be a modest way to get the results. The in silico tools, and database could provide enormous data on the legume of interest additionally docking studies can be promising for considering the peptides of interest. Once the positive outcomes from the in silico study are obtained, the promising peptides can be further validated by in vitro and in vivo studies.

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Chapter 19

Deciphering the scope of in silico screening of novel natural lead molecules against putative molecular targets of multidrug-resistant bacterial pathogens

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Introduction

The past few decades have seen an increase in the incidences of microbial infections, primarily due to the constant utilization of antibiotics, misuse, overuse, or wrongly prescribed drug use. This has in turn triggered the resistance of bacteria against a variety of drugs. Bacterial drug resistance or antibiotic resistance is now a major concern globally. According to the Center of Disease Control (CDC), more than 2.8 million infections involving multidrug-resistant bacteria occur annually in the US and cause around 35,000 plus deaths (https://www.cdc.gov/drugresistance/about.html). Across the world, AMR kills at least 1.27 million people and about five million deaths are AMR linked, as per the 2019 statistics (https://www.cdc.gov/drugresistance/about.html). Apart from persistent antibiotic use, self-diagnosis, self-medication, and exposure to infections in hospitals are also related causes of bacterial drug resistance. Typically, drug resistance can be categorized into three multidrug resistanace (MDR), extensively drug resistant (XDR), and pandrug-resistant bacteria (PDR). MDR is the acquired nonsusceptibility to one antimicrobial agent in three or more drug classes. Some examples include Vancomycin-resistant enterococci (VRE), Methicillin-resistant *Staphylococcus aureus* (MRSA), and beta-lactam resistant *Klebsiella pneumoniae* (Nikaido, 2009). XDR is when the bacteria is nonsusceptible to at least one agent in all but two or lesser categories of anti-icrobials. Similarly, PDR is the nonsusceptibility toward all antimicrobial agents in all categories (Magiorakos et al., 2012). Studies have previously stated that bacteria such as *Pseudomonas aeruginosa, Acinetobacter baumanii, Staphylococcus maltophilia, Bacillus cepacian,* and *Klebsiella pnemoniae* were found to be either XDR or PDR (Abat et al., 2018).

Recently, however, another category has been added to this list, called usual drug resistance (UDR). This pertains to the bacterial isolates that are not completely susceptible to wild-type strains, yet can be easily treated with general/standardized therapies (Rex et al., 2017). UDRs are beneficial when a novel antimicrobial agent is tried against existing standard agents (Rex et al., 2017). Moreover, Kadri et al. (2018), proposed the idea of difficult-to-treat resistance (DTR). The primary reason for this proposal is that MDR, XDR, and PDRs were not easily distinguishable based on the strengths and weaknesses of individual antimicrobial agents/antibiotics, i.e., those with lesser toxicity and greater efficacy are accounted for similarly to those with greater toxicity and lesser efficacy. To address this issue, the researchers re-defined DTRs as resistance to all first-line agents with lower levels of toxicity, such as carbapenems, beta-lactams, and fluoroquinolones. Along similar lines, another group of dangerous pathogens has now recently developed resistance and has fallen under the MDR category. ESKAPE pathogens, a category of clinically significant bacterial species, consist of *Enterococcus faecium*,

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Staphylococcus aureus, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* sp. Certain strains of a Gram-negative pathogen called *Serratia marcescens*, which produces extendedspectrum carbapenemase and beta-lactamase have now been identified, which poses a huge challenge therapeutically (Mulani et al., 2019).

Some studies stated that acceleration of immunocompromised conditions such as diabetes, HIV infections, transplantation of organs, or patients with severe burns, tend to be easier targets for MDR attacks via secondary infections, contributing directly to the further spread of MDR and related nosocomial infections (Tanwar et al., 2014). Therefore, the identification and development of novel alternative therapies that are effective against such drug-resistant pathogens are urgently needed.

Recent aspects of the evolution of antimicrobial resistances

Antimicrobial resistance has now been established as a problem that requires utmost attention. The evolution of drug resistance seems to also be attributed to the various synthetic drugs on the market. For instance, *Mycobacterium tuberculosis* is considered a high-resistance pathogen, with more than 10 million people infected with TB (tuberculosis) every year and 1.5 million of these deaths are due to the same (Dhameliya et al., 2022). Isoniazid came out in 1950 as the first-line antitubercular agent. However, the protein target InhA mutated at the S94A site, rendering the drug ineffective. In 1945, pyrazinamide came into the picture to target the RpsA protein of MTB. However, this protein underwent T5A and T210A mutations, causing the drug to be ineffective. In 1957, clofazimine was introduced but was discontinued due to continued side effects (Cholo et al., 2012). Ethambutol was also a first-line antitubercular developed in 1961, but the MTB rpoB protein mutated at M306P and M306V, rendering it ineffective. This evolution continued with the introduction of rifampicin in 1965, but the organism mutated again at the same sites. This prevalence of multiple drug resistance of *M. tuberculosis* persisted for more than 3 decades until the second line of tuberculars started coming in. Pretomanid in 2000 and bedaquiline in 2012 were developed. However, the mode of action is still unclear and unidentified (Fig. 19.1) (Behr, 2013). As observed, this evolution of drug resistance in MTB could be attributed to its ever-adaptable genome toward synthetic drugs.

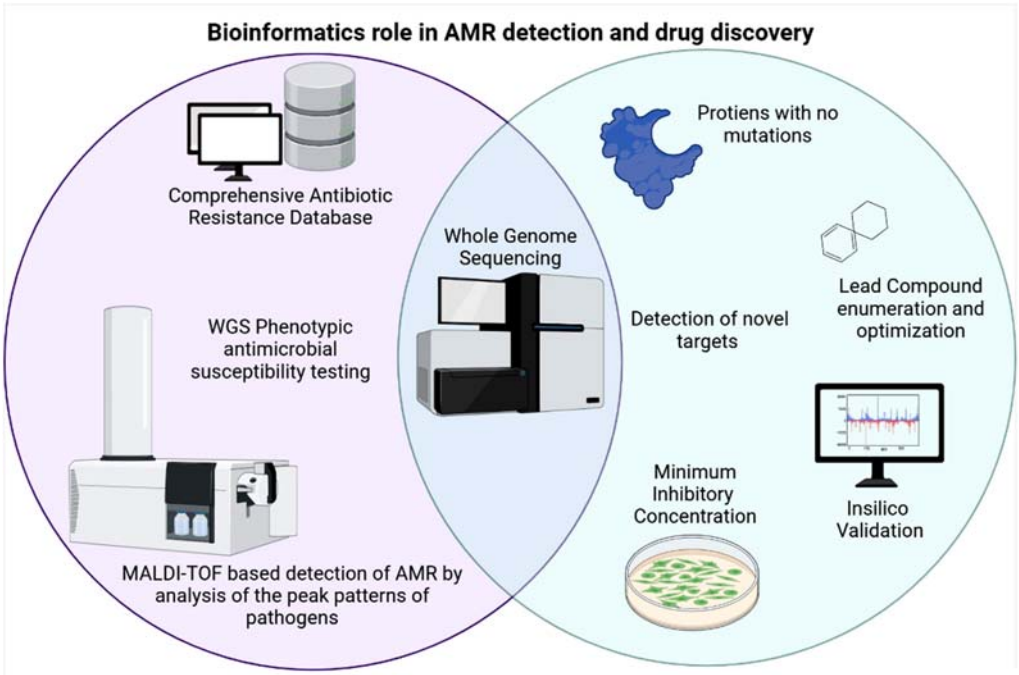

FIGURE 19.1 Role of bioinformatics in the detection of AMR and drug discovery. Whole genome sequencing plays a major role in comprehension of important targets and optimization of lead compounds. Validation using bioinformatics tools help in the identification of the best target-lead complex, that can be taken forward for in-vitro studies such as the identification of the MIC. A comprehensive antibiotic resistance database can either be built or can be used from amongst the existing to gain insights into the various bacterial resistances. This is followed by the whole genome sequencing (WGS) phenotypic antimicrobial susceptibility testing and MALDI-TOF-based detection of AMR by the analysis of the peak patterns of the different pathogens.

Likewise, given the nature of gonorrhea as a disease and the high rate of utilization of antibacterial followed by treatment failures, *Neisseria gonorrheae* has also been gradually evolving, with severe complications. Properly comprehending this evolution and the mechanisms of this evolution may help in the design of newer and better antimicrobials to circumvent this problem (Unemo and Shafer, 2014). Other bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* have also been evolving over decades due to adaptive mutations in the intergenic regions of their genomes (Dunn et a., 2019). This evolution in bacteria makes it extremely challenging to treat, leading to persistent infections.

Conventional antibacterial and their shortfalls

A decrease in the development of new antibacterial over the past several decades has augmented the risk of prevalence and evolution of multidrug-resistant bacteria. Conventionally, there has always been a higher probability of drug success with molecules that already belong to established classes of antibiotics (Silver, 2011). Between the time span of 1981−2005, antibiotic classes such as penicillins, cephalosporins, macrolides, and quinolones accounted for about 73% of all new antibiotics (Newman and Cragg, 2007). However, other issues such as the lack of diversity of the targets of known antibacterials contributed to the reduced development of new ones. Generally, conventional antibiotics tend to work by inhibiting the RNA, DNA, synthesis of the cell wall, and proteins. However, there are fewer than 25 targets that account for their activity well. Targeting cell wall synthesis is the major function of most antibiotics (Singh and Barrett, 2006). Additionally, previous comparative comprehension of bacterial whole genomes has shown that approximately 300 highly conserved proteins could potentially act as novel drug targets for developing antibiotics (Akerley et al., 2002; Forsyth et al., 2002). These studies point us toward the identification of new antibacterial, however, the activity is not sufficient for several of these to be designed without further alterations.

New antibiotics that adhere to the established classes of antibiotics are generally subjected to resistance that has been observed previously in members of the same class. This shortfall of conventional antibiotics has opened avenues to new explorations into alternative treatment approaches. Another notable point is that several conventional antibiotics were developed to treat Gram-positive bacteria inclusive of *Mycobacterium tuberculosis.* These had little to no effect on the Gram-negative resistant bacteria, leading to a huge gap in antibiotic innovation and treatment approaches (Fair and Tor, 2014). This again demands the seeking of alternative approaches to combat drug resistance.

Some new antibiotics introduced chronologically for human usage include dalfopristin in 1999, oxazolidinone linezolid in 2000, daptomycin in 2003, pleuromutilin in 2007, fidaxomicin in 2011, and, bedaquiline in 2012. Among these, bedaquiline and linezolid are completely synthetic while the rest were natural (Jabes, 2011). This demonstrates the shift toward the development of natural as antibacterial than synthetic ones, owing to the fact of the above-mentioned disadvantages of conventional synthetically made antibiotics. Moreover, other drawbacks of conventional antibiotics include the narrow spectrum activity of antibiotics such as bedaquiline and fidaxomicin and the greater toxicity of antibiotics such as polymyxins (Fischbach and Walsh, 2009). These shortfalls suggest and point toward the need for using computational biological resources for the identification of naturals as alternatives for combating MDR.

Need for an alternative strategy for drug screening

Virtual screening of specific libraries of chemical compounds against biological targets for the identification/design of novel drugs has evidenced its dependence on the approach of structure-based drug design. Target-dependent screening, linked to molecular docking studies, allows researchers to analyze and screen through a huge dataset of all probable small molecules/drugs, that can act as ligands to the bacterial targets to interrupt their metabolic signaling pathways. Virtual screening accounts for lower cost and expenditure, with the added value of lesser time consumption to arrive at plausible, potent lead molecules (Breda et al., 2008). The augmented availability of several computational resources for virtually screening high-throughput data enables research laboratories and pharmaceutical industries to contribute vastly to early stage drug discovery. The required databases and tools allow for an understanding of the following:

- Interaction at a molecular level between the target and the natural small molecule
- The specificity, affinity, and strength of binding
- The robustness and stability of the bound complexes
- Pharmacokinetic properties of the compounds
- Drug likeliness of the compounds
- Investigating drug toxicity toward their biological targets
- Identification of off-targets of the drugs
- Pathways that get hindered/interrupted via this binding

These attributes can be easily identifiable through several computational biological tools, algorithms, and perspectives in a shorter period, enabling simpler in vitro and in vivo studies. Since there is currently an urgent need to counter the menace of AMR, in silico drug screening is a promising way to investigate alternative approaches to combat multidrug resistance. Bioinformatic-based approaches offer not only information on the above-mentioned attributes, but also the complex relationships among the drugs, targets, and diseases associated (Liu et al., 2013). These advancements prove that with these developments in technology, counteracting MDR resistance will be much simpler. Moreover, most of the last-line resort antibacterial agents including families of colistin, carbapenems, and high-generation cephalosporins have become resistant to several Gram-negative bacterial pathogens. This situation demands the need for screening novel therapeutic agents that can be used as alternatives to the present generation of antibacterial agents. Computational medicinal chemistry integrated with several in silico tools is one of the most promising approaches to screening novel lead molecules.

Scope of in silico biology and computational resources

The utilization of in silico tools and computational resources serve as novel approaches to combating the problem of AMR. The scope of using these resources stretches far and wide in the discovery of new drugs for fighting resistance. The following approaches and resources stress the same.

Identification of targets

A specific gene or a protein that is primarily responsible for triggering the bacterial infection is known as a target. There can be single or multiple targets. Identification of this target is the primary step in finding alternative methods of reducing incidences of AMR. This begins with the comprehension of the function of the target, and its metabolic and signaling pathways, which are all possible via in silico tools and databases. For this, bioinformatics, chemo-informatics, homology-based approaches, ligand, and structure-based approaches, data mining, high-throughput screening, microarray technologies, text mining, and pattern recognition techniques are beneficial (Rao and Srinivas, 2011).

Validation of targets

The selected targets can be validated using various approaches such as mapping the protein's pathways, understanding interactions between the proteins, mapping the genetic network, mapping the disease loci, and the predictions of subcellular localizations (Bleicher et al., 2003).

Identification of HIT and lead molecules

A thorough high-throughput screening can be used alongside various cellular and biochemical assays, screening of natural molecules from databases, designing of drugs based on the required criteria (structure-based drug design), and using peptidomimetics (Rao and Srinivas, 2011). The list of lead molecules obtained can be taken forward for further studies by optimization.

Optimization of the identified lead molecule

The refinement of the chemical structure is called lead optimization, which is performed to enhance the drug-like features of the molecule. The selected drug candidates undergo attribute checks such as ADME, followed by docking. The optimized molecules will then be tested for their stabilities before in vitro preclinical trials (Olejniczak et al., 2001).

Computer aided drug design (CADD)

This is a specialized area of bioinformatics where computational resources are applied to simulate the interactions between the drugs and the receptors. Some of these key areas include:

Homology modeling

this includes the construction of a comparative model of the target proteins for which structures are not already available, by using the amino acid sequence of the protein and an existing experimental 3D structure of a template (a related homologous protein, whose structure is available) (Rao and Srinivas, 2011).

Interaction analysis

This is typically performed via docking of a small molecule with a target. Protein-protein docking also exists that shows the binding affinities between two proteins. The amino acids that come in contact during binding can be identified, which plays a major role in understanding the capabilities of the drug molecule.

High-throughput screening

in virtual high-throughput screening (vHTS), the identified protein targets are screened against small molecule (natural/synthetic) databases to detect which of these compounds could plausibly bind robustly to the bacterial target. The best-identified "hit" may be extricated from the database for testing purposes (Dutta et al., 2010).

Bioavailability studies

To prevent the failure of the drug during preclinical testing, the lead molecule should pass the ADME and toxicity studies. Using in silico resources such as TOPKAT, CLOGP, AbSolv, GastroPlus, DrugMatrix, BioPrint, etc., the bioactivity and bioavailability of the compound can be predicted before wet lab validation (Rao and Srinivas, 2011).

Role of natural compounds and use of bioinformatics in medicinal chemistry for AMR detection

Plants produce a huge range of compounds that are bioactive in nature and are typically secondary metabolites (Stefanovic and Comic, 2001). These naturals affect cell signaling and protect against oxidative or UV stress, apart from acting as defense mechanisms (Wink et al., 2012). Some important examples of naturals include alkaloids, phenolics, polyphenols, flavonoids, quinones, tannins, coumarins, terpenes, saponins, lectins, and polypeptides (Gupta and Birdi, 2017). The primary advantages of using naturals include:

- Reduced toxicity
- Reduced side effects
- Better acceptance by the body
- Better shelf life and longevity
- No ethical issues
- Easily available
- Easily extractable and lesser upstream/downstream processes
- Reduced costs

Natural compounds from different sources play an important role in pharmaceutical industries as the drug molecules that are on the market are also derivatives of the same. Marine natural products (MNP) and their derivatives such as Salinosporamide A, Depatuxizumab mafodotin, Lurbinectedin, Tetrodotoxin, and Pinabulin are potential FDA-approved drugs as well as drugs that are in clinical trials. These natural marine molecules can be used in the field of medicinal chemistry especially in treating AMR (Jimenez, 2018). Other marine natural compounds are Desotamide, Desotamide B, Anthracimycin, Neofiscalin A, Stachyin B, Stachybocin A & B, Ilicicolin, Etamycin, and Hedonistic are known to have activity against methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermis* (MRSE) and vancomycin-resistant *Staphylococcus aureus* (VRSA). These MNPs are mainly considered potential drug candidates since they obey the drug-likeness property and possess a high degree of bioavailability in nature (Liu et al., 2019; Valdes-Pena et al., 2021). Additionally, essential oils from traditional spice plants such as true cinnamon (*Cinnamomum zeylanicum*), thyme (*Thymus vulgaris*), black pepper (*Piper nigrum*), peppermint (*Mentha piperita*), rosemary (*Rosmarinus officinalis*), basil (*Ocimum basilicum*) and oregano (*Origanum vulgare*) are also known to have potential applications in the field of medicinal chemistry (Diniz do Nascimento et al., 2020) by possessing drug resistance properties.

With naturals being so important and with several such advantages, using these as an alternative approach for tackling multidrug resistance is a feasible solution. As elucidated in previous sections, in silico bioinformatics tools play a major role in the detection of AMR and the discovery of new drugs, be it natural or synthetic. With whole genome sequencing (WSG) playing a critical part in the understanding of the major proteins and bacterial targets, the proteins can be identified with the enumeration and optimization of the lead compounds. Validation using bioinformatics tools will help in the identification of the best target-lead complex, which can be taken forward for in vitro studies such as the identification of

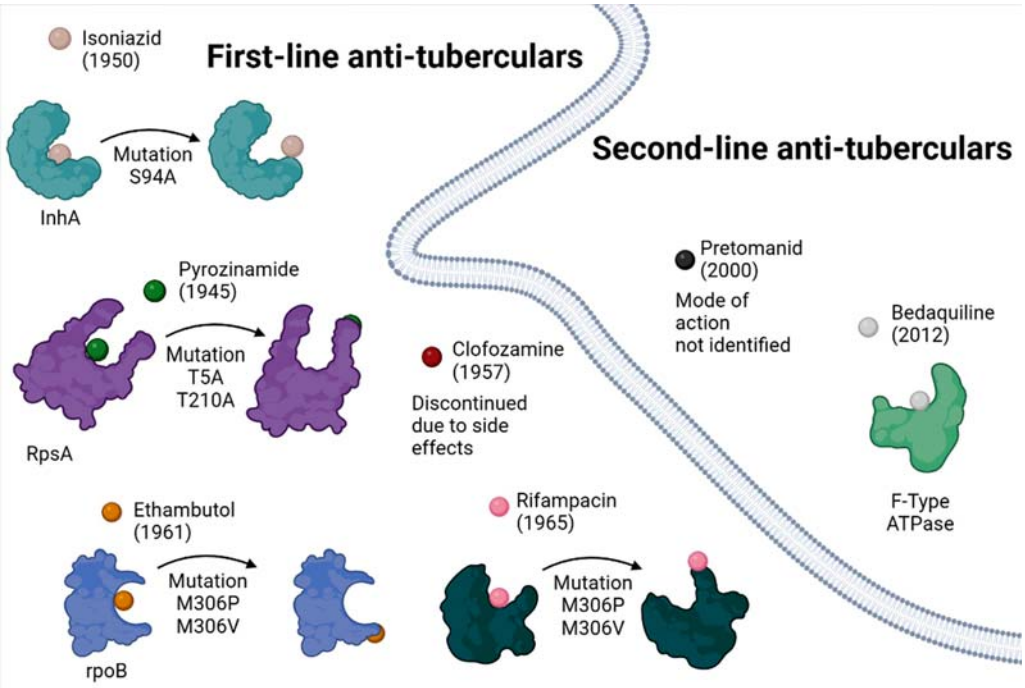

FIGURE 19.2 Evolution of drug resistance explained in tuberculosis. Isoniazid came out in 1950 as the first line antitubercular agent. But target InhA mutated at the S94A site, rendering the drug ineffective. In 1945, pyrazinamide came into the picture to target the RpsA protein of MTB. However, this protein underwent T5A and T210A mutations, causing the drug to be ineffective. In 1957, clofazimine was introduced but was discontinued due to continued side effects (Cholo et al., 2012). Ethambutol was also a first-line anti-tubercular developed in 1961, but the MTB rpoB protein mutated at M306P and M306V, rendering it ineffective. This evolution continued with the introduction of rifampicin in 1965, but the organism mutated again at the same sites. This prevalence of multiple drug resistance of *M. tuberculosis* persisted for more than three decades until the second line of tuberculars started coming in. Pretomanid in 2000 and bedaquiline in 2012 were developed.

the MIC (minimum inhibitory concentration) values. A comprehensive antibiotic resistance database can either be built or can be used from among the existing to gain insights into the various bacterial resistances. This is followed by the WGS phenotypic antimicrobial susceptibility testing and MALDI-TOF-based detection of AMR by the analysis of the peak patterns of the different pathogens (Fig. 19.2).

Major small molecules databases

Searchable databases of natural compounds with their validated sequence and structures allow for easy usage of these compounds for virtual screening. PubChem is one such major database of small molecules (both natural and synthetic) that allow for the downloading of 2D and 3D structures of user-required molecules (Kim et al., 2016). PubChem also portrays information regarding the Globally Harmonized System (GHS) category and criteria for toxicity classifications (Winder et al., 2005). The general properties of the molecules can also be identified from this database. Another natural small molecule database is MolPort (https://www.molport.com/shop/natural-compound-database), wherein, the whole library can be downloaded for easy virtual screening against target proteins. Collection of Open Natural Products Online (COCONUT) is a database developed by Sorokina et al. (2021) that houses bioactive naturals that are produced by living organisms with applications in industry and other pharmaceutical companies (Sorokina et al., 2021). HIT 2.0 (Herbal Ingredients' Targets) hosts 10,031 molecule-target activity pairs from greater than 1250 herbs. These herbal molecules covered PubMed literature from 2000 to 20 (Yan et al., 2022). Another database called Collective Molecular Activities of Useful Plants (CMAUP), by Zeng et al. (2019) houses the molecular activities of 5645 beneficial plants, inclusive of 2567 medicinal herbs that are being used in 79 different countries on 2473 gene ontology (GO) and 646 target proteins of humans. The database also comprises 234 KEGG pathways and their associations with 656 diseases. The biological activities of small molecules can also be identified in this database (Zeng et al., 2019).

TABLE 19.1 List of latest natural small molecules databases with their websites.

Sl. No.	Name of the small molecule database	Website	References
1.	Life chemicals	https://lifechemicals.com/	–
2.	PubChem	https://pubchem.ncbi.nlm.nih.gov/	Kim et al. (2016)
3.	Molport	https://www.molport.com/shop/natural-compound-database	–
4.	ZINC	https://zinc.docking.org/	Sterling and Irwin (2015)
5.	ChemDiv	https://www.chemdiv.com/catalog/	–
6.	COCONUTs (collection of open natural products) online	https://coconut.naturalproducts.net/	Sorokina et al. (2021)
7.	ChEMBL	https://www.ebi.ac.uk/chembl/	Gaulton et al. (2012)
8.	ChemBank	http://chembank.broad.harvard.edu/	Seiler et al. (2007)
9.	ChemDB	http://cdb.ics.uci.edu/	Chen et al. (2005)
10.	HIT (herbal ingredients' targets) 2.0	http://hit2.badd-cao.net/	Yan et al. (2022)
11.	DrugBank 5.0	http://www.drugbank.ca/	Wishart et al. (2018)
12.	Binding DB	https://www.bindingdb.org/bind/index.jsp	Liu et al. (2007)
13.	ChemIDplus	https://chem.nlm.nih.gov/chemidplus/	Tomasulo (2002)
14.	ChemSpider	http://www.chemspider.com/	Pence and Williams (2010)
15.	KEGG COMPOUND database	https://www.genome.jp/kegg/compound/	Kanehisa et al. (2017)
16.	InterBioScreen Ltd (IBS)	https://www.ibscreen.com/	–
17.	CMAUPs (collective molecular activities of useful plants)	http://bidd.group/CMAUP/	Zeng et al. (2019)
18.	REAXYS	https://www.elsevier.com/solutions/reaxys	Goodman (2009)
19.	3DMET	http://www.3dmet.dna.affrc.go.jp/	Maeda and Konda (2013)
20.	ChEBI (chemical entities of biological interest)	https://www.ebi.ac.uk/chebi/	Hastings et al. (2016)

Similarly, other typical small molecule databases include ChEMBL (Gaulton et al., 2012), ChemBank (Seiler et al., 2007), ChemDB (Chen et al., 2005), DrugBank 5.0 (Wishart et al., 2018), REAXYS (Goodman, 2009), 3DMET (Maeda and Kondo, 2013), and ChEBI (Chemical Entities of Biological Interest) (Hastings et al., 2016). Table 19.1 shows some of the major small molecule databases.

Advancements in computational biological perspectives for gaining insights into drug resistance

The following sections show the computational biological perspectives on drug resistance.

Computer-aided virtual screening and compound filtering

Computer-aided virtual screening allows researchers to screen through a complete database or collection of a huge library of molecules and provides the best lead molecules. Ligand and structure-based virtual screening are the two main computer-aided virtual screening processes. If there is a known ligand template for a particular protein target then one can use ligand-based virtual screening and if there is no ligand for protein targets then structure-based virtual screening is

typically employed. Ligand-based virtual screening involves steps such as the generation of 3D structures, conversion of 2D to 3D, and generation of the desired number of conformers. Similarly, the structure-based virtual screening involves different docking methodologies, flexibility in terms of proteins and ligands, different scoring techniques and functions, and force field scoring. One of the advantages of structure-based virtual screening or drug discovery is its cost and rapid, efficient lead optimization and discovery. Consensus docking, ensemble docking, and induced fit are the advanced techniques that have been added recently to structure-based virtual screening to improve its efficiency. Recently, there is an amalgamation of upbringing technology, i.e., artificial intelligence and machine learning for structure-based and ligand-based virtual screening. The algorithms which are used to enhance these virtual screening processes are Artificial Neural Network, Support Vector Machine, Decision tree, k-nearest neighbors, genetic algorithms, and the Monte Carlo approach. One such example of a ligand-based virtual screening tool powered by artificial intelligence is PyRMD (Mason et al., 2007; Kontoyianni, 2021; Maia et al., 2020; Lionta et al., 2014; Yasuo and Sekijima, 2019; Hamza et al., 2012; Gillet, 2013; Amendola and Cosconati, 2021). Moreover, compound filtering is generally carried out using drug likeliness and pharmacokinetic properties of lead molecules as discussed in the following section.

Prediction of drug likeliness and pharmacokinetic features of lead molecules

Various tools which can be used for the prediction of drug likeliness and pharmacokinetic properties include Swiss ADME, Simulation Plus, ADMETlab 2.0, admetSAR, Click2Drug (http://www.click2drug.org/), ADMET Predictor 10.4, Toxtree, VirtualToxLab, BBB predictor (https://github.com/S-A-A-BBB/BBB-Prediction), Vega-QSAR, ACD/I-lab, ADMETopt, FP-ADMET, HobPre, PreADMET, Interpretable-ADMET and many more (Daina et al., 2017; Parrott and Lave, 2002; Xiong et al., 2021; Yang et al., 2019; Ghosh et al., 2016; Bhatia et al., 2015; Vedani et al., 2012; Benfenati et al., 2013; Masunov, 2001; Yang et al., 2018; Venkatraman, 2021; Wei et al., 2022; Kwang, 2005). Pharmacokinetic properties such as Absorption, Distribution, Metabolism, Excretion, and toxicity can be evaluated for the natural compounds using the above-mentioned software, tools, and web servers. These tools evaluate the drug likeliness of the ligands (possibility of a molecule becoming a drug based on bioavailability) based on the famous Lipinski rule of five (Lipinski, 2004). It gives users the output in a detailed manner if there is a violation of any one rule. Further, it provides insights into the other properties such as human gastrointestinal absorption (HIA), BBB (Blood-brain barrier) permeation, skin permeability (Log *Kp*), interaction with cytochrome molecules, and permeability glycoprotein (P-gp).

Molecular docking studies of selected natural molecules toward various bacterial drug targets

Virtual screening, compound filtering, drug likeliness, and pharmacokinetic studies are then followed by molecular docking of selected natural molecules against the identified potential proteins/bacterial drug targets. POAP-Parallelized Open Babel and AutoDock suite Pipeline is one of the recent GNU-based tools used for high throughput virtual screening involving both Open Babel and AutoDock. Advantages of POAP are preparing several ligands together at a time and also performing multiple proteins−multiple ligands docking studies together simultaneously (Samdani and Vetrivel, 2018). Another tool, PyRx, is an open-source virtual screening tool for molecular docking studies that can be used in all OS platforms such as windows, Linux, and Mac OS (Dallakyan and Olson, 2015). Other docking web servers which are available for docking are FireDock (Andrudier et al., 2007), SwissDock (Grosdidier et al., 2011), DockingServer (Hazai et al., 2009), PlayMolecule (Varela-Rial et al., 2020) and furthermore. Few other docking tools/software which is available are AutoDock and AutoDock Vina (Huey et al., 2012), Schrodinger Glide (Friesner et al., 2004), rDOCK (Ruiz-Carmona et al., 2014), VirtualFlow (Gorgulla et al., 2020), LightDock (Jimenez-Garcia et al., 2018), etc.

A recent study has used the phytocompounds (flavonoids) from *Penicillium setosum* against DNA gyrase, beta-ketoacyl ACP reductase (FabG), trans two- enoyl ACP reductase (FabI), 3R-hydroxy acyl ACP dehydratase (FabZ), D-alanine: D-alanine ligase (Ddl) and penicillin-binding protein, elongation factor Tu (George et al., 2019). The docking software used was AutoDock 4, the most commonly used and widely accepted docking software. PEGA-nucleosides have also been previously utilized for Class B one Metallo-beta-lactamases (MBLs) and tools such as MolSoft ICM 3.8, Chem Draw, ICM PocketFinder, SwissADME, and ProTox-II were all employed for virtual screening process (Mendoza et al., 2021). Phytochemicals extracts from *Calotropis gigantea* have been used against targets Tyrosyl-tRNA synthetase proteins from drug-resistant *Staphylococcus aureus* and molecular docking using AutoDock, their binding energies were identified (Beg et al., 2020). Likewise, all-natural compounds from the ZINC15 database were used against diguanylate cyclase PleD protein of *Caulobacter cresentus* (Pestana-Nobles et al., 2020), and natural epiestriol-16 has been tested and validated against target RecA (Protein RecA), PyrE (orotate phosphoribosyltransferase), PyrF (orotidine 5′-phosphate

decarboxylase) and Omp38 (Outer membrane protein) of drug-resistant *Acinetobacter baumannii* (Skariyachan et al., 2020). Additionally, natural compounds from Natural Organism Library (NOL) - N1287 (Skyrin) and N2576 ((4,5-dichloro-1H-pyrrol-2-yl)-[2,4-dihydroxy-3-(4-methyl-pentyl)-phenyl]-methanone) have been used against target sortase A (SrtA) of drug-resistant *Staphylococcus aureus* to test and validate the complex interactions and stabilities (Thappeta et al., 2020). Other such small molecules and their potential targets are further detailed in Table 19.2. All this evidence that molecular docking is the primary step toward identifying the binding capabilities of selected naturals against the bacterial protein targets to asses and estimate the robustness of binding.

Analysis and interpretation of molecular docking and validation

Interpretation of molecular docking and validation can be performed in different ways.

2D and 3D visualization

Interpretation of molecular docking of the top docked complexes is in the form of pictorial representation i.e., 2D and 3D visualization of the protein-ligand complex using various tools and software highlighting the docked regions, residues (amino acids), type of interaction, type of bond formation (hydrogen bond, hydrophobic bond, Pi-Sigma, alkyl, Pi-Alkyl, Van der Waals), number of interactions, number of bonds and bond lengths. Tools that can be used for 2D and 3D visualization include LigPlot+ (Laskowski and Swindells, 2011), protein-ligand profiler (PLIP) (Salentin et al., 2015), PyMOL (Yuan et al., 2017), BIOVIA Discovery Studio (Studio, 2008), Schrodinger 2D Interaction Diagram (Bowers et al., 2006), UCSF-Chimera (Pettersen et al., 2004). A study has previously employed LigPlot tools for 2D visualization of docked complexes between natural molecules, catechin and its derivatives, and glucosamine-6-phosphate synthase (GlmS) (Fikrika et al., 2016).

Scoring functions

To separate the correct poses from the incorrect poses, different scoring functions are used such as knowledge-based scoring functions, empirical scoring functions, and force-field-based scoring functions. One of the best scoring functions which have overcome the drawbacks of affinity prediction (due to inadequate treatment of solvation effects) is physics-based scoring functions such as MM-GB/SA (Molecular Mechanics—Generalized Born/solvent accessible surface area) and MM-PB/SA (Molecular Mechanics—Poisson-Boltzmann/solvent accessible surface area) (Meng et al., 2011; Dar and Mir, 2017). Binding energies also interpret the molecular docking in terms of poor and best-docked complexes through energy values in kcal/mol or joules.

Molecular dynamic simulations

Conventionally, to validate the molecular docking results, molecular dynamics simulation is one of the best techniques to date through the in silico approach. Molecular dynamics simulation is studying the stability of the docked complex at certain molecular conditions under specific temperatures, volume, and pressure for a certain period (usually 100, 200, and 500 ns). Different tools that are available for performing molecular dynamic simulations are NAMD (Philips et al., 2005), Desmond (Bowers et al., 2006), AMBER (Pearlman et al., 1995), GROMACS (Abraham et al., 2015), LAMMPS (Thompson et al., 2022), DL-POLY (Todorov et al., 2011) and many more. It gives researchers a complete understanding of how these protein structures undergo conformational changes in the presence of ligand molecules, the complexity of the structures, and the stability of bound complex through protein root mean square deviations (RMSD) values and graphical plots. Further, molecular dynamic simulations provide an overview of the ligand root mean square fluctuations (RMSF) for its backbone atoms and molecules. It also provides insights into protein-ligand interactions through residue-level interaction information over a period of time (usually 100 ns). More importantly, researchers can visualize the real-time movement of the protein and ligand under specified conditions after simulation and other properties such as radius of gyration (*Rg*) can be estimated to understand how the ligand fits into the cavity of the target protein structure (Hospital et al., 2015; Filipe and Loura, 2022; Hollingsworth and Dror, 2018). A previous study on phytochemicals against the proteins associated with the multidrug-resistant *A. baumannii* employed Nanoscale Molecular Dynamics program v 2.9 and visual molecular dynamics simulation software for carrying out molecular dynamic simulation for the best-docked complexes. The docked complexes which were simulated were found to be stable in terms of protein-ligand binding energies, and conformational stability of the ligand and protein during the simulation step (Skariyachan et al., 2020). In a recent study, the molecular dynamics simulations studies were carried out with Desmond software to determine the

TABLE 19.2 Comprehensive list of previous in silico studies on different bacterial drug targets.

Sl. No.	Ligand molecules	Bacterial drug targets	In silico tools	References
1.	DB04118 (*N*-coeleneterazine), DB04698 (N-(1,4-dihydro-5H-tetrazol-5-ylidene)-9-oxo-9H-xanthene-2-sulfonamide), ZINC 5117079, CID 9809878, ZINC 28356629	dnaE, DNA III alpha of *E. coli* O157:H7 and other gram-positive pathogens such as *Streptococcus, Mycoplasma, Enterococcus* and *Staphylococcus*	DrugBank, binding db	Mondal et al. (2015)
2.	Asinex elite library (http://www.asinex.com/) —100,000 compounds	Topoisomerases I of *Mycobacterium tuberculosis* (MtbTopI)	AutoDock Vina, molecular dynamic simulations (Charmm-GUI web interface)	Sandhaus et al. (2018)
3.	DrugBank (DB01694, DB07861, DB04482, DB02353, DB02731, DB01972)	*Haemophilus influenzae* (3-Deoxy-manno-octulosonate cytidylyltransferase), *Pseudomonas aeruginosa* (UDP-3-O-[3-Hydroxymyristoyl] N-acetyl glucosamine deacetylase), *Helicobacter pylori* (UDP-N-Acetylglucosamine O-acyltransferase), *Arthrospira platensis* (phosphate regulon response regulator OmpR), *Caulobacter crescentus* (chemotaxis family, response regulator CheB) and *Thermotoga maritina* (nitrogen regulation sensor histidine kinase GlnL)	Drug discovery studio 3.0, AutoDock Vina and UCSF chimera tool	Hossain et al. (2017)
4.	Plant extracts from *Boswellia serrata*	AmPC of *Klebsiella pneumoniae*	AutoDock 4, UCSF chimera	Vakayil et al. (2021)
5.	Phytocompounds from Himalayan rhubarb	Pencillin binding protein 3, 3VSL (bacteria)	AutoDock Vina, Swiss ADME, PubChem, RCSB PDB, admetSAR, Protox-II and MDS-Desmond	Rolta et al. (2022)
6.	Antibacterial phytochemicals from *Dictionary of Natural Products*	NAD + -dependent DNA ligase, topoisomerase IV, UDP-galactose mutase, peptide deformylase, cytochrome P450 and tyrosine phosphatase (bacteria)	Molegro virtual Docker version 6.0, RCSB PDB	Snow Setzer et al. (2016)
7.	Acridone and its derivatives	DNA gyrase (*S. aureus*), transcriptional regulator (TtgR)—*Pseudomonas putida*	Surflex-dock module from SYBYL-X 2.0, RCSB PDB, discovery studio	Aarjane et al. (2020)
8.	Phytocompounds (flavonoids) from *Penicillium setosum*	DNA gyrase, beta-ketoacyl ACP reductase (FabG), trans 2- enoyl ACP reductase (FabI), 3R-hydroxyacyl ACP dehydratase (FabZ), D-alanine:D-alanine ligase (Ddl), penicillin binding protein, elongation factor Tu	Chemspider, open babel GUI, RCSB PDB, CASTp web server, AutoDock 4, BIOVIA discovery studio	George et al. (2019)
9.	PEGA-nucleosides	Class B 1 metallo-beta-lactamases (MBLs)	MolSoft ICM 3.8, Chem Draw, ICM Pocket-Finder, SwissADME, ProTox-II	Mendoza et al. (2021)
10.	Beta-lactam-anthraquinone hybrids	Penicillin-binding protein 2a from MRSA	RCSB PDB, Gaussian version 7.0, AutoDock 4.2, chimera and discovery studio	Mohamadzadeh et al. (2020)

11.	Phytochemicals extracts from *Calotropis gigantea*	Tyrosyl-tRNA synthetase protein (*Staphylococcus aureus*)	Mol2, Chem3D ultra, RCSB PDB, CASTp 3.0, AutoDock Vina, PyMOL, Discovery Studio (2019), SwissADME	Beg et al. (2020)
12.	Natural compounds from ZINC15 database	Diguanylate cyclase PleD protein of *Caulobacter cresentus*	ICM software, PDB, AMBER package (MM/PBSA), cpptraj tool	Pestana-Nobles et al. (2020)
13.	Natural epiestriol-16	RecA (protein RecA), PyrE (orotate phosphoribosyltransferase), PyrF (orotidine 5′-phosphate decarboxylase) and Omp38 (outer membrane protein) of *Acinetobacter baumannii*	PDB, STRING, ModRefiner, GROMOS, PROCHECK, STRIDE web server, super natural II, PreADMET, SwissADME, AutoDock Vina, PubChem, Chemspider, PDB, open babel, NAMD v2.9	Skariyachan et al. (2020)
14.	Thiolactomycin	MTB-KasA enzyme of *Mycobacterium tuberculosis*	ADMET, Marvin Sketch, Schrodinger—Glide & LigPrep, GROMACS 5.1.2	Durairaj and Shanmughavel (2019)
15.	Baicalein (baikal skullcap), Luteolin (dandelion), Resveratrol (grape vine), Wogonin, Pyrocide (carrot), Apigenin (coffee)	Aph & dfrA1 (*S. typhi*), dfrA1 (*V. cholerae*), vanH (*S. aureus*)	UniProt database, PSI-BLAST, PDB, T-COFFEE, Modeler 9v11, UCSF-chimera, Pro-Check, ERRAT, PreADMET, ChemSpider, AutoDock Vina	Skariyachan et al. (2014)
16.	Natural compounds from natural organism library (NOL) - N1287 (skyrin) and N2576 ((4,5-dichloro-1H-pyrrol-2-yl)-[2,4-dihydroxy-3-(4-methyl-pentyl)-phenyl]-methanone)	Sortase A (SrtA)—*Staphylococcus aureus*	Dock 3.6	Thappeta et al. (2020)
17.	Natural compounds from Dr. Duke's phytochemical and ethnobotanical database	ArcB & MexB (*Pseudomonas aeruginosa* & *E. coli*)	PubChem, PDB, Schrodinger LigPrep, SiteMap version 2.6, Glide XP-ligand docking, MM-GBSA	Aparna et al. (2014)
18.	Novel lead molecules (ethidium, cetylpyridinium, proflavin, tetraphenylphosphonium, methyl viologen)	Mycobacterium multidrug resistant (MMR) protein of *Mycobacterium tuberculosis*	ExPASy, UniProtKB, BLAST, PSI-BLAST, PHYRE server 0.2, ClustalW, Modeler, Swiss PDB Viewer, Schrodinger suite, PROCHECK, ProSA, PDB, SymmDock, CASTp, Q-SiteFinder, Glide, Pubchem, ChemBank, PyMOL, discovery studio	Malkhed et al. (2014)
19.	Phytoligands	RstA (transcriptional regulatory protein), LpxA, KdsB, ampC, MurA (UDP-N-acetylglucosamine) MDR *A. baumannii*	PubChem, ChemSpider, PreADMET, AutoDock Vina v1.1.2, Desmond	Skariyachan et al. (2019)
20.	ZINC database	L2-beta-lactamase, multi drug resistant bacteria *Stenotrophomonas maltophilia*	RCSB-PDB, UCSF chimera, QMEAN server, ProSA, RAMPAGE web server, PDBsum server, MetaPocket 2.0, COACH online server, AutoDock, GROMACS-4.6.5, PyMOL	Sharma et al. (2021)

stability of the best-docked phytochemicals from a natural source (*Rheum emodi* wall) against proteins (penicillin-binding protein 3, cytochrome p450 14 alpha-sterol Demethylase and N-myristoyl transferase) of drug-resistant bacterial species. From the molecular dynamic simulation studies, many parameters were looked into for analyzing the stability of the best-docked complexes such as RMSD, RMSF, and Rg (Rolta et al., 2022).

