IMPACT OF GREEN-SYNTHESIZED SILVER NANOPARTICLE IN WISTAR RATS: BEHAVIORAL, BIOCHEMICAL, AND HISTOPATHOLOGICAL INSIGHTS FROM ACUTE AND SUB-ACUTE ORAL EXPOSURE

ABSTRACT

Acalypha paniculata (AP) is a subshrub traditionally used in ethnomedicine for treating skin diseases, asthma, and inflammatory conditions. This research 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 general toxicity signs 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 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 acute toxicity studies indicated no evident toxicity signs, including abnormal motor locomotion and behavior. 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) surpassed 2000 mg/kg/day in both male and female Wistar rats, confirming the safety of APSN when administered orally. This research supports the ethnomedicinal claim of APSN, though further clinical studies are necessary to confirm these findings and ensure comprehensive safety validation.

Keywords: *Acalypha paniculata*, silver nanoparticles, acute and sub-acute oral toxicity studies, Wistar rats

1. INTRODUCTION

The utilization of alternative systems of medicine has evolved from ancient times to the modern era, showcasing continual growth. Notably, the widespread use of medicinal plants in various formulations for treating diverse illnesses has captured increased attention across different cultures (3). Despite the common notion that herbal medicines are generally

safe and devoid of untoward effects on the biological system (24), recent advances like silver nanoparticle's screening methods and biological standardization, including bioanalytical assay methods, have revealed the toxic effects of some silver nanoparticles in preclinical models for assessing the IV safety profile (12).

In contrast, the OECD regulations for testing the toxicity of chemicals, encompassing active pharmaceutical ingredients (API), have provided benchmark guidelines for conducting toxicity studies on laboratory rodents (16). According to the World Health Organization (WHO), nearly 80% of Asian populations utilize medicinal plants to address a variety of diseases. Furthermore, the discovery and isolation of bioactive compounds and secondary metabolites have introduced several molecules with fascinating pharmacological properties (14). The global use of medicinal plants continues to escalate, and with many new products entering the market, public health issues and concerns surrounding their safety are gaining recognition. While some herbal extracts derived from the Indian System of Medicine (ISM) show promising potential and enjoy wide usage, many remain untested, lacking adequate monitoring. Furthermore, the absence of standardized testing protocols for extract presents challenges in consistently assessing their safety and efficacy. The variability in extract composition, influenced by factors such as geographical location, climate, and harvesting methods, contributes to the complexity of ensuring their quality, posing a hurdle in establishing universally accepted guidelines for herbal medicine regulation (23).

In recent years, the popularity of herbal silver nanoparticles and remedies has surged, driven by the perception of being natural and, therefore, presumed to be 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 from healthcare professionals. Addressing the growing concerns surrounding the safety of silver nanoparticles requires collaborative efforts from regulatory bodies, healthcare practitioners, and researchers. Rigorous clinical trials and systematic reviews are essential to provide evidence-based information on the safety and efficacy of herbal silver nanoparticles (4). Additionally, promoting awareness among the public about the potential risks associated with herbal silver nanoparticles and encouraging responsible usage are crucial steps towards ensuring the well-being of individuals embracing alternative medicinal practices.

The Acalypha genus is one of the fourth largest genera of 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 in the forested regions of the Eastern Ghats, encompassing the Javvadhu Hills and Parvathamalai Hills in the Tiruvannamalai District (7,15,19,20). Djacbou D. Sylvie et al (6) studied the free radical scavenging activity of the AR and showed 80% of DPPH radical scavenging activity, 70% Nitric oxide scavenging, and Hydroxy radical scavenging of 87% at the concentration of 160µg/mL. Aqueous extract of AR was studied by Iniaghe et al (11) and proved that the 60mg/kg concentration of extract significantly reduces and prevents hepatic necrosis in the rat. Antimicrobial activity against the organisms *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 exposed the 2-log cycle reduction of cell population in 90 minutes at the 6.0mg/mL concentration of the AP extract.

