BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, March 2016. Vol. **13**(1), 95-101

Silver Nanoweapons: A novel Tool against Multidrug Resistant Bacteria

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http://dx.doi.org/10.13005/bbra/2009

(Received: 13 March 2015; accepted: 30 March 2015)

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 multi-drug resistant bacteria. In this study, bio-synthesis of Ag-NPs was attempted using plant extracts of peppermint. Characterization of Ag-NPs was achieved by UV-visible spectrophotometer and TEM. Monodispersed Ag-NPs were obtained with different sizes ranged from 6 to 88 nm. Antibacterial activities of Ag-NPs against pathogenic bacteria were evaluated using disc diffusion method. Our results indicated that Gram positive bacteria were more susceptible than Gram negative. 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: Silver nanoparticles, Pathogenic bacteria, Green synthesis, Silver nanoweapons.

Multidrug resistant (MDR) bacteria continue to threat human beings. Infections caused by MDR bacteria lead to long periods of hospitalization with low chance of treatment[1]. Widely use of antibiotics forces bacteria to resist antibiotics via several mechanisms[2]. Therefore, searching for alternative approaches other than antibiotics is a focus of research[3]. Nanoparticles (NPs) particularly silver nanoparticles (Ag-NPs) open new avenues to develop novel antibacterial drugs[4]. Several reports investigated the use Ag-NPs against a wide array of bacteria. For example, low concentrations of Ag-NPs were found to be more effective against *E. coli* [5], *S. aureus*[4, 6], *Pseudomonas aeruginosa*[7], *Vibrio cholerae*[8] and *Bacillus subtilis*[9]. Moreover, Ag-NPs were reported to be effective against ampicillin-resistant *Escherichia coli*, erythromycin-resistant *Strepococcus pyogenes*, methicillin- resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Staphylococcus aureus* (VRSA)[5, 10-12]. Ag-NPs also have antimicrobial effects against fungi[13, 14] and viruses[15].

The size and morphology of Ag-NPs are two critical issues that determine the efficiency of antibacterial activity[16, 17]. The mechanism of Ag-NPs action is not clearly defined, however, several mechanisms were proposed. Adsorption of Ag-NPs by bacterial cells leads to loss of membrane permeability and toxicity[16, 18]. Ag-NPs were also proposed to exert their activity via reducing DNA replication and interfere with ribosomes[19]. Silver-containing compounds such as silver nitrates serve as a good source to generate Ag-NPs by reductions assays. For this purpose, several chemical, physical and biological methods were described[20, 21]. However, the use of biology-based methods is low cost, safe and ecofriendly[22, 23]. Of these biological sources, plant extracts are rich with bioreducing agents such as terpenoids, amides, carboxylic acids, proteins, DNA, enzymes, etc[24]. In

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the current study, biosynthesis of Ag-nanoparticles was achieved by reduction of silver nitrate. Plant extract was used as bio-reducing agent. Peppermint plant was selected for the reduction process on the base of their availability and ease of extraction. Synthesized Ag-NPs were analyzed by UV-visible spectrophotometer, TEM and SEM. In addition, bactericidal activity of Ag-NPs was tested against two common human pathogenic bacteria from both the Gram-Positive and Gram-Negative bacteria.

MATERIALS AND METHODS

Preparation of Plant Extract

Plant parts of peppermint were rinsed several times with distilled water to remove dusts. 10 g of the plant were cut into small pieces in two separated 250 mL Erlenmeyer flasks. 100 ml of distilled water was added to each flask and boiled for 15 minutes. After boiling has been finished, flasks were 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.

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. 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.

Characterization of Ag-nanoparticles

To confirm the identity of biosynthesized Ag-NPs, dilutions from each Ag-NPs suspension were prepared and scanned by UV-visible spectrophotometry (Lambda 35 UV-VIS spectrophotometer) at different wavelengths (300-720 nm). Absorbencies corresponding to each wavelength were recorded. For further characterization, biosynthesized Ag-NPs were sent to Electron Microscopy Unit, Central Laboratory, King Saud University, Riyadh, KSA. Ag-NPs were also analyzed by scanning and transmission electron microscopy to examine their size and shape. The particle size distribution of Ag-NPs was evaluated using Image J 1.45s software.

