Toxicology Reports 12 (2024) 307–317

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Sub-chronic oral toxicity study of the alkaloid rich fraction from *Luffa cylindrica* fruit in Sprague-Dawley rats

Ankul Singh S, Chitra Vellapandian *

Department of Pharmacology, SRM College of Pharmacy, SRM Institute of Science and Technology, Kattankulathur, 603203, Chengalpattu, Tamil Nadu, India

ARTICLE INFO

Handling Editor: Prof. L.H. Lash

Keywords:
Luffa cylindrica
Sub-chronic toxicity
Alkaloid rich fraction
Sprague-Dawley rat
Natural compound

ABSTRACT

The loofah/sponge gourd *Luffa cylindrica* (L.), a member of the Cucurbitaceae family, is one of the neglected medicinal plants. Traditionally, *Luffa cylindrica* is prescribed for inducing labor. It has a long history of use in China for the treatment of fever, diabetes, dyspnea, and dysentery. This study investigated the toxicity profile of the alkaloid-rich fraction of *Luffa cylindrica* (ARF-LC) for the first time in Sprague Dawley rats. A total of 80 rats (40 male and 40 female rats) aged 13 weeks old and weighing 200–220 g were selected for this study. In SD rats, sub-chronic oral toxicity was investigated at doses of 100, 200, and 400 mg/kg/d for a total of 90 days, followed by a 30-day recovery period. The results showed no variation in body weight among the three dose groups compared to the control group. Treatment-related adverse events, such as alterations in hematology and serum biochemistry parameters and the histology of the liver were sporadic in the high-dose rats but within the reference range. However, these changes disappeared after the doses were withdrawn during the recovery period. In conclusion, the "no observed adverse effect level" (NOAEL) of oral administration of ARF-LC in SD rats was considered 400 mg/kg/d and can be studied for its potential in further *in vivo* chronic investigations.

1. Introduction

The discovery of new natural compounds, particularly those with substantial biological activities and practical value, is tremendously essential for research. In the past, organic compounds were used as the primary therapy option. The countless investigations conducted in the field of herbal medicine have led to the discovery of an enormous number of advantageous and helpful effects of plants [1]. Currently, more research is being done on the natural sources of medicinal plants; *Luffa cylindrica* (L.) Roem has attracted attention due to its studies carried out on memory loss and other complicated diseases [2]. The loofah/sponge gourd *Luffa cylindrica* (L.), a member of the Cucurbitaceae family, is one of the neglected medicinal plants made up of a variety of phytochemicals, including carotenoids, chlorophylls, oleanolic acid, saponin, and triterpenoids [3]. The geographical distribution of Luffa species is widely found in North America, South America, Africa, and the Indian region [4]. Natural products have become a new hope for treating a variety of symptoms from non-alcoholic steatohepatitis [5], Cancer [6,7], Obesity [8], antimicrobial resistance [9], and neurodegeneration [10,11]. *Luffa cylindrica* is used in traditional medicine to treat boils, asthma, tuberculosis, shingles, and generalized body pain. The fruits are beneficial for treating fever, syphilis, tumors, bronchitis, spleenopathy, and leprosy. They are also anthelmintic, carminative, laxative, depurative, emollient, expectorant, tonic, and galactagogue [12,13]. Sponge gourd have not yet explored its highest potential activities [14]. From *Luffa cylindrica*, Straight-chain saturated fatty acids, fatty acid esters, heterocyclic azetidines, furan, and hydroxybenzaldehyde have all been identified as notable bioactive substances with pharmacological activity, including antiviral, antibacterial, antimicrobial, anti-inflammatory, antifungal, antioxidant, and antitumor properties [15]. The main functional elements in water extract were phenolics and flavonoids, whereas the key bioactive elements in ethyl acetate extract were oleanolic acid, carotenoid, and chlorophyll. Additionally, the functions of some additional substances found in Luffa, such as carotenoids and chlorophylls, are still unknown [16].

Scientific reports of the toxicity of *Luffa cylindrica* fruit is minimal and acute toxicity of luffa revealed that they found no death at the dose of 3000 mg/Kg during the evaluation of antihyperglycemic effects [17], and another study performed acute toxicity on luffa leaves at a maximum dose of 4000 mg/Kg respectively to evaluate changes in hematological parameters during the study and also showed no symptoms of toxicity [18]. Most of the plants used to make certified herbal

* Corresponding author.
E-mail address: chitrav@srmist.edu.in (C. Vellapandian).

https://doi.org/10.1016/j.toxrep.2024.03.001
Received 4 January 2024; Received in revised form 23 February 2024; Accepted 2 March 2024
Available online 5 March 2024

products need to possess preclinical evidence of their safety and efficacy by specified bioassays [19]. Since commonly used plants and their compounds should be avoided during the first trimester of pregnancy, preclinical safety evaluations of these substances are urgently needed [20,21]. Notably, the fruit and leaf of *L. echinata* are an important bioresource for natural anti-inflammatory, anti-diabetic, and anticancer medications [22]. Furthermore, no study has paved the way for pathways associated with *Luffa cylindrica* other than memory spatial memory test in oxidative stress-associated disorders especially Alzheimer's disease [2]. Despite its extensive use, no studies have examined the rich fraction's toxicity, which restricts its use in pre-clinical investigation.

Alkaloid rich fractions are known for their traditional use in the management of fever. These extracts have also shown antibacterial efficacy against several pathogenic bacteria, fungi, and viruses [23,24]. Budmunchiamine alkaloids from Albizia spp have been studied recently for their anticholinesterase activity and have shown promising results [25]. Similar studies on acetylcholinesterase and neuroprotection were evaluated by using alkaloid rich fraction on neuronal cell lines and found promising results for acetylcholine esterase inhibition [26]. Such interesting results pave us to retrace the path and evaluate its activity by using alkaloid rich fractions in an *in vivo* animal model. Surprisingly no detailed toxicity studies have been carried out on alkaloid rich fractions which is the need for our study to be carried out to understand its toxicity profile on animals. Therefore, the sub-chronic 90-day oral toxicity experiments of an alkaloid enriched fraction of *Luffa cylindrica* (ARF-LC) presented here were carried out using Sprague Dawley rats as part of an extensive preclinical investigation.

2. Materials and methods

2.1. Analysis, preparation, administration, and characterization of alkaloid-rich fraction

2.1.1. Determination of phytocompounds by LCMS analysis

The analysis of the ARF-LC involved the utilization of Liquid Chromatography coupled with Mass Spectrometry. The investigation utilized a Hypersil GOLD C18 column [100 ×2.1 mm-3 MICRON] and followed the procedure designated as 30 min_+ESI_01112021_MSMS.m. Detection was carried out using a Q-TOF analyzer, specifically the MS Q-TOF [G6550A] module in Dual AJS ESI ion mode. In the AutoMS2 acquisition mode, the parameters were configured with a minimum range of 120 [*m/z*] to a maximum of 1100 *m/z*. The experimental conditions were established using specific parameters: The drying gas temperature at 250°C, Gas Flow set to 10 l/min, and the nebulizer adjusted to 35 psig. The injection model, which featured a needle wash volume of 5 μL, had a wash time of 3 seconds. Channel A used a mixture of 0.1% Formic acid in water [95%], while Channel B was made up of Acetonitrile (90%) + Water (10%) + 0.1% Formic acid [5%]. The apparatus sustained a consistent flow rate of 0.300 mL/min under a stable pressure of 1200 bar, with the temperature consistently maintained at 40°C. To enhance the observation of metabolite ions, the MS analysis was conducted using negative ionization mode. The resultant total ion chromatogram presents a timeline correlated with the area profile, highlighting the comprehensive representation of each component based on the abundance of its molecular ions [27].

2.1.2. Preparation of alkaloid rich fraction

The complete fruits of ripe *Luffa cylindrica* (L.) were harvested in and around Uttar Pradesh. Dr. Jayaraman P, Director of the Plant Anatomy Research Centre (PARC), Tambaram, authenticated the specimen of luffa fruit with the registration number PARC/2021/4528. The fruits of *Luffa cylindrica* (L.) were thoroughly cleaned before being washed and rinsed. The fruits were then ground into crumbs and shade-dried for more than two weeks to properly dry them. The dried crumbles were further crushed to a fine consistency in a blender. The sample was later kept for future use in an airtight container.

Using the method adapted from Guo *et al.*, 2012 [28], the alkaloid-rich fraction was prepared. Ethanol was extracted three times from the shade-dried powder of *L. cylindrica* fruit (1000 g). Following partitioning between 3% tartaric acid and ethyl acetate, the mixed extracts were concentrated under decreased pressure. To extract the aqueous phase, repeated amounts of chloroform were added to a mixture of saturated sodium carbonate (pH 9–10). To obtain the alkaloid-rich fraction (2.5 g), the collected organic layers were concentrated.

2.1.3. Administration of alkaloid rich fraction

Based on the findings of an earlier study in which animals were gavaged 2000 mg/kg for 14 consecutive days by OECD Guideline-423, the dosages employed for this 90-day trial were calculated [29]. Every day, the fraction was calculated, diluted in distilled water, and then administered within four hours of preparation. A magnetic stirrer was used to agitate the dosage formulations continuously to keep them homogeneous. Based on stratified randomization, which used body weight measurements taken before the start of therapy and the number of rats in each group (20 of each sex), rats were divided into four groups. ARF-LC formulation was administered orally (gavage) to rats once daily for 90 days at doses of 0 mg/kg bw (Group I-control), 100 mg/kg bw (Group II-low dose), 200 mg/kg bw (Group III-mid dose), or 400 mg/kg bw (Group IV-high dosage). Every day, fresh dosing solutions were made with sterile water and mixed until the right dose was reached. Animals used as a control were given sterilized water.

2.1.4. Characterization of alkaloid rich fraction

The test article prepared was characterized by LC-MS analysis revealing the presence of various bioactive compounds among which alkaloids were found to be prominently present in the analysis which was traced to work with the rich fraction of alkaloid in understanding its toxicity profile in Sprague Dawley rats. The compounds rich in alkaloids are shown in LCMS analysis along with its chromatogram in Fig. 1 indicating the alkaloids compounds identified with percentages accounting to 97.99~100% respectively.

