Biosynthesis of silver nanoparticles using leaf extract of *Satureja hortensis* treated with NaCl and its antibacterial properties

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**ABSTRACT**

Bio-nanotechnology is a rapidly growing scientific field of producing Nano sized particles by using biological systems. In this study the biosynthesis of silver nanoparticles (Ag NPs) using leaf extract of *Satureja hortensis* treated with different concentration of NaCl (0, 50, 100 and 150 µM) was reported. In addition, the nanoparticles were assessed against two gram-positive and one gram-negative bacteria. The biosynthesized Ag NPs were characterized using FESEM, XRD, UV/vis spectroscopy and related to the size, shape and morphology of the nanoparticles as revealed by FESEM. FTIR spectrum indicated various functional groups effective on Ag NPs biosynthesis. In each treatment, the plant extract showed color change from yellow to brownish-red after Ag NPs biosynthesis. The surface Plasmon resonance found at 450 nm confirmed the formation of Ag NPs. The highest rate of Ag NPs biosynthesis was related to 150 µM treatment. FESEM images revealed relatively spherical shape of Ag NPs. FTIR results expounded the functional groups of plant extract responsible for the bio-reduction of silver ions and their interaction between them. Ag NPs biosynthesis by 150 µM treatment showed the smallest size (2.9 nm) and thus the most antibacterial activity especially against *Bacillus subtilis*. Our results revealed that aromatic bicycle monoterpens have the most effective role in the biosynthesis process in 150 µM treatment. To the best of our knowledge no similar study has been reported.

1. Introduction

Recently, biological methods of nanoparticle bio-synthesis using enzyme [1], microorganism [2], plant or plant extract [3] have been investigated as possible eco-friendly alternatives to chemical and physical procedures. Due to their exclusive properties, Ag NPs have more applications in many areas such as pharmaceutical components [4], chemical sensing and bio-sensing [5], catalysts in chemical reactions [6], optical elements [7], and electrical batteries [8]. Moreover, many of previous studies have shown antibacterial properties of nanoparticles such as silver nanoparticles [9,10]. Although antibiotics are used to kill harmful bacteria nowadays, inappropriate use of these materials has enabled bacteria to increase resistance to them, resulting emergence of resistant bacteria [11]. So to the opinion of the researchers, using of Ag NPs can be a good alternative to antibiotics [12]. Biosynthesis of Ag NPs by plant extracts have already been reported by numerous previous researchers [13–17]. The exact mechanism for this biosynthesis is still unknown. However, there have been several reports that have revealed the presence of biomolecules in plants such as terpenes, phenols and tannins act as effective reducing and capping agents for converting Ag⁺ to Ag⁻ [18–20]. Some environmental and nutritional factors are known to influence the quantity and quality of plants biomolecules such as light, temperature, culture medium compositions and etc. [21]. Salinity is one of the factors that can changes quantity and quality of secondary metabolites and enzyme activity in plants [22]. Many gene networks and metabolic processes in plant are affected by osmotic and ionic components of salt. Such responses depended mainly on the salt concentration, the duration of exposure of the plant to the salt and also innate salt tolerance of the plant [23]. However, numerous of previous studies have shown increase of bioactive compounds in treated plants with different concentration of NaCl [22,24,25]. Science the major biomolecules of plants are involved in nanoparticles biosynthesis [26,27], so the change in the quantity and quality of them can affected the process.

*Satureja hortensis* (*Summer savory*) is annual plant belonging to the lamiaceae family. *S. hortensis* is native to southern Europe and in parts of north America, south and west Asia, including Iran [28]. *S. hortensis* contain a wide range of chemical composition including carvacrol, terpinene cymene, carophyllene and etc. [29]. This plant is used to treat of many diseases such as cramps, muscle aches, nausea,
indigestion and diarrhea [30], also has specified its antifungal, antibacterial and antioxidant properties [31]. Science the medicinal plants contained a high amount of bioactive compounds; there are many reports on the biosynthesis of Ag NPs by using them [32–35]. To the best of our knowledge, there is no report of Ag NPs biosynthesis using aqueous extract of S. hortensis leaf extract treated with different concentration of NaCl. In this study, Ag NPs biosynthesis has been investigated using leaf extract of S. hortensis treated with different concentration of NaCl. Antibacterial property of silver nanoparticles depends on the size of them [36]. So, reduce the size of nanoparticles is one of the reasons for their increased antibacterial properties [37]. Accordingly, the aim of this particular research is providing useful information regarding the mechanism by which nanoparticles are biosynthesized by plants bioactive molecules followed by effective factors on the biosynthesized Ag NPs characteristic such as size, shape, stability and crystallinity. Moreover, we discuss the changes in antibacterial properties of the biosynthesized nanoparticles because of the NaCl treatments, which is different from already reports.

