Original Article

Oral acute and chronic toxicity studies of β, β-dimethylacrylalkannin in mice and rats

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(Received February 17, 2017; Accepted March 2, 2017)

ABSTRACT — β, β-dimethylacrylalkannin is a major active chemical component extracted from *Lithospermum erythrorhizon*, a traditional Chinese medicine that exhibits strong antimicrobial, anti-cancer and anti-inflammatory activities. However, its potential toxicity has not been rigorously studied. To confirm its safety, the oral toxicity of β, β-dimethylacrylalkannin was evaluated *in vivo*. An acute oral toxicity study in mice demonstrated that β, β-dimethylacrylalkannin was practically nontoxic based on its high median lethal dose (LD_{50} > 10 g/kg). No deaths or abnormal responses were observed in the acute toxicity test using Wistar rats, suggesting that the maximum tolerated dose of β, β-dimethylacrylalkannin was greater than 10 g/kg. Chronic toxicity studies also revealed an absence of mortality and clinical symptoms, and no treatment-related adverse effects were detected by hematology, blood biochemistry and urinalysis examinations in all rats treated with 10-160 mg/kg/day β, β-dimethylacrylalkannin during a 6-month period. Increases in the relative organ weight of the lungs of females and the liver of males were observed at 160 mg/kg. Histopathological analyses revealed brown pigmentation in renal tubular epithelial cells at the middle and high doses (40-160 mg/kg/day). The no-observed-adverse-effect level (NOAEL) of β, β-dimethylacrylalkannin is 10 mg/kg/day. These results suggest that β, β-dimethylacrylalkannin is potentially safe for further development as a therapeutic agent in humans.

Key words: *Lithospermum erythrorhizon*, β, β-dimethylacrylalkannin, Acute toxicity, Chronic toxicity, Pigmentation

INTRODUCTION

Lithospermum erythrorhizon is a perennial herb that is widely distributed in Europe and western Asia. *L. erythrorhizon* was initially used in food and fabrics because of its natural red-purple color and has long been used as an effective traditional medicine in wound healing (Papageorgiou *et al*., 2008) and therapy with microorganisms (Brigham *et al*., 1999; Lee *et al*., 2015). Recent *in vitro* and *in vivo* studies have shown beneficial pharmacological effects of *L. erythrorhizon* extract, such as in the treatment of viruses (Chen *et al*., 2003), cancers and inflammation (Haghbeen *et al*., 2011; Han *et al*., 2008). Han *et al*. proved that hexane extracts of the roots of *L. erythrorhizon* are safe in toxicity studies (no-observed-adverse-effect level, NOAEL > 400 mg/kg/day) (Han *et al*., 2015). Because of its value as a pharmaceutical agent and its safety, many studies have focused on the determination of its biological activity, analysis of its active components and synthesis of its natural products (Assimopoulou *et al*., 2009).

The major active chemical components of *L. erythrorhizon*, highly liposoluble naphthoquinone pigments, are β, β-dimethylacrylalkannin with a molecular weight of 370 (Fig. 1) and its enantiomer shikalkin (Damianakos *et al*., 2012; Yazaki *et al*., 1997). β, β-dimethylacrylalkannin has numerous pharmacological properties, including antimicrobial, anti-cancer, anti-inflammatory activities (Shen *et al*., 2002; Wang *et al*., 2015), and is one of the most promising candidates for pharmaceutical and cosmetic preparations due to its biological activity (Assimopoulou and Papageorgiou,

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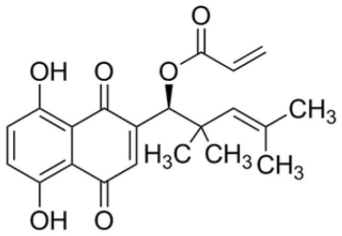

Fig. 1. Chemical structure of β, β-dimethylacrylalkannin.

2005; Sevimli-Gur *et al*., 2010). Previous studies have ensured that β, β-dimethylacrylalkannin is effective in treating hepatitis By inhibiting the replication of the hepatitis virus (unpublished). However, information regarding the toxicity of β, β-dimethylacrylalkannin is very limited. The purpose of this study was to evaluate the safety of β, β-dimethylacrylalkannin through acute and chronic administration in mice and rats and to provide guidance for clinical applications.

MATERIALS AND METHODS

Test material

β, β-dimethylacrylalkannin is a dark red powder that is fat-soluble with a purity of 96%. During the study, the test material was stored in the dark at a temperature of 2-8°C and freshly suspended in 0.5% carboxymethylcellulose sodium (CMC-Na) before use.

Animals and housing

All specific pathogen-free (SPF) KM mice and Wistar rats were obtained from the Chinese Academy of Medical Sciences (China). All animals were housed in groups in an environmental-controlled barrier-sustained animal room in the Peking-Union Pharma animal center. The animals were supplied with a standard commercial diet and drinking water *ad libitum*. The environmental controls for the animal room were set to a temperature of 20 ± 3°C, a relative humidity of 60 ± 20% and a 12 hr light/12 hr dark cycle. The Institutional Animal Ethics Committee of the New Drug Safety Evaluation Center in the Institute of Materia Medica approved the study before it began. Prior to the experiment, the animals were allowed one week to acclimate to the environment.

Oral acute toxicity study

Twenty 5-6-week-old KM mice (19-24 g) of both sexes were used in this study. Prior to the oral administration of the single dose (10 g/kg bw) of β, β-dimethylacrylalkannin, all animals were subjected to a fasting period of 12 hr. All deviations in general behavior associated with the administration of the compound in mice were monitored continuously for 4 hr after dosing. The mice were further observed once per day for up to 14 days for behavioral changes and signs of toxicity and/or death. The body weights were monitored on day 1, 4, 7, 11 and 14. An oral study for calculating the LD_{50} was performed according to the Organization for Economic Co-operation and Development (OECD) Guideline 425 (OECD, 2001).

Twenty 8-week-old Wister rats (170-190 g) of both sexes a received single dose of β, β-dimethylacrylalkannin (10 g/kg, i.g.) only once. Prior to oral administration, the animals were subjected to a fasting period of 12 hr. All drug responses were monitored continuously for 4 hr after administration and observed until14 days post treatment. The body weights were monitored on day 1, 4, 7, 11 and 14. At the end of 14 days, all rats were euthanatized, and anatomical observations were conducted on all organs and tissues. The experimental design was conducted in accordance with the internationally accepted Guide of New Drug and Chinese Medicine and OECD425 (OECD, 2001).

180-Day chronic toxicity study

Eighty adult male and eighty female Wister rats (5-6 weeks old), weighting 70-80 g and 80-90 g, respectively, were housed under the same conditions as described for the acute toxicity study. A total of 160 rats were randomly divided into four groups containing 40 rats each (20 males and 20 females). β, β-dimethylacrylalkannin was administered to rats at dosages of 10, 40 and 160 mg/kg for 6 days every week for 26 weeks. The control group received only the vehicle (1 mL/100 g bw). The rats were closely observed for any behavioral changes every day, and the body weight and the food intake were monitored weekly. At the end of the administration period, the animals were fasted overnight but given water *ad libitum*. After administration for 90 days, 5 animals/sex/group were anesthetized with CO_2 for blood sample collection followed by euthanasia and tissue collection. 10 animals/sex/group were sacrificed 24 hr after the final administration on day 180 for blood collection and histopathological examination. At the end of the 30-day recovery period, the remaining 5 animals/sex/group were treated by the same method (Table 1). Urine and serum samples were kept frozen at -80°C until analyzed. The experimental design was conducted in accordance with the internationally accepted Guide of New Drug and Chinese Medicine.

