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RESEARCH PAPER

Synthesis, Characterization of Silver Nanoparticles Using Rosemary Extract and Their Application as Antioxidant, Antifungal and Antibacterial Agents

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ABSTRACT

Medical applications frequently use nanoparticles nowadays, due to their nanometer size and having a significant surface area to volume ratio, allowing them to absorb large amounts of drugs and move quickly through the blood. In this study, silver nanoparticles were successfully synthesized using rosemary leaf extract. The nanoparticles were fully characterized using UV-Vis and Fourier transform infrared spectroscopy, confirming their stability and identifying their functional groups. The average size of the prepared Ag-NPs was 32.7 nm, and the crystallinity was evaluated using XRD, SEM, and EDX. Based on the characterization results, the UV-Vis absorption spectrum of Ag-NPs was determined at 400 nm. Moreover, the antioxidant method confirmed that silver-nanoparticles have more significant antioxidant activity as compared to vitamin C . The biosynthesized Ag-NPs displayed notable antifungal properties against M. cannonballus at varying concentrations, resulting in remarkable inhibitory effects of 74%, 74%, and 98% respectively. Furthermore, the antibacterial activity of the prepared silver nanoparticles and plant extracts against *S. aureus* and *E.coli* at two different concentrations 2 $mg.mL^{-1}$ and 4 $mg.ml^{-1}$ was investigated using the well diffusion method. The zone of inhibition was utilized as an indicator of antibacterial activity. The findings revealed a potent antibacterial impact of the nano-silver particles on the targeted bacterial strains.The presence of bioactive molecules on the surface of silver nanoparticles indicates that Ag-NPs possess robust antioxidant, antifungal, and antibacterial properties. As a result, Ag-NPs show great potential as biocompatible candidates for various pharmacological and therapeutic uses.

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INTRODUCTION

Nanoparticles (NPs) are microscopic materials that are typically between 1 and 100 nm in size. They are valuable in the consumer goods, pharmaceutical, chemical, environmental, energy, agricultural, and communications sectors due to their remarkable thermal, chemical, optical, physical, and electrical properties [1,2].

They can be produced via chemical, physical, or biological techniques. Biological methods offer greatest opportunities as they are both environmentally friendly and economical, these methods utilize microorganisms or medicinal plants to produce nanoparticles. The therapeutic properties of medical plants transfer to the nanoparticles during the production process, making their use beneficial. Antimicrobial plants enhance nanoparticles' antioxidant and antibacterial effects [3,4].

Nanostructures known as silver nanoparticles have become a very interesting field for researchers because of their exceptional inactivity against a wide range of microorganisms that have a common resistance against traditional antibiotics [5,6]. Silver nanoparticles can be synthesized using various techniques, including electrochemical reduction, photochemical reduction, heat evaporation, and biological methods. However, it has been noted in the literature that these methods can be costly and involve the use of potentially harmful chemicals, increasing the risk to both biological systems and the environment. Recently, silver nanoparticles produced from plant extract are gaining popularity due to their environmentally beneficial properties. [7] Several plant extracts, including M. oleifera (Horseradish), A. indica (Neeem), T. foenum-graecum (Fenugreek), Cydonia oblonga (Quince), and C. colocynthis (Bitter Apple), have been effectively employed for the manufacture of metallic nanoparticles as both a reducing and capping agen [6, 8, 9].

Silver is well-known for its biocidal capabilities; it has bactericidal, fungicidal, and virucidal characteristics independent of the form in which it exists, such as silver ions (Ag^{+}), silver complexes, and metallic silver (Ag^{0}), including silver nanoparticles Ag-NPs. Due to their strong biological action, silver compounds are widely used, particularly in the field of biology and medicine to minimize infection and avoid the generation of more drug-resistant [10,11].

Nanoparticles such as silver nanoparticles Ag-NPs have long been used as nantimicrobial agents and are highly effective against fungal pathogens [12] Ag-NPs act through multiple mechanisms, including to phosphate groups in DNA [13]. and interacting with the plasma membrane, leading toproton diffusion and cell death [14]. They can also interact with sulfhydryl groups inproteins and enzymes, disrupting the electron transport chain and impairing membrane permeability to phosphate groups and protons [15,16].

