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Toxicological Evaluation of *Antrodia cinnamomea* in BALB/c Mice

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Abstract. *Antrodia cinnamomea is a natural component of some herbal medicines used for treatment of abdominal pain, hypertension and hepatocellular carcinoma in Taiwan and other countries. Subchronic oral toxicity studies of A. cinnamomea extracts in male and female BALB/c mice were performed to evaluate its safety. Three different concentrations of A. cinnamomea (16.67, 833.3 and 1666.67 mg/kg/day) were given orally to groups of mice (10 mice/dose) for 90 consecutive days. All animals survived to the end of the study, and there were no significant differences in body weight among the control and treatment groups. No significant differences were found in hematological and serum biochemical parameters among the control and treatment groups. No abnormalities of internal organs were observed in the treated groups.*

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Polysaccharide-rich fungi and plants have been employed for centuries by different societies around the world for their dietary and medicinal benefits (1, 2). They have been reported to aid normal bowel function and blood glucose and lipid levels (3-5), and certain mushrooms have attracted interest for their ability to exert marked effects on immune system function, inflammation and cancer (6-8). Many of these chemically and structurally diverse, non- to poorly-digestible mushrooms or their components have been shown to beneficially affect one or more targeted cellular functions *in vitro* (8-13). However, much of the *in vivo* literature deals with injected polysaccharides (1). For clinicians and scientists interested in immunological effects following dietary intake, the value and food safety of such studies are unclear. Mushroom extracts or contents that elicit effects *in vitro* or by injection may be ineffective or have different effects when taken orally (14).

In Taiwan, *A. cinnamomea* is an endemic, resupinate to pileate perennial, polyporoid mushroom, inhabiting the empty cavity of *C. kanehirae* Hayata using brown-rot of heart-wood. The host *C. kanehirae* is an endemic evergreen broad-leaved tree of Taiwan, found on hillsides at an altitude ranging from 500 to 1500 m (15, 16). *A. cinnamomea* is a popular but expensive medicinal mushroom widely used as a folk remedy for alleviating itching, pain, diarrhoea, inflammation, hangover, and hepatic dysfunction. It also has been used for treating abdominal pain, hypertension and various types of cancer including hepatocellular carcinoma,

leukemia and pancreatic cancer but subchronic data are sparse on the toxicity of *A. cinnamomea* (15-22).

According to the Enforcement Rules of the Health Food Control Act established by the Taiwan Department of Health, health food products should be evaluated for their pharmacological effects and safety by the 90-day subchronic toxicological assessment to examine the blood routine, biochemical activities and pathological assessment including liver, spleen and kidney.

Materials and Methods

Preparation of A. cinnamomea test solution. *A. cinnamomea* (500 mg) and distilled water were mixed thoroughly and filtered (0.22 μm pore size) to provide a solution with a series of concentrations of 2.08, 100.42 and 208.33 mg/ml. *A. cinnamomea* was obtained from Chang Gung Biotechnology Corporation, Ltd. (Taipei, Taiwan, ROC).

Animals. Forty BALB/c male mice and forty BALB/c female mice (10/group) were obtained from the National Taiwan University College of Medicine Animal Medicine Center (our own breeding colony). Mice were four weeks of age and weighing 20-25 g at the beginning of the study. The animals were housed singly in an animal room with a 12-hour light/dark cycle at a temperature and relative humidity range of 20±2˚C and 75±15%, respectively. The animals were acclimated for at least 14 days prior to testing. They were orally fed with Laboratory Rodent Diet 5001 manufactured by PMI Nutrition Internation during the acclimation period and throughout the study.

Study design. Animals used in the present study were maintained in accordance with the guidelines approved by the National Science Council of the Republic of China and the Committee for the Purpose of Control and Supervision of Experiments on Animals. Experiments were performed according to law, regulations and guidelines for animal experiments in Taiwan, which are in agreement with the Helsinki declaration. Male and female mice were randomized and allocated into control (Group 1) and experimental groups of 10 male animals and 10 female animals in each group. Groups 2, 3 and 4 were orally administered with high (1666.67 mg/kg/day), medium (833.3 mg/kg/day) and low (16.67 mg/kg/day) doses of *A. cinnamomea* daily for 90 days. Control animals were orally administered distilled water.

