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# *Salvia officinalis*-mediated synthesis of silver nanoparticles: Characterization and sub-acute toxicity profile in Wistar rats

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## ABSTRACT

Nanotechnology has transformed many scientific domains by providing novel approaches in environmental applications, material science, and medicine. Among the different nanomaterials being explored, silver nanoparticles (AgNPs) have drawn a lot of attention due to their exceptional antioxidant, antimicrobial, and biological qualities. AgNPs synthesized using plant extracts have gained significant interest because of their eco-friendly and biocompatible properties. This study used *Salvia officinalis* aqueous leaf extract to create the *Salvia officinalis* silver nanoparticles (SO-AgNPs). These were characterized using UV-Vis spectrophotometry, FTIR, TEM, and EDX. Interestingly, an absorption peak was found at 434 nm, and functional groups were observed in the FTIR output. The spherical SO-AgNPs ranged in size from 25 to 70 nm, with a strong peak at 3 keV. Investigation of SO-AgNPs' safety was conducted in Wistar rats through sub-acute exposure. Daily oral dosages of SO-AgNPs (3–30 mg/kg body weight) were given to the rats. Clinicals, hematological, and clinical chemistry parameters were monitored to evaluate potential toxicological effects. There were no clinical changes noted. Evaluation of hematological and clinical chemistry parameters showed no appreciable differences between the control and treatment groups ($P > 0.05$). According to the findings, SO-AgNPs may be safe to use up to 30 mg/kg body weight per day. Further investigations on long-term exposure will enhance our understanding of their safety profile.

## INTRODUCTION



Nanoparticles are materials or particles whose dimensions are within the nanoscale range of 1-100 nm. Since nanomaterials are small in size and have a high surface area to volume ratio, they exhibit unique physical and chemical properties [1]. These properties have promoted the emergence of nanoparticles as important players in modern medicine and pharmacology. Their clinical applications range from use as contrast agents in imaging to carriers for drug and gene delivery into tumors and target cells [2]. Nanoparticles have also been used to improve the efficacy of specific therapeutics, offering multiple benefits in treating chronic human diseases by site-specific and target-oriented delivery of medicines [3]. Among noble metal nanoparticles, silver nanoparticles (AgNPs) stand out due to their unique optical, electrical, and magnetic properties, enabling their use in diverse products, including cosmetic products, composite fibers, electronic components, antimicrobial applications, biosensors, and cryogenic superconducting materials in membranes [4-6].

The method used to produce or synthesize the AgNPs influences their functionality and activities. AgNPs can be produced using a variety of physical, chemical, and biological processes. The biological process is known as the green synthesis and makes use of natural compounds like algae, fungi, bacteria, or plant extracts for producing AgNPs [1]. It has been reported that AgNPs derived through the biological process are

biocompatible and have displayed low toxicity, making them a promising candidate for biomedical applications [7]. Plant-based synthesis, in particular, offers several advantages as it eliminates the need for complex downstream processing and requires shorter incubation times for metal reduction [8]. Additional advantages of using plant extracts include the creation of safe biological AgNPs, which are environmentally friendly [9]. These factors make plant-mediated AgNPs a viable option for biomedical applications and sustainable nanotechnology.

*Salvia officinalis*, popularly known as sage, is a plant that occurs in temperate, subtropical, and tropical regions and is of significant acknowledgment in ethnobotany worldwide. Besides its use in ethnobotanical purposes, *S. officinalis* is utilized in the culinary industry and has gained popularity in traditional medicinal systems for its essential oil constituents [10]. Traditional medicine has utilized *S. officinalis* to treat various illnesses, including rheumatism, diarrhea, gout, ulcers, seizures, disorientation, and tremors [11]. Furthermore, its pharmacological properties, like anti-inflammatory [12], anticancer [13], anti-oxidant [14], antimicrobial [15], and antidiabetic [16] effects, have been reported. However, the therapeutic application of plant extracts faces several shortcomings, including variability in active ingredient concentration, poor bioavailability, and limited absorption due to degradation in the gastrointestinal tract. Additionally, the efficacy of these extracts can be influenced by factors such as extraction methods and individual patient responses [17, 18].

According to recent studies on plant-based nano-medicine, nanoparticles offer several advantages, including increased surface area and solubility [19]. Green synthesized AgNPs are considered biocompatible and safe for several therapeutic applications, including as antioxidative, antibacterial, anticancer, antidiabetic agents [20-22], and even as antiviral agents [23]. AgNPs made using *S. officinalis* extract have been shown to exhibit a variety of biological activities, such as anti-inflammatory and antioxidant properties [24], antibacterial activity [25], anti-plasmodial [26], anti-leishmanial [27], and antimalarial [28]. Nevertheless, there is currently no information available regarding the safety of silver nanoparticles made from the aqueous extract of *S. officinalis* leaves.

