Acta Biologica Szegediensis

Volume 67(1):111-122, 2023
Acta Biologica Szegediensis
http://abs.bibl.u-szeged.hu/index.php/abs
DOI:10.14232/abs.2023.1.111-122

ARTICLE

Acute and sub-chronic toxicity studies of the ethanol extract of *Erythrina fusca* Lour. fruit via oral administration in mice

Tran Thi Phuong Nhung and Le Pham Tan Quoc*

Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City, Ho Chi Minh City, Vietnam

ABSTRACT *Erythrina fusca* Lour. is a medicinal plant traditionally used in herbal medicine; however, there are no records of toxicity associated with the ethanol extract of *E. fusca* fruit (EtEF). The objective of this study was to assess the safety of EtEF through toxicity testing. Four groups of Swiss albino mice were employed, including a control group and three groups administered EtEF at doses of 1000, 3000, and 5000 mg/kg (single dose) and 100, 300, and 500 mg/kg (administered repeatedly for 90 days). Various parameters, including body weight, food and water consumption, hematological and biochemical parameters, relative organ weight, urine composition, and histopathology, were evaluated. No significant differences were observed in the tested groups compared to the control group, and there was no evidence of morphological or histopathological damage in the organs of mice treated with EtEF. This study affirms the safety of EtEF and establishes a foundation for further investigations into its utilization in traditional medicine.
Acta Biol Szeged 67(1):111-122 (2023)

KEY WORDS
Erythrina fusca Lour.
acute toxicity
medicine
sub-chronic toxicity

ARTICLE INFORMATION
Submitted
15 May 2023
Accepted
20 October 2023
***Corresponding author**
E-mail: lephamtanquoc@iuh.edu.vn

Introduction

Traditional medicine, a concept deeply rooted in long-standing local practices, has evolved, and found significant application within indigenous communities. For a considerable duration, herbal remedies have been widely employed in traditional medicine for disease prevention and treatment (Pariyani et al. 2015). The use of herbal remedies in healthcare provision, particularly in resource-constrained settings, is on the rise. Approximately 80% of the global population relies on traditional medicine for primary healthcare (Ha et al. 2018). Herbal medicine contributes more than 25% of the total pharmaceuticals available worldwide (Dewick 2002). The presence of compounds such as flavonoids, alkaloids, steroids, glycosides, tannins, saponins, terpenoids, etc. in their composition is the basis of the pharmacological basis of herbal medicines (Tom et al. 2018). Therefore, medicinal plants have become an endless source for traditional medicine to produce herbal medicines for treating diseases in various applications (Mlozi et al. 2020). However, many issues have been raised regarding the use of herbal medicines, such as their therapeutic efficacy and potential adverse effects (Bello et al. 2016). There is ample evidence to suggest that herbal medicines can have negative effects on both animals and humans (Toghueo 2020). Therefore, the belief that herbal remedies are always effective and safe for treating diseases is not always true. It is essential to evaluate the toxicity of plant extracts with pharmacological effects, especially when these herbs are to be developed into new drugs (Mlozi et al. 2020).

Erythrina fusca Lour. has a rich history of traditional use in Vietnam and many other countries. The bark of this tree is used to relieve migraine headaches, while the leaves are used to reduce inflammation. The inner bark is scraped for dressing new wounds. The seeds are used to treat skin itching. Moreover, the flowers and young leaves serve as daily dietary greens. The fruit of the plant is known for its digestive properties, alleviating joint and bone pain, and supporting liver health. Numerous sources have documented that all parts of the plant exhibit various biological activities, including antimicrobial, antiviral, anti-inflammatory, anti-arthritic, central nervous system inhibition, and blood pressure-lowering effects (Azmi et al. 2020). Although traditional use and biological activities have been reported so far, to our knowledge no available data have mentioned the toxic effects of *E. fusca*. Therefore, the purpose of this study was to investigate the acute and subchronic toxicity of ethanol extract from *E. fusca* fruits in Swiss mice to evaluate the safety of the extract.

Material and Methods

Collection and preparation of plant material

In May 2022, fresh *E. fusca* fruits were collected from the farm of a plant seed company located in Buon Ma

Table 1. Preliminary phytochemical tests for the ethanol extract of *E. fusca* fruit (EtEF).

Name of test	Procedure	Observation
Flavonoids test Alkaline reagent test for flavonoids	With the test solution + Few drops of NaOH solution	Yellow color
Phenolic compounds Ferric chloride test	Test solution + Few drops of neutral 5% $FeCl_3$	Green color
Tannins test Braymer's Test	Test solution + 2 mL H_2O + 2-3 drops $FeCl_3$ (5%)	Green precipitate
Saponins test Foam's Test	Test solution + 5 mL H_2O + heat	Froth appears
Gycosides test Liebermann's Test	Test solution 2 mL $CHCl_3$ + 2 mL CH_3COOH	Violet to Blue to Green coloration
Amino acid Ninhydrin test	Test solution + few drops of 5% ninhydrin	Violet color
Protein test Biuret test	Test solution + 4% NaOH + few drops of 1% $CuSO_4$	Violet color
Carbohydrate test Molisch's test	Test solution + few drops alcoholic α napthol + 0.2 mL H_2SO_4	Purple to violet color rings appear
Fats and Oils	Test solution + a drop of phenolphthalein	Formation of soap or partial neutralization

Thuot City, Daklak province, Vietnam. The authenticity of the plant was evaluated, and a voucher specimen (No. EF200522VST) was kept at the Department of Plant Biotechnology under the Institute of Biotechnology and Food Technology at the Industrial University of Ho Chi Minh City for future reference. The fresh fruits were rinsed with water and then cut open, with the seeds removed from the fruits. Subsequently, the *E. fusca* fruits were dried in an oven (Memmert UN75, Germany) at a temperature of 60 °C for 5 h, then ground into a powder, and stored in a desiccator at 25 °C for further studies.

Preparation of the extract

To prepare the ethanol extract of *E. fusca* fruit, 100 g of dried sample (in fine powder form) was soaked in 400 mL of ethanol for 7 days with continuous stirring on the first day. The extract was filtered through a clean muslin cloth, and the extraction process was repeated a second time by adding another 400 mL of ethanol to the residue. The filtrates from each extraction were combined, and the resulting mixture was then vacuum-dried on a rotary evaporator at 50 °C to remove excess ethanol solvent until a dark black ethanol extract was obtained (Total soluble solid: 12.8 °Brix), which was named EtEF. The *E. fusca* fruit extract (EtEF) was stored at 4 °C until further use.

Phytochemical screening of extract

A preliminary qualitative phytochemical analysis was conducted to identify the chemical compounds present in the ethanol extract of *E. fusca* fruit. The phytochemical screening in EtEF followed standard procedures outlined in Table 1, which were based on established methods (Sofowora 1996; Harborne 1998; Edeoga et al. 2005; Ayoola et al. 2008; Evans 2009).

Experimental animals

The healthy female Swiss albino mice weighing 30.5±1.8 g were obtained from the Pasteur Institute in Ho Chi Minh City, Vietnam. The number of animals per group was determined in accordance with the Guidelines for Preclinical and Clinical Trials of Traditional Medicines and Herbal Medicines (No. 141/QĐ-K2ĐT) in 2015 (Vietnam). The mice were housed in standard glass cages with sawdust bedding, which was sterilized using Emina biocidal agent (Japan) and changed twice a week. The mice were maintained in an environment with controlled temperature (26 ± 2 °C) and humidity (55-60%) with a 12 h/12 h light-dark cycle. During the experiment, all mice were provided with clean drinking water and rodent chow. Before exposure, the mice were kept for 7 days to acclimate and adapt to laboratory conditions.

