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Original article

# Green synthesis of silver nanoparticles by *Pisum sativum* L. (pea) pod against multidrug resistant foodborne pathogens

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## ABSTRACT

The consumption of foods contaminated with various foodborne organisms such as, viruses, fungi and bacteria are recognized as the important sources of foodborne illness in animals and humans. Multidrug resistant foodborne bacteria increased morbidity, mortality rates, and severe economic loss and associated with prolong hospitalization. The development of natural and novel antibacterial agent is much needed as there is an increasing concern on multidrug resistant bacteria. This study aimed to synthesize silver nanoparticles using aqueous *Pisum sativum* L. (pea) pod extract and to characterize the nanoparticles. It was further used on various drug resistant foodborne bacterial pathogens and antimicrobial activity was determined. The synergistic antibacterial activity, antioxidant activity and biocompatibility were also assessed. Green synthesized nanoparticles applied in this study were synthesized using aqueous *P. sativum* L. (pea) pod extract with particle size 30 nm. The green synthesized silver nanoparticles showed antimicrobial activity against *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 700802, *Streptococcus gordonii* ATCC 49818, *Staphylococcus aureus* (MRSA) ATCC 4330 and *Staphylococcus aureus* ATCC 25923. Among the pathogenic bacterial strains, silver nanoparticles were highly active against *S. aureus* ATCC 25923 than other bacterial strains ( $22 \pm 2$  mm). The antibacterial activity varied between  $15 \pm 2$  mm and  $22 \pm 2$  mm diameter zone of inhibition. The MIC value of the nanoparticles ranged from 6.25  $\mu\text{g/mL}$  to 75  $\mu\text{g/mL}$  and MBC value ranged from 50  $\mu\text{g/mL}$  to 150  $\mu\text{g/mL}$ . The green synthesized nanoparticles showed antioxidant potential. Normal fibroblast cell lines (L-929) was used to test the cytotoxic activity and the increased  $\text{IC}_{50}$  value ( $750.5 \pm 5.5$   $\mu\text{g/mL}$ ) indicated good biological compatibility. The present findings revealed that green synthesized silver nanoparticles exhibited potent activity against various food borne pathogenic bacteria. These nanoparticles might be well developed as novel antibacterial substances for the treatment of food borne multidrug resistant bacterial infection.

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## 1. Introduction

Green synthesis is a suitable, rapid and highly acceptable method for the biosynthesis of various metal nanoparticles. In recent years, various plant parts such as bark, stem, fruit, seed

and leaf extracts were used effectively for the biosynthesis of metal nanoparticles (Mittal et al., 2013). Among various metal nanoparticles, silver nanoparticles were widely used due to their anti-proliferative, anti-fungal and anti-bacterial activity (Nayak et al., 2015). Silver nanoparticles are widely used in medicines, cosmetics, biofertilizers, seed and food preservation and in food packing (Dipankar and Murugan, 2012). Apart from these applications, these particles are widely used in the field of micro-electronics, catalysis, diagnostics, and bio-molecular detection (Mohanta et al., 2016b). There are several methods used to synthesize silver nanoparticles, including chemical and physical methods, viz, microwave-assisted process, sono-chemical, radiation assisted, thermal decomposition of silver compounds, chemical and photo-

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chemical reactions in reverse micelles, and green chemistry techniques. The green synthesis based nanoparticles has several advantages over the physical and chemical method as it is eco-friendly, cost effective and very simple to manage commercial-scale processes without using much toxic chemicals, elevated temperature, high pressure and applying excessive energy (Song and Kim, 2009).

