



28 Days repeated oral dose toxicity test of aqueous extracts of mahwangyounpae-tang, a polyherbal formula

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ABSTRACT

Mahwangyounpae-tang (MT), consisting of 22 types of herbal extracts has been used for thousands of years in Korean traditional medicine for the oral treatment of respiratory diseases including asthma. As part of a safety evaluation of MT extract for use in asthma, the 28 day repeat oral dose toxicity of an aqueous MT extract was evaluated at 800, 400 and 200 mg/kg per day dose levels. The results showed that no significant toxicological changes were observed when 200 and 400 mg/kg per day of MT extract was administered to rats. But when the dose was increased to 800 mg/kg per day, increases of body weights, food consumptions, and heart and kidney weights were observed with hypertrophy of heart and tubular necrosis of kidney. Besides this, no other signs of toxicity were observed. Based on these results, it can be concluded that the no observed adverse effect level of MT extract is 400 mg/kg per day. Therefore, the use of MT is expected to be safe because 30 mg/kg was shown to be pharmacologically effective in mice and the high dose heart and kidney findings are not considered to represent any safety concern for humans.

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1. Introduction

The use of medicinal plants in therapeutics or as dietary supplements goes back beyond recorded history, but has increased substantially in the last decades (Woods, 1999; Castro et al., 2009). The popularity of herbal medicines is connected with their easy access, therapeutic efficacy, relatively low cost, and assumed absence of toxic side effects. Widespread public opinion is that being a natural product, herbal medicines are harmless and free from adverse effects, and it is believed that even if the expected medical effect is not achieved, their consumption is not dangerous. However, the safety of their use has recently been questioned due to the reports of illness and fatalities (Stewart et al., 1999; Ernst, 2002; Veiga-Junior et al., 2005). In addition, various toxic effects of herbal medicines have been reported including hepatotoxicity (Saad et al., 2006), nephrotoxicity (Cosyns, 2003; Colson and De Broe, 2005; Cheng et al., 2006; Debelle et al., 2008). Considering the complexity of herbals in general and their inherent biological

variation, it is now necessary to evaluate their safety, efficacy and quality (Castro et al., 2009).

Mahwangyounpae-tang (MT) is a polyherbal formula consisting of 22 types of herbs – Ephedrae Herba, Cinnamomi Ramulus, Platycodi Radix, Fritillariae Bulbus, Armeniacae Semen, Schizandrae Fructus, Gelatinum, Aurantii Immaturi Pericarpium, Ponciri Fructus, Amomi Semen, Glycyrrhiza Radix, Crataegi Fructus, Hordei Fructus Germinatus, Massa Medicata Fermentata, Perillae Semen, Raphani Semen, Perillae Herba, Asteris Radix, Magnoliae Cortex, Mori Cortex Radix, Sinapis Semen and Plantaginis Semen, and has been used for thousands of years in Korean traditional medicine for the oral treatment of respiratory diseases including asthma. 30 mg/kg of aqueous extract of MT have been showed inhibitory effects on the ovalbumin-induced asthma in mice; it showed favorable anti-inflammatory effects and relative well preserved the lung and trachea architectures (Kim et al., 2003). MT extract have been shown *in vitro* anti-bacterial effects and against respiration infections (Liu et al., 2005) and it enhanced the anti-bacterial effects of ciprofloxacin (Kim, 2005) and rifloxacin (Seo et al., 2004) in respiratory infected mice. Therefore, MT holds potential as a natural method to control respiratory infections and asthma. As an alternative therapies incorporated into chemical anti-bacterial drugs, MT may provide a convenient way to help manage their asthma and respiratory infections. In addition, we already reported

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that MT extract has not any potential genotoxicity at the standard battery of tests (Seo et al., 2008).

As part of a safety evaluation of MT for use in respiratory diseases, the 28 day repeat oral dose toxicity of an aqueous MT extract was evaluated based on the Korea food and drug administration, 2005 (KFDA) guidelines (2005–60, 2005) at 800, 400 and 200 mg/kg (of body weight) per day dose levels. Because mortalities were recorded after single oral treatment of MT extract 1000 mg/kg in rats at single oral dose toxicity test (Jeon et al., 2006), 800 mg/kg was selected as the highest dosages in this study, and 400 and 200 mg/kg were selected as middle and lower dosages, respectively.

2. Materials and methods

2.1. Animals and husbandry

Each of 40 female and male SD rats (6-wk old upon receipt, SLC, Japan) was used after acclimatization for 7 days. Animals were allocated five per polycarbonate cage in a temperature (20–25 °C) and humidity (45–50%) controlled room. Light:dark cycle was 12 h:12 h, and feed (Samyang, Korea) and water were supplied free to access. All animals were overnight fasted (about 18 h) before first treatment and terminal necropsy. The body weight range of female and male rats on initiation of dosing are 86–108 and 117–155 g, respectively. About 10 male and 10 female rats in each dosing group were used; total four dosing levels including vehicle control were used in this study. Animals were marked by picric acid. All animals were treated according to the Guide for the Care and Use of Laboratory Animals by Institute of Laboratory Animal Resources, Commission on Life Science, National Research Council, USA on 1996, Washington D.C.

