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Research article

Toxicity studies of condensed fuzheng extract in mice and rats

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ABSTRACT

Nutritional supplements have been used to improve immune function. Condensed fuzheng extract (CFE) is a well-known traditional Chinese medicine (TCM) formula that is predominantly made from sheep placenta, *Astragalus mongholicus* Bunge, and *Polygonatum kingianum* Collett & Hemsl. However, the toxicological profile of CFE has not been determined. In this study, we investigated the acute (14 days) and sub-chronic (90 days) oral toxicities of CFE in mice and rats and the phytochemical composition of CFE.

Materials and methods: For the assessment of acute toxicity, 80 ICR mice of both sexes were randomly divided into four groups. Three groups were treated with 4500, 2250 and 1125 mg/kg/d bw CFE daily (n = 10/group per sex) for 14 days; a separate group was used as control. To test the sub-chronic toxicity, male and female Sprague Dawley rats were orally administered 8150, 4075 or 2037 mg/kg bw of CFE for 90 days; a control group was included. Hematological, biochemical, and histopathological markers were tested at the end of the experiment. The chemical composition of CFE was determined by UPLC-HRMS method.

Results: In both acute and sub-chronic toxicity studies, no mortalities, indications of abnormality, or treatment-related adverse effects were observed. The LD50 of CFE was higher than 4500 mg/kg. There were no significant changes in the hematological and biochemical data in the treatment group compared with the control group ($p > 0.05$). Histopathological analyses of the heart, liver, spleen, lungs, kidneys, thymus, testes (male rats) and ovaries (female rats) revealed no anatomical changes of each organ. Phytochemical analysis of CFE revealed the presence of flavonoids (highest abundance), phenols and alkaloids. In conclusion, our results showed that CFE is a safe and non-toxic formula. We also reported phytochemicals in CFE that may possess important pharmacological effects.

Abbreviations: CFE, Condensed fuzheng extract; WBC, White cell count; PLT, Platelet; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BUN, Blood urea nitrogen; Cr, Creatinine; HG, Hemoglobin; HCT, Red blood cell-specific volume; MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; RDW, Red cell volume distribution width; PDW, Platelet distribution width; PCT, Procalcitonin; ALB, Albumin; TG, Triglyceride; TC, Total cholesterol; TBIL, Total bilirubin; TP, Total protein; ALP, Alkaline phosphatase; GLU, Glucose; CK, Creatine kinase; OECD, Organization for Economic Cooperation and Development.

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1. Introduction

As a natural barrier of the body, the immune system has the role of immune surveillance and defense. The immune system includes two types: intrinsic immunity (non-specific immunity) and adaptive immunity (specific immunity), which includes cellular immunity mediated by T lymphocytes and humoral immunity mediated by B lymphocytes. The developmental status of immune organs directly affects the immune function of animals. The thymus is the central immune organ and the site of T-lymphocyte differentiation and maturation. The spleen is the largest peripheral immune organ and is the main site of immune response in the organism. Studies have shown that hundreds of millions of people worldwide are immunocompromised, and certain diseases such as aging and depression can cause immunocompromise. When the body is immunocompromised, there is a higher chance of developing diseases such as diabetes and malignancies. Low immune function thus has a serious impact on the quality of life and health of people.

Traditional Chinese medicine (TCM) has received wide acceptance across Asian countries due to its applications in the prevention and treatment of various diseases [1]. In recent years, the interest in TCM has been growing both in terms of market demand and as a subject of scientific research [2]. Herbal medicines are popular and generally preferred by the general public over other alternative medicine; however, their side effects and concerns over their safety are less frequently addressed by research [3]. Therefore, more research in this direction is needed to address public health questions about the use of TCM. In this direction, the availability of experimental data regarding the toxicity of herbal medications, especially of the various traditional Chinese medicine (TCM) formulations, it is of great importance prove handy and valuable [4]. The assessment of potential toxicity is a necessary step towards promoting routine therapeutic usage of TCM.

CFE is a nutritional supplement consisting of sheep placenta, Bunge (*Astragalus mongholicus*), and Coll et Hemsl (*Polygonatum kingianum*) (Table 1) and has the effect of improving immune deficiency. Modern pharmacological studies have shown that CFE can improve the histopathological morphology of immune organs, immune cell function and immune molecule expression levels in mice with immune hypofunction caused by cyclophosphamide. Placenta Peptide Can Protect from Mitochondrial Dysfunction by inhibiting reactive oxygen species (ROS) and tumor necrosis factor-alpha (TNF-α) generation, by maintaining the mitochondrial dynamic network and increasing interleukin 6 (IL-6) level during chronic fatigue. Several beneficial activities of Bunge (*Astragalus mongholicus*) and Coll et Hemsl (*Polygonatum kingianum*) on human diseases have been reported [5]. Bunge (*Astragalus mongholicus*) [6] contains significant antioxidant, anti-inflammatory, and immune-boosting properties and is widely used to treat cases of compromised immune functions [7]. Coll et Hemsl (*Polygonatum kingianum*) has anti-tumor effects as well as anti-osteoporosis, neuroprotective, immunomodulatory, anti-diabetic, anti-fatigue [8] and other pharmacological effects [9].

Several studies have demonstrated the effects of CFE on therapeutic effects against cancer-induced fatigue and leukopenia after radiotherapy and chemotherapy. However, the toxicological profile of CFE has not been well studied. Hence, in the present study, we performed acute (14 days) and sub-chronic (90 days) oral toxicity studies of CFE in mice and rats. To our knowledge, this report is the first to experimentally assess the safety of CFE and conforms to the Good Laboratory Practice, the Compendium of Technical Guidelines for Veterinary Drug Research and relevant Organisation for Economic Co-operation and Development (OECD) guidelines.

