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Research Article

Technological Study on The Synthesis of Silver Nanoparticles Using Plant Extracts Via Biosynthesis Methods

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ABSTRACT

Introduction: Silver nanoparticles are better than other metal nanoparticles in terms of antimicrobial activity and stability. Recently, the biosynthesis method has been widely used, known for being eco-friendly and having lower toxicity compared to other methods of obtaining metal nanoparticles. This method is characterized by the use of active pharmaceutical ingredients contained in medicinal plants as stabilizers or bio reducers to produce silver nanoparticles. **Methods:** In this study, we synthesized silver nanoparticles using extracts from the upper parts of *Cacalia hastata* L., *Thymus gobicus* Czern., and *Glycyrrhiza uralensis* Fisch., which were collected from various provinces from Mongolia. These medicinal plants are used as bio reducing agents. We determined the most sensitive light absorption of each sample with purified silver nanoparticles using a UV-M51 ultraviolet spectrophotometer. Sizes and distributions were analyzed through Nanophox Particle Size Analysis, while morphological structure was examined using energy dispersive X-ray spectroscopy (EDX). The formations of nanoparticles were determined with instruments such as X-Ray Diffraction (XRD). **Results:** The appropriate formation times for nanoparticles were 24 minutes with *Cacalia hastata* L. extract and 16 minutes with *Thymus gobicus* Czern. extract. XRD analysis revealed characteristic peaks at 38.15°, 44.3°, and 64.55°, indicating the formation of a crystalline structure and confirming the presence of silver nanoparticles. **Conclusion:** Furthermore, these nanoparticles exhibited antibacterial activity against both *S. aureus* and *E. coli*.

Key words: Antimicrobial, Bio reducing, Eco-friendly, Mongolian plants.

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INTRODUCTION

Diseases mostly happen because of harmful changes in cells and molecules. Detecting and treating these problems at such tiny levels are big challenges in modern medicine.[1] Nanotechnology uses special properties of medicine plants at the atomic and molecular levels to build complex systems in a nanostructured environment, which consists of atomic molecules in the primary nanoscale space.[2] In the field of nanotechnology, materials at various nanoscale levels have been extracted and utilized. Currently, nanoparticles are the most widely used in medicine.[3]

In the past few years, more infectious diseases have been spreading. This is affecting the world's economy and public health. This happens because there are too many people, pollution in water and problems with the environment. Due to these factors, researchers are extensively investigating silver nanoparticles owing to their pronounced therapeutic efficacy and distinctive attributes.

Silver nanoparticles have demonstrated greater antimicrobial activity and stability compared to other metal nanoparticles. There are several methods for obtaining silver nanoparticles. Among these methods, the most widely used is biosynthesis, known for its eco-friendly and having lower toxicity. To obtain silver nanoparticles using the biosynthesis method, microorganism or plant extracts are utilized for the deionization of silver. Also, it can be reducing the toxicity of silver particles by coating them with different substances. Researchers expect that by 2030, they will fully understand the human genome, which will allow the development of a new generation of nanotechnology-based medicines.

In Mongolia, nanotechnology development started over a decade ago, but nanomedicine technology has not yet been integrated into our pharmaceutical industry. Consequently, developing modern technology for safe and effective natural silver nanoparticles are the basis for this research, replacing imported medicines.

MATERIALS AND METHODS

Chemicals and Plant Material Collection

All the reagents used were of analytical grade and did not require further purification. Silver nitrate ($AgNO_3$) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) with a ≥99.8% purity. Fresh leaves of *Cacalia hastata* L. were collected from the surroundings of the village of Bugant in the Selenge province and *Thymus gobicus* Czern. was collected from the Delgertsogt region in the Dundgobi province, Mongolia. Fresh roots of *Glycyrrhiza uralensis* Fisch. were sourced from the Nomgon region in the Umnugobi province, Mongolia. (Figure 1) Distilled water was used to prepare all extracted solutions throughout the experiments.

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Figure 1: Leaves and root of *Cacalia hastata* L, *Thymus gobicus* Czern. and *Glycyrrhiza uralensis* Fisch. used for synthesis of Ag NPs.

