Biosynthesis of Silver Nanoparticles Using *Mentha longifolia* (L.) Hudson Leaf Extract and Study its Antibacterial Activity

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ABSTRACT

In the present study, a simple, fast and eco-friendly biosynthesis of silver nanoparticles (AgNPs) using *Mentha langifolia* leaf extract as the reducing agent was investigated. Nanoparticles forming were indicated by the color changes in solution and were confirmed by UV-Vis spectrum, FT-IR analysis and Scanning Electron Microscopic (SEM) micrograph. The average particle size of produced AgNPs was determined by dynamic light scattering (DLS) technique. Further, the antibacterial activity of synthesized AgNPs was evaluated against *Streptococcus pneumonia* and *staphylococcus epidermidis* as Gram-positive bacteria and *Salmonella enterica* and *enterobacter aerogenes* as Gram-negative bacteria. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of biosynthesized silver nanoparticles were determined. UV-Visible spectrophotometer showed absorbance peak in 340 nm. DLS analysis indicated that the average size of AgNPs is 21.1nm. The results of SEM showed that synthesized AgNPs are spherical in shape. The antibacterial activities of the silver nanoparticles were studied against subject bacteria. The present report explores a rapid, simple and economical route, without any hazardous chemicals as reducing or stabilizing agents to synthesis AgNPs and describes the antimicrobial activities of synthetized silver nanoparticles.

Keywords: Silver nanoparticles; *Mentha longifolia*; Green synthesis; Antibacterial activity

INTRODUCTION

Among several noble metal nanoparticles, silver nanoparticles have gained a special focus [1]. Silver has been generally used as an antibacterial agent for centuries, and nowadays the powerful toxicity of silver against wide range of microorganisms is well known; silver nanoparticles have been recently shown to be a promising antimicrobial material [2]. This feature of silver makes it a good choice for various roles in the medical field. Silver is usually used in the nitrate form to cause antimicrobial effect, but when silver is used in the form of silver nanoparticles, a huge increase occurs in the surface area available for microorganisms to be exposed to. Recent attention to this element is mainly because of increasing danger of antibiotic resistance, which is the result of the indiscriminate use of antibiotics [3]. The field of nanotechnology is one of the most important areas of research in modern material science. Nanoparticles have proved to be fascinating in recent years because of their valuable and unusually remarkable properties [4].

A number of methods exist for the synthesis of silver nanoparticles. In traditional chemical methods of synthesizing silver nanoparticles, ethylene glycol, pyridine, and sodium borohydride were used. These chemicals can be toxic for the environment and humans, and the procedures are too expensive to be possible at an industrial scale [5]. So there have been various investigations for inexpensive, reliable, safe, and "green" method to the synthesis of stable metal nanoparticles with controlled size and shape. As the result, some new methods have recently been developed using biologically derived reducing agents such as chitosan [5], glucose [6] and polysaccharides [7], microbes such as bacteria and fungus [8], and a variety of plant (seed, leaf) extracts [9,10] for the synthesis of metal

nanoparticles. Among them, plant leaf extract mediated biological process has gained considerable importance due to its simplicity, speed and cost effectiveness. Plants extract is a rich source of secondary metabolites which can easily reduce silver nitrate to silver nanoparticles [11].

Studies have shown that a number of silver nanoparticles have been synthesized by using various plants such as *Aloe vera* [12], *Azadirachta indica* [13], *Eucalyptus oleosa* [14], *Pimenta dioica* [15], *Acalypha indica* [2], *Coriandrum sativum* [16] and *Mangosteen* [17] as well as various other plant extracts as reducing agent. However, the potential of plants as biological materials for the synthesis of nanoparticles is still under investigation.

Figure 1. Mentha longifolia plant leaves [18].

