Toxicology Reports 9 (2022) 58–63

Contents lists available at ScienceDirect

Toxicology Reports

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

Assessment of the acute and sub-acute toxicity of the ethanolic extract of the aerial parts of *Crassocephalum rabens* (Asteraceae) in rats

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ARTICLE INFO

Handling Editor: Dr. Aristidis Tsatsakis

Keywords:
Crassocephalum rabens
Acute toxicity
Sub-acute toxicity
Food safety

ABSTRACT

This pioneering study was to assess the acute and sub-acute toxicity of the ethanolic extract of the aerial parts of *Crassocephalum rabens* (Asteraceae) in rats. *C. rabens* is a common vegetable and herb for treating inflammation-related syndromes in Taiwan. Pharmacological studies have unveiled that the extracts of *C. rabens* have potential to become hepatoprotective, anti-inflammatory, or anti-cancer agents. The toxicological effects of the aerial parts of *C. rabens* in rodents are still elusive. For the acute toxicity study, rats were administrated with a single dose of 5,000 mg/kg body weight (BW) and observed for 14 days in accordance with the Organization for Economic Cooperation and Development (OECD) guideline No. 420. For the sub-acute toxicity study, animals were orally treated with daily doses of 0, 416.7, 833.3, and 1,666.7 mg/kg BW for 28 days based on the OECD guideline No. 407. The toxicity of the repeated dose was observed with anthropometric, hematological, and biochemical parameters as well as histology. The mortality and critical pathological and biochemical abnormalities were not observed in the acute and/or sub-acute toxicity studies. The oral median lethal dose (LD_{50}) of the extract was greater than 5000 mg/kg BW. The no-observed-adverse-effect-level (NOAEL) in male and female rats was greater than 1,666.7 mg/kg BW. As such, the extract of the aerial parts of *C. rabens* is considered a non-toxic substance.

1. Introduction

Crassocephalum rabens, known as Zhaohe Cao, is a vegetable and ethnomedicinal plant in Taiwan, and it is beneficial for treating a variety of inflammation-related syndromes in folk remedies [1]. Pharmacological studies have revealed that *C. rabens* possesses hepatoprotective, anti-inflammatory, and anti-cancer activities in cells of rodents [1–5]. The therapeutic effects of *C. rabens* are mainly attributed to the bioactive galactolipids, which possess anti-tumor, anti-microbial, anti-viral, immune-suppressive, and anti-inflammatory activities as well as nutritional values [6,7]. 1,2-di-O-linolenoyl-3-O-β-galactopyranosyl-sn-glycerol (dLGG) is the most prominent one in *C. rabens* [4,5]. dLGG enabled to inhibit the expression of nuclear factor-κB (NF-κB) and its downstream inflammatory mediators, such as nitric oxide (NO), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and prostaglandin E2, *in vitro* [8]. Apart from the inhibiting effects on the metastatic lung, skin, and breast tumor cells, the latest research has also revealed that dLGG could suppress the melanoma brain metastasis (MBM) in mice through reprogramming the tumor microenvironment and interacting with melanoma cells and macrophages [9].

As such, *C. rabens* has potential to be developed as anti-cancer and anti-inflammatory agents. Our internal study also discovered the efficacy of *C. rabens* on improving liver function indexes in the lipopolysaccharide/D-galactosamine-induced fulminant hepatitis model [10]. The results inspired us to develop the *C. rabens* as dietary supplements for liver protection. Nevertheless, the safety and toxicity of *C. rabens* are still elusive. Herein, this exploratory study aims to assess the oral acute and sub-acute toxicity of the ethanolic extract of the aerial parts of *C. rabens* in rats.

2. Materials and methods

2.1. Preparation of the ethanolic extract of C. rabens

The aerial parts were collected and washed with running water and distilled water. The cleaned samples were dried by a food dehydrator set at 40 °C followed by crushing with a pulverizer. The powder was ultrasonically extracted with 95 % ethanol (in a ratio of 1:10 w/v) at 40 °C

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https://doi.org/10.1016/j.toxrep.2021.12.005
Received 7 July 2021; Received in revised form 15 September 2021; Accepted 15 December 2021
Available online 16 December 2021

for 3 h, and then the mixture was filtered using the Whatman paper No. 1. The filtrate solution was freeze-dried and stored in a freezer. The dried powder was prepared in reverse osmosis water for the following animal study.