Table1. Experimental design of the 180-day chronic toxicity test on Wister rats.

Group		Drug dose (mg /kg)	Multiple of the proposed clinical dose	Multiple of the effective animal dose	Number of rats
I	Control	0	0	0	40
II	Low dose	10	6	5-10	40
III	Middle dose	40	24	20-40	40
IV	High dose	160	96	80-160	40

Hematological and biochemical analysis

At the end of the administration and recovery periods, blood samples were obtained from the abdominal aorta for the analysis of hematology and serum biochemistry. The blood samples collected in $EDTAK_2$-coated vials were analyzed using an auto-hematology analyzer (MEK-6318K, Nihon Kohden, Japan) for white blood cells (WBCs), red blood cells (RBCs), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean platelet volume (MPV), platelet distribution width (PDW),lymphocyte(LYM), granulocyte (GRAN) and reticulocyte (Reti).

For serum biochemical analysis, blood was centrifuged at 1500 g for 15 min to obtain serum. The biochemistry assays were performed using an automatic biochemistry meter (Selectra-E, Vital Scientific, Netherlands) for the following 15 parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), glucose (GLU), blood urea nitrogen (BUN), total protein (TP), albumin (ALB), albumin-globulin ratio (A/G), total bilirubin (TBIL), total cholesterol (TCHO), triglyceride (TG), creatine kinase (CK), lactate dehydrogenase (LDH) and uric acid (UA). K^+, Na^+ and Cl^- were determined using the ion-selective electrode method with an electrolyte analysis instrument (AVL-9181, Roche, Swiss).

Blood samples were collected in sodium citrate-coated vials, and the plasma was separated to determine the coagulation parameters, including thrombin time (TT), prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen (FIB), using a semi-automated coagulation analyzer (STA-4, Stago, France).

Urinalysis

During the necropsy, urine was collected from bladder and was examined using a test strip for urinalysis (Bayer, Germany) and an automatic urinalysis system (CliniTekGJ2-T-50, Bayer). The urinalysis test items were GLU, bilirubin (BIL), ketone body (KET), specific gravity (SG), occult blood (BLO), pH, protein (PRO), urobilinogen (URO), nitrite (NIT) and leukocyte (LEU).

Histopathological study

After sacrificing the rats, parts of the brain, spinal cord, pituitary gland, sternum, thymus, thyroid, stomach, ileum, duodenum, colon, liver, kidney, adrenal gland, spleen, pancreas, bladder, heart, lung, bone, lymph nodes, testicles, uterus, and ovaries were collected. For the histological examination, all organs and tissues were fixed in 10% formalin, dehydrated with varying grades of alcohol, embedded in paraffin, cut into standard thick sections and stained with hematoxylin-eosin dye for microscopic observation by the relevant professional qualified experts.

In addition, the weight of the liver, kidney, lung, heart, spleen, brain, and thymus tissues were measured and recorded. The organ-body index was calculated according to the following formula (Liu *et al*., 2004): Organ-body index (%)=Wet organ weight/Body weight ×100%.

Statistical analysis

The data are expressed as the mean ± standard deviation (S.D.). All numerical data were analyzed by the F-test to assess the equality of variances. If homoscedasticity of the data was accepted, Dunnett's t-test was conducted to examine the significance between the treatment groups and control. The urinalysis data were ranked and compared using a non-parametric method (Kruskal-Wallis' H-test). Statistical analyses were performed in Excel software. P values of < 0.05 were considered significant.

RESULTS

Acute toxicity in mice

The effects of β, β-dimethylacrylalkannin at a dose of 10 g/kg in mice were locomotor activity reduction and muzzle cyanosis after oral administration for 10 min. One male mouse died on the administration day, and three male mice died the next day. Four female mice died after 3 days. In total, 8 mice died out of the 20 treated with β, β-dimethylacrylalkannin. The observed symptoms and mortality had no gender difference. After 24 hr, the surviving animals improved, and the symptoms disappeared; the animals completely recovered after 14 days. In addition, the stool color was dark red. The treatments had no

effect on the body weight gain or food consumption of the surviving mice. The LD_{50} of β, β-dimethylacrylalkannin in mice was more than 10 g/kg bw. The kidney, spleen, heart, lungs, stomach and intestine of the surviving mice showed no pathological changes in the histological examination.

Acute toxicity in rats

The effects of β, β-dimethylacrylalkannin at a dose of 10 g/kg in rats were locomotor activity reduction and muzzle cyanosis after oral administration for 10 min, which disappeared after 24 hr. The observed symptoms and mortality had no gender difference. No death was observed during the 14-day recovery period. The stool color was dark red. The treatments had no effect on the body weight gain or food consumption of the surviving rats. The MTD of β, β-dimethylacrylalkannin in rats was more than 10 g/kg bw. The kidney, spleen, heart, lungs, stomach and intestine of the surviving mice showed no pathological changes in the histological examination.

180-Day chronic toxicity in rats

Clinical signs

During the experimental period, no treatment-related toxicity or mortality signs were noted in either sex of the rats treated at 10-160 mg/kg. There was no obvious alteration in the behavior or feces of the rats.

Body weight and food consumption

No significant changes were recorded in the body weight gain of control and treated rats (Fig. 2). The food consumption of the β, β-dimethylacrylalkannin-treated groups was insignificant in both the sexes compared to the 0.5% CMC control rats (Fig. 3).

Hematological parameters

All hematological parameters were measured at the end of the administration and recovery periods (Table 2). Compared with the control rats, a significant reduction in RBC, HGB, HCT and LYM and an increase in GRAN were observed in treated females, but the differences dis-

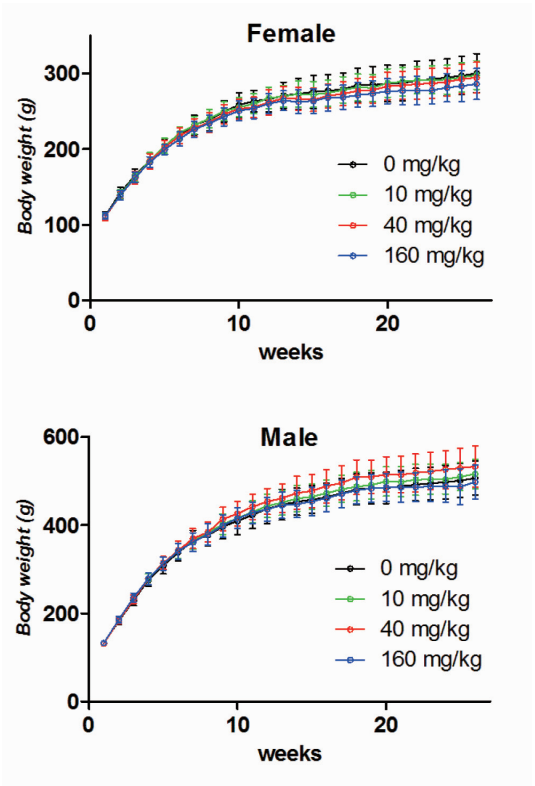

Fig. 2. Effect of β, β-dimethylacrylalkannin on body weight gain in rats in the chronic toxicity study. Values are the means ± S.D. (week 1-13, n = 20; week 14-26, n = 15). No significant differences were observed.

