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RESEARCH PAPER

Antimicrobial effects of green synthesized silver nanoparticles using Melissa officinalis grown under in vitro condition

Saba Pirtarighat [1], Maryam Ghannadnia [1]*, Saeid Baghshahi [2]

[1]Department of Biotechnology, Imam Khomeini International University (IKIU), Qazvin, Iran
[2]Department of Materials Engineering, Imam Khomeini International University (IKIU), Qazvin, Iran

ABSTRACT

Objective(s): To evaluate the biosynthesis of Ag NPs using plant extract of Melissa officinalis (at the eight leaf stage) grown under in vitro (controlled) condition for the first time.

Materials and Methods: Biosynthesis of Ag NPs using plant extract was carried out and formation of Ag NPs confirmed by UV-Visible spectroscopy, X-ray diffraction (XRD), Field Emission Scanning Electron Microscope (FESEM) and Dynamic Light Scattering (DLS). The functional groups of compounds adsorbed on the Ag NPs were identified using Fourier Transform Infrared Spectroscop (FTIR) studies. The antibacterial activity of the Ag NPs was investigated by agar disc diffusion method.

Results: The plant extract showed color change in extract from yellow to brown after formation of Ag NPs. The surface Plasmon resonance found at 450 nm confirmed the formation of Ag NPs. FESEM images revealed relatively spherical- shaped of Ag NPs. The biosynthesized Ag NPs were crystalline in nature with mean diameter about 34.64 nm. FTIR results expounded the functional groups of plant extract responsible for the bio-reduction of silver ions and their interaction between them. The obtained nanoparticles showed good inhibitory activity against the Gram positive and Gram negative bacteria.

Conclusion: These results suggested that with changes in plants culture condition it may be possible to obtain nanoparticles with desired characteristics.

Keywords: *Bactericidal effects, Biosynthesis, In vitro culture, Melissa officinalis, silver nanoparticles*

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INTRODUCTION

Bionanotechnology is a subset of nanotechnology: atom level engineering and manufacturing using biological precedents for guidance. It is also closely joined to biotechnology but adds the ability to design and modify the atomic-level details of the objects created [1]. In the last decades, biosynthesis of nanoparticles using bio nano technological methods has found particular importance [2]. Synthesis of metal nanoparticles (NPs) and their characterization has been an emerging field of nanotechnology since the past few decades because of their unique properties and potential in the fields of physics, chemistry, biology and medicine [3]. Generally, nanomaterials are synthesized using either chemical or physical methods [4] that usually apply toxic chemicals and generate hazardous by products and need specialized equipment [5]. Biological methods have emerged as an alternative methods to the conventional ones for synthesis of NPs. Synthesis of inorganic NPs by biological systems makes nanoparticles more biocompatible and eco-friendly [6]. Moreover, biological methods for NPs synthesis is low-cost than the other methods [4].

Recently, silver nanoparticles (Ag NPs) have emerge as encouraging bactericidal agents, and have been widely applied in various fields of industry such as cosmetic, textile, medical and food industry [7, 8]. Ag NPs are being extensively synthesized using plant extracts, although very little is known about the exact mechanism for this biogenic synthesis. However, recent researches have revealed that the biologically active

* *Corresponding Author Email: ghannadnia_ma@yahoo.com*
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compounds of plant extracts such as phenol, protein and flavonoids play an important role in the reduction of metals ions and capping of the biosynthesized nanoparticles [9, 10].

In vitro culture techniques represent an excellent option for the study and conservation of rare, threatened or endangered medicinal plants. In addition, these techniques reveal an excellent option for the investigation of the effects of culture condition on secondary metabolites in medicinal plants under controlled conditions. Many different in vitro methods have been used for increased biosynthesis and the accumulation of biomolecules in plant grown under in vitro condition. A wide range of environmental and nutritional factors are known to influence the biosynthetic pathways of secondary metabolites such as light, temperature and media composition [11]. Most probably these biomolecules of the plant extract can act as biosynthesizing agent for nanoparticles.