2.2. Study design

The study aims to furnish an assessment of the toxicity concerning exposure over chronic exposure. Different sets of experimental groups underwent daily oral administration of a singular dose of the test substance. The sub-chronic oral toxicity investigation follows the guidelines outlined by the OECD-408. The study was carried out according to the protocol's technical specifications, as well as performed in accordance with CPCSEA and ARRIVE 2.0 guidelines. The protocol was approved on December 06, 2022, number IAEC/293/2022. The study was conducted at the Animal House facility, Department of Pharmacology, SRM College of Pharmacy, SRM Institute of Science and Technology, Kattankulathur, Tamilnadu, India.

Throughout administration, animals were closely monitored to look for any toxicity. Weekly measurements were made of body weight and food intake. Biochemical observations were assessed to observe any changes. The weight of organs, including the testes, ovary, liver, kidney, spleen, stomach, duodenum, and heart, were taken. The tissues from the control and high-dose groups of rats were histologically examined.

2.3. Animals and management

In this study, both genders of Sprague Dawley strain rats were procured from Biogen Laboratory Animal Facility, CPCSEA Reg no-971/PO/RcBIBt/S/2006/CPCSEA and quarantined to acclimatize the experimental environment for one week before the experiments. All animals were housed in the Committee for Control and Supervision of Experiments on Animals (CPCSEA) accredited Institutional Animal Ethics Committee (IAEC) facility of SRM College of Pharmacy located in

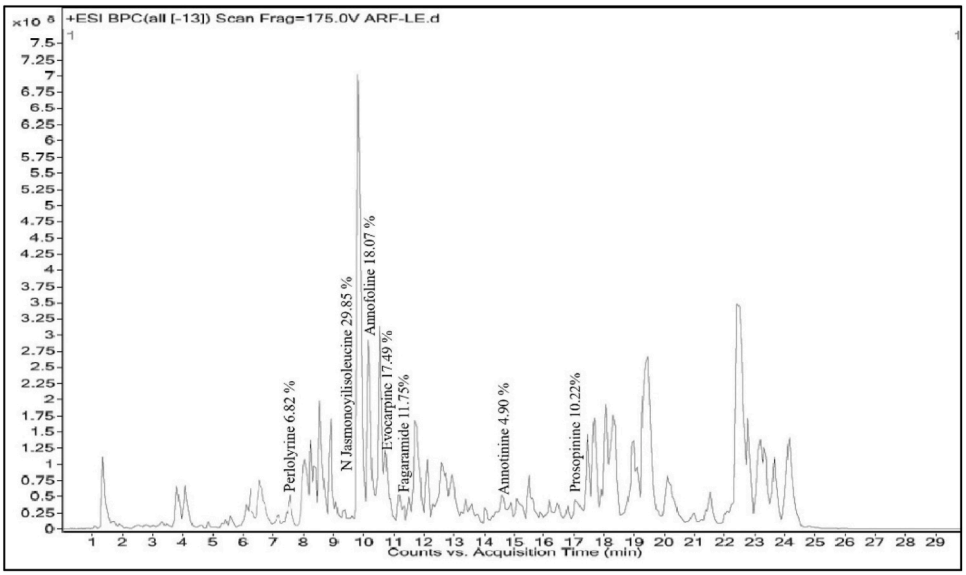

Fig. 1. Chromatogram of LCMS analysis indicating the presence of alkaloids along with their percentage in the test article.

Tamilnadu, India. The animals were allowed to acclimatize for two weeks before the initiation of experiments and all animals were examined for any gross signs of disease or injury. A total of 80 rats (40 male and 40 female rats) aged 13 weeks old and weighing 200–220 g were selected for this study. With a 12-hour light/dark cycle, the room's temperature and relative humidity were kept at 21±2°C and 55±20%, respectively. Throughout the study periods, all animals received Laboratory animal feeds, AMRUT™ (Wheat Flour, Roasted Bengal gram flour, Groundnut flour, Casein, Refined oil, Vitamins, and choline mixture with starch), and drinking water ad libitum, except where fasting was necessary. Rats were placed in four-level groups at random using a computer-based random order generator for sub-chronic oral toxicity after being placed in quarantine. Five rats in each group, per sex, were kept in polypropylene cages with stainless steel lids for all the rodents. Rice husk was utilized as bedding and they were changed weekly.

2.4. Repeated dose oral toxicity study

The sub-chronic oral toxicity study was conducted as mentioned previously [30]. The lab personnel were entirely masked during sample collection and other procedures. The rats were anesthetized using intraperitoneal sodium pentobarbital. The urine samples were collected one day before the euthanasia by cervical dislocation.

2.4.1. Body weight and food intake changes

All animals were examined physically and assessed for behavioral changes, symptoms associated with the medication, and morbidity and death twice daily. Individual body weights were measured and documented before each test, weekly, and at the time of the necropsy. Before the scheduled necropsy, the final body weight and fecal occult blood were observed. Beginning the day before randomization, daily feed intake data were kept, and the mean daily food consumption was computed. The weight gain per 100 g of feed ingested was estimated as feed conversion efficiency. Before the administration of alkaloid rich fraction (study day 0), rectal temperature was observed throughout the 30, 60, and 90 days of drug administration, as well as throughout the subsequent 30-day recovery period.

2.4.2. Biochemical parameters

On days 0, 45, 91, and 120 after fasting for 16–18 h, rats were anesthetized by intraperitoneal injection with 20 mg/kg thiopental sodium, and 1 mL blood was collected from orbital plexus puncture using a fine heparinized capillary tube. Blood for hematology studies was collected into tubes containing ethylenediaminetetraacetic acid- K2 anticoagulant and centrifuged at 3000 rpm for 10 min to obtain plasma. An automatic blood analyzer (ADVIA 2120i, Bayer, Germany) was applied to measure the hematological parameters: red blood cell count (RBC), hemoglobin (HG), platelet count (PLT), white blood cell count (WBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and leukocyte differential counts (LDC). Automatic biochemical analyzer (7180, Hitachi, Japan) was utilized to perform liver function test (SGOT (Serum Glutamic Oxaloacetic Transaminase/ aspartate aminotransferase), SGPT (Serum Glutamic Pyruvic Transaminase/alanine aminotransferase), ALP (alkaline phosphatase), GGT (glutamyl transpeptidase), TP (Total Protein), Bilirubin); electrolytes (sodium, potassium, calcium, magnesium, chloride); Lipid profile (LDL, HDL, TGL (Triglyceride), Total cholesterol), and Renal function test (Uric acid, Creatinine, BUN (Urea nitrogen) from serum samples of non-heparinized blood [31,32].

2.4.3. Urine analysis

Before euthanasia, urine tests were performed on all rats after the dosage and recovery period. The rats were placed in stainless steel metabolic cages for 24 hours in a room temperature without feed (water was allowed) access and the urine collected was evaluated for color, appearance, pH, specific gravity, and volume [33]. A urine analyzer (automated analyzer (Uritest-500B Urine Analyzer, Inc, Kyoto, Japan) was used to measure the following parameters: pH, volume, color, specific gravity, total protein, glucose, occult blood as well as sodium, potassium, chloride, bilirubin, ketones, and urobilinogen. A volume of collected urine from each animal was measured, and centrifuged at 3000×g for 10 min at 4°C, supernatant was collected for estimation, and sediment samples were examined for pus cells, epithelial cells, erythrocytes, sperm, bacteria, and casts under a microscope.

2.4.4. Histopathology

After the trial, all rats were humanely slain, and each animal had a thorough necropsy that included investigations of the intestines, visceral organs, the external surface of the body, and the thoracic and abdominal cavities. Following gross necropsy, all rats had a thorough histopathological evaluation that included measuring the absolute weight of the organs, macroscopic examination, and microscopic examination. The

brain, heart, kidneys, liver, ovaries, pituitary, spleen, and testicles were among the organs that were weighed to calculate the organ weight. Samples of fixed tissue were sectioned, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Samples from the control and high-dose groups were subjected to histopathological analyses. Macroscopic lesions observed at necropsy were also examined from each animal in other dose groups. The examination of tissue integrity and injuries that could be indicators of toxicity were done as part of the histopathological analysis.

2.5. Statistical analysis

All measured parameters were calculated and expressed as mean ± standard deviation. Comparisons of data of body weight, food intake, biochemical parameters, and organ weight were performed by two-way ANOVA, followed by post-hoc Tukey test (Graph pad Prism v 8.4.3)

3. Results

3.1. LCMS chromatogram for alkaloid-rich fraction

A comprehensive examination of the mass spectra and chromatographic peaks of alkaloid-rich fraction identified a total of 69 distinct compounds. The chromatogram of alkaloid compounds from fraction is shown in Fig. 1 respectively.

3.2. General condition and mortality

After the investigation, every animal was alive. Throughout the administration, no changes were noticed. Thus, there were no mortalities reported during the treatment period that were related to alkaloid rich fraction preparation from *Luffa cylindrica* fruit. Throughout the whole trial period, there were no treatment-related aberrant indications in any group. A few symptoms were seen as a result of housing conduct viz., piloerection during the quarantine period and became normal after a few days which was thought to be stress due to change in the facility, Wry neck (inner ear infection): male- 1/10 in Group II; females- 1/10 in Group I during the study which recovered by instilling ear drops in consultancy with veterinarian, redness in the tail: male-3/10 in Group II, mass: female- 1/12 in Group II). The severities of these signs were slight and luckily recovered after isolating them for a week. Rats are clean animals and take care to groom themselves, and grooming behavior was observed in all animals and all animals were active throughout the study and did not show any lethargy behavior or were unable to properly groom their fur. Rat was also palpated frequently to look for any tumor growth for any internal lumps (data not shown). Thus, any such signs were mitigated after isolation and were not considered to be related to the administration of alkaloid rich fraction preparation. Throughout the time of the trial, there was no fatality. In the 400 mg/kg/d group, it showed no abnormalities and treatment-related changes in behavior. Moreover, there was no discernible change in fecal occult blood and rectal temperature between the treatment groups and the control group throughout the experimental period in any of the dosing groups. Throughout the trial period, no additional toxicity-related symptoms, strange behavior, or modifications in motor activity were noticed.

3.3. Body weight gain, and food intake changes

Initially, the body weight of both genders showed no significant differences among all groups till week 5 and showed a considerable increase in the body weight of animals. However, no significant difference was seen in male 100 mg/kg and 400 mg/kg group animals in weeks 6 and 7 which later showed significant differences ($p<0.0001$) among all dosage groups. Finally, in week 13 males, it showed no significant differences in body weight gain compared to other weeks. Meanwhile, the body weights of females in all dosage groups showed no significant differences till week 7 and showed considerable and significant body weight changes ($p<0.0001$ or 0.0291) till week 13 than those in the untreated control group. All statistically significant differences vanished throughout convalescence, and each group's body weight was nearly identical. Fig. 2 summarizes the weekly body weights of SD rats.