There are various reports on green synthesis of silver nanoparticles using plant materials. Moreover, there is need to evaluate most reliable and simple method for environmentally clean route to biosynthesize, commercially viable, economically stable silver nanoparticles to synthesize by using various natural sources, mainly plant based materials (Chung et al., 2016). In recent years, the application of plant based products and in the biosynthesis of silver nanoparticles has been investigated (Arunachalam et al., 2013; Valsalam et al., 2019). Various medicinal plants are used as the traditional medicine for the preparation of nutritional and healthcare product. Green synthesis of nanoparticles using various biological agents including, plant extracts, algae, fungi, and by-products of these sources has attracted much more attention in recent times. Moreover, the application of feed wastes from the legumes in the green synthesis of nanoparticles has not been well documented. The application of food wastes to green synthesize nanoparticles would be an environmentally friendly, low-cost and highly effective to recycle the nutrients for the production of pharmaceuticals and biomedicines. *P. sativum* L. (pea) is edible and the pod is considered as waste and used for the preparation of feed for animals. The pea pod and peel contain bioactive compounds such as, flavonoids, and phenols and it has been widely used as traditional medicines to treat number of diseases and disorders (Hadrich et al., 2014). Pea contains bioactive compounds responsible for antidiabetic, antioxidant and antimicrobial properties. However, peas are widely used to treat diabetes in China, India and other Asian countries. In this study, investigation was carried out to synthesize silver nanoparticles using aqueous extract of pod of pea and to analyze bioactive properties.

## 2. Materials and methods

### 2.1. Plant extract

The half matured *P. sativum* L. pod was collected from the agricultural field and the seeds were removed from the pod. The empty pod was used for the green synthesis of silver nanoparticles. The pod was cut into small pieces and finely ground mechanically. About 1 g of pod powder was mixed in 250 mL Erlenmeyer flask containing 100 mL double distilled water. The Erlenmeyer flask was boiled for 10 min using a water bath and stirred continuously. The water extract of pod was cooled and filtered using Whatman's filter paper number 1 and stored at 4 °C. It was used as the reducing agent for the green synthesis of silver nanoparticles.

### 2.2. Green synthesis of silver nanoparticles

Green synthesis of nanoparticles was performed using the aqueous extract of *P. sativum* L. pod. Initially 100 mL of 1 mM silver nitrate was prepared and stored in an Erlenmeyer flask. To this Erlenmeyer flask, 10 mL of plant pod extract was added slowly under continuous stirring. Stirring was achieved using a magnetic stirrer and continued this process until the solution turns brown or reddish brown. The ratio of aqueous extract and silver nitrate solution was maintained at 1:10 ratio and the size and shape of the nanoparticles were maintained appropriately (Valsalam et al., 2019; Anand et al., 2017).

### 2.3. Characterization of silver nanoparticles

The green synthesized nanoparticles were initially analyzed using UV-Visible spectrophotometer. Scanning electron microscopy (SEM) analysis was performed to determine the size of the nanoparticles. X-ray powder diffraction analysis is one of the standard methods to characterize nanoparticles. SEM and XRD characterization were performed as suggested previously by Ramamurthy et al. (2013). The changes of colour reaction was monitored continuously and checked for every four hours. The morphological characteristics of nanoparticles were analyzed using SEM. Sample was dried in an oven at 60 °C and powdered using a mortar and pestle and the uniformed sample size was used for the determination of XRD (Thomas et al., 2019; Gomathi et al., 2020; Jayaprakash et al., 2017). The diameter of silver nanoparticles was analyzed using Scherer equation as suggested previously by Yousefzadi et al. (2014).

### 2.4. Bacterial inoculums preparation

The pathogenic bacteria such as, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 700802, *Streptococcus gordonii* ATCC 49818, *Staphylococcus aureus* (MRSA) ATCC 4330 and *Staphylococcus aureus* ATCC 25923 were cultured in nutrient broth (Himedia, Mumbai, India) medium and incubated for 18 h at  $37 \pm 1$  °C. The growth was monitored using UV-visible spectrophotometer at 600 nm and the optical density of the culture was maintained as 1.0 and used for further studies (Zhang et al., 2020).