In prior ethnopharmacological investigations, GC-MS analysis revealed that intricate chemical composition in the aerial parts of AP, yielding alkaloids, terpenoids, saponins and flavonoids Elumalai et al., 2024 (8). Notably, pure Alloaromadendrene was isolated through column chromatography, and silver nanoparticles were synthesized by Elumalai et al (9) using a green synthesis approach. Despite these advancements, there remains a notable dearth of information on the pharmacology of AP and the increased rates of their consumption may potentially lead to adverse effects. The existing scientific literature lacks systematic studies concerning the safety of *Acalypha paniculata* herbal silver nanoparticles (APSN), leaving a considerable gap in understanding their toxicity. Recognizing this gap, our research aims to address the lacuna by evaluating both acute and repeated toxicity levels of the APSN in albino Wistar rats.

This exploration seeks to unveil crucial safety insights, imperative for determining appropriate dosages in pre-clinical trials. The primary objective of our study was to assess the acute and subacute oral toxicity of APSN. We aimed to create a thorough toxicity profile, which involved determining the lethal dose (LD50) and identifying the no observed adverse effect level (NOAEL) of APSN. 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 utilization of AP.

2. MATERIALS AND METHODS

2.1. Botanical plant material and silver nanoparticle preparation

The fresh aerial parts of AP were directly collected 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 aided in identifying the plant, while the Department of Pharmacognosy at the Siddha Central Research Institute (CCRS), part of the Ministry of Ayush under the Government of India, authenticated and identified the plant (Reference: H12092201S), located in Chennai 600106.

AP typically blooms from June to January 2023. The plant was shed-dried and coarsely powdered by using a mechanical blender. The coarse powdered material (250 grams) underwent a hot continuous extraction process using an absolute ethanol solution at a 1:5 (w/v) ratio for the extraction. The extraction process was carried out at 60°C over a period of 6 hours. The extract was filtered using 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 combined. The mixture was transferred to a conical flask and covered with aluminium foil. The color of the mixture underwent a change after being exposed to sunlight for 10 minutes, indicating the formation of silver nanoparticles. Using a high-speed centrifuge, the reaction solution was centrifuged three times at 10,000 rpm for 10 minutes subsequently. 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 around (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). Rats were housed in cages to acclimatize to standard laboratory conditions (20-22°C humidity and 12:12 hrs dark and light cycle) for 7 days *ad libitum*.

2.3. Animal Grouping and Drug Administration Schedule

Animals are divided into eight groups of six rats. In which four groups of rats 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 at the dose of (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 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

given on day one and followed by general toxicity observations were made at different time intervals to 14 days (13).

For the subacute 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 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 given continuously for 28 days and at the end of the animals were sacrificed for haematological, biochemical and histopathological studies were done.

2.4. Acute oral Toxicity Study:

Animals were fasted overnight before the commencement of the experiment. The up and down-regulation of OECD guidelines 423 opted 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 observation was monitored at 4hrs, 24hrs, 7^{th} day and 14^{th} 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 were studied using open field tests and rota rod tests to evaluate the spontaneous behavior and locomotor behaviour of the rats (2).

2.5. Sub-acute oral toxicity study:

Animals were fasted overnight before the commencement of the experiment. The OECD guideline 407 was opted for this study, signs and symptoms of toxicity were monitored throughout the 28 days. 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 while they were 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 studies (18).

2.6. Haematological assay

The 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), Platelet distribution width (PDW) were analysed and recorded using autoanalyzer Labomed H-702.

2.7. Biochemical parameters

Blood serum was used to analyse the biochemical parameters. The nonheparinized blood samples were kept in the vacutainer for 12 hrs 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. After fixation of the tissues, thin sections (6 µm thick) were taken using a microtome and processed for standard protocol (10). All the tissue section was stained with Haematoxylin and Eosin (H&E). The stains were subjected to microscopic examination under the magnification of 10x, wherein different tissue structures were systematically evaluated for any abnormalities or lesions. The histopathological evaluation encompassed assessing tissue sections based on predefined criteria and scoring systems, 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) was employed followed by Tukey's post hoc test 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 represents the acute oral toxicity signs tested at 4hr, 24hrs, 7th day, and 14th day administration of APSN in Wistar rats. It is observed that rats treated with a high dose that is 2g/kg dose exhibit salivation in the rats at 4th hour as compared to that of the control and the other two low doses of treated group animal. However, this effect was not observed at 24th hrs and a week. There is an increase in sleeping time was observed in rats at 4hrs after APSN treatment at the dose of 1 and 2 g/kg b.wt indicating the acute CNS depressant effect of APSN. Interestingly, rats treated with all three doses have shown itching behaviour at 4hrs after oral administration. However, neither low dose nor high dose exhibits any morbidity and mortality at 4th hour and till the end of the experimentation period. The NOAEL (no-observed-adverse-effect-level) of APSN was found 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.