2.2. Experimental animals

Sprague-Dawley (SD) rats (acute toxicity: 8 weeks old; sub-acute toxicity: 4 weeks old) were obtained from BioLASCO Taiwan Co., Ltd. The animals were quarantined for around 1 week and acclimated to the animal holding room (temperature: 22 ± 3 °C; relative humidity: 55 ± 15 %; light cycle: 12 h light/dark) before the study. The animals were fed with standard food (ORIENTAL YEAST Co., Ltd.), and water was provided *ad libitum*. The use of animals and the implementation of the study were approved by the International Animal Care and Use Committee of SuperLab Co., Ltd (IACUC No. 105-9 t).

2.3. Acute toxicity test

The acute toxicity study was based on the limit test of the OECD guideline No. 420 [11]. Five male and five female rats were randomly assigned into the experimental and control group. The *C. rabens* extract was administered to the rats by oral gavage with gastric tube, and the testing dose of this study was 5,000 mg/kg BW. After treatment, all rats were observed individually twice a day by a veterinarian for 14 days. All rats were sacrificed by 100 % CO_2 inhalation followed by gross necropsy examination at day 15. The calculation of mortality and LD_{50} followed the OECD methods.

2.4. Sub-acute toxicity study

The sub-acute toxicity study was based on the limit test of the OECD guideline No. 407 [12]. 40 male and 40 female rats were randomly and equally assigned into the experimental [416.7 mg/kg BW (low), 833.3 mg/kg BW (middle), 1,666.7 mg/kg BW (high)] and control (vehicle only) groups. The *C. rabens* extract was administered to the rats by plastic syringes equipped with feeding needles at the daily doses of 0, 416.7, 833.3, and 1,666.7 mg/kg BW for 28 days. Clinical observation, ophthalmological examination, and the records of food consumption were conducted every day. The weights of all animals were measured every week.

All surviving animals were anesthetized with inhalation of CO_2, and sacrificed after blood collecting. The outer appearance, oral cavity, cranial cavity and all tissues and organs in the thoracic and abdominal cavity were examined visually and recorded. The vital organs were collected for histopathological examination including brain, heart, kidney, liver, spleen, adrenal gland, and testes/ovaries. All the collected organs were fixed in 10 % neutral formalin buffer (the testes of the rats were fixed in modified Davidson's solution for 24 h and transferred into 10 % neutral formalin for preservation.

2.5. Hematological analysis

An automatic blood analyzer (XT-1800i; Sysmex) was used for the following analyses: hematocrit, hemoglobin, RBC, WBC, platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), lymphocyte, neutrophil, monocyte, eosinophil, and basophil. A blood coagulation analyzer (CA CA-1500, Sysmex) was used for analyzing prothrombin time (PT).

2.6. Biochemical analysis

An automated analyzer (7070 Autoanalyzer, Hitachi) was used to analyze the following indexes: alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), Gamma-glutamyl transpeptidase(γ-GT), albumin, globulin, total protein, total bilirubin, creatinine, blood urea nitrogen (BUN), glucose, cholesterol, triglyceride, phosphorus, calcium, chloride (Cl), potassium, and sodium.

Table 1
Effect of the single administration of the ethanol extract of the aerial parts of *C. rabens* on body weight of rats ($n = 5$; mean value ± S.D.).

Sex	Day 0	Day 7	Day 14
Weight of male rat (g)	331.7 ± 10.7	352.5 ± 7.2	401.88 ± 13.2
Weight of female rat (g)	232.3 ± 14.1	247.9 ± 13.8	265.26 ± 13.4

Table 2
Effect of the repeated administration of the ethanol extract of the aerial parts of *C. rabens* on food consumption of rats ($n = 10$; mean value ± S.D.).

	Observation time	Average food consumption (g/100 g of body weight of rat/day)			
		0 mg/kg	416.7 mg/kg	833.3 mg/kg	1,666.7 mg/kg
Male	Week 1	7.8 ± 0.3	8.0 ± 0.3	7.9 ± 0.3	7.6 ± 0.2
	Week 2	6.4 ± 0.4	5.9 ± 0.2*	6.3 ± 0.4	6.3 ± 0.4
	Week 3	7.4 ± 0.4	7.4 ± 0.2	7.8 ± 0.4*	7.3 ± 0.3
	Week 4	6.5 ± 0.3	6.6 ± 0.2	6.8 ± 0.4*	6.5 ± 0.4
Female	Week 1	8.0 ± 0.5	7.8 ± 0.4	8.0 ± 0.5	7.7 ± 0.2
	Week 2	8.3 ± 0.5	8.5 ± 0.5	8.6 ± 0.5	8.3 ± 0.2
	Week 3	7.4 ± 0.5	7.6 ± 0.4	7.8 ± 0.5	7.6 ± 0.2
	Week 4	6.7 ± 0.5	6.9 ± 0.2	6.9 ± 0.4	6.8 ± 0.2

* $p < 0.05$; the significance was compared with the control group.