### 2.5. Antimicrobial activity of silver nanoparticles

Disc diffusion method was used for the determination of antimicrobial activity of the silver nanoparticles. The green synthesized nanoparticles were dissolved in dimethyl sulfoxide (DMSO, 5%) and sonicated the extract for 10 min at room temperature ( $28 \pm 2$  °C). Each disc was loaded with 50 µg silver nanoparticles and used for antibacterial assay. The inoculum cultured in nutrient broth medium was diluted individually and the cell density of the inoculum was approximately  $1 \times 10^5$  colony forming unit/mL (CFU/mL) and the zone of inhibition was assayed in mm (Gandhi et al., 2021).

### 2.6. Minimum inhibitory concentration and minimum bactericidal concentration determination

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the sample was determined by broth dilution method as described previously in the literature (Kubo et al., 2004). Silver nanoparticles were diluted at various concentrations (100–1.25%). Initially, silver nanoparticles were serially diluted in nutrient broth containing nanoparticles and continuously the dilution was performed until the dilution reached 1.25%. To the control vial, only nutrient broth was applied. This experiment was performed for all the selected bacterial isolates. The bacterial strains were inoculated and the least concentration which inhibited the bacterial growth at very least doses was considered as MIC. Then the sample was spread on nutrient agar plants and incubated for 37 °C for 24 h. and the CFU value was determined. The values were expressed as µg/mL nanoparticles for MIC and MBC (Kalaiyarasi et al., 2020; Al-Dhabi et al., 2020).

### 2.7. Synergistic activity of silver nanoparticles

Synergistic activity of green synthesized nanoparticles was analyzed as described previously. Briefly, rifampicin was used as the

standard drug and it was diluted with Millipore sterile distilled water. The standard antibiotic, rifampicin was mixed with green synthesized nanoparticles and antibacterial activity was assayed by standard method (Naqvi et al., 2013). The tested bacterial pathogens (*P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *E. faecalis* 700802, *S. gordonii* ATCC 49818, *S. aureus* (MRSA) ATCC 4330 and *S. aureus* ATCC 25923) were cultured in newly prepared nutrient broth medium and the standard antibiotic was mixed with nanoparticles. The antibiotic disc was prepared and placed on bacterial culture plates. Each disc contains 5 µg standard and 10 µg silver nanoparticles and the culture was incubated for 24 h and synergistic antimicrobial potential was observed (Malar et al., 2020; Zhang et al., 2020; Al-Ansari et al., 2020).

## 2.8. Antioxidant activity of green synthesized silver nanoparticles

### 2.8.1. DPPH scavenging activity

The DPPH free radical scavenging activity of nanoparticles assay was carried out as suggested earlier. The nanoparticles were diluted at various concentrations (10–50 µg/mL) and ascorbic acid was used as the external standard. The absorbance of the reaction was performed at 517 nm against control blank and the percentage scavenging activity was calculated (Ezhilarasi et al., 2020).

### 2.8.2. ABTS radical scavenging activity

The ABTS radical scavenging activity of nanoparticles was performed as suggested earlier. Nanoparticles were diluted appropriately (10– 0 µg/mL) and ascorbic acid was used as the external standard. The absorbance of the sample was analyzed at 750 nm against control blank and the percentage radical scavenging potential was obtained (Ezhilarasi et al., 2020).

### 2.8.3. Reducing power assay

The reducing potential of nanoparticles was analyzed as described earlier. Nanoparticles were diluted appropriately (10–50 µg/mL) and the absorbance of the sample was read at 700 nm against the reagent the control blank and the result was expressed as percentage reducing power (Ezhilarasi et al., 2020).

### 2.8.4. Nitric oxide scavenging activity

Nitric oxide (NO) scavenging assay was performed as described previously. Nanoparticles were diluted appropriately (10–50 µg/mL) and the optical density of the reaction mixture was read at 546 nm and the percentage scavenging activity was determined (Ezhilarasi et al., 2020).