The mice were divided into 4 groups (6 mice/group), including 1 control group and 3 groups treated with EtEF. The animals were randomly assigned to the groups using an appropriate randomization method. A blind was implemented on any participant involved in the experiment (Bespalov et al. 2019). Mice within the same group were identified by a permanent marker on their tail (Burn et al. 2021). Laboratory animals are cared for according to the guidelines of the World Health Organization (WHO 2000) and Office of Laboratory Animal Welfare (OLAW 2015). The testing procedures strictly followed the Declaration of Helsinki (Hurst 2014). All possible measures were taken to minimize the distress of animals.

Acute toxicity testing

The acute toxicity study of EtEF was conducted following the protocol of the Organisation for Economic Cooperation and Development (OECD No. 423 2001) with some

slight modifications. The mice were acclimatized in the laboratory conditions for 7 days and then randomly assigned into four groups (n = 6). Prior to the administration of EtEF, all mice have fasted overnight. Subsequently, the body weight of each mouse was measured and the dose of EtEF for each mouse was calculated based on its body weight. A single dose of EtEF was administered orally at 1000, 3000, and 5000 mg/kg body weight to three experimental groups (designated as $EtEF_{1000}$, $EtEF_{3000}$, and $EtEF_{5000}$, respectively) and the mice were monitored for 14 days. The control group was administered physiological saline solution orally. After administration of the EtEF dose on the first day, the mice were observed regularly for 1 h and intermittently for the next 12 h, then their behavior was monitored once every 24 h for the following 14 days. During the observation and evaluation process, behavioral responses (such as running, agitation, salivation, and diarrhea), general appearance (including changes in fur and skin, eye color, nasal mucosa, respiration rate, and heart rate), other abnormalities, and mortality rate were monitored and recorded daily. Body weight as well as urine volume were measured weekly. Food and water consumption were recorded daily, and the weekly average consumption was calculated at the end of each week. Finally, on day 14, after measuring the body weight, blood was collected via retro-orbital puncture. Subsequently, the mouse was anesthetized with a mixture of Xylazine + Ketamine (16 mg + 60 mg i.m/i.p.), underwent surgery, and the important internal organs were harvested. The weight of the organs was measured and recorded. Samples of organ tissues were preserved in 10% formaldehyde for histopathological analysis.

Sub-chronic toxicity testing

The sub-chronic toxicity study of the EtEF was conducted following the Organisation for Economic Cooperation and Development (OECD No. 408 2018) with some slight modifications. Twenty-four mice were weighed and randomly divided into three treatment groups and one control group. The mice in the treatment group were administered the EtEF (at a consistent concentration) via oral gavage at doses of 100, 300, and 500 mg/kg of body weight (referred to as $EtEF_{100}$, $EtEF_{300}$, and $EtEF_{500}$, respectively), using ball-tipped oral gavage needles, while the control group was given food and water without EtEF. The mice were continuously given EtEF for 90 days. The clinical observation was also conducted continuously for 90 days. Body weight, food and water consumption, and urine output were measured weekly for 12 weeks. On day 90 of the experiment, the final body weight of the mice was measured. Blood samples were collected from each mouse by retro-orbital sinus puncture and used for hematological and biochemical analysis. Finally, the mice were anesthetized with a Xylazine + Ketamine mixture (16 mg + 60 mg i.m/i.p.). After surgery, the overall internal organs were observed for any damage. The vital organs were weighed for absolute weight. Samples of organ tissue were preserved in 10% formaldehyde for histopathological analysis.

Clinical observations and survival

All behaviors, mortality rates, and signs of illness in the mice were observed, monitored, and recorded twice daily throughout sub-chronic the testing period. Clinical observations included changes in fur and skin, eye color, changes in nasal mucous membranes, abnormal breathing, and heart rate. Behavioral responses such as tremors, agitation, salivation, diarrhea, changes in gait and posture, and repetitive circling were also observed. The observation and monitoring period one week from started after the start of the EtEF administration and ended on the scheduled sacrifice date.

Bodyweight

The body weight of all mice was measured using Entries (Germany) precision scales and recorded at the baseline, weekly throughout the study, and on the final day prior to sacrifice. The percent increase in body weight was calculated using the following formula:

$$\text{Weight gain (\%)} = (WG_f\text{-}WG_i)/WG_i \times 100$$

where WG_f: final weight; WG_i: initial weight (Ugwah-Oguejiofor et al. 2019).

Food and water consumption

The amount of food and water consumed by mice during the experiment was also recorded. Before each feeding and watering, the amount of food and water for each group was weighed and measured. At the end of each day, the remaining food and water were collected. Then, the amount of food and water consumed was calculated using the formula:

The amount of food intake or water intake original - The amount of food intake or water intake leftover = The amount of food intake or water was consumed

Food and water consumption were recorded daily (expressed in gram/day and mL/day), and the weekly average consumption was calculated at the end of each week (Upadhyay et al. 2019).

Relative organ weights

All mice fasted overnight before sacrifice. Then, they were anesthetized and underwent surgery, and organs such as

Table 2. Effects of EtEF on food and water consumption in acute and sub-chronic toxicity tests.

Acute oral toxicity				
Parameter	**Control group**	**$EtEF_{1000}$**	**$EtEF_{3000}$**	**$EtEF_{5000}$**
The food consumed (g/day)	4.98±0.07	5.08±0.06	5.11±0.09	5.13±0.09
The water consumed (mL/day)	4.65±0.07	4.71±0.08	4.69±0.07	4.72±0.06
Sub-chronic oral toxicity				
Parameter	**Control group**	**$EtEF_{100}$**	**$EtEF_{300}$**	**$EtEF_{500}$**
The food consumed (g/day)	5.02±0.02	5.03±0.07	*5.19±0.03	*5.25±0.03
The water consumed (mL/day)	4.72±0.03	4.79±0.08	*4.85±0.04	*4.83±0.02

Values that are significantly different versus the corresponding control group (*p<0.05) were marked with an asterisk.

the heart, liver, and kidneys were observed and collected. The examination of the histology of these organs was also performed to detect any injuries or other abnormal conditions. The absolute weight of these organs was determined by the electronic scale of Entries (Germany). The relative organ weight (ROW) of each organ was determined using the formula (Ghosh et al. 2019):

ROW (%) = Absolute organ weight (g)/Bodyweight of the mouse on sacrifice day (g) × 100

Hematological and biochemical parameters

Blood samples were collected in EDTA tubes and used for hematological analysis. Hematological parameters such as red blood cells (RBC), hematocrit (HCT), hemoglobin (HGB), white blood cells (WBC), and platelets (PLT) were measured using an automated analyzer (KX-21 Hematology Analyzer, Sysmex Corporation, USA). The blood samples used for biochemical analysis were centrifuged at 3000 rpm at 25 °C for 15 min to collect serum, which was stored at -20 °C. Serum biochemical parameters were measured using the Erbachem 5 semi-automatic analyzer, including total proteins (TP), glucose (GLU), aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine (CRE), and urea (URE).