Sample collection. Animals were fasted for at least 15 h and then placed in metabolism cages one day before the clinical pathology evaluation. Blood was drawn by orbital bleeding at the end of the experiments: 20 μl of whole blood was collected into the EDTA capillary tube for complete blood counts (CBC) test. Cardiac puncture was used to collect blood (0.2 ml with 10 U/ml heparin), which was allowed to clot, and centrifuged (1000 ×*g*, 10 min, room temperature) and used for biochemical tests. At the end of the experiment, liver, spleen and kidney were excised immediately after the animals were sacrificed under anaesthesia by CO_2. Organs were weighed. All three organs were utilized for the following biochemical and histological assessments.

CBC analysis. Haematology parameters included erythrocyte count (CBC), haemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin (MCH), red cell distribution width, platelet count, total white blood cell and differential leukocyte count. Mean corpuscular haemoglobin concentration was also calculated. Blood smears were prepared and evaluated. CBCs, including reticulocytes, were determined on a Medonic CA530 Vet automation instrument produced by Boule Medical AB (Stockholm, Sweden), or determined from microscopic evaluation of the blood smear. Wright-Giemsa-stained blood smears from all animals were examined microscopically for confirmation of automated results and evaluation of cellular morphology.

Serum biomarker analysis. Clinical biochemical values analyzed were: serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), total bilirubin (T-Bil), blood urea nitrogen (BUN), blood creatinine (CRE), total cholesterol (T-Cho), fasting glucose (Glu), total serum protein (T-Pro) and albumin (Alb). These biomarkers were analyzed on an Arkray Spotchen SP-4410 clinical chemical analyzer and reagents used were obtained from Arkray, Inc. (Kyoto, Japan) (23).

Histopathological assessment of organs. Processing of tissue samples including liver, spleen and kidney for histological assessment followed established procedures. In brief, tissue samples were rinsed with 0.9% saline solution and fixed in 10% formalin. Diagonal sections of the liver, the transverse sections of the kidneys and the horizontal sections of the spleen were then processed using a Automatic Tissue Processor (Leica TP1020, Japan) as follows: 10% neutral buffered formalin for 1 h, twice; 70% alcohol for 1.5 h; 80% alcohol for 1.5 h; 90% alcohol for 1.5 h; absolute alcohol for 1.5 h, twice; xylene for 1.5 h, twice; in molten wax at 65˚C for 2.5 h two changes. The processed tissues were embedded in paraffin and sectioned at 4-μm thickness, placed on frosted glass slides and dried on a 70˚C hot plate for 30 min. The tissues were stained using haematoxylin and eosin (H&E) staining. The sections were dewaxed in two changes of xylene (3 min each), hydrated in two changes of 100% ethanol, followed by 90% ethanol and 70% ethanol, for 3 min each, rinsed with water (3 min) and stained. The stained tissues were dehydrated with 70% ethanol followed by 90% ethanol, placed in two changes of 100% ethanol for 3 minutes each and cleaned with two changes of xylene (3 min each). The sectioning and staining were performed by National Taiwan University College of Medicine, Animal Medicine Center, Taipei, Taiwan, R.O.C.

Statistical analysis. Treated and control groups were compared using a one-way analysis of variance (ANOVA). Student's *t*-test was used to compare different treatment groups when one-way ANOVA was significant. Male and female mice were evaluated separately, and differences among groups were judged to be significant at a probability value of $p<0.001$.

Results

Weekly mean body weight and weight gain for all groups that consumed *A. cinnamomea* were comparable to the values of the controls (Figure 1A and B). There were no deaths nor significant weight loss observed during the study.

Toxicity associated with *A. cinnamomea* treatment was assessed at biochemical, haematological and histopathological levels. The serum concentrations of the biochemical markers ALT, AST, BUN, T-Bil and creatinine were obtained to

evaluate liver and renal functions. In addition, the histopathological changes in the target organs, liver, spleen and kidney, were evaluated.

Mice treated with increasing doses of *A. cinnamomea* did not show any gradual elevation of haemoglobin, haematocrit or erythrocyte counts. Differences in these parameters compared to the control group were not statistically significant ($p>0.001$) (Tables I and II). Mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration were not altered by exposure to the three different doses of *A. cinnamomea* (Tables I and II). Platelet count did not significantly increase compare to the control value (Tables I and II). The leukocyte data are also shown in Tables I and II. Mice receiving *A. cinnamomea* did not exhibit any statistically significant decrease or increase in total leukocytes, nor exposure-related changes in differential leukocyte counts.

