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Characterisation of Silver Nanoparticles Synthesized Using *Psidium guajava* Leaves and Their Synergistic Effect with Antibiotics

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Abstract
Synthesis of silver nanoparticles (AgNPs) using plant extracts have been successfully carried out utilizing several economic and eco-friendly processes. Biogenic reduction of metal precursors helps in the synthesis of NPs which are free of chemical contaminants for various medical and biological applications where purity of NPs is most important. The objective of the present study is to synthesize silver nanoparticles using the aqueous extract of *Psidium guajava* leaves and to analyse their synergistic antimicrobial potential when used in combination with the commercially available antibiotics. The silver nanoparticles obtained through this green synthesis method were characterized by UV-visible spectra analysis, Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) studies. The formation of AgNPs was confirmed using the surface plasmon resonance band exhibited in UV–vis spectrophotometry. For the identification of the compounds responsible for the bioreduction of Ag+ ions and the stabilization of AgNPs produced, the functional groups present in AgNPs was investigated using FTIR. The crystalline nature of nanoparticles was confirmed from analysing the XRD pattern. Thereafter, the green synthesized AgNPs were analysed for the antimicrobial efficacy against the clinical isolate, methicillin resistant *Staphylococcus aureus* (MRSA). Furthermore, the synergistic antimicrobial activity of the nanoparticles with four different antibiotics viz. tetracycline, streptomycin, ampicillin and ciprofloxacin were analysed against the MRSA strain. The green synthesized AgNPs proved to be an effective antibacterial agent and exhibited synergistic antibacterial effect when used in combination with the antibiotics.

Keywords
Psidium guajava, silver nanoparticles, Antibacterial synergy, MRSA

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INTRODUCTION
Silver nanoparticles (AgNPs) represent an important nanomedicine-based advance in the fight against multi drug resistant bacteria. Research on nanoparticles have been receiving considerable attention as a result of their unique physical and chemical properties and important applications in optics, electronics, biomedicine, magnetics, mechanics, catalysis, energy science, and so on [1,2,3]. A variety of preparation techniques have been reported for the synthesis of silver nanoparticles. Various chemical approaches, including chemical reduction using a variety of organic and inorganic reducing agents, electrochemical techniques, physicochemical reduction, and radiolysis are widely used for the synthesis of nanoparticles [4]. Nowadays, there is a growing need to develop eco-friendly processes, which do not use toxic chemicals in the synthesis protocols. Synthesis of nanoparticles using biological

methods is very cost effective and utilises eco-friendly processes. These biological methods can be used as an effective alternative for the large-scale production using physical or chemical methods. In the past several years, there are reports of the synthesis of various types of nanoparticles using algae, fungi, bacteria, and plant extracts [5-8]. Water soluble phytochemicals including organic acids, quinones and flavones present in the plant extracts were reported to be responsible for the instantaneous reduction of the silver ions and the formation of nanoparticles [9].

Recent decades have witnessed fast emerging and ever changing multi drug resistant strains causing several recalcitrant infections with disturbing regularity. The mortality rates due to multidrug-resistant bacterial infections are high and, in this context, the discovery of novel and more efficient antimicrobial agents is the need of the hour. A vicious cycle is generated as multidrug resistant pathogens force us to depend on additional broad-spectrum antibiotics to treat these infections, leading to yet more resistance [10]. In order to tackle the situation, research is being focused on various combinations of nanoparticles and antibiotics which could work synergistically to combat drug resistant pathogens [11,12].

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a pathogen with high morbidity and mortality rates [13] and research on new effective drugs against MRSA is an important area of modern medicine. There are only a few reports concerning synergistic antibacterial effects of AgNPs with antibiotics against MRSA. In this study, AgNPs were synthesized using aqueous leaf extract of *P. guajava* and the antibacterial activity of the nanoparticles was evaluated against methicillin resistant *Staphylococcus aureus.* Furthermore, we examined the synergistic antibacterial activity of AgNPs in combination with four different classes of conventional antibiotics viz. 1) β-lactam (ampicillin), 2) quinolone (Ciprofloxacin), 3) aminoglycoside (Streptomycin), and 4) polykeptide (tetracycline).