Since ancient times, silver (Ag) has been used in medicine to treat and control a variety of ailments because of its remarkable antibacterial capabilities along with its therapeutic abilities. The high microbial killing power of Ag ions and Ag-based compounds is widely known [17,18]. However, advancements in technology and a deeper comprehension of how silver prevents disease by eradicating bacteria have made it possible to employ silver in nanomedicine [17]. Likewise, silver is effective against 650 distinct types of bacteria, which makes it appropriate for protecting plants [19]. It exhibits oligodynamic activity against a broad spectrum of cellular targets, in contrast to selective antibiotics [20]. Ag-NPs have the ability to damage a variety of biological organelles, such as the cell membrane's structure, which disrupts electron transport and ultimately cellular metabolism [21]. These nanoparticles also break down DNA, prevent the synthesis of proteins needed to produce ATP, and can produce reactive oxygen species (ROS) causing oxidative stress plus ultimately stop cell division [20].

In this work, Ag-NPs were prepared using the rosemary extract and their application as antioxidant, antifungal and antibacterial agent were investigated.

MATERIALS AND METHODS

Materials and Instrumentation

All chemicals and reagents have been purchased from Sigma-Aldrich , while rosemary leaf has been purchased from the local market.

Many equipment was used in present study, including UV-Vis spectrophotometer from Bioteh engineering management Co.Ltd., UK, centrifuge Laboratory Retirezle from CentrifugeLaboratory Retirezle, magnetic stirrer VF2 from Funkentstört–Germany, heater from IKA,Germany, scanning electron microscope from SEM, Zeiss,Germany, and Fourier transform Infrared spectroscopy from FTIR Perkin Elmer Instrument Japan.

The preparation of plant extract

Rosemary leaves (Rosmarinus officinalis) were collected from an Iraqi local market, 10 g were rinsed three times with distilled water and once with 95% ethanol in water to remove any leftover microbe contamination on the leaf surface. Leaves were dried in an oven at 70 °C for 24 hours. The dried leaves were ground to powder. The aqueous extracts of the sample were produced by boiling the powder leaves in 100 mL of distilled water at 80 °C for 1 hour, stirring constantly with a magnetic stirrer at 600 rpm. The filtered extract was stored for later use [22].

Nanoparticles preparation

Nano-silver was prepared by using the leaf extract of the rosemary plant, following previous research with certain modifications [6]. In brief, 50 mL of rosemary leaf extract, 100 mL of (0.17 mg, 1 mM) $AgNO_3$, and 40 mL of 0.4 M NaOH were added and mixed together. The mixture was then heated to 80 °C to reduce Ag^+ ions and constantly agitated for 20 minutes at 600 rpm with a magnetic stirrer, resulting in the production of a dark brown precipitate. The impurities were then removed from the precipitates by filtering and washing them several times with distilled water and ethanol before drying them at 100 °C for one hour. The resulting powder of dark-brown colour was finely stored for further characterizations.

Antioxidant activity using 2, 2-diphenyl-1-picrylhydrazyl(DPPH) assay

The antioxidant activity of the synthesised nanoparticles was measured by scavenging free radicals from DPPH. Das *et al*. described how to do the technique with a little change [23]. The stable radical DPPH (2, 2-diphenyl-1-picrylhydrazyl) was employed to assess the free radical scavenging properties of Ag-NPs and vitamin C. Various doses (10–100 µg/mL) were mixed with 1mL of freshly prepared DPPH (1 mM in methanol) and extensively vortexing. The solution was then kept at ambient temperature and darkness for 30 minutes. Finally, absorbance was measured at 517 nm. Eq. 1 shows how the free radical scavenging activity was calculated using the percentage of inhibition.

Where Ab is the control's absorbance and Ab test is the absorption of (Ag-NPs) / Vit.C

Antifungal activity

Pathogenic fungi

Monosporascus cannonballus Pathogenic fungus was used in these experiments namely soil-borne fungus. The standard culture of this fungus was obtained from laboratory of fungi in department of biology, collage of science in Babylon university, Iraq.

Assessment of Antifungal Assay

Antifungal activity of Ag-NPs produced using rosemary plants in vitro against *M. cannonballus* was achieved by the agar dilution method [24,25]. The medium agar was supplemented with three different concentrations of Ag-NPs and plant extract separately (4, 2, 1 mg/ml). A disc of 1 cm of mycelial growth of the tested fungi, taken from the edge of 6-day-old fungal culture, was placed in the centre of each plate. The plates with the inoculums were then incubated at 25 °C. The treatment efficacy of Ag-NPs was calculated after 7 days by measuring the radial growth of fungal colonies.

$$\text{Inhibition rate } (\%) = R - r \setminus \mathrm{R}$$

where (R) is the radial growth of fungal hyphae on the control

plate and (r) is the radial growth of fungal hyphae on the plate supplements with Ag-NPs or plant extract.