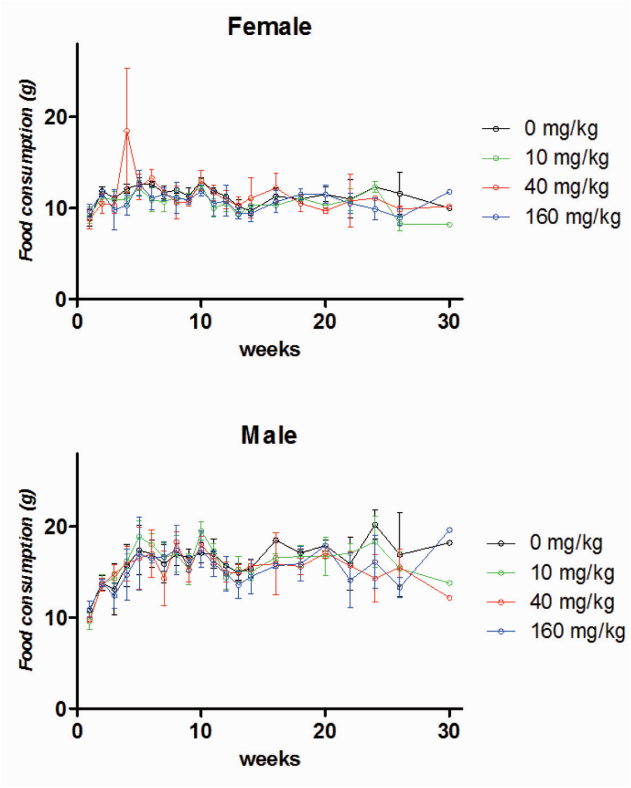

Fig. 3. Effect of β, β-dimethylacrylalkannin on food intake in rats in the chronic toxicity study. Values are the means ± S.D. (week 1-13, n = 20; week 14-26, n = 15; week 30, n = 5). No significant differences were observed.

Table 2. Hematological parameters of β, β-dimethylacrylalkannin at 90 and 180 days of administration and in the recovery period.

Time	Parameters	Female				Male			
		Control	10 (mg/kg)	40(mg/kg)	160(mg/kg)	Control	10(mg/kg)	40(mg/kg)	160(mg/kg)
D90	WBC (10^9/L)	10.1 ± 2.0	11.5 ± 3.5	14.4 ± 3.8	11.7 ± 2.0	15.4 ± 3.0	17.2 ± 8.5	16.5 ± 3.9	19.9 ± 6.3
	RBC (10^{12}/L)	5.7 ± 0.4	4.7 ± 0.2**	5.2 ± 0.0*	4.6 ± 0.1**	5.3 ± 0.4	5.4 ± 0.2	5.7 ± 0.4	5.2 ± 0.4
	HGB (g/L)	112.8 ± 11.4	97.8 ± 4.9*	106.0 ± 2.4	95.0 ± 3.2*	99.0 ± 7.2	100.6 ± 5.3	105.6 ± 11.1	96.4 ± 6.8
	HCT (%)	36.2 ± 2.5	30.8 ± 1.6**	33.9 ± 0.9	30.4 ± 0.9**	32.4 ± 2.6	33.9 ± 1.7	35.5 ± 3.6	32.0 ± 2.7
	MCV (Fl)	63.9 ± 1.3	65.3 ± 0.7	65.1 ± 1.4	65.5 ± 0.8	60.8 ± 0.8	62.5 ± 2.0	61.8 ± 2.7	61.6 ± 0.8
	MCH (pg)	19.9 ± 0.6	20.7 ± 0.5*	20.4 ± 0.3	20.5 ± 0.7	18.6 ± 0.9	18.6 ± 0.4	18.4 ± 0.9	18.6 ± 0.8
	MPV (FL)	10.0 ± 0.4	9.7 ± 0.3	9.8 ± 0.6	9.7 ± 0.2	9.8 ± 0.3	10.0 ± 0.4	10.3 ± 0.2*	10.2 ± 0.2*
	PDW (10/GSD)	13.3 ± 0.4	13.2 ± 0.2	13.4 ± 0.4	13.0 ± 0.4	13.0 ± 0.4	13.4 ± 0.5	13.7 ± 0.2**	13.5 ± 0.3**
	LYM (%)	62.3 ± 2.5	57.5 ± 9.0	58.9 ± 5.0	57.9 ± 4.8	59.8 ± 2.6	51.4 ± 7.7*	59.5 ± 6.7	61.6 ± 4.6
	GRAN (%)	27.4 ± 2.5	31.0 ± 6.9	30.2 ± 4.0	30.6 ± 3.3	26.6 ± 4.4	34.4 ± 10.3	26.2 ± 6.8	20.4 ± 3.8*
	Reti (‰)	6.6 ± 2.9	5.4 ± 1.7	6.0 ± 1.9	6.0 ± 2.0	5.8 ± 1.5	6.4 ± 2.3	6.2 ± 2.8	7.2 ± 3.9
D180	WBC (10^9/L)	11.6 ± 1.6	13.6 ± 3.0	12.9 ± 3.7	13.2 ± 3.7	18.3 ± 6.4	19.3 ± 9.3	18.6 ± 3.3	20.3 ± 4.6
	RBC (10^{12}/L)	5.9 ± 0.5	5.4 ± 0.3*	5.1 ± 0.4**	5.2 ± 0.5**	5.4 ± 0.3	5.6 ± 0.3	5.7 ± 0.5	5.5 ± 0.3
	HGB (g/L)	115.5 ± 9.1	106.0 ± 5.3*	102.3 ± 7.4**	103.7 ± 9.9*	102.4 ± 7.7	104.7 ± 3.8	107.5 ± 9.8	103.0 ± 6.4
	HCT (%)	38.6 ± 3.2	35.6 ± 2.5*	34.5 ± 2.8**	34.7 ± 3.5*	33.5 ± 2.1	35.4 ± 1.6*	35.6 ± 3.4	34.0 ± 2.0
	MCV (Fl)	65.4 ± 1.1	66.3 ± 0.9	67.1 ± 1.5*	66.3 ± 1.1	61.7 ± 1.2	62.8 ± 1.4	61.9 ± 1.1	62.3 ± 1.0
	MCH (pg)	19.6 ± 0.5	19.7 ± 0.6	19.9 ± 0.4	19.8 ± 0.4	18.9 ± 0.9	18.6 ± 0.4	18.7 ± 0.7	18.9 ± 0.6
	MPV (FL)	9.2 ± 0.3	9.2 ± 0.3	9.3 ± 0.3	9.2 ± 0.4	9.5 ± 0.2	9.5 ± 0.3	9.8 ± 0.7	9.8 ± 0.5
	PDW (10/GSD)	13.1 ± 0.5	13.0 ± 0.3	13.0 ± 0.4	12.6 ± 0.4*	13.0 ± 0.5	12.9 ± 0.4	13.1 ± 0.4	13.3 ± 0.3
	LYM (%)	54.1 ± 3.9	46.5 ± 7.0**	48.6 ± 7.1*	47.7 ± 4.9**	51.6 ± 5.9	52.8 ± 11.5	51.8 ± 6.6	55.3 ± 11.2
	GRAN (%)	34.2 ± 4.2	40.8 ± 8.9*	38.9 ± 5.8	40.4 ± 5.4*	33.7 ± 6.0	31.1 ± 12.2	31.9 ± 7.9	27.9 ± 13.0
	Reti (‰)	7.2 ± 2.4	6.5 ± 3.8	5.7 ± 1.2	6.0 ± 2.6	5.8 ± 2.4	6.9 ± 1.9	6.8 ± 2.8	6.4 ± 1.9
Recovery period	WBC (10^9/L)	16.06 ± 2.01	15.22 ± 2.43	16.72 ± 2.78	14.05 ± 4.85	23.18 ± 8.47	24.48 ± 7.50	31.52 ± 7.33	20.90 ± 4.53
	RBC (10^{12}/L)	6.01 ± 0.50	5.75 ± 0.24	5.75 ± 0.29	5.81 ± 0.57	6.13 ± 0.43	6.26 ± 0.49	6.23 ± 0.61	6.31 ± 0.22
	HGB (g/L)	147.4 ± 13.5	134.4 ± 2.5	138.2 ± 11.8	142.0 ± 11.1	140.0 ± 9.7	141.0 ± 6.4	142.0 ± 13.0	141.6 ± 5.8
	HCT (%)	35.62 ± 2.96	33.52 ± 0.92	33.78 ± 2.15	34.85 ± 3.80	34.40 ± 2.37	34.82 ± 2.00	35.20 ± 3.79	34.60 ± 1.35
	MCV (Fl)	59.3 ± 0.8	58.4 ± 1.1	58.8 ± 0.8	59.9 ± 1.2	56.1 ± 0.8	55.7 ± 1.4	56.4 ± 1.3	54.8 ± 1.5
	MCH (pg)	24.5 ± 0.6	23.4 ± 0.8*	24.0 ± 1.0	24.5 ± 0.8	22.9 ± 0.2	22.6 ± 1.1	22.8 ± 0.6	22.4 ± 0.7
	MPV (FL)	9.84 ± 0.31	9.76 ± 0.36	9.64 ± 0.55	9.93 ± 0.69	10.4 ± 0.4	9.8 ± 0.2*	10.6 ± 0.7	10.2 ± 0.4
	PDW (10/GSD)	13.1 ± 0.2	13.1 ± 0.5	13.0 ± 0.4	13.0 ± 0.4	13.2 ± 0.2	13.0 ± 0.4	13.0 ± 0.7	13.1 ± 0.4
	LYM (%)	49.98 ± 4.74	41.86 ± 8.44	37.34 ± 3.83**	49.25 ± 3.19	43.10 ± 6.72	40.94 ± 1.85	39.18 ± 9.22	44.62 ± 4.94
	GRAN (%)	41.14 ± 2.75	46.38 ± 10.46	51.88 ± 4.36**	40.03 ± 5.98	42.88 ± 7.41	45.90 ± 3.15	48.46 ± 8.44	42.72 ± 4.59
	Reti (‰)	6.4 ± 2.3	5.8 ± 2.4	7.0 ± 2.2	7.0 ± 2.9	5.4 ± 1.8	5.4 ± 2.4	6.8 ± 3.1	4.0 ± 2.2