2. Material and methods

2.1. Plant material and growth conditions

Seeds of S. hortensis purchased from the company of Pakanbazr (Isfahan, Iran), were surface sterilized by immersion in 70% ethanol for 2 min and then 5% sodium hypochlorite for 5 min, followed by three times rinsing in sterile water after each step. The seeds were next soaked in a dilute solution of benomyl and transferred to plastic pots containing peat moss (Klasmann-Delima, potgrond H) under equal greenhouse condition at Imam Khomeini International University (Qazvin, Iran) in February 2015. At six leaf stage, plant thinning carried out so that the remaining 20 numbers of the plants in each pot. The remaining plants were subjected to different concentrations of NaCl. Four levels of salinity (0, 50, 100 and 150 μM) were used in this study which were prepared using distilled water. Salt treatment was initiated at six leaf stage every 3 days and continued until early of flowering phase. In each pot, 700 ml saline water was applied and the control plants received 700 ml of distilled water. Every 15 days all of the plants were irrigated with 700 ml distilled water to leach out any accumulated salts in the peat moss. The experiment was organized in a factorial randomized complete block design with three replications. At late vegetative stage (flowering initiation) the leaves were harvested and immediately frozen in liquid nitrogen separately. Then, the samples stored frozen at −80 °C until use.

2.2. Extraction and biosynthesis of Ag NPs

All chemicals were purchased from Merck or Aldrich. Frozen collected leaves were used for preparation of S. hortensis leaf extract. The aqueous extract solutions of plant leaves were prepared separately for each treatment. 10 gr of finely cut plant leaves were boiled in 100 ml of distilled water for 5 min. After cooling, the obtained extract was filtered through Whatman paper No.1 and filtered extract was stored at 4 °C. In order to biosynthesis of Ag NPs, 10 ml of the resulted aqueous extract solution was added to 90 ml of aqueous solution of 1 mM of AgNO₃. The resulted aqueous solution was kept at room temperature with constant rotation. The addition of the plant extract to AgNO₃ aqueous solution leads to turning initially yellowish solution to brownish-red and finally deep brown indicating the formation of Ag NPs.

2.3. Characterization study of biosynthesized Ag NPs

The synthesized nanoparticles indicated by UV–visible spectroscopy, were carried out in a UV–vis spectrophotometer (Labomed, UV-win5, Germany), operating in a wavelength from 300 to 600 nm. Spectroscopy was performed for 150 min with intervals period of 15 min from the beginning time of reaction. To study the effect of salinity on the speed of the biosynthesis of nanoparticles, the charts related to increase of the concentration versus time-dependent were drawn for different treatments and compared. Also for Experimental design and statistical analysis was carried out in the form of factorial experiments according to a completely randomized design and with three replications, and were evaluated statistical analysis related to the effect of salinity on the biosynthesis of silver nanoparticles on the basis of obtained the maximum optical density of the silver nanoparticles by UV–vis spectrophotometer device for 10 times and with 3 replications for each treatment. The data were subjected to ANOVA analysis of variance. Comparison between means to determine significant differences (p ≤ 0.05) was performed using the Duncan’s multiple range test. Correlation between variables was determined with Pearson’s correlation coefficient test, considering a confidence level of 95% (p ≤ 0.05). All statistical analysis was performed using the IBM SPSS software version 21.0. The SOV table and chart were drawn by Microsoft office Excel 2010 software. Bars showing the same letter are not significantly different at p ≤ 0.05.

X-ray powder diffraction patterns of Ag NPs were obtained by X’pert Pro MDI diffractometer made in Holland. The morphology and size of the Ag NPs were determined by Field Emission Scanning Electron Microscopy (HITACHI S-4160, Japan). Fourier Transform Infrared spectroscopy (FTIR) measurements were carried out separately for each treatment to find out the compound responsible for the synthesis of Ag NPs. FTIR spectra of Ag NPs were taken with potassium bromide pellets (1:100) on a Broker Tensor 27 spectrophotometer. The spectra were recorded in the wavenumber range of 400–4000 cm⁻¹ and analyzed by subtracting the spectrum of pure KBr (Potassium Bromide). Average particles size of biosynthesized Ag NPs was determined by Dynamic Light Scattering (DLS) technique (brookhaven, Zetaplus, Canada).