In our study, we synthesized *S. officinalis* silver nanoparticles (SO-AgNPs) using an aqueous extract of *S. officinalis* leaves. The characterization for size, shape, and composition of SO-AgNPs was conducted using UV-visible spectroscopy, Fourier infrared spectroscopy (FTIR), transmission electron microscopy (TEM), and energy dispersive X-ray spectroscopy (EDX). Here, the current study aimed to evaluate the safety of the SO-AgNPs in male Wistar rats through sub-acute exposure.

## MATERIALS AND METHODS

### Collection of plant material

*S. officinalis* whole plants were obtained locally in Kenya from a farm in Kagaa village, Kiambu county. Verification of *S. officinalis* plant specimens was done at the Botany Department in JKUAT. A deposit of *S. officinalis* plant specimens was made in the herbarium under accession number DM-JKUATBH/001/2024.

### Preparation of leaf extract and synthesis of SO-AgNPs

The leaves of *S. officinalis* were thoroughly washed using water and allowed to dry using the air at room temperature for two weeks. The dried leaves of *S. officinalis* were

ground into powder, and aqueous extraction was carried out using the method outlined by Albeladi *et al.* [29]. This involved mixing the ground *S. officinalis* with distilled water in a ratio of 1g: 40 mL and heating the mix for a time of about 30 minutes at a temperature of 50°C. The heating was done with continuous stirring of the solution. After the heating period, the leaf extract was allowed to cool down at room temperature. Upon cooling, the leaf extract was filtered, and the filtrate was kept at 4°C for use in the synthesis. A solution of 1 mM silver nitrate was prepared by dissolving 0.169 g of silver nitrate (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) in a liter of distilled water. The 1 mM solution was then added to the *S. officinalis* leaf aqueous extract in the ratio 1:9 (leaf extract: 1 mM silver nitrate) to synthesize *S. officinalis* silver nanoparticles (SO-AgNPs). This was allowed to stir in the dark at 60°C for 4 h to allow synthesis. A change in color of the solution from pale yellow to dark brown was used as an indicator for synthesis. The solution, after synthesis, was centrifuged for 10 minutes at 5000 rpm to get the SO-AgNPs. The obtained SO-AgNPs pellet was washed with distilled water three times to remove unreacted silver nitrate. The clean pellet was frozen at -80°C for 48 h and then lyophilized for 72 h to obtain dry SO-AgNPs [30].

**Characterization of SO-AgNPs**

Using a UV–Vis spectrophotometer (UV 1800 model, Shimadzu Japan) set to 250–800 nm in wavelength, the *S. officinalis* leaf extract mediated reduction of Ag+ ions to SO-AgNPs was observed [31]. This involved checking for the highest absorbance peak of the SO-AgNPs using UV-vis spectrophotometry. Thereafter, to identify the biomolecules that stabilize the synthesized silver nanoparticles and reduce Ag ions, following a method by Albeladi *et al*. [29], an FTIR spectrophotometer (IRAffinity-1S spectrophotometer, Shimadzu, Japan) using a wavelength of 500 to 4000cm$^{-1}$ was used to obtain an FTIR spectral output of the biosynthesized SO-AgNPs. The instrument was run at a resolution of 4 cm$^{1}$, and the powdered SO-AgNPs were applied directly to the sample stage's glass surface [29]. After employing FTIR for identification of molecules, secondary electron imaging in a Transmission Electron Microscope (JEM-2100 model, New York, USA) with high resolution and voltage of 200kV, was used to ascertain the shape of synthesized nanoparticles and offer valuable information on their morphology. The process involved placing 0.5mg/ml SO-AgNPs on a carbon-coated copper grid for analysis. The TEM was coupled with an Energy-Dispersive X-ray (EDX) (JEOL 733 Superprobe, New York, USA), which was used to gain more information on the elements making up the silver nanoparticles.

**Ethical approval**

The Institutional Committee of Animal Ethics at Jomo Kenyatta University of Agriculture and Technology (JKUAT) permitted the use of the Wistar rats in this study. The protocols were approved under JKU/ISERC/02317/1342, with reference JKU/2/4/896B. The research was carried out according to national ethical guidelines established in Kenya to avoid animal suffering.

**Experimental animals**

Male Wistar albino rats, which were eight weeks old and weighed 270–320g, were reared in the Small Animal Facility for Research and Innovation (SAFARI) house located in JKUAT. The rats were maintained in cleaned plastic cages under good

aeration conditions, having optimum requirements of temperature at 28 ± 2°C; humidity was at 40-45%, had 12 h of exposure to natural light and 12 h spent in darkness. The Wistar rats had unlimited availability of commercial rat feed pellets for their consumption and drinking water ad libitum. Their bedding was changed twice a week to maintain a clean environment.