MATERIALS AND METHODS

Green synthesis of silver nanoparticles (AgNPs)

The aqueous extract of plant material was prepared for the synthesis of nanoparticles using deionized water. Freshly collected leaves (20 gm) were crushed into a paste and mixed with 100 ml of deionized water. The mixture was maintained at 27°C for 12 hrs at 100 rpm in a mechanical shaker. Thereafter, the contents were filtered. For synthesis of AgNPs, 10% (v/v) plant extract was added to 1 mM $AgNO_3$ solution and incubated for 12 hours at room temperature in a mechanical shaker at 100 rpm. The solution of nanoparticles was lyophilized to obtain the nanoparticles in a powder form.

Characterization of Silver nanoparticles

The silver nanoparticles obtained through green synthesis methods were characterized by visual analysis, UV-visible spectra analysis, Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction studies.

UV-visible spectra analysis

Preliminary characterization of the silver nanoparticles was carried out using UV-visible spectroscopy. The metal silver nanoparticles exhibit unique optical properties on account of their surface plasmon resonance (SPR). The spectrum was recorded on Shimatzu spectrophotometer at a wavelength that ranges from 400 to 700 nm.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectrum of silver nanoparticles of the plant extract was recorded to identify the nature of biomolecules which act as capping or stabilizing agents on nanoparticles. The lyophilized powders of the silver nanoparticles were subjected to FTIR spectroscopy measurements. The measurements were carried out on a FTIR-JASCO 4100 spectrophotometer in the diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets.

X-ray Diffraction analysis of silver nanoparticles

The purified silver nanoparticles were characterized with an X-ray diffractometer. Lyophilized nanoparticles were coated on XRD grid, and the spectra were recorded by using Rigaku miniflex 600. X-ray generator was operated at a voltage of 40 k V and a current of 30 mA with CuKα radiation (1.5405 Å).

Antibacterial assay of silver nanoparticles

Antibacterial activity of synthesized silver nanoparticles was analysed by agar well diffusion and MIC/MBC assays. Screening of antimicrobial activity of silver nanoparticles was done using Agar well diffusion method [14]. Wells were bored into the agar medium with heat sterilized 6 mm cork borer. Then fifty microliters of solution of silver nanoparticles (10 mg/ml) were filled in each of the wells. After incubation at 37°C for 24 hours, the plates were examined for zones of inhibition. The diameter of zones of inhibition was measured and recorded.

Quantitative antimicrobial activity assay was carried out for silver nanoparticles of plant extracts to find Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of silver nanoparticles using the microtitre plate method [15]. The 100 µl (10 mg/ml) of the silver nanoparticle solution was diluted through double fold serial dilution to obtain different concentrations of the nanoparticles. The wells were inoculated with 0.01 ml of the standardized bacterial cell suspension (10^7 CFU/ml) and incubation was done at 37˚C for 24 hours. After incubation, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were calculated.

Analysis of synergistic antimicrobial effect of silver nanoparticles with antibiotics

The cumulative antimicrobial effect of silver nanoparticles and antibiotics was tested using broth micro dilution checkerboard method in 96-well microtitre plates [16]. The first antimicrobial agent of the combination was serially diluted along the ordinate, while the second was diluted along the abscissa. The initial concentration of the antimicrobial agent taken in the first well was equal to ½ MBC. Finally, each well contained a unique combination of different concentrations of the two agents. Each microtiter well was inoculated with a standardized bacterial inoculum. The pattern of growth was recorded after incubating the plates for 24 hrs at 37˚C.

To evaluate the effectiveness of the combinations, the fractional bactericidal concentration index (FBCI) was calculated for AgNPs and each of the antibiotics in all the combinations using the formula: FBCI index = (MBC of drug A in combination/MBC of drug A alone) + (MBC of drug B in combination/MBC of drug B alone). The FBCI index was interpreted as follows: synergy, <0.5; partial synergy, 0.5-0.75; additive effect, 0.76-1.0; indifference, >1.0-4.0; and antagonism, >4.0[17, 18].

