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Evaluation of acute and sub-chronic toxicity of bitter melon seed extract in Wistar rats

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ABSTRACT

Pharmacological studies have revealed the potential antidiabetic effects of bitter melon seeds (*Momordica charantia*) in animals and humans. However, the sub-chronic safety of bitter melon seeds remains elusive. This exploratory study aimed to assess the acute and sub-chronic toxicity of a bitter melon seed extract from supercritical carbon dioxide ($scCO_2$) extraction in Wistar rats based on the Organization for Economic Co-operation and Development (OECD) guidelines No. 423 and 408. No mortality and toxicity were observed in rats treated with a single dose of the extract during the 14-day observation period. The median lethal dose (LD_{50}) of the extract was considered greater than 2000 mg/kg body weight (BW). For the sub-chronic toxicity study, male and female rats were orally administered daily doses of 0, 250, 500, and 1000 mg/kg BW for 90 days. No mortality, morbidity, and abnormal pathological and biochemical alterations were observed. The no-observed-adverse-effect-level (NOAEL) of the bitter melon seed extract was greater than 1000 mg/kg BW. Accordingly, bitter melon seed extract from $scCO_2$ extraction may be considered a non-toxic dietary ingredient.

1. Introduction

Bitter melon (*Momordica charantia*, Cucurbitacea) is a common vegetable and well-known ethnomedicinal herb in subtropical regions [1]. The whole plant of bitter melon (e.g., fruit pulp, leaves, and seeds) possesses several pharmacological effects, such as anti-diabetic, anti--hyperlipidemic, contraceptive, laxative, and anti-bacterial effects [2]. In particular, bitter melon is considered a complementary and alternative medicine (i.e., dietary supplement) for glycemic control [3–7]. According to the report by the International Diabetes Federation (IDF), the global diabetes prevalence in adults in 2021 is around 10% (537 million), and the number of patients is projected to increase to 643 million by 2030 [8]. Diabetes causes an increase in healthcare expenditures; for example, the total cost of diabetes diagnosis increased from $327 billion in 2017 to $245 billion in 2012 [9].

The hypoglycemic effects of bitter melon seed extracts have been demonstrated in rodents and patients with diabetes/prediabetes [10–16]. The bitter melon seeds mainly consist of (37.6%, dry basis) and protein (30.4%, dry basis), and their phytochemicals mainly contain flavonoids, phenolics, triterpenes (e.g., cucurbitacins), and saponins (e.g., charatin) [17–20]. According to prior studies, charatin, polypeptide-p, polypeptide-k, momordins, mcIRBP-19, and associated derivatives (e.g., alpha-momorcharin) isolated from the seeds might serve as insulin-like compounds for glucose modulation in muscle and fat cells [1,12–16,21]. For instance, mcIRBP-19 interacts with the insulin receptor and enhances the expression of glucose transporter 4 (GLUT4) in 3T3-L1 adipocytes [21]. Nevertheless, bitter melon seeds are not officially recognized as a dietary ingredient by most countries owing to their possible adverse effects (e.g., hemolytic anemia, stomach pain, headache, abortion, and antifertility) [22]. Alpha-eleostearic acid and lectin purified from the seeds may induce apoptosis and cytotoxicity in leukemia cells [23,24]. Bitter melon seed extracts with the major active component methoxy-phenyl oxime also demonstrated cytotoxicity in human tumor cells [25]. The immune, hepatic, and reproductive toxicity of bitter melon seed extracts was observed in zebrafish embryo and rodent models [26–31]. Rats treated with alpha-momorcharin for five weeks had obvious immunogenicity, immunotoxicity, and liver lesions [26]. Several studies revealed that bitter melon seeds caused histological alterations in the prostate and testes of male rodents [29–31]. Nonetheless, to the best of our knowledge, the long-term safety of bitter melon seed extracts in rodent models is elusive.

In this study, we assessed the acute and sub-chronic oral toxicity of

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bitter melon seed extract from $scCO_2$ extraction in rats in compliance with OECD guidelines No. 423 and 408 [32,33]. This research may provide a scientific perspective on the safety of bitter melon seed supplements during the evaluation of novel foods.

2. Materials and methods

2.1. Preparation of the bitter melon seed extract

Ripe bitter melon was purchased from Xi'an, Shaanxi, China. The ripe yellow fruits were cleaned with running water and cut to gather the seeds manually, and the skin with pulp inside the fruit was disposed of. The red arils surrounding bitter melon seeds were removed. The processed seeds (230 kg) were washed with running and distilled water. Afterwards, the seeds were dehydrated using food dehydrators at 40 °C and ground using a blender. The dried bitter melon seeds were extracted using supercritical carbon dioxide (pressure: 26–27 MPa; temperature 50–53 ℃). Subsequently, the extract was evaporated by freeze-drying for 3 days.

2.2. Animal and dose administration

Wistar rats (*Rattus norvegicus*) were obtained from an animal breeding facility (Jai Research Foundation, JRF). Animals for the acute and sub-chronic toxicity studies were 10–11 and 7–8 weeks old, respectively. The animals were kept in an experimental room (temperature: 19–22 ℃; relative humidity: 55–66%; light cycle: 12 h light/dark) and acclimatized for a period of 5–15 days before the main studies. Rats were housed in groups of two rats/sex/cage during the study period in sterilized polypropylene rat cages. Feed (rodent diet, Envigo) and water were provided ad libitum. The study was approved by the institutional animal ethics committee of JRF.

The bitter melon seed extract was dissolved in reverse osmosis (RO) water. Individual dose volumes were adjusted according to the body weight and dose levels. All rats were administrated via oral gavage (day 0) using a BD 1 mL disposable syringe. The rats were fasted overnight prior to dosing until three hours post-dosing.

2.3. Acute toxicity study

The acute toxicity study complied with OECD guideline no. 423 [32]. The first set of three female rats was administered a single dose of 300 mg/kg body weight (BW) of the bitter melon seed extract. As no mortality was observed at this dose level, the second set of three female rats was administered the same dose of the extract. No mortality was observed at this dose level; therefore, the third set of three female rats was administered 2000 mg/kg BW of the extract. As no mortality was observed at this dose level, the fourth set of three female rats was administered the same dose of the extract. No mortality was observed at this dose. Accordingly, the endpoint was achieved and a further study was not required. The LD_{50} value was determined based on the principle that at least two doses result in mortality rates higher than 0% and lower than 100%.

2.4. Sub-chronic toxicity study

The sub-chronic toxicity study complied with OECD guideline no. 408 [33]. Forty male and forty female rats were randomly assigned into the control (0 mg/kg BW), low-dose (250 mg/kg BW), middle-dose (500 mg/kg BW), or high-dose (1000 mg/kg BW) group. The prepared dose formulations of the bitter melon seed extract in RO water were administered orally by gavage to rats for 90 days. During the experimental period, all rats were observed for signs of toxicity, morbidity, and mortality. Body weight was recorded weekly. Body weight and food consumption were measured weekly. Ophthalmological examinations were performed before and after the study. Neurobehavioral observations were performed weekly. A functional observational battery was performed during week 12 in the main groups. At the end of the treatment, clinical pathology evaluations were performed in all rats. Microscopic examination was performed using the tissues and organs in the control and high-dose groups.

