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GREEN SYNTHESIS OF SILVER NANOPARTICLES USING LEAF EXTRACTS OF *MENTHA PIPERITA* AND ITS ANTIBACTERIAL ACTIVITY

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ABSTRACT

The emergence of antibiotic resistance bacteria represents a serious threat to human beings. Antibiotic resistance phenomenon can be attributed to the wide use of antibiotic. Moreover, multidrug resistant bacteria (MDR) are more difficult to be treated and are responsible for long-term hospitalization. In addition, MDR-infected patients have to be administered broad-spectrum antibiotics, which have low efficacy, toxic and expensive. Introducing of nanotechnology and particularly silver nanoparticles to fight MDR bacteria is the focus of the current study. In this study, bio-synthesis of Ag-NPs was attempted using plant extracts of *Mentha piperita*. Characterization of Ag-NPs was examined by UV-visible spectrophotometer, TEM, SEM and EDS. Monodispersed Ag-NPs were obtained with different sizes ranged from 6 to 44 nm. Antibacterial activities of Ag-NPs against seven pathogenic bacteria were evaluated using disc diffusion method. Our results indicated that Gram positive bacteria were more susceptible than Gram negative. Minimum inhibitory concentration (MIC) values of biosynthesized Ag-NPs were measured for each bacterium. The average MIC value of Ag-NPs against Gram positive bacteria was (4.5 µg/ml) and (5.85 µg/ml) against Gram negative bacteria. The current study offers a cost-effective and eco-friendly method for biosynthesis of potent bactericidal Ag-NPs and their use against human pathogenic bacteria.

Keywords: Green synthesis, *Mentha piperita*, Silver nanoparticles, Characterization, Antimicrobial activity

INTRODUCTION

The spread and emergence of Multi-Drug Resistance (MDR) Pathogenic Bacteria become a great danger and increases day by day and represents a serious threat to human beings. Therefore the search and looking for new methods to overcome such danger as well as these big problems is of interest. The use of particles in the nanoscale becomes a very important and advanced approach in the nanotechnology. One of these most common nanoparticles is the silver. It has been known that silver and its compounds have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities for bacteria, fungi, and virus since ancient times (1). The using of silver nanoparticles (AgNPs) as an antimicrobial agent has been widely recorded and large number of significant studies have been published recently (2).

Antibacterial activities of Ag-NPs have received much attention due to their effective killing and cost-effectiveness. Biosynthesis is an attractive and eco-friendly method to produce silver nanoparticles (Ag-NPs). Ag-NPs are considered a promising tool to overcome the emergence of multidrug resistant bacteria (MDR).The antimicrobial potential of silver was recorded since ancient times. It was used for treatment of burns, chronic wounds and to make water potable (3, 4). In the end of the seventeenth century, silver nitrate was used for the treatment of genital diseases, salivary glands fistulae and abscesses (5).Silver ions were found to be effective against more than 12 species of bacteria including *E. coli* (6). Ag-nanoparticles are widely used in different formats. They are involved in fabrication of medical devices to inhibit bacterial colonization of prostheses, catheters, vascular grafts, dental materials and stainless steel materials (7). In addition, Ag-nanoparticles are effective in protecting water filters, air sanitizer sprays, pillows, respirators, socks, wet wipes, detergents, soaps and many other consumer products (8). In the last decade, formulation of silver at the nanolevel was found to be more effective against many pathogenic bacteria. This approach raised as an alternative approach to antibiotics to fight Multidrug Resistant Bacteria (MDR) which threat the life of individuals (9). Moreover, Ag-nanoparticles have wide-spectrum activities, cost effective and lower tendency to induce microbial resistance (10). Multidrug resistant bacteria (MDR) continue to threat human beings. Infections caused by MDR bacteria lead to long periods of hospitalization with low chance of treatment (1).