Melissa officinalis L. is a valuable medicinal plant in herbal medicine, native to the eastern Mediterranean Region and western Asia. Citral (geranial and neral), citronellal and geraniol are main composition in the essential oil of the plant. M. officinalis has been traditionally used for different medical purposes as tonic, antispasmodic, carminative, diaphoretic, surgical dressing for wounds, sedative-hypnotic strengthening the memory and relief of stress induced headache, but in modern pharmacology is value in the management of mild to moderate Alzheimer's, against migraine and rheumatism, antitumel and antioxidant activities [12].

Thus far, the bio-produced nanomaterials were usually poly-size and poly-shape [13]. Synthesis of NPs to have a better control over particle size, distribution, morphology, purity, quantity and quality by employing environment friendly economical processes has always been a challenge for the researchers [14]. Furthermore, fabrication of NPs with controllable size and shape is a requirement for the goals of many particle applications. To the best of our knowledge, there is no report of Ag NPs biosynthesis by utilizing of aqueous extract of M. officinalis grown under cotrolled conditions (in vitro). Thus this study aims at biosynthesis of Ag NPs using M. officinalis extract grown in Murashige and Skoog (MS) medium and investigation of their antibacterial effects for the first time. In this way, by altering the culture condition, biosynthesis of nanoparticles with controlled size and shape will be possible.

MATERIALS AND METHODS

Plant culture and growth conditions

Seeds of M. officinalis purchased from the Pakanbazr company (Isfahan, Iran), were surfaced sterilized by immersion 70% ethanol for 1 min, and then 2.5 % sodium hypochlorite for 15 min followed by three times rising in sterile water after each step. The seeds were placed in petri dishes contained an autoclaved basal medium of murashige and Skoog's (MS) mineral salts, 3% sucrose and 4.5% agar (3 replicates). The pH of the medium was adjusted to 5.8 prior to autoclaving. After germination, the seedlings were transferred into jars containing the same medium. All cultures were kept under equal growth chamber conditions (period of day/night: 16/8, at 25±2 ˚C). The harvested samples protected from light and stored in capped bottles.

Extraction of plant materials

All the chemicals and reagents were purchased from Merk or Aldrich. The dried samples were ground to fine powder and stored at 37 ˚C separately. 2 g of each sample was weighted and dissolved in 10 ml doubled-distilled water and was boiled for 5 min. After cooling the aqueous extracts were filtered through Whatman No.1 filter papers. Filtered extracts were stored at 4 ˚C for further studies.

Biosynthesis of Ag NPs

1 ml of M. officinalis extract was added into the 9 ml of aqueous of 1 mM of silver nitrate and kept at room temperature with constant rotation. The color change of the solution revealed the formation of Ag NPs.

Characterization of the synthesized Ag NPs

Ag NPs were characterized by following measurements. An UVD 3200, LABOMED INC UV-VIS spectrophotometer was used for the spectrometric analysis to confirm silver nanoparticle formation. X-ray powder diffraction patterns of Ag NPs were obtained by Equinox 3000 diffractometer. Field Emission Scanning Electron Microscopy (FESEM, HITACHI, S-4160) was used for visualization of Ag NPs. Particles size distributions of Ag NPs were determined using

Dynamic Light Scattering Malvern-Zetasizer (Nno-z 590). FTIR spectra of Ag NPs were taken with potassium bromide (1:80) on a Bruker Tensor 27 spectrophotometer. The pellets were used for FTIR analysis in the range of 500-4000 cm^{-1}.

Assays for bactericidal activity

The antibacterial activity of the biosynthesized Ag NPs was analyzed by using disc diffusion method against bacterial species Bacillus subtilis supsp. inaquosorum strain (accession number: M59 KP406766), Bacillus vallismortis strain (accession number: M92 KP406765) and Escherchia coli (PTCC 1276). Luria-Bertani medium was prepared and sterilized at 121 ˚C. About 25 ml of the medium was transferred aseptically into each sterilized petri plates. The bacterial strains were spread on the petri plates using pipette. Later, the soaked discs of 6 mm diameter with different samples (distilled water, plant extract and the biosynthesized Ag NPs) were placed on agar plates and the plates were incubated at 37 ˚C for 18 h. Zone of inhibition was measured with a meter ruler around each disc in mm and recorded.