Preparation of Extract

Extracted solutions were prepared by the following procedure: The sand, dirt and other residues from the collected plant materials were carefully cleaned, washed with cold water and protected from excessive drying, moisture absorption and pest contamination. They were then dried in compliance with the specified technological requirements in a cool, dry location with adequate air circulation. After drying and preparing the upper and lower parts of *Cacalia hastata* L., *Thymus gobicus* Czern., and *Glycyrrhiza uralensis* Fisch., we crushed the upper part into 5-7 mm pieces using a Bio Base Bioindustry plant crusher from China. The lower part was cut into 2 mm pieces. We then took 5 g of each type of plant material, added 120 ml of distilled water, and heated it to 80°C for 30 minutes. Finally, we filtered and purified the resulting extract using Whatman №1 membrane filter.

Synthesis of Ag Nanoparticles

First, accurately weighed 17 g of 99.8% pure silver nitrate powder and dissolved it in 1 liter of distilled water to make a 10 mM $AgNO_3$ solution. Then, created 5 different $AgNO_3$ solutions with concentrations of 1 mM, 2 mM, 3 mM, 4 mM and 5 mM by diluting the 10 mM solution. Measured 20 ml of plant extracts and add them to 180 ml of a 1-5 mM $AgNO_3$ solution prepared earlier. Stir the mixture continuously at 60°C using a magnetic stirrer with a heater for the deionization reaction. Additionally, the mixture was stored in the refrigerator for the antibacterial activity test and further analyzed using a UV-Vis spectrophotometer, Nanophox Particle Size Analysis and Energy-dispersive X-ray analysis (EDX).

Characterization of Ag Nanoparticles

To find out the size and distribution of Ag NPs, we first measured and put 1 ml of the nanoparticle sample into a cuvette glass. Then, we used Nanophox Particle Size Analyzer, which uses a 630 nm laser light to figure out how big the nanoparticles are and how they're spread in the sample. UV-Vis spectral analysis was done by using UV-Vis spectrophotometer UV-M51 at the wavelength of 300-800 nm.

During the sample preparation, each sample was cleaned and subjected to rotation at 5000 rpm for 15 minutes, after which the precipitates were collected and dried at room temperature. The prepared samples were then examined using Energy Dispersive X-ray Spectroscopy (EDX).

X-ray diffraction (XRD) was employed to ascertain the formation of Ag NPs. A thin layer of the sample was deposited on silicon sheet, and monochromatic X-rays were irradiated onto it using an Enraf- Nonius Delft device at an angle ranging from 0° to 80°(2Θ) with a 30kV voltage and 30 mA current at a 4 degrees/minute scanning speed to determine the crystal structure formation. Research results will be processed using the Origin 2022 program.

Antimicrobial Study

The antimicrobial activity has been investigated against *S. aureus* as a model for Gram-positive bacteria and *E. Coli* as a model for Gram-negative bacteria. The antimicrobial activity was evaluated by the disc diffusion method. *S. aureus* was cultured on Mannitol Salt agar, and *E. Coli* was cultured on Endo agar for 24 hours. Two solutions with a concentration of 0.5 MacFarland units were prepared. This concentration is often used to standardize bacterial suspensions. To test the antibacterial activity, Ag NPs (likely a bacterial extract) was prepared in Muller-Hinton agar. This agar is commonly used for antibiotic susceptibility testing. The Ag NPs were then impregnated onto discs in five different solutions with concentrations of 25μg, 50μg, 75μg, 100μg and 150μg, with each solution containing 20μl. Antibacterial activity was determined by incubating the samples in a thermostat for 24 hours and comparing them with gentamicin and vancomycin antibiotics.

Statistical Analysis

Statistical Analysis Data are expressed as the mean ± standard deviation. Differences between groups were analyzed using one-way analysis of variance. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Color reaction for Ag NP formation

The formation of Ag NPs was evaluated by comparing the color change, which depended on the concentration of silver nitrate. At the beginning of the experiment, when the extracts of *Cacalia hastata* L. and *Thymus gobicus* Czern. were straw-yellow in color, adding silver nitrate at concentrations of 1-5 mM caused the solution's color to change to gray-green during the experiment. A silvery surface formed on the walls of the test flasks, and both the surface area and the color changed, indicating the formation of silver nanoparticles. However, no color change occurred when *Glycyrrhiza uralensis* Fisch. extract and silver nitrate solution with a concentration of 1-5 mM were stirred in a magnetic stirrer with a heater. (Figure 2)

When the plant extract is mixed with an AgNO3 solution, it undergoes a color change, indicating the formation of nanoparticles.[4] Kirithiga S, Santhanalakshmi J (2015) studied the antibacterial activity of silver nanoparticles extracted from Clitoria ternatea and Solanum nigrum plant extracts through biosynthesis.[5] The color of the extracted silver nanoparticles changed from brown to gray-green. Similarly, the color of our prepared silver nanoparticles changed from straw yellow to gray-green as the concentration of AgNO3 increased, indicating not only the formation of nanoparticles but also aligning with the results of the aforementioned researchers.