During the study period, food consumption among animals in each cage per group was observed where most animals in male gender showed significant differences $p<0.0001$. Moreover, in weeks 2, 3,5, 7,8, 9,10, and 12 observation of the male gender showed no statistically significant difference >0.9999 in the low and high dose group respectively. Similarly, weeks 3,4,5,6,7,9,10,11 showed no statistically significant difference >0.9999 in the low and mid-dose groups respectively whereas, weeks 1 and 12 showed no statistically significant differences in high doses respectively. Whereas, females in the 400 mg/kg group had a slightly higher food consumption than those in the 0 mg/kg group, 100 mg/kg group, and 200 mg/kg respectively (Fig. 3).

3.4. Haematology and serum chemistry

The results of hematologic tests for hemoglobin level, total red blood

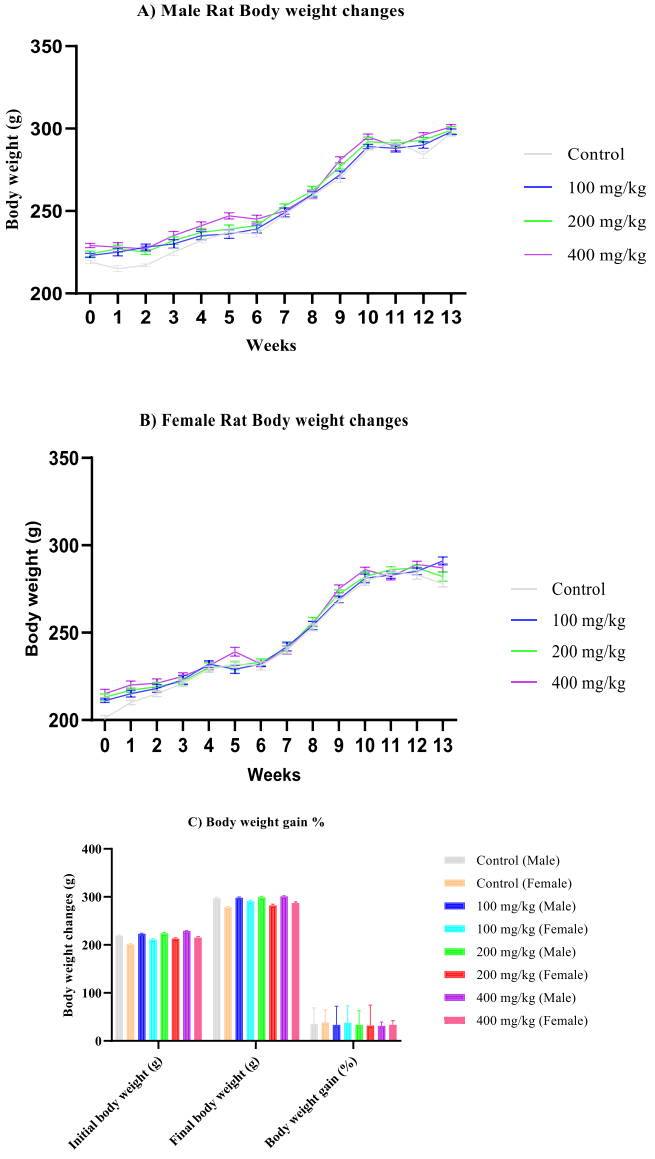

Fig. 2. Mean body weight increase of rats per cage in each week in the sub-chronic toxicity study of SD rats from weeks 0–13 (n=5 animals per cage). $p>0.9999$ as non-significant results for body weight gain %.

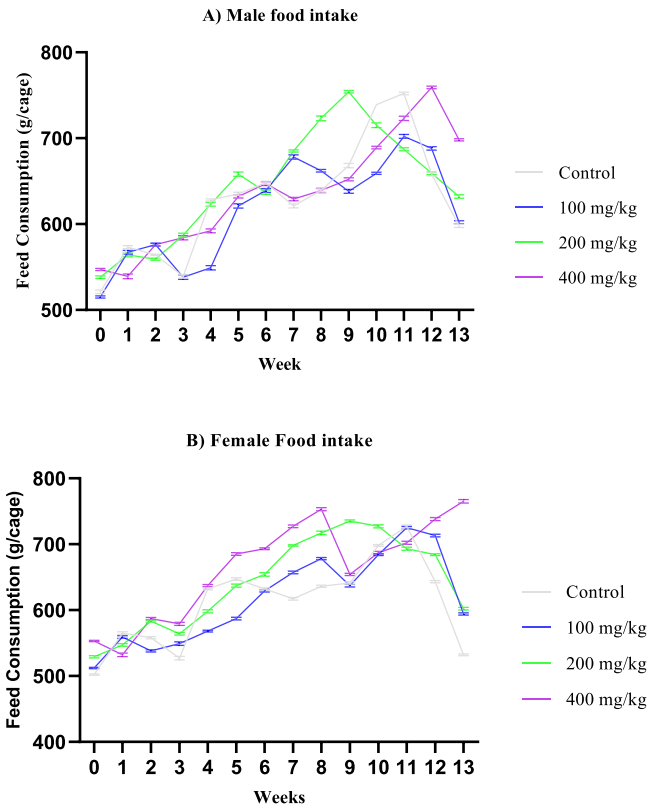

Fig. 3. Mean feed consumption of rats per cage in each week in the sub-chronic toxicity study of SD rats from weeks 0–13 (n=5 animals per cage).

cell (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet, mean platelet volume (MPV), red cell width (RDW), total white blood cell (WBC), eosinophils, basophils, neutrophils, lymphocytes, and monocytes in WBC differential are listed in Table 1. The hematological parameter in the male and female rat groups, which received the ARF fraction before and after the 45th day, showed no significant changes when compared with the control animal, except that animal in the 400 mg/kg group of Total WBC from Day 45, Day 91, and Day 120 showed significant differences when compared with control animals in both sexes. Haemoglobin showed a significant difference in the 100 mg/kg group of Day 120 in the female population, indicating the lack of a dose-dependent trend. It was also noteworthy that there were no significant differences observed in the RBS level of all groups in both genders. At first, only females showed a marked increase in packed cell volume (PCV) in the group receiving 400 mg/Kg (0.0021) of ARF-LC in Day 45 i.e., within the group compared to Day 45 control animals and mean corpuscular volume (MCV) showed significant differences in 100 mg/Kg male and 400 mg/kg female group (54.13±2.14) (54.38 ±0.49) ($p < 0.0001$) at the 45th day of the test when compared with that of control (58.17 ±1.06 and 58.12±0.09) respectively. It was also noteworthy that the data showed no significant differences in mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell width (RDW), and mean platelet volume (MPV), respectively. The aforementioned significant change after administration of the ARF-LC was not observed in both sexes, lacked accompanying changes in other red cell parameters, remained within the physiological range, was of small magnitude, and/or was not noted in a dose-related manner; consequently, this change was considered an incidental variation and not a treatment-related adverse effect. The above-described changes were well within the normal laboratory control or physiological range at the end of the study and hence considered incidental changes or biological variations and not treatment-related effects. In the Liver function test results (Table 2), there was no statistically significant difference between the groups and the control group from day 1 to day 45 and from day 120 during recovery except SGOT levels in both genders of 200 mg/kg group 70.34±0.84 and 64.24±0.34 and in 100 mg/kg group male (64.13±0.47) respectively within the group. Similarly, there was a significant difference in the 400 mg/kg group (25.27±0.34) in the female gender and in all groups and both genders from Day 91, which subsided until Day 120, within the normal range. Similar observations were made in both SGPT, ALP, and Total Cholesterol groups which were also found to lie in the physiological range. Nonetheless, other data, including total Bilirubin, Total Protein, Albumin, and Globulin levels, showed no statistically significant differences among the groups. Similar observations were also observed in the kidney function test (Table 3) with no statistically significant differences in both BUN and Creatinine levels. The treatment-related changes in hematological markers tended to dissipate away after the recovery period. The fact that these modifications were not consistent, statistically different from the concurrent controls, dose-independent, and detected only in one sex led experts to conclude that they were not toxicologically relevant.

3.5. Urine analysis

The assessed urine parameters showed no test article-related effects. When all the groups were compared between the control and ARF-LC treated groups at the planned analysis, there were no statistically significant differences. Urine analysis revealed yellow coloration with a characteristic odor. Not all rats had glucose found in their urine. The volume and specific gravity of the urine analysis did not significantly differ from the corresponding control groups (Table 4). Between the control and high-dose groups, there were no treatment-related changes. Males in the low-dose group had greater urine pH, and females had lower urine potassium concentrations than those in the control group. These modifications, however slight, were unrelated to the medication because additional tests turned up no signs of linked changes. Thus, consumption of ARF-LC at levels up to 400 mg/kg/day for 90 days does not affect urine parameters in rats. The microscopic analysis of urine samples obtained from ARF-LC treated rats did not reveal the presence of blood cells, casts, crystals, bacteria, or epithelial cells (data not shown).

3.6. Macroscopic examination, organ weight

Macroscopic analysis performed during necropsy did not reveal significant systemic changes in the organs or tissues between the animals given 400 mg/kg/d oral treatment and the control group. When the control group and the other test substance-treated groups were compared, however, some differences were found (Table 5). Some animals in the Mid dosage groups developed enlarged, hard, and yellowish livers along with abnormalities to their sexual organs, such as a uterus that was more slender and smaller testicles and prostates as compared to the control group. The observation, however, was not consistent and could not be linked to the test article's long-term effects. No statistically significant differences were found in the other treatment groups, although the 200 mg/kg group had a higher absolute weight of the liver when compared to the control group. At the maximum tested dose (400 mg/kg), there was a statistically significant rise in the weight of the pancreas, spleen, lung, and kidney in females ($P<0.05$). Similar large reductions in brain organ weight were observed in both sexes at 100 mg/kg. The liver in the 200 mg/kg males showed a significant increase in organ weights in both sexes.

3.7. Histopathology

After macroscopic observations and organ weight, histopathology of visceral organs was observed for any abnormalities among control and

Table 1
Summary of selected hematology values of SD rats orally administered with ARF-LC for 90 days repeated oral toxicity and 30 days post-treatment study.

