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Original Article

Phyto-mediated silver nanoparticles via *Melissa officinalis* aqueous and methanolic extracts: synthesis, characterization and biological properties against infectious bacterial strains

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ABSTRACT

Background: The present study was aimed to examine the influence of extraction method on the morphology, physico-chemical characteristics and antimicrobial properties of AgNPs synthesized from Melissa officinalis.
Methods: AgNPs were prepared by two extraction methods. The properties of obtained nanoparticles were characterized by SEM, UV-Vis, XRD and FTIR techniques. Accordingly, SEM images showed different shape, size and morphology of AgNPs using two different extracts types.
Results: The UV-Vis spectroscopy confirmed the formation of AgNPs by observing a distinct surface Plasmon resonance band around 450 nm. SEM images showed different shape, size and morphology of AgNPs using two different extracts types. AgNPs derived from the aqueous extract were rod-shaped with a diameter of 19 to 40 nm whereas spherical particles were synthesized by the methanolic extract found smaller with size distribution ranging from 13 to 35 nm. The XRD pattern indicated that AgNPs formed by the reduction of Ag+ ions using the methanolic extract were crystal-like in nature. The functional groups of the methanolic extract involved in synthesis and stabilization of AgNPs were investigated by FTIR. In addition, AgNPs containing methanolic extract showed higher antioxidant activity.
Conclusion: In this respect, the biosynthesized particles showed potential bio-functionality through which higher antioxidant property and antimicrobial profile against infectious strains were observed. By and large, these results have shown that

using medicinal plants for biosynthesis of AgNPs can boost the quality of prepared particles for exhibiting trustworthy functionalities.

Keywords: Bio-reducing agent; green synthesis; plant extract.

1. Introduction

Nanomaterials are being used as smart material in almost every field of human activities. Among all types of nanoparticles, silver nanoparticles (AgNPs**)** have been used for a wide variety of applications in human life [1]. AgNPs have more application in the medical field as antimicrobials and sterilizers. The antimicrobial property of AgNPs is well known and it is mediated by the silver cations released from the $AgNO_3$ (silver nitrate) [2]. These particles have been synthesized by different approaches including chemical [1] and biological methods from microorganisms and plants [3].

The chemical methods often generate highly toxic compounds that remain adsorbed on the particle surface and pose adverse effects on human health. This warrants the use of safe, biocompatible and eco-friendly green biological methods for the synthesis of AgNPs [4, 5]. The green synthesis is based on the use of non-toxic and eco-friendly ingredients moreover; it is cost-effective, low energy consumption, less complex and less time-consuming process [6].

Medicinal plants have important roles in the development of biochemical methods due to the existence of a wide variety of secondary metabolites that presented within their tissues [7, 8]. Since ancient times, human being has been used medicinal plants for different proposes so that until now the methods for processing of medicinal plants for extraction of trustworthy metabolites have been advanced [7, 9]. In this respect, because of the development of plant-based biosynthesis of nanoparticles, a wide range of plant resources have been explored by researchers to synthesize NPs such as *Berberis vulgaris* (Berberidaceae) [10], *Coriandrum sativum* (Apiaceae) [11], *Vaccinium arctostaphylos* L. (Ericaceae) [12] and so on. *Melissa officinalis* is a perennial and medicinal herb from the Lamiaceae family. Due to its lemon-like fragrance, it is also known as lemon balm [13]. Different organic compounds present in lemon balm extract including protein, essential oils, flavonoids, rosmarinic, caffeic and gallic acids and phenolic contents are responsible for bio-reduction of Ag^+ ions [14]. Therefore, this plant has been used to synthesize bio-AgNPs for reaching higher chemical yields and bio-activity.

This study aimed to examine the impact of aqueous and methanolic extracts of *Melissa officinalis* on the size, shape and morphology of synthesized silver nanoparticles and their biological properties against infectious microbial strains.

2. Material and methods

2.1. Plant extraction

2.1.1. Aqueous extract

The aerial parts of *Melissa officinalis* were washed with distilled water and shade dried for 7 days at 28 °C. The dried plants were ground into a fine powder. For preparation of the aqueous extract 150 mL of sterile distilled water was added to 6 g of powdered samples and boiled for 10 min. The suspension was left for 3 h and then filtered through Whatman filter paper No. 1. The samples were centrifuged at 20000 rpm for 15 min and stored at 4 °C until further analysis.

2.1.2. Methanolic extract

For this, about 6 g of powdered sample was added into a thimble, and extracted in Soxhlet with methanol for the period until the solvent in siphon tube of extractor becomes colorless. The extracts were filtered using filter paper and the solvent was allowed to evaporate from the extract in a rotary evaporator to have a syrupy consistency. The extract was stored at 4 °C for further experiments.

2.2. Biosynthesis of silver nanoparticles

The AgNPs were prepared according to the method of Savithramma *et al* [30] with slight modification. A, 1 mM $AgNO_3$ (silver nitrate) solution was prepared and stored in amber colored bottle at 4 °C. A 50 mL of $AgNO_3$ solution (1 mM) was added into 5 mL of lemon balm extracts with constant stirring at 28 °C until the color change as an indication of the formation of AgNPs. The extract content was then centrifuged at 14000 rpm for 30 min and the reduction of Ag^+ ions and the formation of AgNPs was studied by using UV-Vis spectroscopy.