Antibacterial activity

Agar disc diffusion was used to quantitatively assess the antimicrobial activity of Ag-NPs synthesized from rosemary aqueous extract against *S. aureus* (Gram-positive bacteria); and *E. coli* (Gram-negative bacteria). Mueller-Hinton agar plates were swabbed with the bacterial suspensions, the wells were cut, and the concentrations of 2 and 4 mg ml^{-1} of the synthesized Ag-NPs and plant extract were loaded in the wells. The plates were then incubated at

$$\text{Percentage of DPPH Scavenged} = \frac{\text{Ab of control} - \text{Ab of test}}{\text{Ab of control}} \times 100 \quad (1)$$

37 °C for 24 hours. After that, the diameter of the inhibition zones around the wells was measured and calculated.

RESULTS AND DISCUSSION

UV-Visible Analysis

The UV spectrum revealed a broad peak at 400 nm, demonstrating nanoparticle formation (Fig. 1), which is consistent with reliable research [26]. A change in the colour of the reaction mixture indicated the synthesis of Ag-NPs.

FT-IR analysis

FT-IR analysis was performed to show the differences between rosemary extract, which served as a capping and reducing agent, and Ag-NPs. The spectra of rosemary extract and silver nanoparticles are shown in (Figs. 2A and 2B). On one side, the FT-IR spectra of plant extract consist of multiple peaks, indicating its complex composition.

A medium peak was noticed at 3412.19 and clear once at 3549.14 cm^{-1} corresponding to –OH of alcohol or phenol stretching vibration, carboxylic acid –OH stretch and N-H stretching of amine respectively. The aromatic and unsaturated hydrocarbons' C-H (=C-H stretch) is represented by the plant extracts broad peak at 2931.90 cm^{-1}. A sharp peak was noticeable at 1512.24 cm^{-1} representing NH_2 in amino acids (NH_2 deformation). The strong peak at 1587.47-1608.69 cm^{-1} is characterized by –NH stretch of primary amines. Moreover, one peak presented at 1423.51 cm^{-1} can be assigned to $–CH_2$. Two sharp peaks were noticed at 1257.63 and 1072.46 cm^{-1}, which represent–C-N stretching vibration. C-N-C, N-C=O, and O-C=O bends were all observed at 600-650 cm^{-1}, which is the peak of O-C=O bending in carboxylic acids.

On the other hand, the obtained FT-IR data of Ag-NPs revealed various absorption peaks, given the various functional group of phytochemicals. The absorption peaks at 3556.58, 3477.77, 3414.12, 2928.04, 2400.01, were assigned the O-H stretch of phenolic compounds, N-H stretch of primary and secondary amines and amides, C-H stretch of methyl groups, H-C=O: stretch of aldehydes. A sharp peak was presented at 1687.77 and 1550.82 cm-1 indicating amide N-H as a bending vibration. A peak at 1708.99 cm-1 presented the C=O stretch of carbonyl groups of flavonoids and tannins respectively.

Furthermore, one peak was obtainable at 1464.02 cm^{-1},which can be assigned to CH_2. A notable peak was presented at 1259.56 cm^{-1}, indicating C-N stretching vibration. Two medium

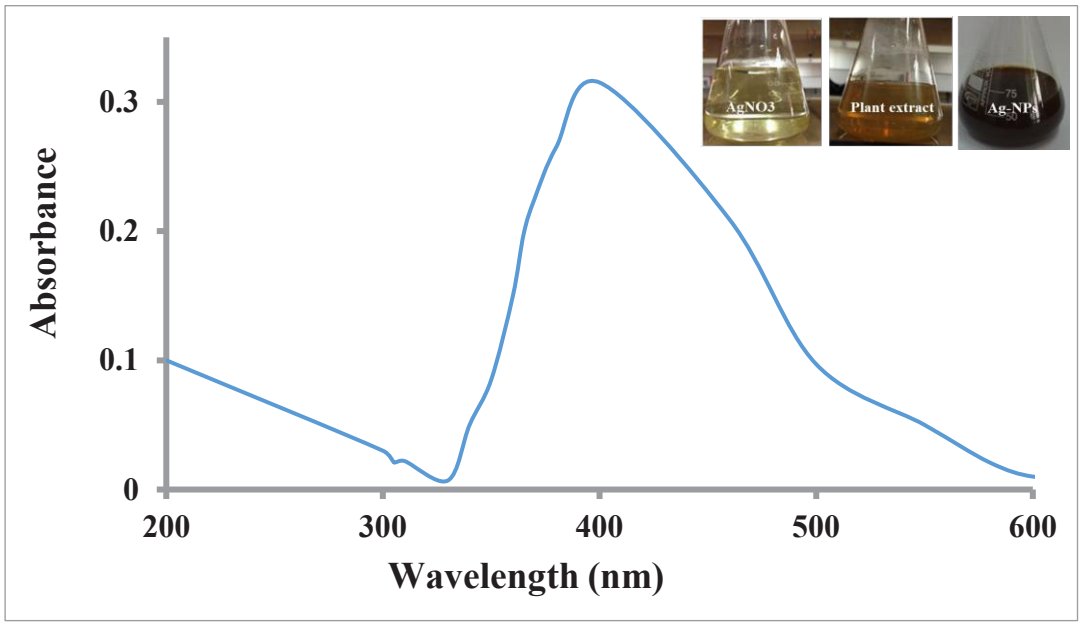

Fig. 1. UV-Vis spectra represent the formation of Ag-NPs using rosemary extract.