Values are expressed as the means ± S.D. *$p < 0.05$ and **$p < 0.01$, versus the vehicle group.

appeared at the end of the recovery period. Furthermore, a lower fluctuation range of some changes (MCH, MPV, PDW) in female and male rats during the treatment and recovery periods lacked a dose-effect relationship and were not considered as obvious treatment-related toxicity.

Biochemical and blood electrolytic parameters

In the blood chemistry examination, there was no significant difference related to toxicity in female rats at the end of the 90-day treatment. The TBIL level was higher in the females that received β, β-dimethylacrylalkannin (160 mg/kg), but the differences disappeared at the end of the recovery period. GLU levels were higher in all treated males at the end of the recovery period. Furthermore, there were significant reductions in GPT, GOT, CK, LDH, TG and UA, which were not related to signs of toxicity. Some parameters (TP, ALB, BUN) displayed significant alterations in both female and male rats without a dose-effect relationship (Table 3).

No significant differences in blood electrolytic parameters were observed in all rats of any treatment group (Fig. 4).

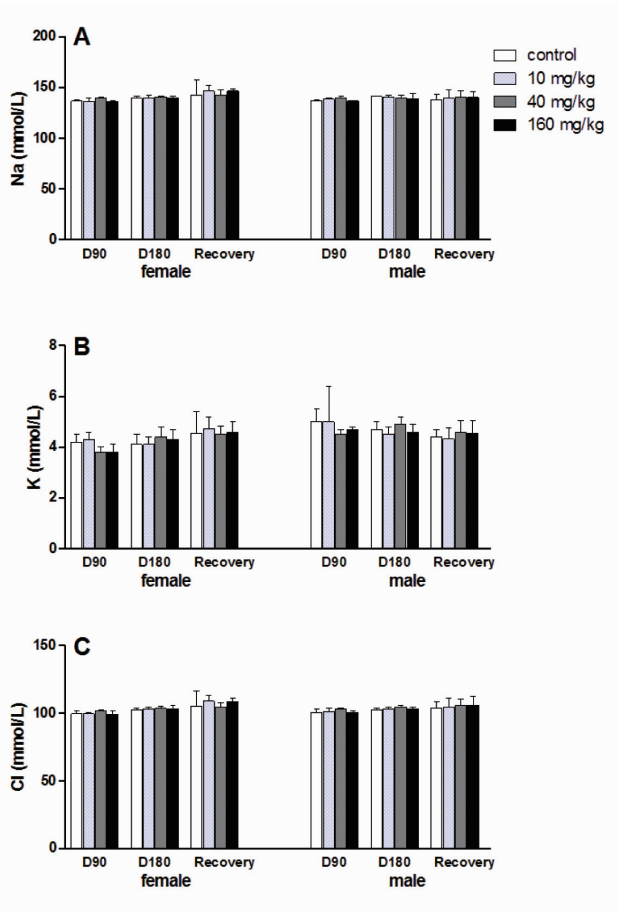

Fig. 4. Effect of β, β-dimethylacrylalkannin on blood electrolytic parameters in rats in the chronic toxicity study. Panel A: Na^+; Panel B: K^+, Panel C: Cl^-. Values are expressed as the means ± S.D. No significant differences were observed.

Coagulation parameters

The blood coagulation parameter values are summarized in Fig. 5. The TT values in female rats (10-40 mg/kg) on D90 and D180 increased significantly (Fig. 5A). On the contrary, the TT values in males were not significantly different, except in the recovery period when the values in males (10-40 mg/kg) increased significantly. However, the differences disappeared at the end of the recovery period (Fig. 5A). Furthermore, the coagulation parameters (PT, APTT, Fib) did not display any significant alterations in any of the treated rats (Fig. 5B, C, D).

Urinalysis

Positive results were obtained in BLO and PRO in the treatment group and control group in both sexes. Males receiving a dose of 40 mg/kg had a positive reaction in GLU on D180. Moreover, positive reactions in BIL, KET, NIH and LEU were observed in males and females receiving β, β-dimethylacrylalkannin (10-160 mg/kg) or the vehicle during the administration and recovery periods. However, these effects were not dose-dependent. Furthermore, no other unique changes were observed (Table 4). Therefore, these effects were not related to β, β-dimethylacrylalkannin administration.