A variety of techniques were described for preparation of metallic nanoparticles. These techniques involve chemical, physical and biological methods. For example, reduction by chemical reducing agents (organic and inorganic) (11), microwave-assisted dielectric heating reduction, gamma irradiation, thermal decomposition of silver oxalate in water and in ethylene glycol, and photochemical methods (12). Due to the fact that chemical methods are expensive, time consuming, adversely affect the ecosystem and require specialized instruments (13). An alternative approach that overcomes limitations of the chemical approach is the biological or green synthesis technique. The principle of the biological approach is to exploit natural compounds for reducing, capping, and stabilizing silver nanoparticles. This technique is less time consuming, cost-effective, and more ecofriendly (13, 14).

A variety of sources can serve as biological agents for Ag-nanoparticles synthesis. These agents could be enzymes/proteins, amino acids, polysaccharides and vitamins. In addition, microorganisms such as bacteria and fungi were successfully employed in bio-reduction of silver nitrate. The most powerful bio-reducing agent is the use of plant extract as it contains phytochemicals. These phytochemicals involve a wide array of biomolecules (terpenoids, flavones, ketones, aldehydes, amides, carboxylic acids and quinones) that mediate the reduction process (15). Several literatures reported the use of different plant extracts such as *Emblica officinalis* (14), *Parthenium* (16), *Aloe vera* (17), *Pisonia grandis* (18), *Jatropha curcas* (19), *Justicia genderussa* (20).

Properties of synthesized Ag-nanoparticles can be characterized by UV-Visible spectroscopy, Photoluminescence studies, FT-IR (fourier transform infrared) spectroscopy, XRD (X- ray diffraction), SEM (scanning electron microscopy), EDX (energy dispersive X-ray spectroscopy), TEM (transmission electron microscopy) and AAS (atomic absorption spectrophotometer) analyses. In this study, the plant extract of *Mentha piperita* was used as bio-reducing agents to biosynthesis the Ag-nanoparticles. This plant was used because of its availability and eases of extraction as well as it a well-known plant at our Arab Countries. The bio-synthesized Ag-NPs were analyzed by UV-visible spectrophotometer, TEM and SEM. On the other hand, antibacterial activity of Ag-NPs was tested against seven human pathogenic bacteria. Assays involved determination of MIC and MBC for each bacterium.

MATERIALS AND METHODS

2.1. Materials

Fresh leaves of *Mentha piperita* (family: *Lamiaceae*) was collected from available commercial sources. Seven bacterial strains; three represent Gram positive bacteria (*Bacillus cereus*, *Enterococcus faecalis* and*Staphylococcus aureus*) and four for Gram negative bacteria (*Escherichia coli*, *Salmonella typhi*, *Shigella sp.* and *Acinetobacter baumannii)*. Silver nitrate was purchased from Sigma-Aldrich (Chemie GmbH).

2.2. Preparation of Plant Extract

Fresh leaves of *Mentha piperita* were rinsed several times with distilled water to remove dust and other attached particles. 10 g of the plant's leaves were then cut into small pieces in 250 mL Erlenmeyer flasks. 100 ml of double distilled water was added to the flask and boiled for 15 minutes. After boiling has been finished, the flask was cooled down at room temperature and their contents dispensed into 50 ml falcon tubes. To remove large particles and cellular debris, tubes were centrifuged at 10,000 rpm for 30 minutes and supernatants were filtered via 0.2µm Millipore filter into new sterile 50ml tubes. Tubes were kept in the refrigerator for further use.

2.3. Biosynthesis of Silver Nanoparticles

Biosynthesis of Ag-NPs was attempted by mixing an aqueous solution of silver nitrate (1 mM) with pre-formed plant extracts. For quick and complete reduction, the mixture was irradiated using a microwave oven at low power 300 W for 5 minutes. The mixture was then left overnight at room temperature in dark condition. Change in color was observed which refer to Ag-NPs formation. The reaction was performed in dark conditions with two flasks as controls; the first contains aqueous solution of $AgNO_3$ and the second have only the plant extract. Reaction products containing biosynthesized Ag-NPs were centrifuged at low speed (3000 rpm/20 min) to remove unwanted particles. Supernatants were transferred into sterile tubes and centrifuged again at high speed (20,000 rpm/40 min). Pellets were then collected by reconstitution in 100 µl deionized water and air dried.