The Labiataes family includes 220 genera of aromatic plants which are widely used for different purposes. *Mentha langifolia L.(M. longifolia),* usually known as a wild mint named Puneh (fig. 1) grows fast and has creeps along an underground rootstock which can grow to 1-2 m tall. Its stem is white or grey-villous, and sometimes sparsely hairy. Various species of Mentha have been used for treating many diseases because of their anti-inflammatory, antiemetic, diaphoretic, antispasmodic and analgesic activities [19].

In this study, Silver nanoparticles were prepared using *Mentha longifolia* extract as a reducing agent without the presence of hazardous and toxic solvents. To our knowledge, green synthesis of AgNPs by this plant for the reduction of silver salts to nanosilver has not been reported so far. The antibacterial activities of AgNPs against gram-positive and gram-negative bacteria were also studied.

MATERIALS AND METHODS

Materials

Silver nitrate was obtained from Merck Company. Fresh and healthy leaves of plant were purchased from local market Tajrish, Iran and authenticated as *M. longifolia* by a botanist. Four standard strain (*Staphylococcus epidermidis* (ATCC 49461), *Streptococcus pneumonia* (ATCC 49619), *Enterobacter aerogenes* (ATCC 13048) and *Salmonella enterica* (ATCC 9270) were prepared from Iran microbial collection. Nutrient agar and Muller Hinton agar were purchased from Merck Company.

Preparation of the extract

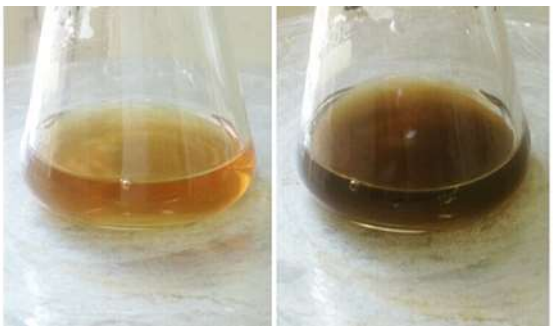
The leaves were first rinsed perfectly with tap water. Thereafter, distilled water was used to remove all the dust and unwanted visible particles. Then, they were cut into small pieces and dried at room temperature. The aqueous extract solutions of plant leaves were collected by weighting 3 gr of finely cut leaves in a beaker containing 97 mL distilled water and then boiling the obtained mixture at 100 $^{\circ}$C for about 15 min. The aqueous extracts were filtered through Whatman paper No. 1 pore size 125mm and then by 25 mm to remove particulate matter and to get clear solutions which were then collected in 250 mL Erlenmeyer flasks and stored in refrigerator (4°C) for further experiments.

Synthesis of silver nanoparticles

Aqueous solution of 10 mM $AgNO_3$ was prepared for the synthesis of silver nanoparticles. 0.5 mL of the $AgNO_3$ aqueous solution was drop-wise added to 7 mL of the resulted aqueous extract solution. The volume was adjusted to 10 mL by de-ionized water and heated with stirrer in dark condition at 150 rpm and 60°C for 2 hours. The pH of the solution was adjusted to 9. The observed color change of reaction mixture from yellow to dark brown indicates the formation of silver nanoparticles. The formation of AgNPs was furthermore confirmed by spectrophotometric analysis. Then, the obtained solution was centrifuged at 10,000 rpm for 10 min, and subsequently was washed three times with deionized water to remove the non-coordinated materials. Finally, The biosynthesized AgNPs were dried at room temperature and stored properly for future use.

Characterization of green synthesis silver nanoparticles visual observation of solution

The color change of solution was recorded through visual observation. The color of the reaction mixture changed from yellow to dark brown which indicated that the silver nanoparticles were yielded (fig. 2).



(a) **(b)**

Figure 2. a) Aqueous solution of leaf extract. **b)** Aqueous solution of Ag nanoparticles in leaf extract after 30 min.

UV-Visible spectral analysis

UV–Vis spectral analysis was done by using a Double Beam spectrophotometer (SHIMADZU Prestige-21, Japan) in a wavelength range between 200 and 800 nm. The reduction of silver ions (Ag+) to silver Nanoparticles was monitored through measuring the UV–Vis spectrum of the reaction medium after diluting a small amount of the sample into deionized water, using deionized water as a reference.