Relevant case studies

Antimicrobial resistant bacteria have previously been attempted to be treated with natural compounds such as DB04118 (*N*-coeleneterazine), DB04698 (*N*-(1,4-dihydro-5H-tetrazol-5-ylidene)-9-oxo-9H-xanthene-2-sulfonamide), ZINC 5117079, CID 9809878 and, ZINC 28356629 against dnaE, DNA III alpha of resistant *E. coli* O157:H7 and other gram-positive resistant pathogens such as *Streptococcus, Mycoplasma, Enterococcus,* and *Staphylococcus.* The study used DrugBank and BindingDB as in silico resources for testing and validating the studies (Mondal et al., 2015). Likewise, Sandhaus et al. (2018) utilized the Asinex Elite library (http://www.asinex.com/) comprising 100,000 natural compounds to target the topoisomerases I of multidrug-resistant *Mycobacterium tuberculosis* (MtbTopI) with good outcomes. Databases and tools such as AutoDock Vina, and Molecular Dynamic Simulations (Charmm- GUI Web interface) were employed in this study. More recently, multidrug-resistant *Haemophilus influenzae* (3-Deoxy-Manno-octulosonate cytidylyltransferase), *Pseudomonas aeruginosa* (UDP-3-O-[3-Hydroxymyristoyl] N-acetyl glucosamine deacetylase), *Helicobacter pylori* (UDP-N-Acetylglucosamine O-acyltransferase), *Arthrospira platensis* (Phosphate regulon response regulator OmpR), *Caulobacter crescentus* (Chemotaxis family, response regulator CheB) and *Thermotoga maritina* (Nitrogen regulation sensor histidine kinase GlnL) were targeted by DrugBank molecules such as DB01694, DB07861, DB04482, DB02353, DB02731 and DB01972 (Hossain et al., 2017). For this study, tools such as Drug Discovery Studio 3.0, AutoDock Vina, and the UCSF Chimera tool were employed. This study used small molecules to treat the above-mentioned multidrug-resistant bacteria.

Likewise, another recent study employed plant extracts of *Boswellia serrata* to target the AmPC of resistant *Klebsiella pneumoniae* (Vakayil et al., 2021). The study performed molecular docking and visualization using AutoDock four and UCSF Chimera respectively to test the binding efficacy against multidrug resistance bacteria with satisfactory results. Studies have also employed phytocompounds from Himalayan rhubarb against Penicillin Binding protein 3, 3VSL, common to most bacteria. Computational tools such as AutoDock Vina, Swiss ADME, PubChem, RCSB PDB, admetSAR, Protox-II, and MDS-Desmond were employed in this study to perform molecular docking, find the binding efficacy, understand the pharmacokinetic properties, and to visualize the interactions (Rolta et al., 2022). Acridone and its derivatives have also been used against DNA gyrase of drug-resistant *S. aureus* and transcriptional regulator (TtgR) of *Pseudomonas putida* (Aarjane et al., 2020). All these case studies provide corroboratory results with robust evidence that natural molecules act as potential alternatives for combating the menace of multidrug resistance.

Conclusion

Today's world is riddled with several harmful diseases, with antibacterial resistance being the most problematic. The emergence of multidrug-resistant bacteria is one of the main causes of death worldwide. The evolution of these perilous organisms has led to an increase in fatality rates, and thus, with conventional methods not solving these issues, newer and better alternatives are the need of the hour. *In-silico* computational resources fill the existing dearth in the traditional techniques, with advanced technology, novel approaches, faster identification of relevant and required plausible drug molecules, and better ideologies for tackling various MDR infections. The modern-day drug design and development provides us with predictions of the best lead hits after a virtual screening of a library of thousands of molecules. The use of natural has an even better advantage since it counteracts all the disadvantages of synthetically designed drugs. The screened natural can then be predicted for these pharmacokinetic and drug-like properties with an insight into its toxicity as well. Bioinformatic tools help gain insights into the binding capabilities of the natural molecule and provide us with a real-time simulation of the compound stability via dynamic simulations. With these advancements, predictions have also improved with the use of advanced artificial intelligence and machine learning algorithms to improve the prediction accuracy in pharmacophore models. Therefore, this chapter has focused on providing immense and detailed insights into the possibilities of using in silico tools for better and more accurate solutions to the problem of multidrug resistance.

Future perspectives

The future of using in silico approaches has great potential in combating multidrug resistance in bacteria. Advancements in computational techniques open large avenues that can be explored for attaining better solutions. The use of naturals offers a much safer way of tackling resistance.

- The combination of several computational approaches to study various aspects of resistance, their mechanisms, and understanding their pathways to know which protein can be targeted is the first step in the identification of newer natural-based antibiotics.
- The first steps of predictions provide the way for other in vitro and in vivo technologies to take over for validating the predictions. CRISPR is the latest technology for which simulated drugs can be used to reach specific targets.
- Using bioinformatics, a mechanism to reduce cross-resistance can be identified and reduced.
- Innovative structures of the chemicals/naturals can be identified or modeled or designed with specific binding to the active sites of bacterial targets so that there is robustly attached to the targets.
- Existing naturals can be made better by the addition of side chains so that their natural qualities can be retained with the added benefits of better properties.
- Using computational biological methods, the concepts in medicinal chemistry can be utilized to their fullest potential by advanced machine learning and deep learning algorithms.

Thus, the improvement of research in the coming years will be vital to take a significant step forward to decipher the scope of using advanced tools and techniques for managing the resistance menace.

Abbreviations

ADME Absorption, Distribution, Metabolism and excretion
AMR Antimicrobial resistance
CADD Computer-Aided Drug Design
MDR Multi-drug resistance
PDR Pan-drug resistant
XDR Extensively drug resistant

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Chapter 20

A computational approach to identify natural putative inhibitors to combat monkeypox

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Introduction

Monkeypox disease

Monkeypox is a disease that is caused by infection with the monkeypox virus. This virus belongs to the Orthopoxvirus genus in the own family of Poxviridae. The Orthopoxvirus genus also consists of the vaccinia virus, variola virus, and cowpox virus (Lum et al., 2022). Monkeypox was first identified in 1958 when two outbreaks of a pox-like disease happened in colonies of monkeys kept for research, hence the name "monkeypox." The genetic material of the monkeypox virus is double-stranded DNA. The poxviruses virions have a brick-shaped structure measuring 200−400 nm long (Louten, 2016).

Epidemiology

The first human case of monkeypox was recorded in 1970 in the Democratic Republic of Congo during a period of intensified effort to eliminate smallpox (Dhawan et al., 2022). For decades, Monkeypox has frequently been reported in Africa. However, it is occasionally observed in different nations, including the USA. Within the spring of 2003, the first outbreak of monkeypox occurred in the United States Texas imported cargo of infected animals from Ghana. The infected animal spread the virus to pet prairie dogs, which then affected 47 human beings within the Midwest (Thornhill et al., 2022). In this era, the foreign journey becomes easier, and viruses that were once relatively confined to definite places can easily be transmitted around people.

Re-emergence of virus

In the season of summer (2021), a case of monkeypox disease was determined in a U.S. citizen who had traveled from Nigeria. The year 2022 brought outbreaks to areas outside of Africa, which include Australia, Europe, and the Americas. On May 18, 2022, 14, seven, and 13 cases were stated in Canada, Spain, and Portugal respectively. On May 19, 2022, Italy, Sweden, and Belgium reported their first monkeypox disease. On May 20, Australia confirmed two cases. The first was from Melbourne and the second was from Sydney. These individuals came back from Europe. Netherlands, Germany, and France showed their first cases on May 20. Additionally, the UK identified more than eight cases of monkeypox with a total number of 71 on this date (WHO, 2022). Belgium was the first nation to implement 21-day compulsory quarantine for MPV. Spain identified the first case on May 18, 2022, Israel and Switzerland showed their first cases on May 21. On June 6, Spain identified a surge in the number of 20 cases bringing the cases to 186 (WHO, 2022). Moreover, 61,282 total real cases were reported in numerous patients in 104 nations (CDC, 2022).

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The Table 20.1 shows the entire number of cases and deaths in each nation till September 9, 2022 worldwide.

Monkeypox in India

On July 24, 2022, four cases of monkeypox have been identified, the first case was identified on July 14, 2022. All four have been male. The three people were from Kerala with a travel history from international countries. However, the last case reported from the national capital (Delhi) had no history of an overseas tour (Ministry of Health and Family Welfare, Government of India, March 2022).

On August 3, 2022, in Delhi, a Nigerian female of the age of 31 was observed to have effective test results for monkeypox. This is the first monkeypox ever reported among a woman-affected person in India. With this new case, the number of Indian monkeypox cases rises to 9. In India, incidences have been recorded in several areas in the states of Tamil Nadu, Kerala, Himachal Pradesh, Andhra Pradesh, and Uttar Pradesh (MPNRC India, 2022) (Table 20.2).

Transmission of the virus

The experimental research performed at the Kinshasa laboratory confirmed that the monkeypox virus becomes very effectively transmitted through air and excreta (vomit and feces) among captive squirrels, and by skin scarification. Ants (*Crematogaster* spp.) were additionally examined because, in nature, they represent a significant part of the weight loss plan of squirrels, and they consume the tissues of dead mammals. No virus was observed in ants that consumed and devoured the infected tissues of squirrels (Khodakevich et al., 1988). The infection is transmitted from animals to people and human beings to individuals (Wilson et al., 2014). The natural reservoirs are nonhuman primates, dormice, monkeys, and squirrels. Human beings are affected via close contact, bite/scratch, and through consuming improperly cooked meat of infected animals. Transmission among people is transmitted by contaminated fomites, direct touch, and large respiratory droplets. The secondary attack rate among household contacts is much less than 10%, in contrast to smallpox, where it became 35%−88% (Khodakevich et al., 1988). The role of direct sexual transmission is unknown. However, mucosal contact and intimate skin for the duration of intercourse facilitate outspread. Vertical transmission of information from female parent to the newborn leading to congenital monkeypox has also been opined (Mbala et al., 2018).

Signs and symptoms of monkeypox disease

The symptoms of monkeypox are remarkable to the ones of smallpox sufferers, but not as extreme. The incubation duration for monkeypox is usually two 2 weeks, with a maximum of 21 days. Patients often have records of exposure to human beings and animals diseased with the monkeypox virus, the primary signs and symptoms are much like "influenza," followed by experiencing scarring after scabs, pustules, and herpes on the skin. Based on the manner monkeypox virus infection is specifically classified explicitly into levels. The prodromal section involves symptoms such as muscle aches, fever, extreme lymphadenopathy headache, and fatigue, and the rash section (lasting 7−21 days). The rash usually begins to seem within 1−5 days after fever, and the patient is contagious when the rash appears. The rash appears on the conjunctiva, and oral mucosa, affecting the face and extremities, fingers, soles of the feet, and genitals. The rash lasts 2−4 weeks and evolves from scabs, pustules, blisters, and plaque to papules, and then shedding. Lesions can arise in locations ranging from some to numerous thousands (Macalino et al., 2015). In extreme cases, the regions of lesions can merge and cause large patches of skin to fall off. Sufferers frequently present with symptoms of lymphadenopathy, maximum frequently in the groin, and can also be observed through several complications, which include encephalitis, bronchopneumonia, corneal infection, respiratory distress, secondary bacterial infection, respiratory distress, a disease with eye blindness, and dehydration due to diarrhea as well as vomiting (Brown and Leggat 2016) (Fig. 20.1).

Pathogenesis of monkeypox infection

Poxviruses are huge, linear, double-stranded DNA viruses with a genome size ranging from 130 to 360 kbp that replicate in the cytosol of host cells (Okyay et al., 2022). They are unique in the way that poxviruses depend closely on virus-encoded proteins that allow them to duplicate within the cytosol (Moss, 2012, 2013). A significant portion of the genome includes genes concerned with key critical functions, consisting of virus assembly and transcription, whereas those placed at the end have functioned in virus-host interactivity (Stanford et al., 2007; Upton et al., 2003). The larger size of poxviruses makes it more difficult for viruses, including monkeypox, to breach host defense by using passing through gap junctions. The bigger size of the virus also makes it difficult for the virus to duplicate rapidly, and Orthopoxvirus needs a more comprehensive

TABLE 20.1 Monkeypox virus global outbreak 2022.

Sr.no	Country	Cases	Deaths	Sr.no	Country	Cases	Deaths
1	Belgium	726	1	53	Ireland	160	0
2	Brazil	5726	2	54	Israel	241	0
3	The Central African Republic	8	2	55	Italy	805	0
4	Cuba	2	1	56	Jamaica	9	0
5	Ecuador	59	1	57	Japan	4	0
6	Ghana	76	4	58	Latvia	4	0
7	India	10	1	59	Lebanon	8	0
8	Nigeria	220	4	60	Liberia	2	0
9	Spain	6749	2	61	Lithuania	5	0
10	Andorra	4	0	62	Luxembourg	53	0
11	Argentina	221	0	63	Malta	33	0
12	Aruba	2	0	64	Martinique	1	0
13	Australia	129	0	65	Mexico	788	0
14	Austria	286	0	66	Moldova	2	0
15	Bahamas	2	0	67	Monaco	3	0
16	Barbados	1	0	68	Montenegro	2	0
17	Benin	3	0	69	Morocco	3	0
18	Bermuda	1	0	70	Netherlands	1195	0
19	Bolivia	103	0	71	New Caledonia	1	0
20	Bosnia and Herzegovina	3	0	72	New Zealand	5	0
21	Bulgaria	5	0	73	Norway	82	0
22	Cameroon	7	0	74	Panama	12	0
23	Canada	1321	0	75	Paraguay	1	0
24	Chile	486	0	76	Peru	1760	0
25	Colombia	938	0	77	Philippines	4	0
26	Costa Rica	3	0	78	Poland	145	0
27	Croatia	27	0	79	Portugal	871	0
28	Curaçao	1	0	80	Qatar	3	0
29	Cyprus	5	0	81	Republic of the Congo	3	0
30	Czechia	58	0	82	Romania	36	0
31	The democratic Republic of the Congo	195	0	83	Russia	1	0
32	Denmark	181	0	84	Saint Martin	1	0
33	Dominican Republic	7	0	85	Saudi Arabia	8	0
34	Egypt	1	0	86	Serbia	31	0
35	El Salvador	1	0	87	Singapore	16	0
36	Estonia	10	0	88	Slovakia	14	0
37	Finland	30	0	89	Slovenia	45	0
38	France	3785	0	90	South Africa	5	0
39	Georgia	2	0	91	South Korea	2	0

Continued

TABLE 20.1 Monkeypox virus global outbreak 2022.—cont'd

Sr.no	Country	Cases	Deaths	Sr.no	Country	Cases	Deaths
40	Germany	3530	0	92	South Sudan	2	0
41	Gibraltar	6	0	93	Sudan	2	0
42	Greece	66	0	94	Sweden	165	0
43	Greenland	2	0	95	Switzerland	480	0
44	Guadeloupe	1	0	96	Taiwan	3	0
45	Guatemala	11	0	97	Thailand	7	0
46	Guyana	2	0	98	Turkey	1	0
47	Honduras	4	0	99	United Arab Emirates	16	0
48	Hong Kong	1	0	100	United Kingdom	3484	0
49	Hungary	71	0	101	United States	21,893	0
50	Iceland	12	0	102	Uruguay	5	0
51	Indonesia	1	0	103	Venezuela	3	0
52	Iran	1	0				

Resource: Centers for disease control and prevention.

approach to survive within the host (Okyay et al., 2022). The large Orthopoxvirus generates an immune system to combat the immune response of the host, several proteins act as modulators against components of the host's immune system (Kaler et al., 2022). Additionally, based on their mechanism of action, proteins are divided into two groups. 1. Extracellular proteins 2. Intracellular proteins (Fig. 20.2).

Life cycle of monkeypox virus

Orthopoxviruses are characterized by means of using their cytoplasmic life cycle, big, huge size, and complicated composition (Fig. 20.3). They synthesize two kinds of infectious particles such as extracellular virions and mature virions. Both MVs and EVs of vaccinia virus, the model poxvirus, take benefit of host cellular endocytosis to enter the host cell. They activate micropinocytosis, the most appropriate form of endocytosis for large, massive particles. Monkeypox virus can get into its host through the intradermal routes and oropharynx. The virus replicates at the inoculation site and proliferates, spreading to nearby lymph nodes. Following a duration of initial viremia, the MPV proliferates to some body organs. It has an identical structure to other recognized poxviruses. Monkeypox viruses are oval-shaped and are surrounded by a lipoprotein present on the outer surface of the cell. The life cycle takes place within the cytoplasm. There are various protein factors are required for virion assembly, DNA duplication, and transcription (Kugelman et al., 2014) (Fig. 20.4).

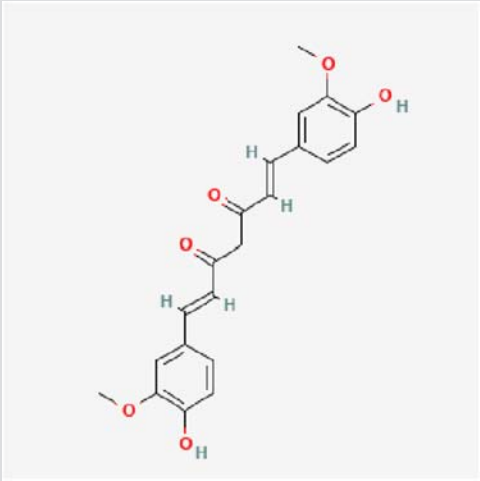
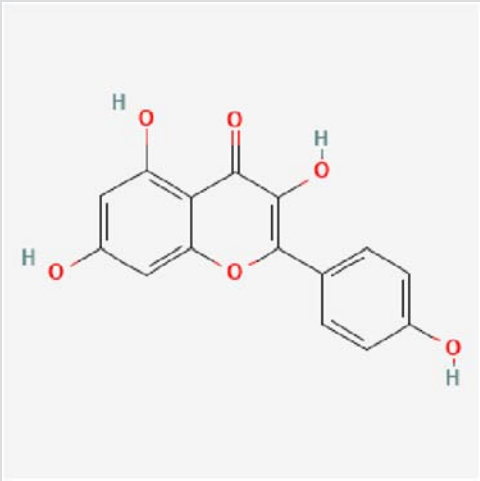
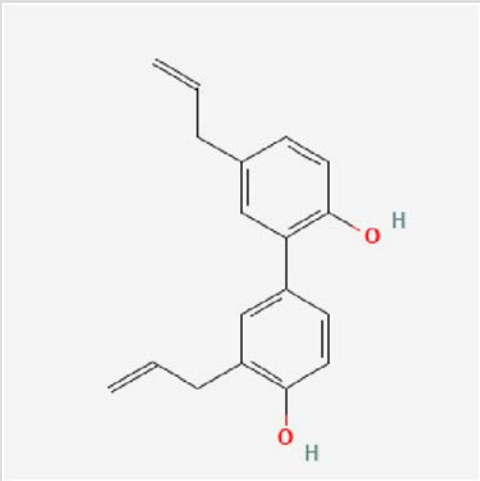
Similarities between human monkeypox and smallpox viruses

For the determination of the biological relationship among the orthopoxviruses inflicting these two diseases. Shchelkunov et al. carried out sequencing of the genome of the monkeypox virus isolated from the sufferer during the human monkeypox outbreak in Zaire. The series of nucleotides inside the relevant location of the monkeypox virus DNA, which encodes important structural proteins and enzymes become 96.3% similar to that of the smallpox virus. In opposition, there was a substantial variance between VAR and MPV inside host-range elements and the domain encoding virulence nearby the termini of the genome. Sergei et al. suggested that the monkeypox virus is not the direct ancestor of the variola virus and is not going to naturally acquire all properties of the variola virus (Shchelkunov et al., 2001).

Intracellular proteins

There are various types of intracellular proteins such as virotransducer and virostealth proteins. These virotransducer proteins play a significant part in preventing the host cell's potential to combat the disease, which includes apoptotic

TABLE 20.2 Reported antiviral phytochemicals that can be used to target the monkeypox virus.

Sr. No	Plant nane	Phytochemical	Viruses	2D-structure	References
1	*Curcuma longa*	Curcumin	Influenza, RSV, HBV, HCV, ZIKV, CHIKV, norovirus, HIV, HPV, CMV, EV71, DENV type-2		(Mathew and Hsu, 2018)
2	*Lilium Candidum*	Kaempferol	HSV-1, HSV-2		(Yarmolinsky et al., 2012)
3	*Magnolia officinalis*	Honokiol	Dengue virus type 2		(Fang et al., 2015)

Continued

TABLE 20.2 Reported antiviral phytochemicals that can be used to target the monkeypox virus.—cont'd

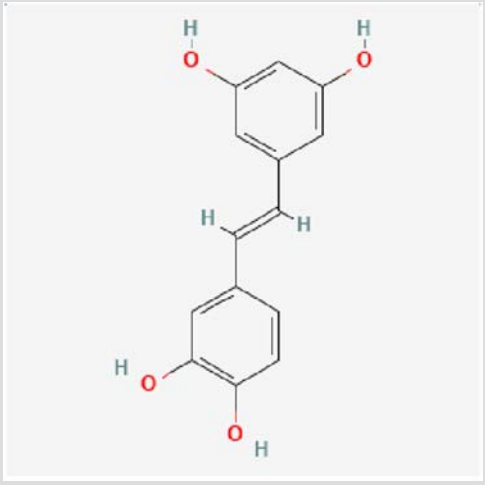
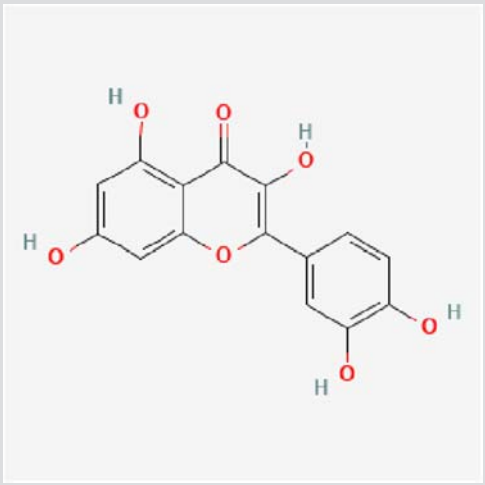
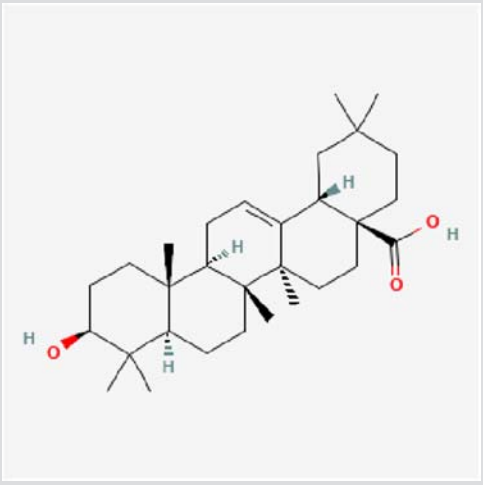
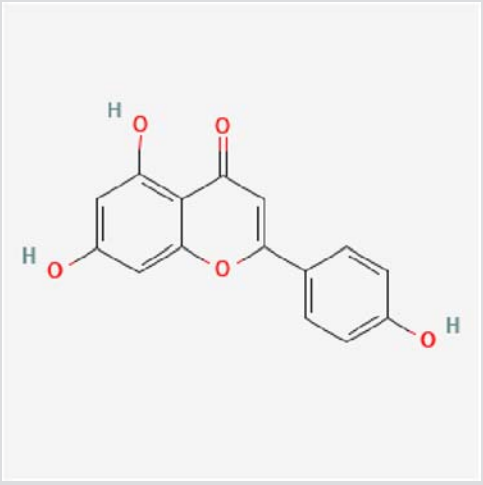
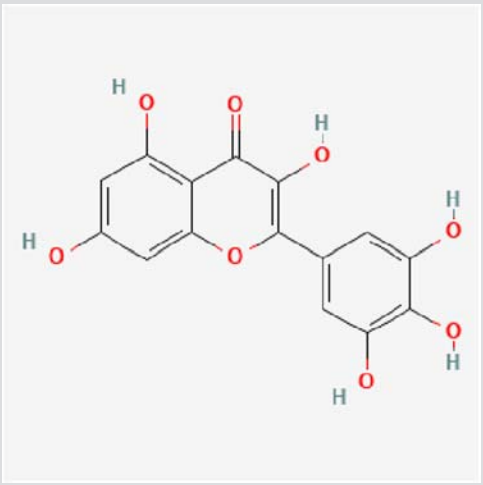
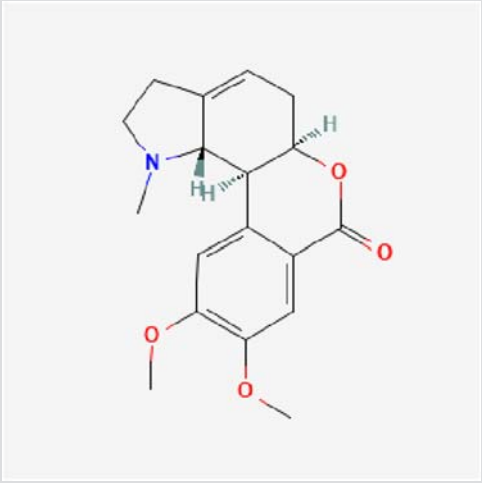
Sr. No	Plant nane	Phytochemical	Viruses	2D-structure	References
4	*Panax ginseng*	Theaflavin monglayes	Human rotavirus		(Clark et al., 1998)
5	*Vitis labrusca*	Piceatannol	Human cytome-galo virus		(Wang et al., 2022)
6	*Red grapes*	Quercetin	JEV, influenza A, EBV, MAYV, RV, HCV		(Anand David et al., 2016)

TABLE 20.2 Reported antiviral phytochemicals that can be used to target the monkeypox virus.—cont'd

Sr. No	Plant nane	Phytochemical	Viruses	2D-structure	References
7	*Olive plant*	Oleanolic acid	Acute and chronic hepatitis		(Somova et al., 2004)
8	Parsley	Apigenin	Enterovirus 71, FMDV, HCV, ASFV, influenza A		(Shibata et al., 2014)
9	Tea	Myricetin	HIV, RLV, influenza		(Peng et al., 2022)

Continued

TABLE 20.2 Reported antiviral phytochemicals that can be used to target the monkeypox virus.—cont'd

Sr. No	Plant nane	Phytochemical	Viruses	2D-structure	References
10	*Leucojum vernum*	Homolycorine	HIV-1		(Szlávik et al., 2004)

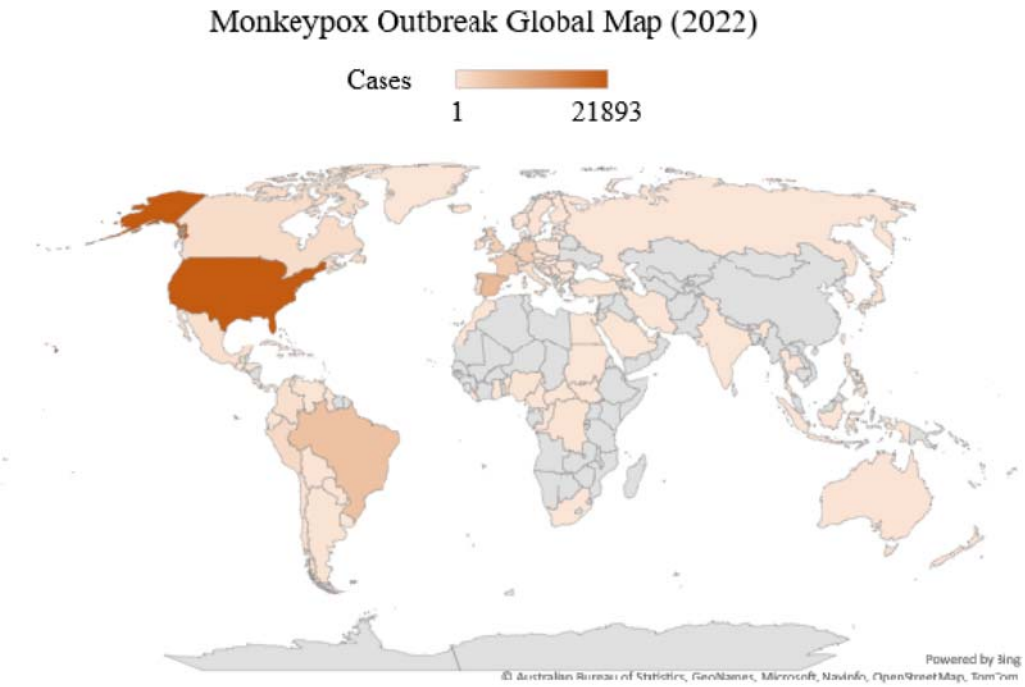


FIGURE 20.1 Monkeypox outbreak global Map (2022).

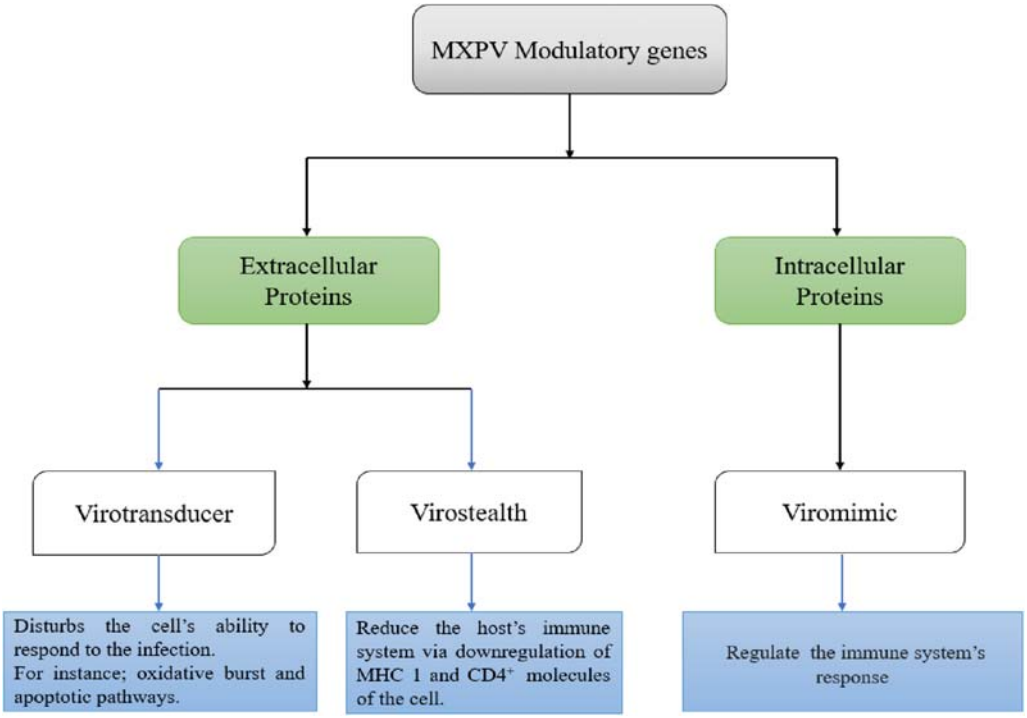


FIGURE 20.2 Intracellular and extracellular modulatory proteins of MXPV.

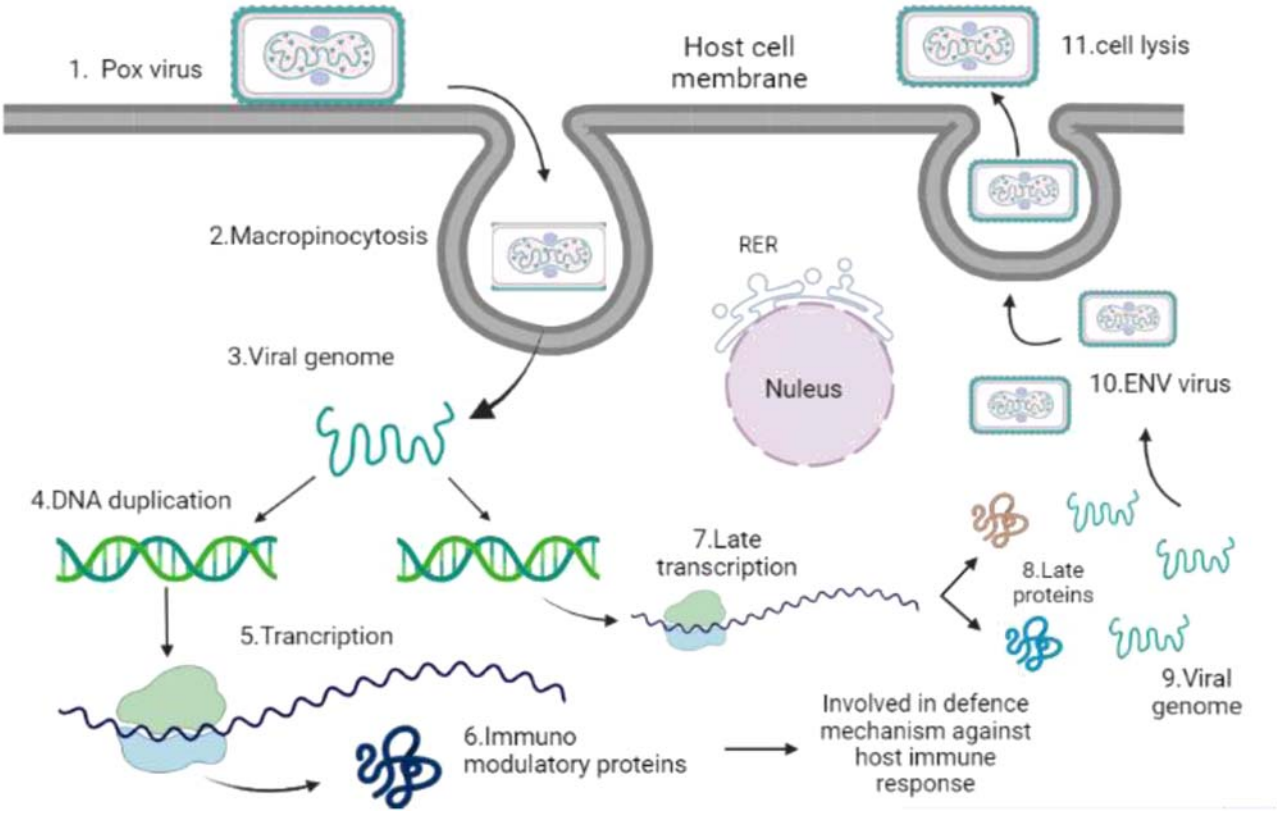


FIGURE 20.3 Life cycle of poxvirus. *Created using Bio Render program.*

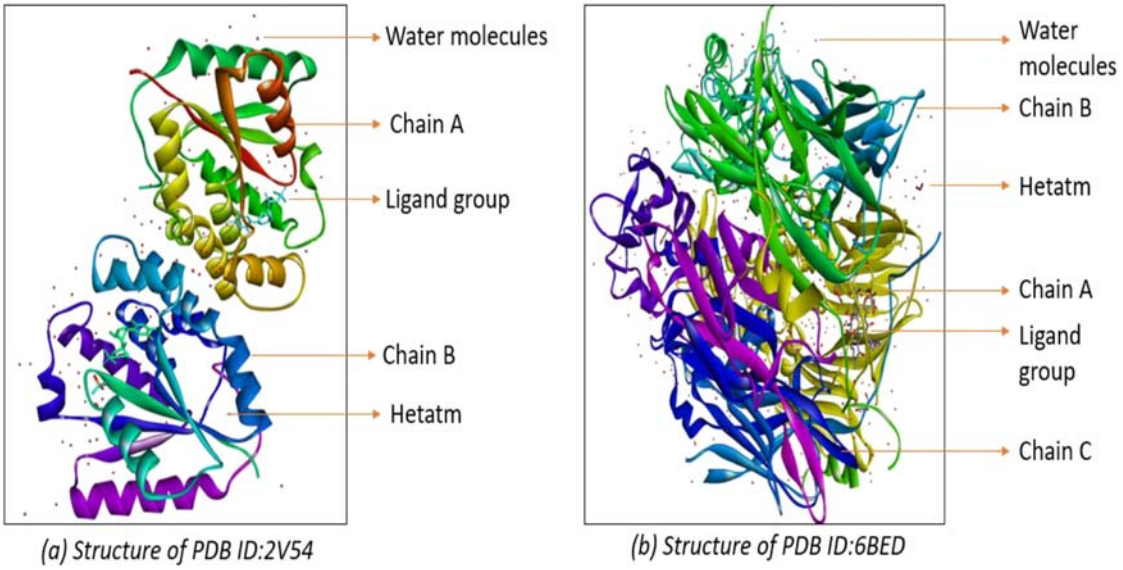


FIGURE 20.4 Representative images of (A) thymidylate kinase and (B) D13 viral protein.

pathways and oxidative burst (Stanford et al., 2007). The virostealth proteins perform their function intracellularly, decreasing the chance of virus identification by using the host's immune system through the holding of MHC molecules that are present in the cell (Petersen et al., 2019; Stanford et al., 2007) (Fig. 20.5 and 20.6).

Extracellular proteins

Viriomic proteins are type of extracellular proteins. Further, viriomic proteins are classified into two categories. The main function of these proteins is to regulate the immune reaction. The viroreceptors are cellular glycoproteins that bind to chemokines and cytokines which are released by the host cell, for this reason, inhibit their mechanism (Kaler et al., 2022;

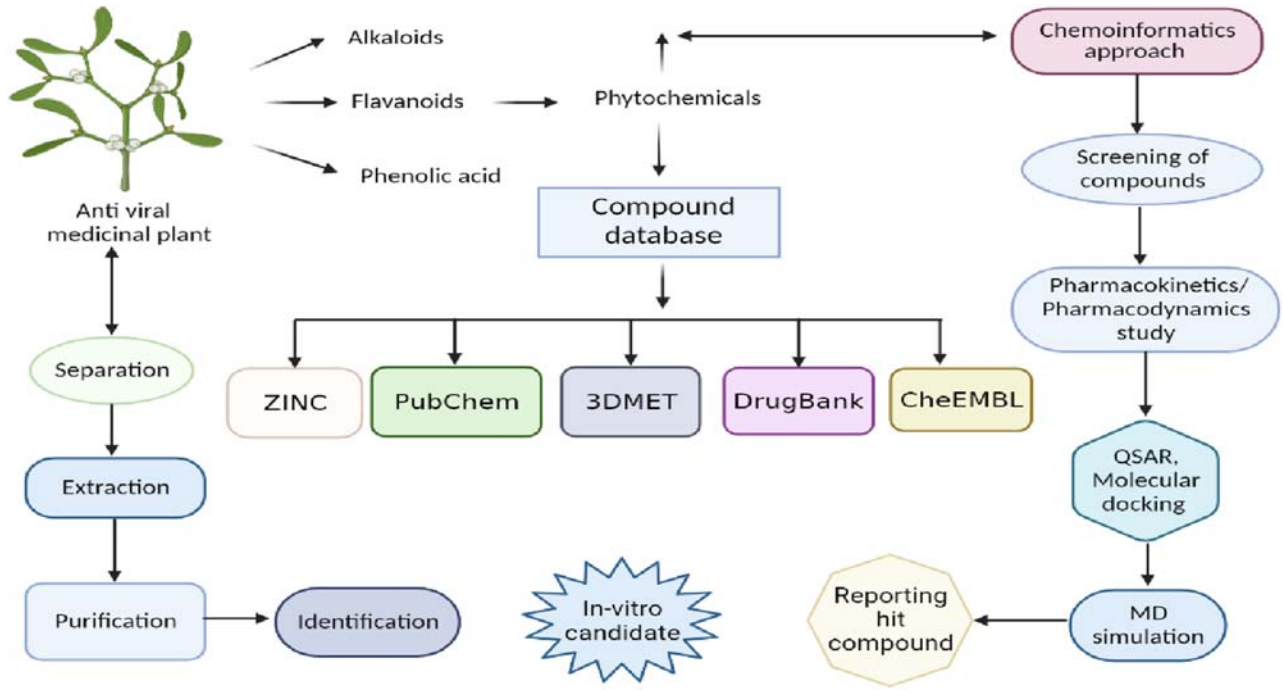


FIGURE 20.5 Process flow diagram to identify/report hit compound by in silico approach.

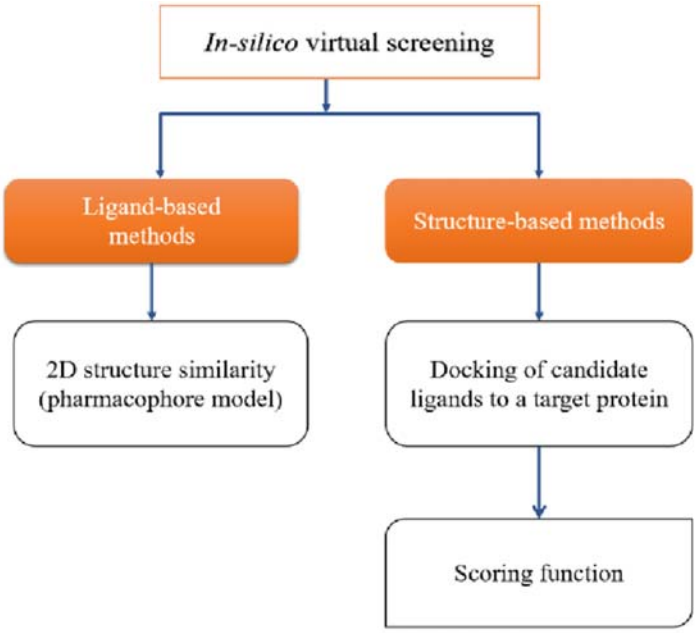


FIGURE 20.6 Flow chart of In-silico virtual screening methods.