2.4. Characterization of Ag-nanoparticles

2.4.1. UV-visible spectrophotometry

To confirm the identity of biosynthesized Ag-NPs, 1 ml from each Ag-NPs suspensions was diluted with 2ml deionized water and scanned by UV-visible spectrophotometry (Beckman-Model No.

DU-50, Fullerton, CA, USA). Wavelengths of 300, 350, 400, 410, 4120, 430, 450, 500, 550, 600, and 700 nm in a spectrophotometer having a resolution of 1 nm. UV-vis spectra were recorded at intervals of 0 min, 15 min, 30 min, 45 min, 60 min and 24 h.

2.4.2. TEM and Energy Dispersive X-ray (EDS)

Energy-dispersive X-ray spectroscopy (EDS) is an analytical method for elemental analysis. Characterization of an element is based on the fact that each element has a unique atomic structure and hence unique set of peaks on its X-ray spectrum. A lyophilized form of Ag-NPs was analyzed by SEM and EDS using JEOL-MODEL JED-2200 series machine (Electron Microscopy Unit, Central Laboratory, King Saud University, Riyadh, Saudi Arabia). Thin films of the Ag-NPS were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid and then the films on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min. Size and morphology of Ag-NPs were examined using Transmission Electron Microscopy (JEM 1011) (Electron Microscopy Unit, Central Laboratory, King Saud University, Riyadh, Saudi Arabia).

2.5. Antibacterial Activities of Ag-NPs

Bacterial strains were streaked over nutrient agar plates and then transferred to nutrient broth medium to restore their physiological activities. To investigate the antibacterial activities of Ag-NPs, bacterial strains were spread as a lawn over Muller Hinton agar plates and allowed to dry for 15 minutes at room temperature. Ag-NPs-loaded disks with three different concentrations (50 µg/ml, 5 µg/ml and 0.5 µg/ml) were placed over the agar plates and the plates were kept for incubation at 37°C for 24 h. At the end of incubation, inhibition zones formed around the disc were measured. Disks soaked in sterilized distilled water were used as negative controls.

2.6. Determination of MIC value

The minimum inhibitory concentration (MIC) was determined using 96-well plate method. Briefly, bacterial strains were grown in liquid LB medium at 37°C and vigorous shaking for 9 hours. Then, bacterial strains were diluted in fresh LB liquid medium using 5×10^8 colony forming units (CFU)/ml. 100 µl of Ag-NP's solution was added to the first two rows and then a two-fold serial dilution was performed. Thus, gradient concentrations of Ag-NPs; 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.4 µg/ml were obtained. 100 µl of the diluted suspension of each bacterium was added to the assigned rows. The second two rows were left as negative control (i.e. without Ag-NPs) and the third row was left as blank. The plates were incubated at 37°C for 24 h. Plates were read by ELIZA reader at 595 nm wavelength and MICs were recorded as the lowest concentration of Ag-nanoparticles showing no visible growth in the broth.Samples from wells used in broth microdilution assay that did not exhibit any visible growth after the 24 h incubation was subculture onto MHA plates. The MBC was recorded as the lowest concentration of the Ag nanoparticles that did not allow a single colony to grow on the agar plate after 24 hr incubation**.**

RESULTS

3.1. Biosynthesis and Characterization of Ag-NPs

The formation of Ag-NPs was indicated from color change by mixing 1 mM of silver nitrate and *M. piperita*'s extract. Bioreduction is accelerated by microwave irradiation at low power. The color of the solution changed from pale green to yellowish brown to reddish brown and to colloidal brown indicating AgNPs precipitation (Figure 1). The UV-vis spectra of biosynthesized Ag-NPs were recorded after time intervals and the maximum absorption peak of Ag-NPs formed in this study was at 420 nm (Figure 2), a characteristic plasmon resonance of silver nanoparticles. Stable and monodispersed Ag-NPs were obtained as visualized by TEM with size ranged from 6 to 44 nm (Figure 4). EDS spectra recorded from the silver nanoparticles were shown in Figure 3. Silver nanoparticles have the weight percentage of silver as 81.8%.