Purification and separation of Ag NPs

The extracted Ag NPs were purified using the centrifugation method. The results demonstrated that Ag NPs completely settled at the bottom of the test tube within 15-18 minutes. Therefore, for subsequent experiments, we determined that a centrifugation speed of 5000 rpm for 15 minutes was suitable for purifying Ag NPs. (Figure 3)

Determination of the size of Ag NPs

Cacalia hastata L. extract was combined with a 3 mM $AgNO_3$ solution, resulting in Ag NPs with an average size of 129.93±0.293 nm. Similarly, Ag NPs produced using *Thymus gobicus* Czern. extract and a 2 mM $AgNO_3$ solution had an average size of 104.97±0.052 nm. However, the average size of Ag NPs obtained using *Glycyrrhiza uralensis* Fisch. extract and a 3 mM $AgNO_3$ solution were 219.98 nm, which was relatively larger than the Ag NPs obtained using the other two plant extracts ($p<0.001$). Consequently, we excluded Ag NPs extracted using *Glycyrrhiza uralensis* Fisch. extract from further research. (Figure 4)

Mongolian scientists Erelt and Bum-erdene (2021) extracted silver nanoparticles of 145.1 nm from Carduus crispus plant extract and studied their antibacterial activity.[6] Silver nanoparticles of 129.93 nm

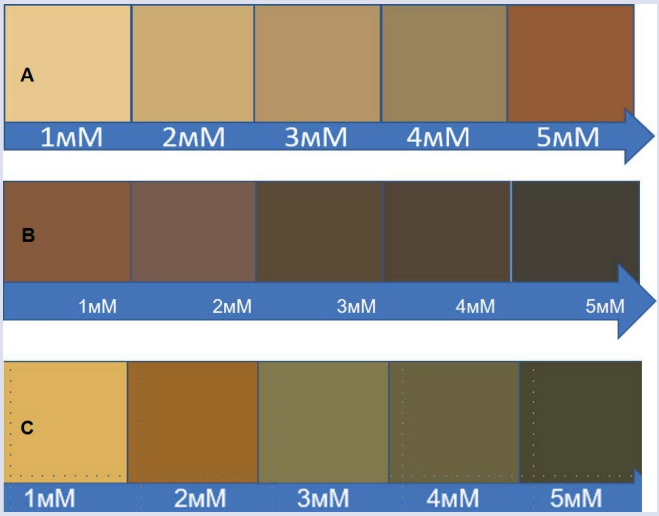

Figure 2: Color reactions of *Cacalia hastata* L, *Thymus gobicus* Czern. and *Glycyrrhiza uralensis* Fisch. for Ag NP formation.

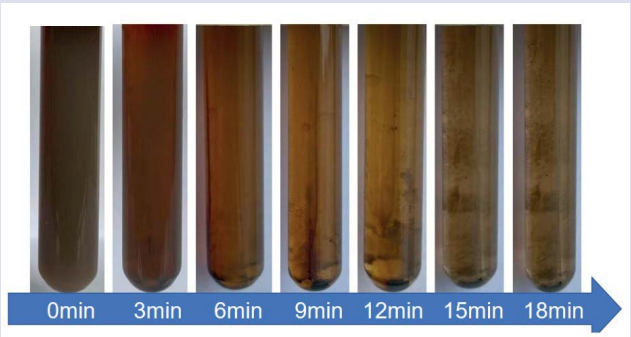

Figure 3: The Purification and separation time of Ag NPs .

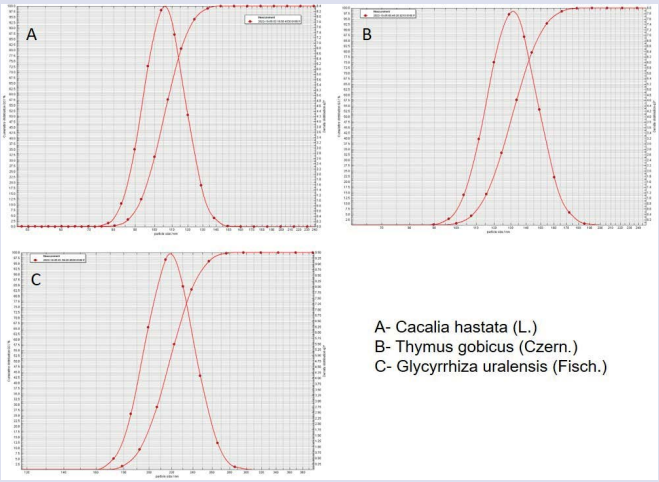

Figure 4: Determination of the size of Ag NPs .

