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Antibacterial activity of silver nanoparticles produced by *Plantago ovata* seed extract against antibiotic resistant *Klebsiella pneumoniae*

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Abstract

The synthesis of nanoparticles has become the matter of great interest in recent years due to its various advantageous properties and applications in various fields. Though physical and chemical methods are more popular for nanoparticle synthesis, the biogenic production is a better option due to eco-friendliness.The purpose of this study is to synthesis of silver nanoparticles by using green method on extract from *Plantago ovata* and determine its potential antibacterial effects against antibiotic resistant *Klebsiella pneumoniae* isolates.A total of 30 *K.pneumoniae* strains were isolated from urine cultures of hospitalized patients suffering from urinary tract infections in three hospitals in Zahedan during the years 2011- 2012. Isolated bacteria were identified by Gram's stain and standard biochemical tests. The susceptibility of used antibiotics was carried out using standard disc diffusion method. The seeds of *Plantago ovata* were used for silver nanoparticle sunthesis. UV–vis spectral and Transmission Electron Microscopy analysis were used in order to confirm the formation of silver nanoparticles. The broth micro-dilution method was used to determine MIC of silver nanoparticles. The antibiotic resistance profile of K. *pneumoniae* isolates was as follow: Penicillin (93.3%), Erythromycin and Ampicillin (76.6%), Tetracycline and Cefixime (53.3%), Ceftazidime (40%) and Nalidixic acid (36.6%). The highest and the least MIC of *P. ovate* seed extract values were found to be 200 and 12.5 ppm respectively. The present study concludes that at a specific dose, chitosan-based AgNPs kill bacteria without harming the host cells, thus representing a potential template for the design of antibacterial agents to decrease bacterial colonization and to overcome the problem of drug resistance.

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Introduction

Human beings are often infected by micro-organisms such as bacteria, molds, yeasts, and viruses present in their living environments. Because of the emergence and increase in the number of multiple antibiotic-resistant bacteria and the continuing emphasis on health-care costs, many scientists have researched methods to develop new effective antimicrobial agents that overcome the resistances of these organisms. The silver metal has a great toxicity against a wide range of micro-organisms, particularly gram negative bacteria. Silver nanoparticles (Ag-NPs) are found to be effective as anti-inflammatory, anti-angiogenesis, anti-platelet activity and against cancer cells which makes them vital (Sotiriou, 2010). Besides that, Ag-NPs were also being reported in the literature to exhibit a strong cyto-protective activity towards human immunodeficiency virus (HIV) infections (Sun *et al*., 2005). Considering the well-documented crucial importance of the transmembrane proton gradient in overall microbial metabolism, it seems inevitable that the elimination of proton motive force should result in cell death (Dibrov *et al*., 2002). Ag+ also forms complexes with bases contained in DNA and is a potent inhibitor of fungal DNases (Ahearn *et al*., 1995; Ghandour *et al*., 1988).

However, green synthesis approaches of producing Ag NPs are an alternative source of conventional method and possess excellent antimicrobial activity (Sharma *et al*., 2009). Researchers showed that *Plantago ovata* have hypo-cholesterolemic (Salas-Salvado *et al*., 2007), anti-diarrheal (Washington *et al*., 1999), anti-diabetic (Hannan *et al*., 2006) and low anti-oxidant (Souri *et al*., 2008) effects.

The aim of present study was synthesis of silver nano-particles by using green method on seed extract from *Plantago ovata* and determination of its potential antibacterial effects against antibiotic resistant *Klebsiella pneumoniae.*

Material and methods

Isolation of bacteria

A total of 30 *K.pneumoniae* strains were isolated from urine cultures of hospitalized patients suffering from urinary tract infections in three hospitals in Zahedan (south-eastern Iran) during 2011- 2012. Isolated bacteria were identified by Gram's stain and standard biochemical tests (Forbes *et al*., 2007).

