Review

A review on anti-bacterials to combat resistance: From ancient era of plants and metals to present and future perspectives of green nano technological combinations

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ABSTRACT

In the primitive era, humans benefited partially from plants and metals to treat microbial infections. Later these infections were cured with antibiotics but further suffered from resistance issues. In searching of an alternative, researchers developed an adjuvant therapy but were hampered by spreading resistance. Subsequently, nanoparticles (NPs) were proposed to cease the multi-drug resistant bacteria but were hindered due to toxicity issues. Recently, a novel adjuvant therapy employed metals and botanicals into innovative nanotechnology as nano-antibiotics. The combination of green synthesized metallic NPs with antibiotics seems to be a viable platform to combat against MDR bacteria by alleviating resistance and toxicity. This review focuses on the primitive to present era dealings with bacterial resistance mechanisms, newer innovations of nanotechnology and their multiple mechanisms to combat resistance. In addition, special focus is paid on greener NPs as antibiotic carriers, and their future prospects of controlled release and toxicity study.

1. Introduction

Antimicrobial resistance has become a cosmopolitan problem and it has been a challenge in the medical and pharmaceutical fields from 20th century. With major advances in medicine, huge surgical procedures, such as heart surgery and kidney transplantation, are being victorious; but the infection after the surgeries is a major issue due to microbial resistance. Thus, our competitive medical world is

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deadlocked in the evolutionary arms of microbes. As we are developing newer approaches to treat microbial infections, microbes are using their own mechanisms to develop resistance [1].

An emergency prevailed in the 20th century to fight against microbes, and led to the discovery of first chemotherapeutic agent salvarsan against syphilis by Paul Ehrlich. Nevertheless, the toxicity of salvarsan made it less ideal for its further existence. The later discovery was optochin by Morgenroth and Levy to treat pneumococci infection. However, it was one of the first cases where antimicrobial resistance was spotted [2,3]. By the mid-20th century, a phenomenal layout of true antibiotic discoveries advanced with sulfonamides and penicillin, followed by anti-tubercular and antifungal agents, and went on with the entire sequel of antimicrobials [4]. These marvelous discoveries improved the quality of life, but very shortly, new and emerging expedient nosocomial and community-acquired pathogenic resistance disturbed the positive functioning of antimicrobial agents [5], and is demonstrated in Fig. 1. For instance, ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) have developed resistance globally in hospitals and were isolated from community-acquired infections [6].

Once the bacteria attain resistance to the antibiotic, they consign that characteristic feature to other cells and offspring's through a horizontal or vertical transmission [7]. Multi-Drug Resistance (MDR) has been a long time concern for the government and health agencies including WHO, in the process of producing antibiotics in a safe and effective way. But clinicians and all health organizations have failed to date as the resistance proof-antibiotic has not been entered into the market. Therefore, antibiotic pipeline is drying out and efficiency of existing antibiotics against MDR strains has been diminishing [8]. At least 2 million people in the United States, and about 400 000 patients in the European Union are developing resistant bacterial strains annually, among them at least 23 000 and 25 000 people respectively are at mortality zone in this decade [9]. Over the past 30 years, a few antimicrobials were approved by FDA and in particular 2018, 2017, 2016, and 2015 constitute 0, 3, 2, 1 antibacterial drugs respectively. Moreover, these new anti-bacterials mostly

work against gram-positive bacteria [10]. It is much overwhelming as these newer antibiotics fit in known class and are a source of spreading further resistance. Some bacteria, vancomycin-resistant *Enterococcus* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem-resistant *Enterobacteriaceae* (CRE), multi-drug-resistant *Mycobacterium tuberculosis* (MDR-TB) have developed resistance to the majority of known antibiotics, which is a serious health concern [11]. Thus, our world is at threat in the post-antibiotic phase, where antibiotics are no longer effective against infectious diseases. The center for disease control and prevention treats this situation as the world's acute health problems of the 21st century. Therefore, it is high time to gather the root causes of the problem in order to find alternate solutions to the failing antibiotics.

Modifications in target sites, alteration of influx/ efflux of drugs, enzymatic degradation are the common strategies utilized by bacteria to initiate and enhance intrinsic resistance to antibiotics [12]. The reason behind resistance involves not only newer mechanisms exhibited by the microbes, but also due to the activities of humans such as arbitrary prescribing, inappropriate and overdosing (self-medication and counterfeit drugs), using broad-spectrum antimicrobials, and unnecessary usage of antibiotics in agriculture and cattle that end up in mobilization of antimicrobial resistance [13]. Therefore, it is time to eliminate antimicrobial resistance as no world exists without diseases and antibiotics. There is a persistent requirement for newer antimicrobials with different chemical composition and novel mechanisms to fight against MDR bacteria [14]. In a struggle to find new and alternate sources of antimicrobials, plants and metals seem to be viable options to combat drug resistance with immunomodulatory action [15].

Botanicals and metals are known to have medicinal value from thousands of years. Healing power in plants and metals is a primitive belief even before any conventional drugs existed. The Ayurvedic system (Materia Medica), which is a 5000 year old natural healing system, contains drugs derived from plants, animals, metals and mineral sources [16]. Botanicals occupied the ancient records of herbs from ancient times to the present era. Ayurvedic medicine explored several pharmacologically active herbs and spices such as turmeric, tulsi, liquorice, cardamom and many more [17]. The Chinese pharmacopeia discussed various medicinal plants and plant medicines, which became the prototype of modern pharmacopeias for 1500 years. Out of 250 000 to 500 000 known plant species, very few have been examined phytochemically, and a few have been biologically screened. Salicylic acid was the first synthetic substance discovered from medicinal plant in 1853 [18].

Plants are renewable and economical sources of antimicrobials with least toxicity and rich chemical diversity. Metabolites (alkaloids, polyphenols, terpenes, glycosides, etc.) in plant extracts are mainly phenolic derivatives that can stop bacterial growth by binding to bacterial proteins or through reducing pH, which alters the bacterial cellular process and kill bacteria [19]. These plant-based antimicrobials (PBA) possess certain clinical value, as the bioactivity does not impose any resistance [20,21]. Till date, there has been no claim on
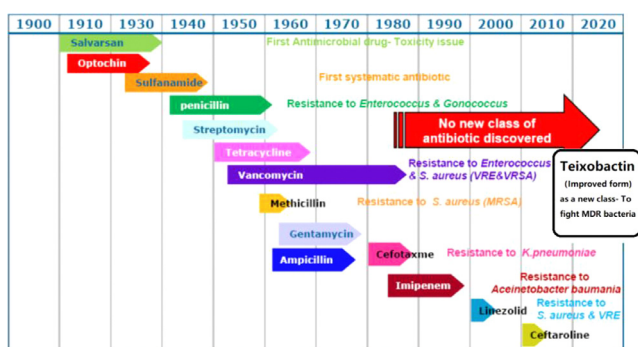

Fig. 1 – Antibacterial drug discovery and their resistance development- Journey through 20th and 21st centuries.

identification of bacteria, which developed resistance to plant antimicrobials. There are also recent reviews on plant extract's antibacterial activity describing their effective action and the defense mechanisms of plants to bacterial resistance [3,21]. Nevertheless, the traditional medicine (Ayurveda, Traditional Chinese medicine, Kampo, Unani, and Siddha) has not entered into modern medicinal framework due to the chemical complexity of plant extracts, the requirement of higher concentrations for activity, poor water solubility and the lack of standardization [22]. Beyond these minute limitations, their true chemical diversity (extensive functional group and chirality) has encouraged phytochemicals for industrial biological applications [14,21].

Metals have been used from the ancient times in *Ayurvedic* classics (*Charaka Samhita* and *Sushruta Samhita* etc.) and *Rasa Shastra* (8th century A.D.). These ancient classics deal with the drugs of metals and the mineral origin. Within a short span of time, herbo-mineral and metallic preparations grabbed attention in *Ayurvedic* pharmacopeia with ensured safety and efficacy [23]. Even in recent times, a few metals such as silver and copper are the first-line drugs as antimicrobials and are used in consumer products, agriculture, and medicine. Metals target through the pleiotropic process even on dormant strains resulting in lethal effects of MDR pathogens [24]. As per these ancient classics, purification of micro fine powders (*Ayaskriti*) should be performed with great caution to avoid toxicity. There are several documented studies on the performance of metals as antimicrobials [25]. Recent studies on metals have revealed the mechanisms against bacterial pathogens might be due to oxidative stress, protein dysfunction or membrane damage [25,26].