2.7. Histopathological examination

The fixed organs of control group and high dose group were subjected to histopathological examination. These fixed organs were dehydrated, clarified, infiltrated with paraffin and embedded after trimming, forming paraffin tissue blocks, being cut into 2 μm thickness of a tissue slice using paraffin tissue slicing machine, stained with Hematoxylin & Eosin (H&E).

2.8. Statistical analysis

The SPSS statistical software was used for the analysis. The body weight, feed intake, organ weight, hematology, and clinical biochemistry analysis were analyzed by one-way ANOVA followed by Duncan's multiple range test to determine the significant difference between the treatment and control group, with $p < 0.05$ considered significant.

3. Results

3.1. Acute toxicity study

The limit test of acute oral toxicity study was under the OECD guideline No. 420. The testing animals were treated with a single administration of the extract of the aerial parts of *C. rabens* at a dose of 5000 mg/kg BW. Table 1 shows that the average body weights of male and female rats were normally increased within the 14-day observation period. In addition, there were no mortality, abnormal clinical sign, and significant gross lesions discovered after the study. The LD_{50} of the extract was considered greater than 5,000 mg/kg BW.

3.2. Sub-acute toxicity

All animals survived after the 28 days of treatment. There were no obvious toxicity signs in skin fur, eyes, and mucous membranes and abnormal behaviors were observed in the experimental and control groups.

Table 3
Effect of the repeated administration of the ethanol extract of the aerial parts of *C. rabens* on body weight of rats ($n = 10$; mean value ± S.D.).

	Observation time	Average body weight (g)			
		0 mg/kg	416.7 mg/kg	833.3 mg/kg	1,666.7 mg/kg
Male	Baseline	219.4 ± 17.4	218.4 ± 12.8	219.4 ± 12.8	218.5 ± 8.2
	Week 1	273.9 ± 18.6	274.9 ± 11.7	273.2 ± 11.0	269.8 ± 10.9
	Week 2	323.8 ± 23.9	319.6 ± 13.2	324.1 ± 13.2	316.9 ± 16.2
	Week 3	365.4 ± 30.5	354.8 ± 16.7	369.2 ± 17.5	354.7 ± 22.6
	Week 4	408.3 ± 35.6	399.7 ± 21.4	420.2 ± 17.9	405.4 ± 25.3
Female	Baseline	179.6 ± 7.4	179.0 ± 10.0	180.0 ± 8.1	178.8 ± 7.9
	Week 1	202.1 ± 14.5	200.9 ± 6.6	202.2 ± 14.0	200.5 ± 7.3
	Week 2	224.9 ± 17.9	221.3 ± 9.1	218.2 ± 17.7	217.9 ± 5.0
	Week 3	241.0 ± 19.4	239.1 ± 7.5	238.1 ± 14.3	238.3 ± 7.7
	Week 4	256.7 ± 22.1	253.9 ± 7.4	253.3 ± 15.4	252.3 ± 11.2

Table 4
Effect of the repeated administration of the ethanol extract of the aerial parts of *C. rabens* on the relative organ weight of rats ($n = 10$; mean value ± S.D.).

	Organ	Relative organ weight (g/100 g BW)			
		0 mg/kg	416.7 mg/kg	833.3 mg/kg	1,666.7 mg/kg
Male	Testis	0.869 ± 0.920	0.961 ± 0.100	0.876 ± 0.161	0.860 ± 0.101
	Adrenal gland	0.016 ± 0.001	0.018 ± 0.002*	0.019 ± 0.005*	0.017 ± 0.002
	Spleen	0.187 ± 0.023	0.181 ± 0.031	0.198 ± 0.031	0.187 ± 0.100
	Kidney	0.865 ± 0.089	0.918 ± 0.086	0.904 ± 0.111	0.895 ± 0.061
	Heart	0.410 ± 0.032	0.402 ± 0.021	0.416 ± 0.061	0.394 ± 0.029
	Brain	0.547 ± 0.039	0.594 ± 0.032	0.560 ± 0.087	0.568 ± 0.031
	Liver	3.420 ± 0.398	3.857 ± 0.475*	3.813 ± 0.493*	3.606 ± 0.176
Female	Testis	0.036 ± 0.006	0.037 ± 0.004	0.043 ± 0.011	0.039 ± 0.008
	Adrenal gland	0.030 ± 0.004	0.035 ± 0.010	0.033 ± 0.005	0.031 ± 0.003
	Spleen	0.196 ± 0.009	0.222 ± 0.018*	0.220 ± 0.019*	0.216 ± 0.026*
	Kidney	0.907 ± 0.070	0.941 ± 0.067	0.939 ± 0.035	0.947 ± 0.049
	Heart	0.417 ± 0.017	0.423 ± 0.029	0.405 ± 0.023	0.405 ± 0.031
	Brain	0.830 ± 0.064	0.852 ± 0.029	0.843 ± 0.035	0.855 ± 0.043
	Liver	3.553 ± 0.308	3.769 ± 0.303	3.696 ± 0.369	3.75 ± 0.239

* $p < 0.05$; the significance was compared with the control group.