were extracted using *Thymus gobicus* Czern. and silver nanoparticles of 104.97 nm were extracted using *Glycyrrhiza uralensis* Fisch. plant extract. Silvered nanoparticles were formed with a size of 219.98 nm, respectively. It can be observed that, except for the silver nanoparticles extracted using *Glycyrrhiza uralensis* Fisch. extract, the results are similar to those of the aforementioned researchers. In the case of *Glycyrrhiza uralensis* Fisch. plants, it is hypothesized that glycyrrhizic acid, the main active ingredient, may lead to excessive clustering of nanoparticles due to its sweet and sticky properties.

UV-Vis Spectra Analysis

Spectrophotometric determination of the optimal $AgNO_3$ concentration for Ag NP formation

The UV-Vis spectrophotometer was used to determine and compare the absorption peak wavelengths for a total of 10 samples, which included Ag NPs extracted using 2 different plant extracts and $AgNO_3$ concentrations ranging from 1-5 mM. The characteristics of Ag NPs normally appear at a wavelength interval of 300-800 nm.

Ag NPs made with *Cacalia hastata* L. extract and 3 mM $AgNO_3$ had their peak light absorption at 454 nm, and those made with *Thymus gobicus* Czern. extract and 2 mM $AgNO_3$ had their peak absorption at 465 nm, with a statistically significant difference ($p<0.001$). (Figure 5)

Ag NPs typically exhibit optimal light absorption in the wavelength range of 400-600 nm.[7] Vasireddy R, Paul R, and Mitra AK (2012) employed a biosynthesis method to produce silver nanoparticles of varying sizes (100 nm and 90 nm) using different molar concentrations of NaOH. They also prepared 80 nm silver nanoparticles and studied their light absorption using a UV-visible light spectrophotometer.[8] According to their research findings, the nanoparticles exhibited light absorption peaks at wavelengths of 478 nm, 496 nm, and 504 nm within the 400-600 nm range. It was further concluded that nanoparticles of different sizes absorb light to varying degrees, with larger nanoparticles demonstrating increased light absorption due to their larger surface area.

The optimal duration for the formation of Ag NPs were determined using spectrophotometric analysis

The light absorption of Ag NPs, obtained using the extract of *Cacalia hastata* L. and $AgNO_3$ solution with a concentration of 3mM, continuously increased from 2 to 26 minutes. This happened because Ag NPs were forming well. However, the formation of particles started to decrease after 28 minutes. The light absorption of Ag NPs, produced using *Thymus gobicus* Czern. extract and a 2mM AgNO3 solution, steadily increased from 4 to 18 minutes. However, the formation of Ag NPs started to decrease after 20 minutes. (Figure 6)

Results of spectrophotometric determination of the suitable ratio of $AgNO_3$ and plant extracts for the formation of Ag NPs

To find the suitable ratio of $AgNO_3$, plant extracts for Ag NP formation in ratios of 45:5, 40:10, 35:15, 30:20, and 25:25, respectively. We used suitable concentration of $AgNO_3$ with the most sensitive light absorption, *Cacalia hastata* L. and *Thymus gobicus* Czern. were used as a plant extract. After 10 minutes of mixing by magnetic stirrer with hot plate, we determined and compared the most sensitive light absorption wavelengths.

The 40:10 ratio of *Cacalia hastata* L. extract and 3mM AgNO3 exhibited a continuous increase at 470 nm, and similarly, the extract of *Thymus gobicus* Czern. with 2mM AgNO3 also showed an increase at 440 nm. (Figure 7)

Spectrophotometric determination of whether the mixture ratio of plant extracts affects the formation of silvered nanoparticles

Silvered nanoparticles obtained by using a 60:40 mixture of *Thymus gobicus* Czern. and *Cacalia hastata* L. extracts and 3 mM AgNO3 solution had the most sensitive light absorption at 432 nm, while the silver nanoparticles obtained using a 60:40 mixture of *Cacalia hastata* L. and *Thymus gobicus* Czern. extracts and 3 mM AgNO3 solution had the