2.3. Effect of the concentration of extracts on the biosynthesis of silver nanoparticles

To study the effect of the different concentration of extracts, various volumes of aqueous extract *(*7, 10, 15 and 20 mL) and methanolic extract (0.1, 0.5, 1, and 5 mg /50 mL) were used to prepare AgNPs.

2.4. Characterization of synthesized silver nanoparticles

2.4.1. UV-Vis spectrophotometry

UV–visible spectroscopy was carried out using a UV–Vis spectrophotometer (Labomed, UV-win5, Germany), in a wavelength range of 300–600 nm. Silver ions reduction was monitored by measuring the UV–Vis spectra of solutions after dilution a small aliquot of the sample by distilled water [15].

2.4.2. Scanning Electron Microscopy (SEM)

The size and shape of AgNPs synthesized by *M. officinalis* were determined by scanning electron microscopy using high-performance MIRA-3 SEM machine (TESCAN, Brno, Czech Republic).

2.4.3. X-Ray Diffraction analysis

The XRD analysis of synthesized AgNPs was conducted by using Bruker D8 Advance X-ray diffractometer (D8-Advance Bruker, Germany) and the XRD patterns were recorded with a Cu K_α radiation in the range of 20 °C to 280 °C at 40 keV [16].

2.4.4. Fourier Transform Infrared spectroscopy

The nanoparticles were characterized using the lyophilized samples by potassium bromide pellet technique in the range of 500 to 4000 cm^{-1} on a Bruker Tensor 27 FTIR spectrometer (Karlsruhe, Germany). FTIR was used to characterize the nanoparticles using the lyophilized samples by potassium bromide pellet technique in the range of 500 to 4000 cm^{-1}.

2.5. DPPH free radical scavenging assay

Antioxidant activity of AgNPs was quantitatively studied by using DPPH method [17]. In this, a 150 µl of 1 mM NPs solution and ascorbic acid (positive control) were separately mixed with 2.8 mL of a methanolic solution of DPPH, shaken and incubated in a dark place 4 °C for 24 h. After incubation, the absorbance of the samples (reduction of DPPH radical) was measured by UV–Vis spectrophotometer at 517 nm against methanol as blank. The methanolic

solution of DPPH without the sample served as control. The DPPH free radical scavenging activity (percentage of inhibition) was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

2.6. Antimicrobial assays of the biosynthesized AgNPs

The antimicrobial efficacy of AgNPs was directed against these bacterial and one fungal cultures. Microbial cultures namely *Staphylococcus aureus* PTCC 1113, *Escherichia coli* PTCC 1330 and *Bacillus subtilis* NCTC 5398 and fungus *Saccharomyces cerevisiae* IBRCM 30069 were provided from Iranian Biological Resourece Center IBRC, Iran. Bacterial cultures wre mainatianed on LB agar and fungal culture was preserved on potato dextrose agar PDA at 4 °C.

The antimicrobial assay performed as according to the Kirby–Bauer disc diffusion [18] and agar well diffusion methods [19]. In disc diffusion method, NA and PDA plates were inoculated with 100 µl of bacterial (1×10^6 cells/mL) and fungal spore suspension (1×10^6) respectively using spread-plating technique. After drying the plates, filter paper discs of 6-mm diameter were soaked in 30 and 40 µl of AgNPs solution (1 mM), plant extract and distilled water as control and placed on LB agar plates. Plates were incubated at 28 °C for 24-48 h. Following the incubation, the susceptibility of the test organisms was determined by measuring diameter of zone of inhibition (mm).

For agar well diffusion test, the inoculated plates after drying, were punched to make wells of 8-mm diameter, and aseptically filled with 20, 30 and 40 µl of biosynthesized 1 mM solution of NPs and plant extract. Plates were allowed to diffuse for 2 h at 8 °C followed by incubation 28 °C for 24-48 h. The antimicrobial activity was analyzed by measuring the diameter of inhibition zone (mm) around the wells.

3. Results and discussion

3.1.1. Extraction and biosynthesis of AgNPs

The reduction of silver ions into silver nanoparticles can be followed by color change. The fresh extract of *M. officinalis* was pale yellow in color and after the addition of $AgNO_3$ and stirring at 28 °C, the color of solution changed into pale pink (within 30 min in case of aqueous extract) and light red (within 15 min in case of methanolic extract). The emulsion turned dark brown after 24 and 36 h for methanolic and aqueous extracts respectively.

3.1.2. Effect of different concentrations of extracts on the biosynthesis of AgNPs

Different volumes of aqueous extract (7, 10, 15 and 20 mL) and methanolic extract 0.1, 0.5, 1, and 5 mg /50 mL were used to study the formation of AgNPs. Change in color in the aqueous extract of lemon balm during biosynthesis of AgNPs began within 30 min. The color change occurred first in the lowest concentration (7 mL) followed by color changes in samples having higher concentrations. These color changes indicated the reduction of silver particles into silver nanoparticles. Thus, velocity of the formation of AgNPs was inversely proportional to the concentration of the aqueous extract. It began first with lowest concentration and then with increasing concentrations of the aqueous extract.