Morphology and histological of organs

At the end of the experimental period, mice were dissected to collect the heart, liver, and kidneys. Subsequently, they were fixed in 10% formalin for specimen preservation. Prior to processing, the tissue samples were washed using water to remove formalin. Next, they were processed for paraffin embedding. The paraffin blocks were sectioned into 4-6 μm thick segments using a Thermo Scientific HM525 NX cryo-microtome (USA) and stained with hematoxylin and eosin (H&E). Finally, histopathological changes were identified by observation under a light microscope.

Urinalysis

Once a week, each mouse was placed in a glass cage with water available but no food for 16 h to collect urine samples. The analysis of urinary parameters such as pH, specific gravity (SG), and glucose (GLU) was performed using an automated urine analyzer (Roche Urisys 2400, Switzerland). An automatic electrolyte analyzer (Easy-Lyte EXPAND, China) was used to analyze electrolyte levels in urine such as sodium (Na^+), potassium (K^+), and chloride (Cl^-).

Statistical analysis

The results of the study are presented as Mean ± SD. Differences between groups were determined by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test when using the Stagraphics Centurion XVI software (Statpoint Technologies, Warrenton, Virginia, USA), with a significance criterion set at $p < 0.05$.

Results

Qualitative phytochemical analysis

Preliminary screening of phytochemicals from *E. fusca* fruit indicated the presence of flavonoids, saponins, tannins, phenolic compounds, carbohydrates, and the absence of cardiac glycosides, proteins, amino acids, fats, and oils in the extract. The use of ethanol as the solvent in the study did not adversely affect experimental outcomes.

Behavioral responses and general appearance of the treated mice

Using EtEF orally showed no mortality rate in mice in both the acute and sub-chronic toxicity tests during the study period. Clinical observations of mice during the experiment showed no abnormal signs such as changes in skin color, fur, eye color, and nasal mucosa. There were no changes in behavior, tremors, salivation or diarrhea,

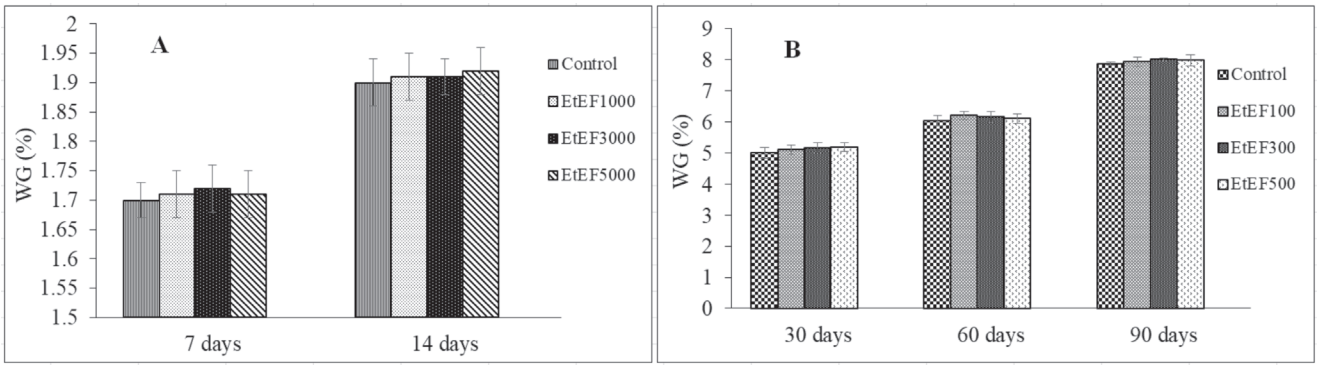

Figure 1. The average extent of body weight gain in mice orally administered EtEF in acute (A) and sub-chronic toxicity (B) tests. Values that are significantly different from the corresponding control group (*p<0.05) are marked with an asterisk.

and no cases of unconsciousness occurred. Therefore, no pathological abnormalities appeared in all observed groups of mice. This demonstrates the safety of EtEF at the administered doses.

Body weight, food intake, and water consumption

Figure 1. depicts that there was no significant change in the average body weight of mice between day 0 and day 14 during the acute toxicity test. In the sub-chronic toxicity test, the average body weight of mice on day 90 showed a significant increase compared to day 0 ($p < 0.05$). However, there was no significant difference observed between the extract group and the control group ($p > 0.05$).

The use of the extract during the experimental period led to an increase in food and water consumption in the EtEF-treated groups (Table 2). In the sub-acute toxicity test, food and water consumption significantly increased in the $EtEF_{300}$ and $EtEF_{500}$ groups compared to the control group (p<0.05); however, no significant differences were observed in the other groups (p>0.05).

Hematological and biochemical effects

In the current study, hematological data indicated that EtEF did not affect hematopoiesis as there were no significant differences among groups in acute and sub-chronic oral toxicity tests (Table 3). Table 4 presents the results of renal and hepatic function tests based on serum levels of URE, CRE, AST, ALT, ALP, TP, and GLU. In both acute and sub-chronic toxicity testing, there were no significant differences observed in the levels of urea and creatinine between the control group and the groups treated with EtEF. There were also no significant differences in the biochemical analysis of AST, ALT, ALP, PT, and GLU compared to the control group.

Relative organ weights

The relative organ weights of mice in acute and sub-chronic toxicity of ethanol extract from *E. fusca* fruits are shown in Figure 2. The relative weights of heart, liver, and kidney recorded in EtEF-treated groups at all doses were not significantly different from the control group. The observed difference is a result of natural variability within the data samples (Fig. 1 and Fig. 2). Environmental conditions during the experiment (diet, lighting, nutritional conditions) were kept consistent between both the experimental and control groups, thus potentially

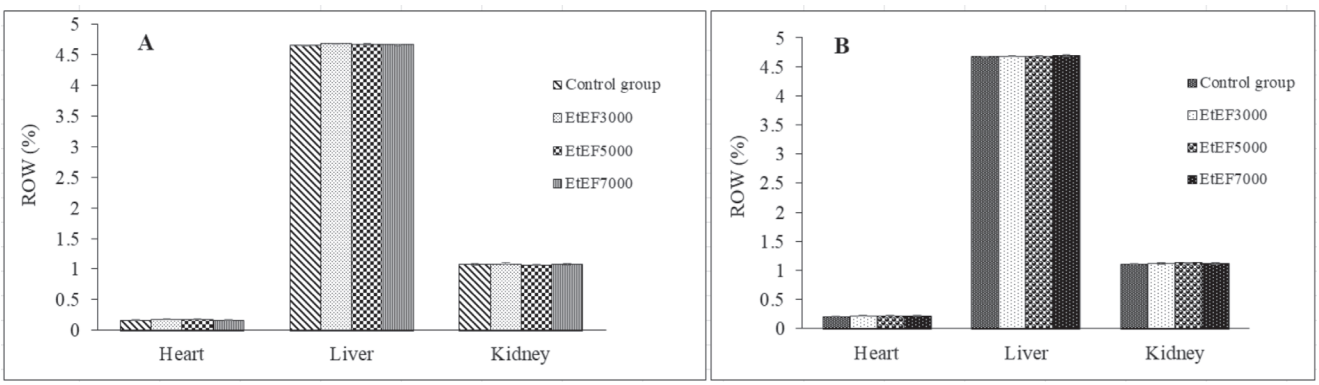

Figure 2. Average relative weights of the heart, liver, and kidneys of mice orally administered EtEF in acute (A) and sub-chronic toxicity tests (B).

Table 3. Hematology parameters of mice orally administrated with EtEF for acute toxicity test.

