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Biosynthesis of Silver Nanoparticles using *Ajuga iva* leaf extract and evaluating their antibacterial activity against *E. coli* and *Streptococcus* Bacteria

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Abstract

Silver nanoparticle synthesis from plant extracts has been widely used in medicine. Potential applications of silver nanoparticles include wound dressings, prosthetic and surgical instrument coatings, drug delivery, and an antimicrobial agent. The present study prepared and tested an aqueous extract of *Ajuga iva* leaves for its phytochemical components. The results of the phytochemical analysis of *Ajuga iva* leaf extract revealed the presence of alkaloids, carbohydrates, proteins, flavonoids, phenols, saponins, and coumarins. Silver nanoparticles were synthesized using the *Ajuga iva* leaf extract in a 1 mM solution of silver nitrate**.** The synthesized silver nanoparticles were characterized with UV-Vis spectroscopy, X-ray diffraction (XRD), Scanning Electron Microscopy (SEM), Energy-dispersive X-ray spectroscopy (EDX), and Fourier Transform Infra-Red (FTIR) spectrum. SEM analysis revealed the size of the AgNPs of 36 nm, 55 nm, 70 nm, 100 nm, and 300 nm. The EDX study showed that the optical absorption peak was detected

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at 3 keV, the characteristic peak for the absorbed metallic silver nanoparticles. FTIR analysis identified the possible functional group involved in the reduction of silver metal ions into silver nanoparticles. In addition, the antibacterial activity of synthesized silver nanoparticles was also examined and the results showed good antibacterial activities against *E. coli* and *Streptococcus* bacteria.

Keywords: *Ajuga ive* Leaf, Silver Nanoparticles, Antibacterial Activity, *E. coli*

Introduction

Nanoparticles (NPs) and nanotechnology have garnered significant attention from the scientific community globally, emerging as a rapidly developing and intriguing area of research, with NPs being the fundamental component in the fabrication of nanostructures, typically defined as materials with at least one dimension less than 100 nm, ranging in size from 1 to 100 nm (Cushing *et al*., 2004, Khan *et al*., 2017). NPs exhibit unique physical and chemical properties due to their high surface area and nanoscale size. These characteristics make them suitable candidates for various applications, including biomedicine, cosmetics, healthcare, food, drug-gene delivery, environmental science, mechanics, optics, chemical industries, electronics, space industries, and energy science (Xu *et al*., 2006). NPs can generally be divided into two categories: organic nanoparticles, which consist of carbon nanoparticles (fullerenes), and inorganic nanoparticles, which include semiconductor nanoparticles (such as TiO2 and ZnO2), magnetic nanoparticles, and noble metal nanoparticles (like Au and Ag). There is increasing interest in inorganic nanoparticles, particularly noble metal nanoparticles, due to their superior material properties and versatility (Gurunathan *et al*., 2014, Gurunathan *et al*., 2015). Silver nanoparticles (AgNPs) have attracted considerable attention from researchers because of their significant properties, including good conductivity, chemical stability, catalytic activity, surface-enhanced Raman scattering, and antimicrobial activity (Chen *et al*., 2005, Li *et al*., 2006). They are considered safer than their chemically synthesized counterparts (Khatoon *et al*., 2018). Additionally, silver nanoparticles are used in various fields, including food, healthcare, medical applications, consumer products, and industrial uses, due to their remarkable physical, chemical, and biological properties. (Mukherjee *et al*., 2001). The production of silver nanoparticles of varying sizes through a variety of biological systems, including bacteria, fungi, and plant extracts, has been reported (Gurunathan· *et al*., 2009). Plants can accumulate various heavy metals in their different parts. In addition, the green synthesis of nanoparticles using plant extracts seems to be a highly efficient method for developing