	Dose (mg/Kg)	PCV %		MCV fL		MCH pg		MPV fl		PLT 103 Cu.mm	
		M	F	M	F	M	F	M	F	M	F
Day 0	Baseline	45.72±1.72	44.57±0.91	57.32±2.17	58.32±1.07	19.29±0.85	18.39±0.73	6.27±0.75	6.31±0.37	878.2±75.41	861±42.35
Day 45	Control	44.32±0.79	44.47±0.04	58.17 ±1.06	58.12±0.09	19.12±1.5	18.37±0.67	7.32±0.32	7.28±0.46	1047.2±65.3	998±32.31
	100	45.39±1.27	45.89±0.97	54.13±2.14 ****	57.38±0.64	20.12±0.58	18.38±1.4	7.41±0.03	7.39±0.14	1083±11.54	1023±18.64
	200	45.78±0.65	46.57±0.32	56.73±1.85	56.18±0.65	19.73±1.23	18.37±1.64	7.45±0.07	7.83±0.17	1058±23.65	1047±13.45
	400	46.17±0.53	47.32±0.03 **	56.38±1.64	54.38±0.49 ****	19.78±1.26	18.27±0.86	7.39±0.34	7.71±0.46	1099±18.63	1128±21.33 ####
Day 91	Control	44.28±0.93	45.13±0.72	58.46±1.4	58.17±1.07	19.3±0.85	18.93±1.73	7.59±1.47	7.34±0.6	1031.6±25.3	1012±28.37
	100	44.87±0.62	45.78±1.27	58.39±1.07	57.37±0.37	19.37±0.64	18.16±0.3	7.45±1.83	7.45±1.37	1169±12.62####	1028±18.64
	200	45.37±0.41	46.21±0.82	56.15±0.55	57.08±0.05	19.18±0.52	18.37±0.64	7.59±1.37	7.38±0.46	1138±33.41##	1057±13.45
	400	46.28±0.87	45.17±0.63	57.39±0.42	57.98±0.64	19.39±0.38	20.27±0.54	8.14±0.86	7.47±0.38	1152±48.41###	1139±20.68 ####
Day 120	Control	45.13±1.72	45.93±0.56	58.32±0.47	58.32±1.07	19.18±0.43	18.15±0.41	7.37±0.67	7.39±0.37	1061.2±45.3	1017±36.21
	100	45.16±1.12	45.62±1.38	56.16±0.81	57.17±0.37	19.15±0.64	18.36±0.3	7.49±1.83	7.89 ±0.37	1152±18.62###	987±18.64
	200	45.36±0.98	45.29±0.13	57.38±0.37	57.18±0.05	19.28±0.52	18.29±0.64	7.39±1.37	7.85±0.46	1141±33.41##	1043±13.45
	400	45.84±0.92	46.31±1.14	58.18±0.61	57.38±0.64	19.73±0.38	18.47±0.54	8.10±0.86	7.68±0.38	1163±18.41####	1132±20.68 ####
Ref Range		**43.6–48.6%**		**55.8–62.2 fl**		**17.7–20.1pg/mL**		**6.2–9.8 fl**		**250 – 1200 10^3 Cu.mm**	

Days	Dose (mg/Kg)	Total WBC 103 Cu.mm		Ne%		Ly %		Mo%		Eo %		Ba %		RBC Million/Cu. mm		Hb g/dL	
		M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Day 0	Baseline	9.17±0.99	8.98±0.29	18.12 ±0.34	18.15 ±0.85	85.36 ±0.91	75.15 ±3.95	3.45 ±0.08	3.75 ±0.12	3±0.01	2±0.2	1 ±0.06	1 ±0.06	7.58 ±0.54	7.45 ±0.04	15.84 ±.57	15.75 ±.52
Day 45	Control	9.57±0.84	9.18±0.48	20.21 ±0.74	19.75 ±0.58	82.42 ±1.09	77.42 ±1.84	3.85 ±0.03	2.48 ±0.02	3±0.06	3±0.06	1 ±0.06	1 ±0.06	8.30 ±0.14	8.35 ±.05	15.75 ±0.46	15.42 ±0.62
	100	10.27±0.74	9.29±0.89	14.34 ±0.98 ****	15.31 ±1.21 ****	75.23 ±1.21 ****	75.25 ±0.91	2.87 ±0.03 ****	2.85 ±0.06 *	2±0.04 ****	3±0.06	1 ±0.06	1 ±0.06	7.45 ±0.6	7.25 ±0.65	14.87 ±0.96	14.42 ±0.74
	200	10.17±0.54	10.84±0.26	15.67 ±1.86 ****	15.59 ±1.24 ****	78.47±3.1	77.38 ±1.1	2.93 ±0.01 ****	3.96 ±0.02 ****	2±0.02 ****	3±0.06	1 ±0.06	1 ±0.06	8.22 ±1.07	7.32 ±0.61	14.35 ±0.87	14.86 ±0.42
	400	12.37 ±0.29****	11.39±0.31 ***	18.39 ±1.56	17.38 ±0.87	85.39 ±4.31	80.15 ±2.31	2.84 ±0.04 ****	3.84 ±0.01 ****	2±0.01 ****	1±0.01 ****	1 ±0.06	1 ±0.06	8.25 ±0.56	7.40 ±0.59	14.42 ±0.54	14.46 ±0.71
Day 91	Control	10.17±1.46	9.37±0.09	19.98 ±1.24	19.59 ±0.66	84.16±0.9	75.38 ±1.2	3.80 ±0.02	1.98 ±0.02 ###	3±0.02	1±0.03 ####	1 ±0.06	1 ±0.06	8.32 ±0.67	8.32 ±1.04	15.70 ±.32	15.55 ±0.41
	100	10.31±0.04	9.68±1.34	15.29 ±0.46 ####	15.39 ±0.31 ####	78.17 ±0.46	75.34 ±0.42	2.85 ±0.03 ####	1.84 ±0.03 ####	2±0.02 ####	3±0.06	1 ±0.06	1 ±0.06	8.05 ±0.83	7.85 ±0.42	14.84 ±0.54	14.67 ±0.46
	200	10.39±0.34	11.32 ±0.21##	16.03 ±1.86 ####	15.62 ±0.37 ####	78.39 ±2.01	76.72 ±0.61	2.82 ±0.01 ####	2.76 ±0.02	2±0.03 ####	3±0.06 ####	1 ±0.06	1 ±0.06	8.12 ±0.62	7.45 ±0.41	14.52 ±0.27	14.92 ±0.25
	400	12.35 ±0.06####	12.17±0.34 ####	18.89 ±0.49	17.54 ±0.47	82.25 ±1.01	79.55 ±0.46	2.78 ±0.04 ####	3.87 ±0.01 ####	2±0.01 ####	1±0.01 ####	1 ±0.01	1 ±0.06	8.24 ±1.23	7.40 ±0.07	14.75 ±0.32	15.34 ±0.82
Day 120	Control	10.14±0.72	9.06±0.76	20.17 ±0.68	19.48 ±0.42	82.16 ±0.87	76.37 ±0.84	3.83 ±0.02	2.23 ±0.62	3±0.01	1±0.01 ####	1 ±0.06	1 ±0.06	8.35 ±0.54	8.38 ±.04	15.48 ±0.36	15.28 ±0.33
	100	10.32±0.74	10.26±0.39	15.12 ±0.43 ####	14.79 ±1.21 ####	75.57 ±0.31 ####	76.18 ±0.47	2.84 ±0.03 ####	2.14 ±0.01	2±0.02 ####	3±0.06	1 ±0.06	1 ±0.06	8.54 ±0.41	7.95 ±0.65	14.74 ±0.62	13.45 ±0.54 #
	200	10.27±0.54	10.54±0.41	16.28 ±0.46 ####	15.37 ±0.34 ####	76.98 ±1.41 ##	77.49 ±1.07	2.92 ±0.01 ####	2.98 ±0.02 ###	2±0.02 ####	3±0.06	1 ±0.06	1 ±0.06	8.23 ±0.47	7.85 ±0.47	14.52 ±1.67	13.75 ±0.28
	400	11.89±0.19 ###	11.37±0.12 ###	18.19±0.3	17.42 ±0.46	81.67 ±2.53	78.25 ±0.46	2.97 ±0.04 ####	3.84 ±0.01 ####	2±0.01 ####	1±0.01 ####	1 ±0.06	1 ±0.06	8.28 ±0.64	7.58 ±0.03	14.48 ±0.36	14.25 ±0.82
Ref Range		**7.2–12.6 10^3 Cu.mm**		**6–27%**		**66–91%**		**1–4%**		**< 4%**		**< 1%**		**7.21–8.45 million/Cu.mm**		**13.2–16.4 g/dL**	

Note: PCV- Packed Cell Volume, MCV- Mean Corpuscular Volume, MCH- Mean Corpuscular Haemoglobin, MPV- Mean Platelet Volume, PLT-Platelet count, WBC-White Blood Cells, Ne-Neutrophils, Ly-Lymphocytes, Mo-Monocytes, Eo-Eosinophils, Ba-Basophils, RBC-Red Blood Corpuscles, Hb-Haemoglobin, Cu.mm- cubic millimeter, g/dL-gram per decilitre. All the values are indicated in mean ±SD. 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), < 0.0001 (****) was kept as observation within the group, and 0.1234 (ns), 0.0332 (#), 0.0021 (##), 0.0002 (###), < 0.0001 (####) was observed for comparison between the groups.

Table 2
Summary of significant changes of Liver Function Test of SD rats orally administered with ARF-LC for 90 days and a 30 days post-treatment study.