Antibacterial Activities of Ag-NPs

To investigate the antibacterial activities of Ag-NPs, a representative one Gram-Positive and one Gram-Negative bacterial strains were spread as a lawn over Muller Hinton Agar plates and allowed to dry 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.

RESULTS

Biosynthesis of Ag-NPs

Biosynthesis of Ag-NPs was attempted by mixing an aqueous solution of $AgNO_3$ and extracts of peppermint. Color turned to yellow as compared with the original color (pale green) (Figure 1A). After 1h the color turned to brownish (Figure 1B). Change in color indicating that the bioreduction process occurred and formation of Ag-NPs.

Characterization of Ag-NPs

Ag-NPs biosynthesized by peppermint extract was characterized by UV-visible spectrophotometry (Figure 2), TEM (Figure 3) and SEM (Figure 4). The sizes of biosynthesized Ag-NPs are in the range of 6 to 88 nm. As shown in (Figures 3, 4), the absorbance peak of Ag-NPs is at 420 nm wavelength which is characteristic for silver nanoparticles.

Antimicrobial Activities of Ag-nanoparticles

Inhibition zones created by Ag-NPs-loaded disks were measured and recorded (Table 1). The inhibition effect was greater at concentration of 50 µg/ml and decreased gradually at 5 µg/ml and 0.5 µg/ml. The bactericidal effect was more effective against Gram positive bacteria

than Gram negative (Table 1). The inhibition zone for Gram negative bacteria *E. coli* recorded the largest size of inhibition zone; 5.7 mm with 50 µg/ml concentration of silver nanoparticles, however, in case of *B. cereus* the inhibition zone was 10.3 mm at the same concentration.

DISCUSSION

Historically, silver vessels have long been used to preserve foods and drinks. In recent years, silver is introduced in the field of clinical applications to fight pathogenic bacteria. Although silver metal is able to inhibit microbial growth, its nano-form is more effective. Several chemical, physiological and biological methods were reported to synthesize Ag-NPs. Of these methods, biosynthesis of Ag-NPs using biological techniques is the most common methods. Biological methods secure safe, cost effective, ecofriendly and easy ways of Ag-NPs formation[25]. Extracts from plants, bacteria and fungi can be used to biosynthesize Ag-NPs by reducing silver salts[26, 27].

Several literatures reported the use of plant extracts in Ag-NPs synthesis. For example, extracts from *Musa balbisiana*, *Azadirachta indica* and *Ocimum tenuiflorum*[28], *Terminalia arjuna*[29] and *Clitoria ternatea* and *Solanum nigrum*[30]. In the current study Ag-NPs of peppermint was synthesized (Figure 1). Selection of this plant is based on its permanent availability and ease of extraction. Plant extract is rich with carbohydrates, proteins, nucleic acids, and other molecules which act as strong bioreducing agents[24]. In *in vitro* reaction, mixing aqueous solution of silver nitrate with plant extract is associated with color change and Ag-NPs precipitation.

Table 1. Antibacterial activities of Ag-nanoparticles synthesized from peppermint

Bacterial strains	Type	Inhibition zone (mm diameter)		
		50 µg/ml	5 µg/ml	0.5 µg/ml
Bacillus cereus	Gram-positive bacteria	10.3	7.5	3.8
Escherichia coli	Gram-negative bacteria	5.7	2.8	1.5

Fig. 1. Biosynthesis of Ag-NPs. A) Preparation of plant extract. B) Color change after 1h. C) Controls: right flask, peppermint's extract and left flask, aqueous solution of Ag-NPs.