3.2.1. Open field test

The rats were introduced to an open-field behavioural assessment model and positioned at the centre 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, while also observing potential alterations in their behavior. The evaluation involved tallying the number of quadrants traversed by the 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.

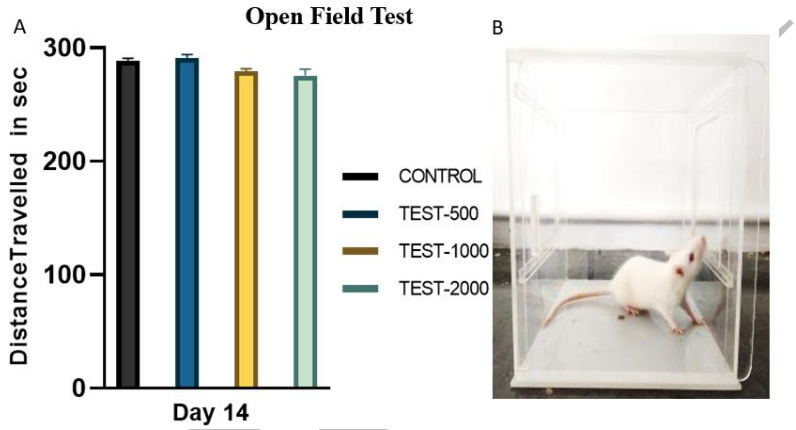


Fig. 1. Open-field behavioural assessment. A. The values of distance travelled by the tested rat. B. Open-field cage model.

Fig 1 represents 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 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 given at different dose intervals.

3.2.2. Rota rod test

In the Rota rod experiment, rats were positioned beneath elevated cylinders rotating at speeds ranging from 4 to 40 rpm. The duration for which the rats stayed beneath the cylinder without tumbling was recorded within a 60-second timeframe. This assessment aims to scrutinize potential neurological impairments impacting the animals' motor abilities, encompassing factors like 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.

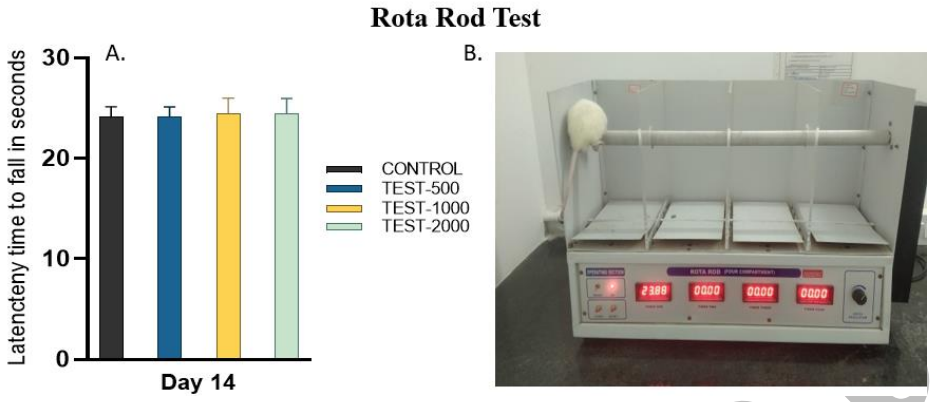


Fig. 2. Rota rod test for motor abilities**.** A. The values of latency time to fall from the rota rod. B. Rota rod model.

The fig 2 represents the effect of APSN treated 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 (secs) for the animal to fall from the rotating rod treated with different doses of APSN as 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.

3.3. Repeated oral toxicity study

3.3.1. Feed intake and water consumption analysis

Throughout the 28-day treatment period, all rats thrived 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 and body weight.

