Received: 11 September 2023 | Accepted: 7 November 2023

DOI: 10.1002/fsh3.12026

RESEARCH ARTICLE

FOOD SAFETY AND HEALTH

WILEY

Acute, subacute, and sub-chronic toxicity evaluation of *Bletilla formosana* tubers in rats

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Funding information

Yunnan Provincial Basic Research Project, Grant/Award Number: CB23078C159A; Yunnan Province in 2023 Food Safety Local Standard Project, Grant/Award Number: Yunwei[2023]DB002; High-level Talent Promotion and Training Project of Kunming, Grant/Award Number: 2022SCP003; Yunnan Major Natural Science Foundation, Grant/Award Number: 202202AG050009

Abstract

Bletilla formosana (Hayata) Schltr. is an important economic medicinal and edible plant in China. Its tubers (BFTs) are traditionally consumed as a beverage and canned foods for promoting human health. In this paper, the nutrients, the contents of total phenol, flavonoids, and polysaccharides, and the safety and toxicity of BFT are determined. The chemical analysis reported that BFT is rich in proteins, polysaccharides, polyphenols, flavonoids, and trace elements. The acute (14 days), subacute (28 days), and sub-chronic (90 days) toxicities of BFT were evaluated for the first time in female and male rats. An acute toxicity study showed that the female rats by single oral administrations of BFT with 10,000 mg/kg body weight (BW) did not have behavioral changes and mortality, indicating that 50% lethal dose (LD50) of BFT was over 10,000 mg/kg BW. Similarly, BFT at 5000 mg/kg BW for consecutive 28 and 90 days in rats was also not toxic for rats after the analysis of physiological and biochemical parameters. Additionally, there are no significant differences on the histopathological changes of the heart, liver, spleen, lung, kidney, ovary, or testis between the control and BFT groups. Our findings revealed that BFT with concentration of less than 5000 mg/kg BW are safe for the female or male rats, indicating that BFT could be regarded as a safe food source.

KEYWORDS

acute toxicity, *Bletilla formosana*, histopathological examination, sub-acute toxicity, sub-chronic toxicity

1 | INTRODUCTION

Bletilla formosana (Hayata) Schltr. (family Orchidaceae) is a traditional medical and edible plant and is mainly distributed in Western and Southwestern China (X. Wu et al., 2019). Its tuber (BFT) is the main medicinal and edible part. Traditionally, BFT is processed as soup, beverage, and canned food by "Yi", "Miao", "Lisu", and other ethnic minorities. Particularly, the co-fermentation product by fresh BFT and honey could moisten the lung and smoothen it to breathe heavily and relieve cough, which has a long edible history in Yunnan province, China (Dong et al., 2014). BFT has been also used as an astringent hemostatic medicine in Traditional Chinese Medicine for treating various bleeding disorders, such as hemoptysis, traumatic bleeding, ulcer bleeding, gastrointestinal bleeding, and so on (Jiang et al., 2021). In addition, BFT also had the potential benefit for the treatment of incised wounds, ulcerative carbuncle, lung diseases, and

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cough (Jiang et al., 2021). Besides, BFT is used as cosmetics materials due to its moisturizing activity. Therefore, BFT could be served as an important industrial crop for the development of health foods.

Although BFT had a long edible history, it is not listed in the common foods, medicinal and food homologous plants, and Chinese medicinal herbs in the development of health food. According to the guideline of development of health foods, phytochemicals, biological activities together with the potential molecular mechanism, and the safety and toxicological evaluation should be investigated for declaring new food raw materials in China. Recently, some phytochemical studies on BFT resulted in the isolation and identification of a series of polysaccharides, phenanthrenes, phenolics, flavonoids, terpenoids, steroids, and bibenzyl compounds, and polysaccharides are the abundant compounds with the contents of about 51% fresh weight (He et al., 2017; Lin et al., 2016; Song et al., 2019). Its extracts or isolated phytochemicals from BFT have antioxidative, anti-inflammatory, cytotoxic, immunomodulatory, and antibacterial effects (Liao et al., 2019; Niu et al., 2020; M. Zhang et al., 2014). Our previous study reported that two polysaccharides from BFT could significantly inhibit inflammatory response on the expressions of pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α via the regulation of NF-κB signaling pathway (Gu et al., 2022). Many studies reported that its polysaccharides from natural plants are usually applied in the commercial products as embedding medium, emulsifiers, adhesives, ultrasonic coupling agents, eye drops, ointments, and so on (Ren et al., 2022; Uccello-Barretta et al., 2010; Y. Yang et al., 2023). To date, the phytochemical and pharmacological studies on BFT had been investigated. However, the research on its safety and toxicity is not carried out.

In order to better understand the comprehensive application of BFT and the development of its health foods, there is an urgent need to explain its nutrition and edible security. This paper firstly determined the safety and toxicity of BFT by acute (14 days), subacute (28 days), and sub-chronic (90 days) toxicological experiments to fill the gap.

2 | MATERIALS AND METHODS

2.1 | Plant material

The BFT were collected in September 2021 from Puer, Yunnan Province, China. It was identified by Prof J. X. Cao, Kunming University of Science and Technology. A voucher specimen (No. cheng20211110-01) was preserved at the above university. The BFT were crushed and passed through a 60-mesh sieve.

2.2 | Determinations of total polyphenol content and total flavonoid content

The methods for the determinations of total polyphenol content (TPC) and total flavonoid content (TFC) were employed as described in our previous study (J. Zhang et al., 2022). Briefly, the BFT powder was extracted by methanol aqueous solution at a ratio of 1:10 (*w/v*). After evaporation, the extract was dissolved in methanol to prepare sample solution. The sample solution (0.1 mL) was further mixed with 0.5 mL Folin–Ciocalteu reagent for 1 min and then diluted with 7.0 mL water and incubated in a water bath at 70°C for 10 min. The absorbance of the mixture was measured at 765 nm using a microplate reader. The value of TPC was expressed as mg gallic acid equivalent (GAE)/g extract (mg GAE/extract) (Fan et al., 2022).

For the determination of TFC, the sample or rutin solution (1.0 mL) was mixed with 10% $NaNO_2$ solution (0.15 mL) for 5 min. Then, the mixture was orderly mixed with 0.15 mL of $Al(NO_3)_3$, 2.0 mL of 1 M NaOH, and 6.7 mL of 70% aqueous ethanol solution. After incubation for 30 min, the absorbance of the sample was measured at 510 nm. The value of TFC was expressed as mg rutin equivalent (RE) per gram of extract (mg rutin/g extract) (Wei & Zhang, 2023).

2.3 | Determination of total polysaccharide content

Total polysaccharide content of BFT was measured using the phenol sulfuric acid method (Z. Wu et al., 2018). Briefly, the dried BFT powder (1.0 g) was mixed with 20 mL of double distilled water and then extracted twice at 90°C for 2 h. After filtration, the solution was volumed to 50 mL by double distilled water. The diluted sample (1 mL) was mixed with phenol solution (1 mL, 5%) and 5 mL concentrated sulfuric acid and was incubated in a water bath at 40°C for 30 min. The absorbance of the sample was measured at 490 nm.

2.4 | Investigations of BET for essential nutrients

Nutrient analysis can effectively assess the impact of food intake on the body (Picó et al., 2019). The determinations of essential nutrients in BFT were carried out using the methods described in the Standardization Administration of the People's Republic of China, which is as follows:

1. Determination of water content: GB 5009.3-2016, the direct drying method;
2. Determination of ash content: GB 5009.4-2016, the muffle furnace high-temperature cautery method;
3. Determination of crude protein: GB 5009.5-2016, the trace Kjeldahl method; and
4. Determination of crude fat: GB 5009.6-2016, the Soxhlet extraction method.

The determinations of mineral elements: potassium and sodium (GB 5009.91-2017), calcium (GB 5009.92-2016), iron (GB 5009.90-2016), selenium (GB 5009.93-2016), selenium (GB 5009.93-2017), zinc (GB 5009.14-2017), magnesium (GB 5009.241-2017), phosphorus (GB 5009.87-2016), copper (GB 5009.13-2017), manganese (GB 5009.242-2017), and ascorbic acid (GB 5009.86-2016) were measured according to the Standardization Administration of the People's Republic of China.

2.5 | Animals and experimental design

SPF male and female SD rats (body weight 200–220 g) were purchased from the Department of Laboratory Animal Science, Kunming Medical University (Yunnan Province) (Certificate number: SYXK (Yunnan), 2019-0004). All rats were housed in a room at 23°C–27°C and 40%–60% humidity. The light was limited to 12 h (8:00–20:00 for light). The animal experiments were strictly carried out following the Guidance of the Care and Use of Laboratory Animals. The toxicological evaluation of BFT on SD rats was approved by the Animal Experiment Ethics Committee of Kunming University of Science and Technology with the approval number PZWH (Dian) K2021-0025. The schematic representation of the experimental design is shown in Figure 1.