2.5. Clinical pathology

At the end of the treatment, blood samples were collected from rats under light isoflurane anesthesia by orbital plexus puncture using a fine heparinized capillary tube. Rats were fasted overnight (ad libitum supply of drinking water) before blood collection. Blood samples were collected for hematology (in vials containing 4% EDTA anticoagulant for whole blood), coagulation parameters (in vials containing 3.2% sodium citrate anticoagulant for plasma separation), and biochemical analysis (in plain vials for serum separation). The thyroid hormones, triiodothyronine (T3) and thyroxine (T4), were analyzed using liquid chromatography tandem-mass spectrometry (LC-MS/MS), and thyroid-stimulating hormone (TSH) levels were determined with ELISA.

2.6. Pathological examination

All rats were euthanized by carbon dioxide asphyxiation and subjected to full gross necropsy under the supervision of a veterinary pathologist. All rats were carefully examined for external abnormalities. The cranial, thoracic, and abdominal cavities were cut and opened, and a thorough examination of the organs was performed to detect abnormalities. All macroscopic abnormalities observed during necropsy were recorded.

Organs and tissues from both sexes were collected, weighed, and preserved. Adherent adipose tissue from the organs was removed via trimming, and the wet weights of the organs were recorded. All organs were preserved in 10% neutral buffered formalin solution, except for the testes and eyes, which were collected in modified Davidson's fluid and Davidson's fluid, respectively. The paired organs were weighed together, and the combined weights presented. The parathyroid and pituitary weights of the thyroid gland were determined after fixation. The organ weight ratio was determined as a percentage of terminal body weight.

Histopathological examination was carried out on the preserved organs and tissues of rats from the control and high-dose groups. The vital organs and tissue samples were processed, embedded, cut into 3–5 μm thick sections, and stained with hematoxylin and eosin.

2.7. Animal welfare

The acute and sub-chronic toxicity studies complied with the guidelines of the "Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International" and "Guidelines for Laboratory Animals Facility" issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Compliance with these guidelines ensured humane care of the animals throughout the experiment and further enhanced the well-being of animals, which subsequently promoted the quality of outcomes of the experiments for the advancement of biological knowledge relevant to humans and animals. The project proposals were approved by the "Institutional Animal Ethics Committee (IAEC)," JRF. The ethics proposal numbers for the acute and subchronic studies were JRF/IAEC/2020/471 and JRF/IAEC/2020/461, respectively.

2.8. Statistical analysis

Data were presented as the group mean and standard deviation. The Kolmogorov-Smirnov test or Shapiro–Wilk test was first employed for normality checks of all data [34]. Subsequently, based on the analysis of homogeneity of variance [F-test (comparison of two groups), Bartlett's

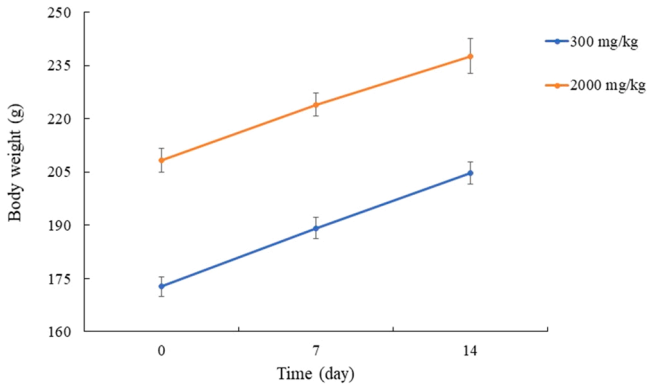

Fig. 1. Effect of the single administration of bitter melon seed extract on the body weight of rats ($n = 6$; mean value ± SEM).

test (comparison of three groups), Levene's test (comparison of three groups), or Brown–Forsythe test (comparison of three groups)], significant analyses of body weight, food consumption, absolute organ weight, relative organ weight, hematology, and clinical chemistry were determined using Student's *t*-test, Satterthwaite method, or analysis of variance (ANOVA) with Dunnett's test [35]. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Acute toxicity assessment of the bitter melon seed extract

The animals were orally administered a single dose of 300 or 2000 mg/kg BW of the bitter melon seed extract. The mean body weights of male and female rats in the low-and high-dose groups increased during the 14-day observation period (Fig. 1). The food consumption and water intake patterns of animals did not show significant changes (data not shown). Further, mortality, abnormal clinical signs, or obvious pathological lesions were not observed. Accordingly, the LD_{50} of the bitter melon seed extract in rats was greater than 2000 mg/kg BW.

3.2. Sub-chronic toxicity assessment of bitter melon seed extract

No mortality or morbidity was observed in this study. Clinical and neurobehavioral observations and ophthalmological examination of the skin, fur, mucous membranes, occurrence of secretions and excretions, and autonomic activity did not reveal any abnormal effects in the animals during the 90-day study period.

3.3. Influence of bitter melon seed extract on body weight and food consumption

There was no statistical difference in weekly body weight between the control and experimental groups for either sex (Fig. 2). The weight gain of male and female rats displayed similar trends. Food consumption by female rats in the experimental groups was comparable with that of females in the control group; however, feed intake by male rats in the low-dose group at week 1 and male rats in the middle-dose group at weeks 1 and 7 was significantly decreased relative to that consumed by rats in the control group.

3.4. Influence of bitter melon seed extract on biochemical indices

Hematological examination did not reveal any noticeable findings or treatment-associated effects in all experimental groups (Table 1). Table 2 shows the results of the biochemical parameters. The liver and kidney indexes in the experimental groups did not reveal any obvious findings except a significant increase in γ-glutamyltransferase (γ-GT) in female rats in the high-dose group. In male rats, the albumin/globulin (A/G) ratios in all treatment groups were significantly lower than those in the control group. In addition, there was no statistically significant difference in the levels of T3, T4, and TSH between the control and experimental groups (Table 3).

3.5. Influence of bitter melon seed extract on pathological parameters

Gross findings did not reveal abnormal alterations or palpable lesions in any of the animals (Tables S1 and S2). In female rats, the absolute and relative weights of the liver, heart, spleen, brain, and thymus were