2. Material and methods

2.1. Materials and preparation of CFE

CFE was kindly provided by the Mongolian Medicine Hospital of Alxa League in Inner Mongolia. The batch number of the product was 20200615. CFE was synthesized using a patented methodology (national patent number 202110881011.5), and the three medicinal herbs and proportions in CFE are presented in Table 1. The method of preparation of CFE is briefly outlined below. First, 35 kg of sheep placenta and 35 kg of water were combined followed by protease fermentation for 24 h. Then, a mixture of 2 kg of Bunge (*Astragalus mongholicus*) and 2 kg Coll et Hemsl (*Polygonatum kingianum*) were added to 120 kg of water and kept under boiling condition for 4 h. The fermentation broth was added to the mixture composed of Bunge (*Astragalus mongholicus*) and Coll et Hemsl (*Polygonatum kingianum*) and the mixture was centrifuged at 9000 rpm for 30 min. After isolation, the supernatant was isolated (approximately 500 mL), 2 kg brown of sugar were added and the mixture was kept under boiling condition for 2 h. A final volume of 3 L of CFE was obtained for subsequent experiments.

2.2. Experimental animals

The animal testing procedures were approved by the Tianjin University of Chinese Medicine Laboratory Animal Welfare Ethics Committee (Nos. TCM-LAEC2020047 and TCM-LAEC2020048). Male (n = 40) and female (n = 40) mice of Cancer Research (ICR) and

Table 1
The composition of CFE.

Chinese name	Full name	Medicinal part	Proportion (%)	data supplied
Yangtaipan	–	Dried Sheep Placenta	35kg/39 kg	–
Huangqi	*Astragalus mongholicus* Bunge.	Radix Astragali	2kg/39 kg	2010-07-14
Huangjin	*Polygonatum kingianum* Coll.et Hemsl.	Rhizoma Polygonati	2kg/39 kg	2012-03-23

Note: The plant name has been checked with http://www.theplantlist.org.

male (n = 40) and female (n = 40) Sprague-Dawley rats (SD) were orally exposed to CFE to investigate its acute and sub-chronic toxicity profiles. Male and female ICR mice (19–21 g; SCXK (JING) 2019-0010) and male and female SD rats (130–150 g, 120–140 g; SCXK (JING) 2016-0006) were purchased from the SPF (Beijing) Biotechnology Co., Ltd. Animals were housed at 24.5 ± 0.5 °C with a 12-h light/dark cycle and free access to food and water (males and females housed separately); the animals underwent seven days of acclimatation before experiments.

2.3. HPLC fingerprints of CFE

The high-performance liquid chromatography-diode array detection (HPLC-DAD) was quantified using Waters 2695 series HPLC system linked to the autosampler, diode array detector, quaternary pump and DAD detector. The Waters Spherisorb ODS column (250 mm × 4.6 mm, 5 μm) was kept at 30 °C. The mobile phase consisted of solvent A (water with 0.5 % phosphoric acid) and solvent B (acetonitrile). The running duration was 30 min with the following gradient: 0–15 min, 5–15 % B, 15–30 min, 15–40 % B. 10 μL was injected used for the final washing and equilibration. The flow rate was set to 1 mL min− 1, with an injection volume set to 10 μL [10].

2.4. Acute toxicity study

Acute oral toxicity studies were carried out in accordance with the Technical Guidelines for Veterinary Drug Research (Veterinary Drug Assessment Centre of the Ministry of Agriculture of the Peoples Republic of China, 2012). Eighty mice weighing 19–21 g were randomly divided into four groups: a control group (CG, n = 10 males and 10 females/group), a high-dose group (CFE-H, n = 10 males and 10 females/group), a medium-dose group (CFE-Z, n = 10 males and 10 females/group) and a low-dose group (CFE-L, n = 10 males and 10 females/group). The mice were administered 4500, 2250 and 1125 mg/kg/d CFE by gavage with 0.2 ml per animal orally for 14 days. The dose selection for the CFE-H group was the maximum dose recommended for mice, and the dose for the CFE-Z group was half of the maximum dose; the control group received an equivalent volume of physiological saline [11,12].

2.5. Sub-chronic toxicity study

The sub-chronic oral toxicity study was conducted in accordance with guidelines 408 of the Organization of Economic Co-operation and Development [11]. Eighty SD rats weighing 170–240 g were randomly divided into four groups: a control group (CG, n = 10 males and 10 females/group), a high-dose group (CFE-H, n = 10 males and 10 females/group), a medium-dose group (CFE-Z, n = 10 males and 10 females/group) and a low-dose group (CFE-L, n = 10 males and 10 females/group). Treatment groups were given CFE orally at doses of 8150, 4075 and 2037 mg/kg in 4 ml per animal orally for three months. The dose selection for the CFE-H group was the maximum dose recommended for rats, and the dose for the CFE-Z group was half of the maximum dose; the control group was treated with the same volume of physiological saline. The weight of each animal was recorded weekly during the study. Treatment-induced changes in animal behaviors and physical characteristics were monitored throughout the experiment. Upon completion of the treatment schedule, rats were administered an intraperitoneal injection of a polyurethane anesthetic (2 ml/100 g) after overnight fasting (12–16 h) [13].

2.5.1. Hematology

Blood samples were collected from anaesthetized rats by puncturing the abdominal aorta. Complete blood was drawn into an EDTA test tube (containing EDTA potassium salt). Counts of white blood cells (WBC), lymphocytes (LYMPH#) and monocytes (MONO#) were recorded. Hematological analysis was performed using an automatic blood cell analyzer (Mindray® BC-2800Vet) [14].

2.5.2. Serum biochemistry

Blood samples were collected in empty tubes and centrifuged (5 °C, 3000 rpm) for 15 min. Sera were analyzed using an automatic biochemical analyzer (Mindray®BS-240VET). Clinical biochemistry parameters assessed in serum included alanine amino-transferase (ALT), aspartate aminotransferase (AST), albumin (ALB), triglyceride (TG), total cholesterol (TC), total bilirubin (TBIL), total serum protein (TP), alkaline phosphatase (ALP), glucose (GLU), creatinine (Cr), blood urea nitrogen (BUN) and creatine kinase (CK) [15].

2.5.3. Organic index

The animals were dissected. The heart, liver, kidneys, lungs, spleen, thymus, epididymis, testes, uterus, ovary and other vital organs were harvested, rinsed in cold saline and wiped using an absorbent paper before weighing the organ (in grams) [16]. The relative organ weight (ROW) was computed for each animal:

$$\text{ROW} = [\text{absolute organ weight (g)} \div \text{weight of animal on the day of sacrifice (g)}] \times 100$$

2.5.4. Histopathology

After measuring the weights, the organs were quickly fixed in 4 % paraformaldehyde. Thin sections (5 mm) of each organ were prepared using a micro-cutting machine (Leica RM2235) followed by hematoxylin and eosin (H&E) staining. Histopathological changes in the control and treatment groups were observed using a microscope and recorded [17].