2.2. Preparation and administration of MT extract

Individual 22 type's herbs consisting of MT were purchased from Cho-Heung Pharmaceutical Ind. Co. (Daegu, Korea) after confirming the morphology under microscopy. The composition of MT was listed in Table 1. The prepared 22 types of herbs (1020 g) was boiled in 4 L of distilled water for 2 h and filtrated. The filtrate was decompressed using a rotary vacuum evaporator (Lab. Camp, Korea) and lyophilized in a programmable freeze dryer (Ilshin Lab., Korea). Total acquired MT extract was 30.5 g (yield 2.99%). Powders of the MT extract were stored in a desiccator to protect against light and moisture.

MT extract was well dissolved at least 160 mg/mL concentrations in distilled water. The test article was orally administered at a dosage volume of 5 mL/kg using distilled water as vehicle at 800, 400 and 200 mg/kg dose levels, once a day for 28 days.

Table 1

Composition of Mawhangyounpae-tang used in this study.

Herbs	Amounts (g)
Ephedrae Herba	80
Cinnamomi ramulus	80
Platycodi radix	60
Fritillariae bulbus	60
Armeniacae semen	60
Schizandrae fructus	40
Gelatinum	40
Aurantii immaturi pericarpium	40
Ponciri fructus	40
Amomi semen	40
Glycyrrhiza radix	40
Crataegi fructus	40
Hordei fructus germinatus	40
Massa medicata fermentata	40
Perillae semen	40
Raphani semen	40
Perillae herba	40
Asteris radix	40
Magnoliae cortex	40
Mori cotex radicis	40
Sinapis semen	40
Plantaginis semen	40
Total (22 types)	1020

2.3. Abnormal behavior, clinical sign and body weights

All abnormal clinical signs and behaviors were recorded before and after dosing at least twice a day based on the functional observational battery test (Irwin, 1968; Dourish, 1987). Body weights were measured on the day of the administration (Day 0) prior to treatment, 1, 7, 14, 21, 27 and 28 days after start of administration. The day of first treatment, Day 0, means immediately before first treatment in the present study.

2.4. Food consumption measurement

Food consumption was measured per cage at the initiation of treatment and once a week thereafter. Measured amounts of food and water were supplied to each cage once a week and their remnants were measured on the next day to calculate the difference which was regarded as the daily food consumption (g/rat/day).

2.5. Ophthalmological examinations

External eye examination of all males and females per group was conducted shortly before the initiation of treatment and the scheduled termination, respectively. The ocular fundus of all males and females per group was examined shortly before scheduled termination using an indirect binocular ophthalmoscope (IO-H, Neitz Instruments Co., Japan).

2.6. Clinical examinations

Blood samples were drawn from posterior vena cava using a syringe with a 23 gauge needle under ether anesthesia. The animals had been 18 h overnight fasted (water was not restricted) prior to necropsy and blood collecting. The blood samples were collected into CBC bottles containing EDTA-2 K (1.8 mg/mL of blood). Red blood cell count (RBC), hemoglobin concentration (HB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), fibrinogen (FB), reticulocyte cell count, white blood cell count (WBC) and white blood cell differential count (neutrophils, lymphocytes, monocytes, eosinophils and basophils) were conducted using automated hematology cell counter (MS9–5 V; Melet Schloesing Lab., France). In addition, prothrombin time (PT) was measured using a blood clotting analyzer (ACL300 plus, Instrumentation Laboratory, Italy) treated with 3.2% sodium citrate.

To get the sera for blood chemistry, blood samples on a separation tube were centrifuged at 3000 rpm for 10 min on the day of necropsy. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine phosphokinase (CPK), glucose (GLU), total protein (TP), albumin (ALB), albumin/globulin ratio (A/G ratio), blood urea nitrogen (BUN), creatinine (CRE), triglyceride (TG), phospholipid (PL), total cholesterol (TC), total bilirubin (TB), calcium (Ca), inorganic phosphorous (P) levels were conducted using autoanalyzer (Hemagen Analyst, Hemagen Diagnostic, USA; SP-4410, Spotochem, Japan). In addition, chloride (Cl), sodium (Na) and potassium (P) were measured using an ion autoanalyzer (Dri-Chem 800, Fuji, Japan).

2.7. Necropsy and organ weight measurements

All animals were subjected to terminal necropsy at the end of experiment at Day 28 after overnight fasting (about 18 h, water was not restricted). Animals were euthanized by carbon dioxide and gross necropsy was performed. The absolute organ weights of lung, liver, heart, kidney, spleen, testis/ovary, brain and thymus was measured, and then relative organ weight (% for body weight) was calculated.

2.8. Histopathology

Samples from lung, liver, heart, kidney, spleen, pancreas, adrenal gland, salivary gland, lymph node, femoral muscle, sciatic nerve, and skin, prostate were fixed in 10% neutral buffered formalin. Testis/ovary, epididymis, brain, thymus, stomach, duodenum, jejunum, ileum, cecum, colon and rectum were fixed in Bouin solution, and eye was fixed in Davidson solution. After 18 h of fixation, paraffin embedding was conducted and 4 µm sections were prepared by routine histological methods. Representative sections of each specified organs were stained with hematoxylin-eosin for light microscopical examination and body weight was calculated.