**Administration of SO-AgNPs to Wistar albino rats**

For the sub-acute toxicity investigation, a sum of 16 male Wistar rats were split into four groups each having four animals. The groups were according to dosage of SO-AgNPs: Group 4 received 30 mg/kg of SO-AgNPs, Group 3 was administered 10 mg/kg of SO-AgNPs, Group 2 was administered 3 mg/kg of SO-AgNPs, and Group 1 was the standard control group, which was given distilled water. For 21 days, a final volume of 2 milliliters of the drug was given orally once daily to evaluate sub-acute toxicity. Prior to giving the animals the drug orally, they were fasted overnight for 8 h and weighed first before the SO-AgNPs were administered orally using oral gavage [32].

**Monitoring of clinical signs and measuring the weight of rats**

Clinical signs were observed before as well as 3 h post administration of SO-AgNPs. Throughout the experiment, animals were monitored twice a day (6 am and 6 pm) for any indicators of toxicity, such as any change or abnormality in appearance, behavior, fur color, feeding habits, and mortality [32].

**Collection of organs and blood samples**

On the 22$^{nd}$ day, following an 8 h overnight fast, the animals were first weighed before being euthanized, in accordance with a procedure by Shanker *et al.* [33]. Samples of blood were drawn via cardiac puncture and placed in sterile collecting containers for hematological and clinical chemistry analysis. The collecting tubes for hematological analysis were embedded with EDTA to prevent the blood from clotting, whereas the tubes for clinical chemistry were plain to allow clotting of the blood. The heart, spleen, liver, lungs, and kidneys were meticulously removed from the dissected rats. Gross pathology analysis was done by a trained Pathologist to physically assess any damage to the organs, including any hemorrhages, ecchymosis, and petechial appearances. The organs were washed using saline and their weights checked. The organ weights were used to establish the relative organ weight (ROW) using the following formula:

ROW = (Fresh organ weight in grams / Live weight of rat in grams) × 100

After macroscopy, harvested organ tissues were preserved in 10% formalin solution [34].

**Biochemical analysis**

Blood collected was given an hour to clot and thereafter centrifuged for 10 minutes at a speed of 5000 rpm to separate the serum from blood cells and obtain serum. The obtained sera were used for sub-acute toxicity biochemical assay. This was conducted using an automated Roche-Reflotron dry chemistry analyzer (Roche Diagnostics, Mannheim, Germany) to assess serum concentration of urea, creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST). The method involved

pipetting 10µL of the serum on commercial dry reagent strips and inserting them into the analyzer [35]. The analyzer automatically calculated and displayed the concentrations for the tested parameters.

### Hematology analysis

Blood collected into EDTA blood collecting tubes, which prevented the blood from clotting, was used for hematological analysis. Following a method by Hauwa *et al.* [36] , hematology analysis of the blood was conducted. The blood analysis was performed using a Mindray BC-5000 hematological automatic analyzer from Shenzhen, China [36]. The probe of the machine was allowed to suck the blood from the collecting tube for automated analysis, giving output readings. The hematology parameters that were analyzed included red blood cell count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCH-C), white blood cell count, neutrophils, lymphocytes, monocytes, basophils, eosinophils, and platelet count.

### Statistical analysis

The statistical analysis of collected data was conducted in a student trial version of the software GraphPad Prism 8$^{th}$ Version. The inhibition of the enzymes was expressed as a percentage, and $IC_{50}$ values were calculated and presented as mean ± SD. All data collected, including the weights of the animals, organ weights, clinical chemistry, and hematological analysis, were recorded as mean ± Standard error (SEM). Analysis of Variance (ANOVA) and Dunnett's test for multiple comparisons were used to look for significant differences between the control and treatment groups in all assessed parameters ($p<0.05$) [37].

## RESULTS

### Characterization of SO-AgNPs using Spectrophotometry

Upon employing UV Vis spectroscopy on the 1 mM $AgNO_3$ solution and SO extract, the solutions did not give a peak between 400 and 450 nm. After the synthesis period, the mixture of silver nitrate and SO extract changed from a light-yellow color to a dark brown color, showing the creation of SO-AgNPs. Presence of SO-AgNPs was verified by obtaining a high absorption peak at 434 nm (Figure 1).