2.5.1 | The 14-day acute toxicity experiment

The acute toxicity test of BFT was measured on female SD rats based on GB15193.3-2014, China's Ministry of Health (M. Yang et al., 2019). In this investigation, the "up and down technique" was used to calculate the median lethal dose (LD_{50}). The concentration of BFT with 10,000 mg/kg BW was applied in this study. The rats were divided into two groups, the control group (G1) and the BFT group (G2), each of which had 10 rats. After fasting for 12 h, the G1 group was fed with basal pellet feed, and the G2 group was treated with 10,000 mg/kg BW of BFT. The body weight and food intake of the rats were recorded, and the changes in general behavior were observed at 30 min, 1, 2, and 4 h after administration with the BFT. The mortality of rats was recorded during the 14-day experimental period. After the experiment, the rats were euthanized under anesthesia with pentobarbital sodium (40 mg/kg body weight). The LD_{50} and organ coefficients were calculated by Karn's approach (Y. Wang et al., 2021).

2.5.2 | 28-Day subacute toxicity experiment

The 28-day subacute toxicity experiment was performed strictly according to GB15193.22-2014, China's Ministry of Health (M. Yang et al., 2019). Sixty rats (200 ± 20 g) were randomly divided into five groups (6 male and 6 female rats per group). The control group (G3) was fed with basic feed, and other four groups were orally treated with different doses of BFT: 625 mg/kg BW (G4), 1250 mg/kg BW (G5), 2500 mg/kg BW (G6), and 5000 mg/kg BW (G7), respectively. The body weight, food intake, anomalous behavior, and toxicological side effects of rats including hair, skin, sports situation, nervous reflex, death, and so on, were recorded daily. The rats were euthanized under anesthesia with pentobarbital sodium (40 mg/kg body weight) after feeding for 28 days. The blood and the organs (the liver, lung, heart, spleen, kidney, testis, and ovary) were collected to analyze the biochemical parameters and calculate the organ coefficients (Tian et al., 2022).

2.5.3 | 90-Day sub-chronic toxicity experiment

The sub-chronic toxicity test was performed according to GB15193.13-2015, China's Ministry of Health, by feeding the SD rats for 90 days. After acclimatization, the rats were divided into four groups: control group (G8, basic feed) and BFT groups (G9, 1250 mg/kg BW; G10, 2500 mg/kg BW; G11, 5000 mg/kg BW), and each group had 12 rats (6 male and 6 female rats). During the 90-day experiment, the body weight, food intake, anomalous behavior, and toxicological side effects (the same as 28-Day subacute toxicity experiment) were recorded daily. After feeding for 90 days, the rats were euthanized under anesthesia with pentobarbital sodium (40 mg/kg body weight). The blood and organs (the liver, lung, heart, spleen, kidney, testis, and ovary) were obtained for the determination of biochemical indexes and organ coefficients (Zhou et al., 2011).

2.6 | Hematological analysis

The whole blood was stored in the tubes containing heparin lithium for hematological analysis. The indexes in the test included red blood cells (RBCs), hemoglobin (HGB), red blood cell volume (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), leukocyte (WBC), lymphocyte (LYM), percentage of lymphocytes (LYM percent), monocytes (MON), percentage of granulocytes (GRA percent), platelet (PLT), platelet pressure (PCT), and mean platelet volume (MPV). The measurements of these indexes were performed

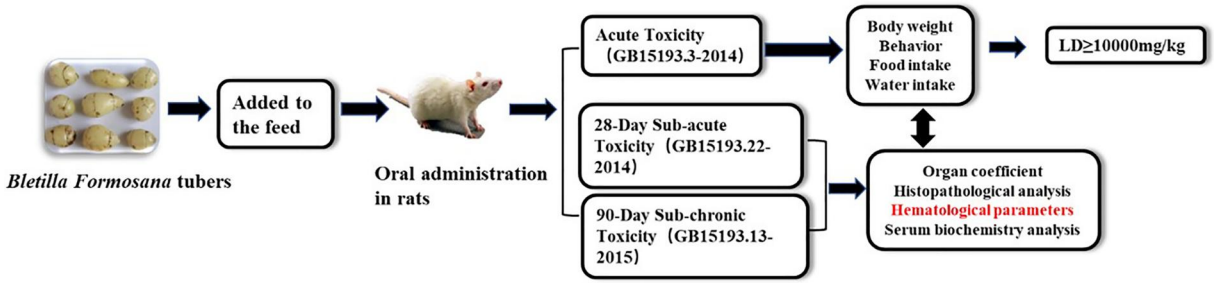


FIGURE 1 The experimental design of acute, subacute, and sub-chronic toxicity experiments.

according to the instructions of the commercial kits (Mellado-García et al., 2016) (HF-3800, HANFANG Ltd.).

2.7 | The analysis of bioactive parameters in the serum

The blood was centrifuged at 1800 g at 4°C to obtain the serum for the analysis of bioactive parameters, including total protein (TP), albumin (ALB), triglyceride (TG), total cholesterol (T-CHO), alanine aminotransferase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN), sodium (Na), potassium (K), and chlorine (Cl). The determination methods of these indexes were in accordance with instructions of commercial kits (Xu et al., 2012) (Nanjing Jiancheng Biological Co., Ltd.).

2.8 | Histopathological examination

The organ tissues of the liver, heart, lung, spleen, kidney, testis, and ovary were collected for fixing in 4% paraformaldehyde solution. These tissues were dehydrated using gradient ethanol solutions from 50% to 100% ethanol, and then soaked in xylene to be embedded with wax. The tissues were sliced into 4 μm sections, which were then stained with hematoxylin-eosin (H&E) solution. After being dehydrated with 95%–100% ethanol solution, the slices were observed using a microscope (Hu et al., 2023) (Olympus IX83, Japan).

2.9 | Statistical analysis

The data were expressed as mean ± standard deviation (Mean ± SD). The one-way analysis of variance was conducted for statistical significance by Origin 8.5 (Origin Lab). The $p < 0.05$ represented significance (M. Yang et al., 2019).

3 | RESULTS

3.1 | The values of total polyphenol content, total flavonoid content, and total polysaccharide content in BFT

Polyphenols, flavonoids, and polysaccharides are important bioactive ingredients of plants which are reported to have antioxidant and anti-inflammatory effects (Rakha et al., 2022; Su et al., 2023; Teng et al., 2022). Thereby, the values of TPC, TFC, and total polysaccharide content (TP) in the BFT were measured in this study. The results showed that levels of TPC, TFC, and TP were 1.60 ± 0.39 mg GAE/g, 0.84 ± 0.02 mg RE/g, and 281.25 ± 0.42 mg/g, respectively. Our findings are in accordance with the results reported in the literature (Dong et al., 2014; Gu et al., 2022).

3.2 | Nutritional composition analysis

To completely understand the basic composition of BFT, the nutritional composition was analyzed in this study. As shown in Table 1, except for moisture, the carbohydrates accounted for most of the ingredients in *B. formosana* tuber. The protein content in BFT was 4.42 g/100 g. Among trace elements, K level (1617 mg/kg) was the highest in the BFT, followed by Ca with 669 mg/kg.

3.3 | Acute toxicity analysis of BFT

The acute toxicity test is a vital process for toxicological evaluation to obtain the LD_{50} value, which further guides the follow-up toxicity tests. In this study, acute toxicity results implied that neither mortality nor unusual behavioral alterations were observed with a dose of 10,000 mg/kg BW of BFT for 14 days. As shown in Table 2, the body weight and food intake of BFT groups showed no appreciable differences in comparison with those of the control group. Meanwhile, there was no significant difference in organ coefficients in various BFT groups and no obvious difference between the control group and BFT groups. Moreover, no apparent lesions were found during autopsy. The histological results of the heart, liver, spleen, lung, kidney, ovary, and stomach of female rats showed that there were no obvious damages in the G1 (control) and G2 groups (BFT) (Figure 2). Thereby, the LD_{50} value of BFT was higher than 10,000 mg/kg/day.

TABLE 1 Nutritional composition analysis of *Bletilla formosana* tuber.

Nutritional components	Contents
Moisture (g/100 g)	40.9 ± 0.32
Ash (g/100 g)	0.66 ± 0.01
Protein (g/100 g)	4.42 ± 0.17
Fat (g/100 g)	0.60 ± 0.02
Carbohydrates (g/100 g)	53.18 ± 3.20
K (mg/kg)	1617 ± 18.44
Ga (mg/kg)	669 ± 16.81
Fe (mg/kg)	81.67 ± 1.62
Se (mg/kg)	<0.1
Zn (mg/kg)	5.61 ± 0.37
Mg (mg/kg)	323 ± 8.05
P (mg/kg)	322 ± 6.90
Cu (mg/kg)	1.12 ± 0.16
Mn (mg/kg)	17.43 ± 0.25
Vitamin C (mg/kg)	0.0028 ± 0.0001

3.4 | The analysis of 28 days subacute toxicity of BFT

3.4.1 | Body weight, food consumption, water intake, and organ coefficients of rats fed with the BFT for 28 days

The subacute toxicity analysis of 28 days was performed in this study to further investigate the toxicity of BFT. Figure 3 and Table 3 exhibited that no death in rats fed with BFT (5000 mg/kg BW) were observed in 28 days. The rats treated with BFT showed no significant differences in the behavior and status as compared to the control group. The body weight, feed intake, and water intake of rats in 625 and 1250 mg/kg body weight BFT groups (G4 and G5) exhibited a slight increase, while the 2500 or 5000 mg/kg BFT groups (G6 and G7) showed a slight decrease in most indexes. The results demonstrated that the organ coefficients (the heart, liver, spleen, lung, kidney, testis/ovary, and stomach) in BFT groups showed no significant difference compared with the control group.