RESULTS AND DISCUSSION

Biosynthesis and characterization of the biosynthesized AgNPs

The fresh extract of M. officinalis was yellowish-green in color. However, after addition of $AgNO_3$ and stirring for 1 h at room temperature, the solution color change into purple red (Fig. 1) which indicated the formation of Ag NPs [15]. The change in color is an attribute to excitation of surface plasmon vibration of
Ag NPs [16].

Fig. 2 displays UV-Vis absorption spectrum of Ag NPs. Though the plasmon band is broad due to the presence of components in M. officinalis extract which are also being read in the spectrophotometric range. The formation of Ag NPs was monitored through UV-visible spectroscopy (excitation band near 450 nm for silver) with increase in absorbance upon increasing time until 360 min. According to Mie theory, only a single SPR band is expected in the absorption spectra of spherical nanoparticles, the number of peaks increases as anisotropy increases [17]. The Ag NPs biosynthesis process was completed in 6 h of initiation. This was due to the increasing concentration of Ag NPs as well as the particles growth in size [18]. The intensity of color was increased when the time of incubation was increased. At the end of biosynthesis process, there was a dark purple color of Ag NPs that settled at the bottom conical flask.

FTIR was carried out to identify the possible bio-molecules responsible for the reduction of Ag ions and capping of Ag NPs biosynthesized by M. officinalis extract. The FTIR spectra of aqueous leaf extract (Fig. 3a) and the biosynthesized Ag NPs (Fig. 3b) were analyzed and are shown in Fig. 3. The images revealed strong peaks at (3414 and 3423), (2928 and 2926), (2241 and 2239), (2138.65 and 2138.78), (2039 and -), (1635 and 1632), (1124 and 1150), (674 and 675) and (604 and 603) Cm^{-1} also the peak at 1410.67 Cm^{-1} disappeared in Ag NO_3 treated M. officinalis extract. The strong band at 3414 and 3423 Cm^{-1} is assigned to the OH group from alcohols /phenols [19] and NH stretching of amid A [20].

The peaks at 2928 and 2926 Cm^{-1} indicate CH stretch of alkanes [19]. The peaks at 2039 to 2241 Cm^{-1} represent C≡C and C≡N groups in aliphatic/ aromatic compounds [21]. The sharp peaks at 1635 and 1632 Cm^{-1} indicate C=C group from aromatic compounds [22]. Some of the other research has assigned these peaks show

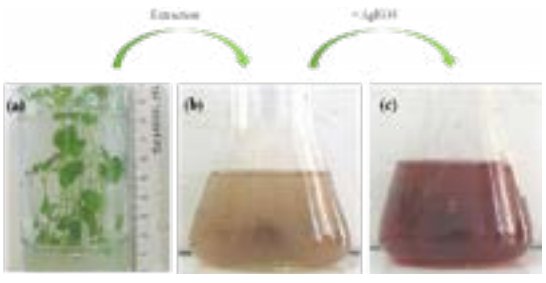

Fig. 1. color change in solution, a) M. officinalis cultured under in vitrio conditions b) 5 minutes, and c) 6 hours after reaction

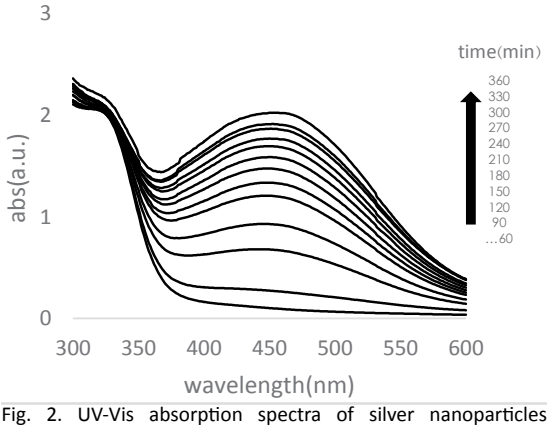

Fig. 2. UV-Vis absorption spectra of silver nanoparticles synthesized from M. officinalis leaf extract at different times

