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**Original Article**

# Impact of Green-Synthesized Silver Nanoparticle in Wistar Rats: Behavioral, Biochemical, And Histopathological Insights from Acute and Sub-Acute Oral Exposure

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## ABSTRACT

Acalypha paniculata (AP) is a subshrub traditionally used in ethnomedicine for treating skin diseases, asthma, and inflammatory conditions. This study focuses on the eco-friendly synthesis and characterization of silver nanoparticles derived from the Acalypha paniculata herb. The safety profile of Acalypha paniculata-based silver nanoparticles (APSN), particularly regarding behavioral, biochemical, and histopathological aspects, has not been thoroughly investigated. This study evaluated the acute and sub-acute toxicity of APSN in rats, adhering to OECD guidelines. Four groups of six rats each received a single oral dose of APSN at 500, 1000, and 2000 mg/kg. Post-administration, the rats were monitored for thirteen signs of general toxicity over four hours and assessed for motor and locomotive behavior using a rota rod and open field test on the 14th day. In repeated- dose toxicity studies, four groups of six rats each were administered 500, 1000, and 2000 mg/kg APSN daily for 28 days. Parameters such as feed intake, body weight, biochemical and hematological profiles, and organ histopathology were studied. The results of the acute toxicity studies indicated no evident signs of toxicity, including abnormal motor locomotion and behavior. The rats exhibited good tolerance across the three doses. However, sub-acute exposure at 2g/kg showed minor morphological changes in liver histopathology, evidenced by minimal hepatic cell infiltration. The oral no-observed-adverse-effect-level (NOAEL) exceeded 2000 mg/kg/day in both male and female Wistar rats, confirming the safety of APSN when administered orally. This study supports the ethnomedicinal claim of APSN, though further clinical studies are necessary to confirm these findings and ensure comprehensive safety validation.

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## 1. Introduction

The utilization of alternative systems of medicine has evolved from ancient times to the modern era, demonstrating continual growth. Notably, the widespread use of medicinal plants in various formulations for treating diverse illnesses has captured increased attention across different cultures (1). Despite the common notion that herbal medicines are generally safe and free from adverse effects on the biological system (2), recent advances like silver nanoparticle screening methods and biological standardization, including bioanalytical assays, have revealed toxic effects of some silver nanoparticles in preclinical models assessing the IV safety profile (3).

In contrast, OECD regulations for testing chemical toxicity, including active pharmaceutical ingredients (API), have provided benchmark guidelines for conducting toxicity studies on laboratory rodents (4). According to World Health Organization (WHO), nearly 80% of the Asian population use medicinal plants to address a variety of diseases. Furthermore, the discovery and isolation of bioactive compounds and secondary metabolites have led to the development of several molecules with fascinating pharmacological properties (5). The global use of medicinal plants continues to escalate, and with many new products entering the market, public health concerns and safety issues are gaining recognition. While some herbal extracts derived from the Indian System of Medicine (ISM) show promising potential and enjoy widespread usage, many remain untested, lacking adequate monitoring. Furthermore, the absence of standardized testing protocols for herbal extract poses challenges for consistent safety and efficacy assessment Variability in extract composition, due to factors such as geographical location, climate, and harvesting methodscomplicates quality assurance and hinders the establishment of universally accepted guidelines for herbal medicine regulation (6).

In recent years, the popularity of herbal silver nanoparticles and remedies has surged, driven by the perception that they are natural and, therefore, harmless. However, misconceptions about the safety of silver nanoparticles can lead to adverse effects, especially when individuals self-prescribe or combine them with conventional medications without proper guidance. Addressing these growing concerns about the safety of silver nanoparticles requires collaborative efforts from regulatory agencies, healthcare practitioners, and researchers. Rigorous clinical trials and systematic reviews are essential to provide evidence-based information regarding the safety and efficacy of herbal silver nanoparticles (7). Additionally, promoting public awareness about potential risks associated with herbal silver nanoparticles and encouraging responsible usage are crucial steps toward safeguarding individuals using alternative medicinal practices.

The *Acalypha* genus is one of the largest within the Euphorbiaceae family, commonly utilized in Indian traditional medicine, especially in Tamil Nadu, for treating various ailments. The *Acalypha* genus, which includes *Acalypha paniculata* (AP) (Synonym: *Acalypha racemosa)* (AR), is extensively distributed across the forested regions of the Eastern Ghats, including the Javvadhu Hills and Parvathamalai Hills in the Tiruvannamalai District (7,15,19,20). Djacbou D. Sylvie et al. (8) studied the free radical scavenging activity of AR and showed 80% of DPPH radical scavenging activity, 70% Nitric oxide scavenging, and87% Hydroxy radical scavenging at a concentration of 160µg/mL. Iniaghe et al. (9) examined aqueous extracts of AR and demonstrated that the 60mg/kg concentration of extract significantly reduces and prevents hepatic necrosis in rats. The extract also exhibited antimicrobial activity against *E. coli* NCTC 10418, *Staphylococcus aureus* NCTC 6571, and a clinical isolate of *Candida albicans*. The cold maceration extract of AP showed *Staphylococcus aureus* bacteriostatic activity with the MIC range of 3.0 mg/mL to 4.0 mg/mL. The MBC results revealed a 2-log cycle reduction in cell population within 90 minutes at the concentration of 6.0mg/mL of the AP extract.

In prior ethnopharmacological investigations, GC-MS analysis revealed an intricate chemical composition in the aerial parts of AP, yielding alkaloids, terpenoids, saponins and flavonoids. Notably, pure Alloaromadendrene was isolated through column chromatography and silver nanoparticles were synthesized using a green synthesis approach by Elumalai. Despite these advancements, there remains a notable dearth of information on the pharmacology of AP, and the increasing consumption may potentially lead to adverse effects. The existing scientific literature lacks systematic studies on the safety of *Acalypha paniculata* herbal silver nanoparticles (APSN), leaving a considerable gap in understanding their toxicity. Recognizing this gap, our study aims to address

the lacuna by evaluating both acute and repeated toxicity levels of the APSN in albino Wistar rats.

This objective is to reveal crucial safety insights necessary for determining appropriate dosages in pre-clinical trials. The primary objective of our study was to assess the acute and sub-acute oral toxicity of APSN and to create a thorough toxicity profile, which includes determining the lethal dose (LD50) and identifying the no –observed- adverse- effect level (NOAEL). Through this research, we aimed to contribute valuable information to the existing knowledge base, fostering a better understanding of the safety aspects associated with the use of AP.

## 2. Materials and Methods

### 2.1. Botanical Plant Material and Silver Nanoparticle Preparation

The fresh aerial parts of AP were collected directly from primary and secondary forests in the Parvathamalai hill region (2.4352°N, 78.9684°E), Thiruvannamalai District, TN state, India. Assistance from a local herbalist helped identify the plant, which was authenticated and identified by the Department of Pharmacognosy at the Siddha Central Research Institute (CCRS), under the Ministry of Ayush , Government of India, (Reference: H12092201S), located in Chennai 600106.