From the prehistoric era to date, medicinal plants and metals have their own medicinal identity. Nevertheless, focus on them took a downturn with interference of conventional antibiotics. However, it took less time for the scientists to realize that the effective lifetime of any antibiotic to develop resistance is finite. Owing to the multifaceted effects of medicinal plants and metals, they are known to strengthen the immune system with prophylaxis potential against infectious diseases [27]. Metal and plant-based antimicrobial therapies need a better perceptual view for a rational design of metal and plant-based antimicrobials. Expectantly, green-nanotechnology and their combinational therapies with antibiotics have recently been employed as modern methodology in standardized protocols, to provide alternate mechanisms against MDR bacteria [19]. Green NPs with antibiotics carry numerous moieties in one scaffold; where the bacteria cannot develop multiple gene mutations simultaneously in the same microbial cell [28]. Thus, this combinational approach can command and modify molecular structures at the nano-scale to attain smart, targeted and controlled delivery in an economical way through securing natural microbiome.

Hopefully, this review can find a scope to inhibit MDR pathogens through innovative and combinational strategies in the bio-nanofield. Moreover, greener nanotechnology as an adjuvant to antibiotics can prioritize disease-specific bactericidal treatment at low dosage to ensure safety with sustaining immunity.
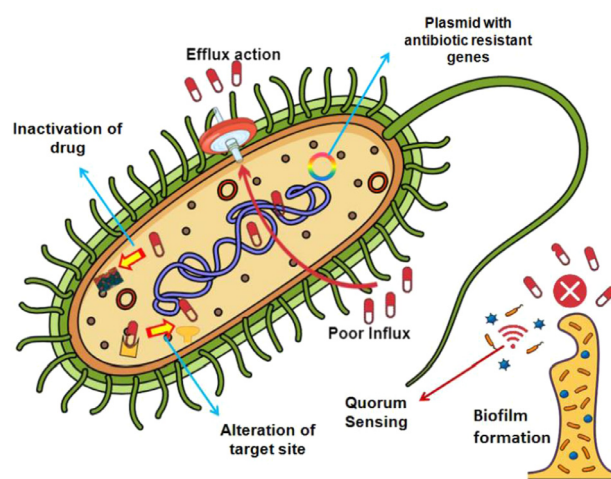

Fig. 2 – Multiple resistance mechanisms of bacteria that turns to MDR bacteria.

2. Resistant developmental strategies of bacteria towards conventional antibiotics

The majority of existing antibiotics that are used for attenuating or killing harmful bacteria act on three targets namely; cell wall, translational activity and DNA replication. Unfortunately, there are various strategies of bacteria to develop resistance either by being intrinsically resistant to certain antibiotics or by inducing mutation in bacterial chromosome. Soon after the bacterial resistance attained, the normal type gets terminated by the drug and the resistant mutant can survive and spread the resistance through horizontal or vertical transfer [7].

Bacteria may develop resistance towards antibiotics and non-antibiotics (Fig. 2) through following mechanisms:

(i) Poor drug influx through porin channel. For instance, resistance of *P. aeruginosa* to imipenem through a mutation that modifies the outer membrane permeability [3,12].

(ii) Excessive drug efflux from the target cell through efflux pumps. For Example, *P. aeruginosa*'s conversion into a MDR pathogen through mutation in the regulatory protein that enhances the drug efflux. These poor influx and excessive drug efflux mechanisms hinder effective concentration at the site of action. These mechanisms result in a sub-lethal concentration of the drug at the active site, which causes elevated target-based resistance. Many bacterial strains follow both of these mechanisms and induce resistance to drugs such as tetracyclines, sulfonamides, quinolones, aminoglycosides, chloramphenicol, macrolides, and streptogramins [12].

(iii) Genetic alterations that modify/protect target sites. This amendment may be due to constitutive and inducible enzymes produced by bacteria. For instance, mutation of erythromycin resistance methylase (*erm*) in group C and G streptococci that inhibits binding of MLS (macrolide, lincosamide and streptogramin B) antibiotics to target site [29].

Other notable instances are the ribosomal point mutation in *streptococcus pneumonia* that cause resistance to tetracyclines, macrolides, clindamycin, streptogramin, and telithromycin [30], altered DNA gyrase and topoisomerase IV mutations in *proteus species* that show resistance to fluoroquinolones [31], modified penicillin-binding proteins in *streptococcus pneumoniae,* which show resistance to penicillins [32], and rpoB gene mutations in *mycobacterium tuberculosis* that causes rifampin resistance [12,33].

(iv) Drug molecule inactivation through covalent binding is a common mechanism to develop resistance. For instance, New Delhi Metallo beta-lactamase 1 (NDM-1) is a recently discovered β-lactamase with carbapenemase activity, which is resistant to all antibiotics that are used to treat serious infections [34]. Another notable example is inactivation of β-lactam antibiotics (penicillin, ampicillin, amoxicillin, imipenem, piperacillin, ceftazidime etc.) by cleavage of the β-lactam ring in *S. aureus*, *N. gonorrhea* and *H. influenza*. Another instance is the inactivation of aminoglycosides by acetyl, phospho- and adenylyl transferases [12].

(v) R plasmids play a major role in transmitting resistance by carrying multiple resistant genes in a single plasmid. This process is facilitated by a mobile genetic element (transposons) through horizontal gene transfer in case of antibiotics and bulk metal ions [35]. Bacterial species may exchange their genetic material through the transformation process. For example, enterococcal pheromone-responsive plasmids possess MGE called pheromone, which aids in resistance development in *E. faecalis*. The plasmid-borne kpc gene is associated with a dominant clone in *K. pneumonia* [12].

(vi) Formation of biofilms. Bacterial biofilms are surface-associated microbial communities hidden in a self-generated 3D film exo-polysaccharide matrix that protects bacteria from antimicrobial agents. The gene mutation is enormous in biofilm forming strains. Through quorum sensing, bacterial cells express genes to synthesize and secrete matrix of extracellular polymeric substance (EPS). EPS matrix surrounds the bacterial cells and protects them from high concentrations of antibiotic agents (recalcitrance) resulting in chronic infections such as cystic fibrosis, periodontitis, native valve endocarditis, prostatitis and otitis media. Biofilm matrix reduces the bioavailability of antibiotics by slowing down the drug diffusion process with its higher viscosity or the genetic material exchange between biofilms. Strength of biofilm strains against antimicrobials is due to molecular mechanism differences in planktonically growing isogenic bacteria. There are several reports on biofilms as a resistance inducer in numerous bacteria [36].

These resistance development strategies of bacteria are hindering the antibiotics from their effective action against harmful pathogens. This necessitates increased dose of antibiotics, or change in the class of antibiotics. Moreover, there is scarcity in discovery of new antibiotic classes in the last two decades. Furthermore, bacteria invariably attained resistance to all the known antibiotic classes soon after their introductory period (Fig. 1) [5].

3. Adjuvant therapy to alter resistance

In an era of sustainable resistance and with the lack of novel antimicrobials, combination therapy of antibiotic-antibiotic and antibiotic-adjunct has been developed. Adjuvant therapy is capable of extending the lifetime of existing antibiotics through synergistic action or inhibiting antibacterial resistance.

3.1. Antibiotic-Antibiotic combination strategy

Antibiotic-antibiotic is a combination of two or more antimicrobial drugs as a treatment regimen. This combination may result in synergism ($C>a+b$)/ antagonism ($C<a+b$) /additivity ($C=a+b$)/ autonomy (C~a or C~b) [37]. Where, 'a' is antibiotic1, 'b' is antibiotic2, 'C' is combination of antibiotic1 and 2. This result can be calculated *in-vitro* by checkerboard method, Kirby-Bauer disc method or agar-well diffusion method.

This combination may act by any of the following mechanisms: (a) Targeting through different pathways as in case of isoniazid, rifampicin, ethambutol, and pyrazinamide for Mtb and other *mycobacteria*. (b) Inhibition of different targets through a single pathway. For instance, combination of sulfamethoxazole and trimethoprim inhibits sequential steps in the folic acid biosynthesis pathway. (c) Inhibition of the same target with multiple mechanisms as in case of streptogramins and virginiamycin. There are a wide variety of antibiotic combinations, such as aminoglycosides and penicillins, bacitracin, vancomycin and cycloserine, which inhibit various steps in cell-wall synthesis. Other notable example is vancomycin and oxacillin that synergistically acts against MRSA strains [38].