Agar disk diffusion assay

The susceptibility of all antibiotics was carried out using standard disc diffusion method on Mueller-Hinton agar as recommended by CLSI. Briefly, *K.pneumoniae* isolated plates were grown overnight on blood agar, and colony suspension was prepared using the sterile saline water equivalent to a 0.5 McFarland standard. Suspension (10 µl) was spread over the Mueller-Hinton plates and antibiotic discs were transferred aseptically on the surface of inoculated media plates. The antibiotics and their potencies were as follow: Ceftazidime (30 µg), Tetracycline (30 µg), Erythromycin (15 µg), Cefixime (30 µg), Penicillin (10 µg), Ampicillin (25 µg), Nalidixic acid (30 µg).

Plant materials

The seeds of *Plantago ovata* were collected in the region of Iran (Zabol, south-eastern, Iran) and were identified by Zabol university herbarium. The seeds were dried at room temperature and transferred into glass containers and preserved until extraction procedure was performed in the laboratory.

Preparation of seed extract

Seed samples (50g) were sterilized using 30% sodium hypochlorite for 5 minutes and then rinsed three times with sterile distilled water. The process was followed by soaking in 70% alcohol for two minutes and then rinsed five times with sterile distilled water. Sterile water was added to disinfected seeds (2:1 V/V) and incubated 25°C temperature for 7 days. The prepared seed extract was filtered through 40 whattman filter paper and was kept in refrigerator for further studies.

Synthesis of silver nanoparticles

Silver nitrate ($AgNO_3$) was used as the source for synthesis of silver nanoparticles. Briefly, 5ml of the obtained seed extract was diluted by 15ml sterile water and was added to a concentration of 2mM silver nitrate for the reduction of Ag+ to Ag0.

The biosynthesis of silver nanoparticles using the extract of *P. ovata* was preliminary confirmed by the change of the color of the solution from yellow to brown. The synthesis was further confirmed by the absorption peak between 400-450 nm due to surface plasma resonance (Manikprabhu and Lingappa, 2013).

MIC determination of silver nanoparticles

The MIC (Minimum Inhibitory Concentration) is defined as the lowest concentration of the extract at which the microorganism does not demonstrate the visible growth. The broth micro-dilution method was used to determine MIC. Briefly, serial doubling dilutions of the silver nanoparticles produced in the plant *P. ovata* seed extract were prepared in a 96-well micro-titer plate ranged from 12.5ppm to 200ppm. To each well, 10 µl of indicator solution and 10 µl of Mueller Hinton Broth were added. Finally, 10 µl of bacterial suspension (10^6 CFU/ml) was added to each well to achieve a concentration of 10^4 CFU/ml. The plates were wrapped loosely with cling film to ensure that the bacteria did not get dehydrated. The plates were prepared in triplicates, and then they were placed in an incubator at 37°C for 18-24 hours. The color change was then assessed visually. The microorganism growth was indicated by turbidity.

Results

UV–vis spectral and Transmission Electron Microscopy (TEM) analysis were used in order to confirm the formation of silver nanoparticles from 2mM solution of silver nitrate (Chart no.1). Fig. 1 is TEM image of *P. ovata* seed extract containing 2mM AgNO3 solution at 30 °C. *P. ovata* seed extract produced silver nanoparticles which were often semi spherical. The silver nanoparticles showed Gaussian distributions with average diameter of 13 nm with some deviations.