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technology that is quick, safe, non-toxic, and environmentally friendly (Akhtar *et al*., 2013). The green synthesis of silver nanoparticles using phytochemicals as bioreductants is gaining significant momentum (Rai & Yadav, 2013; Dubey *et al*., 2009). Phytochemicals in plant extracts, such as terpenoids, flavonoids, phenols, and alkaloids, are believed to be responsible for the reduction and stability of nanoparticles. However, little is still known about the behavior of AgNPs in the environment (Sehnal *et al*., 2019). This study focused on the potential for synthesizing silver nanoparticles using *Ajuga iva* (L) extract. *Ajuga iva* is a member of the Lamiaceae family and is locally known in Libya as Chendghoura; it also has the colloquial name Toutoulba (Miara *et al*., 2013). *Ajuga iva* is widely distributed across the temperate regions of Asia, North America, Europe, and Africa (Boran *et al*., 2015). This plant possesses various biological activities, including antidiabetic and anti-inflammatory properties, and is commonly used in traditional medicine to treat a variety of diseases (Medjeldi *et al*., 2018). Moreover, *Ajuga* has been used to treat joint pain, gout, and jaundice (Naghibi *et al*., 2005). Moreover, a decoction was made from the aerial parts of *Ajuga iva* (leaves and flowers), which have been used to treat many diseases, such as kidney and digestive disorders, diabetes, hypertension, and painful menstruation (Benkhnigue *et al*., 2014). *Ajuga iva* extracts have demonstrated significant antibacterial, anticancer, antioxidant, and antidiabetic activities (Bouyahya *et al*., 2020). Furthermore, a wide range of chemical constituents, including ajugarine, neoclerodane diterpenoids, anthocyanins, essential oils, flavonoids, tannins, terpenoids, steroids, fatty acids, and phenolic acids, have been identified in *Ajuga iva* extracts (Medjeldi *et al*., 2018). Thus, all the primary and secondary metabolites found in the leaves and fruits of *Ajuga iva* account for the majority of its pharmacological and biological activities (Diafat *et al*., 2016). There have been numerous reports on the synthesis of silver nanoparticles from Ajuga species, such as *Ajuga* parviflora (Mehdi *et al*., 2020), *Ajuga macrosperma* (Parveen *et al*., 2022), and *Ajuga bracteosa* (Andleeb *et al*., 2022). However, so far, there has been one report on the green synthesis of silver nanoparticles using *Ajuga iva* leaf extract, which studied their toxicity and photocatalytic activities (Al Moudani *et al*., 2023). Therefore, the present study aims to investigate the synthesis of silver nanoparticles of *Ajuga iva* leaf extract and to examine their antibacterial activity.

Materials and Methods

Material

Silver nitrate was purchased from BDH Chemicals ltd, and double-distilled deionized water was used.

Ajuga iva plant was collected from the Lamamra area, Al-Khums, Libya. The leaves of the plant were cleaned and washed well with distilled

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water to remove any remaining dust. Then they were left to dry under shade for about 8-10 days. After that, they were put in an oven at 40°C for 3 hours, and then they were ground into fine powder by the grinder.

Preparation of leaf extract

To prepare the leaf extract solution, 5 g of fine powder was taken into a 250 ml conical flask and mixed with 100 ml of distilled water, followed by boiling at 100 °C for 10 minutes. After that, the leaf extract was filtered using filter paper (Whatman no. 1), and the solution was collected in a separate conical flask.

Photochemical screening

A preliminary phytochemical analysis was performed on the aqueous extract of *Ajuga iva* leaves to evaluate the naturally occurring chemical compounds in this plant, following the standard methods outlined by Harborne (Harborne, 1987).

Test for Alkaloids

A few drops of Wagner's reagent were added to 2-3 ml of plant leaf extract. The formation of a brown-reddish precipitate indicated the presence of alkaloids

Test for Carbohydrates

2 drops of alcoholic α-naphthol were added to 2 ml of leaf extract followed by the addition of a few drops of concentrated H_2SO_4, resulting in a purplish-red ring indicating the presence of carbohydrate.

Test for Flavonoids

A few drops of H_2SO_4 were added to 2 ml of leaf extract, the orange color indicated the presence of flavonoids in the leaf extract.

Test for Proteins

1ml of leaf extract was mixed with a few drops of concentrated HNO_3 and the result confirmed the presence of proteins by changing the colour to yellow.

Test of Phenols

2 drops of 5% of $FeCl_3$ solution were added to 1 ml of leaf extract, changing the colour into blue or black showed phenols contents in the leaf extract.

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Test of Tannins

1 ml of leaf extract mixed with 2 ml of 10 % NaOH showed a positive result and tannins's existence.

Test for Saponins

3 ml of distilled water was mixed with 3 ml of plant extract and shaken vigorously for 15 mins layer of 1 cm of foam was formed, indicating the presence of saponins in the plant extract.