2.4. Assessment of antibacterial activity of Ag NPs

In order to examine the antibacterial activity of the biosynthesized Ag NPs Kirby-Bauer disc diffusion method against bacterial species Bacillus subtilis, Bacillus vallismortis (gram-positive) and Escherichia coli (gram-negative) was used. Luria-Bertani medium was prepared and sterilized at 121 °C. About 25 ml of the medium was transferred aseptically into each sterilized plate. The bacterial strains were spread on the petri plates using pipette. Later, the soaked discs of 6 mm diameter with different samples (AgNO₃, distilled water and the biosynthesized Ag NPs) were placed on agar plates, followed by incubation for 24 h in 37 °C. Zone of inhibition was measured with a meter ruler around each disc in mm and recorded.

3. Results and discussion

3.1. Biosynthesis and characterization Ag NPs

Biosynthesis of Ag NPs using 1 mM AgNO₃ was added to leaf extract of the treated plants is shown in Fig. 1. Color change from yellow to brownish-red after addition of AgNO₃ and stirring at room temperature was due to excitation of the surface Plasmon resonance [38,39].

The Characteristics silver surface Plasmon resonance (SPR) bands were detected at the wavelength of 450 nm (Fig. 2) that is in good agreement with the reported spectra in the literature for the silver nanoparticles [40]. The results revealed that incubation time affects the Ag NPs formation [41]. As the time duration increased, the nanoparticle synthesis also increased. Ag NPs biosynthesis was initiated nearly within 15 min. The completion of Ag NPs biosynthesis was reduced after 2.5 h as identified in Fig. 2. After this time, the precipitation of Ag NPs occurred due to the instability of the nanoparticles. Agglomeration of Ag NPs showed the larger size of nanoparticles. So the optimum time duration for the formation of Ag NPs was 2.5 h. It was also found that the treatments had affected processes of Ag NPs.
biosynthesis. Our obtained results showed that the highest and lowest rate of Ag NPs biosynthesis was related to 100 and 150 μM of NaCl treatments, respectively (Fig. 2).

The speed of biosynthesis process was almost identical initial 45 min in all of the treatments (Fig. 3). After this time speed of the Ag NPs biosynthesis was increased in 100 μM treatment and reduced in 50 μM treatment in comparison to control.

Statistically analysis revealed that the salinity treatments and time duration have significant different on Ag NPs biosynthesis process (Table 1). The highest concentration of Ag NPs was observed in 100 μM treatment while the lowest was recorded for the 50 μM treatment (Fig. 4). There was no significant difference between 100 μM treatments compared to the control. However, Ag NPs biosynthesis significantly reduced at 50 and 150 μM treatments compared to the control.

XRD pattern obtained for the Ag NPs (Fig. 5) shows that the crystalline structure of silver is face centered cubic. In XRD, silver has similar diffraction profile with intense peaks at 2θ of 38°, 44°, 64° and 77°, respectively. This indicates that the synthesized Ag NPs by using leaf extract of *S. hortensis* had crystalline nature similar results have been reported by previous researchers by using *Cissus quadrangularis* and *Erythrina indica* extracts [20,41].

FTIR was carried out to identify the possible potential functional groups for biosynthesis and stabilizing of Ag NPs using *S. hortensis* leaf extract treated with different concentration of NaCl. The FTIR spectra of aqueous leaf extract and the biosynthesized Ag NPs were analyzed and are shown in Fig. 6. FTIR spectra showed that leaf extract alone of the treated plants were different from the control. These results show that the different concentration of NaCl might have considerable influence on biochemical constituents of the samples. The strong peaks at 3379 Cm⁻¹ to 3402 Cm⁻¹ in all the samples is assigned to the OH group.
from alcohols/phenols \[41\] NH stretching of Amide A \[42\]. The weak peaks at 2925 Cm\(^{-1}\) to 2927 Cm\(^{-1}\) indicate CH stretch of alkanes \[41\]. The peaks at 2140 Cm\(^{-1}\) to 2243 Cm\(^{-1}\) represent C≡C and C≡N groups in aliphatic/aromatic compounds \[43\]. The peaks at 1608 Cm\(^{-1}\) to 1613 Cm\(^{-1}\) indicate C=C group from aromatic compounds \[44\]. Another report has assigned these peaks show the amid I (C=O group) protein \[42\]. The peaks at 1404 Cm\(^{-1}\) to 1406 Cm\(^{-1}\) indicating the presence of CH\(_2\) group from carbohydrates \[45\]. The peaks at 1121 Cm\(^{-1}\) to 1144 Cm\(^{-1}\) correspond to the C-O in plane bending of alkanes, alcohols, carboxylic acids, esters and ethers. Finally, the peak at 843 Cm\(^{-1}\) may represent CH group from aromatic bicyclic monoterpenes \[46\]. In plants, the most sensitive mechanism to abiotic stress is photosynthesis, and metabolic adjustment is one of adaptation processes in response to environmental stresses \[23\].