3.2. Antibiotic-adjunct combination strategy

Adjuncts are the non-antibiotic compounds with little/no antibacterial activity. However, they aid in the enhancement of antibiotic potency or resistance suppression. This combination offers the most successful way to combat multidrug resistance through a number of mechanisms and can be effectively used as an alternation to antibiotic-antibiotic combinations which often suffer from certain pitfalls such as drug-drug interactions. The antibiotic-adjunct combos that attained clinical success include inhibitor adjuncts (β-lactamase inhibitors; efflux pump inhibitors), natural and biological adjuncts, and outer membrane permeabilizers.

3.2.1. Inhibitor adjuncts
These adjuncts normally do not have any antimicrobial potency, but they may improve the delivery efficacy of antibiotic with simultaneous inhibition of resistance mechanisms. For example, β-lactamases are enzymes found in demanding species (*E. coli, P. aeruginosa and Proteus mirabilis*) that inactivate β-lactam antibiotics (penicillins, cephalosporins, carbapenems and monobactams) through hydrolysis. The paradigm β-lactamase inhibitor is augmentin (amoxicillin+clavulanic acid) that inhibits lactamase activity and enables amoxicillin to inhibit cell wall synthesis. The other inhibitors are timentin

(ticarcillin+ clavulanic acid), unasyn (sulbactam (sulfones) +ampicillin) and zosyn (tazobactam (sulfones) + piperacillin). Clavulanic acid in combination with sulfones works through the inhibition of ser-β-lactamases. Avibactam is a non-β-lactam β-lactamase inhibitor that binds covalently with enzyme and works against cephalosporin resistance in gram-negative bacteria (*E. coli* and *K. pneumonia*) [38,39]. Unfortunately, many β–lactamases (oxacillinases (type D), metallo-β-lactamases-NDM-1) are not inhibited by clavulanic acid and other inhibitors, due to numerous resistant enzymes, distinct mechanisms and substrate profiles [34].

Efflux pump inhibitors serve as a major promising strategy in suppressing resistance through intracellular accumulation of antibiotics. Most of the gram positive and gram negative bacteria acquire resistance through major drug efflux transporters as described in the above section. An example is PAβN that inhibits efflux pumps in gram-negative MDR bacteria and helps levofloxacin free from bacterial resistance. Another example is celecoxib that inhibits the MDR1 efflux pump through inhibition of COX-2, which prevents antibiotic (chloramphenicol, ampicillin, ciprofloxacin, and kanamycin) resistance from *S. aureus*. It has been a challenge with the toxicity of PAβN and other efflux inhibitors which hinder their clinical usage. However, inhibitor adjunct therapy is a splendid approach to consider [38,39].

3.2.2. Outer membrane permeabilizers

Gram-negative bacteria possess complex outer membrane with lipopolysaccharides which restricts the entry of antibiotics. With the aid of permeabilizers (cationic or amphiphilic), antibiotics can weaken the polyanionic outer membrane, and increase the drug uptake. Examples of permeabilizers are colistin, aminoglycosides, cationic peptides, and polyamines. Colistin toxicity can be reduced by using a combination of colistin with rifampin or vancomycin. PAβN also possess membrane permeabilizer property similar to polymyxin B, and this was proven in case of *E. coli* and *P.aeruginosa* with enhanced efficacy to β-lactams [39,40].

3.2.3. Natural and biological adjuncts

The advantage of using natural and biological adjuncts is the enhancement of antimicrobial efficacy in a natural way. Examples of biological adjunct are PhagoBioDerm (lytic phage cocktail and ciprofloxacin in a biodegradable polymer matrix) and IgG antibodies that refine immunity and prevent antibiotic resistance. Other notable example is plant-derived thymol in combination with vancomycin and EDTA [41]. A detailed review of plant phenolics and terpenoids as adjuncts to antibiotics has been given by Zacchino et al. in recent times [42]. Apart from these combinations, drugs but non-antibiotics also exhibit synergistic anti-bacterial action. Examples of such strategy include synergy between diclofenac and streptomycin [43] and synergy between azole antifungals and citridones [44].

Adjuvant therapy can prolong the lifetime of antibiotic and inhibit resistance mechanisms. But they often suffer from adverse effects due to drug-drug interactions. Moreover, complex studies are needed to formulate dosing regimens with compatible pharmacokinetic and pharmacodynamic properties. Extensive care is required to avoid antagonistic effects. As the antibiotics have gained noticeable resistance in monotherapy, adjuvant combinations may often develop double resistant mutants. Simultaneous administration of drugs not only alters pharmacokinetics but may also disturb host microbiome, as they act on the broad spectrum of microbes. To overcome this ultimatum, the feasible option for the targeted delivery with reduced toxicity can be attained through nanotechnology with a special focus on green-NPs and their combinations.

4. Dealing of nanotechnology with MDR microbes

Nanomaterials are defined as the materials with at least one of its dimension lesser than 100 nm. Nano materials are the primary building blocks of nanotechnology [45]. NPs serve as "magic bullets" with the aim of targeting the drug delivery at right place, with right concentration and for appropriate time [28]. Nanotechnology has vast opportunity to command and modify molecular structures at nanoscale to attain specific target action. Nano-bullet targeting is advantageous over conventional systems as they enhance therapeutic capacity by preventing microbial resistance. Furthermore, nano targeting often prevents frequent drug intake and reduces side effects. Therefore, nanoscience enhances patient compliance through protecting natural microbiome . NPs have the capability to overcome drug resistance due to their multi-functionality, as bacteria cannot develop multiple gene mutations simultaneously.

4.1. Organic versus inorganic NPs

NPs for the desired action may be organic or inorganic. Organic NPs include liposomes, polymeric NPs, polymeric micelles and solid lipid NPs (SLNs) that are used in major therapeutics. They possess major advantages such as handling either hydrophilic or hydrophobic drugs, biodegradability, compatibility and low systemic toxicity. However, they suffer from certain limitations such as short shelf life, low encapsulation efficiency and poor stability at high temperature and cannot withstand harsh processing conditions [46]. A detailed review on organic NPs along with their combination therapy and controlled release was described by Huh, et al. [47]. In contrast, metallic NPs procure small size with high loading capacity and high stability [48]. With the introduction of nanotechnology, particles are manipulated at atomic level to form metallic NPs [49]. The distinctive size-dependent properties assist their various applications of catalysis, biotechnology, medical and pharmaceutical fields as therapeutic agents, medical diagnostic imaging and drug delivery systems [49]. Inorganic NPs hold unique physicochemical properties (optical, chemical, electronic, catalytic, mechanical and magnetic) due to their high surface-volume ratio that can transcend barriers and ensure higher activity in biological systems when compared bulk materials. Metal-based NPs are highly promising therapeutics as anti-bacterials by overcoming the disadvantages of antibiotics and bulk metals [50]. Metallic NPs have versatile applications in diagnostic assays, radiotherapy, drug and gene delivery, and thermal ablation techniques [51].
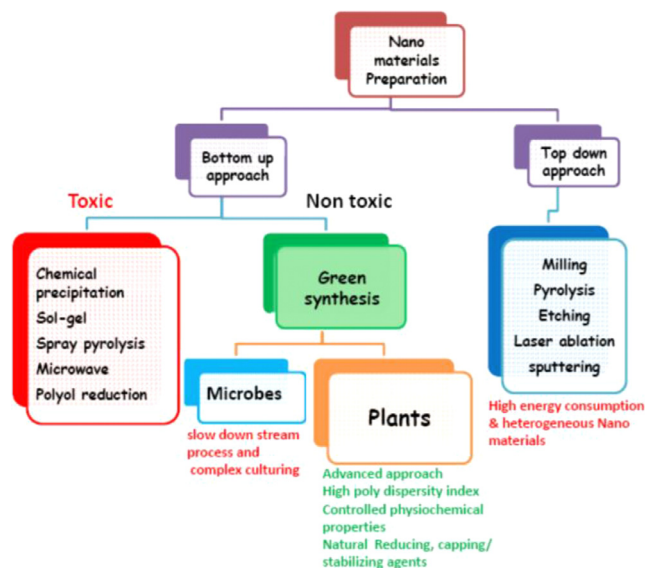
Fig. 3 – Different approaches for fabrication of inorganic NPs.