3.4.2 | The analysis of hematological parameters

The determination of hematological parameters is a very important clinical method for revealing the potential injury of the body. Thereby, the measurements of these hematological indexes were performed in the subacute toxicity test. The data of hematological parameters are summarized in Table 4. Slight changes were found in various hematological parameters among the BTF groups with different concentrations when compared with those of the control group. In addition, after treatment with various doses of BFT, some hematological parameters were higher than those of the control group in both male and female rats, such as MCH, PLT, PCT %, and MCV. In all groups, the toxicity of BFT was not found in the hematological parameters of rats after 28 days' feeding.

TABLE 2 Body weight, food and water consumption, and organ coefficients of rats fed with *Bletilla formosana* tubers for 14 days.

Classes	G1 (control)	G2 (10,000 mg/kg BFT)
Initial weight (g)	213.17 ± 15.67	218.20 ± 16.24
One week (g)	242.33 ± 18.95	236.00 ± 19.16
Two weeks (g)	258.33 ± 18.34	249.60 ± 17.05
Body weight gain (g/14 days)	35.16	31.40
Food intake (g/day)	113.65 ± 17.05	108.48 ± 11.04
Water intake (g/day)	200.18 ± 15.24	192.04 ± 14.20
Heart (g/100 g)	0.39 ± 0.03	0.39 ± 0.02
Liver (g/100 g)	3.04 ± 0.12	2.98 ± 0.18
Spleen (g/100 g)	0.25 ± 0.02	0.24 ± 0.02
Lung (g/100 g)	0.59 ± 0.02	0.57 ± 0.02
Kidney (g/100 g)	0.70 ± 0.03	0.74 ± 0.05
Ovary (g/100 g)	0.10 ± 0.02	0.09 ± 0.01
Stomach (g/100 g)	0.68 ± 0.51	0.67 ± 0.35

Note: Results are expressed as mean ± SD (male/female: $n = 5$; food and water intake: 28 day). G1, control; G2, 10,000 mg/kg BFT.
Abbreviation: BFT, *Bletilla formosana* tuber.

3.4.3 | Analysis of serum biochemical indexes

The serum biochemical indexes, including ALT, AST, TC, TG, and so on, reflect potential organic damages. For example, the activities of ALT and AST are important indicators for liver damage. Hence, these plasma biochemical indexes were also measured in this study. Table 5 implies that there is no significant difference for most biochemical indexes between the control group and BFT groups. Meanwhile, the changes of these biochemical indexes (TP, ALB, TG, TC, ALT, AST, Na, K, and Cl) were not dose dependent in the BFT group. Interestingly, the levels of ALT, AST, TC, and TG with the treatment of the BFT were lower than those with the control, indicating that BFT had hepatoprotective effect and a potential effect on regulating lipid metabolism. Moreover, BUN levels in male and female rats were lower than those in the control group, which suggested that BFT intake might have an influence on improving the kidney health. The above results demonstrated that the BFT with various concentrations (625, 1250, 2500, and 5000 mg/kg body weight) had no obvious toxicity on rats after 28 days' feeding.

3.4.4 | Histopathological analysis

The histopathological analysis by H&E staining of eight main organs (the heart, liver, spleen, lung, kidney, ovary or testis, and stomach) in male (Figure 4a) and female (Figure 4b) rats displayed that there were almost no organic damages in the BFT groups (G4–G7) in comparison to the control group. The cells in these tissues of various organs were arranged neatly, and the nucleus aggregation, cell atrophy, and nucleus enlargement did not exist in these tissues. The indicators reflecting the organ damages, such as ALT, AST, and BUN levels, were not significantly increased compared with those of the control group, which were consistent with the results of plasma biochemical indexes.

3.5 | Analysis of 90-day sub-chronic toxicity

3.5.1 | Body weight, food consumption, water intake, and organ coefficients of rats treated with BFT for 90 days

To investigate the toxicity characteristics and possible target organs of BFT under a long-term exposure, a 90-day sub-chronic toxicity

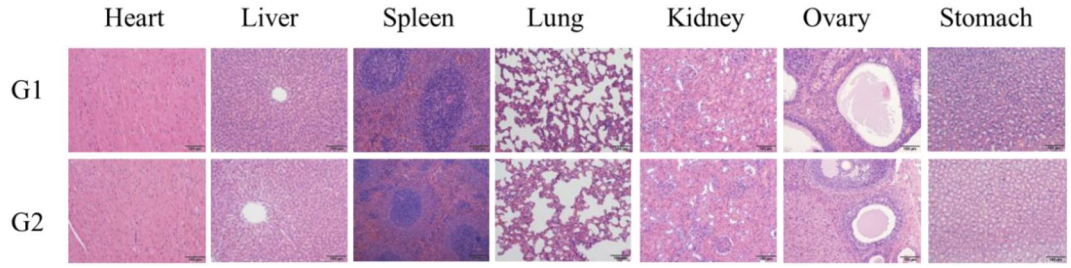


FIGURE 2 Histopathological results of seven organs of female rats after 14 days' oral administration with BFT (H&E staining, 200×). G1 and G2, means control group, and 10,000 mg/kg BFT group of female rats, respectively. BFT, *Bletilla formosana* tuber.

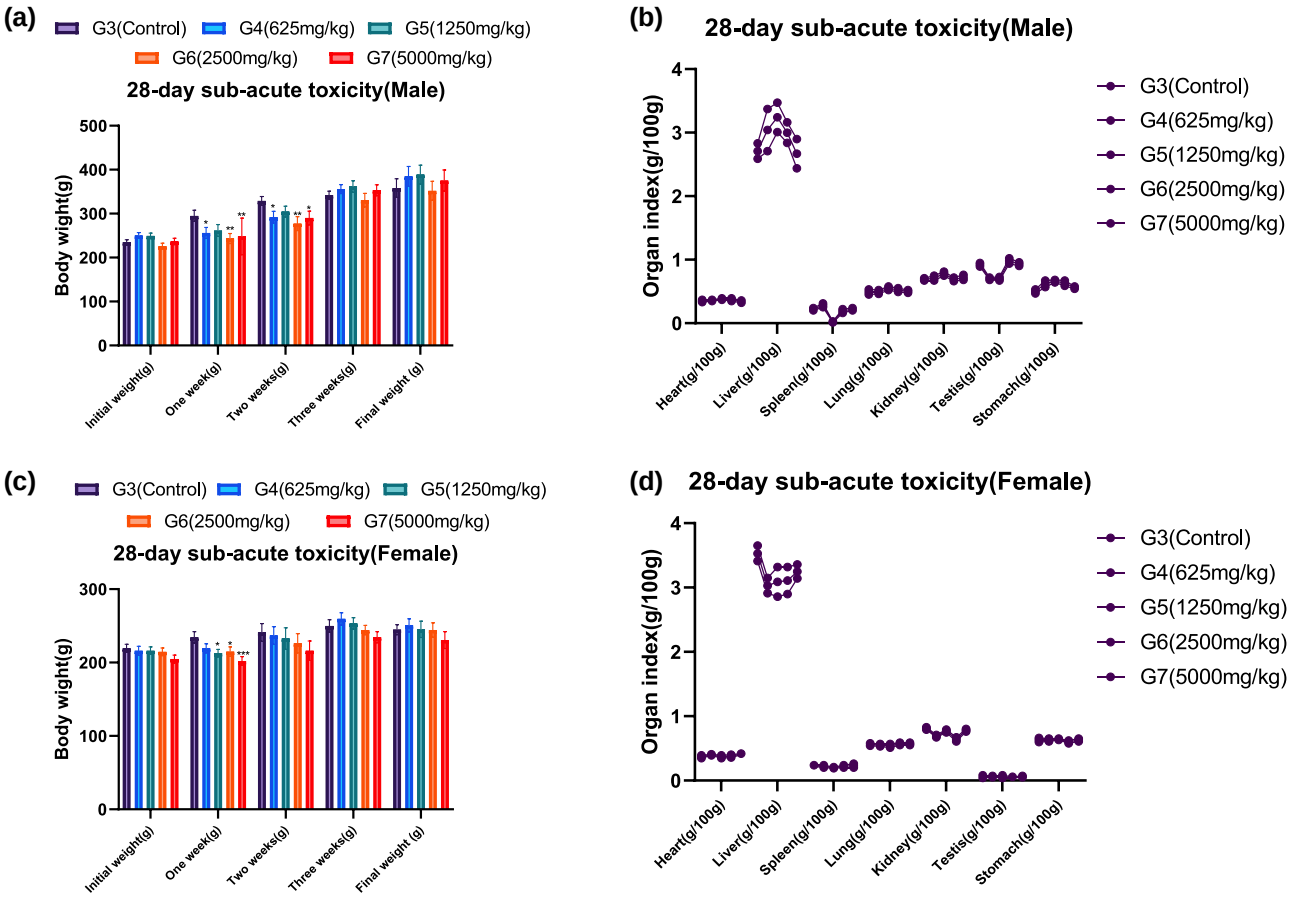


FIGURE 3 Effect of BFT on body weight and organ coefficients in 28-day subacute toxicity experiments. (a) Body weight of male rats; (b) organ index of male rats; (c) body weight of female rats; and (d) organ index of female rats. BFT, *Bletilla formosana* tuber.

test was carried out in this study. After feeding with various doses of BFT (1250, 2500, or 5000 mg/kg BW) for 90 days, the body weight gain and daily food or water intake of BFT groups (G9–G11) were higher than those of the control group; especially, the body weight of female rats significantly increased by 13.85%, 38.89%, and 59.44% ($p < 0.05$), respectively, exhibiting a great growth-promoting effect (Figure 5, Table 6). The organ coefficients of the heart, liver, spleen, lung, kidney, ovary or testis, and stomach represented no significant difference between the control group and BFT groups. The present results demonstrated that 90-day feeding with BFT might not cause toxicity in various organs of male and female rats.

