
M. Azizi¹*, H. Kaboli Farshchi¹, F. Oroojalian², and H. Orafaee³

**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 (Mashreghi et al., 2014). This plant locally called ‘‘Karafse-Koohi’’, 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
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(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:

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

Abs<sub>sample</sub> is the absorbance in presence of all extract samples.
Abs<sub>blank</sub> is the absorbance of samples without DPPH.
Abs<sub>control</sub> is the absorbance of DPPH.

EC<sub>50</sub> 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 EC<sub>50</sub> 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<sub>50</sub>index as the ratio of reference standard (α-tocopherol) EC<sub>50</sub> to samples EC<sub>50</sub> (Table 1).

### Synthesis of Silver Nanoparticles

The 10<sup>3</sup> 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 AgNO<sub>3</sub> 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>
<thead>
<tr>
<th>Extract type/ Standards compounds.</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;(μg/ml)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>693</td>
<td>0.42</td>
</tr>
<tr>
<td>Ethanolic</td>
<td>733</td>
<td>0.40</td>
</tr>
<tr>
<td>Methanolic</td>
<td>861</td>
<td>0.34</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>294</td>
<td>1</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>367</td>
<td>0.80</td>
</tr>
</tbody>
</table>

EC<sub>50</sub>: The concentration of samples required to inhibit 50% of the DPPH free radicals. EC<sub>50</sub>index: The ratio of reference standard (α-tocopherol) EC<sub>50</sub> to samples EC<sub>50</sub>.
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 Zetasizer 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 Zetasizer 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
results correlate with the reports of Ahmadi et al., 2007.

The EC\textsubscript{50} value is a common parameter to measure the free radical scavenging activity. A lower EC\textsubscript{50} indicates a higher antioxidant activity. The lowest EC\textsubscript{50} belonged to α-tocopherol (EC\textsubscript{50}=294 µg/mL), then ascorbic acid (EC\textsubscript{50}=367 µg/mL), aqueous extract (EC\textsubscript{50}=693 µg/mL), ethanolic extract (EC\textsubscript{50}=733 µg/mL) and methanolic extract (EC\textsubscript{50}=861 µg/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\textsubscript{50} value basis) of extracts was: aqueous extract (EC\textsubscript{50}=693 µg/mL) > ethanolic extract (EC\textsubscript{50}=733 µg/mL) > methanolic extract (EC\textsubscript{50}=861 µg/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 \textit{K. odoratissima} is the best extract for bio-reduction of AgNO\textsubscript{3} to SNPs.

\section*{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 \textit{K. odoratissima} implying that the bio-reduction of the silver nitrate has taken place following incubation of the AgNO\textsubscript{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\textsubscript{3} (3×10\textsuperscript{-3} mM) and aqueous extract (10\% v/v) of \textit{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 AgNO$_3$ ($10^{-3}$ mM). (b) 10ml plant extract plus 90 ml AgNO$_3$ ($10^{-3}$ mM).

Figure 3. Size distribution of AgNPs in solution containing (a) 5% plant extract plus 95% AgNO$_3$. Z-Average(d.nm):100.8 ; Zeta Potential(mV):-17.0 (b) 10% plant extract plus 90% AgNO$_3$ 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 $R$. 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 (Rodriguez-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 $S$. 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.

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

$^a$ 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.

REFERENCES


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سندرز نانوذرات نقره با استفاده از عصاره کروش کوهی و بررسی اثرات ضد باکتریایی آن

م. عزیزی، ج. کابلی فرشچی، ف. عروجی‌یان، و ح. عرفایی

چکیده

در این تحقیق عصاره خوشگل کروش کوهی برای سندرز نانوذرات نقره (AgNPs) استفاده شد. در ابتدا فعالیت آنتی‌اکسیدانی عصاره های مختلف کروش کوهی را مقایسه نمودد. سپس محلول حاوی نتیجه نقره با عصاره آتی که بیشترین فعالیت آنتی‌اکسیدانی را نشان داد با یکدیگر مخلوط و آنکوبه شدند. سندرز نانوذرات نقره از طریق تجزیه و تحلیل تهیه رزونانس سپتیم فوریت مورد بررسی قرار گرفت. آنالیز میکروسکوپ الکترونی (TEM) نیز برای توصیف نانوذرات سندرز شده مورد استفاده قرار گرفت. فعالیت ضد میکروبی محلول حاوی نانوذرات نقره با استفاده از آزمون میکروآبیولوژی انداده‌‌گیری شد. باکتری‌های اولین مبتنی مواد غذایی مانند باکتری‌های گرم منفی (استافیلکوکوس اورنوس، باسیلوس سرنوس، لیستریا مونوسپوروزیز) و باکتری‌های گرم منفی (اشتریا کلی 7، O157: H7، سالسومونا و سودومونا آتروژنوزا) برای ارزیابی استفاده شدند. عصاره آتی کروش کوهی بالاترین فعالیت آنتی‌اکسیدانی را نشان داد و این عصاره آتی برای سندرز نانوذرات نقره استفاده شد. قطر نانوذرات سندرز شده برابر 20-40 نانومتر تعیین شد و پتانسیل زنا که آنها برای 17-19 میلی ولت ثب‌ت گردید. تصاویر میکروسکوپ الکترونی (میکروگراف) نشان داد که نانوذرات نقره منفی اکریلی فلکل و سپار مونودispers هستند. حاداف غلظت بازارندگی (MIC) نانوذرات نقره سندرز شده به روش سپی علیه باکتری‌های گرم منفی و گرم منفی بسته در دامنه ۰.۰۱۲-۰.۰۵ و ۰.۰۶-۰.۱ بسته گرم / میلی لیتر بود.