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Research Article

Determination of the Effective Dose of Biogenic Silver Nanoparticles Synthesized Using *Mentha spicata* Leaf Extract in Albino Rats

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Abstract | Background: Mentha species is widely used in complementary therapies, food, and cosmetics, mostly to treat digestive problems such ulcerative colitis, indigestion, nausea, vomiting, and anorexia. The purpose of this work is to determine the optimal dosage of biosynthesised silver nanoparticles (AgNPs) in albino rats. AgNPs were synthesised using extract from *Mentha spicata* and characterised by UV-Vis spectroscopy, XRD, FTIR, SEM, and AFM. Methodology: The method designed to determine the effective dose, doses were given to 30 female albino rats. They were separated into five identical groups (6/group). The control group gavage distilled water orally. As for the four remaining groups, they were dosed AgMSNPs orally and with doses of (T1: 6.25, T2: 12.5, T3: 25 and T4:50) µg/ k.g B.W respectively and for a period of 14 days. Blood sample were collected and serum was isolated to estimate Urea, Creatinine, GSH, MDA and ATPase as biomarkers for determining the effective dose of AgMSNPs on kidney and it was 12.5 µg/k.g of the body weight. Results: These nanoparticles have been studied by UV-visible spectra analysis where the peak appeared at 245 nm and the Fourier Transform Infrared the presence of effective totals (3404.36, 3390.86, 2956.87, 2927.94, 1620.21, 1516.05, 1357.89, 1062.07, 831.32, 777.31, 615.29, 547.78). By using X-ray diffraction technique it has been detected that there is crystal structure of silver nanoparticles at spectral range (110, 111, 120, 220, 300, 311). The scanning microscope images show a hexagonal shape of nanoparticles with a diameter of (33.17- 60.06), In order to analyses and view materials at the atomic and nanoscales, the Atomic Force Microscope—a high resolution kind of scanning probe microscopy—uses a sharp point in a raster motion that the range diameter of obtained silver Nano particle was (3.53- 28.2nm), the results shown a significant increase in serum urea, creatinine and ATPase concentrations after 14 days of treatment with AgMSNPs. Serum levels of reduced glutathione and malondialdehyde did not significantly differ between any of the groups when compared to the control group. Conclusion: the study indicated that aqueous leaf mint extract works as a good reducing agent of silver nanoparticle and the effective dose of AgMSNPs on kidney was 12.5 µg/k.g BW.

Keywords | Atomic force microscopy, Herbal medicine, Fourier transform infrared, Nano-biotechnology, Oxidative stress, Scanning electron microscope, Silver nanoparticle, UV-visible, X-Ray diffraction

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INTRODUCTION

The term "Nano biotechnology" refers to the study and enhancement of biotechnological processes and their resulting products via the use of nanotechnology (Sumathi *et al*., 2024). Nano biotechnology is a rapidly developing topic that draws upon many different academic disciplines including those of biology, chemistry, physics and medicine (Tiquia-Arashiro and Rodrigues, 2016). Accordingly, a wider range of plant materials including leaf extracts, fruit, bark, fruit peels, root and callus have recently been added to the green synthesis of nanoparticles employing phytocompounds as bio-reductants (Abedini *et al*., 2024). Alkaloids, flavonoids and terpenoids were discovered to be the components in plant extracts that reduced and stabilized nanoparticles (Khattak *et al*., 2024). Green chemistry can be employed to make silver nanoparticles from the extract of *Mentha Spicata* leaves. This procedure is environmentally friendly, reduces costs and nontoxic (Moosavy *et al*., 2023). Medicinal plants are an important component of medicinal products and frequently serve as the foundation for indigenous or traditional healing practices that are still employed by the majority of people in many nations (Sharifi-rad *et al*., 2018). Mentha plants belong to the Lamiaceae family which is extensively cultivated in Europe, Asia, Africa, Australia and North America (Mamadalieva *et al*., 2017). Extracts from this genus have been used as food for a long time and are very valuable because they contain a lot of antioxidants, such as phenolic bioactive compounds (Salehi *et al*., 2018).

There are biologically active parts in Mentha species that are used in traditional medicines. Mint species can also be used to treat common health problems like colds, coughs, sinusitis, fever, bronchitis, and nausea (Dhifi *et al*., 2013). Also, the mint plant has been said to kill insects, fight germs, stop muscle spasms, fight fungi, and protect antioxidant (Öztürk-Yalçın *et al*., 2024).