In case of methanolic extract (0.1, 0.5, 1, and 5 mg/50 mL) the color changing occurred with higher concentrations of the extract. The speed of the reaction was increasing with increase of the concentration of methanolic extract. The concentration 2.5 mg/100 mL responded

best compared to 0.5 and 0.25 mg/100 mL while 0.05 mg/100 mL concentration did not show any color change indicating no formation of AgNPs in this lowest concentration.

The results also showed that the size of colloidal particles was between 1 to 1000 nm. The formation of colloidal solutions from the reduction of silver ions occurs in two steps (nucleation and growth) (Figure 1). Reducing and stabilizing agents are known as the major components in the formation of metal nanoparticles [20]. Biologically, plant metabolites such as sugars, proteins, terpenoids, polyphenols, alkaloids, flavonoids and phenolic acids are responsible for the reduction and stabilization of nanoparticles. It has been reported that the *OH* groups present in the backbone flavonoids play a critical role in the reduction of silver ions to AgNPs [21]. Since 7 mL aqueous and 2.5 mg/100 mL methanolic extracts resulted in the faster formation of AgNPs, they were used for further analysis.

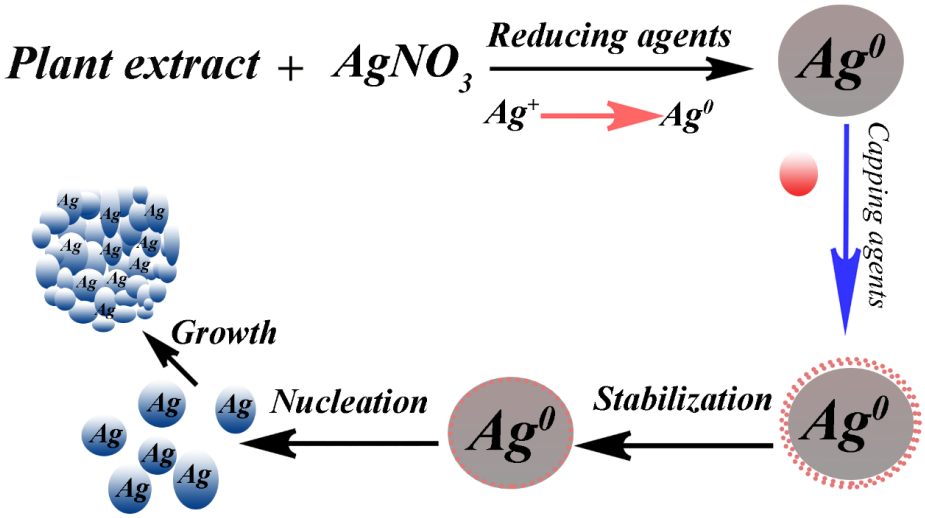

Figure 1. The reducing agents (in plant extract) donate electrons to Ag^+ ions lead them as neutral Ag atoms (Ag^0). These atoms due to van der Waal's forces of attractions come closer and combine to form cluster of Ag atoms of diameter 1 to 100 nm, called Ag nanoparticle. A silver nanoparticle in nano scale may contain about hundreds of atoms of silver. If these clusters (nanoparticles) come closer they agglomerate first and then aggregate to form bulky particles, which is not AgNPs [20].

3.2. Characterization of NPs

3.2.1. Ultraviolet-visible spectroscopy

Color change from yellow to brownish-red and dark brown is the first sign of nanoparticle production and is due to excitation of the surface Plasmon resonance [22]. The biosynthesis of nano particle should be confirmed via physical methods like UV-Vis spectrophotometer, SEM, XRD and FTIR [23].

The biosynthesis of AgNPs and the reduction of Ag^+ ions to Ag atoms were recorded by UV-Vis spectroscopy. The reaction was conducted for 2 h. The UV-Vis absorption spectra of colloidal solutions of AgNPs using *M. officinalis* methanolic extract had absorbance near 450 nm. The broadening peak is a sign of the poly-dispersed particles formation (Figure 2). Hafez *et al.* [24] produced AgNPs that showed UV-Vis absorbance at 425 nm and Keshari *et al.* [25] reported the absorption band of AgNPs at 442 nm. Also, the absorption of the AgNPs was observed near 430 nm [26] and 420 nm

[27] in the UV–Vis spectrum The absorbance wavelength depends on the concentration of plant extract, different times [28], fresh and freeze-dried samples [29] and particle size [23] .