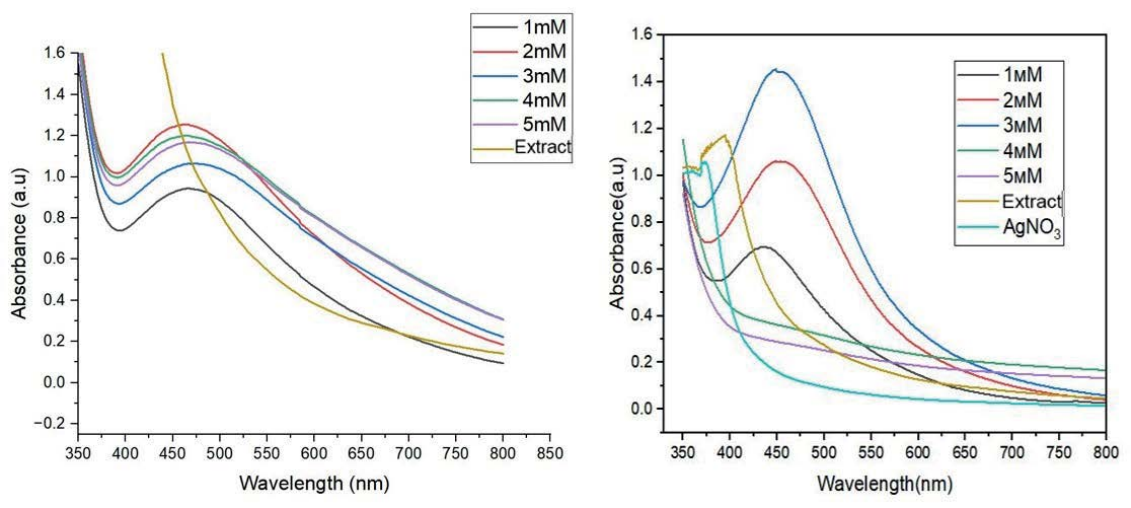

Figure 5: Spectrophotometric determination of the optimal $AgNO_3$ concentration for Ag NP formation.

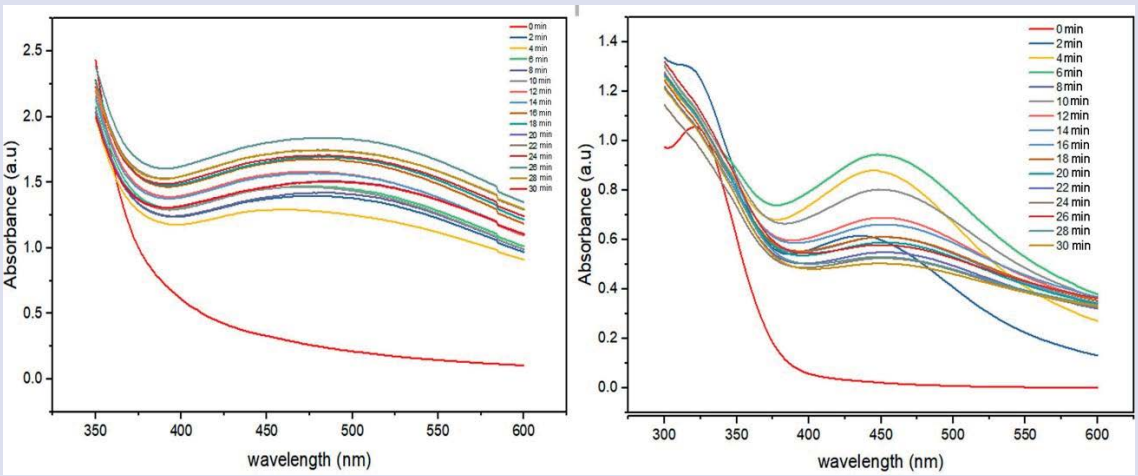

Figure 6: The optimal duration for the formation of Ag NPs were determined using spectrophotometric analysis.