Table 5
Effect of the repeated administration of the ethanol extract of the aerial parts of *C. rabens* on hematological parameters of rats ($n = 10$; mean value ± S.D.).

Parameter	0 mg/kg		416.7 mg/kg		833.3 mg/kg		1,666.7 mg/kg	
	Male	Female	Male	Female	Male	Female	Male	Female
WBC (10^3/μL)	13.26 ± 2.69	10.19 ± 2.91	13.98 ± 6.43	9.40 ± 2.50	12.18 ± 3.60	9.71 ± 1.86	13.6 ± 2.90	10.52 ± 2.27
RBC (10^6/μL)	8.93 ± 0.54	8.77 ± 0.37	9.24 ± 0.76	8.80 ± 0.58	8.74 ± 0.36	8.46 ± 0.53	9.02 ± 0.32	8.53 ± 0.48
Hemoglobin (g/dL)	17.20 ± 0.54	16.76 ± 0.74	17.49 ± 1.34	16.79 ± 0.87	16.83 ± 0.42	16.63 ± 0.93	17.42 ± 0.65	16.38 ± 1.08
Hematocrit (%)	51.05 ± 1.46	48.77 ± 1.52	52.00 ± 3.79	49.48 ± 2.14	51.26 ± 1.63	49.12 ± 2.34	53.2 ± 2.16	48.61 ± 2.61
MCV (fL)	57.33 ± 2.70	55.64 ± 1.36	56.38 ± 2.42	56.36 ± 2.79	58.73 ± 2.07	58.15 ± 2.21*	58.96 ± 1.75	57.01 ± 1.88
MCH (pg)	19.31 ± 0.78	19.11 ± 0.56	18.97 ± 0.53	19.10 ± 0.78	19.29 ± 0.59	19.67 ± 0.48	19.30 ± 0.52	19.20 ± 0.5
MCHC (g/dL)	33.70 ± 0.70	34.34 ± 0.56	33.63 ± 0.89	33.92 ± 0.55	32.84 ± 0.34*	33.85 ± 0.59	32.75 ± 0.36*	33.69 ± 0.67*
Platelet (10^3/μL)	995 ± 150	984 ± 135	974 ± 150	940 ± 67	941 ± 62	933 ± 142	955 ± 104	988 ± 133
Neutrophil (%)	12.14 ± 2.8	11.01 ± 2.32	13.05 ± 3.02	14.37 ± 3.70*	12.53 ± 2.75	13.10 ± 3.22	12.8 ± 4.01	12.40 ± 3.30
Lymphocyte (%)	82.00 ± 3.65	83.50 ± 2.33	80.53 ± 4.58	79.79 ± 4.19*	81.95 ± 3.06	81.34 ± 3.14	81.8 ± 4.49	81.98 ± 3.71
Monocyte (%)	4.61 ± 0.82	4.21 ± 1.00	5.34 ± 1.97	4.51 ± 1.18	4.54 ± 0.88	4.23 ± 0.86	4.35 ± 0.74	4.27 ± 0.73
Eosinophil (%)	1.02 ± 0.46	1.03 ± 0.36	0.76 ± 0.38	1.05 ± 0.35	0.70 ± 0.23	1.01 ± 0.34	0.87 ± 0.34	1.02 ± 0.39
Basophil (%)	0.23 ± 0.07	0.25 ± 0.08	0.32 ± 0.42	0.28 ± 0.17	0.28 ± 0.12	0.32 ± 0.17	0.18 ± 0.08	0.33 ± 0.21
PT (sec.)	13.59 ± 12.42	9.67 ± 0.18	12.42 ± 4.16	9.60 ± 0.40	12.46 ± 0.91	9.49 ± 0.19	12.24 ± 1.30	9.80 ± 0.34

WBC: white blood cells; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PT: prothrombin Time.

* $p < 0.05$; the significance was compared with the control group.

3.3. Effect of the extract on body weight and food consumption

In male rats, food consumption at low dose group was significantly lower than that control group at week 2, and food consumption at middle dose group was significantly higher than control group at weeks 3 and 4 (Table 2). By contrast, there was no significant difference of food consumption between the treatment and control groups in female animals during the study (Table 2). The average body weights of male and female rats are shown in Table 3. There was no significant differences in body weight between the treatment and control group groups in male and female rats within 28 days of the study.