2.9. Statistical analyses

Multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test. If the Levene test indicated no significant deviations from variance homogeneity, the obtain data were analyzed by one way ANOVA test followed by Scheffe test to determine which pairs of group comparison were significantly different. In case of significant deviations from variance homogeneity were observed at Levene test, a non-parametric comparison test, the Mann–Whitney U–Wilcoxon Rank Sum W test was conducted to determine the specific pairs of group comparison, which are significantly different. Statistical

analyses were conducted using SPSS for Windows (Release 12.0 K, SPSS Inc., USA) and a p -value of less than 0.05 was considered to be a significant difference. In addition, degree of clinical signs, gross and histopathological findings were subdivided into 3 degrees: 3 + Severe, 2 + moderate, 1 + slight.

3. Results

3.1. Mortalities

No MT extract-treatment related mortalities were recorded in all three different dosages levels tested; all of animals in all MT extract treated female and male rats were survived until 24 h after the end of 28th treatment and all rats subjected to the terminal necropsy.

3.2. Abnormal clinical signs

No MT extract-treatment related clinical signs were detected in the present study regardless of genders except for slight (1+) soft stools detected in 800 mg/kg per day treated female and male rats and sporadically detected salivation and hair loss throughout all experimental groups tested in the present including both gender of vehicle control (Table 2).

Table 2

Clinical signs in rats treated orally with MT extract for 28 days.

Group	Hair loss	Soft stool	Salivation
Male			
Vehicle control	2/10	0/10	3/10
800 mg/kg	2/10	5/10	5/10
400 mg/kg	2/10	0/10	1/10
200 mg/kg	1/10	0/10	1/10
Female			
Vehicle control	2/10	0/10	2/10
800 mg/kg	2/10	3/10	2/10
400 mg/kg	1/10	0/10	0/10
200 mg/kg	2/10	0/10	1/10

Values are expressed as observed animals/total observed animals.

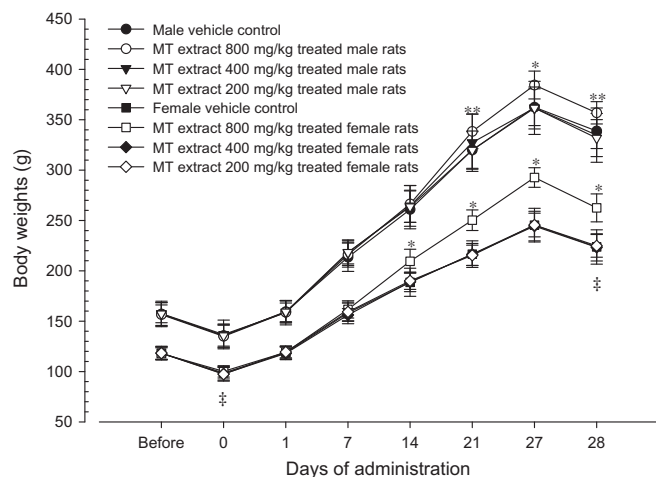


Fig. 1. Body weight changes in rats during 28 days repeat oral administration of MT extract. No meaningful changes on body weight were detected as compared with vehicle control in all dose levels tested except for significant ($*P < 0.01$ or $**P < 0.05$) increases of body weights detected from 14th to 21st treatment days in MT extract 800 mg/kg treated female and male rats, respectively. †, all rats were overnight fasted; Values are expressed as mean $\pm$ SD of ten rats; before means 1 day before treatment day; 0 means just before first treatment; 28 means at sacrifice day, 24 h after end of 14 treatments.

3.3. Changes of body weights

No meaningful changes on body weight and gains were detected as compared with vehicle control in all dose levels tested regardless of animal genders except for significant ($P < 0.01$ or $P < 0.05$) increases of body weights detected from 21st treatment days in MT extract 800 mg/kg per day treated male rats and from 14th treatment in MT extract 800 mg/kg per day treated female rats as compared with equal genders of vehicle control. As a consequence, mean body weight of 800 mg/kg per day treated female and male rats at termination was 17.34% (females) and 5.31% (males) higher than control (Figs. 1 and 2).

3.4. Changes of food consumptions

No meaningful changes on the food consumptions were detected as compared with vehicle control in all dose levels tested

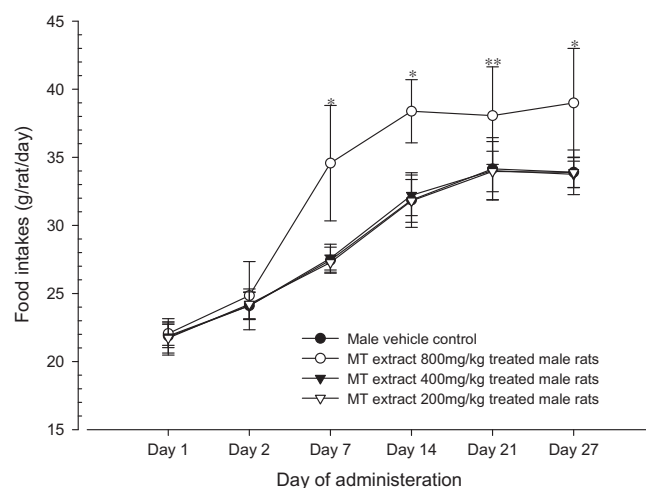


Fig. 2. Food intakes in male rats during 28 days repeat oral administration of MT extract. No meaningful changes on food intakes were detected as compared with vehicle control in all dose levels tested except for significant ($*P < 0.01$ or $**P < 0.05$) increases of food intakes from 7th treatment days in MT extract 800 mg/kg treated male rats. Values are expressed as mean $\pm$ SD of 10 rats.

