

Green Synthesis of Silver Nano-particles Using *Kelussia odoratissima* Mozaff. Extract and Evaluation of its Antibacterial Activity

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ABSTRACT

In this research *Kelussia odoratissima* Mozaff. leaf extract was used for the green synthesis of silver nanoparticles (AgNPs). At first we compared antioxidant activity of different extracts of *K. odoratissima*. Then solution containing silver nitrate was treated with the extract which showed high antioxidant activity. Synthesized AgNPs were evaluated by analyzing the excitation of surface plasmon resonance. TEM analysis was also used for nanoparticle characterization. Antibacterial activity of the solution containing AgNPs was measured by microdilution test. Common food contaminant bacteria such as gram-positive (*Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*) and gram-negative (*Escherichia coli* O157: H7, *Salmonella enterica* and *Pseudomonas aeruginosa*) were used for the evaluation. The aqueous extract showed the highest antioxidant activity and the solution was used for the green synthesis of AgNPs. The particle diameters were calculated to be 20-40nm with -17 to -19.9 mV zeta potential. The TEM micrographs showed that the AgNPs are nearly spherical in shape and highly monodispersed. MIC of the AgNPs against gram-positive and gram-negative bacteria was between 0.012-0.025 and 0.006-0.012 mg/ml respectively.

Keywords: *Kelussia odoratissima*, Green synthesis, Silver nanoparticles, Antibacterial activity, Electron microscopy

INTRODUCTION

One of the major aspects of nanotechnology science is concerned with production and development of toxicity-free synthesis of metal nanoparticles. Application of nanoscale materials and structures, usually ranging from 1 to 100 nanometers (nm), is an emerging area of nanoscience and nanotechnology. Noble metal nanoparticles (NPs) are famous to have important applications in the fields of electronic, catalytic properties, magnetic,

optoelectronics, information storage and biomedicine especially drug delivery (Jin and Ye, 2007). Among the various metal nanoparticles, silver nanoparticles have been widely investigated because they exhibit unusual optical, electronic and chemical properties, depending on their size and so in preparation of nanoparticles, focus on controlling the size and shape is essential. Silver nanoparticles display unique physical, chemical, (Jin *et al.*, 2003) and biological properties such as high antibacterial activity toward a large number of bacterial strains (Zeng *et al.*, 2007). A number of approaches

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are available for the synthesis of silver NPs. For example, silver ions are reduced by chemical, radiation, photochemical methods (Callegari and Chergui, 2003) and Langmuir–Blodgett (Ahmad *et al.*, 2010). In the global efforts to reduce generated hazardous waste, “green” chemistry and chemical processes are progressively integrating with modern developments in science and industry. Chemical synthesis methods use toxic compounds and may be a hazard to the environment and have adverse effects in the medical application (Virender *et al.*, 2009). Therefore biosynthesis of silver nanoparticles by green synthesis method has advantages over physical and chemical approaches as it is ecofriendly, cost effective and high temperature, pressure, energy and toxic compounds are not required in this approach. Use of microorganisms (Banu *et al.*, 2011) and plants in producing metal nanoparticles is accelerated after the raising of economic and environmental concerns on physical and chemical methods, opening a new era. Various plants such as *Aloe vera* (Chandran *et al.*, 2006), *Anacardium occidentale* (Mukunthan, 2012), *Hibiscus rosa-sinensis* (Philip, 2010), *Dillenia indica* (Singh *et al.*, 2013) *Hevea brasiliensis* (Guidelli *et al.*, 2011), and *Cocos nucifera* coir (Roopan *et al.*, 2013) have successfully been employed for the extracellular synthesis of silver nanoparticles (SNPs) (Yilmaz *et al.*, 2011).

Kelussia odoratissima Mozaff. belongs to the Apiaceae family and is a sweet-smelling, self-growing plant which is endemic to Iran (Mashregi *et al.*, 2014). This plant locally called “Karafse-Koochi”, is a wild, erect, glabrous, perennial aromatic herb, which grows to a height of 120 to 200 cm. *K. odoratissima* also has anti-inflammatory, sedative and anti-tussive properties. However, it is known for its potential antioxidant activity (Ahmadi *et al.*, 2007). The aim of this study was green synthesis of SNPs by using *K. odoratissima* extract and evaluation of the particle properties and their antibacterial activity using micro-dilution assay.