2.6. Statistical analysis

Data are expressed as mean ± standard deviation. Statistical differences between the control and treatment groups were evaluated by a single-factor analysis of variance (ANOVA) followed by Dunnett's test. GraphPad Prism 11.0 was used to compare the findings between sexes across all experimental groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Analysis of CFE

The chromatographic peaks with higher abundance in the base peak chromatogram (BPC) diagram were examined for peak shape and confirmed by secondary spectrogram; the chromatographic peak numbers were marked by positive and negative ion graphs in numerical order (Fig. 1A, B). As shown in Table 2, the 32 peaks marked in CFE mainly contain carboxylic acids, indoles, pyrrolidines, quinolines, benzenes, flavonoids, lipids and other compounds. The components of sheep placenta, Bunge (*Astragalus mongholicus*) and Coll et Hemsl (*Polygonatum kingianum*) were all measured separately [18–20].

3.2. Acute toxicity study

In the acute toxicity studies, no casualties or physical changes were observed in the mice treated with 4500, 2250 and 1125 mg/kg/d CFE compared with controls. Similarly, no noticeable differences in body weights based on sex were evident between the treatment and control groups (Fig. 2A, B).

3.3. Sub-chronic toxicity study

3.3.1. Mortality and body weight

In comparing the body weights of control and treatment groups (Fig. 3A, B), no statistical differences across the groups were found throughout the study. All treatment groups showed similar levels of body activities as the control group. Additionally, no signs of toxicity or mortality were evident after 13 weeks in any of the experimental groups (CG, CFE-H, CFE-Z, CFE-L).

3.3.2. Hematology analysis

The effects of treatments (CG, CFE-H, CF-Z, CFE-L) on the blood index of male and female SD rats were examined (Table 3). Hematological analysis showed no changes in counts of white blood cells, red blood cells, lymphocytes and other indicators in response to CFE treatment.

3.3.3. Clinical biochemistry

The treatment effects of CFE treatments (CG, CFE-H, CFE-Z, CFE-L) on serum AST, ALT, ALP, total bilirubin, total protein and albumin were examined, with no noticeable variability in the treatment groups compared with controls (Table 4). Similarly, serum electrolyte parameters (Na^+, Cl^-, K^+) were comparable between different treatment groups.

3.3.4. Organic index

Organic index is a valuable tool for screening the internal organs for physical abnormality from chronic treatment exposure (Table 5). Evidence of treatment-induced changes to internal organs was too hard to be deciphered. Thus, we speculate that CFE

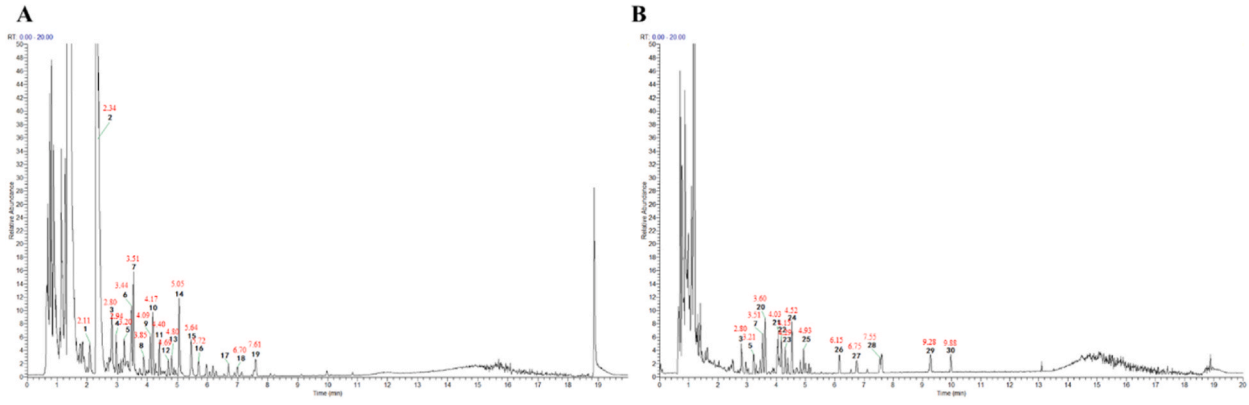

Fig. 1. Analysis of the components of CFE. (A) BPC diagram in positive ion mode-standard peak, (B) BPC diagram in negative ion mode-standard peak.

Table 2
Primary chemical ingredients analysis of CFE ((μmol/g).