3.4. Effect of the extract on relative organ weigh

There were no significant findings on the ophthalmological inspection and incidence of gross lesion in male or female rats (Tables S1, S2). Table 4 indicates the results of the relative organ weight of the male and female rats. In male rats, the relative weights of the adrenal gland and liver at low and middle dose groups were significantly higher than those of the control group, but the relative weights of other organs did not

Table 6
Effect of the repeated administration of the extract of leaves and stem of *C. rabens* on biochemical parameters of rats ($n = 10$; mean value ± S.D.).

Parameter	0 mg/kg		416.7 mg/kg		833.3 mg/kg		1,666.7 mg/kg	
	Male	Female	Male	Female	Male	Female	Male	Female
BUN (mg/dL)	14.76 ± 1.40	14.05 ± 1.78	14.97 ± 1.52	14.82 ± 1.46	14.58 ± 1.50	13.46 ± 1.89	15.2 ± 1.55	14.03 ± 1.79
Creatinine (mg/dL)	0.71 ± 0.06	0.31 ± 0.03	0.68 ± 0.04	0.32 ± 0.04	0.67 ± 0.05	0.29 ± 0.06	0.69 ± 0.06	0.29 ± 0.03
AST (U/L)	90.30 ± 19.96	103.70 ± 33.09	109.60 ± 32.48	126.00 ± 89.50	91.10 ± 13.12	116.60 ± 43.96	106.40 ± 38.44	121.90 ± 35.44
ALT (U/L)	38.6 ± 7.62	33.90 ± 16.67	41.30 ± 11.90	31.40 ± 9.44	37.80 ± 7.07	31.60 ± 9.13	46.00 ± 18.18	32.90 ± 10.31
Total protein (g/dL)	6.68 ± 0.37	7.34 ± 0.32	6.63 ± 0.25	7.39 ± 0.52	6.65 ± 0.18	7.25 ± 0.31	6.55 ± 0.28	7.39 ± 0.33
Albumin (g/dL)	4.70 ± 0.22	5.50 ± 0.35	4.65 ± 0.45	5.69 ± 0.46	4.63 ± 0.19	5.48 ± 0.19	4.67 ± 0.15	5.62 ± 0.34
ALP (U/L)	181.60 ± 50.30	77.70 ± 14.18	152.8 ± 37.80	79.30 ± 21.59	160.40 ± 49.90	80.00 ± 21.82	169.00 ± 34.90	71.20 ± 15.48
γ-GT (U/L)	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
Cholesterol (mg/dL)	72.00 ± 14.22	75.00 ± 16.85	61.20 ± 14.86	71.80 ± 10.02	65.10 ± 11.33	72.50 ± 14.31	66.10 ± 10.20	75.50 ± 9.98
Triglyceride (mg/dL)	90.70 ± 54.29	41.70 ± 14.09	65.00 ± 24.25	33.70 ± 15.60	108.00 ± 19.14	36.90 ± 16.66	71.3 ± 24.03	35.40 ± 12.57
Calcium (mg/dL)	12.2 ± 1.11	12.48 ± 0.59	12.39 ± 1.03	13.27 ± 0.45	12.72 ± 0.83	13.08 ± 0.50	12.17 ± 0.71	13.64 ± 0.94
Phosphorus (mg/dL)	12.12 ± 1.06	11.66 ± 1.39	12.82 ± 1.12	12.01 ± 1.10	12.85 ± 1.30	11.87 ± 1.12	13.4 ± 1.34	12.74 ± 1.50
Sodium (meq/L)	146.60 ± 1.07	143.70 ± 1.83	147.20 ± 1.03	143.30 ± 1.42	147.10 ± 0.88	144.50 ± 1.08	147.10 ± 1.60	143.20 ± 1.62
Potassium (meq/L)	7.11 ± 0.94	9.10 ± 1.68	8.31 ± 1.09*	9.71 ± 1.71	7.55 ± 1.19	8.96 ± 1.35	8.59 ± 1.49*	10.91 ± 2.80
Chloride (meq/L)	99.70 ± 1.77	102.80 ± 1.62	100.40 ± 1.43	100.80 ± 1.81*	99.20 ± 1.55	101.00 ± 1.76*	99.70 ± 1.49	100.60 ± 1.43*
Globulin (g/dL)	1.98 ± 0.25	1.84 ± 0.18	1.98 ± 0.31	1.70 ± 017	2.02 ± 0.16	1.77 ± 0.21	1.88 ± 0.15	1.77 ± 0.16
Total bilirubin (μg/dL)	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04

BUN: Blood urea nitrogen; AST: aspartate Transaminase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; γ-GT:γ-Glutamyltransferase.
* $p < 0.05$; the significance was compared with the control group.