3.2. Antibacterial Activities of Ag-nanoparticles

The antimicrobial activity was measured by the inhibition zone assay and the results were shown in Table 1. The clear zone was greater at concentration of 50 µg/ml and decreased gradually at 5 µg/ml and 0.5 µg/ml. The effect of biosynthesized Ag-NPs varied among tested bacteria (Figure 5B). However, the bactericidal effect was more effective against Gram positive bacteria than Gram negative (Table 1). Among Gram positive bacteria, inhibition was more pronounced against *S. aureus* where the diameter of inhibition zone was (15.8 mm). In a similar fashion, for Gram negative bacteria, *E. coli* recorded the largest size of inhibition zone; 5.7 mm (Figure 5 A).

3.3. MIC values

MIC values of different bacterial strains were measured by microtiter plate method. The plate was measured by ELIZA reader at 690 nm wavelength and results recorded as µg (Ag)/ml) (Table 1). Higher concentrations of Ag-nanoparticles completely inhibited bacterial growth. The effect gradually decreased with lower concentrations. The average MIC value of Ag-NPs against Gram positive bacteria was (4.5 µg/ml) and (5.85 µg/ml) against Gram negative bacteria (Table 1).

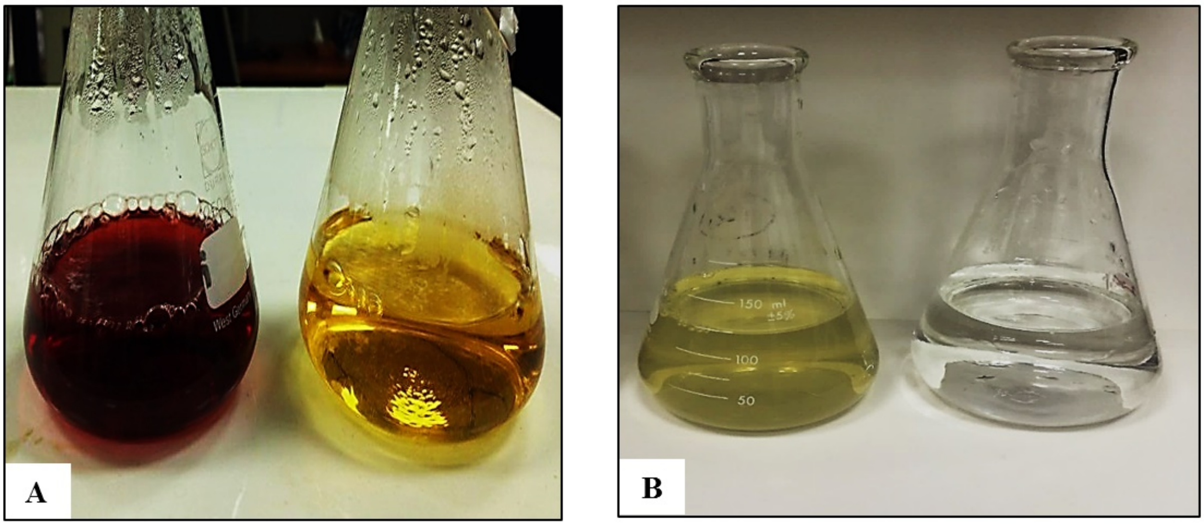

Figure 1. Preparation of plant extracts and biosynthesis of Ag-nanoparticles. Plant extract of *M. piperita* was prepared by boiling fresh leaves and then purified extract was mixed with 1 mM $AgNO_3$. Color change was observed 30 minutes after mixing (Panel A right flask). The bio-reduction process was mediated by microwave irradiation (Panel A left flask). $AgNO_3$ solution and plant extract was left as controls as shown in Panel B right and left flasks, respectively