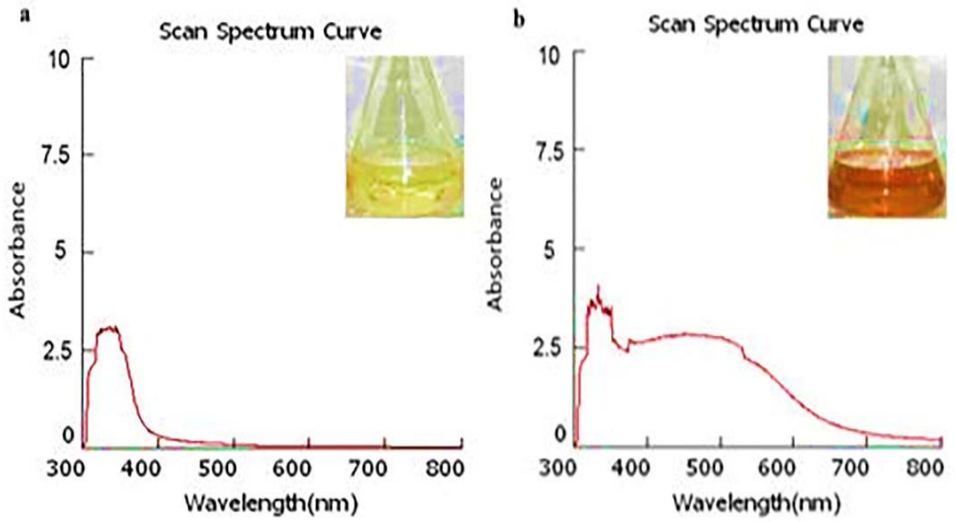

Figure 2. UV–Vis absorption spectrum of the photosynthesized AgNPs. **a)** The reaction at time zero showed no absorption in 400–500 nm region. **b)** Spectra represented the formation of silver nanoparticles with the help of *Melissa officinalis* methanolic extract. The absorption of the AgNPs was observed near 450 nm which is due to surface Plasmon resonance of AgNPs.

3.2.2. Scanning electron microscope

To determine the morphological characters nanoparticles synthesized by lemon balm extracts, SEM assay was used. The SEM images showed rod-shaped nanoparticles formed with diameter in the range of 19-40 nm from the aqueous extract. Also, the nanoparticles derived from the methanolic extract were spherical with diameter of 13-35 nm (Figure 3).

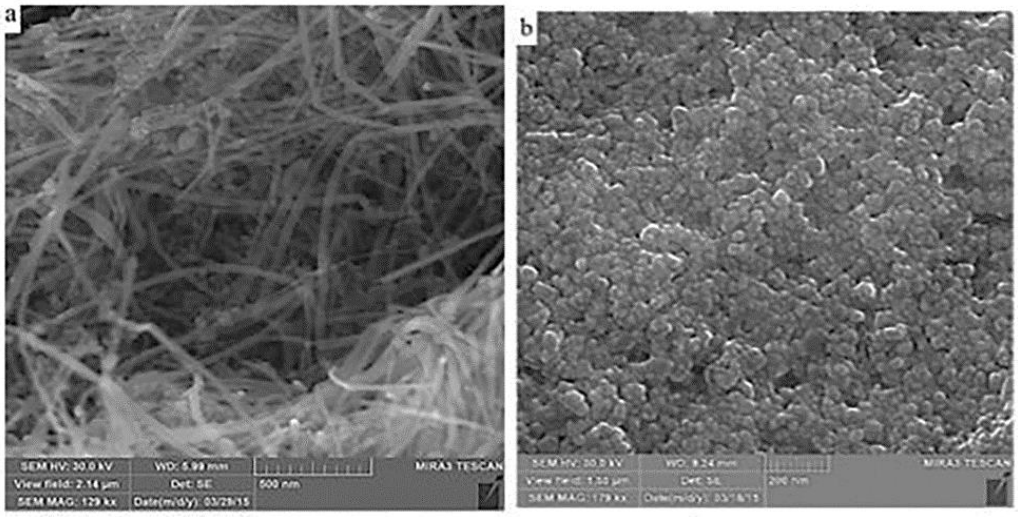

Figure 3. SEM images of biosynthesized silver nanoparticles by using different extracts of *Melissa officinalis*; **a)** rod-shaped nanoparticles from aqueous extract and **b)** spherical shape nanoparticles from methanolic extract.

It has been reported that the nanoparticles deformation as different shapes and diameters is the result of altering the plant extract and/or the reaction conditions depending on the application [21]. The surface areas and the shapes of nanoparticles effect on their antimicrobial activity [30] due to different effective surface areas and active facets [30]. It was suggested that *Melissa officinalis* scavenged DPPH radical in a concentration-dependent manner [31] that is in agreement with the result of our study. DPPH assay revealed the sample of 5 mg/50 mL methanolic extract had the highest antioxidant activity compared to the 7 mL aqueous extract.

Based on the SEM results, the particles produced by methanolic extract were spherical and smaller in size in comparison to the rod-shaped particles derived from the aqueous extract. Therefore, further experiments were conducted using the methanolic sample.

3.2.3. X-ray diffraction (XRD) analysis

The XRD is one of the most widely used techniques to characterize the structural properties of NPs. In order to gain the structural information, the resulting diffraction patterns obtained from the penetration of X-rays into the nanomaterials is compared with standards [32]. Figure 4 shows the XRD pattern of the AgNPs synthesized by using the methanolic extract of lemon balm. The AgNPs diffractogram displayed several sharp intense peaks at 2 theta angles, which indicated the crystalline structure of the AgNPs and confirmed the formation of the AgNPs. Four distinct reflections at 37.5° (111), 44.37° (200), 64.56° (220) and 76.58° (311) evidently indicated the formation of the face-centered cubic structure of the AgNPs in the sample.