RESULTS AND DISCUSSION

Characterization of Silver nanoparticles

1. **UV-visible spectrophotometric analysis of silver nanoparticles.**

AgNPs using the extract of *Psidium guajava* leaves were synthesized at room temperature using 1mM silver nitrate solution. Visually observable change in the colour of the reaction solution from light green to dirty reddish brown indicated the formation of silver nanoparticles. No colour change was observed in the absence of plant extract. The synthesis of the AgNPs in aqueous solution was then confirmed by recording the absorption spectra at a wavelength range of 400-700 nm. In UV-Vis spectrum, a single, strong and broad surface plasmon resonance (SPR) was observed at 405 nm. The typical absorption spectrum obtained for the silver nanoparticles clearly indicated the formation AgNPs in aqueous solution.

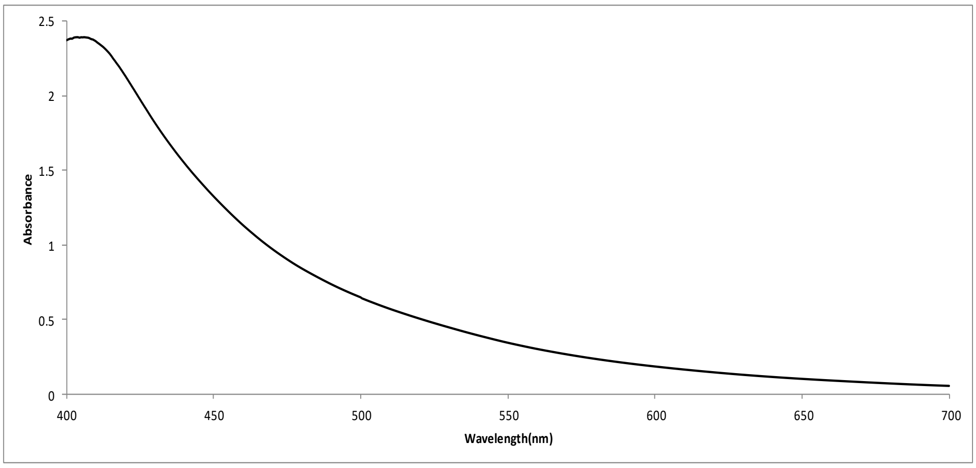

Figure 1: UV–visible spectrum of silver nanoparticles of *Psidium guajava*

2. **FTIR spectral analysis of silver nanoparticles**

FTIR spectrum of silver nanoparticles of the leaf extract was recorded to identify the nature of biomolecules which act as capping or stabilizing agents on nanoparticles. FTIR is a technique that provides information about the chemical bonding or molecular structure of materials. The technique works on the fact that bonds and groups of bonds vibrate at characteristic frequencies. During FTIR analysis, a spot on the specimen is subjected to a modulated IR beam. The specimen's transmittance and reflectance of the infrared rays at different frequencies is translated into an IR absorption plot consisting of reverse peaks.

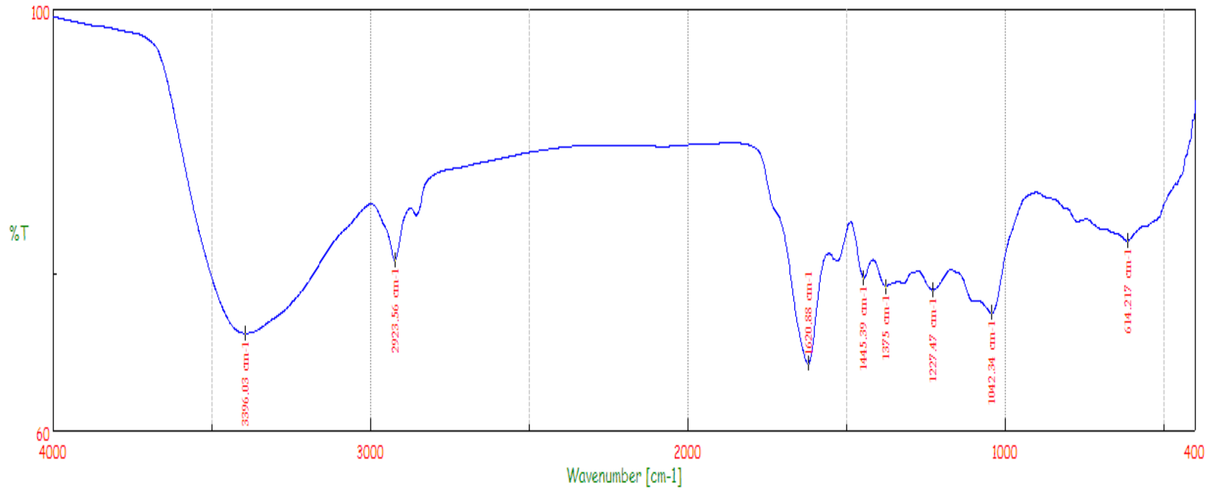

Figure 2: FTIR spectra of silver nanoparticles synthesized from *Psidium guajava*