4.2. Fabrication of inorganic NPs

Most common ways to prepare inorganic NPs are top-down and bottom-up approaches. These methods fine-tune the physicochemical properties of metals to attain nanostructures with specific functionalities, which are depicted in Fig. 3 [52]. In top-down approach, metallic NPs are prepared by trimming bulk material into fine particles. This approach has particular limitations such as heterogeneous nanostructures, high energy consumption and complexity in synthesis. On the other hand, bottom-up approach deals with self-assembly of atoms and molecules into cluster, which eventually format desired homogenous metallic NPs through chemical or biological methods [53]. Functionalization of these metallic NPs aids in targeted delivery with several pharmaceutical theranostic applications [54]. The synthetic methods of inorganic NPs via top down and bottom up approach have been provided in a recent review [55]. In bottom up approach (chemical methods), formation of inorganic NPs utilize reducing and capping agents for the reduction of metal salts, attaining desired size and functionalization of NPs. For example, sodium borohydride, sodium citrate, ascorbate, elemental hydrogen, tollen's reagent, N, N-dimethyl formamide (DMF) and poly (ethylene glycol) block copolymers are used for reduction of metal salts in aqueous or non-aqueous solutions [55]. Capping agents (citrate, chitosan, polyvinyl acetate (PVA), polyvinyl pyrrolidone (PVP), 11-mercaptoundecanoic acid) are used to increase the stability for even dispersion of the NPs [56]. Thus, physical methods produce heterogeneous NPs with high consumption of energy, and chemical methods utilize synthetic capping, reducing and stabilizing agents, and eliminate non-ecofriendly byproducts. Moreover, these methods utilize complex process in synthesizing NPs. Metallic NPs are usually optimized by different parameters such as reducing agent concentration, temperature, time and pH. In case of metal oxide NPs, optimization is further followed by thermal treatment in the

ambient air and/or oxygen atmosphere [48,57]. In addition, capping agent plays a crucial role, as it acts as a bridge between NP surface and biomolecule, which further influences loading capacity, releasing and aggregation [58]. However, assessment of toxicity of nano particles fabricated by these methods is difficult due to the involvement of chemicals and byproducts, as each chemical compound has different level of toxicity [56,57].

To reduce toxicity and improve efficacy of chemically synthesized NPs as antibacterials, some researchers have employed plant metabolites as adjuvants. For example, combination effects of Ag-NPs with cinnamaldehyde and eugenol, Se-NPs with quercetin, Au-NPs and Ag-NPs with resveratrol have been reported with irreversible membrane damage against gram-positive and gram-negative bacteria [59–62]. When metallic NPs are administered at therapeutic dose, they are not toxic by themselves, as there is daily requirement of metals for our body [63]. But toxicity may arise due to agents in the chemical synthesis. There is a recent review by Khan et al., focusing on the toxicity associated with chemical methods and properties of NPs [64]. To overcome the toxicity issue, green nanotechnology can be an undeniable approach as they do not employ any synthetic reagents [56,65–67]. In the present review, biosynthesis of inorganic NPs has been explored as the main focus is on less toxic antimicrobials.

4.2.1. Biosynthesis of metallic NPs

The employment of natural resources (microorganisms and plants) as potential reducing agents in the synthesis of metallic NPs is favorable to the environment and human health [68]. Moreover, this method is simple, reproducible and economical as it involves one step synthetic process [69]. Biosynthesis involves biological extract from microorganisms (bacteria, fungi, yeast, algae, and virus) or plants as reducing agent to reduce metal ions either extra-cellularly or intra-cellularly [70]. Components of natural sources (phenols, terpenoids, alkaloids, amides, proteins, pigments, flavones) act as reducing agent, which makes exact mechanism of metallic NPs biosynthesis difficult to understand [71]. These components further act as capping agents and help in the production of stable NPs through prevention of aggregation [58]. Therefore, the most critical criteria of non-toxicity, reproducibility, easy scale up with uniform size and morphology, targeted delivery and resistance free anti-microbials can be attained through bio-fabrication of metallic NPs [72].

4.2.1.1. *Microbial components as a reducing agent* Microbes have the capability to detoxify metals ions. Therefore, utilization of microbial components as reducing agents in the preparation of metallic NPs can minimize toxicity associated with the respective metal. Extracellular method of NPs synthesis is most preferred than intracellular method, as it is rapid process and bypasses the downstream processing [73]. Bacteria are ubiquitous and are more favorable as bio reducing agents due to their simple culturing feasibility [74]. It's an outstanding approach of using microbes as a reducing agent in synthesizing NPs to kill harmful pathogens. Various species of *Bacillus*, *Pseudomonas*, *Aeromonas*, *Escherichia*, *Klebsiella*, *Enterobacter*, *Rhodobacter* and many more have been used in synthesizing metal (Ag, Au, Cu, Pt, Pd) and metal oxide NPs (CuO, ZnO, TiO$_2$,

Fe_2O_3) [75,76]. Even dead bacteria have the capability to reduce metal ions for fabrication of NPs because of organic functional groups on their cell wall. The main mechanism of NP synthesis via microbes lies on the reductase enzyme or protein or biochemical pathway in that respective bacterium [77].

Compared to bacteria, synthesis of NPs through fungi is a straightforward and easy culturing process, which produces stable and less toxic mono-dispersed metallic NPs [78]. In addition, fungi have higher metal bioaccumulation capability along with high tolerance capacity. It is an efficient secretor of intracellular or extracellular enzyme that helps in the production of metal, metal oxide and sulfide NPs [79]. *Aspergillus*, *Penicillium*, *Fusarium*, *Trichothecium*, *Colletotrichum*, *Trichoderma* etc. are most important species for bio-reduction and stabilization. Besides many advantages with fungi, its limitation is time span [79]. Other microbes useful for NPs synthesis include yeast, actinomycetes, algae and virus [80–83].

Along with core profit of large-scale synthesis and absence of toxic chemicals [84], microbial based syntheses involves certain drawbacks such as complexity in culturing techniques, size control and slow processing with difficulty in recovery [52]. Since the last decade, there have been several reviews available solely on metallic NPs using microbes to go through in detail [67,74]. Metal oxide NPs synthesis was also reported with microbial components but with limited literature available [52,53,57,71,73,76,77,79,84].

4.2.1.2. Plant components as a reducing agent In ancient period, medicinal plants were used primarily for medicinal purpose. Domination of antibiotic era hindered focus on botanicals due to the large requirement of plant material for medicinal activity, which is burdensome despite of its non-toxic nature. At present, to utilize the major benefits of Mother Nature, plant source have been employed as reducing agents in the preparation of metallic NPs, which is described as phyto-nanotechnology. It is a boon these days with a salutation to ancient knowledge of metals and botanicals [85,86].

Phyto-nanotechnology has drawn significant attention due to its rapid, eco-friendly, non-toxic, cost-effective protocol in a single step synthetic process without the use of high pressure, energy, temperature or toxic chemicals. In plant-mediated synthesis, the extract is mixed with a metal precursor solution at room temperature and particular pH for a certain period of time. Role of plant extract as reducing, stabilizing and capping agent in the synthesis of metallic NPs might be due to carbonyl and hydroxyl groups as described by Mohamad et al. [87]. This synthetic process is used for large-scale production of stable NPs with biocompatibility, scalability and medical applicability. Almost all parts of medicinal plants (leaves, stem, root, latex) have been used as they contain one or more components (proteins, phenols, vitamins, organic acid, flavonoids, terpenoids, glycosides, and polysaccharides) to act as a reducing agent [88]. In addition, these bio-molecules can be easily extracted and can further act as stabilizing and capping agents to prevent agglomeration. Process of attaining stabilized NPs is shown in Fig. 4. Furthermore, metabolites that are used for reduction process are hypothesized to bind to the surface of NP and further enhance activity [89]. Several plants possess metal accumulation property that can be later

reduced intra-cellularly as NPs [90]. More plants must be investigated as they are indispensable sources to produce fascinating homogenous NPs for antibacterial activity without resistance and toxicity issues. A vast research focused on green synthesis and many articles and reviews were reported in the recent past for detailed study of the synthesis, mechanism, characteristic techniques and applications of green synthesized metallic NPs [91]. T. M. Abdelghany et al., outlined a review on green synthesis of Ag NPs along with their applications [92]. Soumyamenon et al., marked a review on Au NPs [93]. There are many discussions with reviews regarding the green synthesis of CuO, ZnO, Fe_2O_3 NPs for knowledge on respective synthesis, mechanism, and their applications [94–96]. In addition, with limited literature, plant components are also used as reducing agents in the synthesis of bimetallic NPs for effective antibacterial activity [97].