Test for Coumarins

2 mL of 10% NaOH was added to the plant extract sample, and a yellow color was observed, confirming the presence of coumarins.

Synthesis of silver nanoparticles

A 1 mM $AgNO_3$ solution was prepared and stored in an amber colour bottle. 50 ml of this solution was taken in a flask containing a magnetic bead, and then it was put on a magnetic stirrer. After that, 5 mL of leaf extract was added dropwise to the $AgNO_3$ solution with a little heat at 50-60 °C. A change of colour from yellow to dark brown indicated the formation of silver nanoparticles. Next, the conical flask was incubated at room temperature for 48 hours followed by centrifuged at 6,000 rpm for 15 minutes.

Characterization techniques

Ultraviolet-visible spectroscopy (UV-Vis) was carried out using a HACH LANGE DR-3900. The size, morphology, and composition of the silver nanoparticles were measured using scanning electron microscopy (SEM), energy dispersive X-ray (EDX) analysis with a JEOL instrument, and X-ray diffraction (XRD) analysis using a Bruker D8 Discover. Functional groups of the silver nanoparticles were analyzed using an FT-IR (Perkin Elmer FT-IR Spectrometer Frontier)

Anti microbial activity

The antibacterial activity of silver nanoparticles against two types of bacteria, *Escherichia coli* (negative gram) and *Streptococcus* (positive gram), was studied. The disk diffusion method was used to examine the antibacterial activity of silver nanoparticles. Strained bacteria were spread on the Petri dishes. After that, the disks soaked in distilled water as a control, Ag NO_3 solution, and silver nanoparticles (100 µg/ml) were separately put on Petri dishes and were incubated at 37 °C. The zone inhibition of each disk was determined by a ruler after 24 hrs.

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Results and Discussion

Phytochemical screening of *Ajuga iva* leaf extract

Phytochemical screening of *Ajuga iva* leaf extract was carried out using color-changing and precipitating chemical reagents for examining plant constituent's extract. The results of the testing extract possessed constituents such as alkaloids, carbohydrates, proteins, flavonoids, phenols, saponins, and coumarins, as shown in **Table 1.** This result was comparable with previous reports (Kuria *et al*., 2002). In addition, it was found that phytochemical constituents might be attributed to its various antimicrobial activities (Pramila *et al*., 2012). Furthermore, it is noteworthy that phytochemical components could be responsible for reducing silver ion solution to metallic silver to synthesize silver nanoparticles (Mogomotsi *et al*., 2019).

Table 1. Phytochemical constituents of *Ajuga iva* leaf extract

Chemical Groups	**Observations**
Alkaloids	+
Carbohydrate	+
Proteins	+++
Flavonoids	++
Phenols	+
Tannins	_
Saponins	+++
Coumarins	+++

Synthesis of silver nanoparticles

The synthesis of silver nanoparticles using *Ajuga iva* leaf extract was performed according to the Linga Rao procedure (Rao & Savithramma, 2011). The formed silver nanoparticles were indicated by a color change from yellowish to dark brown after the addition of *Ajuga iva* leaf extract to the silver nitrate solution, as shown in **Figure 1**. The color change is due to the reduction of ionic silver into metallic silver (Yousaf *et al*., 2020).

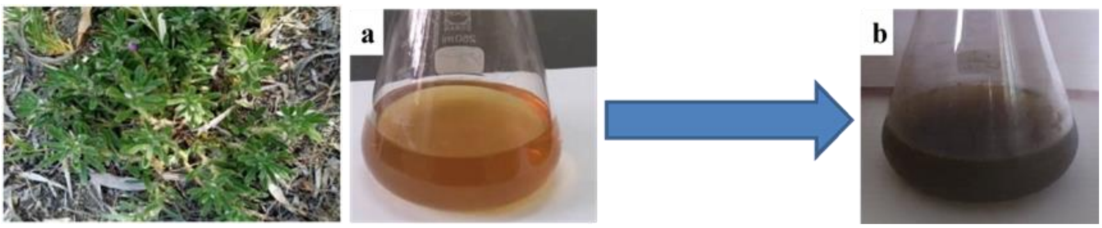

Figure 1. *Ajuga iva* leaf extract **(a)** and synthesized silver nanoparticles **(b)**

Characterization of silver nanoparticles

UV-Vis Spectroscopy

The synthesized silver nanoparticle was confirmed by UV-visible spectrum that showed the maximum absorbance peak at 430 nm as seen in **Figure 2**. A similar result has been obtained by Thiyagarajan and Kanchana,

 ISSN: 1857-7881 (Print) e - ISSN 1857-7431

who recorded a characteristic absorption band for silver nanoparticles at 430 nm (Thiyagarajan & Kanchana., 2022). This peak occurs due to its specific surface plasmon resonance (SPR) excitation (Das *et al.*, 2014). Moreover, the absorbance peak indicated the formation of spherical and uniform silver nanoparticles (Ben Salah *et al.*, 2021).