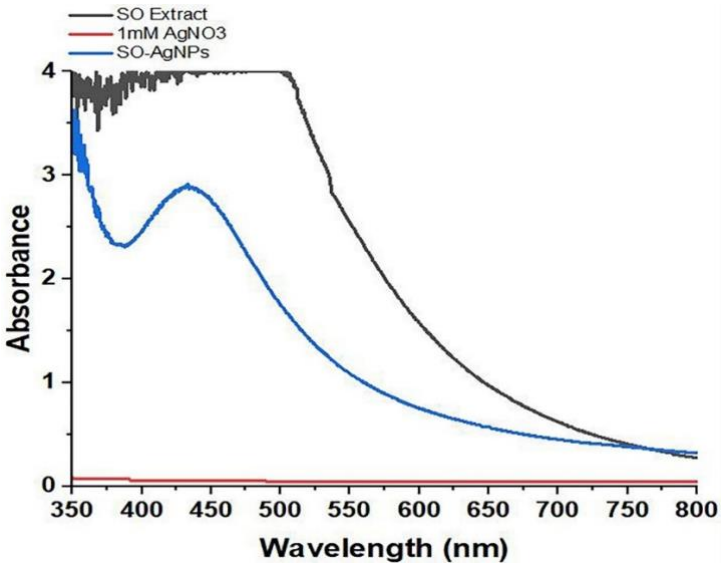


**Figure 1.** UV vis spectrophotometry spectral absorption of the *S. officinalis* aqueous extract (SO extract), 1 mM silver nitrate ($AgNO_3$), and biosynthesized *S. officinalis* silver nanoparticles (SO-AgNPs). The $AgNO_3$ served as a control and had no absorption peak. A distinct highest absorption peak is given by the SO-AgNPs at a wavelength of 434nm.

### Functional groups of the biosynthesized SO-AgNPs

After investigating the functional groups of the biosynthesized SO-AgNPs and SO Extract, we observed an FTIR spectrum with vibrations (Figure 2). The FTIR spectra, which ranged from 4000 to 500 $cm^1$, captured the transmittance bands of various chemical functional groups. The presence of IR absorption bands was observed at 3248, 2962, 1741, 1589, 1388, 1224, 1047, and 534 $cm^1$. Each of the vibrations represented a different functional group, including amines (C-N), ester stretches (C=O), nitro compounds (N-O), alkanes (C-H), primary alcohol stretches (C-O), and halo compounds (C-X).

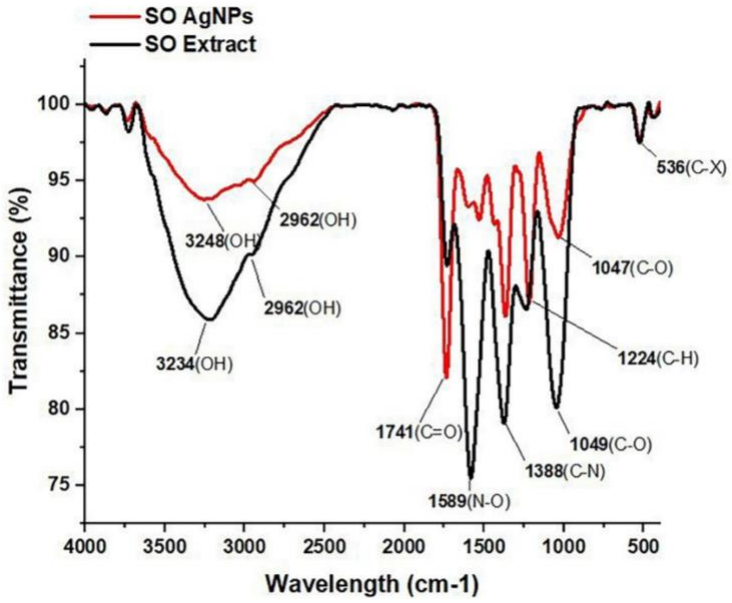


**Figure 2.** FTIR spectrum of *S. officinalis* aqueous extract (SO Extract) and biosynthesized *S. officinalis* silver nanoparticles (SO-AgNPs). The characteristic peaks correspond to vibrations at different wavelengths. These peaks indicate the presence of specific functional groups, providing insight into the biomolecules involved in the synthesis and stabilization of the SO-AgNPs.

### Size, shape, and composition of SO-AgNPs

Biosynthesized SO-AgNPs with diameters ranging from 25 to 70 nm were shown to have a spherical shape by TEM (Figure 3A and B). Furthermore, the silver element was identified by a strong peak in the EDX spectrum at 3 keV [31]. The EDX showed the purity of the SO-AgNPs as 75.1%, as well as the presence of some residual elements, including 17.5% carbon, 6.8% oxygen, and 0.7% chlorine.