Day	Dose (mg/kg)	SGOT/AST (U/L)		SGPT/ALT (U/L)		ALP (U/L)		GGT (U/L)		Total Cholesterol	
		M	F	M	F	M	F	M	F	M	F
Day 0	Baseline	72.28±0.43	71.34 ±0.32	28.24±0.23	28.13±0.43	113±1.7	111.98±2.5	4.8± 0.23	5.1±0.35	84±1.28	82±0.91
Day 45	Control	68.72±0.27	67.31 ±0.54	27.31±0.27	27.82±0.37	102±1.4	105.28±2.3	4.5±0.21 ***	4.9±0.24	87±1.51	85±1.73
	100	64.13±0.47 ***	66.28 ±0.31	26.26±0.19	27.46±0.48	98±1.2	99.32±2.4	3.8±0.17 ***	3.7±0.21 *	78±1.25 ****	79±1.46 ***
	200	70.34±0.84 **	64.24 ±0.34 ***	27.34±0.24	26.43±0.63 **	94±1.5 *	93.87±1.7 ****	3.6±0.42 ***	3.8±0.82	69±1.53 ****	71±1.28 ****
	400	69.23±0.28	66.21 ±0.28	26.29	25.27±0.34 ***	108±4.7	97.25±4.7	3.7±0.42	4.8±0.91	72±1.48 ****	73±1.69 ****
Day 91	Control	68.12±0.58	67.98 ±0.34	25.34±0.14 ###	28.21±0.16	125±3.9 ####	123±2.8 ####	4.8±0.26 ###	4.6±0.21	85±1.48	84±1.87
	100	68.62±0.38	68.16 ±0.34	23.28±0.18 ###	27.32±0.23	147±4.3 ####	144±2.3 ####	4.5±0.24 ###	4.2±0.37	83±1.91	84±1.58
	200	65.86±0.81 ###	68.42 ±0.39	24.15±0.21 ###	25.17±0.38 ###	128±2.7 ####	126±2.1 ####	4.1±0.32 ###	3.9±0.35	81±1.74 ###	79±1.39 ###
	400	67.34±0.17	67.43 ±0.18	21.72±0.27 ###	26.26±0.29 ###	132±1.8 ####	127±3.8 ####	3.8±0.37 ###	4.1±0.47	85±2.68	77±2.83 ####
Day 120	Control	67.27±0.75	66.72 ±0.77	27.45±0.48	26.97±0.19	120±1.5 ####	118±1.8 ####	4.6±0.41 ###	4.7±0.35	86±1.47	85±1.04
	100	66.23±0.46 ###	67.25 ±0.64	27.73±0.57	27.43±0.64	115±2.5 ####	119±1.7 ####	4.1±0.35 ###	3.9±0.63	81±1.42 ###	82±1.45
	200	68.72±0.23	67.82 ±0.82	27.39±0.68	27.12±0.24	115±1.5 ####	118±2.3 ####	3.8±0.12 ###	3.7±0.56 #	57±1.67 ####	75±1.28 ####
	400	79.37±0.49 ###	65.73 ±0.87 #	27.28±0.8	27.14±0.41	112±1.3 ###	115±1.2 ###	3.5±0.18 ###	4.5±0.22	62±1.67 ####	74±1.23 ####
Reference		**45.7–80.8 U/L**		**17.5–30.2 U/L**		**56.8–128.0 U/L**		**0.5–5.3 U/L**		**50–100 mg/dL**	

Day	Dose (mg/kg)	Total Bilirubin (mg/dL)		Total Protein (g/dL)		Albumin (g/dL)		Globulin (g/dL)	
		M	F	M	F	M	F	M	F
Day 0	Baseline	0.48±0.12	0.47±0.17	7.3±0.4	7.5±0.2	4.6±0.4	4.5±0.3	5.3±0.2	5.2±0.1
Day 45	Control	0.45±0.11	0.46±0.23	7.2±0.2	7.3±0.4	4.4±0.3	4.6±0.4	5.1±0.3	4.9±0.2
	100	0.38±0.17	0.34±0.27	6.9±0.3	6.7±0.3	4.3±0.1	4.4±0.3	4.9±0.5	4.5±0.3
	200	0.37±0.23	0.32±0.39	7.1±0.1	6.9±0.1	3.9±0.2	4.1±0.5	4.6±0.2	4.2±0.4
	400	0.42±0.38	0.43±0.47	6.8±0.2	6.5±0.8	4.2±0.3	4.3±0.4	4.8±0.2	4.4±0.3
Day 91	Control	0.47±0.3	0.45±0.1	7.1±0.4	6.9±0.1	4.2±0.3	4.1±0.5	4.7±0.7	4.5±0.2
	100	0.45±0.1	0.56±0.1	6.8±0.1	6.7±0.5	4.4±0.2	4.3±0.2	4.5±0.5	4.1±0.2
	200	0.46±0.4	0.54±0.4	6.5±0.3	6.9±0.3	4.5±0.1	4.4±0.3	4.9±0.4	4.7±0.2
	400	0.47±0.2	0.38±0.3	5.9±0.2 ###	5.5±0.5 ###	4.3±0.2	3.9±0.1	4.5±0.1	4.1±0.4
Day 120	Control	0.46±0.1	0.46±0.3	7.2±0.2	7.1±0.5	4.3±0.1	4.5±0.2	4.2±0.2	4.7±0.3
	100	0.43±0.2	0.48±0.2	6.5±0.1	6.7±0.3	4.1±0.5	4.2±0.3	4.1±0.3 #	4.3±0.2
	200	0.45±0.1	0.49±0.1	6.8±0.2	6.5±0.3	4.2±0.2	4.3±0.2	4.3±0.1	3.9±0.3 #
	400	0.43±0.3	0.41±0.2	6.5±0.1	6.9±0.1	4.1±0.3	4.1±0.2	4.6±0.2	4.3±0.6
Reference		**0.2–0.55 mg/dl**		**5.6–7.6 g/dl**		**3.8–4.8 g/dL**		**1.5–5.5 g/dL**	

Note: SGOT- Serum Glutamic Oxaloacetic Transaminase, AST- Aspartate Aminotransferase, SGPT- Serum Glutamic Pyruvic Transaminase, ALT- Alanine Aminotransferase, ALP- Alkaline Phosphatase, GGT- Gamma-Glutamyl Transferase. All the values are indicated in mean ±SD. 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), < 0.0001 (****) was kept as observation within the group, and 0.1234 (ns), 0.0332 (#), 0.0021 (##), 0.0002 (###), < 0.0001 (####) was observed for comparison between the groups.

high-dose groups for both genders. Each organ was prepared for histological testing so that microscopic inspection could be performed. Research on the sub-chronic oral toxicity of a high-dose alkaloid rich fraction and a control group of rats (Fig. 4), showed light microphotographs of the transverse sections of the visceral organs. The basic architecture of all major organs was seen, and the absence of cell structure modification, and the absence of any unfavorable effect in organs were all revealed by histopathological analysis of the control groups. However, there were slight changes observed in a few organs of either sex which were not detrimental as they occurred in either sex. The brain showed neural parenchyma with predominantly normal areas and no evidence of congestion or edema in the heart. The kidney showed mild passive congestion of periglomerular vessels and liver parenchyma with preserved architecture. Lung parenchyma showed a peribronchiolar chronic inflammation-like structure and other organs showed basic architecture in high dosage groups besides the control group. Testis and ovary also revealed no pathological changes in tissue regions for the control and high-dose groups. Based on these results, there appears to be no toxicity to the organs tested upon administration of alkaloid rich fraction daily for 91 days.

4. Discussion

Plants have been used for medicinal purposes long before the prehistoric period and continued to be used for various purposes including pharmacological research and drug development as ethnobotanicals [34]. Studies on toxicity are essential to medicinal Plants to identify the harmful effects of medicinal plants that have been employed in a variety of traditional practices [35]. *Luffa cylindrica* preliminary phytochemical screening reveals the presence of anthocyanins, glycosides, flavonoids, triterpenoids, cardiac glycosides, saponins, carbohydrates, proteins, alkaloids, and tannins [36]. It is thereby found to possess various pharmacological and biological properties against inflammation, fungus, hypertriglyceridemia, immunity, asthma, and others [37]. Controlling food safety requires research to ascertain the amount of alkaloids in foods, and quality has a significant impact. A greater understanding of food's alkaloids can result in a healthier diet and better benefits for people. To assess the potential harmful effects that can result from exposure over an extended period, repeated dosage toxicity studies are carried out. The computation of the dose at which the test material can be constantly exposed without suffering any detrimental effects is also

Table 3
Summary of Renal/kidney function test of SD rats orally administered with ARF-LC for 90 days and a 30 days post-treatment study.

Days	Dose	BUN (mg/dL)		Creatinine (mg/dL)	
		M	F	M	F
Day 0	Baseline	22.17±1.13	21.31±1.21	1.9±1.17	1.9±1.13
Day 45	Control	15.32±2.11	15.28±1.32	1.75±1.12	1.87±1.11
	100	14.84±1.39	14.73±2.46	1.89±1.14	1.73±1.24
	200	12.37±2.17	13.29±2.87	1.73±1.13	1.68±1.21
	400	13.62±1.23	12.23±2.19	1.83±1.27	1.82±1.13
Day 91	Control	15.42±1.38	15.32±1.67	1.65±1.17	1.63±1.23
	100	14.91±1.18	14.85±1.28	1.82±1.16	1.79±1.19
	200	14.38±1.36	14.61±1.46	1.76±1.31	1.68±1.25
	400	13.75±1.62	13.74±1.32	1.82±1.23	1.78±1.42
Day 120	Control	15.73±2.48	15.46±1.26	1.62±1.26	1.58±1.26
	100	14.48±1.34	14.27±1.27	1.79±1.17	0.97±1.32
	200	12.61±2.4	12.34±1.44	1.75±1.21	1.32±1.21
	400	13.28±1.4	12.27±1.61	1.87±1.15	1.82±1.15
Reference		**10.00–33.00 mg/dl**		**0.5–2.2 mg/dl**	

Note: BUN-Blood Urea Nitrogen. All the values are indicated in mean ±SD. All the values are indicated in mean ±SD. 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), < 0.0001 (****) was kept as observation within the group, and 0.1234 (ns), 0.0332 (#), 0.0021 (##), 0.0002 (###), < 0.0001 (####) was observed for comparison between the groups.

provided, together with details on the potential for cumulative effects [38].

Moreover, toxicological studies carried out on this plant are minimal and limited till acute toxicity, and no sub-chronic toxicity has been executed so far and provides useful scientific merit about the administration of ARF-LC in the diet [39]. Given the chemical's potential utility, the paucity of in-depth toxicological studies, and the variety of experimental animals, more evidence of its pharmacology and safety is required. The present study firstly represents a comprehensive toxicological evaluation of ARF-LC for the first time by performing sub-chronic oral toxicity assessments in SD rats. The capacity to change the genome in rodent species has increased their usage in toxicity research, particularly for determining carcinogenicity and for exploratory investigations motivated by a hypothesis [40]. The discovery of scaffolds with a variety of bioactivities that can either be developed directly or used as building blocks for the development of innovative drugs is still possible using natural products [41]. Given its potential utility, the paucity of a comprehensive toxicological analysis, and the variety of experimental animals, more proof of the pharmacology and safety is required. As a result, a thorough preclinical program that included sub-chronic trials was carried out.