5.   Antibacterial mechanisms of NPs

The massive use of nanomaterials in biomedical applications enhanced research interest to explore the antibacterial mechanisms of NPs [46]. NPs can alter the metabolic activity of bacteria by maintaining contact with bacterial cells via electrostatic interaction, vanderwaals forces, receptor-ligand and hydrophobic interactions [98]. Further, these NPs can cross the bacterial membrane and assemble along the metabolic pathway influencing the shape and functioning of the cell membrane. Finally, NPs interact with the bacterial cell basic components causing oxidative stress, permeability and gene expression changes, diverse alterations, electrolyte balance disorders, protein deactivation, and enzyme inhibition [26]. In order to study the toxicity of NPs against bacterial and mammalian cells, it is important to understand the antibacterial mechanism of NPs. The following mechanisms that are most frequently proposed in current research include oxidative stress, metal ion release, and non-oxidative mechanisms. The bactericidal effects on harmful bacteria based on the stated mechanisms are demonstrated in Fig. 5.

5.1.   *Dissolved metal ions*

Metal ions gently release from metal and metal oxide NPs and are absorbed through the cell membrane. Each metal ion has its own sensitivity to different microorganisms. For instance, Ag^+ ions react with sulfhydryl groups in enzymes and other cellular constituents leading to cellular dysfunction. Ag^+ also prevents cell wall synthesis in gram-positive bacteria. In addition, Ag^+ ions can also interact with microbial DNA inhibiting growth by obstructing DNA replication and cell division [99,100]. In the case of copper-based NPs, Cu^{++} interacts with amine and carboxyl groups on the surfaces of microbial cells. Moreover, copper ions can penetrate into the bacterial cell and can bind with DNA molecules affecting inter and intra nucleic acid strand cross linking, which causes disorganization of the helical structure [101]. This is the same mechanistic approach in case of Au NPs that attenuates bacteria through bacterial membrane disruption [102]. However, metal ion-mediated inhibition is insignificant due to the pH of lipid vesicles, which
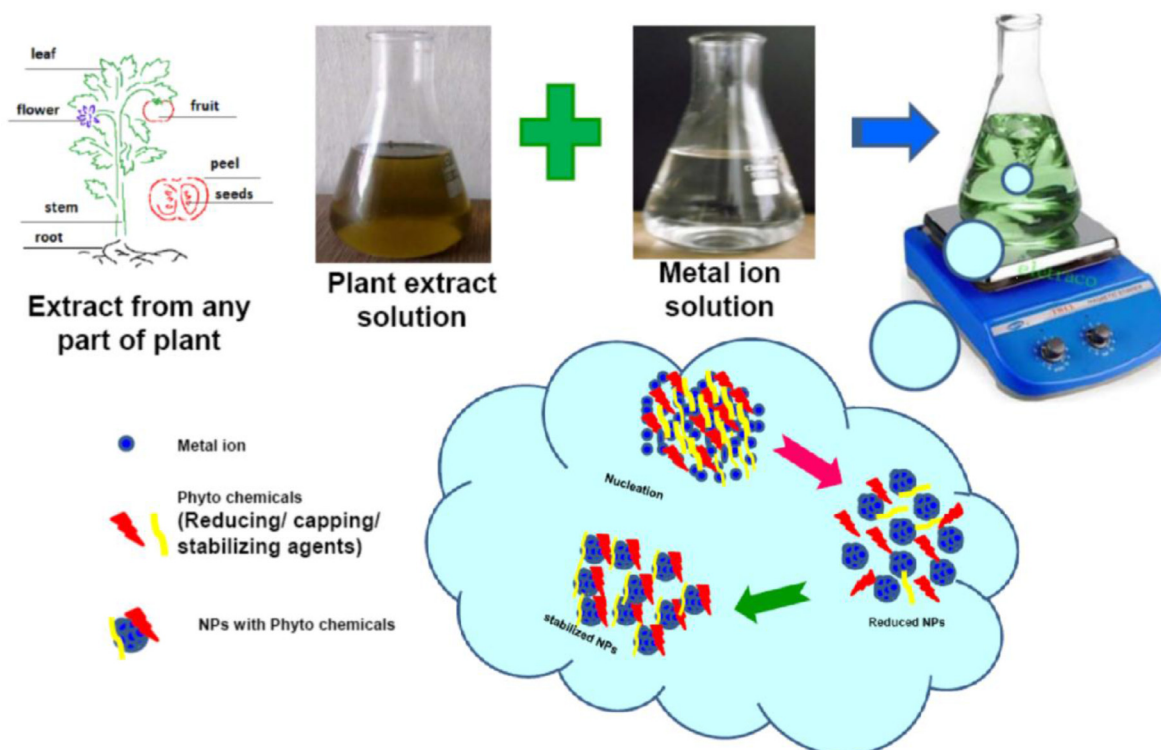
Fig. 4 – Green synthesis of metal and metal oxide NPs with elucidation of synthetic mechanism.
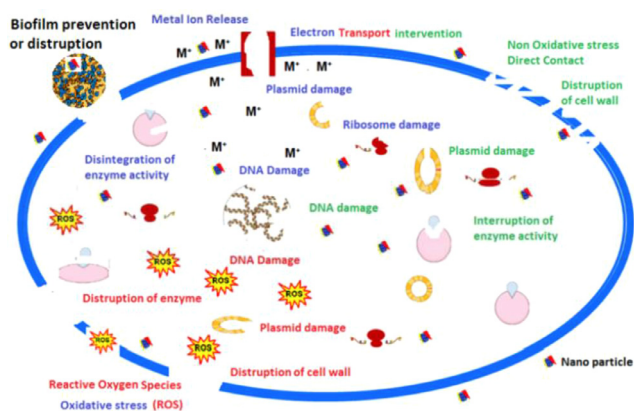

Fig. 5 – Antibacterial mechanisms of inorganic NPs.

results in weak antimicrobial activity. Therefore, metal ion release contribution to antibacterial activity may not be the dominant mechanism for bacterial inhibition.

For example, the metal ion release from Cu based NPs is 253 times more than that of ion release from Ag-NPs. This is attributed to higher oxidation states of Cu than Ag. If metal ion release plays a key role in killing bacteria, Cu^{2+} is expected to exhibit higher antibacterial activity than Ag-NPs. Nevertheless, Ag-NPs have been more efficient in bacterial growth inhibition than Cu-NPs [103].

There have been conflicting reports in the literature on dominant mechanism with metal ion release and reactive oxygen species. Some reports have claimed that the dominant mechanism for bacterial inhibition is metal ion release, whereas, other studies have demonstrated alternative mechanisms for the same NPs [104]. These conflicting studies emphasize the importance of absolute evaluation of each type of NP under standardized testing conditions.

According to the guidelines of World Health Organization (WHO), the presence of metal ions; Zn, Ag, and Cu at concentrations 3 mg, 0.1 mg, and 2–3 mg respectively per day is acceptable for adults [105]. If the metal ion consumption is more than the therapeutic dose, it could be harmful to human health as it exhibits adverse effects on normal cells. Unfortunately, proteomics investigation of toxicity for NPs is rare to evaluate the toxic effect of NPs. In order to reduce the metal ion release and/ or slow release from metal and metal oxide NPs, there have been many attempts such as introducing biocompatible capping or stabilizing agents or employing coreshell and doped NPs for controlled release, or preparation of composites with inorganic/ organic compounds. These strategies may aid in enhanced antibacterial activity with low toxicity [98,106].