Petersen et al., 2019). The virally secreted proteins (Virokines) from viral mimics of host growth factors, chemokine, and cytokines which are potent in preventing host responses. Also, they enhance the reaction of viral replication and proliferation (Kaler et al., 2022; Chen et al., 2005).

Mutations in the viral genome

DNA virus does not indicate numerous mutations in contrast to RNA virus. In the recent pandemic, SARS-CoV has shown mutation modifications and become more lethal to individuals (Sanjuán and Domingo-Calop, 2016). However, as reported, genetic analysis results showed that the virus isolated from the current outbreak of Monkeypox virus appears to have many mutations. Interestingly, these isolates showed 40 mutations that differentiate them from their closest variant. In the ordinary standard evolutionary period of time, one would expect a virus-like MPXV to grasp more mutations that can take over years. But, it seems like MPV has mutated due to its potential to spread among human beings (Siggs, 2014). Mutations in the viral genome have an adverse effect on the virus but not all mutations are harmful to the virus. Since 2017, that has been transmitted to human beings and advised that the virus has a mutation rate of around 10 times the standard mutation rate. Presently, records are not sufficient on how novel mutations in the monkeypox virus have affected the host. However, various mutations in nucleotide sequences of the monkeypox virus from the recent outbreak are alarming to researchers, and additional research is required to recognize the mode of action of these newly developed mutations (Kumar et al., 2022).

Diagnosis

Diagnosis tests are performed to determine the presence of disease in an individual suspected of having a disease. The diagnostic assays are important to detect the monkeypox virus and require to be coordinated with epidemiological and scientific data. Diagnosis of the disease is standing totally on signs and symptoms, history, and laboratory tests. The diagnostics consist of ELISA, PCR, immunohistochemistry, and western blot. The conformational analysis is essential to rule out different feasible infectious diseases like smallpox (MacNeil et al., 2009). Lesion exudate is collected on a swab to carry out the genome diagnose. The genome (DNA) is then amplified by the real-time polymerase chain method. Alternatively, Monkeypox virus proteins are utilized for western blot investigation to verify the infection of monkeypox virus (Adalja and Inglesby, 2022). As consistent with WHO, this method (RT-PCR) is the desired test to diagnose monkey poxvirus disease.

Treatment

Antiviral drugs authorized to treat smallpox can be used to treat monkeypox infection. Cidofovir is a medicinal drug that prevents the activity of viral DNA polymerase and is effective against all poxviruses during research. As per the recent guidelines of CDC, this medicine may be used to cure significantly ill monkeypox sufferers. However, the research outcome stays unknown. Tecovirimat is an antiviral medicinal drug utilized to cure human smallpox disease in pediatric and adult sufferers. This drug is authorized by the FDA (Food and drug administration) and may be utilized to cure monkeypox during this outbreak situation. Vaccinia Immune Globulin Intravenous is used to treat complications due to vaccination of vaccinia, including severe generalized, vaccinia eczema vaccinium, and infections induced by vaccinia virus. It can be used for the treatment of Monkeypox through an outbreak. Brincidofovir is an antiviral drug that the FDA approved to deal with human smallpox disease in person and pediatric patients (Kugelman et al., 2014).

As researchers are more focused more on treatment using traditional synthetic drugs, the scale of improvisation using phytochemicals through bioinformatics approaches is in demand. People are more diverted to make use of traditional medicinal plants and nosode through Ayurveda and Homeopath approaches. The later discussed part in this chapter is proof that the use of natural remedies has a major application and opportunity.

Targets of monkeypox virus

Crystal structure of vaccinia virus thymidylate kinase

Orthopoxviruses duplicate in the cytoplasm of the host cell, like most DNA viruses. They contain their own thymidine and thymidylate kinases in addition to other enzymes needed for the transcription and duplication of the gene. Few herpes viruses code only one enzyme catalyzing both reactions, it is used to accelerate the prodrug. Caillat et al. determined the crystal structures of thymidylate kinase. Even though the virus and human enzymes have identical nucleotide sequences, they differ in their homodimeric association and active-site conformation. The vaccinia thymidylate kinase dimer arrangement is orthogonal and not antiparallel as in human enzymes. This different monomer exposure is related to the presence of a canal connecting the edge of the dimer interface to the TMP base binding pocket. Accordingly, the pox enzyme accommodates nucleotides with bulkier bases such as at dGMP and brivudin monophosphate; these are efficiently phosphorylated and stabilize the enzyme. The BMP-bound structure determines the structural basis for this specificity, opening the gate to developing antipox inhibitors (Caillat et al., 2008).

Crystal structure of VACV D13 viral protein

Poxviruses are giant DNA viruses that create infections in human beings. They differ from enveloped viruses due to their cell membrane is obtained from precursors of membranes congregating onto a viral protein scaffold formed by the D13 protein rather than budding through cellular compartments. Many years ago, it has been shown that rifampicin medicine prevents this process and inhibits the formation of the scaffold. Analyzing the rifampicin action, Garriga et al. identified the clear structures of six D13-rifamycin complexes. This drug binds to an F- ring, at the central channel of the D13 trimer by nuclear magnetic resonance, site-directed mutagenesis, and surface plasmon resonance that A17, a membrane link viral protein, liaises the recruitment of the D13 scaffold by also binding to the F- ring. This rifampicin drug inhibits A17 binding and elucidates the prevention of virus growth. The F- ring of D13 is both conserved in sequence in mammalian poxviruses and crucial for interaction with A17, establishing a target for the making of inhibitor for virus assembly (Garriga et al., 2018).

Such listed compound has numerous applications reported in various viral diseases and is already proven in other sorts of infections, viz. bacterial and fungal. Out of which, reported potential antiviral compounds can be studied with the sophisticated computational approach that will aid in this research on monkeypox. Enlisted compounds can be utilized for in silico analysis.

Potential anti-viral compounds to treat monkeypox

Curcumin

It has been observed that curcumin utilized widely in traditional Chinese medicine, Ayurveda, and Siddha for many years. As it has numerous medicinal properties including anticarcinogenic, antiinflammatory, antioxidant, and antiseptic activity. Furthermore, it is noticed as safe by FDA (Food and Drug Administration). It also functions as an antiviral agent and has

been examined closely in the case of viruses like Ebola virus, HIV, influenza type A virus, and Herpes simplex virus. Since curcumin's advantageous effects outweigh the harmful effects and their role in focusing numerous cell signaling pathways, further preventing the replication of viruses makes it a representative aspirant for an antiviral drug (Mathew and Hsu, 2018).

Kaempferol

Crude ethanol extracts from Ficus benjamina leaves have a strong ability to suppress Varicella Zoster Virus as well as Herpes simplex virus (HSV one and 2) infection in a wet lab by several Bioassays. Also, these assays guided the fractionation of the crude extract and showed that the most effective growth prevention of HSV-1 and HSV-2 was got with the flavonoid fraction. Further, Yarmolinsky et al. (2012) identified this substance with significant antiviral activity from the flavonoid fraction of F. benjamina extracts.

Honokiol

Honokiol is a natural compound isolated from the magnolia plant. To it wide extent utilized in traditional medicine as distension, analgesic, or anxiety relief. Furthermore, it has different biological actions, which include antithrombosis, antitumorigenic, and neuroprotective effects. Moreover, this phytochemical can cure hepatitis C disease in cell models by targeting viral entry. As a result, honokiol is an effective medicinal compound with excellent bioavailability (Fang et al., 2015).

Theaflavin monglayes

Tea is quite popular throughout the world. The healthful effects of tea have been mostly credited to its catechin. The Black tea is produced from the leaf of the Camellia sinensis plant, and it is rich in 30 theaflavin polyphenols, for instance, theaflavin-3-monogallate. Vero and A549 cells were used to examine the effect of individual black tea theaflavins as an antiherpes simplex virus one agent. With the rise 33 of HSV-resistant strains, there is a critical need to develop new antiherpes viral remedies. Analysis of 34 of the cytotoxicity assays examined by MTS showed that theaflavins do not show toxicity toward the Vero cell line (Clark et al., 1998).

Quercetin

The meaning of "Quercetum" is Oak Forest, which belongs to the class called flavonoids that cannot be synthesized in the individual's body. Quercetin is known to reveal antibacterial effects against all strains of bacteria, particularly affecting the gastrointestinal and respiratory systems. Their antiinfective and antireplicative ability give the antiviral properties. Viruses that respond to flavonoids are adenovirus, Japanese encephalitis virus, and respiratory syncytial virus (Anand et al., 2016).

Piceatannol

Piceatannol is also known as trans-3,4,3′,5′-tetrahydroxy-stilbene, which is a natural derivative of polyphenol and resveratrol present in sugar cane, red wine, and grape. It has been proven that it has several activities such as anticancer, antiinflammatory, immunomodulatory, and antiproliferative. Resveratrol has demonstrated a potent antiviral effect on the number of animal and human viruses, which includes both RNA and DNA viruses in the entry, replication, or transcription stage (Fang et al., 2015).

Apigenin

Our study revealed that apigenin inhibits HCV replication. A liver-specific miRNA and miR122 have been reported to be linked with pleiotropic physiological functions such as liver development, and fatty acid metabolism. A particularly intriguing function of miR122 is involved in enhancing HCV replication (Shibata et al., 2014).

Myricetin

It can surge the ubiquitin modification level on TNF receptor-associated factors 3 and 6 reduced by infectious bronchitis virus PLpro. In conclusion, these analyses indicated that myricetin showed antiviral activity against the virus by

suppressing the deubiquitinating activity of PLpro, which can provide new evidence for the prevention and treatment of Ebola virus (Peng et al., 2022).

Homolycorine

Three alkaloids, lycorine, homolycorine, and 2-*O*-acetyllycorine, were purified from the bulbs of *Leucojum vernum* and identified by nuclear magnetic analysis. The alkaloids isolated from *L. vernum* and from other Amaryllidaceae species were studied in vitro for HIV-1 replication inhibitory activity on MT4 cells (Szlávik et al., 2004).

Various online pipelines are in working mode in the field of Bioinformatics that helps to design an in vitro experiments. It will save time to design experiments in vitro and help to reduce cost in terms of materials used in the formation of drugs. The subsequent discussion is more crucial and will act as a pilot scale for the in vivo design of drug and experiments.

In silico tools and techniques in natural products research

Compound database

Bioinformatics databases are computerized and systematized storehouses of biological data that provide a standardized way for searching and updating data. They can be defined as libraries containing data collected from scientific tests, published literature, and computational analysis.

PubChem

PubChem is a public chemical database created by the National Library of Medicine (NLM), an institute within the U.S. National Institutes of Health (NIH). With millions of specific users every month, PubChem is a widely popular chemistry information resource or biomedical research communities in many areas, which includes cheminformatics, chemical biology, medicinal chemistry, and drug discovery. PubChem contains the structure of compounds; they can be downloaded in a variety of formats (SDF, JSON, XML, ASN.1). Importantly, PubChem additionally serves as a source of big information in chemistry, used in information technology science projects for virtual screening, computational toxicology, drug repurposing, etc (Bolton et al., 2011).

PubChem was first released in 2004 as a large repository of three connected databases, covering Substance; emulsion, and Bio-assay. The Substance database contains chemical information further than 200 million entries, while emulsion contains the factual chemical structure data(more than 90 million entries) deduced from the Substance database. Bio-assay contains all of the natural exertion data that has been deposited with PubChem, presently over one million entries, with further than 230 million bioactivities. The data are handed to PubChem by well over 500 contributors (Kim et al., 2016).

ZINC

ZINC is a free public resource for ligand discovery. The database contains over 20 million commercially available molecules in biologically relative representations that may be obtained in popular ready-to-dock formats and subsets. They are annotated with chemical properties like as molecular weight, calculated LogP, and the number of rotatable bonds. This database is available in several file formats which include SMILES, 3D SDF, mol2, and DOCK formats. This database will bring virtual screening libraries to individuals of structural biologists and medicinal chemists (Irwin et al., 2012).

ChEMBL

ChEMBL is an open large-scale bioactivity database, earlier described in the 2012 and 2014 Nucleic Acids Research Database Issues. The content of this database continues to; allow 22 of the database to carry data obtained from more than 65,000 kinds of literature, together with 50 deposited data sets, and data drawn from other databases. In total, there are more than 1.6 million distinct molecules described in it, with 14 million activity values from $>$1.2 million biological assays. These assays are mapped to approximately 11,000 targets, involving 9052 proteins. ChEMBL pursues to collect information sets from both not-for-profit and commercial organizations that want to deliver information to the research society. These deposited data sets carry numerous new molecules as well as their bioactivity data. The library of natural compounds was developed at the University of Dundee. This library is being screened in several neglected disease assays. To date, data about the compound structures and cytotoxicity analyses is available in ChEMBL database (Gaulton et al., 2017).

COCONUT online

Natural compounds have obtained constant attention from researchers because of their applicability in molecular biology, drug discovery, and chemical ecology. They are small compounds synthesized by biological organisms with effective functions in industries and pharmacology because numerous of them are bioactive. This potentiality raised attentiveness interest in natural compounds investigation in the world and in various operation fields, thus, over the time addition of generalistic and thematic natural product databases has been detected. However, at this time, no online resource regrouping all known natural product databases in just one place, would highly simplify these compounds' exploration and permit virtual in-silico screening (Sorokina et al., 2021).

KEGG database

KEGG stands for Kyoto Encyclopedia of Genes and Genomes. It is an information domain for systematic analysis of gene functions in terms of the networks of genes and molecules. The crucial element of KEGG is the pathway database which includes biochemical pathways involving most of the known metabolic pathways and regulatory pathways. KEGG maintains the gene database of all organisms with entire genomes and selected organisms with partial genomes, which are continuous lyre-annotated, as well as the database for chemical structure and proteins. The KEGG molecular catalogs are meant to deliver structural and functional groups of enzymes, natural and chemical compounds as well as RNAs (Kanehisa et al., 2022).

Super Natural II

Super Natural II is an online web server and easy-to-access database of natural compounds. It includes the data for each compound, involving toxicity prediction, medium of action, and pathways information. Additionally, it provides toxicity caution for the use of a particular natural compound. It is the first openly accessible dataset of natural compounds with as numerous as 326,000 compounds and the corresponding wealth of other information. It also gives data about the mode of action of compounds with respect to structurally identical drugs and their respective targets (Banerjee et al., 2015).

Chemoinformatics

Cheminformatics is an area of IT that emphasizes the repository collection, analysis, and modification of chemical data. The chemical data of interest generally cover data on small compound structure formulas, properties, structures, activities, and spectra (natural or artificial). It primarily emerged as a vehicle to aid the drug design and discovery process. However, cheminformatics today take part a progressively significant part in numerous fields of biology and biochemistry.

Virtual screening of compounds through computational techniques

Despite the significant improvements in the field of pharmaceutical chemistry during the past decades, the infection caused by viruses has remained a serious issue for human health. In silico virtual screening utilizes in silico approaches to evaluate the interactions of protein-ligand. Various approaches exist for this aim, which can substantially be classified into two methods (Murgueitio et al., 2012). 1. Ligand-based methods. 2. Structure-based methods.

Ligand-based methods

From known information on ligands with structures, binding to a particular protein target, a protein model may be developed from collected data of the ligands which is known as the pharmacophore model. Ligand-based 2D methods illustrate lower calculation times and therefore are mostly preferred as first classification filters to eliminate the number of compounds to screen in the last stages. Additionally, another method is the use of the 2D chemical similarity analysis procedure which is also a ligand-based method to appropriate compound databases against numerous ligands (Murgueitio et al., 2012).

Structure-based methods

Protein(macromolecule)-ligand docking is a significant structure-based 3D method, which focuses on estimating the ligand binding mode for a target protein with a known 3D structure, predicting the target binding site to be mostly nonflexible and the ligand to be flexible. Furthermore, the scoring function is applied for calculating higher binding affinity of the ligand to the target. Pharmacophores are also utilized as the beginning phase for constructing models of 3D QSAR. Both ligand and

structure-based software for pharmacophore creation are accessible to recognize putative targets or ligands (Kroemer, 2007).

QSAR study

QSAR stands for a qualitative structure−activity relationship that interlinks biological activities with physicochemical parameters. This analysis statistically joins one or multiple molecular descriptors with the structure-property. The statistical models hence designed are utilized for the prediction of the pharmacological activity of recently discovered chemical molecules yet to be tested experimentally (Zhang et al., 2016).

It is also very significant to the study of the pharmacokinetic properties of natural compounds utilized for drug design and development.

ADMET analysis

Experimental assessment of small compound absorption, distribution, metabolism, excretion, and toxicity properties is both costly and time-consuming. These analyses do not always scale in animal and human models. The evolution of computational strategies to optimize pharmacokinetic and toxicity properties may allow the development of discovery leads successfully and swiftly to drug candidates. The pharmacokinetic profile of a compound defines its ADMET properties. Appreciation of the significance of pharmacokinetics has brought about their attention in early-level drug manufacturing, leading to a significant decrease in the range of compounds that are not passed in clinical trials because of inappropriate properties (Pires et al., 2015).

Open access tools to predict the ADMET parameters

ADMETlab It gives pharmacokinetics profiling of query chemicals based on the comprehensive database of chemical compounds with characterized QSAR models. It is an open-access server suitable for the rapid identification of ADMET parameters. It includes several tools like Drug-likeness and systemic Evaluation, pharmacokinetics and aggregator prediction, and Application Domain (Kar and Leszczynski, 2020). SMILES or SDF format of molecules is accepted as input and obtained output is exported as a.csv file.

admetSAR The admetSAR can predict more than 40 significant pharmacokinetics along with numerous environmental toxicity endpoints employing QSAR models. Currently, the updated tool admetSAR2.0 is developed based on 47 models which are used for drug development and ecotoxicity predictions (Cheng et al., 2012). For input, the individuals can give canonical SMILES or can draw the structure of the molecule. Qualitative and quantitative outcomes are obtainable along with the appropriate information (Fig. 20.7).

PreADMET PreADMET is a web-based application for predicting ADME data and building drug-like library using in silico method. Also, it calculates the toxicity of the compound and provides guidance for drug discovery (Source: PreADMET Web) (Figs. 20.8−20.10).

SwissADME SwissADME is an online freely available web server that gives data on water solubility, lipophilicity, drug-likeness, and medicinal chemistry of compounds. It also provides data about Boiled-egg, ilogP, and Bioavailability Radar. To be powerful as a medicine, a potent molecule must reach its location of a target in the body in a proper quantity, and stay there in a bioactive form long enough for biological activity. Drug development involves the evaluation of ADME parameters in the drug discovery process (Daina et al., 2017).

ADMET lab2.0 ADMETlab 2.0 server is freely available. It is a completely updated model of the considerably used AMDETlab online server for the predictions of ADMET parameters of chemical compounds, of which the supported pharmacokinetics associated endpoints are about twice the range of the endpoints withinside the preceding model, which include molecular and physiochemical properties, medicinal chemistry properties, ADME, and eight toxicity parameters (Xiong et al., 2021).

pkCSM pkCSM is a free approachable in silico online tool for the estimation of pharmacokinetics parameters that use graph-based signatures to develop predictive models. This platform can rapidly determine major pharmacokinetics parameters essential for lead-likeness and bioavailability just by giving the query compound as an input in the form of SMILES. It also gives data on molecular properties such as the number of rotatable bonds, toxicophoric

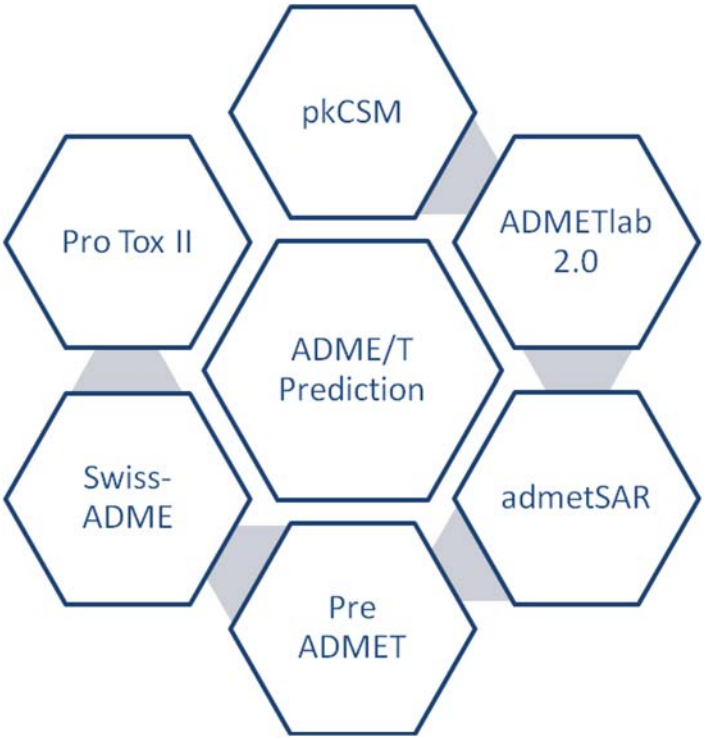


FIGURE 20.7 ADMET prediction tools and webservers.

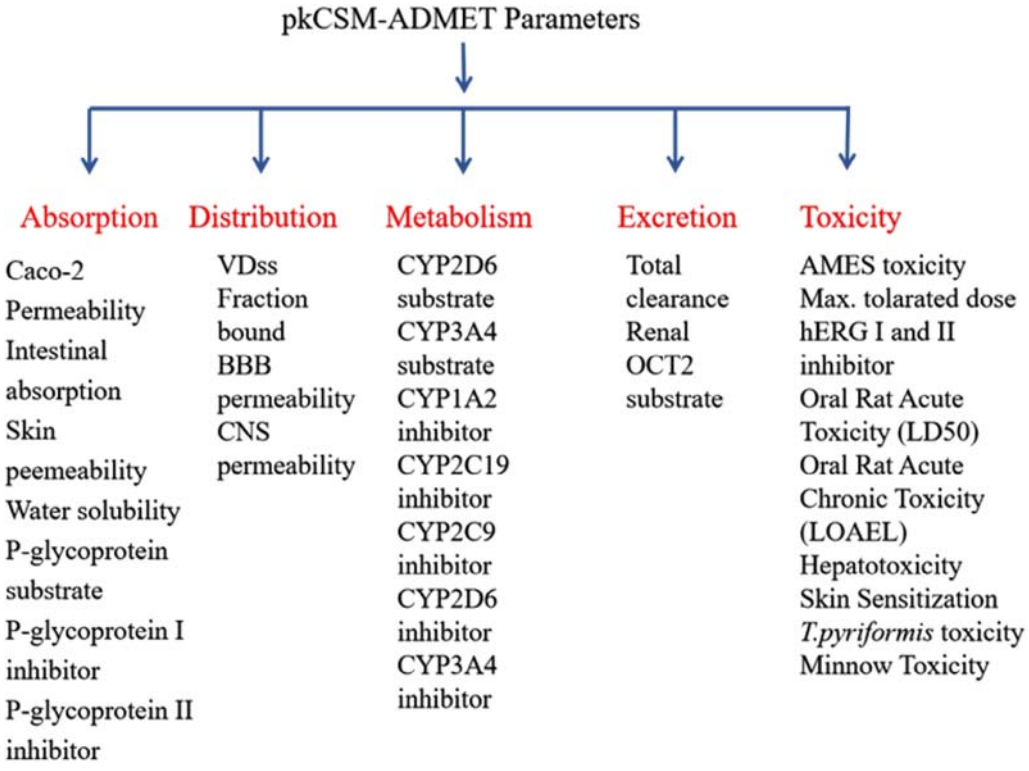


FIGURE 20.8 Workflow of pkCSM webserver.

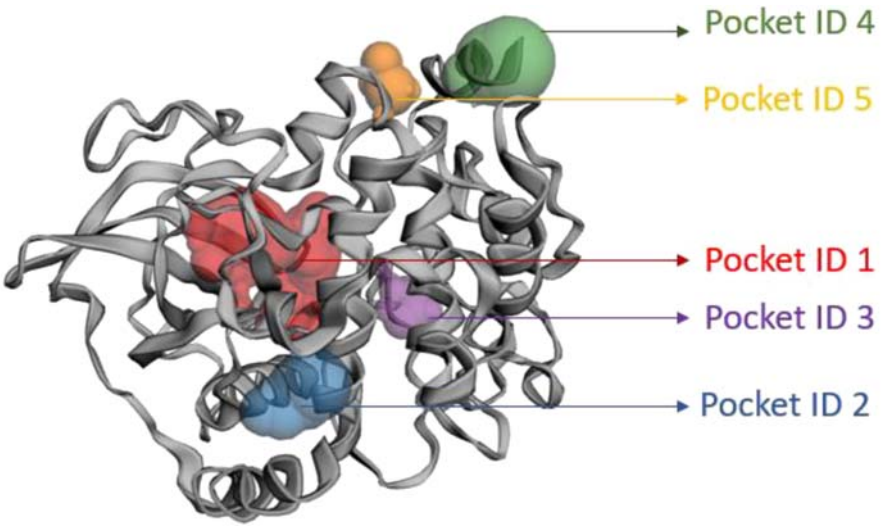


FIGURE 20.9 Visualization of binding pockets in protein through CASTp.

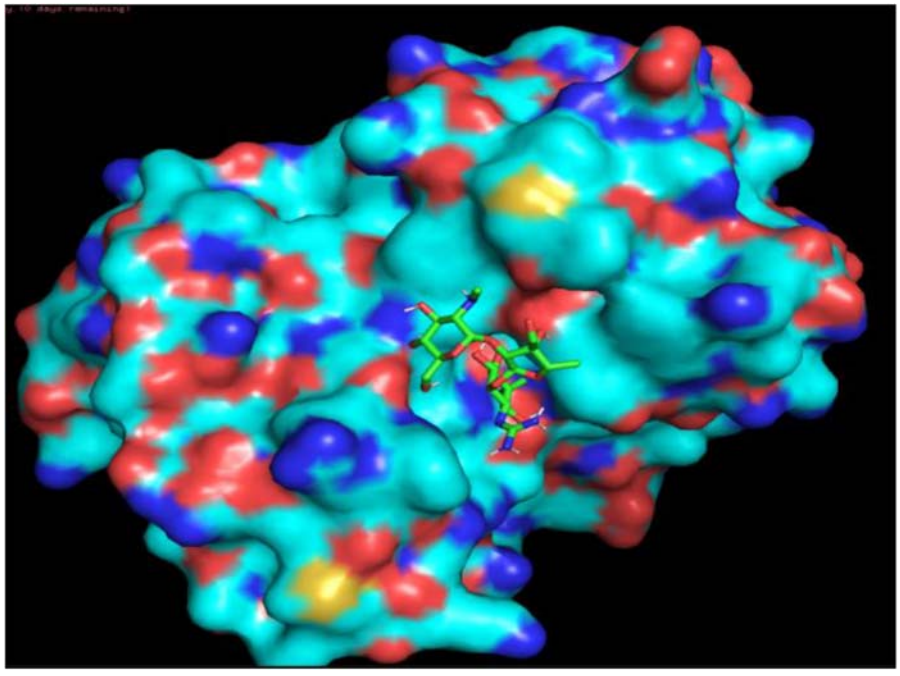


FIGURE 20.10 3D docking representation of ligand with the protein.

fingerprint, lipophilicity, Molecular weight, atomic pharmacophore frequency count, lipophilicity, and surface area. The pkCSM platform considers 14 regression-based models for the identification of pharmacokinetics parameters. Individuals can provide SMILES as input. The outcome data can be collected into an excel or a text file (Pires et al., 2015).

pkCSM: absorption Absorption of the drug is the movement of the drug from the site of administration to the target site and has the following attributes:

- Water solubility: A drug to be absorbed should be in an aqueous solution form at the site of absorption. Water solubility of poorly soluble drugs can be enhanced by physical and chemical modifications of the drug. Water solubility <1 is very soluble.

- Caco-2 permeability: The Caco-2 monolayer cell line is highly utilized as an in-vitro model for the prediction of the absorption of the orally administered drugs. The predicted value is the logarithm of the apparent permeability coefficient (log Papp). Log Papp $>.90$ states high Caco-2 permeability of the drug.
- Intestinal absorption: Intestine is the main site for the absorption of the orally administered drugs. Maximum absorption takes place through the lumen of the small intestine. Intestinal absorption $<30\%$ is poor.
- Skin permeability: It is a significant consideration for the drugs which are delivered transdermally. It is expressed as the skin permeability constant logKp. LogKp > -2.5 states low skin permeability.
- P-glycoprotein substrate: P-glycoprotein, is an ATP-binding cassette carrier. This is present in the inner epithelium lining of the small and large intestines. It is highly expressed in tumor cells. It acts as a barrier by pushing xenobiotics and toxins from the cell. It reduces the bioavailability of the drugs which are its substrates. So, during chemotherapy, it confers multidrug resistance.
- P-glycoprotein I and II inhibitors: The drugs acting as inhibitors of P-glycoprotein can increase the bioavailability of the susceptible.

pkCSM: distribution It is the reversible transfer of the drug from one location to another within the body. Once the drug enters the systemic circulation it must be distributed into interstitial and intracellular fluids. The attributes defining it are given below.

- VDss: It stands for volume of distribution and also is the volume of the drug dose which is needed to be uniformly distributed into the body. The high value of the VD means the drug is delivered in the tissue rather than plasma. Log VDss $< -0.15 \rightarrow$ low and Log VDss $>0.45 \rightarrow$ high.
- Fraction unbound: The medicines in the plasma live in a bound and unbound state. The drug efficacy gets affected by the degree to which it binds to plasma protein, as the more it is in the bound state the less freely it can cross the membrane.
- BBB permeability: The potentiality of a drug to cross the blood-brain barrier is significant parameter to believe. BBB permeability is measured as logBB which is the logarithmic ratio of brain to plasma drug concentration. logBB $>0.3 \rightarrow$ readily crosses the brain logBB < -1 poorly crosses the brain.
- CNS permeability: It is measured as the BBB permeability surface area product (logPS) which is a more direct measurement. logPS $> -2 \rightarrow$ penetrates CNS while logPS $< -3 \rightarrow$ unable to penetrate CNS.

pkCSM: metabolism Drug metabolism is the breakdown of drugs by the various enzymatic system of the body. It is a set of metabolic pathways that chemically modify the structure of the drug and make them vulnerable to excretion. Mostly the lipophilic drugs are converted to hydrophilic products. It generally has two phases phase I reactions and phase II reactions. The tool pkCSM gives information about phase I reactions.

pkCSM: excretion It is the removal of drugs from the body as metabolites or as an unchanged drug. There are several routes like urine, bile sweat, saliva, etc. Most of the excretion takes place through the kidney or liver. The attributes of pkCSM predicting excretion are given below.

- Renal OCT2 substrate: OCT2 stands for organic cation transporter 2, which is a renal uptake transporter that does renal clearance of drugs. It is in the membrane of tubular epithelial cells of PCT. If a drug is a substrate of OCT2 it means that the drug will be cleared out of the body through it.
- Total clearance: Drug clearance is estimated by the proportionality constant CLtot, and occurs primarily as the combining of hepatic and renal clearance. It is necessary to calculate it as it is significant for examining the dosing rates to achieve steady-state concentrations.

pkCSM: toxicity Drug toxicity is the level of damage a drug can cause to an organism. The toxic effects are dose-dependent.

- AMES toxicity: It is a procedure that utilizes bacteria to evaluate whether a particular chemical compound can cause mutations in the DNA of the test organism. It measures the mutagenic potentiality of the drug. The compound showing a positive Ames test is mutagenic.
- Maximum tolerated dose: MTD provides an estimate of the toxic dose threshold of chemicals in humans. It is important for deciding the starting dose for pharmaceuticals in phase I clinical trials. MTD ≤ 0.477 log(mg/kg/day) $\rightarrow$low, MTD > 0.477 log(mg/kg/day).

- hERG I and II inhibitors: The prevention of the potassium channels encoded by the hERG gene is the principal cause of the occurrence of long QT syndrome—leading to fatal ventricular arrhythmia. The Suppression of hERG channels has led to the withdrawal of many drugs from the pharmaceutical market.
- Oral rat acute toxicity (LD50): It is the amount of the drug that kills 50% of the test organism. It is expressed in mg/kg. A lower LD50 is indicative of increased toxicity.
- Oral rat chronic toxicity: Exposure to low mild doses of chemical substances over a long time period is a substantial concern. It aims to identify the lowest dose of a compound that results in an observed adverse effect (LOAEL). The predicted log LOAEL in log value is predicted. These values then require to be concluded relative to the bioactive concentration and remedy lengths required.
- Hepatotoxicity: Drug-induced liver damage is the main safety reason for drug development. In pkCSM, a substance was classed as hepatotoxic if it had a harmful effect on the liver.

Retrieval of a target protein

RCSB Protein Data Bank

RCSB PDB Structure Summary page, which depicts summary data for each PDB entry. These entries are assigned with an alphanumeric PDB ID and contain at least one polymer entity, which refers to chemically unique molecules in an entry. The data, obtained by X-ray crystallography, NMR spectroscopy, or cryoelectron microscopy, and submitted by biologists and biochemists from around the world, are freely accessible on the Internet. Coverage of cell surface proteins in the internet portal progressed significantly and visitors now have access to new 1-D and 3-D visualizations for proteins. Recent improvements and the Mol* 3-D viewer, collaboratively evolved by the Protein Data Bank in Europe and RCSB PDB (Burley et al., 2021).

Identification of binding sites

A binding site is a region on a protein, DNA or RNA, to which a ligand can bind. CASTp measures area and volume of pocket. It may be used to observe surface features and important areas of proteins. It is updated each day and can be accessed at CASTp 3.0: Computed Atlas of Surface Topography of proteins (uic.edu). The Geometric and topological parameters of protein molecules, which include inner cavities, surface pockets, and cross channels, which are of essential significance for proteins to behavior their application. It is a web server that provides online services for locating, delineating, and measuring these geometric and topological properties of protein structures. Besides this, it provides a detailed delineation of all atoms dealing in their formation. It also calculates the exact sizes of the mouth openings as well as volumes and areas if it is there (Tian et al., 2018).

Docking programs

Docking is a method that predicts the preferred orientation of one molecule to a second when a ligand and a target are bound to each other to form a stable complex. The utilization of docking in pharmaceutical research is of great significance, to screening the chemical ligands for their drug likeliness. There are several docking software available that identify the site, geometry, and energy of small ligands interacting with the target protein. Molecular docking is the most frequently used method in structure-based drug design, due to its ability to predict the binding conformation of small ligand compounds to the appropriate binding site. Characterization of the binding behavior plays a crucial role in drug development as well as to explain fundamental biological processes. There are many docking programs available for instance, Flex X, Gold, Gide, Zdock, Rdock, Dock, Autodock, and Autodock Vina (Flores-Holguín et al., 2019).

AutoDock 4 and AutoDockVina

AutoDock 4 and AutoDockVina currently employ several simplifications that affect the results that are obtained. AutoDock is a computational docking program based on an empirical free energy force field and rapid Lamarckian genetic algorithm search method. The AutoDock Vina scoring function is highly appropriate, with no electrostatic contribution, spherically symmetric hydrogen bond potentials, and implicit hydrogens. AutoDock has been demonstrated to perform well with ligands with typical composition and biological size (Flores-Holguín et al., 2019).

It is an open-source program for performing molecular docking. It applies an easier scoring function that permits a hasty pursuit method and gives reproducible results to bigger frameworks with upwards of 20 chemical bonds (Forli et al., 2019).

PyRx

PyRx is a virtual screening software used for computational drug discovery which is used to screen libraries of compounds with the potential drug targets. It enables the user to run virtual screening of the ligands being made and analysis of the results. It is open-source software with an intuitive user interface that runs on all major operating systems (Ubani et al., 2020).

PyRx includes the tools like Auto Dock four and AutoDock Vina for docking, AutoDock tools for generating input files, Python as the programming language, and Matplotlib for 2-D plotting.

GOLD

The Chemscore role was delivered as a scoring function for the macromolecule-small molecule docking program GOLD, and its presentation compared to the primary Goldscore function and two consensus docking protocols, "Goldscore- CS" as well as "Chemscore- GS," in terms of docking perfection, estimating the binding affections, and speed. In the "Goldscore-CS" protocol, dockings created with the Chemscore function (Verdonk et al., 2003).

Molegro virtual docker

MVD is docking simulation program that permits researchers to do simulations. It has been successfully applied to numerous receptors, with docking functions like other docking programs. For instance, AutoDock Vina and Autodock 4. The Molegro Virtual Docker has four algorithms and scoring functions. It also opens the prospect of a detailed statistical examination of docking outcomes.

Docking result visualization

The understanding and optimization of protein-ligand interactions are instrumental to medicinal chemists identifying potential lead compounds. Over the past couple of decades, many powerful standalone tools for computer-aided drug discovery have been developed in research providing insight into protein-ligand interactions. It is an open-source and most utilized docking visualization software. The aim to use PyMOL is because it is well developed, widely used, user-friendly has good quality performance capacities, and in particular, has great importance to allow for access and modification of the structure of molecules stored in PyMOL using Python plugins (Lill and Danilelson, 2011).

Molinspiration

The bioavailability of pharmaceuticals involves the concept of drug-likeness for which Lipinski has proposed myriad rules. The resulting descriptors can be easily determined by applying canonical SMILES to the quickly available online Molinspiration server. The Lipinski rule of five calculates the oral bioavailability of a capable drug and it is usual that compounds fail to pass it, mostly due to their molecular weight and volume. Molinspiration was asses again for the evaluation of the bioactivity scores which are estimates of the ability of the potential drug to function as kinase inhibitors, GPCR ligands, act as Ion Channel modulators, or interact with nuclear receptors and enzymes (Mohan and Geetha, 2017).

Molecular properties of compounds

The drug-likeness property scores have been calculated by considering LogP, nAtoms TPSA, nOHNH, nON, and Molecular Weight. These parameters are determined and mentioned on basis of Lipinski's rule of 5. The compounds which follow this rule and confirmed good drug-likeness scores are considered (Brüstle et al., 2002).

MiLogP: Logarithm of partition coefficient. It is the measure of the lipophilicity or hydrophobicity of the compound. LogP > 0, states the drug is lipophilic and LogP < 0 states the drug being hydrophilic.

TPSA: Topological Polar Surface Area. It is the surface sum over all polar atoms. The results which are below 90 $Å^2$ show good candidate.

nON: nON stands for number of hydrogen bond acceptors. nON ≤ 10 is acceptable.

nONH: nOHNH stands for number of hydrogen bond donors. nOHNH ≤ 5 is acceptable.

Molecular Weight (MW): The low molecular weight of candidates (<500 KDa) means they are easily diffused, absorbed, and transported compared to other large molecules.

N violations: The violations equal to 0 mean that all compounds can smoothly bind to the target receptor.

nAtoms: It is the entire number of atoms in the compound.

Thus, bioinformatics approach in form of in silico methods to run simulations before conducting a major experiment of drug design and development has always been crucial in the research field. Such approaches and the use of these tools will

save time and clear ideas of what to focus on and what to not while the development of drugs against this viral infection. Globally we succeeded in the Covid-19 situation but we are not sure about what will come again in the future in terms of infections and epidemic or pandemic conditions.

Conclusion

One of the most important steps in virtual screening is target selection. One can identify targets mostly in two ways. One is by literature mining and the other is by using target prediction tools. In this chapter, the target for the monkeypox virus is selected from a literature survey. Additionally, the antiviral phytochemicals were selected from published articles. These antiviral compounds can be scrutinized based on molecular and pharmacokinetics properties based on Lipinski's rule of 5 (Absorption, Distribution, Metabolism, Excretion, and Toxicity). Upon docking, the lead compound can be reported based on the binding affinity of compounds with the target protein. The compound with a given docking pose and binding score can be used as a lead compound. And this lead compound can further be used to evaluate in vitro and in vivo assays as monkeypox virus inhibitors.

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Conflict of interest

The authors have no conflict of interest.

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Chapter 21

In silico identification of natural product inhibitor for multidrug resistance proteins from selected gram-positive bacteria

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Introduction

The search for remedies against several human ailments such as pain, illness, and diseases dates back years before, even though their causes were unknown. History validates the use of a wide range of natural products available for the treatment of infections, such as herbs, honey, and even the crumbs of moldy, wheaten loaf of bread, which was said to have beneficiary effects in treating infections related to the skin, particularly in Egypt, China, Serbia, Greece, and Rome (Dhingra et al., 2020).

In the second half of the nineteenth century, Robert Koch observed the inhibitory effect of the microorganism, which Louis Pasteur later confirmed, suggesting that it might be used as medicine. By the end of the nineteenth century, Paul Ehrlich, a German Bacteriologist, started seeking new compounds with antimicrobial activity (Keyes et al., 2008). This marked the need to search for the infections' treatments, and a series of experiments finally led to the introduction of the first antimicrobial Salvarsan in 1910, which was used to treat syphilis (Hutchings et al., 2019; Zaffiri et al., 2012). The initiation of the antibiotic era was then marked by the discovery of penicillin by Alexander Fleming, also termed a fortunate accident (Zaffiri et al., 2012).

Evidence has always been documented about the increasing rate of bacterial resistance to therapeutic drugs (Davies, 1996). Hence, because of rising resistance in the microbes, the golden era of antibiotics (1940−1980s) could not last long (Talebi Bezmin Abadi et al., 2019).

Resistance to therapeutic drugs, most commonly known as antimicrobial resistance, happens when the pathogens develop the ability to overcome the drugs applied to eradicate them. The extent of bacterial survival in an environment is highly facilitated by the ability to adapt and evolve. Therefore, it can be said that natural selection plays a significant role in bacterial evolution, where inadequate exposure to antimicrobial drugs has acted as a stressor, favoring resistance (Guevara Salazar et al., 2021).

Through time, bacterial strains have now developed resistance to numerous antibiotics with the help of overexpression of proteins and genes that can eliminate or modify the antibiotics conferring them to be multidrug-resistant (Mwangi et al., 2019). Multidrug resistance presents a severe problem in the treatment of bacterial infections. Increased infections due to multidrug-resistant pathogens impose a severe demand for alternative approaches to control and cure.

Over the years, there have been developments of antimicrobial agents that are said to be effective against several Gram-positive multidrug-resistant organisms. *Staphylococcus aureus* is one of the most common multidrug-resistant Gram-positive

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pathogens and is observed to be one of the major causes of morbidity and mortality worldwide and has been assigned under the list of serious threats by Center for Disease Prevention and Control (CDC), 2019. Methicillin-resistant *Staphylococcus aureus* has caused 323,700 cases & 10,600 deaths as per the CDC threat report 2019. Another Gram-positive multidrug-resistant bacterium under the severe threat list is *Streptococcus pneumonia*, which causes several community-acquired infections. Studies on some strains of *Streptococcus pneumonia* show it is resistant to antibiotics such as clindamycin, erythromycin, cotrimaxazole, and penicillin (Koulenti et al., 2019).