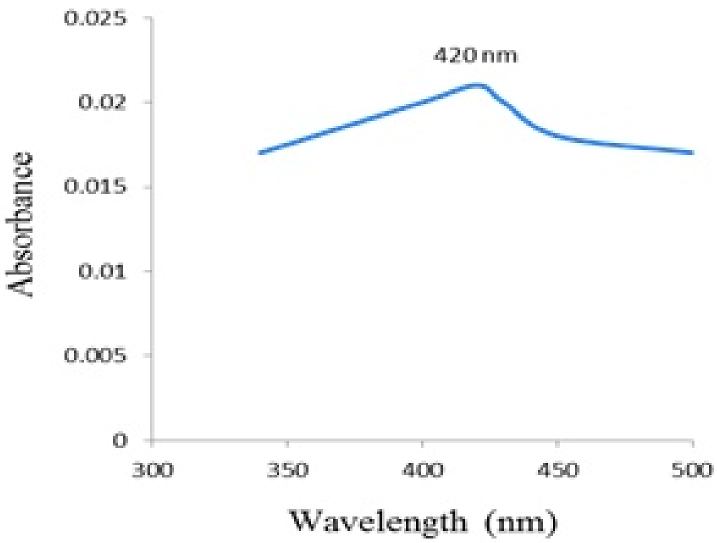

Figure 2: UV–vis absorption spectrum of Ag-NPs

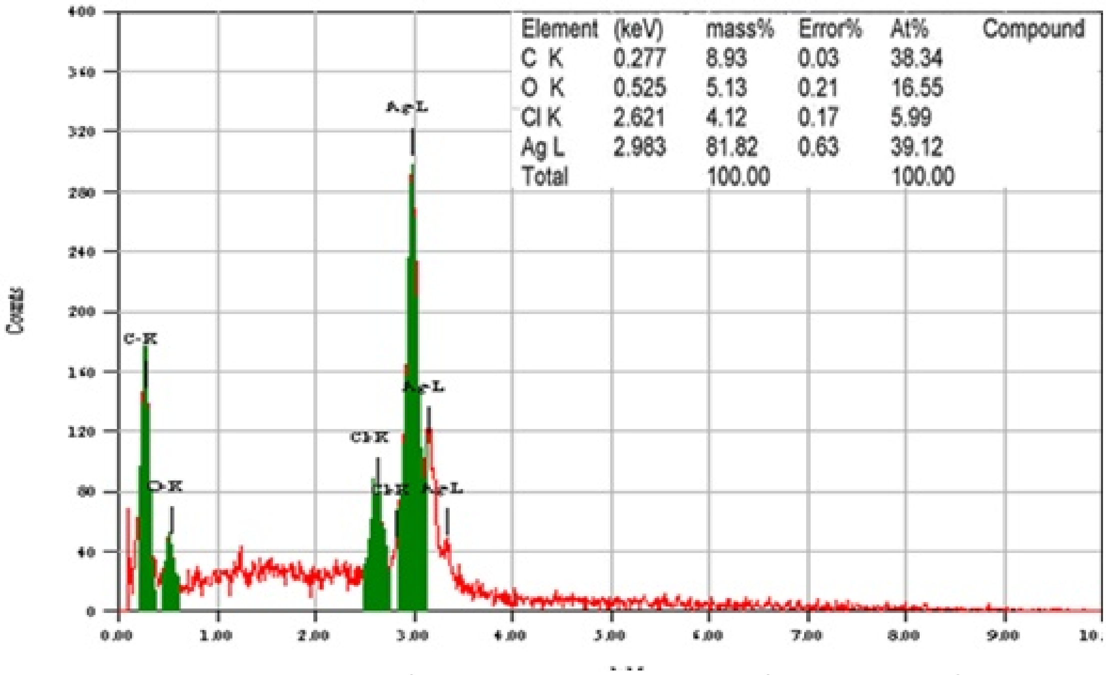

Figure 3: EDS characterization spectrum of Ag-NPs powder. Visible peaks confirm the presence of silver, oxygen and silicon dioxide substances in the tested sample.

