Toxicology Reports 13 (2024) 101682

Contents lists available at ScienceDirect

Toxicology Reports

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

Sub-chronic toxicity of the active fraction of a modified Huang-Lian-Jie-Du Decoction

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ARTICLE INFO

Handling editor: Prof. L.H. Lash

Keywords:
Modified Huang-Lian-Jie-Du Decoction
Safety
Sub-chronic toxicity
Rats

ABSTRACT

A traditional Chinese herbal medicine formula named Huang-Lian-Jie-Du Decoction (HLJDD) has been used to cure various inflammatory diseases with a long history. However, one component of HLJDD *Gardeniae fructus* has remarkable liver and kidney toxicities. Therefore, it was altered with *Dictamni cortex* to form a modified HLJDD (MHLJDD). In this study, we aimed to evaluate the sub-chronic toxicity of the active fraction of MHLJDD (MHLJDD-F) in rats. Adult rats of both sexes were intragastrically administered with vehicle or MHLJDD-F (at the dose of 170, 340, and 680 mg/kg/day) once daily for 90 days. Half of the rats from each group were kept for an additional 30-day period to observe the drug withdrawal effect. The signs of toxicity and mortality of the rats were observed, and the body weight and food consumption were recorded. Blood was collected for hematological and biochemical analyses and major organs were weighed and harvested for histopathological examinations. The results revealed that no systemic toxicity of MHLJDD-F was found during the experiments. Organ coefficients and pathological alterations of major organs were comparable to the control rats. The no-observed adverse effect level (NOAEL) of MHLJDD-F was found up to 680 mg/kg/day. All these results demonstrated that long-term oral administration of MHLJDD-F did not cause significant toxicity, which is worthy to be widely applied as a new herbal medicine in pre-clinical and clinical studies.

1. Introduction

Huang-Lian-Jie-Du Decoction (HLJDD) contains four Chinese herb medicines including *Coptidis rhizoma*, *Phellodendri chinensis cortex*, *Scutellariae radix* and *Gardeniae fructus*. HLJDD is a well-known written prescription for alleviating inflammatory conditions in the theory and practices of Chinese medicine because of the function of clearing body fire and removing heat toxicity [1]. Previous clinical studies have documented that HLJDD showed potent therapeutic effects on various inflammatory diseases including atopic dermatitis (AD) [2–4]. In addition, our previous work [5] also demonstrated that HLJDD has strong anti-inflammatory and anti-allergic activities in *in vivo* and *in vitro* models of AD. However, one herbal medicine of HLJDD *Gardenia fructus* (GF) was reported to cause remarkable liver and kidney injuries in long-term treatment [6], which extremely limited the application of HLJDD in patients with AD.

Fortunately, another herbal medicine named *Dictamni cortex* (DC), has been traditionally used to eliminate the influences made by damp and hot weather on the body in the theory of Traditional Chinese Medicine. Also, DC is commonly used to resolve different kinds of inflammatory situations, especially of the skin in the clinics. DC was also found to have strong anti-allergic activity in a mouse model of AD [7]. Since the pathogenesis of AD is involved with both inflammation and allergic reactions [8], GF was altered with DC to form a modified HLJDD (MHLJDD). MHLJDD (1480 mg/kg/day) was found to be more efficient than HLJDD (600 mg/kg/day) in relieving 2,4-Dinitrobenzene (DNCB)-induced AD-like skin lesions in mice in our previous work [5,9], and the extracted active fraction of MHLJDD (MHLJDD-F, at the dose of 520 and 1040 mg/kg/day) possessed the most potent anti-AD efficacy [9]. Now that pharmacologic treatments for AD generally last for a long time, it is necessary to reveal the toxicological profile of MHLJDD-F for drug safety. The acute toxicity of MHLJDD-F has been evaluated and the

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https://doi.org/10.1016/j.toxrep.2024.101682
Received 28 April 2024; Received in revised form 11 June 2024; Accepted 12 June 2024
Available online 13 June 2024

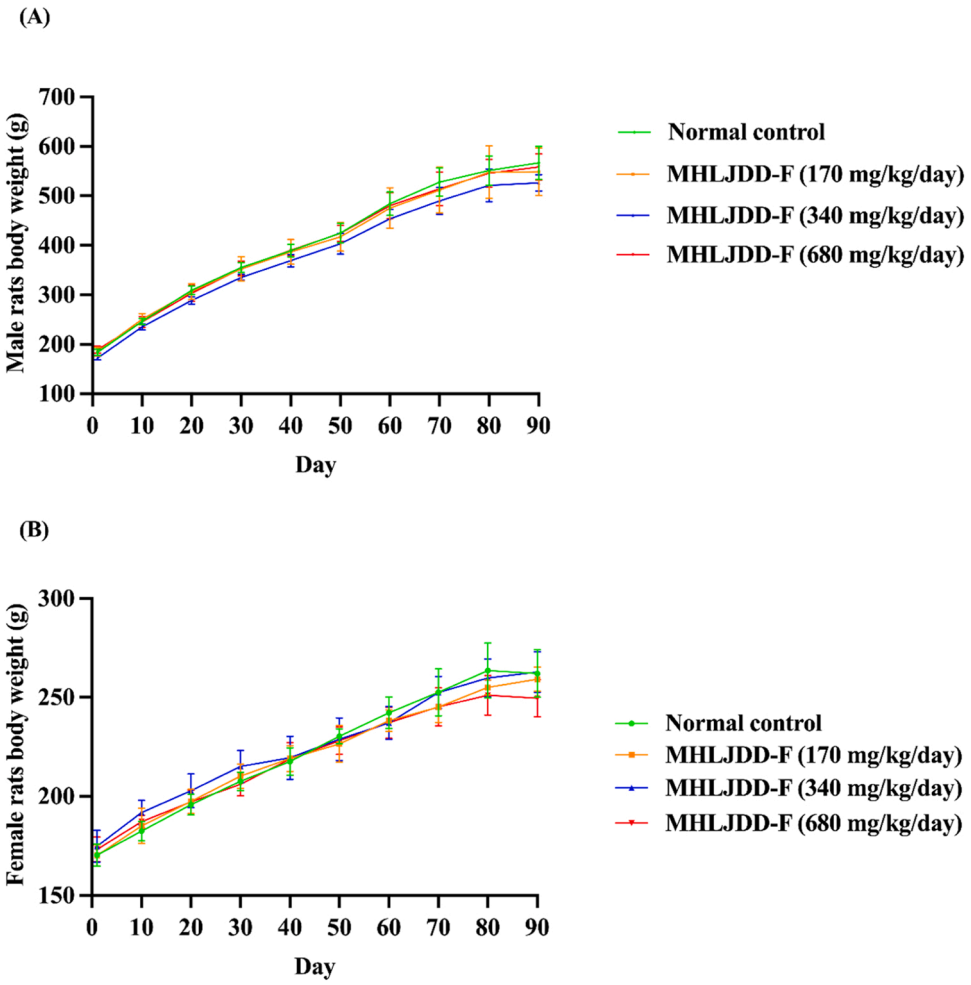

Fig. 1. Effects of MHLJDD-F on the body weight of male (A) and female (B) rats after 90-day drug treatment experiment. Data were presented as mean ± SEM (n = 10).