Mentha was used in the past in the same way that it is used today in medicinal plants. Common colds, coughs, sinusitis, fevers, bronchitis, nausea, vomiting, indigestion, intestinal colic, and appetite loss can all be treated with mentha. It can also be used to add flavor to chewing gum, toothpaste, confectionery and pharmaceutical preparations (Zamljen *et al*., 2024). Silver nanoparticles (AgNPs) are known for their diverse biological properties, including antifungal (Deweer *et al*., 2023), anti-inflammatory (Li *et al*., 2024), antiviral (Mahendran and Rahman, 2020), anti-angiogenic (Rogers *et al*., 2008), and antiplatelet activities (Gurunathan *et al*., 2009). In this study, we synthesized silver nanoparticles by reducing silver ions from silver nitrate using an aqueous extract of *Mentha spicata* leaves. The biosynthesized nanoparticles were then evaluated for safety and toxicity in female albino rats over 14 days of daily oral administration.

Mentha spicata, a medicinal plant from the Lamiaceae family, is rich in secondary metabolites, particularly essential oils. Traditionally, its aerial parts are used to prepare herbal tea, which has been associated with various therapeutic effects, as documented in ethnopharmacological studies worldwide. Recent experimental studies have confirmed the biological activity of its bioactive compounds. Extracts and essential oils from *M. spicata* have demonstrated multiple pharmacological benefits, such as antioxidant, anticancer, antiparasitic, antimicrobial, and antidiabetic effects (El Menyiy *et al*., 2022).

The primary objective of this research was to synthesize AgNPs using *Mentha spicata* extract and assess their safety and toxicity profile in kidneys albino rats following 14 days of administration.

MATERIALS AND METHODS

Ethical Approval

All experimental procedures were reviewed and approved by the Ethical Committee of the College of Pharmacy, University of Kerbala, Iraq (Approval No.: COPh-6381) that aligns with international guidelines.

Green Synthesis and Characterization of Silver Nanoparticles (AgNPs)

Silver nanoparticles (AgNPs) were synthesized following the method described by Alwan *et al*. (2021). A 1 mM aqueous solution of silver nitrate ($AgNO_3$) was prepared and used as the precursor for nanoparticle synthesis. 5 mL of filtered aqueous leaf extract of *Mentha spicata* was added dropwise to 200 mL of the $AgNO_3$ solution under ultrasonication for 30 minutes to facilitate the reduction of Ag^+ ions to Ag^0.

The reaction mixture was then incubated at room temperature for 20 minutes under vigorous magnetic stirring, during which a rapid color change was observed, indicating the formation of AgNPs. The resulting mixture was allowed to react for 72 hours under gentle stirring to ensure complete nanoparticle formation. Aliquots were collected at predetermined intervals to monitor the synthesis process. The biosynthesized AgNPs were characterized using:

UV-Vis spectroscopy (Shimadzu UV-1600, Japan).
X-ray diffraction (XRD) (Shimadzu, Japan).
Fourier-transform infrared spectroscopy (FTIR) (Shimadzu, Japan).
Scanning electron microscopy (SEM) (CYAN, Belgium).
Atomic force microscopy (AFM) (TOSHIBA, Japan).

All chemicals used were of analytical grade, sourced from Sigma-Aldrich (Germany). Fresh *Mentha spicata* leaves were obtained from the local market for extract preparation.

Experimental Animals and Study Design

Thirty healthy albino rats (aged 10-12 weeks, weighing 175-200g) were obtained for this investigation. The animals were housed in the animal care facility of the College of Pharmacy, University of Kerbala, Iraq, under controlled environmental conditions with free access to standard rodent chow and water.

The rats were randomly allocated into five experimental groups (n=6 per group):

Control group: Received distilled water only.
T1 group: Administered 6.25 µg/kg AgNPs daily.
T2 group: Administered 12.5 µg/kg AgNPs daily.
T3 group: Administered 25 µg/kg AgNPs daily.
T4 group: Administered 50 µg/kg AgNPs daily.

All treatments were administered orally once daily for 14 consecutive days.