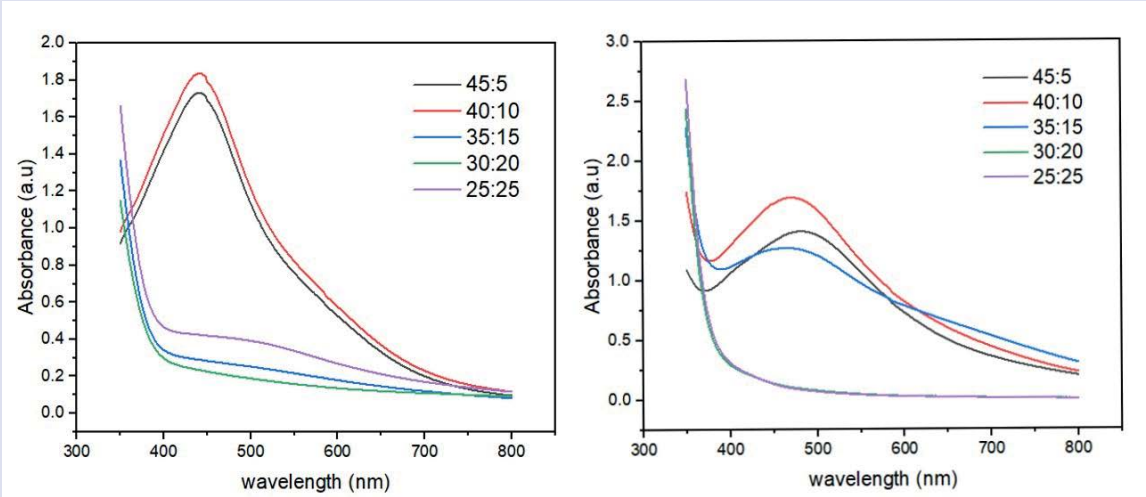

Figure 7: Results of spectrophotometric determination of the suitable ratio of AgNO3 and plant extracts for the formation of Ag NPs.

most sensitive light absorption at 417 nm ($p<0.001$). Nevertheless, the silver nanoparticles obtained using a 50:50 mixture of *Cacalia hastata* L. and *Thymus gobicus* Czern. extracts and a 3mM AgNO3 solution exhibited the most sensitive light absorption at 406 nm. (Figure 8)

We determined the optimal time for the formation of Ag NPs by mixing the two plant extracts in the following ratio. The light absorption of silver nanoparticles, obtained by using a 60:40 mixture of extracts from *Thymus gobicus* Czern. and *Cacalia hastata* L. with a 3mM AgNO3 solution, continuously increased from 2 to 18 minutes. However, the formation of silver nanoparticles began to decrease starting from 20 minutes. (Figure 9)

Results of determining the elemental composition of Ag NPs

In determining the composition of elements, the nanoparticles extracted using the extract of *Cacalia hastata* L. were uniformly well distributed, with 99.8% silver and 0.2% silicon detected, while the silver nanoparticles extracted using the extract of *Thymus gobicus* Czern. were evenly distributed, with 98.9% silver, 0.2% aluminum, 0.3% iron, and 0.6% silicon detected. (Figure 10)

Silicon plays a crucial role in plant metabolism, particularly in grasses. It is also associated with the organic compounds present on the surface of silver nanoparticles, playing a significant role in discharge reactions and stability during biosynthesis. The absence of a nitrogen peak in the sample suggests the lack of $NO3^-$ ions, indicating the sample's complete purification.

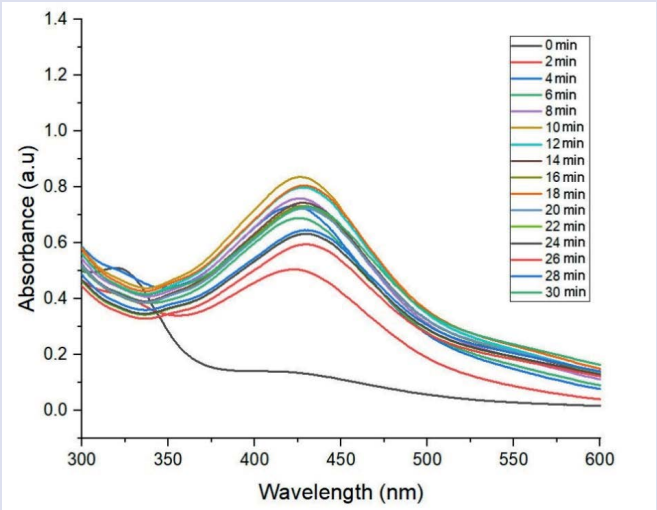

Figure 8: Spectrophotometric determination of whether the mixture ratio of plant extracts affects the formation of silvered nanoparticles.