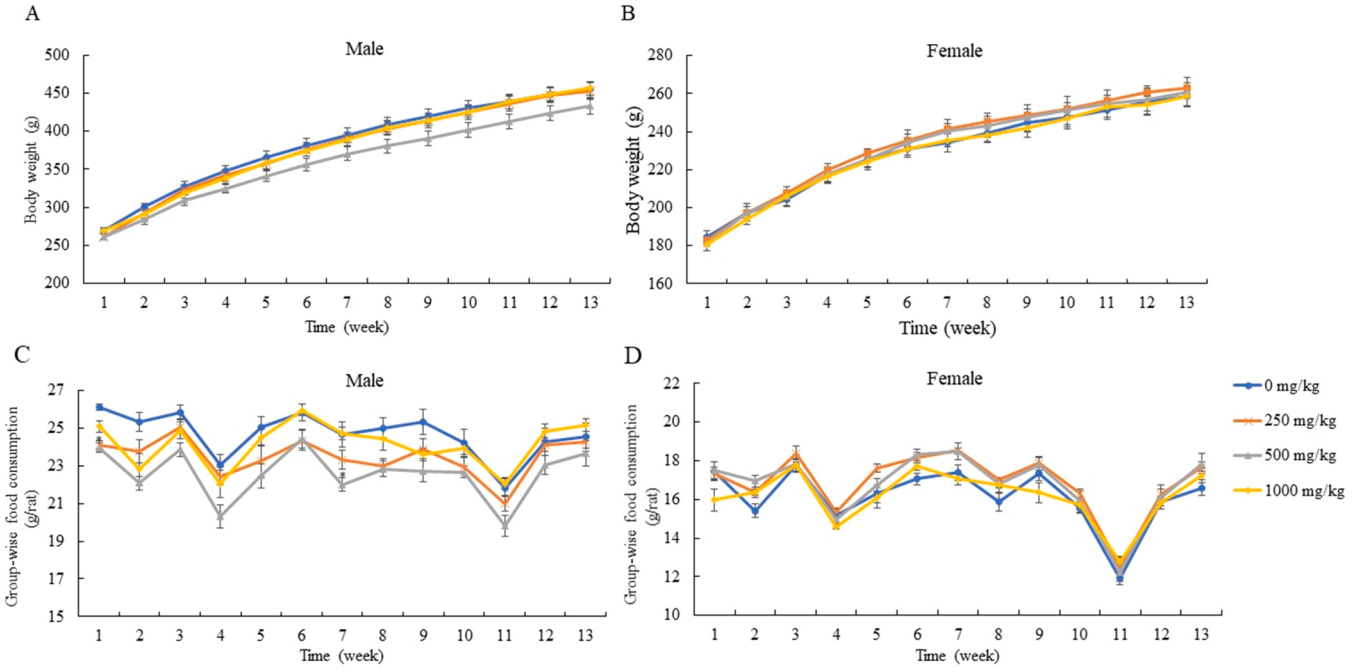

Fig. 2. Effects of the repeated administration of bitter melon seed extract on the body weight and food consumption of rats. (A, B) Body weight of both sexes ($n = 10$; mean value ± SEM). (C, D) Group-wise food consumption for both sexes ($n = 5$; mean value ± SEM).

Table 1
Results of the hematological analysis for rats ($n = 10$; mean value ± S.D.).

Parameter	0 mg/kg		250 mg/kg		500 mg/kg		1000 mg/kg	
Sex	Male	Female	Male	Female	Male	Female	Male	Female
WBC (10^3/μL)	5.12 ± 1.06	3.68 ± 1.00	5.96 ± 1.58	3.83 ± 1.24	5.49 ± 1.11	3.37 ± 0.80	6.21 ± 1.03	3.48 ± 0.92
RBC (10^6/μL)	9.35 ± 0.55	8.52 ± 0.29	9.16 ± 0.60	8.38 ± 0.25	9.09 ± 0.45	8.47 ± 0.44	9.27 ± 0.37	8.56 ± 0.17
Hemoglobin (g/dL)	16.38 ± 0.40	15.77 ± 0.39	16.54 ± 0.51	15.57 ± 0.39	16.44 ± 0.45	15.76 ± 0.51	16.38 ± 0.53	15.89 ± 0.38
Hematocrit (%)	46.26 ± 1.72	44.51 ± 0.80	46.26 ± 1.76	43.41 ± 0.91	45.55 ± 1.82	44.26 ± 1.53	46.42 ± 1.42	44.92 ± 1.21
MCV (fL)	49.59 ± 2.04	52.30 ± 1.26	50.58 ± 1.77	51.83 ± 1.34	50.15 ± 1.05	52.28 ± 1.56	50.12 ± 0.87	52.51 ± 0.91
MCH (pg)	17.57 ± 1.01	18.53 ± 0.47	18.12 ± 1.14	18.58 ± 0.53	18.09 ± 0.64	18.65 ± 0.95	17.69 ± 0.55	18.57 ± 0.44
MCHC (g/dL)	35.40 ± 0.95	35.46 ± 0.46	35.78 ± 1.19	35.87 ± 0.67	36.12 ± 0.73	35.60 ± 0.99	35.31 ± 0.57	35.37 ± 0.69
Platelet (10^3/μL)	978.20 ± 93.29	992.70 ± 121.02	953.40 ± 51.41	934.30 ± 63.56	943.60 ± 71.36	986.10 ± 64.71	998.10 ± 63.67	1018.50 ± 58.70
Neutrophil (10^3/μL)	1.37 ± 0.25	0.70 ± 0.13	1.73 ± 0.82	0.85 ± 0.31	1.40 ± 0.44	0.64 ± 0.16	1.38 ± 0.17	0.91 ± 0.43
Lymphocyte (10^3/μL)	3.32 ± 1.04	2.68 ± 0.81	3.75 ± 0.98	2.71 ± 1.00	3.63 ± 0.80	2.50 ± 0.62	4.34 ± 0.83	2.29 ± 0.65
Monocyte (10^3/μL)	0.17 ± 0.05	0.11 ± 0.04	0.19 ± 0.09	0.11 ± 0.03	0.17 ± 0.05	0.09 ± 0.04	0.17 ± 0.05	0.10 ± 0.05
Eosinophil (10^3/μL)	0.12 ± 0.04	0.10 ± 0.08	0.12 ± 0.04	0.07 ± 0.03	0.13 ± 0.04	0.06 ± 0.02	0.11 ± 0.03	0.08 ± 0.04
Basophil (10^3/μL)	0.08 ± 0.03	0.05 ± 0.03	0.11 ± 0.03	0.05 ± 0.02	0.09 ± 0.03	0.05 ± 0.03	0.10 ± 0.02	0.05 ± 0.02
Retic count ($\times 10^9$/L)	180.33 ± 28.01	176.35 ± 30.77	175.49 ± 28.06	189.49 ± 26.90	188.60 ± 34.45	187.58 ± 35.08	194.42 ± 34.00	175.43 ± 30.99
PT (sec.)	12.18 ± 0.53	9.58 ± 0.82	12.04 ± 0.38	10.26 ± 0.69	12.22 ± 0.57	10.59 ± 1.44	12.16 ± 0.63	9.82 ± 0.99
APTT (sec.)	16.26 ± 2.08	18.36 ± 1.32	17.46 ± 1.71	19.03 ± 1.58	16.70 ± 1.51	19.73 ± 1.31	16.45 ± 1.75	17.65 ± 2.08

WBC, white blood cells; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PT, prothrombin time; APTT, activated partial thromboplastin time.

Table 2
Results of the biochemical analysis for rats ($n = 10$; mean value ± S.D.).