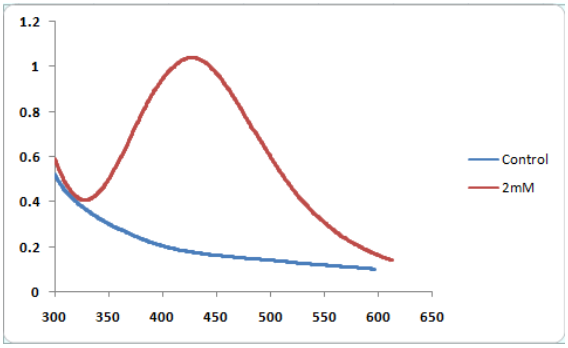
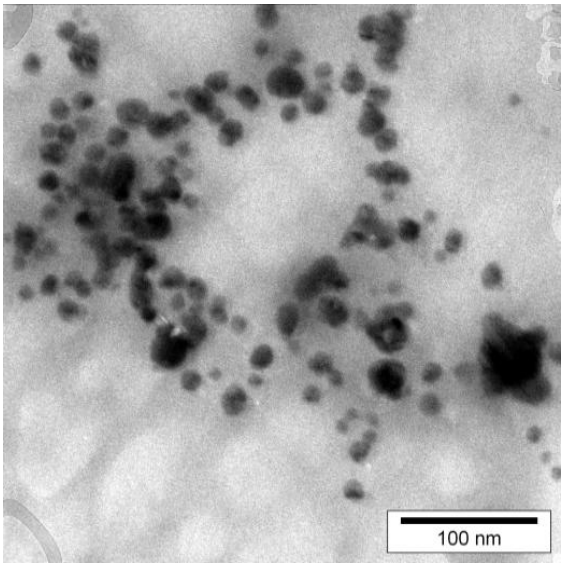


Chart 1. UV-vis spectrum of Ag nanoparticles produced by *Plantago ovata* seed extract



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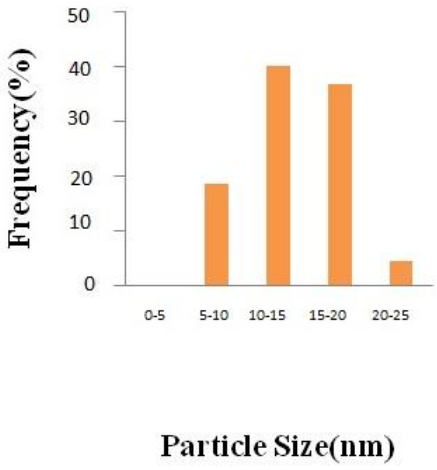

B

Fig. 1. (A) TEM image and (B) particles size distribution of Ag nanoparticles synthesized by *Plantago ovata* seed extract.

The antibiotic resistance profile of *K. pneumoniae* isolates were as follow: Penicillin (93.3%), Erythromycin and Ampicillin (76.6%), Tetracycline and Cefixime (53.3%), Ceftazidime (40%) and Nalidixic acid (36.6%) (Table1). The highest and the least MIC of *P. ovata* seed extract values were found to be 200 and 12.5 ppm respectively (Table 2).

Table 1. Percentage of antimicrobial resistance of 30 isolatess of *Klebsiella pneumoniae*

	CAZ	CN	E	TE	P	AM	NA
S	53.3	36.6	6.6	40	0	0	50
I	6.6	10	16.6	6.6	6.6	23.3	13.3
R	40	53.3	76.6	53.3	93.3	76.6	36.6

S= Sensitive, I= Intermediate, R= Resistant, CAZ= Ceftazidime, TE= Tetracycline, E= Erythromycin, CN= Cefixime, P=Penicillin, AM=Ampicillin, NA=Nalidixic acid

Table 2. Antimicrobial susceptibility and MIC

Bacterial code	MIC(ppm)	CAZ	CN	E	TE	P	AM	NA
1	50	I	R	R	R	R	R	R
2	50	S	R	R	I	I	R	R
3	50	S	S	R	R	R	I	I
4	25	S	S	I	S	R	R	R
5	50	I	I	R	I	R	R	S
6	100	R	R	R	R	R	I	S
7	200	R	R	R	R	R	R	I
8	25	R	R	R	R	R	R	R
9	25	S	S	I	S	R	R	R
10	100	R	R	R	R	R	R	R
11	50	S	S	R	R	R	I	S
12	100	S	R	S	S	R	R	S
13	100	S	S	R	S	R	I	R
14	200	S	I	R	R	R	R	S
15	25	R	R	R	R	R	R	I
16	25	R	R	R	R	R	R	R
17	12.5	S	I	R	R	R	R	R
18	25	R	R	R	S	R	R	S
19	25	R	R	R	R	R	R	S
20	100	S	S	R	S	R	R	S
21	Any grow	R	R	R	R	R	R	S
22	Any grow	S	S	R	S	R	R	S
23	25	S	S	R	S	R	R	S
24	Any grow	S	S	R	S	I	I	R
25	Any grow	R	R	R	R	R	R	R
26	25	S	R	I	S	R	I	R
27	100	S	S	S	S	R	R	S
28	25	R	R	I	R	R	R	S
29	12.5	R	R	R	R	R	R	I
30	25	S	S	I	S	R	R	S