MATERIALS AND METHODS

Preparation of Leaf Methanolic and Ethanolic Extract

Dried herb of *K. odoratissima* Mozaff. was supplied from Isfahan Research Center. The sample was authenticated in FUM herbarium by Jouharchi and voucher specimen (No. 77242 FUMH) was deposited in the herbarium.

Dry leaves of *K. odoratissima* were used for preparation of the extracts according to Ahmadi *et al.*, (2007). The plant materials (20 g of powdered leaf of *K. odoratissima*) were extracted with 200 ml methanol or ethanol for 24 hours. This was repeated thrice. The obtained methanolic and ethanolic extracts were passed through Whatman filter paper No.1 (Whatman Ltd., England). The filtrates were concentrated under vacuum using rotary evaporator at 40°C to give crude dried extract. The extract dried using oven at 50°C. Then 0.5 g of the dried extract was dissolved in 50ml methanol and ethanol and the extract evaluated for antioxidant activity.

Preparation of Aqueous Leaf Extract

The aqueous extract of leaf powder of *K. odoratissima* was prepared according to Ahmad *et al.*, (2010) with some modification. Briefly twenty g of the leaf powder was added in 200 mL deionized water in 500 mL round shape flask and then it was connected to rotary evaporator using 50°C and reduced pressure for five minutes. The extracts were filtered with socks then followed by milli-pore filter (0.45 µm). Then the extract was freeze-dried and 0.5 g of the dried extract was dissolved in 50ml distilled water and the extract was evaluated for antioxidant activity.

Antioxidant Activity Assay

Because different extracts of medicinal plants show various antioxidant activity

(Soria *et al.*, 2008) and green synthesis of SNPs using the extract depend on its antioxidant activity potential, at first we evaluated antioxidant activity of different *K. odoratissima* extracts for choosing the best extract type. In order to measure antioxidant activity, DPPH (2, 2'-diphenyl-1-picrylhydrazyl) free radical scavenging assay was used. It is one of the most extensively used antioxidant assay methods for plant samples (Duduku Krishnaiah and Nithyanandam, 2010). In this assay, the purple chromogen radical DPPH is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine. In this method, a 0.1mM solution of DPPH in methanol was prepared. Then different volumes (100, 200, 400, 500, 600, 800, 1000 µl) of each plant extract were added to 1.0 mL DPPH solution (0.1mM) in a series of test tubes and the final volume adjusted to 5 mL by adding absolute methanol. The mixture was shaken vigorously and allowed to stand at room temperature for 60min in the dark. Then absorbance of the reaction mixture was measured at 515 nm using UV–visible spectrophotometer (Cecil 1000 Series; Cecil Instruments, Cambridge, UK). A large decrease in the absorbance of the reaction mixture indicates significant free antioxidant activity of the compound. The ascorbic acid and α-tocopherol was used as a positive control of reference.

The antioxidant activity percentage (AA%) was determined according to Duduku Krishnaiah and Nithyanandam, 2010:

AA% = 100 -
$$\left[\frac{(Abs_{sample} - Abs_{blank}) \times 100}{Abs_{control}} \right]$$

Abs_{sample} is the absorbance in presence of all extract samples.

Abs_{blank} is the absorbance of samples without DPPH.

Abs_{control} is the absorbance of DPPH.

EC₅₀ value of the samples was calculated using Log dose inhibition curve. For this purpose a percentage inhibition versus concentration curve was plotted and the concentration of sample required for 50% inhibition was determined as EC₅₀ value for each of the test solutions. Comparison of the different extracts with reference standards (α-tocopherol and Ascorbic acid) was carried out by calculating EC_{50index} as the ratio of reference standard (α-tocopherol) EC₅₀ to samples EC₅₀ (Table 1).

Synthesis of Silver Nanoparticles

The 10⁻³ M silver nitrate solution was prepared and stored in brown bottles. The five or 10mL of *K. odoratissima* aqueous extract was added to 95 or 90 mL AgNO₃ respectively and were incubated at 37°C in dark and stationary condition. The synthesis of SNPs was monitored periodically for 2 days by using a UV–visible spectrophotometer (Cecil 1000 Series; Cecil Instruments, Cambridge, UK).