peaks were observed at 513.09 and 424.35 cm^{-1}, representing the formation of silver nanoparticles [3].

SEM and EDX analysis

Scanning electron microscopy (SEM), EDX, and energy dispersive X-ray analysis were used to determine the surface morphology, size, and shape of Ag-NPs, as presented in (Figs. 3 and 4).

XRD analysis

XRD analysis displayed distinct diffraction peaks at 2θ values of 38.2415, 46.3537, 64.5608 , 77.4157 degrees in the experimental diffractogram. These peaks correspond to hkl values in the (111), (200), (220) and (311) confirmed the formation of silver , thus having a big agreement with previous research [27] patents are marked 1-4, as shown in (Fig. 4).

The X-ray diffraction (XRD) pattern of silver nanoparticles was recorded using Cu as an anode material, K-α1 as intended wavelength (λ = 1.54060 Å), 8 mA-40 kV, and 2θ/θ scanning mode. Data was collected over the 2θ range (30 to 80 degrees). Table1 shows the XRD data along with the details of the most significant peaks.

Additionally, the diffractogram exhibits an additional five peaks at 32.3723, 44.4036, 54.94

57.5866, 64.5608 degrees. These peaks may be attributed to $AgNO_3$, suggesting that a small

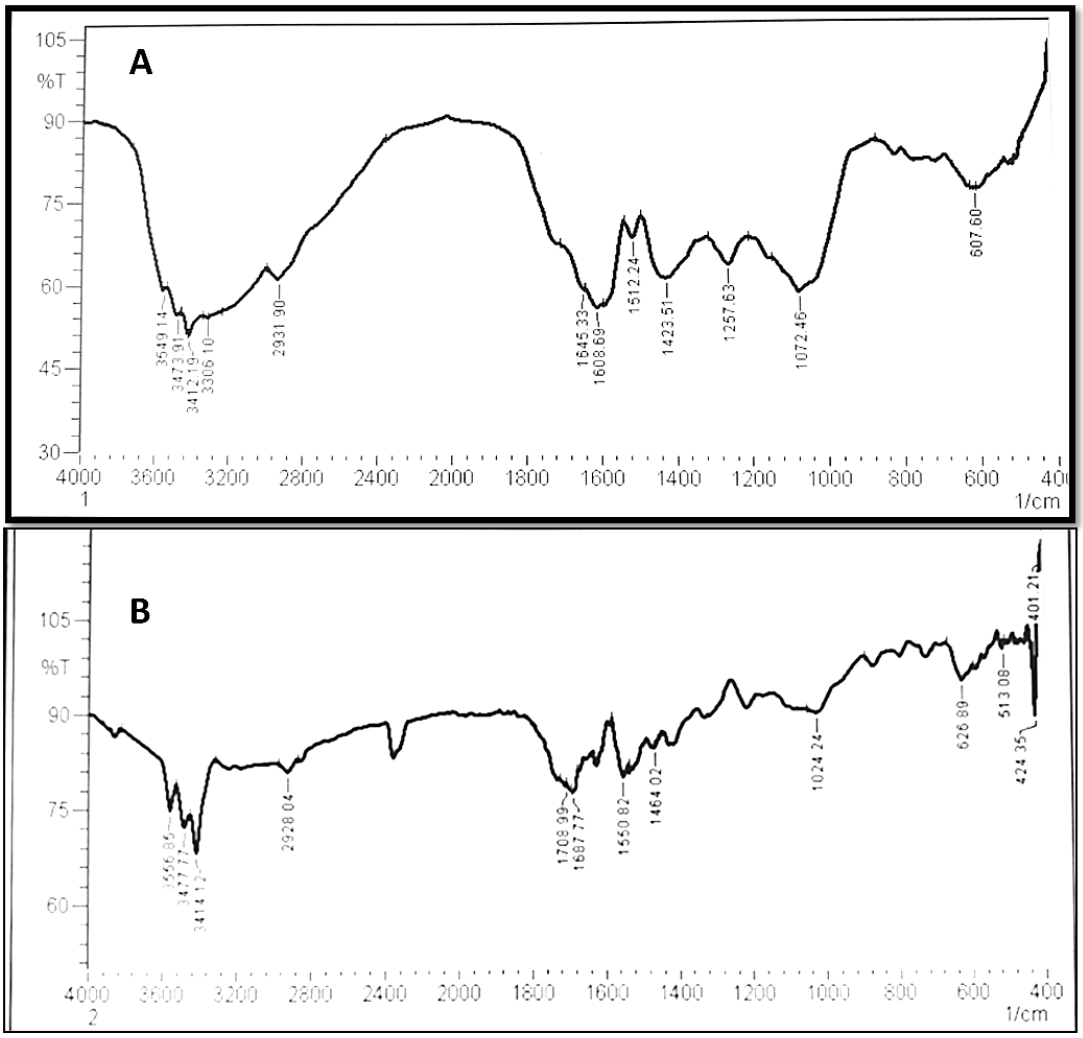

Fig. 2. FT-IR spectrum of A, rosemary extract and B, synthesised Ag-NPs.