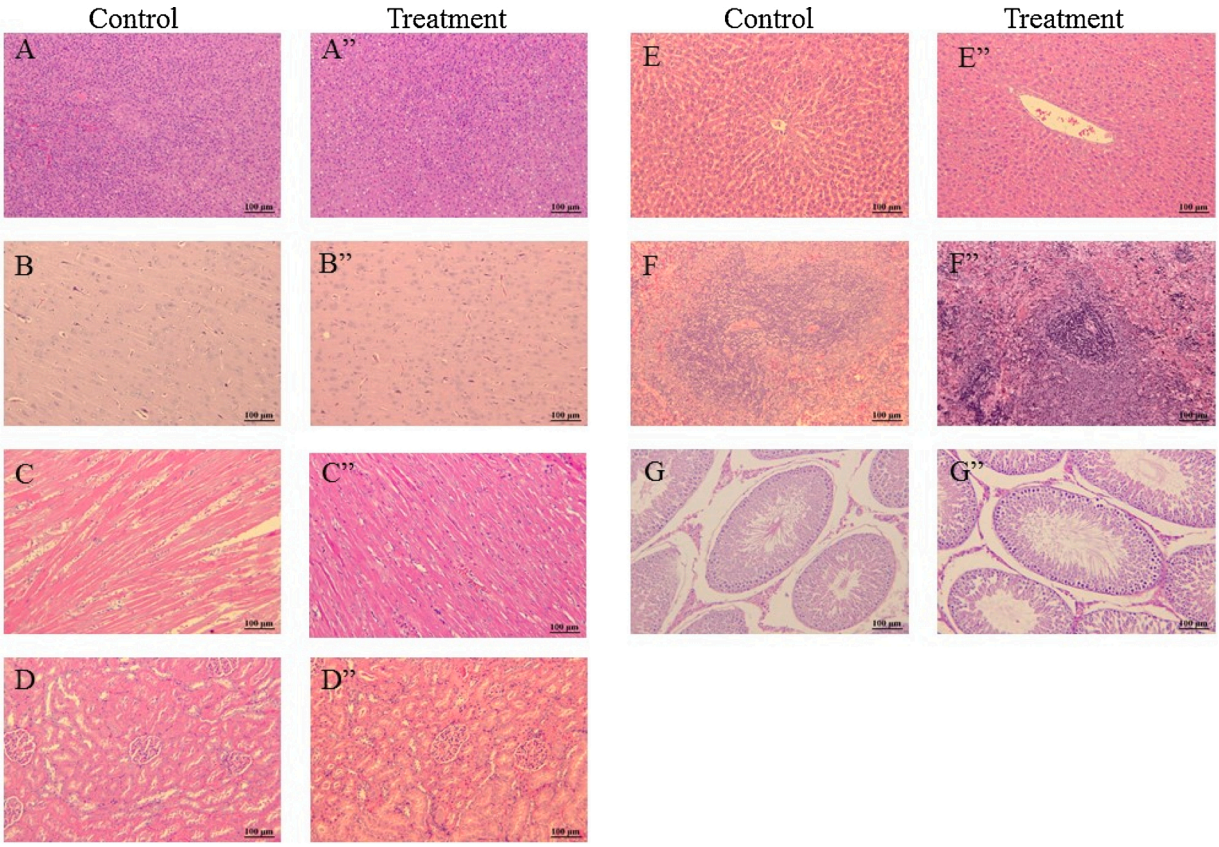

Fig. 1. Histopathologic results of sections of adrenal gland (A, A”), brain (B, B”), heart (C, C”), kidney (D, D”), liver (E, E”), spleen (F, F”), and testis (G, G”) in male rats. A-F represented the results of the control group. A”- F” represented the results of the high dose group.

show any obvious difference in comparison with the control group. On the other hand, the relative weights of the spleen at all experimental groups in female rats were significantly higher than those of the control group. Besides, the results of the relative weights of other organs did not show abnormal findings in any of the rats tested.