Multidrug resistance imposes a significant difficulty in treating diseases caused by them. Studies on these resistances have suggested multiple mechanisms exhibited by the microbes to confer resistance. A microbe can be intrinsically resistant to antibiotics before prior exposure to the antibiotic or can be gained via spontaneous mutation, DNA transfer or uptake of new genes by horizontal gene transfer (Blair et al., 2015).

Microbes use one defense strategy to overcome the antibiotic provided. The defense strategies used by them are also known as a resistance mechanism. Mechanisms that are observed that help them survive are (a) enzyme inactivation or degradation, (b) decreased cell membrane, (c) efflux, (d) altered target site, (e) altered target enzymes, and (f) protection of the target site (Ndagi et al., 2020; Nikaido, 2009).

Streptococcus genus comprises Gram-positive organisms, spherical or oval, typically arranged in chains. Most streptococci are oxidase- and catalase-negative, and many are facultative anaerobes. Numerous species of *Streptococcus* have been recognized as opportunistic pathogens for humans and/or animals (Georrge and Umrania, 2012; Lannes-Costa et al., 2021). The pathogenic strains of *Streptococcus* are Erythromycin-resistant Group A *Streptococcus* and Clindamycin-resistant Group B *Streptococcus* has been reported to be on the list of concerning threats due to the increase in lethality and emergence of antibiotic resistance (Peraman et al., 2021).

Staphylococcus is a genus of Gram-positive bacteria. Under the microscope, they appear round (cocci) and form grape-like clusters. The *Staphylococcus* genus includes 45 species and 24 subspecies (Gherardi et al., 2018). *Staphylococcus* can cause various diseases in humans and other animals through either toxin production or penetration. Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Staphylococcus aureus* (VRSA) are considered to be concerning threats due to their nature being antibiotic-resistant (George, 2016; Peraman et al., 2021).

Bioinformatics plays an important role in the design of new drug compounds. Rational drug design is used in the biopharmaceutical industry to discover and develop new drug compounds. The development of new molecular biology and computer science methods has significantly improved drug design tools. More and more new drugs are developed with the help of computer techniques. The field of bioinformatics has become a major part of drug design that plays a key role in the validation of drug targets. Bioinformatics can help understand complex biological processes and help improve drug discovery (Gajipara and John. J, 2018).

This chapter focuses on identifying natural product inhibitors for multidrug resistance proteins from multi-resistant gram-positive genus *Streptococcus* and *Staphylococcus* with the help of in silico virtual screening approach. These comprise computational methodologies in which a compound subset is selected and retrieved from a database to predict its binding mode with a target protein. This employed identification and retrieval of multidrug-resistant proteins using various steps followed by three-dimensional structure prediction and structure assessment. The ligand library was constructed, the docking procedure was followed, and the docking score was analyzed.

Materials and methods

Pathogenic strains of the genus *Streptococcus* and *Staphylococcus* were laid open to the antimicrobial target prediction strategy. The methods and strategies used in this study have been summarized in Fig. 21.1.

Retrieval of multidrug resistance proteins

Protein sequences associated with multidrug resistance were retrieved from (a) a Database of multidrug resistance genes (DbMDR), (b) a Database of drug targets for resistance for resistant pathogens (DDTRP), and (c) Antibiotic resistance genes database (ARDB).

Clustering by CD-HIT

Cluster Database at High Identity with Tolerance (CD-HIT), this program is used to produce a "non-redundant" (nr) cluster file by removing "redundant" or highly similar sequences. The multidrug protein sequences retrieved from the above databases were clustered using the CD-HIT program (https://www.bioinformatics.org/cd-hit/) to produce a closely related protein family by removing redundancy.

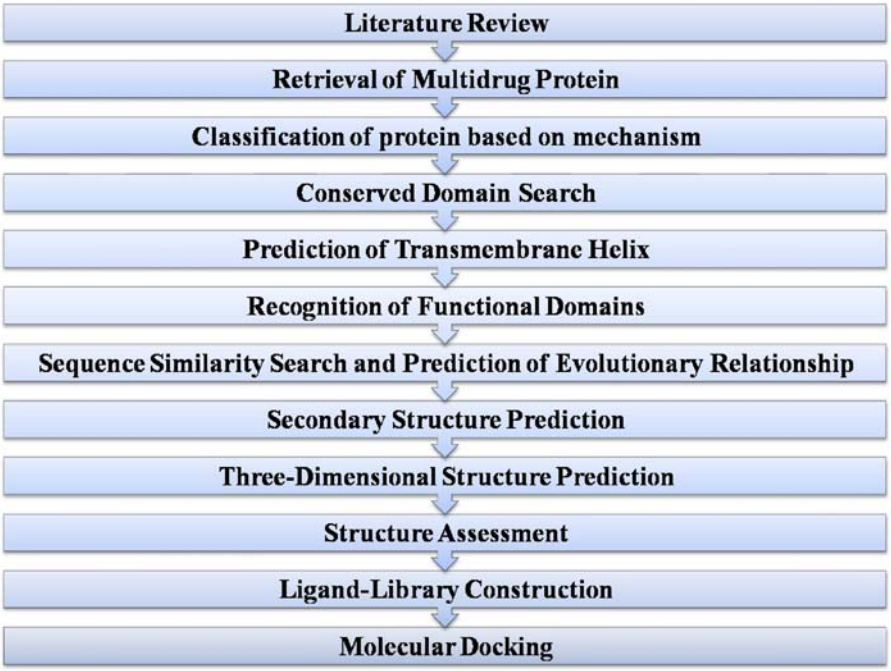

FIGURE 21.1 Flow chart of the methodology.

Mechanism-based protein classification

The clustered proteins obtained from the above steps were then classified based on the involvement of the proteins in the various mechanisms that infer multidrug resistance. Bacterial proteins play a major role in developing resistance through several mechanisms. These proteins also act as a direct target for antibiotics as they are involved in the organism's key mechanism, such as the synthesis of the cell wall and nucleic acids. Hence, the resistance is linked with changes in these proteins, such as modification of the proteins. The bacterial protein that assures pathogens' resistance is said to belong to large superfamilies (Egorov et al., 2018). One of the most frequently employed strategies in biological systems that show resistance to cytotoxic drugs is the extrusion or efflux of these compounds from the cell via membrane proteins. These membrane proteins act like bilge pumps, decreasing the drug's intracellular concentration to subtoxic levels. These efflux pump proteins are classified into seven families/superfamilies based on substrate specificity and energy source (Chitsaz and Brown, 2017). Hence, efflux pump proteins were given more importance in this study.

Conserved domain search

Using the Conserved Domain Database (CDD), the conserved domain in the protein sequences was analyzed. CDD is a resource for protein annotation that uses RPS-BLAST, a PSI-BLAST variant for identifying conserved domains (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

Prediction of transmembrane helix

To predict the region of the transmembrane helix in the protein TMHMM server (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) was used. TMHMM is a membrane protein topology prediction method based on a hidden Markov model. Additionally, TMHMM can discriminate between soluble and membrane proteins with specificity and sensitivity better than 99%, although the accuracy drops when signal peptides are present. This high accuracy allowed us to predict reliably integral membrane proteins in an extensive collection of genomes (Krogh et al., 2001).

Recognition of functional domains

For the recognition of the functional and conserved domains present in the protein sequences, BLOCK Database (http://blocks.fhcrc.org/) was employed. The Blocks Database has a collection of blocks representing protein families and can find conserved blocks. Blocks are multiple aligned ungapped segments corresponding to the most highly conserved regions of proteins.

Sequence similarity search and prediction of evolutionary relationship

Phylogenetic tree construction was performed to predict the evolutionary relationship among the protein sequences. The protein sequences were also aligned using multiple sequence alignment tools to find their sequence similarity. For Multiple Sequence Alignment, ClustalW was used. Phylogenetic analysis was conducted from the Multiple Sequence Alignment to predict the protein sequences' shared evolutionary origins. PhyloDraw was used as a visualizing tool for viewing the phylogenetic tree.

Secondary structure prediction

For the prediction of secondary structure, Jpred (http://www.compbio.dundee.ac.uk/jpred/) was used. Jpred is a secondary structure prediction software used to predict accurate secondary structures using the Jn*et al*gorithm (Cole et al., 2008).

Three-dimensional structure prediction

For the prediction of the three-dimensional structure, the following web servers were used; SWISS-MODEL (https://swissmodel.expasy.org/); it is a web-based expert system dedicated to homology modeling of protein 3D structures, Iterative Threading ASSEmbly Refinement (I-TASSER: https://zhanggroup.org/I-TASSER/); I-TASSER server is an internet service for protein structure and function predictions, and Local Meta-Threading Server (LOMETS: https://zhanggroup.org/LOMETS/); a local threading meta-server, that generates quick and automated predictions for protein three-dimensional structure prediction.

Structure assessment

The accuracy of the predicted three-dimensional structure of the protein was checked by using; (a) Atomic Nonlocal Environment Assessment (ANOLEA: http://melolab.org/anolea/), it is an online web server that performs energy calculations at the atomic level in protein structures and (b) PROCHECK (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/), it is an online program to check the stereochemical quality of protein structures.

Ligand-library construction

Ligand-library was constructed to perform molecular docking studies to identify the potent inhibitor for the multidrug-resistant protein. The DrugBank database (https://go.drugbank.com/) was used to retrieve 13 (13) molecules, and the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) was used to retrieve 3D structures of the molecules that were selected as to be ligand.

Molecular docking

Molecular docking is a drug-design approach that would describe the "best-fit" orientation of a ligand that binds to a particular protein of interest. Molecular docking uses a matching technique that describes the protein and the ligand as complementary surfaces. The second approach simulates the docking process, in which the ligand−protein pairwise interaction energies are calculated. Four targets were selected for the docking procedure. Molecular docking aims to achieve an optimized conformation for both the protein and ligand and relative orientation between protein and ligand such that the free energy of the overall system is minimized.

Results and discussion

Results by CD-HIT

Protein sequences were retrieved from three databases: (a) Database of multidrug resistance genes (DbMDR); (b) Database of drug targets for resistance for resistant pathogens (DDTRP), and; (c) Antibiotic resistance genes database (ARDB), which CD-HIT clustered. The CD-HIT cut-off value was 0.9 to group 90% of the similar sequence together. This analysis found 58 representative sequences for *Staphylococcus* and 36 for *Streptococcus.*

Classification of proteins

Based on the mechanism of multidrug resistance, the proteins were manually classified. Among all the proteins involved in multidrug resistance, efflux pump-related proteins have been given preference.

Results by Conserved Domain Database (CDD)

CDD was performed to find domains of selected proteins. From both genus, only two domains, namely MFS and NorM were selected as they were common to both, as summarized in Table 21.1.

Multiple sequence alignment and phylogenetic analysis

Multiple Sequence alignment and phylogenetic tree construction were done using the following selected proteins:

a. Putative MDR permease; transmembrane efflux protein
b. Putative MDR permease; multidrug efflux pump
c. Major facilitator transporter one
d. Major facilitator transporter two
e. MATE efflux family protein one
f. MATE efflux family protein two
g. MATE efflux family protein three
h. unnamed protein product
i. Na+-driven multidrug efflux pump

Multiple sequence alignment shows the similarity among the sequences based on which the phylogenetic tree was constructed. The phylogenetic tree shows selected nine proteins' evolutionary relationships (Fig. 21.2).

Findings from TMHMM

TMHMM is used to find the number of the inside, outside, and transmembrane helix. Most of the regions of the efflux pump were found to fall under the transmembrane helix.

Three-dimensional structure prediction

The three-dimensional structure was predicted for the selected four targets, as depicted in Fig. 21.3.

Ligand-library construction

The list of molecules tested for the docking study is depicted in Table 21.2.

Docking results

All these 13 molecules were docked against the selected multidrug conferring efflux pump proteins. Four targets for the docking study as summarized in Table 21.3.

All the molecules showed good binding with the efflux pump proteins. The docking studies show that natural products can be better inhibitors than already available antibiotics and chemical molecules. Table 21.4 represents the docking results.

TABLE 21.1 Total Number of Domains in both the genus for Efflux Pump Mechanism (the highlighted part has been selected for further analysis).

Domain	*Streptococcus*	*Staphylococcus*	Total
EmrB	0	2	2
2A0121 (H+ antiporter protein)	1	0	1
MFS	2	2	4
NorM	3	2	5
PRK14995 (methyl viologen resistance protein SmvA)	0	1	1
MatE	0	2	2
MdlB	0	2	2

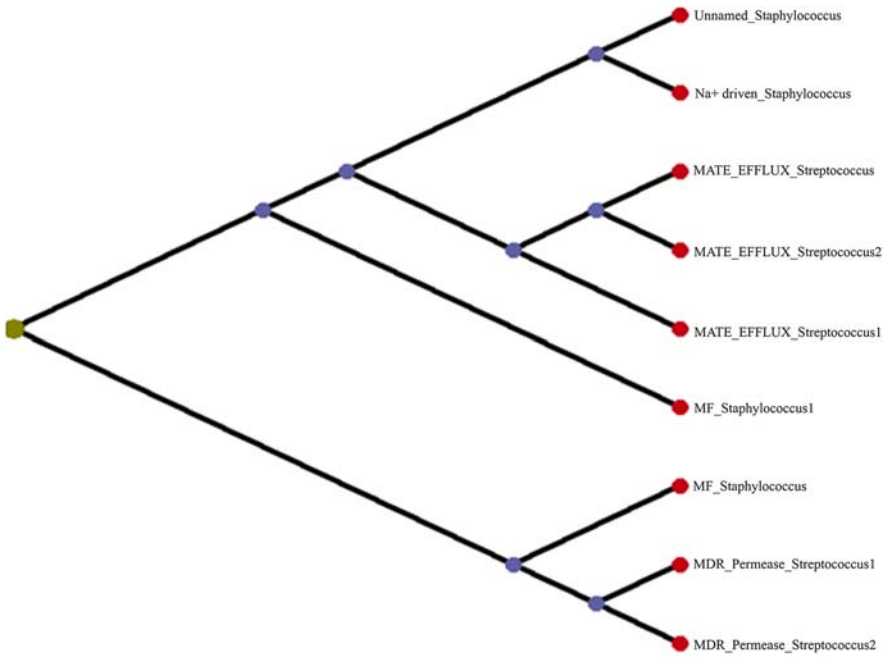

FIGURE 21.2 Phylogenetic tree of the protein sequences from *Staphylococcus* and *Streptococcus* genus.

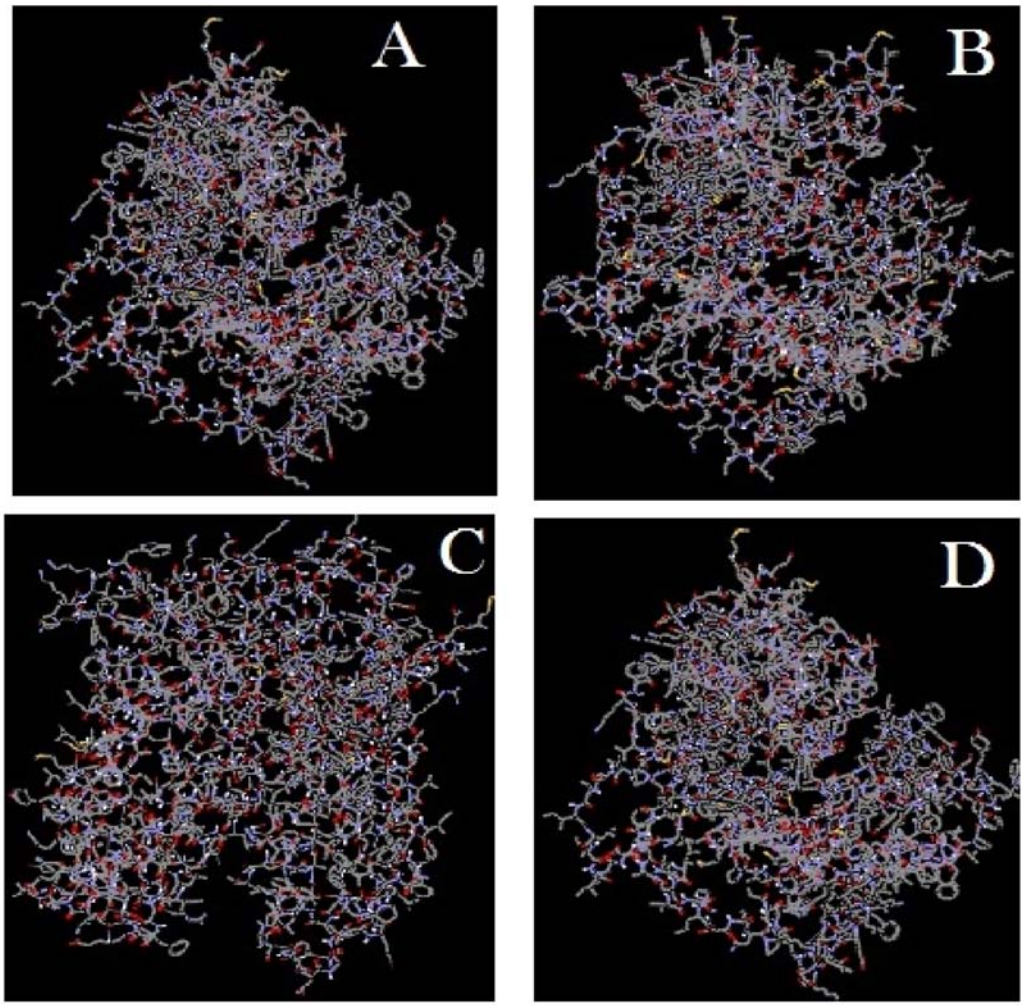

FIGURE 21.3 The predicted three-dimensional structure of the selected four targets. (A) Target-1: (WP_011074691.1) multidrug efflux MFS transporter MdeA; (B) target-2: (WP_001006445.1) MULTISPECIES: multidrug efflux MFS transporter NorC; (C) target-3: (WP_001036273.1) MATE family efflux transporter and; (D) target-4 (WP_000278,524.1) Sodium-coupled multidrug efflux MATE transporter PdrM.

TABLE 21.2 List of molecules selected for docking studies. (Natural products, chemical molecules, and antibiotics have been selected.)

Sl. No.	IUPAC name	PubChem ID
1.	Delphinidin 3-rhamnoglucoside	192919
2.	Tamarixetin 7-rutinoside	44258036
3.	Globulin G	74329879
4.	1pf8	23586025
5.	STK722046	3127896
6.	Echinacoside	5281771
7.	Acteoside	5281800
8.	MLS001110119	23603139
9.	2bdy	11663836
10.	NSC204856	5458426
11.	Nadifloxacin	4410
12.	6-demethyl-9-(N,N-dimethylglycylamido)-6-deoxytetracycline	54692950
13.	Flumequine	3374

TABLE 21.3 List of targets selected for docking studies.

Target NO.	NCBIID	Protein name
Target 1	WP_011074691.1	Multidrug efflux MFS transporter MdeA
Target 2	WP_001006445.1	MULTISPECIES: multidrug efflux MFS transporter NorC
Target 3	WP_001036273.1	MATE family efflux transporter
Target 4	WP_000278,524.1	Sodium-coupled multidrug efflux MATE transporter PdrM

TABLE 21.4 Docking study results.

IUPAC name	PubMed ID	Docking score			
		Target1	Target2	Target3	Target4
Delphinidin 3-rhamnoglucoside	25244897	−6.237	−7.960	−6.358	−10.176
Tamarixetin 7-rutinoside	5483811	−6.625	−8.240	−8.914	−9.775
Globulin G	24839946	−6.190	−8.169	−7.216	−9.745
1pf8	23586025	−7.036	−5.459	−7.250	−9.609
STK722046	3127896	−6.036	−8.069	−7.079	−9.386
Echinacoside	5281771	−4.503	−6.683	−8.409	−8.976
Acteoside	5281800	−6.362	−5.894	−8.346	−8.952
MLS001110119	23603139	−6.296	−6.714	−5.820	−8.493
2bdy	11663836	−4.572	−6.990	−5.563	−7.727
NSC204856	5458426	−6.871	−6.709	−4.665	−7.611
Nadifloxacin	4410	−4.518	−6.380	−6.667	−6.884
6-demethyl-9-(N,N-dimethylglycylamido)-6-deoxytetracycline	54692950	−5.826	−3.236	−7.829	−6.474
Flumequine	3374	−5.468	−6.141	−6.036	−6.282

Conclusion

Studies are finding new opportunities to inhibit its resistance. Undoubtedly, antibiotic therapies have transformed the treatments of infections since it was discovered to overcome life-threatening multidrug-resistant pathogenic infection. However, an increase in resistance to antibiotics is posing a threat. Hence, it is now the need for a condition that new antimicrobials have to be brought up. Bacteria being versatile and adaptive have imposed a series of threats by their capability to handle toxic substances. The current in silico study aims to help to treat MDR infections. The efflux proteins of *Staphylococcus* and *Streptococcus* have a higher identity in the sense of sequence structure and function. All efflux proteins fall under only NorM, efflux_EmrB, MatE, MdlB, MFS, Na+ -driven multidrug efflux pump family. Most of the regions fall under the transmembrane helix. The docking show that natural products can be better inhibitors than already available antibiotics. Only Nadifloxacin, 6-demethyl-9-(N,N-dimethylglycylamido)-6-deoxytetracycline, and Flumequine binds with the target; it shows that only these three antibiotics can efflux through NorM family of efflux proteins. Other molecules with a better docking score have a high affinity toward the target; they may bind irreversibly with NorM proteins and block the efflux mechanism. Hence, it is now essential to rewire the thoughts of developing antimicrobial agents and start looking to natural sources for tracing the availability of molecules that can fight resistance. More biological confirmation is required to validate this in silico work.

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Chapter 22

Virtual screening and molecular docking for the identification of potential antibreast cancer agents targeting estrogen receptor

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Introduction

Currently, cancer is regarded as a significant global public health concern. Breast cancer is one of the most common malignancies to affect women worldwide. It is complicated to treat cancer (Mutazah et al., 2020). Even though supplementary medicines have only a little evidence supporting their safety and efficacy, most people in underdeveloped nations rely on these treatments to cure various illnesses. Traditional medicine is more widely accepted by people in places like India because there is greater trust in evidence-based research (Rathinavel et al., 2020). A useful approach for identifying new therapeutic leads is provided by purified phytochemicals derived from medicinal plants. Natural remedies have long been used to cure a variety of ailments in traditional medicine (Alamri et al., 2020). Natural products are the primary sources of molecular structure information for drug discovery. The reservoir of natural products has a significant degree of chemical originality and diversity; about 40% of the reported natural compounds have chemical scaffolds that are original and not the result of synthetic chemistry. This is a significant motivating factor that pharmaceutical researchers and corporations focus on while looking for and developing new drugs from natural substances. It is impractical to separate and gather all the intriguing products and randomly screen them because it would be excessively costly and time-consuming (Shen et al., 2003).

What is virtual screening

In order to speed up the drug development process, researchers often apply computer tools for virtual screening, which involves comparing virtual datasets, such as those used in silico lab, against virtual targets. Through computational analysis, virtual screening fundamentally aims to explore enormous virtual databases of chemical structures or virtual libraries and choose a small subset of candidate compounds most likely to be active against a particular biological receptor. For the purpose of developing new drugs, it is crucial to have a strong knowledge of molecular recognition mechanisms because the majority of pharmaceuticals interact with protein targets like receptors or enzymes. Docking simulates the ligand-receptor binding process and can be used in virtual screening methods. On the other hand, a pharmacophore is a condensed 3D model of the primary structural features of a collection with well compounds or the targeted receptor associated with the biological activity. Thus, using a pharmacophore as a query structure in a database search for virtual screening, one can indirectly map the recognition of ligands and receptors (Waszkowycz et al., 2001).

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Why do virtual screening

The efficacy of this approach for discovering active compounds has been demonstrated by recent promising improvements in virtual screening. But even utilizing more complex computational techniques like flexible docking, it is currently possible to examine up to several hundred (10^5) molecules daily. As a result, the medicinal chemists' ambition of going "from structure to hit in weeks" has come true. This is one of the reasons why most pharmaceutical companies have embraced virtual screening. Virtual screening is currently an important step in generating leads for several businesses, such as Locus Discovery, Inc. [http://www.locusdiscovery.com/] and Structural Bioinformatics, Inc [http://www.strubix.com/] (Muegge and Rarey, 2001).

How to do virtual screening

Two strategies have been applied to virtual screening:

(1) Using pharmacophore-based database searching to find probable hits in databases, typically when the targets' 3D structures are unknown.
(2) Docking is used to ranking the databases, with the assumption that the targets' 3D structures are available.

These two methods are typically used in tandem because the first can swiftly filter out chemicals, and the second can more precisely assess ligand-receptor binding (Fig. 22.1) (Shen et al., 2003).

Pharmacophore-based screening

A 3D-pharmacophore structure is created by looking at the correlation between structure and activity, a number of active analogs, or the X-ray crystal structure of a ligand-receptor complex. In a 3D database search, this 3D-pharmacophore structure can be used as a query structure to choose small molecules from molecular databases that have the pharmacophore components and may be able to achieve the geometric pharmacophore requirements. For the actual pharmacologic experiment, the chosen compounds are taken from a commercial or organic synthesis. The method of pharmacophore-based virtual screening has produced a large number of successful hits (Eringis and Goldman, 2002).

Docking-based virtual screening

It requires knowledge of the receptor and ligand basic features. The process consists of four steps: receptor modeling, compound database generation, computer screening, and hit molecules postprocessing. Most docking programs can take ligand flexibility into consideration. However, the flexibility of receptor proteins poses a challenge for docking simulation, especially for docking-based virtual screening (Joseph-McCarthy, 1999).

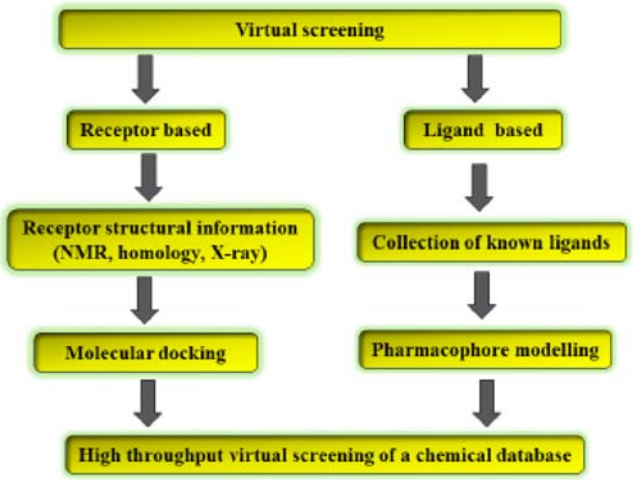

FIGURE 22.1 A flowchart of two main in silico virtual screening techniques.

Molecular docking

Ligand preparation

From DrugBank (http://www.drugbank.ca/), ChemSpider (http://chemspider.com), and PubChem (http://pubchem.ncbi.nlm.nih.gov/), the structure of phytochemicals was retrieved. The 3D structure generator CORINA generated the 3D structures of the molecules using compounds' canonical smiles. All compounds performed energy minimization and molecular optimization using Arguslab 4.0.1. The geometry of Arguslab 4.0.1 was optimized using the Austin Model one semiempirical quantum mechanics force field. In order to obtain the ideal conformer, energy minimization and geometry optimization were used. The resulting structures with the lowest energy were stored in.pdb format (Arba et al., 2020). Most of these sizable databases, which include commercially accessible substances, include the NCBI PubChem, eMolecules (www.emolecules.com), and ZINC. AutoDock input files from manufacturers like ChemBridge, Otava, and Asinex are now available by ZINC for several different libraries (Karaman et al., 2018). A protein target's initial structure can also be created using homology modeling, which uses structural and sequencing information from known protein families as a template.

Target preparation

Preparing the protein structure is crucial in virtual screening (Shen et al., 2003). Proteins were prepared for virtual screening and molecular docking by removing cocrystallized ligands and water molecules and storing them in PDB formats as monomers (Alamri et al., 2020). Currently, there are more than 19,900 macromolecule structures in the Protein Data Bank (PDB), the majority of which are proteins. The PDB (http://www.resh.org/pdb/intex.html) receives 50−100 new structures every week. The structures of numerous important targets or their homologous proteins have been established due to the rapid advancements in genomics and structural biology. The main structure of a protein target can frequently be acquired from the PDB, where the protein structures were created using nuclear magnetic resonance (NMR) spectroscopy or X-ray crystallography (Shen et al., 2003).

Compounds' 3D structures were either manually created in ChemDraw Ultra 7.0 and saved in a single SDF file, or they were obtained from the PubChem database, which is available online at https://pubchem.ncbi.nlm.nih.gov (Alamri et al., 2020). The target molecule often has an easily identifiable active site. In these situations, it is optimal to have a target structure attached to an inhibitor or substrate, which compels the target to adopt a conformation more appropriate for binding additional substances. In certain circumstances, we are presented with a target molecule that has never been studied before and for which we have no idea where ligands might be able to bind. In these circumstances, we can do blind docking to the complete protein to find the regions that bind ligands tightly (Cosconati et al., 2010).

Docking score

Protonation state calculations are the next stage, and a suitable force field should be used to optimize the protein. Identifying a binding site's space volume is the next step. This is vital since a tiny binding site restricts the search space that may be used, whereas a more significant receptor site requires more computational effort. In this step, two approaches are possible. To directly select a binding site, a ligand-receptor combination's 3D structure is often used. Other times, a biological function study such as mutagenesis must be used to manually select the binding site, especially for receptors for which ligand-bound structures are not yet available. Some algorithms exist for determining the binding location (e.g., SiteID) (Zhao et al., 2013). Building the grid of the binding site is another step that is occasionally required for docking. The docking software will determine whether this step is necessary; some programs already contain this step (e.g., in DOCK and AutoDock). The peptide, cofactor, and all water molecules were eliminated. Any protein residue within 6 Å of any heavy atom of the various ligands was considered the binding site, which was confirmed by the location of the native ligand. To score all docking configurations, GoldScore was applied as the fitness function. A few compounds were chosen to be tested in vitro after all docking poses had been visually inspected and assessed. The docking poses that resulted were saved. By analyzing protein-ligand interactions in the obtained docking poses, drugs were chosen for testing. Before docking, all molecules from the structures were taken out except for the zinc ion (Zn^{2+}). Before docking, structurally bringing water molecules were inserted into the binding location of the protein structures (Fig. 22.2) (Friesner et al., 2004).

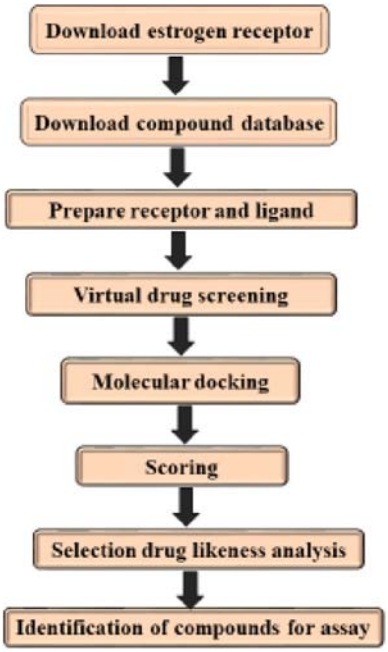

FIGURE 22.2 Flowchart of an in silico method for identifying potential breast cancer drug candidates.

Types of a scoring function

For instance, the FlexX score, PMF score, DOCK energy score, and GOLD score are the four scoring functions used by the consensus scoring spreadsheet Cscore in Sybyl. Molecules with higher scores across all four scoring components are candidates for further experimental screening (Shen et al., 2003).

- Force-field based
- Empirical
- Knowledge-based
- Consensus scoring

Analysis of results

Analyzing the docking data and selecting the compounds that will be ordered and tested during virtual screening is one of the trickiest and most arbitrary steps. Due to the scoring functions' imperfections, which produce ranking errors, the process is challenging. To enhance the success rate, we have employed various strategies. It is frequently beneficial to evaluate docking performance on the investigated system before performing virtual screening calculations. If a ligand is present, this is often accomplished by redocking its cocrystallized conformation. This has numerous advantages, including validating the target preparation, adjusting the docking calculation's parameters, and validating the technique for predicting the known binding pose (Cosconati et al., 2010).

Various docking programs

Dock

We try to fit every molecule from a database into the binding site by accurately defining the geometries of ligands and binding sites by sets of spheres. By applying an accurate clique-detection approach, the spheres can overlap. The ligand−receptor complex, steric matching scores, electrostatic interaction energies, and molecular−mechanics interaction energies are all recognized in the most recent DOCK version. The atomic hydrophobicity descriptors are identified while rating the docked orientations. Scheming the fit measure for each record conformation and grading the molecules according to the assessed scores causes the database search (Reddy et al., 2007).

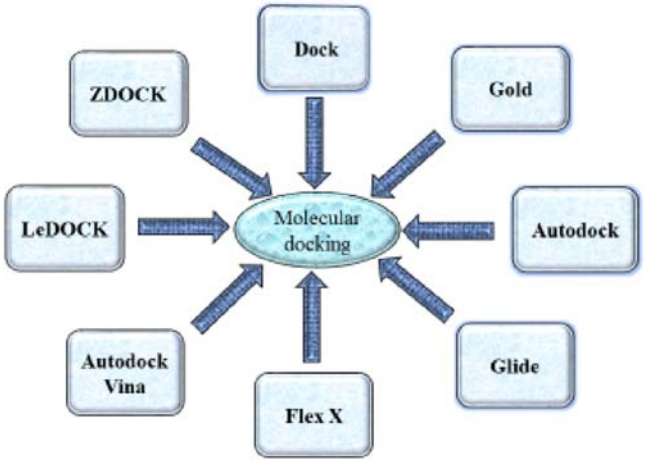

FIGURE 22.3 Various tools used in molecular docking.

Gold

To search for a protein−ligand complex, GOLD uses a genetic algorithm search approach in which different molecular characteristics of the complex are encoded as chromosomes. Both the complex configuration and ligand conformation are searched. The ranking of dockings is based on an atom-based fitness function that incorporates terms for van der Waals forces, hydrogen bonds between proteins and ligands, and ligand internal energy (Fig. 22.3) (Cummings et al., 2005).

Autodock vina

It is a free platform made to be much quicker than AutoDock four and more accurate in predicting binding pockets. In contrast to AutoDock 4, it automatically produces grid maps and clusters, and because multithreading is used on multicore systems, results are obtained more quickly (Srivastava et al., 2022). To conduct the docking analysis, the iDock program was used. While adding additional capabilities that allow automatic docking of huge compound libraries, the iDock uses the AutoDock Vina machine (Arba et al., 2020). Individual virtual screenings of phytochemical databases against the structures of target proteins were carried out using the Autodock Vina virtual screening tool in PyRx 8.0. Compounds were imported into the OpenBabel software, which is a PyRx implementation, at the list, where they were transformed into the Autodock PDBQT format and had their energy reduced using the MMFF94 force field. The 3D grid box parameters covered the active site cavity within each protein. Top-ranking substances with the highest ratings for binding energy to each protein were chosen as hit substances and subjected to various testing (Alamri et al., 2020). Other docking parameters, such as rate of generations (maximum number 27,000), cross-over (0.8), rate of mutations (0.02), docking assessment (10 times), population size (150), and energy evaluation (maximum number 250,000), were set to default values using the autotor utility of the auto dock tool (Rathinavel et al., 2020).

iGMDOCK

The National Chiao Tung University's Institute of Bioinformatics developed an automated, graphical application for integrated docking, screening, and postanalysis. Its current version is iGEMDOCK 2.1. In order to identify the binding sites for a certain ligand, software was used. The evolutionary genetic approach is used by iGEMDOCK to determine the conformation and direction of the ligand about the target protein binding site. Solution number of 2, generations of 70, population size of 200, and the docking feature of "standard docking" were the parameters chosen for GA. The software updates the energy calculations for each position after generating a collection of poses. The interaction data now includes each posture's energy individually and the set's total energy. The best fit is chosen to reflect all of the energy associated with the expected pose at the protein binding site, including the energy associated with electrostatic interactions, hydrogen bonds, and van der Waals interactions (Yang and Chen, 2004).

Pharmacokinetic (PK) parameters prediction

The determination of prospective therapeutic compounds' ADMET characteristics is a crucial step in the drug development process. The fate of a therapeutic agent in an organism can be predicted using a user-friendly interface provided by SwissADME (http://www.swissadme.ch). Along with other characteristics like total prostate-specific antigen, lipophilicity (LIPO), flexibility (FLEX), and insolubility, the server predicts essential characteristics including size, unsaturation, and bioavailability (INSOLU). The online tool admetSAR v1.0 (https://immd.ecust.edu.cn/admetsar2/) uses the known logP value of a reference drug as a starting point to compute and estimate physiochemical parameters like lipophilicity (LIPO) of a query molecule. The entire carbon count should contain at least 0.25% of sp-hybridized carbons for the saturation percentage. Log S (estimated using the ESOL model) for solubility shouldn't be greater than 6.

P-glycoprotein inhibitor, Ames test-based mutagenesis, acute oral toxicity, human intestinal absorption (HIA), Caco-2 penetration, subcellular localization, biodegradation, and are just a few of the physiochemical and biochemical characteristics that can be predicted using admetSAR for a potential drug candidate. Oral medication is predominantly absorbed in the intestines (HIA). Positive results were seen for all phytoconstituents and baseline reference medications, demonstrating their assimilation and absorption in the human gut. A model for the digestion of medicines and other substances by the human digestive tract is the human colon epithelial cancerous cell line (Caco-2). The ability of medication candidates to cross the BBB is a significant factor (Tian et al., 2015).

Drug likeliness and ADME calculations

On the Swiss ADME website, criteria for phytochemical drug likeliness were tested. Pharmacokinetic properties of ligands with high docking scores and ligands with no Lipinski violation are determined using the PreADMET web-based tool. The preclinical and clinical screening phases of drug development will not require as much time or money because of drug likeness (Rathinavel et al., 2020). The hit list postprocessing is the final stage of virtual screening. Based on the features of their ADMET (absorption, distribution, metabolism, excretion, and toxicity) processes, the compounds without drug-like properties are filtered out in this step. In the past, issues with absorption, metabolism, and toxicity led to a failure rate of over 40% for drugs and therapeutic candidates. Therefore, this step is essential. Computing techniques can be used to precisely determine a wide range of physical properties, such as solubility (in terms of the number of hydrogen bond donors and acceptors and molecular polarity), molecular size, and molecular weight hydrophilicity (in terms of the partition coefficient, log P). Indicators of developmental issues, such as toxicity and metabolic outcome, can also be provided by these methods. Virtual screening can rapidly clear out compounds unsuitable for further drug development based on this data and some empirical criteria like the rule-of-five (Shen et al., 2003). Using the SwissADME web server (http://www.swissadme.ch), the two best compounds from the ZINC database and one compound from the HerbalDB database had their ADME properties determined. Each compound's SMILE file was uploaded to the web server in order to produce the ADME characteristics (Kadioglu et al., 2021).

Lipinski's rule of five

Based on violations of the Lipinski rule of five, the phytocompounds' drug-likeliness was analyzed (Mutazah et al., 2020). Molecular weight 500, number of hydrogen bond acceptor sites >10, and logP 5, topological polar surface area (TPSA) (140 Å2), the number of rotatable bonds (10) were determined to be the parameters of drug-likeness.

Veber rule

Membrane permeability is a critical element of oral bioavailability. Two essential factors for a molecule to behave as a therapeutic candidate are its number of rotatable bonds and polar surface area. Reduced polar surface area causes permeation to increase, while an increase in the number of rotatable bonds causes permeation to increase significantly.

Ghose filter

Molar refractivity, molecular lipophilicity, and pharmacological molecules significantly impact receptor binding, cellular absorption, and bioavailability. In 3D-QSAR research, they are both applied to assess how drug-like the molecules under research are by representing the hydrophobic and van der Waals interactions of a drug molecule.

According to the Ghose filter, the following criteria are required by a potential drug candidate:

1. The average value of clogP is 2.52, with a range of −0.4 to 5.6.
2. The average value of molecular weight is 357, with a range of 160−480.
3. The average value of molar refractivity is 97, with a range of 40−130.
4. The average value of the number of atoms is 48, with a range of 20 and 70.

Before any in vitro and in vivo testing of hypothetically proposed drugs, the before-mentioned considerations must be taken.

Leadlikeness

Compounds with an XLOGP3 value of 3.5, a molecular weight of 250−350, and seven rotatable bonds match the lead likeness requirements.

Egan rule

Compounds with log $P > 5.88$ or TPSA $>$ 131.6 Å are identified by the existence of drug-like properties and their characteristics.

Muegge rule

It states that substances that exhibit the following properties are shown to conform to the Muegge rule and function as possible drugs: Number of rotatable bonds is 15, number of H-bond donors is 5, number of rings is 7, number of carbon atoms is greater than 4, number of H-bond acceptors is 10, number of heteroatoms is greater than 1, and TPSA is 150 Å, XLogP between −2 and 5 (Srivastava et al., 2022).

Pharmacophore concept in natural product research

One of the essential steps in early drug development is the quick identification and elimination of substances with undesirable physicochemical and pharmacokinetic features. Studies on natural products have shown that secondary metabolites have a wide variety of scaffolds and that biosynthesized compounds also exhibit spatial and structural traits that are more similar to therapeutic leads than those of synthetic molecules. In contrast to synthesized molecules, natural products typically have a higher number of chiral centers and a far more sophisticated stereochemical structure. Additionally, compared to synthetic molecules, they often have a higher proportion of carbon, hydrogen, and oxygen but a lower proportion of nitrogen and other elements. Lipinski's rule of five is frequently exhibited by the high polarity and molecular weight of many natural compounds, which are more than 500 Da. However, only around 10% of natural products had two or more violations of Lipinski's criteria.

In conclusion, it is possible to think of natural chemistry as a wide range of scaffolds that are endowed with pharmacophores that could be used as drugs (Rollinger et al., 2008). Understanding the chemical interaction between a smaller molecule and its macromolecular targets, such as a receptor, enzyme, or ion channel, is the purpose of pharmacophore models. The purpose of creating pharmacophore models is to understand protein-ligand interactions better and determine whether novel chemicals can be included in the model. These substances are considered to show biological activity if they fulfill the criteria of the pharmacophore. There are two techniques generally accepted for determining a pharmacophore: the ligand-based or indirect approach, which attempts to align active molecules and assess their similarity, and the structure-based or direct approach, which includes 3D information about the protein (Schuster and Wolber, 2010).