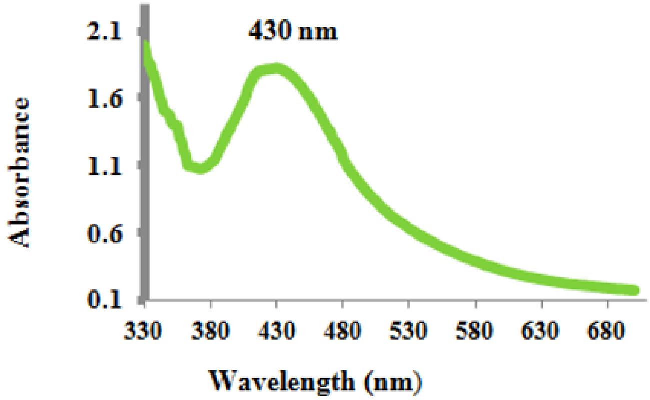

Figure 2. UV-Vis absorption spectrum of silver nanoparticles synthesized by *Ajuga iva* leaf extract treated with 1mM silver nitrate

XRD analysis of silver nanoparticles

The X-ray diffraction (XRD) was analyzed to examine the crystalline silver of AgNPs of *Ajuca ive* leaf extract. **Figure 3** shows the diffraction peaks at angles of 2θ at 38°, 44°, 67°, and 77°, which are related to (111), (200), (220), and (311). These finding results correspond to the face–centered cubic structure of silver metal silver (JCPDS file no. 84-0713 and 04-0783). Whereas other unmarked peaks might be due to the crystallizing of biological organic phases happening on the silver nanoparticles' surface. Similar results were reported by Willian and his group (Willian *et al.*, 2022).

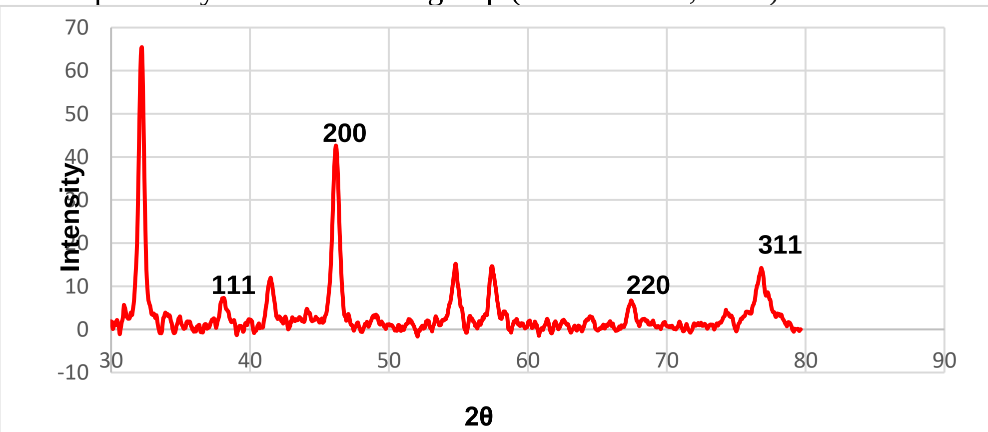

Figure 3. XRD pattern of silver nanoparticles

 ISSN: 1857-7881 (Print) e - ISSN 1857-7431

SEM of silver nanoparticles

The morphology and size of the AgNPs were analyzed using SEM.as seen in **Figure 4**. The images of SEM showed a spherical shape of AgNPs. In addition, the SEM image showed the different sizes of the AgNPs (36 nm, 55 nm, 70 nm, 100 nm, and 300 nm). These differences in the shape of the AgNPs might be due to the variety in the chemical structure of phytochemical constituents, which may be responsible for the reduction and stabilization of silver ions into silver metal. Moreover, the increase in the size of AgNPs (300 nm) might be because the two particles came together, so in the SEM image appeared one particle. These findings of SEM' sizes were parallel with Al Moudani *et al.*, who found the size of silver nanoparticles of *Ajuga iva* leaf extract were 100-300 nm (Al Moudani *et al*., 2023).