### 2.8.5. Biocompatibility of silver nanoparticles

The normal fibroblast (L-929) cells were used for the determination of biocompatibility by analyzing the viability of the cells. The culture flask was seeded with Dulbecco's Modified Eagle's Medium (DMEM), M-199 medium, fetal bovine serum (10%) and incubated for 24 h at 37 °C with 5% CO<sub>2</sub>. After 24 h incubation, the adhered mammalian cells were trypsinized (5 min) and the individual cells were separated by centrifugation (1000 rpm, 5 min). The cells were further placed in 96-well microtiter plate with approximately 5000 cells/well and incubated for 12 h to achieve a monolayer with ~80 to 90% confluence (Song and Kim, 2009). Silver nanoparticles reduced ATP content of the cell and involved in the mitochondrial damage and increased the generation of reactive oxygen species (ROS) and the increase of ROS was based on nanoparticles concentrations. In this study nanoparticles were added at various concentrations ranging from 200 to 1200 µg/mL. To assay the viability of L-929 cells, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution was added and incubated for 5–6 h. After reaction, MTT reagent was completely discarded and DMSO (250 µL) was added

to the wells and incubated for 20 min under dark condition. The developed formazan was determined at 595 nm using a microtiter plate reader (Al-Ansari et al., 2021; Wu et al., 2020).

## 3. Results

### 3.1. Green synthesis of silver nanoparticles

The pea pod was used for the green synthesis of silver nanoparticles and 1 mM silver nitrate solution was used initially. The colour change was observed and the synthesis of nanoparticles was observed. The colour of the solution was turned dark brown after 2 h of incubation. The colour intensity was improved further and no colour change was observed after 4 h of treatment. The development of silver nanoparticles was observed using various characterization methods.

### 3.2. Characterization of nanoparticles

The green synthesized nanoparticles were tested and the absorption spectra were monitored continuously to confirm nanoparticles in the sample. The absorbance spectrum indicated the development of silver nanoparticles. The UV-spectrum of the sample was monitored continuously for 36 h and no visible colour change in the nanoparticles after 24 h incubation. The morphology of the green synthesized nanoparticles was further characterized using HRTEM. The synthesized nanoparticles were approximately 30 nm and observed uniform sized particle (Fig. 1). SAED analysis of green synthesized nanoparticles was described in Fig. 2. Fig. 3 shows EDX spectrum and it revealed the chemical composition and purity of the silver nanoparticles. The percentage silver metal observed in this study was more appropriate. The other elements such as Cu, Mg, and Na were detected at lower percentages.

### 3.3. Antibacterial activity of intestinal pathogens

The human intestinal pathogens such as, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *E. faecalis* 700802, *S. gordonii* ATCC 49818, *S. aureus* (MRSA) ATCC 4330 and *S. aureus* ATCC 25923 were used for antibacterial assay and the pea pod synthesized nanoparticles showed activity against these tested bacterial pathogens. The antibacterial activity of silver nanoparticles was described in the Table 1. Among the pathogenic bacterial strains, silver nanoparticles were highly active against *S. aureus* ATCC 25923 (22 ± 2 mm) than other bacterial strains. The antibacterial activity varied between 15 ± 2 mm and 22 ± 2 mm zone of inhibition. The

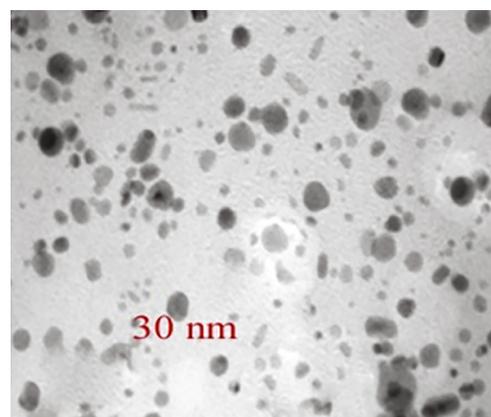
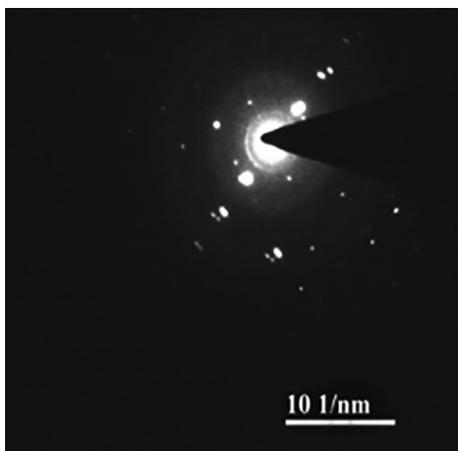


Fig. 1. HRTEM analysis of green synthesized silver nanoparticles.