Structure-based pharmacophore modeling

Knowledge of the ligand-target interaction, as well as the availability of the target's 3D structure through X-ray crystallography or NMR or created based on the structure of homologous proteins, are essential for creating a structure-based model. The three-dimensional coordinates of protein structures that have been determined through experiments are individually stored in the Brookhaven PDB. An effective starting point for a structure-based 3D model is a crystalline complex that has a ligand bound to the active site of a protein. In this case, it may be beneficial to use precise information about the

ligand's bioactive conformation that is kept in the binding site of the crystalline complex. On a new software tool for the successful development of such chemical features-based models, it was recently revealed: LIGAND-SCOUT is a tool for PDB data mining and ligand interpretation. This program's effectiveness enables the identification of crucial protein-ligand interaction sites. It is possible to visualize the complex binding process of the ligand at the protein's active site. The LIGANDSCOUT algorithms interpret the ligand molecules in four steps: planar ring detection, functional group pattern assignment, hybridization state determination, and finally, Kekule pattern assignment. The evaluation of the ligand molecules provides the basis for the subsequent phase, which involves the automated development of pharmacophore models using data from a crystalline complex of the PDB. A pharmacophore model is generated by automatically identifying and classifying protein−ligand interactions into hydrogen bonds, charge transfer, and lipophilic domains. The graphical user interface can display a combined view of the interaction lines, pharmacophore model, protein, and ligand. LIGANDSCOUT was employed in a previously published work to identify and evaluate critical patterns of ligand-protein structural interactions. A quick virtual screening of databases, including multiple-conformational 3D structures, was carried out in a subsequent phase using the cutting-edge virtual screening CATALYST (Rella et al., 2006).

Ligand-based pharmacophore

Modern molecular biology methods combined with biophysics and computational methods are known to increase the likelihood of successfully obtaining detailed atomic structure information, but lead discovery projects frequently move to a later stage before detailed structural information on the protein target becomes available. One effect is that researchers frequently choose and create novel compounds for a target using theoretical interaction models and preliminary structure-activity data. By making it possible to perceive and comprehend meaningful interactions between a receptor and a ligand, the chemical feature-based pharmacophore approach has proven successful within this framework. An ensemble of steric and electrostatic characteristics shared by various compounds that are essential for those compounds to interact with a specific biological target structure is represented by a function-based pharmacophore. *In silico* screening trials, these pharmacophore models have proven to be incredibly helpful when combined with substantial 3D structure databases derived from either internal chemical collections, commercial vendors, or natural product databases. When ligand-based pharmacophore models rather than protein 3D structures are employed as screening filters, affinity estimate is entirely dependent on the geometric fit of compound atoms or groups to the properties of the model. When this happens, the estimated values are frequently very far from reality, but they are still helpful for separating potential hits from nonbinding compounds.

The ligand-based chemical feature pharmacophore technique can give medicinal chemists crucial information because there are currently no experimental data on either the natural conformation of the ligand or the target protein in the majority of investigations. Additionally, the number of compounds that may be processed at once is higher than even for high throughput docking because pharmacophore fitting processes have far lower computation requirements than docking algorithms. One of the best software suites for chemical feature-based pharmacophore modeling, the CATALYST program, has been employed in a number of successful applications in this area (Schuster et al., 2006).

Virtual screening in drug discovery

The primary sources of bioactive agents have historically been natural products, which naturally have a more excellent range of structural variety than synthesized molecules. Natural products will continue to play a vital role in developing novel medications. However, the intriguing problem for medicinal chemists and pharmacologists is how to efficiently and effectively access this wide chemical range. Identification of bioactive components in natural products will be facilitated by virtual screening, which has shown considerable promise in the drug discovery process (Fig. 22.4) (Gani et al., 2021). To evaluate relatively large chemical databases in silico and choose a small number of candidates that are expected to have the necessary biological activity is the core concept behind all computational techniques used in the early stages of drug development. Virtual screening tools have significantly increased the effect of computational chemistry in the lead discovery process, and chemoinformatics now dominates early phase drug research. By focusing experimental efforts on the most promising candidates, such strategies aim to lower the overall cost associated with discovering and developing a new medicine (Rollinger et al., 2008). To create new drugs, prevent multidrug resistance, and use precision medicine to choose medications for personalized therapies, recent advancements have provided a new dimension by coupling virtual drug screening techniques with machine learning methodologies (Arba et al., 2020). The ligand−receptor complex structure can be predicted via ligand docking in the lack of experimental 3-D structures of the receptor-ligand complex. This strategy can help with lead optimization, the creation of virtual combinatorial libraries, and the improvement of understanding of the

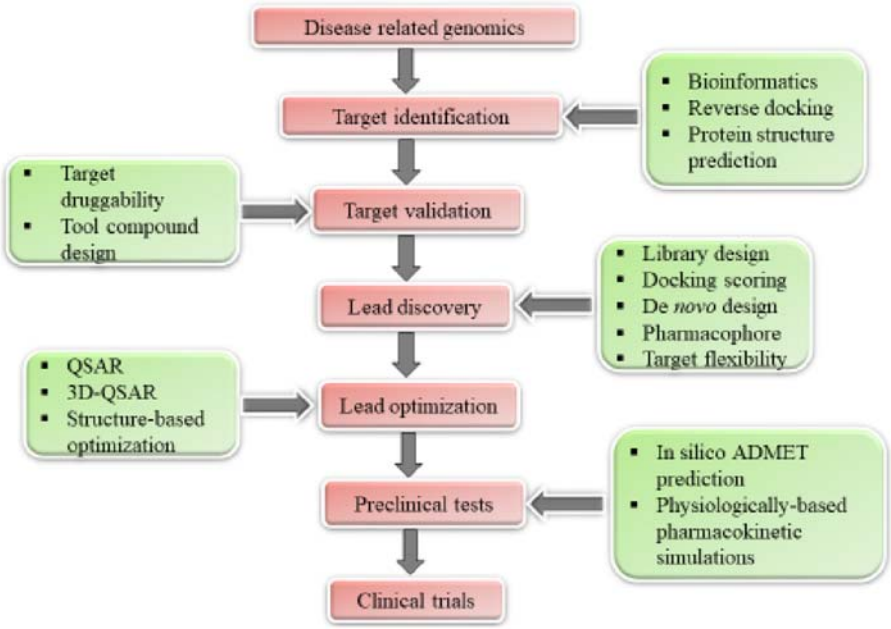

FIGURE 22.4 Process of drug discovery.

ligand binding determinants (Cavasotto and W orry, 2007). By determining the essential interactions between the small compounds and the receptor and conducting calculations on those interactions, some computer tools, like LIGANDSC-OUT, can build a pharmacophore model automatically. Often, screening of isolated active substances and natural product extracts has been viewed as being too expensive, requiring additional time and labor-intensive processes such as isolation, fractionation, and characterization (Ma et al., 2011).

Two facts about the development of drugs from natural sources are in play:

1. According to statistics, the plethora of structurally unique natural chemicals are the most frequently used source of novel medications for clinical usage.
2. Based on a growing understanding of the molecular principles governing protein-ligand interactions, the drug development process has shifted toward more logical concepts.

The advantages of a virtual screening cycle over an in vitro screening procedure are:

(i) Reduced experimental testing effort, (ii) less requirement for isolated molecules, (iii) higher capacity, (iv) by adding more drug-like filters and virtually limiting ADME features, it is possible to increase the quality of hit compounds, which lowers failure rates in the early phases of drug development, (v) it is possible to compute and predict interactions between all known natural compounds and all structurally defined targets (Rollinger et al., 2008).

Conclusion

The impact of virtual screening on drug discovery is significant. The main source for developing new medicines will be natural substances. Combining the chemical information of natural products with virtual screening will become increasingly crucial in drug discovery in the postgenomic era as more and more potential targets are revealed by functional genomic research. A useful tool for explaining the biological action of natural products is the combination of molecular modeling techniques, particularly 3D pharmacophore modeling. The chemical understanding of small molecule natural product binding to macromolecular targets is a suitable complement to big-guided methods.

When the necessary data about the target protein is available, choosing a specific docking technique for a target protein could often be possible. Today's medicinal chemists can quickly and effectively find lead compounds against biomolecular targets using a variety of modeling methodologies. A valuable supply of bioactive scaffolds that exhibit remarkable chemical diversity in both structure and function is reemerging from natural products at this time. Virtual screening is now a crucial component of modern pharmacological development. In order to efficiently use quick screening techniques and produce powerful hits, a variety of computational tools are being created and improved.

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Chapter 23

In silico techniques for screening of key secondary metabolites of medicinal plants

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Introduction

Medicinal plants are important sources of pharmaceuticals that are utilized all over the world as alternatives to conventional treatment. Traditional medicine uses natural ingredients. In some countries, 70%−80% of people use herbal medications for basic therapy (https://www.nmpb.nic.in/content/medicinal-plants-fact-sheet). India contributes significantly to global biodiversity and has over 7000 medicinal plant species. India's alternative medical system, AYUSH (Ayurveda, Yoga, Unani, and Siddha), is being used today (Hamilton, 2004) Herbal medicinal preparations are used by one-third of the adult population in industrialized areas and more than 80% of the population in developing countries to improve health and to cure common ailments such as acute viral nasopharyngitis, cardiac infarction, diabetes, and central nervous system problems. Many scientists believe that plants may adapt to environmental changes via direct contact and mutual interaction (Li et al., 2006). Most of the substance's plants generate don't appear to affect growth and development. Secondary metabolites are chemicals that vary among plant taxonomies. The scientific community is interested in using in silico technologies for drug discovery, design, development, toxicological end objectives, ADMET analysis, and pharmaceutical chemical metabolism (Sharanabasappa et al., 2007). Drug discovery and development is widely acknowledged to be a time and resource-intensive process. Pharmaceutical and other xenobiotic product development and safety may be improved using computing power applied to a chemical-and-biological space, and in silico methods to toxicology and pharmacology can be guaranteed to be accurate and reliable. The ability of industries and scientists to employ in silico tools to assist in risk assessments of drug-induced toxicities and in safety evaluation is one of the main goals for using these software programs (Vanjari et al., 2012).

Overview of in silico and other researches

"In silicio," is the proper Latin word for "in silicon," temporarily refuted "in silico." Beginning in the first decade of the 19th century, a Swedish scientist named Jons Jacob Berzelizus used the Latin word silicium to describe silicon. In silico, a notion used by Miramontes, refers to biological investigations conducted using specific software. The European Community Commission employed in silico in white papers (Danchin et al., 1991) that were published to help the development of bacterial genome projects. Drugs were formerly discovered by a series of time-consuming, multi-step methods that

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included evaluating compounds against a battery of in vivo biological screens. The potential toxicity, metabolism, and pharmacokinetics of promising substances were then examined during subsequent development research. If in a study, negative results were obtained, the study would have to be terminated or restarted in pursuit of a new clinical candidate, putting an unnecessary strain on a pharmaceutical company's research and development budget. In the modern age, this paradigm has undergone several revisions (Kennedy, 1997). Before a chemical is put into clinical trials, it is subjected to extensive testing for its metabolism, pharmacokinetics, and toxicity. Biotech's and major pharmaceutical corporations alike now routinely use (ultra) high-throughput screening (HTS) facilities to rapidly gather data on a wide range of potential drugs. Chemical compounds are screened for pharmacological action using HTS. According to the screening paradigm, a molecule that has reached the first step toward becoming a medication is that it should successfully interact with a target. Compounds that fail to pass this first screening are put back into the library where they will be subsequently tested against other targets (Kennedy, 1997).

Phytochemicals are nonnutritive plant compounds that have anticancer and chemopreventive capabilities. Around a 1000 different forms of phytochemicals, or plant byproducts known as secondary metabolites, have been identified. Certain phytochemicals include lycopene (tomato), flavonoids (fruits), genistein (soyabean) and catechins (green tea), 6-gingerol (ginger), eugenol (clove), and other well-known phytochemicals. All of them have a wide range of health-promoting activities including antioxidant, antiviral, antibacterial, antiatherosclerotic, antiinflammatory properties, spasmolytic properties, hepatoprotective qualities, etc. Additionally, they have an impact on a wide range of cell functions, including proliferation, apoptosis, cell cycle regulation, angiogenesis, inflammation, and DNA repair. They are also used in chemopreventive agents as well as modulators of miRNA and, as a result, of their target genes' expression (Lançon et al., 2013). A class of phytochemicals known as isoflavones controls the amount of estrogen in the body; other phytochemicals known as terpenes act as protease inhibitors and interfere with enzyme activity. Through the control of several signaling pathways, dietary polyphenols are natural medicines with the potential to improve the effectiveness of chemotherapy and radiation (Petric et al., 2015).

Natural coumarinolignoid compounds isolated from *Cleome viscosa* seeds were studied using molecular docking and QSAR. The QSAR model created by forward stepwise multiple linear regression using physicochemical descriptors from Scigress Explorer was used to examine immuno-stimulatory activity (Hardy et al., 2010). Coumarinolignoids seem to bind to a wide range of immunomodulatory receptors, including COX-1, COX-2, TLR-4, iNOS, CD14, CD86, and IKK b, according to docking studies (Meena et al., 2011; Yadav et al., 2010). Some instances of successful molecular docking utilizing secondary metabolites are shown in Table 23.1.

TABLE 23.1 Molecular docking analysis of some studied secondary metabolites compounds with their targets.

Compound	Source	Biological effect	Target	References
ALKALOIDS	Norbelladine	Alzheimer's disease	AChE enzyme(rhAChE)	Muhammad and Fatima (2015)
	Crinine			
	Pretazetine			
	Galanthamine			
	Montanine			
GLYCOSIDES	Quercetin glycosides	Hypertension and congestive heart failures	ACE (PDB: 1O86) (Peptidyl-dipeptidase A) Angiotensin receptor Enzyme-2	Perera et al. (2021)
	Momordicoside A	Diabetes mellitus Antiinflammatory activity	α-amylase isomaltase	Gupta et al. (2021)
	Karaviloside VI			
	Charantoside XV			
	Kuguaglycoside C			

TABLE 23.1 Molecular docking analysis of some studied secondary metabolites compounds with their targets.—cont'd

Compound	Source	Biological effect	Target	References
STEROIDS (STEROIDAL alkaloids)	Kurchessine,	Gastrointestinal disorder	ECD $_{GC\text{-}C}$ Epigallocatechin-3-gallate	Gyebi et al. (2021)
	Kurchine			
	Holadysenterine			
	Conessimine			
	Holanamine			
	Holadienine			
	Pubesciene			
	Isoconessimine			
	Conessine			
TERPENOIDS	Nelfinavir mesylates	Coronavirus disease-19 (COVID-19)	SARS-Cov-2	Khalifa et al. (2020)
	3-Benzoylhosloppone			
	Cucurbitacin B			
	7-Deacetoxy-7-oxogedunin		SARS-CoV	
	3-Friedelanone			
	3-Benzoylhosloppone		MERS-CoV	
TANNINS	Pedunculagin	Coronavirus (SARS-CoV-2)	3CLpro 3C-like-protease	Yu et al. (2012)
	Tercatain			
	Castalin			
	Bicornin			
	Potentillin			

Software and hardware

Computational approaches are the methods of choice in natural product drug development since there are thousands of pharmacological targets and most natural substances demonstrate pleiotropic effects by interacting with many targets (Rollinger et al., 2009). With medicinal plant databases, chemo- and bioinformatics may be used to explore medicinal plants' pharmacological potential. This comprises chemical structures and therapeutic applications of medicinal plant phytoconstituents. The identification of complementary leads and targets is aided by chemo informatics and bioinformatics tools. Molecular docking pharmacophores, quantitative or qualitative "structure−activity" correlations ((Q)SAR), and machine learning algorithms are all used in many of these methodologies to aid lead development against specific targets (Sharma et al., 2012). Combinatorial techniques perform parallel searches against each individual target to find virtual hits that interact with several targets at the same time. Even while 3D target-based techniques are widely used, they have limits in terms of the number of targets with 3D structures. As a result, the usage of 2D structures seems to be fairer in terms of allowing numerous target leads. Plant phytoconstituents are studied either by bioactivity-guided fractionation or by screening plant extracts at random. Only the bioactive principles for conventional activities have been employed as templates for novel drug development employing molecular docking for recognized bioactivities to date. As a result, phytochemicals with uncertain biological action are mainly unexplored. Multi-targeted in silico techniques may be used to explore their potential. To research the therapeutic potential of medicinal plants, bioinformatics and systems biology techniques, as well as the chemoinformatics methodologies listed above, are becoming more essential (Barlow et al., 2012). They are used in the selection of targets for

docking as well as the identification of correlations between the disclosed activities of phytochemicals on targets and the well-established curative benefits of medicinal plants. the traditional methods of (Q)SAR (quantitative or qualitative "structure−activity" relationships), molecular modeling, and virtual screening (VS), which are widely used in drug discovery for synthetic compounds, can be used to investigate the biological activity of medicinal plants.

All of these strategies are founded on the idea that a compound's activity is determined by its structure. For the construction of (Q)SAR models, three main components are required: (1) noncontradictory data on the structures and biological activity of studied compounds; (2) descriptors for the presentation of structures (structural fragments, fingerprints, constitutional, topological, electro-topological, quantum-chemical, and physicochemical descriptors); (3) machine learning methods for the identification of the relationship between descriptors (multiple linear regressions, neural networks, support vector machines, random forest, similarity, etc.) (Q)SAR models based on heterogeneous data are termed global models with a broad application area, and they may be utilized for VS, biological activity prediction, and target shing. Local models are (Q)SAR models that are built based on homogenous data. Traditionally, they've been employed to improve hit or lead compounds. Pharmacophore creation may also be done using local 3D-QSAR models. A pharmacophore is a collection of atoms in a molecule that are thought to be responsible for a pharmacological activity. Docking is a technique for predicting the preferred orientation of one molecule relative to another when they are bonded together to create a stable complex (Lengauer et al., 1993). It is widely used to evaluate the affinity and forecast activity of tiny drug-like compounds by predicting the binding orientation of the molecules to their protein targets. A docking process requires the 3D structures of targets.

Receptor preparation

The chemical interactions between a ligand and a particular receptor (target/protein) in a live organism provide the basis for biomolecular recognition. Proteins have evolved to use "high specificity" to bind ligands that have a propensity to fulfill the particular needs of the cell (Heifetz et al., 2016). The structural knowledge of proteins enables a novel use of diverse technologies, such as VS, to discover further hits and move toward an effective medicine (Abdolmaleki et al., 2018). Identifying a target, synthesizing an active chemical with relevant properties such as minimum toxicity, high bioavailability, cost-effective synthesis, etc., and then preparing it for market introduction is a time-consuming, exceedingly difficult, and serious venture (Sethi et al., 2019). Docking success relies on both the quantity and quality of structural information about the target, as well as those small molecules being docked, that are known to have a role in the illness progression. After that, the next step is to check the target for the existence of a binding pocket (Hajduk et al., 2005; Fauman et al., 2011). Typically, this is done by analyzing existing target-ligand cocrystal structures or by employing in silico techniques to find new binding locations (Laurie and Jackson, 2006).

The optimal starting point for docking is a target structure empirically established by X-ray crystallography or NMR methods and placed in the PDB. Target structures are identified at an accelerated pace due to structural genomics. Several VS campaigns have been described that work well even though the target protein structures haven't been determined experimentally (Becker et al., 2006; Warner et al., 2006).

The target structure or protein must undergo target preparation prior to the docking procedure. To prevent steric encounters, this involves the introduction of hydrogen atoms and energy minimization of the target protein. A major issue in molecular docking is the lack of an appropriate target for the process. Modeling the protein's 3D structure using homology modeling is a frequent option. This approach uses the target protein's amino acid sequence as a template. Multiple sequence alignment identifies similar protein structures with more than 20% identity, and the 3D model of the target is built using fragment assembly, segment matching, or spatial restrictions. Fragment-based approaches use template structures to detect conserved sections and build a model. Segment matching matches protein segments to a template. In this procedure, comparable areas are aligned to choose protein fragments. The fourth approach involves spatial restrictions and employs templates to produce probability density functions (PDFs) for restraints derived from geometric data.

Ligand preparation

Once a relationship between the target and illness is established, viable candidates that may halt or reverse disease progression are identified (https://www.fda.gov/forpatients/approvals/drugs/ucm405382.htm). This procedure begins with the identification of effective compounds, termed "hits." The "hits" are then chemically changed to enhance their medicinal qualities, creating "leads." But, it is quite apparent that the method stated above for thediscovery of a drug has a number of pitfalls. HTS is impractical, expensive, and time-consuming from a research standpoint. Molecular docking helps solve these obstacles. VS is a fully valid alternative or supplementary strategy to HTS for screening hundreds of

millions of compounds in a few days. Molecular docking needs conformational care. The ligand's flexibility determines the conformational space it will occupy in the protein cavity. In circumstances when the target binding site or the cavity is unknown, certain tools like POCKET (Levitt and Banaszak, 1992), LIGSITE (Hendlich et al., 1997), and SURFNET (Laskowski, 1995) can be useD. Various programs such as Marvin Sketch (https://chemaxon.com/products/marvin/download) and ChemDraw (https://www.perkinelmer.com/category/chemdraw) may be used to draw the ligand structures. Docking algorithms estimate a ligand's 3D binding orientation (its 'pose') and binding energy. After years of usage, it became obvious that these docking systems' scoring methods could not reliably estimate binding-free energies and did not rank molecules by their expected affinities (Pantsar and Poso, 2018). Most docking tools utilize a scoring function to determine ligand-target binding affinity. Using a scoring algorithm, docked ligands are sorted and the best are picked for further study and testing.

The approach identifies ligand locations in a protein's binding pocket and predicts ligand-protein affinity. VS is ligand- and structure-based. Structure-based VS uses the protein cavity shape, whereas ligand-based uses the natural ligand. A succession of weak connections and favorable contacts allows the receptor to bind with high specificity and affinity to its ligand. The interaction between the ligand and its receptor generally results in the formation of weak and reversible forces like (1) hydrogen bonds (10−40 kJ/mol), (2) hydrophobic interactions, which act as the "driving force" to promote bond formation, (3) van der Waals forces (0.03−0.1 kcal/mol), (4) electrostatic interactions (0.3−4 kcal/mol), (5) pep interactions, and (6) coordination with metals. Electrostatic interactions and hydrogen bonding govern protein-ligand complementarity. During complex formation, protein and ligand interact enthalpically and entropically in concert. Free energy varies owing to intra- and intermolecular noncovalent bond formation and desolvation. Even though protein-ligand interactions are critical for binding enthalpy, water must also be considered. Molecular recognition occurs in water. Water molecules are structured to make as many hydrogen bonds as possible, reducing entropy. As numerous broken bonds are rebuilt between the ligand and the receptor and water molecules rearrange around the newly formed complex, the free energy difference is typically near zero (Fersht, 1987; Salari and Chong, 2010).

Ligand-receptor docking

Molecular docking examines how ligands connect to receptors via intermolecular interactions (Hoque et al., 2017). Docking score or binding energy measures intermolecular interactions. Docking software has two key components: the sampling method, which looks for probable molecular positions, and the scoring function, which analyses interaction energy. Ligand and receptor conformers may be provided with the corresponding active site coordinates of their target receptors in the input (Tang et al., 2006). After input, the software runs various programs. In the output component of the program, the binding affinity cum energy may be auto-computed for too many ligand-receptor poses (Kumar and Pandey, 2013). Docking analysis is done using binding energy output data. The docking study validates the accurate docking of ligand to receptor's active site. Several docking options are now available, including Rigid, Flexible, and Semi-flexible docking (Tang et al., 2006; Kola and Landis, 2004; Kumar and Pandey, 2013).

1. Rigid docking: This kind of molecular docking ignores the flexibility of the ligand and protein molecules. In this situation, the docking simulation keeps the side chains and backbones of the two molecules constant, with no torsion angles or atom distance changes. Docking does not change ligand bond length, bond angles, or torsion angles.
2. Semiflexible docking: The receptor stays unaltered as the ligand conforms. It focuses on ligand structural modifications and is utilized to dock tiny ligands and macromolecules.
3. Flexible docking: Flexible docking addresses every conformational change in the protein and ligand. Strong intermolecular interactions owing to flexible attachment. Flex dock is one of the flexible docking applications (Anderson, 2003; Hoque et al., 2017; Prieto-Martínez et al., 2018; Veselovsky and Ivanov, 2003; Vyas et al., 2012).

The scoring function ranks ligand locations. The score should match precisely to the ligand's protein binding affinity, so the best binders get the highest scores. Scoring functions fall into three categories: (1) force field-based, based on the sum of intermolecular (van der Waals and electrostatic) interactions between all atoms of the two molecules; (2) empirical, based on the number of various types of interactions; (3) knowledge-based, based on a statistical analysis of observed pairwise distributions. AutoDock (Morris et al., 2009), DOCK (Allen et al., 2015), GOLD (Verdonk et al., 2003), FlexX (Schellhammer and Rarey, 2004), Glide (Friesner et al., 2004), Surflex (Jain, 2003) are among the more than 60 docking software documented in the literature. Docking-scoring combinations must be assessed depending on the target's properties. The stiff receptor/flexible ligand concept is the most widely used. Monte Carlo, genetic algorithm, fragment-based, and molecular dynamics are widely used docking approaches. Small molecule ligands may activate or inhibit enzymes. If the protein is a receptor, its binding may result in either antagonism or agonism (Eweas et al., 2014).

Secondary metabolites

Overview of secondary metabolites

Primary metabolites perform important metabolic roles in eating and reproduction. Secondary metabolites (SM) aren't required for a cell's survival but are crucial for how it interacts with its environment. In protecting plants from biotic or abiotic stressors, several substances are often implicated. Secondary metabolites are derived from many metabolite families and may be strongly induced in response to stressors. A selected number of secondary metabolites are employed as chemical compounds, such as medicines, flavors, scents, insecticides, and dyes, and as a result, they are very valuable economically. The utilization of higher plants as a source of novel chemicals, particularly therapeutic compounds, will be extended and enhanced by these new technologies. It is anticipated that continued and increased efforts in this area would result in the effective biotechnological creation of particular, worthwhile, and as of yet unidentified plant compounds (Pagare et al., 2015).

Plant metabolites and docking

With a better knowledge of structural and functional molecules, new methodologies and strategies have been used in drug development programs (Yi et al., 2017). A rapid and simple strategy for accurate prediction of huge chemical compounds followed by in-vitro and in-vivo pharmacological studies for validation would increase the efficacy of evaluating medicinal plant biological activities. Many FDA-approved natural medications have been found utilizing in silico methods (Yi et al., 2017). To provide originality for the search, design, and optimization of new medications intended to cure human illnesses, computational approaches are put into practise. However, in silico tools can reduce medication erosion rates when used in conjunction with therapeutic bioinformatics strategies, pharmacovigilance computational data mining techniques, and prognostic computer models for identifying potential drug toxicity (Valerio, 2012). Today, in silico modeling is an essential tool in the drug development process. Most molecular docking algorithms treat proteins as rigid objects, resulting in erroneous correlations between docking findings and actual protein structure. In molecular interfaces, no docking technique can precisely predict the ligand's binding affinity (Lakshmi et al., 2013). In-silico techniques help speed up the manufacturing and screening of drugs based on the examination of intended assets and prediction models for drug remedial objectives and the identification of protective responsibility. All the while reducing the need for time- and money-consuming animal testing and in-vitro assays. Due to the discovery of semisynthetic pharmaceuticals and new bioactive molecules, plant secondary metabolites, also known as natural products, have gotten a lot of interest across the world. Currently, a big percentage of the worldwide population depends on natural products to heal disorders and chronic diseases and boost their immune system. Commonly used medications for cancer, ulcer, TB, asthma, etc., are claimed to be of plant origin and to elicit therapeutic effects in vivo and in vitro. The in-vitro analytical approach extracts, isolates, and purifies secondary metabolites. Organic and aqueous solvents are used for extraction depending on solubility and polarity. Column chromatography purifies, identifies, and analyses extract components. Thin-layer chromatography (TLC), LCMS, and HPLC are kinds of column chromatography (HPLC). LCMS and HPLC are the best ways to detect and evaluate secondary metabolites in plant samples (Zhang et al., 2018). An in-silico molecular docking bioinformatics analysis of secondary metabolites found by extraction and separation procedures may determine the potential therapeutic target in extract plant samples.

Plant secondary metabolites may be separated by biosynthetic principle (Agostini-Costa et al., 2012). A basic categorization has three groups.

1. **Nitrogen-containing compounds** such as alkaloids and glucosinolates
2. **Terpenes** such as mono-, di-, tri-, sesqui-, and tetraterpenes, saponins, steroids, cardiac glycosides, and sterols
3. **Phenolic compounds** such as phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins, and lignins.

Computational techniques provide novel medication candidates faster and cheaper. VS, de novo design, in silico ADME/T prediction, and enhanced protein-ligand binding techniques are major roles of computing in drug development. Bioinformatics has helped identify secondary metabolites in silico. There are several techniques and databases to search, discover, and describe secondary metabolites, biosynthetic enzymes, and genes. Web portals are being built to give connectivity to all bioinformatics sources. The Secondary Metabolite Bioinformatics Portal is one of the examples of such portals (Weber and Kim, 2016). Bioinformatics databases and algorithms may identify structural similarities between an uncharacterized secondary metabolite and described bioactive chemicals. Unknown structure (query) is submitted to the tool, which searches small molecule databases for comparable structures.

Predicting ADMET characteristics is critical in drug design since they cause 60% of clinical medication failures. Poor pharmacokinetics and toxicity are the primary reasons for high drug development dropouts. In order to prevent such a setback, these issues should be addressed early on in drug development in order to boost efficiency and save costs. Developers struggle to resolve drug candidates' pharmacokinetic and toxicological features. Intestinal permeability, human intestinal absorption, human oral bioavailability, aqueous solubility, active transport, blood-brain barrier permeation, P-glycoprotein efflux, plasma protein binding, metabolic stability, interactions with cytochrome P450s, and toxicity are evaluated using in silico models. Simulations plus ADME/T predictor and PK-Map from Accerlary company (Willmann et al., 2005) can perform ADME/T analysis. Although natural compounds have better ADME/T qualities than synthetic drugs, studying phytochemical ADME/T features is vital in medication development (Dhiman et al., 2017).

High throughput screening and docking

To find new therapeutic targets, HTS is used. HTS has become a staple strategy in the drug development toolkit for screening huge libraries of chemicals against a protein target (Wu et al., 2015). HTS may be replaced with, or aided by, VS since it is more cost-effective than the latter. For example, in HTS, VS is used to narrow down a vast library of compounds before moving on to more expensive HTS. As VS does not need the physical production of chemicals, it is not constrained by the experimentally available chemical space, as does HTS. VS, on the other hand, requires experimental data, either a protein structure for structure-based VS or a list of known actives for ligand-based VS.

It's not out of the question that the two approaches may be useful in tandem. Docking's shortcomings (Moore et al., 1999) are orthogonal to those of HTS, therefore one may anticipate that compounds that fit well into a protein structure, as revealed by docking, and are also active in an HTS campaign would be the best candidates for early consideration.

HTS is used to find novel lead compounds for drug discovery using compound libraries (Dunn et al., 2000; Haggarty et al., 2000; Hamasaki and Rando, 1998; Moore et al., 1999; Wu et al., 2015). VS employing molecular docking may also be utilized to find novel lead compounds when the three-dimensional structure of the target is known or can be modeled (Gschwend et al., 1996; Jones et al., 1997; Lengauer and Rarey, 1996; Nussinov and Wolfson, 1999; Olson and Goodsell, 1998). While computer-based docking screen predictions are not always accurate, HTS is expected to find all the intriguing ligands in the database in theory. There are many false positives and false negatives in docking and high throughput screens. In HTS, several substances are screened quickly, and inhibition may be detected or overlooked because of assay errors (false negatives) (Ferry and Boutin, 2000; Zhang et al., 2000). Some types of substances seem to inhibit because they interact with the assay's characteristics or inhibit with low selectivity, which causes them to be overrepresented in HTS hit lists (false-positives). Numerous issues with molecular docking as a screening technique have been raised, including erroneous scoring functions, imprecise conformational state sampling, and a general disregard for many solvent-related variables (Bissantz et al., 2000; Charifson et al., 1999; Gohlke and Klebe, 2001; Knegtel and Wagener, 1999; Kuntz et al., 1994; Morris et al., 1996; Schnecke et al., 1998; Stahl and Rarey, 2001). However, molecular docking offers certain useful benefits. The capacity to accurately filter out substances that do not fit in a binding site or that have blatantly incorrect electrostatic characteristics is greater than its limited ability to discriminate between two substances that both fit in an active site. This makes it possible to conduct thorough experimental tests on a limited set of database chemicals. Additionally, substances for which there are no available physical samples may be screened via docking. When choosing a smaller section of a collection of commercially available chemistry to buy, this is very helpful. Finally, a docking hit includes a geometry prediction that enables the optimization of an inhibitor within the context of a binding site. Such potential benefits are only intriguing if molecular docking screens, despite all their flaws, are still able to find new inhibitors often enough to compete with HTS.

Introduction of various classes of secondary metabolites

Secondary metabolites are able to be categorized on the basis of their chemical structure (for instance, having rings, including a sugar), composition (containing nitrogen or not), their solubility in a variety of solvents, or the metabolic process that leads to their formation (e.g., phenylpropanoid, which produces tannins). And often arranged according to the routes that were used in their biosynthesis (Harborne and Walton, 1996). There are three major compound families: phenolics, terpenes, and steroids, as well as alkaloids, flavanoids, and terpenoids (Bourgaud et al., 2001).

Phenolics compounds

The term "phenolic compound" refers to a broad category of plant compounds that share an aromatic ring with one or more hydroxyl substituents. Since phenolic compounds are often found in the cell vacuole and commonly occur in combination with sugar as glycosides, they tend to be water-soluble. There are several thousand known structures for natural phenolic compounds. Flavonoids are the largest group, but there are also a lot of simple monocyclic phenols, phenylpropanoids, and phenolic quinones. Lignins, melanins, and tannins, which are all important plant polymers, have phenolic units. Sometimes, phenolic units can also be found in proteins, alkaloids, and terpenoids. While certain classes of phenolic compounds (e.g., lignin's, anthocyanins, etc.) have well-established functions (e.g., cell wall structure, flower pigmentation), the purpose of other groups is still an enigma. When plant cell components come together and the membranes are disrupted during isolation processes, phenols readily bond with protein and often limit the activity of enzymes in crude plant extracts. The phenols themselves are very susceptible to enzymic oxidation, and phenolic material may be lost through separation processes since all plants contain the specialized "phenolase" enzymes. Since all phenolic chemicals are aromatic, they all exhibit strong absorption in the UV portion of the spectrum (Harborne et al., 1998).

Flavonoids

The flavanoids are a huge family of phenolic natural compounds, with over 4500 distinct examples discovered so far. Flavonoids may be found as monomers, dimers, and higher oligomers in most plant tissues, especially in vacuoles. Flavonoids are a broad group of chemicals that serve a variety of activities. Plants may also be protected from UV-B irradiation by certain flavonoids. The flavonoids are made up of chalcones, aurones, flavanones, isoflavonoids, flavones, flavonols, leucoanthocyanidins, catechins, and anthocyanins, among other plant metabolites (Tiwari and Rana, 2015).

Tannins

Tannins, also called tannic acid, are polyphenols that dissolve in water and are found in many plant foods. It has been said that they cause experimental animals to eat less food, grow slower, use food less efficiently, have less net metabolizable energy, and have a harder time digesting protein. So, foods that are high in tannins are thought to have less nutritional value (Chung et al., 1998). Numerous plants that are consumed by humans and used as animal feed have been shown to contain tannins. grains used as food, such as sorghum, millets, barley, dry beans, faba beans, peas, carobs, pigeon peas, winged beans, and other legumes (Chavan et al., 1997; Deshpande et al., 1984; Hulse, 1979; Price et al., 1980; Salunkhe et al., 1983). A significant amount of tannins are also present in fruits such apples, bananas, blackberries, cranberries, dates, grapes, hawthorn, peaches, pears, persimmons, plums, raspberries, and strawberries (Barnell and Barnell, 1945; Goldstein and Swain, 1963; Haslam, 1977; Hoff and Singleton, 1977; Lloyd, 1911; Reeve, 1959; Thompson et al., 1972). There are additional reports of tannins in forages such crownvetch, lespedeza, lotus, sainfoin, and trefoil [94,95]. Tannins may be divided into two distinct groups: hydrolyzable tannins and nonhydrolyzable tannins, often known as condensed tannins. Tannins that may be hydrolyzed have a central core made of a polyhydric alcohol like glucose, as well as hydroxyl groups that are esterified either partly or entirely by gallic acid (gallotannins) or hexahydroxy diphenic acid. Hydrolyzable tannins are found in a variety of plants (ellagitannins) Contrary to hydrolyzable tannins, condensed tannins have a more complicated structural makeup; nonetheless, their exact structures are yet unknown. They mostly consist of flavan-3-ols, flavan-3,4-diols, or a combination of the two polymerized derivatives. The "flavolans," also known as condensed tannins, are the polymers. Condensed tannins are found in large quantities in a variety of foods, including fruits, vegetables, forage plants, chocolate, red wine, and certain grains including sorghum, finger millets, and legumes (Freudenberg , 1920).

Alkaloids

The wide class of organic bases known as alkaloids includes substances that contain secondary, tertiary, or cyclic amines. The biggest single family of secondary plant substances—about 5500 alkaloids—are now understood. Alkaloids are defined as "those fundamental chemicals which include one or more nitrogen atoms, frequently in combination as part of a cyclic system." There isn't a single definition of the word that is entirely adequate (Harborne and Walton, 1998). Alkaloids are pharmacologically active, nitrogen-containing basic chemicals that are found in plants. They may also cause hallucinations, loss of coordination, seizures, vomiting, and death by blocking ion channels, inhibiting enzymes, or interfering with neurotransmission. Phenolics may inhibit development, impair enzyme function, and prevent cell division, or just taste bad (Chung et al., 1998). In plant metabolism, plant catabolism, or plant physiology, the roles of alkaloids are

proposed as end products of metabolism or waste products, nitrogen storage reservoirs, protective agents for the plant against predator attack, growth regulators, and substitutes for minerals, such as potassium and calcium, in plants. The concept that at least some alkaloids may serve as plant growth regulators was encouraged by the structural resemblance of certain alkaloid structures to plant growth hormones (Nagel et al., 2019).

Terpenes

Terpenes are a chemically varied collection of natural compounds that are widely used. Terpenes are a unique category of hydrocarbon-based natural compounds with isoprene-like structures. The number of 5-carbon units in terpenes is used to classify them. Allelophathy, Insecticidal, Insect Pollinators, Plant Hormones are some of the ecological and physiological functions of terpenes in plants (Abscisic acid, giberrellin) (Tiwari and Rana, 2015). The precursor units with five carbon atoms, isopentenyl pyrophosphate (IPP), and its functional isomer, dimethylallyl pyrophosphate (DMAPP), are used to create these chemicals by the synthesis process. The subsequent condensation of IPP and DMAPP by the action of isoprenyl diphosphate synthase (IDS), which is a type of prenyltransferase (PT), produces acyclic and achiral isoprenyl diphosphate/pyrophosphate (ID, C5n) intermediates. These intermediates include geranyl pyrophosphate (GPP, C10), farnesyl pyrophosphate (FPP, C15), and geranylgeranyl pyrophosphate (GGPP, C20), which are considered universal terpenoid precursors (Cole et al., 2019). To create a combinatorial variety of terpenes, terpene synthases (TPSs) operate on one or more of these universal precursors, including DMAPP. Terpenoids, often referred to as isoprenoids, are terpenes having an oxygen moiety and other structural rearrangements, while terpenes are simple hydrocarbons based on combinations of DMAPP and ID.

Steroids

Steroids are a class of lipophilic, low-molecular weight chemicals produced from cholesterol that may be found in or derived from a wide range of marine, terrestrial, and synthetic sources. The sterols, bile acids, several hormones (including gonadal and adrenal cortical hormones), and certain hydrocarbons are all members of the steroid family. The physiology and biochemistry of the living creatures in which they are present are significantly influenced by all steroid groups and their metabolites. Numerous synthetic steroids are used widely as antihormone, contraceptive, cardiovascular, osteoporosis, antibiotic, anesthetic, antiinflammatories, and antiasthmatic medications (Bartnik and Facey, 2017).

Phytosterols (also called plant sterols)

These belong to a class of steroid alcohols that may be found in their natural environment in plants. They are a white powder that is insoluble in water but soluble in alcohols, and they have a distinctive smell that is not overpowering. They have a wide range of uses, including as additions in food, in medicine, and in cosmetics. The ability to decrease cholesterol levels in human subjects by up to 15%, as well as their potential to prevent cancer, make phytosterols (such as ergosterol 48) popular dietary supplements (Bartnik and Facey, 2017).

Glycosides

Most secondary metabolites found in plants are glycosides. Glycosides have a wide range of structural characteristics, and because of their established bioactivities and long history of usage, they are crucial to the discipline of pharmacognosy. Regular bond attachment between the other nonsugar moiety or aglycone and the condensed form of the sugar moiety or glycone, which is often a polysaccharide, results in the formation of glycosides. In general, carbon, hydrogen, oxygen, sulfur, and nitrogen make up the crystalline structure of the colorless alkaloid compounds known as glycosides. Most of the glycosides that have accumulated in plants are inert substances. The inactive glycosides were hydrolyzed by the enzyme to produce active glycosides, which may be important for plant cells' defense mechanism (Panche et al., 2016).

Examples of molecular docking on various flavonoidal compounds

Flavonoids are polyphenols found in fruits, vegetables, cereals, barks, roots, stems, flowers, tea, and wine (Sohrabi et al., 2020; Tanwar and Modgil, 2012; Yao et al., 2004). These phenylalanine-derived plant pigments give flowers their vibrant hues. Flavonoids are benzo-4-pyrone-structured polyphenolic chemicals found in plants and fruits. Flavonoids are found in vegetables, fruits, cereals, legumes, beans, herbs, roots, leaves, seeds, etc. Flavonoids have a 15-carbon, three-ring

diphenyl-propane (C6−C3−C6) skeleton. A C3 moiety connects the flavonoid's A and B benzene rings. C3 creates a six-membered ring linked to ring A.