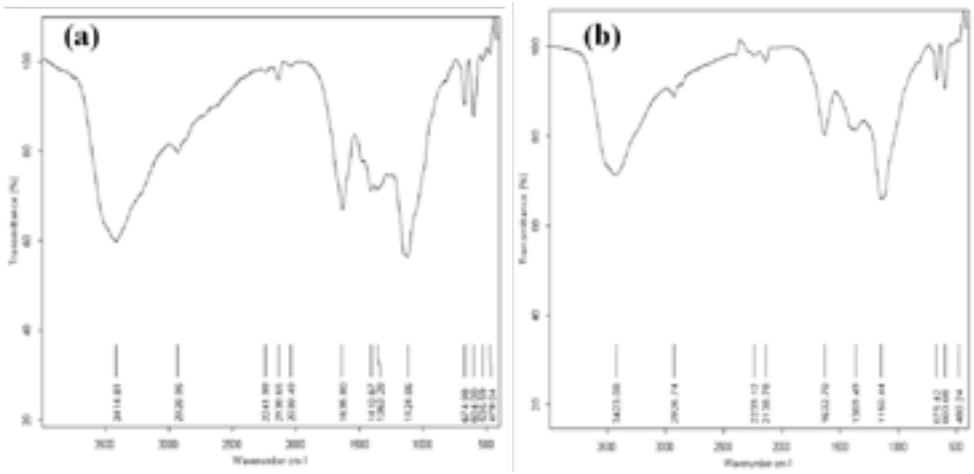

Fig. 3. FTIR spectra of (a) M. officinalis leaf extract and (b) synthesized Ag NPs from M. officinalis leaf extract

the amid I (C=O group) protein [20]. The peak at 1363 to 1400 Cm^{-1} indicating the presence of CH_3 group from carbohydrates [23]. The sharp peaks at 1124 and 1150 Cm^{-1} correspond to the C-O in-plane bending of alkanes, alcohols, carboxylic acid, esters and ethers. Finally, the peaks at 603 to 675 Cm^{-1} may represent =CH group from aromatic bicyclic monoterpenes [24].

The comparison of FTIR spectrum between the plant extract and prepared Ag NPs evidenced only minor changes in the position and absorption bands also. Shifting of these peaks indicates the possible involvement of all functional groups of plant extract in nanoparticle synthesis. Phyto-chemical screening of the lamiaceae family extract showed the presence phenolic content, glycosides, terpenoids, tannins, flavonoids as chemical constituents [25]. The flavonoids present in the extract are powerful reducing agents which may be suggestive for the formation of Ag NPs by reduction of silver nitrate. On the other hand, it is well known that proteins can bind to metal nanoparticles through free carboxylate group and act as surfactant to attach on the surface of nanoparticles and results in Ag NPs stabilization [26]. It can be said that, M. officinalis extract is likely to play dual role as reducing and stabilizing agents of Ag NPs. It seems that the functional groups such as OH and CO groups present in the sample play an important role for biosynthesis of Ag NPs [27].

XRD of biosynthesized Ag NPs is confirmed by the characteristic peak observed in Fig. 4. Minor but broad peaks at lower 2 θ values can be assigned to the organic content of the M. officinalis extract. The silver nanoparticles had a similar

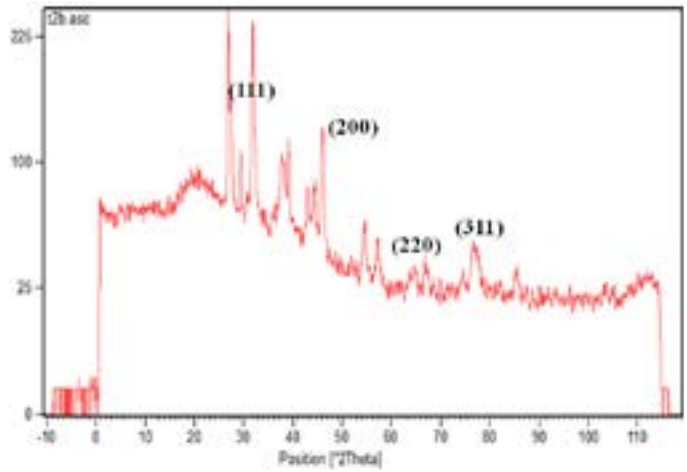

Fig. 4. X-ray diffraction profile of biosynthesized Ag NPs in M. officinalis extract