Characterization of Nanoparticles

Synthesized nanoparticles were evaluated by measuring the absorption spectra of the solution, size and zeta potential of nanoparticles. Transmission Electron Microscopy (TEM) was also used for

Table 1. EC₅₀ of α-tocopherol, Ascorbic acid and *Kelussia odoratissima* extracts.

Extract type/ Standards compounds.	EC ₅₀ (µg/ml)	EC _{50 index}
Aqueous	693	0.42
Ethanollic	733	0.40
Methanollic	861	0.34
α-tocopherol	294	1
Ascorbic acid	367	0.80

EC₅₀: The concentration of samples required to inhibit 50% of the DPPH free radicals. EC_{50 index}: The ratio of reference standard (α-tocopherol) EC₅₀ to samples EC₅₀.



studying the synthesized nanoparticles.

Absorption spectroscopy in UV-visible region has long been an important tool for nanoparticle characterization. Color transition arises due to molecular and structural changes in the substances being examined, leading to corresponding changes in the ability to absorb light in the visible region of the electromagnetic spectrum (Banu *et al.*, 2011). To determine the time point of maximum production of silver nanoparticles, the absorption spectra of the samples were taken at 340 to 540 nm using a UV-vis spectrophotometer (Cecil 1000 Series; Cecil Instruments, Cambridge, UK).

In this research particle size distribution of AgNPs was evaluated using Malvern Zeta-sizer Nanoseries compact scattering spectrometer (Malvern Instruments Ltd, Malvern, UK).

Usually after nanoparticles preparation surface charge is created, because of ionized groups or absorption of ions from external phase. Creating this electric atmosphere around the dispersed particles and also their disposal, has a major role in the stability of the dispersion nanoparticles and prevention of aggregation. In this research zeta potential (mV) of AgNPs was evaluated using Malvern Zeta-sizer Nanoseries compact scattering spectrometer.

Transmission electron microscopy (TEM) as a powerful tool has been extensively used to investigate the morphologies and size of the synthesized AgNPs. For the observation a drop of aqueous solution containing the silver nanoparticles were prepared by placing on carbon-coated copper grids and films on the grids were allowed to stand for 2 min. The samples were studied using a Philips CM 20 instrument with a LaB6 cathode operating at 200 kV.

Antibacterial Activity

Antibacterial activities of the solution after formation of silver nanoparticles were determined by micro-dilution methods as described earlier (Oroojalian *et al.*, 2010).

Briefly sterile 96-well microplates were used for the assay (0.34 ml volume, Orange Scientific). Bacterial strains were cultured overnight at 37°C in Muller Hinton Broth (MHB, Oxoid). Dilution series of the extract solutions and also chloramphenicol as an antimicrobial standard were prepared using Mueller Hinton Broth (MHB). Then 70 µl of each dilution was transferred into each well, followed by adding 70 µl of MHB, and inoculating 70 µl of respected standardized microorganism suspensions containing 10⁸ colony forming units (cfu/ml) (according to McFarland turbidity standards). After incubation at 37°C for 22–24 h the first well without turbidity was assigned as minimum inhibitory concentration (MIC, µg/ml). The microorganism growth inhibition was evaluated by measuring absorbance at 630 nm using an ELISA reader (Statfax-2100, Awareness Technology Inc, USA). The minimum bactericidal concentrations (MBC) of the extracts were determined according to the MIC values. Of each well showing complete absence of growth, 5 µl was transferred to agar plates (MHA) and incubated at 37°C for 24 h. The lowest concentration of extract where no viable bacteria were identified was the MBC. This experiment was replicated for three times and the results expressed as MIC and MBC.

RESULTS AND DISCUSSION

DPPH Radical-Scavenging Activities

In our study DPPH radical-scavenging activities of the three extracts were evaluated and the results were presented as relative activities against ascorbic acid and α -tocopherol as positive control. Then the results were compared with positive control and were expressed as percent reduction of the initial DPPH absorption by the test compound (Figure 1). The results showed that α -tocopherol (500, 600 µL/5 ml) had excellent DPPH radical scavenger activities as about 90 and 92% of DPPH scavenged under these experimental conditions. Our