3.5.2 | Hematological parameters

As shown in Figures 6 and 7, there was no significant difference in hematological parameters in both male and female rats between control group and the BFT groups, such as RBC, HGB, HCT, MCH,

TABLE 3 The body weight gain, food, and water consumption of rats fed with *Bletilla formosana* tuber for 28 days.

		B. formosana tuber			
	G3 (control)	G4 (625 mg/kg)	G5 (1250 mg/kg)	G6 (2500 mg/kg)	G7 (5000 mg/kg)
Male					
Body weight gain (g) (28 days)	123.71	134.40	139.60	125.80	138.40
Food intake (g/day)	103.19 ± 11.32	106.53 ± 10.21	102.50 ± 11.83	101.51 ± 8.10	102.50 ± 10.23
Water intake (mL/day)	129.54 ± 71.51	189.92 ± 76.50	181.41 ± 97.53	194.88 ± 65.96	179.37 ± 78.32
Female					
Body weight gain (g) (28 days)	25.40	34.60	29.00	29.80	25.80
Food intake (g/day)	96.75 ± 17.55	102.54 ± 12.31	102.56 ± 14.08	102.53 ± 12.60	102.54 ± 11.57
Water intake (mL/day)	185.91 ± 83.75	199.12 ± 94.06	196.65 ± 101.12	214.86 ± 105.25	206.77 ± 110.09

Note: Results are expressed as mean ± SD (male/female: $n = 6$). G3, control; G4–G7, different doses of BFT.

Abbreviation: BFT, *Bletilla formosana* tuber.

TABLE 4 Hematological analysis of rats treated with *Bletilla formosana* tuber for 28 days.

		B. formosana tuber			
	G3 (control)	G4 (625 mg/kg)	G5 (1250 mg/kg)	G6 (2500 mg/kg)	G7 (5000 mg/kg)
Male					
WBC (10^9/L)	4.20 ± 0.43	4.52 ± 0.21	3.66 ± 0.31	4.0 ± 0.13	3.9 ± 0.24
LYM (10^9/L)	3.517 ± 0.32	3.12 ± 0.19	3.36 ± 0.09	3.21 ± 0.08	2.55 ± 0.23
LYM (%)	65.20 ± 3.58	64.9 ± 4.21	67.18 ± 3.15	64.12 ± 5.12	62.8 ± 4.21
MON (10^9/L)	0.29 ± 0.03	0.28 ± 0.06	0.24 ± 0.09	0.24 ± 0.05	0.24 ± 0.07
GRA (%)	90.4 ± 4.69	88.4 ± 7.26	87.1 ± 2.74	90.52 ± 6.24	89.3 ± 5.16
RBC (10^{12}/L)	6.03 ± 0.60	5.84 ± 0.51	5.34 ± 0.24	5.56 ± 0.35	5.21 ± 0.48
HGB (g/L)	152.46 ± 9.28	149.04 ± 11.67	143.76 ± 10.39	148.18 ± 13.10	141.86 ± 11.94
MCH (pg)	25.20 ± 0.94	25.48 ± 1.31	26.88 ± 2.84	26.66 ± 1.36	27.22 ± 0.89
MCV (fL)	64.78 ± 3.28	65.78 ± 4.03	66.43 ± 3.75	65.72 ± 2.46	65.26 ± 2.97
HCT (%)	39.15 ± 3.15	38.36 ± 2.87	35.42 ± 4.15	35.88 ± 3.20	33.92 ± 2.69
PLT (10^9/L)	479.30 ± 28.94	543.23 ± 30.24	526.26 ± 31.54	513.75 ± 33.48	546.6 ± 31.08
PCT (%)	0.42 ± 0.07	0.50 ± 0.05	0.48 ± 0.10	0.47 ± 0.08	0.49 ± 0.09
Female					
WBC (10^9/L)	4.32 ± 0.41	5.3 ± 0.43	3.28 ± 0.38	4.78 ± 0.31	4.96 ± 0.4
LYM (10^9/L)	1.96 ± 0.22	1.924 ± 0.34	1.9 ± 0.24	2.38 ± 0.38	2.62 ± 0.17
LYM (%)	64.70 ± 4.61	69.7 ± 4.88	68.4 ± 3.16	75.4 ± 2.87	61.4 ± 5.66
MON (10^9/L)	0.29 ± 0.07	0.31 ± 0.09	0.22 ± 0.05	0.33 ± 0.10	0.2 ± 0.06
GRA (%)	84.74 ± 3.15	79.73 ± 1.84	80.10 ± 2.74	74.71 ± 2.18	83.14 ± 3.06
RBC (10^{12}/L)	5.00 ± 0.39	5.68 ± 0.32	5.1 ± 0.41	5.2 ± 0.29	5.41 ± 0.35
HGB (g/L)	115.20 ± 10.53	143.32 ± 9.48	133.91 ± 9.35	134.06 ± 8.17	137.86 ± 9.24
MCH (pg)	23.62 ± 1.25	25.16 ± 0.84	26.53 ± 0.34	25.74 ± 0.88	25.44 ± 0.91
MCV (fL)	59.70 ± 3.24	62.28 ± 4.21	64.42 ± 2.87	61.71 ± 4.16	61.22 ± 3.21
HCT (%)	29.82 ± 1.78	35.18 ± 1.49	32.96 ± 2.14	32.04 ± 1.68	32.08 ± 2.44
PLT (10^9/L)	421.04 ± 34.15	471.42 ± 36.82	452.74 ± 33.12	443.31 ± 35.44	497.75 ± 38.42
PCT (%)	0.32 ± 0.09	0.35 ± 0.12	0.36 ± 0.14	0.39 ± 0.08	0.47 ± 0.09

Note: Results are expressed as mean ± SD (male/female: $n = 6$). G3, control; G4–G7, different doses of BFT.

Abbreviations: BFT, *Bletilla formosana* tuber; GRA, granulocytes; HCT, red blood cell volume; HGB, hemoglobin; LYM, lymphocyte; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MON, monocytes; PCT, platelet pressure; PLT, platelet; RBC, red blood cell; WBC, leukocyte.

TABLE 5 Effect of *Bletilla formosana* tuber on plasma biochemical indexes in rats for 28 days.

		B. formosana tuber			
	G3 (control)	G4 (625 mg/kg)	G5 (1250 mg/kg)	G6 (2500 mg/kg)	G7 (5000 mg/kg)
Male					
TP (g/L)	71.31 ± 3.28	74.50 ± 2.28	68.47 ± 3.76	71.98 ± 3.24	72.43 ± 2.28
ALB (g/L)	22.93 ± 2.29	25.28 ± 3.02	23.55 ± 3.72	23.55 ± 3.54	23.06 ± 4.38
TG (mmol/L)	0.58 ± 0.05	0.53 ± 0.06	0.52 ± 0.05	0.42 ± 0.07	0.52 ± 0.05
TC (mmol/L)	1.46 ± 0.15	1.36 ± 0.13	1.39 ± 0.14	1.42 ± 0.14	1.61 ± 0.16
ALT (U/L)	36.63 ± 3.56	30.33 ± 2.49	30.27 ± 3.35	33.17 ± 2.85	33.49 ± 2.51
AST (U/L)	141.11 ± 5.39	133.42 ± 3.03	134.57 ± 4.37	137.60 ± 6.07	139.31 ± 4.36
BUN (mmol/L)	9.58 ± 1.28	7.02 ± 1.47	7.31 ± 1.07	7.61 ± 1.44	6.30 ± 0.65
Na (mmol/L)	143.45 ± 10.33	130.14 ± 12.78	137.17 ± 8.36	139.87 ± 9.77	130.36 ± 10.06
K (mmol/L)	6.49 ± 0.66	6.38 ± 0.42	6.45 ± 0.43	6.87 ± 0.57	6.59 ± 1.02
Cl (mmol/L)	102.66 ± 0.44	105.19 ± 0.38	108.68 ± 0.43	106.54 ± 0.84	111.92 ± 0.96
Female					
TP (g/L)	66.37 ± 2.62	60.42 ± 3.85	63.09 ± 3.09	62.20 ± 7.03	68.37 ± 4.32
ALB (g/L)	30.70 ± 4.05	27.06 ± 3.91	27.25 ± 4.95	29.65 ± 2.93	27.62 ± 3.61
TG (mmol/L)	0.44 ± 0.03	0.51 ± 0.03	0.38 ± 0.04	0.47 ± 0.05	0.49 ± 0.04
TC (mmol/L)	1.38 ± 0.15	1.32 ± 0.26	1.30 ± 0.13	1.44 ± 0.18	1.41 ± 0.15
ALT (U/L)	61.40 ± 3.43	47.70 ± 2.23	40.26 ± 2.74	41.04 ± 2.41	46.72 ± 1.47
AST (U/L)	149.95 ± 5.23	138.32 ± 5.09	135.99 ± 2.78	143.93 ± 3.83	146.27 ± 4.45
BUN (mmol/L)	10.28 ± 1.47	9.64 ± 1.34	8.74 ± 1.81	9.30 ± 1.26	9.22 ± 1.37
Na (mmol/L)	140.37 ± 11.21	108.52 ± 12.28	112.03 ± 9.41	106.59 ± 9.66	116.91 ± 10.51
K (mmol/L)	6.84 ± 0.32	6.63 ± 0.29	6.87 ± 0.25	6.44 ± 0.49	6.68 ± 0.38
Cl (mmol/L)	96.16 ± 0.34	107.72 ± 0.35	108.96 ± 0.43	108.99 ± 1.34	103.31 ± 0.66

Note: Results are expressed as mean ± SD (male/female: $n = 6$). G3, control; G4–G7, different doses of BFT.