amount of it may not have undergone reduction and still exists in the sample. It is worth noting that these peaks have not been accounted for in Table 1. The average size of the Ag nanoparticles produced through the bio-reduction technique was calculated to be 33.61 nm using the equation provided below.

$$D = k\lambda / \beta \theta \cos$$

Antioxidant activity

NPs' antioxidant properties are determined by their chemical composition, nature, stability, surface-to-volume ratio, size, surface coating, and charge [28]. The results revealed that both Ag nanoparticles and Vitamin C have an antioxidant properties, but Ag-NPs had the maximum radical scavenging activity. Increasing nanoparticle concentration from 10 to 100 µg.mL^{-1} resulted in a significant increase in antioxidant activity from 40% to 92%. (Fig. 5)

The results showed that Ag-NPs have higher antioxidant activity than Vitamin C when DPPH is used. This might be attributed to the change of DPPH on the surface of Ag-NPs to scavenge radicals. Furthermore, DPPH-(Ag-NPs) may improve the efficiency of DPPH in scavenging radicals created by the oxidation process. It was reported that

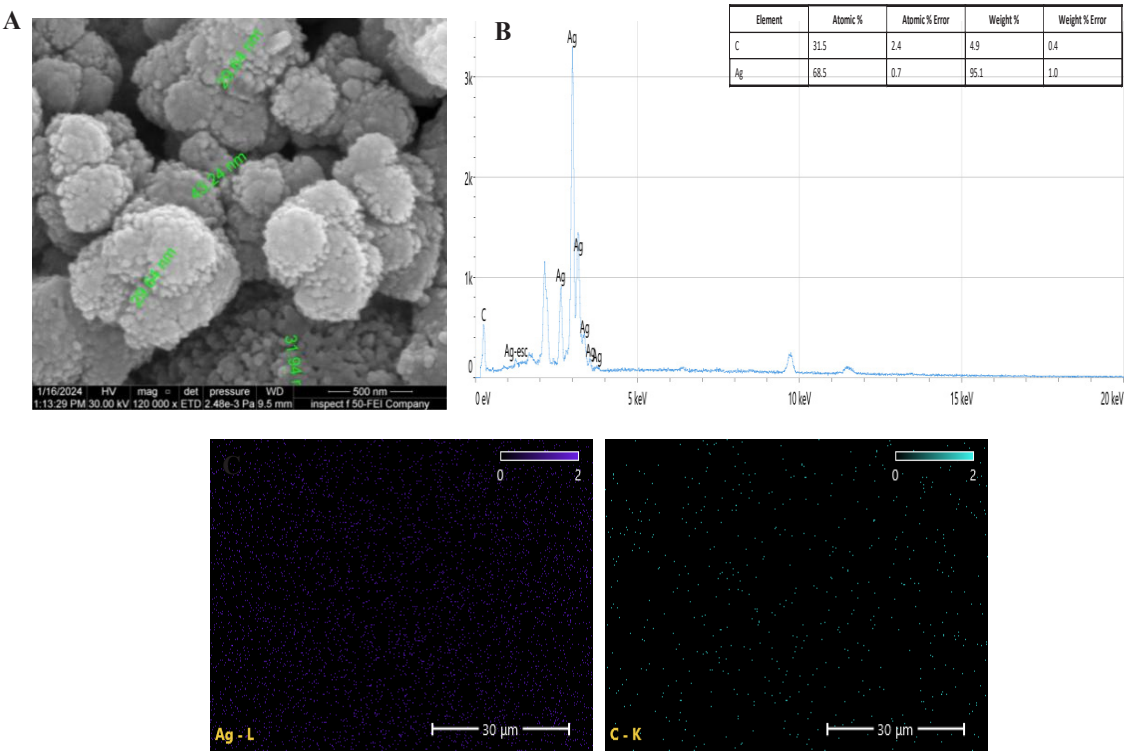

Fig. 3. A, SEM image analysis of Ag-NPs B, EDX spectrum analysis of Ag-NPs and C, map contents.