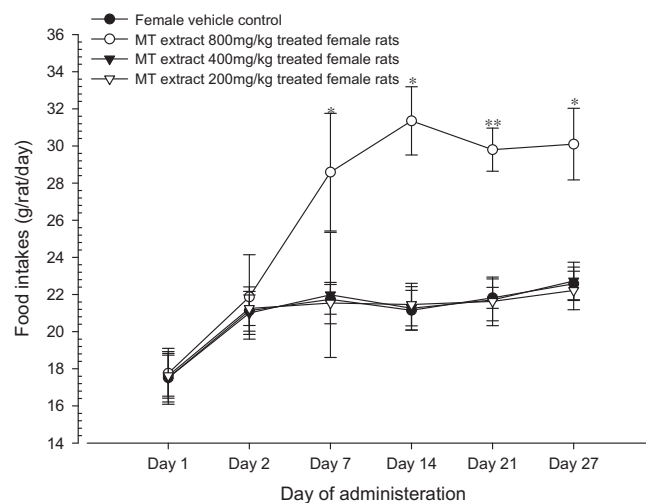


Fig. 3. Food intakes in female rats during 28 days repeat oral administration of MT extract. No meaningful changes on food intakes were detected as compared with vehicle control in all dose levels tested except for significant ($*P < 0.01$) increases of food intakes from 7th treatment days in MT extract 800 mg/kg treated female rats. Values are expressed as mean $\pm$ SD of 10 rats.

regardless of animal genders except for significant ($P < 0.01$ or $P < 0.05$) increases in MT extract 800 mg/kg per day treated female and male rats from 7th treatment days as compared with equal genders of vehicle control, respectively. As a consequence, mean food consumption of 800 mg/kg per day treated female and male rats at 1 day before termination was 33.30% (females) and 15.01% (males) higher than control (Fig. 3).

3.5. Ophthalmological examinations

No meaningful changes on the ophthalmological examinations were detected as compared with vehicle control in all dose levels tested regardless of animal genders in the present study.

3.6. Changes on the clinical examinations

No meaningful changes on the 17 hematological and 18 serum biochemical items were observed in all MT extract treated rats as compared with each gender of vehicle controls in the present study, respectively (Tables 3 and 4).

Table 3

Hematological parameters in rats treated orally with MT extract for 28 days.