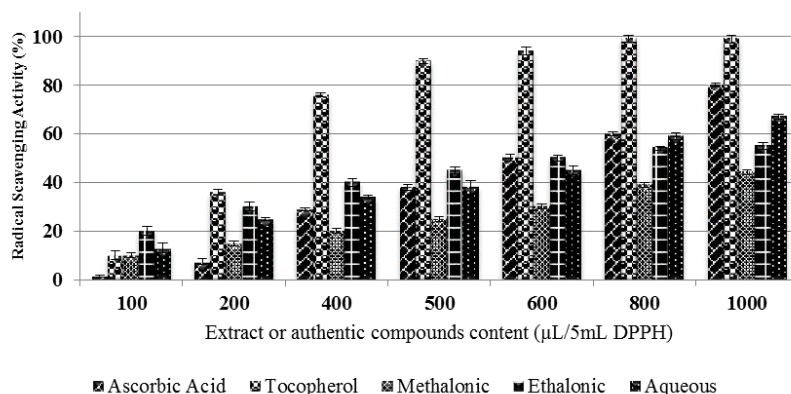


Figure 1. Radical-scavenging activities of different plant extracts, ascorbic acid and α -tocopherol by DPPH method at different ratio ($\mu\text{L}/5\text{mL}$).

results correlate with the reports of Ahmadi *et al.*, 2007.

The EC_{50} value is a common parameter to measure the free radical scavenging activity. A lower EC_{50} indicates a higher antioxidant activity. The lowest EC_{50} belonged to α -tocopherol ($\text{EC}_{50}=294 \mu\text{g}/\text{mL}$), then ascorbic acid ($\text{EC}_{50}=367 \mu\text{g}/\text{mL}$), aqueous extract ($\text{EC}_{50}=693 \mu\text{g}/\text{mL}$), ethanolic extract ($\text{EC}_{50}=733 \mu\text{g}/\text{mL}$) and methanolic extract ($\text{EC}_{50}=861 \mu\text{g}/\text{mL}$). Amongst these three types of plant extracts, the yield of aqueous extract was higher than yield of ethanolic and methanolic extract. The order of effectiveness (EC_{50} value basis) of extracts was: aqueous extract ($\text{EC}_{50}=693 \mu\text{g}/\text{mL}$) > ethanolic extract ($\text{EC}_{50}=733 \mu\text{g}/\text{mL}$) > methanolic extract ($\text{EC}_{50}=861 \mu\text{g}/\text{mL}$) (Table1). Our results were in accordance with the results of other researches such as (Stanojevic *et al.*, 2009). These findings confirm that water extract of *K. odoratissima* is the best extract for bio-reduction of AgNO_3 to SNPs.

Evaluation of Ag Nanoparticles Synthesized

The mixture of plant extract and silver nitrate solution was incubated at 37°C for 2 days, and silver nanoparticles were synthesized gradually. The color change was

caused by the surface plasmon resonance of silver nanoparticles in visible region. Silver nanoparticles are known to exhibit size and shape dependent surface plasmon resonance bands which are characterized by UV-visible absorption spectroscopy. Figure 2(a-b) show the characteristic surface plasmon resonance (SPR) absorption band at 400 to 460nm. Silver nanoparticles are synthesized by the water extract of *K. odoratissima* implying that the bio-reduction of the silver nitrate has taken place following incubation of the AgNO_3 solution in presence of the cell-free extract. Our results are in agreement with other reports (Banu *et al.*, 2011, Mukunthan, 2012, Sadowski *et al.*, 2008). It has been reported that the absorption spectrum of spherical silver nanoparticles presents a maximum between 420nm and 450nm (Rathod, 2011).

The higher intensity of SPR band shows increasing concentration of NPs by time. To optimize the reaction time, a time variation study was carried out using the optimized concentration of AgNO_3 ($3 \times 10^{-3} \text{ mM}$) and aqueous extract (10% v/v) of *K. odoratissima*.

The size distribution and Zeta potential of the synthesized nanoparticles are shown in Figures 3 (a-b). The results confirmed that the solution which contained 10% of plant extracts (Figure 3-b) produced nanoparticles smaller than the solution containing 5% of plant extracts (Figure 3-a).