The observed peak values were compared with the standard values to recognize various functional groups present in the nanoparticles. FTIR spectrum showed absorption bands at 3396, 2923, 1620, 1445, 1375, 1227, 1042 and 614 cm-1 indicating the presence of different capping agents with the nanoparticles. The band at 3396 cm-1 in the spectra corresponds to O-H stretching vibration indicating the presence of alcohol and phenol. The band at 1620 cm-1 in the spectra corresponds to C=C stretching indicating the presence of conjugated alkenes. The band at 1042 cm-1 in the spectra corresponds to CO-O-CO stretching indicating the presence of an anhydride. These functional groups have role in stabilizing the AgNPs as previously reported in many studies [19, 20]. Aqueous leaves extract of *Psidium guajava* may contain primary and secondary metabolites, mainly of polar in nature. Hence, polar secondary metabolites including glycosides and polyphenolics like flavonoids and tannins could be responsible for reduction of metal ions to nanoparticles [21]. The exact mechanism of reducing the Ag+ ion and capping the Ag nanoparticles by flavonoids is not clearly understood. It has been believed that the different -OH groups present in flavonoids play a crucial role in reducing the silver ion to silver metal [22]. This is in line with the observations of the FTIR peaks obtained in the present study.

3. X-ray diffraction studies of silver nanoparticles

X-ray Diffraction analysis of the silver nanoparticles of *Psidium guajava* extracts were carried out to find out the approximate dimensions of the silver nanoparticles and to confirm whether they fall in the specific range.

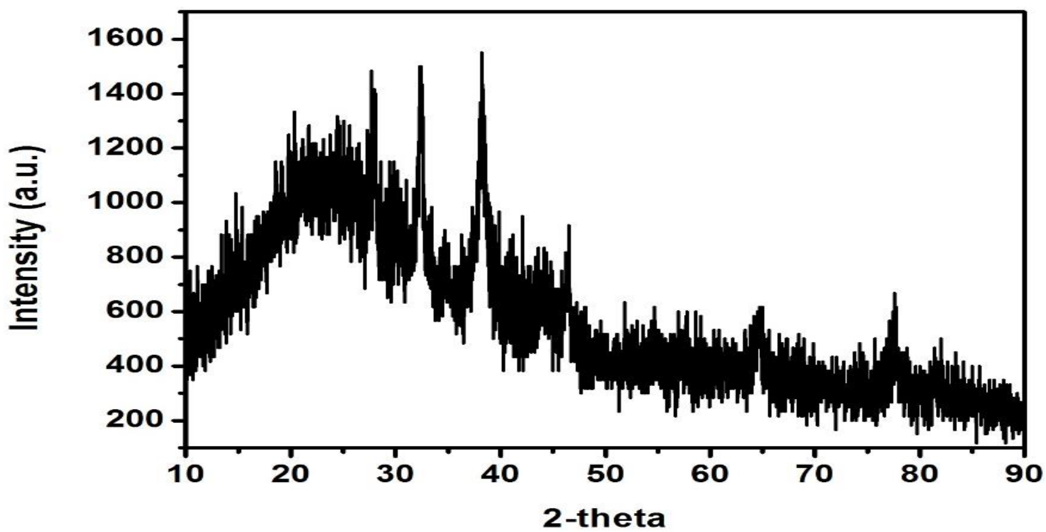

Figure 3: XRD pattern of nanoparticles of *Psidium guajava*

Analysis of XRD data obtained for the lyophilized silver nanoparticles revealed that the particle sizes of the extracts are in the nanometer range.