Parameters	Male (mg/kg)				Female (mg/kg)			
	0	800	400	200	0	800	400	200
WBC ($\times 10^3/\mu\text{L}$)	10.43 $\pm$ 2.20	10.49 $\pm$ 2.08	10.92 $\pm$ 2.30	10.53 $\pm$ 1.09	7.46 $\pm$ 1.36	7.30 $\pm$ 1.54	7.53 $\pm$ 1.13	7.65 $\pm$ 1.47
RBC ($\times 10^6/\mu\text{L}$)	7.81 $\pm$ 1.33	8.16 $\pm$ 1.93	7.72 $\pm$ 1.12	7.72 $\pm$ 1.09	7.34 $\pm$ 0.78	7.32 $\pm$ 1.17	7.39 $\pm$ 0.94	7.26 $\pm$ 0.69
HGB (g/dl)	14.83 $\pm$ 1.88	14.57 $\pm$ 1.77	14.53 $\pm$ 2.23	14.41 $\pm$ 1.79	14.42 $\pm$ 0.45	14.26 $\pm$ 0.82	14.46 $\pm$ 0.57	14.48 $\pm$ 0.71
HCT (%)	46.71 $\pm$ 3.55	47.66 $\pm$ 4.00	45.92 $\pm$ 4.00	45.84 $\pm$ 2.94	45.26 $\pm$ 2.30	45.28 $\pm$ 2.87	44.18 $\pm$ 2.27	44.52 $\pm$ 2.75
MCV (fl)	61.89 $\pm$ 3.23	61.35 $\pm$ 2.42	62.11 $\pm$ 1.89	61.47 $\pm$ 2.16	61.69 $\pm$ 3.15	61.72 $\pm$ 2.15	61.82 $\pm$ 1.93	61.86 $\pm$ 2.37
MCH (pg)	19.38 $\pm$ 0.35	19.41 $\pm$ 0.71	19.27 $\pm$ 0.54	19.28 $\pm$ 0.55	19.91 $\pm$ 0.71	19.86 $\pm$ 0.61	19.70 $\pm$ 0.65	19.85 $\pm$ 0.98
MCHC (g/dl)	31.59 $\pm$ 0.76	31.78 $\pm$ 0.73	31.40 $\pm$ 0.75	31.63 $\pm$ 0.90	31.92 $\pm$ 1.36	31.94 $\pm$ 1.12	31.86 $\pm$ 1.04	31.92 $\pm$ 0.87
PLT ($\times 10^3/\mu\text{L}$)	13.27 $\pm$ 0.61	13.29 $\pm$ 0.38	13.24 $\pm$ 0.79	13.20 $\pm$ 0.79	12.68 $\pm$ 0.51	12.63 $\pm$ 0.54	12.68 $\pm$ 0.67	12.64 $\pm$ 0.55
Neutrophil (%)	8.79 $\pm$ 1.82	8.67 $\pm$ 0.75	8.61 $\pm$ 1.08	8.74 $\pm$ 0.81	7.10 $\pm$ 1.47	6.84 $\pm$ 0.47	7.01 $\pm$ 1.10	7.20 $\pm$ 1.41
Lymphocyte (%)	86.26 $\pm$ 1.91	86.44 $\pm$ 0.93	86.45 $\pm$ 1.11	86.23 $\pm$ 1.39	89.12 $\pm$ 1.81	89.40 $\pm$ 1.03	89.12 $\pm$ 1.43	88.97 $\pm$ 1.51
Monocyte (%)	2.09 $\pm$ 0.63	2.04 $\pm$ 0.53	1.91 $\pm$ 0.68	2.04 $\pm$ 0.75	1.37 $\pm$ 0.37	1.42 $\pm$ 0.55	1.35 $\pm$ 0.37	1.36 $\pm$ 0.48
Eosinophil (%)	0.94 $\pm$ 0.56	0.91 $\pm$ 0.49	0.96 $\pm$ 0.63	0.93 $\pm$ 0.27	0.87 $\pm$ 0.38	0.79 $\pm$ 0.34	0.93 $\pm$ 0.12	0.89 $\pm$ 0.17
Basophil (%)	1.12 $\pm$ 0.59	1.10 $\pm$ 0.48	1.22 $\pm$ 0.36	1.20 $\pm$ 0.32	1.09 $\pm$ 0.19	1.10 $\pm$ 0.23	1.12 $\pm$ 0.34	1.13 $\pm$ 0.22
Others (%)	0.80 $\pm$ 0.19	0.84 $\pm$ 0.20	0.85 $\pm$ 0.16	0.86 $\pm$ 0.20	0.45 $\pm$ 0.26	0.45 $\pm$ 0.38	0.47 $\pm$ 0.36	0.45 $\pm$ 0.26
Reticulocyte (%)	3.13 $\pm$ 0.65	3.06 $\pm$ 0.28	3.09 $\pm$ 0.37	3.26 $\pm$ 0.52	2.58 $\pm$ 0.64	2.57 $\pm$ 0.68	2.57 $\pm$ 0.60	2.59 $\pm$ 0.53
PT (sec)	13.68 $\pm$ 0.68	13.75 $\pm$ 0.97	13.66 $\pm$ 0.39	13.75 $\pm$ 0.46	14.46 $\pm$ 0.42	14.37 $\pm$ 0.39	14.48 $\pm$ 0.38	14.44 $\pm$ 0.49
FB (g/dl)	0.32 $\pm$ 0.04	0.32 $\pm$ 0.01	0.32 $\pm$ 0.03	0.32 $\pm$ 0.01	0.26 $\pm$ 0.03	0.26 $\pm$ 0.04	0.26 $\pm$ 0.05	0.26 $\pm$ 0.03

Values are expressed as mean $\pm$ SD of ten rats.

Table 4

Serum biochemical parameters in rats treated orally with MT extract for 28 days.