Relative organ weights

The relative organ weights of the rats are shown in Fig. 6. After 90 days of treatment, the heart and brain index of females (40 mg/kg) showed significant differences ($P < 0.05$) compared with the control group, and the thymus index in males (10-160 mg/kg) decreased significantly compared with the control (Fig. 6A). After 180 days of treatment, an increasing lung index compared to the control was observed only in female rats of the 160 mg/kg group, and the liver index in males of the 160 mg/kg groups increased significantly (Fig. 6B). However, these differences disappeared at the end of the recovery period. In addition, the heart and kidney indexes in females of the 10 mg/kg group, the brain index in females of the 40-160 mg/kg group, and the heart index in males of the 160 mg/kg group displayed significant alterations compared with the control group (Fig. 6C).

Table 3. Biochemical parameters of β, β-dimethylacrylalkannin at 90 and 180 days of administration and in the recovery period.

Time	Parameters	Female				Male			
		Control	10 (mg/kg)	40 (mg/kg)	160 (mg/kg)	Control	10 (mg/kg)	40 (mg/kg)	160 (mg/kg)
D90	GPT (U/L)	33.2 ± 3.1	33.0 ± 7.2	26.6 ± 3.2*	35.8 ± 4.4	44.5 ± 5.2	47.0 ± 11.1	41.2 ± 7.9	46.6 ± 16.3
	GOT (U/L)	149.8 ± 16.9	104.6 ± 3.4**	104.4 ± 9.8**	178.8 ± 53.8	158.6 ± 18.8	115.2 ± 7.6**	109.2 ± 11.4**	152.6 ± 26.8
	TP (g/L)	62.4 ± 2.0	62.2 ± 1.1	66.3 ± 2.3*	64.8 ± 3.9	60.8 ± 3.6	65.1 ± 5.0	62.6 ± 1.9	58.8 ± 6.9
	ALB (g/L)	38.3 ± 1.0	38.1 ± 0.5	40.2 ± 1.2*	39.5 ± 1.8	36.4 ± 1.2	38.1 ± 2.7	37.3 ± 0.8	35.4 ± 4.6
	TBIL (μmol/L)	8.90 ± 0.35	9.10 ± 0.69	9.53 ± 1.12	10.18 ± 1.50	8.60 ± 0.58	9.15 ± 0.80	8.86 ± 1.07	9.32 ± 1.12
	ALP (U/L)	44.6 ± 8.6	37.4 ± 9.2	40.8 ± 7.2	43.0 ± 4.7	60.8 ± 3.1	58.2 ± 8.3	64.0 ± 11.9	61.8 ± 14.8
	CK (U/L)	844.6 ± 189.9	440.2 ± 64.4**	453.6 ± 65.7**	629.8 ± 80.3*	767.8 ± 58.2	448.0 ± 184.2	391.4 ± 66.0	777.2 ± 280.0
	LDH (U/L)	1102.0 ± 164.8	628.8 ± 131.5**	680.4 ± 129.7**	984.4 ± 78.9	1150.0 ± 154.1	612.4 ± 185.9**	638.0 ± 103.6**	1035.0 ± 185.8
	GLU (mmol/L)	5.49 ± 0.44	6.10 ± 0.28*	6.38 ± 0.75*	5.98 ± 0.80	5.60 ± 0.89	9.15 ± 4.50	6.49 ± 0.42	6.27 ± 1.44
	BUN (mmol/L)	9.26 ± 0.67	8.59 ± 0.51	8.48 ± 0.72	9.64 ± 0.62	8.75 ± 1.48	7.99 ± 0.87	7.97 ± 0.56	8.56 ± 1.53
	UA (μmol/L)	69.1 ± 18.9	43.5 ± 5.6*	57.0 ± 13.9	55.5 ± 8.8	56.6 ± 26.1	93.1 ± 130.0	33.2 ± 4.9	51.5 ± 14.0
	TG (mmol/L)	1.67 ± 0.32	1.59 ± 0.60	2.52 ± 0.80	1.43 ± 0.66	1.27 ± 0.34	1.27 ± 0.45	1.07 ± 0.35	1.27 ± 0.33
	A/G	1.59 ± 0.03	1.58 ± 0.03	1.53 ± 0.04*	1.56 ± 0.07	1.50 ± 0.11	1.41 ± 0.05	1.47 ± 0.05	1.50 ± 0.08
	CHO (mmol/L)	1.35 ± 0.07	1.41 ± 0.13	1.28 ± 0.05	1.31 ± 0.12	1.80 ± 0.17	1.70 ± 0.12	1.71 ± 0.23	1.57 ± 0.28
D180	GPT (U/L)	35.3 ± 9.3	30.2 ± 4.8	32.6 ± 5.1*	28.1 ± 3.6*	71.6 ± 62.4	45.5 ± 7.6	66.4 ± 56.2	43.0 ± 13.0
	GOT (U/L)	136.2 ± 22.8	113.7 ± 22.2*	115.1 ± 12.2	111.4 ± 15.9*	178.5 ± 44.0	147.9 ± 39.5	168.8 ± 100.0	162.4 ± 27.2
	TP (g/L)	61.7 ± 1.6	62.5 ± 1.5	63.3 ± 3.7	62.9 ± 2.9	61.5 ± 1.9	62.6 ± 1.7	61.9 ± 3.9	62.5 ± 1.7
	ALB (g/L)	38.6 ± 1.1	39.1 ± 1.0	39.2 ± 2.1	39.0 ± 1.3	36.3 ± 1.1	37.5 ± 0.8*	37.0 ± 1.8	37.5 ± 0.8
	TBIL (μmol/L)	9.35 ± 0.59	9.74 ± 0.77	10.18 ± 0.82	10.29 ± 0.82**	7.19 ± 1.95	8.23 ± 1.40	7.91 ± 2.69	8.67 ± 1.65
	ALP (U/L)	25.0 ± 6.4	21.0 ± 3.8	23.6 ± 8.2	21.2 ± 3.7	45.7 ± 6.7	38.0 ± 4.0**	43.1 ± 7.3	38.9 ± 8.1
	CK (U/L)	583.7 ± 146.7	596.0 ± 351.2	518.0 ± 102.6	472.0 ± 102.3	748.5 ± 158.7	674.5 ± 255.6	493.0 ± 155.7**	808.1 ± 239.5
	LDH (U/L)	918.1 ± 149.8	753.0 ± 228.6	826.8 ± 134.4	786.4 ± 128.6	1231.0 ± 205.9	1050.1 ± 329.7	858.9 ± 188.0**	1300.3 ± 319.3
	GLU (mmol/L)	4.88 ± 1.00	5.47 ± 0.83	5.00 ± 0.65	5.03 ± 0.80	5.79 ± 1.39	7.65 ± 1.55*	9.28 ± 2.39*	6.64 ± 1.00
	BUN (mmol/L)	7.05 ± 1.01	7.08 ± 0.60	7.25 ± 0.68	7.28 ± 0.56	6.43 ± 0.58	6.71 ± 0.97	6.46 ± 0.72	6.77 ± 0.30
	UA (μmol/L)	58.9 ± 16.6	63.2 ± 33.2	50.0 ± 8.5	66.1 ± 32.1	50.5 ± 8.4	84.1 ± 75.4	148.6 ± 116.5	69.6 ± 13.6**
	TG (mmol/L)	1.52 ± 0.39	1.26 ± 0.43	1.48 ± 0.74	1.09 ± 0.48*	1.11 ± 0.43	1.62 ± 0.45*	1.46 ± 0.39	1.30 ± 0.65
	A/G	1.66 ± 0.05	1.66 ± 0.03	1.63 ± 0.07	1.63 ± 0.10	1.44 ± 0.07	1.49 ± 0.04	1.48 ± 0.07	1.50 ± 0.07
	CHO (mmol/L)	1.33 ± 0.13	1.23 ± 0.23	1.29 ± 0.13	1.30 ± 0.13	1.71 ± 0.21	1.75 ± 0.13	1.69 ± 0.23	1.76 ± 0.24
Recovery period	GPT (U/L)	38.2 ± 7.9	33.6 ± 4.0	33.8 ± 3.5	40.5 ± 9.0	57.6 ± 11.5	48.6 ± 2.9	55.8 ± 4.0	44.0 ± 3.0*
	GOT (U/L)	141.2 ± 27.2	108.6 ± 15.5*	117.2 ± 8.9	149.8 ± 25.6	205.6 ± 10.5	135.6 ± 17.8**	183.2 ± 20.7	160.0 ± 25.4**
	TP (g/L)	63.5 ± 1.7	63.5 ± 2.2	55.4 ± 1.5**	63.8 ± 1.7	61.5 ± 3.3	59.5 ± 2.0	61.8 ± 1.0	62.1 ± 1.5
	ALB (g/L)	39.6 ± 1.0	39.8 ± 1.0	36.8 ± 0.9**	39.7 ± 0.9	37.0 ± 1.2	36.5 ± 1.2	36.9 ± 0.6	37.4 ± 0.7
	TBIL (μmol/L)	7.67 ± 2.35	6.76 ± 2.12	6.07 ± 1.59	6.92 ± 1.44	3.80 ± 1.37	3.09 ± 0.35	3.38 ± 0.57	3.66 ± 1.34
	ALP (U/L)	24.2 ± 4.1	24.8 ± 5.1	24.0 ± 4.8	25.5 ± 4.0	57.6 ± 17.6	48.6 ± 8.3	47.4 ± 4.8	41.8 ± 4.9
	CK (U/L)	762.8 ± 201.2	423.0 ± 106.5*	622.0 ± 104.8	726.3 ± 61.1	1206.2 ± 139.2	620.2 ± 166.7**	713.2 ± 128.4**	880.6 ± 189.1*
	LDH (U/L)	1177.0 ± 258.3	666.8 ± 164.2**	950.6 ± 132.1	1088.8 ± 172.2	1826.2 ± 234.1	1022.0 ± 195.9**	1126.6 ± 156.0**	1332.0 ± 247.5*
	GLU (mmol/L)	4.77 ± 0.97	5.61 ± 0.76	5.37 ± 0.55	4.67 ± 0.51	4.77 ± 0.41	7.23 ± 1.77*	5.85 ± 0.87*	5.77 ± 0.65*
	BUN (mmol/L)	7.00 ± 0.81	7.23 ± 0.75	7.44 ± 0.75	9.46 ± 0.59**	6.63 ± 0.57	7.40 ± 0.51	7.18 ± 0.71	7.22 ± 0.64
	UA (μmol/L)	66.6 ± 14.0	41.7 ± 12.5*	51.0 ± 12.9	59.2 ± 21.4	45.3 ± 22.0	53.3 ± 41.3	32.2 ± 6.2	38.8 ± 5.1
	TG (mmol/L)	3.43 ± 1.58	3.25 ± 1.53	3.07 ± 0.64	2.91 ± 1.16	2.02 ± 0.40	1.96 ± 0.19	2.31 ± 0.41	2.59 ± 0.98
	A/G	1.65 ± 0.03	1.67 ± 0.06	1.70 ± 0.05	1.64 ± 0.03	1.52 ± 0.11	1.58 ± 0.03	1.48 ± 0.03	1.51 ± 0.03
	CHO (mmol/L)	1.37 ± 0.18	1.17 ± 0.10	1.24 ± 0.10	1.25 ± 0.15	1.86 ± 0.26	1.59 ± 0.17	1.92 ± 0.10	1.93 ± 0.11