Parameter	0 mg/kg		250 mg/kg		500 mg/kg		1000 mg/kg	
	Male	Female	Male	Female	Male	Female	Male	Female
Glucose (mg/dL)	119.41 ± 14.41	120.98 ± 14.69	115.37 ± 16.27	117.32 ± 17.66	118.99 ± 12.30	120.97 ± 23.20	124.30 ± 12.66	109.24 ± 18.14
Total cholesterol (mg/dL)	104.60 ± 19.49	85.37 ± 12.10	92.60 ± 15.08	81.86 ± 14.57	96.80 ± 16.92	79.91 ± 9.01	97.40 ± 11.72	88.18 ± 21.46
Triglycerides (mg/dL)	80.80 ± 21.91	42.83 ± 9.45	84.63 ± 43.97	40.60 ± 7.74	73.03 ± 21.21	47.67 ± 8.99	69.07 ± 29.71	43.23 ± 8.02
Creatinine (mg/dL)	0.65 ± 0.08	0.63 ± 0.04	0.64 ± 0.06	0.67 ± 0.05	0.64 ± 0.04	0.64 ± 0.03	0.66 ± 0.07	0.64 ± 0.04
γ-GT (U/L)	0.98 ± 1.18	0.32 ± 0.35	0.72 ± 0.62	1.30 ± 2.04	1.63 ± 3.43	0.52 ± 0.49	1.35 ± 1.35	0.69 ± 0.42 *
ALP (U/L)	82.23 ± 16.90	31.24 ± 3.18	89.35 ± 14.23	36.43 ± 11.27	80.34 ± 10.20	40.69 ± 21.40	83.44 ± 17.56	35.63 ± 13.78
ALT (U/L)	57.89 ± 20.35	31.45 ± 5.41	61.87 ± 13.84	32.32 ± 6.61	67.78 ± 18.05	33.67 ± 6.19	56.64 ± 9.38	38.99 ± 8.52
AST (U/L)	133.66 ± 29.77	101.13 ± 13.83	127.31 ± 29.17	112.66 ± 34.18	124.87 ± 41.38	96.66 ± 20.66	139.31 ± 46.34	113.38 ± 21.69
Calcium (mg/dL)	10.86 ± 0.38	10.72 ± 0.18	10.71 ± 0.16	10.69 ± 0.22	10.85 ± 0.42	10.37 ± 0.68	10.86 ± 0.21	10.73 ± 0.45
Phosphorus (mg/dL)	5.44 ± 0.62	4.61 ± 0.73	5.75 ± 0.83	5.05 ± 0.82	5.48 ± 0.51	4.45 ± 0.66	5.66 ± 0.49	4.90 ± 1.11
Total protein (g/dL)	6.71 ± 0.27	7.00 ± 0.25	6.79 ± 0.28	6.89 ± 0.29	6.94 ± 0.32	6.94 ± 0.47	6.91 ± 0.24	7.17 ± 0.37
Albumin (g/dL)	3.92 ± 0.14	4.21 ± 0.10	3.88 ± 0.12	4.10 ± 0.22	3.96 ± 0.15	4.13 ± 0.35	3.94 ± 0.11	4.24 ± 0.23
Globulin (g/dL)	2.79 ± 0.18	2.79 ± 0.22	2.91 ± 0.17	2.79 ± 0.12	2.98 ± 0.18	2.81 ± 0.16	2.96 ± 0.17	2.93 ± 0.16
A/G	1.41 ± 0.07	1.52 ± 0.12	1.34 ± 0.04 *	1.47 ± 0.07	1.33 ± 0.05 *	1.47 ± 0.09	1.33 ± 0.07 *	1.45 ± 0.06
Urea (mg/dL)	37.17 ± 4.29	43.04 ± 6.31	37.02 ± 5.43	48.91 ± 7.95	39.29 ± 3.96	44.98 ± 4.93	39.19 ± 4.89	45.24 ± 6.07
BUN (mg/dL)	17.36 ± 2.00	20.10 ± 2.94	17.29 ± 2.54	22.84 ± 3.71	18.35 ± 1.85	21.01 ± 2.30	18.30 ± 2.28	21.13 ± 2.84
Total bilirubin (μmol/L)	0.78 ± 0.34	0.58 ± 0.55	0.77 ± 0.18	0.84 ± 0.58	0.85 ± 0.22	0.57 ± 0.39	0.72 ± 0.32	0.29 ± 0.27
HDL-C (mg/dL)	19.63 ± 1.06	18.55 ± 2.99	17.83 ± 2.22	18.25 ± 3.20	18.42 ± 2.30	17.45 ± 2.09	18.19 ± 2.26	19.33 ± 5.06
LDL-C (mg/dL)	3.20 ± 1.06	2.91 ± 0.81	3.46 ± 1.58	3.17 ± 1.06	3.60 ± 0.89	3.62 ± 1.29	1.88 ± 0.15	3.23 ± 1.17
Sodium (meq/L)	144.54 ± 0.84	142.79 ± 0.61	144.21 ± 1.54	142.96 ± 1.11	144.13 ± 1.11	143.04 ± 1.39	147.10 ± 1.60	143.50 ± 0.91
Potassium (meq/L)	4.43 ± 0.22	4.29 ± 0.29	4.41 ± 0.22	4.25 ± 0.42	4.38 ± 0.25	4.18 ± 0.28	8.59 ± 1.49 *	4.34 ± 0.34
Chloride (meq/L)	109.10 ± 1.11	107.69 ± 1.12	108.35 ± 1.47	108.01 ± 2.04	108.19 ± 0.99	107.72 ± 2.18	99.70 ± 1.49	108.62 ± 1.36

γ-GT, γ-glutamyltransferase; ALP, alkaline phosphatase; AST, aspartate transaminase; ALT, alanine aminotransferase; A/G, albumin/globulin; BUN, blood urea nitrogen; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. * $p < 0.05$; significance compared to the control group.

Table 3
Results of thyroid hormone analysis for rats ($n = 10$; mean value ± S.D.).

	T3 (ng/mL)		T4 (ng/mL)		TSH (ng/mL)	
Group	Male	Female	Male	Female	Male	Female
0 mg/kg	0.497 ± 0.091	0.532 ± 0.089	66.648 ± 5.457	41.965 ± 6.793	1.200 ± 0.595	0.837 ± 0.524
250 mg/kg	0.492 ± 0.085	0.556 ± 0.096	70.218 ± 10.685	47.374 ± 8.809	0.921 ± 0.301	1.484 ± 1.609
500 mg/kg	0.509 ± 0.098	0.440 ± 0.150	75.909 ± 4.644	38.036 ± 13.254	1.048 ± 0.734	1.187 ± 0.683
1000 mg/kg	0.507 ± 0.087	0.390 ± 0.180	73.361 ± 11.495	30.228 ± 12.351	1.139 ± 0.672	1.023 ± 0.433

T3, triiodothyronine; T4, thyroxine (T4); TSH, thyroid-stimulating hormone.

Table 4
Results of the absolute and relative organ weights of rats ($n = 10$; mean value ± S.D.).

