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A Thirteen-week Oral Dose Subchronic Toxicity Study of *Isaria sinclairii* in Rats

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Isaria sinclairii (IS) was orally administered at doses of 0, 0.04, 0.2, and 1 g/kg/day over a 13-week period. There were no observed clinical signs or deaths related to treatment in all the groups tested. Therefore, the approximate lethal oral dose of *I. sinclairii* was considered to be higher than 1 g/kg in rats. Throughout the administration periods, no significant changes in diet consumption, ophthalmologic findings, organ weight, clinical pathology (hematology, clinical chemistry, coagulation, and urinalysis) or gross pathology were detected. Minor changes were found in hematological parameters for the 0.04 g/kg/day and 0.2 g/kg/day IS treated groups (triglyceride reductions of 20.1~46.6% and platelet increases), but all changes were within physiological range. Microscopic examination failed to identify any treatment-related histopathologic changes in the organs of the IS-treated rats other than nuclear enlargement (cellular atypia) of the tubular regions in the medulla of the kidney in the high dose group. From these results, one can conclude that the no-observed effect level (NOAEL) of *I. sinclarii* is less than 0.04 g/kg/day in rats.

Key words: *Isaria sinclairii*, 13- week toxicity.

INTRODUCTION

Medicinal mushrooms, which are recognized as exerting many health benefits, have recently attracted the attention of the pharmaceutical industry for the development of nutraceuticals (Wang *et al.*, 2003). Cicada Dongchunghacho (a powdered form of *Isaria sinclairii* grown on silkworms) was recently introduced in Korea as a crude drug for the treatment of cancer and diabetes (Ahn *et al.*, 2004a). Dongchunghacho contains the fruiting bodies of IS and the larva of its parasitized host (silkworm). FTY720, a semi-synthetic derivative of Myriocin (ISP-1) obtained from the *I. sinclairii* cell supernatant, was found to act as a safe and potent immunomodulator by inducing reductions in peripheral lymphocyte numbers, especially for CD4 positive cells and IL-2R positive cells (Ueda *et al.*, 2005). Moreover, this Dongchunghacho was found to possess selective antihypertensive activity in a spontaneously hypertensive rat (SHR) model (Ahn *et al.*, 2007a), as well as antiobesity activity in Zucker fat/fat rats (Ahn *et al.*, 2007b).

However, safety evaluation data are scare, with just some published toxicology data: including a genotoxicity test of IS extract using the Ames test, a chromosome aberration (CA) test in Chinese hamster ovary (CHO) cells *in vitro,* and a micronucleus (MN) test *in vivo* (Ahn *et al.*, 2004a); also, an acute oral toxicity test in beagle dogs (Ahn *et al.*, 2003) and a sub-acute (13-week) toxicity study of administered *ad libitum* feedings at percentage levels of 0, 1.25, 2.5, 5 and 10 percent (calculated at about 8 g/kg/feed) (Ahn *et al.*, 2004b). However, for the accumulated toxicity evaluation of IS, exact ingested dose reports corresponding to systematically based data and results were necessary.

Hence, the present investigation was undertaken to assess the sub-acute toxicity of IS by orally administering doses of 0, 0.04, 0.2 and 1 g/kg/day over a 13-week period.

MATERIALS AND METHODS

Materials. The dried *Isaria sinclairii* (IS) was collected in Mountain Halla located in Cheju-do, South

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Korea. This fungus endophytically parasitizes dead or living *Cicadae* subspecies. This strain used was isolated from conidiospores and cultured in potato dextrose agar (PDA) medium and then sprayed (inoculated) onto silkworms. By evading host defensive mechanisms either during the penetration of the cuticle or upon reaching the hemocoel, *I. sinclairii* proliferated inside larvae to form fruiting bodies. These were cultivated in the Department of Agricultural Biology, National Institute of Agricultural Science and Technology, Korea.

Test preparation of IS. Dried IS was homogenized in a blender to a powder, stored at 4°C, dissolved in phosphate Buffered saline (Sigma-Aldrich Inc., St. Louis, MO), and then orally administered at doses of 0, 0.04, 0.2 and 1 g/kg/day /day over a 13-week period.

Animals. Specific pathogen free SD rats (4 weeks old, weighing 165 ± 5 g, male and female), purchased from Samtako Co. Ltd. (Osan, Korea), were housed in an environmentally-controlled room with 23 ± 1°C, relative humidity of 55 ± 10%, air ventilation of 10~18 times/hr, a 12-ht light/dark cycle of 150~300 lux, and feed and water available *ad libitum*.

All procedures were conducted in accordance with the Korean Food and Drug Administration (KFDA) *Guidelines for Toxicity Tests of drugs and related Materials* (KFDA, 1999). Rats were kept for one week under normal physical conditions (23 ± 2°C, 55 ± 10%, humidity and a regular day/night cycle, air ventilation of 10~18 times/hr, a 12-ht light/dark cycle of 150~300 lux) and fed with standard diet (Samtako Co. Ltd., Osan, Korea), and water *ad libitum* before repeated-dose toxicity study testing began.