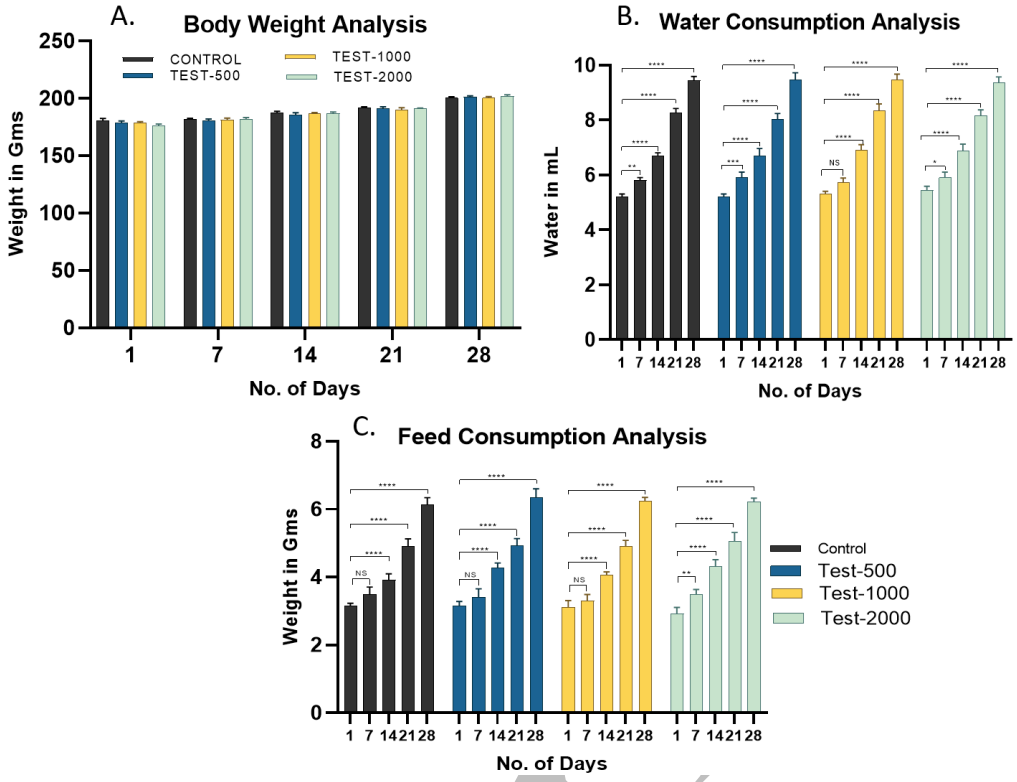


Fig. 3 A. Body weight analysis of tested rats. B. Water consumption analysis. C. Feed consumption analysis

Fig 3A represents the body weight analysis of different groups of rats treated with normal saline and APSN of different doses. The body weight changes were measured at different time intervals (day 1 to day 28). Two-way ANOVA analysis suggests that there is a 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 that there is a significant ($p < 0.001$) increase in body weight changes observed in the control and APSN-treated rats group. The mean weight of body weight at the end of the experimentation period (on the 28th day) was found to be Control (187.68gm), APSN 500 (187.51gm), APSN 1000 (187.34) and APSN 2000 (187.85) respectively.

Fig 3B represents the water intake analysis of different groups of rats treated with normal saline and APSN of different doses. The water intake changes were measured at different time intervals (day 1 to day 28). Two-way ANOVA analysis suggests that there is a 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 there is a significant (p<0.001) increase 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 28th day) was found to be Control (7.08ml), APSN 500 (7.06ml), APSN 1000 (7.14ml) and APSN 2000 (7.14ml) respectively.

Fig 3C represents the feed intake analysis of different groups of rats treated with normal saline and APSN of different doses. The feed consumption changes were measured at different time intervals (day 1 to day 28). Two-way ANOVA analysis suggests that there is 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 that there was a significant (p<0.001) increase 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 28th day) was fond to be Control (4.34gms), APSN 500 (4.42 gms), APSN 1000 (4.32 gms) and APSN 2000 (4.39 gms) respectively.

3.3.2. Organ weight analysis

Relative organ weight serves as a crucial index frequently employed in toxicological assessments, offering 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.