**Fig. 2.** Selected area for electron diffraction (SAED) analysis of green synthesized nanoparticles.

MIC value ranged from 6.25  $\mu\text{g}/\text{mL}$  to 75  $\mu\text{g}/\text{mL}$  and MBC value ranged between 50  $\mu\text{g}/\text{mL}$  and 150  $\mu\text{g}/\text{mL}$ .

### 3.4. Synergistic activity of silver nanoparticles

Synergistic activity of green synthesized nanoparticles was analyzed with rifampicin. The zone of inhibition was  $18 \pm 2$  mm against *P. aeruginosa* ATCC 27,853 and silver nanoparticles treated antibiotic increased zone of inhibition ( $21 \pm 2$  mm). Synergistic activity was observed against *S. aureus* ATCC 25923 ( $24 \pm 2$  mm). Moreover, supplementation of nanoparticles with antibiotics did not improve antimicrobial activity and the result was described in Table 2. Both rifampicin and  $\text{AgNO}_3$  incorporated disc showed synergistic antibacterial activity against drug resistant bacteria.

### 3.5. Antioxidant activity

The antioxidant properties of the silver nanoparticles were analyzed and the result was described in Fig. 4. The DPPH activity of green synthesized nanoparticles was described in Fig. 4a and showed antioxidant activity of  $68 \pm 3.1\%$  at 50  $\mu\text{g}/\text{mL}$  concentration. Nanoparticles showed increased NO activity at 50  $\mu\text{g}/\text{mL}$  con-

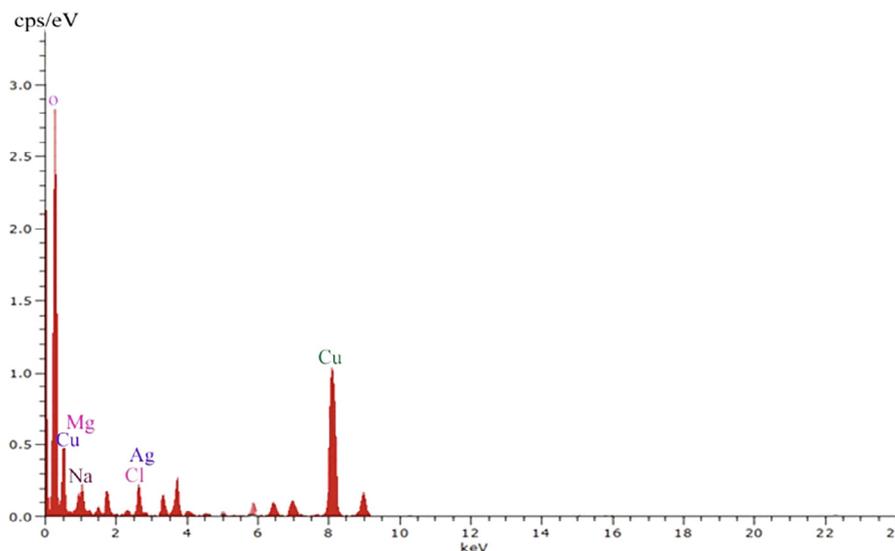
centration ( $86 \pm 2.8\%$ ) (Fig. 4b). The ABTS activity of nanoparticles was described in Fig. 4c and attained  $68 \pm 2.7\%$  activities at 50  $\mu\text{g}/\text{mL}$ . The reducing activity was  $27 \pm 21\%$  at 10  $\mu\text{g}/\text{mL}$  concentrations and it increased at 50  $\mu\text{g}/\text{mL}$  ( $74 \pm 1.5\%$ ) (Fig. 4d).