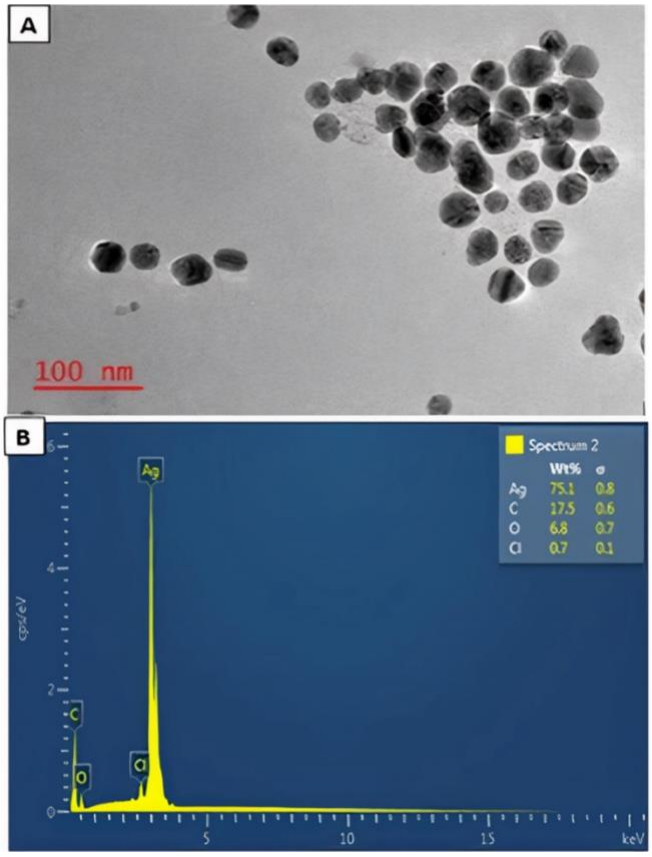


**Figure 3.** A) TEM analysis using a scale of 100nm, the biosynthesized *S. officinalis* silver nanoparticles (SO-AgNPs) are spherical in shape and are nano-sized, ranging from 25-70nm. B) EDX spectra of the biosynthesized SO-AgNPs give a sharp silver (Ag) peak at 3 keV and show residual elements including Carbon C), Oxygen (O), and Chlorine (Cl).

### Effect of SO-AgNPs on body weight, gross pathology, and organ weight in rats

The subacute toxicity study of SO-AgNPs revealed no mortality or adverse clinical signs in treated groups. The rats maintained regular food and water intake and exhibited healthy skin, fur, and normal urination throughout the treatment. There were no signs of diarrhea, fatigue, excessive salivation, or other symptoms of distress. The normal clinical signs observed promoted weight gain in all groups throughout the treatment period of 21 days. The percentage weight gain for all the Wistar rats was calculated using the weekly weights (Figure 4). Upon doing one-way ANOVA, the difference was not significant at $p<0.05$ in mean weights among the different treatment groups as well as the percentage body weight gain. The calculated percentage of body weight gain showed that the animals continued to gain weight each week throughout the 21 days. After the first 7 days, the different groups' weight gain was between 7 and 8%. On the 14$^{th}$ day, the animals had gained 14.96 to 17.31%. After 21 days of treatment, the animals gained weights of 22.96 and 24.61%.

Gross pathology analysis of organs, including the heart, kidneys, liver, and spleen, showed no inflammation upon examination; the color and texture of the organs were found to be good among all the treatment groups compared with the control. Upon checking the ROW of organs (liver, kidney, heart, lungs, and spleen), there was no significant difference between the control and different treatment groups as P>0.05 (Table 1).

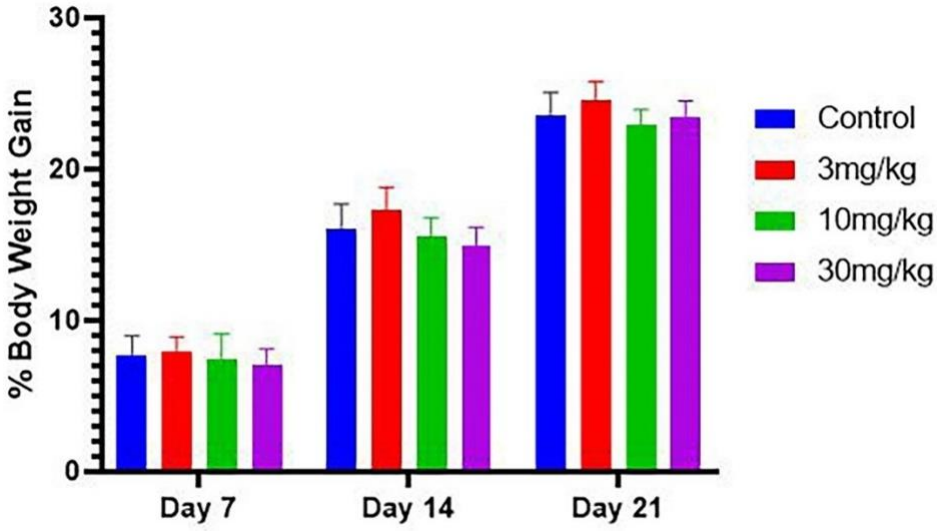


**Figure 4.** Percentage weight gain in male Wistar rats throughout 21 days following oral administration of SO-AgNPs. The control group received distilled water, while the treatment groups were administered SO-AgNPs at doses of 3 mg/kg, 10 mg/kg, and 30 mg/kg, adjusted according to their body weights. The percentage (%) body weight gain is given as mean ± standard error (SEM), where n=4 rats/group.