Table 1. The XRD data along with the details of major peaks and their crystalline size.

Pos. [°2θ]	FWHM Left [°2θ]	Crystallite Size only [Å]	d-spacing [Å]	Area calc.	Area [cts*°2θ]	Backgr.[cts]
38.2415	0.522	134	2.35163	250.49	250.49	126.59
46.3537	0.6878	109	1.95721	124.48	124.48	109.2
67.5399	0.4162	143	1.38578	16.64	16.64	91.32
77.4157	0.6024	106	1.23179	186.86	186.86	90.75

conventional coupling of the antioxidant functional moieties or entrapping the functional bio-activates on the surface of the inorganic nanoparticles could be one of the fantastic strategies for combining the surface activities of the nano-scaled particles and the antioxidant effects of incorporated functional moieties [29, 30].

The mechanisms of nanoparticle antioxidant

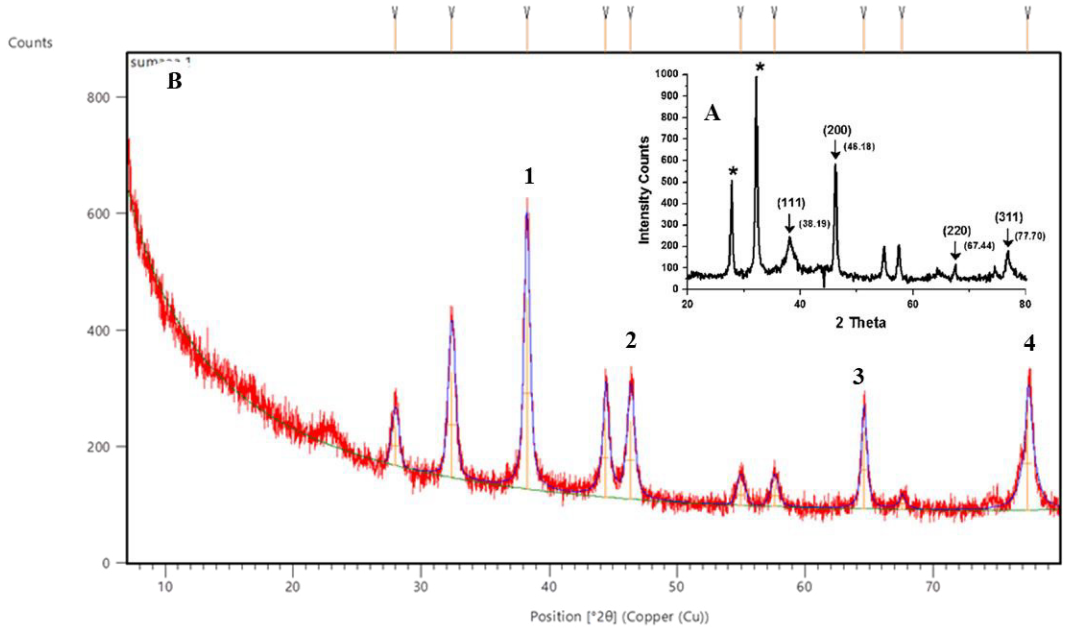

Fig.4. X-ray Diffraction analysis of, B. synthesised (Ag-NPs) where the peaks pointed out at 2θ values and A. synthesised (Ag-NPs) from previous study [27].

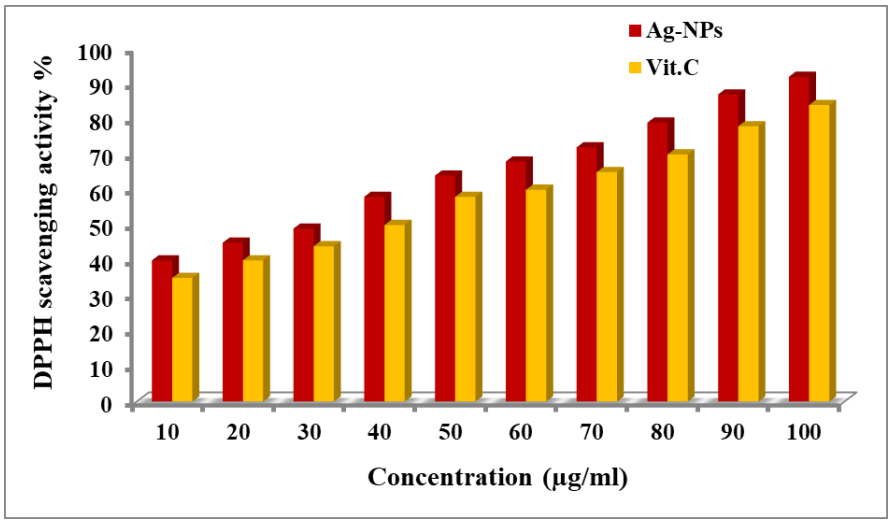

Fig. 5. DPPH scavenging activity of Ag-NPs in comparison with Vitamin C using at a range of different concentrations.