Acute oral toxicity				
Parameter	**Control group**	**$EtEF_{1000}$**	**$EtEF_{3000}$**	**$EtEF_{5000}$**
RBC (×10^6 cell/mm^3)	7.62 ± 0.07	7.69 ± 0.11	7.72 ± 0.09	7.86 ± 0.07
HGB (g/dL)	13.93 ± 0.08	14.01 ± 0.07	14.03 ± 0.06	14.02 ± 0.09
HCT (%)	0.45 ± 0.08	0.43 ± 0.08	0.42 ± 0.07	0.48 ± 0.05
WBC (×10^3 cell/mm)	3.41 ± 0.08	3.44 ± 0.09	3.52 ± 0.06	3.48 ± 0.05
PLT (×10^3 cell/mm^3)	626.25 ± 7.83	639.78 ± 7.99	644.78 ± 5.43	645.28 ± 7.63
Sub-chronic oral toxicity				
Parameter	**Control group**	**$EtEF_{100}$**	**$EtEF_{300}$**	**$EtEF_{500}$**
RBC (×10^6 cell/mm^3)	7.97 ± 0.08	8.01 ± 0.08	8.08 ± 0.07	8.11 ± 0.07
HGB (g/dL)	14.85 ± 0.08	14.92 ± 0.08	14.95 ± 0.11	14.97 ± 0.06
HCT (%)	0.54 ± 0.05	0.55 ± 0.06	0.58 ± 0.06	0.53 ± 0.05
WBC (×10^3 cell/mm)	3.55 ± 0.07	3.59 ± 0.07	3.63 ± 0.09	3.75 ± 0.07
PLT (×10^3 cell/mm^3)	657.35 ± 10.19	653.19 ± 13.06	655.85 ± 9.08	661.73 ± 9.18

Values that are significantly different versus the corresponding control group (*p<0.05) were marked with an asterisk.

diminishing the observed differences.

Morphology and histological features

The morphology of the heart, liver, and kidney of mice did not differ between the control and EtEF-exposed groups. A comparison between the groups using the highest doses of EtEF in the acute and chronic toxicity experiments with the control group reveals the liver morphology of mice in the control group (Fig. 3A), the $EtEF_{5000}$ group (Fig. 3D), and the $EtEF_{500}$ group (Fig. 3G) all exhibit a soft, dark red surface. The liver consists of four lobes, including the right lobe, left lobe, square lobe, and caudal lobe. The morphology of the diaphragm surface and visceral surface remained unchanged. The kidney morphology of female mice in the saline solution group (Fig. 3B), the $EtEF_{5000}$ group (Fig. 3E), and the $EtEF_{500}$ group (Fig. 3H) consisted of two pea-shaped, crimson organs with a smooth anterior surface and a rough posterior surface. The renal hilum was concave, with renal blood vessels visible. The heart appeared as a three-sided pyramid with a crimson color and smooth surface. The base of the heart corresponded to the posterior surface of the two atria, and there was an interatrial fissure in the middle. The apex of the heart corresponded to the underside of the ventricles. The heart morphology of mice in the $EtEF_{5000}$ (Fig. 3F) and $EtEF_{500}$ (Fig. 3K) groups did not change and was not significantly different from the control group (Fig. 3C).

The histology evaluation of the heart, liver, and kidney of mice showed no changes when compared to the EtEF experimental and control groups. Histological examination of the liver sections from mice treated with EtEF at a dose of 5000 mg/kg (Fig. 4E) and 500 mg/kg (Fig. 4I) revealed the normal structure of the central vein and hepatic sinusoids lined by a similar endothelium to that of the control group (Fig. 4A). Hepatocytes had normal size and shape, and no necrotic cells were observed in their parenchyma. Moreover, the normal lobular architecture of the liver was not affected. The histopathology evaluation of the kidneys of mice treated with EtEF at doses of 5000 mg/kg (Fig. 4F) and 500 mg/kg (Fig. 4H) showed no abnormal changes compared to the control group. The kidney histology of the control group and the group treated with the extract exhibited normal characteristics: intact renal corpuscle and tubules, normal glomerular tuft, and Bowman's space size. No abnormal changes were observed in the EtEF-treated groups compared to the control group (Fig. 4B). The cardiac muscle tissue is composed of fused or individual cardiac muscle cells. The cardiac muscle cells are interconnected by intercalated discs, which are surrounded by collagen fibers and other substances that form the extracellular matrix. The cardiac muscle cells are rectangular in shape and contain a nucleus and organelles. This is the common structure of the mouse heart in the $EtEF_{5000}$ group (Fig. 4G), the $EtEF_{500}$ group (Fig. 4M), and the control group (Fig. 4C).

Urinalysis

The results of urine analysis showed slight changes in color (pale gray, yellow, or brown) (Table 5). There was no presence of factors such as GLU in urine. The concentrations of Na^+, K^+ and Cl^- ions did not significantly differ (p>0.05) between the groups treated with EtEF and the control group.