Mice treated with increasing doses of *A. cinnamomea* did not exhibit any gradual elevation of serum AST, ALT and T-Bil concentrations (Tables III and IV). Although the ALT in the female control (80±39 IU/l) and high-dose treatment group (88±40 IU/l) were different, this was not statistically significant. The T-Bil concentrations for males were increased (1.70±0.91 mg/dl) after administration of only low-dose extract in comparison to those of the control group (1.05±0.86 mg/dl) but did not reach statistical significance ($p>0.001$). The renal biomarkers BUN and creatinine were mostly unaffected by increasing doses of *A. cinnamomea*. Female mice treated with a low dose had significantly ($p<0.001$) higher BUN levels (30.1±4.3 mg/dl) in comparison with the control group (24.1±6.7 mg/dl) (Tables III and IV). Total cholesterol, fasting glucose, total serum protein, and albumin concentrations were not altered by *A. cinnamomea* treatment. Neither body weight or weights of different organs were affected by *A. cinnamomea* treatment (Table V). Liver, spleen and kidney tissue sections were stained with H&E. The histopathology assessment in liver, spleen and kidney were performed for control and experimental groups. Mice in the control and experimental groups exhibited normal, well-defined histological structures without any signs of vascular or inflammatory changes (Figure 2). The histopathological analysis of the liver revealed no signs of toxicity after administration of *A. cinnamomea* (Figure 2). The histological assessment of the spleen did not reveal any vascular changes in any of the *A. cinnamomea*-treated groups. Furthermore, no inflammatory changes were observed in the control and experimental groups (Figure 2). Normal histology of the glomerulus and tubules was found in kidney tissue of control mice and those that received *A. cinnamomea* treatment. *A. cinnamomea* did not induce any vascular or inflammatory changes such as signs of vascular congestion, tubular necrosis and glomerular atrophy, which is a degenerative phenomenon. Photomicrographs also showed no areas of red blood cell extravasation into the interstitium and amidst the spaces between the tubules (Figure 2). Based on our findings, no infiltration, aggregation, necrosis and atrophy were found in control and experimental sections of liver, spleen and kidney.

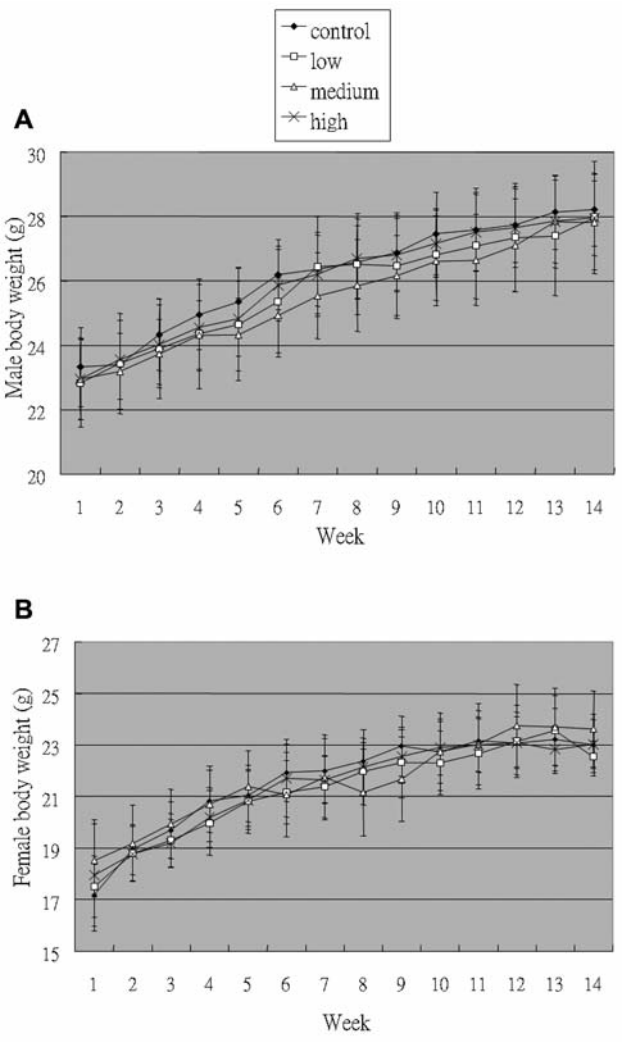

Figure 1. *Food consumption for male (A) and female (B) mice exposed for 90 days to Antrodia cinnamomea. Values represent the mean±SD of 10 mice/sex/group.*

Discussion

A. cinnamomea, a medicinally important fungus, plays an important role in traditional Chinese medical practice and it has been proven to be effective in treating liver diseases and tumours (17). Further characterization of this fungal species may therefore yield medicinal benefits. However, its slow growth rate and exclusive host requirement render large-scale production of this fungus for medicinal purposes difficult (24-26).