### 3.6. Biocompatibility of green synthesized nanoparticles

Biocompatibility analysis is widely used to analyze the possible application in biomedicine and drug use or food additives. Normal fibroblast cell lines (L-929) was used to test the cytotoxic activity using nanoparticles. The toxicity of silver nanoparticles is one of the important concerns on cell lines which significantly influenced on biological uses. At lower concentrations of silver nanoparticles, no inhibitory effect was observed. Moreover, the cell viability declined at increasing concentration of nanoparticles and the result was depicted in Fig. 5. The  $\text{IC}_{50}$  value of the green synthesized nanoparticles showed  $750.5 \pm 5.5$   $\mu\text{g}/\text{mL}$  which indicated maximum biological compatibility (Fig. 5). The pod extract did not induced toxicity against L-929 cell lines and indicated potential application in medicine and food.

## 4. Discussion

Medicinal plants are widely used to treat various diseases and disorders. The secondary metabolites produced by the medicinal plants have therapeutic applications. The bioactive potential of medicinal plants varied based on extraction methods, plant parts, growth phases, climatic condition and seasons. The consumption of vegetables has been earlier proven to minimize the risk of various diseases and disorders. *P. sativum* L. is widely used as astringent, laxative, refrigerant, appetizer, and also used in treating phlegm, acne, diabetes, skin and intestinal inflammation (Yusuf et al., 2007; Angels and Joan, 2007). Hypoglycemic, antimicrobial and antioxidant activities have been observed from this plant. Interestingly, the pod contains potential antihyperglycemic activity (Taha et al., 2011). Petiole and tendril showed the presence of chemical compounds such as, p-coumaric esters, quercetin-3-triglucoside, and kaempferol-3-triglucoside. Plant mediated nanoparticles synthesis is highly efficient than microbial biosynthesis of nanoparticles. Plants have various phytochemicals, including polyphenols that can effectively act as stabilizing agent in the green synthesis of nanoparticles. In this study, pea pod



**Fig. 3.** EDX analysis of green synthesized silver nanoparticles.

**Table 1**  
Antibacterial activity of green synthesized silver nanoparticles against human bacterial pathogens.

Bacteria	Zone of inhibition (mm)	MIC (µg/mL)	MBC (µg/mL)
<i>P. aeruginosa</i> ATCC 27853	15 ± 2	50	100
<i>E. coli</i> ATCC 25922	17 ± 1	50	150
<i>E. faecalis</i> 700802	15 ± 3	75	125
<i>S. gordonii</i> ATCC 49818	18 ± 2	12.5	75
<i>S. aureus</i> (MRSA) ATCC 4330	17 ± 1	12.5	75
<i>S. aureus</i> ATCC 25923	22 ± 2	6.25	50