LD_{50} was 825 mg/kg/day (562–1210 mg/kg/day, 95 % CI) in mice [9]. In this study, we aimed to evaluate the sub-chronic toxicity of MHLJDD-F in rats. The presented investigation was a preliminary non-Good Laboratory Practice (GLP) toxicity study which followed most of the testing requirements given in OECD 408 [10].

2. Methods and materials

2.1. Preparation of MHLJDD-F

The detailed procedure to extract and fractionation of MHLJDD has been reported in our published work [9]. Considering the dose of this formula used in patients (MHLJDD, 30 g/65 kg/day) and the extraction rate (about 12 %) of MHLJDD-F, and the equivalent dose calculation based on body surface area from human to rats is 6.2 [11], therefore the dose levels used in rats in this study was 170, 340, and 680 mg/kg/day.

2.2. Experimental animals and housing conditions

Male and female Sprague-Dawley (SD) rats were provided by Laboratory Animal Services Center, CUHK. All the experimental procedures were reviewed and approved by the Animal Experimentation Ethics Committee of CUHK (Reference no. 21/109/ITF).

2.3. Grouping and drug treatment

Six to eight-week-old Sprague-Dawley rats (weighing 140–180 g) of both sexes were randomly divided into eight groups of 20 each. After the 90-day drug treatment, 10 rats from each group were sacrificed, and the remaining 10 rats were kept for the 30-day withdrawal observation. Different doses of MHLJDD-F (170, 340, and 680 mg/kg/day) were suspended in 0.5 % CMC-Na solution and given by gavage once daily to rats for 90 days. The rats of the normal control (NC) were given the vehicle. The signs of toxicity and mortality of the animals were observed throughout the experiments. The rat body weight and food consumption were also recorded every 10 days. In the 30-day drug withdrawal study, the left rats had free access to food and distilled water, without application of MHLJDD-F or vehicle.

2.4. Sample collection and organ weights

At the last day of the 90-day and the 30-day withdrawal experiments, rats of each group were sacrificed by decapitation under anesthesia. Blood from the heart was collected in vacutainer respectively with and without ethylenediaminetetraacetic acid (EDTA) for hematological and biochemical analyses. After sacrifice, the major organs were weighed (brain, heart, thymus gland, spleen, adrenal gland, kidney, lung, liver, pancreas, ovary, uterus, testes, epididymis), and the relative organ weight was calculated [12].

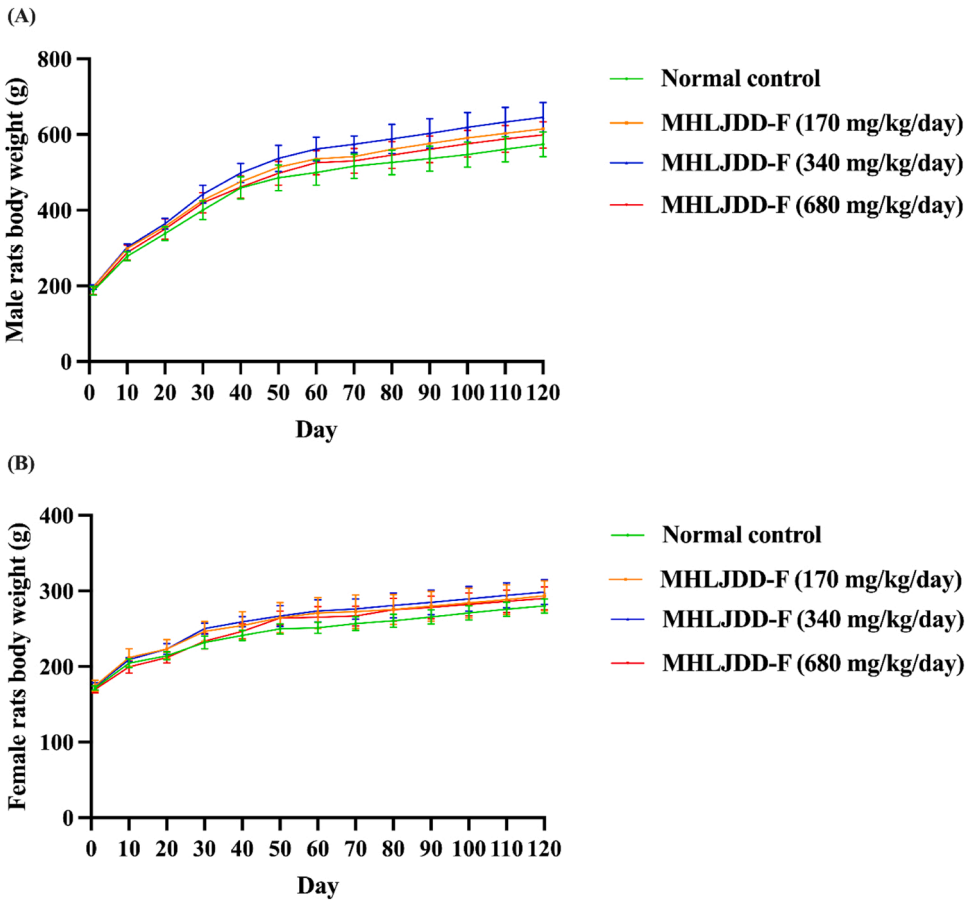

Fig. 2. Effects of MHLJDD-F on the body weight of male (A) and female (B) rats after 30-day drug withdrawal experiment. Data were presented as mean ± SEM (n = 10).

Table 1
Effects of MHLJDD-F on the feed intake (g/day/per rat) of rats after 90-day drug treatment experiment.

	Female				Male			
Day	NC	MHLJDD-F (170 mg/kg/day)	MHLJDD-F (340 mg/kg/day)	MHLJDD-F (680 mg/kg/day)	NC	MHLJDD-F (170 mg/kg/day)	MHLJDD-F (340 mg/kg/day)	MHLJDD-F (680 mg/kg/day)
1	16.00 ±1.41	16.60±1.52	16.00±1.22	16.20±0.45	20.00 ±1.41	19.40±0.55	19.40±1.14	19.80±1.79
10	16.20 ±1.30	16.80±0.84	16.80±1.30	16.60±0.89	21.20 ±0.84	21.20±1.64	21.40±1.34	21.60±1.52
20	16.60 ±1.14	16.60±1.34	16.20±1.30	16.40±1.52	22.00 ±1.00	22.4±0.55	22.20±1.30	21.80±0.84
30	15.60 ±0.55	15.80±1.30	16.80±0.84	16.80±1.10	22.20 ±0.84	21.80±1.10	21.20±0.84	22.60±0.89
40	17.00 ±1.22	16.20±1.30	16.00±1.22	16.60±1.14	21.60 ±1.14	21.80±0.84	21.40±1.52	22.00±1.41
50	17.80 ±1.10	17.60±1.14	17.20±1.10	18.40±0.89	22.00 ±1.22	22.40±0.89	21.80±1.30	21.40±1.34
60	17.00 ±1.41	18.40±0.89	17.40±0.89	17.60±1.14	24.20 ±1.30	24.20±1.64	24.60±1.52	23.60±0.55
70	17.40 ±1.14	18.00±0.55	17.20±0.84	17.40±1.34	24.00 ±0.71	24.20±1.10	24.80±1.10	24.60±1.52
80	16.80 ±1.30	18.40±0.55	17.80±0.84	18.20±1.30	24.80 ±1.64	23.20±0.45	24.20±1.30	25.00±1.58
90	16.80 ±0.84	18.00±1.22	17.00±1.00	16.80±0.84	24.80 ±1.30	23.80±0.84	24.80±1.30	24.40±1.52

Data were presented as mean ± SD (n = 10).