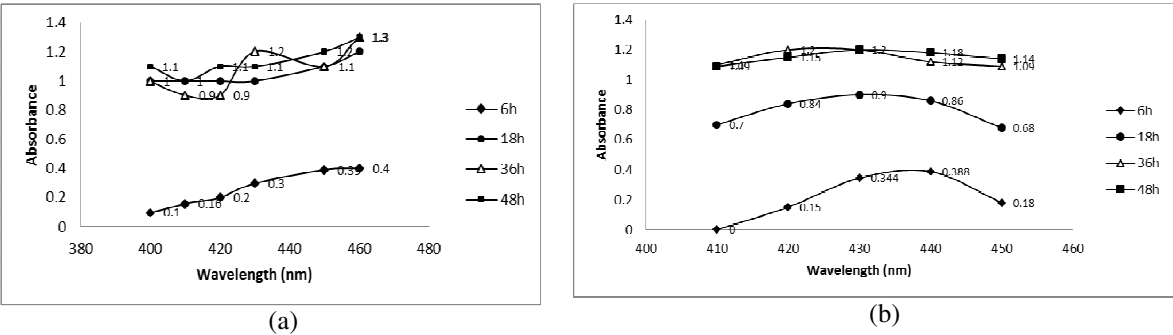


Figure 2. UV spectral peak of AgNPs synthesized at different time. The solution contained (a) 5ml plant extract plus 95 ml AgNO₃ (10⁻³ mM). (b) 10ml plant extract plus 90 ml AgNO₃ (10⁻³ mM).

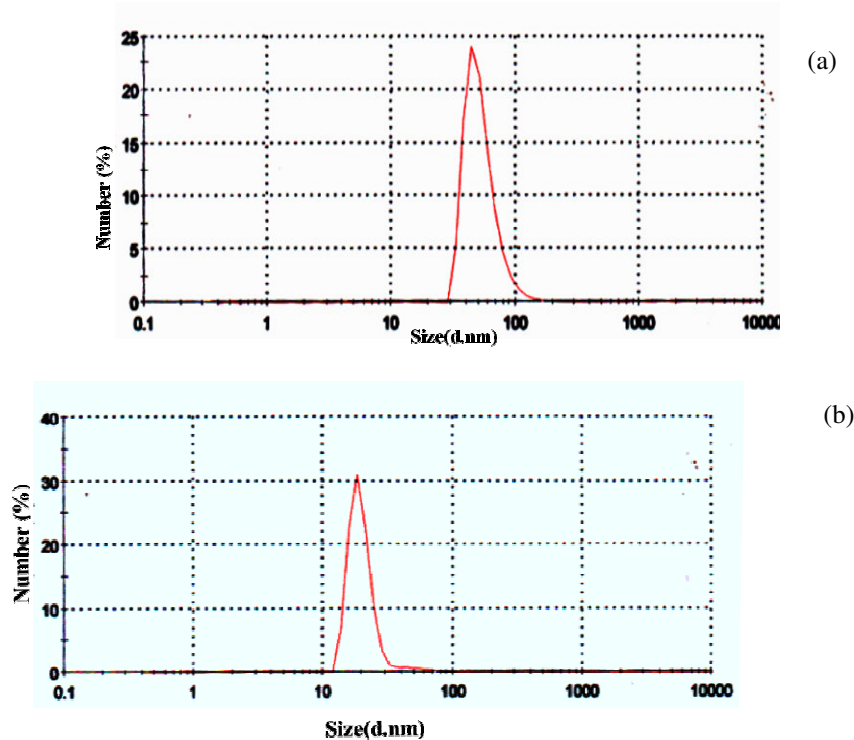


Figure 3. Size distribution of AgNPs in solution containing (a) 5% plant extract plus 95% AgNO₃. Z-Average(d.nm):100.8 ; Zeta Potential(mV):-17.0 (b) 10% plant extract plus 90% AgNO₃ Z-Average(d.nm):90.61 Zeta Potential(mV):-19.9

In (Figure 3-b), size distribution of AgNPs with maximum intensity at 20-40nm in solution containing 10% (V/V) of the sample extracts has been shown but in (Figure 3-a), the maximum intensity was at 100 nm, so it has been observed that 5% of *K. odoratissima* extract is insufficient for nanoparticles synthesis. Our results were in accordance with a research by Sukirtha *et al.*, 2011. We observed that 10% *K.*

odoratissima solution is suitable for the green synthesis of Ag nanoparticles.