Peak neam	RT min	*m/z*	adduct	compound name	score	Amount
1	2.11	169.0966	[M+H]+	3-Methylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione	0.9977	1.28
2	2.34	166.0860	[M+H]+	Phenylalanine	0.9998	3.25
3	2.80	203.1388	[M+H]+	Alanyl-Leucine	0.9921	5.12
4	2.94	231.1700	[M+H]+	DL-Leu-DL-Val	0.996	2.12
5	3.20	231.1700	[M+H]+	Ile-Val	0.879	2.08
6	3.44	205.0969	[M+H]+	L-Tryptophan	0.9929	3.14
7	3.51	231.1699	[M+H]+	Val-Leu	0.9979	3.55
8	3.85	197.1282	[M+H]+	Cyclo(val-pro)	0.9064	4.58
10	4.17	245.1863	[M+H]+	Ile-Leu	0.9977	14.23
11	4.40	245.1855	[M+H]+	Ile-Ile	0.9994	3.47
12	4.69	279.1699	[M+H]+	Ile-Phe	0.9968	5.25
13	4.80	211.1439	[M+H]+	Cyclo(isoleucylprolyl)	0.9816	12.2
14	5.05	211.1438	[M+H]+	L,L-Cyclo(leucylprolyl)	0.975	15.2
15	5.46	245.1279	[M+H]+	DL-Prolylphenylalanine	0.9542	12.3
16	5.72	284.1388	[M+H]+	Brevianamide F	0.8868	8.45
18	6.70	227.1750	[M+H]+	Crotetamide	0.7164	5.63
19	7.61	309.0863	[M+H]+	Alprazolam	0.7433	1.21
3	2.80	201.1234	[M − H]-	Alanyl-Leucine	0.9836	2.36
5	3.21	229.1549	[M − H]-	Ile-Val	0.9961	14.2
7	3.51	229.1548	[M − H]-	Val-Leu	0.9952	14.1
20	3.60	227.1028	[M − H]-	PyroGlu-Val	0.9846	13.8
21	4.03	261.0873	[2M − H]-	5-[(4-Ethoxyphenoxy)methyl]-2-furoic acid	0.8222	14.4
22	4.15	243.1706	[M − H]-	Leu-Leu	0.9952	12.8
23	4.29	130.0861	[M − H]-	Leucine	0.9995	18.6
24	4.52	483.2450	[M − H]-	2-Piperazinepropionic acid, 5-isobutyl-3,6-dioxo-	0.9942	17.6
25	4.93	275.1021	[M − H]-	Ofloxacin	0.7124	14.7
26	6.15	187.0965	[M − H]-	Azelaic acid	0.9865	18.5
27	6.75	137.0228	[M − H]-	3-Hydroxybenzoic acid	0.9985	16.1
28	7.55	283.0604	[M − H]-	Acacetin	0.9936	1.71
29	9.28	329.2325	[M − H]-	9-Octadecenoic acid, 5,8,11-trihydroxy-	0.984	0.85
30	9.98	267.0654	[M − H]-	Formononetin	0.9924	1.22

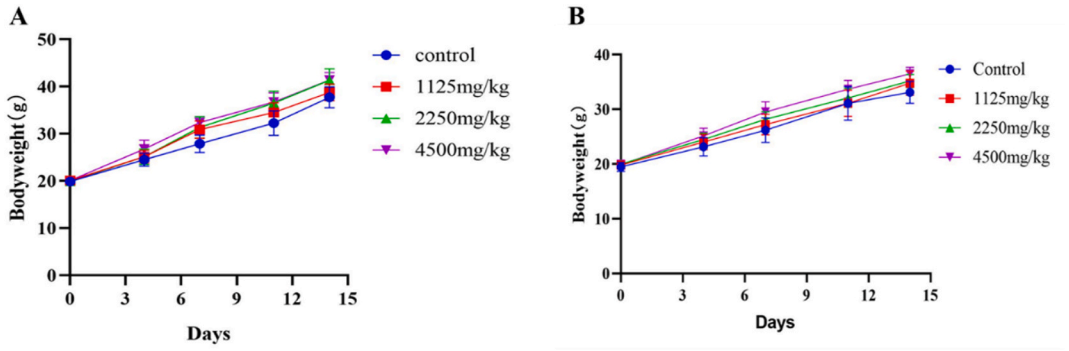

Fig. 2. (A) Body weights of male ICR mice measured for the 14 days of oral CFE exposure (n = 10). (B) Body weights of female ICR mice measured for the 14 days of oral CFE exposure (n = 10). No statistically significant differences based on sex were evident.

exposure may be safe on the key internal organs, including the heart, spleen, liver, thymus, lungs, spleen, adrenal glands, kidneys, testes (male), epididymis (male), uterus (female), and ovaries (female) if used in routine medical care of patients. No significant differences in absolute and relative organ weights between different treatment groups based on sex were found.

3.3.5. Histopathology analyses

Even after 90 days of chronic exposure to CFE, histopathological studies of sliced organs revealed no statistically significant variations that can be attributed to treatments. There were no significant changes in cardiac, hepatic, splenic, renal, thymic, testicular (male), and uterine (male) pathology in CFE-L, CFE-M, and CFE-H groups compared with controls. In the heart, myocardial fibers were neatly aligned and well defined, with no breaks in the cardiac machinery fibers (Figs. 4A and 5A). In the liver, liver cells were structurally normal in morphology and there was no necrosis and inflammatory cell infiltration (Figs. 4B and 5B). In the spleen, splenic tissues were normal in morphology, and peritubes, trabeculae, white medulla, and red medulla structures were clear; the inner wall of the splenic corpuscle was not enlarged. Red medulla oblongata were clear, the splenic corpuscle was not enlarged, the germinal center was obvious, the inner wall of the splenic sinusoid was smooth, and no congestion and lymphocytes were seen (Figs. 4C and 5C). In the

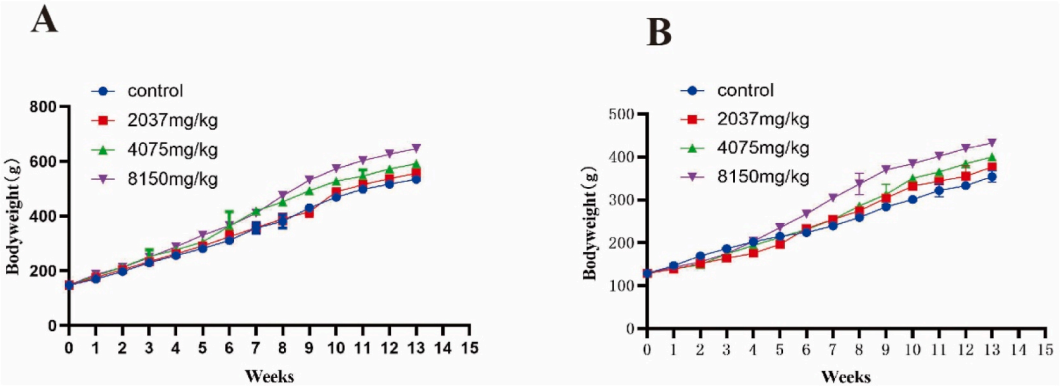

Fig. 3. (A) Body weights of male SD rats measured for the 90 days of oral CFE exposure (n = 10). (B) Body weights of female SD rats measured for the 90 days of oral CFE exposure (n = 10). No statistically significant differences based on sex were evident.

Table 3
Hematological indexes of male rats treated with CFE for 90 days.