action are unknown; however, one theory is based on their intrinsic physicochemical features, which can improve scavenging reactive nitrogen and oxygen species while mimicking antioxidant molecules or antioxidant enzymes [31, 32]. Another way nanoparticles reduce free radicals is by converting alkyl peroxyl radicals to hydroperoxides [31].

Antifungal activity

Silver nanoparticles (Ag-NPs) occupy a prominent place as potential antifungal agents for clinical use due to their broad spectrum of antimicrobial activity. The agar-well diffusion technique was used to assess the activity of Ag-NPs on *M. cannonballus* species, and the results showed that synthesised Ag-NPs had potential antifungal action against the tested fungal strains. This may be due to their ability to limit fungal development by separating the cell membrane from the cell wall and intruding into the cytoplasm, resulting in the loss of cell wall integrity and homogeneity [33].

Various concentrations (1, 2, and 4 mg.mL^{-1})

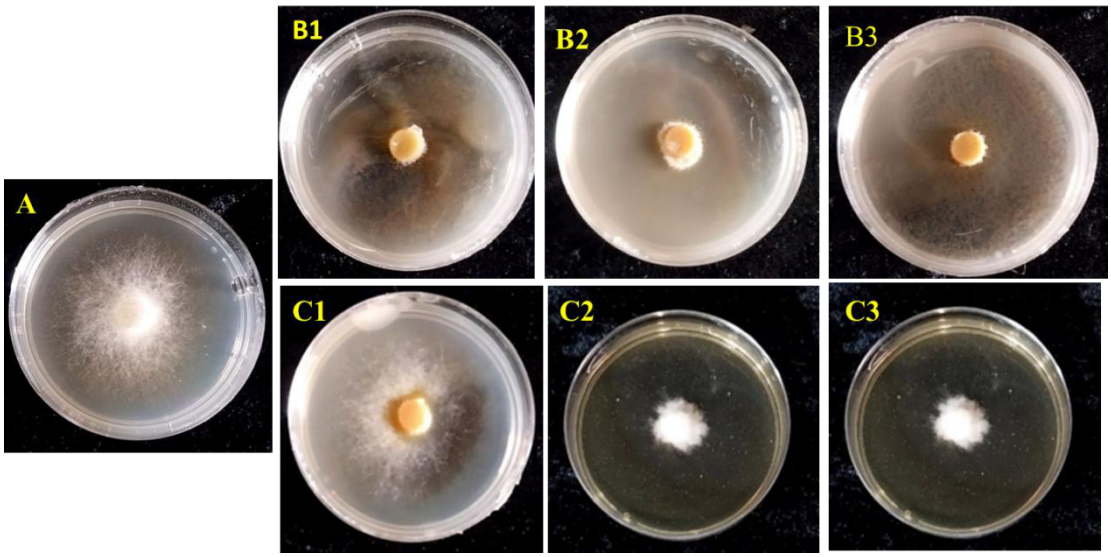

Fig. 6. Antifungal activity of Ag-NPs and rosemary extract against M.C. growth at different concentrations (1,2 and 4 mg.mL^{-1}). B1, B2 and B3 Ag-NPs, while, C1, C2 and C3 rosemary extract. A is M.C. without treatment as a control.

Table 2. The radial growth of fungi pathogen M.C. in different concentration of rosemary extract and Ag-NPs.

Concentrations	R1 (cm)	R2 (cm)	R3 (cm)	Mean (cm)	Inhibition %
Control	5	4.8	5	5	0 %
Ag 1 mg.mL^{-1}	1.3	1.5	1.2	1.3	74 %
Ag 2 mg.mL^{-1}	1.2	1.3	1.4	1.3	74 %
Ag 4 mg.mL^{-1}	0.2	0.1	0.2	0.1	98 %
Ex 1 mg.mL^{-1}	4.5	5	4	4.5	10 %
Ex 2 mg.mL^{-1}	4.5	4	4	4.1	18 %
Ex 4 mg.mL^{-1}	4	4	4.5	4.1	18 %

of Ag-NPs and rosemary extract (Ex) were tested against this fungi. The results were significantly inhibited of *M. cannonballus* growth, resulting in effective fungus growth control. (Fig. 6; Table 2)

Nano-silver was shown remarkable inhibition of *M.C.* in compassion to plant extract especially at the highest concentration possibly due to the increased permeability of the cell membrane. This leads to the structural destruction of cells and organelles [34]. It was confirmed that small-diameter Ag-NPs may penetrate pathogen cell walls and enter their cytoplasm [35].