Values are expressed as the means ± S.D. *$p < 0.05$ and **$p < 0.01$, versus the vehicle group.

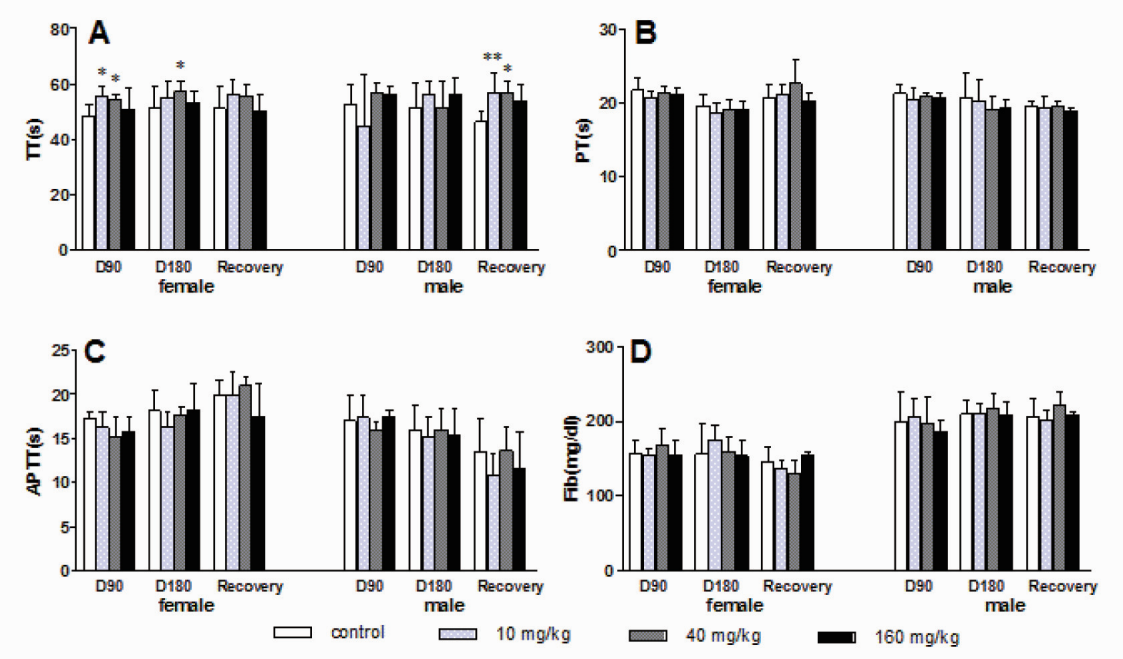

Fig. 5. Effect of β, β-dimethylacrylalkannin on coagulation parameters in rats in the chronic toxicity study. Values are expressed as the means ± S.D. *$p < 0.05$, versus the vehicle group.

Histopathology

No treatment-related macroscopic findings were observed in the treated animals during the necropsy. In the histological examination, changes in the kidneys of the β, β-dimethylacrylalkannin-treated group were dose-related. Brown pigmentation in the renal tubular epithelial cells of the male and female rats in the 40-160 mg/kg and slight pigmentation in rats in the 10 mg/kg group were observed during the administration and recovery periods (Fig. 7, arrow). Consequently, this pigmentation was considered a β, β-dimethylacrylalkannin-related change. Histopathological examinations of the other examined organs did not reveal any treatment-related changes.