AP typically blooms from June to January 2023. The plant was shed-dried and coarsely powdered using a mechanical blender. A total of 250 grams of the coarse powdered materialunderwent a hot continuous extraction process using an absolute ethanol solution at a ratio of 1:5 (w/v). The extraction process was carried out at 60°C for 6 hours. The extract was filtered through Whatman No.1 paper, and the resultant filtrate evaporated using a rotary vacuum evaporator under reduced pressure at 25°C and 115 rpm. A mixture of 0.016g of silver nitrate and 90 mL of water was prepared, and then 10 mL of AP extract was added. The mixture was transferred to a conical flask and covered with aluminium foil. The color change of the mixture after exposure to sunlight for 10 minutes indicated the formation of silver nanoparticles. The reaction solution was centrifuged three times at 10,000 rpm for 10 minutes each.

The precipitate obtained from the process was vacuum freeze-dried to acquire APSN powder, which was then preserved in anhydrous ethanol.

### 2.2. Experimental Animals

Inbreed Adult Wistar rats of either sex, weighing approximately 170-180 ± 2.5g, were obtained from the Cape Biolab animal breeding and animal experimentation facility. This animal study protocol was approved by CPSEA (approval number: CBLRC/IAEC/02/01-2023). The rats were housed in cages to acclimatize to standard laboratory conditions (20-22°C humidity, and 12:12- hour dark - light cycle) for 7 days, *ad libitum*.

### 2.3. Animal Grouping and Drug Administration Schedule

Animals are divided into eight groups of six rats each. Four groups were used for acute oral toxicity studies as per the OECD guidelines 423, and the remaining groups were for sub-acute toxicity studies as per the OECD guidelines 407. For the acute oral toxicity study, Group 1 rats (n=6) served as control and were administered with normal saline 5mL/kg b.wt p.o. route. Group 2 rats (n=6) served as the test drug-treated group and were administered with a low dose of APSN (500mg/kg b.wt, p.o.), Group 3 rats (n=6) served as the test drug-treated group and were administered with a high dose of APSN (1000mg/kg, b.wt, p.o, and Group 4 rats (n=6) served as test drug-treated group and were administered with high dose of APSN (2000mg/kg b.wt. p.o.). The test drugs were administered on day one, and general toxicity observations were made at different time intervals over the following 14 days (10).

For the sub-acute toxicity study, Group 1 rats (n=6) served as control and were administered with normal saline 5mL/kg b.wt p.o. route, group 2 rats (n=6) served as test drug treated group and were administered with low dose of APSN at the dose of (500mg/kg b.wt, p.o.), group 3 rats (n=6) served as test drug treated group and were administered with high dose of APSN at the dose of (1000mg/kg, b.wt, p.o and group 4 rats (n=6) served as test drug treated group and were administered with high dose of APSN (2000mg/kg b.wt. p.o.). The test drugs were administered continuously for 28 days.t the end of the study period ,animals were sacrificed for haematological, biochemical and histopathological examinations.

### 2.4. Acute Oral Toxicity Study

Animals were fasted overnight before the experiment commenced. The up and down-regulation of OECD guidelines 423 was followed and the starting test dose was 500 mg/kg, b.w. Further, the test doses (1000 & 2000 g/k.g. b.wt,) are escalated depending on the animal mortality rate, that is ,if there is no mortality in the low test

dose-treated animals group, then the second high test dose is administered into another group of animals. The general observations were monitored at 4hours, 24hours, 7th day and 14th day of experimentation. We documented various observations, including alterations in skin, fur, eyes, mucous membranes, changes in respiration, the occurrence of tremors, convulsions, salivation, diarrhoea, lethargy, sleep, and coma. In addition, observations on food and water intake were made. Behavioural assessments included open field tests and rota rod tests to evaluate spontaneous behavior and locomotor behaviour of the rats (11).

**2.5. Sub-acute Oral Toxicity Study**

Animals were fasted overnight before the experiment commenced. The OECD guideline 407 was folloed for this study, signs and symptoms of toxicity were monitored throughout the 28- day period. The following parameters such as body weight, food and water intake were recorded on a daily basis. At the end of the experimental period, following the final test dosing schedule, blood samples were collected from the rats using both heparinized and non-heparinized vacutainer tubes under isoflurane anesthesia. All rats were euthanized using carbon dioxide inhalation, and the brain, visceral organs and sexual gonads were excised and weighed. Subsequently, all organs were preserved in 10% buffered neutral formalin for histopathological examination (12).

**2.6. Haematological Assay**

Heparinized blood samples were used for assaying various haematological parameters such as White blood cell count (WBC), Red blood cell count (RBC), Haemoglobin (Hb), Haematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC), Red cell distribution width (RDW), Platelets (PLT), Mean platelet volume (MPV), and Platelet distribution width (PDW) were analysed and recorded using autoanalyzer Labomed H-702.

**2.7. Biochemical Parameters**

Blood serum was used to analyze the biochemical parameters. The non-heparinized blood samples were kept in the vacutainer for 12 hours and serum was separated. Subsequently, the serum biochemistry analyzer (Micro lab Rx50V) is used to measure the following parameters: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), High-density Cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), Gamma-glutamyl transferase (c-GT), Serum Total Protein (STP), Albumin (ALB), Urea and Creatinine, Total Bilirubin (TB), Triglyceride (TG), Sodium ions (Na), Potassium ions (K), and Chloride ions (Cl).

**2.8. Histopathological Studies:**

For histopathological examination, organs such as the brain, oesophagus, heart, lungs, liver, spleen, kidneys, testes, and ovaries were excised. Following excision, the organs were preserved in 10% v/v neutral buffered formalin. Once fixed , thin sections (6 µm thick) were taken using a microtome and processed for standard protocol (13). All sections wete stained with Haematoxylin and Eosin (H&E). The stained slides were examined under a microscope at 10x magnification, and different tissue structures were systematically evaluated for any abnormalities or lesions. The histopathological evaluation involved scoring tissue sections based on predefined criteria, documenting findings related to cellular architecture, inflammation, necrosis, and other pertinent indicators.

**2.9. Data Analysis**

All data were expressed as mean±SEM. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to discern specific mean values. Two-way ANOVA was employed for a few parameters followed by multiple comparison tests. The p-value ($p<0.05$) was considered statistically significant. All the data were analyzed using GraphPad Prism software (Version 9.0).