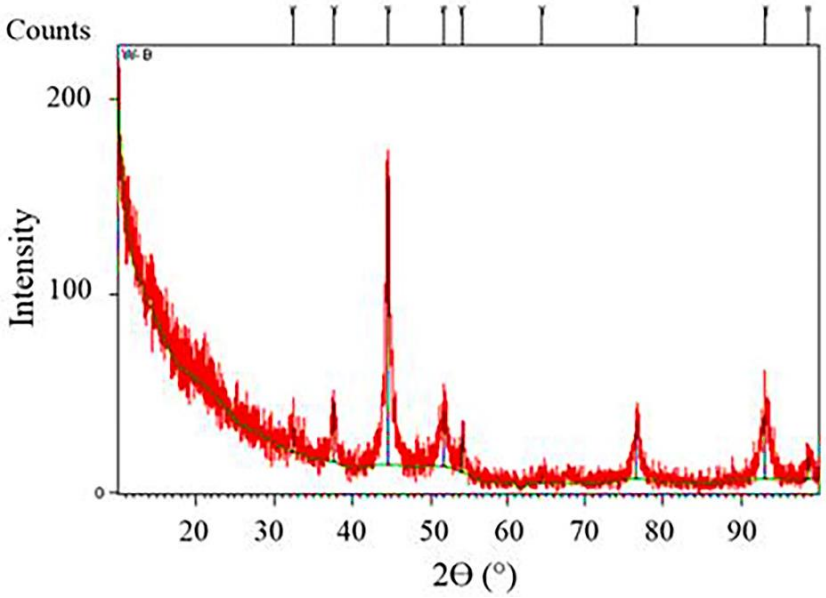

Figure 4. XRD diffractogram of biosynthesized AgNPs by *Melissa officinalis* methanolic extract.

The XRD outline displayed that the AgNPs formed by the reduction of Ag^+ ions by *M. officinalis* extract are crystal-like in nature. This result is in accordance with the XRD analysis of Shaik *et al*. [33]. Additional peaks at 32.25° and 54.62° were observed on the preparation of AgNPs using *M. officinalis* methanolic extract (Figure 4). These peaks are attributed to the existence of some

bioorganic compounds in *M. officinalis* leaf broth[34]or related to unreduced and left over residues of $AgNO_3$ in the sample [35]. It has been suggested that magnesium of the chlorophyll is the center of X-ray diffraction in the bioorganic crystalline phase [36].

3.2.4. Fourier transform infrared (FTIR) spectroscopic analysis

Here, FTIR was used to analyze the chemical composition of lemon balm responsible for reduction of Ag ions. Using this technique organic functional groups (COO^-, OH, …) attached to the Ag and other chemical residues surface can be detected. The methanolic extract of aerial parts of lemon balm displayed a number of absorption peaks, reflecting its complex nature. The results of FTIR analysis showed different stretches of bonds shown at different peaks including 3446.51 cm^{-1} could be assigned to O-H stretch, H-bonded corresponded to alcohols and phenols, 2357.56 cm^{-1} assigned for single aldehyde, 1151.92 cm^{-1} indicates the fingerprint region of C-O stretching, 674.99 cm^{-1} could be attributed to the presence of C-H bend alkynes and 674.99 cm^{-1} and 599.95 cm^{-1} correspond to halo compound.

Figure 5 shows the peaks near 3000 cm^{-1} assigned to O-H stretching and aldehyde C-H stretching. The absorption peaks between 1500 to 2000 cm^{-1} can be attributed to the presence of C-O stretching in carboxyl coupled to the amide linkage in amide I. These are the characteristic of the presence of protein and enzymes in the supernatant and it confirms the extracellular formation of AgNPs [37]. Consequently, the occurrence of these peaks in the FTIR spectrum evidently indicates the dual role of the *M. officinalis* extract, *i.e.* both as a green reducing as well as stabilizing agents.