diffraction profile and the XRD peaks at 2θ of 37.9 °, 45.1 °, 65.1 ° and 76.8 ° could be attributed to the 111, 200, 220, and 311 crystallographic planes of the face-centered cubic (fcc) silver crystals, respectively [28].In order to study the morphology and size of biosynthesized Ag NPs, FESEM images were recorded (Fig. 5). FESEM images enumerate the formation of homogenous and relatively spherical nanoparticles with average particle size ~ 34.64 nm. The average size distribution of Ag NPs were found to be 35 nm and well dispersed in the solution (Fig. 6).

Antibacterial activity of Ag NPs

Bio-synthesized Ag NPs have shown bactericidal activity against all of the tested bacteria (Fig. 7). The zone of inhibition in millimeters of respective discs loaded with the biosynthesized Ag NPs, M. officinalis extract and distilled water as control was determined. Antibacterial studies elucidate that B. subtilis and B. vallismortis are more sensitive to Ag NPs and it has been eliminated potentially with zone of inhibition of 15 mm whereas E. coli has shown least zone of inhibition of 12 mm. in general, the antibacterial property

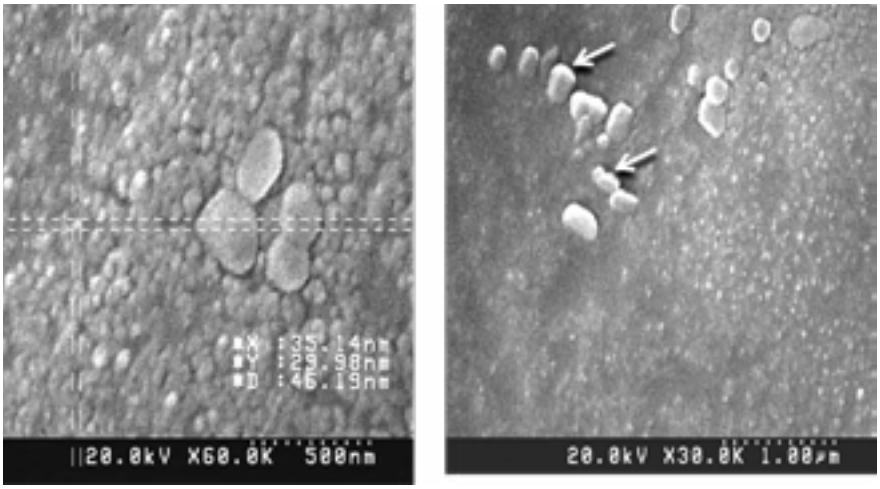

Fig. 5. FESEM images of biosynthesized Ag NPs, the arrows show thin layer of some capping organic material from plant leaf extract

