Original Article

A 4-week Repeated Dose Oral Toxicity and Cytotoxicity Study of Gumiganghwaltang in Crl:CD (SD) Rats

Mee-Young Lee, In-Sik Shin, Chang-Seob Seo, Jung-Hoon Kim, Heykyung Ha, Jung-Im Huh[1], Hyeun-Kyoo Shin

Depatment of Herbal Medicine EBM Research Center, Korea Institute of Oriental Medicine, Yuseong-gu, Daejeon 305 811, [1]Division of Nonclinical Studies, Korea Institute of Toxicology, Yuseong-gu, Daejeon 305 343, Korea

ABSTRACT

Gumiganghwaltang (GGT) is a traditional oriental herbal prescription commonly used to treat colds and inflammatory diseases in Korea. This study reports the first evaluation of the oral toxicity and cytotoxicity effects of repeat doses of GGT. GGT was orally administered daily at doses of 0, 500, 1000, and 2000 mg/kg for 4 weeks. Analysis of body weight gain, mortality, clinical observations, urinalysis, blood biochemistry, hematology, organ weight, and histopathological data revealed no significant differences between the V.CONTROL and GGT-treated groups. In addition, we investigated the cytotoxicity of GGT against LNCaP, RBL-1, and BEAS-2B cell lines, and splenocytes. Based on the results, we conclude that GGT orally administered to rats is safe with no drug-related toxicity, even at the highest dose, in 4-week repeated dose studies. Thus, this concentration is considered the non-observable effect dose in rats.

Key words: Gumiganghwaltang, oriental herbal prescription, toxicity

INTRODUCTION

Herbal medicines are becoming increasingly popular in modern society, and physicians have begun to prescribe herbal medicine along with Western therapies.[1] Medicinal plants are popular remedies used by the vast majority of the global population. The efficacy of medicinal plants in disease management is established, and the Word Health Organization has recognized their use in the primary healthcare delivery system. Medical therapeutics are currently evaluated via a series of scientific studies. However, herbal medicines are often not subjected to toxicity tests before being administered to humans. In view of the complexity of herbal medicines and their inherent biological variations, it is necessary to evaluate their safety, efficacy, and quality.[2] With the increasing usage of herbal therapies, significant concerns have been raised over the lack of quality control and scientific evidence of the efficacy and safety of these agents.[3,4] In particular, safety of herbal prescriptions has become an increasingly important issue[5] and is generally evaluated via toxicological assessment of all medicinal plant materials.

Access this article online

Quick Response Code:

Website: www.toxicologyinternational.com

DOI: 10.4103/0971-6580.84268

Gumiganghwaltang (GGT; Jiu Wei Qianghuo tang in Chinese, Kumi-Kyokatsu-to in Japanese), a polyherbal formula consisting of nine different herbs [Table 1], is a Korean prescription commonly used for the treatment of cold and fever in Asian countries. GGT is one of the most widely prescribed herbal formulae in Korea, China, and Japan. Indeed, GGT was recently ranked fourth in terms of consumption in the Korea herbal markets.[6] GGT has been shown to exert an anti-inflammatory effect in peritoneal macrophages.[7] However, little is known about the toxicity and safety of GGT, and standard toxicity assessments have

Address for correspondence: Mr. Hyeun-Kyoo Shin, Herbal Medicine EBM Research Center, Korea Institute of Oriental Medicine, Exporo 483, Yusung-gu, Daejeon 305 811, Korea. E-mail: cozy11@kiom.re.kr

Table 1: Composition of Gumiganghwaltang

Scientific name	Amount (g)	Company of purchase	Source
Ostericum koreanum	5.625	HMAX	China
Saposhnikovia divaricata	5.625	HMAX	China
Cnidium officinale	4.5	Omniherb	Yeongcheon, Korea
Angelica dahurica	4.5	Omniherb	Yeongcheon, Korea
Atractylodes japonica	4.5	HMAX	China
Scutellaria baicalensis	4.5	HMAX	Jeongseon, Korea
Rehmannia glutinosa	4.5	Omniherb	Kunwi, Korea
Asiasarum sieboldi	1.875	HMAX	China
Glycyrrhiza uralensis	1.875	HMAX	China
Total	37.5		

not been performed to date. To address this issue, we performed a 4-week repeated oral dose toxicity study of GGT in Crl:CD (SD) rats. The cytotoxicity of GGT was additionally examined in LNCaP, rat basophilic leukemia (RBL)-1, BEAS-2B cell lines, and splenocytes.

As part of a safety evaluation of GGT, an evaluation of the cytotoxicity and 4-week repeated-dose toxicity of an aqueous extract of GGT was conducted according to guidelines established by the Organization for Economic Cooperation and Development for the testing of chemicals in accordance with modern Good Laboratory Practice Regulations.