Antimicrobial activities of silver nanoparticles against MRSA-I isolate

After the synthesis of silver nanoparticles and confirmation of the same through various techniques, these was subjected for antibacterial activity. The silver nanoparticles exhibited antibacterial activity against a methicillin resistant *Staphylococcus aureus* strain by agar well diffusion and MBC assays. The silver nanoparticles displayed an inhibition zone of 23 ± 1.0 mm in agar well diffusion assay and MBC value of 1.25 mg/ml. Several studies have reported the antimicrobial efficacy of silver nanoparticles against pathogenic microorganisms [23]. In an earlier work, Inhibitory effect of biosynthesized silver nanoparticles of *Psidium guajava* against *Pseudomonas aeruginosa* was demonstrated [24]. Antibacterial effect of silver nanoparticles could be based on the fact that smaller sized particles of AgNPs provides better contact and interaction with the microbial cell surface than the larger ones [25]. Besides, Silver ions released from AgNPs may also contribute to the antibacterial activity by penetrating the cell membrane and interacting with the sulphur and phosphorus containing compounds like proteins and DNA which ultimately leads to cell death [26]. However, the mechanism of the antimicrobial action of AgNPs is still not clearly elucidated. There are reports proposing that (i) the electrostatic attraction between the negatively charged bacterial cell and the positively charged nanoparticles causes the deposition of nanoparticles on the bacterial membrane, and thus enhancing cell permeability, (ii) the association of silver with oxygen and its reaction with sulfhydryl (S-H) groups on the cell wall leads to the formation of R-S-S-R bonds, thereby obstructing respiration, (iii) the silver nanoparticles interact with and thereby inhibits bacterial proteins, which ultimately leads to cell death, and (iv) the nanoparticles release silver ion which exerts further bactericidal effect [27, 28].

Synergistic effect of combinations of nanoparticles and antibiotics

The synergistic antibacterial effect of silver nanoparticles in combination with four antibiotics has been investigated against MRSA using checkerboard assay. The antibacterial effect exerted by each of the antibiotics used in this study was found to be enhanced when used in combination with AgNPs. A fourfold reduction was observed in the MBC of all the antibiotics when combined with AgNPs against the methicillin resistant *Staphylococcus aureus* strain used in the present study. The bactericidal concentrations of the silver nanoparticles were also found to be reduced considerably in all the combinations. This clearly indicated the effect of biosynthesised anisotropic silver nanoparticles in enhancing the antimicrobial action of antibiotics. All the combinations of silver nanoparticles with antibiotics exhibited FBCI values within the range prescribed for a synergistic relationship between the members. Our results are also in line with the observations of previous studies in which synergistic effect of AgNPs in combination with conventional antibiotics were tested [29-31]. Moreover, this research work paves the way for the development of new combinations of antimicrobial agents which can be used against drug resistant strains. This kind of combinational therapy may also prevent the development of drug resistance among pathogens [32]. Furthermore, the observations of the present study suggest that nanoparticles synthesized from plant extracts have the potential to work in combination with antibiotics against MDR strains like MRSA at very low concentrations of each of the components.

Table 1: FBCI indexes of combinations of nanoparticles and antibiotics

Bacterial isolate	Antibacterial agents	MBC(µg/ml)		FBCI index	Outcome
		Alone	Combination		
MRSA-I	Guava AgNP Tetracycline	1250 32	313 8	0.5	Synergistic
MRSA-I	Guava AgNP Ampicillin	1250 63	313 15	0.48	Synergistic
MRSA-I	Guava AgNP Streptomycin	1250 39	313 10	0.5	Synergistic
MRSA-I	Guava AgNP Ciprofloxacin	1250 20	313 5.0	0.5	Synergistic

CONCLUSIONS

In conclusion, this study revealed a cost-effective and environment friendly route for green synthesis of Ag nanoparticles and evaluated the antibacterial effect of the biosynthesized Ag nanoparticles against methicillin resistant *Staphylococcus aureus*. The synthesized nanoparticles were analysed through various techniques like UV-visible spectroscopy, Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction analysis (XRD) to confirm their successful synthesis as well as characterization. The synthesized silver nanoparticles using aqueous leaf extract of *Psidium guajava* proved outstanding antimicrobial efficacy against multi drug resistant clinical isolate MRSA. Moreover, these nanoparticles also displayed the ability to work in combination with antibiotics to exert a synergistic antibacterial activity against MDR strains like MRSA. Further studies to analyse the mechanism of action, surface engineering, specific targeting, and conjugation may be helpful to make novel nanoparticles for various applications.