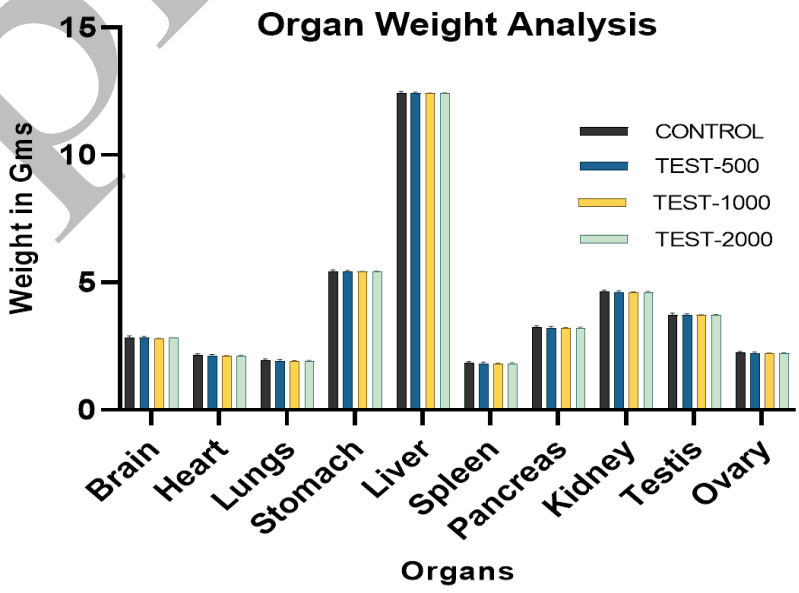


Fig. 4. Organ weight analysis of APSN treated animals

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 treatment during the 28 days, exhibited no notable changes compared to the control group. This outcome aligns with and supports the findings of our investigation. Fig 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 that there were 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 as compared to that of control rats. One-way ANOVA reveals that there are no significant brain weight changes weight among all the APSN treated groups as compared to that of the control group [$F(3, 8) = 0.9077; p<0.4790$].

3.3.3. Haematological and Biochemical Analysis

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

The AST level notably increased in males subjected to the 2,000 mg/kg/day dose in comparison to the vehicle control group ($p < 0.05$). However, no significant distinctions were noted among these groups. Additionally, at the conclusion 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 in comparison to the control group.

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 ($10^6/\mu L$)	8.63±4.27	7.73±5.12	7.73±1.27	8.91±2.51
	WBC ($10^3/\mu L$)	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

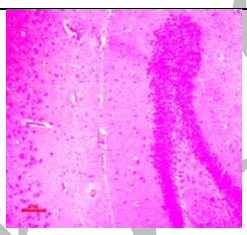
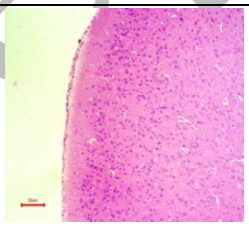
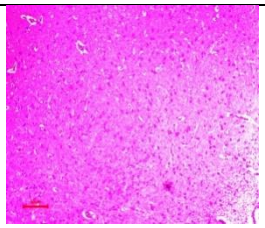
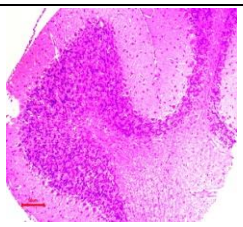
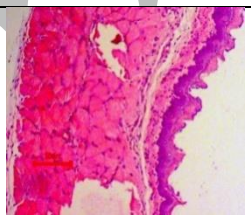
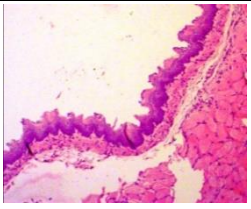
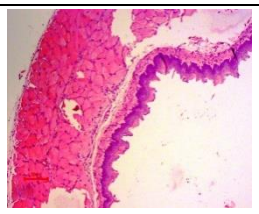
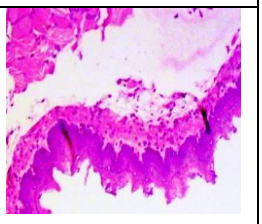
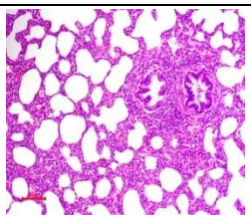
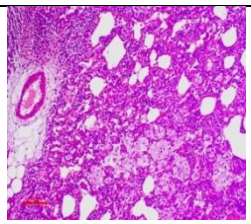
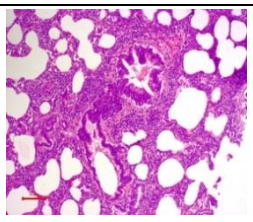
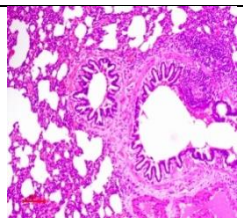
	ALP (U/L)	411±0.47	382±2.09	393±0.67	401±0.29
	CRE (mg/dl)	0.85±0.14	0.72±0.71	0.75±0.44	0.77±0.15
	BUN (mg/dl)	56.8±2.31	56.1±1.91	55.9±1.92	56.1±2.44
	Glucose (mg/dL)	94±2.74	92.1±3.82	92.7±4.13	93.8±1.92
	Total Cholesterol (mg/dl)	77.2± 14.52	79.91±37.14	81.53±4812	86.91±26.75
	Total Protein (g/dl)	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