Parameter	Male				Female			
	Control	CFE-L	CFE-M	CFE-H	Control	CFE-L	CFE-M	CFE-H
WBC# ($\times 10^9$/L)	10.56 ± 2.64	11.61 ± 1.72	10.67 ± 1.95	10.56 ± 1.70	6.95 ± 2.37	6.53 ± 2.90	7.57 ± 2.02	5.74 ± 1.48
Lymph# ($\times 10^9$/L)	8.3 ± 2.39	9.01 ± 1.37	7.87 ± 0.75	7.59 ± 0.79	5.48 ± 1.96	4.76 ± 1.35	5.97 ± 1.53	4.31 ± 1.32
Mon# ($\times 10^9$/L)	0.26 ± 0.07	0.29 ± 0.07	0.26 ± 0.08	0.23 ± 0.64	0.14 ± 0.07	0.17 ± 0.06	0.14 ± 0.04	0.14 ± 0.05
Gran# ($\times 10^9$/L)	2.04 ± 0.58	2.03 ± 0.59	2.10 ± 0.38	2.24 ± 0.33	0.88 ± 1.17	1.03 ± 0.34	1.05 ± 0.18	1.2 ± 0.42
Lymph% (%)	75.61 ± 11.50	80.74 ± 4.03	77.22 ± 1.52	69.83 ± 5.25	79.93 ± 12.01	80.52 ± 3.61	80.45 ± 8.43	76.27 ± 3.55
Mon% (%)	2.47 ± 0.35	2.65 ± 0.52	2.67 ± 0.24	2.82 ± 0.32	2.23 ± 0.35	2.28 ± 0.47	2.1 ± 0.39	2.15 ± 0.32
Gran% (%)	17.19 ± 1.16	16.61 ± 3.66	16.8 ± 0.68	17.38 ± 1.26	14.52 ± 3.27	16.9 ± 3.20	16.83 ± 6.66	18.1 ± 1.52
RBC ($\times 10^{12}$/L)	6.76 ± 0.31	6.67 ± 0.81	6.33 ± 0.49	6.26 ± 0.39	7.91 ± 0.37	7.91 ± 0.65	7.45 ± 1.20	7.55 ± 0.53
HGB (g/L)	139.00 ± 4.58	139.10 ± 4.03	136.9 ± 6.11	135.00 ± 3.16	145.4 ± 7.77	147.8 ± 8.28	142.9 ± 20.62	140.6 ± 4.74
HCT (%)	40.47 ± 3.75	40.94 ± 2.28	40.71 ± 2.20	41.18 ± 2.42	42.82 ± 2.10	43.58 ± 1.67	42.61 ± 4.65	42.69 ± 1.74
MCV (fL)	54.72 ± 0.77	56.70 ± 1.65	55.29 ± 1.58	56.73 ± 1.53	60.26 ± 1.31	58.78 ± 1.77	61.26 ± 2.68	61.79 ± 1.88
MCH (pg)	17.32 ± 0.50	18.29 ± 0.55	17.99 ± 0.55	18.32 ± 0.41	18.34 ± 0.34	18.82 ± 0.82	19.17 ± 0.89	19.26 ± 0.54
MCHC (g/L)	317.20 ± 5.81	323.80 ± 4.04	325.70 ± 3.61	323.60 ± 3.44	304.8 ± 3.76	320.8 ± 5.06	314.1 ± 3.56	312.3 ± 3.41
RDW (%)	12.15 ± 1.25	12.04 ± 0.71	11.56 ± 0.42	11.97 ± 0.67	12.76 ± 1.01	12.3 ± 1.19	13.09 ± 1.06	12.52 ± 0.77
PLT ($\times 10^9$/L)	1279.50 ± 274.24	1135.10 ± 224.62	1186.0 ± 104.17	1227.80 ± 208.03	1261.8 ± 93.34	1166.5 ± 154.02	1316.5 ± 172.69	1358.7 ± 127.27
MPV (fL)	5.88 ± 0.24	5.86 ± 0.35	6.02 ± 0.21	6.18 ± 0.33	5.58 ± 0.16	5.57 ± 0.24	5.87 ± 0.32	5.93 ± 0.16
PDW	15.92 ± 0.19	16.01 ± 0.18	15.90 ± 0.10	15.96 ± 0.16	16.04 ± 0.11	15.869 ± 0.12	15.99 ± 0.18	16.05 ± 0.92
PCT (%)	0.63 ± 0.04	0.60 ± 0.10	0.68 ± 0.02	0.63 ± 0.02	0.65 ± 0.03	0.60 ± 0.05	0.67 ± 0.02	0.66 ± 0.01

Note: The values in the table represent mean ± SEM, n = 10. No noticeable difference between control group and CFE-treated groups, White blood cell count (WBC), lymphocyte (Lymph), Monocytes (Mon), Neutrophil granulocyte (Gran), Red blood cell (RBC), Hemoglobin (HGB), Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Red Blood Cell distributionwidth (RDW), Platelet Count (PLT), Mean Platelet Volume (MPV), Platelet Distribution Width (PDW), Platelet Hematocrit (PCT).

lungs, the lung tissue structure was normal, the alveolar septum and alveolar lumen were intact, and the interstitium of the lungs was uniformly distributed (Figs. 4D and 5D). In the kidney, glomerular vascular collaterals were thin and clear, the surrounding renal tubules were structurally intact, the lumen was clear, basement membrane was intact, epithelial cells of the renal tubules were neatly arranged, and there was no vacuolated cell formation and no inflammatory cell infiltration under the microscope (Figs. 4E and 5E). In the thymus, the thymus peritoneum was complete, cortex was thicker, and the border of medulla was clear (Figs. 4F and 5F). In the

Table 4
Biochemical indices of males and female rats treated with CFE for 90 days.