Table 2
Effects of MHLJDD-F on feed intake (g/day/per rat) of rats after 30-day drug withdrawal experiment.

Day	Female				Male			
	NC	MHLJDD-F (170 mg/kg/day)	MHLJDD-F (340 mg/kg/day)	MHLJDD-F (680 mg/kg/day)	NC	MHLJDD-F (170 mg/kg/day)	MHLJDD-F (340 mg/kg/day)	MHLJDD-F (680 mg/kg/day)
1	15.60 ±0.55	16.20±0.84	16.00±1.41	17.20±0.84	20.20 ±1.30	19.80±1.79	20.60±1.34	21.00±1.00
10	17.00 ±1.00	16.00±0.71	15.60±0.89	16.20±1.10	21.80 ±1.64	22.00±1.22	22.40±2.19	23.00±2.00
20	16.40 ±1.34	16.80±1.30	17.00±1.00	16.00±1.00	23.00 ±1.41	21.60±2.30	21.40±1.34	23.60±1.52
30	16.00 ±1.00	16.40±1.14	15.80±1.30	16.60±0.55	23.20 ±2.05	21.40±1.52	22.40±1.82	23.00±1.22
40	16.80 ±0.84	16.20±1.30	16.00±0.71	17.20±0.84	22.20 ±1.92	21.20±1.79	21.60±1.52	22.40±1.82
50	16.60 ±0.89	18.00±1.00	18.00±0.71	18.00±1.22	20.80 ±1.92	22.60±1.82	23.60±1.34	22.40±1.95
60	18.20 ±1.30	17.40±1.52	17.60±0.55	17.60±1.14	21.60 ±1.52	21.20±2.17	22.60±1.34	24.00±2.24
70	17.00 ±0.71	17.80±1.30	17.60±1.14	18.20±0.45	24.20 ±1.10	22.40±2.07	22.40±2.30	21.60±0.89
80	16.20 ±0.45	18.00±1.22	17.20±1.92	17.00±0.71	22.40 ±1.52	22.40±1.52	22.00±1.41	21.20±1.30
90	18.80 ±1.79	19.60±0.89	17.80±1.64	17.60±2.41	23.20 ±1.64	23.00±2.00	22.20±2.28	21.60±1.52
100	18.40 ±1.67	16.60±2.07	16.20±1.30	16.40±1.52	24.80 ±1.92	24.20±2.17	25.80±1.30	24.40±1.82
110	15.80 ±1.79	16.60±2.30	17.20±1.92	16.60±1.14	24.80 ±1.48	26.00±0.71	24.80±1.79	24.00±2.35
120	15.80 ±1.10	17.60±1.34	16.40±1.67	17.00±1.00	24.00 ±1.58	23.40±2.19	24.60±1.14	23.80±2.05

Data were presented as mean ± SD (n = 10).

Table 3
Effects of MHLJDD-F on organ coefficients of rats after 90-day drug treatment experiment.

Organ	Female				Male			
	NC	MHLJDD-F (170 mg/kg/day)	MHLJDD-F (340 mg/kg/day)	MHLJDD-F (680 mg/kg/day)	NC	MHLJDD-F (170 mg/kg/day)	MHLJDD-F (340 mg/kg/day)	MHLJDD-F (680 mg/kg/day)
Brain	0.73 ±0.10	0.64±0.06	0.64±0.03	0.64±0.10	0.39 ±0.01	0.33±0.04*	0.35±0.01	0.37±0.02
Heart	0.34 ±0.02	0.33±0.03	0.31±0.04	0.35±0.06	0.27 ±0.07	0.27±0.03	0.24±0.03	0.27±0.04
Thymus Gland	0.10 ±0.06	0.13±0.10	0.19±0.09	0.12±0.01	0.13 ±0.07	0.09±0.03	0.09±0.04	0.08±0.03
Spleen	0.16 ±0.02	0.17±0.01	0.17±0.01	0.17±0.01	016 ±0.00	0.14±0.01	0.15±0.01	0.14±0.01
Adrenal Gland	0.02 ±0.01	0.02±0.00	0.02±0.00	0.02±0.00	0.01 ±0.00	0.01±0.00	0.01±0.00	0.01±0.00
Kidney	0.72 ±0.06	0.70±0.04	0.69±0.02	0.69±0.02	0.58 ±0.06	0.58±0.05	0.58±0.04	0.56±0.02
Lung	0.54 ±0.08	0.54±0.10	0.58±0.12	0.58±0.04	0.40 ±0.08	0.44±0.13	0.38±0.13	0.53±0.14
Liver	3.21 ±0.24	3.29±0.24	3.37±0.23	3.25±0.23	3.24 ±0.26	3.18±0.39	3.30±0.21	3.27±0.21
Pancreas	0.29 ±0.11	0.26±0.03	0.20±0.05	0.30±0.08	0.14 ±0.02	0.15±0.05	0.14±0.02	0.20±0.08
Ovary	0.04 ±0.01	0.04±0.01	0.03±0.01	0.06±0.01**	/	/	/	/
Uterus	0.19 ±0.03	0.20±0.02	0.19±0.03	0.23±0.05	/	/	/	/
Testes	/	/	/	/	0.59 ±0.05	0.57±0.02	0.56±0.02	0.59±0.02
Epididymis	/	/	/	/	0.32 ±0.07	0.24±0.05	0.29±0.09	0.23±0.02

Data were expressed as mean ± SD (% of the fasting body weight) (n = 10). * $p < 0.05$ and ** $p < 0.01$ compared with the NC group.

Table 4
Effects of MHLJDD-F on organ coefficients of rats after 30-day drug withdrawal experiment.