Different types of plants can react differently under salinity stress. The effect of the salinity on plants arises as a result of interaction of morphological, physiological and biochemical processes available in the plant \[47\].

Given the complex biological matrix, the changes of a specific functional group cannot be assigned to a particular molecule in the cells. However, assessment of changes in these groups can reflect somewhat the changes in chemical composition and followed by metabolic processes \[48\]. Results of this study revealed that treatment with 100 \(\mu\)M salinity caused relative increase in bioactive compounds including phenols, alcohols, proteins, alkanes, lipids and aromatic compounds compared to control. These results are in accordance with those of several previous similar investigation \[22,49\]. In addition, there was a gradual increase in the components such as carbohydrates, alcohols, ethers, esters, carboxylic acid and alkaloids under the increasing salinity concentration. These results of our study are consistent with the observations by Gengmao et al., who showed that salinity treatment increases secondary metabolites in sunflower \[22\]. Although the differences among the peaks of nitrile compound are negligible, the minimum absorption occurred in control followed by 150, 50 and 100 \(\mu\)M cyanide compounds production in plants considered as a chemical stress response \[50\]. In general, among all primary metabolites: amino acids, sugars and sugar alcohols are the most important metabolite which concentration in plant tissues is affected by stress \[51\]. On the other hand, variations occurring in the secondary metabolites under salinity stress are thought to be associated with the plant defense mechanism against harmful effects of salt \[52\].

The FESEM images show the morphological character of Ag NPs synthesized by using leaf extract of \textit{S. hortensis} cultured under different treatments of NaCl (Fig. 7). Most of the biosynthesized Ag NPs were nearly spherical in shape. However, few Ag NPs with triangle shape were observed in 50 and 100 \(\mu\)M treatments. According to the previous reports NPs synthesized by plants extracts show different shapes (spherical, hexagonal, triangle) which depends on the extract chemical compositions, concentration, and pH of media \[33,53,54\].

The particle size distribution study revealed that the average size of the biosynthesized Ag NPs were 3.4, 3.2, 3.2 and 2.9 nm for control, 50, 100 and 150 \(\mu\)M treatments, respectively (Fig. 8). In addition, DLS results showed that Ag NPs average size reduced with increase of salinity at the treatments as it was 2.9 nm at 150 \(\mu\)M treatment.

The comparison of FTIR spectrum between the aqueous leaf extract and prepared Ag NPs in each treatments, revealed changes in the position and adsorption bands also. Shifting of these peaks indicates the

![Fig. 3. Biosynthesis reaction rate by leaf extract of different treatment.](image1)

![Fig. 4. Mean comparison for effect of different level of salinity on the Optical Density of silver nanoparticles at the 5% probability using Duncan’s Multiple Range Test.](image2)

![Fig. 5. XRD pattern obtained for the Ag NPs biosynthesized.](image3)

![Table 1](image4)
possible involvement of the assessed functional groups of leaf extract in Ag NPs biosynthesis and also the effect of treatments on this process (Fig. 6). The phytochemical analysis of S. hortensis as an aromatic plant reveals the presence of hydrocarbons, phenols/polyphenols, flavonoids/flavonoids, alkaloids and polypeptides [29,55,56]. FTIR spectroscopy revealed the involvement of carboxyl, hydroxyl and amid groups in NPs biosynthesize [57]. In addition, flavonoids and phenolic compounds present in the leaf extract are powerful reducing agents which may be suggestive for the biosynthesis Ag NPs of Ag ions [10,41]. The carboxylate group present in proteins can play as surfactant to attach on the surface of biosynthesized NPs and results in Ag NPs stabilization [10]. In fact, the plant extracts maybe plays dual role as reducing and stabilizing agents of biosynthesized NPs. The FTIR spectrum of different treatments in the prepared Ag NPs was almost the same except for the minor changes in the position and absorption intensity of the bands.