## 3. Results

**3. 1. Oral Acute Toxicity Studies**

Table 1 summarizes the acute oral toxicity signs ob at 4hours, 24hours, 7th day, and 14th day following APSN administration in *Wistar rats*. *It is* observed that rats treated with a high dose of 2g/kg dose exhibited salivation in the rats at 4th hour as compared to control animals and those treated with lower doses . However, this effect was not observed at 24th hourrs and a week. An increase in sleeping time was observed in rats at 4hours after APSN treatment with doses of 1 and 2 g/kg b.w, indicating an acute CNS depressant effect of APSN. Interestingly, rats treated with all three doses have shown itching behaviour at 4 hours after oral administration. However, neither low dose nor high dose exhibits any morbidity and mortality at 4th hour throughout the observation period . The NOAEL (no-observed-adverse-effect-level) of APSN was determined to be 2000 mg/kg.

**Table 1.** Effect of APSN on the behaviour of rats in acute toxicity studies.

| Parameters | 4 hr | | | | 24 hr | | | 7th day | | | 14th day | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | G1 | G2 | G3 | G4 | G2 | G3 | G4 | G2 | G3 | G4 | G2 | G3 | G4 |
| Fur & Skin | * | * | * | * | * | * | * | * | * | * | * | * | * |
| Eyes | * | * | * | * | * | * | * | * | * | * | * | * | * |
| Salivation | * | * | * | ** | * | * | * | * | * | * | * | * | * |
| Respiration | * | * | * | * | * | * | * | * | * | * | * | * | * |
| Urination (color) | * | * | * | * | * | * | * | * | * | * | * | * | * |
| Somatomotor Activity | * | * | * | *** | * | * | * | * | * | * | * | * | * |
| Faeces Consistency | * | * | * | * | * | * | * | * | * | * | * | * | * |
| Sleep | * | * | *** | *** | * | * | * | * | * | * | * | * | * |
| Mucous Membrane | * | * | * | * | * | * | * | * | * | * | * | * | * |
| Convulsions & tremors | * | * | * | ** | * | * | * | * | * | * | * | * | * |
| Itching | * | **** | **** | **** | * | * | * | * | * | * | * | * | * |
| Coma | # | # | # | # | # | # | # | # | # | # | # | # | # |
| Mortality | # | # | # | # | # | # | # | # | # | # | # | # | # |

*-Nill, **-Slightly Found, #-Not Found, ***-Increased. ****-Present.

### 3.1.1. Open Field Test

The rats were subjected to an open-field behavioural assessment model and placed at the center of an apparatus featuring an acrylic floor divided into quadrants and illuminated by a singular overhead white light. This test was conducted to assess both the exploratory tendencies and locomotor activity of the animals, as well as to observe any potential behavioral alterations. The evaluation involved tallying the number of quadrants traversed by each animals and recording instances of elevation, defined as rearing behavior, where the animal assumes a vertical stance on its hind limbs, with the front limbs either suspended in the air or braced against the enclosure wall. These observations were made over a duration of six minutes.

Figure 1 illustrates the effect of APSN treated at different dose levels (500-2000mg/kg b. wt) tested in an activity cage. It is observed that there are no significant changes in the total distance travelled in control and APSN-treated groups [one way ANOVA F (3,8)=4.250; $p$<0.0452] ,suggesting no putative CNS effect of the test drugs administered at different dose intervals.

### 3.1.2. Rota Rod Test

In the Rota rod experiment, rats were placed beneath elevated cylinders rotating at speeds ranging from 4 to 40 rpm. The duration each rat remained on the cylinder without tumbling was recorded within a 60-second timeframe. This assessment aims to scrutinize potential neurological impairments impacting motor abilities, such as sedation, hyper-excitability, ataxia and muscle relaxation. The rats were acclimated to this setup for two days before conducting the test. The results from the Rota Rod apparatus exhibited a substantial improvement in the motor coordination of treated animals in comparison to the control group.

The figure 2 represents the effect of APSN administered at different dose levels (500-2000mg/kg b. wt) tested in rota rod test. It is observed that there are no significant changes in latency time (seconds) for the animal to fall from the rotating rod across different doses of APSN compared to that of saline-treated control group rats [one way ANOVA F(3,8)=4.784; $p$<0.0341] ,suggesting no effect on the motor behaviour of the rats.

## 3.2. Repeated Oral Toxicity Study

### 3.2.1. Feed Intake and Water Consumption Analysis

Throughout the 28-day treatment period, all rats remained healthy without exhibiting any evident signs of toxicity. Comparison between the group treated with APSN and the control group revealed no noteworthy alterations in either feed, water intake or body weight.

Figure 3A illustrates the body weight analysis of different groups of rats treated with normal saline and APSN at different doses. Body weight changes were measured at different time intervals (day 1 to day 28). Two-way ANOVA analysis suggests significant body weight changes among the rat groups [an interaction between groups F (12,40) =3.132; MS=0.5625;p< 0.0033]. The row and column factors for the groups are as follows [Row factor F (4,40)=3688; MS = 662.3;p<0.0001 and Column factor F (3,40) = 4.187; MS = 0.752; p<0.0114). Tukey's multiple comparison tests revealed a significant increase (p<0.001) in body weight changes observed in both the control and APSN-treated rats group. The mean body weight oat the end of the experimentation period (on the 28th day) were as follows:

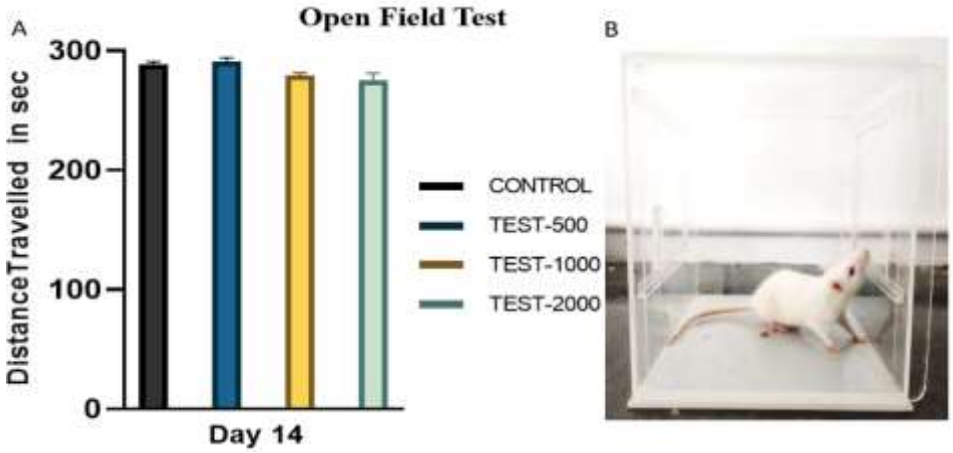


**Figure 1.** Open-field behavioural assessment. A. The values of distance travelled by the tested rat. B. Open-field cage model.