DISCUSSION

Studies have indicated that *L. erythrorhizon* has promising therapeutic effects, with β, β-dimethylacrylalkannin as the main chemical component. However, few reports have focused on the effects and safety of β, β-dimethylacrylalkannin. In this study, we systemically evaluated the *in vivo* toxicity of β, β-dimethylacrylalkannin.

The acute toxicity tests indicate that mice administered β, β-dimethylacrylalkannin at a dose of 10 g/kg exhibited toxic effects and mortality. The oral $LD_{50}$of β, β-dimethylacrylalkannin is more than 10 g/kg. In the acute toxicity test in rats, the MTD was more than 10 g/kg, which is an equivalent dosage for rats of approximately 5988 times that used in clinics. Moreover, β, β-dimethylacrylalkannin may be considered practically non-toxic according to Loomis and Hayes (1996).

In the 180-day chronic toxicity test, we used doses that were 6 times (10 mg/kg), 24 times (40 mg/kg) and 96 times (160 mg/kg) higher than the highest dose used in clinical applications. During the administration and recovery periods, no deaths occurred. β, β-dimethylacrylalkannin had no effect on the normal growth of the rats. Combined with the changes observed in the hematological analysis, serum biochemical examinations and urinalysis, we found that, compared with the control group, RBC, HGB, HCT and LYM were reduced, and GRAN was increased in females after administration for 90 and 180 days. The changes were reversible and disappeared during the recovery period. The thymus-body ratios also decreased in males after the 90-day administration. These changes may be related to the effect of β, β-dimethylacrylalkannin on immunologic function (Wang *et al.*, 2015). β, β-dimethylacrylalkannin could suppress the function of activated DCs and the expression and secretion of proinflammatory cytokines in a psoriasis mouse model (Wang

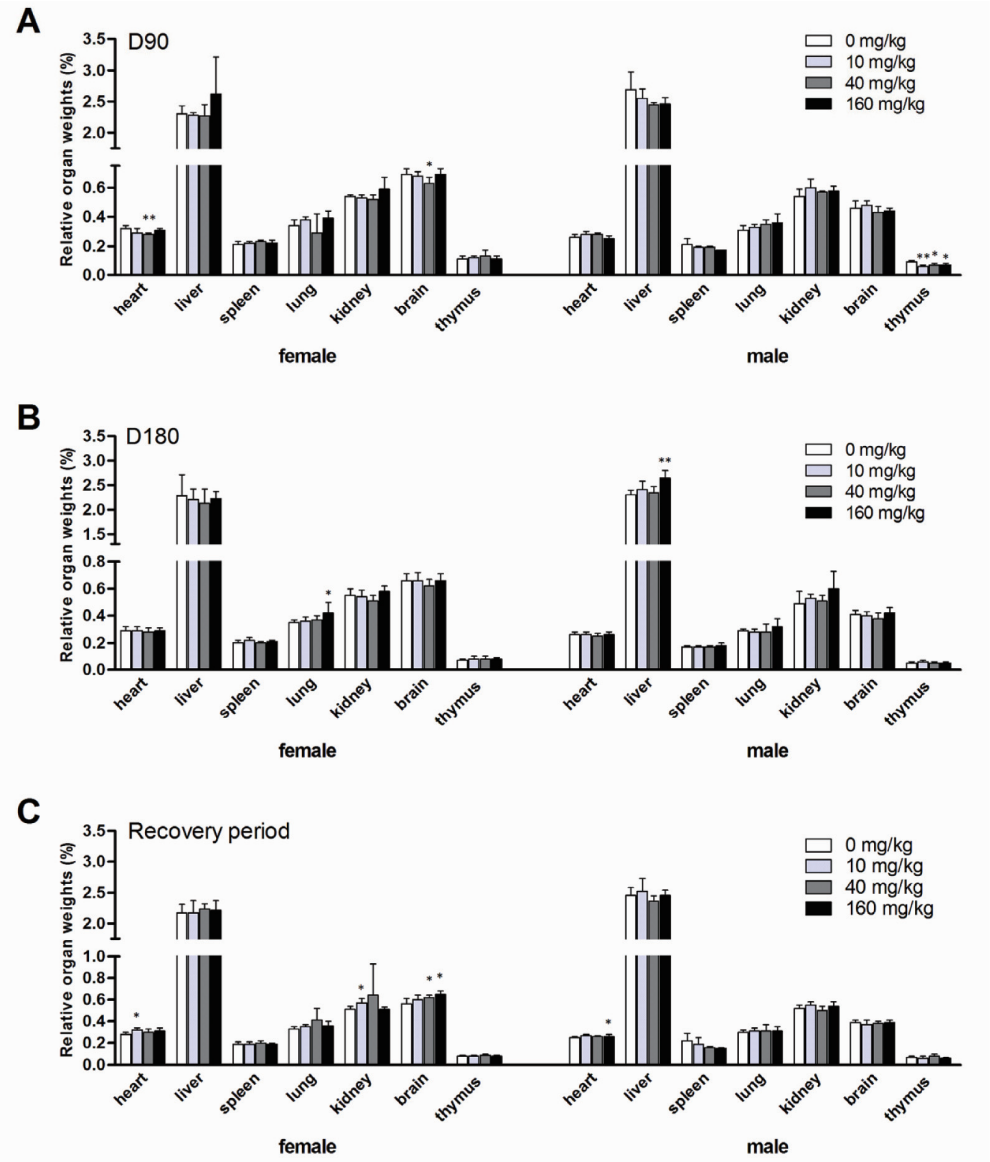

Fig. 6. Effect of β, β-dimethylacrylalkannin on the relative organ weights of rats in the chronic toxicity study. Values are expressed as the means ± S.D. *$p < 0.05$, ** $p < 0.05$ versus the vehicle group.

et al., 2015). Thus, β, β-dimethylacrylalkannin had no effects on the circulatory system, except for a temporary inhibition of the immune system. Moreover, the effect of β, β-dimethylacrylalkannin on TBIL in females (160 mg/kg) and GLU in males (10-160 mg/kg) should be considered in long-term medication.

The absolute and relative weights of the heart, brain and kidney changed significantly compared with the control group. However, such changes were not consistent over time, were dose-related or only appeared at the low dose, and no pathological changes were observed in the histological examination. Therefore, these changes cannot be considered a toxic effect of β, β-dimethylacrylalkannin without any other convincing evidence. Increases in the lung-body ratio in females and the liver-body weight ratio in males were observed in the high-dose group at the end of administration and disappeared during the recovery period. These increases in organ weight and relative organ weight without histologic or clinical pathology alterations are considered an adaptive and non-adverse reaction (Hall

Table 4. Urinalysis parameters of β, β-dimethylacrylalkannin at 90 and 180 days of administration and in the recovery period.