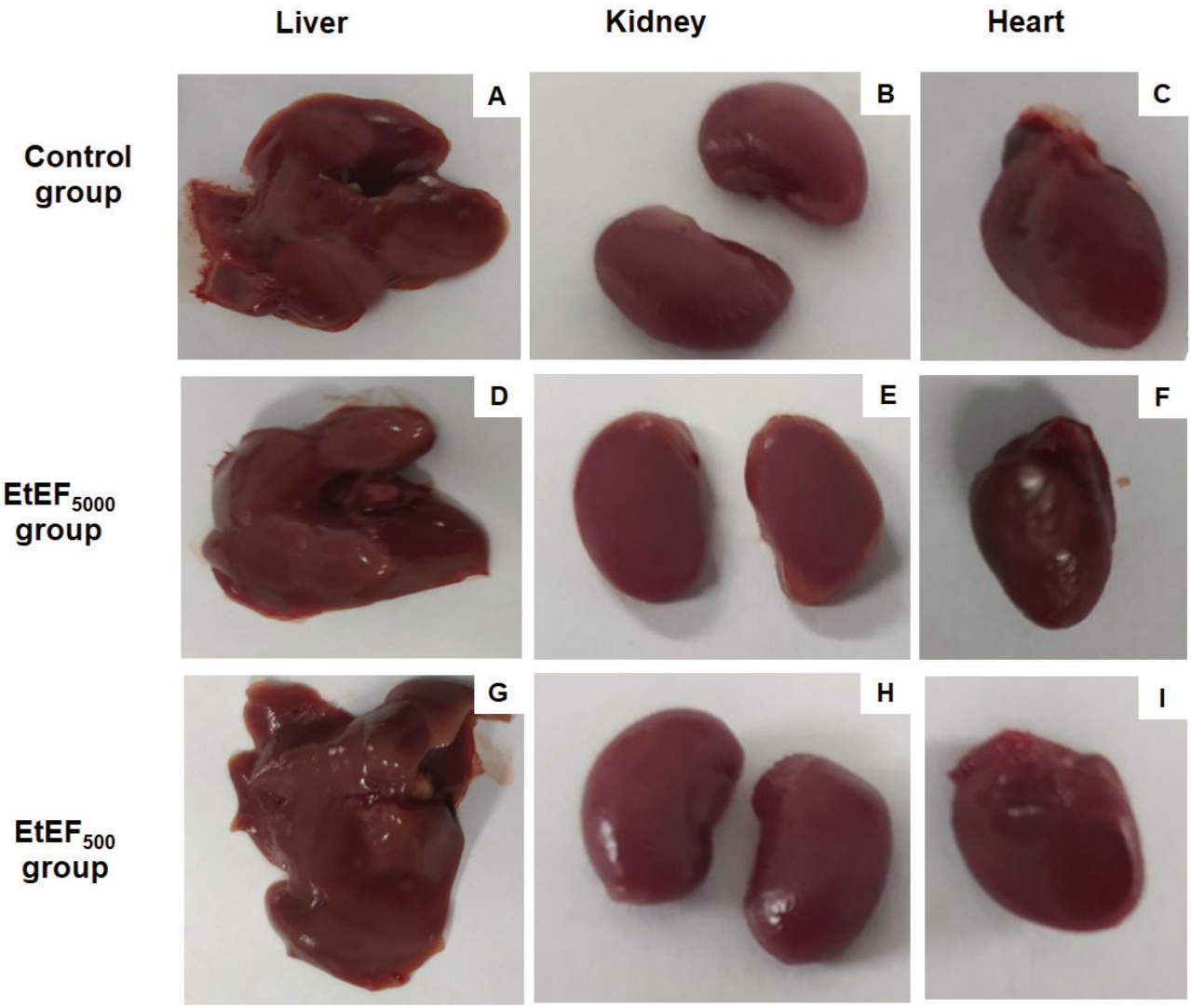

Figure 3. Photographs of the hearts, livers, and kidneys of mice from the acute and sub-chronic toxicity study.

Discussion

E. fusca has long been employed in traditional medicine to treat various digestive disorders, malaria, liver ailments, joint pain, etc. However, there is a lack of scientific reports regarding the safety of *E. fusca*. In this study, an ethanol extract of *E. fusca* fruit was utilized to assess its safety, serving as a scientific basis for future investigations. Ethanol was selected due to its high safety profile, precise control of concentration, excellent solubility support for essential organic compounds, minimal harm to animals, environmental friendliness, and biodegradability. Given the ethanol's solubility characteristics with the organic constituents in *E. fusca* flowers, it proved to be an effective solvent for enhancing the extraction efficiency. The ethanol concentration in the EtEF was meticulously controlled to ensure negligible impact on mouse health. Furthermore, the health of the mice used in the study was continuously monitored, including assessments of their demeanor, behavior, cognition, and overall well-being throughout the EtEF exposure period. Swiss mice were the preferred choice for ethanol-related extraction studies due to their genetic suitability, age, individual physical characteristics, and living environment. Therefore, Swiss mice served as an appropriate reference source for studies related to ethanol-based treatment protocols, especially concerning EtEF. The results of these plant chemical screenings provide supplementary information for subsequent experiments aimed at elucidating additional pharmacological activities of ethanol extracts from *E. fusca* products.

The safety assessment of herbal medicine aims to in-

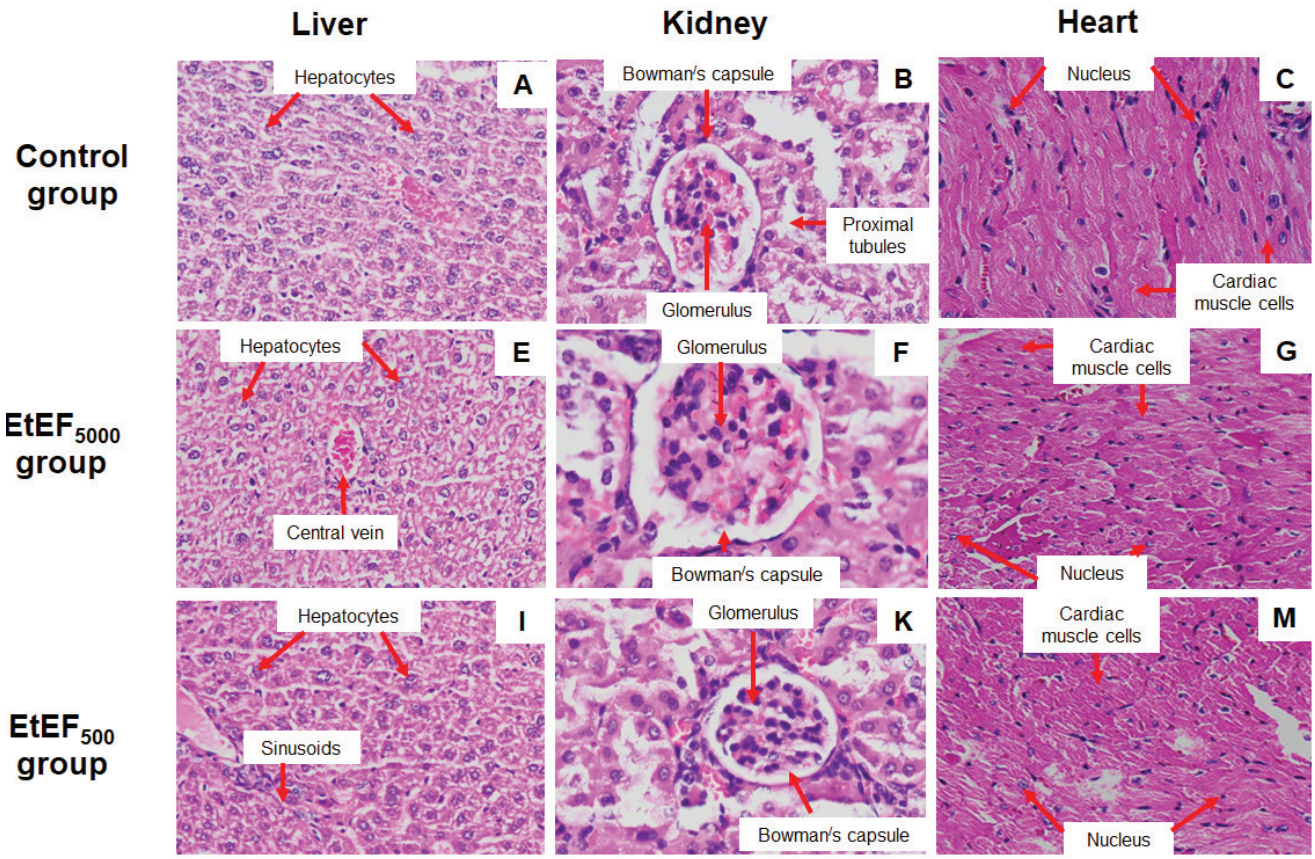

Figure 4. Photomicrographs (magnification 200X) of heart, liver, and kidney tissues demonstrating normal features in mice treated with EtEF orally in acute and sub-chronic toxicity models. The sections were stained with Hematoxylin and Eosin (H&E).

vestigate the characteristics, significance, potential side effects, and extent of exposure of the medicinal plant, particularly in the context of its observed effects (Ugwah-Oguejiofor et al. 2019). The findings from the acute toxicity study revealed that the oral administration of the ethanol extract of *E. fusca* fruits at doses of 1000, 3000, and 5000 mg/kg to mice did not result in any adverse effects, signs of toxicity, or mortality among the animals. Additionally, the acute and subacute toxicity profiles of EtEF were assessed in mice by evaluating parameters such as body weight, food and water consumption, hematological and biochemical indicators, relative organ weights, urine volume, and organ histopathology. The study outcomes demonstrated no significant distinctions between the treatment groups and the control group, leading to the conclusion that EtEF is safe at the tested dosage levels.