	Organ	0 mg/kg		250 mg/kg		500 mg/kg		1000 mg/kg	
		Absolute organ weight (g)	Relative organ weight (%)	Absolute organ weight (g)	Relative organ weight (%)	Absolute organ weight (g)	Relative organ weight (%)	Absolute organ weight (g)	Relative organ weight (%)
Male	Liver	11.624 ± 0.681	2.649 ± 0.124	11.688 ± 1.543	2.662 ± 0.177	11.167 ± 1.249	2.668 ± 0.164	11.733 ± 1.267	2.664 ± 0.246
	Heart	1.178 ± 0.089	0.269 ± 0.024	1.167 ± 0.088	0.267 ± 0.019	1.133 ± 0.104	0.271 ± 0.012	1.165 ± 0.121	0.264 ± 0.021
	Spleen	0.632 ± 0.078	0.144 ± 0.014	0.622 ± 0.086	0.142 ± 0.014	0.621 ± 0.084	0.149 ± 0.019	0.664 ± 0.087	0.151 ± 0.016
	Brain	2.151 ± 0.109	0.491 ± 0.035	2.105 ± 0.092	0.482 ± 0.032	2.080 ± 0.109	0.499 ± 0.030	2.104 ± 0.110	0.478 ± 0.023
	Thymus	0.289 ± 0.059	0.066 ± 0.013	0.351 ± 0.084	0.080 ± 0.015	0.297 ± 0.040	0.071 ± 0.010	0.349 ± 0.062	0.080 ± 0.016
	Kidneys	2.327 ± 0.225	0.529 ± 0.0.34	2.333 ± 0.202	0.533 ± 0.032	2.223 ± 0.182	0.532 ± 0.024	2.357 ± 0.247	0.536 ± 0.056
	Adrenals	0.065 ± 0.005	0.015 ± 0.001	0.067 ± 0.012	0.015 ± 0.002	0.066 ± 0.008	0.016 ± 0.002	0.067 ± 0.010	0.015 ± 0.003
	Testes	4.035 ± 0.325	0.919 ± 0.069	3.863 ± 0.252	0.885 ± 0.072	3.983 ± 0.337	0.958 ± 0.102	3.960 ± 0.529	0.898 ± 0.100
	Epididymides	1.322 ± 0.099	0.301 ± 0.019	1.299 ± 0.120	0.297 ± 0.024	1.286 ± 0.072	0.309 ± 0.025	1.225 ± 0.193	0.278 ± 0.039
	Seminal vesicles with coagulating glands and prostate	2.184 ± 0.388	0.495 ± 0.069	2.291 ± 0.403	0.522 ± 0.073	2.309 ± 0.259	0.554 ± 0.065	2.349 ± 0.312	0.533 ± 0.059
	Thyroid with parathyroid	0.0216 ± 0.0029	0.0049 ± 0.0005	0.0234 ± 0.0038	0.0053 ± 0.0007	0.0216 ± 0.0025	0.0052 ± 0.0007	0.0254 ± 0.0029[a]	0.0058 ± 0.0009
	Pituitary gland	0.0104 ± 0.0016	0.0024 ± 0.0003	0.0104 ± 0.0018	0.0024 ± 0.0003	0.0102 ± 0.0012	0.0025 ± 0.0002	0.0107 ± 0.0016	0.0024 ± 0.0003
Female	Liver	7.015 ± 0.404	2.856 ± 0.267	7.305 ± 0.520	2.929 ± 0.252	7.157 ± 0.919	2.880 ± 0.173	7.174 ± 0.617	2.927 ± 0.200
	Heart	0.836 ± 0.038	0.340 ± 0.017	0.837 ± 0.068	0.335 ± 0.028	0.848 ± 0.072	0.343 ± 0.032	0.820 ± 0.058	0.335 ± 0.020
	Spleen	0.440 ± 0.039	0.179 ± 0.021	0.454 ± 0.042	0.182 ± 0.020	0.450 ± 0.063	0.182 ± 0.026	0.425 ± 0.055	0.174 ± 0.023
	Brain	1.969 ± 0.073	0.801 ± 0.055	1.981 ± 0.048	0.794 ± 0.044	1.916 ± 0.114	0.776 ± 0.064	1.907 ± 0.083	0.780 ± 0.048
	Thymus	0.308 ± 0.033	0.125 ± 0.011	0.337 ± 0.073	0.135 ± 0.028	0.326 ± 0.064	0.132 ± 0.027	0.303 ± 0.054	0.123 ± 0.020
	Kidneys	1.552 ± 0.076	0.631 ± 0.033	1.587 ± 0.075	0.636 ± 0.045	1.514 ± 0.144	0.611 ± 0.038	1.507 ± 0.093	0.615 ± 0.040
	Adrenals	0.076 ± 0.009	0.031 ± 0.005	0.081 ± 0.004	0.033 ± 0.003	0.080 ± 0.014	0.032 ± 0.004	0.078 ± 0.009	0.032 ± 0.003
	Ovaries	0.089 ± 0.010	0.036 ± 0.005	0.095 ± 0.022	0.038 ± 0.008	0.104 ± 0.011	0.042 ± 0.003	0.092 ± 0.017	0.038 ± 0.006
	Uterus with cervix	0.628 ± 0.130	0.255 ± 0.049	0.708 ± 0.114	0.284 ± 0.049	0.742 ± 0.166	0.304 ± 0.084	0.641 ± 0.151	0.262 ± 0.064
	Thyroid with parathyroid	0.0159 ± 0.0019	0.0065 ± 0.0007	0.0165 ± 0.0023	0.0066 ± 0.0011	0.0166 ± 0.0026	0.0067 ± 0.0011	0.0149 ± 0.0015	0.0061 ± 0.0009
	Pituitary gland	0.0164 ± 0.0020	0.0067 ± 0.0010	0.0159 ± 0.0022	0.0064 ± 0.0010	0.0150 ± 0.0016	0.0061 ± 0.0008	1.0151 ± 0.0020	0.0062 ± 0.0008

[a] $p < 0.05$; significant compared to the control group.

determined. The kidneys, adrenals, testes/ovaries, epididymides, seminal vesicles with coagulating glands and prostate, thyroid with parathyroid glands, and pituitary gland in the treatment groups were similar with those of the control group (Table 4). On the other hand, in male rats, the absolute weight of the thyroid with parathyroid in the high-dose group was significantly higher than that of the control group, but other organ weights in the treatment groups were similar with those in the control group.

The microscopic lesions observed in various organs were of low occurrence, non-specific, and insignificant (Tables S1, S2). These lesions were considered spontaneous or incidental in nature, and were not related to treatment with the test item. Figs. 3 and 4 show the histopathological results of vital organs in men and women. There were no treatment-related histopathological changes in the liver, kidneys, heart, spleen, adrenals, brain, or testes/ovaries in the control and high-dose groups. Non-specific lesions of the kidneys, heart, and lungs in male rats and adrenals, kidneys, liver, and spleen in female rats were incidentally observed in the control or high-dose groups; however, these lesions were not associated with treatment-related effects or dose-related toxicity. As a result, the no observed adverse effect level (NOAEL) of male and female rats was greater than 1000 mg/kg BW.

4. Discussion

In this study, bitter melon seed extract was prepared by $scCO_2$ extraction rather than aqueous or organic extraction. $scCO_2$ is a green and nontoxic solvent for separating hydrophobic components from liquid solutions [36]. The bitter melon seed extract contained 29.88% of protein, its lipid content was less than 8%, and the levels of mcIRBP-19, charantin, vicine, and cucurbitacin B were 969 ppm, 645.48 ppm, 21.82 mg/g, and 0.94 mg/g, respectively, in this extract (data not shown). Charantin is a peptide that resembles insulin; however, its toxicity remains elusive [37]. Vicine may cause favism in individuals with genetically inherited deficiencies in glucose-6-phosphate dehydrogenase (G6PD) [38]. According to a prior study, 50–150 mg/kg BW of vicine exerted good anti-inflammatory effects and no hepatotoxic