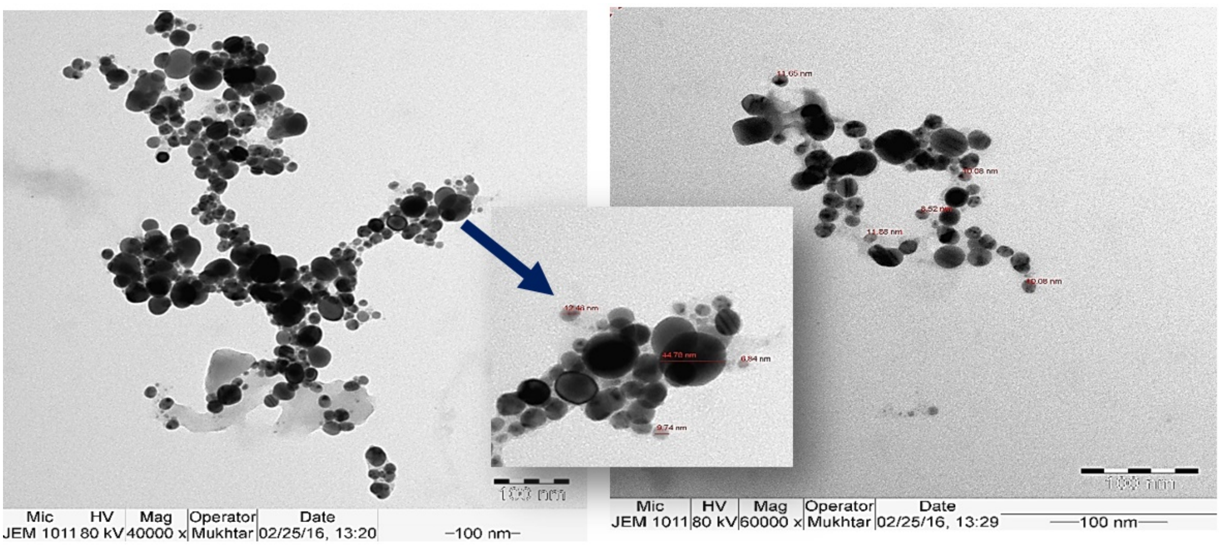

Figure 4: TEM images of Ag-NPs formed by extracts of *M. piperita* with size ranged from 6 to 44nm.