Figure 4 shows a typical TEM image of AgNPs synthesized by *K. odoratissima* extract. The micrograph shows individual silver particles. The morphology of the AgNPs was variable, with a spherical majority. It can be seen that the particles range in size from 17 to 57 nm with mean size of 38.7 nm. TEM analysis of

the solution indicated the formation of monodisperse spherical particles with 50 nm diameters (Figure 4).

During the experiment development, the color of solution changed from light yellow to brown. Increased color is indicative of the reaction progress. Figure 5 Shows color intensity of biosynthesized AgNPs after 24h.

In this experiment, the absorption value of *K. odoratissima* extract (10% extract + 90% silver nitrate) reached the constant level after two days which showed the end of SNPs green synthesis. Other researchers reported that green synthesis of SNPs using *Rhizopus stolonifer* need more time (Banu *et al.*, 2011). Therefore if the saving time and the velocity of SNPs synthesis is important then green synthesis of SNPs using *K. odoratissima* extract is recommended. Essential oils analysis of the plant in other researches confirm the presence of (Z)-3-butylidene-phthalide (14.37%), (Z)-ligustilide (54.11%), limonene + β -phellandrene (6.36%) which show antioxidant activity (Data not shown). Phenolic form of medicinal plant compounds change to quinone form during this process and the higher the phenolic compounds the lower time required for the Ag nanoparticle synthesis. Some other researcher reported that if the phenolic compounds of the extract were very high this process would need a few minutes (Rodríguez-León *et al.*, 2013).

Antibacterial Activity

The antibacterial properties of the green synthesized SNPs are shown in Table 2. The results indicated strong anti-bacterial properties of the green synthesized SNPs. MIC of the SNPs against gram-positive bacteria was between 0.012 and 0.025 mg/ml. MIC of the SNPs against gram-negative bacteria was between 0.006 to 0.012 mg/ml. Lkhagvajav *et al.*, (2011) investigated the antibacterial effects of colloidal silver nanoparticles against *S.*

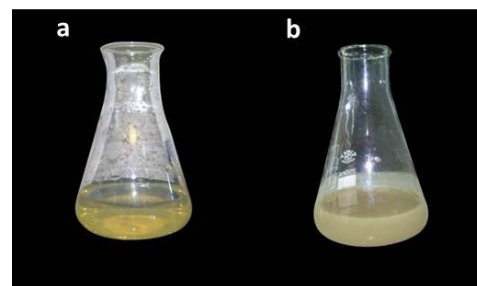


Figure 4. Color intensity of biosynthesized AgNPs (a: Before b: After 24 h).

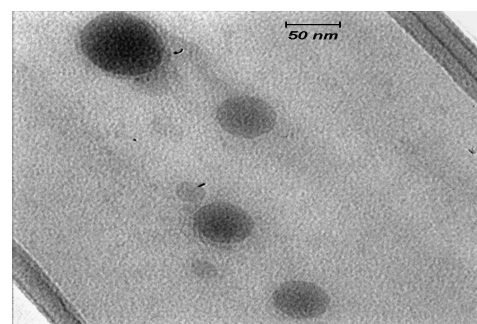


Figure 5. TEM image of AgNPs synthesized by using leaves extract of *Kelussia odoratissima*.

aureus and *E. coli* and obtained the MIC of it as 0.004 and 0.003 mg/ml, respectively (Lkhagvajav *et al.*, 2011). Perhaps the reason for this difference is the difference of bacterial strain or presence of subsidiary compounds in *K. odoratissima* extract. SNPs are surrounded by a thin layer of organic material, suggesting that biomolecules, in *K. odoratissima* extract, capped the silver nanoparticles which appear to be a characteristic of AgNPs prepared by plant extracts (Rathod, 2011, Sadowski *et al.*, 2008). Antibacterial activity of biomolecules in other medicinal plants of Apiaceae family was confirmed previously (Ali *et al.*, 2014, Goudarzi *et al.*, 2011, Oroojalian *et al.*, 2010). Rai and Gade (2009) and Martinez-Castanon *et al.* (2008) showed that the antibacterial properties of nanoparticles is highly dependent on particle size and shape (Rai and Gade, 2009, Martinez-Castanon *et al.*, 2008).



Table 2. Antibacterial activities of synthesized silver nanoparticle by using micro-dilution assay test. Data in parenthesis show the MIC of Chloramphenicol for comparison.