Abbreviations: ALB, albumin; ALT, alanine aminotransferase; AST, aspartate transaminase; BFT, *Bletilla formosana* tuber; BUN, blood urea nitrogen; Cl, chlorine; K, potassium; Na, sodium; TG, triglyceride; TP, total protein.

MCV, WBC, LYM, and so on. In comparison with the results of the sub-acute toxicity test above, the long-term exposure to BFT had more stable results of hematological parameters without larger fluctuations among G9 (1250 mg/kg BW of BFT), G10 (2500 mg/kg BW of BFT), and G11 (5000 mg/kg BW of BFT) groups.

3.5.3 | Effect of BFT on the plasma biochemical indexes

To assess the effect of BFT on organ toxic symptoms, some organ bioindicators were examined in this work. Figure 8 indicated that BFT administration could exert hepatoprotective, kidney-protective, and hypolipidemic effects by reducing the activities of ALT and AST and the contents of BUN, TC, and TG compared to the control group. In addition, there was no dose dependence of various BFT doses on these bioactive indexes. Meanwhile, the G9 (1250 mg/kg BW) group showed better protective effects on organs than the other groups.

3.5.4 | Histopathological analysis

The histopathological results in Figure 9 demonstrated that there were no damages in various organs (the heart, liver, spleen, lung, kidney, ovary or testis, and stomach); in addition, the abnormal cellular morphology, disappearance of the cell membrane, aggregation of the cells nuclear, and so on were not observed. The results illustrated that 90-day feeding with BFT did not cause organ injury in both male and female rats.

4 | DISCUSSION

The *B. formosana* tuber (BFT) is a traditional Chinese medicinal and edible material with some excellent benefits on human health. It has wide applications in the form of food and medicinal or other health products in many provinces of China. To date, the safety of BFT has not been fully investigated. Therefore, the composition and acute,

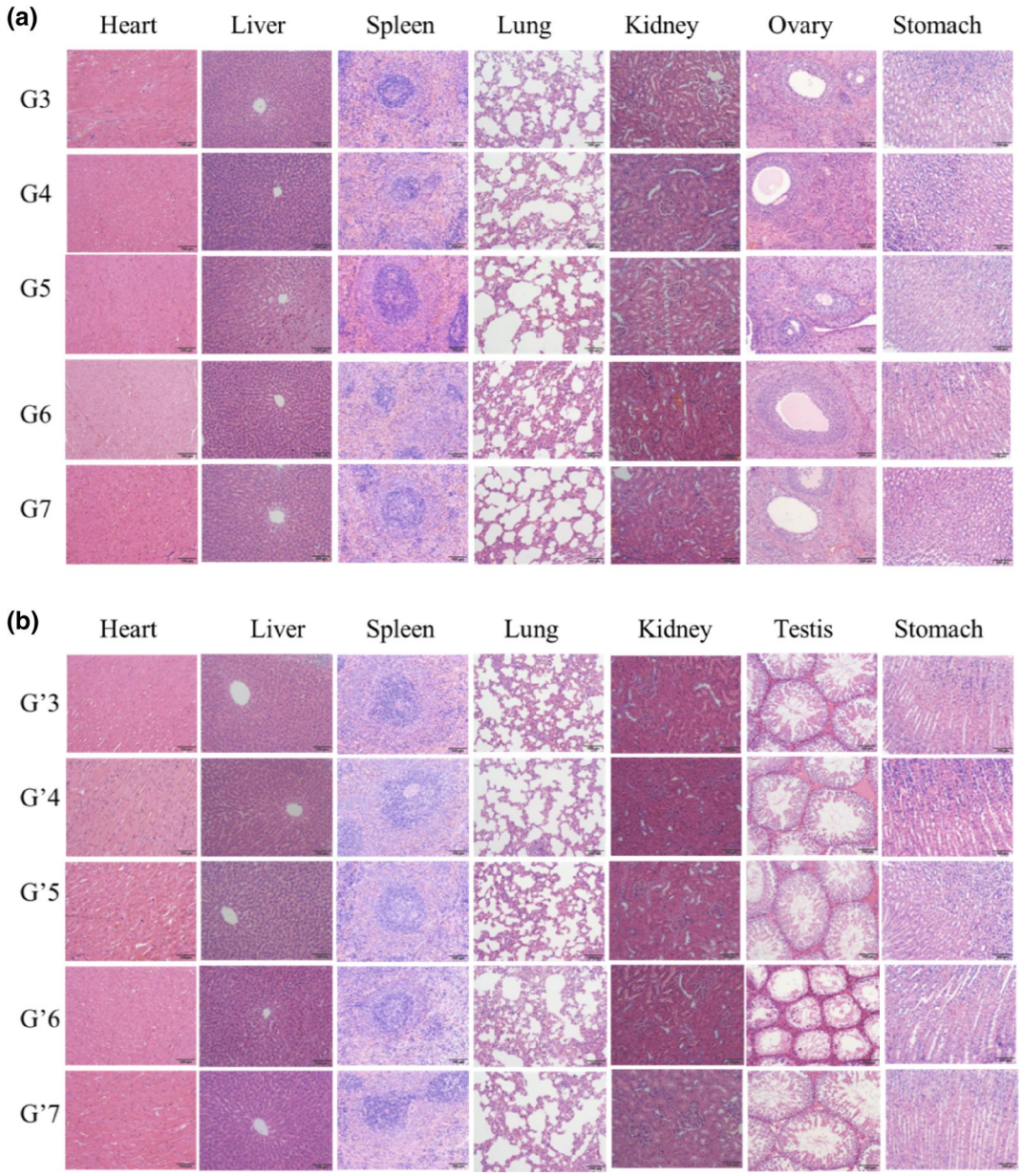


FIGURE 4 Histopathological results of seven organs in female (a) and male (b) rats after oral administration of BFT for 28 days (H&E stain, 200×). G3, G4, G5, G6, and G7 represent the control group, 625 mg/kg BFT group, 1250 mg/kg BFT group, 2500 mg/kg BFT group, and 5000 mg/kg BFT group of female rats, respectively. G′3, G′4, G′5, G′6, and G′7 represent the control group, 625 mg/kg BFT group, 1250 mg/kg BFT group, 2500 mg/kg BFT group, and 5000 mg/kg BFT group of male rats, respectively. BFT, *Bletilla formosana* tuber.

subacute, and sub-chronic toxicity tests of BFT were performed in this study to investigate its safety for further applications.

The results of nutrient analysis showed that the main component of BFT was polysaccharide, and it could be speculated that the active ingredient was mainly polysaccharide, which was also proved in our previous experiments. We have a comprehensive understanding of the microcomponent composition of BFT, which has a relatively rich microcomponent, which can provide guidance for the future development of healthy food.

The present study of the acute toxicity test revealed that there was no toxicity effect on rats fed with 10,000 mg/kg/day of BFT. Additionally, there was no significant difference between the control group and BFT groups in terms of body weight, food and water intake, or organ coefficients. The findings suggested that BFT might be nontoxic because its single oral LD_{50} was larger than 10,000 mg/kg body weight (Wei & Zhang, 2023). To further verify the nontoxic results of the acute toxicity assay, a 28-day subacute toxicity test and a 90-day sub-chronic toxicity test were carried out in this study. The results revealed that there were no significant toxicities in rats fed with 625, 1250, 2500, and 5000 mg/kg BW of BFT for 28 days, as well as 1250, 2500, and 5000 mg/kg BW of BFT for 90 days. Daily oral administration of different doses of BFT in rats had no difference in food and water intake compared to the control group. An interesting finding was that