Organ	Female				Male			
	NC	MHLJDD-F (170 mg/kg/day)	MHLJDD-F (340 mg/kg/day)	MHLJDD-F (680 mg/kg/day)	NC	MHLJDD-F (170 mg/kg/day)	MHLJDD-F (340 mg/kg/day)	MHLJDD-F (680 mg/kg/day)
Brain	0.75 ±0.04	0.70±0.04	0.71±0.04	0.72±0.04	0.34 ±0.03	0.34±0.02	0.35±0.03	0.34±0.02
Heart	0.32 ±0.03	0.33±0.01	0.31±0.01	0.33±0.02	0.25 ±0.01	0.26±0.01	0.25±0.01	0.25±0.01
Thymus Gland	0.14 ±0.08	0.12±0.02	0.25±0.02	0.16±0.03	0.10 ±0.06	0.12±0.02	0.09±0.02	0.13±0.05
Spleen	0.17 ±0.02	0.15±0.00	0.15±0.02	0.16±0.01	0.13 ±0.01	0.12±0.01	0.12±0.01	0.13±0.01
Adrenal Gland	0.02 ±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.01 ±0.01	0.01±0.01	0.01±0.00	0.01±0.00
Kidney	0.70 ±0.08	0.66±0.04	0.69±0.05	0.68±0.06	0.57 ±0.04	0.60±0.04	0.56±0.09	0.58±0.04
Lung	0.51 ±0.07	0.51±0.04	0.50±0.05	0.51±0.04	0.29 ±0.04	0.30±0.01	0.32±0.05	0.28±0.02
Liver	3.28 ±0.34	3.24±0.13	3.19±0.42	3202±0.23	3.23 ±0.24	3.07±0.18	3.21±0.14	3.20±0.12
Pancreas	0.13 ±0.05	0.19±0.09	0.20±0.06	0.19±0.05	0.10 ±0.04	0.12±0.01	0.12±0.02	0.12±0.04
Ovary	0.04 ±0.01	0.03±0.01	0.04±0.01	0.04±0.01	/	/	/	/
Uterus	0.20 ±0.05	0.19±0.03	0.20±0.04	0.19±0.02	/	/	/	/
Testes	/	/	/	/	0.61 ±0.03	0.61±0.04	0.58±0.07	0.61±0.04
Epididymis	/	/	/	/	0.32 ±0.04	0.31±0.01	0.33±0.01	0.30±0.01

Data were expressed as mean ± SD (% of the fasting body weight) (n = 10).

2.5. Hematological examination and biochemical analysis

Hematological examinations were estimated by an autoanalyzer (ADVIA® 2120i, Siemens Healthcare Diagnostics, Germany). The differential leukocyte count (%) was performed using Leishman's stain on blood smears. Blood without EDTA was collected and stood at room temperature for an hour, and then centrifuged at 5000 ×g for 20 min. The serum was collected and stored for the following biochemical analysis using ELISA kits (Nanjing Jian Cheng, China).

2.6. Histopathological analysis

Tissue samples from kidney, pancreas, lung, and liver were collected for histopathological examination, which were fixed in 10 % formalin overnight and then embedded in paraffin, followed by sectioned at 10 μm thickness. And then the sections were stained with hematoxylin and eosin (H & E) solution as described in our published studies [7,13].

2.7. Statistical analysis

All data were showed as mean ± standard deviation (SD)/ standard error of mean (SEM). Difference comparisons between multiple groups were performed using one-way ANOVA followed by the Bonferroni test. The statistical significance was considered when $p < 0.05$.

3. Results

3.1. Effects of MHLJDD-F on body weight and feed intake of rats

We found no mortality and clinical signs of toxicity during the period of 90-day and the 30-day withdrawal experiments. As shown in Figs. 1 and 2, the body weight of both female and male rats increased gradually during the entire period of the 90-day experiment and the 30-day withdrawal experiment. At the end of the 90-day experiment, the body weight of MHLJDD-F treated groups did not show statistical differences, as compared with the NC group, either in the male or female rats. At the end of the 30-day drug withdrawal experiment, the body weight of rats of both sexes showed similar changes to the 90-day drug treatment experiment, except that the body weight of male rats in the MHLJDD-F (340 mg/kg/day)-treated group was higher ($p < 0.05$), as compared with the NC group. These results suggested that long-term treatment with MHLJDD-F did not show significant side effects on the rat's body weight.

In addition, as shown in Table 1 and Table 2, there lack of differences in feed intake in all MHLJDD-F treated groups in both sexes, as compared to the corresponding control group.

3.2. Effects of MHLJDD-F on organ coefficients of rats

As shown in Table 3, after 90-day oral administration of MHLJDD-F (680 mg/kg/day), the relative organ coefficient of ovary of female rats outstandingly raised ($p < 0.01$), when compared with the NC female rats. In the 90-day experiment, the relative organ coefficient of brain was observably decreased in the MHLJDD-F (170 mg/kg/day)-treated male rats ($p < 0.05$), when compared with the male NC rats. However, in case of 30-day withdrawal experiment, these changes were reversed in the MHLJDD-F-treated male and female rats (Table 4).

3.3. Effects of MHLJDD-F on hematological parameters of rats

After 90-day oral administration of MHLJDD-F, only a few hematological parameters of both female and male rats were found to change (Table 5). As shown in Table 5, MHLJDD-F (680 mg/kg/day) treatment significant decreased the MCV in the female ($p < 0.01$) and male rats ($p < 0.05$) and MCH in the male rats ($p < 0.05$), when compared with the NC group. MHLJDD-F (680 mg/kg/day) also signally increased the MCHC in the female rats ($p < 0.05$) (Table 5). However, these changes were reversed after 30-day drug withdrawal experiment (Table 6).

Table 5
Effects of MHLJDD-F on hematological parameters (mean ± SD) in rats after 90-day drug treatment experiment.