Ten animals of both sexes in each group were weighed, and administered with IS at a dose of 0.04, 0.2 or 1 g/kg/day or its vehicle over a 13-week period.

The parameters examined included clinical signs and mortality, body weight, food consumption, urine analysis, hematological analysis, serum biochemical analysis, organ weight, ophthalmic observation and histopathological findings (Song *et al.*, 2006).

Body weight. Animals were observed three times daily for clinical signs. Changes in body weight were recorded weekly and group means were calculated. Rat body weights were measured at the initiation of treatment and then weekly until autopsy at 13 weeks post-treatment initiation.

Food consumption. Daily food consumption was determined by subtracting leftover feed from provided feed. Food consumption was measured daily for the 1st week and weekly thereafter. Again differences between that food supplied and remaining were regarded as daily consumption (g/rat/day).

Urine sampling. During the final week of testing (week 13), rats were transferred to metabolic cages for 24 hr and urine was collected to determine specific gravity, pH, leukocyte content, nitrite, protein, glucose, ketone, uro-bilinogen, bilirubin and hemoglobin levels using commercial kits (Roche Diagonisctics GmbH, Mannheim, Germany).

Blood sampling and plasma assay. After 13 weeks of treatment, blood (~3 ml) was collected from posterior vena cava under light CO_2 inhalation and used for serum chemistry measurements. The parameters examined included total protein, albumin, total bilirubin, glucose, glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), γ-glutamyl transferase (GGT), alkaline phosphatase (ALP), lactic dehydrogenase (LDH), total cholesterol, blood urea nitrogen (BUN), creatinine, triglyceride, uric acid, sodium, potassium and chloride. All were evaluated using an autoanalyzer (Hitachi 7060 automatic clinical analyzer, Tokyo).

Organ weights. Absolute and relative (organ-to-body weight ratios) weights were determined after sacrifice at 13 weeks of the following; brain, pituitary gland, adrenal glands, liver, spleen, kidneys, heart, thymus, lung, salivary glands, thyroid gland, testes and epididymis.

Pathology and histopathology. The organs, tissues, in the cranial, thoracic, and abdominal cavities of euthanized rats, were examined grossly for ophthalmic observation. Each organ was excised and fixed in phosphate-buffered formalin. After paraffin embedding, the excised organs and tissues were prepared for microscopic examination by sectioning and staining with hematoxylin and eosin.

Statistical analysis. Mean and standard errors of all parameters were determined for each of the 4 groups. The Student's *t*-test was used to establish the significances of differences between the control and treatment groups. $p < 0.05$ was considered statistically significant.

RESULTS

Clinical signs and food consumption. No deaths or adverse clinical signs were observed due to the

Table 1. Mortality of Sprague-Dawley rats treated orally with *I. sinclairii* for 13 weeks

Sex	Dosage (g/kg)	Weeks													Mortality
		Start	1	2	3	4	5	6	7	8	9	10	11	12	13	
Male	CON[a]	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	0.04	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	0.2	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	1.0	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Female	CON	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	0.04	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	0.2	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	1.0	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

[a]CON: treated with PBS buffer.

ingestion of *IS* at a dose of 0.04, 0.2, 1.0 g/kg (Table 1). Food consumption was similar for all study groups, though it was more or less differences between control and treated group, especially IS 1.0 g/kg treated male and female group (Fig. 1). It could be concerned with leptin hormone (Ahn *et al.*, 2007b).

Body weight changes. There were no toxicologically significant differences in mean body weight between any of the treatment groups (Fig. 2). During the 13-week administration period, the body weights of the male and female SD rats in the 3 treatment groups were comparable across the control and treated groups.

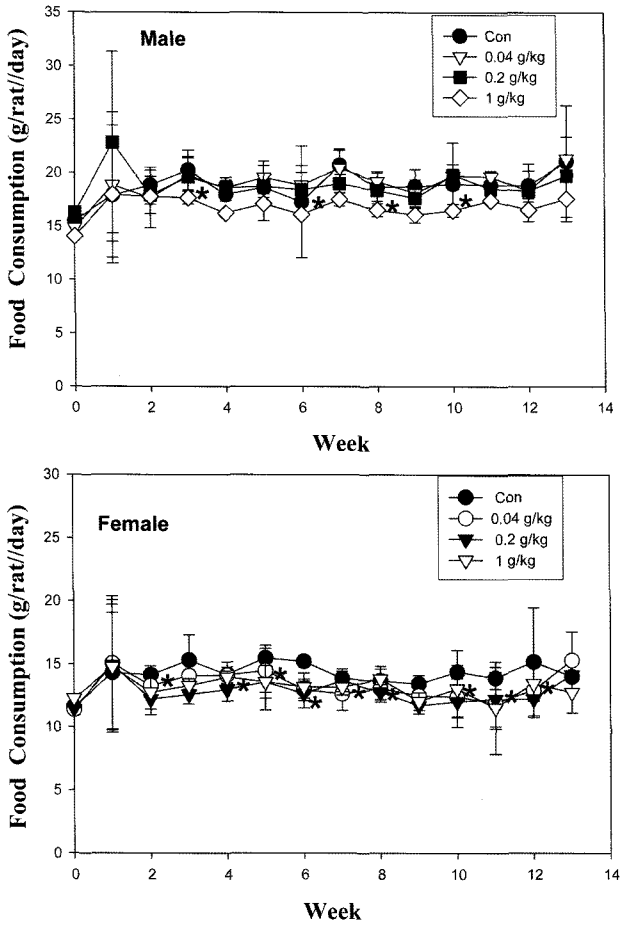

Fig. 1. Food consumption of male and female SD rats, treated orally with *I. sinclairii* over a 13-week period. *Significantly different from the untreated controls ($P < 0.05$).