REFERENCES

1. Khan I., Saeed K., Khan I., Nanoparticles: Properties, applications, and toxicities. *Arab J Chem*, *12*(*7*): 908-931, (2019).
2. Maruthi G., Smith A. A., Manavalan R., Nanoparticles--A Review. *J Adv Sci Res*, *2:4*, (2011).
3. Aanchal S., Biosynthesis of Titanium Dioxide Nanoparticles Using Eco-Friendly Approach: A Review, *Int J Pharm Biol Sci, 11(3)*: 58-63, (2021).
4. Iravani S., Korbekandi H., Mirmohammadi S.V., Zolfaghari B., Synthesis of silver nanoparticles: chemical, physical and biological methods. *Res pharma sci*, *9*(*6*):385, (2014).
5. Dubey S.P., Lahtinen M., Sillanpää M., Tansy fruit mediated greener synthesis of silver and gold nanoparticles. *Process Biochem*, *45*(*7*);1065-1071, (2010).
6. Vanaja M., Gurusamy A., Coleus aromaticus leaf extract mediated synthesis of silver nanoparticles and its bactericidal activity. *Appl Nanosci, 3*: 217-223, (2013).
7. Upadhyay L.S.B., Verma N., Recent developments, and applications in plant-extract mediated synthesis of silver nanoparticles. *Anal. Lett. 48(17):* 2676-2692, (2015).
8. Rajesh Kumar S., and Bharath L.V., Mechanism of plant-mediated synthesis of silver nanoparticles–a review on biomolecules involved, characterisation and antibacterial activity. *Chem Biol Interact*, *273:* 219-227, (2017).
9. Afreen A, Ahmed R, Mehboob S, Tariq M, Alghamdi H.A., Zahid A.A., Ali I., Malik K., Hasan A., Phytochemical-assisted biosynthesis of silver nanoparticles from *Ajuga bracteosa* for biomedical applications. *Mater Res Express, 7(7):*075404, (2020).
10. Tamma P.D., Cosgrove S.E., Maragakis L.L., Combination therapy for treatment of infections with gram-negative bacteria. *Clin Microbiol Rev*, *25(3):*450-70, (2012).
11. Jyoti K., Baunthiyal M., Singh A., Characterization of silver nanoparticles synthesized using Urtica dioica Linn. leaves and their synergistic effects with antibiotics. *J Radiat Res Appl Sci*, *9(3)*:217-27, (2016).
12. Sanhueza L., Melo R., Montero R., Maisey K., Mendoza L., Wilkens M., Synergistic interactions between phenolic compounds identified in grape pomace extract with antibiotics of different classes against Staphylococcus aureus and Escherichia coli. *PloS one*, *12(2)*: e0172273, (2017).
13. Turner N.A., Sharma-Kuinkel B.K., Maskarinec S.A., Eichenberger E.M., Shah P.P., Carugati M., Holland T.L., Fowler V.G., methicillin-resistant Staphylococcus aureus: an overview of basic and clinical research *Nat Rev Microbiol*, *17(4)*:203-18, (2019).
14. Kalishwaralal K., BarathManiKanth S., Pandian S.R., Deepak V., Gurunathan S., Silver nanoparticles impede the biofilm formation by Pseudomonas aeruginosa and Staphylococcus epidermidis. *Colloids Surf B Biointerfaces*, *79(2)*:340-4, (2010).
15. Li W.R., Xie X.B., Shi Q.S., Zeng H.Y., You-Sheng O.Y., Chen Y.B., Antibacterial activity and mechanism of silver nanoparticles on Escherichia coli. Applied microbiology and biotechnology *Appl Microbiol Biotechnol*, *85(4)*:1115-22, (2010).
16. Hwang I.S., Hwang J.H., Choi H., Kim K.J., Lee D.G., Synergistic effects between silver nanoparticles and antibiotics and the mechanisms involved. *J Med Microbiol*, *61(12)*:1719-26, (2012).
17. Seesom W., Jaratrungtawee A., Suksamrarn S., Mekseepralard C., Ratananukul P., Sukhumsirichart W., Antileptospiral activity of xanthones from Garcinia mangostana and synergy of gamma-mangostin with penicillin G. *BMC Complement Altern Med*, *13(1)*:1-6, (2013).
18. Odds F.C., Synergy, antagonism, and what the chequerboard puts between them *J Antimicrob Chemother*, *52(1)*:1-1, (2003).
19. Niraimathi K.L., Sudha V., Lavanya R., Brindha P., Biosynthesis of silver nanoparticles using Alternanthera sessilis (Linn.) extract and their antimicrobial, antioxidant activities. *Colloids Surf B Biointerfaces, 102*:288-91, (2013).
20. Prakash P., Gnanaprakasam P., Emmanuel R., Arokiyaraj S., Saravanan M., Green synthesis of silver nanoparticles from leaf extract of Mimusops elengi, Linn. for enhanced antibacterial activity against multi drug resistant clinical isolates. *Colloids Surf B Biointerfaces*, *108*:255-9, (2013).
21. Patil S.P., Rane P.M., Psidium guajava leaves assisted green synthesis of metallic nanoparticles: a review. *Beni Suef Univ J Basic Appl Sci, 9(1)*:1-7, (2020).
22. Makarov V.V., Love A.J., Sinitsyna O.V., Makarova S.S., Yaminsky I.V., Taliansky M.E., Kalinina N.O., "Green" nanotechnologies: synthesis of metal nanoparticles using plants. *Acta Naturae*, *6(1 (20))*:35-44, (2014).