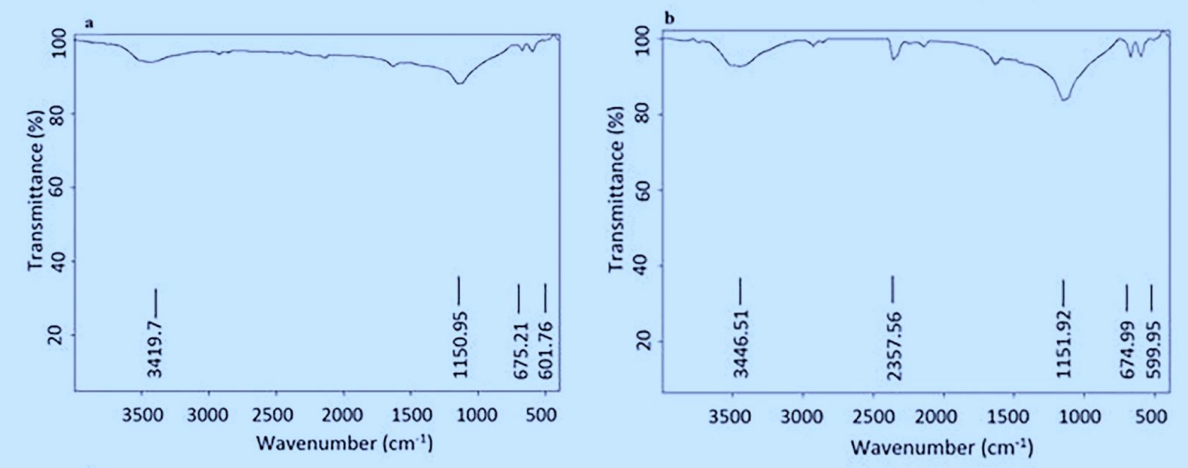

Figure 5. FTIR spectra of **(a)** *Melissa officinalis* methanolic extract and **(b)** biosynthesized AgNPs from *Melissa officinalis* methanolic extract.

Interactions between metabolites in the extract and metal ions cause the bio-reduction of metal salts and synthesis of nanoparticles. The functional groups in the plant extract act as reducing, capping, and stabilizing agents. Negatively charged (COO^-) and polar (OH and CO) groups presented in the plant extract attach on the Ag surface with high tendency and contribute in both reduction and stabilization of AgNPs [32].

3.3. Antioxidant capacity-DPPH radical scavenging assay

The DPPH free radical scavenging assay was determined by measuring the ability of plant extracts to capture the stable radical DPPH [15, 21]. The initial

violet color of DPPH solution changed to yellow as soon as the AgNPs were added. This may be because of the presence of antioxidant in the medium. However, the process of yellowing may slow down and the target substance exhibits poor antioxidant activity or no color change. The inhibitory concentration of the material is investigated based on the absorbance of DPPH radiation at a wavelength of 517 nm. The color change rate in the methanolic extract was also higher than that of the aqueous extract and AgNPs (Figure 6). The methanolic extract of lemon balm and AgNPs exhibited highest antioxidant activity compared to the aqueous extract.

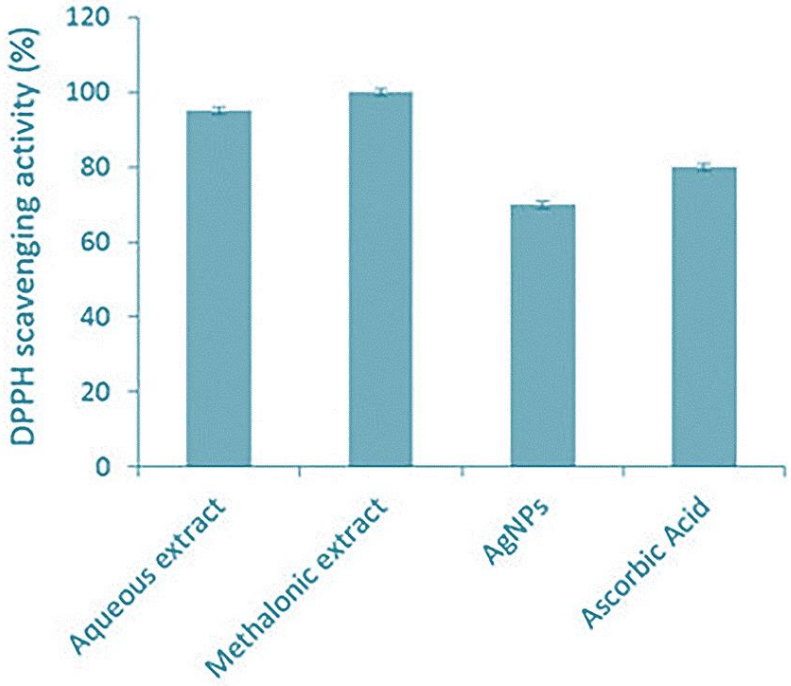

Figure 6. DPPH radical scavenging activity of two extracts of Melissa officinalis and the synthesized AgNPs using methanolic extract. The methanolic extract had the highest antioxidant activity among the samples.

Various studies have reported the DPPH scavenging activity by AgNPs from plant extracts [24]. The production of iron NPs using aqueous extracts obtained from 26 tree species and antioxidant properties of these have been reported [25]. These researchers interestingly found dried leaves (higher concentration) produce extracts with higher antioxidant capacities than non-dried leaves (lower concentration). This is probably due to evaporation of water from leaves that result in the concentration of antioxidants [25].

3.4. Antimicrobial property of AgNPs

The antimicrobial effect of spherical silver nanoparticles was investigated by agar well diffusion and disc diffusion methods. Green synthesis of the AgNPs using this lemon balm extract showed an effective antimicrobial activity against Gram-positive bacteria such as *B. subtilis* and *S. aureus* and Gram-negative bacterium namely *E. coli* and against yeast, *S. cerevisiae* (Figure 7 and 8).

In disc diffusion method the diameter of inhibition zone for *B. subtilis*, *S. aureus*, *E. coli* and *S. cerevisiae* was 5.7, 5.6, 7 and 4 mm respectively, while in agar well diffusion method it was 10, 10, 11.3 and 9.25 mm, respectively (Figure 7 and 8).