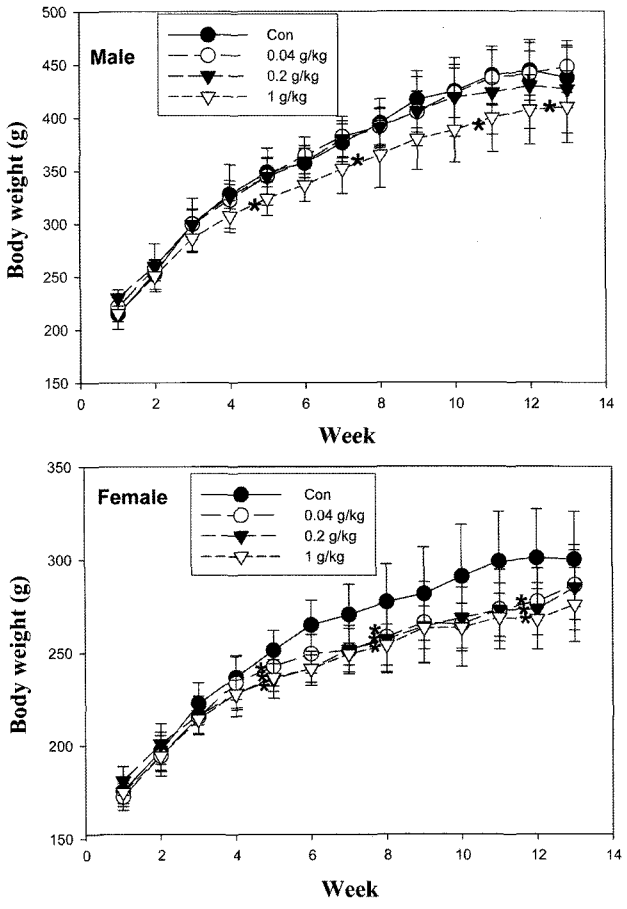

Fig. 2. Body weight increases of male and female SD rats, treated orally with *I. sinclairii* over a 13-week period. *Significantly different from the untreated controls ($P < 0.05$).

Table 2. Urinalysis data of rats (n = 10) in the IS treated groups at the end of the administration period

Sex		Male				Female			
Dosage (g/kg)		CON[a]	0.04	0.2	1	CON[a]	0.04	0.2	1
Specific gravity	1.000	10	7	9	3	7	8	9	6
	1.005	0	0	1	0	2	1	1	0
	1.010	0	3	0	1	1	1	0	1
	1.015	0	0	0	2	1	0	0	2
	1.020	0	0	0	4	1	0	0	1
	1.025	0	0	0	0	0	0	0	0
PH	6.0	0	0	0	2	0	0	0	1
	6.5	0	0	0	2	0	0	0	0
	7.0	0	0	0	0	2	1	0	2
	7.5	4	4	5	4	5	1	3	0
	8.0	6	6	5	2	3	8	7	5
Leucocyte	10~25 mg/dl	8	8	4	1	10	10	9	8
	75	1	2	6	7	0	0	1	2
	500	1	0	0	2	0	0	0	0
Nitrite	-	3	6	5	10	10	0	2	4
	+	7	4	5	0	0	10	8	6
Protein	25 mg/dl	10	10	10	10	10	10	10	10
	50	0	0	0	0	0	0	0	0
	75	0	0	0	0	0	0	0	0
Glucose	normal	10	10	10	10	10	10	10	10
	50 mg/dl	0	0	0	0	0	0	0	0
	100 mg/dl	0	0	0	0	0	0	0	0
Ketone	-	10	10	10	10	10	10	10	10
	5 mg/dl	0	0	0	0	0	0	0	0
	10 mg/dl	0	0	0	0	0	0	0	0
Urobili Nogen	normal	10	10	10	10	10	10	10	10
	1 mg/dl	0	0	0	0	0	0	0	0
	4 mg/dl	0	0	0	0	0	0	0	0
Bilirubin	-	10	10	10	10	10	10	10	10
	1 mg/dl	0	0	0	0	0	0	0	0
	5 mg/dl	0	0	0	0	0	0	0	0
Blood	-	10	10	10	10	10	10	10	10
	1+	0	0	0	0	0	0	0	0
	2+	0	0	0	0	0	0	0	0
Hemoglobin	-	10	10	10	10	10	10	10	10
	1+	0	0	0	0	0	0	0	0
	2+	0	0	0	0	0	0	0	0

[a]CON: treated with PBS buffer.