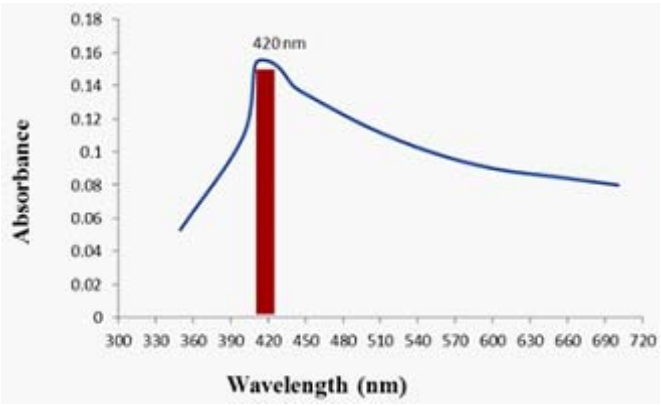

Fig. 2. UV–vis absorption spectrum of Ag-NPs

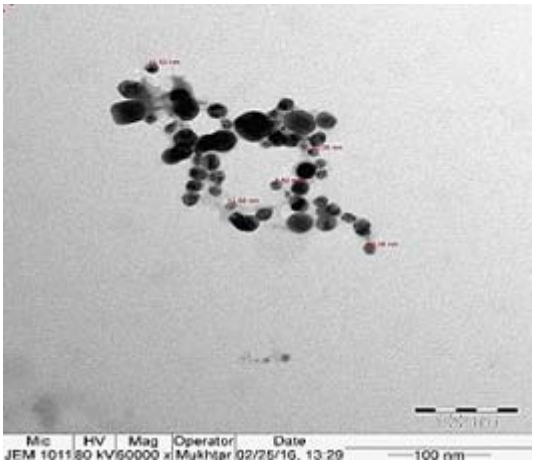

Fig. 3. TEM image of Ag-NPs formed by extract of peppermint

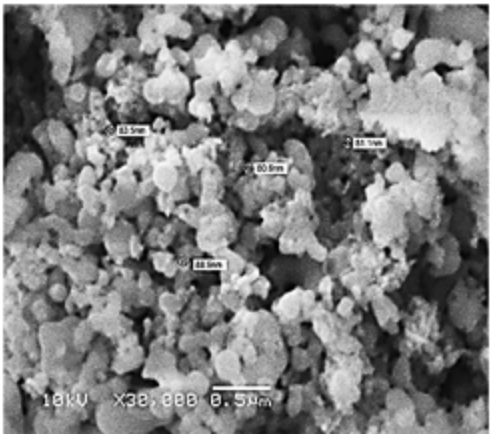

Fig. 4. SEM image of Ag-NPs synthesized by extract of peppermint

The morphology and resonance properties of Ag-NPs were examined by SEM and UV-visible spectrophotometer, respectively. The size, distribution and stability of Ag-NPs vary with the plant extract or the organism used for the biosynthesis process. Surface plasmon resonance of Ag-NPs reported in previous studies displayed different peak values*; E. coli* (400 nm)[31], *Aspergillus niger* (420 nm)[32], *Pisonia grandis* (420 nm)[33], *Merrimia tridendata* (440 nm)[34], *Kappaphycus alvarezii* (420 nm)[35], *Citrullus colocynthis* (440 nm)[36], *Allium cepa* (412 nm)[37]. Ag-NPs synthesized in our study have resonance peak values of 420 nm for peppermint (Figure 2). Ag-NPs obtained in this study are uniform spherical with size ranged from 6 to 88 nm (Figures 3 and 4). Other investigators synthesized different sizes of Ag-NPs; 30-40 nm by *Boswellia ovalifoliolata*[38]; 30-50nm by *Merremia tridendata*[34], *Carcia papaya*[39], and *Emblica officinalis*[40].

The antibacterial potential of Ag-NPs was assayed using disc diffusion method. Ag-NPs exhibited antibacterial activity against *B. cereus* and *E. coli* (Table 1). Similar antibacterial activity of Ag-NPs was reported against *E. coli, Bacillus cereus* and *Pseudomonas aeruginosa*[41]; *Pseudomonas aeruginosa, E. coli, Streptococcus pyrogens, Samonella enteritis*[36]; *Proteus vulgaris, Vibrio cholera*[42]; *Staphylococcus aureus, Bacillus subtilis, E. coli* and *Klebsiella pneumonia*[43], *Samonella typhi* and *E. coli*[44]; *Proteus morgani* and *Staphylococcus aureus*[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). 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.

The exact mechanism of Ag-NPs action is not clearly defined. However, several hypotheses were proposed to reveal their bactericidal mechanisms. Of these, Gogoi *et al*, proposed that Ag-NPs have no direct effect on cellular DNA and proteins[47]. 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[6, 48].

ACKNOWLEDGEMENT

Hussam H. Arafat, Mohammed H. El-Sayed and Askora A., are supported with the research grant No (5/1436/17/8), Deanship of Scientific Research, Northern Border University, Arar, KSA.

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