Parameters	Female				Male			
	NC	MHLJDD-F (170 mg/kg/day)	MHLJDD-F (340 mg/kg/day)	MHLJDD-F (680 mg/kg/day)	NC	MHLJDD-F (170 mg/kg/day)	MHLJDD-F (340 mg/kg/day)	MHLJDD-F (680 mg/kg/day)
RBC ($\times10^{12}$/L)	7.07±0.71	7.49±0.17	7.60±0.19	7.32±0.66	7.50±0.21	7.66±0.34	7.48±0.36	8.06±0.57
Haemoglobin (g/L)	124.40 ±10.81	132.60±1.82	134.75±2.22	127.00±10.12	129.50 ±2.12	126.60±4.22	126.75±6.45	131.60±6.88
Haematocrit (%)	40.52 ±4.31	42.48±0.29	42.70±1.52	39.36±3.49	42.55 ±0.07	41.72±1.20	40.63±2.34	42.34±1.86
MCV (fL)	57.32 ±0.83	56.78±1.11	56.20±0.73	53.80±1.04**	56.75 ±1.63	54.48±1.23	54.25±1.07	52.66±1.86*
MCH (pg)	17.62 ±0.32	17.70±0.21	17.73±0.24	17.42±0.38	17.25 ±0.21	16.52±0.35	16.93±0.36	16.38±0.39*
MCHC (g/L)	307.60 ±8.05	312.00±3.74	315.75±6.08	323.80±3.70*	303.50 ±4.95	303.00±2.92	312.25±2.63	311.20±5.40
Reticulocyte (%)	2.36±0.21	2.52±0.37	2.95±0.64	2.56±0.17	4.60±1.27	3.56±0.67	3.98±0.50	3.52±0.38
Reticulocyte count ($\times10^9$/L)	166.86 ±14.05	187.76±27.15	192.95±34.58	185.76±13.64	342.05 ±84.22	272.08±53.84	297.30±47.12	281.26±19.15
WBC ($\times10^9$/L)	1.71±0.90	1.05±0.42	1.89±1.01	2.18±0.57	6.28±1.77	4.86±2.90	3.91±2.25	6.86±1.31
Neutrophil (%)	19.80 ±12.78	16.90±2.79	16.50±7.44	14.20±1.50	17.40 ±0.99	22.30±9.50	20.85±4.24	15.78±2.88
Neutrophil count ($\times10^9$/L)	0.30±0.16	0.17±0.06	0.28±0.11	0.31±0.09	1.09±0.25	1.01±0.59	0.76±0.35	1.09±0.34
Lymphocyte (%)	74.20 ±14.44	78.38±2.14	78.53±6.46	81.46±1.92	78.25 ±1.34	73.68±9.99	75.25±5.19	80.16±3.48
Lymphocyte count ($\times10^9$/L)	1.32±0.79	0.83±0.34	1.52±0.90	1.78±0.47	4.93±1.46	3.65±1.87	3.02±1.87	5.49±0.98
Monocyte (%)	2.34±1.12	1.42±0.51	2.10±0.29	1.56±0.25	2.05±0.21	1.40±0.44	1.48±0.43	1.60±0.37
Monocyte count ($\times10^9$/L)	0.03±0.01	0.02±0.01	0.04±0.03	004±0.01	0.14±0.05	0.06±0.03	0.06±0.04	0.11±0.04
Eosinophil (%)	2.04±0.80	2.04±0.75	1.40±0.37	1.28±0.34	0.90±0.28	1.36±0.59	1.18±0.64	1.08±0.73
Eosinophil count ($\times10^9$/L)	0.04±0.03	0.02±0.00	0.03±0.02	0.03±0.01	0.06±0.01	0.07±0.06	0.04±0.03	0.07±0.04
Basophil (%)	1.20±1.17	0.40±0.12	0.55±0.42	0.32±0.16	0.15±0.07	0.28±0.18	0.25±0.10	0.30±0.14
Basophil count ($\times10^9$/L)	0.016 ±0.01	0.002±0.00*	0.010±0.01	0.006±0.01	0.02±0.01	0.01±0.01	0.01±0.01	0.02±0.01
Large unstained cells (%)	0.98±0.57	0.84±0.40	0.93±0.39	1.24±0.11	1.25±0.35	0.96±0.43	0.98±0.53	1.12±0.24
Large unstained cells count ($\times10^9$/L)	0.02±0.01	0.01±0.01	0.02±0.02	0.03±0.01	0.08±0.00	0.05±0.04	0.03±0.02	0.08±0.02
Platelet ($\times10^9$/L)	773.40 ±433.25	1130.60±53.94	1101.50±23.22	883.80±350.21	1383.00 ±226.27	1111.40±152.00	1161.00±62.59	1140.00±164.18

Data were presented as mean ± SD (n = 10). * $p < 0.05$ and ** $p < 0.01$ compared with the NC group.

3.4. Effects of MHLJDD-F on serum parameters of rats

After 90-day oral administration of MHLJDD-F, results of the biochemical analysis showed very few statistical differences in the parameters of the MHLJDD-F treated groups compared to their respective control group, both in the 90-day drug treatment experiment (Fig. 3) and 30-day drug withdrawal experiment (Fig. 4). Significant decreases were observed in the serum level of AST in the female rats treated by MHLJDD-F (680 mg/kg/day) in the 90-day drug treatment experiment, and 30-day drug withdrawal experiment (both $p < 0.05$). And the level of CRE in serum also significantly decreased in the female rats treated with MHLJDD-F (170 and 680 mg/kg/day) ($p < 0.01$ and $p < 0.05$) in the 30-day drug withdrawal experiment. However, no notable changes were observed in male rats treated with MHLJDD-F, ether in the 90-day drug treatment and 30-day drug withdrawal experiments.

3.5. Effects of MHLJDD-F on histopathologic evaluation of rats

Results of histopathologic evaluation of lung, pancreas, liver, and kidney showed that rats treated with the 90-day oral application of MHLJDD-F up to the dose of 680 mg/kg/day had few pathological changes in both sexes, when compared with the NC rats (Fig. 5). These pathological changes such as lymphocytic infiltration in the liver and kidney were incidental on the basis that the frequency and severity were comparable to the untreated rats and no dose relationship was observed. The same situations were also observed in the 30-day drug withdrawal experiment (Fig. 6).

4. Discussion

HLJDD was reported to have *in vitro* genotoxicity [14] and *in vivo* hepatic and renal toxicity [15]. In preclinical studies and clinical practices, HLJDD was also found to produce some adverse effects on the stomach, gastrointestinal tract, liver, and kidney [16,17]. The toxicity of HLJDD was mainly due to the GF, which was reported to cause noticeable liver and kidney injury in the rats after 12-week application [6]. The hepatotoxicity and renal toxicity of GF extremely restricted the long-term use of HLJDD in clinics. Therefore, many attempts have been made to reduce the toxicity of HLJDD. In the present study, we replaced GF with DC, with the hope of arriving at a safer and more resultful therapy to be utilized in inflammatory skin diseases.

Results of hematological analysis present the toxicity of test substances on coagulation function and immunologic function, which presented a few marked variations in MCV, MCH, and MCHC of rats receiving MHLJDD-F at the dose level of 680 mg/kg/day, in the 90-day drug treatment experiment. In the 90-day experiment, MCV level significantly decreased by 6.14 % and 7.20 %, respectively in the female and male rats that received MHLJDD-F at the dose level of 680 mg/kg/

Table 6
Effects of MHLJDD-F on hematological parameters of rats after 30-day drug withdrawal experiment.