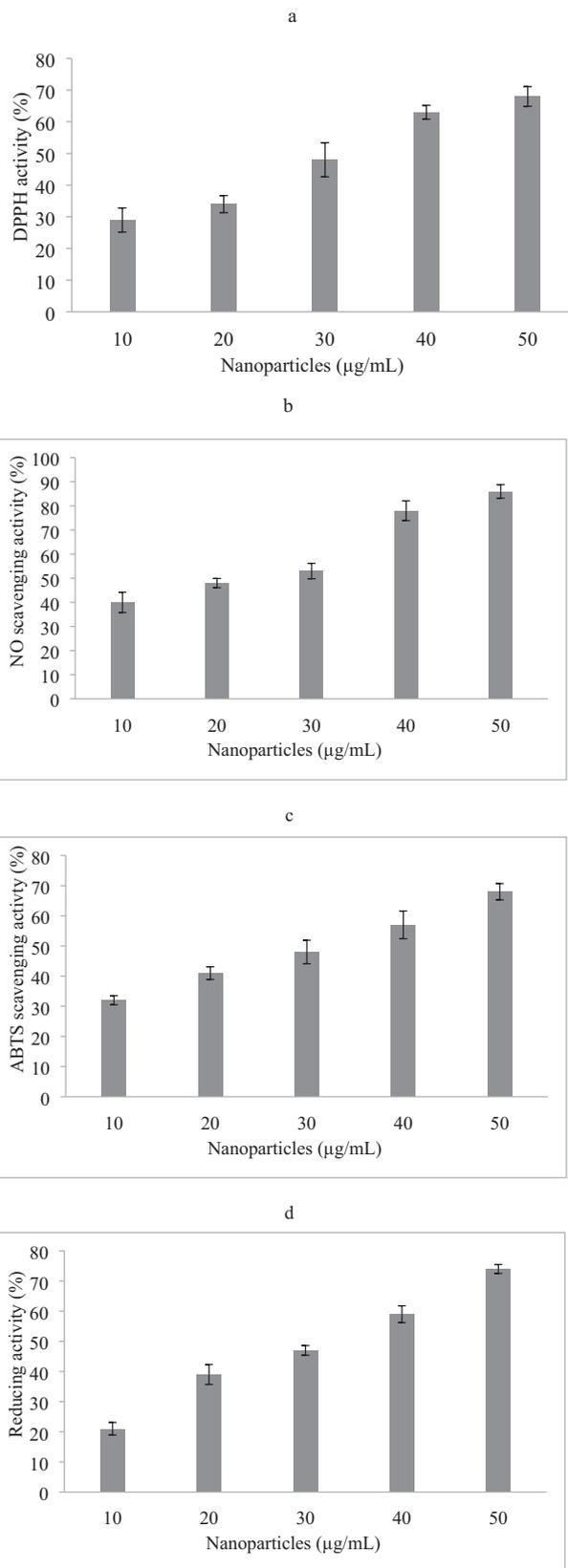
**Table 2**  
Antibacterial activity of green synthesized silver nanoparticles.

Bacteria	Zone of inhibition (mm)	
	Rifampicin	AgNO <sub>3</sub> + Rifampicin
<i>P. aeruginosa</i> ATCC 27853	18 ± 2	21 ± 2
<i>E. coli</i> ATCC 25922	13 ± 1	12 ± 1
<i>E. faecalis</i> 700802	15 ± 2	17 ± 3
<i>S. gordonii</i> ATCC 49818	17 ± 1	19 ± 2
<i>S. aureus</i> (MRSA) ATCC 4330	19 ± 3	21 ± 1
<i>S. aureus</i> ATCC 25923	21 ± 1	24 ± 2

was used as the reducing agent for the green synthesis of nanoparticles. Unlike chemical methods, plant based green synthesis of nanoparticles is economical and eco-friendly. Also, nanoparticles synthesized using plant sources have been highly stable than fungi and microbes (Gomathi et al., 2020).