Blood Sample Collection Protocol

Following the 14-day experimental period, blood samples were collected from the albino rats under fasting and anesthesia. The animals were first anesthetized via intramuscular injection of a ketamine (60 mg/kg) and xylazine (12 mg/kg) combination. Once deep anesthesia was confirmed (absence of pedal reflex), cardiac puncture was performed to obtain blood samples (Patlolla *et al*., 2015), sample were placed in non-heparinized gel tubes and allowed to stand for 30 minutes before being centrifuged (for 15 minutes at 3000 rpm) and stored in firmly sealed tubes for further analysis at -20 C°, to assess the malondialdehyde MDA (Biotech –China), Reduce glutathione (GSH) (Biosciences –China), urea (Linear- Spain), creatinine (Linear- Spain) and ATPase (Sun long –China).

Statistical Analysis

The experimental data are presented as mean ± standard error (S.E.) in tables. Statistical analyses were performed using SPSS software (version 22). Intergroup comparisons were conducted using one-way analysis of variance (ANOVA), with a p-value ≤ 0.001 considered statistically significant (Snedecor and Cochran, 1980).

RESULTS

AgMSNP characterization the first stage of AgNPs' creation was distinguished by a noticeable color shift, with the color turning yellowish after 20 minutes. The color then began to alter after an hour to brown then to dark brown after 72 hours, remaining constant at dark brown. The optical absorbance of the synthesized *Mentha spicata* leaf extract-mediated silver nanoparticles was characterized by UV-Vis spectroscopy across the wavelength range of 200-800 nm, with a spectral resolution of 2 nm. The analysis revealed distinct absorption peaks occurring between 205-665 nm, confirming the formation of nanoparticles, Figure 1. UV-Vis spectroscopy absorbance of AgMSNPs. The FT-IR spectra of dried powdered AgNPs reduced by *Mentha Spicata* was obtained at various peak ranges ranging from 500 to 4000 cm 1.

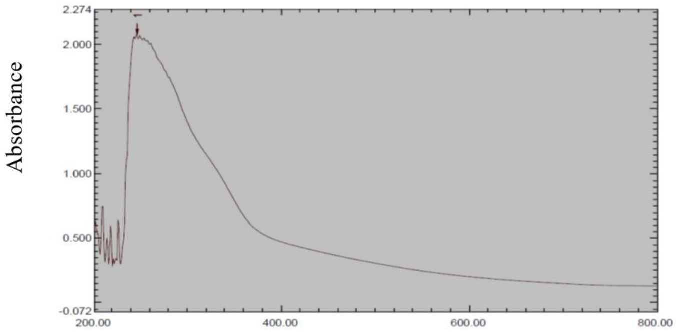

Figure 1: UV-Vis spectroscopy absorbance of AgMSNPs.

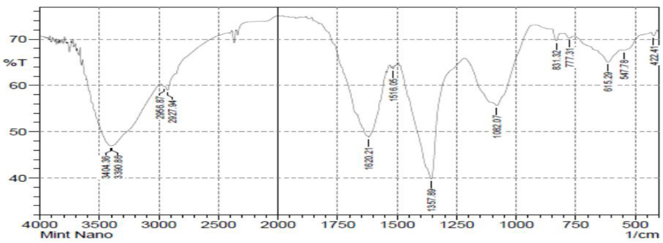

Figure 2: FT-IR spectrum of Biogenic AgNPs.

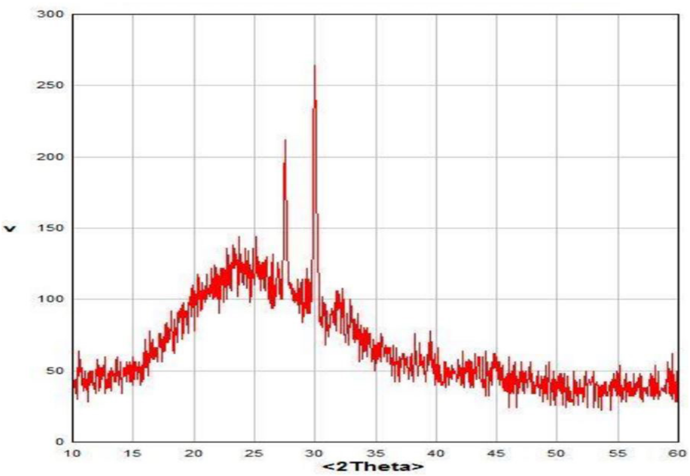

Figure 3: The XRD pattern of biosynthesized AgNPs.