23. Dar M.A., Ingle A., Rai M., Enhanced antimicrobial activity of silver nanoparticles synthesized by Cryphonectria sp. evaluated singly and in combination with antibiotics. *Nanomedicine, 9(1)*:105-10, (2013).
24. Bose D., Chatterjee S., Biogenic synthesis of silver nanoparticles using guava (Psidium guajava) leaf extract and its antibacterial activity against Pseudomonas aeruginosa. *Appl Nanosci*, (*6*):895-901, (2016).
25. Kvítek L., Panáček A., Soukupova J., Kolář M., Večeřová R., Prucek R., Holecová M., Zbořil R., Effect of surfactants and polymers on stability and antibacterial activity of silver nanoparticles (NPs). *J Phys Chem C, 112*(*15*):5825-34, (2008).
26. Matsumura Y., Yoshikata K., Kunisaki S.I., Tsuchido T., Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate. *Appl Environ Microbiol*, *69*(*7*):4278-81, (2003).
27. Morones J.R., Elechiguerra J.L., Camacho A., Holt K., Kouri J.B., Ramírez J.T., Yacaman M.J., The bactericidal effect of silver nanoparticles. *Nanotechnology*, *16*(*10*):2346, (2005).
28. Shrivastava S., Bera T., Roy A., Singh G., Ramachandrarao P., Dash D., Characterization of enhanced antibacterial effects of novel silver nanoparticles. *Nanotechnology*.18(22):225103, (2007).
29. Fayaz A.M., Balaji K., Girilal M., Yadav R., Kalaichelvan P.T., Venketesan R., Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against gram-positive and gram-negative bacteria. *Nanomedicine*, *1*;*6*(*1*):103-9, (2010).
30. Saratale G.D., Saratale R.G., Benelli G., Kumar G., Pugazhendhi A., Kim D.S., Shin H.S., Anti-diabetic potential of silver nanoparticles synthesized with Argyreia nervosa leaf extract high synergistic antibacterial activity with standard antibiotics against foodborne bacteria. *J Clust Sci*, *28*(*3*):1709-27, (2017).
31. Deng H., McShan D., Zhang Y., Sinha S.S., Arslan Z., Ray P.C., Yu H., Mechanistic study of the synergistic antibacterial activity of combined silver nanoparticles and common antibiotics. *Environ Sci Technol, 50(16)*:8840-8, (2016).
32. Hill J.A., Cowen L.E., Using combination therapy to thwart drug resistance. *Future microbiol,* 10(11):1719-26, (2015).