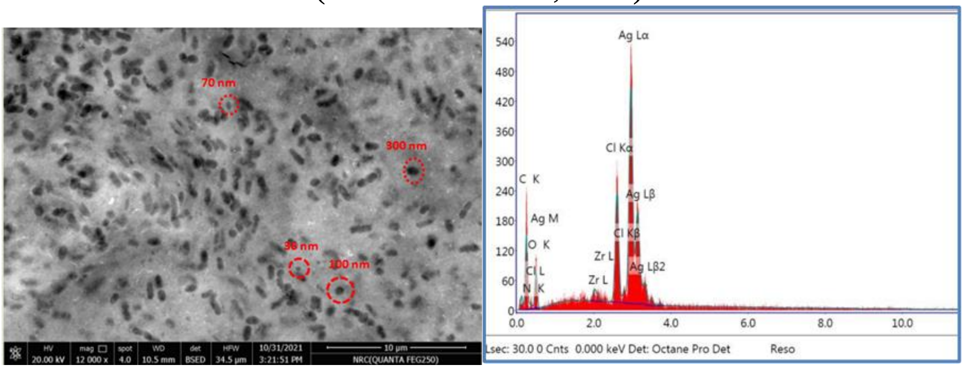

Figure 4. Scanning Electron Micrograph of *Ajuga iva* leaf extract silver nanoparticles (left) and EDX spectrum analysis demonstrating the main peak of silver nanoparticles at 3 keV, corresponding to silver nanoparticles

EDX analysis of silver nanoparticles

EDX analysis also showed a very strong signal in the silver region, confirming the formation of silver nanoparticles, as seen in **Figure 4**. The optical absorption peak was observed at 3 keV, which is typical for the absorption of metallic silver nanoparticles because of surface Plasmon resonance (Magudapathy *et al*., 2001). In addition, other peaks for Cl, O, C, and N were observed, which may be due to proteins or enzymes present in the culture supernatant (Mandal & Sastry, 2005).

IR of silver nanoparticles

FTIR spectra were conducted to identify the biosynthesized silver nanoparticles from *Ajuga iva* leaf extract. The IR spectra of the *Ajuga iva* extract and the biosynthesized silver nanoparticles were compared. The IR spectrum of the *Ajuga iva* leaf extract shows absorption bands in the range of 3286.53 to 878.90 cm^{-1}, as illustrated in **Figure 5**. In contrast, the AgNPs from the *Ajuga iva* leaf extract exhibit absorption bands in the range of 3287.88 to 824.73 cm^{-1}, as seen in **Figure 6**. The broad band at 3286.53 cm^{-1} in the *Ajuga*

 ISSN: 1857-7881 (Print) e - ISSN 1857-7431

iva leaf extract shifts to 3287.88 cm^{-1} and becomes less intense in the AgNPs. Previous reports indicate that this absorbed band corresponds to the OH group of alcohols and phenolic compounds (Vanaja *et al*., 2013). The vibration band at 2918.87 cm^{-1} shifts to 2921.14 cm^{-1}, which is attributed to the stretching vibration of C-H in aliphatic compounds. The sharp peak located at 2850.45 cm^{-1} in the *Ajuga iva* extract is assigned to the C=C stretching mode; after the formation of AgNPs, this band diminishes and loses intensity. In the free extract, the absorption band of C=O appears at 1730.88 cm^{-1}, which is associated with carbonyl-containing compounds. However, in the AgNPs, this band disappears, suggesting the involvement of C=O compounds in the bioreduction of silver ions (Sadeghi & Gholamhoseinpoor., 2015). The bands at 1603.01 cm^{-1} and 1416.54 cm^{-1} are primarily attributed to the stretching vibrations of aromatic C=C bonds and C–N bonds associated with amine functional groups (Yan-Yu *et al*., 2016). In the AgNPs, a new band at 1372.62 cm^{-1} is formed, which may be associated with the N-O symmetry stretching of the nitro compound (Balaji *et al*., 2009). Similarly, the small bands at 1240.47 cm^{-1} and 1008.56 cm^{-1} in the free extract are related to the presence of C–O and C–H stretching vibrations of polysaccharides (Jena *et al*., 2016). These bands shifted to a single absorption band at 1023.07 cm^{-1}. The bands at 878.90 cm^{-1} and 824.73 cm^{-1} indicate the presence of C–H bonds in aromatic compounds found in the *Ajuga iva* extract and the biosynthesized AgNPs. The results of the infrared spectra of the obtained AgNPs demonstrate the presence of characteristic functional groups, such as alcohols, phenols, aldehydes, flavonoids, and amines, which may be responsible for the bioreduction and stabilization of the synthesized silver nanoparticles (Kaviya *et al*., 2011; Nayak *et al*., 2015).