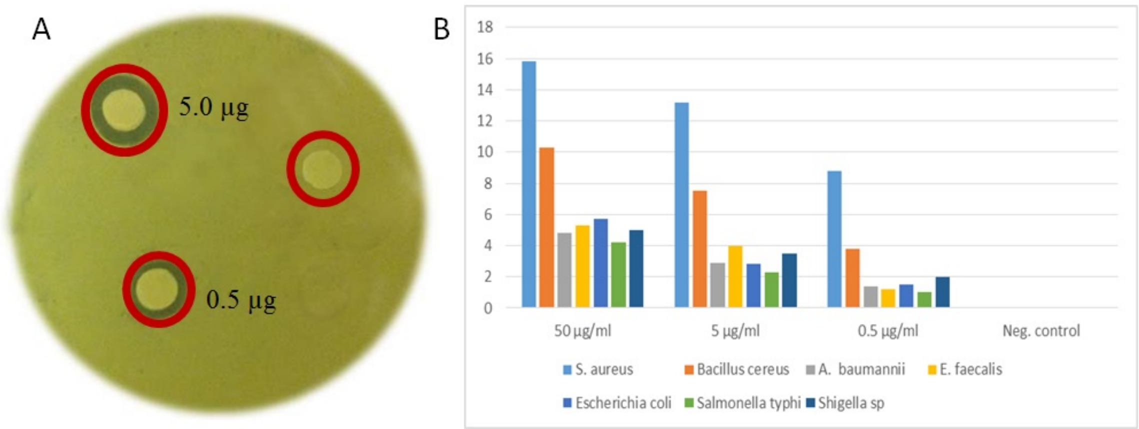

Figure 5: Ag-NPs antibacterial activities measured by disk diffusion method against *E. coli*; concentrations 50 µg and 0.5 µg (A) and the inhibition zone measured for all tested bacteria (B).

Table 1: Antibacterial activities of Ag-nanoparticles biosynthesized by extracts from *M. piperita*

Bacterial strains	Inhibition zone (mm diameter)				MIC Value µg (Ag)/l)
	50 µg/ml	5 µg/ml	0.5 µg/ml	Neg. Control	
Gram Positive strains					
Bacillus cereus	10.3	7.5	3.8	0.0	4.5
Staphylococcus aureus	15.8	13.2	8.8	0.0	5
Enterococcus faecalis	8.3	5.0	2.2	0.0	4
Gram Negative strains					
Acinetobacter baumannii	4.8	2.9	1.4	0.0	5.4
Salmonella typhi	4.2	2.3	1.0	0.0	6
Escherichia coli	5.7	2.8	1.5	0.0	5.5
Shigella sp	5.0	3.5	2.0	0.0	6.5

DISCUSSION

The use of plant extracts to synthesis Ag-NPs was reported in several literatures. For example, extracts from *Clitoria ternatea* and *Solanum nigrum* (21), *Terminalia arjuna* (22) as well as *Musa balbisiana*, *Azadirachta indica* and *Ocimum tenuiflorum* (23). In this study, plant extracts from *Mentha piperita* was used to reduce silver nitrate with the help of microwave irradiation (Figure 1). We used *Mentha piperita* because of its availability and ease of extraction. In *in vitro* reaction, mixing diluted aqueous solution of silver nitrate with plant extract is associated with color change and Ag-NPs precipitation. Jha et al. (24) reported that; plant extract is rich with carbohydrates, proteins, nucleic acids and other molecules which act as strong bioreducing agents.

The morphology and resonance properties of Ag-NPs were examined by TEM, SEM and UV-visible spectrophotometer. UV- spectra due to surface plasmon resonance of Ag-NPs reported in previous studies displayed different peak values*; E. coli* (400 nm) **(**25), *Allium cepa* (412 nm) **(**25), *Kappaphycus alvarezii* (420 nm) **(**26), *Pisonia grandis* (420 nm) **(**27), *Aspergillus niger* (420 nm) **(**28), *Citrullus colocynthis* (440 nm) **(**29) and *Merrimia tridendata* (440 nm) **(**30). Ag-NPs synthesized in our study have resonance peak values of 420 nm *Mentha piperita* (Figure 2). For further characterization, EDS spectra were obtained by analyzing the energy and intensity distribution of X-ray signals produced by an electron beam on Ag-NPS (Figure 4). EDS spectra recorded from the Ag-NPs have the weight percentage of silver as 81.8%. Similar result was reported previously **(**31). Ag-NPs obtained in this study are uniform spherical with size ranged from 6 to 44 nm (Figure 3). Other investigators synthesized different sizes of Ag-NPs; 30-40 nm by *Boswellia ovalifoliolata* **(**32); 30-50nm by *Emblica officinalis* **(**33), *Carcia papaya* **(**17) and *Merremia tridendata* **(**30).