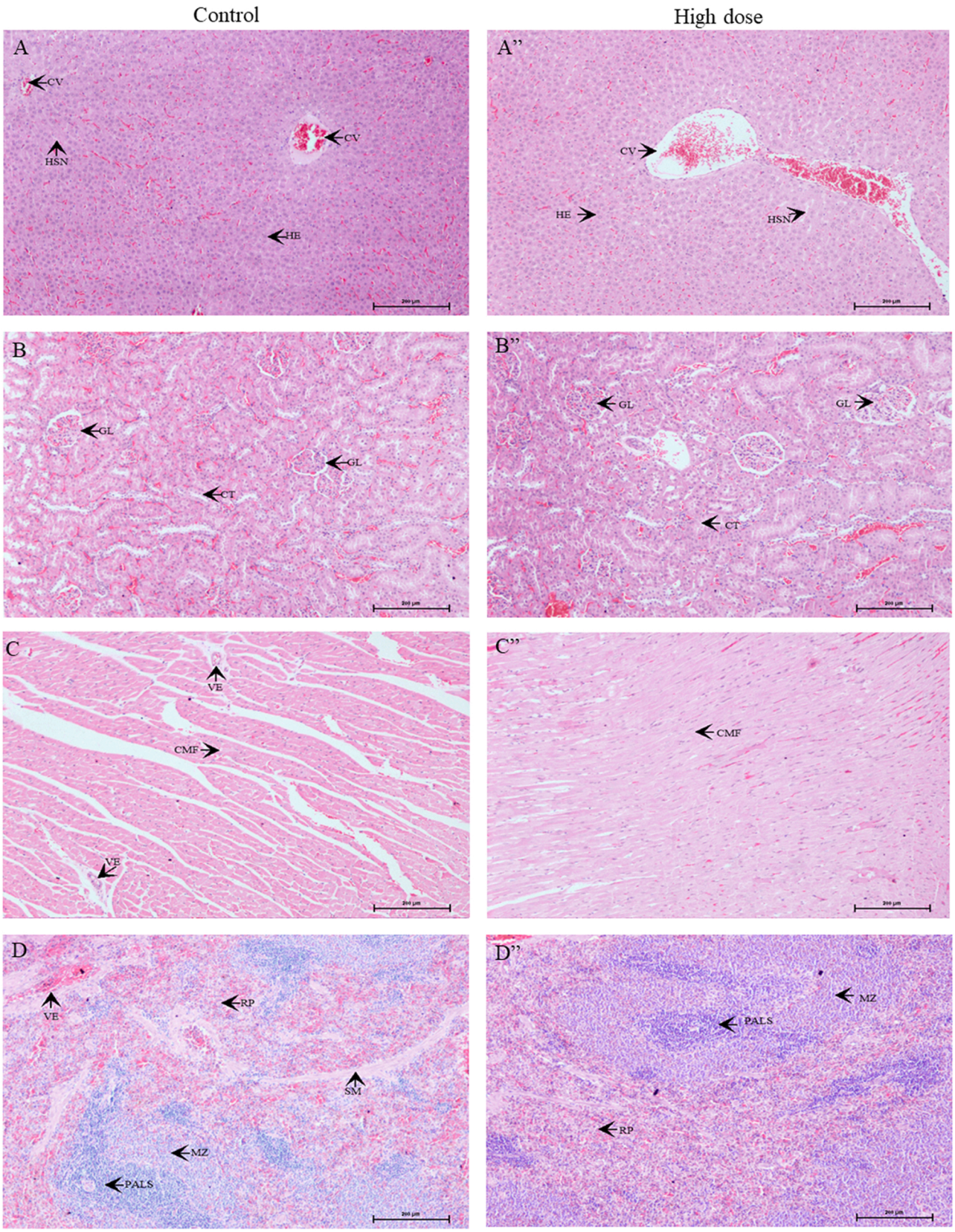

Fig. 3. Microscopic sections of the liver (A, A”), kidneys (B, B”), heart (C, C”), spleen (D, D”), adrenals (E, E”), brain (F, F”), and testes (G, G”) in male rats. A-F represent the results of the control group. A”- F” represent the results of the high dose group. CV: central vein, HSN: hepatic sinusoids; HE: hepatocytes; GL: glomerulus; CT: convoluted tubules; CMF: cardiomyofiber; VE: vessel; PALS: periarteriolar lymphoid sheath; MZ: marginal zone, RP: red pulp; SM: smooth muscles; ME: medulla; CO: cortex; DG: dentate gyrus; HP: hippocampus; FPC: fronto parietal cortex; ST: seminiferous tubules, LC: Leydig cells; GC: germ cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

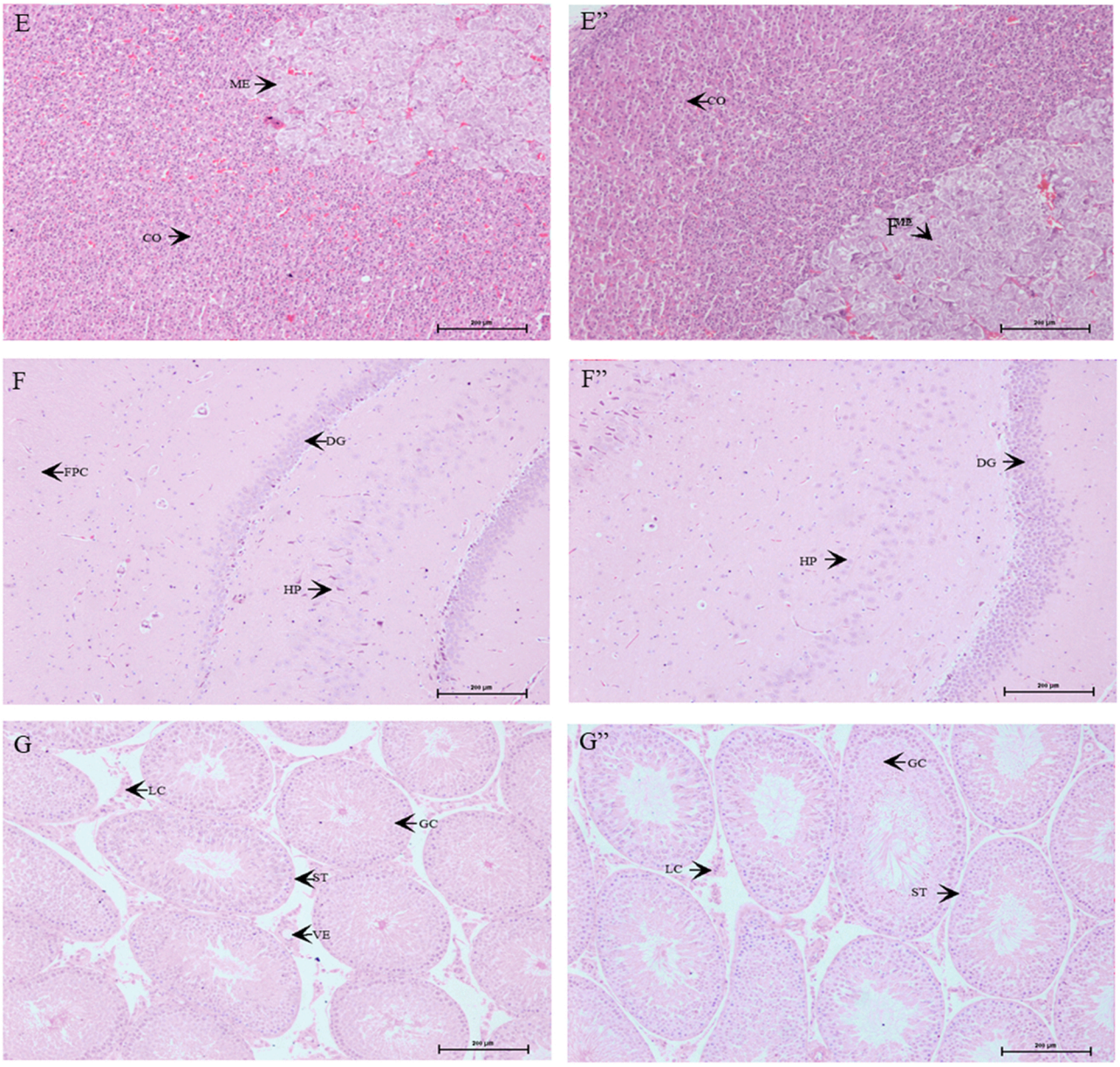

Fig. 3. (*continued*).