The mean weekly body weights versus time are presented in Fig. 2. In the male rats, there were statistically significant differences in body weight between the IS (0.04 g/kg: body weight gain increase; 1.0 g/kg: body weight gain decrease compared control) treated groups and the control group (group, $p > 0.05$); however, no statistically significant differences were observed between the 0.2 g/kg IS-treated group and the control group. Furthermore, such a decrease in body weight gain was also found in the female IS treated rats.

Urinalysis. No significant difference was observed between the treatments and the control groups (Table 2).

Hematology and blood chemistry. Some significant differences were observed between the treated and control groups with respect to the hematological parameters at the end of the experiment. At the end of the administration period, as a coagulation parameter, an increase in platelet count was observed in the female

Table 3. Hematological finding of the IS treated groups for 13 weeks

Dosage (g/kg)	Item	Unit	CON[a]	0.04	0.2	1
Male	WBC	$10^3/mm^3$	8.4 ± 3.2	6.1 ± 2.0	5.7 ± 1.7*	6.4 ± 1.7
	RBC	$10^6/mm^3$	9.2 ± 0.7	9.7 ± 0.5	9.0 ± 0.7	9.0 ± 0.7
	Hgb	g/dl	16.3 ± 1.0	17.0 ± 1.2	16.2 ± 1.2	15.6 ± 1.5
	Hct	%	56.5 ± 3.4	57.1 ± 3.5	53.9 ± 4.2	52.5 ± 5.9
	MCV	fl	61.3 ± 2.2	59.0 ± 1.9*	59.7 ± 1.8	58.3 ± 4.3
	MCH	pg	17.7 ± 0.7	17.5 ± 08	18.0 ± 0.6	17.3 ± 1.1
	MCHC	g/dl	28.8 ± 1.0	29.7 ± 0.5*	25.3 ± 6.2	29.6 ± 0.9*
	PLT	$10^3/mm^3$	347.3 ± 164.9	685.9 ± 61.8*	721.0 ± 178.4*	716.9 ± 253.7*
Female	WBC	$10^3/mm^3$	5.1 ± 1.5	7.3 ± 2.7	5.0 ± 1.5	6.0 ± 1.9
	RBC	$10^6/mm^3$	7.9 ± 0.6	8.2 ± 1.2	8.4 ± 0.9	8.6 ± 0.5*
	Hgb	g/dl	14.9 ± 1.3	14.7 ± 1.0	15.4 ± 1.2	15.4 ± 1.0
	Hct	%	48.3 ± 4.6	47.6 ± 4.4	50.9 ± 4.5	52.1 ± 3.6*
	MCV	fl	60.9 ± 1.8	58.4 ± 3.4*	60.3 ± 1.5	60.7 ± 1.8
	MCH	pg	18.8 ± 0.5	18.1 ± 1.6	18.3 ± 0.6*	17.9 ± 0.5
	MCHC	g/dl	30.9 ± 0.8	30.9 ± 1.2	30.3 ± 0.6	29.5 ± 0.9*
	PLT	$10^3/mm^3$	636.1 ± 148.7	679.3 ± 185.1	735.4 ± 167.8	806.0 ± 122.8*

Abbreviations: WBC, white blood cell; RBC, red blood cell; Hgb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet.
[a]CON: PBS (as a vehicle) treated with murine normal diet.
Each value represents mean ± S.D. Statistically significant from control (*P < 0.05).

rats in all of the treated groups; (controls, 636.1 ± 148.4 $10^3/mm^3$, 0.04 g/kg, 679.3 ± 185.1 $10^3/mm^3$; 0.20 g/kg, 735.4 ± 167.8 $10^3/mm^3$; 1.0 g/kg, 806.0 ± 122.8 $10^3/mm^3$) and the same result was shown within the males (Table 3). As indicators of RBC function, we found that MCV, MCH and MCHC were not significantly different between the treated groups and the control group.

Serum biochemistry. In the sera of the IS treated groups, trigyceride and bilirubin were significantly lower than in the control after 13 weeks. A significant decrease in the triglyceride was observed in the 0.04 g/kg

Table 4. Biochemical serum values of male rats treated orally with *I. sinclairii* for 13 weeks