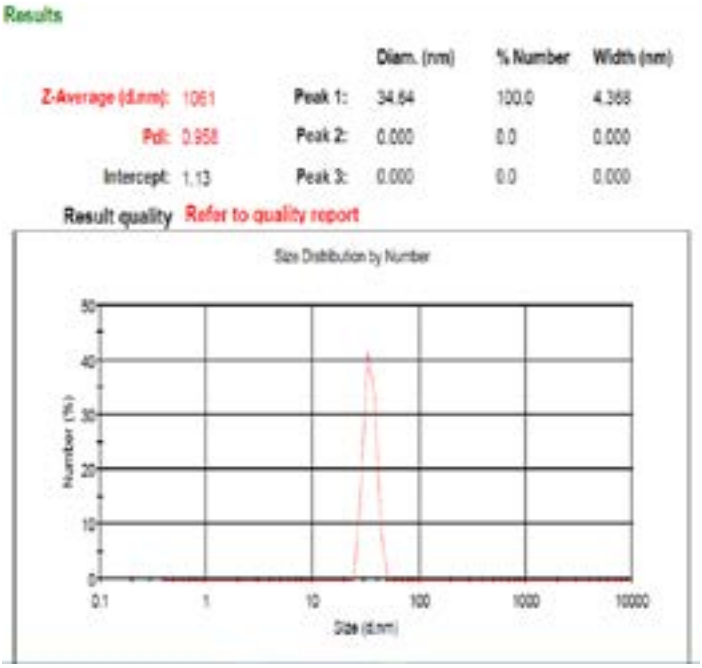

Fig. 6. Particle diameter analysis of silver nanoparticles

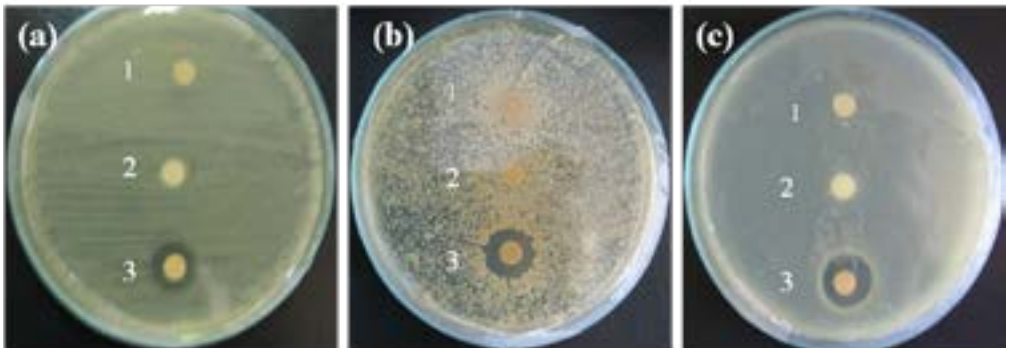

Fig. 7. Antibacterial activity of Ag NPs determined by disk-diffusion technique, (a) E. coli, (b) B. subtilis, (c) B. vallismortis, 1. M. officinalis extract, 2. distilled water as control and 3. Ag NPs biosynthesized by M

of Ag NPs is mainly due to the release of silver cations from Ag NPs that acts as reservoir for them [26]. Most likely, antibacterial activity of Ag NPs is due to mechanisms other than associated with antibiotics [29]. Specific mechanisms for antibacterial properties of Ag NPs are reported. some of putative mechanisms include attach to the bacteria cell wall, destabilizing the outer membrane and splitting of plasma membrane thereby causing reduction of intracellular ATP [26, 28], high affinity to react with sulfur or phosphorus containing biomolecules in the cell [28], blocking bacteria respiration [30], and interacting with DNA and losing its ability to replicate [31].

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 [32].

CONCLUSION

Silver nanoparticles were prepared by using M. officinalis extract grown under in vitro condition. The biosynthesized Ag NPs were relatively spherical in shape with average particle size ~ 34.64 nm. Results of FTIR revealed the possible bio-molecules of the plant extract responsible for the reduction of Ag ions and capping of the biosynthesized Ag NPs including proteins, phenolic compounds, glycosides, terpenoids, tannins and flavonoids. Antibacterial studies elucidate that the gram positive bacteria are more sensitive to the gram negative. Some of plant biomolecules are responsible for the formation of nanoparticles [33]. On the other hand, the production of secondary metabolites in plants is affected by different environmental factors. In vitro plant culture and controlled environment production systems offer an excellent opportunity for the production of specific bioactive molecules on them [34]. Due to the unknown exact mechanism of nanoparticle biosynthesis by plants [26, 35], it seems that study of the relation between change in culture condition, secondary metabolites production in the cultured plants and followed by characteristics of the biosynthesized nanoparticles may reveals some mechanisms involved in this process. Thus, our preliminary results may open an interesting area for further work such as create changes in culture condition of the plants and evaluation their relation with the characrteristics of the biosynthesized nanoparticles. Certainly, more homogeneous silver bio-nanoparticles can be a good alternative to antibiotics in various medical applications.

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CONFLICT OF INTEREST

The authors confirm that this article content has not any conflicts of interest.

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