Body weight encompasses the weight of muscles, fat, bones, organs, and fluids. An increase in body weight can result from muscle mass gain, fat accumulation, or excess fluid retention. Prior research has indicated that an elevated metabolic rate can lead to weight gain (Anthanont and Jensen 2016). Food supplies energy to the body, and when an ample quantity of food is provided, it elevates energy expenditure, resulting in an excess of energy stored as fat and subsequent weight gain. Nutrients in food such as proteins, carbohydrates, and fats aid in muscle maintenance, support growth, and provide energy. Increased insulin secretion can also contribute to fat accumulation, thereby contributing to weight gain (Wu et al. 2022).

Chemical compounds such as flavonoids, saponins, tannins, phenolic compounds, carbohydrates, and others found in EtEF may influence body weight through various mechanisms. For instance, flavonoids can inhibit the absorption and utilization of fats, saponins can affect lipid and protein absorption by loosening the gastrointestinal mucosa, tannins can interfere with nutrient absorption and digestion, phenolic compounds may inhibit fat formation in the body, and carbohydrates can provide energy to the body (Tucci 2010). In the initial days of EtEF usage, slight weight loss was observed. However, after 14 and 90 days of observation, significant weight gain was noted in mice treated with EtEF. This indicates that EtEF does not significantly affect metabolic processes and body

Table 4. Serum biochemical parameters of mice orally administrated with EtEF for acute and sub-chronic toxicity test.

Acute oral toxicity				
Parameter	**Control group**	**$EtEF_{1000}$**	**$EtEF_{3000}$**	**$EtEF_{5000}$**
Total proteins (g/dL)	5.54 ± 0.23	5.59 ± 0.24	5.62 ± 0.28	5.67 ± 0.19
Glucose (mg/dL)	63.42 ± 0.66	63.52 ± 0.21	63.46 ± 0.23	63.54 ± 0.27
AST (U/L)	17.69 ± 0.27	17.72 ± 0.38	17.77 ± 0.25	17.74 ± 0.32
ALT (U/L)	18.67 ± 0.16	18.71 ± 0.23	*19.51 ± 0.21	19.27 ± 0.37
ALP (U/L)	131.85 ± 0.32	131.91 ± 0.37	131.83 ± 0.34	131.95 ± 0.33
Creatinine (μmol/L)	0.38 ± 0.12	0.44 ± 0.13	0.41 ± 0.11	0.47 ± 0.12
Ure (mg/L)	30.84 ± 0.27	30.81 ± 0.28	30.86 ± 0.31	30.78 ± 0.27
Sub-chronic oral toxicity				
Parameter	**Control group**	**$EtEF_{100}$**	**$EtEF_{300}$**	**$EtEF_{500}$**
Total proteins (g/dL)	6.71 ± 0.22	6.69 ± 0.16	6.71 ± 0.13	6.75 ± 0.24
Glucose (mg/dL)	65.73 ± 0.25	65.78 ± 0.21	65.81 ± 0.24	65.83 ± 0.26
AST (U/L)	18.26 ± 0.23	18.33 ± 0.34	18.25 ± 0.24	18.31 ± 0.26
ALT (U/L)	19.51 ± 0.18	19.49 ± 0.15	19.55 ± 0.15	19.57 ± 0.17
ALP (U/L)	132.53 ± 0.36	132.61 ± 0.27	132.63 ± 0.22	132.56 ± 0.14
Creatinine (μmol/L)	0.45 ± 0.13	0.49 ± 0.11	0.53 ± 0.13	0.56 ± 0.16
Ure (mg/L)	31.75 ± 0.33	31.79 ± 0.34	31.83 ± 0.37	*32.36 ± 0.47

Values that are significantly different versus the corresponding control group (*p<0.05) were marked with an asterisk.

weight in mice.

Changes in food and water consumption were used as indicators to evaluate the health status of the experimental mice (El-Hilaly et al. 2004). Food and water consumption are adjusted through various biological processes to ensure stable body weight over time (Kuriyan et al. 2007). Appetite regulation plays an important role in weight regulation as it adjusts the body's needs for food (Ugwah-Oguejiofor et al. 2019). The use of EtEF resulted in an increased food intake by the mice. Consequently, the observed increase in body weight may be attributed to the higher amount of food consumed by the mice. Some studies have indicated that increased appetite leads to weight gain (Kuriyan et al. 2007). Flavonoids are a group of plant chemicals extensively metabolized by the gut microbiota and host tissues. They enhance the gut immune system, promote digestive activity, and stimulate appetite (Pei et al. 2020). Typically, dietary intake goes hand in hand with fluid consumption. When food intake rises, water consumption follows suit (Kurtz and Feeney 2019). The groups treated with EtEF exhibited higher water intake compared to the control group during the treatment period. This outcome might be attributed to the vasodilatory effect (lowering blood pressure) of EtEF on smooth muscle cells (Al-Awthan et al., 2010), stimulating thirst and increasing fluid intake (Thunhorst et al. 2010).

However, at doses of 300 and 500 mg/kg, both food and water intake increased significantly, but the mice did not gain excessive weight. This phenomenon can be explained as follows: EtEF might affect the nutrient absorption process in the digestive tract, leading to reduced absorption of nutrients from food, thereby preventing the mice from accumulating enough nutrients to cause significant weight gain. Additionally, EtEF might directly influence the fat storage process in the body by inhibiting the conversion of nutrients into fat or promoting fat utilization. Therefore, when mice consume more food, their bodies may utilize energy from fat storage rather than storing it. Furthermore, EtEF contains compounds with antioxidant and anti-inflammatory properties, thus influencing metabolic processes and mitigating weight gain by reducing inflammation and oxidative stress in the mice's bodies.

The hematological system has high predictive value for toxicity, therefore hematological analysis is used to evaluate the toxicity and risk of plant extracts. Blood components are exposed to a significant amount of potentially toxic compounds (Olson et al. 2000). Red blood cells (RBCs), white blood cells (WBCs), and platelets (PLTs) all originate from pluripotent stem cells (Cavanaugh 2003). Lymphocytes, a subtype of white blood cells, play a pivotal role in the immune system, serving as intermediaries for immune responses against foreign substances and generating antibodies against invading agents such as bacteria, etc. (Ugwah-Oguejiofor et al. 2019). The increased number of lymphocytes indicates the immunostimulatory effects of the ethanol extract from *E. fusca* fruits. The elevation in PLT count contributes to the regulation of bleeding

Table 5. Urine analysis parameters of mice treated with EtEF in acute and sub-chronic toxicity tests.