Parameters	Female				Male			
	NC	MHLJDD-F (170 mg/kg/day)	MHLJDD-F (340 mg/kg/day)	MHLJDD-F (680 mg/kg/day)	NC	MHLJDD-F (170 mg/kg/day)	MHLJDD-F (340 mg/kg/day)	MHLJDD-F (680 mg/kg/day)
RBC ($\times10^{12}$/L)	7.98 ±0.22	7.91±0.22	7.59±0.32	8.17±0.7	7.61 ±0.28	7.54±0.23	7.46±0.45	7.59±0.28
Haemoglobin (g/L)	139.75 ±5.12	136.60±4.34	133.80±4.87	136.60±4.83	129.40 ±6.69	131.8±4.44	130.00±4.32	132.20±4.60
Haematocrit (%)	46.48 ±2.44	45.02±1.41	44.76±1.37	45.52±0.94	43.78 ±2.98	47.36±1.50	45.80±1.70	45.54±1.01
MCV (fL)	58.30 ±4.04	56.90±0.75	56.42±0.68	55.50±4.09	57.52 ±2.65	55.46±1.29	54.23±1.17	54.32±1.18
MCH (pg)	17.53 ±0.44	17.30±0.33	17.68±0.25	16.80±1.64	17.00 ±0.56	16.60±0.50	16.43±0.43	16.58±0.41
MCHC (g/L)	301.0 ±15.9	304.0±5.5	313.0±4.6	303.8±132.6	295.4 ±5.8	296.0±3.8	297.2±1.2	295.60±4.4
Reticulocyte (%)	2.33 ±0.56	2.40±0.20	2.80±1.04	2.58±0.56	3.54 ±0.55	3.48±0.38	3.58±0.75	3.68±0.44
Reticulocyte count ($\times10^9$/L)	183.7 ±40.1	188.2±17.8	211.1±74.6	210.6±43.7	269.7 ±43.1	279.6±31.5	280.3±48.2	245.7±132.8
WBC ($\times10^9$/L)	4.82 ±1.10	4.68±1.61	4.77±0.96	4.63±0.66	6.55 ±2.15	7.10±1.64	7.18±1.24	7.01±1.57
Neutrophil (%)	18.65 ±6.79	18.76±7.65	16.30±4.20	17.44±2.96	21.86 ±4.28	19.90±3.91	20.35±4.29	20.32±1.92
Neutrophil count ($\times10^9$/L)	0.85 ±0.15	0.87±0.47	0.78±0.21	0.74±0.14	1.43 ±0.55	1.56±0.21	1.39±0.31	1.48±0.26
Lymphocyte (%)	77.00 ±6.87	76.72±7.89	80.68±4.12	81.66±3.56	73.78 ±4.51	78.66±5.08	78.45±5.00	76.80±1.89
Lymphocyte count ($\times10^9$/L)	3.76 ±1.13	3.60±1.38	2.46±0.76	3.26±0.58	3.82 ±1.57	5.10±1.76	4.44±1.29	4.80±1.32
Monocyte (%)	1.73 ±0.45	2.18±0.48	1.94±0.51	2.18±0.69	1.98 ±0.49	1.72±0.87	1.98±0.57	1.80±0.39
Monocyte count ($\times10^9$/L)	0.08 ±0.02	0.11±0.05	0.06±0.03	0.08±0.02	0.14 ±0.08	0.27±0.06	0.16±0.05	0.15±0.05
Eosinophil (%)	1.55 ±0.56	1.04±0.36	1.12±0.22	1.28±0.31	1.16 ±0.22	1.06±0.57	0.78±0.39	0.78±0.19
Eosinophil count ($\times10^9$/L)	0.08 ±0.04	0.05±0.02	0.06±0.01	0.05±0.01	0.07 ±0.02	0.11±0.05	0.06±0.03	0.06±0.02
Basophil (%)	0.38±0.1	0.32±0.13	0.40±0.23	0.32±0.04	0.24 ±0.05	0.31±0.04	0.35±0.10	0.33±0.08
Basophil count ($\times10^9$/L)	0.0175 ±0.00	0.012±0.00	0.012±0.00	0.014±0.00	0.021 ±0.01	0.017±0.01	0.023±0.01	0.024±0.01
Large unstained cells (%)	0.70 ±0.08	1.00±0.56	1.12±0.45	1.06±0.18	1.00 ±0.07	1.24±0.33	1.13±0.43	0.88±0.08
Large unstained cells count ($\times10^9$/L)	0.03 ±0.01	0.05±0.04	0.03±0.01	0.04±0.01	0.06 ±0.02	0.07±0.02	0.09±0.03	0.07±0.01
Platelet ($\times10^9$/L)	868.8 ±216.5	1045.2±159.1	1037.4±243.7	1095.6±106.7	1086.6 ±439.3	1265.8±191.8	1125.3±145.3	1280.6±205.5

Data were presented as mean ± SD (n = 10).

day, but the values were within the background range [18,19]. These minor changes could reverse at the end of the 30-day withdrawal experiment, and no abnormal pathologic changes were observed in the histopathologic examinations. Likely, these changes in MCH and MCHC were also reversible and showed no association with histopathological examination. In addition, the AST level was significantly reduced by 41 % and 27 % in the female rats treated with MHLJDD-F (680 mg/kg/day), and the CRE level was reduced by 38 % and 52 % in the female rats treated with MHLJDD-F (170 and 680 mg/kg/day), which compared to the untreated rats.

In the 90-day experiment, the ovary weight of the MHLJDD-F treated group (680 mg/kg/day) increased by about 50 % and the relative brain weight of the MHLJDD-F treated group (170 mg/kg/day, male) decreased by about 15 % as compared to the untreated normal rats but within the normal range [20]. These changes were also recovered during the 30-day drug withdrawal observation. The results showed no correlation with histopathological examination and thus were considered of no toxicological significance. Also, an increase in the relative epididymis weight of males in the 30-day withdrawal experiment was observed, but these differences observed in rats treated with MHLJDD-F were not dose-dependent. No significant pathological changes were observed in MHLJDD-F treated rats, though some clinical changes such as lymphocytic infiltration were observed in the liver and kidney. These observations were considered incidental and thought not related to MHLJDD-F administration, as the frequency and severity of pathological changes were comparable to the untreated rats and no dose relationship was observed. Compared with other published data of assessment on the toxicity of HLJDD [21], MHLJDD-F did not adversely affect the RBC and reticulocyte, as well as any specific organ.

5. Conclusion

Oral administration of the rats with MHLJDD-F in both sexes rats for 90 consecutive days did not produce any evident toxicity or adverse effects to organs. In the evaluation of the sub-chronic toxicity, NOAEL of MHLJDD-F was found up to 680 mg/kg/day in both sexes of rats.