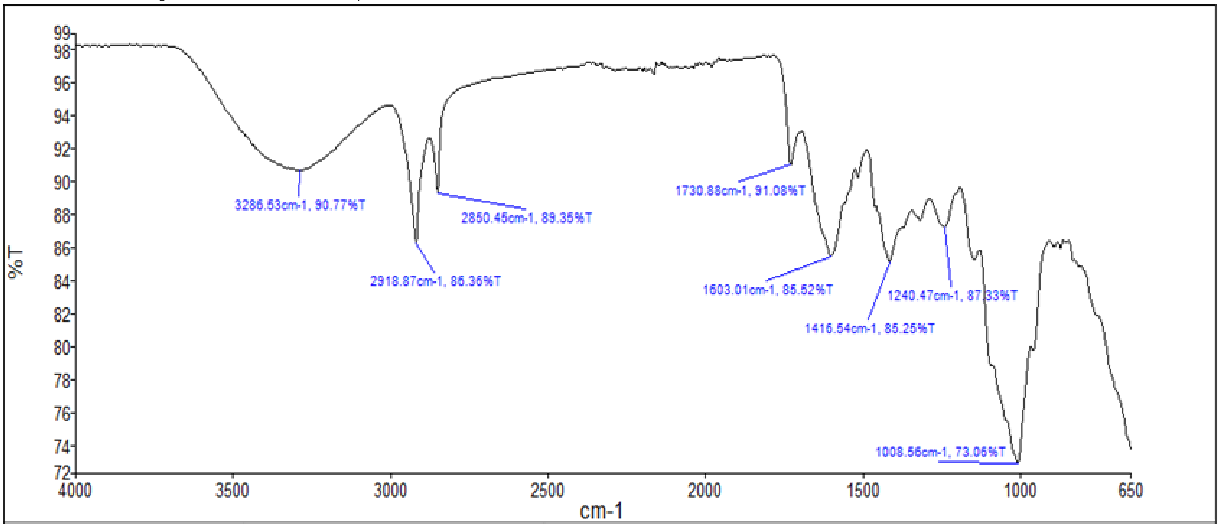

Figure 5. FTIR Spectrum of *Ajuga iva* leaf extract

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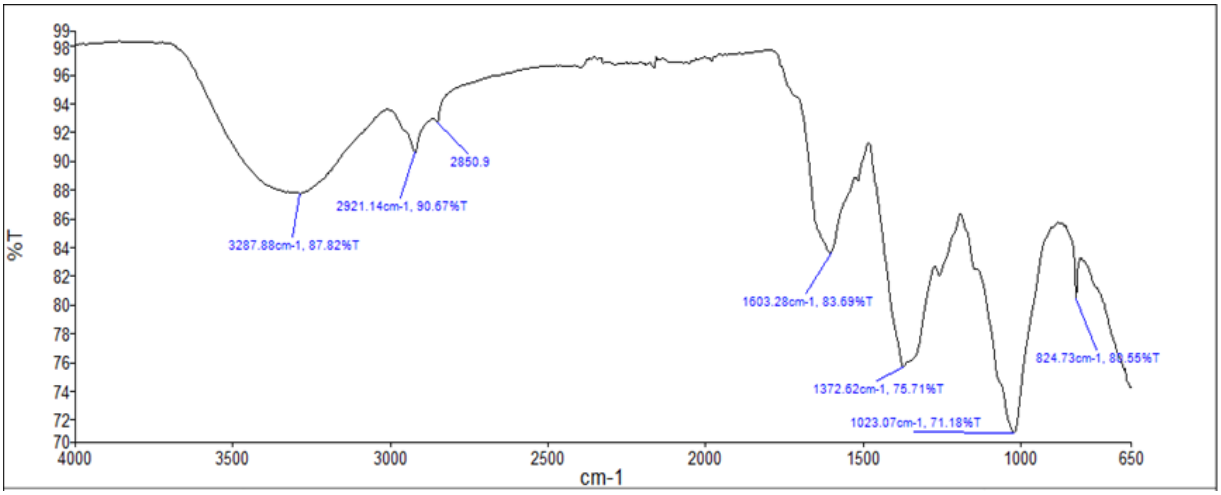