Item	g/kg	CON[a]	0.04	0.2	1
Toal protein	g/dl	8.0 ± 0.5	7.9 ± 0.3	7.4 ± 0.2**	7.6 ± 0.3*
GGT	g/dl	-2.3 ± 3.2	-0.5 ± 3.7	-0.3 ± 1.8*	1.4 ± 1.2*
GPT	IU/l	44.9 ± 15.8	49.8 ± 23.9	38.8 ± 8.1	54.5 ± 26.9
GOT	IU/l	159.5 ± 48.8	177.3 ± 91.3	128.3 ± 28.9	142.5 ± 49.3
ALP	IU/l	96.4 ± 61.9	56.3 ± 34.6*	54.8 ± 30.9*	133.2 ± 78.2*
LDH	IU/l	3229.9 ± 1758.9	2890.0 ± 1923.3	1980.1 ± 789.6*	2604.3 ± 1685.3
Glucose	mg/dl	95.4 ± 80.3	64.0 ± 44.0	63.7 ± 31.1	130.3 ± 48.3
Cholesterol	mg/dl	69.6 ± 9.4	63.6 ± 6.7	69.9 ± 10.4	73.4 ± 6.9
Bilirubin	mg/dl	1.3 ± 0.7	1.0 ± 0.9	0.8 ± 0.4	0.5 ± 0.2*
BUN	mg/dl	21.4 ± 2.3	17.2 ± 1.9*	15.1 ± 1.9*	23.0 ± 2.4
Creatine	mg/dl	0.7 ± 0.1	0.8 ± 0.1*	0.7 ± 0.1	0.8 ± 0.1
Triglyceride	mg/dl	110.9 ± 32.7	60.6 ± 14.7*	73.8 ± 28.8	124.7 ± 44.0
Uric acid	mg/dl	4.2 ± 1.4	3.7 ± 1.8	3.7 ± 1.4	4.7 ± 1.2
K	mmol/l	25.9 ± 3.9	20.1 ± 2.1*	17.7 ± 3.1*	15.6 ± 2.6*
Na	mmol/l	137.3 ± 2.9	142.8 ± 3.2*	143.5 ± 3.1*	146.8 ± 1.8*
Ca	mmol/l	11.1 ± 3.2	10.4 ± 5.8	9.4 ± 3.8	8.5 ± 3.0
Cl	mmol/l	92.4 ± 2.8	95.3 ± 1.8	95.5 ± 1.7*	96.1 ± 1.1*
P	mmol/l	24.4 ± 6.8	19.6 ± 6.3	19.1 ± 5.0	17.9 ± 9.1
CPK	IU/l	854.9 ± 399.6	821.5 ± 594.9	672.5 ± 307.9	725.8 ± 376.8
HDL	IU/l	15.9 ± 2.6	15.7 ± 3.1	15.9 ± 2.5	24.6 ± 4.6*

Abbreviations: GGT, γ-glutamyl transferase; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; ALP: alkaline phosphatase; LDH, lactate dehydrogenase; BUN, blood urea nitrogen; K, potassium; Na, sodium; Ca, calcium; Cl, chloride; p, phosphorus; CK: creatinine phosphokinase; HDL: High Density lipoproten.
[a]CON: PBS (vehicle) treated with murine normal diet.
Each value represents mean ± S.D. Statistically significant from control (*P < 0.05).

Table 5. Biochemical serum values of female rats treated orally with *I. sinclairii* for 13 weeks

Item	g/kg	CON[a]	0.04	0.2	1
Toal protein	g/dl	7.8 ± 0.3	7.7 ± 0.2	7.9 ± 0.3	7.8 ± 0.5
GGT	g/dl	0.2 ± 1.9	0.7 ± 1.5	-0.4 ± 2.1	1.1 ± 1.8
GPT	IU/l	35.7 ± 6.6	35.3 ± 7.3	38.9 ± 16.1	38.9 ± 12.2
GOT	IU/l	146.9 ± 51.1	133.5 ± 30.9	189.8 ± 78.3	157.5 ± 92.1
ALP	IU/l	118.0 ± 84.8	102.2 ± 84.6	40.9 ± 25.2*	79.7 ± 57.2
LDH	IU/l	2334.4 ± 1638.2	1532.4 ± 1092.3	1411.9 ± 778.6	974.5 ± 921.1*
Glucose	mg/dl	223.9 ± 120.8	146.2 ± 95.2	75.0 ± 30.3*	199.7 ± 226.1
Cholesterol	mg/dl	77.2 ± 13.4	76.1 ± 13.0	74.6 ± 7.4	79.2 ± 15.8
Bilirubin	mg/dl	0.9 ± 0.5	0.8 ± 0.4	0.8 ± 0.3	0.9 ± 0.7
BUN	mg/dl	19.8 ± 3.3	19.7 ± 4.2	21.4 ± 1.9	18.9 ± 2.8
Creatine	mg/dl	0.9 ± 0.1	0.8 ± 0.1*	0.8 ± 0.1	0.9 ± 0.1
Triglyceride	mg/dl	152.4 ± 95.7	122.0 ± 42.3*	82.9 ± 36.0*	108.8 ± 47.9
Uric acid	mg/dl	6.7 ± 3.2	4.0 ± 2.5	3.1 ± 1.2*	4.7 ± 2.7
K	mmol/l	15.4 ± 2.2	12.0 ± 1.5*	13.0 ± 3.4*	10.3 ± 2.5*
Na	mmol/l	143.0 ± 2.3	145.2 ± 1.4*	144.5 ± 3.1	146.2 ± 3.3*
Ca	mmol/l	9.9 ± 2.5	11.4 ± 3.4	10.1 ± 2.7	10.5 ± 3.4
Cl	mmol/l	98.3 ± 2.2	98.0 ± 2.8	97.3 ± 2.3	99.2 ± 2.8
P	mmol/l	16.0 ± 5.7	14.9 ± 6.5	17.1 ± 3.8	14.5 ± 3.9
CPK	IU/l	852.5 ± 560.5	972.2 ± 614.6	1411.9 ± 778.6	974.5 ± 921.1
HDL	IU/l	30.5 ± 4.7	28.4 ± 5.7	25.3 ± 3.2*	30.0 ± 6.0

[a]CON: PBS treated with murine normal diet.
Each value represents mean ± S.D. Statistically significant from control (*P < 0.05).