5.2. Reactive oxygen species

Toxicity of nanomaterials can be mainly attributed to the production of reactive oxygen species (ROS) that inhibits bacterial growth by restricting amino acid synthesis, lipid peroxidation and DNA replication [107]. The production and liberation of ROS in bacterial cells are balanced under normal conditions [102]. In contrast, in the presence of NPs, excessive production of ROS leads to an unbalanced state, which results in oxidative stress, creating damage to the basic individual

components of bacterial cells [108]. There are 4 types of ROS, namely superoxide radical (O^{2-}), hydroxyl radical ($\cdot OH$), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2), that are generated by NPs with different levels of activity and toxicity. Reasons behind the ROS production are oxygen vacancies and defective sites in crystal structures [109]. Literature has proved that O^{2-} and H_2O_2 can cause moderate stress reactions which can be neutralized by endogenous antioxidants, whereas $\cdot OH$ and O_2 cause acute microbial death [110]. Further, oxidative stress can be a potential contributor to alter cell membrane permeability and cause irreversible membrane damage. However, various NPs produce different types of ROS by reducing oxygen molecules. These free radicals increase ROS level to restrict respiratory enzymes and evoke inflammatory responses, which ultimately induce apoptosis. The induced toxicity through ROS generation and different toxicological pathways has been described well in recent reviews [63,104].

When ROS mechanism of metal oxide NPs (ZnO- and TiO$_2$–NPs) is considered, ROS interfere with cell functions through photo catalysis. Electrons (e^-) in the valence band are stimulated and transmitted to conduction band when illuminated with energy greater than or equal to the band gap of particular NP [111,112]. In the case of ZnO-NPs, H_2O_2 and $\cdot OH$ species are generated but no O^{2-}, whereas TiO$_2$-NPs can generate electron-hole pairs after absorbing light to exhibit antibacterial potency [113]. Electron-hole pairs will react with water and air on the surface of NPs to produce highly chemically active ROS. In contrast, ZnO-NPs produce minimal amounts of $\cdot OH$ in the dark which can contribute to antibacterial activity [114]. However, the generation of all four types of ROS has been proposed in the case of copper oxide NPs [115,116]. It has been found that changes in cell membrane permeability primarily occur due to oxidative stress. Once after NPs enter the cell, intracellular ROS induce loss of membrane integrity, attacks proteins and enzymes that are crucial for cell morphology and disturbs the normal physiological processes of bacterial cells. Moreover, it causes elevated expression of oxidative proteins that lead to cell apoptosis and death [117]. However, excessive ROS stress might be harmful to humans as cells can trigger their defence mechanism. Further, these protective mechanisms fail to restore cellular redox balance and create irreparable damage of DNA, lipids and proteins, leading to necrosis and/or apoptosis. However, this concept is valid only if ROS production is dominant mechanism of toxicity [118]. By contrast, NPs surface and composition can be manipulated to reduce excess production of ROS induced stress (toxicity) by controlling the oxygen vacancies at the surface or through doping.

5.3. Direct contact/Non-oxidative mechanism

The non-oxidative mechanism involves direct interaction of NPs with cell walls. There are several possible interaction mechanisms of NPs with bacterial surface including electrostatic, vanderwaals forces, hydrophobic and receptor-ligand interactions. Multi-layered structure enveloping bacterial cell acts as defensive barriers and creates hostile environment. Gram-positive and gram-negative bacteria produce different adsorption pathways for NPs as they have different structure in terms of cell membrane components. In

gram-negative bacteria, lipopolysaccharides (LPS) and phospholipid layers are responsible for highly negative-charged cell surface, which attracts positively charged NPs. In contrast, in gram-positive bacteria, teichoic acids linked with plasma membrane or peptidoglycan layer impart negative charge to the surface. Phosphate present in teichoic acid attracts and distributes NPs along the molecular chain of phosphate, avoiding any aggregation. Moreover, it has been validated that antibacterial effect of NPs is more pronounced for gram-positive than gram-negative bacteria due to porosity difference [104]. In addition, surface charge alone is not the dominant factor for direct interaction of NPs and bacterial cell. For example, positive surface charge of Ag-NPs is the dominant factor for adhesion [119]. The positive charge of Ag-NPs provides electrostatic interaction with negatively charged cell membrane of microorganisms, which promote the attachment to cell membrane. Due to interactions, morphological changes in cells can be examined by shrinkage of cytoplasm and membrane detachment that leads to cell wall rupture. Besides electrostatic attraction, interaction between sulphur-containing bio-molecules and Ag-NPs provides irreversible changes enabling cell wall disruption [120]. This in turn affects the integrity of lipid bilayer and permeability of cell membrane. The attachment of negatively charged ZnO-NPs with both gram-positive and gram-negative bacterial membrane is due to predominant interactions of receptor-ligand than weak electrostatic repulsion [121].

Thus, the attachment between nanomaterial and cell membrane is through different types of physicochemical interactions. Electrostatic forces might contribute greatly to the adhesion of positively charged particles, but other mechanisms cannot be excluded. In the case of weak electrostatic repulsions between NPs and bacterias, receptor-ligand interactions may be dominate interactions between oxides and bacteria. Such a bond formation was reported in bacterial adhesion to natural mineral surfaces [122]. Carboxyl, amide, phosphate, hydroxyl groups and carbohydrate related moieties in the bacterial cell wall may provide sites for the molecular scale interactions with oxide NPs [122]. Once the particle gets attached to cell surface, re-dox reactions take place to create oxidative stress in bacteria. However, attachment without cell damage can occur for certain nanomaterials, and there were studies showing that toxicity still remains even if NPs and bacteria are separated by membrane. Thus, direct contact is often but not in all cases proposed as necessary mechanism of toxicity. Direct contact inhibits enzymes and proteins involved in cell metabolism, thus disrupting normal functioning of bacterial cell [107]. In addition, we can tune the surface charge of NPs using biocompatible ligands or capping agents [123,124].

In short, to get rid of toxicity issue and for enhanced bactericidal effect, tuning of metal size, composition, surface properties and employing bio reducing agent can be done by optimizing synthetic process [125]. Furthermore, the mechanism associated with toxicity is much of a concern to deal with each type of nanoparticle. If the predominant mechanism of antibacterial activity is metal ion release accompanying toxicity, the metal release and its concentration must be assessed and precursors should be modified accordingly in synthetic process through doping. For instance, metal ion release in metal-

lic NPs can be reduced by preparing a core-shell with metal oxides, metal ion release in metal oxide NPs can be controlled by doping or preparing a composite with metals. If the chief mechanism of antibacterial action is ROS, toxicity can be abolished by controlling oxygen vacancies and defective crystal sites in its structure.

6.　The necessity of phytoNPs as a carrier of antibiotics

NPs (liposomal NPs, solid lipid (SL) NPs, polymer-based NPs, inorganic nanodrug carriers, terpenoid-based NPs, and dendrimer NPs) effectively combat microbial resistance by overcoming massive resistance mechanisms of bacteria and also play a role as a carrier to antibiotics and target bacteria by protecting the antibiotic from resistance [126]. Furthermore, the release of antibiotic can be controlled by maintaining optimum concentration at the infection site for prolonged time, which reduces the frequency of medication along with inhibitory effect on cell growth [127]. Moreover, the combination of drugs can also be carried on a single NP and kill MDR microbes through multiple mechanisms with least resistance [128]. In addition, NPs can be operated through external stimuli such as magnetic field, light, pH, chemical agents and heat [129].

Thus, NPs can be an assured paradigm as drug vehicles for effective therapy against MDR bacteria either by passive targeting or active targeting (through ligand functionalization) [130]. NPs are being preferred as 1st choice drugs for RES illness based on their distribution sites, extended lifetime (slow elimination) [131], broader therapeutics, low side effects and least resistance. Instead, NPs are known to induce cytotoxicity, genotoxicity, immunotoxicity depending on the synthetic procedure and their size, concentration and the mechanism of action through which they target bacteria [131,132]. Toxicity associated with NPs is overviewed in recent studies along with mechanisms of toxicity as a compulsory education in the nano field [133,134]. Thus, nontoxic (bio) nanosystems as a carrier to antibiotics can be a probable option for delivery of antibiotics to steer clear the toxicity problem. The synopsis of combination approaches of NPs with antibiotics via physical and chemical methods are described in some reports and reviews but no report exists with a special focus on biosynthesized NP combinations, which is crucial in resistance era to admit toxic free antimicrobials [135,136].

7.　Combinational strategies of biosynthesized NPs with antibiotics

Green synthesized NPs are known to inhibit popular resistance mechanisms exhibited by bacteria such as excessive and poor influx, biofilm etc. [137]. The strategy of using green NPs as an adjuvant to antibiotics is to acquire toxic free antimicrobials as utmost criteria [56,65–67]. Toxicity issues associated with NPs may create a major threat to humans rather than original infection caused by microbes. Method of NPs preparation and dosage level of NPs and antibiotics make a bigger hand in rising toxicity. Thus, functionalization of

greener NPs with drugs is an alluring strategy in nano field to combat resistance at lower dose with least toxicity, and the same is portrayed in Fig. 6. It is encouraging to know that research is focused recently on microbial and plant assisted NPs in combination with antibiotics, considering them as least toxic to mammalian cells. Formerly, many reports and reviews are available on green synthesized NPs using various plant and microbial sources [92,95,138], but very few are available solely on combinational therapy of green synthesized NPs as an adjuvant to antibiotics, and so are listed in Tables 1 and 2.