SEM analysis

The green synthesized silver nanoparticles were dried to powder form after centrifugation and were analyzed by KYKY (EM3200) Scanning Electron Microscopy for morphology identification.

Dynamic light scattering

DLS is a useful technique to investigate the size of distribution pattern and average particles size of the biosynthesis silver nanoparticles present in the solution. Silver nanoparticles dispersed in deionized water followed by ultra-sonication and Dynamic light scattering measurements were acquired by a Malvern zeta size analyzer instrument (Malvern3600 Instruments Ltd., Malvern, UK).

FTIR analysis

FTIR analysis was used to detect the possible biomolecules responsible for the reduction of the silver ions into silver nanoparticles. A Nicolet IR100 instrument was used for FTIR spectroscopies.

Antimicrobial Sensitivity Assay

Disk Diffusion

The synthesized silver nanoparticles were tested for antimicrobial activity by disc diffusion method against *Staphylococcus epidermidis*, *Streptococcus pneumonia*, *Enterobacter aerogenes* and *Salmonella enterica*. Approximately 25ml of molten and cooled media (Muller-Hinton agar) was poured into sterile Petri plates. The plates were left for one night at room temperature to consider for any contamination to appear. The organisms were grown in nutrient broth. 0.1ml from 10^{+8} dilution of different bacteria suspension was spread on Muller-Hinton agar plates. Four different concentrations (25%, 50%, 100% and 200% (µg/ml)) of AgNPs were prepared in distilled water. Blank discs had been impregnated with them and placed on the plates. Ampicillin and Gentamicin discs were used for gram-positive bacteria and gram-negative bacteria respectively, as positive controls. Water was used as a negative control. The plates were then incubated at 37°C for 24 hours and the mean diameter of inhibition zones around the disc were measured with a ruler in millimeter.

Minimal inhibitory concentration of silver nanoparticles and minimum bactericidal concentration

MIC of the AgNPs was determined in multi-well culture plates of 96 wells according to standard protocols previously published. 1/100 dilution of Bacterial suspension of 0.5 McFarland's Standard was prepared and 100 µl of it was pipetted into a 96 well-plate with different concentrations in a row of AgNPs (400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19 µg/ml) and row of gentamicin and ampicillin (128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.12, 0.06 µg/ml) for gram-negative bacteria and gram-positive bacteria. 100 µl of broth and 100 µl of suspension were pipetted into a well as a positive control. Also 200 µl broth was pipetted into a well as the medium control. Micro plates were incubated for 24 hr at 37°C. Following 24-hour incubation, the suspension was visualized for clarity. The MBC was recorded as the lowest concentration of the Ag nanoparticles that did not allow a single colony to grow on the agar after further 24 hr incubation.

RESULTS

The plant *Mentha langifolia* belonging to the family Labiatae was selected and used in this report on the synthesis of silver nanoparticles from its leaf extracts. The nanoparticle solution showed maximum absorbance at 340 nm (fig. 3).

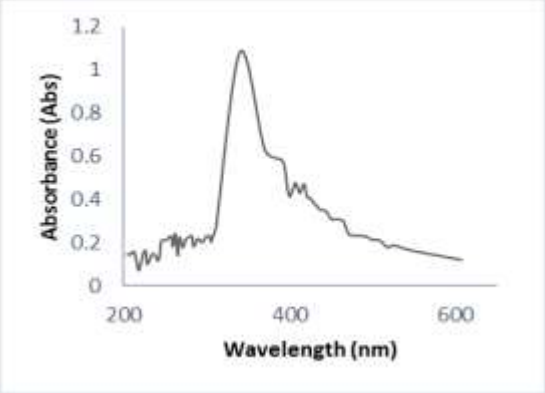


Figure 3. UV–Vis absorption spectrum of silver nanoparticles.