Parameter	Male				Female			
	Control	CFE-L	CFE-M	CFE-H	CFE-L	CFE-M	CFE-H	8150 mg/kg
ALT (U/L)	30.05 ± 1.71	33.85 ± 2.44	32.60 ± 1.91	32.23 ± 1.66	31.34 ± 1.39	30.98 ± 1.25	32.55 ± 2.49	32.77 ± 3.21
AST (U/L)	70.97 ± 13.28	70.78 ± 3.95	68.58 ± 14.27	69.63 ± 3.75	69.49 ± 15.67	67.08 ± 8.41	68.84 ± 5.23	67.6 ± 3.51
ALB (U/L)	33.91 ± 3.76	34.57 ± 4.49	35.68 ± 5.71	32.73 ± 3.89	43.57 ± 9.10	43.46 ± 6.61	42.69 ± 5.44	41.25 ± 4.29
TG (mmol/L)	0.78 ± 0.19	0.77 ± 0.27	0.72 ± 0.13	0.79 ± 0.11	0.54 ± 0.14	0.55 ± 0.12	0.52 ± 0.12	0.52 ± 0.19
TC (mmol/L)	1.62 ± 0.14	1.66 ± 0.07	1.68 ± 0.09	1.62 ± 0.13	1.33 ± 0.08	1.38 ± 0.11	1.36 ± 0.13	1.38 ± 0.13
TBIL (μmol/L)	3.41 ± 0.23	3.42 ± 0.12	3.48 ± 0.17	3.45 ± 0.15	3.07 ± 0.25	2.96 ± 0.21	3.06 ± 0.22	3.03 ± 0.17
TP (g/L)	69.23 ± 7.49	66.17 ± 4.84	70.93 ± 7.15	68.09 ± 5.17	76.53 ± 5.52	78.09 ± 5.15	77.62 ± 7.29	77.59 ± 5.39
ALP (U/L)	38.62 ± 2.27	38.06 ± 3.47	38.53 ± 2.70	37.57 ± 3.11	45.33 ± 4.98	44.35 ± 4.24	45.03 ± 4.57	46.70 ± 2.14
GLU (mmol/L)	7.05 ± 0.35	7.27 ± 0.45	7.33 ± 0.44	6.93 ± 0.49	7.3 ± 0.33	6.96 ± 0.42	6.95 ± 0.42	7.11 ± 0.48
Cr (μmol/L)	33.47 ± 3.62	36.36 ± 2.49	33.71 ± 4.04	34.17 ± 3.55	30.75 ± 2.53	32.96 ± 2.14	32.69 ± 1.90	31.30 ± 2.88
BUN (mmol/L)	5.41 ± 0.52	5.37 ± 0.24	5.27 ± 0.31	5.45 ± 0.20	6.29 ± 0.26	6.27 ± 0.49	6.34 ± 0.41	6.29 ± 0.42
CK (U/L)	247.42 ± 20.52	253.85 ± 19.18	253.64 ± 29.39	252.64 ± 20.95	255.23 ± 46.82	256.76 ± 31.62	260.88 ± 23.60	252.98 ± 8.46
Na+ (mmol/L)	147.09 ± 5.35	148.95 ± 6.42	148.75 ± 4.73	147.13 ± 3.91	147.63 ± 5.13	143.66 ± 5.50	146.91 ± 4.08	147.36 ± 2.11
K+ (mmol/L)	4.56 ± 0.26	4.45 ± 0.40	4.58 ± 0.28	4.16 ± 0.54	5.11 ± 0.40	5.03 ± 0.56	4.96 ± 0.62	5.03 ± 0.32
Cl^- (mmol/L)	94.56 ± 6.54	93.26 ± 4.08	94.54 ± 3.89	96.04 ± 2.62	97.48 ± 7.61	96.55 ± 3.91	99.56 ± 5.55	98.44 ± 4.39

Note: The values in the table represent mean ± SD, n = 10. No noticeable difference between control group and CFE-treated group. Alanine Transaminase (ALT), Aspartate Transaminase (AST), Albumin (ALB), Triglyceride (TG), Total Cholesterol (TC), Total Bilirubin (TBIL), Total Protein (TP), Alkaline Phosphatase (ALP), Glucose (GLU), Creatinine (Cr), Blood Urea Nitrogen (BUN), Creatine Kinase (CK), Natrium (Na), Kalium (K), Chlorine (Cl).

Table 5
Organic index of males and female rats treated with CFE for 90 days.

	Male				Female			
	Control	CFE-L	CFE-M	CFE-H	Control	CFE-L	CFE-M	CFE-H
Heart	0.34 ± 0.02	0.33 ± 0.02	0.35 ± 0.03	0.36 ± 0.03	0.36 ± 0.01	0.36 ± 0.01	0.36 ± 0.02	0.37 ± 0.01
Liver	2.58 ± 0.20	2.79 ± 0.18	2.78 ± 0.15	2.93 ± 0.15	2.71 ± 0.09	2.70 ± 0.06	2.79 ± 0.08	2.72 ± 0.02
Spleen	0.21 ± 0.01	0.21 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.14 ± 0.07	0.17 ± 0.06	0.14 ± 0.04	0.14 ± 0.05
Lung	0.41 ± 0.03	0.42 ± 0.01	0.43 ± 0.01	0.42 ± 0.01	0.51 ± 0.02	0.56 ± 0.01	0.52 ± 0.01	0.49 ± 0.01
Kidney	0.35 ± 0.01	0.35 ± 0.01	0.36 ± 0.01	0.35 ± 0.01	0.35 ± 0.01	0.33 ± 0.01	0.33 ± 0.01	0.33 ± 0.01
Thymus	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
Adrenal glands	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
Brian	0.45 ± 0.02	0.47 ± 0.01	0.46 ± 0.01	0.46 ± 0.01	0.68 ± 0.03	0.65 ± 0.01	0.64 ± 0.01	0.66 ± 0.01
Testis	0.44 ± 0.01	0.44 ± 0.01	0.45 ± 0.01	0.43 ± 0.01				
Epididymis	0.20 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.20 ± 0.01				
Uterus					0.24 ± 0.01	0.24 ± 0.01	0.25 ± 0.01	0.26 ± 0.01
Ovary					0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01

Note: The values in the table represent mean ± SD, n = 10. No noticeable difference between control group and CFE-treated group.

testis, the nuclei of spermatogonia in spermatogonial tubules were large and rounded, adhered to basement membranes, and were neatly arranged; the number of spermatogonial layers at all levels was clearly visible and a large number of spermatocytes could be seen on the side of near lumen of tubules and the lumen was full of spermatozoa (Fig. 4G). In the ovary, cellular arrangement, good oocyte maturity, high number of primary and secondary oocytes (Fig. 5G).