Apart from a few insignificant indicators in the 400 mg/kg dose group, observations during the research period did not reveal any abnormalities or behavioral changes connected to the medication. There were no statistically significant changes in body weight variations throughout the study but few statistically significant changes in several weeks in both low dose and high dose respectively. Because the alterations were so slight compared to those in the vehicle control group, as reported in an earlier study, these variations were not thought to be toxicologically significant [42]. The reduced body weight gain could also be attributed to the report of an oral toxicity test from an ethanolic root extract of *Caesalpinia bonduc* (L.) Roxb. in Wistar rats [43]. There was also food variation observation seen throughout the study in several weeks which could also be linked with different gonadal hormone productions at least partially responsible for variations in food intake and body weight in the male gender which could be the attributed reason where male gender showed significant differences in food intake [44]. To evaluate the potential toxicologic consequences, the biochemical parameters, and histopathology can provide information [45], where in our study showed no toxicological significance.

The hematological changes showed significant changes in a few parameters but were later found to be within the range and showed no significant changes when compared with the control animal as observed in a sub-chronic study of dietary alpha linoleic acid enriched

Table 4
Effect of ARF-LC on Urinary parameters of male and female rats in sub-chronic toxicity study.

Parameters	Control		100 mg/kg		200 mg/kg		400 mg/kg	
	Male	Female	Male	Female	Male	Female	Male	Female
Colour	Dark yellow	Dark Yellow	Pale yellow	Dark yellow	Dark yellow	Dark yellow	Amber	Dark yellow
Appearance	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
Volume (mL/24 hr)	5.78 ± 1.5	6.23 ± 2.15	5.84 ± 1.73	5.87 ± 1.36	4.36 ± 1.56	5.79 ± 2.85	5.82 ± 1.37	6.17 ± 1.79
pH	5.4 ± 0.25	6.8 ± 0.3	5.6 ± 0.37	6.5 ± 0.37	6.2 ± 0.57	6.7 ± 0.39	5.8 ± 0.46	6.9 ± 0.42
Glucose	-	-	-	-	-	-	-	-
Bilirubin	-	-	-	-	-	-	-	-
Ketone	-	-	-	-	-	-	-	-
Specific gravity	1.02 ± 0.0057	1.02 ± 0.0046	1.02 ± 0.0038	1.02 ± 0.0042	1.02 ± 0.0049	1.02 ± 0.0053	1.02 ± 0.0051	1.02 ± 0.0059
Protein (mg/dL)	30.256 ± 2.542	32.173 ± 1.361	30.256 ± 3.684	34.173 ± 1.569	102 ± 3.739	111 ± 1.275	123 ± 2.763	132 ± 1.479
Leukocytes	-	-	-	-	-	-	-	-
Nitrite	-	-	-	-	-	-	-	-
Urobilinogen (mg/dL)	0.9 ± 0.0032	0.7 ± 0.0067	0.42 ± 0.0039	0.37 ± 0.0068	0.54 ± 0.0082	0.43 ± 0.0072	0.58 ± 0.0069	0.49 ± 0.0037

Values are represented in mean ± SD, n=10 rats/sex/group.

Table 5
Absolute organ weight of Sprague Dawley rat in sub-chronic toxicity study.

Dose (mg/kg)	Heart (g)		Liver (g)		Lung (g)		Stomach (g)		Pancreas (g)		Kidney (g)		Brain (g)		Spleen (g)	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Control	0.68 ±0.12	0.58 ±0.12	2.50 ±0.2	2.48 ±0.6	1.23 ±0.13	0.98 ±0.12	0.90 ±0.2	0.92± 0.3	0.62 ± 0.1	0.59 ± 0.4	0.55± 0.2	0.42 ± 0.1	1.38 ±0.4	1.37 ± 0.6	0.25 ± 0.2	0.28 ± 0.1
100	0.59 ±0.15	0.52 ±0.13	2.49 ±0.7	2.46 ±0.2	1.08 ±0.23	0.89 ±0.17	0.84± 0.4	0.87± 0.6	0.64 ± 0.3	0.53 ± 0.6	0.53 ± 0.2	0.58 ± 0.3	1.31± 0.8	1.29 ± 0.4	0.23 ± 0.2	0.25 ± 0.1
200	0.57 ±0.18	0.54 ±0.11	2.57 ±0.3	2.55 ±0.5	0.98 ±0.12	0.87 ±0.14	0.87± 0.3	0.89 ±0.4	0.67 ± 0.2	0.54 ± 0.5	0.52 ± 0.1	0.57± 0.5	1.33± 0.6	1.32 ± 0.7	0.25 ± 0.1	0.23 ± 0.3
400	0.55 ±0.12	0.53 ±0.14	2.48 ±0.2	2.47 ±0.3	0.95 ±0.27	1.23 ±0.17	0.85± 0.6	0.86± 0.2	0.65 ± 0.4	0.56 ± 0.7	0.54 ± 0.2	0.55 ± 0.4	1.35± 0.6	1.35± 0.5	0.24 ± 0.1	0.32 ± 0.1

The non-significant (ns) data was considered when compared with the control group in both genders. All the values are indicated in mean ±SD.

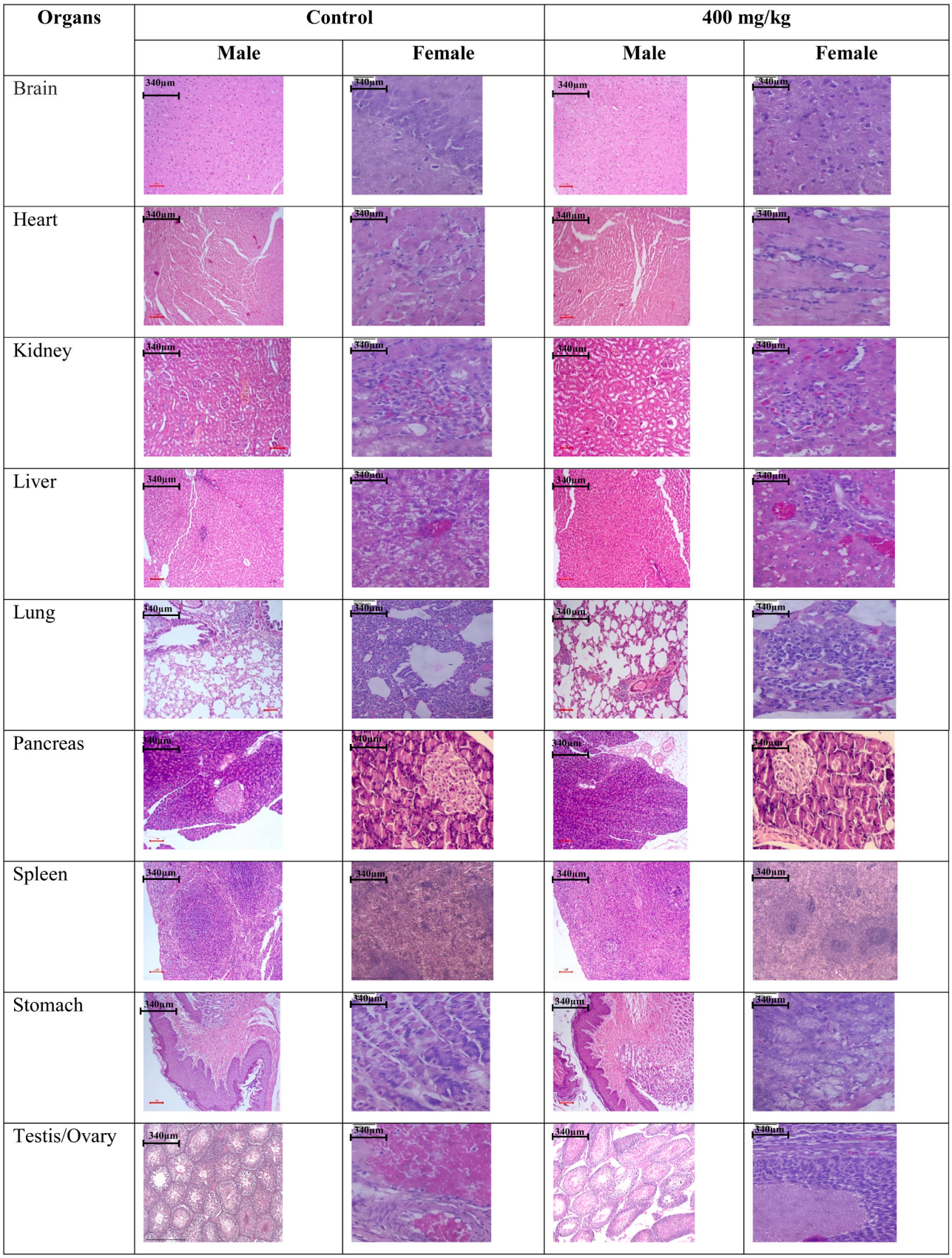

Fig. 4. Microphotographs of stained section of Liver, pancreas, kidney, lung, brain, spleen, stomach, and heart. Note: H&E (Haemoxylin and Eosin) stain was used for this study results and evaluated under 10X magnification with a scale of 340 µm.

diacylglycerol in rats [46]. Although in the 400 mg/kg group WBC count was found to be increased, it was inferred that WBC data showed fluctuation and instability in the highest tested doses irrespective of sexes whose differences are not highly understood but were also observed in a study that such changes might be attributed to the inflammatory response [47], host immune demand in various blood collection locations i.e., tail, eye, and heart [48,49]. Following treatment of 200 mg/kg of ARF-LC, hematological examinations of rats showed no changes in lymphocyte and neutrophil counts, as observed in a different rodent investigation [50]. It was later inferred that such changes tend to reverse normal values after withdrawal of the test drug and were considered incidental and normal. However, such changes observed in our study were found to be within the range and considered dose-dependent. Similar observations were also observed in the liver function test except SGOT, SGPT levels in both genders [47], and kidney function test with no statistically significant differences in both BUN and Creatinine levels according to a previous study [51]. The above-noted significant change was not observed in both sexes, was not accompanied by changes in other red cell parameters, was of small magnitude, and/or was not noted in a dose-related manner, making this change not considered to be a side effect of the medication.