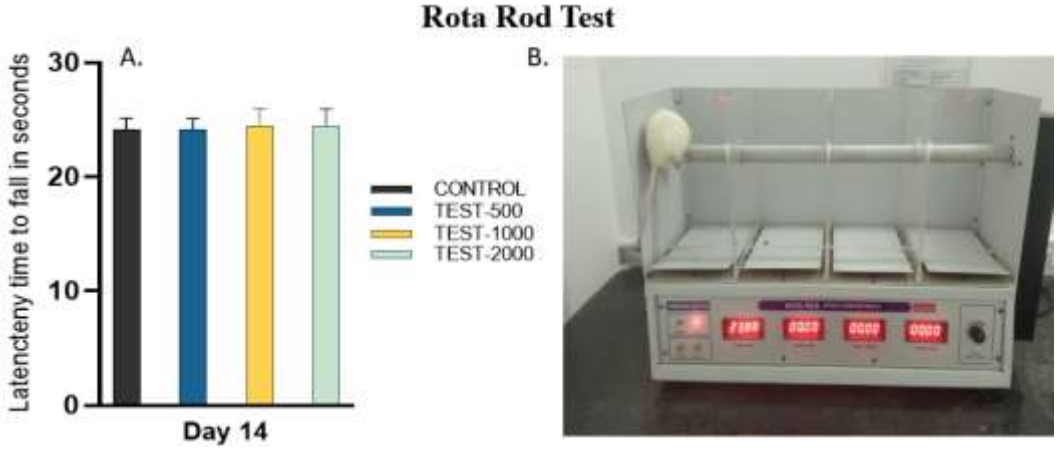


**Figure 2.** Rota rod test for motor abilities**.** A. The values of latency time to fall from the rota rod. B. Rota rod model.

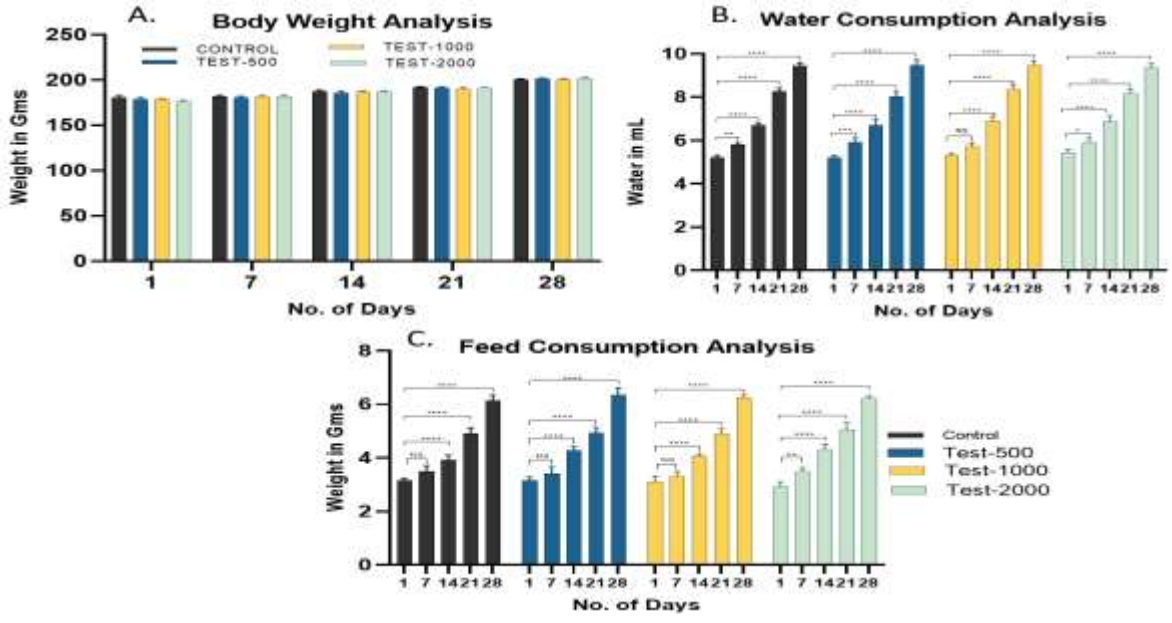


**Figure 3** A. Body weight analysis of tested rats. B. Water consumption analysis. C. Feed consumption analysis.

Control (187.68gm), APSN 500 (187.51gm), APSN 1000 (187.34) and APSN 2000 (187.85) respectively. Figure 3B represents the water intake analysis of different groups of rats treated with normal saline and APSN of different doses. Water intake changes were measured at different time intervals (day 1 to day 28). Two-way ANOVA analysis suggests that there are significant water intake changes among the rat groups [an interaction between groups F (12,40) =08485; MS=0.2956;p< 0.6025]. The row and column factors for the groups are as follows: [Row factor F (4,40)=1044; MS = 34.96;p<0.0001 and Column factor F(3,40) = 0.8724; MS = 0.03039; p<0.463). Tukey's multiple comparison tests revealed that a significant increase (p<0.001) in body weight changes observed in the control and APSN treated rats group. The mean volume of water at the end of the experimentation period (on the 28$^{th}$ day) was: Control (7.08ml), APSN 500 (7.06ml), APSN 1000 (7.14ml) and APSN 2000 (7.14ml).

Figure 3C shows the feed intake analysis of different groups of rats treated with normal saline and APSN at different doses. Feed consumption changes were measured at different time intervals (day 1 to day 28). Two-way ANOVA analysis suggests a significant feed intake changes among the rat groups [an interaction between groups F (12, 40) =0.8246; MS= 0.0303; p< 0.6249]. The row and column factor for the groups are as follows: [Row factor F (4, 40) = 523.5; MS = 19.25; p< 0.0001 and Column factor F (3,40) = 0.9197; MS = 0.0382; p<0.4410). Tukey's multiple comparison tests revealed a significant increase (p<0.001) in feed intake changes observed in the control and APSN treated rats group. The mean value of feed intake at the end of the experimentation period (at 28$^{th}$ day) was: Control (4.34gms), APSN 500 (4.42 gms), APSN 1000 (4.32 gms) ,and APSN 2000 (4.39 gms).

**3.2.2. Organ Weight Analysis**

Relative organ weight serves as a crucial index frequently employed in toxicological assessments, providing a more precise parameter than absolute weight when evaluating toxicity in rats. Typically, a decrease in the internal organ weight signifies potential toxicity after exposure to harmful substances.

In our study, the relative weights of various organs, such as the brain, heart, lungs, liver, spleen, stomach, kidneys, and testes in male rats, as well as the ovary in female rats following 28 days of treatment, exhibited no notable changes compared to the control group. This outcome aligns with and supports the findings of our investigation. Figure 4 represents the organ weight analysis of rats treated with saline and different doses of APSN. Administration of saline and APSN for 28 days revealed no significant weight changes in visceral organs such as the brain, heart, lungs, liver, spleen, stomach, and kidneys as well as sexual gonads of the rats when compared to control rats. One-way ANOVA reveals no significant brain weight changes among all the APSN treated groups compared to that of the control group [F(3, 8) = 0.9077;p<0.4790].