The mean average size of Silver nanoparticles were 21.1 nm as shown in DLS histogram (fig.4).

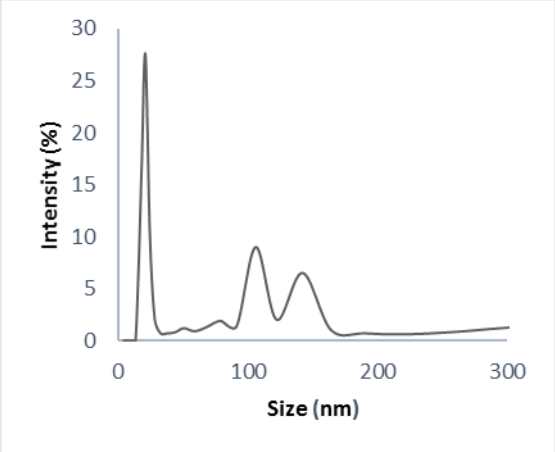
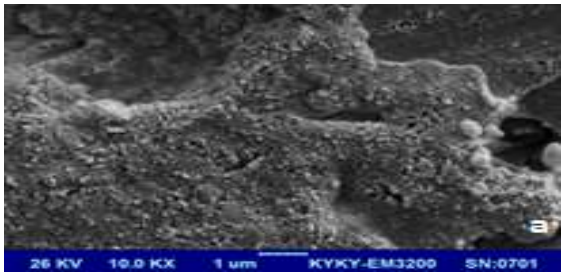


Figure 4. Histogram of size distribution report by intensity.

The images of Scanning Electron Microscopy showed that synthesized AgNPs are spherical in shape. (Fig. 5 a and b)



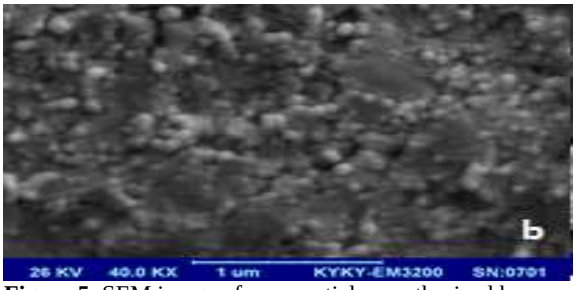


Figure 5. SEM image of nanoparticles synthesized by aqueous extract of *Mentha langifolia* L. with a) 10KX and b) 40KX magnification.

FTIR spectrum indicated the major peak at 3388 cm^{-1} and other peaks were obtained at 2922, 1598, 1404 and 1257 cm^{-1} respectively. The band at 3388 cm^{-1} was assigned to the stretching vibration of OH groups of alcohol. The peak at 2922 cm^{-1} related to the stretching vibration of aliphatic C-H groups available in extract, as well as peaks in 1598 cm^{-1}, 1404 cm^{-1} and 1257 cm^{-1} related to the stretching vibration of alkenes and bending vibration of C-H (fig. 6).

Further study confirmed the antibacterial activities of the synthesized nanoparticles against both Gram positive and negative bacteria. Antibacterial activity of silver nanoparticles against *Enterobacter aerogenes*, *Salmonella enterica*, *S. epidermidis* and *S. pneumonia* were investigated and compared with the standard drug. For *E. aerogenes*, the zone of inhibition of gentamycin (10 µg/ml) was 21±0 mm (Sensitive), while the zones of inhibition of silver nanoparticles (25, 50, 100 µg/ml) were 9.5±0.5, 14.5±0.5 and 17±0 mm respectively. For *Salmonella enterica* the zone of inhibition of gentamycin (10 µg/ml) was 22±0 mm (Sensitive), while the zones of inhibition of silver nanoparticles (25, 50, 100 µg/ml) were 11±1, 16.5±1 and 16.5±1 mm respectively. For *S. epidermidis* the zone of inhibition of ampicillin (10 µg/ml) was 23±0 mm (Sensitive), while the zones of inhibition of silver nanoparticles (25, 50, 100 µg/ml) were 9.5±0.5, 12±0 and 13.5±0.5 mm respectively. For *S. pneumonia* the zone of inhibition of ampicillin (10 µg/ml) was 45±0 mm (Sensitive), while the zones of inhibition of silver nanoparticles (25, 50, 100 µg/ml) were 10.5±0.5, 15±0 and 17±0 mm respectively. Table 1 shows the inhibition zones of antibiotics and 25, 50 and 100 µg/ml of silver nanoparticles. No zone of inhibition was obtained in case of control group.