Parameters	Male (mg/kg)				Female (mg/kg)			
	0	800	400	200	0	800	400	200
AST (IU/L)	121.02 $\pm$ 18.74	121.25 $\pm$ 19.15	116.63 $\pm$ 13.75	119.25 $\pm$ 16.07	120.23 $\pm$ 15.97	121.79 $\pm$ 15.15	119.19 $\pm$ 20.48	121.95 $\pm$ 22.15
ALT (IU/L)	33.37 $\pm$ 6.83	35.65 $\pm$ 5.32	32.63 $\pm$ 4.77	34.66 $\pm$ 5.18	33.09 $\pm$ 8.00	33.59 $\pm$ 11.56	33.68 $\pm$ 8.53	34.37 $\pm$ 8.38
ALP (IU/L)	874.01 $\pm$ 184.84	878.60 $\pm$ 174.94	893.40 $\pm$ 186.75	876.00 $\pm$ 144.68	527.50 $\pm$ 150.39	523.50 $\pm$ 112.27	521.20 $\pm$ 95.55	529.30 $\pm$ 60.49
BUN (mg/dl)	15.86 $\pm$ 2.77	15.94 $\pm$ 2.21	15.49 $\pm$ 2.18	15.96 $\pm$ 3.42	17.39 $\pm$ 2.43	16.98 $\pm$ 2.75	17.59 $\pm$ 2.33	17.32 $\pm$ 1.07
CRE (mg/dl)	0.44 $\pm$ 0.07	0.43 $\pm$ 0.08	0.43 $\pm$ 0.07	0.44 $\pm$ 0.06	0.48 $\pm$ 0.02	0.47 $\pm$ 0.04	0.47 $\pm$ 0.06	0.49 $\pm$ 0.07
GLU (mg/dl)	134.00 $\pm$ 24.95	130.91 $\pm$ 25.81	128.90 $\pm$ 14.32	130.40 $\pm$ 22.94	118.30 $\pm$ 9.44	119.30 $\pm$ 30.74	117.40 $\pm$ 6.90	117.10 $\pm$ 11.13
TC (mg/dl)	64.71 $\pm$ 12.01	65.36 $\pm$ 12.86	63.08 $\pm$ 10.71	63.58 $\pm$ 7.45	67.97 $\pm$ 12.77	66.84 $\pm$ 7.58	65.83 $\pm$ 9.29	71.50 $\pm$ 13.16
A/G ratio	2.57 $\pm$ 0.25	2.75 $\pm$ 1.15	2.64 $\pm$ 0.93	2.84 $\pm$ 1.16	3.12 $\pm$ 1.10	3.06 $\pm$ 0.64	3.08 $\pm$ 1.26	3.59 $\pm$ 2.18
TP (g/dl)	5.90 $\pm$ 0.15	5.89 $\pm$ 0.22	5.94 $\pm$ 0.21	5.93 $\pm$ 0.23	5.90 $\pm$ 0.22	5.80 $\pm$ 0.29	5.92 $\pm$ 0.27	5.89 $\pm$ 0.26
ALB (g/dl)	4.24 $\pm$ 0.11	4.23 $\pm$ 0.24	4.23 $\pm$ 0.34	4.29 $\pm$ 0.39	4.37 $\pm$ 0.34	4.34 $\pm$ 0.26	4.36 $\pm$ 0.30	4.37 $\pm$ 0.58
CPK (IU/L)	847.30 $\pm$ 147.22	851.80 $\pm$ 195.40	854.20 $\pm$ 195.48	838.60 $\pm$ 144.87	799.30 $\pm$ 137.33	761.90 $\pm$ 197.30	815.80 $\pm$ 167.37	791.40 $\pm$ 182.65
TG (mg/dl)	36.63 $\pm$ 14.40	37.44 $\pm$ 10.88	35.35 $\pm$ 15.09	36.84 $\pm$ 11.95	15.38 $\pm$ 3.10	16.12 $\pm$ 2.09	15.92 $\pm$ 1.85	15.29 $\pm$ 3.16
Ca (mg/dl)	10.01 $\pm$ 0.78	9.90 $\pm$ 0.65	10.03 $\pm$ 0.52	10.05 $\pm$ 0.76	9.35 $\pm$ 0.23	9.30 $\pm$ 0.32	9.32 $\pm$ 0.33	9.31 $\pm$ 0.44
P (mg/dl)	9.57 $\pm$ 0.97	9.50 $\pm$ 0.95	9.58 $\pm$ 1.19	9.36 $\pm$ 0.87	7.80 $\pm$ 0.48	7.84 $\pm$ 0.28	7.89 $\pm$ 0.29	7.82 $\pm$ 0.34
PL (mg/dl)	88.30 $\pm$ 8.07	89.40 $\pm$ 9.13	88.00 $\pm$ 8.35	87.30 $\pm$ 11.61	99.80 $\pm$ 7.96	98.30 $\pm$ 8.39	96.96 $\pm$ 7.48	96.81 $\pm$ 5.77
Na (mmol/L)	141.50 $\pm$ 1.35	142.90 $\pm$ 5.43	141.90 $\pm$ 5.17	141.40 $\pm$ 1.78	142.90 $\pm$ 3.00	141.60 $\pm$ 2.37	140.60 $\pm$ 3.92	142.80 $\pm$ 4.32
K (mmol/L)	5.13 $\pm$ 0.59	5.17 $\pm$ 0.96	5.19 $\pm$ 0.97	5.11 $\pm$ 0.95	4.41 $\pm$ 0.68	4.56 $\pm$ 0.37	4.43 $\pm$ 0.48	4.45 $\pm$ 0.41
Cl (mmol/L)	102.10 $\pm$ 2.64	100.70 $\pm$ 5.12	101.20 $\pm$ 1.81	101.30 $\pm$ 2.50	103.30 $\pm$ 7.96	102.00 $\pm$ 6.73	104.50 $\pm$ 3.72	104.60 $\pm$ 6.62

Values are expressed as mean $\pm$ SD of ten rats.

3.7. Necropsy findings

No MT extract-treatment related changes on the gross findings were observed as compared with equal gender of vehicle control except for 800 mg/kg per day treated female and male rats. Enlargement of heart was detected in 800 mg/kg per day treated 3/10 male and female rats. Although high dose female showed quite similar frequency of pale appearance of kidney, but it was more numerous encountered in high dose male (3/10) as compared with male vehicle control (0/10) (Table 5).

3.8. Changes on the organ weights

No meaningful changes on the organ weights were observed in all MT extract treated rats as compared with each gender of vehicle controls except for 800 mg/kg per day treated female and male rats. Significantly increases of heart weights were detected in 800 mg/kg per day treated female and male rats as compared with equal genders of vehicle control, respectively and significant increases of absolute kidney weight was detected in 800 mg/kg per

Table 5

Necropsy findings in rats treated orally with MT extract for 28 days.

Findings (degrees)	Male (mg/kg)				Female (mg/kg)			
	0	800	400	200	0	800	400	200
Heart enlargement 1+	0/10	3/10	0/10	0/10	0/10	2/10	0/10	0/10
Enlargement 2+	0/10	0/10	0/10	0/10	0/10	1/10	0/10	0/10
Kidney pale appearance 1+	0/10	3/10	0/10	0/10	1/10	1/10	1/10	0/10

Values are expressed as observed animals/total observed animals.