They have biochemical and antioxidant effects on cancer, Alzheimer's, atherosclerosis, etc. Flavonoids are widely employed in nutraceutical, pharmacological, medicinal, and cosmetic goods across the globe because of their vast array of health-enhancing characteristics. It's because of their ability to influence critical cellular enzyme activities (antioxidative, antiinflammatory, antimutagenic, and anticarcinogenic). Some of these enzymes, such as xanthine oxidation (XO), lipoxygenation, and phosphoinositide 3-kinase (P3K), are known to be powerful inhibitors of these compounds. Table 23.2 displays some of the most current flavanoids molecular docking studies. Low-molecular-weight phenolic compounds are found throughout the plant world and have been shown to provide a variety of health benefits. In higher plants, they are one of the most well-known types of chemicals. Chalcones, flavones, flavonols, and isoflavones are among the many subclasses of flavonoids. Each of these subgroups has its own distinct primary source. Flavonols and flavones, for example, may be found in onions and tea.

Molecular docking studies are needed to uncover flavonoids' potential compounds for use in the human health system. Flavonoids' interactions with receptor molecules in the treatment of acute and chronic disorders are a significant topic of future study. Research is required to find novel flavonoids from nature's bounty so that they may be used in lieu of manufactured drugs that are damaging to the body.

Experimental design, materials, and methods

Protein selection and preparation

Protein data bank was queried to acquire the crystal structures of the chosen proteins (Database of the PDB, www.rcsb.org). The structures were preprocessed by adding hydrogens, filling in missing loops or side chains, capping uncapped C and N termini, adjusting bonds and formal charges for metals, removing water molecules, removing unwanted chains, optimizing hydrogen-bonded structures, and then minimizing the structures.

TABLE 23.2 Molecular docking analysis of some important flavonoidal compounds against SARS-CoV-2.

Flavanoids	Papyriflavonol A	Coronavirus (SARS-CoV-2)	SARS-CoV PLpro	Park et al. (2016)
	Xanthoangelol E		SARS-CoV PLpro	Jo et al. (2020)
	Herbacetin		SARS-CoV 3CLpro	Soukhova et al. (2004)
	Pectolinarin			
	Rhoifolin			
	Hesperetin			Ryu et al. (2010)
	Amentoflavone			Roh (2012)
	Gallocatechin gallate			Jo et al. (2019)
	Helichrysetin		MERS-CoV 3CLpro	Kim et al. (2014)
	Herbacetin			
	Isobavachalcone			Yu et al. (2012)
	Myricetin		SARS-CoV NTPase/helicase	Cho et al. (2013)
	Scutellarein		ATPase activity	
	Catechin gallate		N protein	
	Gallocatechin gallate			Jo et al. (2019)
	Geranylated flavonoids (tomentin A−E)		SARS-CoV PLpro	Kim et al. (2014)
	Neobavaisoflavone			
	Psoralidin			

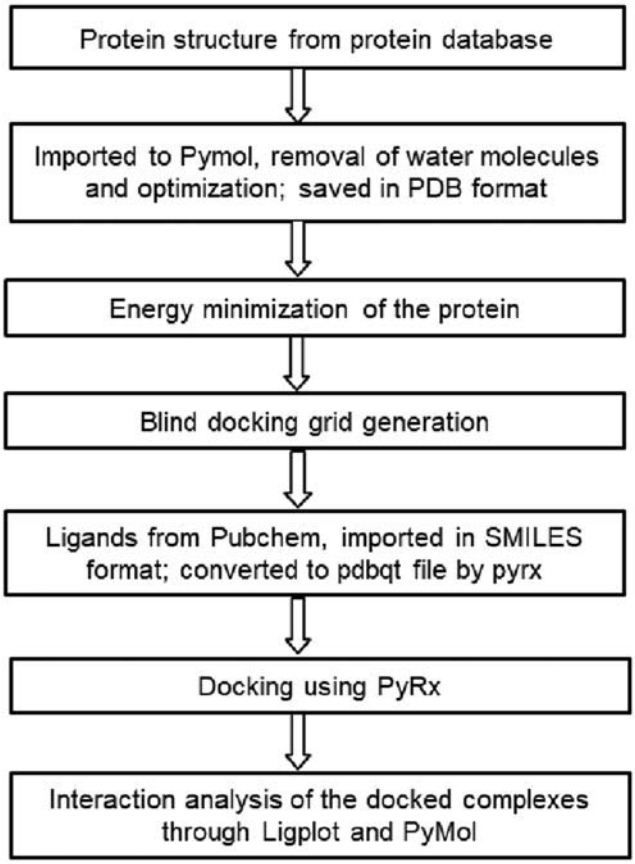

FIGURE 23.1 Flowchart of the methodology followed while docking target protein to the ligands.

Ligand preparation and molecular docking

The structures of the eight chosen flavonoids i.e., Apigenin, Genistein, Glabridin, Glycyrrhizin, Kaempferol, Liquiritigenin, Naringenin, and Quercetin were retrieved from Pubchem (https://pubchem.ncbi.nlm.nih.gov/) in canonical smiling notation and converted to PDB format using Corina (https://demos.mn-am.com/corina.html). Utilizing Ligprep, energy reduction was performed. Docking the reduced structures to the produced protein. Based on the binding energy and interaction with amino acid residues of each protein, the optimal flavonoid was selected. A flowchart of the methodology is depicted in Fig. 23.1.

Result and discussion

Two molecules out of eight demonstrated the most effective and dependable strikes against the Covid19 target. Docking result of the flavonoids with 6W63 receptor showed an excellent result with Glabridin to be the best docked among other flavonoids followed by Glycyrrhizin and Quercetin with the docked value of −8.7 kcal/mol, −8.5 kcal/mol and −7.6 kcal/mol respectively in PyRx (Table 23.3). Interactions were further examined for bond lengths and Hydrogen bonds, as illustrated in Ligplot, as shown in Fig. 23.2.

Molecular docking experiments reveal that the affinity of flavonoid Glabridin with the amino acids of the viral protein 6W63 was considerably strong enough in comparison with other flavonoidal compounds (Fig. 23.3). For the successful treatment of the new coronavirus COVID-19, the effectiveness of the above-stated flavonoids may further be tested for safety and efficacy characteristics at both preclinical and clinical stages.

TABLE 23.3 Binding energy values (docking score) and interactions between the eight flavonoids.

Flavonoids	Binding energy (kcal/mol)
Apigenin	−7.4
Genistein	−7.3
Glabridin	**−8.7**
Glycyrrhizin	−8.5
Kaempferol	−7.1
Liquiritigenin	−7.1
Naringenin	−7
Quercetin	−7.6

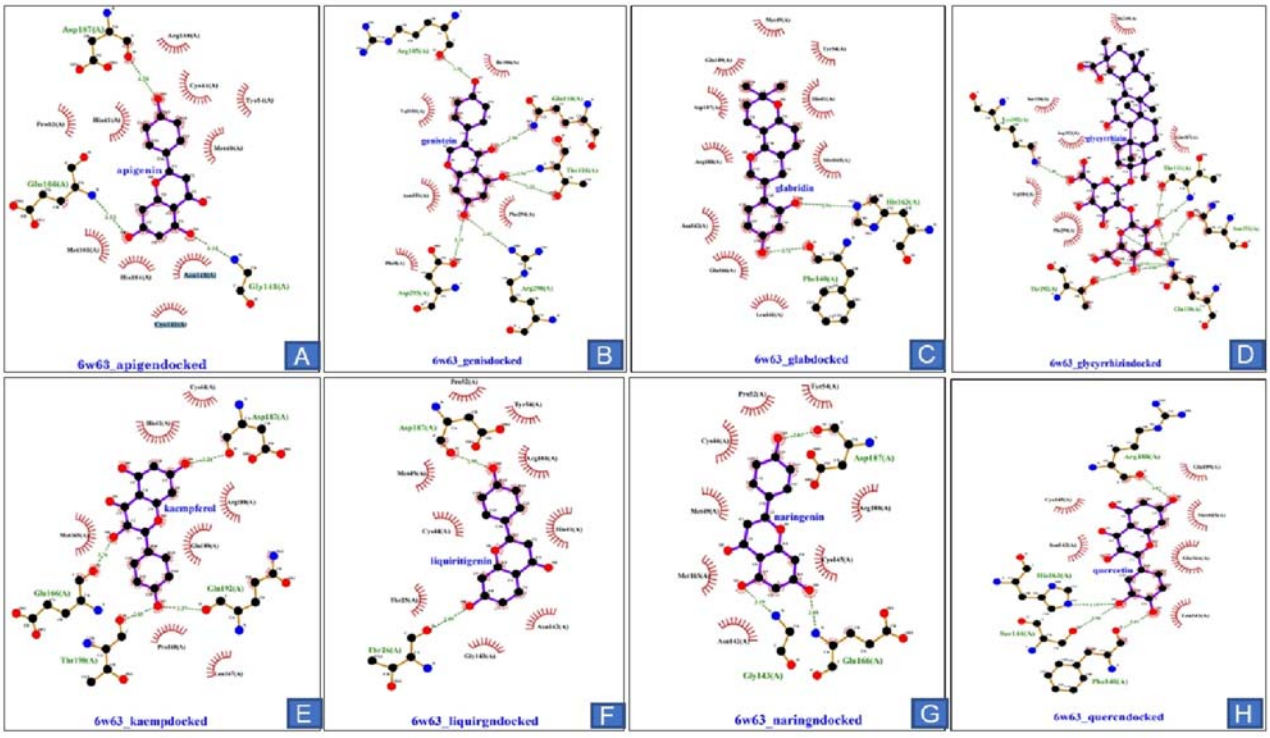

FIGURE 23.2 Diagrammatic sketch illustrating the interaction between binding site residues (docking results of eight ligands) flavonoids: (A) Apigenin (B) genistein (C) Glabridin (D) Glycyrrhizin (E) Kaempferol (F) Liquiritigenin (G) Naringenin and (H) Quercetin by LigPlot. Ligand is shown in purple and: green dashed lines indicate hydrogen bonds with distance in angstrom (Å), spoked red arcs indicate hydrophobic contacts, atoms are shown in black for carbon, ligand is shown in purple, and: green dashed lines indicate hydrogen blue for nitrogen and red represents oxygen. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

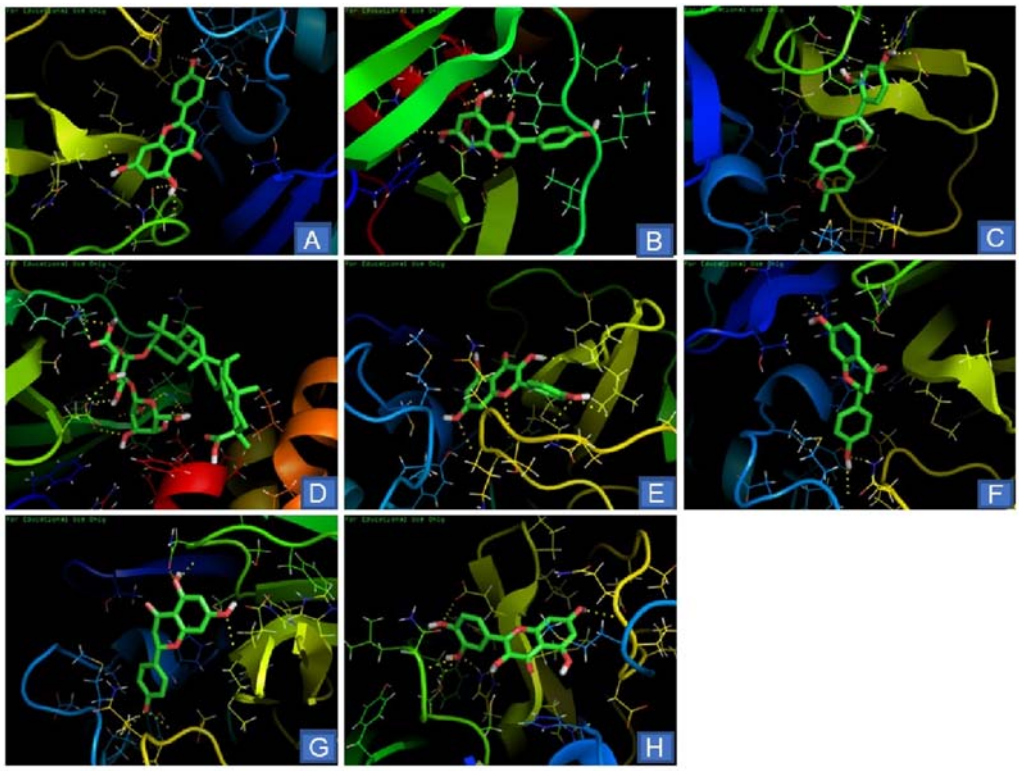

FIGURE 23.3 Pymol visualization of interaction (docking results of eight ligands) flavonoids: (A) Apigenin (B) genistein (C) Glabridin (D) Glycyrrhizin (E) Kaempferol (F) Liquiritigenin (G) Naringenin and (H) Quercetin.

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Chapter 24

Comprehensive resources for ligand-based drug discovery

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Introduction

Plants have been necessities for livelihood, ranging from infrastructure to native sources of energy and nutrition. Apart from this, it also plays a significant role in human health as a primary source of medicine. According to the World health organization, 80% of the population in around 174 countries directly or indirectly depend upon herbal and plant-based medicine. WHO defines herbal medicine as herbal remedies containing active ingredients from plant or plant-based derivates (Pranskuniene et al., 2022). Traditional herbal medicine has been used as primary medicine for any disease condition for thousands of years, ranging from various parts of the world (Solanki et al., 2020). The population has their preparation and prescribed use based on the availability and diversity of the human population and plants (Zhang et al., 2021). Based on th culture of the situated population, herbal medicine, and natural product-based medicine also evolved as part of daily life as rituals in history, ranging from Ayurveda in India, Traditional Chinese Herbal medicine (So et al., 2019), Kampo medicine in Japan, Aboriginal Medicine in Australia, TeRongoa in New Zealand and Yunan medicine. Herbal medicine advances our conventional therapies by providing cures for some rare disease conditions that were not possible before. Some mental conditions and diverse cancer types can be cured with herbal medicine (Zhang et al., 2021).

Natural products or herbal medicine are plant derivatives derived from different parts of plants ranging from leaves, roots, branches, flours, etc. Plants produce an enormous number of metabolites and secondary metabolites because of biodiversity in the plant population, which is highly significant with the unique medicinal properties that give several essential properties that are like a high number of SP^3-carbon atoms, a higher number of the h-bond donor, and accepter group and greater molecular rigidity provide greater drug likeliness to produce biological activity in a particular disease. Conventional synthetic compounds come with several challenges with a low success rate and involve tremendous costs and a time-consuming screening process. Still, there is a significant toxicity level and low efficacy. On the other hand, most natural product-based molecules are from edible plant sources that provide safe uses and low toxicity levels. Also, low-cost screening and high success rate make it a promising method for drug discovery (Yuan et al., 2016). Natural products and plant-based derivate molecules that are essential and primary metabolites are becoming the favorite choice of study with daily publishing peer review journal articles with new ethnomedical properties ranging from Antibiotic and Antifungal also 50% of new drugs for the treatment of various types of cancer, cardiovascular, diabetes, etc., in last 3 decade coming from natural products. Natural products-based small molecule drug discovery or ligand-based drug discovery is a complex process in the absence of a three-dimensional structure of target protein is a challenging task to get information and particular tools designed for the same. Therefore, it's essential to have comprehensive databases and tools for natural product-based drug discovery (Gajipara & Georrge, 2018). Here we discussed comprehensive databases and tools for natural product-based drug discovery that include 3d QSAR, pharmacophore, and ligand-based screening, as well as target fishing methods. Lack of databases and tools information hamper innovation and technological advancement in this area by restricting the implementation of new ideas (Sorokina & Steinbeck, 2020).

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Databases

A natural product database provides foundational information for ligand-based drug discovery with structural, taxonomic, and literature data about specific ligands. There are diverse databases available with relevant information for natural products and ligand compounds based on specific needs for development and maintenance. There are databases with structural information, like the ZINC database, that provide entry of over 80,000 compounds with structural information that are synthetically possible entities and available for purchase but lack insight into literature, taxonomy, and plant sources (Sorokina et al., 2021). And some databases aim to provide information regarding natural product-derived small compounds but lack updated data and proper maintenance with accurate information. Super Natural II is one of the large databases but lacks information about the origin of the compound and plant taxonomy data with this concern. There are databases with sufficient relevant data and well-maintained that are specifically focused on a particular category of compounds. NPAtlas (Natural Product Atlas) only contain data on microbial compounds (Van Santen et al., 2019). Also, there are databases such as StreptomeDB, AfroDB, and NuBBEDB with comprehensive information for ligand compounds that are not easily downloadable, and sometimes the information lacks the proper references and first source of the data. This database can lead to confusion that demands further validation with other resources (Sorokina & Steinbeck, 2020).

To provide relevant resources, we have classified databases based on three categories. The first is open-access databases, which are public repositories of easily accessible validated data worldwide. Users can access them without registration or login credentials. Still, there is also a concern about the accuracy of this content, as its public repositories sometimes lead to false-positive information. Hence, it is always recommended to go for relevant databases, as mentioned in Table 24.1 (Federhen, 2011). The second is commercial databases that provide relevant information through proper login IDs and credentials. The advantages of these databases are that they are well maintained and updated daily, and there are experts to validate the data before it gets available in this database (Sorokina & Steinbeck, 2020). Commercial databases also provide additional information and user-friendly, more advanced search engines with various filters that help to enhance user experiences. Also, more diverse data is available with the information of its origin and plant sources illustrated in Table 24.2. And the third category consists of databases for natural products or ligand molecules with specific information, specifically designing databases to achieve exact motives. These databases contain precise information and are of both types, open access or commercial, based on the data and features of the databases provided. That is specific to the organism or microbial natural product data basis that are commercial or subscription-based databases such as Antibase, MarinLit, The Dictionary of Natural Products, NPEdia, and NPAtlas. Specific database like NPBS provides manually curated detailed information about ligand and relational information with biological sources from literature. This database is significant in linking ligand compounds with specific species from which it derived (Xu et al., 2020). Moraine organisms are also considered potential sources of secondary metabolite and ligand compounds with considerable drug-likeliness properties because biodiversity is responsible for significant medicinal potential. Very few databases are available with a specific focus on marine natural products. CMNPD is one of the large databases with 31,000 chemical structure entry-specific collections from marine natural products or ligand compound data (Lyu et al., 2021). Also, well-maintained phytochemical and secondary metabolites from plants such as IMPPAT, NuBBEDB, KnapSack, CMAUP, Dr. Duke, and TCM@Taiwan are some of the well-known databases with a collection of ligand compounds have therapeutic potential as mentioned in Table 24.3 (Sorokina et al., 2021).

Tools and resources

3D QSAR

In ligand-based drug discovery, QSAR (Quantitative Structure-Activity relationship) model is applied to identify potential ligand molecules or sometimes molecules with virtual screening. QSAR model is based on the principle that a similar compound has similar chemical properties and produces similar biological activity (Vyas and Georrge, 2015). It aims to correlate structural features with the functional properties of a ligand (Roy et al., 2015). It uses linear statistic methods such as Multiple Linear Regression, Partial Least Square, or nonlinear methods like Support Vector Machines (SVM), Artificial Neural Network (Pradeep et al., 2016), Decision Trees, Bayesian Classifiers, *etc* (Du et al., 2022; Kausar and Falcao, 2018). To predict the biological function of a novel compound, it uses existing known ligand compounds as a training set, and novel compounds as a test set, chemical properties which directly involved in the function of a compound are calculated for both the known and unknown at the same time with chemical properties we also know the biological activity of training set, so based on various statistic method, it aims to achieve correlation between chemical properties and biological properties so that later with the use of this formula we able to predict the possible biological activity of unknown

TABLE 24.1 Open-source existing database resources for natural products and ligands (Sorokina and Steinbeck, 2020).

Sr no	Database name	NP type	Compounds	Stereochemistry (%)	URL
1.	3DMET	Generalistic	18,248	N/A	http://www.3dmet.dna.affrc.go.jp/
2.	AfroCancer	TM, plants, Africa	390	69.76%	http://www.afrocancer.org/
3.	AfroDB	TM, plants, Africa	954	70.73%	https://doi.org/10.1371/journal.pone.0078085
4.	AfroMalariaDB	TM, plants, Africa	265	70.93%	https://doi.org/10.1186/s13588-014-0006-x
5.	Alkamid database	Plants, structure	300	N/A	https://alkamid.ugent.be/
6.	BioPhytMol	Drug-like, plants, Asia	633	N/A	http://abopenlab.csir.res.in/biophytmol
7.	CamMedNP	TM, plants, Africa	>2500	N/A	https://doi.org/10.1186/1472-6882-13-88
8.	Carotenoids database	Structure	1174	57.63%	http://carotenoiddb.jp/
9.	CEMTDD—Chinese Ethnic Minority traditional drug database	TM, plants, Asia	4060	N/A	http://www.cemtdd.com/
10.	ChEBI	Chemicals	15,736	71.33%	https://www.ebi.ac.uk/chebi/
11.	ChEMBL	Chemicals	1899	91.59%	https://www.ebi.ac.uk/chembl/
12.	ChemIDplus	Drug-like, to000ins	9042	N/A	https://chem.nlm.nih.gov/chemidplus/
13.	ChemSpider	Chemicals	9732	29.50%	http://www.chemspider.com/
14.	CHMIS-C	Plants, TM, Asia	>8000	N/A	https://doi.org/10.1021/jm049838d
15.	CMAUP	Plants	47,645	72.37%	http://bidd.group/CMAUP/
16.	ConMedNP	Plants, TM, Africa	3118	69.59%	https://doi.org/10.1039/C3RA43754J
17.	Database of Indonesian medicinal plants	Plants, TM, Asia	6776	N/A	http://herbaldb.farmasi.ui.ac.id/v3/
18.	Drugbank NPs	Drug-like	2617	51.32%	https://go.drugbank.com/
19.	ETCM (Encyclopedia of traditional Chinese medicine)	TM, Asia	7274	N/A	http://www.tcmip.cn/ETCM/
20.	ETM-DB	TM, plants, Africa	1795	40.46%	http://biosoft.kaist.ac.kr/etm/home.php/
21.	GNPS	Dereplication	7619	31.08%	https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp
22.	HIM (herbal Ingridients in vivo Metabolism database)	Drug-like, TM, plants	1261	41.62%	http://www.bioinformatics.org.cn/
23.	HIT (herbal Ingridients targets)	Drug-like, TM, plants	524	44.03%	http://hit2.badd-cao.net
24.	IMPPAT	TM, plants, Asia	9596	N/A	https://cb.imsc.res.in/imppat/

Continued

TABLE 24.1 Open-source existing database resources for natural products and ligands (Sorokina and Steinbeck, 2020).—cont'd

Sr no	Database name	NP type	Compounds	Stereochemistry (%)	URL
25.	InflamNat	Drug-like	552	63.75%	http://www.inflamnat.com/
26.	InPACdb	Drug-like, plants, Asia	124	62.10%	http://www.inpacdb.org
27.	KNApSaCK	Plants	10,265	74.76%	http://www.knapsackfamily.com/KNApSAcK/
28.	MedPServer	Plants, TM, Asia, drug-like	1124	N/A	http://bif.uohyd.ac.in/medserver/
29.	Mitishamba database	Plants, Africa	1102	23.84%	http://erepository.uonbi.ac.ke/handle/11295/92273
30.	NANPDB	Plants, Africa	6832	75.02%	https://doi.org/10.1021/acs.jnatprod.7b00283
31.	NaprAlert	Generalistic	>15,5000	N/A	https://napralert.org/
32.	NAPROC-13	Dereplication	>18,000	N/A	https://c13.materia-medica.net/
33.	NeMedPlant	TM, plants, Asia	100	N/A	http://bif.uohyd.ac.in/nemedplant/
34.	NMRShiftDB	Dereplication	1875	N/A	https://nmrshiftdb.nmr.uni-koeln.de/

TABLE 24.2 Commercial database or subscription base databases resources for natural products and ligand (Sorokina & Steinbeck, 2020).

Sr no	Database name	NP type	Compounds	URL
1.	Ambinter-Greenpharma natural compound library (GPNCL)	Generalistic, industrial	>150,000	https://www.ambinter.com/
2.	AntiBase	Drug-like	>40,000	https://sciencesolutions.wiley.com/antibase
3.	Berdy's Bioactive natural products database	Generalistic	N/A	https://doi.org/10.1007/978-3-642-75165-3_23
4.	CAS registry	Chemicals	>300,000	https://www.cas.org/cas-data/cas-registry
5.	ChemBridge diversity datasets	Generalistic, industrial	N/A	https://www.chembridge.com/
6.	DNP (Dictionary of natural products) by Chapman and Hall (also known as CHEMnetBase)	Generalistic	>230,000	https://dnp.chemnetbase.com/
7.	LOPAC1280 by Merck	Drug-like	1280	https://www.sigmaaldrich.com/IN/en/product/sigma/lo1280
8.	NADI	TM, plants	3000	http://www.nadi-discovery.com/
9.	Prestwick	Plants, industrial	320	https://www.prestwickchemical.com/
10.	TargetMol natural compound Library	Generalistic, industrial	1680	https://www.targetmol.com/

TABLE 24.3 Specific or unique database resources for Natural products and ligand (Sorokina and Steinbeck, 2020).

Sr no.	Database	NP type	Compounds	URL
1.	AntiMarin	Marine, drug-like	>60,000	https://www.scienceopen.com/document?vid=03a1a98e-434c-4255-a287-5a900f59d024
2.	DFC (Dictionary of Food COmpounds)	Food	>41,000	https://dfc.chemnetbase.com/
3.	DMNP (Dictionary of marine natural products)	Marine	>30,000	https://dmnp.chemnetbase.com/
4.	eBasis	Food		https://www.eurofir.org/our-tools/ebasis/
5.	MarineLit	Marine	>29,000	https://marinlit.rsc.org/
6.	ATBD (Animal To000in database)	to000ins	1000	https://doi.org/10.1093/nar/gkm832
7.	AnalytiCon discovery MEG000	Bacteria, plants, industrial	5147	https://ac-discovery.com/screening-libraries/
8.	BitterDB	Food	654	https://bitterdb.agri.huji.ac.il/
9.	FooDB	Food	24,215	https://foodb.ca/
10.	Marine compound database (MCDB)	Marine	182	http://www.bioinformation.net/003/003000032008.htm
11.	Marine natural product database (MNPD)	Marine	6000	https://doi.org/10.1021/ci010111x
12.	PAMDB	Metabolites, bacteria	N/A	https://doi.org/10.1021/ci010111x
13.	Phenol-explorer	Food	862	https://doi.org/10.1093/database/bat070
14.	PhytoHub	Food, plants	1200	https://phytohub.eu/
15.	Seaweed metabolite database (SWMD)	Marine	1110	https://doi.org/10.6026/97320630005361
16.	StreptomeDB	Bacteria	6415	https://doi.org/10.6026/97320630005361
17.	Super Sweet	Food, metabolites	15,000	https://doi.org/10.1093/nar/gkq917

ligand compound. Several tools are available for QSAR modeling as it involves many steps, so there are two sets of tools mentioned in Table 24.4. One is for calculating molecular descriptors. In other words tool for the calculation of chemical properties that are drug likeliness of compounds ranging from Lipinski Rules to pharmacokinetic properties of given ligand compound, and the second one is for QSAR model building that involves various statistic methods (De et al., 2022).

Pharmacophore modeling

Pharmacophore is a molecular modeling method that deploys to achieve the potential component of a compound responsible for the biological activity of a ligand molecule in a particular case when no information or unclear structural information about the target protein is available (Patel et al., 2018) in this case, pharmacophore model used to define an active center of ligand compound and predict possible binding pose and physiochemical behavior of ligand compound in case of interaction with a target protein. The most common feature of the active center in ligand molecules for the pharmacophore model is H-bond acceptor, H-bond donor acidic and basic groups, aliphatic hydrophobic moieties, and aromatic hydrophobic moieties introduced in spheres of a specific resistance radius for virtual screening of small molecule. It results in possible interaction between ligand and target protein by utilizing structural information of ligand molecule. It is also used as an alternative or pretool before going further with docking. Several tools and web-based resources are available for pharmacophore modeling in Table 24.5 (Tyagi et al., 2021).

TABLE 24.4 Tools and resources for QSAR (Pradeep et al., 2016).

Sr no	Name of tool	Tool type	URL
1.	Vega QSAR	Web tool	https://www.vegahub.eu/about-qsar/
2.	3D-QSAR	Web tool	https://www.3d-qsar.com/
3.	DPubChem	Web tool	https://www.cbrc.kaust.edu.sa/dpubchem/
4.	SMIREP	Stand alone	http://www.karwath.org/systems/smirep.html
5.	ChemDes	Stand alone	http://www.scbdd.com/chemdes/
6.	Codessa-pro	Online	http://www.codessa-pro.com/
7.	Danish (Q)SAR	Online	http://qsar.food.dtu.dk/
8.	QSAR-ME Profiler	stand alone	http://www.qsar.it/
9.	Xternal validation plus	Stand alone	https://sites.google.com/site/dtclabxvplus/
10.	Classification Based QSAR	Stand alone	https://drive.google.com/file/d/1I5D82KDh5430oC2z2DYHFfBNNiZQ9du_/view
11.	BioPPSy	Stand alone	https://sourceforge.net/projects/bioppsy/
12.	SEABED	Online	http://www.bsc.es/SEABED
13.	Strip-it	Stand alone	http://ww1.silicos-it.com/
14.	OECD QSAR Toolbox	Stand alone	https://qsartoolbox.org/
15.	Build QSAR	Stand alone	https://buildqsar.software.informer.com/download/
16.	SwissADME	Online	http://www.swissadme.ch/

TABLE 24.5 Tools and resources for pharmacophore (Muhammed and Aki-Yalcin, 2021).

Sr no	Name of tool	Tool type	URL
1.	CATALYST-HipHop	Web tool	doi/10.1021/ci020368a
2.	CATALYST-HypoGen	Web tool	https://doi.org/10.1007/s00894-012-1381-8
3.	GALAHAD	Web tool	https://doi.org/10.1007/s10822-006-9082-y
4.	GASP	Stand alone	https://doi.org/10.1021/ci100194k
5.	LigandScout	Stand alone	https://docs.inteligand.com/ligandscout/
6.	MOE	Stand alone	https://www.chemcomp.com/Products.htm
7.	Pharmer	Stand alone	https://sourceforge.net/projects/pharmer/
8.	PharmaGist	Web tool	https://bioinfo3d.cs.tau.ac.il/PharmaGist/
9.	PharmMapper	Web tool	http://www.lilab-ecust.cn/pharmmapper/
10.	PHASE	Stand alone	https://www.schrodinger.com/products/phase

Reverse docking or target identification

In silico Reverse Docking or target, fishing is a significant method for *In silico* target identification in the absence of three-dimension structure information of the target protein. In the case of in silico, drug discovery in the absence of three-dimension structural information of protein target is not available. Still, a huge ligand compound gets deposited in a public repository with relational literature with ethnomedical uses (Cockroft et al., 2019). Experimental methods for target identification are expressive and time-consuming and involve a very complex methodology. Conversely, Reverse docking or target fishing is a promising approach to feed this gap by identifying suitable target proteins involved in disease-curing

mechanisms. This method is categorized into two based on application and available data: ligand-based and receptor-based (Galati et al., 2021; Wang et al., 2019). A ligand-based method is more suitable when we have ligands with structural similarity to the known compounds. In this case, huge ligands can be screened in one go based on computational power. Further, to improve accuracy and speed, the machine learning model can also reduce commuting time. On the other hand, receptor-based method demands structural information of the target protein should be present then we can screen the molecule based on binding affinity with the known active site of the available protein target. In some cases, we have used both the approach ligand-based and receptor-based reverse docking for the prediction and validation of biological activity (Galati et al., 2021).

Discussion

Many ligand compounds and small molecule structures are deposited in public repositories with technological advancement and computational power. It is possible to convert this data into knowledge by correlating the compound with the target protein, which is possible with the ligand-based drug discovery method. Nowadays, it is very much possible to test the hypothesis of the traditional use of plant-based remedies and medicine. Still, there is a scope for improvement in this area. There is no tool for the aggregation of all data sets, and it requires tools for various ligand-based target identification methods in one place. Also, new methods and algorithms, such as artificial intelligence and deep learning, can apply to improve existing methods.

Conclusion

A natural product is a significant source of medicine around the world. In most cases, traditional medicine lacks target information. Ligand-based drug discovery methods are important in reaching suitable lead compounds for natural product-based medicine. For that, it's necessary to have enough resources for easy asses and analysis of structural compounds. Ligand-based drug discovery resources can speed up drug discovery and innovation in this area for possible cures.

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Chapter 25

Molecular docking of phytochemicals targeting ER, PR, and HER2 receptors as therapeutic sites for breast cancer: An in silico study

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Introduction

Cancer is an uncontrollable and immortal disease that affects aberrant cells over an extended period of time. Cancerous cells can be aggressive, invasive, and metastatic, and they frequently invade other organs. Breast cancer is quite varied in nature and interferes with the normal mammary epithelial cells' ability to function. In the entire world, breast cancer is one of the most common diseases affecting women (Jemal et al., 2011). from wealthy nations, around 25% of breast cancer patients were reported (Park et al., 2005). Breast cancer is one of the more frequent cancers, accounting for more than 27% of all cancer patients in India (Torre et al., 2012). More than 144,937 instances of breast cancer have been documented in 2012 alone, with a death rate of 48.45%. Patients between the ages of 50 and 64 experienced a sharp increase in the incidence of breast cancer cases (Nandakumar, 2001). One breast cancer case was reported for every 22 women in urban areas, and for every 60 women in rural areas. Rarely, though, have males also been known to have breast cancer. Each year, more than 1500 new cases are reported in the United States (Giordano et al., 2002). This cancer is among the second most common causes of death worldwide in western nations. More than 60% of breast cancer diagnoses in Asian nations are estrogen receptor alpha positive (ER-) tumors. Although ER-a specifically mediates the proliferation of estrogen-induced cells in ER a breast cancer cell lines in vitro via an autocrine mode of action, ER-a plays a crucial role in the development of breast cancer in normal mammary gland development (Tanet al., 2009). Progesterone hormone receptor and mammaglobin testing are breast cancer diagnostic techniques with the highest positive predicted value (88%) with estrogen (Maria et al., 2012).

One of the main factors contributing to the occurrence of breast cancer is the overproduction of estrogen. According to a report, the 17 b-estradiol ligands, which is sometimes referred to as estrogen, effectively activates the estrogen receptor, a nuclear receptor. Both ER- and ER-estrogen receptors are found in the human population naturally, while ER-is more commonly expressed in the uterus and mammary glands. Breast cancer in women is significantly influenced by the estrogen receptor in terms of apoptosis, inflammation, homeostasis, differentiation, metabolism, maturation, and proliferation (Bai and Gust, 2009). It is well known that the receptor, ER-, participates in immune surveillance, resistance to apoptosis, metastasis, and cell proliferation (Jiang et al., 2006). In addition to holding several molecular targets for the exploration of cancer drugs, the hyperactivity of the estrogen hormone may potentially cause the multiplication of the ER-in mammalian cells, which contributes to the maintenance and growth of different types of breast cancer.

In recent years, radiation, chemotherapy, and hormone therapy have formed a variety of combinations that require meticulous planning for each individual therapy. Around 60% of premenopausal women and nearly 75% of post-menopausal women have estrogen-dependent breast cancer, and cancer therapy has been successful in inhibiting ER

Nanotechnology and In Silico Tools. https://doi.org/10.1016/B978-0-443-15457-7.00013-7

activity (Peng et al., 2009). Endocrine therapy is frequently advised for metastatic/recurrent breast cancer or the early stages of the disease in the case of breast cancer. Letrozole, an aromatase inhibitor, generally lowers the production of estrogen in peripheral cells and the tumor by inhibiting the activity of aromatase. Fulvestrant, a selective estrogen receptor down regulator and selective estrogen receptor modulators that induce ER degradation and destabilization, is currently the only treatments available for estrogen-based breast cancer. Although there are several anticancer medications and potential inhibitors for a wide range of targets, the effective rise in resistance and the variety of side effects point to the urgent need for innovative cancer therapies. Because ER-receptors are present, virtual screening (VS) can be a useful method for locating and screening prospective chemicals from a variety of natural sources. For the purpose of identifying ligands against the ER-receptor, numerous VS techniques including negative image-based screening, molecular docking, and generic pharmacophore hypothesis have been applied (Niinivehmasa et al., 2016). About 50% of people who have been diagnosed with cancer that involves both the progesterone and ER-receptors respond well to most medicines, including tamoxifen, which primarily targets ER-function. Cancer is known to lack expression of ER-coregulatory proteins, which are considered to be highly tightly regulated proteins under natural conditions (Suganya et al., 2014). It is possible for the estrogen receptor to bind to DNA. Numerous researches have concentrated on the estrogen receptors alpha and beta in humans (McDonnell et al., 2015). Estrogen is a crucial steroid hormone that regulates the differentiation, development, and functionality of the particular organ. The estrogen binds to its receptor in the nucleus, according to the Hellenic mechanism of ERs. Estrogen receptor elements (EREs) and ERs interact specifically, and EREs are primarily found in target gene promoters (Nilsson. et al., 2001). When EREs and ERs interact, it effectively induces conformational changes in the receptors' ligand-binding domains, which are found in the promoters of particular target genes (Nilsson et al., 2001). Effectively stimulating conformational changes in the receptors' ligand-binding domains, EREs bind with ERs.

Animal trials and in-vitro analysis are too time-consuming, expensive, and labor-intensive classic drug development techniques. The currently available antibreast cancer treatments are either ineffective or unsuccessful and suffer from numerous drawbacks over time. The initial-Line therapy might or might not be effective; it might help for a while but then halt, or could have very negative consequences. According to estimates, on average, it takes 12 years and 2.7 billion USD to build a new home. Drug discovery using conventional techniques. Investigating computation-based prediction tools can help screen a bulk number of molecules.

For their anticancer potential in a remarkably brief period, without significant investment, and offering up animals. This can give the initial information for more research (Li et al., 2020).

Depending on the goal and relevant systems, computer-aided drug discovery methods have been widely employed to improve the efficiency of the drug discovery and development pipeline in drug research (Acharya et al., 2019). To analyze the molecular behavior of molecules that attach to target proteins, one such technology utilized frequently in drug discovery is called molecular docking (Aftab et al., 2021).

The main aim of this study is to find suitable and effective bioactive compounds by screening numerous phytochemical molecules using a bioinformatic approach for the development of an effective treatment for breast cancer. In addition, marketed anticancer standard drugs were used for comparative evaluation.

Material and methodology

Protein preparation

The three-dimensional structure of the proteins involved in breast cancer such as PR (4OAR), ER (3ERT), HER2 (3PPO), and Aromatase receptor (3S7S) was obtained from the RCSB Protein Data Bank (PDB) (https://www.rcsb.org/). The description of various proteins involved in the study is mentioned in Table 25.1.

Preprocessing and analyzing the structures

This preprocessing is important for subsequent structure-preparation procedures including creating heteroamorous states, assigning H-bonds, and minimizing the level of the structure. The preprocessing stage in the protein preparation wizard has a number of choices. They are filling loops, adding hydrogen, deleting water molecules, and setting bond ordering.

TABLE 25.1 Target proteins and their respective functions.

Protein	Protein description	Protein function	PDB ID
PR	Progesterone receptor	Cellular proliferation, differentiation, gene expression, and transcriptional activator and repressor	4OAR
ER	Estrogen receptor	An important regulator of development, growth, and differentiation of the normal mammary gland.	3ERT
HER2	Human epidermal growth factor receptor 2	Help control how a healthy breast cell grows, divides, and repairs itself	3PPO
AR	Aromatase receptor	Aromatase is an enzyme involved in the conversion of androgen (such as testosterone) to estrogen (such as 17β-estradiol). It is also a very effective therapeutic target for the treatment of endocrine-responsive breast cancer.	3S7S

Ligand preparation

The list of phytochemicals with proven anticancer properties was retrieved from Dr. Dukes Phytochemical and Ethnobotanical Database.

(https://phytochem.nal.usda.gov/phytochem/search). Further, the canonical smiles and three-dimensional structure of phytochemicals were obtained from the PubChem library (https://pubchem.ncbi.nlm.nih.gov/).

Pharmacological properties of compounds

For initial screening, SwissADME (http://www.swissadme.ch/), an online web-based platform, was used, which evaluates the pharmaceutical fidelity of the drug candidates.

Various attributes such as molecular weight, lipophilicity, number of hydrogen bond acceptors, and donors were analyzed using this tool. As these attributes form the foundation for the Lipinski rule of five, any molecule deviating from the threshold values was eliminated from further analysis.

Molecular docking

Prior to molecular docking analysis, proteins were preprocessed using Discovery Studio 2020. This step includes the removal of any hetero-groups and the addition of Hydrogen atoms and different charges. Further, the active sites for each protein were identified using the CASTp server (http://sts.bioe.uic.edu/castp/index.html?1ycs). Further, the preparation of ligands and receptors in the PDBQT file format was carried out in the Auto Dock tool. The molecular docking was carried out using Auto Dock Vina and discovery studio to understand the interaction between receptors and ligands.

Bioavailability radar and toxicity

Drug-likelihood was comprehensively evaluated for candidates, considering six physiochemical properties such as solubility, molecular size, polarity, lipophilicity, saturation, and flexibility and bioavailability radar was obtained using the SwissADME tool (http://www.swissadme.ch/). At the same time, the ADMETlab 2.0 webserver (https://admetmesh.scbdd.com/service/screening/cal) was used to predict the toxicity of the ligands.

ADME and drug-likeness properties

For the assessment of the drug-likeness and ADMET properties of the substances under study, accessible online web servers like SwissADME (http://www.swissadme.ch/index.php) are used. The websites help scientists find new medication candidates, reduce the number of empirical tests, and increase the success rate (Bello Umar et al., 2020; Sagiru Hamza et al., 2021). In this study, the key ADMET properties—measures of the pharmacokinetics of the compounds under investigation—are computed after Lipinski's rule of five (ROF) is used as the primary screening step for the drug-likeness features.

Result and discussion

Breast cancer continues to be a severe public health concern despite technological advances and intensive study, and it is given top attention in medical studies (Aftab et al., 2021). This study primarily focuses on using various computational approaches to identify possible inhibitors of breast cancer. An effort was made to investigate different phytochemicals against a number of proteins thought to be potential therapeutic targets and involved in the progression of breast cancer.