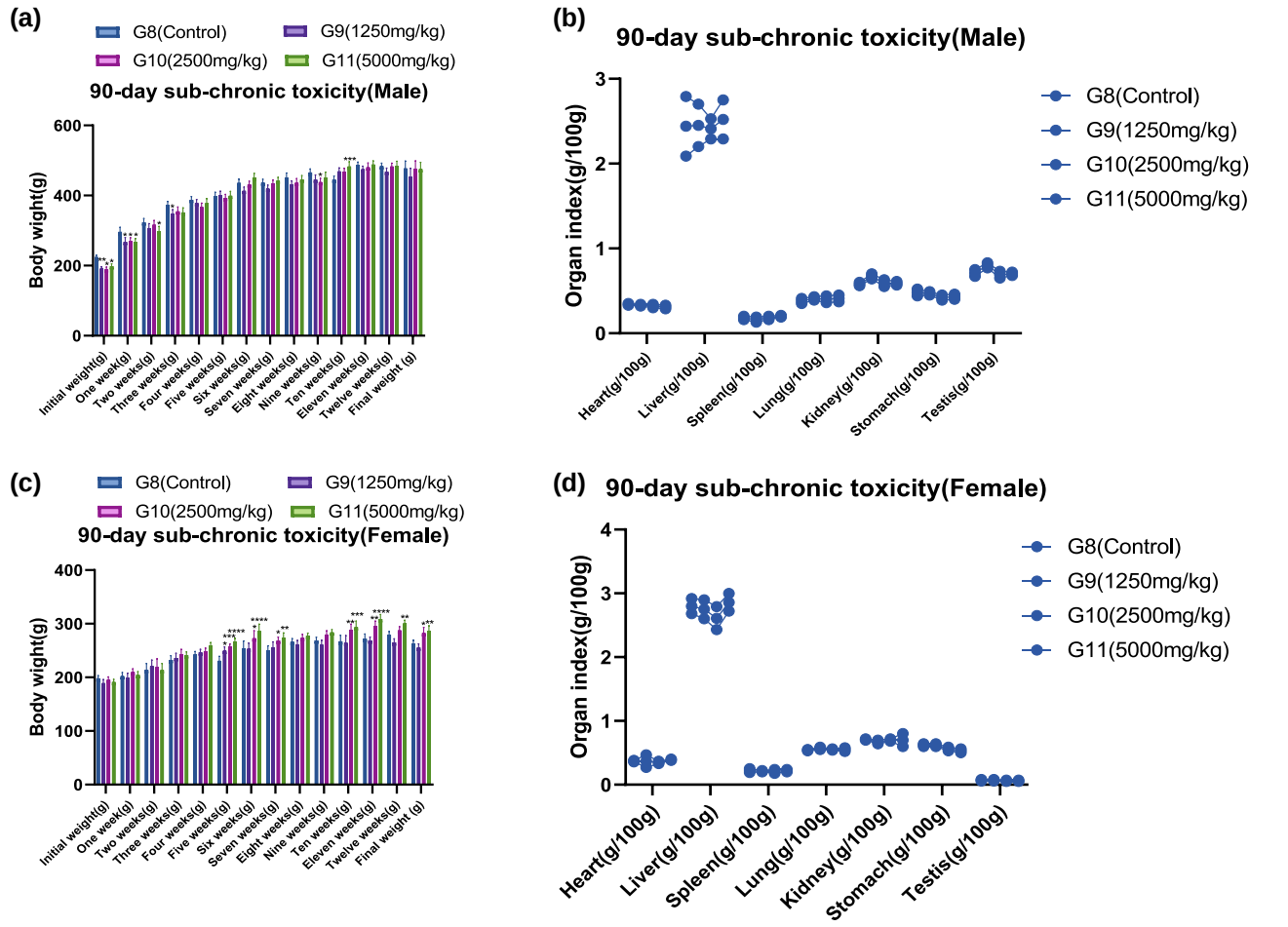


FIGURE 5 Effect of BFT on body weight and organ coefficients in 90-day sub-chronic toxicity experimental rats. (a) Body weight (male 90-day sub-chronic toxicity); (b) organ index (male 90-day sub-chronic toxicity); (c) body weight (female 90-day sub-chronic toxicity); and (d) organ index (female 90-day sub-chronic toxicity). BFT, *Bletilla formosana* tuber.

TABLE 6 The body weight gain, food, and water consumption of rats fed with *Bletilla formosana* tuber for 90 days.

		B. formosana tuber		
	G8 (control)	G9 (1250 mg/kg)	G10 (2500 mg/kg)	G11 (5000 mg/kg)
Male				
Body weight gain (g)	253.08	263.40	287.80	277.00
Food intake (g/day)	95.18 ± 11.31	105.09 ± 19.11	105.14 ± 10.83	104.01 ± 16.11
Water intake (mL/day)	174.62 ± 91.01	195.87 ± 105.30	189.69 ± 86.73	197.34 ± 67.24
Female				
Body weight gain (g)	84.670	96.40	117.60	135.40
Food intake (g/day)	100.31 ± 17.55	110.61 ± 15.72	108.61 ± 16.06	110.04 ± 13.90
Water intake (mL/day)	182.51 ± 78.45	204.59 ± 102.06	208.85 ± 101.13	201.41 ± 93.45

Note: Results are expressed as mean ± SD (male/female: $n = 6$). G8, control; G9–G11, different doses of BFT.
Abbreviation: BFT, *Bletilla formosana* tuber.

BFT administration could promote the growth of rats, but the organ weights and organ coefficients were not abnormal, indicating that BFT could promote the healthy growth of rats.

Blood biochemical analysis is also important for evaluating the safety of test samples (Lulekal et al., 2019). In this study, the administration of BFT exerted hepatoprotective effect by reducing

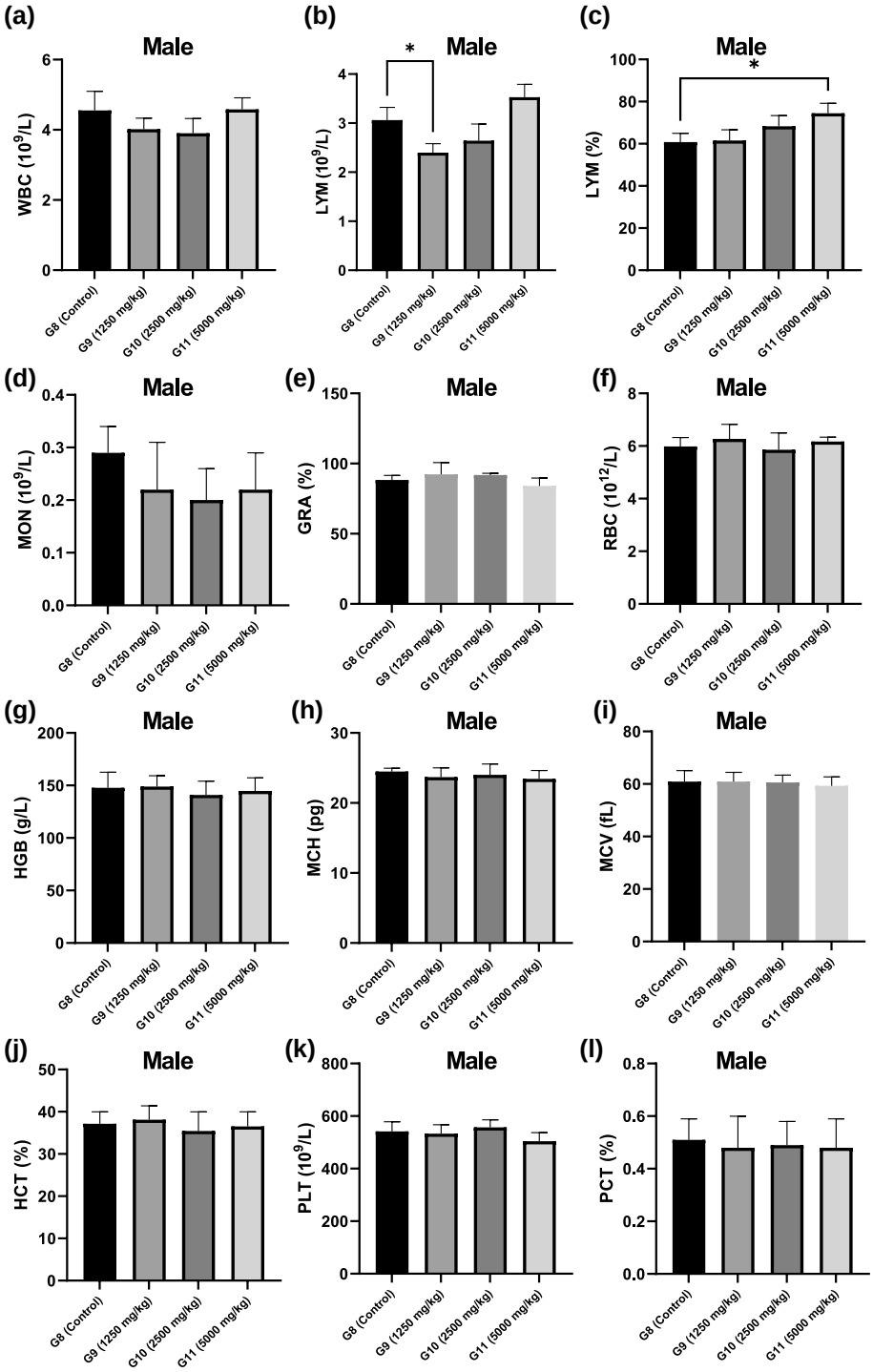


FIGURE 6 Hematological analysis of male rats fed with *Bletilla formosana* tuber for 90 days. (a) WBC (10^9/L); (b) LYM (10^9/L); (c) LYM (%); (d) MON (10^9/L); (e) GRA (%); (f) RBC (10^{12}/L); (g) HGB (g/L); (h) MCH (pg); (i) MCV (fL); (j) HCT (%); (k) PLT (109/L); and (l) PCT (%). GRA, granulocytes; HCT, red blood cell volume; HGB, hemoglobin; LYM, lymphocyte; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MON, monocytes; PCT, platelet pressure; PLT, platelet; RBC, red blood cell; WBC, leukocyte.