Figure 2, FT-IR spectrum of Biogenic AgNPs; showed the FT-IR spectra with distinct peaks bands at band (3404.36, 3390.86, 2956.87, 2927.94, 1620.21, 1516.05, 1357.89, 1062.07, 831.32, 777.31, 615.29, 547.78). XRD analysis showed three distinct diffraction peaks of 20°,36.8° 43°, 58.5°,63.5°,75.5° and 78.5 ° at 2θ values indexed to (110), (111), (200), (220), (300) and (311) peak which were shown in Figure 3. The XRD pattern of biosynthesized Ag-

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NPs. SEM of AgMSNPs induced by reduction of AgNO3 with *Mentha Spicata* leaf extract in the current study showed spherical shape nanoparticles with a dimeter range of 33.5-60.06 nm and 1.526- 2.059 um (Figure 4). Scanning Electron Microscope image for Biogenic AgNPs). Surface topography analysis was performed using atomic force microscopy (AFM). Figure 5a presents the two-dimensional AFM image of silver nanoparticles synthesized using *Mentha spicata* aqueous leaf extract (AgMSNPs), revealing aggregated nanoparticle formations. The corresponding three-dimensional representation in Figure 5b demonstrates a homogeneous population of nanoparticles with uniform surface morphology and consistent particle distribution.

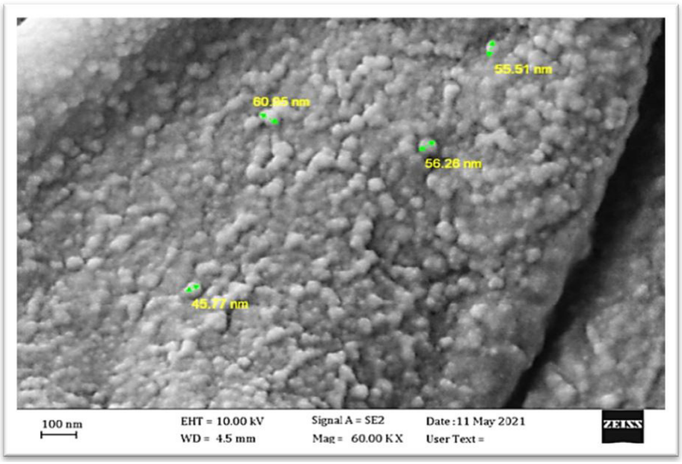

Figure 4: Scanning Electron Microscope image for Biogenic AgNPs.

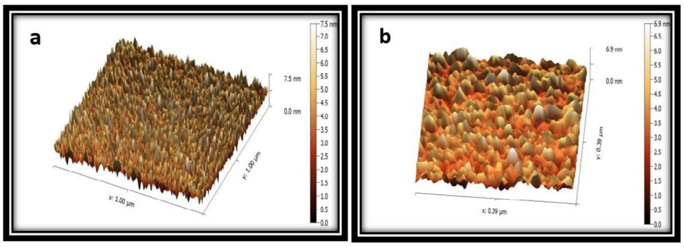

Figure 5: Atomic force microscopy (AFM) for silver nanoparticles was synthesized by aqueous leaf extract of *M. Spicata*.(AgMSNPs); **a:** Two-dimensional image; **b:** three-dimensional image.

Figures 6A, 6B, and 6C demonstrate the dose-dependent effects of *Mentha spicata*-synthesized silver nanoparticles (AgMSNPs) on renal and metabolic parameters following 14-day administration in adult female rats. Figure 6A, Serum urea concentration variations across treatment groups, Figure 6B, Serum creatinine level alterations, Figure 6C, Changes in ATPase enzyme activity, which showed a significant ($p \leq 0.001$) increase in serum urea and creatinine with a significant ($p \leq 0.05$) increase in ATPase concentrations after 14 days of treatment with AgMSNPs. These results were used as a response for estimation of ED as follows: Serum Urea concentration: The results in Figure 6A, showed a positive relationship between serum urea concentration and four successive doses of AgMSNPs. The correlation coefficient was equal (0.9563). From the equation, the estimated ED of AgMSNPs that cause a marked elevation in serum urea concentration was found to be equal to 12.5 µg/Kg B.W. 4.5.2 Serum Creatinine concentration: The results in (Figure 6B) revealed of positive linear relationship between four successive doses of AgMSNPs and serum creatinine concentration. The correlation coefficient was equal to (0.9164) whereas the estimated ED of AgMSNPs was equal to 12.5 µg/Kg B.W. 4.5.3 Serum ATPase concentration: The results in Figure 6C showed a positive relationship between serum ATPase concentration and four successive doses of AgMSNPs. The correlation coefficient was equal (0.9296). From the equation, the dose ED of AgMSNPs in serum concentration was found to be equal to 12.5 µg/Kg B.W.