3.5. *Effect of the extract on hematological parameters*

Table 5 shows the hematological results of male and female rats. In the male rat group, the MCHC values at medium and high dose levels were obviously lower than those of the control group. In the female rat group, the neutrophil and lymphocyte values at low dose group were significantly higher than those of the control group; moreover, the MCV value at middle dose group and the MCHC value were significantly

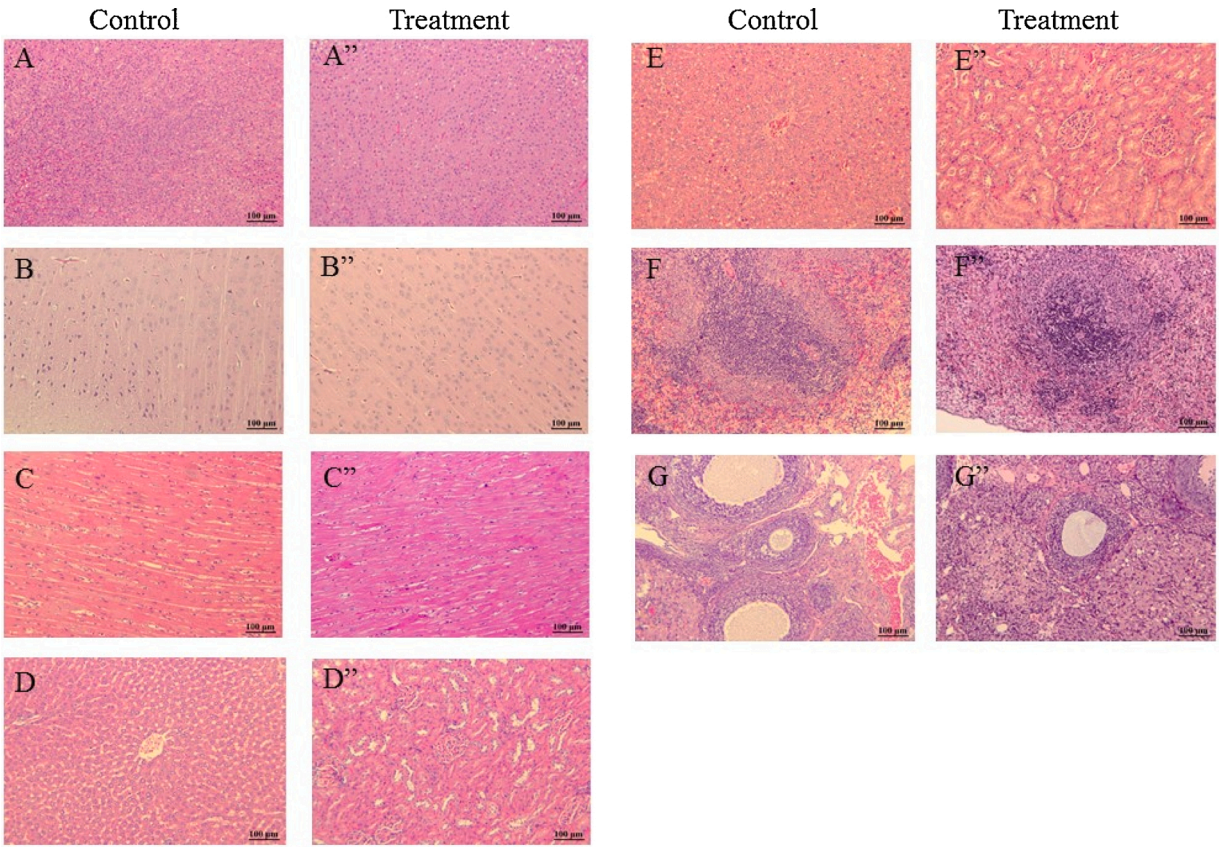

Fig. 2. Histopathologic results of sections of adrenal gland (A, A”), brain (B, B”), heart (C, C”), kidney (D, D”), liver (E, E”), spleen (F, F”), and ovary (G, G”) in female rats. A-F represented the results of the control group. A”- F” represented the results of the high dose group.

higher than the results of the control group. Other hematological values did not show statistically significant differences from the outcomes of the control group.

3.6. Effect of the extract on biochemical parameters

The results of most biochemical indexes of the animals in the experimental groups did not display abnormal values in comparison with the control groups (Table 6). However, the potassium levels of the male rats at low and high dose groups were significantly higher than those of the control group. In the female rat group, the chloride levels of all treatment groups were lower than those of the control group.

3.7. Effect of the extract on histopathological change

Figs. 1 and 2 and Table S3 show the histopathological results of vital organs examined in this study. There were no treatment-related histopathology changes of the adrenal glands, heart, kidneys, liver, ovaries, spleen, and testes in all testing animals. The incidences of the lesions of adrenal gland, heart, and liver in all animals of high dose group showed no significant treatment-related effects in comparison with those of the control group, and there was no obvious dose-associated toxicity finding for the incidence of the lesion of testes in high dose group. Based on these results, the NOAEL in male and female rats was determined to be greater than 1,666.7 mg/kg BW.