8.　Toxicity associated with green NPs

Possible administration routes of NPs include oral, parenteral, topical or through inhalation by avoiding physical and chemical barriers faced by conventional drugs. The exact route of administration that works for greener NPs rather than conventional drugs was interpreted in clear by Pik ling et al. [161]. NPs are rapidly absorbed due to their small size and distributed in liver, spleen and lymph nodes [162]. They may aggregate and accumulate in body for prolonged periods causing toxicity depending on metal, reducing agents, mechanism of antibacterial activity, NP size and concentration [163]. Toxicity problems can be reduced to a certain extent through changes on the surface of NPs, chemical transformations or decreasing the concentration of NPs [125, 132]. Of course, some studies have revealed that there is no apparent toxicity with NPs [163,132]. It can be assured that bio-mediated NPs show the least toxicity to mammalian cells when compared to physical and chemical methods [56,65–67]. Bio involves microbes as well as plants that are assumed to provide least-toxic NPs. Owing to strenuous cell cultural procedures and contamination due to microbes, plant-based NPs are mainly focused to describe the toxicity. Vicario pares et al., performed a toxicity study of metal oxide NPs (CuO-, ZnO-, TiO$_2$-NPs) on zebra fish embryo. Out of that, ZnO-NPs has shown more toxicity but lesser toxicity than bulk Zn [164]. The same toxicity test was performed by Jeyabharathi et al. on zebra fish embryo through *Amaranthus caudatus* leaf extract mediated ZnO-NPs. This green mediated experimentation has shown no toxicity, and displayed excellent antibacterial activity towards *Staphylococcus epidermidis* and *Enterobacter aerogenes* at a similar concentration employed by Vicario-Pares et al. [164,165]. Hazarika et al., tested the cytotoxic effect of *Thalictrum foliolosum* root extract synthesized Ag-NPs on RAW 264.7 cells at bactericidal concentration (5 μM), which has proved no toxicity in cells and shown similar antibacterial activity to standard antibiotics [166]. Other noteworthy instances include core-shell supramolecular gelatin NPs loaded with vancomycin inhibiting *S. aureus* and *Staphylococcus epidermidis* with negligible cytotoxicity and elevated biocompatibility on human embryonic kidney (293T) and human hepatocyte (LO2) cells [167]. Biosynthesized Ag-NPs predominantly exhibited least cytotoxicity and genotoxicity *in vivo* in comparison with chemically synthesized NPs [67]. Another report on negative cytotoxicity is the synthesis of silver NPs using *Aloe vera* plant extract against *S. epidermidis* and *P. aeruginosa*, which proved no obvious cytotoxicity on human PBMCs [168]. Excretion of NPs is usually slow and by bile [131].

Table 1 – The synergy of plant-based NP and antibiotic for antibacterial activity.

Nano particle	Source of reducing agent	Combination of antibiotic	Targeted bacteria	Ref
Ag NP	Corn leaf waste of *Zea mays* extract	Kanamycin and rifampicin	*Bacillus cereus* ATCC 13061 19115, *Escherichia coli* ATCC 43890, *Staphylococcus aureus* ATCC 49444, *Listeria monocytogenes* ATCC, and *Salmonella Typhimurium* ATCC 43174	[139]
Ag NP	Gum kondagogu	Ciprofloxacin, streptomycin, and gentamicin	Gram-positive (*Staphylococcus aureus*25923, *Staphylococcus aureus* 49834) and Gram-negative (*E. coli* 25922, *Pseudomonas aeruginosa* 27853)	[140]
Ag NP	Flower broth of *Tagetes erecta*	Commercial antibiotics(15)	Gram positive (*Staphylococcus aureus and Bacillus cereus*), Gram negative (*E. coli* and *Pseudomonas aeruginosa*) bacteria	[141]
Ag NP	*Adiantum philippense* extract	Amoxicillin	MRSA	[142]
Ag NP	Leaf extracts of *Ficus virens*	streptomycin	Gram-positive (*Bacillus subtilis, Staphylococcus epidermidis, Enterococcus faecalis*) and three gram-negative (*Klebsiella pneumoniae, Vibrio cholera* and *Vibrio vulnificus*)	[143]
Ag NP	*Cassia roxburghii* leaf extract	Ampicillin, polymyxin, gentamicin, chloramphenicol, penicillin-G, amikacin, tetracycline, cephalothin, amoxiclav, cefpirome, clotrimazole	Gram-positive bacteria (*S. aureus* and *B. cereus*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*)	[144]
Ag NP	*Citrullus lanatus rind* extract	Kanamycin, rifampicin	*Bacillus cereus, E. coli, Listeria monocytogenes, S. aureus, S. typhi*	[145]
Ag NP	*Dioscorea bulbifera* tuber extract	beta-lactam (piperacillin) and macrolide (erythromycin)	*A. baumannii*	[120]
Ag NP	*Eichhornia crassipes*	vancomycin, penicillin, streptomycin and tetracycline	*E. coli, S. aureus, K. pneumonia, enterococcus*	[146]
Ag NP	Silky hairs of corn aqueous extract	Kanamycin and rifampicin	*E. coli, S. aureus*	[147]
Ag NP	Leaf extract of *Typha angustifolia*	Gentamicin, cefotaxime, meropenem	*E. coli, K. pneumoniae*	[148]
Ag NP (Citrate-capped)	*Allium sativum*	Cephalothin, cefazolin, chloramphenicol	*M. luteus, Bacillus subtilis, P. aeruginosa, E. coli*	[149]
Au NP	Aqueous extracts of outer oriental melon peel (OMP) and peach	Kanamycin and rifampicin	*E. coli, S. aureus*	[150]
Starch NP	Corn starch powder	Penicillin and/or streptomycin	*Streptococcus pyrogenes*	[151]
Cu NP	Green tea (*Camellia sinensis*) and β-cyclodextrin	Ampicillin, amoxicillin, gentamicin and ciprofloxacin	*E. coli* (Gram negative rods), *S. typhi*(Gram negative rods), *Micrococcus luteus*(Gram positive cocci) and *Streptococcus mutans*	[152]

9. Deficiency in current research

Though green NPs as antibiotic carriers fulfill the needs of effective antimicrobials, it is essential to overcome some pitfalls associated with them. At present, though many types of green NPs are synthesized through various sources, only limited data is available on clinical applications and toxicity restricts NPs and nano-antibiotics to enter the mainstream of antibiotic pipeline as a new class. Though some bio-nanoantibiotics have proven their performance *in vitro* and in experimental animal models with respect to pharmacodynamics, pharmacokinetics and toxicity, evidences from well-designed *in vivo* assays and clinical trials are inadequate with many unanswered questions on neurotoxicity, genotoxicity, and cytotoxicity. Thus, *in vivo* assays and clinical trials must be performed on every nano-antibiotic to assess toxicity associated, site-specific drug delivery, preterm drug release, elimination, and stability study.

Controlled release analysis of nano-antibiotics is not in serious focus comparing to in cancer therapy. But, it is paramount in this case as people are in death zone due to infections after surgeries. A comparative study is mandatory for every type of nanoparticle (through all methods) against each type of microbe to get an analysis of drug loading efficiency, site-specific delivery, safety, and toxicity associated. This comparative study helps health professionals pick the appropriate NP for effective antibacterial activity.