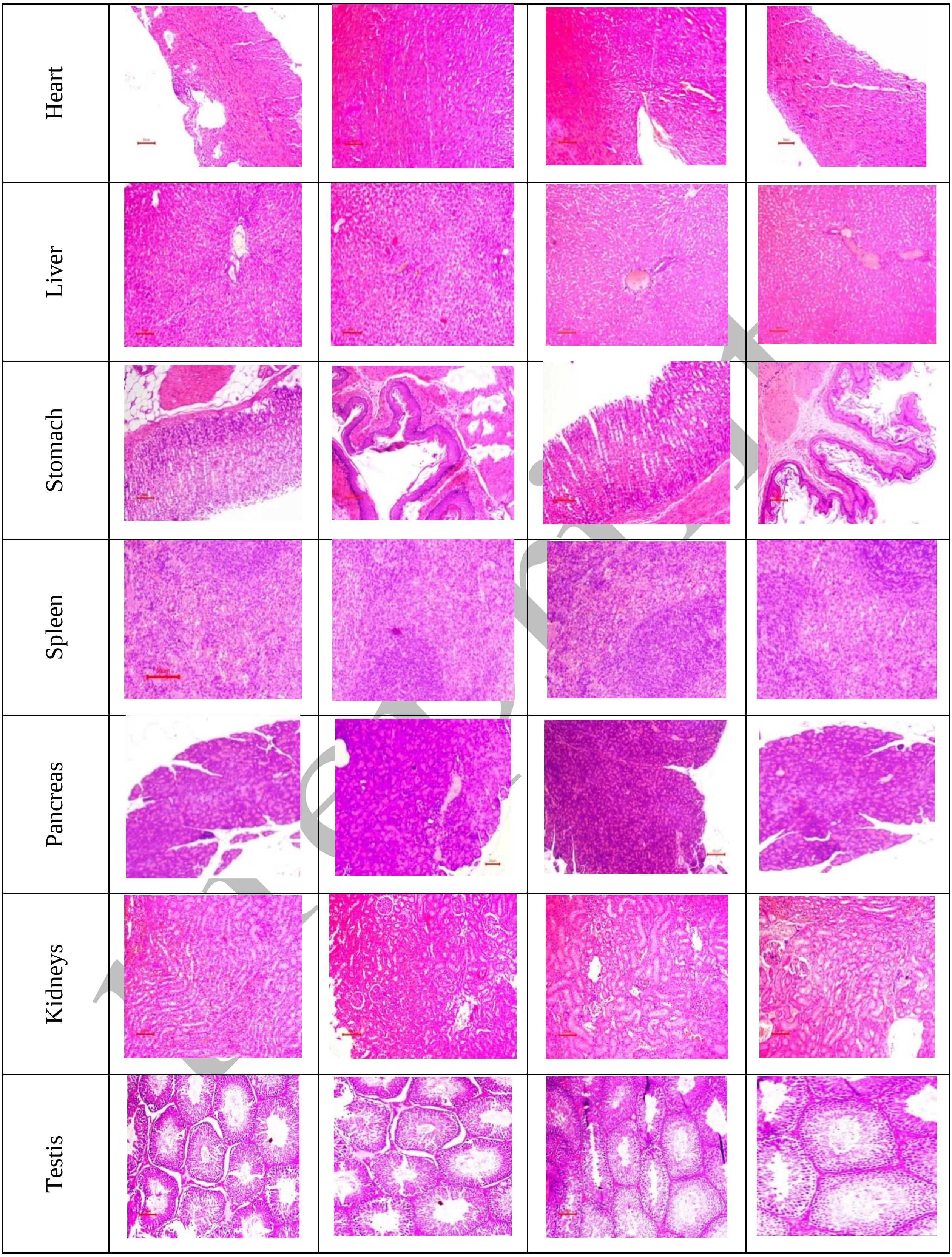
AST- Aspartate Aminotransferase, ALT- Alanine Aminotransferase, ALP- Alkaline Phosphatase, CRE- Creatinine, BUN-Blood Urea Nitrogen. Values were expressed as Mean ± SEM. Statistical comparison was made across the rows, and values were not significantly distinct by ANOVA ($p>0.05$).