Urine toxicology testing is used to confirm the changes in parameters to observe the effects of tested drugs. There were no statistically significant differences when the control and test article-treated groups were compared among all the groups at the scheduled analysis as observed in a previous study which showed no changes in all tested doses during the study in both sexes [52]. When compared to the control group, macroscopic inspection during necropsy did not show any systemic alterations in the organs or tissues. The liver in the 200 mg/kg males showed a significant increase in organ weights in both sexes. Although liver weight gain and microsomal enzyme induction are associated, it should be emphasized that in rats, dogs, and monkeys, the degree of induction may not be directly proportional to either the size of the liver weight increase or the degree of hepatocyte hypertrophy [53]. Histopathological studies revealed a kidney with mild passive congestion of periglomerular vessels and lung parenchyma with a peribronchiolar chronic inflammation-like structure which was due to a sub-chronic study. Histological alterations, characterized by the presence of multinucleated giant cells in the spleen and periportal lymphocytic inflammation in the liver, were observed at varied doses [54].

5. Conclusion

Overall, when the Sprague Dawley rats were exposed to ARF-LC from *L cylindrica*l at doses of 400 mg/Kg in sub-chronic toxicity studies for 90 days and with subsequent recovery tests for 30 days, respectively, no mortality, adverse changes in general behaviors, body weight, as well as alterations in hematological, biochemical, and histopathological analysis, didn't appear and remained as the variables in the normal range. According to this sub-chronic investigation, the NOAEL (No-observed Adverse Effect Level) of ARF-LC in Sprague Dawley rats is 400 mg/kg. According to the sub-chronic 90-day oral toxicity trial, the treatment with ARF-LC at a dose of 400 mg/kg/d showed no adverse effects and no substantial change in any of the following indices. However, these changes were either sex-specific or dose-independent. This ARF-LC toxicity profile can be used to conduct additional in vivo tests and assess the effectiveness of numerous research, including neurotoxicological studies. When applying ARF-LC to human applications, these findings may be a crucial guide for choosing a safe dosage. The health implications of lifelong exposure to ARF-LC on animals and their offspring will be further investigated, which will aid humans in the controlled and efficient exploitation of this limited resource. The safety of ARF-LC as a prospective functional food and nutritional supplement was supported by our results.

Ethical conduct of research

The study was conducted ethically following CPCSEA norms and OECD guidelines

CRediT authorship contribution statement

Chitra Vellapandian: Writing – review & editing, Visualization, Supervision, Investigation, Conceptualization. **Ankul Singh S:** Writing – original draft, Resources, Methodology, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

Acknowledgment

The authors would like to express sincere gratitude to the management of SRM College of Pharmacy, SRM Institute of Science and Technology for extending their support in the completion of this work.

Statement of human and animal rights

The IAEC committee has approved the animal study to be carried out with reference number IAEC/293/2022.

Statement of informed consent

Not applicable

References

[1] T. Noori, S. Shirooie, A. Sureda, E. Sobarzo-Sanchez, A.R. Dehpour, M. Saldías, E. K. Akkol, Regulation of DAPK1 by natural products: an important target in treatment of stroke, Neurochem. Res. 47 (2022) 2142–2157, https://doi.org/10.1007/s11064-022-03628-7.
[2] L. Dong, C. Liu, Effects of Loofah cylindrica extract on learning and memory ability, brain tissue morphology, and immune function of aging mice, Open Life Sci. 16 (2021) 399–407, https://doi.org/10.1515/biol-2021-0046.
[3] M. Aboh, P. Oladosu, G. Adeshina, B. Olayinka, OlonitolaSO, Phytochemical screening and antifungal activity of leaves extracts of Luffa cylindrica (Roem), Afr. J. Microbiol. Res. 11 (2017) 1681–1687, https://doi.org/10.5897/AJMR2017.8715.
[4] A. Avecilla-Ramírez, M.D. López-Cuellar, B. Porras, A. Rodríguez Hernández, Characterization of poly-hydroxybutyrate/luffa fibers composite material, Bioresources 15 (2020) 7159–7177.
[5] Y.-R. LENG, M.-H. ZHANG, J.-G. LUO, H. ZHANG, Pathogenesis of NASH and promising natural products, Chin. J. Nat. Med. 19 (2021) 12–27, https://doi.org/10.1016/S1875-5364(21)60002-X.
[6] M. He, L. Xia, J. Li, Potential mechanisms of plant-derived natural products in the treatment of cervical cancer, Biomolecules 11 (2021) 1539, https://doi.org/10.3390/biom11101539.
[7] N. Fares Amer, T. Luzzatto Knaan, Natural products of marine origin for the treatment of colorectal and pancreatic cancers: mechanisms and potential, Int. J. Mol. Sci. 23 (2022) 8048, https://doi.org/10.3390/ijms23148048.
[8] W.W. D'silva, P.R. Biradar, A. Patil, Luffa cylindrica: a promising herbal treatment in progesterone induced obesity in mice, J. Diabetes Metab. Disord. 20 (2021) 329–340, https://doi.org/10.1007/s40200-021-00749-8.
[9] L. Huang, S. Ahmed, Y. Gu, J. Huang, B. An, C. Wu, Y. Zhou, G. Cheng, The effects of natural products and environmental conditions on antimicrobial resistance, Molecules 26 (2021) 4277, https://doi.org/10.3390/molecules26144277.
[10] A.S.S., C. Vellapandian, The role of plant-based products in the prevention of neurological complications, Drug Metab. Bioanal. Lett. 15 (2022) 81–92, https://doi.org/10.2174/1872312815666220413095159.
[11] S. Fakhri, A. Iranpanah, M.M. Gravandi, S.Z. Moradi, M. Ranjbari, M.B. Majnooni, J. Echeverría, Y. Qi, M. Wang, P. Liao, M.H. Farzaei, J. Xiao, Natural products attenuate PI3K/Akt/mTOR signaling pathway: a promising strategy in regulating