Acute oral toxicity				
Parameter	**Control group**	**$EtEF_{1000}$**	**$EtEF_{3000}$**	**$EtEF_{5000}$**
GLU (mg/dL)	Absent	Absent	Absent	Absent
pH	7.2 ± 0.26	7.4 ± 0.31	7.3 ± 0.42	7.1 ± 0.27
SG	1.012 ± 0.002	1.014 ± 0.001	1.011 ± 0.002	1.013 ± 0.002
Na^+ (mmol/L)	65.09 ± 2.09	64.39 ± 3.45	64.63 ± 2.66	65.45 ± 2.05
K^+ (mmol/L)	44.01 ± 1.02	45.18 ± 2.66	*46.47 ± 1.01	45.61 ± 1.78
Cl^- (mmol/L)	72.12 ± 2.56	70.94 ± 2.65	71.41 ± 3.98	72.39 ± 2.64
Sub-chronic oral toxicity				
Parameter	**Control group**	**$EtEF_{100}$**	**$EtEF_{300}$**	**$EtEF_{500}$**
GLU (mg/dL)	Absent	Absent	Absent	Absent
pH	7.3 ± 0.34	7.4 ± 0.29	7.3 ± 0.27	7.2 ± 0.36
SG	1.012 ± 0.001	1.013 ± 0.001	1.012 ± 0.002	1.014 ± 0.001
Na^+ (mmol/L)	65.42 ± 3.41	65.05 ± 2.47	66.11 ± 2.54	65.59 ± 3.43
K^+ (mmol/L)	45.04 ± 2.24	46.11 ± 2.36	45.52 ± 2.43	46.29 ± 1.96
Cl^- (mmol/L)	72.96 ± 2.41	72.23 ± 2.19	74.29 ± 2.26	75.01 ± 2.23

Note: glucose (GLU), pH, specific gravity (SG), sodium (Na+), potassium (K+), chloride (Cl−). Values that are significantly different versus the corresponding control group (*p<0.05) were marked with an asterisk.

and blood clotting in cases of injury (Santos et al. 2016).

After the administration of EtEF at high doses or for an extended period, significant fluctuations in RBC (red blood cells) and WBC (white blood cells) counts were observed. These changes can be attributed to neural responses or adaptive reactions to alterations in the endocrine milieu. The enhanced production of RBCs and WBCs serves as a response to counteract the effects of the extract. However, following the cessation of EtEF intake, these indices gradually returned to stable levels. This phenomenon may be attributed to the body's capacity to adapt and regulate blood cell levels over time. After the initial response to EtEF, the body adapts and reverts to its natural equilibrium, which includes reducing blood cell production or enhancing its elimination. Once the initial response has occurred, automatic regulatory mechanisms aid in reestablishing equilibrium. This is an integral part of the body's self-regulatory process to maintain functional stability.

The primary function of the liver is to detoxify and prevent harmful substances from entering the bloodstream, whereas the kidneys are responsible for filtering and excreting toxins from the body. Consequently, investigating the roles of the liver and kidneys is crucial in assessing the toxicity of botanical extracts. The assessment of liver and kidney function can be conducted through the analysis of serum biochemistry (Bariweni et al. 2018). In toxicity studies using animal models, the serum concentrations of AST, ALT, and ALP are employed to assess liver function, while serum creatinine and urea levels are used to evaluate kidney function (Al-Afifi et al., 2018). AST and ALT are enzymes produced by liver cells, and any insult to the liver can lead to an elevation in the levels of these enzymes in the bloodstream (Adedapo et al. 2004). The hepatic enzyme levels were found to fall within the expected normal range for Swiss mice utilized in this study (Santos et al. 2016). Our study also revealed no significant differences in total protein and glucose levels in the serum between the groups receiving EtEF doses and the control group. The hematological and biochemical findings of our research demonstrated that EtEF did not induce acute or subacute toxicity in the mouse model. Both the liver and kidneys are highly vulnerable to damage when exposed to high concentrations or prolonged exposure to toxic substances (Asif 2012). Upon exposure to toxic agents, an increase in liver weight is observed due to hepatocellular degeneration and necrosis, leading to enhanced cell membrane permeability and the release of enzymes into the bloodstream (Arfat et al. 2014). In cases of renal toxicity, fluid and waste retention occurs as a result of nephron damage, leading to reduced filtration capacity and a relative increase in kidney weight (Craig et al., 2015).

In the current study, the relative weights of the liver and kidney in the EtEF treatment group were not significantly different from those in the control group. Additionally, biochemical markers of liver and kidney function (AST, ALT, creatinine, and urea) did not change much. This indicates that EtEF does not cause liver and kidney damage at the tested dosage levels.

In most animals, there is an excretory system to eliminate excess substances, maintain chemical homeostasis, and prevent harm to the body. Dissolved waste is mainly excreted through urine (Lee et al. 2019). When animals are exposed to high levels or prolonged exposure to toxic xenobiotics, their internal organs can be seriously affected. Therefore, the heart, liver, and kidneys become the main detoxifying organs of the body. During the process of detoxifying xenobiotics, the toxins are eliminated through the liver and kidneys by hydrolysis, oxidation-reduction, dissolution, and excretion of the toxins through urine (Hodges and Minich 2015). The composition and concentration of substances in the urine of mice treated with EtEF did not show any abnormal changes and were not significantly different from the control group. This indicates that EtEF is safe at the evaluated dosage level.

Conclusions

The toxicity study of ethanol extract from *E. fusca* fruits did not produce adverse effects on the behavior and overall response of Swiss albino mice at the tested doses. Therefore, the oral LD_{50} of EtEF was greater than 5000 mg/kg. The acute and sub-acute toxicity investigations showed that EtEF did not negatively affect body weight, hematological parameters, blood biochemistry, relative organ weights, cardiac, hepatic, and renal histology. No signs of toxicity were observed in experimental mice. The study results clearly indicate the safety profile of ethanol extract from *E. fusca* fruit.

Acknowledgments

The authors are very grateful to the colleagues from the Department of Pathological Anatomy, Department of Biochemistry and Department of Hematology, 175 Military Hospital, the Pauster Institute of Ho Chi Minh city, the Institute of Biotechnology and Food-technology, Industrial University of Ho Chi Minh City, for their support in services and facilities during this project.

References

Adedapo AA, Abatan MO, Olorunsogo OO (2004) Toxic effects of some plants in the genus *Euphorbia* on haematological and biochemical parameters of rats. Vet Arh 74(1):53-62.

Al-Afifi NA, Alabsi AM, Bakri MM, Ramanath A (2018) Acute and sub-acute oral toxicity of *Dracaena cinnabari* resin methanol extract in rats. BMC Complementary Altern Med 18(1):50.

Al-Awthan YS, Zarga MA, Abdalla S (2010) Flavonoids content of *Dracaena cinnabari* resin and effects of the aqueous extract on isolated smooth muscle preparations, perfused heart, blood pressure and diuresis in the rat. Jordan J Pharm Sci 3(1):8-16.

Anthanont P, Jensen MD (2016) Does basal metabolic rate predict weight gain? Am J Clin Nutr 104(4):959-963.

Arfat Y, Mahmood N, Tahir MU, Rashid M, Anjum S, Zhao F, Li DJ, Sun YL, Hu L, Zhihao C, Yin C, Shang P, Qian AR (2014) Effect of imidacloprid on hepatotoxicity and nephrotoxicity in male albino mice. Toxicol Rep 1:554-561.

Asif M (2012) A brief study of toxic effects of some medicinal herbs on kidney. Adv Biomed Res 1:44.

Ayoola GA, Coker HA, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC, Atangbayila TO (2008) Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in Southwestern Nigeria. Trop J Pharm Res 7(3):1019-1024.

Azmi AS, Rahim NA, Zahari Z, Salim F (2020) Cytotoxic activity of *Erythrina fusca* Lour. leaf, twig and flower extracts. Malays J Anal Sci 24(3):313-319.

Bariweni MW, Yibala OI, Ozolua RI (2018) Toxicological studies on the aqueous leaf extract of *Pavetta crassipes* (K. Schum) in rodents. J Pharm Pharmacogn Res 6(1):1-16.