Table 6. Absolute organ weight of Sprague-Dawley rats treated orally with *I. sinclairii* for 13 weeks

Sex	Organs	Unit (g)			
	Dosage (g/kg)	CON	0.04	0.2	1
Male	Adernal gland R.	0.026 ± 0.012	0.031 ± 0.014	0.028 ± 0.010	0.023 ± 0.011
	L.	0.031 ± 0.005	0.028 ± 0.012	0.030 ± 0.012	0.029 ± 0.013
	Kidney R.	1.459 ± 0.150	1.523 ± 0.133	1.763 ± 0.339	1.704 ± 0.351
	L.	1.434 ± 0.134	1.546 ± 0.145*	1.709 ± 0.298*	1.688 ± 0.322*
	Heart	1.364 ± 0.118	1.314 ± 0.109	1.412 ± 0.179*	1.255 ± 0.097*
	Liver	13.026 ± 1.212	11.740 ± 1.150*	12.277 ± 1.268	13.802 ± 2.322
	Lung	1.474 ± 0.343	1.649 ± 0.223	1.782 ± 0.242	1.514 ± 0.155
	Spleen	0.716 ± 0.064	0.858 ± 0.381	0.714 ± 0.069	0.721 ± 0.170
	Testis R.	1.879 ± 0.396	1.627 ± 0.423	1.887 ± 0.152	1.685 ± 0.215*
	L.	1.879 ± 0.286	1.773 ± 0.188	1.901 ± 0.205	1.715 ± 0.168
	Stomach	1.581 ± 0.123	1.372 ± 0.181	1.587 ± 0.158	1.582 ± 0.175
	Pancreas	0.693 ± 0.143	0.828 ± 0.263	0.663 ± 0.168	0.715 ± 0.078
	Thymus	0.388 ± 0.134	0.399 ± 0.078	0.427 ± 0.061	0.420 ± 0.098
Female	Adernal gland R.	0.050 ± 0.015	0.044 ± 0.009	0.039 ± 0.007*	0.033 ± 0.006*
	L.	0.048 ± 0.010	0.045 ± 0.008	0.040 ± 0.010*	0.037 ± 0.011*
	Kidney R.	0.944 ± 0.064	0.959 ± 0.129	0.904 ± 0.093	0.920 ± 0.100
	L.	0.927 ± 0.074	0.979 ± 0.128	0.911 ± 0.080	0.924 ± 0.078
	Heart	0.969 ± 0.075	0.926 ± 0.086	0.916 ± 0.086	0.932 ± 0.061
	Liver	8.745 ± 1.494	8.976 ± 2.210	7.192 ± 0.963*	7.604 ± 0.837*
	Lung	1.393 ± 0.152	1.498 ± 0.152	1.442 ± 0.188	1.287 ± 0.109
	Spleen	0.636 ± 0.091	0.682 ± 0.100	0.589 ± 0.094	0.636 ± 0.097
	Ovary R.	0.077 ± 0.025	0.081 ± 0.021	0.082 ± 0.019	0.059 ± 0.021
	L.	0.086 ± 0.023	0.078 ± 0.012	0.074 ± 0.018	0.050 ± 0.026*
	Stomach	1.559 ± 0.303	1.341 ± 0.141*	1.311 ± 0.201*	1.293 ± 0.264*
	Pancreas	0.610 ± 0.101	0.530 ± 0.107	0.580 ± 0.086	0.571 ± 0.103
	Thymus	0.370 ± 0.117	0.350 ± 0.101	0.318 ± 0.061	0.347 ± 0.066

Each value represents mean ± S.D.
Statistically significant from control (*P < 0.05).

and 0.2 g/kg group for both the males and females (male: controls, 110.9 ± 32 mg/dl; 0.04 g/kg, 60.1 ± 15.8 mg/dl; 0.2 g/kg, 73.6 ± 31.8 mg/dl; female: controls, 160.1 ± 97.2 mg/dl; 0.04 g/kg, 122.0 ± 42.3 mg/dl; 0.2 g/kg, 84.1 ± 37.7 mg/dl).

The potassium ion levels of the treated groups were reduced in dose dependent manners in the males (control, 25.9 ± 3.9 nmol/l; 1.0 g/kg, 15.6 ± 2.6 nmol/l), and females (controls, 15.5 ± 2.3 nmol/l; 1.0 g/kg, 9.8 ± 1.7 nmol/l), whereas the natrium ion level increased (male: control, 137,3 ± 2.9 nmol/l; 1.0 g/kg, 146.8 ± 1.8 nmol/l, female: control. 142.8 ± 2.3 nmol/l; 1.0 g/kg, 146.9 ± 2.4 nmol/l) (Table 4 and Table 5).