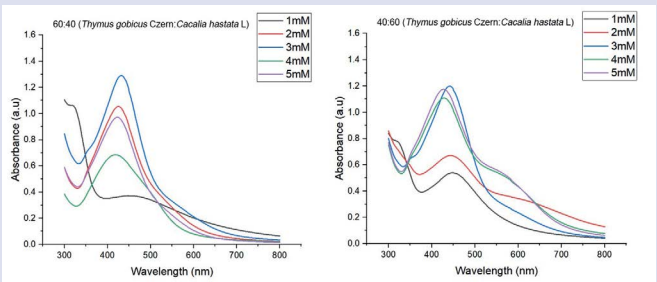

Figure 9: Spectrophotometric determination of whether the mixture ratio of plant extracts affects the formation of silvered nanoparticles.

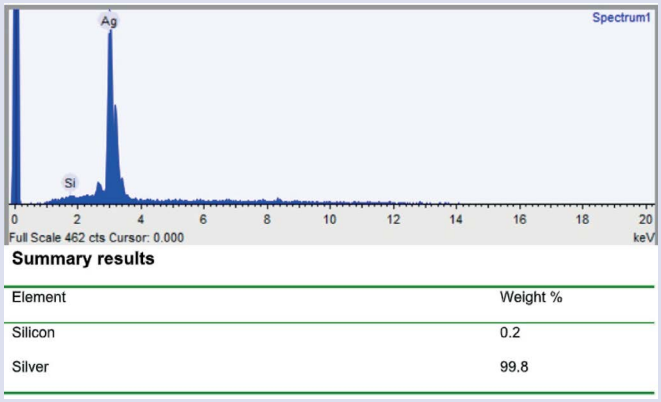

Figure 10: Results of determining the elemental composition of Ag NPs.

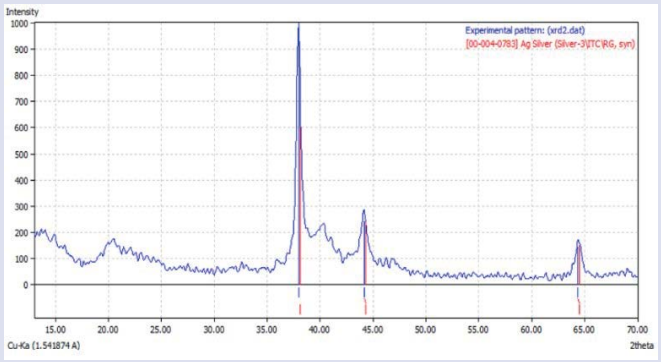

Figure 11: The results of determining the formation of silver nanoparticles using an X-Ray Diffraction (XRD) instrument.

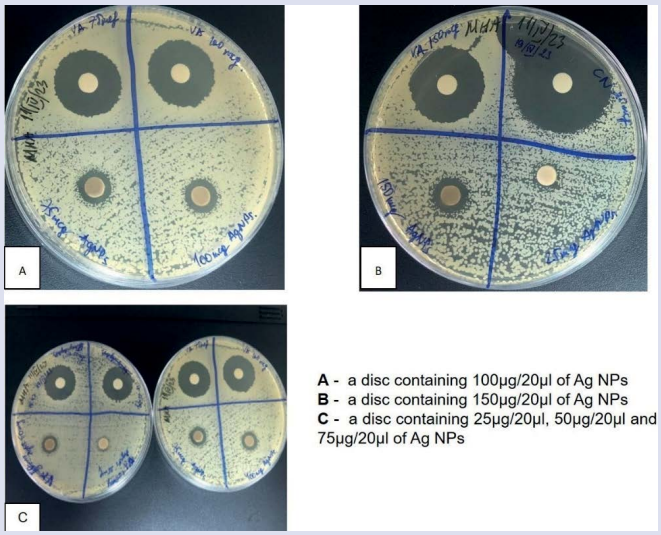

Figure 12: Results of the antibacterial activity determination of Ag NPs using disc diffusion method.

The results of determining the formation of silver nanoparticles using an X-Ray Diffraction (XRD) instrument

XRD analysis of the crystal structure of silver nanoparticles obtained using the extracts of *Cacalia hastata* L. and *Thymus gobicus* Czern. plants showed peaks at 38.15°, 44.3°, and 64.55°. These peaks are sharp, indicating that the nanoparticles are spherical in shape. (Figure 11)

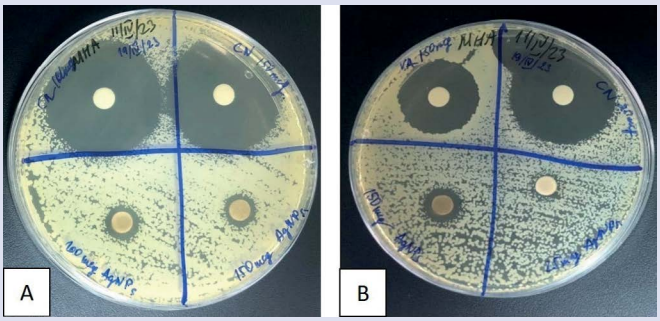

Figure 13: In Escherichia coli, compared to Gentamicin antibiotic.