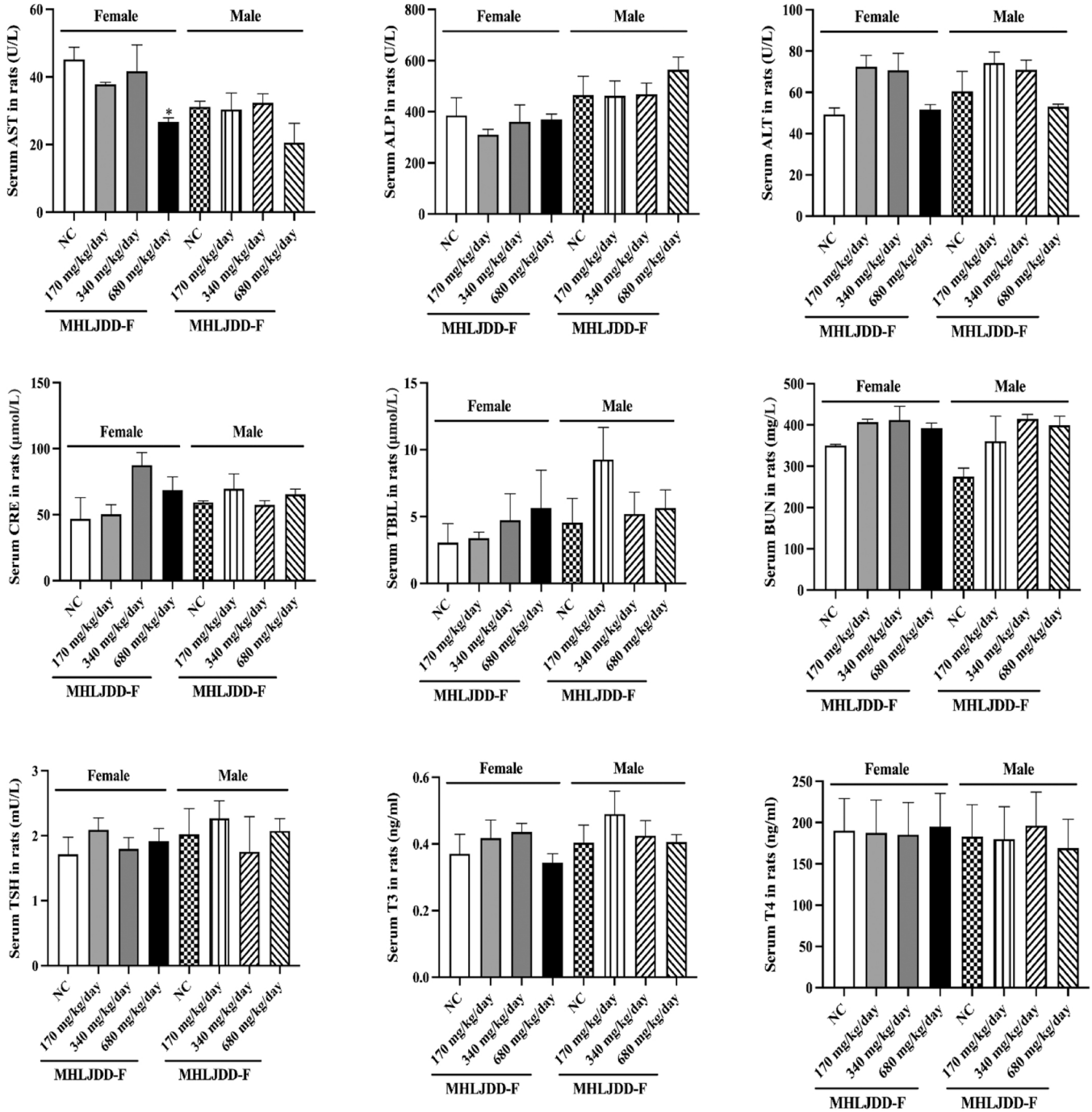

Fig. 3. Effects of MHLJDD-F on the serum parameters of rats after 90-day drug treatment experiment. Data were presented as mean ± SEM (n = 10). * $p < 0.05$ compared with the NC group.

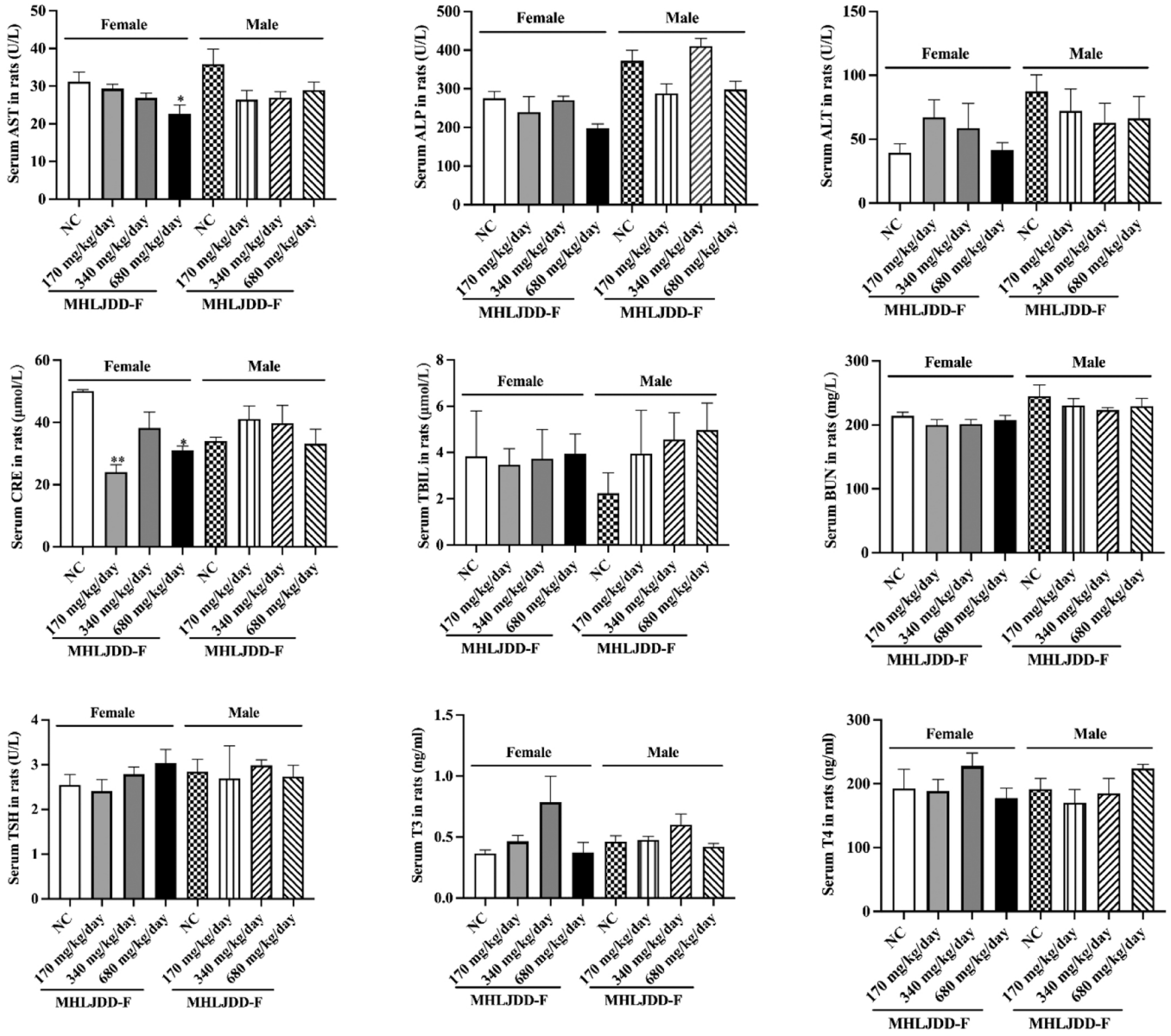

Fig. 4. Effects of MHLJDD-F on serum parameters of rats after 30-day drug withdrawal experiment. Data were presented as mean ± SEM (n = 10). * $p < 0.05$ and ** $p < 0.01$ compared with the NC group.