**Table 1.** Effect of SO-AgNPs on relative organ weight in sub-acute toxicity in rats.

| Organ | Control | 3mg/kg | 10mg/kg | 30mg/kg | p value |
|---|---|---|---|---|---|
| Liver | 3.48 ± 0.05 | 3.52 ± 0.06 | 3.58 ± 0.11 | 3.54 ± 0.08 | 0.6327 |
| Kidney | 0.34 ± 0.00 | 0.32 ± 0.00 | 0.34 ± 0.00 | 0.33 ± 0.02 | 0.7695 |
| Heart | 0.26 ± 0.01 | 0.24 ± 0.01 | 0.23 ± 0.01 | 0.25 ± 0.01 | 0.1696 |
| Spleen | 0.65 ± 0.03 | 0.60 ± 0.03 | 0.62 ± 0.01 | 0.64 ± 0.02 | 0.4185 |
| Lungs | 0.47 ± 0.02 | 0.44 ± 0.02 | 0.52 ± 0.02 | 0.50 ± 0.02 | 0.1113 |

All values are given as mean ± standard error (SEM) and P value set at 0.05. (n=4 rats/group)

### Effect of SO-AgNPs on clinical chemistry in rats

The obtained P values showed that the difference was not significant between the control and treatment groups' levels of AST, ALT, urea, and creatinine, according to clinical chemistry analysis (p>0.05) (Figure 5). The significance levels on the tested parameters were as follows: A) ALT (p= 0.4403), B) AST (p= 0.2597, C) urea (p=0.4095), and D) creatinine (p=0.3794). The results indicated that the different dosages of SO-AgNPs (3mg/kg, 10mg/kg, and 30mg/kg b.w.t) caused no significant changes in the tested kidney products (creatinine and urea) and enzymes of the liver, namely ALT and AST.

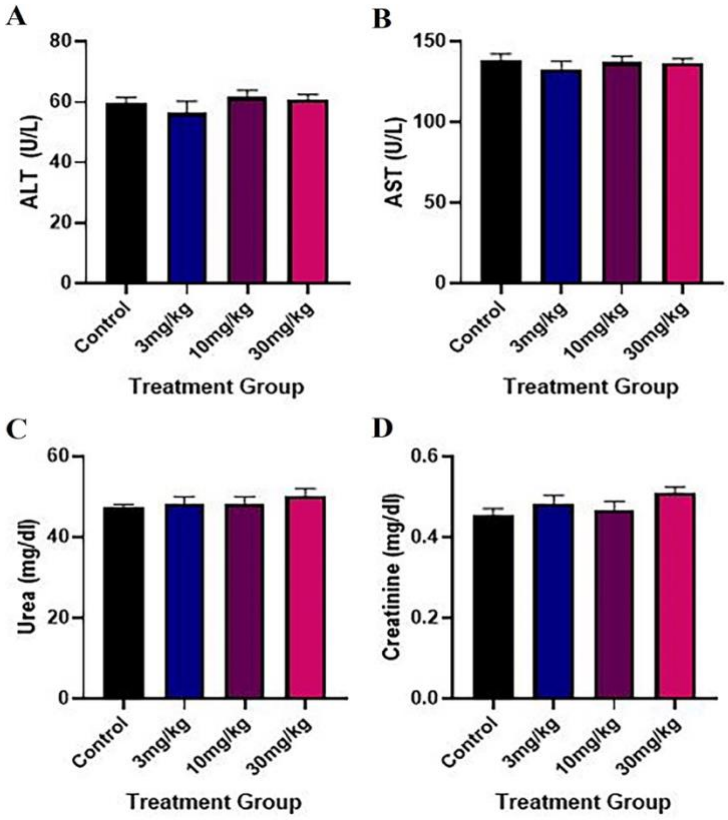


**Figure 5.** Wistar rats' clinical chemistry findings after a 21-day SO-AgNPs treatment period using different doses of 3mg/kg; 10mg/kg, and 30mg/kg b.w.t. The group that served as the control was administered distilled water. The levels of the liver enzymes (A) alanine transaminase (ALT), (B) aspartate transaminase (AST), and kidney function markers (C) urea, and (D) creatinine are given as mean and standard error (shown as error bars). Each group consists of four rats.