Figure 6. FTIR Spectrum of synthesized silver nanoparticles of *Ajuga iva* leaf extract

Anti-bacterial activity

The antibacterial activity of the synthesized AgNPs from *Ajuga iva* leaf extract has been investigated. The antibacterial effect of synthesized silver nanoparticles was studied on two types of bacteria, *E. coli* (Gram-negative) and *Streptococcus* (Gram-positive). The disc diffusion method was used as an antibacterial susceptibility testing method (Thiruvengadam & Bansod, 2020). The AgNPs of *Ajuga iva* leaf extract **(A1**) were compared with $AgNO_3$ (Ag), as shown in **Figure 7.**

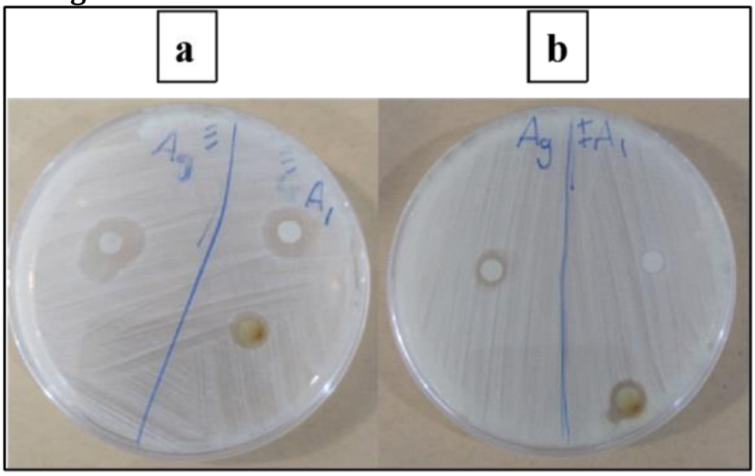

Figure 7. Anti-bacterial activity of formed silver nanoparticles from *Ajuga iva* leaf extract against *E coli* (a), *Streptococcus (b)*, Ag (AgNO3 as a controller), A1 (AgNPs)

The findings illustrated that the disc diffusion method proved the antibacterial activity of the AgNPs. The inhibition zones showed a maximum value of 12 mm **(A1)** against *E. coli* and 7 mm **(A1)** against *Streptococcus*, as

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seen in **Table 2.** In this study, the AgNPs of *Ajuga iva* leaf extract **(A1)** show better antibacterial activity for both types of bacteria. Similar results were observed for AgNPs against *E coli* (Vu *et al*., 2018). It was reported that the silver nanoparticles' size affects the inhibition to a better extent (Murugan & Natarajan., 2018).

Table 2. Effect of AgNPs on bacterial activity

Bacterial strain	Concentration of AgNPs	Zone of inhibition (mm)
E. Coli	100 µg/ml	12 mm
Streptococcus	100 µg/ml	7 mm

Conclusion

In summary, the green synthesis of silver nanoparticles of *Ajuga iva* leaf extract was performed. The formation of silver nanoparticles was characterized by UV-Vis spectroscopy, X-ray diffraction (XRD), Scanning Electron Microscopy (SEM), Energy-dispersive X-ray spectroscopy (EDX), and Fourier Transform Infrared (FTIR). Additionally, the small size of silver nanoparticles contributes to their impressive antibacterial activity, making them potential candidates for future antibacterial drugs. In addition, it is recommended to pursue additional research to determine the specific compounds responsible for the antibacterial properties of silver nanoparticles derived from *Ajuga iva* leaves. Gaining insight into the exact compounds that demonstrate antibacterial activity will aid in the development of more targeted and effective silver nanoparticles.

Conflict of Interest: The authors reported no conflict of interest.

Data Availability: All data are included in the content of the paper.

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