the activities of ALT and AST, protective effect on the kidney via decreasing BUN level, and antilipemic effect by reducing TC and TG levels. This may be related to high levels of polysaccharides in BFT. Studies have shown that polysaccharides from natural sources play a crucial role in the health of the human body due to their excellent antioxidant or anti-inflammatory abilities (Fernandes & Coimbra, 2023; Huang et al., 2023). It has been demonstrated that phenolics, derived from natural sources, play a vital role in benefiting health of the body due to their excellent antioxidant or anti-inflammatory abilities (Abd El-Hack et al., 2023; Narayanankutty et al., 2022). TPC and TFC are rich in plants, herbs, and flowers, which have antioxidative, anti-inflammatory, and lipid-lowering

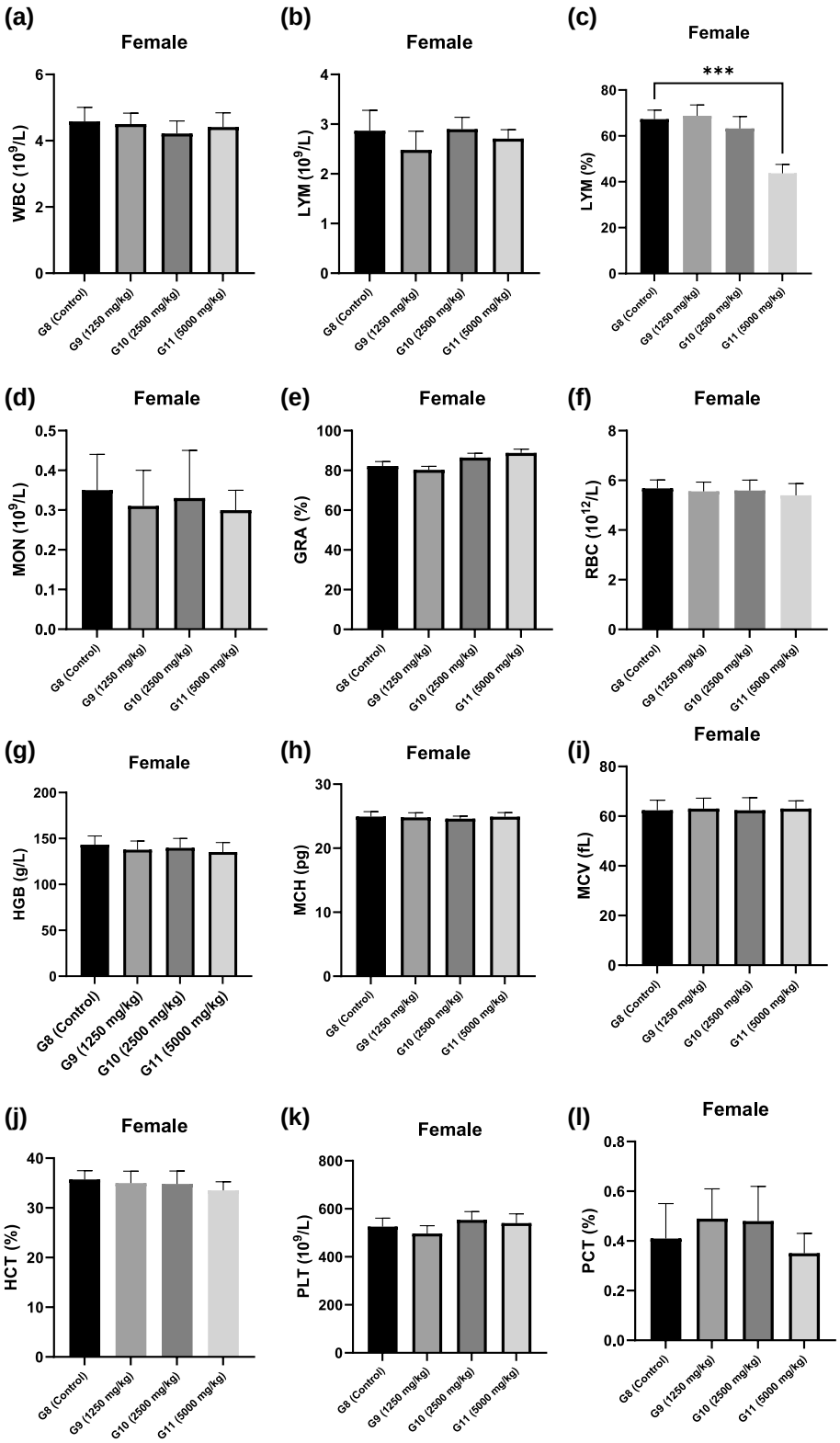


FIGURE 7 Hematological analysis of female rats fed with *Bletilla formosana* tuber for 90 days. (a) WBC (10^9/L); (b) LYM (10^9/L); (c) LYM (%); (d) MON (10^9/L); (e) GRA (%); (f) RBC (10^{12}/L); (g) HGB (g/L); (h) MCH (pg); (i) MCV (fL); (j) HCT (%); (k) PLT (109/L); and (l) PCT (%). GRA, granulocytes; HCT, red blood cell volume; HGB, hemoglobin; LYM, lymphocyte; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MON, monocytes; PCT, platelet pressure; PLT, platelet; RBC, red blood cell; WBC, leukocyte.

effects (Chandrasekara & Shahidi, 2018; Purohit et al., 2021). For example, the extracts of E Se tea, Que Zui tea, *Anneslea fragrans* leaves, or *Dendrobium fimbriatum* Hook have antioxidative, anti-inflammatory, or hepatoprotective effects because of high TPC and TFC levels (Hu et al., 2023; Y. Wang et al., 2023; Z. Wang et al., 2023; Zhao, Dong, et al., 2023; Zhao, Zheng, et al., 2023). Furthermore, our previous studies illustrated that the polysaccharide of BFT had anti-inflammatory effect via suppressing the NF-κB pathway. Thereby, the bioactive chemical composition might be of primary importance for the protective effect of BFT on the body.

The value of organ coefficient can reflect the health degree of rats (Li et al., 2023). Compared with growth of the body, the