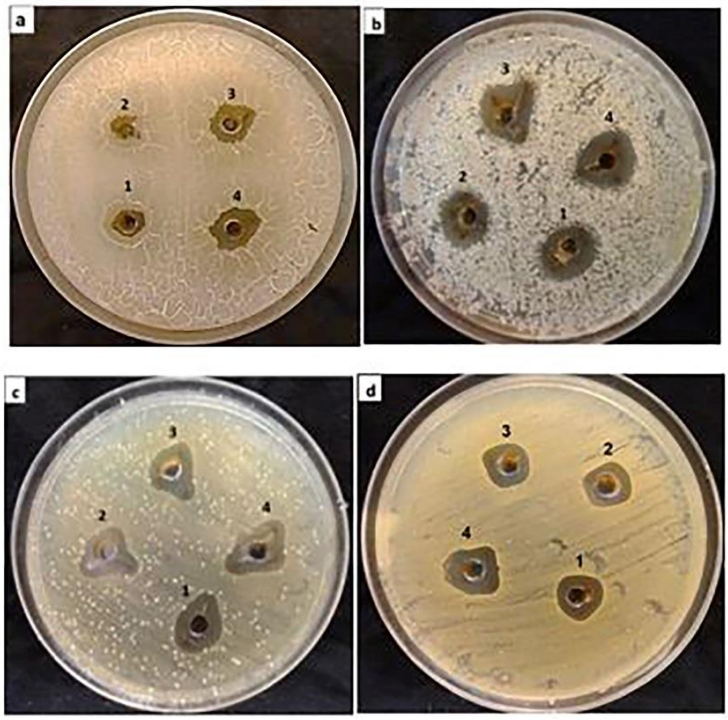

Figure 7. Agar well method showing antimicrobial activity of biosynthesized AgNPs at different volumes; **1)** *Melissa officinalis* methanolic extract, **2)** 20, **3)** 30 and **4)** 40 µl of biosynthesized AgNPs solution evaluated against **a)** *Saccharomyces cerevisiae,* **b)** *Bacillus subtilis,* **c)** *Escherichia coli* and **d)** *Staphylococcus aureus*.

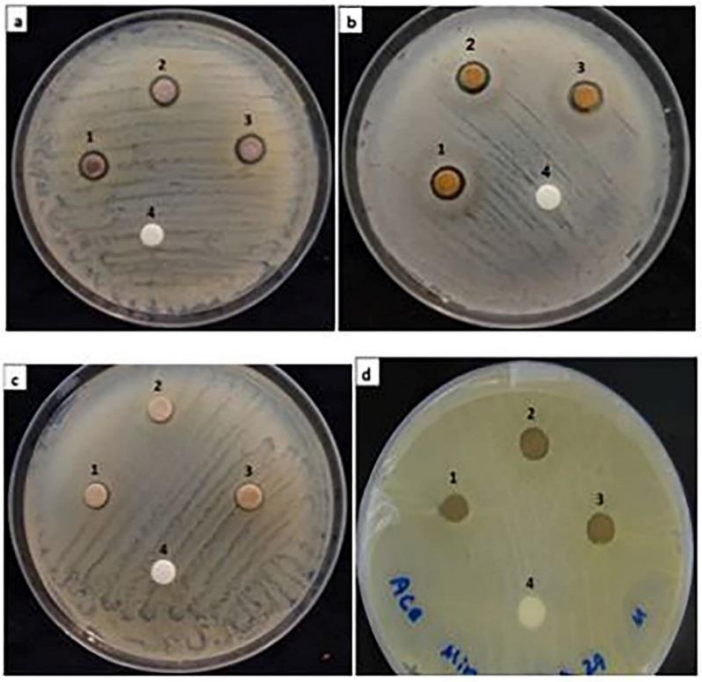

Figure 8. Disc diffusion method showing inhibition zones produced by biosynthesized AgNPs at different volumes; **1)** *Melissa officinalis* methanolic extract, **2)** 30 and **3)** 40 µl of biosynthesized AgNPs solution plus **4)** distilled water evaluated by against **a)** *Escherichia coli,* **b)** *Staphylococcus aureus*, **c)** *Bacillus subtilis* and **d)** *Saccharomyces cerevisiae*

In both methods, Gram-negative bacterium, *E. coli* exhibited the highest sensitivity to the AgNPs compared to other test organisms. The results of the present study were similar to those of others [38, 39] and elucidated that AgNPs from methanolic extract of lemon balm inhibited bacterial and fungal growth. The antimicrobial effect of AgNPs was found to be dependent on the size, shape and concentration of AGNPs, smaller and spherical particles produced higher inhibition compared to larger and non-spherical particles. This may be due to the availability of more surface area in smaller and spherical particles [40]. Similarly, higher concentrations of AgNPs resulted in more inhibition of the bacterial and fungal growth. Antibacterial property of AgNPs derived from aqueous extract of lemon balm leaves against *S. aureus* and *E. coli* have been reported by Ruíz-Baltazar et al [14]. AgNPs mainly exert their antimicrobial effects through the disruption of the cell wall and cell membrane that finally leading to bacterial cell death. Additionally, there are some other mechanisms including attack on bacterial surface and membrane through interaction with sulfur-containing proteins [40], disruption of cell permeability and respiration, form the pits on the cell surface and induce the proton leakage that causes cell death [41], inhibition of respiratory enzymes of bacterial cells by combining with the thiol group [42] as well as cell retention of DNA replication and preventing cellular division [43] might widely contributed to the antibacterial effects of AgNPs. The entrance of AgNPs also produce higher amount of ROS molecules that is linked to deactivation of the respiratory enzymes and disrupt the cellular membrane and damage DNA molecule [44]. Figure 9 illustrates some possible molecular mechanisms might link to antimicrobial action of AgNPs.