Protein preparation of ER, PR, HER2, and aromatase protein

The crystal structure of the estrogen receptor protein was created using the auto dock model and retrieved from the Protein Data Bank (ID: 3ERT, 4OAR, 3PPO, and 3S7S) given in Fig. 25.1. The protein-heavy atoms received hydrogen atoms, which were then tuned. Cofactors and water molecules were eliminated. Using an OPLS-3 force field with attached atoms in the backbone, protein minimization was also accomplished successfully.

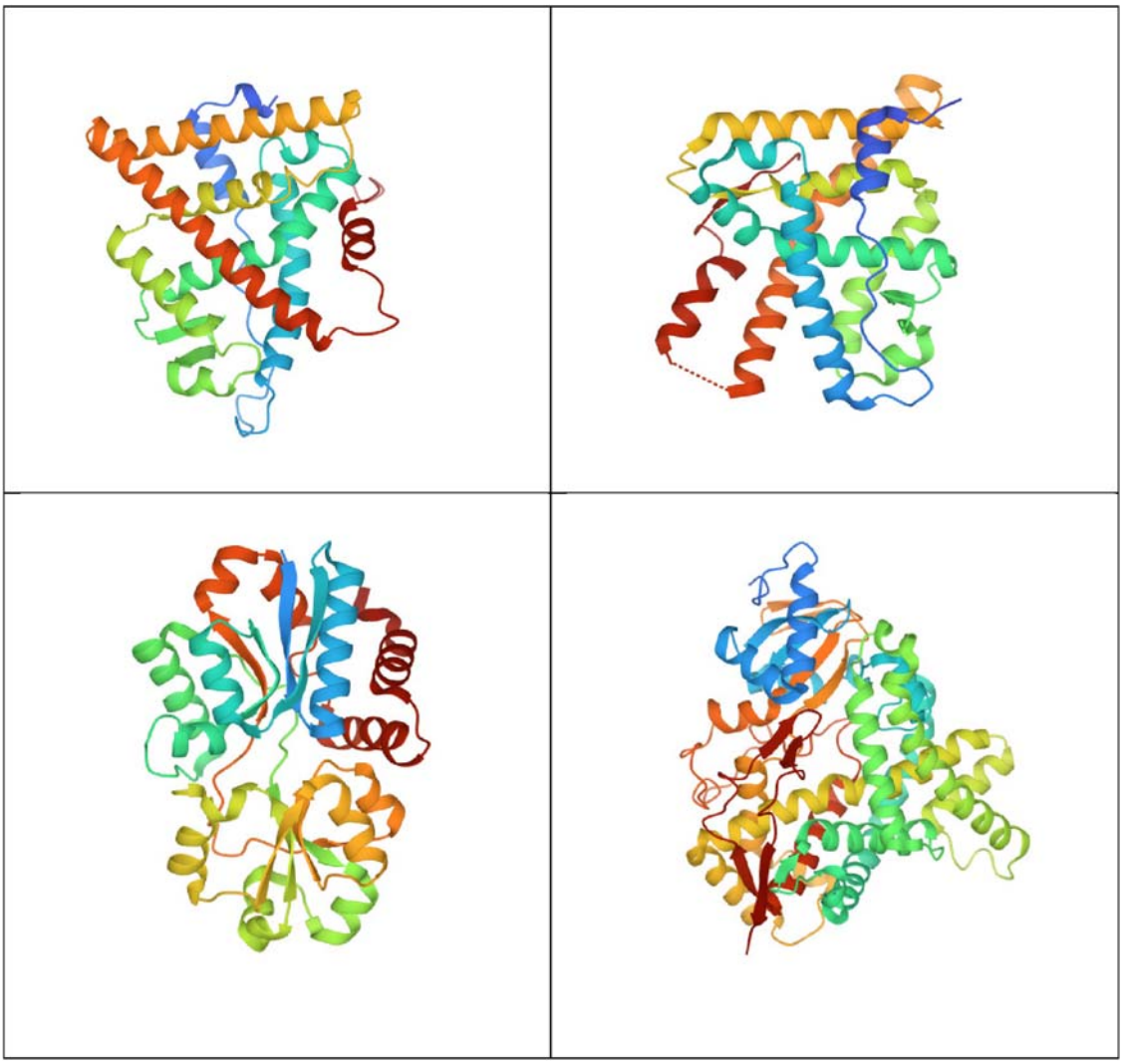


FIGURE 25.1 Three dimensional structure of prepared ER, PR, HER2, and aromatase protein (PDB ID: 3ERT, 4OAR, 3PPO, and 3S7S).

Ligand selection

The molecules examined in this study were chosen from a wide variety of phytochemical classes, including triterpenoids, alkaloids, flavonoids, etc. A few of the compounds have already been mentioned as cancer-related enzyme inhibitors in various works of literature. Nine phytochemicals that have already been recognized as anticancer agents were evaluated in the current investigation. To begin with, pharmacokinetic characteristics were assessed, and Lipinski's rule of five was taken into account when determining how similar a given molecule was to a given medicine. All of the compounds from the chosen phytochemicals that satisfied the Lipinski rule were taken into consideration for further study.

Molecular docking

One of the most popular virtual screening techniques for predicting the interaction between receptors and ligands is molecular docking. This approach may forecast both the protein-ligand complex's structure and binding affinity, which is crucial knowledge for lead optimization (Singh and Konwar, 2012). To determine the binding affinity in the current investigation, the chosen receptors were docked to the screened compounds.

Screening of phytochemicals for estrogen receptor (3ERT)

The binding affinity of cucurbitacine B, D, and E, Echinatin, Beta-sitosterol,16-deoxycucurbitacin B, 18-deoxycucurbitacin B, Oleanolic acid, and Flavonoids with estrogen receptor given in Table 25.2, and important interaction with amino acid given in Figs. 25.2 and 25.3.

Screening of phytochemicals for progesterone receptor (4OAR)

The binding affinity of cucurbitacine B, D, and E, Echinatin, Beta-sitosterol,16-deoxycucurbitacin B, 18-deoxycucurbitacin B, Oleanolic acid, and Flavonoids with progesterone receptor given in Table 25.3, and important interaction with amino acid given in Fig. 25.4.

TABLE 25.2 Estrogen receptor binding affinity docking score.

Name of ligand	Docking score affinity (kcal/mol)	Important interaction with amino acid residue
Cucurbitacin B	−8.2	Hydrogen bond: LEU A:536, MET A:522, Alkyl: VAL A:533, Pi Alkyl: LYS A:529, TYR A:526
Cucurbitacin D	−9.1	Hydrogen bond: ASP A:351, VAL A:533, Pi-Sigma: TRP A:383, Alkyl: LEU A: 354, LYS A:529, Pi Alkyl: ALA A:350, LEU A:536
Cucurbitacin E	−8.7	Hydrogen bond: TRP A:383, Carbon hydrogen bond: PRO A:535, Pi-Sigma: TRP A:383, Pi Alkyl: TYR A:526
Echinatin	−8.3	Hydrogen bond: LEU A: 387, Pi-Sigma: LEU A: 525, Pi-Sulfur: MET A: 343, Alkyl: ALA A:350, Pi Alkyl: LEU A:391, MET A:388, LEU A:428
Beta-sitosterol	−7.6	Pi sigma: TRP A: 383 Alkyl: VAL A:533 Pi Alkyl: LYS A:529, TYR A:526, LEU A:536, LEU A:525
16-Deoxycucurbitacin B	−8.8	Carbon hydrogen bond: LYS A:529 Pi Alkyl: TYR A:526
18-Deoxycucurbitacin B	−7.9	Pi-Sigma: TRP A:383 Alkyl: LEU A:354, LEU A:539 Pi Alkyl: LEU A:536, MET A:522, TYR A:526
Oleanolic acid	−9.7	Hydrogen bond: GLU A:380 Alkyl: ALA A:350, TRP A:383, LEU A:525, LYS A: 529, LEU A:536, TYR A: 526 Pi Alkyl MET A: 522
Flavonoids	−7.6	Hydrogen bond: ASP A:351, Pi Sulfur: MET A:522, Pi-Pi Stacked: TRP A:383 Pi Alkyl: LEU A:536, LEU A: 525, MET A:522

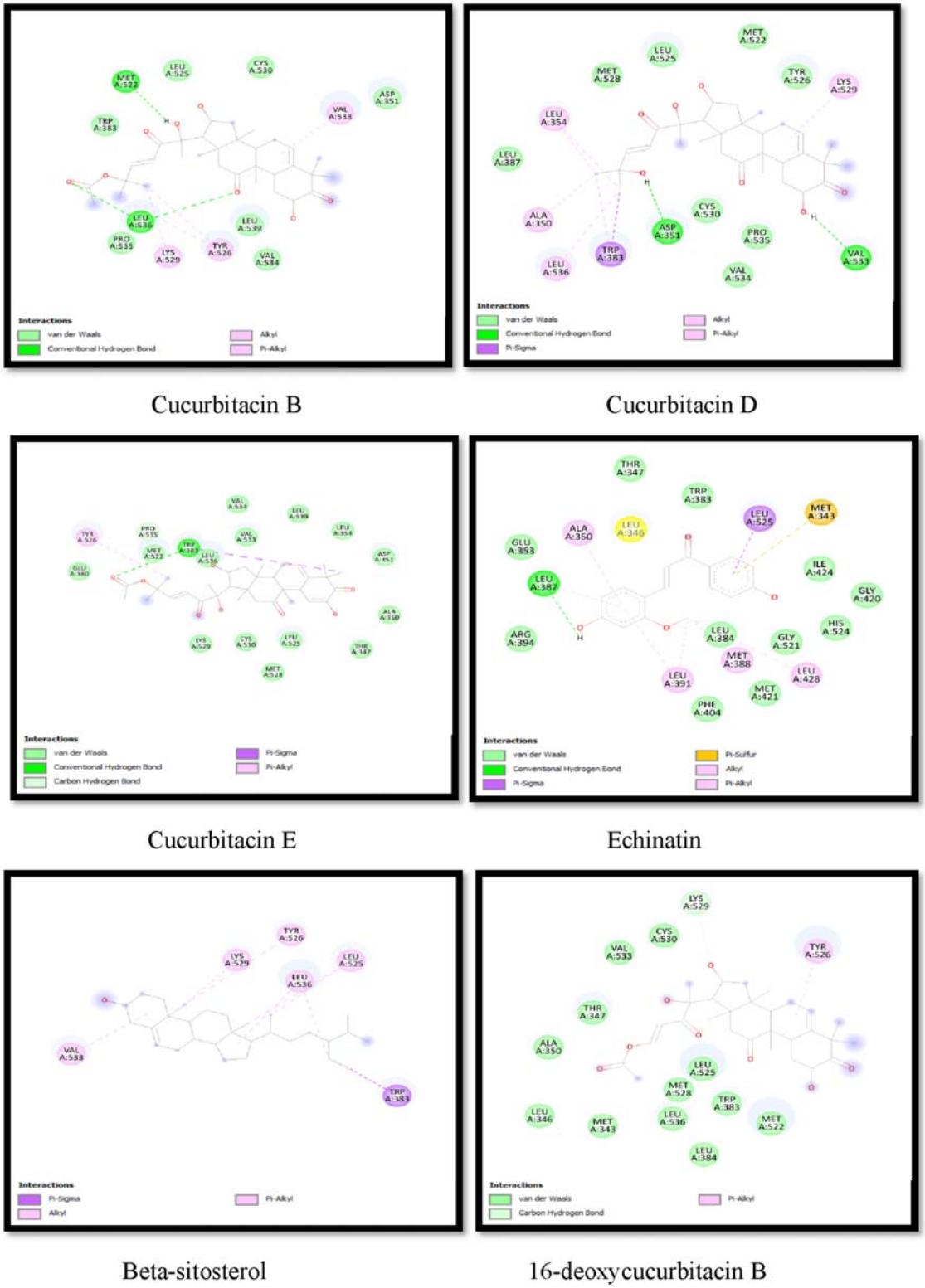
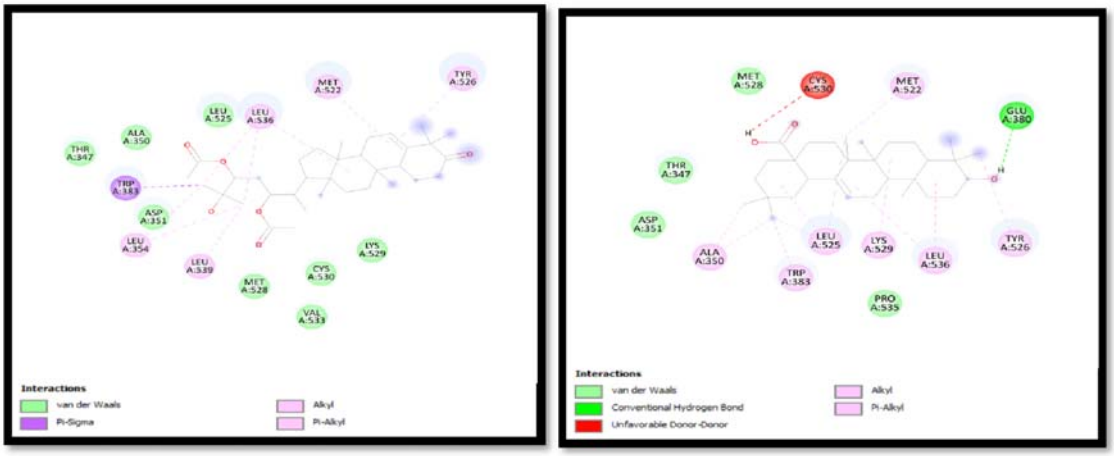
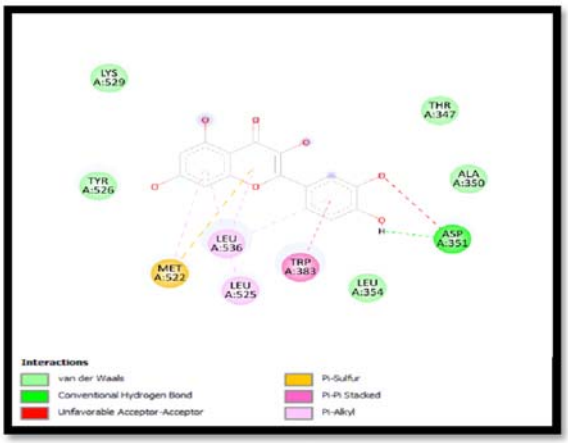


FIGURE 25.2 Two-dimensional interaction diagrams of the ligand with estrogen receptor.



18-deoxycucurbitacin A

Oleanolic acid



Flavonoids

FIGURE 25.2 cont'd

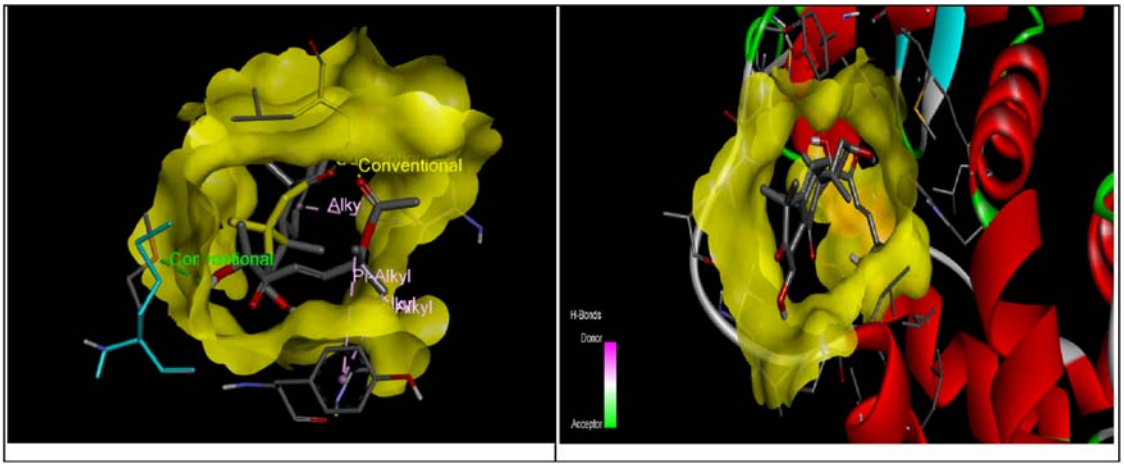


FIGURE 25.3 3D interaction diagrams of the ligand with estrogen receptor.

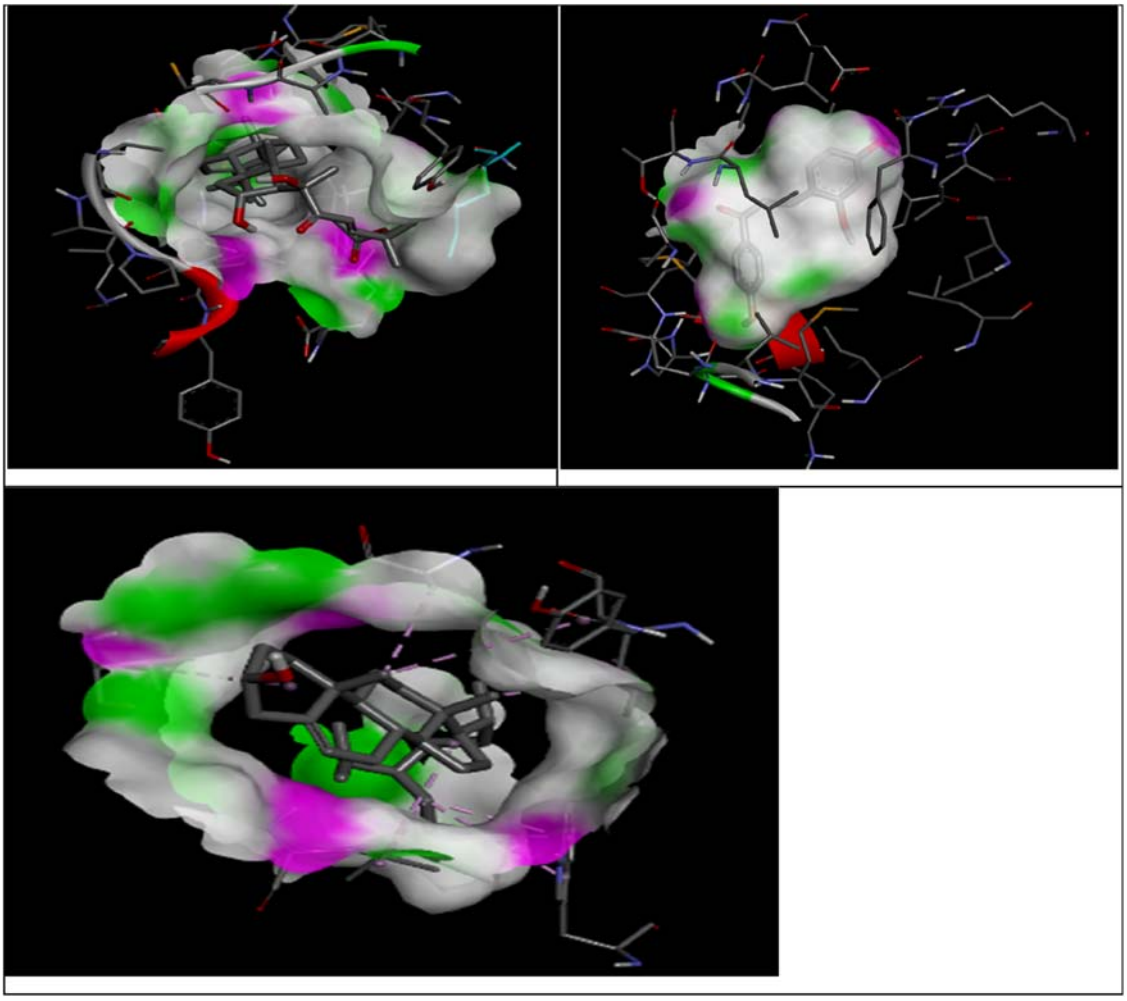


FIGURE 25.3 cont'd

Screening of phytochemicals for HER2 (3PPO)

The binding affinity of cucurbitacine B, D, and E, Echinatin, Beta-sitosterol,16-deoxycucurbitacin B, 18-deoxycucurbitacin B, Oleanolic acid, and Flavonoids with HER2 receptor given in Table 25.4, and important interaction with amino acid given in Fig. 25.5.

Screening of phytochemicals for aromatase (3S7S)

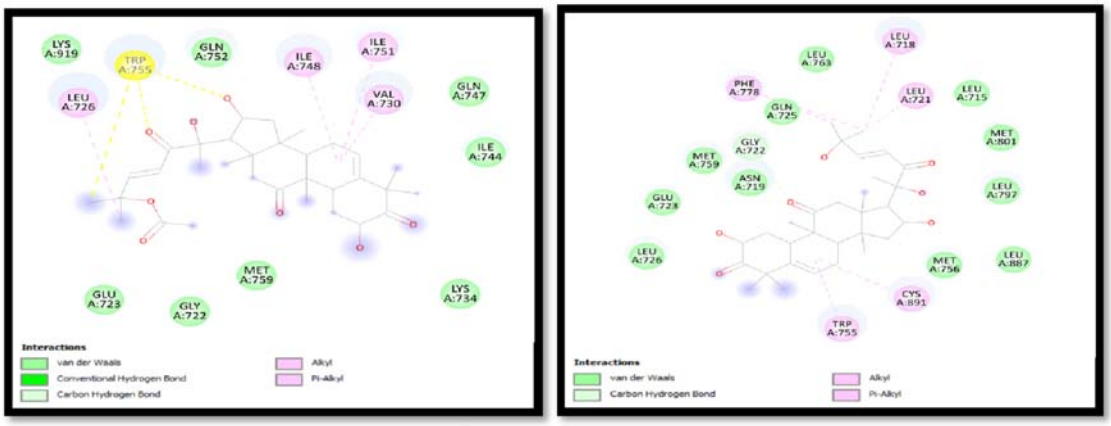
The binding affinity of cucurbitacine B, D, and E, Echinatin, Beta-sitosterol,16-deoxycucurbitacin B, 18-deoxycucurbitacin B, Oleanolic acid, and Flavonoids with aromatase receptor given in Table 25.5, and important interaction with amino acid given in Fig. 25.6.

Bioavailability radar and toxicity prediction

Even if a medicine has a considerable effect, it may not be able to provide the required therapeutic effect within the body without careful consideration of bioavailability during the drug development process. The SwissADME tool's bioavailability radar map is one tool that may be used to forecast the drug's bioavailability in silico (Lipinski et al., 2001). Bioavailability prediction, *Salmonella typhimurium* reverse mutation assay, oral acute toxicity, and carcinogenicity are given in Table 25.6.

TABLE 25.3 Progesterone receptor binding affinity docking score.

Name of ligand	Docking score affinity (kcal/mol)	Important interaction with amino acid residue
Cucurbitacin B	−7.9	Alkyl: LEU A:726, ILE A:748, ILE A:751, VAL A:730
Cucurbitacin D	−7.4	Carbon hydrogen bond: GLY A:722, Alkyl: PHE A:778, LEU A:718, LEU A:721, Pi Alkyl: TRP A:755, CYS A:891
Cucurbitacin E	−8.1	Hydrogen bong: ASN A:719, THR A:894, Pi-Sigma: PHE A:895, Alkyl: LEU A:715, LEU A:718
Echinatin	−7	Hydrogen bond: CYS A:891, ASN A:719, Carbon hydrogen bond: ASN A:719, Alkyl: MET A:759, MET A:756, Pi Alkyl: LEU A:797, LEU A:887, LEU A:718, LEU A:721, PHE A:778, MET A:801
Beta-sitosterol	−6.9	Alkyl: LYS A:769, HIS A:770, TRP A:765, ARG A:766, Pi-Alkyl: PRO A:696
16-Deoxycucurbitacin B	−7.7	Hydrogen bond: GLN A:916
18-Deoxycucurbitacin B	−8.5	Alkyl: LEU A:718, CYS A:891, TRP A:755, Pi Alkyl: LEU A:721, PHE A:778
Oleanolic acid	−7.4	Alkyl: MET A:759, ALA A:915, Pi Alkyl: TRP A:755, PHE A:895
Flavonoids	−7.4	Hydrogen bond: GLN A:725, Pi-Cation: ARG A:766, Pi-Anion: GLU A:695, Pi Alkyl: VAL A:698, ILE A:699, PRO A:696

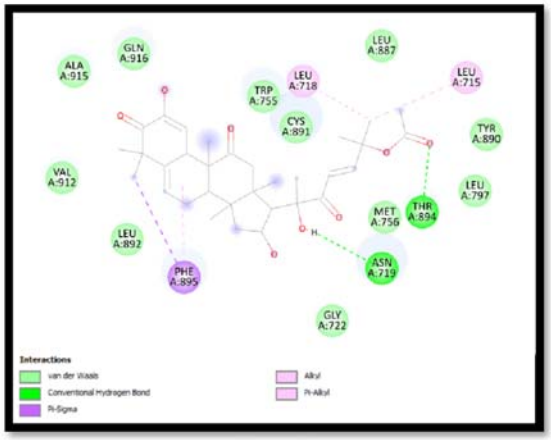


Cucurbitacin B Cucurbitacin D

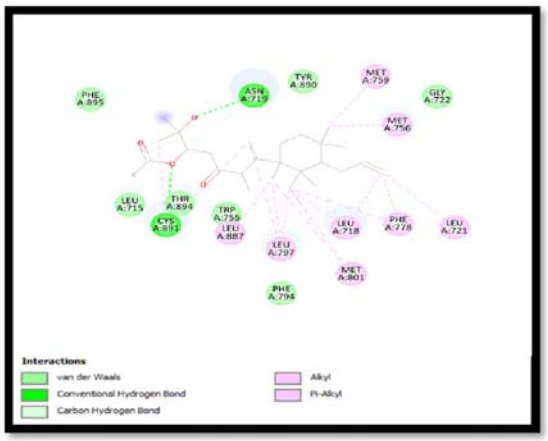
FIGURE 25.4 Two-dimensional interaction diagrams of the ligand with progesterone receptor.

ADME and pharmacokinetics study

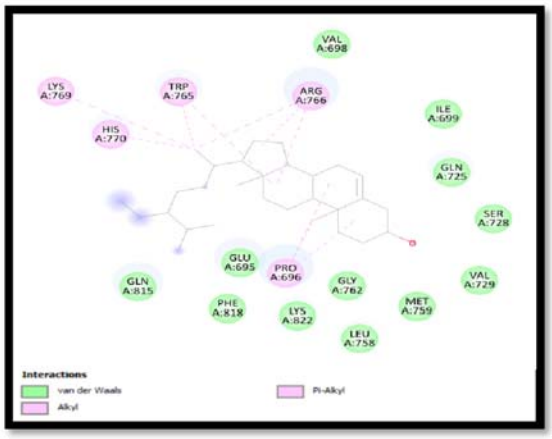
With a medicine that had been proven to be effective against breast cancer, the ADMET and pharmacokinetics features of the target hit compounds were evaluated. The drug-likeness properties of the chosen compounds were computed using the online program SwissADME (Daina et al., 2017), and their ADME properties were formulated using the pkCSM online web page. The evaluation of their drug-likeness parameters serves as the major criterion for choosing drug candidates in the early stages of the drug discovery process. This is accomplished by linking a given molecule's physicochemical characteristics to its biopharmaceutical aspect within the human body, primarily its effect on oral bioavailability (Bickerton et al., 2012).



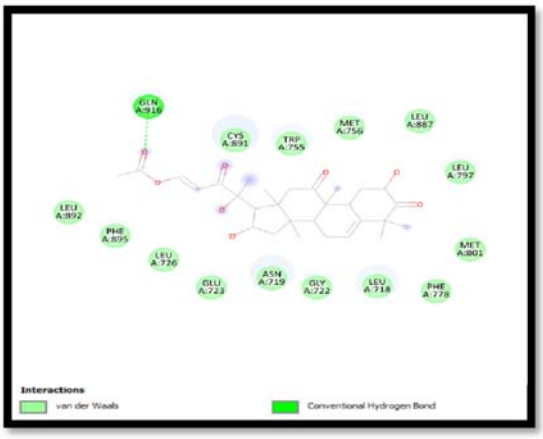
Cucurbitacin E



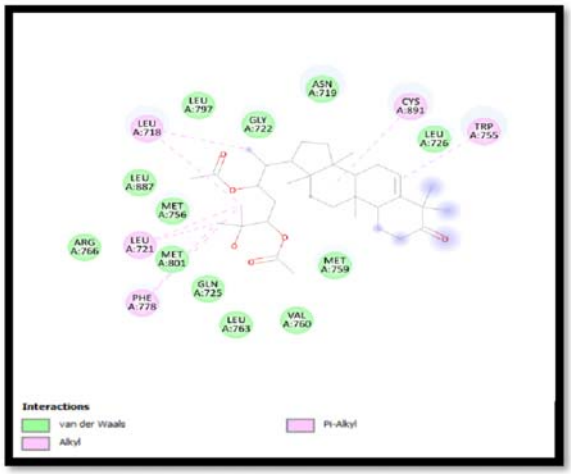
Echinatin



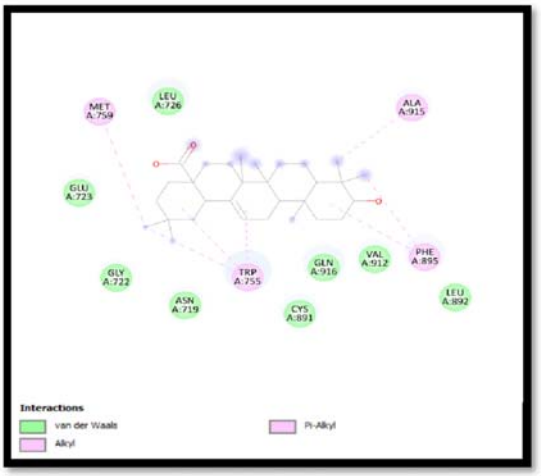
Beta-Sitosterol



16-deoxycucurbitacin B

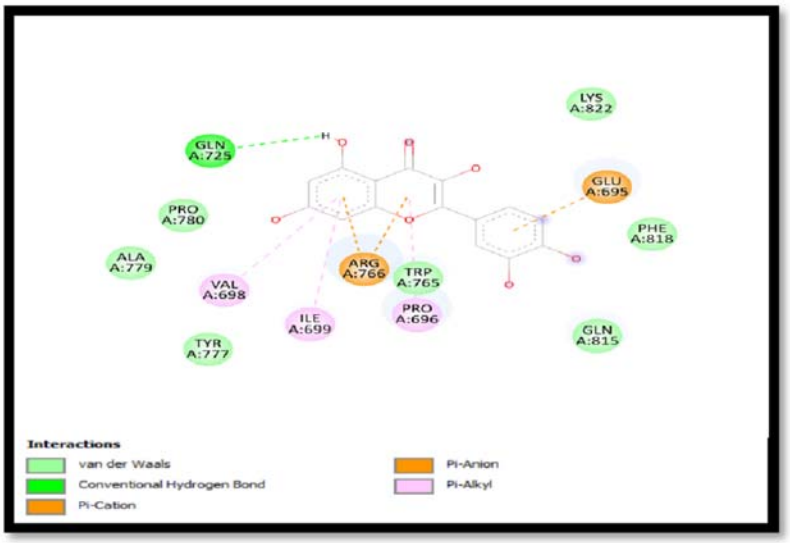


18-deoxycucurbitacin A



Oleanolic acid

FIGURE 25.4 cont'd



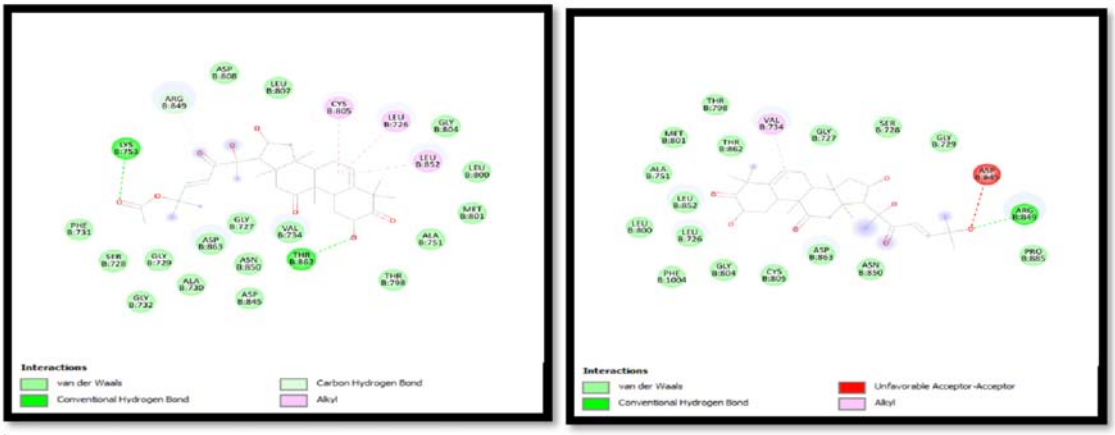
Flavonoids

FIGURE 25.4 cont'd

TABLE 25.4 HER2 receptor binding affinity docking score.

Name of ligand	Docking score affinity (kcal/mol)	Important interaction with amino acid residue
Cucurbitacin B	−7.6	Hydrogen bond: LYS B:753, THR B:862 Carbon hydrogen bond: ARG B:849, Alkyl: CYS B:805, LEU B:726, LEU B:852
Cucurbitacin D	−7.8	Hydrogen bond: ARG B:849 Alkyl: VAL B:734
Cucurbitacin E	−7.7	Hydrogen bong: LYS: A:887, VAL A:884, ARG A:756 Alkyl: LYS A:883, PHE A:731 LYS A:992 Pi Alkyl: PRO A:992, TRP A:888
Echinatin	−6.1	Hydrogen bond: ILE B:886, LYS B:887, VAL B:884 Alkyl: PRO B:885, Pi Alkyl: PRO B:992, TRP B:888, LYS B:992, LEU B:807
Beta-sitosterol	−7.7	Alkyl: PRO B:885, LEU B:726, ARG B:849, VAL B:734, ALA B:751, LEU B:852
16-Deoxycucurbitacin B	−7.6	Hydrogen bond: PHE A: 731, Carbon hydrogen bond: ALA A:730, Alkyl: LYS A:883
18-Deoxycucurbitacin A	−8.1	Hydrogen bond: CYS B: 805 Alkyl: LEU B:852
Oleanolic acid	−8.2	Hydrogen bond: ASP A:871, Pi-Sigma: TYR A:772, Alkyl: MET B:953, Pi Alkyl LEU A:869, ILE A:872
Flavonoids	−8.8	Hydrogen bond: MET B:801, THR B:862, Pi-sigma: LEU B:852, LEU B:726, Pi-Pi Stacked: PHE B:1004, Pi Alkyl: ALA A:751, LYS B:753

The "rule of 5," which states that a compound will be well absorbed or permeated only when its molecular weight (mol. wt.) 500, its number of hydrogen bond donors (HBD) 5, its number of hydrogen bond acceptors (HBA) 10, and its partition coefficient octanol/water Log P 5, was developed by Lipinski (Lipinski et al., 1997) who also performed the most innovative and thorough investigation of drug-likeness properties. If a compound does not break more than two (2) of the conditions, it is considered to satisfy the rule of five. Table 25.7 displays the findings of the possible hit compounds' drug-likeness qualities.



Cucurbitacin B Cucurbitacin D

FIGURE 25.5 Two-dimensional interaction diagrams of the ligand with HER2 receptor.

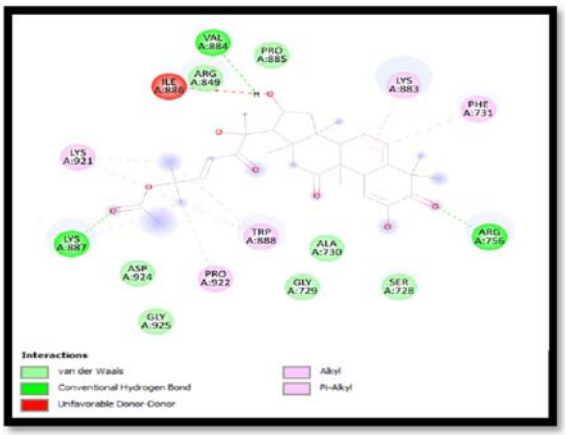
According to the findings of the pharmacokinetic experiments, all of the chosen compounds are well absorbed, permeable, and have drug-like qualities because they all do not break any rules, while the remaining compounds only break one (1) rule because their molecular weight is greater than 500.

Additional evaluation was conducted using bioavailability score (ABS) standards (Martin, 2005), with a computed value of 0.55 for each of the chosen substances. This method was based on the chance that a molecule would have an ideal profile of permeability and bioavailability, where 0.55 signifies compliance with the Lipinski rule of five and the rat bioavailability value is 55%, which is higher than the 10% probable value. Additionally, the chosen compounds were rated on a scale from 1 (extremely simple to synthesize) to 10 for their accessibility to synthetic processes (very difficult to synthesize). All of the selected compounds have a synthetic accessibility range of 2.68−6.81 (Table 25.7), making it simple to synthesize the compounds.

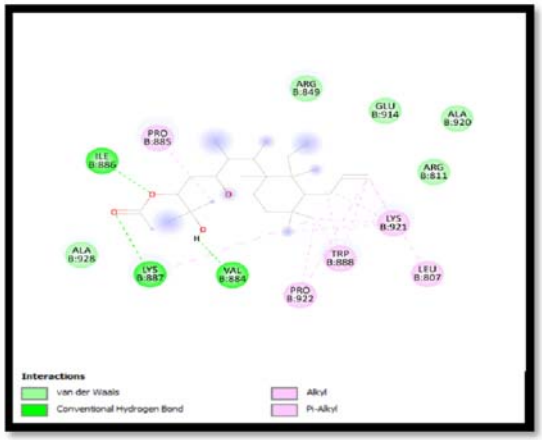
Furthermore, as these values are higher than the minimum suggested values of 30%, all of the chosen compounds have outstanding human intestinal absorbance values ranging from 76.69% to 100%. The central nervous system (CNS) permeability and the blood−brain barrier (BBB) permeability are specified as >0.3−1 log BB and >2−3 log PS, respectively. Therefore, for any chemical, a value of log BB more than 0.3 indicates that the medicine can pass the BBB, whereas a value of log BB less than 1 depicts undesirable distribution of the drug to the brain (BBB). In terms of log PS, a score more than two indicates that the compound can enter the CNS, whereas a value less than 3 suggests that the molecule may find it difficult to induce into the CNS (Martin, 2005). The results given in Table 25.8 indicated that the selected compounds had a good chance of passing through the barriers. It is crucial to take the drug's metabolism into consideration since it reveals how a drug is bio-transformed in the body through its enzymatic metabolism. The cytochrome P450 super enzyme family plays a significant part in the metabolism of drugs. The CYP families responsible for medication metabolism include 1A2, 2C9, 2C19, 2D6, and 3A4, with the 3A4 enzyme being the most significant and containing the majority of the chosen drugs as both a substrate and an inhibitor.

Total clearance is a measure that illustrates the relationship between a drug's rate of removal and its concentration in the body. The chosen compounds showed greater total clearance values, which fall within the permissible range for a drug candidate in the body. Finally, the chosen drugs demonstrated positive pharmacokinetic and physicochemical ADMET properties. Therefore, based on these findings, it can be said that these substances can be used as ER, PR, HER2 inhibitors, and breast cancer medications in the years to come.

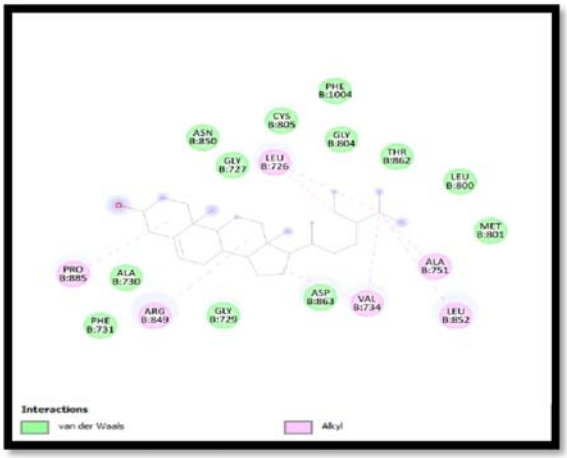
The main conclusions of this study show that several phytochemicals have the capacity to block numerous breast cancer-related genes. The better lead molecules include those like cucurbitacin B, Cucurbitacin D, Cucurbitacin E, 16-deoxycucurbitacin B, 18- deoxycucurbitacin A. More analogs of these prospective compounds can be created using drug designing and pharmacophore modeling techniques. The in silico study can be validated by in vitro and in vivo testing, and if the outcomes support our findings, the phytochemicals can then be chemically synthesized. For the purpose of extending and further testing the study we have produced here, more research, including realism evaluation, would be beneficial to our findings.