**3.2.3. Haematological and Biochemical Analysis**

In male rats administered a dose of 2,000 mg/kg/day, there was a significant increase in Neutrophils, Haemoglobin, Haematocrit, and Mean Corpuscular Haemoglobin Concentration compared to the vehicle control group (p<0.05). However, there were no discernible differences observed in females between these groups. Moreover, at the end of the recovery period, no significant disparities in any of the hematological parameters were detected between the groups of either gender (Table 2).

The AST level notably increased in males subjected to the 2,000 mg/kg/day dose compared to the vehicle control group (p<0.05). However, no significant distinctions were noted among these groups. Additionally, at the end of the recovery period, there were no significant differences observed in any of the serum biochemical values between the groups in either gender (Table 3).

The hematological parameters (Table 2) and biochemical parameters (Table 3) for both female and male rats exhibited no notable alterations following the 28-day toxicity test compared to the control group.

**3.2.4. Histological Findings**

Histopathological examination of vital organs revealed no gross microscopic change or lesions observed in tissues, suggesting an absence of organ-specific toxicity from the administration of different doses of APSN. The cortical and subcortical structure of the brain showed normal configuration, with no evidence of fibrosis, inflammation and cortical, or cerebellar degeneration.

The microscopic appearance oesophagus, lungs and heart revealed normal architecture with no signs of oedema, congestion or hypertrophy. No abnormality was detected in the stomach, spleen, pancreas,or kidney. The microscopic appearance of sexual gonads appears normal.

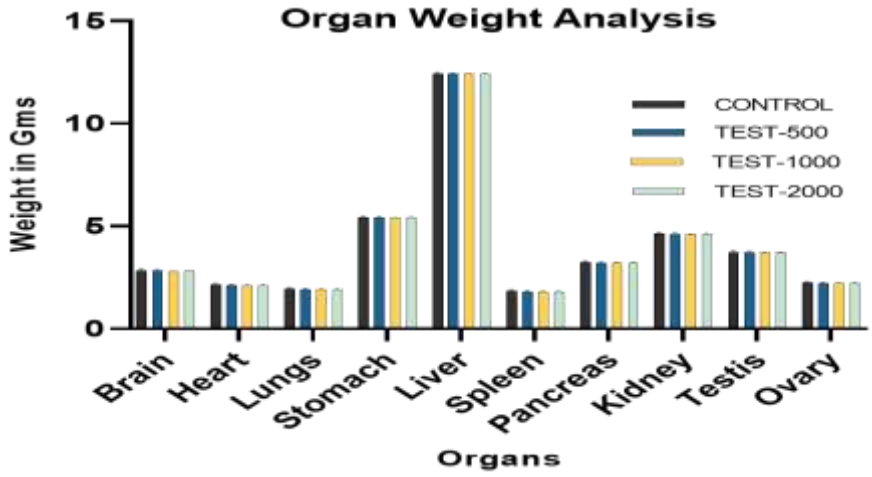


**Figure 4.** Organ weight analysis of APSN treated animals.

**Table 2.** Effects of haematological parameters in repeated oral toxicity of APSN for 28 days in rats.

| Gender | Parameters | Control | Test | | |
|---|---|---|---|---|---|
| | | | 500 | 1000 | 2000 |
| Male | RBC ($10^6$/µL) | 8.63±4.27 | 7.73±5.12 | 7.73±1.27 | 8.91±2.51 |
| | WBC ($10^3$/µL) | 9.11±2.71 | 9.02±5.71 | 8.81±5.41 | 8.93±3.11 |
| | Neutrophils (%) | 11.2±1.24 | 12.1±3.41 | 12.9±4.12 | 13.7±2.31 |
| | Haemoglobin (g/dL) | 15.9±2.14 | 15.4±3.15 | 15.8±4.57 | 16.1±1.52 |
| | Hematocrit (%) | 48.1±3.59 | 48.2±2.87 | 49.1±1.02 | 49.1±3.14 |
| | Eosinophils (%) | 2.8±2.51 | 2.7±4.17 | 2.8±4.21 | 3.1±5.17 |
| | Basophils (%) | 0.3±6.34 | 0.3±0.24 | 0.3±3.72 | 0.4±1.35 |
| | Monocytes (%) | 2.0±5.03 | 1.9±4.91 | 1.9±6.17 | 1.9±8.13 |
| | Lymphocytes (%) | 72±0.41 | 71±0.24 | 71±0.34 | 73±0.15 |
| | Platelets ($10^3$/µL) | 1570±1.41 | 1560±2.13 | 1565±4.71 | 1572±3.14 |
| | MCV (fL) | 61±0.34 | 61±0.41 | 60±0.53 | 61±2.13 |
| | MCH (pg) | 17.03±0.41 | 17.02±0.24 | 16.9±0.51 | 17.4±0.32 |
| | MCHC (g/dL) | 30±1.04 | 29.7±0.31 | 31.4±0.42 | 32.2±0.14 |
| Female | RBC ($10^6$/µL) | 7.73±6.37 | 7.73±6.37 | 7.73±6.37 | 7.72±4.28 |
| | WBC ($10^3$/µL) | 8.77±1.64 | 8.71±3.41 | 8.75±0.24 | 8.81±4.12 |
| | Neutrophils (%) | 8.2±1.41 | 8.1±2.15 | 8.2±3.21 | 8.1±1.15 |
| | Haemoglobin (g/dL) | 14.2±0.21 | 14.3±3.01 | 14.4±0.21 | 14.5±1.02 |
| | Hematocrit (%) | 49.1±2.11 | 49.4±4.15 | 49.8±1.08 | 49.4±1.40 |
| | Eosinophils (%) | 1.7±1.01 | 1.6±1.42 | 1.7±2.14 | 1.7±1.27 |
| | Basophils (%) | 0.3±1.24 | 0.3±3.14 | 0.3±1.24 | 0.4±0.32 |
| | Monocytes (%) | 2.2±2.11 | 2.1±1.52 | 2.2±0.51 | 2.1±2.12 |
| | Lymphocytes (%) | 80.4±1.01 | 81.2±2.01 | 80.9±1.43 | 79.4±2.42 |
| | Platelets ($10^3$/µL) | 1470±4.01 | 1482±3.11 | 1491±1.41 | 1496.21±2.31 |
| | MCV (fL) | 62±1.51 | 61±1.71 | 62±3.61 | 62±5.11 |
| | MCH: (pg) | 17.2±3.34 | 18.1±1.21 | 17.2±1.42 | 17.5±1.71 |
| | MCHC (g/dL) | 30±0.23 | 31±0.14 | 31±4.01 | 31±1.45 |

*RBC-Erythrocyte Count, WBC-Leukocyte Count, MCV-Mean Cell Volume, MCH-Mean Corpuscular Haemoglobin, MCHC-Mean Corpuscular Haemoglobin Concentration. Values were expressed as Mean ± SEM. Statistical comparison was made across the rows, and values were not significantly distinct by ANOVA ($p>0.05$).