Pathogens	Strain ATCC	MIC ^a (µg/ml)	MBC ^b (µg /ml)
<i>S. aureus</i>	25923	25 (93)	50
<i>B. cereus</i>	11778	25 (24)	50
<i>L. monocytogenes</i>	19112	12 (124)	25
<i>E. coli O157:H7</i>	700728	6 (182)	12
<i>S. enterica</i>	49416	12 (184)	25
<i>Ps. aeruginosa</i>	15442	6 (121)	12

^a Minimum Inhibitory Concentration, ^b Minimum Bactericidal Concentration

Although the precise mechanism of the inhibitory effects of silver nanoparticles on bacteria is not fully understood, but possible mechanisms have been proposed by some researchers. In general, it is believed that silver nanoparticles are attached to the cell wall of bacteria and lead to protein denaturation and ultimately cell death. Oxygen also reacts with silver nanoparticles affecting thiol groups (SH) in the bacterial cell wall that leads to the formation of RSSR which will stop breathing and cell death (Kim *et al.*, 2007).

CONCLUSION

To the best of our knowledge, this is the first report in the literature on nanoparticle synthesis using extracts of *Kelussia odoratissima*, an endemic medicinal plant of Iran. The rapid synthesis of SNPs using *K. odoratissima* was demonstrated. Silver nanoparticles were prepared with suitable size using extract of the plant. The results confirm application potential of plants in the

field of nano-science. The particles can be applied in the appropriate fields, including medical research, therapeutic applications and agriculture. The biomolecules responsible for the green synthesis are phenols. Antioxidant activity of different medicinal plants contributed to the phenol content (Maisuthisakul and Pongsawatmanit, 2007). Water extract of the plants showed the highest antioxidant activity especially in high concentration and with respect to the traditional consumption is a suitable result that recommends higher consumption of the plants in food diet.

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سنتز سبز نانوذرات نقره با استفاده از عصاره کرفس کوهی و بررسی اثرات ضد باکتریایی آن

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چکیده

در این تحقیق عصاره برگ کرفس کوهی برای سنتز سبز نانوذرات نقره (AgNPs) استفاده شد. در ابتدا با فعالیت آنتی اکسیدانی عصاره های مختلف کرفس کوهی را مقایسه نمودیم. سپس محلول حاوی نیترات نقره با عصاره ای که بیشترین فعالیت آنتی اکسیدانی را نشان داد با یکدیگر مخلوط و آنکوبه شدند. سنتز نانو ذرات نقره از طریق تجزیه و تحلیل تهییج رزونانس پلاسمون سطحی مورد بررسی قرار گرفت. آنالیز میکروسکوپ الکترونی (TEM) نیز برای توصیف نانوذرات سنتز شده مورد استفاده قرار گرفت. فعالیت ضد میکروبی محلول حاوی نانو ذرات نقره با استفاده از آزمون میکرودايلوشن اندازه گیری شد. باکتریهای رایج آلاینده مواد غذایی مانند باکتریهای گرم مثبت (استافیلوکوکوس اورئوس، باسیلوس سرئوس، لیستریا مونوسیژنوز) و باکتریهای گرم منفی (اشریشیا کلی O157: H7، سالمونلا و سودوموناس آئروژینوزا) برای ارزیابی استفاده شدند. عصاره آبی کرفس کوهی بالاترین فعالیت آنتی اکسیدانی را نشان داد و این عصاره آبی برای سنتز سبز نانو ذرات نقره استفاده شد. قطر نانو ذرات سنتز شده برابر ۲۰-۴۰ نانومتر تعیین شد و پتانسیل زتا آنها برابر ۱۷- تا ۱۹.۹ میلی ولت ثبت گردید. تصاویر میکروسکپ الکترونی (میکروگراف) نشان داد که نانو ذرات نقره تقریباً کروی شکل و بسیار مونودیسپرس هستند. حداقل غلظت بازدارندگی (MIC) نانوذرات نقره سنتز شده به روش سبز علیه باکتریهای گرم مثبت و گرم منفی بترتیب در دامنه ۰.۰۱۲-۰.۰۲۵ و ۰.۰۰۶-۰.۰۱۲ و میلی گرم / میلی لیتر بود.