Among the different concentrations of silver nanoparticles tested, 0/39 and 0/78 µg/ml proved to be MIC and MBC respectively for *Streptococcus pneumonia* and 0/19 and 1/56 µg/ml was recorded as the MIC and MBC for *Staphylococcus epidermidis*. The MIC of ampicillin in both bacteria was 0.06. For *Enterobacter aerogenes*, MIC and MBC were 3/12 and 25 µg/ml respectively and the MIC of gentamycin was 0.06. Also 1/56 µg/ml proved to be MIC and MBC for *Salmonella* while 0.12 µg/ml was reported for the MIC of gentamicin. Also no turbidity was observed in the well of medium control while the positive control well had the opacity which is a sign of bacterial growth.

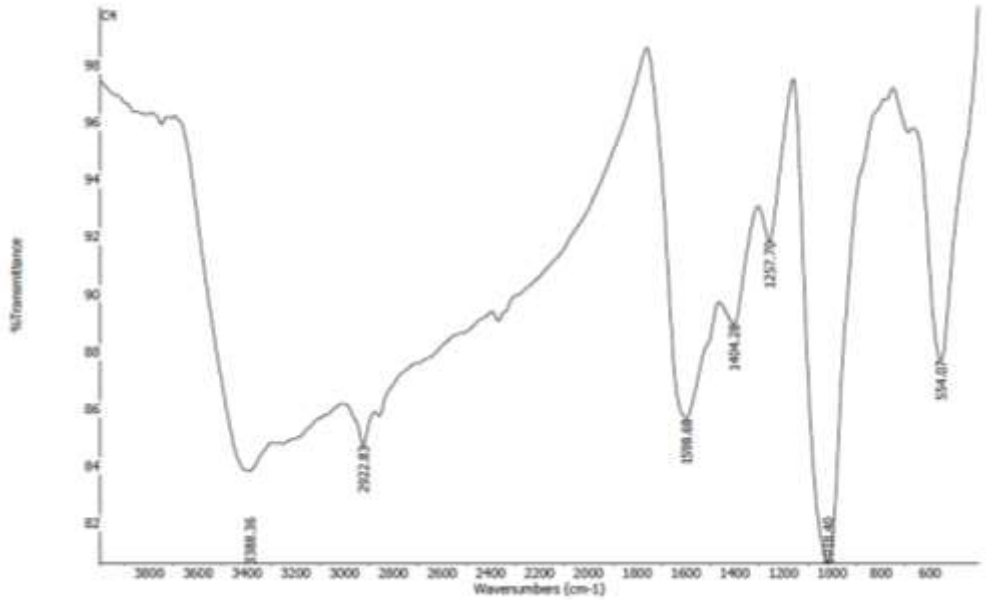


Figure 6. FT-IR spectra for silver nanoparticles.

Table 1. Inhibition zone of antibiotics and 25, 50 and 100 µg/ml of silver nanoparticles.