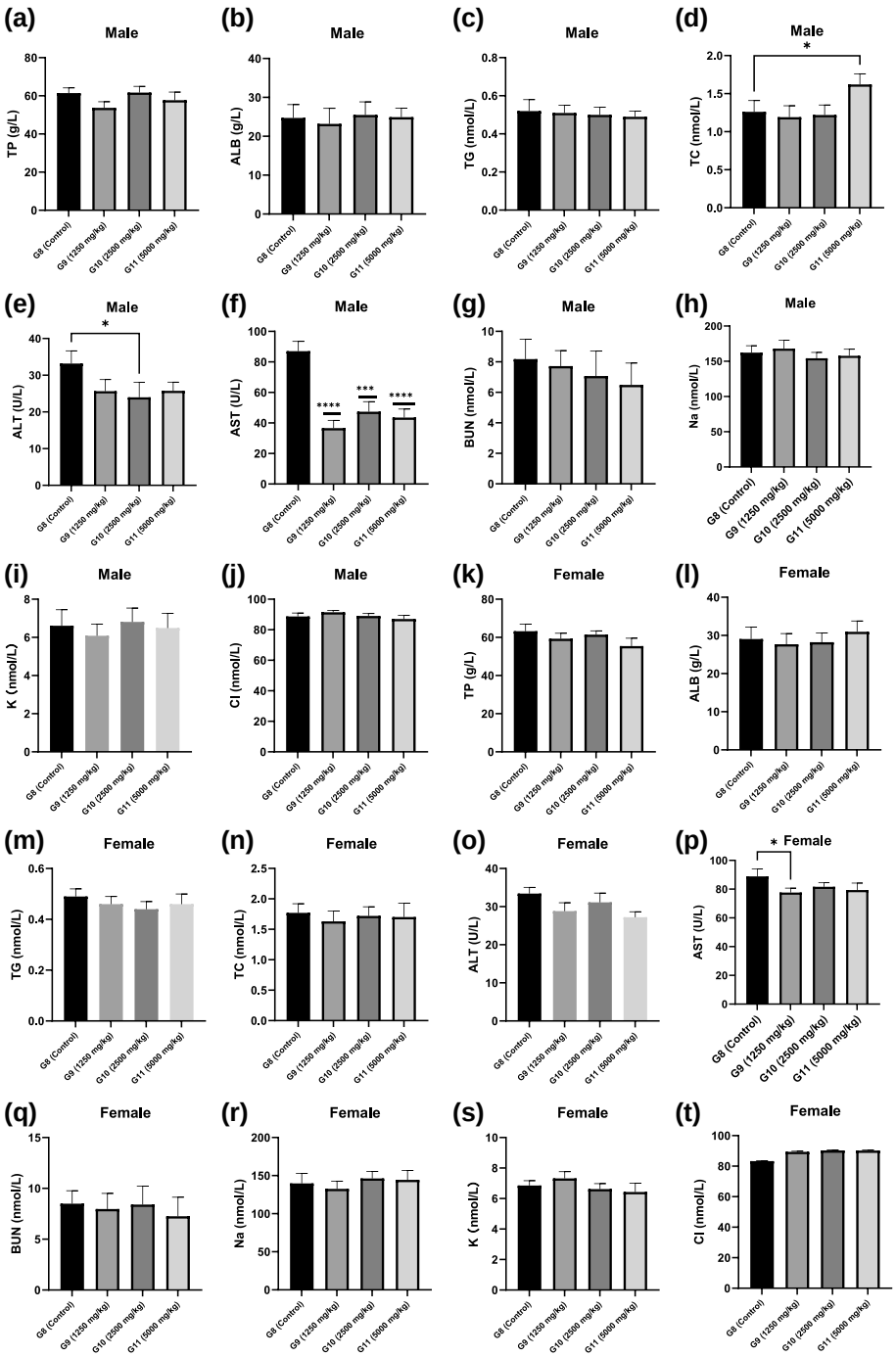


FIGURE 8 The effects of *Bletilla formosana* tuber on plasma biochemical indexes of rats in 90 days. Male: (a) TP (g/L); (b) ALB (g/L); (c) TG (mmol/L); (d) TC (mmol/L); (e) ALT (U/L); (f) AST (U/L); (g) BUN (mmol/L); (h) Na (mmol/L); (i) K (mmol/L); and (j) Cl (mmol/L). Female: (k) TP (g/L); (l) ALB (g/L); (m) TG (mmol/L); (n) TC (mmol/L); (o) ALT (U/L); (p) AST (U/L); (q) BUN (mmol/L); (r) Na (mmol/L); (s) K (mmol/L); and (t) Cl (mmol/L). ALB, albumin; ALT, alanine aminotransferase; AST, aspartate transaminase; BUN, blood urea nitrogen; Cl, chlorine; K, potassium; Na, sodium; TG, triglyceride; TP, total protein.

increasing values of different organ (the liver, heart, kidney, spleen, ovary, and stomach) coefficients might refer to unhealthy or even damaged of organs, including hypertrophy, edema, congestion, etc. (Min et al., 2020). In this study, in comparison with the control group, the BFT groups with various doses showed no obvious differences on internal organ coefficients. Moreover, even high doses (5000 mg/kg or 10,000 mg/kg BW) of BFT had no significant changes in organ coefficients.

Besides the values of organ coefficients, the histological examination of organs is another very effective and important method for assessing pathological alteration (Ognik et al., 2021). These findings of this study revealed that BFT had no toxic or adverse effects on the liver, kidney, or other organs of rats. There were no appreciable differences between male and female rats in plasma biochemical indexes. In addition, the damages of the liver, kidneys, and other organs were not significantly observed in rats fed with different doses of BFT. In each experimental group, there were no macroscopic indications of edema, congestion, or apoptosis in various organs. Moreover, the results of histological investigations indicated that neither the experimental group nor the control group had any detectable pathological

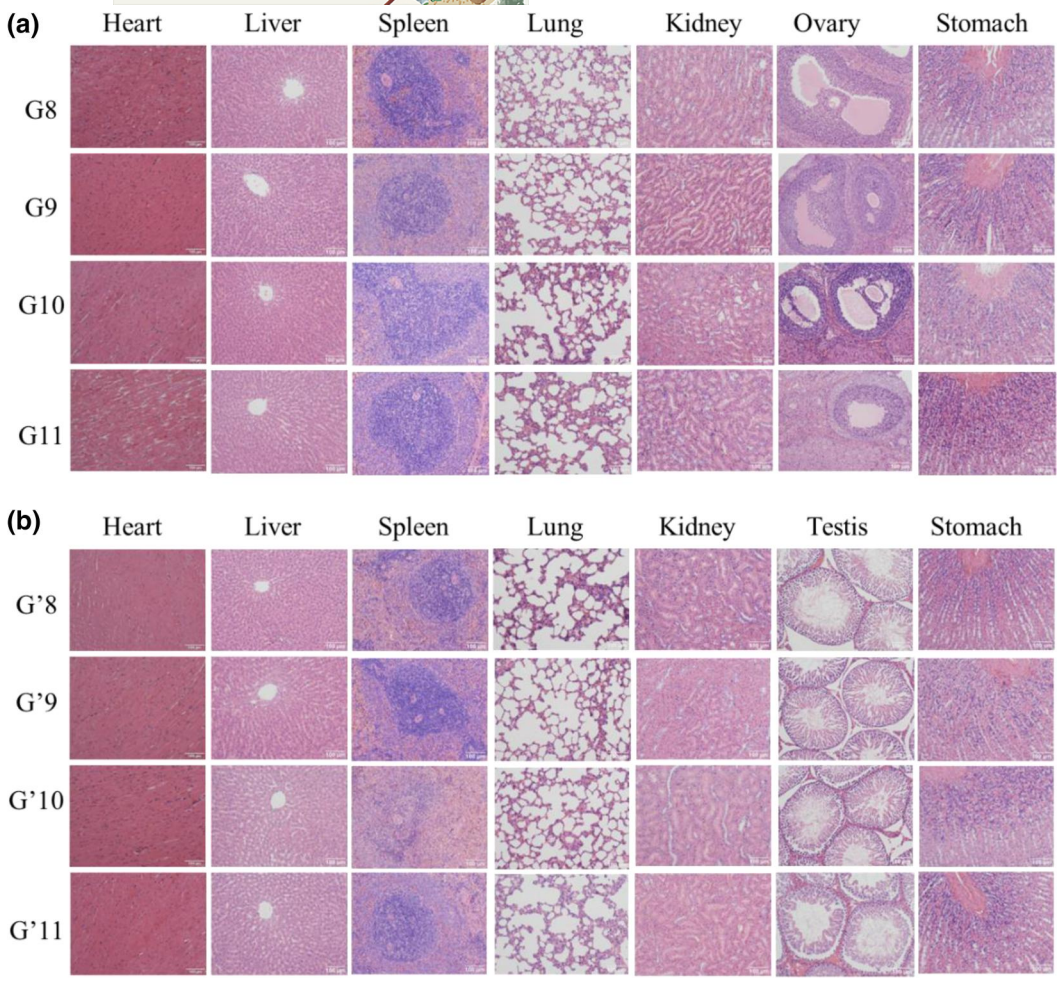


FIGURE 9 Histopathological results of seven organs in female (a) and male (b) rats after oral administration of BFT for 90 days (H&E stain, 200×). G8, G9, G10, and G11 mean control group, 1250 mg/kg BFT group, 2500 mg/kg BFT group, and 5000 mg/kg BFT group of female rats, respectively. G′8, G′9, G′10, and G′11 mean control group, 1250 mg/kg BFT group, 2500 mg/kg BFT group, and 5000 mg/kg BFT group of male rats, respectively. BFT, *Bletilla formosana* tuber.

alterations. This finding was in accordance with the results of hematological and serum biochemical analyses, showing a safety of BFT in mice.

5 | CONCLUSION

The nutritional composition of BFT showed that the main component of BFT was polysaccharide (281.25 ± 0.42 mg/g), and it was rich in trace elements such as potassium and calcium. The results of the acute toxicity test showed that the LD_{50} values of BFT on SD rats were greater than 10,000 mg/kg BW, indicating that it was basically nontoxic. The subacute toxicity study demonstrated that the BFT groups treated with a maximum daily dose of 5000 mg/kg BW for 28 consecutive days had no adverse reactions. In the 90-day sub-chronic toxicity test, administration of 5000 mg/kg BW did not cause any mortality to the rats; in addition, it could be considered nontoxic. It is worth noting that the administration of BFT had an antilipemic effect and exerted protective effects on the liver and the kidney. But these protective mechanisms of BFT ought to be further explored. In conclusion, the present study showed that BFT is a safe and edible natural source that should be used for further development and utilization of functional foods.

AUTHOR CONTRIBUTIONS

Yongchao Wang: Methodology; Validation; Visualization; Writing—original draft. **Jian Zhang**: Methodology; Validation; Visualization. **Tianrui Zhao**: Conceptualization; Visualization; Project administration. **Jianxin Cao**: Conceptualization; Visualization; Project administration. **Afsar Khan:** Writing—review. **Zhengxuan Wang**: Project administration; Supervision; Funding acquisition; Writing—review and editing. **Yaping Liu**: Methodology; Visualization. **Guiguang Cheng**: Project administration; Supervision; Writing—review and editing

ACKNOWLEDGMENTS

This research was funded by the Local Standard of Food Safety in Yunnan Province (Yunwei [2023] DB002), the Yunnan Provincial Basic Research Project (CB23078C159A), the Yunnan Major Natural Science Foundation (202202AG050009), the High-level Talent Promotion and Training Project of Kunming (2022SCP003).

CONFLICT OF INTEREST STATEMENT

The authors declare that they do not have competing financial interests.

DATA AVAILABILITY STATEMENT

All the required and necessary data has been provided.

ETHICS STATEMENT

On behalf of, and having obtained permission from all the authors, I declare that:

1. The paper has not been published in whole or in part elsewhere.
2. The paper is not currently being considered published elsewhere.
3. All authors have been personally and actively involved in substantive work leading to the report and will hold themselves jointly and individually responsible for its content.
4. All animal procedures were conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethical Committee for Animal Experimentation of Kunming University of Science and Technology.

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How to cite this article: Wang, Y., Zhang, J., Zhao, T., Cao, J., Khan, A., Wang, Z., Liu, Y., & Cheng, G. (2024). Acute, subacute, and sub-chronic toxicity evaluation of *Bletilla formosana* tubers in rats. *Food Safety and Health*, *2*(1), 117–132. https://doi.org/10.1002/fsh3.12026