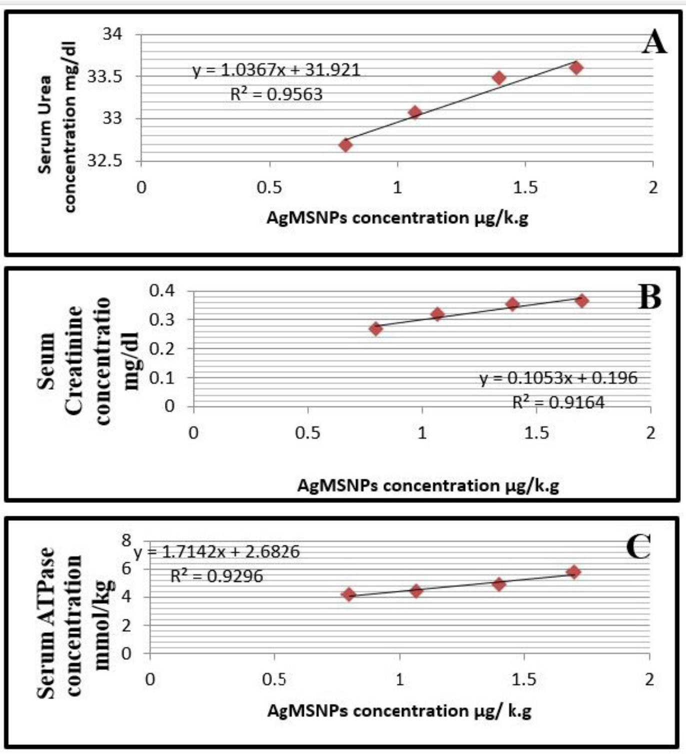

Figure 6: Reveals the effect of different successive doses of AgMSNPs on serum; **A:** urea; **B:** creatinine; **C:** ATPase concentrations after 14 days in adult female rats.

In Table 1, Effect of different doses of AgMSNPs on serum urea (mg/dl), serum creatinine (mg/dl), showed significant ($p \leq 0.001$) increase in serum urea in T1, T2, T3 and T4 compare with control, the serum creatinine showed no significant ($p > 0.001$) different between T2 and T3 compare with control, while a significant ($p \leq 0.001$) increase in T3 and T4 compare with control, also the serum concentration of ATPase showed significant ($p > 0.001$) differences between T2, T3 and T4 compare with T1 and control groups. When comparing every population to the control group, there were no appreciable variations in the blood levels of the oxidative stress indicators MDA and GSH, this result showed in Table 2. Effect of different doses of AgMSNPs on MDA and GSH in adult female rats after 14 days of experiment.

Table 1: Effect of different doses of AgMSNPs on serum urea (mg/dl), serum creatinine (mg/dl), in adult female rats after 14 days of experiment.

Groups	Urea mg/dl	Creatinine Mg/dl	ATPase(μmol/g)
Control	31.25±0.25c	0.3115±0.0405 bc	4.2476±0.2162b
T1	32.25±0.337b	0.2855±0.0322 c	4.1975±0.3518b
T2	33.70 ±0.413b	0.31775±0.00297abc	4.3875±0.2891b
T3	33.5±0.042a	0.35225±0.00201ab	4.8875±0.2104a
T4	33.60±0.253a	0.3615±0.00379a	5.7548±0.2308a
P value	< 0.0001	0.0143	0.0143

Table 2: Effect of different doses of AgMSNPs on MDA and GSH in adult female rats after 14 days of experiment.