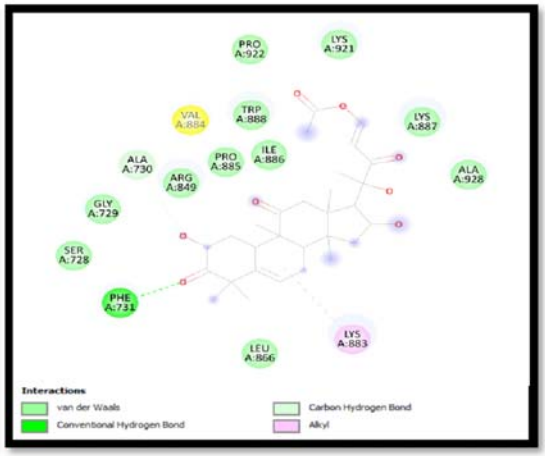
Cucurbitacin E



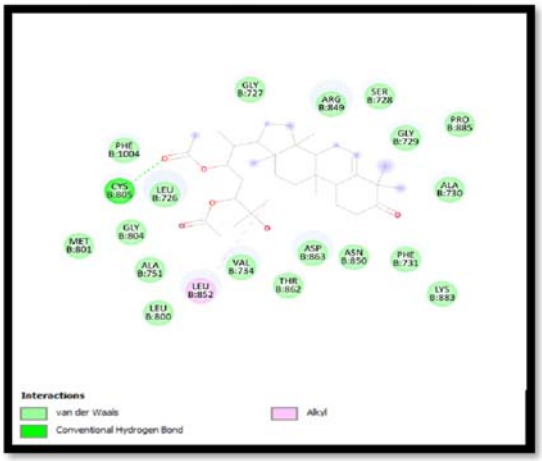
Echinatin



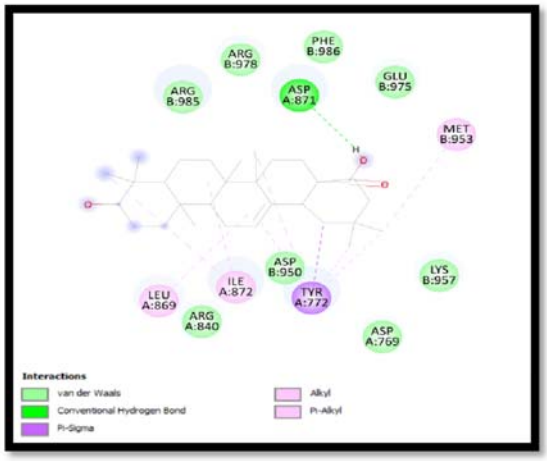
Beta-Sitosterol



16-deoxycucurbitacin B

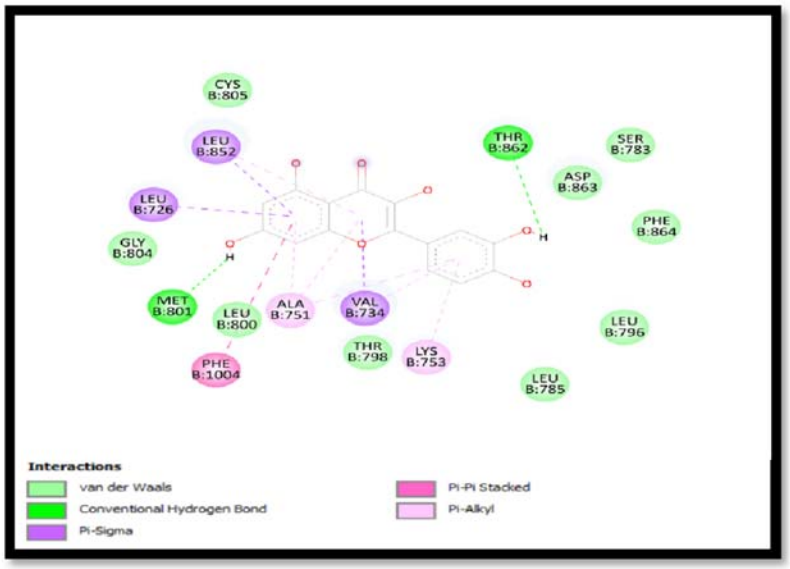


18-deoxycucurbitacin A



Oleanolic acid

FIGURE 25.5 cont'd



Flavonoids

FIGURE 25.5 cont'd

TABLE 25.5 Aromatase receptor binding affinity docking score.

Name of ligand	Docking score affinity (kcal/mol)	Important interaction with amino acid residue
Cucurbitacin B	−7.6	Hydrogen bond: GLY A:154, TYR A:164, Carbon hydrogen bond: SER A:153, Pi-Sigma: TYR A:441
Cucurbitacin D	−7.8	Hydrogen bond: TYR A:361 Pi-Sigma:TYR A:441, Alkyl: LEU A:157
Cucurbitacin E	−7.7	Hydrogen bond: LYS A:440, GLN A:428 Carbon hydrogen bond: TYR A:144 Pi-Sigma: TYR A:424
Echinatin	−6.1	Hydrogen bond: ALA A:438, Alkyl: CYS A:437, TRP A:224, LEU A:477, Pi Alkyl: LEU A:152, MET A:303, PHE A:148, ALA A:306, ILE A:133, VAL A:370, PHE A:134,
Beta-sitosterol	−7.7	Hydrogen bond: GLN A:428, Alkyl: LEU A:157, ILE A:350, MET A:444
16-Deoxycucurbitacin B	−7.6	Hydrogen bond: TYR A:361, Carbon hydrogen bond: SER A:153, Pi-Sigma: TYR A:441
18-Deoxycucurbitacin A	–8.1	Pi-Sigma: TYR A:361, Alkyl: PRO A:429, Pi Alkyl: PHE A:430, TYR A:424
Oleanolic acid	−8.2	Hydrogen bond: ARG A:115, Alkyl: LEU A: 152, ALA A:306, Pi-Alkyl: ALA A:438, CYS A:437, ILE A:133, PHE A:134, VAL A:370, LEU A:477, MET A:374
Flavonoids	−8.8	Hydrogen bond: TYR A:336, ASN A:75, ARG A:403, GLN A:367Carbon hydrogen bond: PRO A:368, Pi-Cation: LYS A:473, Pi-Sigma: LEU A:479, Pi-Pi T-Shaped: HIS A:475, Pi-Alkyl: PRO A:368

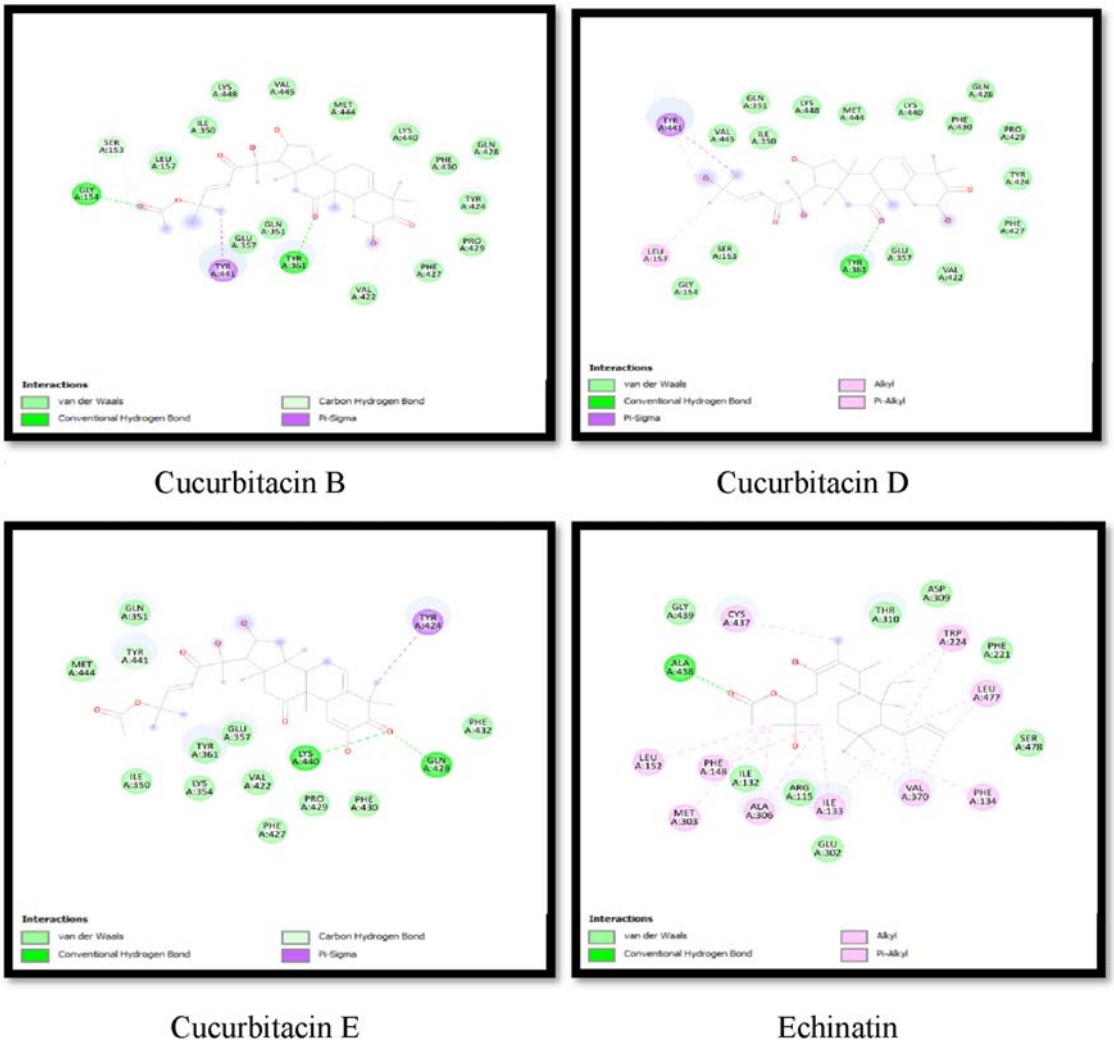
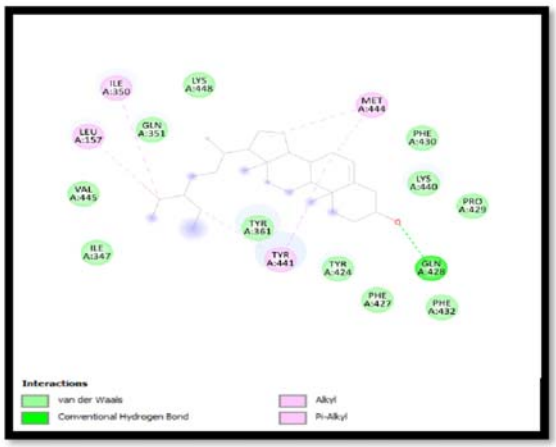


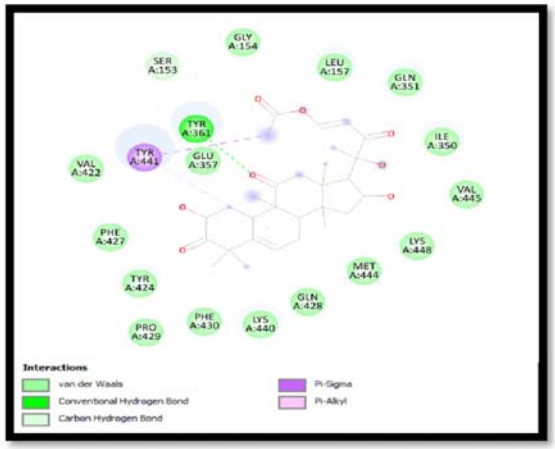
FIGURE 25.6 Two-dimensional interaction diagrams of the ligand with aromatase receptor.

Conclusion

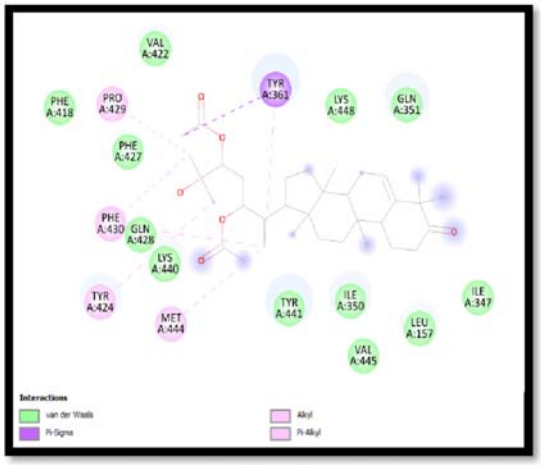
In this investigation, the rational method combined with in silico prediction capability was used to target 3ERT, 4OAR, 3PPO, and 3S7S for limiting the growth of breast cancer cells. The research took advantage of the power of pharmacophore modeling to investigate compounds with high binding affinities and interactions with biological importance. After comparing the screened compounds to the recorded compounds and the data set used to create the pharmacophore sample, docking tests were conducted. In order to confirm the consistency of the protein complex architectures, molecular dynamics simulation is used. The complex's high degree of consistency throughout the simulation period is indicated by the hydrogen bond interaction outcome, which supports the complex's stability and supports the docking result. To further investigate their drug-likeness and pharmacokinetic features, the potential hit compounds were further put through ADMET and pharmacokinetic profile investigations. Since the majority of the compounds broke Lipinski's rule of five (ROF), they are well absorbed, permeable, and have features that are similar to those of drugs. Their synthetic accessibility value ranges from 2.68 to 3.81, indicating that they are easily synthesized, and their bioavailability score of 0.55 indicated that they are orally bioavailable. Additionally, the results of the ADMET studies suggested that the compounds have excellent human intestinal absorbance values between 76.69% and 100%, which are higher than the minimal recommended values of 30% and indicate that they can cross the BBB and reach the CNS. They are primarily substrates and inhibitors of the cytochrome P450 enzymes responsible for drug metabolism, and they are quickly removed from the body. Only three



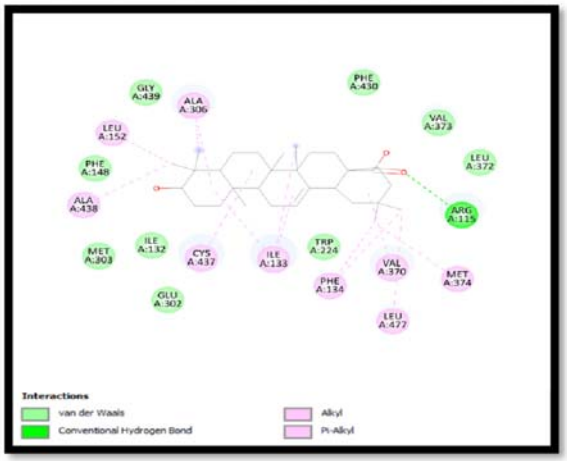
Beta-sitosterol



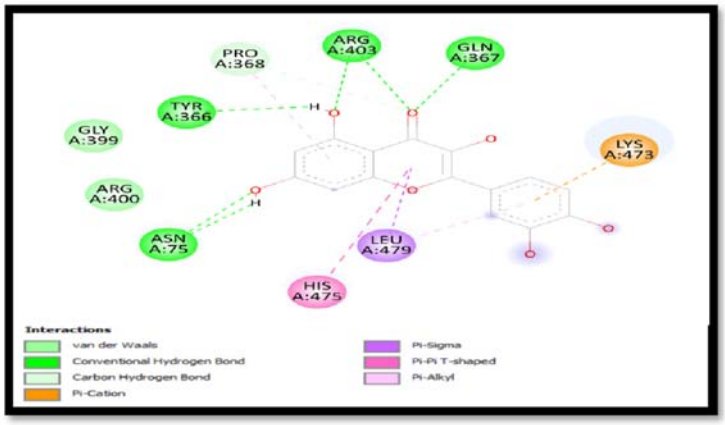
16-deoxycucurbitacin B



18-deoxycucurbitacin A



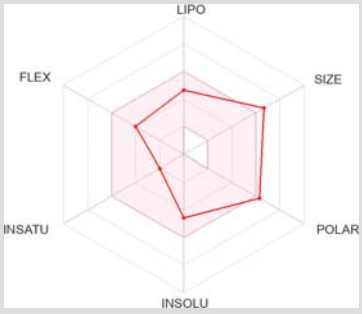
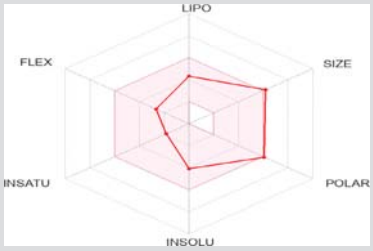
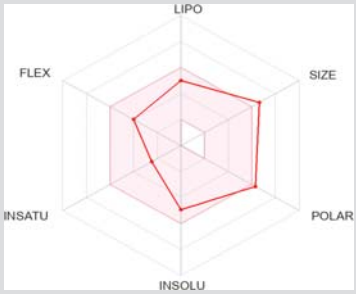
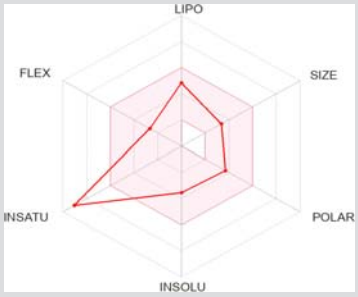
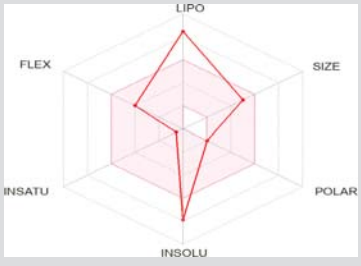
Oleanolic acid



Flavonoids

FIGURE 25.6 cont'd

TABLE 25.6 Bioavailability radar plot along with the toxicity profile of the molecule.

Name	Bioavailability radar	AMES toxicity	Oral acute toxicity	Carcinogenicity
Cucurbitacin B		Negative	Negative	Negative
Cucurbitacin D		Negative	Negative	Positive
Cucurbitacin E		Negative	Negative	Negative
Echinatin		Negative	Positive	Positive
Beta-sitosterol		Negative	Negative	Negative

Continued

TABLE 25.6 Bioavailability radar plot along with the toxicity profile of the molecule.—cont'd

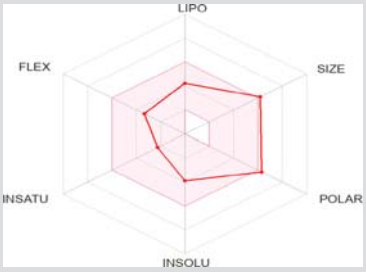
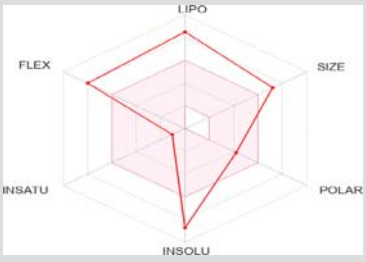
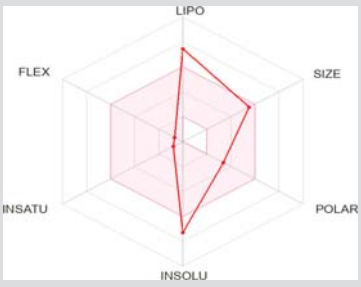
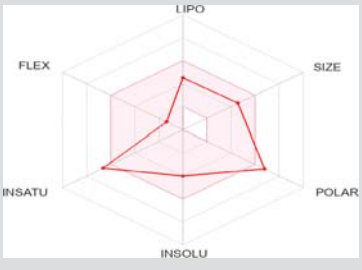
Name	Bioavailability radar	AMES toxicity	Oral acute toxicity	Carcinogenicity
16-Deoxycucurbitacin B		Negative	Negative	Positive
18-Deoxycucurbitacin A		Negative	Negative	Negative
Oleanolic acid		Negative	Negative	Negative
Flavonoids		Positive	Negative	Negative

TABLE 25.7 Predicted drug-likeness properties of the selected compounds.

Compound name	Molecular weight (g/mol)	HBA	HBD	mlogP	Synthetic accessibility	Bioavailability score	Lipinski violation	Drug likeness
Cucurbitacin B	558.70	8	3	1.76	6.79	0.55	1	Yes
Cucurbitacin D	516.67	7	4	1.44	6.65	0.55	1	Yes
Cucurbitacin E	556.69	8	3	1.68	6.74	0.55	1	Yes
Echinatin	270.28	4	2	1.83	2.68	0.55	0	Yes
Beta-sitosterol	414.71	1	1	6.73	6.30	0.55	1	Yes
16-Deoxycucurbitacin B	516.62	8	3	1.19	6.61	0.56	1	Yes
18-Deoxycucurbitacin A	604.94	5	1	5.41	6.81	0.17	2	No
Oleanolic acid	465.70	3	2	5.82	6.08	0.85	1	Yes
Flavonoids	376.36	8	6	−0.38	4.13	0.55	1	Yes

TABLE 25.8 Predicted ADME properties of the selected compounds.

Compound name	Absorption Intestinal human absorption	Distribution logBB, logPS		Metabolism substrate inhibition CYP 2D6, 3A4, 1A2, 2C19, 2C9, 2D6, 3A4							Total clearance
Cucurbitacin B	82.73%	−1.13	−2.954	No	Yes	No	No	No	No	Yes	0.145
Cucurbitacin D	80.96%	−0.845	−3.45	No	Yes	No	No	No	No	No	0.276
Cucurbitacin E	85.95%	−1.225	−2.937	No	Yes	No	No	No	No	Yes	0.104
Echinatin	90.79%	−0.39	−2.238	No	No	Yes	Yes	Yes	No	Yes	0.677
Beta-sitosterol	94.86%	0.797	−1.754	No	Yes	No	No	No	No	No	0.628
16-Deoxycucurbitacin B	76.69%	−1.089	−2.982	No	Yes	No	No	No	No	No	0.352
18-Deoxycucurbitacin A	93.49%	−0.442	−1.576	No	Yes	No	No	No	No	No	0.469
Oleanolic acid	100%	−0.198	−1.188	No	Yes	No	No	No	No	No	−0.081
Flavonoids	69.92%	−1.55	−3.362	No	No	Yes	No	No	No	No	0.112

of the probable contaminant chemicals are hazardous to some extent. Therefore, the chosen compounds may be employed as future treatments to improve the activity of the ER, PR, HER2, and aromatase receptors in breast cancer.

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Chapter 26

In silico identification of novel drug target and its natural product inhibitors for herpes simplex virus

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Introduction

Herpes simplex virus (HSV) are double-stranded DNA virus that belongs to the Herpesviridae family. In the current scenario, due to this virus, approximately 67% population is suffering from it (http://www.who.int/mediacentre/factsheets/fs400/en/). Herpes simplex virus type 1 (HSV-1) is a widespread human herpes virus responsible for several clinically important diseases, including recurrent orolabial lesions, corneal blindness, and encephalitis (Piret & Boivin, 2020; Rivera-Caraballo et al., 2022). After infection, the 152 kb genome, five encoding at least 80 genes, six circularizes and is transcribed by the host RNA polymerase II (Jellinge et al., 2021; Jiao et al., 2019). The viral genes are expressed in a temporarily coordinated fashion. They can be divided into three classes: α (immediate-early (IE)), β (delayed early), and γ (late) (Gruffat et al., 2016). The expression of immediate-early genes initiates a cascade of viral gene expression. Transcription of early (E) genes, which primarily encode enzymes involved in DNA replication, is followed by an expression of late (L) genes, mainly encoding structural components of the virion. In the IE gene products, only ICP4 and ICP27 are essential for expressing E and L genes; hence, viral replication occurs (Packard & Dembowski, 2021). HSV-1 shares with other herpes viruses the ability to establish the lifelong latent infection of the host. Periodic reactivation from latency is responsible for most of the clinical disease burden of HSV infection (Greenan et al., 2021; Kennedy, 2021). Periodic reactivation of this latent infection allows for subsequent infection of other hosts. The latent infection is characterized by a shutdown of virus replicative functions and the inability to detect the infectious virus. The process of latent infection and reactivation has been subject to continuing investigation in animal models and, more recently, in cultured cells (Kotadiya & Georrge, 2015). The initiation and maintenance of latent infection in neurons are passive phenomena in that no virus gene products need to be expressed or are required (Whitford & Cliffe, 2022; Wilson, 2022). Currently, there are three classes of drugs licensed (acyclovir, famciclovir, and valacyclovir) (https://www.who.int/news-room/fact-sheets/detail/herpes-simplex-virus) to treat HSV infection, all of which target Thymidine kinase and DNA polymerase indispensable for viral DNA replication (Sadowski et al., 2021). During latency, transcription of the genome is limited to the Latency-Associated Transcript (Padariya and Jg, 2015), while the lytic genes are maintained in a transcriptionally repressed state. This partitioning of the genome into active and inactive transcription areas suggests epigenetic control of HSV-1 latent gene expression. During latency, viral transcription is not regulated by DNA methylation but is likely by post-translational histone modifications (Whitford & Cliffe, 2022). Literature studies are based on the strategy that an essential survival gene nonhomologous to any human host gene is a candidate drug target for a pathogen (Majewska & Mlynarczyk-Bonikowska, 2022). ICP27, also called IE63 (immediate-early protein 63), plays an essential regulatory function in virus replication required after the onset of early gene expression and viral DNA synthesis. ICP27 is necessary to modulate early and late gene expression, mainly at the posttranscriptional level, possessing various positive and negative effects on the

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expression of viral genes during infection. ICP27 shuttles between the nucleus and cytoplasm and regulates late gene protein synthesis by facilitating the export of late RNAs (Fontaine-Rodriguez & Knipe, 2008; Park et al., 2013; Wang et al., 2020). Studies have also revealed that ICP27 inhibits host cell splicing by interacting with spliceosome-associated protein 145 before the first catalytic step. Further researchers have also reported that ICP27 is required to prevent apoptosis in infected human cells (Guo et al., 2022). Cheminformatics has always played a key role in antiviral drug development. When a virus becomes a threat, the challenges and the quickly rising public interest have significantly influenced computational drug discovery. Structure-based computational drug design methods mainly focus on the molecular targets with known three-dimensional structures, followed by determining their affinity to ligand (drug), based on which hits are obtained. Knowledge of these targets and corresponding drugs, particularly in clinical uses and trials, is beneficial for drug discovery.

Materials and methods

Target identification and model building

From the studies done earlier, ICP27 has been reported to have a multifunctional posttranscriptional regulatory mechanism and hence has been inevitable for replication during the viral lytic phase (Vijayakumar et al., 2022; Wang et al., 2022). Protein sequences were obtained from GenBank, and Blast against Human was performed to obtain nonorthologous sequences. All the critical proteins were searched against PDTD, TTD, Drug Bank, and HIT to check for the novelty of the protein. Due to the absence of Crystallographic structure and similar structure in PDB, Ab initio structure prediction was performed for the target protein using freely available I-Tasser. The stereochemical properties of the model were verified using Anolea and Procheck of the Swiss model Server (Altschul et al., 1990; Berman et al., 2000; Guex et al., 2009; Laskowski et al., 2006; Yang & Zhang, 2015).

Active site prediction

The active site of the target was obtained from the I-Tasser predicted binding site. Moreover, a Q-site finder was also used to predict the binding site. The active site residues were analyzed and considered based on their presence in both tools (Laurie & Jackson, 2005).

Molecular docking

Molecular docking studies were performed using Glide and Molegro Virtual Docker against a prepared library of 133,992 chemical molecules. The target protein was prepared for subsequent grid generation by the Protein Preparation Wizard tool supplied with Glide (Bitencourt-Ferreira & de Azevedo, 2019; Friesner et al., 2004). The in silico structure-based high-throughput virtual screening (HTVS) method of Glide was used to identify potential ligand molecules interacting with at least one residue on the 3D structure. For subsequent molecular docking of compounds in the binding site of the target, the ligand molecules from the HTVS mode results were prepared using the "LigPrep" module and were subsequently subjected to Glide "Ligand docking" protocol with "XP mode." The ligands were docked with the active site using the "Extra precision" Glide algorithm. Glide generates conformations internally and passes these through a series of filters. The final energy evaluation is done with the Glide Score, and a single best pose is generated as the output for a particular ligand. After performing HTVS with Glide, the ligands were subjected to Molegro virtual docker with two detected cavities shown in Fig. 26.1. The docked results were evaluated based on binding affinity, moldock score, and reranking score (Patel et al., 2018; Thomsen & Christensen, 2006).

Screening of ADME properties

The QikProp program was used to obtain the ADME properties of the analogs obtained after docking with Glide "XP mode" and with "molegro virtual docker" (Laoui & Polyakov, 2011). It predicts both physically significant descriptors and pharmaceutically relevant properties. The program was processed in normal mode and predicted 31 properties for all molecules, comprising principal descriptors and physicochemical properties with a detailed analysis of the log P (Octanol/Water), QP%, and log HERG. It also evaluates the acceptability of the analogs based on Lipinski's rule of 5, which is essential for rational drug design. The molecules were sorted based on the appropriate range for a particular descriptor. The finally screened molecules common in Glide and MVD cav1 and cav2 were exported to an excel file. The ligand molecules present in common in both Glide results and MVD results in both cavities were considered based on their respective G-score, Energy values, and Re-rank score (Nirajkumar et al., 2020). The finally selected molecules were checked for toxicity using Topkat (Prival, 2001).

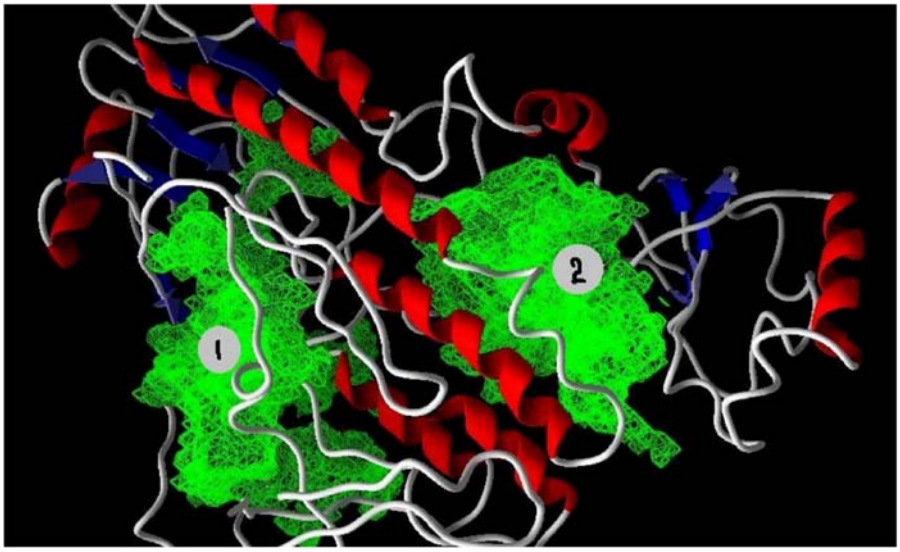


FIGURE 26.1 Cavities detected by molegro.

Multiple sequence alignment and phylogenetic analysis

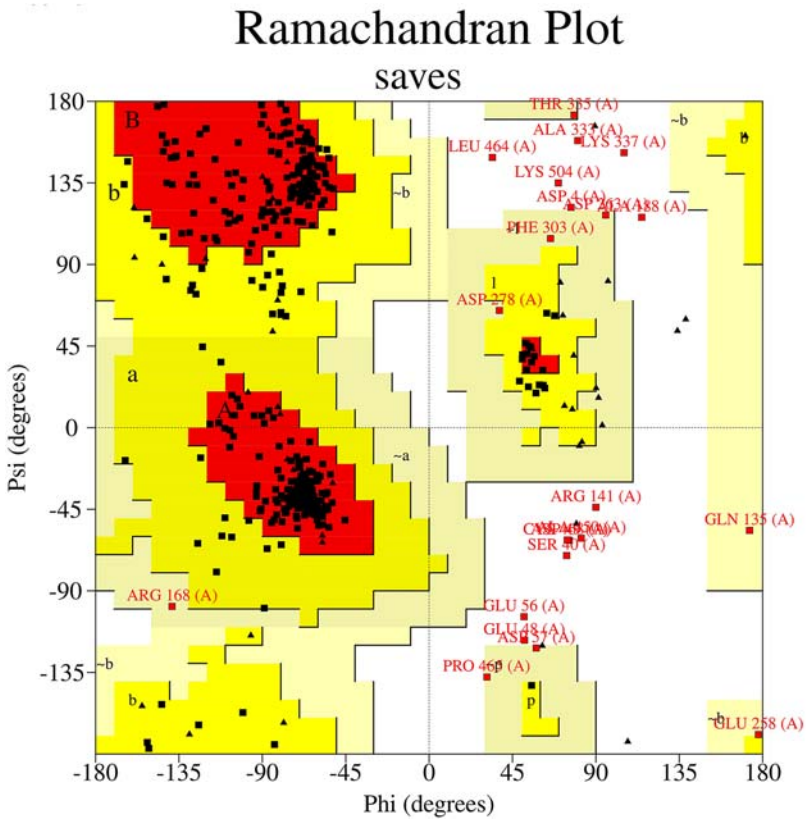
Phylogenetic studies efficiently understand the orthology among different proteins and reveal high conservation concerning the residues involved. Evolutionary theory provides a unifying framework for analyzing genomics data and studying the various molecular, cell, or developmental biology phenomena. Studies have found that evolutionary-based inference systems play an increasingly important role in diverse areas, such as the elucidation of the tree of life, studies of epidemiology and virulence, drug design, human genetics, cancer or biodiversity (Ahmad et al., 2022; Georrge & Umrania, 2011; Vanchurin et al., 2022). This analytical theory is applied to the Herpes family to find the conservation of proteins.

Results and discussion

Blast against Human sequence for ICP27 showed no significant similarity and was not used as a drug target to date, so it was selected as a novel drug target. The predicted 3-D structure of ICP27 using I-Tasser showed 84.4% residues in the core region, 10.6% in allowed regions, and 2.7% in the generously allowed region. These scores have been proven structured to have a better resolution (Gopalakrishnan et al., 2007). The Ramachandran Plot for ICP27 is shown in Fig. 26.2 ICP27 and was docked with the library of compounds using Glide and Molegro Virtual Docker docking tools. The preliminary stage included a total no of 133,992 compounds, followed by continuous screening with HTVS, XP, and QikProp shown in Fig. 26.3. The Qikprop screening included 31 parameters: oral absorption, molecular weight, Blood-Brain barrier, H-bond donors-acceptors, and many more.

The three best are found to be potent inhibitors for ICP 27 (Table 26.1). All three ligands have shown lower energy when docked with Glide. 4,5-diacetyloxy-6-(2-amino-6-oxo-3H-purin-9-yl) oxan-3-yl acetate, has shown the lowest binding energy. This ligand candidate belongs to an NCI, where an in-vivo investigation found that it showed anticancer activity in the rat. The other two ligands were from DrugBank-approved drugs. Didanosine is a potent inhibitor of HIV replication, acting as a chain-terminator of viral DNA by binding to reverse transcriptase. It has been used for viral target GAG-Pol Polyprotein (Kryštůfek et al., 2021). Rimantidine, an RNA synthesis inhibitor, is used as an antiviral agent in the prophylaxis and treatment of influenza. It has shown the interaction with Matrix protein 2 (Nguyen & Le, 2015). All three ligands have also shown lower energies when docked with MVD, shown in Table 26.1, with both cavities as predicted by the Q-site finder.

Evolutionary studies using multiple sequence alignment showed various domains RRM Domain/RNA recognition motif (103−138 Amino acids), RGG box Domain (138−152), Herpes virus transcriptional regulator family domain (303−509), and Protease resistant DNA binding domain (N-terminal 229−292, C-terminal 495−512). Based on interactions, the Arginine residue is common in the RRM and RGG domains. The RRM motif is probably diagnostic of an RNA-binding protein. RRMs are found in various RNA binding proteins, including hnRNP proteins, proteins implicated in regulating alternative splicing, and protein components of snRNPs. A putative RRM in these proteins, which are implicated in various cellular processes, strongly suggests that their function involves binding to RNA (Knezic et al., 2022). RGG box Domain is associated with the RNA-binding domains. It contains repeats of Arg-Gly-Gly, often interspersed with aromatic



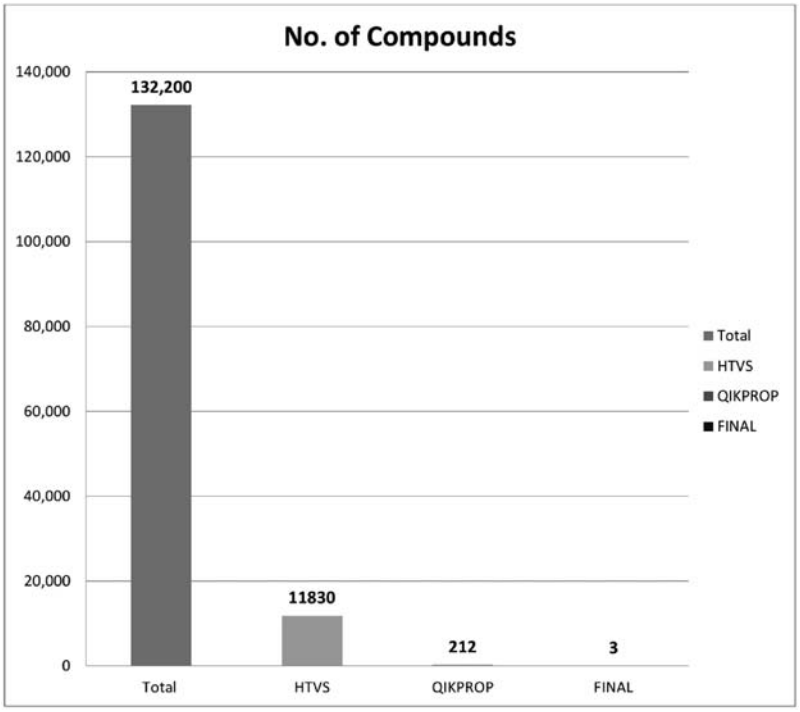


FIGURE 26.2 Ramachandran plot for ICP27.

FIGURE 26.3 Screening of molecule by glide.

TABLE 26.1 Docking Score of best-selected Ligands in Glide and MVD.

Ligand	Glide G-score	MVD		Rerank score
		Cavity 1	Cavity 2	
Didanosine	−4.621692	−91.7631	−96.6896	−77.8328
Rimantidine	−4.343060	−48.1456	−58.107	−49.5828
[4,5-diacetyloxy-6-(2-amino-6-oxo-3H-purin-9-yl) oxan-3-yl] acetate	−6.917391	−64.9811	−80.2084	−67.2084

residues. The RGG box motif of the HSV ICP27 protein mediates an RNA-binding activity and determines in vivo methylation (Seyffert et al., 2021).

Previous studies showed these arginines were methylated during infection and that the methylation of these arginines regulates ICP27 export to the cytoplasm and its interaction with two cellular proteins (Wu et al., 2021). Recently, it was shown that the Arginine methylation of Aly/REF promotes the efficient handover of mRNA to TAP/NXF1, suggesting that Arginine methylation can regulate the RNA-binding activity of Aly/REF (Wang et al., 2021). Herpesvirus transcriptional regulator family domain includes amino acid residues from 303 to 509. This domain is mostly conserved throughout the herpes family. The major transcriptional regulatory protein of HSV-1 includes a protease-resistant DNA binding domain with an amino terminus between residues 229−292, while its carboxyl terminus is between residues 495−512. Mutations outside this region that affect DNA binding by the intact protein do not eliminate the binding of the proteinase K-resistant domain. This domain overlaps with the transcriptional family regulator domain.

The inhibitors have mostly been shown to bind with various residues within this protein domain. The phylogenetic analysis of ICP27 with that of the Herpes family indicates that the Immediate Early Protein of Human herpes virus two is highly similar to ICP27 of HSV-1. The pair distance between these two protein sequences is 0.205820, which gave us some clues to their functional similarity. ICP27 of CHV2 and UL54 of CHV16, CHV1, and SHV-1 also showed (Fig. 26.4) a similar resemblance with ICP27 of HSV-1. The multifunctional expression regulator of human herpes virus one and the transcriptional regulator IE63 of human herpesvirus 1 (strain HFEM) were closer degrees of similarity.

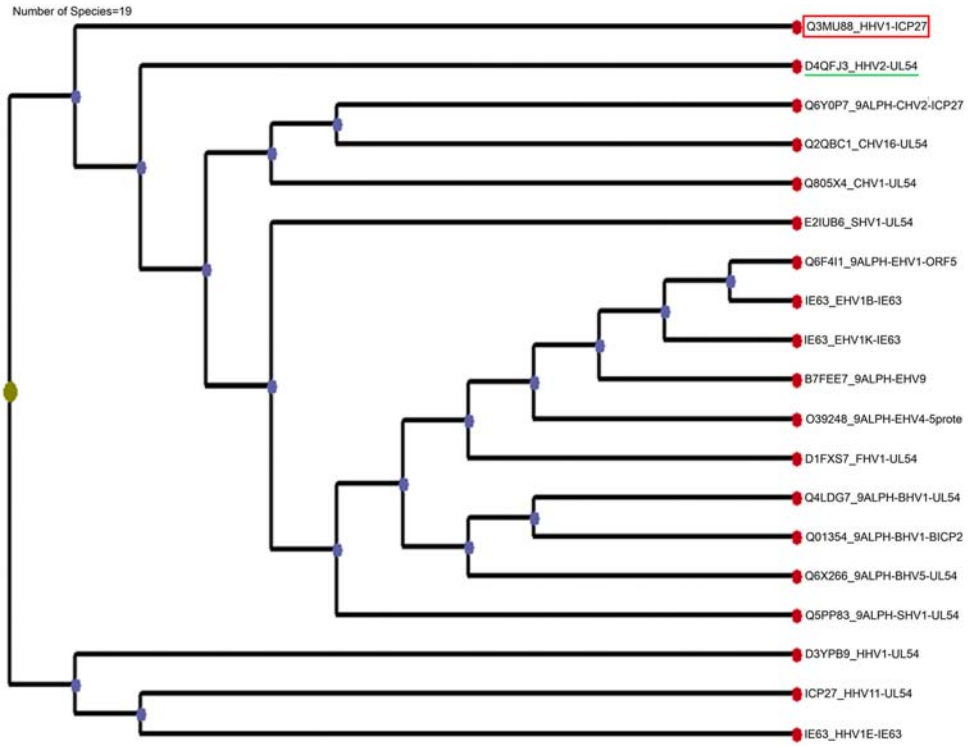


FIGURE 26.4 Phylogenetic representation of the herpes family.

This study concludes that the molecule from NCI 4,5-diacetyloxy-6-(2-amino-6-oxo-3H-purin-9-yl) oxan-3-yl acetate, which screened as an anti-cancer agent, can be used as a putative drug. As Rimantidine and Didanosine are FDA-approved, they can be used to treat Herpes Simplex -1 in reproductive and latent stages effectively. The amino acid residues interacting with ligands were conserved throughout the Herpes family. Biological confirmation is required for the obtained ligand to treat the disease caused by the Herpes family.

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DRUG DISCOVERY **UPDATE**

SERIES EDITOR: **DR. CHUKWUEBUKA EGBUNA**

NANOTECHNOLOGY AND IN SILICO TOOLS

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Edited by Mital Kaneria and Kalpna Rakholiya

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Mital Kaneria has received his MSc and PhD degrees in Plant Science. During his PhD, he was awarded a BSR Fellowship from UGC, India, for 3 years and later awarded young scientist award by DST-SERB to participate in an international conference at Institute of Pasture, Paris, France. He has published more than 65 research articles in journals, book chapters, and conference proceedings, with notable citation indices. He is a reviewer and editorial board member of many journals, is a member of many scientific societies, has presented number of papers in conferences, and has received best paper awards, as well as recently filed one patent. He is working as Assistant Professor in the Department of Biosciences, Saurashtra University, India, since 2012. He is working in the field of medicinal plants, herbal technology, pharmacology, and drug discovery since more than 14 years. His doctoral research was based on the phytochemical and pharmacological potency of a selected medicinal plant from Gujarat region. His group is working on various aspects of phytochemistry, metabolomics, antiageing, drug discovery, and designing.

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