**Table 3.** Effects of serum biochemical parameters in repeated oral toxicity of APSN for 28 days in rats.

| Sex | Parameters | Control | Test 250 | Test 750 | Test 2000 |
|---|---|---|---|---|---|
| Male | AST (U/L) | 112±1.41 | 112±2.13 | 112±3.61 | 112±4.52 |
| | ALT (U/L) | 77±2.76 | 75±3.24 | 76±0.61 | 76±1.73 |
| | ALP (U/L) | 411±0.47 | 382±2.09 | 393±0.67 | 401±0.29 |
| | CRE (mg/dl) | 0.85±0.14 | 0.72±0.71 | 0.75±0.44 | 0.77±0.15 |
| | BUN (mg/dl) | 56.8±2.31 | 56.1±1.91 | 55.9±1.92 | 56.1±2.44 |
| | Glucose (mg/dL) | 94±2.74 | 92.1±3.82 | 92.7±4.13 | 93.8±1.92 |
| | Total Cholesterol (mg/dl) | 77.2± 14.52 | 79.91±37.14 | 81.53±4812 | 86.91±26.75 |
| | Total Protein (g/dl) | 5.98±3.72 | 5.41±2.17 | 5.79±3.51 | 6.02±2.08 |
| | Albumin (g/dl) | 4.2±0.02 | 3.8±0.03 | 3.7±0.02 | 3.6±0.03 |
| | Urea (mg/dl) | 49±2.81 | 46±2.47 | 47±1.05 | 48±2.61 |
| | Triglyceride (mg/dl) | 170±4.07 | 166±3.58 | 167±4.15 | 170±1.53 |
| | Calcium (mg/dL) | 11±1.03 | 10.1±2.14 | 10.4±3.47 | 10.9±2.04 |
| | Potassium (mg/dL) | 3.7±1.42 | 3.61±1.57 | 3.6±1.62 | 3.69±1.51 |
| | Sodium (mg/dL) | 143±2.01 | 142±1.34 | 142±2.18 | 143±2.71 |
| | Phosphorus (mg/dL) | 5.4±0.04 | 5.3±0.03 | 5.4±0.02 | 5.3±0.01 |
| | Globulin (g/L) | 3.6±1.06 | 3.7±1.05 | 3.9±1.06 | 3.9±1.08 |
| | Total Bilirubin (µmol/L) | 3.1±1.41 | 3.1±1.02 | 3.2±1.34 | 3.3±1.26 |
| Female | AST (U/L) | 103±1.23 | 102±2.14 | 101±1.25 | 102±1.46 |
| | ALT (U/L) | 56±2.04 | 57±0.41 | 58±0.62 | 59±1.35 |
| | ALP (U/L) | 76±0.47 | 71±0.15 | 72±0.07 | 73±0.02 |
| | CRE (mg/dl) | 0.79±0.02 | 0.76±0.03 | 0.79±0.04 | 0.81±0.01 |
| | BUN (mg/dl) | 49±2.14 | 48±1.25 | 48±0.31 | 48±1.04 |
| | Glucose (mg/dL) | 84±1.56 | 85±1.42 | 86±1.62 | 89±1.81 |
| | Total Cholesterol (mg/dl) | 55.2±15.26 | 53.1±24.15 | 53.9±37.13 | 54.7±19.41 |
| | Total Protein (g/dl) | 6.1±2.02 | 6.1±2.04 | 6.2±2.14 | 6.2±2.31 |
| | Albumin (g/dl) | 2.7±0.01 | 2.6±0.03 | 2.8±0.01 | 2.9±0.02 |
| | Urea (mg/dl) | 47±1.9 | 47±2.5 | 47±3.7 | 48±3.7 |
| | Triglyceride (mg/dl) | 168±2.01 | 167±1.81 | 169±2.17 | 171±1.92 |
| | Calcium (mg/dL) | 10±1.42 | 10±2.31 | 10±1.4 | 11±2.6 |
| | Potassium (mg/dL) | 5.1±1.31 | 4.9±1.42 | 5.1±2.14 | 5.2±1.02 |
| | Sodium (mg/dL) | 140±2.03 | 138±1.04 | 141±2.12 | 142±1.24 |
| | Phosphorus (mg/dL) | 5.1±0.01 | 5.2±0.02 | 5.2±0.01 | 5.3±0.02 |
| | Globulin (g/L) | 3.5±1.04 | 3.4±1.06 | 3.7±1.08 | 3.9±1.05 |
| | Total Bilirubin (µmol/L) | 2.9±1.47 | 2.8±1.13 | 2.9±1.04 | 3.0±1.12 |

AST- Aspartate Aminotransferase, ALT- Alanine Aminotransferase, ALP- Alkaline Phosphatase, CRE- Creatinine, BUN-Blood Urea Nitrogen. Values were expressed as Mean ± SEM. Statistical comparison was made across the rows, and values were not significantly distinct by ANOVA ($p>0.05$).

However, there is a mild infiltration of liver was observed in APSN treated rats with 2gm/kg b.wt (Figure 5).

## 4. Discussion

In recent years, the global acceptance of medicinal herbs as complementary medicine has grown; however, concerns about the toxicity and safety of commonly used medicinal plants persist. Previous reports emphasize the importance of evaluating the toxicity of herbal products, especially considering potential adverse effects from short-term to long-term usages of doses.

In developing countries, herbal plants are widely used, and the misconception that phytoconstituents are harmless highlights the necessity for clinical investigations into the toxicity profiles of phytopharmaceutical preparations (14).

These discussions underscored the need for safety tests, standardization, and regulation of herbal medicines,