4. Discussion

The idea of herbs as complementary medicine has recently become a prevalent approach to health care around the world. Consumers usually have little concerned with the toxicity and safety of their frequently used dietary supplements, but some studies have observed adverse effects of some herbs [13–15]. The toxicity of the extract of the aerial parts of *C. rabens* in mammals is unclear. Hence, in this present study, we conducted the acute and sub-acute oral toxicity to assess the ethanol extract of the aerial parts of *C. rabens* in rats. Rat is a ubiquitous animal model to investigate pharmacological effects and toxicity of drugs and botanical extracts [16,17].

There were no observations of mortality or clinical abnormality in the acute toxicity study. The oral LD_{50} of the extract was higher than 5,000 mg/kg BW, which is classified into category 5 or unclassified in the Globally Harmonized System [11]. The LD_{50} result presents that the extract is non-toxic [17].

In the oral sub-acute study, there was no significant difference of body weight between all experimental and the control groups during the study period despite some notable changes of feed consumption in the low and middle dose groups. There were no observations of mortality, physical abnormalities, or irrational behaviors in any of the treatment groups. The alteration of relative organ weight is commonly associated with organ enlargement (*e.g.*, congestion, edema) or reduction in organ size (*e.g.*, necrosis, atrophy) caused by toxics [18]. Although the relative adrenal gland, liver, and spleen weights in experimental groups demonstrated the obvious variations as compared with the control groups, the values were still in the normal reference ranges as evidenced by the organ weight data at different ages in SD rats (relative adrenal gland weight: 0.00–0.02 g; relative liver weight: 2.22–2.68 g; relative spleen weight: 0.19–0.25 g) [19].

Changes in hematological parameters in animals provide more accurate information for assessing the toxicity effects in humans [20]. The repeated administration of the extract of stem and leaves of *C. rabens* imposed no significant change on most indexes as compared with the control groups, except for the remarkable increase in MCV and the decreases in MCHC, neutrophils, and lymphocytes. Nonetheless, these alternations were still in the acceptable reference ranges as reported in the

previous study [MCHC: 32.80–35.00 (male), 33.30–35.30 (female) g/dL; MCV: 51.70–55.00 fL, netrophil: 7.90–15.90 %, lymphocyte: 77.80–86.60 %] [21]. The changes of MCHC an MCV are subject to the morphology and osmotic fragility of RBC [22]. Namely, the swelling of RBC leads to a decrease of MCHC. Moreover, lymphocytes and neutrophils are responsible for the broad immune response and antimicrobial activity, respectively [23,24].

Liver and kidney functions are essential in the survival for animals [21]. Herein, liver and kidney function indexes were in the normal reference ranges in animals at all dose levels. The variations of potassium (K) and chloride (Cl) ions levels were in our normal reference ranges based on the historical records measured by SuperLab (an accredited laboratory in New Taipei City, Taiwan) (K: 6.90–9.10 meq/L; Cl: 100.10–103.30 meq/L). These two ions are associated with the heart, muscle, and neural functions. These outcomes also corresponded with the normal findings observed in the histopathological examination. The representative histopathological images were obtained from the similar anatomical locations of the organs, and there were no obvious tissue structure changes observed in these images. Note that the display differences in the images were contributed by the use of fields of view in image acquisition. The macroscopic and microscopic inspection for all animals in the experimental and control groups did not discover significant histopathologic changes due to treatment-related effects caused by the extract on organs. A small number of sporadic lesions in adrenal gland, heart, liver, and testis were observed in the control and high dose groups, which is considered to be clinically insignificant and sporadic in nature. The NOAEL in male and female rats was determined to be > 1, 666.7 mg/kg BW.

5. Conclusion

In summary, there was no significant toxicological findings for the ethanol extract of the aerial parts of *C. rabens* in this research. There were no observations of mortality, or critical pathological and biochemical abnormalities in any of the treatment groups from both the acute and sub-acute toxicity studies. The oral LD_{50} of the extract was greater than 5000 mg/kg BW, which is regarded as a practically non-toxic substance. The NOAEL of the extract in male and female SD rats was greater than 1,666.7 mg/kg BW. Accordingly, our data suggests that the ethanolic extract of the aerial parts of *C. rabens* is non-toxic and safe for human use.

Author contribution

Pang-Kuei Hsu: Project administration, Funding acquisition, Data curation, Manuscript preparation. Yueh-Ting Tsai: Study implementation, Data analysis, Software. Yu-Cheng Lin: Sample preparation, Manuscript preparation. Chen-Meng Kuan: Project administration, Data analysis, Manuscript preparation.

Conflict of interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgment

We are truly grateful to the whole team of SuperLab Co., Ltd. (New Taipei City, Taiwan) for providing the fully technical support.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2021.12.005.

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