Antibacterial activity

The antibacterial activity of Ag-NPs and plant extract was assessed against two different types of bacteria including gram-positive and gram-negative bacteria using well diffusion method. The results showed that Ag-nanoparticles have significant zone of inhibition in case of both types of these bacteria, and this inhibition increased significantly when the concentration increased too as presented in (Table3).

No zones inhibition were observed when rosemary extract has been tested against *E.coli, S. aureus* at the concentration of (2mg/ml). While, the antibacterial activity of plant extract has increased slightly at the highest concentration to be 7 mm. (Fig. 7)

The inhibition zone around each well was calculated to identify the antibacterial activity of both Ag-NPs and plant extract respectively.

It was reported that the antimicrobial activity of Ag-NPs differing to numerous bacteria was shape dependent [9].

Researchers who investigated the affectivity of silver nanoparticles on Gram-negative and Gram-positive bacterial strains, Khan *et al*. [36], observed that the toxicity of silver nanoparticles has the following order of effectiveness: *E. coli > P. aeruginosa > M. luteus > S. aureus*. This indicates that the synthesised Ag-NPs exhibit greater activity against Gram-negative bacteria compared to Gram-positive bacteria. Our results support this finding, as *E. coli* showed higher susceptibility even at lower concentrations of Ag-NPs. (Fig. 7, Table 3)

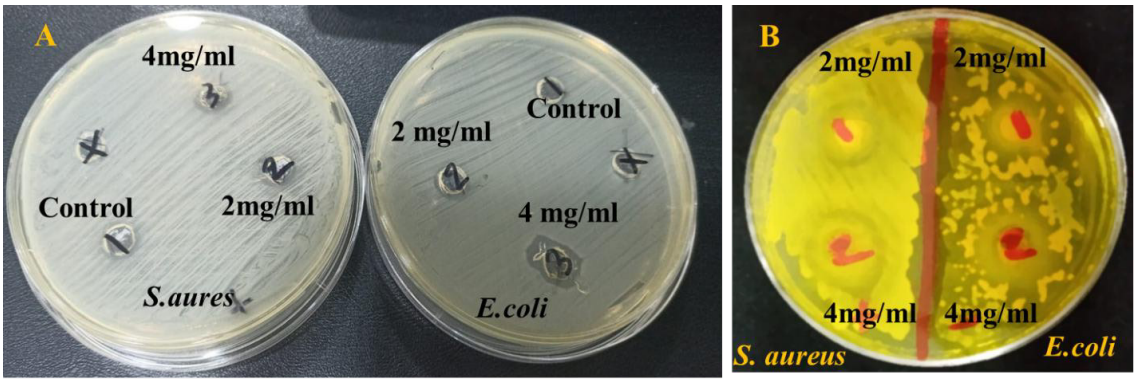

Fig. 7. Antibacterial activity of Ag-NPs and rosemary extract against both gram-positive (*S.aureeus*) and gram-negative bacteria(*E. coli*) , A. plant extract and B. Ag-NPs.

Tabe3. Antibacterial activity of Ag-NPs and rosemary extract against gram-positive and gram-negative bacteria.

Samples	Concentrations ($mg.mL^{-1}$)	Microorganism	Zone inhibition (mm)
Ag-NPs	2, 4	*Escherichia coli*	25, 30
	2, 4	*Staphylococcus aureus*	20, 22
Plant extract	2, 4	*Escherichia coli*	0 , 7
	2, 4	*Staphylococcus aureus*	0 ,0

CONCLUSION

Developing a biosynthetic approach for Ag-NPs with high efficiency, environmental friendliness, and low cost is becoming necessary today for nanotechnology research and applications. The current study indicated that rosemary leaf extract can be used to produce Ag-NPs with enhanced functionality. The generated Ag-NPs have a spherical shape and homogeneous size. Furthermore, the biosynthesized Ag-NPs had a strong inhibitory effect on the proliferation of M.C and bacteria. They have also shown exceptional antioxidant activity. Overall, the results suggest that Ag-NPs are an excellent option for viral disease treatment, and they could be used in the development of new antifungal and antibacterial agents. Further studies may be needed to confirm these findings and explore other potential treatment options for different diseases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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