### Effect of SO-AgNPs on blood hematological parameters in rats

The hematology assay showed that the difference between the tested parameters of treatment groups and the control was not significant since P>0.05, as shown in Table 2. Results showed that all the dosages of *S. officinalis* silver nanoparticles, ranging from 3mg/kg to 30mg/kg, did not cause or promote any significant changes in all tested hematology components. This suggests that SO-AgNPs do not contain toxic substances capable of causing hematological abnormalities.

**Table 2.** Effect of SO-AgNPs on hematological parameters in the rat sub-acute toxicity.

| Parameters | Units | Control | 3mg/kg | 10mg/kg | 30mg/kg | p value |
|---|---|---|---|---|---|---|
| Red Blood Cells | $10^6/\mu L$ | 8.0 ± 0.18 | 8.70 ± 0.20 | 8.37 ± 0.12 | 7.89 ± 0.39 | 0.1419 |
| Hemoglobin | g/dl | 14.0 ± 0.10 | 13.53 ± 0.13 | 13.80 ± 0.12 | 13.37± 0.29 | 0.097 |
| Hematocrit | % | 49.2 ± 0.72 | 47.73 ± 0.50 | 48.2 ± 0.84 | 49.70 ± 1.21 | 0.3817 |
| MC-Volume | Fl | 65.6 ± 1.97 | 68.60 ± 0.37 | 66.63 ± 0.63 | 69.03 ± 0.40 | 0.1247 |
| MC-Hemoglobin | Pg | 21.9 ± 0.35 | 21.43 ± 0.41 | 21.23 ± 0.90 | 20.77 ± 0.51 | 0.5768 |
| MCH-Concentration | g/dl | 34.90 ± 0.39 | 34.73 ± 0.35 | 34.57 ± 0.27 | 35.5 ± 0.23 | 0.2242 |
| Platelet | $10^3/\mu l$ | 918.7 ± 3.32 | 923.67 ± 2.95 | 924.33 ± 2.87 | 913.67 ± 3.17 | 0.1009 |
| White Blood Cells | $10^3/\mu L$ | 5.67 ± 0.10 | 5.73 ± 0.08 | 5.73 ± 0.17 | 5.93 ± 0.13 | 0.5346 |
| Neutrophils | % | 23.70 ± 1.21 | 23.30 ± 1.69 | 20.17 ± 0.70 | 22.10 ± 0.13 | 0.1584 |
| Lymphocytes | % | 67.83 ± 3.81 | 69.73 ± 2.23 | 71.77 ± 2.44 | 68.5± 1.91 | 0.743 |
| Monocytes | % | 0.32 ± 0.01 | 0.36 ± 0.01 | 0.34 ± 0.00 | 0.32 ± 0.03 | 0.4033 |
| Eosinophils | % | 0.01 ± 0 | 0.01 ± 0 | 0.01 ± 0 | 0.01± 0 | 0 |
| Basophils | % | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 |

All values are given as mean and standard error (SEM); each group had 4 rats, and the P value was set at 0.05. MC- mean corpuscular, MCH - mean corpuscular hemoglobin

## DISCUSSION

This present study involved green synthesis of SO-AgNPs, assessing their safety using an *in vivo* model of male Wistar rats. A distinctive peak seen in UV vis spectrophotometry served as confirmation of the successful synthesis. The peak matches the results obtained by Sharifi *et al.,* [27] when they characterized silver nanoparticles from *S. officinalis*. Based on an earlier study by Martínez-Castañón *et al.* [38], AgNPs that are spherical when they absorb light present the highest absorption peak between a wavelength of 420 and 450 nm. More information on the composition of the SO-AgNPs was obtained through other techniques. Our FTIR analysis confirmed the participation of molecules in *S. officinalis* leaf extract in capping and reducing silver ions of SO-AgNPs, evident from IR absorption bands similar to those reported in previously conducted studies by Sharifi *et al.* [27] and Okaiyeto *et al.* [26]. It is the hydroxyl functional groups found in phytochemicals, particularly in phenols and sterols, that are regarded as key players in the metal reduction reaction [39].

In addition to FTIR spectra, the EDX peak obtained at 3 keV is known as a typical characteristic of nano-crystalline silver and shows that the ions of silver have been completely converted to silver nanoparticles [40]. The content of silver in our biosynthesized nanoparticles was below that reported earlier [29-41], but more than what was reported by Okaiyeto *et al.* [26] in their SO-AgNPs. The variation in the composition of silver nanoparticles can be attributed to differences in particle size [42] and fluctuations in the concentration of the biomolecules or phytochemicals present in the plant extract [39].