CAZ= Ceftazidime, TE= Tetracycline, E= Erythromycin, CN= Cefixime , P=Penicillin, AM=Ampicillin, NA=Nalidixic acid

Discussion

In the present study, *K.pneumoniae isolates* were resistanct to penicillin (93.3%), erythromycin and ampicillin (76.6%), tetracycline and cefixime (53.3%), ceftazidime (40%) and nalidixic acid (36.6%). As our pervious study, overall *K. pneumoniae* were resistanct to ampicillin (65%), gentamicin (30%), trimethoprim-sulfamethoxazol (25%), ciprofloxacin (20%), nitrofurantoin (15%) and nalidixic acid (15%) (Saeidi *et al*., 2014). In contrast, Zamani *et al.* showed that the most effective antibiotics against the bacterial isolates are tobramycin and ceftazidime (79%), ceftizoxime (78%), ciprofloxacin (76.1%), ceftriaxone (76.2%) and amikacin (74.2%) (Zamani *et al*., 2013). Results of another study showed that 28 to 76 % of isolates are resistant to ceftizoxime and cefotaxime (Sikarwar and Batra, 2011). The study of Feizabadi on *Klebsiella pneumoniae* isolates showed the highest resistance to amoxicillin-clavulanic acid (81.8%), cefixime and ceftazidime (72.7%) (Feizabadi *et al*., 2007).

In our study, the highest and the least MIC of *P. ovata* seed extract values were found to be 200 and 12.5 ppm respectively. In the study of Soo-Hwan the results show that *S. aureus* and *E. coli* were substantially inhibited by Ag-NPs (Soo-Hwan *et al*., 2011). Sondi and Salopek-Sondi reported that silver nanoparticles have excellent antibacterial activity against *E. coli* (Sondi and Salopek-Sondi, 2004). As the results of Guzman *et al* study, the nanoparticles of silver showed high antimicrobial and bactericidal activity against gram positive bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* which is a highly methicillin resistant strain (Guzman *et al*., 2009). Silver nanoparticles have been shown to be effective biocides against different bacteria such as *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermis, Leuconostoc mesenteroides, Bacillus subtilis, and Klebsiella pneumoniae* among others (Benn and Westerhoff, 2008; Chen and Chiang, 2008; Falletta *et al*., 2008; Hernandez-Sierra *et al*., 2008; Ingle *et al*., 2008; Jung *et al*., 2008; Kim, 2007; Kim *et al*., 2008; Kvitek *et al*., 2008). Gopinath *et al* reported that *Staphylococcus aureus* and *Streptococcus pneumoniae* exhibit similar zone of inhibition for all three silver nanoparticle concentrations. They concluded that the most significant effect of silver nanoparticles at low concentration of 10 µL per disc against *Shigella dysenteriae* produces a 2-mm inhibition zone for gram-negative bacteria, and at the same concentration, the nanoparticles did not show any significant effect on *Bacillus subtilis, Pseudomonas aeruginosa*, and *Proteus vulgaris* (Gopinath *et al*., 2013). The study of Das showed that these nanoparticles can be used as effective growth inhibitors against *Staphylococcus, Basillus* and *Pseudimonas* species (Das *et al*., 2011).

The present study concludes that at a specific dose, chitosan-based AgNPs kill bacteria without harming the host cells, thus representing a potential template for the design of antibacterial agents to decrease bacterial colonization and to overcome the problem of drug resistance. Silver nanoparticles have a potent antimicrobial activity against antibiotic resistant *K.pneumoniae* strains. However, further research is required to evaluate the practical value of these particles before therapeutic usage.

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