Degrees = 1+, slight; 2+, moderate; 3+, severe.

Table 6

Absolute organ weights in rats treated orally with MT extract for 28 days.

	Male (mg/kg)				Female (mg/kg)			
	0	800	400	200	0	800	400	200
Final body weight	338.90 ± 17.38	356.90 ± 11.07	334.80 ± 26.98	331.80 ± 18.32	223.70 ± 17.01	262.50 ± 13.88	223.30 ± 13.46	224.80 ± 11.30
<i>Organs</i>								
Lung	1.408 ± 0.118	1.473 ± 0.083	1.419 ± 0.180	1.432 ± 0.195	1.105 ± 0.079	1.216 ± 0.138	1.112 ± 0.094	1.101 ± 0.105
Liver	11.106 ± 0.742	11.488 ± 0.903	11.206 ± 0.786	11.207 ± 0.435	6.953 ± 0.821	7.588 ± 0.952	6.971 ± 0.245	6.928 ± 0.625
Heart	1.248 ± 0.089	1.483 ± 0.178 [*]	1.242 ± 0.119	1.249 ± 0.104	0.865 ± 0.074	1.205 ± 0.190 [*]	0.863 ± 0.049	0.866 ± 0.168
Kidney ^a	2.614 ± 0.336	2.702 ± 0.419	2.649 ± 0.329	2.588 ± 0.378	1.691 ± 0.099	1.843 ± 0.185	1.690 ± 0.106	1.694 ± 0.099
Spleen	0.697 ± 0.073	0.685 ± 0.124	0.699 ± 0.117	0.696 ± 0.049	0.526 ± 0.086	0.524 ± 0.113	0.529 ± 0.081	0.528 ± 0.089
Testis/ovary ^a	3.053 ± 0.272	3.112 ± 0.187	3.062 ± 0.240	3.060 ± 0.227	0.089 ± 0.023	0.096 ± 0.022	0.090 ± 0.018	0.091 ± 0.017
Brain	1.991 ± 0.079	2.038 ± 0.057	1.987 ± 0.093	1.989 ± 0.055	1.846 ± 0.129	1.970 ± 0.220	1.843 ± 0.078	1.846 ± 0.077
Thymus	0.651 ± 0.093	0.661 ± 0.083	0.654 ± 0.095	0.653 ± 0.107	0.607 ± 0.119	0.647 ± 0.173	0.604 ± 0.164	0.604 ± 0.155

Values are expressed as mean ± SD of ten rats, g.

^a Sum of bilateral sides of organs.^{*} $P < 0.01$ as compared with equal genders of vehicle control.**Table 7**

Relative organ weights in rats treated orally with MT extract for 28 days.

Organs	Male (mg/kg)				Female (mg/kg)			
	0	800	400	200	0	800	400	200
Lung	0.417 ± 0.047	0.413 ± 0.016	0.426 ± 0.064	0.434 ± 0.071	0.496 ± 0.043	0.465 ± 0.066	0.500 ± 0.053	0.492 ± 0.060
Liver	3.281 ± 0.227	3.221 ± 0.269	3.367 ± 0.352	3.385 ± 0.184	3.127 ± 0.447	2.886 ± 0.276	3.134 ± 0.256	3.087 ± 0.290
Heart	0.369 ± 0.030	0.417 ± 0.057 [*]	0.372 ± 0.041	0.377 ± 0.035	0.389 ± 0.045	0.460 ± 0.070 [*]	0.388 ± 0.032	0.388 ± 0.089
Kidney ^a	0.773 ± 0.108	0.759 ± 0.133	0.798 ± 0.129	0.786 ± 0.147	0.760 ± 0.074	0.703 ± 0.075	0.758 ± 0.053	0.755 ± 0.050
Spleen	0.207 ± 0.030	0.192 ± 0.038	0.210 ± 0.039	0.211 ± 0.022	0.237 ± 0.049	0.201 ± 0.047	0.237 ± 0.035	0.235 ± 0.043
Testis/ovary ^a	0.900 ± 0.054	0.872 ± 0.046	0.919 ± 0.097	0.924 ± 0.075	0.040 ± 0.012	0.037 ± 0.008	0.041 ± 0.010	0.040 ± 0.008
Brain	0.589 ± 0.043	0.572 ± 0.027	0.596 ± 0.050	0.601 ± 0.029	0.833 ± 0.113	0.754 ± 0.105	0.828 ± 0.062	0.824 ± 0.070
Thymus	0.193 ± 0.033	0.185 ± 0.025	0.196 ± 0.027	0.198 ± 0.038	0.274 ± 0.063	0.247 ± 0.070	0.274 ± 0.083	0.270 ± 0.075

Values are expressed as mean ± SD of ten rats, % of body weight.

^a Sum of bilateral sides of organs.^{*} $P < 0.05$ as compared with equal genders of vehicle control.

day treated female rats as compared with female vehicle control. Relative heart weights of 800 mg/kg per day treated female and male rats being up to 18.21% and 12.91% higher, respectively than control (Tables 6 and 7).

Table 8

Microscopical findings in rats treated orally with MT extract for 28 days.