Recently, silver is introduced in the field of clinical applications to fight pathogenic bacteria. They are effective against a wide array of Gram positive and Gram negative bacteria. For example, Ag-NPs were of great killing potential against *E. coli* **(**34), *Pseudomonas aeruginosa*, *Strepococcus pyogenes*, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) **(**35), *Vibrio cholerae*, *Pseudomonas aeruginosa* and *Salmonella typhus* **(**36), *Bacillus subtilis* and *Staphylococcus epidermidis* **(**37) *Enterococcus faecalis* and *Streptococcus mutans* **(**38). In addition, incorporation of Ag-NPs in cotton fabrics had efficient and strong bactericidal potential. Another interesting application of Ag-NPs is to protect wounds and burns through what wound dressing. In this format, polyvinyl alcohol nanofibres are impregnated with silver nanoparticles have bactericidal activities against *E. coli* and *Staph. aureus* **(**39). Other studies investigate the synergistic effect of silver nanoparticles and antibiotics **(**40).

The antibacterial potential of Ag-NPs was assayed using disc diffusion method. Ag-NPs exhibited antibacterial activity against *B. cereus*, *S. aureus*, *E. faecalis*, *S. typhi*, *Shigella sp*, *E. coli*, and *A. baumannii* (Table 1, Figure 5). Similar antibacterial activity of Ag-NPs was reported against

Proteus morgani and *Staphylococcus aureus* (41); *Samonella enteritis, Pseudomonas aeruginosa, E. coli,*and *Streptococcus pyrogens,* (29); *Staphylococcus aureus,Bacillus subtilis, E. coli* and *Klebsiella pneumonia* (42), *Samonella typhi* and *E. coli* (43); *E. coli, Bacillus cereus* and *Pseudomonas aeruginosa* (44) and *Vibrio cholera, Proteus vulgaris* (45).

Although the bactericidal activities of Ag-NPs vary among bacterial strains, the effect was greater against Gram positive than Gram negative bacteria (Table 1, Figure 5). This is due to the fact that more Ag-NPs accumulate over cell walls of Gram positive bacteria of metals than that of Gram negative bacteria (46). Another factor that plays a major bactericidal role is the size of Ag-NPs where small size Ag-NPs is more effective than larger one. To investigate the relation between the size of Ag-NPs and their bactericidal activities, MIC values were measured using micro-dilution method. The average MIC value of Ag-NPs against Gram positive bacteria was (4.5 µg/ml) and (5.85 µg/ml) against Gram negative bacteria (Table 1). Our results are similar to that obtained by other investigators (47).

Although, the exact mechanisms of Ag-NPs action is not clearly defined. However, several hypotheses were proposed to reveal their bactericidal mechanisms. Of these, Gogoi *et al.*, (47) proposed that Ag-NPs have no direct effect on cellular DNA and proteins. Adherence of Ag-NPs to bacterial cell wall results in the formation of pits which in turn lead to permeability loss and cell death (4). Free radicals generation and release of silver ions by Ag-NPs are possible mechanisms that may lead to cell death (47). The bactericidal mechanism of Ag-nanoparticles can be attributed to several effects. It was suggested that Ag-NPs affect cell membrane permeability through the formation membrane pores, inactivating bacterial enzymes by the interaction of silver ions with the thiol groups of several vital enzymes, damage of cellular DNA and inhibition of signal transduction (47).

Though the mode of action of Ag-NPs on the bacteria is still unknown, it's possible mechanism of action has been suggested according to the morphological and structural changes in the bacterial cells (35). Many mechanisms have been suggested to explain the mode of bactericidal action of silver ions previously. They are known to inhibit proteins by binding to their thiol groups and denaturing them (48) and prevent replication of DNA by its condensation (48). Consequently, the nanoparticles preferably attack the respiratory chain, cell division finally leading to cell death (35). In addition, Sureshbabu *et al*., (49) reported a new mechanism for the antimicrobial effect of the Ag-NPs, which is Programmed Cell Death (Apoptosis).

CONCLUSION

So, we can conclude that the green synthesis of *M.piperita*-Ag-nanoparticles had a strong cidal effect on the pathogenic Gram positive and Gram negative bacterial cell types and then we can use to fight the MDR bacterial cells.

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