4. Discussion

For centuries, natural medicines have played a critical role in the prevention and treatment of disease [21]. Both chemically synthesized drugs and natural drugs are required to undergo long-term toxicity studies and acute toxicity studies before use in patients. While CFE was previously showed to exhibit pharmacological effects, its toxicity has not been well examined. To address this gap, we performed acute and subacute toxicity study of CFE in mice and rats following OECD guidelines (Co-operation & Development, 2008). Many studies have shown that females are more sensitive to drugs than males [22], However, for the drug to produce adverse reactions in males and for mice to be able to take higher concentrations of the drug orally according to the human to mouse drug concentration conversion formula, mice are more suitable for acute toxicity experiments, rats were used for long-term toxicity experiments. We used both females and males in long-term toxicity studies and acute toxicity studies [23].

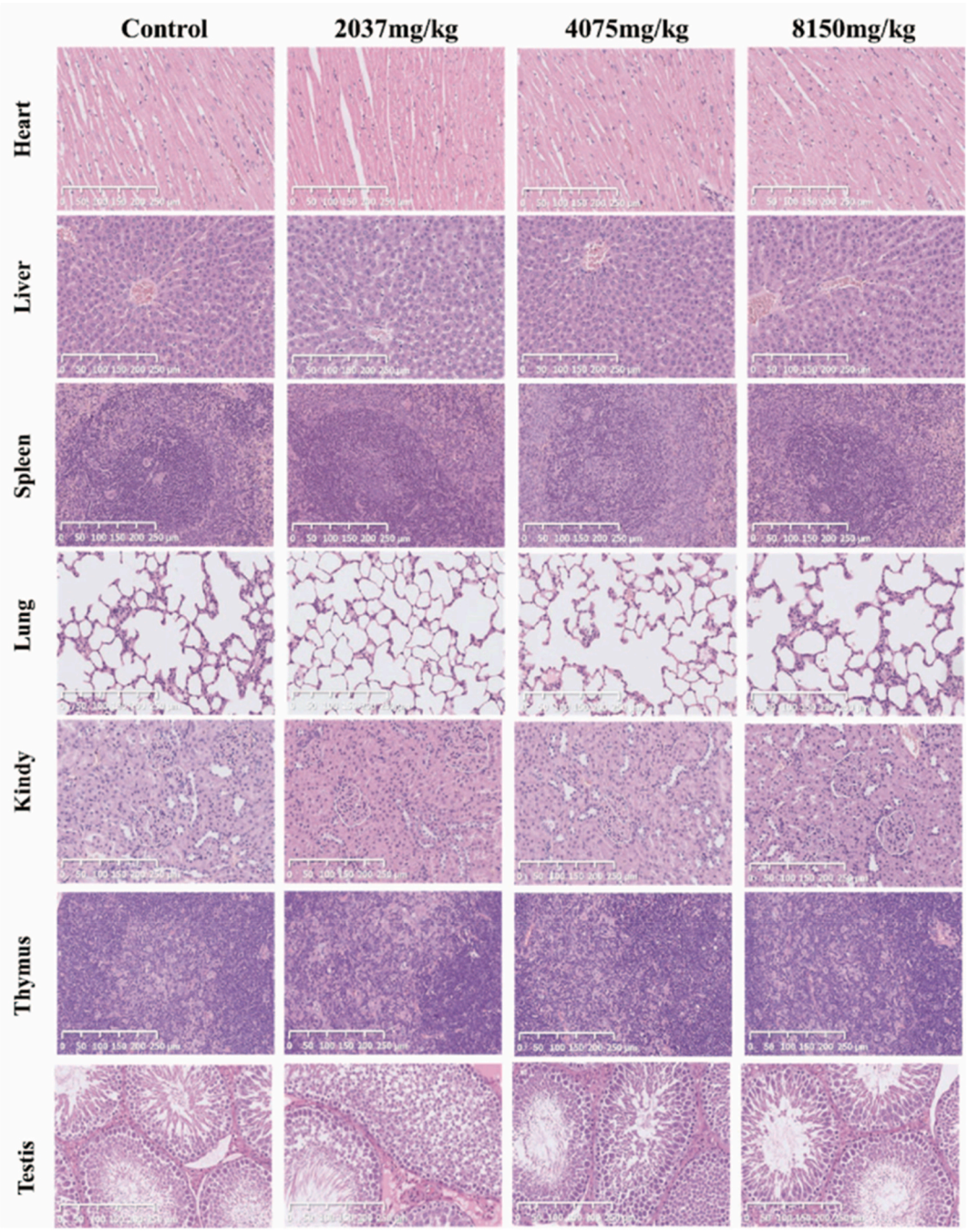

Fig. 4. Histopathological examination of organs of male rats after chronic exposure to CFE.

Acute toxicity is a toxic reaction that occurs when an animal receives a certain dose of a test substance at one time or multiple times within a 24-h period. The main purpose of the acute toxicity test is to determine the LD50 level of the drug [24]. In the current investigation, the oral LD50 value of the CFE was determined to be 4500 mg/kg based on the acute toxicity evaluation, also known as the limit test. Our study had no mouse mortalities for all treatments over 14 days of continuous administration of the maximum dose. Thus, the LD50 of CFE was estimated be higher than 4500 mg/kg [25].

Subacute toxicity tests are designed to detect organ damage caused by different doses of a substance. This type of study, as opposed to an acute toxicity study, is designed to determine the dose level of toxicity in the actual environment. Typically, three doses are used: 1) a high dose, sufficient to trigger specific signs of toxicity but not sufficient to harm the test species; 2) a low dose, in which harmless effects are expected; and 3) an intermediate dose, which is half of the high dose [26].