Findings (degrees)	Male (mg/kg)				Female (mg/kg)			
	0	800	400	200	0	800	400	200
<i>Heart</i>								
Hypertrophy 1+	0/10	3/10	0/10	0/10	0/10	3/10	0/10	0/10
Hypertrophy 2+	0/10	0/10	0/10	0/10	0/10	1/10	0/10	0/10
<i>Kidney</i>								
Tubular necrosis 1+	0/10	3/10	0/10	0/10	0/10	2/10	0/10	0/10

Values are expressed as observed animals/total observed animals.

Degrees = 1+, slight; 2+, moderate; 3+, severe.

3.9. Histopathological findings

No MT extract-treatment related changes on the histopathological findings were observed as compared with equal gender of vehicle control except for 800 mg/kg per day treated female and male rats. Tubular necrosis of kidney was detected in 800 mg/kg per day treated 3/10 male and 2/10 female rats, and hypertrophy of heart was detected in 3/10 high dose male and 4/10 high dose female rats, respectively (Table 8).

4. Discussion

The safety of herbal medicine use has recently been questioned due to the reports of illness and fatalities (Stewart et al., 1999; Ernst, 2002; Veiga-Junior et al., 2005), hepatotoxicity (Saad et al., 2006) and nephrotoxicity (Cosyns, 2003; Colson and De Broe, 2005; Cheng et al., 2006; Debelle et al., 2008). Considering the complexity of herbals in general and their inherent biological variation, it is now necessary to evaluate their safety, efficacy and quality

(Castro et al., 2009). In the present study, we investigated the 28 day repeat oral dose toxicity of MT extract, a poly herbal formula, in female and male rats. MT extract was orally administered to female and male rats, once a day for 28 days at dose levels of 800, 400, 200 and 0 mg/kg. The mortality and changes on body weight, food consumptions, clinical signs, ophthalmological examinations, hematology, serum biochemistry and gross observation were monitored according to the KFDA Guidelines (2005–60, 2005) with organ weight and histopathology of principle organs.

Salivation and hair loss signs detected in the present study were considered as not MT extract-treatment related clinical signs because these signs were also observed in the vehicle control too. Although no obvious changes in gastrointestinal tract were observed at gross and histopathological observations, some evidence that high doses of MT extract may induce digestive tract disturbance was noted based on the presence of soft stools at MT extract 800 mg/kg per day treated female and male rats.

Increases of body weights detected in MT extract 800 mg/kg per day treated female and male rats as compared with equal genders of vehicle control well corresponded to the food intake increases are considered as evidences that higher dosage of MT extract than 800 mg/kg per day facilitated the food intakes and induced body weight increases. These changes on the body weight considered as a part of pharmacological effects of herbs not as toxicological signs. Generally, many herbal extracts have been showed body weight increases as pharmacological aspects related to their anti-oxidative effects (Ahlem et al., 2009; Mangathayaru et al., 2009; Pandikumar et al., 2009).

As shown in Tables 3 and 4, no abnormal changes were detected on the hematological and serum biochemical analysis up to the highest dosages of MT extract, 800 mg/kg per day, and test in this study in the both genders of rats as compared with equal genders of vehicle control, respectively. In addition, there no meaningful changes were also demonstrated at the ophthalmological observations in this study.

Increases of heart and kidney weights restrictly detected in 800 mg/kg per day treated female and male rats, were observed as MT extract-treatment related toxicological signs, and they were revealed as cases of hypertrophy of the heart and tubular necrosis of the kidney at histopathological examination. Thus, the primary target organ of MT extract was considered as heart and kidney in the present study. However, these abnormal finding on heart and kidney were restrictly detected to some high dose rats, not all animals, perhaps as a result of individual sensitivity differences or higher inter-animal systemic exposure. No obvious serum biochemistry correlate, especially in BUN and creatinine levels, was seen. Damage to the heart and kidney after treatment of Ephedrae Herba or it's main chemical component, ephedrine have been reported related to the overstimulation of the adrenergic system (Lyon and Turney, 1996; Zahn et al., 1999; Schier et al., 2003) and Ephedrae Herba is one of main component of MT extract. Therefore, the abnormal changes on the heart and kidney detected in the present study may be related to the overstimulation of the adrenergic system by Ephedrae Herba.

In conclusion, the present study showed that when MT extract was administered to rats at the dose of 400 and 200 mg/kg per day, there were no significant toxicological changes. But when the dose was increased to 800 mg/kg per day, the increases of body weights, food consumptions, and heart and kidney weights were observed with hypertrophy of heart and tubular necrosis of kidney were detected as MT extract treatment possibly related signs. Based on these results, it can be concluded that the no observed adverse effect level of MT extract is 400 mg/kg per day. Although treatment of MT extract 800 mg/kg per day also showed heart and kidney toxicities related to the overstimulation of the adrenergic system

like herb ephedra (Lyon and Turney, 1996; Zahn et al., 1999; Schier et al., 2003), the use of MT is expected to be safe and not considered to represent any safety concern for humans because anticipated intake to treat asthma is small with efficacy demonstrated at 30 mg/kg in mice (Kim et al., 2003) and there are no evidences of cardiovascular or nephrotoxicity have been seen from the long herbal use of MT.

Conflict of Interest

The authors declare that there are no conflicts of interest

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