Table I. *Mean haematology counts for male mice administered different doses of Antrodia cinnamomea for 90 days.*

Parameter	Dose (mg/kg/day)			
	Control	16.67	833.3	1666.67
Erythrocyte count (10^6/mm^3)	11.35±0.91	12.10±1.30	11.64±1.14	11.72±0.98
Haemoglobin concentration (g/dl)	16.39±1.28	17.38±1.79	16.74±1.51	16.66±1.29
Haematocrit (%)	57.55±4.52	62.00±7.05	60.05±5.73	59.65±5.02
Mean corpuscular volume (μm^3)	50.71±1.35	51.14±1.08	51.60±1.61	50.90±1.36
Mean corpuscular haemoglobin (pg)	14.33±0.23	14.37±0.28	14.38±0.44	14.23±0.49
Mean corpuscular haemoglobin concentration (g/dl)	28.50±0.61	28.08±0.49	27.92±0.42	27.96±0.43
Red cell distribution width (%)	15.49±0.60	16.06±0.81	16.00±0.69	16.01±0.69
Platelet count (10^3/mm^3)	642.5±119.2	676.8±191.8	633.4±184.9	729.2±196.8
White blood cells (10^3/mm^3)	2.19±0.93	2.03±1.00	2.42±0.86	2.25±1.61
Differential leukocyte count				
Lymphocytes (%)	18±13	30±13	26±14	22±12
Granulocytes (%)	67±15	56±26	59±27	63±20
Reticulocytes (%)	0.9±0.3	0.9±0.7	0.8±0.4	1±0.6
Others (%)	15±6	17±9	15±9	16±7

Table II. *Mean haematology counts for female mice administered different doses of Antrodia cinnamomea for 90 days.*

Parameter	Dose (mg/kg/day)			
	Control	16.67	833.3	1666.67
Erythrocyte count (10^6/mm^3)	10.99±0.56	12.06±1.02	11.52±0.88	11.30±0.39
Haemoglobin concentration (g/dl)	16.18±0.77	17.37±1.46	16.79±1.03	16.24±0.71
Haematocrit (%)	57.58±2.95	63.55±5.10	60.96±4.71	60.39±2.71
Mean corpuscular volume (μm^3)	52.37±0.96	52.68±0.75	52.72±0.74	53.37±0.92
Mean corpuscular haemoglobin (pg)	14.72±0.27	14.38±0.33	14.58±0.28	14.36±0.28
Mean corpuscular haemoglobin concentration (g/dl)	28.13±0.35	27.33±0.63	27.58±0.49	26.91±0.37
Red cell distribution width (%)	15.23±0.36	15.56±0.82	14.88±0.43	15.11±0.43
Platelet count (10^3/mm^3)	546.6±45.7	692.6±117.8	512.6±144.7	545.1±131.3
White blood cells (10^3/mm^3)	1.88±0.84	2.13±0.75	1.90±0.47	1.64±0.49
Differential leukocyte count				
Lymphocytes (%)	33±18	33±25	25±17	27±26
Granulocytes (%)	55±18	54±22	57±17	57±24
Reticulocytes (%)	1±0.5	0.8±0.7	0.7±0.8	1±0.6
Others (%)	13±6	16±6	19±5	16±9

Plants produce a great diversity of substances that can have therapeutic benefits for maintaining health and improving the quality of life, thus justifying their use in traditional medicine (23-27). However, many plant extracts may have undesirable effects, which must be determined. In the present study, we determined if *A. cinnamomea* would have toxic effects when chronically administered to mice. We present here 90-day studies in mice, to assess *A. cinnamomea* from the viewpoint of the toxicologist. Defined exposures are useful biological test models for investigative toxicology and mechanistic studies, and serve as an important element used in pre-clinical safety assessment and the evaluation of environmental agents for toxic risk to humans. Compared with *in vitro* tests, *in vivo* tests may provide more relevant data for the assessment of DNA damage potential in humans since they take into account dynamic whole-animal physiological processes such as uptake and systemic distribution by the circulatory system, phase I and II metabolism, and intact elimination/excretory systems that cannot be entirely recreated *in vitro*. The histopathological evaluation is considered to be the primary assay to assess *in vivo* toxic potential across multiple species, including humans. After acute toxicity studies, which provide a basis for dose levels and potential target organs, subchronic 90-day studies are conducted.