Pathology and organ weight. No significant treatment-related pathologies were observed. Any minor changes were few and dose independent. At the end of the administration period, there were no treatment-related changes in the absolute (Table 6) or relative organ weights (Table 7). There were some histopathological findings to be observed, however the histopathological alterations at the end of the administration period were not related to treatment dose increment (Table 8).

Epithelioid granulomas of the lungs were found at the end of the administration period in the 0.04 g/ kg and 0.2 g/kg treated male and female. However, at the high dose level in 1.0 g/kg group, there were no detected any pathological finding for either sex. Furthermore, although there was no essential factor that presented in the 13-week repeated toxicity test, we observed cellular atypia and nuclear enlargement of the kidney. For example, some scattered atypical tubular epithelial cells with nuclear enlargement in the medulla occurred in the daily oral dose of 0.2 g/kg and 1 g/kg treated females and in the 0.04 g/kg, 0.2 g/kg, and 1.0 g/kg treated males over a 13-week period, in dose responsive manners [low dose level: obscure nuclear abnormality; high dose level: nuclear enlargement in the kidney tubular epithelial cells (Table 8)].

In some treated groups (male 0.04 g/kg: 4 cases; male, 0.2 g/kg: 1 case; female 0.04 g/kg: 2 cases: female 0.2 g/kg: 4 cases) focal foam cell aggregation was observed (by infectious tubercular inflammation), but there was no evidence in 1.0 g/kg in high dose level. This suggests that high dose level IS treated rats recovered from the unknown disease germ.

Table 7. Relative organ weight of Sprague-Dawley rats treated orally with *I. sinclairii* for 13 weeks

Sex	Organs	Unit (g)			
	Dosage (g/kg)	CON	0.04	0.2	1
Male	Adernal gland R.	0.006 ± 0.003	0.007 ± 0.003	0.006 ± 0.002	0.005 ± 0.003
	L.	0.007 ± 0.002	0.006 ± 0.003	0.007 ± 0.003	0.007 ± 0.004
	Kidney R.	0.349 ± 0.042	0.360 ± 0.028	0.415 ± 0.072*	0.395 ± 0.112
	L.	0.342 ± 0.035	0.365 ± 0.028	0.402 ± 0.060*	0.418 ± 0.061*
	Heart	0.326 ± 0.034	0.304 ± 0.033	0.333 ± 0.040	0.313 ± 0.026
	Liver	3.108 ± 0.230	2.801 ± 0.206*	2.897 ± 0.255	3.418 ± 0.361*
	Lung	0.354 ± 0.088	0.356 ± 0.121	0.424 ± 0.066	0.378 ± 0.044
	Spleen	0.171 ± 0.017	0.201 ± 0.079	0.168 ± 0.014	0.182 ± 0.039
	Testis R.	0.449 ± 0.097	0.387 ± 0.103	0.445 ± 0.031	0.418 ± 0.033
	L.	0.449 ± 0.075	0.419 ± 0.039	0.449 ± 0.048	0.427 ± 0.029
	Stomach	0.354 ± 0.082	0.323 ± 0.035	0.372 ± 0.036	0.394 ± 0.036
	Pancreas	0.165 ± 0.034	0.185 ± 0.037	0.154 ± 0.040	0.179 ± 0.027
	Thymus	0.094 ± 0.039	0.094 ± 0.019	0.101 ± 0.014	0.102 ± 0.017
Female	Adernal gland R.	0.017 ± 0.006	0.015 ± 0.003	0.014 ± 0.002	0.012 ± 0.003*
	L.	0.016 ± 0.004	0.016 ± 0.005	0.014 ± 0.004	0.014 ± 0.005*
	Kidney R.	0.316 ± 0.020	0.326 ± 0.030	0.311 ± 0.056	0.342 ± 0.049
	L.	0.313 ± 0.026	0.337 ± 0.028	0.332 ± 0.022	0.347 ± 0.041*
	Heart	0.314 ± 0.027	0.312 ± 0.030	0.325 ± 0.039	0.346 ± 0.036*
	Liver	2.939 ± 0.436	3.046 ± 0.642*	2.624 ± 0.256	2.844 ± 0.240
	Lung	0.427 ± 0.130	0.511 ± 0.054	0.533 ± 0.059*	0.479 ± 0.061
	Spleen	0.214 ± 0.028	0.232 ± 0.032	0.215 ± 0.033	0.235 ± 0.038
	Ovary R.	0.025 ± 0.008	0.027 ± 0.007	0.030 ± 0.006	0.022 ± 0.009
	L.	0.028 ± 0.008	0.047 ± 0.066	0.026 ± 0.006	0.020 ± 0.010
	Stomach	0.519 ± 0.109	0.467 ± 0.047	0.480 ± 0.079	0.479 ± 0.095
	Pancreas	0.206 ± 0.038	0.211 ± 0.091	0.211 ± 0.026	0.213 ± 0.048
	Thymus	0.124 ± 0.037	0.117 ± 0.028	0.116 ± 0.023	0.127 ± 0.022

Each value represents mean ± S.D.
Statistically significant from control (*P < 0.05).