MATERIALS AND METHODS

Preparation of the GGT extract

The GGT extract was prepared in our laboratory from a mixture of chopped crude herbs purchased from Omniherb (Yeongcheon, Korea) and HMAX (Chungbuk, Korea). Before preparing GGT, the plant materials were confirmed taxonomically by Professor Je-Hyun Lee of Dongguk University, Gyeongju, Republic of Korea. A voucher specimen was deposited at the Korea Institute of Oriental Medicine, Daejeon, Republic of Korea. GGT was prepared as described in Table 1 and extracted in distilled water at 100°C for 2 h. The extract was evaporated to dryness and freeze-dried (yield; 22.21%). Lyophilized GGT extract was weighed (500 mg) into a 100-ml flask and distilled water was added to the volumetric mark. The mixture was shaken for 10 min at room temperature. After extraction, the mixture was passed through a 0.2-μm membrane filter, and 10 μl aliquots of filtrate were injected into an high-performance liquid chromatography (HPLC) system.

HPLC analysis of GGT

Liquiritin, ferulic acid, baicalin, baicalein, glycyrrhizin, and wogonin were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The purity of each compound was determined to be greater than 98% by HPLC analysis. HPLC-grade reagents, methanol, acetonitrile, and water were obtained from J.T. Baker (Phillipsburg, NJ, USA). Glacial acetic acid was procured from Junsei Chemical Co. (Tokyo, Japan). A methanol standard stock solution containing compounds liquiritin, ferulic acid, baicalin, baicalein, glycyrrhizin, and wogonin was prepared and diluted to the appropriate concentration range for the establishment of calibration curves.

Analysis was performed using a Shimadzu LC-20A HPLC system (Shimadzu Co., Kyoto, Japan) consisting of a solvent delivery unit, on-line degasser, column oven, autosampler, and PDA detector. The data processor used LC solution software (Version 1.24). The analytical column used was Gemini C18 (250×4.6 mm; particle size 5 μm; Phenomenex, Torrance, CA, USA). The mobile phases consisted of solvents A (1.0%, v/v, aqueous acetic acid) and B (acetonitrile with 1.0%, v/v, acetic acid). Gradient flow was as follows: (A)/(B) = 95/5 (0 min) → (A)/(B) = 30/70 (40 min) → (A)/(B) = 0/100 (45 min; hold for 5 min) → (A)/(B) = 95/5 (55 min; hold for 15 min). The column temperature was maintained at 40°C. Analysis was carried out at a flow rate of 1.0 ml/min, with PDA detection at 254, 280, and 320 nm. The injection volume was 10 μl.

Cell culture and viability

BEAS-2B, LNCaP, and RBL-1 cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). BEAS-2B (1×10^4 cell/ml) cells were maintained in Dulbecco's modified Eagle's medium supplemented with glutamine (1 mM), 10% (v/v) heat-inactivated fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) at 37°C in 5% (v/v) CO_2. RBL-1 (5×10^4 cell/ml) and LNCaP (5×10^3 cell/ml) cells were cultured in RPMI 1640 with 10% (v/v) fetal bovine serum, 2 mM glutamine, and 1% (v/v) antibiotic mixture under 5% (v/v) CO_2 at 37°C. Cells were seeded onto 96-well plates and incubated in serum-free medium in the presence of different concentrations of GGT (below 200 μg/ml) for 24 or 48 h, respectively. The cell counting kit-8 (CCK-8) reagent (Dojindo, Japan) was added at 4 h. Thereafter, absorbance at 450 nm was measured using an enzyme-linked immunosorbent assay microplate reader (Benchmark; Bio-Rad Laboratories, CA, USA). Cell viability was calculated relative to that of the untreated control group. All experiments were performed in triplicate. Cells treated with PBS were used as vehicle controls.

Preparation and treatment of splenocyte suspensions

Spleens from BALB/c mice were aseptically removed and a single-cell suspension obtained by passing cells twice through a needle after suspension in RPMI 1640 containing 10% (v/v) fetal bovine serum, 25 mM HEPES, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (GibcoBRL, Grand Island, NY, USA). Red blood cells (RBCs) were lysed with lysis buffer (Sigma, St Louis, MO, USA) at 37°C for 10 min. After washing with PBS, cells were cultured in 100 mm dishes for 4 h. Splenocytes were plated onto 96-well plates at a density of 1×10^6 cells/ml and treated with different concentrations of GGT (50, 100, and 200 μg/ml) for 1 h, followed by treatment with concanavalin A (ConA, 1 μg/ml) for a further 3 days. Next, splenocyte viability was assessed using the CCK-8 assay. All experimental procedures were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and animal handling followed the dictates of the National Animal Welfare Law of Korea.

Animals

Each of 20 female and male specific pathogen-free Sprague Dawley rats (4 weeks old upon receipt; OrientBio, Republic of Korea) were used after acclimatization for 14 days before study initiation with an evaluation of health status. Five animals were used per group. Three animals were allocated per polycarbonate cage stainless-steel wire-mesh cage and allowed sterilized tap water and commercial rodent chow (Purina Co., Pyeongtaek, Republic of Korea) *ad libitum*. Animals were maintained in environmentally controlled rooms at 22 ± 3°C under relative humidity of 50 ± 20% with a 12 h light–dark cycle (artificial lighting from 08:00 to 20:00) and 12–15 air changes per hour. This study was performed at the Korea Testing and Research Institute, and the study protocol was approved by the institutional Animal Care and Use Committee according to the "Guidelines for Toxicity Tests of Drugs and Related Materials, Document #2009-116" prepared by Korea Food and Drug Administration (2009).