Additionally, the sharp peaks indicate that the nanoparticle is spherical in shape. We confirmed the crystalline nature and formation of the silver nanoparticles by X-ray diffraction (XRD). The Bragg reflections of the 2θ peaks for both extracted Ag NPs were observed at 38.15°, 44.3°, and 64.55°, consistent with the face-centered cubic (fcc) crystal structure typical of silver nanoparticles, as reported by scientists Chen Yu and Jingchun Tang (2017).[9,10]

According to the study's findings, silver nanoparticles were synthesized from plants grown in our country and verified using instrumental analysis methods, yielding results similar to those of other researchers.

Results of the antibacterial activity determination of Ag NPs using disc diffusion method

It has been proven in many studies that Ag NPs obtained by biosynthesis is effective against bacteria, viruses, fungi, and peroxidation.[11,12] The antibacterial activity of silver nanoparticles was determined using the disc diffusion method in *S. aureus* and compared to Vancomycin antibiotic. The most sensitive disc, with a 100 µg dose of Ag NP in 20 µl, exhibited a zone of inhibition measuring 10.2 mm, while the disc with a 150 µg dose measured 10.3 mm, respectively.

In 20 µl, the disc with 25 µg dose of Ag NP's zone of inhibition was 6 mm, the disc with 50 µg dose was 9.2 mm, and the disc with 75 µg dose was 9.6 mm, respectively. (Figure 12)

In *E. coli*, compared to Gentamicin antibiotic, the most sensitive disc with 100 µg of Ag NP in 20 µL produced a range of 8.9 mm, disc with a dose of 150 µg produced a range of 9.4 mm, while a disc with a dose of 25 µg produced a range of 6 mm. (Figure 13)

Researchers have extensively studied the antimicrobial activity of AgNPs against bacteria, fungi, viruses, and a wide range of microorganisms. The ability of AgNPs to combat pathogenic bacteria has proven to be a promising alternative to antibiotic therapy.[13] For instance, Marslin G and Selvakesavan RK (2015) investigated the antibacterial activity of silver nanoparticles biosynthesized from Withania somnifera plant extract through topical application. They compared the effectiveness of these AgNPs with AgNO3 prepared by oil application against human pathogens including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Escherichia coli*, *Candida albicans*, and plant pathogen *Agrobacterium tumefaciens*. It was found that AgNPs prepared by oil application was 200 times more effective than AgNO3.[14] Similarly, Aritonang HF and Koleangan H (2019) assessed the antibacterial activity of AgNPs against gram-positive *Staphylococcus aureus* (11.03-13.8 mm) and gram-negative *Escherichia coli* (10.2-8.9 mm).[15] In our study, the antibacterial activity of silver nanoparticles measured 10.2-10.3 mm for *Staphylococcus aureus* and 8.9-9.4 mm for *Escherichia coli*, which closely aligns with the findings of these researchers.

CONCLUSION

It was suitable for preparing silver nanoparticles using plant extracts stirring them by magnetic stirrer with hot plate at 600 rpm and centrifuging them at 5000 rpm for 15 minutes. When determining the size of the Ag NPs, obtained using *Thymus gobicus* Czern. extract measured 104.97 nm, which was the smallest.

It was convenient to use 3mM $AgNO_3$ for the *Cacalia hastata* L. extract and 2mM $AgNO_3$ for the *Thymus gobicus* Czern. extract. The optimal nanoparticle formation time was 24 minutes for the *Cacalia hastata* L. extract and 16 minutes for the *Thymus gobicus* Czern. extract.

Purity of Ag NPs was high, no nitrogen was detected in either of them. As determined by XRD analysis, both have peaks at 38.15°, 44.3°, and 64.55°, confirming that the Ag NPs are spherical in shape.

In determining the antibacterial activity of Ag NP using the disc diffusion method, the activity against *S. aureus* bacteria was relatively higher.

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