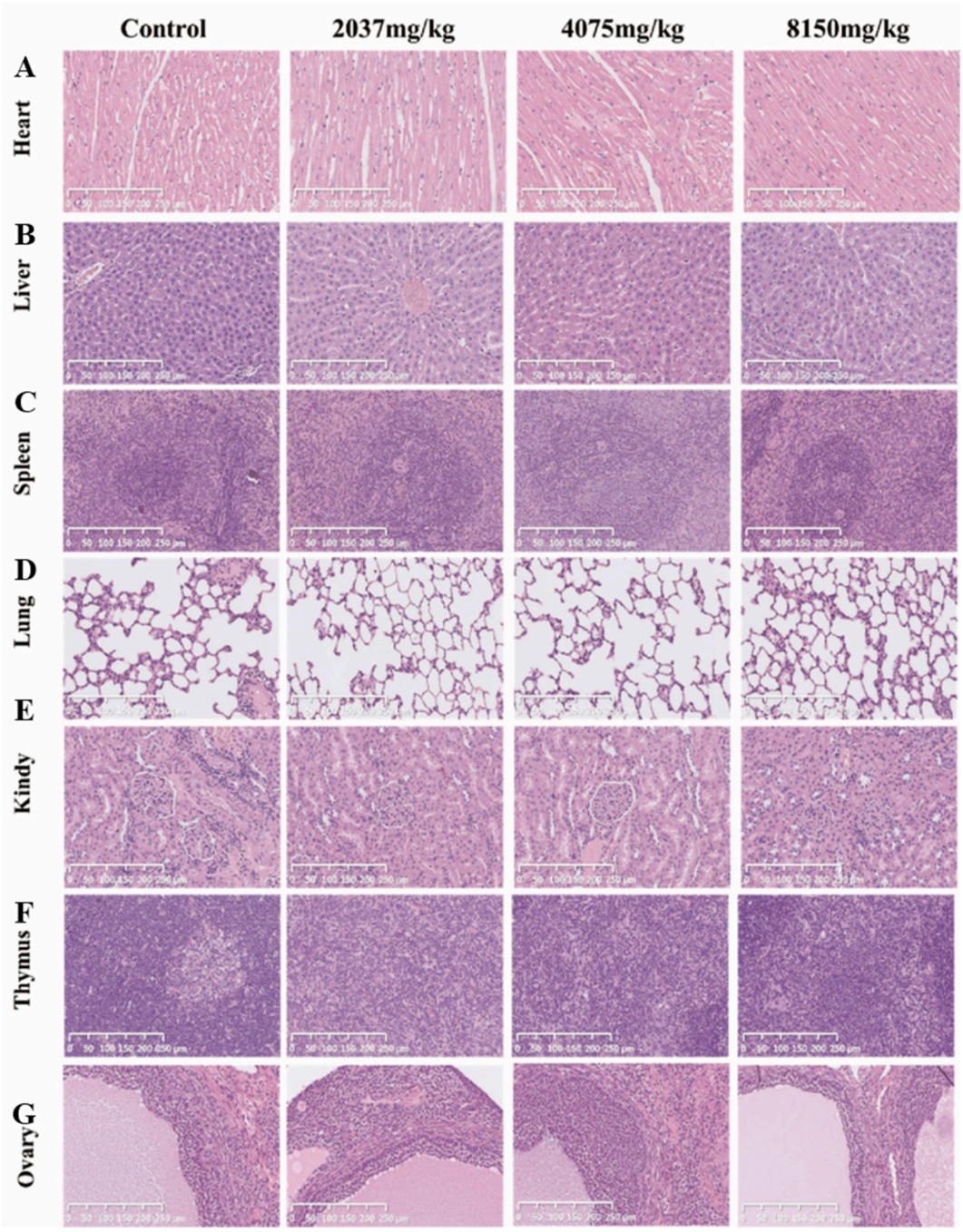

Fig. 5. Histopathological examination of organs of female rats after chronic exposure to CFE.

Changes in body weight and behavior are often early signs of harmful effects of chemicals. While the body weight of mice in all groups increased after CFE treatment in acute toxicity and long-term toxicity experiments, the increase was not significant ($P > 0.05$). The cause of weight gain may be from the high protein content of CFE, which provides many small molecule peptides to promote growth and development [27]. Studies have shown that certain diseases such as cancer and immune deficiency will lead to weight loss, and weight loss will lead to further decline in immune function. Thus, when weight loss occurs, the supplementation of protein substances and polysaccharides are required to maintain weight and enhance immunity [28].

Blood is a major system of the body with feedback mechanisms and homeostatic control. Red blood cells, white blood cells, and platelets all come from the bone marrow to transport the oxygen to locations in the body, clear pathogenic microorganisms, and participate in blood clotting [29]. The results of long-term toxicity experiments showed that CFE did not have significant effects on the

hematological system. AST, ALT, Cr, and BUN reflect liver function and kidney function, TG and TC reflect lipid levels, and Na^+, K^+, and Cl^- ions reflect body electrolyte levels. CFE does not significantly change these biochemical indicators [30].

Our results show that the main chemical composition of CFE was mainly carboxylic acids, indoles, benzodiazepines, pyrrolidines, quinolines, benzenes, flavonoids, lipids and other compounds. CFE contains a high quantity of amino acids such as phenylalanine, and studies have shown that phenylalanine can be used as a carrier for anti-cancer drugs to introduce drug molecules directly into the tumor area and is three to five times more effective than other amino acids. This approach can restrain the growth of cancer tumors and reduce the toxic side effects of drugs. Benzodiazepines such as alprazolam are used to treat anxiety, depression, and insomnia. Flavonoids such as prickly mangosteen have anti-inflammatory, anti-oxidant, anti-apoptotic, lipid regulating, anti-thrombotic and tumor inhibiting effects [31].

5. Conclusion

Our results showed that the LD50 of CFE is greater than 4500 mg/kg; no signs of mortality and morbidity were observed in the animal experiments. Repeated administration and long-term use of CFE did not reveal toxic effects on selected organs as determined by hematological, biochemical indices and histopathological analyses. This toxicity study concluded that CFE was safe for animals and may be used for the analysis of different pharmacological activities.

Data availability statement

All data are available in the main text or the supplementary materials.

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CRediT authorship contribution statement

JiDa Wang: Conceptualization. **Li Wang:** Conceptualization. **Junzhen Tan:** Validation. **RunDong Chai:** Conceptualization. **Ying Wang:** Conceptualization. **Yue Wang:** Investigation. **ShuWu Zhao:** Supervision. **XiangLing Wang:** Conceptualization. **YuHong Bian:** Funding acquisition. **JianWei Liu:** Supervision.

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.

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