activity in rats. Further, the LD_{50} of vicine was 2100 mg/kg BW in rats [39]. The dose levels of 300 and 2000 mg/kg/BW of the bitter melon seed extract represented 6.55 and 43.64 mg/kg BW in this acute toxicity study; thus, the utilized dosages appeared within the safe range [40]. Moreover, the LD_{50} of cucurbitacin B was 1.1 mg/kg BW in mice [41]. The bioavailability of a chemical administered intraperitoneally was approximately 6-fold higher than that administered orally; thus, we deduced that cucurbitacin B had a minimal impact in this study [42].

No obvious findings of mortality or abnormal clinical signs were observed in the acute toxicity study. The oral LD_{50} of the extract was greater than 2000 mg/kg BW. According to the Globally Harmonized System of Classification, bitter melon seed extract is classified as category 5 or unclassified [32]. Abalaka et al. and Husna et al. reported that the LD_{50} values of the ethanolic extracts of bitter melon were 1200 and $<$ 2000 mg/kg BW, respectively, in rats [31,34]. In addition, a methanolic extract of bitter melon seeds was found to have a lethal effect in zebrafish embryos [28]. These results were not consistent with our observations; this discrepancy might be in part due to the differences in the extraction method, the bitter melon used, and the study model.

In the repeated-dose 90-day study, there was no statistically significant difference in body weight between both sexes in the control and treatment groups. The decline in food intake in male rats was marginal, dose-independent, and inconsistent; therefore, the change in food consumption was not considered a treatment-related effect. Hematological parameters in animals enable the identification of concordant target organ toxicities in humans [43]. The hematological parameters in all animals were within the reference ranges, and there were no noticeable changes in hematological parameters between the control and treatment groups. Liver and kidney functions are essential for survival and play important roles in detoxification in animals [44]. Although a significant increase in γ-GT was noted in female rats in the high-dose group, this phenomenon was not caused by treatment-related effects due to lack of consistency between sexes and the absence of concomitant effects on ALT, AST, and total bilirubin. This outcome might be explained by the presence of hepatotoxins in bitter melon seeds [27]. Tennekoon et al. observed elevated γ-GT and ALP levels in rats after oral treatment with a bitter melon seed extract; however, no noticeable histopathological changes were observed in the liver [27]. The kidney function indices in

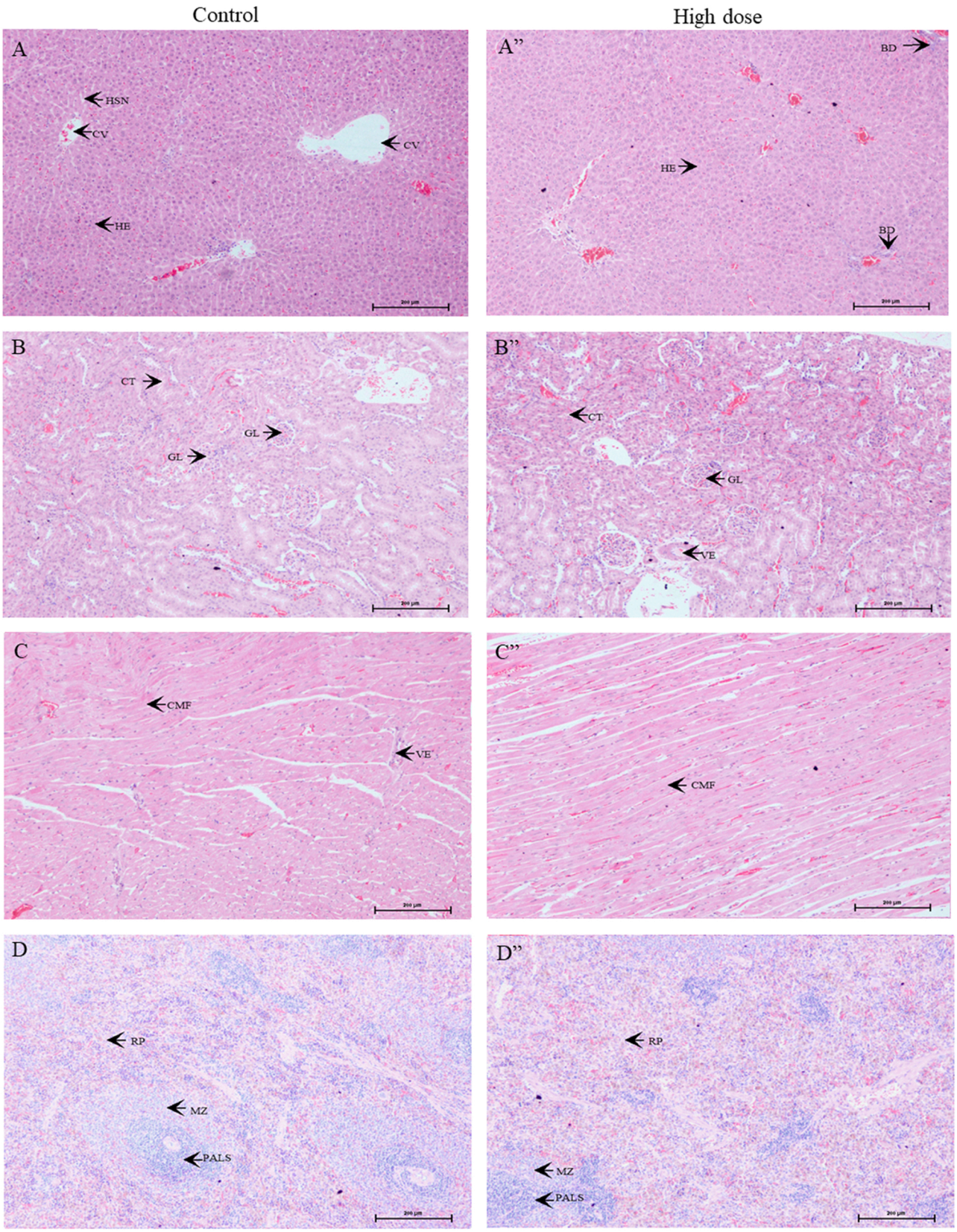

Fig. 4. Microscopic sections of the liver (A, A”), kidneys (B, B”), heart (C, C”), spleen (D, D”), adrenals (E, E”), brain (F, F”), and ovaries (G, G”) in female rats. A-F represent the results of the control group. A”- F” represent the results of the high dose group. CV: central vein, HSN: hepatic sinusoids; HE: hepatocytes; BD: bile duct; GL: glomerulus; CT: convoluted tubules; VE: vessel; CMF: cardiomyofiber; PALS: periarteriolar lymphoid sheath; MZ: marginal zone, RP: red pulp; ME: medulla; CO: cortex; DG: dentate gyrus; HP: hippocampus; FPC: fronto parietal cortex; CL: corpus luteum; IC: interstitial cells; GF: graafian follicle; GRF: growing follicle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