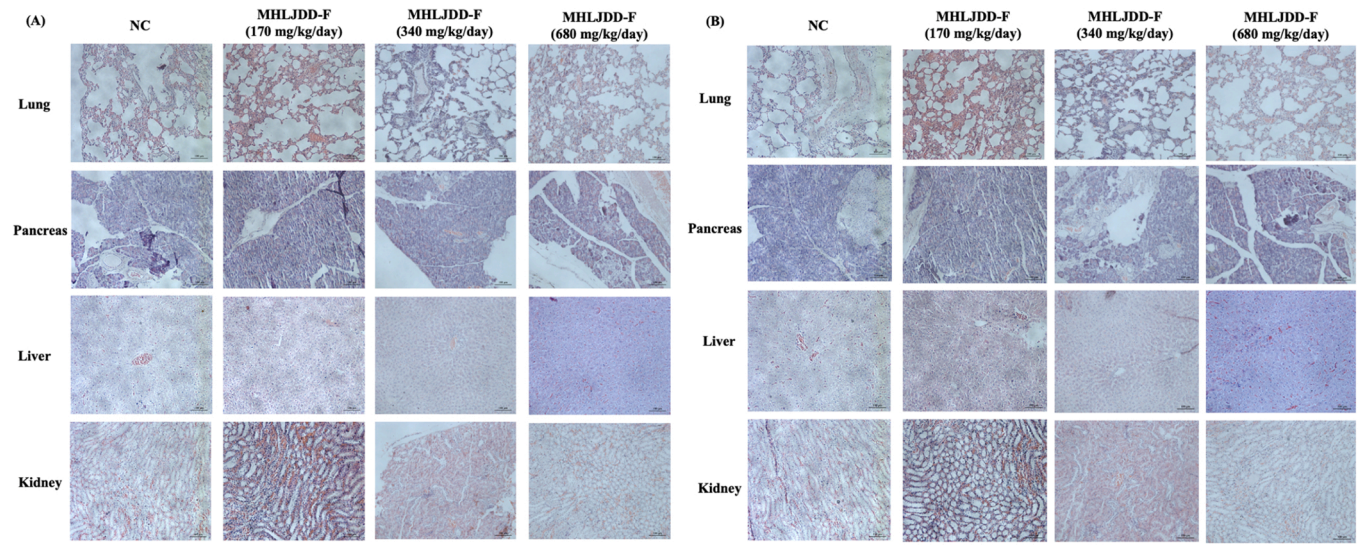

Fig. 5. Effects of MHLJDD-F on histopathologic evaluation of lung, pancreas, liver, and kidney tissues of rats. Respective photos of histopathologic evaluations (magnification 100 ×) on lung, pancreas, liver, and kidney of rats treated with MHLJDD-F and the normal control rats after 90-day drug treatment experiment. (A) Male. (B) Female.

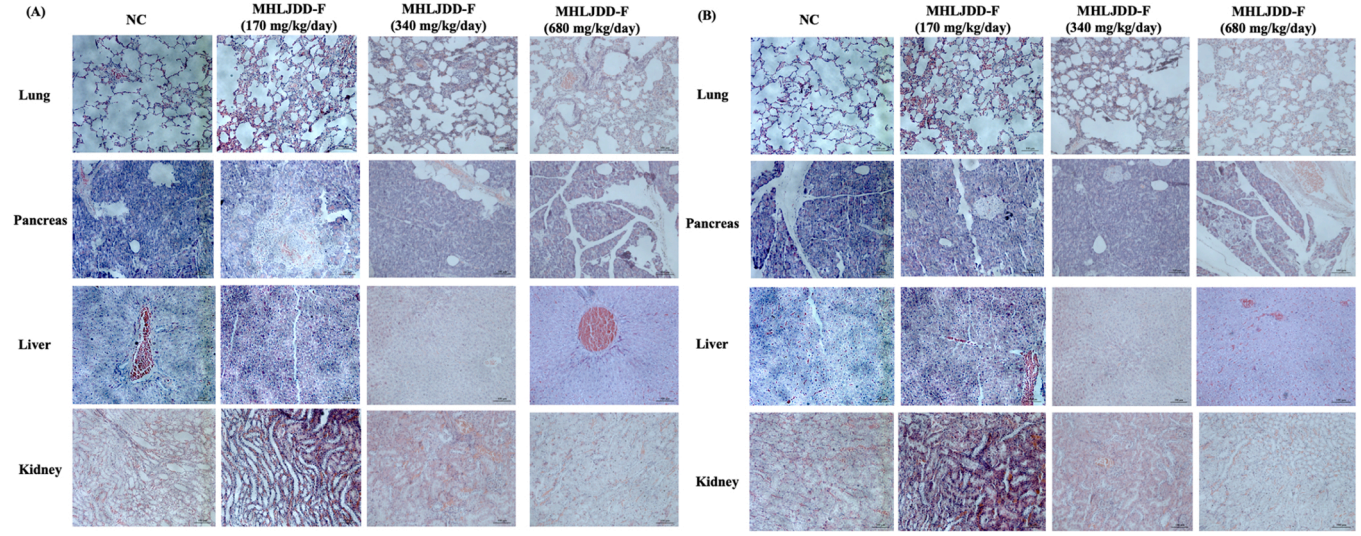

Fig. 6. Effects of MHLJDD-F on histopathologic evaluation of lung, pancreas, liver, and kidney tissues of rats. Respective photos of histopathologic evaluations (magnification 100 ×) on lung, pancreas, liver, and kidney of rats treated with MHLJDD-F and the normal control rats after 30-day drug withdrawal experiment. (A) Male. (B) Female.

Author statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We understand that the Corresponding Author is the sole contact for the Editorial process. She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

CRediT authorship contribution statement

Steven King Fan Loo: Resources, Formal analysis, Data curation. **Siu-Po Ip:** Validation, Resources, Data curation. **Yanfang XIAN:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Zhi-Xiu Lin:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Lan Wang:** Writing – original draft, Software, Investigation. **Wen Yang:** Methodology, Data curation. **Jiaqian Zhu:** Software, Investigation, Data curation. **Yanfeng Huang:** Methodology, Formal analysis. **Mei Zhong:** Software, Data curation.

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.

Data availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments

This work was supported by the Innovation and Technology Commission Funding Administrative System of Hong Kong (Project No.: ITS/122/19FP).

Author contributions

LIN ZX and XIAN YF conceived and designed the study. WANG L, YAN W, HUANG YF, ZHU JQ, ZHONG M and IP SP performed the experiments and recorded the data. WANG L analyzed the data and drafted the manuscript. LIN ZX and XIAN YF reviewed and revised the manuscript. All authors have read and approved the final manuscript.

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