neurodegeneration, Phytomedicine 91 (2021) 153664, https://doi.org/10.1016/j.phymed.2021.153664.
[12] T.Ben Hlel, F. Belhadj, F. Gül, M. Altun, A. Yağlıoğlu, I. Demirtaş, M.N. Marzouki, Variations in the bioactive compounds composition and biological activities of Loofah (Luffa cylindrica) fruits in relation to maturation stages, Chem. Biodivers. 14 (2017) e1700178, https://doi.org/10.1002/cbdv.201700178.
[13] L. Zhang, P. Wang, M. Shi, Z. Fang, J. Ji, X. Liao, X. Hu, F. Chen, The modulation of Luffa cylindrica (L.) Roem supplementation on gene expression and amino acid profiles in liver for alleviating hepatic steatosis via gut microbiota in high-fat diet-fed mice: insight from hepatic transcriptome analysis, J. Nutr. Biochem. 80 (2020) 108365, https://doi.org/10.1016/j.jnutbio.2020.108365.
[14] I.O. Oboh, E. Aluyor, Luffa cylindrica - an emerging cash crop, Afr. J. Agric. Res 4 (2009) 684–688.
[15] A.S.S., C. Vellapandian, Phytochemical studies, antioxidant potential, and identification of bioactive compounds using GC–MS of the ethanolic extract of Luffa cylindrica (L.) fruit, Appl. Biochem. Biotechnol. (2022), https://doi.org/10.1007/s12010-022-03961-1.
[16] T.H. Kao, C.W. Huang, B.H. Chen, Functional components in Luffa cylindrica and their effects on anti-inflammation of macrophage cells, Food Chem. 135 (2012) 386–395, https://doi.org/10.1016/j.foodchem.2012.04.128.
[17] M. Hazra, S. Kundusen, S. Bhattacharya, P.K. Haldar, M. Gupta, U.K. Mazumder, Evaluation of hypoglycemic and antihyperglycemic effects of Luffa, Cylind. Fruit. Extr. Rats., J. Adv. Pharm. Educ. Res 2 (2011) 138–146.
[18] E. Asuquo Etim, Y.A. Adebayo, O.E. Ifeanyi, Effect of Luffa cylindrica leaf extract on hematological parameters of Swiss Albino Mice, Med. Aromat. Plants 07 (2018), https://doi.org/10.4172/2167-0412.1000318.
[19] M. L.K. Mensah, G. Komlaga, A. D. Forkuo, C. Firempong, A. K. Anning, R. A. Dickson, Toxicity and Safety Implications of Herbal Medicines Used in Africa. in: Herb. Med., IntechOpen, 2019, https://doi.org/10.5772/intechopen.72437.
[20] B. Kaggwa, H. Kyeyune, E.I. Munanura, G. Anywar, S. Lutoti, J. Aber, L. K. Bagoloire, A. Weisheit, C.U. Tolo, P.F. Kamba, P.E. Ogwang, Safety and efficacy of medicinal plants used to manufacture herbal products with regulatory approval in uganda: a cross-sectional study, Evid. -Based Complement. Altern. Med. 2022 (2022) 1–21, https://doi.org/10.1155/2022/1304839.
[21] C.C. Wang, L. Li, L.Y. Tang, P.C. Leung, Safety evaluation of commonly used Chinese herbal medicines during pregnancy in mice, Hum. Reprod. 27 (2012) 2448–2456, https://doi.org/10.1093/humrep/des180.
[22] S.B. Patel, S.G. Ghane, Phyto-constituents profiling of Luffa echinata and in vitro assessment of antioxidant, anti-diabetic, anticancer and anti-acetylcholine esterase activities, Saudi J. Biol. Sci. 28 (2021) 3835–3846, https://doi.org/10.1016/j.sjbs.2021.03.050.
[23] C. Sarkar, C. Quispe, M.T. Islam, S. Jamaddar, M. Akram, N. Munior, M. Martorell, M. Kumar, J. Sharifi-Rad, N. Cruz-Martins, Plant-derived alkaloids acting on dengue virus and their vectors: from chemistry to pharmacology, Future Microbiol 17 (2022) 143–155, https://doi.org/10.2217/fmb-2021-0002.
[24] E. Mgbeahuruike, P. Fyhrquist, H. Vuorela, R. Julkunen-Tiitto, Y. Holm, Alkaloid-rich crude extracts, fractions and piperamide alkaloids of Piper guineense possess promising antibacterial effects, Antibiotics 7 (2018) 98, https://doi.org/10.3390/antibiotics7040098.
[25] M.E. Hussein, O.G. Mohamed, A.M. El-Fishawy, H.I. El-Askary, A.A. Hamed, M. M. Abdel-Aziz, R. Alnajjar, A. Belal, A.M. Naglah, A.A. Almehizia, A.A. Al-Karmalawy, A. Tripathi, A.S. El Senousy, Anticholinesterase activity of budmunchiamine alkaloids revealed by comparative chemical profiling of two Albizia spp., molecular docking and dynamic studies, Plants 11 (2022) 3286, https://doi.org/10.3390/plants11233286.
[26] C.A. Gasca, N.C.S. Moreira, F.C. de Almeida, J.V. Dutra Gomes, W.O. Castillo, C. W. Fagg, P.O. Magalhães, Y.M. Fonseca-Bazzo, E. Sakamoto-Hojo, Y.K. de Medeiros, W. de Souza Borges, D. Silveira, Acetylcholinesterase inhibitory activity, anti-inflammatory, and neuroprotective potential of Hippeastrum psittacinum (Ker Gawl.) herb (Amaryllidaceae), Food Chem. Toxicol. 145 (2020) 111703, https://doi.org/10.1016/j.fct.2020.111703.
[27] R.P. Grant, B.A. Rappold, Development and Validation of Small Molecule Analytes by Liquid Chromatography-Tandem Mass Spectrometry. in: Princ. Appl. Clin. Mass Spectrom., Elsevier,, 2018, pp. 115–179, https://doi.org/10.1016/B978-0-12-816063-3.00005-0.
[28] L.-L. Guo, H.-P. He, Y.-T. Di, S.-F. Li, Y.-Y. Cheng, W. Yang, Y. Li, J.-P. Yu, Y. Zhang, X.-J. Hao, Indole alkaloids from Ervatamia chinensis, Phytochemistry 74 (2012) 140–145, https://doi.org/10.1016/j.phytochem.2011.11.002.
[29] N.N. Ibekwe, N.N. Ibekwe, L.B. John-Africa, Anti-inflammatory Effect of the Alkaloid-rich Fraction of Landolphia owarensis, Niger. J. Pharm. Res. 17 (2021) 39–44, https://doi.org/10.4314/njpr.v17i1.4.
[30] M. Hong, M. Lu, Y. Qian, L. Wei, Y. Zhang, X. Pan, H. Li, H. Chen, N. Tang, A 90-day sub-chronic oral toxicity assessment of mulberry extract in Sprague Dawley rats, Inq. J. Heal. Care Organ. Provis., Financ. 58 (2021), 004695802110560. https://doi.org/10.1177/00469580211056044.
[31] M. Yimam, P. Jiao, M. Hong, L. Brownell, Hyun-Jin Kim, Y.-C. Lee, Q. Jia, Repeated dose 28-day oral toxicity study of a botanical composition composed of Morus alba and Acacia catechu in rats, Regul. Toxicol. Pharmacol. 94 (2018) 115–123, https://doi.org/10.1016/j.yrtph.2018.01.024.
[32] A. Nakajima, K. Nemoto, Y. Ohizumi, An evaluation of the genotoxicity and subchronic toxicity of the peel extract of Ponkan cultivar 'Ohta ponkan' (Citrus reticulata Blanco) that is rich in nobiletin and tangeretin with anti-dementia activity, Regul. Toxicol. Pharmacol. 114 (2020) 104670, https://doi.org/10.1016/j.yrtph.2020.104670.
[33] J.-W. Yun, J.-R. You, Y.-S. Kim, S.-H. Kim, E.-Y. Cho, J.-H. Yoon, E. Kwon, J.-J. Jang, J.-S. Park, H.-C. Kim, J.-H. Che, B.-C. Kang, In vitro and in vivo safety studies of cinnamon extract (Cinnamomum cassia) on general and genetic toxicology, Regul. Toxicol. Pharmacol. 95 (2018) 115–123, https://doi.org/10.1016/j.yrtph.2018.02.017.
[34] S. Wachtel-Galor, I.F.F. Benzie, Herbal medicine: an introduction to its history, usage, regulation, current trends, Res. Needs (2011) http://www.ncbi.nlm.nih.gov/pubmed/19754380.
[35] K. Subramanian, D. Sankaramourthy, M. Gunasekaran, Toxicity studies related to medicinal plants. in: Nat. Prod. Drug Discov., Elsevier,, 2018, pp. 491–505, https://doi.org/10.1016/B978-0-08-102081-4.00018-6.
[36] A.-S. Esmail, Constituents and pharmacology of Luffa cylindrica- a review, IOSR, J. Pharm. 9 (2019) 68–79.
[37] S. Partap, A. Kumar, N. Sharma, K. Jha, Luffa Cylindrica: An important medicinal plant, J. Nat. Prod. Plant Resour. 2 (2012) 127–134.
[38] R.M. Bhide, B. Bethapudi, N.S.S. Chalichem, M. Nithyanantham, S.K. Murugan, D. Mundkinajeddu, Acute and subchronic toxicity study of flavonoid rich extract of Glycyrrhiza glabra (GutGard®) in Sprague Dawley Rats, J. Toxicol. 2022 (2022) 1–12, https://doi.org/10.1155/2022/8517603.
[39] Y. Sanjaya Kumar, Genus Luffa- an ethnopharmacological and phytochemical review, Int. J. Pharma Sci. Res. 7 (2016) 240–244.
[40] P.J.M. Clements, B. Bolon, E. McInnes, S. Mukaratirwa, C. Scudamore, Animal Models in Toxicologic Research: Rodents. in: Haschek Rousseaux's Handb. Toxicol. Pathol., Elsevier, 2022, pp. 653–694, https://doi.org/10.1016/B978-0-12-821044-4.00011-X.
[41] A.G. Atanasov, S.B. Zotchev, V.M. Dirsch, C.T. Supuran, Natural products in drug discovery: advances and opportunities, Nat. Rev. Drug Discov. 20 (2021) 200–216, https://doi.org/10.1038/s41573-020-00114-z.
[42] S. Deng, E. AGA, H. Xie, H. Xiong, B. Ye, Evaluation of the acute toxicity and 28-days subacute toxicity of the alcoholic extract from Ganoderma leucocontextum, Food Sci. Nutr. 11 (2023) 434–442, https://doi.org/10.1002/fsn3.3075.
[43] M. Sindete, A. Gbankoto, R. Osseni, N.D. Tossavi, S. Azonbakin, L. Baba-Moussa, A. Laleye, A 90-Day Oral Toxicity Study of an Ethanolic Root Extract of Caesalpinia bonduc (L.) Roxb. in Wistar Rats, Evid. -Based Complement. Altern. Med. 2021 (2021) 1–11, https://doi.org/10.1155/2021/6620026.
[44] A. van Hest, F. van Haaren, N.E. van de Poll, The behavior of male and female Wistar rats pressing a lever for food is not affected by sex differences in food motivation, Behav. Brain Res. 27 (1988) 215–221, https://doi.org/10.1016/0166-4328(88)90118-0.
[45] D.A. Wilkie, B.C. Gilger, J.T. Bartoe, Standards for Conducting Ophthalmic Examinations in Laboratory Animals. in: Stand. Ocul. Toxicol. Inflamm., Springer International Publishing, Cham, 2018, pp. 1–25, https://doi.org/10.1007/978-3-319-78364-2_1.
[46] H. Bushita, Y. Ito, T. Saito, Y. Nukada, N. Ikeda, H. Nakagiri, K. Saito, O. Morita, A 90-day repeated-dose toxicity study of dietary alpha linolenic acid-enriched diacylglycerol oil in rats, Regul. Toxicol. Pharmacol. 97 (2018) 33–47, https://doi.org/10.1016/j.yrtph.2018.05.017.
[47] Y. Wu, X. Tang, W. Yang, J. Fan, L. Tang, C. Wang, Z. Yu, X.-D. Jia, B. Fan, Subchronic toxicity of cerium nitrate by 90-day oral exposure in wistar rats, Regul. Toxicol. Pharmacol. 108 (2019) 104474, https://doi.org/10.1016/j.yrtph.2019.104474.
[48] W. Jacob Filho, C.C. Lima, M.R.R. Paunksnis, A.A. Silva, M.S. Perilhão, M. Caldeira, D. Bocalini, R.R. de Souza, Reference database of hematological parameters for growing and aging rats, Aging Male 21 (2018) 145–148, https://doi.org/10.1080/13685538.2017.1350156.
[49] A.L.D. Northern, S.M. Rutter, C.M. Peterson, Cyclic changes in the concentrations of peripheral blood immune cells during the normal menstrual cycle, Exp. Biol. Med. 207 (1994) 81–88, https://doi.org/10.3181/00379727-207-43795.
[50] V.D. Oliviera Araujo, C.E. Linhares Andreotti, M.D. Paula Reis, D. Alves Di LIma, K. B. Pauli, B.C. Nunes, C. Gomes, R.D. Melo Germano, E.L. Cardozo Junior, A. G. Junior, E.L. Botelho Lourenço, 90-Day Oral Toxicity Assessment of Tropaeolum majus L. in Rodents and Lagomorphs, J. Med. Food 21 (2018) 823–831.
[51] E.A. Ugbogu, C.D. Nwoku, V.C. Ude, O. Emmanuel, Evaluating bioactive constituents and toxicological effects of aqueous extract of fermented Pentaclethra macrophylla seeds in rats, Avicenna J. Phytomed. 10 (2020) 101–113, http://www.ncbi.nlm.nih.gov/pubmed/31921612.
[52] H. Chen, X. Sha, Y. Luo, J. Chen, X. Li, J. Wang, G. Cao, X. Peng, Acute and subacute toxicity evaluation of Houttuynia cordata ethanol extract and plasma metabolic profiling analysis in both male and female rats, J. Appl. Toxicol. 41 (2021) 2068–2082, https://doi.org/10.1002/jat.4198.
[53] D. Ennulat, D. Walker, F. Clemo, M. Magid-Slav, D. Ledieu, M. Graham, S. Botts, L. Boone, Effects of hepatic drug-metabolizing enzyme induction on clinical pathology parameters in animals and man, Toxicol. Pathol. 38 (2010) 810–828, https://doi.org/10.1177/0192623310374332.
[54] A.O. Saliu, M. AkanjiA and Idowu O.A, ENZYME ACTIVITIES AND HISTOPATHOLOGICAL STUDIES OF SELECTED TISSUES IN ALBINO MICE TREATED WITH METHANOLIC LEAVES EXTRACT OF LUFFA CYLINDRICA (AN ANTIMALARIAL HERB) 4 J. Malar. Res. Phytomedicine. 2020, 7–19.