Time	Parameters	Female				Male			
		Control	10 (mg/kg)	40 (mg/kg)	160 (mg/kg)	Control	10 (mg/kg)	40 (mg/kg)	160 (mg/kg)
D90	GLU	-	-	-	-	-	-	-	-
	BIL	-	-	1/5(+)	2/5(+)	-	-	3/5(+)	-
	KET	-	-	2/5(+)	2/5(+)	-	-	1/5(+)	-
	SG	1.005 ± 0.00	1.005 ± 0.00	1.009 ± 0.00	1.007 ± 0.00	1.007 ± 0.00	1.005 ± 0.00	0.822 ± 0.451	1.005 ± 0.00
	BLO	4/5(+)	2/5(+)	4/5(+)	4/5(+)	5/5(+)	3/5(+)	5/5(+)	5/5(+)
	PH	7.5 ± 0.4	6.9 ± 0.4*	7.5 ± 0.9	7.1 ± 0.2	7.1 ± 0.2	6.9 ± 0.2	6.9 ± 0.4	7.0 ± 0.0
	PRO	-	1/5(+)	3/5(+)	2/5(+)	1/5(+)	-	5/5(+)*	-
	URO	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
	NIH	-	-	-	-	-	-	-	-
	LEU	-	1/5(+)	2/5(+)	-	2/5(+)	-	2/5(+)	1/5(+)
D180	GLU	-	-	-	-	-	-	1/8(+)	-
	BIL	-	-	-	-	-	-	-	-
	KET	-	-	-	-	-	-	2/8(+)	-
	SG	1.005 ± 0.00	1.005 ± 0.00	1.006 ± 0.00	1.005 ± 0.00	1.009 ± 0.01	1.006 ± 0.00	1.009 ± 0.00	1.009 ± 0.01
	BLO	5/10(+)	+	9/10(+)	8/9(+)	8/10(+)	10/10(+)	8/8(+)	7/7(+)
	PH	7.1 ± 0.5	6.7 ± 1.0	7.3 ± 0.5	7.4 ± 0.5	7.3 ± 0.4	7.1 ± 0.4	7.1 ± 0.3	7.5 ± 0.5
	PRO	-	1/10(+)	2/10(+)	3/9(+)	4/10(+)	7/10(+)	6/8(+)	4/7(+)
	URO	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
	NIH	-	-	-	2/9(+)	2/10(+)	-	-	-
	LEU	1/10(+)	2/10(+)	4/10(+)	1/9(+)	5/10(+)	3/10(+)	8/8(+)	5/7(+)
Recovery period	GLU	-	-	-	-	-	-	-	-
	BIL	-	-	-	-	-	-	-	-
	KET	-	-	-	1/4(+)	1/5(+)	1/5(+)	3/5(+)	-
	SG	1.006 ± 0.00	1.006 ± 0.00	1.005 ± 0.00	1.005 ± 0.00	1.007 ± 0.00	1.006 ± 0.00	1.007 ± 0.00	1.005 ± 0.00
	BLO	4/5(+)	5/5(+)	5/5(+)	4/4(+)	4/5(+)	4/5(+)	5/5(+)	5/5(+)
	PH	7.3 ± 0.6	7.0 ± 0.4	6.8 ± 0.3	7.3 ± 0.3	7.0 ± 0.4	6.9 ± 0.2	6.7 ± 0.3	6.9 ± 0.2
	PRO	4/5(+)	1/5(+)	-	2/4(+)	5/5(+)	5/5(+)	5/5(+)	5/5(+)
	URO	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
	NIH	-	-	-	-	-	-	-	-
	LEU	4/5(+)	2/5(+)	2/5(+)	-	5/5(+)	5/5(+)	5/5(+)	5/5(+)

Values are expressed as the means ± S.D. *$p < 0.05$ and **$p < 0.01$, versus the vehicle group.

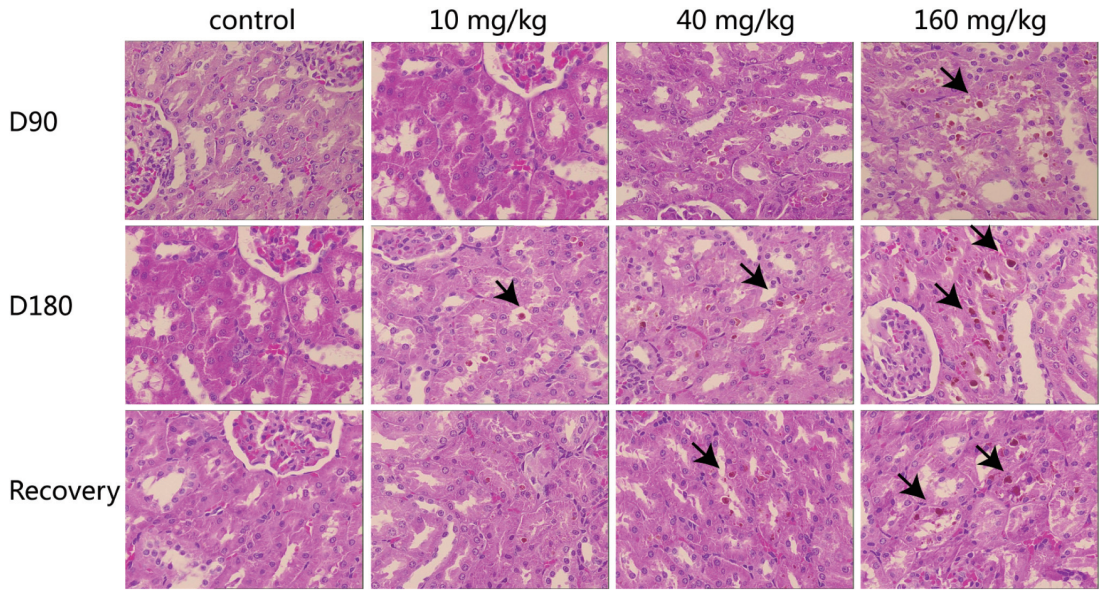

Fig. 7. Histological changes in the kidney after β, β-dimethylacrylalkannin treatment in rats (40 ×, HE). Brown pigmentation appears in the renal tubular epithelial cells (arrow).

et al., 2012).

The dose-related brown pigment in the renal tubule epithelial cells is considered to be caused by β, β-dimethylacrylalkannin. However, no abnormal changes related to the function of the kidneys were observed in the hematological, serum biochemical or urinalysis determinations. In addition, morphological changes were not discovered in the histopathological examination. The pigmentation partially decreased during the recovery period. One study suggests that the pigmentation is possibly associated with hemosiderin, lipofuscin, or BIL (Han *et al*., 2015). However, our research suggests that the pigmentation is possibly associated with drug absorption and that the cells do not readily excrete the pigmentation. Therefore, β, β-dimethylacrylalkannin does not have an effect on kidney function in a short-term prognosis, but the long-term effect should be considered in clinical tests.

In conclusion, the estimated LD_{50} of β, β-dimethylacrylalkannin is more than 10 g/kg body weight in mice, and the MTD is more than 10 g/kg in rats. Administration of β, β-dimethylacrylalkannin by i.g. for 180 days caused no obvious toxicities in organs in rats, except for the brown pigment in the renal tubules. Based on the serum biochemistry and hematology results, the NOAEL of β, β-dimethylacrylalkannin is 10 mg/kg under the present experimental condition in rats.

ACKNOWLEDGMENTS

This work was supported by funds from the National Science and Technology Major Projects for "Major New Drugs Innovation and Development" (no. 2012ZX09301002-001-009; no. 2013ZX09302302).

Conflict of interest---- The authors declare that there is no conflict of interest.

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