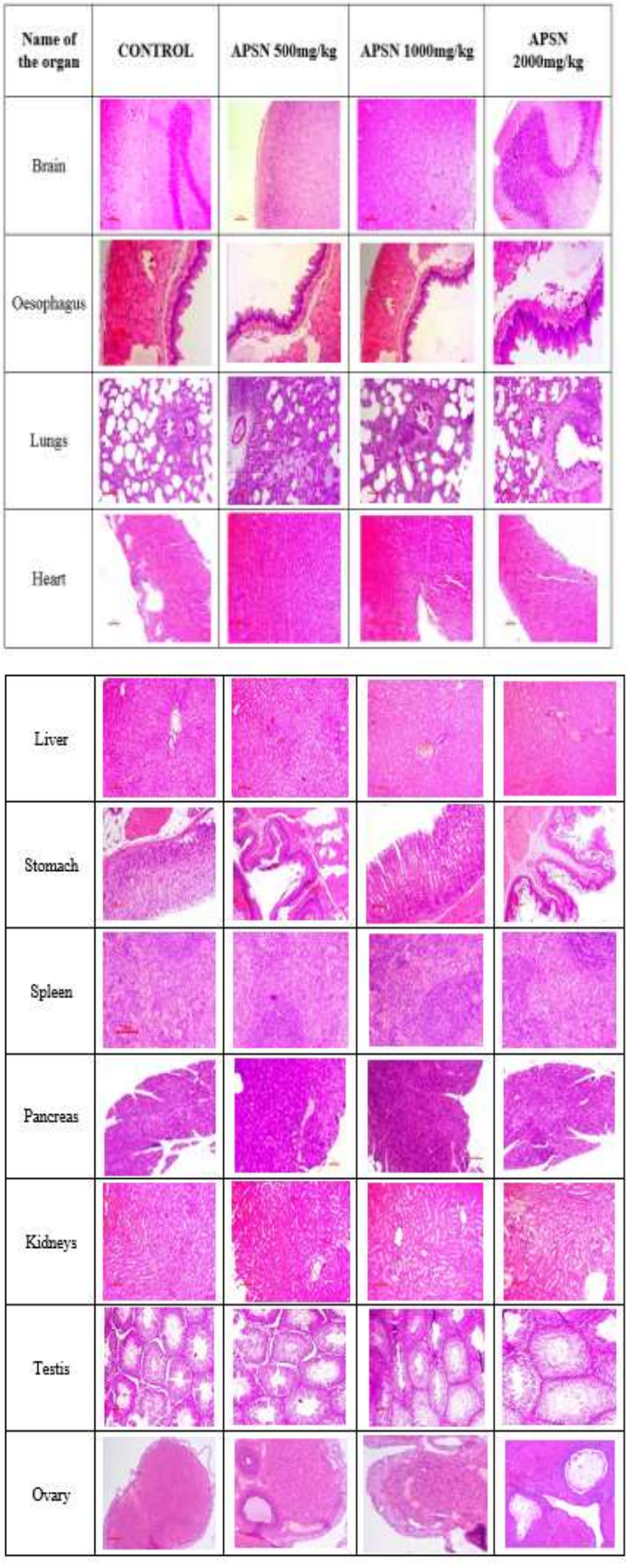


**Figure 5.** Histopathological observations of Sub-acute toxicity studies at the end of 28th day (10x).

emphasizing that even medicinal plants require toxicity evaluation before widespread use. Toxicological research, particularly *in-vivo* studies, is crucial for providing scientific evidence regarding the safety and efficacy of herbal products.

The genus *Acalypha* has been traditionally used to treat various gastrointestinal disorders such as dysentery, severe diarrhoea, and for treating neonatal jaundice (15). AP, an unexplored species, has a long history of traditional use for treating and preventing certain diseases, as highlighted earlier. However, to date, there is no scientific data available regarding the toxicity evaluation of APSN in rats.

DLS analysis showed that the AP extract reduced the size of silver ions, with 94% of the particles averaging 54.7 nm and a PDI of 0.732. The colloidal stability was confirmed by a prominent zeta potential peak at -27.0 mV. Both DLS and zeta potential measurements highlighted effective electrostatic repulsion among the nanoparticles, preventing aggregation. TEM images confirmed the quasi-spherical shape of the nanoparticles, further supporting their morphology (16).

According to OECD guidelines, rats are the primary predictive models for human effects in toxicity assessments; hence, this study was conducted using rats. Acute toxicity involves assessing mortality within 24 hours after the administration of a single high dose of APSN. In contrast, sub-acute toxicity assesses the adverse effects following repeated administration of APSN in small doses over 28 days. In our study, rats administered with different doses of APSN did not exhibit any salivary secretion, indicating no direct effect of the varying doses of APSN on the salivary gland or toxic effect on the cholinergic nerve or inhibition of cholinesterase enzyme. A high dose of APSN (2g/kg b. wt) showed mild convulsions in rats within 4 hours of administration, with the magnitude of the seizure lasting 1 minute, without inducing rigor mortis in rats. Moreover, the convulsions ceased after 4 hours, and all animals behaved normally at the end of the observation period. Additionally, itching was observed by scratching the skin using the fore and hind paws of rats treated with 500 mg/kg – 2 g/kg of b.wt, suggesting an acute allergic response mediated by histamine degranulation. It has been reported that the APSN contains pleiotropic polyvalent phytochemicals, particularly alkaloids, which may be contribute to this itching effect. However, the itching effect subsided after 4

hours and till the end of the experimentation period. It is interesting to note that there is no signs of coma or mortality were observed in acute and sub-acute toxicity studies.

The Locomotor and muscle grip strength of the rats were assessed at the end of the acute toxicity studies. Observations indicate no impaired locomotor activity or muscle strength, as evidenced by equal time spent in the open field and more retention time noted in rats treated with various doses of APSN compared to the control group. The LD50 of APSN exceeds 2000 mg/kg and can be identified as a Class 4 drug according to the acute toxicity classification criteria for substances.

In sub-acute toxicity assessment, the impact of APSN through repeated administration is evaluated based on OECD 407 guidelines. These include body weight, feed intake, and water consumption, exhibiting a gradual daily increase throughout the 28-day period. In this study, the observed normal body weight, feed intake, and water consumption in rats across all groups offer substantial assurance regarding the safety of APSN. The increase in feed consumption among APSN-treated rats,is likely influenced by bioactive constituents such as alkaloids and saponins ,known for their appetite-binding properties, aligns with normal metabolic processes. The concurrent rise in body weight and relative organ weight suggests increased adiposity, which may influence blood glucose levels and is consistent with previous findings (17).

Acknowledging that alterations in organ weight alone may not conclusively indicate normalcy, our evaluation, in line with Pang-Kuei et al. (18) investigated haematological, serum biochemical, and histopathological parameters to comprehensively assess APSN toxicity and identify potential major effects on organs. In the APSN treated groups, various hematological parameters such as RBC, WBC, Neutrophils, Haemoglobin, Hematocrit, Eosinophils, Basophils, Monocytes, Lymphocytes, Platelets, Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), and Mean Corpuscular Haemoglobin concentration (MCHC) did not display any significant changes compared to the control group. All indicators pertaining to blood composition were observed to be within the normal range, indicating no deviation from the normal functional processes during growth.Conversely, the non-significant changes ($p<0.05$) observed in total cholesterol, albumin, bilirubin, creatinine, triglycerides, and protein levels may suggest that the impact of APSN on animals could be either beneficial or harmful, depending on the specific alterations involved. Previous research has indicated that plant extracts can lead to heightened bilirubin and creatinine levels, coupled with decreased tissue protein levels in rats (19). The reduced serum albumin observed in this study may indicate underlying hepatic injury. The noteworthy increase ($p<0.05$) in ALT, AST, and ALP levels in rats treated with 2000 mg/kg compared to other treated groups and the control suggests potential liver abnormalities. While higher doses of other plant extract typically resulted in elevated serum urea and creatinine, due to kidney toxicity, our study did not observe an increase in these markers at higher doses of APSN. The observed non-significant ($p>0.05$) rise in serum electrolyte levels among the treated male Wistar rats could imply that the APSN has minimal to no impact on the electrolyte profile of rats. These results may reflect the high antioxidant capacity of the APSN, a characteristic also documented by our earlier research. Histological studies of the brain, oesophagus, lungs, heart, stomach spleen and kidney revealed no significant changes, which is consistent with biochemical and hematological parameters. Differences in histopathology occurred only in the liver with mild infiltration.