GSH U/mol	MDA μmol/L	Groups
2.612± 0.756 a	0.135± 0.513 a	Control
2.60± 0.77 a	0.154± 0.25 a	T1
2.672 ± 0.738 a	0.0962± 0.25 a	T2
2.655± 0.102 a	0.148± 0.029 a	T3
2.722± 0.804 a	0.169± 0.139 a	T4

Values are expressed as mean ± SE. n=6 rats / each group. Small letter denote significant differences between groups (p< 0.001).Group 1: Control negative Animals received normal saline. T1: Animals received AgMSNPs (6.25μg/kg B.W) orally. T2: Animals received AgMSNPs (12.5μg/kg B.W) orally.T3: Animals received AgMSNPs (25μg/kg B.W) orally. T4:Animals received AgMSNPs (50 μg/kg B.W) orally.

DISCUSSION

The UV-visible spectra study done for detection of phyto constituents contained in extract of M. Spicata. The average crystallite size of the biosynthesized silver nanoparticles (AgNPs), calculated using the Debye–Scherrer equation, was found to be 36.8 nm. This result aligns with previous findings reported by Reddy *et al.* (2018). UV-Vis spectroscopy analysis after 72 hours of incubation revealed a distinct peak in the range of 205–665 nm (Figure 1). The pale yellow silver colloids synthesized using *Mentha spicata* leaf extract exhibited a surface plasmon resonance (SPR) band within this range, suggesting the formation of spherical or nearly spherical AgNPs (Sathishkumar *et al.*, 2009). The appearance of this peak at 205–665 nm confirms the reduction of silver ions, consistent with studies by Awwad *et al.* (2014) and Nasrollahzadeh *et al.* (2015). It is important to note that the SPR band position is influenced by factors such as nanoparticle size and shape. The spectra were recorded once the sample color and absorption intensity stabilized, providing preliminary insights into the size distribution of the AgNPs (Sulaiman *et al.*, 2013).

FTIR analysis (Figure 2) indicated shifts in absorption bands, suggesting changes in vibrational frequencies. The spectra confirmed the presence of various functional groups in the *Mentha spicata* extract, including those associated with phenols, alcohols, alkanes, alkynes, and aldehydes. Similar observations were reported by Jha *et al.* (2009).

This study aims to evaluate the effective dose of biosynthesized silver nanoparticles (AgNPs) derived from *Mentha spicata* leaf extract in experimental albino rats. The AgNPs were synthesized through a green synthesis approach, utilizing aqueous *M. spicata* extract as a reducing agent to convert silver ions (Ag^{+}) into metallic silver (Ag^{0}). This reduction process is facilitated by the bioactive phytochemicals present in the extract. The color change observed during AgNP formation is attributed to the excitation of surface plasmon vibrations in the nanoparticles (Nawfal *et al.*, 2019; Islam *et al.*, 2024).

The XRD analysis (Figure 3) confirmed the crystalline structure of the synthesized silver nanoparticles (AgNPs), with peak broadening indicating their small particle size (Ali and Khudair, 2019). These findings demonstrate that *Mentha spicata* leaf extract serves as an efficient and rapid green synthesis route for producing stable AgNPs. SEM and AFM imaging (Figures 4 and 5) revealed spherical AgNPs with smooth surfaces and nanoscale dimensions, corroborating previous reports by Gauthami *et al.* (2015). SEM analysis further showed well-dispersed nanoparticles (60–80 nm) with minor aggregation, attributed to surface interactions with bioactive cellular compounds acting as capping/reducing agents (Bélteky *et al.*, 2019). Notably, AFM measurements in this study (~60 nm) aligned closely with recent work by Alsamhary (2020).

Throughout the 14-day experimental period, none of the groups treated with varying doses of biosynthesized AgNPs exhibited any mortality (0% mortality rate) or signs of toxicity. This study was specifically designed to evaluate the effective dose of *Mentha spicata*-derived AgNPs in albino rats, with the aim of assessing their safety and toxicological profile through multiple exposures (Makarov *et al.*, 2014). In an ideal effective dose study, adverse effects typically manifest within 14 days of administration (Reddy *et al.*, 2018); however, no toxicity indicators—including mortality, tremors, convulsions, writhing, analgesia, increased motor activity, cyanosis, or anesthesia—were observed at any administered dose (6.25, 12.5, 25, or 50 μg/kg).