Table III. *Mean clinical biochemistry values for male mice administered different doses of Antrodia cinnamomea for 90 days.*

Parameter	Dose (mg/kg/day)			
	Control	16.67	833.3	1666.67
Aspartate aminotransferase (IU/l)	282±171	308±132	253±130	281±166
Alanine aminotransferase (IU/l)	111±104	102±53	88±66	92±62
Total bilirubin (mg/dl)	1.05±0.86	1.70±0.91	1.03±0.6	1.05±0.75
Blood urea nitrogen (mg/dl)	21.5±5.7	26.8±5.2	26.3±4.9	23.1±3.7
Blood creatinine (mg/dl)	0.59±0.08	0.87±0.19	0.70±0.15	0.69±0.13
Total cholesterol (mg/dl)	94.6±11.5	93.7±9.1	86.7±13.0	93.1±7.4
Fasting glucose (mg/dl)	103.1±21.2	93.4±7.6	78.7±14.6	97.5±11.8
Total serum protein (g/dl)	5.51±0.17	2.53±0.25	2.39±0.14	2.41±0.17
Albumin (g/dl)	2.46±0.49	2.84±0.64	2.14±0.93	2.43±0.48

Table IV. *Mean clinical biochemistry values for female mice administered different doses of Antrodia cinnamomea for 90 days.*

Parameter	Dose (mg/kg/day)			
	Control	16.67	833.3	1666.67
Aspartate aminotransferase (IU/l)	159±56	165±56	163±77	160±83
Alanine aminotransferase (IU/l)	80±39	74±17	82±20	88±40
Total bilirubin (mg/dl)	0.49±0.34	0.40±0.37	0.53±0.44	0.44±0.39
Blood urea nitrogen (mg/dl)	24.1±6.7	30.1±4.3*	25.9±3.0	27.9±4.2
Blood creatinine (mg/dl)	0.50±0.12	0.58±0.13	0.51±0.17	0.44±0.09
Total cholesterol (mg/dl)	82.5±13.9	83.9±17.7	92.8±17.1	95.5±12.5
Fasting glucose (mg/dl)	109.9±29.5	108.4±27.5	127.5±27.9	126.2±20.8
Total serum protein (g/dl)	5.71±0.41	5.63±0.39	5.90±0.64	5.59±0.28
Albumin (g/dl)	2.38±0.13	2.29±0.19	2.38±0.21	2.51±0.13

*Significant difference between control and treatment group at $p<0.001$.

The lack of change in AST, ALT aminotransferases and T-Bil levels found in the male and female mice correlated well with lack of histopathological changes in liver morphology and relative organ weight. We did not find any changes in liver function-related enzymes or adverse histopathological changes. Although we demonstrate the safety of *A. cinnamomea*, we were not able to establish a correlation between toxicology and biomarkers that can provide early detection of toxicity. No significant correlation was found between the plasma BUN levels and kidney morphological changes.

In the present study, *A. cinnamomea* did not have any significant effects on histopathology of various organs, nor on haematology and serum biochemistry. It is not known at what dose *A. cinnamomea* may have toxic effects in mice. Chen and co-workers indicated that no significant differences were found in urinalysis, haematological and serum biochemical parameters between the treatment and control groups, and reported that necropsy and histopathological examination indicated that there were no treatment-related changes. The "no observed adverse effect level" NOAEL in that study in Sprague-Dawley rats was 3000 mg/kg BW/day, which is markedly higher than the maximum dose used in the present study (1666.67 mg/kg/day) (20).

Table V. *Mean organ weights of male and female mice that consumed Antrodia cinnamomea for 90 days.*

		Organ weight		
		Liver (g)	Spleen (g)	Kidney(g)
Dose (mg/kg/day)	Control	0.89±0.07	0.08±0.01	0.30±0.02
	16.67	0.85±0.08	0.08±0.01	0.29±0.03
	833.3	0.82±0.08	0.07±0.01	0.27±0.03
	1666.67	0.84±0.08	0.08±0.01	0.29±0.03

We conclude that administration to male and female mice of up to 1666.67 mg/kg/day for 90 days does not produce any changes in blood cell counts and serum chemistry.