Generally, in FTIR spectrum, intensity of a transmittance peak is in contrast with its absorbance peak intensity. In other words, enhancement of a functional groups transmittance peak intensity reveals decrease of its absorbance peak and there by concentration of it in the solution. On the other hand, reduce the concentration of any functional group in the solution can reveals its more effective role in the NPs biosynthesis process. Accordingly, our results showed that OH and NH adsorption increased with increase in NaCl concentration in the treatments up to 150 mM. This result reveals negative effect of salinity on contribution the compounds such as alcohols, phenols and also proteins on Ag NPs biosynthesis. The negative effects of salinity for alkanes (~ 2925 Cm^{-1}), nitrile and cyanide groups (2240 Cm^{-1}) were negligible. Given that the lowest absorption of C=C, CH₃ and CO groups were observed in 50 mM treatment, it seems likely that aromatic compounds, carbohydrates, alcohols, carboxylic acids, esters and ethers have important effects on the Ag NPs biosynthesis process in 50 mM treatment. The relatively low absorption of = CH group in 150 mM could suggest that aromatic bicyclic monoterpenes in this treatment have effective function on process of Ag NPs biosynthesis.

3.2. Antimicrobial efficacy

Apparently, treatment with 150 mM of salinity enhanced the amount of some bioactive molecules such as aromatic bicyclic monoterpenes in the plant extract and Ag NPs biosynthesis process followed by with the smallest size (2.9 nm). So, the highest antibacterial property of the biosynthesized Ag NPs was related to 150 mM treatment of salinity. The Biosynthesized Ag NPs have shown antibacterial activity against all of the tested bacteria (Fig. 9). Antibacterial studies elucidate that B. subtilis and B. vallismortis are more sensitive to Ag NPs with zone of inhibition of 8 and 7.5 mm respectively, whereas E. coli has shown least zone of inhibition of 7 mm. Generally the gram-positive bacteria are more susceptible than gram-negative bacteria due to having only an outer peptidoglycan layer which is not an effective permeability barrier while the gram-negative bacteria possesses an outer phospholipidic membrane carrying the structural lipopolysaccharide compound. Thus, the cell wall is impermeable to drug constituent in gram negative
bacteria because of the presence of multilayered of peptidoglycan and phospholipidic bilayer [58].

The mechanism of antibacterial activity of Ag NPs was not understood clearly. Most likely, antibacterial activity of Ag NPs is due to mechanisms other than associated with antibiotics [59]. In general, the antibacterial property of silver nanoparticles is mainly due to the release of silver cations from Ag NPs that acts as reservoir for them [10]. Anyway some of the researchers explained the possible mechanisms of antibacterial activity of silver nanoparticles including: 1- Ag NPs attach with the cell wall of bacteria by electrostatic attraction and disrupt the cell permeability and respiration due to the generation of the reactive oxygen species. 2- Ag NPs bind with thiol groups of DNA and RNA and affect the protein synthesis of the bacteria [51,52]. 3- Silver nanoparticles form the pits on the cell surface and induce the proton leakage resulting in cell death [55].

The antimicrobial activity of silver nanoparticles depends on the...
size and shape of the nanoparticles. Smaller particles having the larger surface area available for interaction will give more bactericidal effect than the larger particles and since they easily penetrate into the cell [37,60–63].

4. Conclusions

The present study reports biosynthesis of Ag NPs using S. hortensis leaf extract cultured under different treatments of salinity. The results revealed that NaCl treatment affected active biomolecules of the plants and followed by characteristics of NPs biosynthesized by them such as size, shape and morphology. FESEM image revealed that the Ag NPs were predominantly spherical in shape ranged from 2.9 to 3.4 nm. The relative size of the Ag NPs was decreased with increasing salinity concentration so that 150 μM treatment showed the smallest size of the Ag NPs (2.9 nm). FTIR results revealed aromatic bicyclic monoterpenes concentration so that 150 μM treatment showed the smallest size of the Ag NPs (2.9 nm). FTIR results revealed aromatic bicyclic monoterpenes in 150 μM treatment have the highest effective function on process of Ag NPs biosynthesis. Synthesized Ag NPs in 150 μM treatment exhibited the most antibacterial activity because of the smallest size in this regard, B. subtilis showed the most sensitivity. Conclusively, the utilization of treated plant extract for the synthesis of silver nanoparticles. Overall, our preliminary results may open an interesting area for further investigations such as create changes in culture condition of the plants using different treatments and evaluation their relation with the characteristics of the biosynthesized nanoparticles. Certainly, nanoparticle production with desired characteristics can be a good alternative to antibiotics in various medical applications. In addition, more homogeneous Ag NPs have the potential for use in the agricultural, cosmetics and the related other industries. It seems different treatments affect components of the plant extract and nanoparticle biosynthesis process followed by, although more investigations should take place in this field.

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References