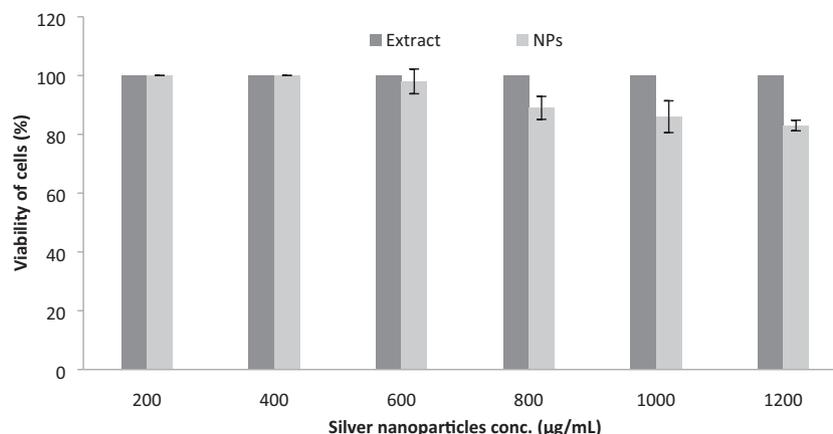
Phytochemicals are widely used to prepare silver nanoparticles and it generally requires current up to date knowledge on the pharmacological properties (Valsalam et al., 2019). The leaf extract of *A. indica* has been used previously for the biosynthesis of nanoparticles and spherical shaped nanoparticles were observed. The phytochemical components involved in reducing and capping nanoparticles. Green synthesized nanoparticles have been showed antimicrobial activity against various drug resistant bacterial pathogens (Ahmed et al., 2016). The unrestricted use of commercial antibiotics induced the development of drug resistance among bacteria. This drug resistance may be the result of defence mechanism by bacteria due to horizontal gene transfer. The enzymes synthesized by the bacteria degrade or inactivate the administered drugs (Poole, 2002). Silver is widely used as antimicrobial agents and the synthesized nanoparticles were effective against various diseases. In this study, the green synthesized nanoparticles were effective against *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *E. faecalis* 700802, *S. gordonii* ATCC 49818, *S. aureus* (MRSA) ATCC 4330 and *S. aureus* ATCC 25923. Likewise, antibiotics incorporated with nanoparticles improved synergistic activity. The pattern of antimicrobial activity varied from previous results indicated varying shape, size and strains used for antimicrobial testing. Rifampicin was used to test the synergistic activity with silver nanoparticles and results revealed improved zone of inhibition (18 ± 2 mm against *P. aeruginosa* ATCC 27853) and silver nanoparticle treated antibiotic increased zone of inhibition (21 ± 2 mm). The nanoparticles showed DPPH scavenging, reducing power, ABTS radical scavenging, and nitric oxide scavenging activities. The antioxidant potential of nanoparticles has been previously described (Thomas et al., 2019).

The green synthesized silver nanoparticles have been showed antibacterial activity against multi-drug bacterial strains (Mani et al., 2021). Likewise, the green synthesized zinc oxide nanoparticles prepared using *Tabernaemontana divaricata* showed improved antimicrobial potential (Raja et al., 2018). Anand et al. (2020) have



**Fig. 4.** Antioxidant activity of nanoparticles (A) DPPH radical scavenging activity, (b) Nitric oxide scavenging activity; (c) ABTS radical scavenging activity, (d) Reducing power activity.

been characterized nickel oxide nanoparticles and reported antimicrobial activities against drug resistant bacterial strains. Silver



**Fig. 5.** Biocompatibility analysis of pod extract and green synthesized nanoparticles on L929 fibroblast cell lines. Pod extract was considered as control. Error bar indicates standard deviation.

nanoparticles have been synthesized using *Moringa oleifera* flower (Bindhu et al., 2020), rizhome extract of *Curcuma longa* and *Zingiber officinale* (Venkatadri et al., 2020), floral extract of *Chrysanthemum indicum* L. (Arokiyaraj et al., 2015), *M. oleifera* (Surendra et al., 2016), *Acorus calamus* extract (Arasu et al., 2019), and these nanoparticles showed antioxidant, antimicrobial and dye degrading properties. *Prunus dulcis* (Almond Gum) extract has been used for the green synthesis of ZnO nanoparticles and showed potential activity against antibiotic resistant bacteria (Anand et al., 2019). NiO nanoparticles green synthesized using *Aegle marmelos* leaf extract have been showed antibacterial activity (Ezhilarasi et al., 2018). The green synthesized nanoparticles showed less toxicity to cell lines. In this study, we analyzed the biological properties of nanoparticles in human fibroblast cell lines (L929). The cell viability was not declined significantly than control cell lines.

## 5. Conclusions

The green synthesized silver nanoparticles showed potent activity against the foodborne bacterial pathogens. Nanoparticles have the potential to kill both Gram-negative and Gram-positive bacteria. Hence, the green synthesized nanoparticles could be formulated as novel antibacterial agent against both multi-drug resistant Gram-positive and Gram-negative bacteria. Silver nanoparticles may lead to develop various products including food packing materials, coating in medical devices and to develop antimicrobial drugs.

## Conflict of interest

We have no conflict of interest for publishing this paper.

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