3.3.4. Histological findings

Histopathological examination of vital organs revealed that there is no gross microscopical change or lesions observed in tissues suggesting no organ-specific toxicity exhibited by 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 there are no signs of oedema, congestion and hypertrophy. No abnormality was detected in the stomach, spleen, pancreas and kidney. The microscopical appearance of sexual gonads is normal. However, there is a mild infiltration of liver was observed in APSN treated rats with 2gm/kg b.wt.

Name of the organ	CONTROL	APSN 500mg/kg	APSN 1000mg/kg	APSN 2000mg/kg
Brain				
Oesophagus				
Lungs				


Heart
Liver
Stomach
Spleen
Pancreas
Kidneys
Testis

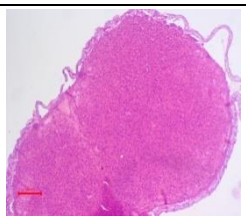
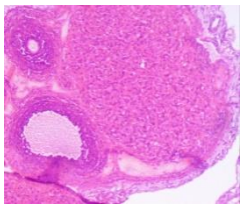
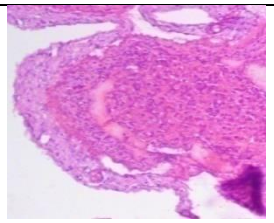
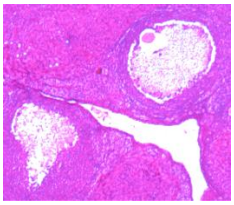
Ovary				

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

4. DISCUSSION

In recent times, the global acceptance of medicinal herbs as complementary medicine has grown, but 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 dose usage.

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 (25). The discussions underscored the need for safety tests, standardization, and regulation of herbal medicines, emphasizing that even medicinal plants require toxicity evaluation before widespread use. Toxicological research, particularly *in-vivo* studies, is crucial to providing scientific evidence on the safety and efficacy of herbal products.

The genus Acalypha has been known for various gastrointestinal disorders such as dysentery, severe diarrhoea and for treating neonatal jaundice (21). AP, an unexplored species, has a long history of traditional use for treating and preventing certain diseases, as highlighted in the introduction. 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 indicated 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 (22).

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

refers to the mortality observed in animals within 24 hours after the administration of a single high dose of APSN. In contrast, subacute toxicity encompasses the adverse effects experienced by animals 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 there is 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. Likewise, 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 has pleiotropic polyvalent phytochemicals, particularly alkaloids, which may be responsible for the itching effect. However, the itching effect elapsed after 4 hours and till the end of the experimentation period. It is interesting to note that there is no sign of coma, and mortality was not 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 and less muscle weakness, 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 that of the control group. The LD50 of APSN is therefore greater than 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, which exhibited a gradual daily increase throughout the 28-day study 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, 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, potentially influencing an increase in blood glucose levels, consistent with previous findings (5).

Acknowledging that alterations in organ weight alone may not conclusively indicate normalcy, our evaluation, in line with Pang-Kuei et al (17) 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 when compared to the control group. All indicators pertaining to blood composition were observed to be within the normal range. These findings imply a lack of deviation from the normal functional processes during growth.

Conversely, the observed non-significant changes ($p<0.05$) 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 (1). The reduced serum albumin observed in this study may be linked to 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 provide insight into the high antioxidant capacity of the APSN, a characteristic also documented by our earlier research (9). 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, HNA and IN.; Drafting of the manuscript, EA.; Critical revision of the manuscript for important intellectual content, HNA and IN.; Statistical analysis, E.A.; Administrative, technical, and material support, M.VVP, IY, AK.P, P.D; Study supervision, IN.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence this research article.

Data availability statement

Data associated with the study hasn't been deposited into a publicly available repository. Data will be made available on request.

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

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