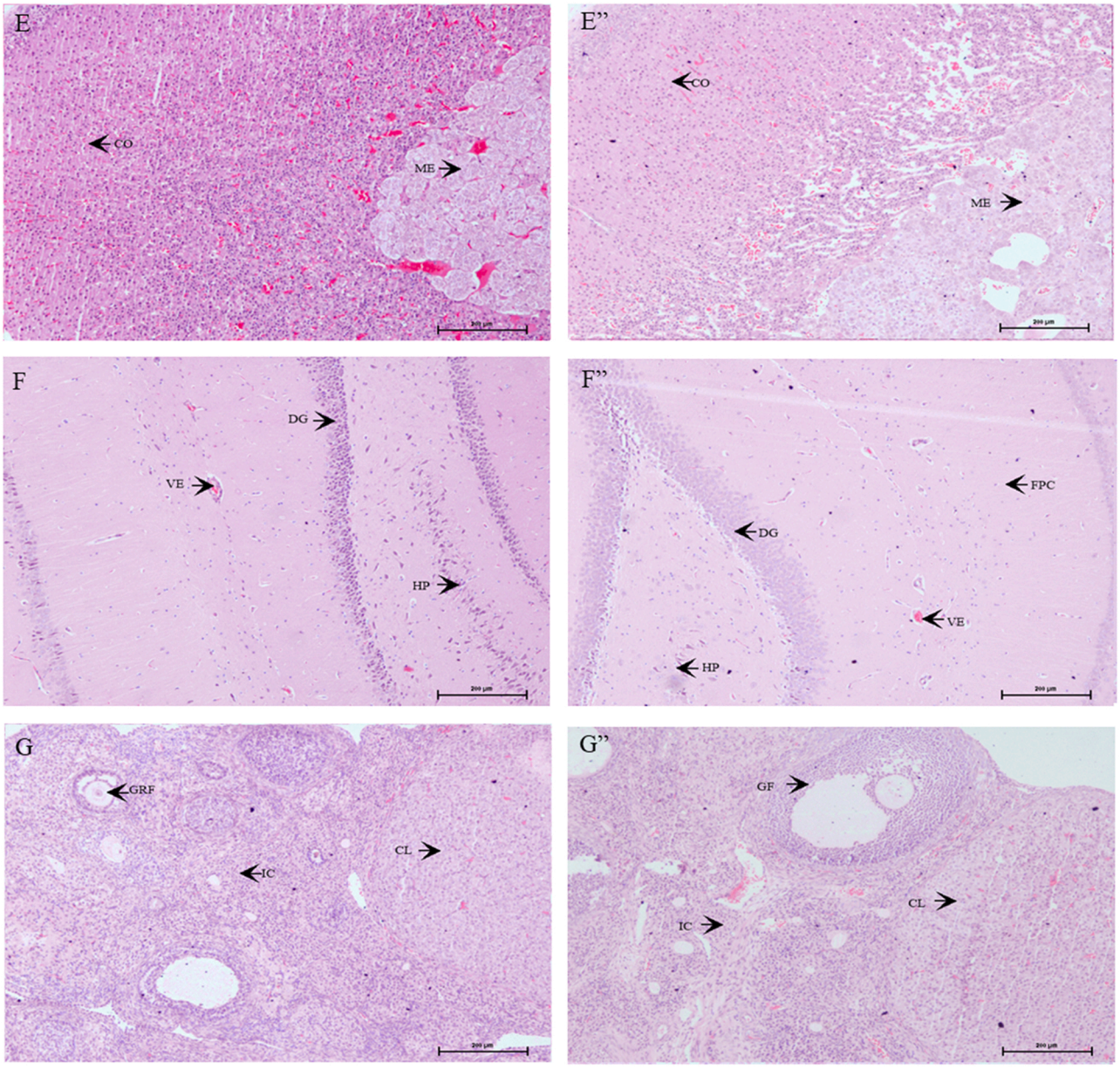

Fig. 4. (*continued*).

all animals did not show any abnormalities. The decrease in A/G ratios in male rats in the treatment groups was still within the historical reference ranges (male: 1.01 −2.43; female: 1.08 −3.54) and might not be considered treatment-related effects, owing to the absence of similar findings in female rats. The thyroid gland is a vital endocrine system that secretes thyroid hormones that modulate the basal metabolic rate and affect diverse bodily functions [45]. The extract did not interfere with the function of the thyroid gland, as the levels of TSH, T3, and T4 in both sexes in the treatment groups were comparable to those in the control groups. To our knowledge, this is the first study to report the thyroid toxicity of bitter melon seeds.

An increase in the absolute weight of the thyroid gland in male rats in the high-dose group did not occur in female rats, which may be attributed to non-treatment-related effects. Pathological examination did not reveal any significant findings, and microscopic lesions were considered spontaneous and incidental. The results correlated with the outcomes of biochemical parameters. Importantly, pathological examination did not reveal histomorphological alterations in the seminiferous tubules and prostate of male rats observed in previous reproductive studies [29–31].

Regarding the teratogenic effect and miscarriage caused by bitter melon seeds, we conducted a prenatal developmental toxicity study based on the OECD guideline No. 414 to clarify the concerns of the sample [46]. The results of body weight, weight gain, reproductive parameters (e.g., live/dead fetuses, sex ratio, anogenital distance), fetal examination (i.e., external and visceral observations), organ weight, and thyroid hormone analysis in all treatment groups (250, 500, and 1000 mg/kg BW of the bitter melon seed extract from $scCO_2$ extraction) were comparable with those of the control group. The toxic effects of bitter melon seed extract, especially hepatotoxicity, nephrotoxicity, and reproductive toxicity, were not observed in this study. The NOAEL of bitter melon seed extract was greater than 1000 mg/kg BW, which is equivalent to 100–200-fold of the recommended daily dosage of the product (300–600 mg/day for adults). The detailed chemical composition of bitter melon seeds is unclear. Further, most toxicity studies on bitter melon seeds were conducted using extracts instead of their specific components. To clearly identify the toxicity of the sample, we will conduct further studies to profile the explicit chemical composition and then perform a toxicity analysis. Notably, although this pioneering research does not discover

the developmental toxicity of the bitter melon seed extract for pregnant rats, we do not encourage pregnant women to use such a product because the safety of bitte melon seeds is required more further investigation. We believe that these findings may provide a different perspective on the novel food evaluation of bitter melon seed extracts.

5. Conclusion

This study revealed the acute and sub-chronic toxicity of bitter melon seed extract from $scCO_2$ extraction in rats. There was no significant difference in mortality, morbidity, biochemical parameters, and abnormal tissue alterations in all animals examined. The oral LD_{50} of the extract was greater than 2000 mg/kg BW. Accordingly, the extract is regarded as a practically non-toxic substance. The NOAEL of the extract was greater than 1000 mg/kg BW. Collectively, these results suggest that the bitter melon seed extract is safe for long-term use.

CRediT authorship contribution statement

Wan-Yu Chung: Project administration, Data curation, Manuscript preparation. **Sudhakar Jadhav:** Study implementation, Data analysis, Software. **Pang-Kuei Hsu:** Funding acquisition, Manuscript preparation. **Chen-Meng Kuan:** Project administration, Data analysis, Manuscript preparation and revision.

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.04.024.

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