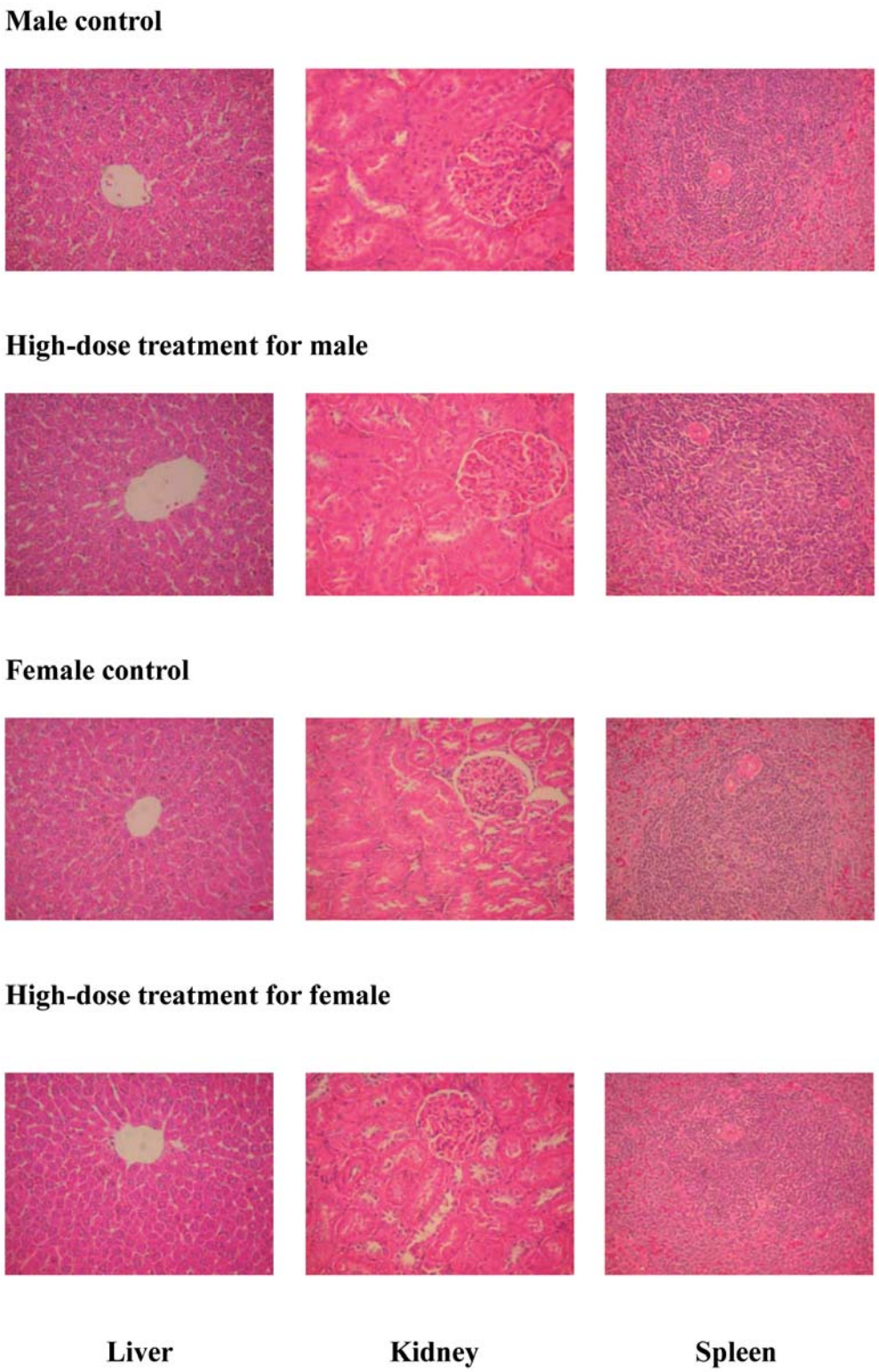

Figure 2. *Histopathology of liver, kidney and spleen after high-dose administration of H&E staining. Photomicrograph x200 of liver section after administration of H&E staining showed no vascular congestion in the central veins and no red blood cells pooling in the sinusoids. We also found no microabscesses. Photomicrographs of kidney tissue sections after administration showed normal orientation of nephrons with adequate glomeruli and well-spaced tubules. There were no signs of toxicity in the spleen in all groups. Photomicrographs from the high-dose group showed normal morphology of the spleen including the red and white pulp areas and areas of cellularity admixed without any mild congestion.*

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