The current study demonstrated that SO-AgNPs were safe at the administered dosages as observed in the behavior of the animals, which remained normal; there were no changes in the physical appearance, as well as the feeding habits. Normal feeding promoted weight gain in treatment groups. In toxicity assessment, body weight can be used as a parameter in the assessment of an animal's health status [43]. The weight gain observed in our study indicated that the administered SO-AgNPs did not have a toxic effect on the animals. This was supported by morphology observations of the internal organs (liver, kidney, heart, and spleen). Gross pathology provides a macroscopic assessment of organ systems and tissues, allowing the identification of visible lesions, abnormalities, or morphological changes resulting from exposure to test substances. This initial evaluation is essential for determining the potential toxic effects of a test substance. Changes in organ morphology affect relative organ weight (ROW). The ROW of the organs was not significantly different from the control, indicating normal size. Our findings are in accordance with Rhaimi *et al.,* [44] who observed no significant difference in ROW. Since organ weight is impacted by the reduction of body weight, Loha *et al.* [45] state that a notable change in ROW between treated and untreated animals may be a sign of toxicity. Moreover, ROW observation is important in evaluating the safety of a drug or substance administered to animal models [46].

The clinical chemistry analysis suggests that the SO-AgNPs had no impact on kidney function. This was observed through the normal concentration of urea and creatinine. Creatinine levels remained in the normal test range of 0.1-0.8 mg/dL [47], suggesting the safety of the SO-AgNPs for the tested doses. It was reported by Saud et al.[48] that SO-AgNPs have a renal protective effect, which can explain why urea and creatinine levels remained normal, suggesting the safety of SO-AgNPs in the kidneys. In addition, the concentration of AST and ALT in all the treatment groups had no significant difference when compared to the control group. The liver is considered to be a major metabolic organ, aiding in the regulation and maintenance of homeostasis [49]. Elevated concentrations of AST and ALT are crucial indices for liver impairment [50].

There has been a report on the hepatoprotective effect of *S. officinalis* [51]. This could explain why the AST and ALT levels were not affected by the SO-AgNPs.

In hematological analysis, all three treatment groups and the control showed no significant difference in tested blood parameters, including red blood cells, hemoglobin, hematocrit, mean corpuscular hemoglobin, white blood cells, and platelet count. Since the hematopoietic system is susceptible to toxic compounds, hematological analysis is a key indicator for assessing treatment effects and potential toxicity of administered substances [52]. The results reported in our study showed that the SO-AgNPs had no toxic effect on the hematopoietic system. In general, AgNPs synthesized using plant extract are regarded as much safer compared to synthetic nanoparticles that have been linked to toxicity and immunological responses [53]. Furthermore, AgNPs synthesized from plants can frequently possess or maintain bioactive compounds and beneficial characteristics of their source plants [54]. Consequently, the bioactive substances in *S. officinalis* leaf extract are responsible for the safety of the SO-AgNPs that we observed in our investigation [28]. *S. officinalis* aqueous leaf extract is considered nontoxic, as confirmed by Qnais *et al*., [55] who reported a lethal dose 50 ($LD_{50}$) above 1000mg/kg. Rhaimi et al., [44] also reported an $LD_{50}$ above 3000mg/kg for *S. officinalis* essential oil and concluded that the level of no observed adverse effect was 1000 mg/kg.

Several studies have investigated various benefits and activities of SO-AgNPs, including antibacterial, antiplasmodial, antioxidant, and anti-inflammatory. This study is the first to evaluate the safety of SO-AgNPs *in vivo* using Wistar rats. Our findings indicate that the biosynthesized SO-AgNP were found to be safe within the tested dosage range (3–30 mg/kg b.w.t). The main limitation of our study is that we did not perform histopathology analysis of the harvested organ tissues to fully assess the effect of SO-AgNPs on cell morphology. Further toxicity studies are also necessary to assess the long-term safety following prolonged exposure of animals to SO-AgNPs.

## CONCLUSIONS

In the synthesis and stabilization of SO-AgNPs, the aqueous extract of *S. officinalis* leaves served as both a reducing and capping agent. This was verified through UV-vis spectrophotometry, FTIR, EDX, and TEM, which detected spherical silver nanoparticles. Subacute toxicity assessment showed no mortality, no clinical toxicity, or adverse effects on hematology and biochemical parameters (AST, ALT, urea, creatinine) at dosages of 3–30 mg/kg b.w.t., indicating the safety of the SO-AgNPs. Histopathology analysis of vital organs and studies involving longer periods of SO-AgNPs administration are needed to fully evaluate their safety.

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## AUTHOR CONTRIBUTIONS

DM did the experimental work, gathered, recorded, and analyzed the data, and did the drafting of the manuscript. NM and DK oversaw and supervised the whole study. They also evaluated the scientific information presented in the publication. All authors have reviewed and approved the submission of the final manuscript.

## CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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