Microorganisms	Antibiotics	Inhibition zone of antibiotic	Inhibition zone of 100 µg/ml silver nanoparticles	Inhibition zone of 50 µg/ml silver nanoparticles	Inhibition zone of 25 µg/ml silver nanoparticles
Enterobacter aerogenes	**Gentamycin**	22±0	16.5±0.5	16.5±0.5	11±1
Salmonella enterica	**Gentamycin**	21±0	17±0	14.5±0.5	9.5±0.5
Staphylococcus epidermidis	**Ampicillin**	23±0	13.5±0.5	12±0	9.5±0.5
Streptococcus pneumonia	**Ampicillin**	45±0	17±0	15±0	10.5±0.5

DISCUSSION

Nowadays, synthesis of silver nanoparticles using plant extracts is getting more attention [2]. In the present study silver nanoparticles were synthesized using leaves extract of *M. Longifolia*. Earlier reports about the green synthesized silver nanoparticles showed that reduction of silver ion into silver nanoparticles during the reaction could be followed by characteristic color changes [12]. Results of biosynthesis of AgNPs in this study were indicated with color change from yellow to brown in solution during the course of reaction. Velhal et *al.* observed absorbance peak at 290 nm during their study on Taguchi Design for Parameter Optimization of Size-Controlled Synthesis of Silver Nanoparticles [1]. Krishnaraj et al studied on synthesis of silver nanoparticles using *Acalypha indica* leaf extracts was indicated absorbance peak at 420

nm [2]. Shanmugavadivu et al results showed absorbance peak at 370 nm [21]. The results of this study showed maximum absorbance at 340 nm. UV-Visible spectroscopy can be used as a simple and reliable method for studying the stability of nanoparticle solutions. The optical properties of silver nanoparticles change when particles aggregate and the conduction electrons near each particle surface become delocalized and are shared amongst neighboring particles [22]. Further, the shape of silver nanoparticles was studied using SEM showed that silver nanoparticles are spherical in shape and the mean average size of them which is indicated with DLS was 21.1 nm. AgNPs which have been biosynthesized by various plants are in different sizes from about 5 nm to about 350 nm and different shapes (spherical, triangle, pentagon and hexagon and …) [4]. For example Poinern et *al* in 2013 synthesized spherical AgNPs with 10-100 nm size using leaf extract of *Eucalyptus macrocarpa* [20]. The synthesized nanoparticles were characterized by FTIR to identify functional groups and organic compounds in the surface of particles. Peaks obtained were consistent with results of Jayapria & Lalitha in 2013 [11]. Synthesized AgNPs showed effective antibacterial activity against the test bacteria. According to the results, the sensitivity of *Streptococcus pneumoniae* and *enterobacter aerogenes* to silver nanoparticles 100µg / ml was identical (mm 17). *Salmonella enterica* had also almost similar sensitivities (16/5 mm), while *S. epidermidis* had the least sensitivity (13/5 mm). Ahmed et *al* (2016) studied the antibacterial effect of nanoparticles synthesized by the plant *Azadirachta indica* leaf extract on *Staphylococcus aureus* and *Escherichia coli* and 9 mm reported as inhibition zone diameter in both bacteria. The MIC and MBC of the Ag nanoparticles were determined by broth micro dilution method. Results showed that Silver nanoparticles at concentrations 0/19 µg / ml inhibit the growth of *S. epidermidis*. Krishnaraj et *al* in 2010 investigated the antimicrobial activity of silver nanoparticles synthesized by *Acalypha indica* extract on *E.coli* and *V. cholerae*, where the MIC for both were reported as 10 µg / ml.

CONCLUSION

The present study proves the use of medicinal plant for biosynthesis of silver nanoparticles which is rapid, cost effective and environmentally safe with potential use against microbes. This method can be used in different biotechnological applications. The results showed the silver nanoparticles to be effective against the four tested bacterial isolates at 25, 50 and 100 µl concentrations. Results obtained in previous studies [4, 10] also support the antibacterial potential of AgNPs. The zone of bacterial inhibition by AgNPs collected from *Mentha longifolia* leaf extract show maximum inhibition against *Streptococcus pneumonia* and *Enterobacter aerogenes*.

"The authors declare no conflict of interest"

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