In the overall study, both acute and sub-acute toxicity studies didn't show any significant changes comparable with control and this research also supports the statement that the APSN is safe to use. As our study continues, ongoing investigations seek to further unravel its implications. Our comprehensive findings following acute and subacute oral administrations of APSN unveiled no instances of mortality, unfavourable shifts in behavior, or significant changes in biochemical, and hematological parameters. Furthermore, these administrations exhibited no apparent impact on the histology of vital organs in both male and female Wistar rats. Nevertheless, to gain a more profound understanding of the silver nanoparticles potential effects on critical bodily functions, such as hormone levels, essential enzymes, and the nervous system, additional comprehensive research remains crucial. Hence, our collected data provides a robust scientific foundation, supporting and validating the traditional use of AP in folk medicine, and highlighting its potential in the pharmaceutical industry. While our acute toxicity testing revealed potential toxic effects in the APSN, our data suggests that the silver nanoparticles

sourced from the aerial parts of AP holds promise as a non-toxic and safe option for potential human use.

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## Authors' Contribution

Study concept and design: E.A.
Acquisition of data: E. A.
Analysis and interpretation of data: H. NA, I. N.
Drafting of the manuscript: E. A.
Critical revision of the manuscript for important intellectual content: H. NA, I. N.
Statistical analysis: E. A.
Administrative, technical, and material support: M. VVP, I. Y, A. K.P, P. D.
Study supervision, I. N.

## Ethics

Cape Biolab animal breeding and animal experimentation facility. This animal study protocol was approved by CPSEA (approval number: CBLRC/IAEC/02/01-2023).

## Conflict of Interest

The authors declare that they have no competing financial interests or personal relationships that could have influenced this research article.

## Data Availability

Data related to this study hasn't been deposited into a publicly available repository but will be made available upon request.

## References

1. Aggarwal G, Sharma M, Singh R, Sharma U. Ethnopharmacologically important highly subsidized Indian medicinal plants: Systematic review on their traditional uses, phytochemistry, pharmacology, quality control, conservation status and future prospective. Journal of Ethnopharmacology. 2024;320:117385.
2. Wang H, Chen Y, Wang L, Liu Q, Yang S, Wang C. Advancing herbal medicine: enhancing product quality and safety through robust quality control practices. Frontiers in pharmacology. 2023;14:1265178.
3. Jităreanu A, Trifan A, Vieriu M, Caba I-C, Mârțu I, Agoroaei L. Current trends in toxicity assessment of herbal medicines: A narrative review. Processes. 2022;11(1):83.
4. Puhlmann N, Vidaurre R, Kümmerer K. Designing greener active pharmaceutical ingredients: Insights from pharmaceutical industry into drug discovery and development. European Journal of Pharmaceutical Sciences. 2024;192:106614.
5. Opuni KF, Kretchy J-P, Agyabeng K, Boadu JA, Adanu T, Ankamah S, et al. Contamination of herbal medicinal products in low-and-middle-income countries: A systematic review. Heliyon. 2023;9(9).
6. Singh S, Shukla VK. Current regulations for herbal medicines in India. International Journal of Drug Regulatory Affairs. 2021;9(2):30-4.
7. Bhatt A. Phytopharmaceuticals: A new drug class regulated in India. Medknow. 2016. p. 59-61.
8. Sylvie DD, Anatole PC, Cabral BP, Veronique PB. Comparison of in vitro antioxidant properties of extracts from three plants used for medical purpose in Cameroon: Acalypha racemosa, Garcinia lucida and Hymenocardia lyrata. Asian Pacific Journal of Tropical Biomedicine. 2014;4:S625-S32.
9. Iniaghe O, Malomo S, Adebayo J, Arise R. Evaluation of the antioxidant and hepatoprotective properties of the methanolic extract of Acalypha racemosa leaf in carbon tetrachloride-treated rats. African Journal of Biotechnology. 2008;7(11).
10. Kalaivani P, Siva R, Gayathri V, Langade D. Mutagenicity and safety evaluation of Ashwagandha (Withania somnifera) root aqueous extract in different models. Toxicology Reports. 2024;12:41-7.
11. Amudha P, Vanitha V. Toxicological, biochemical and histopathological evaluation of the ethanolic extract of seagrass-Enhalus acoroides in albino wistar rats. Biocatalysis and Agricultural Biotechnology. 2019;18:101082.
12. Suvarna R, Suryakanth VB, Bakthavatchalam P, Kalthur G, Nayak D, Prabhu MM, et al. Acute and sub-chronic toxicity of Liberin, an anti-diabetic polyherbal formulation in rats. Journal of Ayurveda and Integrative Medicine. 2023;14(6):100804.
13. Amoateng EO, Amoateng P, Ossei PPS, Fenteng EA, Amponsah IK, Ayibor WG, et al. An acute and sub-acute toxicological assessment of Reissantia indica plant extract in male Sprague-Dawley rats: Hematological, serum biochemical and histopathology. Scientific African. 2024;23:e02089.

14. Xia Y, Zhu J, Qu M, Yang W, Fu S, Yu L, et al. Acute and subchronic oral toxicity study of Camelina sativa oil in Wistar rats. Heliyon. 2024;10(1).

15. Seebaluck R, Gurib-Fakim A, Mahomoodally F. Medicinal plants from the genus Acalypha (Euphorbiaceae)–A review of their ethnopharmacology and phytochemistry. Journal of ethnopharmacology. 2015;159:137-57.

16. Aliya S, Rethinasabapathy M, Yoo J, Kim E, Chung J-Y, Cha J-H, et al. Phytogenic fabrication of iron oxide nanoparticles and evaluation of their in vitro antibacterial and cytotoxic activity. Arabian Journal of Chemistry. 2023;16(6):104703.

17. Chen D-H, Mao P-J, Diao W-J, Li Q. Toxicity study of compound granules of Hedyotis diffusa: Acute toxicity and long-term toxicity. Journal of Ethnopharmacology. 2024;321:117434.

18. Hsu P-K, Tsai Y-T, Lin Y-C, Kuan C-M. Assessment of the acute and sub-acute toxicity of the ethanolic extract of the aerial parts of Crassocephalum rabens (Asteraceae) in rats. Toxicology reports. 2022;9:58-63.

19. Bemidinezhad A, Zojaji SA, Jamshidi ST, Mohammadi M, Alavi MS, Ghorbani A. Evaluation of acute, subacute, and subchronic toxicity of a hepatoprotective herbal formulation. Toxicology Reports. 2023;11:452-9.