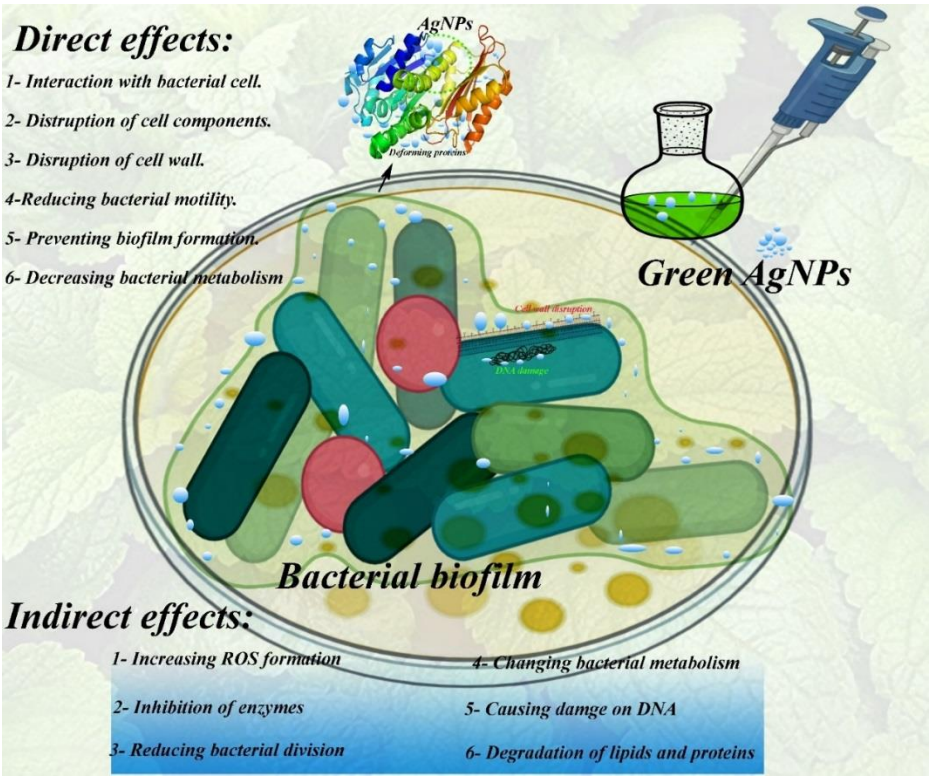

Figure 9. Proposed mechanism of AgNPs formation and antimicrobial activities exerted by Melissa officinalis. (by Photoshop software).

Gram-negative and Gram-positive bacteria were more susceptible to AgNPs compared to the fungal strain [37]. These differences in bactericidal and fungicidal effects of the AgNPs are due to the differences in structural organization of the bacterial and fungal cell walls. The bacteria are evolutionarily prokaryotic types and are less complex. Therefore, they are more susceptible to the toxic effects of AgNPs compared to the eukaryotic fungi. Fungi being the eukaryotic organisms, possess superior detoxification system that makes them resistant to higher concentrations of AgNPs [37].

4. Conclusion

Green production of AgNPs is a low-cost and an eco-friendly method and could provide lots of benefits for researchers. Therefore, it is a good alternative for the industrial production of NPs. The biosynthesis of AgNPs using lemon balm extracts and their antibacterial, antifungal and antioxidant activities were studied. The nanoparticles produced by the methanolic extract of the lemon balm resulted in quicker formation of AgNPs and exhibited potent antimicrobial and antioxidant activities. Due to the antimicrobial properties of the green AgNPs, they can be used as an antiseptic, sterilant and antimicrobial agents. The antioxidant properties of green AgNPs also make them ideal candidate for food industries to eliminate the development of infectious pathogens within food cans or similar products.

Abbreviation:

AgNO3: Silver nitrate
AgNPs: Silver nanoparticles
DPPH: 2,2-diphenyl-1-picrylhydrazyl
FTIR: Fourier Transform Infrared
SEM: Scanning Electron Microscope
XRD: X-Ray Diffraction
UV: Ultraviolet
UV-Vis: Ultraviolet-visible spectroscopy

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Conflict of Interest Statement:

The authors declare no competing interests.

Consent for publications

The authors have read and approved the submitted manuscript.

Availability of data and material

The authors declare that all the data is embedded in the manuscript.

Authors' contributions:

F.D.N designed the experiment, analyzed and interpreted the data and drafted the manuscript, M.M performed experiments, collected the data and prepared figures, S.M. helped in performing experiments and R.Z.S revised and edited the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate:

This article does not contain any studies with human participants or animals performed by any of the authors.

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