10. Future perspective of bio-nanotechnological combinations

Through observing Tables 1 and 2, it can be acclaimed that there is only a few researches focused on antibiotics loaded with bio mediated NPs, and majority of reports are in combination with Ag-NPs. There is many more metallic NPs syn-
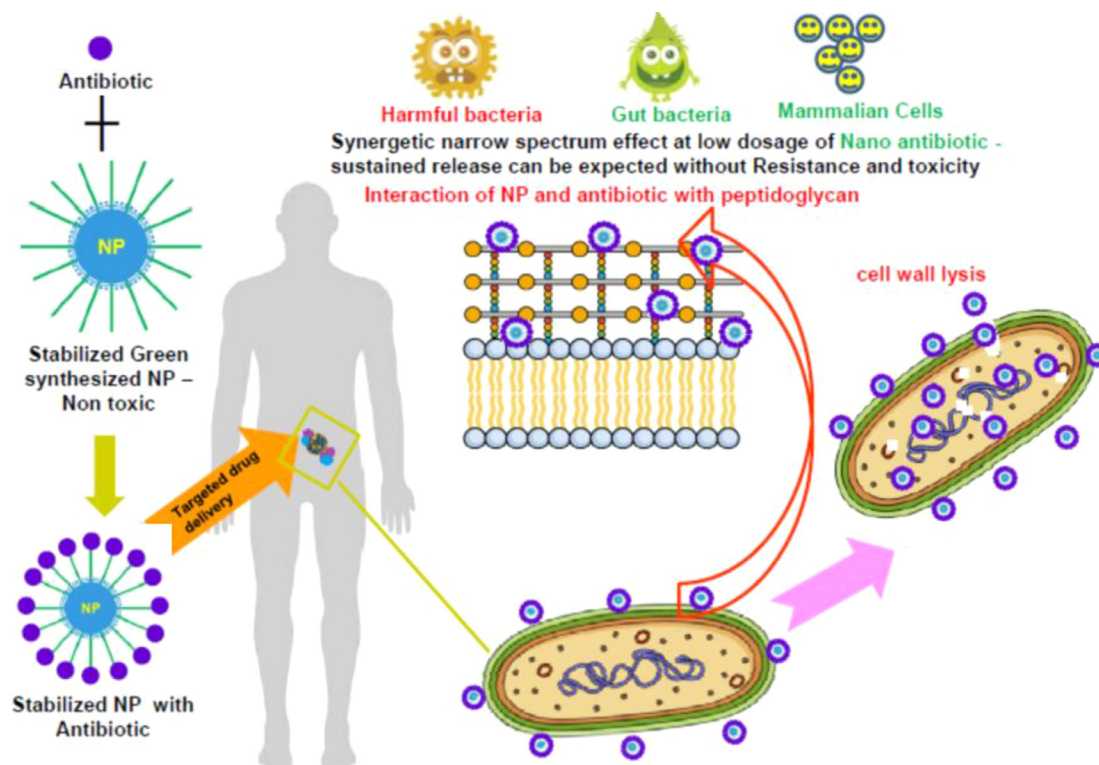
Fig. 6 – Synergistic effect of nano-antibiotic through narrow spectrum targeted drug delivery.

Table 2 – The synergy of microbial-based NP and antibiotic for antibacterial activity.

Nanoparticle	Source of reducing agent	Combination of antibiotic	Targeted bacteria	Ref.
Ag NP	*Acinetobacter calcoaceticus*	Aminoglycosides, β-lactams, cephalosporins, glycoproteins, quinolones, tetracyclines	*A. baumannii, P. aeruginosa, S. mutans, E. aerogenes, S. aureus, s. typhi, shigellasonnie.*	[153]
Ag NP	*Aspergillus flavus*	Ciprofloxacin, imipenem, gentamycin, vancomycin, trimethoprim.	Gram positive and gram negative bacteria	[154]
Ag NP	*Streptomyces xinghaiensis* OF1 strain	Ampicillin, kanamycin and tetracycline	*P. aeruginosa, C. albicans, M. furfur, B. subtilis, E. coli S. aureus* and *Klebsiella pneumoniae*	[155]
Ag NP	*E. coli*	Bacitracin, Ampicillin, Kanamycin	*E. coli, Salmonella paratyphiB, Corynebacterium diphtheria, K. pneumoniae*	[156]
Ag NP	*Aspergillus flavus* and *Emericella nidulans*	Amikacin, kanamycin, oxytetracycline and streptomycin	*E. coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*	[157]
Ag NP	Bacteria from petroleum soil	Doxycycline	*Klebsiella pneumonia*	[158]
Ag NP	*Trichoderma viride*	Ampicillin, kanamycin, erythromycin, and chloramphenicol	Gram-positive and gram-negative bacteria.	[159]
bimetallic Ag-Au NP	Cell free supernatant of *Pseudomonas veronii* strain AS41G on *Annona squamosa* L.	Bacitracin, kanamycin, gentamicin, streptomycin, erythromycin, chloramphenicol	*Bacillus subtilis, E. coli and Klebsiella pneumoniae*	[160]

thesized through microbial and plant extract mediation that must be studied in combinational approaches. Besides this, biosynthesized bimetallic NPs and their combinations, and controlled release pattern of biological NPs must be assessed. As of now, there is no report on green synthesized bimetallic NPs as a carrier and a few reports exist on controlled release of

antibiotics with bio-based NPs as a carrier. For instance, a report by Shibani Basu et al. examined the release of drug from hydrogel through green Ag nanocomposite obtained from *Dolichos biflorus Linn*. This study has shown that modification of acrylamide and silver ion can improve swelling properties of nanocomposite hydrogel and pH response which makes it

suitable for drug delivery applications [169] and another report on controlled release by Avnesh kumari et al. shows the controlled and sustained release of Quecertin from PLA NPs for healthy immune system [170]. Considering the attractive antibacterial activity towards MDR pathogens and toxic free nature of green synthesized NPs, more study is required with respect to combinational approaches along with targeted and controlled release aspects to achieve resistance free drugs.

11. Conclusion

In primitive era, metals and plants were used to treat infections, but due to their random use without proper diagnostics, numerous deaths took place even in case of minor infections. Fortunately with the discovery of antibiotics, many lives were saved from several infectious diseases. New categories of antibiotics were discovered subsequently for half a century, but antibiotics attained resistance soon after their introductory period. Later on, no new classes have entered the antibiotic pipeline in the past 30 years. On the other hand, all renowned antibiotic classes have earned notable resistance and monotherapy approaches have become limited in the landscape of MDR pathogens. As an alternative, researches have focused on the resistance mechanisms of bacteria and developed adjuvant therapy with antibiotics. This combinational approach can bypass bacterial resistance mechanisms, but due to adjuvant-antibiotic interactions, resistance is spreading further. In addition, broad spectrum activity of these combinations is affecting natural microbiome. Therefore, scientists have shifted their focus on nanotechnology as an interdisciplinary approach to target MDR bacteria.

NPs have the ability to modulate their physicochemical properties that grant novel molecules for effective bactericidal activity with feasible administration routes. Several methods are employed (physical, chemical and biological) to prepare NPs. Each method has its own pros and cons with the foremost merit of resistance-free and principal demerits of heterogeneous (physical) and toxicity (chemical). Of all methods, biological method may satisfy the criteria of resistance-free and homogenous NPs with limited toxicity. Thus, it is a reverse brain drain of plants and metals in nano forms, as green NPs works against MDR bacteria through targeted delivery with no resistance and toxicity. In spite of NPs efficacy as antimicrobials with targeted approach, our world cannot ignore antibiotics as they have proved their outstanding performance in antibacterial activity from the 20th century. Thus, a combination of antibiotic with greener NPs can be a paradigm shift to deliver the optimum concentration of drug at the target place, by bypassing resistance mechanisms of bacteria. In addition, adopting greener NPs as drug delivery vehicles can be a toxic-free approach with fewer administration frequencies along with biocompatibility.

It is difficult for nano-antibiotics to enter the medicinal framework unless they cross the pre-clinical and clinical trials effectively. All researches must put maximum attention in assessing NPs from their preparation step to clinical studies. Since the last decade, numerous plant sources have been reported with bactericidal activity, but till date none of them has entered antibiotic pipeline as a new class. This might be due to lack of efficient in vivo studies. Thus, every NP report should provide its full data on pharmacodynamic and pharmacokinetic properties including toxicity and comparative studies of each NP on each class of bacteria. The government must support with funding the clinical trials to make the fabulous nano to enter the drug pipeline. With the hand in hand approach of government and researchers of various fields (pharmacists, biologists, physicians, biochemists etc.), a new class of nano-drugs that fulfills all the requirements of an ideal antimicrobial is no longer away. Once nano-drugs enter official drug pipeline, no further research might be needed to find any alternate solutions, as green-nano antibiotics can fulfill all the requirements of an effective antibacterial, and save millions of lives.

Conflict of Interest

The authors declare that there is no conflicts of interest.

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