Kim and Ryu (2013) suggest that the toxicity of AgNPs cannot be attributed solely to the presence of Ag^{+} ions in the nanoparticle solution. The safety of these AgNPs may instead be linked to the bioactive compounds in the *M. spicata* methanol extract, which serve as reducing agents (converting Ag^+ to Ag^0), as well as to factors such as nanoparticle size and administered dose. Additionally, the kidneys—being primary target organs for numerous substances due to their vital roles in detoxification and excretion (Mahmoodi *et al*., 2018; Mohammed *et al*., 2021)—showed no evidence of toxicity in this study.

The ATPase enzyme functions as a primary transporter, actively pumping protons out of the cell. The notable rise in ATPase activity observed in rats treated with spearmint (*Mentha spicata*)-derived silver nanoparticles (AgNPs) may result from multiple biochemical and physiological processes. ATPases, including Na+/K+-ATPase and Ca^{2+}-ATPase, play a vital role in maintaining ion balance and cellular homeostasis. One possible explanation is that AgNPs stimulate ion transport by interacting with cell membranes (Mikhailova, 2020). Additionally, low concentrations of AgNPs might modulate oxidative stress responses by boosting antioxidant enzymes, thereby protecting ATPase function (Ahamed *et al*., 2010). *Mentha spicata* contains polyphenols and flavonoids, which could help reduce oxidative damage to ATPases (Ferrentino *et al*., 2020). Furthermore, the biogenic synthesis of AgNPs using spearmint may improve their biocompatibility, preventing severe oxidative inhibition of ATPases (Mittal *et al*., 2013). Interestingly, low doses of AgNPs might enhance ATPase activity by activating cellular repair mechanisms, whereas higher concentrations could suppress it due to excessive reactive oxygen species (ROS) production (Muhamad *et al*., 2022).

Given the critical role of oxidative stress in organ toxicity—marked by the production of malondialdehyde (MDA), 4-hydroxynonenal, and altered antioxidant enzyme activity (Alwan *et al*., 2021; Nawfal and Al-Okaily, 2022)—this study evaluated the biochemical impact of *M. spicata*-derived AgNPs. Notably, no significant changes in MDA or glutathione (GSH) levels were observed compared to controls, aligning with findings by Nasir *et al*. (2014) and Sood and Khudiar (2018), who reported stable oxidative stress biomarkers after 14 days of AgNP exposure.

Renal function, assessed via urea and creatinine levels, remained unaffected across all groups (control G1 and treated T1–T4) during the 14-day observation period. No mortality or behavioral abnormalities (e.g., lethargy, tremors) were recorded, and biochemical parameters in T1–T3 (6.25–25 µg/kg) showed no significant divergence from controls. These results, consistent with Alwan *et al*. (2021), suggest that biogenic AgNPs are safe for oral administration in albino rats at tested doses. However, the high-dose group (T4; 50 µg/kg) exhibited significant biochemical alterations, corroborating reports by Patlolla *et al*. (2015) and Muhamad *et al*. (2022) on AgNP-induced cytotoxicity at elevated concentrations. Based on dose-response proximity to control data, 12.5 µg/kg was identified as the effective dose (ED) for subsequent studies.

CONCLUSIONS AND RECOMMENDATIONS

This study demonstrates that *Mentha spicata* leaf extract serves as an effective reducing agent for green synthesis of silver nanoparticles (AgNPs). Toxicity evaluation in albino rats revealed no mortality, oxidative stress (stable MDA/GSH levels), or renal dysfunction across tested doses. The effective dose (ED) was determined to be 12.5 µg/kg body weight, as it elicited no adverse effects while maintaining biochemical and physiological stability.

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NOVELTY STATEMENTS

The primary objective of this research was to synthesize AgNPs using Mentha spicata extract and assess their safety and toxicity profile in kidneys albino rats following 14 days of administration.

AUTHOR'S CONTRIBUTIONS

Alaa Hashim Ali and Luma Walid Khaleel were responsible for designing and conducting the experiments, analyzing the data, providing reagents, materials, and analysis tools, as well as writing the manuscript.

Conflict of Interest

The authors affirm that they have no actual or potential conflicts of interest, whether financial, personal, or professional, that might be construed as influencing the research or its outcomes.

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