Table 8. Microscopic findings of Sprague-Dawley rats treated with *I. sinclairii* for 13 weeks

Sex	Organs	Dose (g/kg)			
		CON[a]	0.04	0.2	1
Male	Adernal gland	0	0	0	0
	Kidney	0	10c	10c	10c
	Heart	0	0	0	0
	Liver	0	0	0	0
	Lung	0	5b	1b	0
	Spleen	0	0	0	0
	Testis R.	0	0	0	0
	L.	0	0	0	0
	Stomach	0	0	0	0
	Pancreas	5a	0	0	5a
	Thymus	0	0	0	0
Female	Adernal gland	0	0	0	0
	Kidney	0	0	10c	10c
	Heart	0	0	0	0
	Liver	0	0	0	0
	Lung	0	2b	2b1d	1d
	Spleen	0	0	0	0
	Testis R.	0	0	0	0
	L.	0	0	0	0
	Stomach	0	0	0	0
	Pancreas	1a	1a	1a	0
	Thymus	0	0	0	0

a: Focal enzymatic fat necrosis. b: small epithelioid granulomas. c: some sacttered atypical tubular epithelial cell with nuclear enlargement in the medulla. d: focal foam cell aggregation.

DISCUSSION

Recently, it was reported that the primary pharmacological activities of *I. sinclairii* include selective antihypertensive activity (Ahn *et al.*, 2007a) and anti-obesity activity in rats (Ahn *et al.*, 2007B; Ahn *et al.*, 2007c). However, *I. sinclairii* contains polyhydroxylated alkaloids such as 1-deoxynojirimycin (DNJ) that are metabolized from silkworm nutrients (Asano *et al.*, 2001), and a synthetic long-chain N-alkylated immini sugar, N-buthyldeoxynojirimycin, was reported, where its membrane disruption and cytotoxicity were dependent on the inhibition of protein and lipid glycosylation, reducing hepatitis virus (Mellor *et al.*, 1998).

The safety of IS has been evaluated systematically by a series of acute and sub-acute toxicological tests. For example, in an acute oral toxicity study of IS in beagle dogs (Ahn *et al.*, 2003), IS did not induce any remarkable acute toxic responses, and the LD_{50} was greater than 10 g/kg (Kim *et al.*, 2003). In a genotoxicity study, IS extract showed no mutagenicity, as judged by an Ames test and a chromosome aberration (CA) test in Chinese hamster ovary (CHO) cells *in vitro*, and a micronucleus (MN) test *in vivo* (Ahn *et al.*, 2004a). In a 13 week feeding study, IS was administered *ad libitum* to rats at levels of 0, 1.25, 5, and 10% feed (calculated at approximately 8 g/kg/day) for a period of 3 months. Here, the no-observed-adverse-effect level (NOAEL) of IS was found to be less than 1.25% (1 g/kg/day) (Ahn *et al.*, 2004b). However, the treatments above 1.25% (1 g/kg/day) resulted in cell alterations of the kidney as well as edema, in dose responsive manners in both sexes; but this was not consistent with the kidney biochemical serum analysis data, which was normal (Ahn *et al.*, 2004b). In a repeated oral toxicity study, similar to our present study, SD rats received orally administered doses of 0, 0.04, 0.2, and 1 g/kg/day of IS for over a 13-week period. There were no observed clinical signs or deaths related to treatment for all the groups tested. Therefore, the approximate lethal oral dose of *I. sinclairii* was considered to be higher than 1 g/kg/day in rats. Throughout the administration period, no significant changes in diet consumption, ophthalmologic findings, organ weight, clinical pathology (hematology, clinical chemistry, coagulation, and urinalysis), and gross pathology were detected. Minor changes were found in the hematological parameters for the 0.04 g/kg/day and 0.2 g/kg/day IS-treated groups (triglyceride reductions of 20.1~46.6% and platelet increases), but all changes were within physiological range. Microscopic examination failed to identify any treatment-related histopathologic changes in the organs of the IS-treated rats other than nuclear enlargement (cellular atypia) of the tubular regions in the medulla of the kidney in the high dose group. In general, for the genotoxicity study, nuclear aberration, including nuclear diploidy, etc., was not detected. A rise in permeability of the kidney tubular cells alone could cause cell enlargement, and might not be related to mutagenic and genotoxic damage by *I. sinclairii*.

Currently, change in nuclear size is not a parameter for toxicity assessment in terms of food approval. In the case of Glivec treatment, an anaplastic agent for leukemia, nuclear enlargement may take place (Smetana *et al.*, 2007).

Overall, from these results, one can conclude that the no-observed effect level (NOAEL) of *I. sinclairii* is less than 0.04 g/kg/day in rats.

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