Bello I, Bakkouri AS, Tabana YM, Al-Hindi B, Al-Mansoub MA, Mahmud R, Asmawi MZ (2016) Acute and sub-acute toxicity evaluation of the methanolic extract of *Alstonia scholaris* stem bark. Med Sci 4(1):4.

Bespalov A, Wicke, K, Castagné V (2019) Blinding and randomization. In Bespalov A, Michel M, Steckler T, Ed., Good Research Practice in Non-Clinical Pharmacology and Biomedicine. Springer Nature, Switzerland, 81-100.

Burn CC, Mazlan NHB, Chancellor N, Wells DJ (2021) The pen is milder than the blade: identification marking mice using ink on the tail appears more humane than ear-punching even with local anaesthetic. Animals 11(6):1664.

Cavanaugh BM (2002) Nurse's manual of laboratory and diagnostic tests, 4th ed. F.A. Davis Company, Philadelphia.

Craig EA, Yan Z, Zhao QJ (2015) The relationship between chemical-induced kidney weight increases and kidney histopathology in rats. J Appl Toxicol 35(7):729-736.

Dewick PM (2002) Medicinal Natural Products: A Biosynthetic Approach, 3rd ed. John Wiley & Sons, West Sussex.

Edeoga HO, Okwu DE, Mbaebie BO (2005) Phytochemical constituents of some Nigerian medicinal plants. Afr J Biotechnol 4(7):685-688.

El-Hilaly J, Israili ZH, Lyoussi B (2004) Acute and chronic toxicological studies of *Ajuga iva* in experimental animals. J Ethnopharmacol 91(1):43-50.

Evans W (2009) Trease and Evans' pharmacognosy, 16th ed. Elsevier, London.

Ghosh D, Mondal S, Ramakrishna K (2019) Acute and sub-

acute (30-day) toxicity studies of *Aegialitis rotundifolia* Roxb., leaves extract in Wistar rats: safety assessment of a rare mangrove traditionally utilized as pain antidote. Clin Phytosci 5(1):13.

Guidelines for preclinical and clinical trials of traditional medicines and herbal medicines (No. 141/QĐ-K2ĐT) (2015) Ministry of Health, Department of Science, Technology and Training. Hanoi. (In Vietnamese)

Ha AW, Kang HJ, Kim SL, Kim MH, Kim WK (2018) Acute and subacute toxicity evaluation of corn silk extract. Prev Nutr Food Sci 23(1):70-76.

Harborne JB (1998) Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis, 3rd ed. Springer Dordrecht, London.

Hodges RE, Minich DM (2015) Modulation of metabolic detoxification pathways using foods and food-derived components: a scientific review with clinical application. J Nutr Metab 2015:760689.

Hurst SA (2014) Declaration of Helsinki and protection for vulnerable research participants. Jama 311(12):1252.

Kuriyan R, Raj T, Srinivas SK, Vaz M, Rajendran R, Kurpad AV (2007) Effect of *Caralluma fimbriata* extract on appetite, food intake and anthropometry in adult Indian men and women. Appetite 48(3):338-344.

Kurtz DM, Feeney WP (2019) The influence of feed and drinking water on terrestrial animal research and study replicability. ILAR J 60(2):175-196.

Lawrence TS, Robertson JM, Anscher MS, Jirtle RL, Ensminger WD, Fajardo LF (1995) Hepatic toxicity resulting from cancer treatment. Int J Radiat Oncol Biol Phys 31(5):1237-1248.

Lee MJ, Jung HK, Lee KH, Jang JH, Sim MO, Seong TG, Ahn BK, Shon JH, Ham SH, Cho HW, Kim YM, Park SJ, Yoon JY, Ko JW, Kim JC (2019) A 90-day repeated oral dose toxicity study of *Alismatis rhizoma* aqueous extract in rats. Toxicol Res 35(2):191-200.

Michael B, Yano B, Sellers RS, Perry R, Morton D, Roome N, Johnson JK, Schafer K, Pitsch S (2007) Evaluation of organ weights for rodent and non-rodent toxicity studies: a review of regulatory guidelines and a survey of current practices. Toxicol Pathol 35(5):742-750.

Mlozi SH, Mmongoyo JA, Chacha M (2020) The in vivo toxicity evaluation of leaf and root methanolic extracts of *Tephrosia vogelii* Hook.f using animal model. Clin Phytosci 6(1):73.

Office of Laboratory Animal Welfare (OLAW) (2015) Public Health Service Policy on Human Care and Use of Laboratory Animals. Department of Health and Human Services, NIH Publication, USA.

Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, Lilly P, Sanders J, Sipes G, Bracken W, Dorato M, Deun KV, Smith P, Berger B, Heller A (2000) Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul Toxicol Pharmacol 32(1):56-67.

Organisation for Economic Cooperation and Development (OECD No. 423) (2001) Acute Oral Toxicity – Acute Toxic Class Method.

Organisation for Economic Cooperation and Development (OECD No. 408) (2018) Repeated Dose 90-day Oral Toxicity Study in Rodents.

Pariyani R, Ismail IS, Azam AA, Abas F, Shaari K, Sulaiman MR (2015) Phytochemical screening and acute oral toxicity study of Java tea leaf extracts. BioMed Res Int 2015:742420.

Pei R, Liu X, Bolling B (2020) Flavonoids and gut health. Curr Opin Biotechnol 61:153-159.

Santos EW, Oliveira DC, Hastreiter A, Silva GB, Beltran JSO, Tsujita M, Crisma AR, Neves SMP, Fock RA, Borelli P (2016) Hematological and biochemical reference values for C57BL/6, Swiss Webster and BALB/c mice. Braz J Vet Res Anim Sci 53(2):138-145.

Sofowora A (1993) Medicinal Plants and Traditional Medicine in Africa. Spectrum Books, Ibadan.

Thunhorst RL, Beltz TG, Johnson AK (2010) Drinking and arterial blood pressure responses to ANG II in young and old rats. Am J Physiol Regul Integr Comp Physiol 299(5):1135-1141.

Toghueo RMK (2020) Bioprospecting endophytic fungi from *Fusarium* genus as sources of bioactive metabolites. Mycology 11(1):1-21.

Tom ENL, Nyunai N, Djaouro KG, Medou FM, Nankia FD, Dimo T (2018) Acute and subacute toxicity evaluation of the stem bark aqueous extract of *Harungana madagascariensis* in rodents. J Adv Pharm Sci Technol 1(4):1-12.

Tucci SA (2010) Phytochemicals in the control of human appetite and body weight. Pharmaceuticals (Basel) 3(3):748-763.

Ugwah-Oguejiofor CJ, Okoli CO, Ugwah MO, Umaru ML, Ogbulie CS, Mshelia HE, Umar M, Njan AA (2019) Acute and sub-acute toxicity of aqueous extract of aerial parts of *Caralluma dalzielii* NE brown in mice and rats. Heliyon 5(1):01179.

Upadhyay P, Shukla R, Tiwari KN, Dubey GP, Mishra SK (2019) Toxicity assessment of the alcoholic leaves extract of *Reinwardtia indica*. Braz J Pharm Sci 55:18224.

World Health Organization (WHO) (2000) General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine.

Wu Y, Hu S, Yang D, Li L, Li B, Wang L, Li M, Wang G, Li J, Xu Y, Zhang X, Niu C, Speakman JR (2022) Increased variation in body weight and food intake is related to increased dietary fat but not increased carbohydrate or protein in mice. Front Nutr 9:835536.