Dose selection and animal treatment

In a previous single oral dose study of GGT, no treatment-related toxic changes were observed at the highest dose (2000 mg/kg). Therefore, the highest dose level (2000 mg/kg) with a common ratio of 2 was selected for our 4-week repeated dose study. Healthy male and female rats were randomly assigned to four experimental groups: GGT 500, 1000, and 2000 mg/kg/day and a vehicle control group. Each group consisted of five rats of each sex. Because the oral route is the clinically intended route for GGT, oral administration was selected in this study. The GGT was suspended in distilled water and freshly prepared daily before treatment. The daily application volume (5 ml/kg body weight) of GGT was calculated in advance based on the most recently recorded body weights of individual animals. The GGT was administered orally to rats for 4 weeks. Distilled water was given to the animals in the vehicle control group.

Animal data

All animals were examined twice daily for general appearance, behavior, and signs of toxicity, morbidity, and mortality. Body weights were recorded weekly, and at the end of the observation periods the rats were examined by necropsy and the weights of organs recorded. On day 28, the animals were fasted overnight and on day 29 they were killed. Food and water consumption was measured per cage at the initiation of treatment and weekly thereafter. Measured amounts of food and water were supplied to each cage, and the remnant was measured on the next day to calculate the difference, which was regarded as the daily food and water consumption.

Clinical test parameters

All animals in each group were subjected to an external eye examination during the pretest period and both an external and fundus examination using an indirect binocular Ophthalmoscope (IO-H, Neitz Instruments, Japan) during the last week of dosing. During the last week of treatment, urinalysis of all animals was conducted to assess urine volume (Vol), specific gravity (SG), pH, protein (PRO), ketone body (KET), glucose (GLU), bilirubin (BIL), nitrite (NIT), urobilinogen (URO), bilirubin (BIL), nitrite (NIT), casts, epithelial cells (EPI), erythrocytes (RBC), leucocytes (WBC), and occult blood (BLO) using a Multistix 10SG (Bayer, USA) and urine chemical analyzer (Clinitek 500, USA). Blood samples were drawn from the posterior vena cava of animals under the isoflurane anesthesia, collected into CBC bottles containing EDTA-2K (Sewon Medical, Republic of Korea), and analyzed to determine RBC count, hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular cell volume (MCV), mean corpuscular cell hemoglobin (MCH), mean corpuscular cell hemoglobin concentration (MCHC), platelet (PLT), large unstained cell (LUC), white blood cell count (WBC), and differential WBC count. All parameters were measured using an ADVIA 120 Hematology System (Bayer). Prothrombin time (PT) and activated partial thromboplastin time (APTT) were determined in blood samples treated with 3.2% sodium citrate using a coagulometer (Coagrez-100s, Japan).

For serum biochemistry analysis, blood samples were centrifuged at 3000 rpm for 10 min and analyzed using a Toshiba 200FR NEO analyzer (Toshiba, Japan). Serum biochemistry parameters were examined, including blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine (CREA), glucose (GLU), total cholesterol

(TCHO), albumin/globulin ratio (A/G), total protein (TP), albumin (ALB), creatine kinase (CK), triglyceride (TG), total bilirubin (TBIL), phospholipid (PL), gamma glutamyl transferase (GGT), calcium (Ca), inorganic phosphorus (IP), chloride (Cl), sodium (Na), and potassium (K). The absolute organ weight of the brain, pituitary gland, adrenal gland, liver, spleen, kidneys, heart, thymus, lung, salivary gland, thyroid glands, testis, epididymides, seminal vesicle, prostate, uterus, and ovaries was measured, and then relative organ weight (% for body weight) was calculated.

Histopathology

Samples from liver and kidney were fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin, sectioned at 4-μm thickness and stained with H&E solution (hematoxylin, Sigma MHS-16; Eosin, Sigma HT110-1-32). Tissues were subsequently mounted and coverslipped using mounting medium (Invitrogen, USA).

Statistical analysis

Data collected during the study were examined for variance homogeneity using the Bartlett's test. In cases where no significant deviations from variance homogeneity were observed, one-way analysis of variance was performed at $\alpha = 0.05$. In case of significance, a multiple comparison test (Dunnett's test) was conducted to determine which pairs of groups were significantly different. In case of significant deviations from variance homogeneity, a non-parametric comparison (Kruskal–Wallis test) was conducted. The Dunn's Rank Sum test was conducted to determine the specific pairs when significant differences were observed in the Kruskal–Wallis test. Statistical analyses were performed using the Path/Tox System (Ver. 4.2.2). The level of significance was taken as $P < 0.05$ or 0.01. Because treatment-related animal deaths were not observed, LD_{50} values were not determined.

RESULTS

Characterization of the GGT extract

Three-dimensional chromatograms were obtained using the HPLC-PDA detector. Under optimized chromatography conditions, six components were eluted before 35 min in sample analysis using mobile phases consisting of solvent A (1.0%, v/v, aqueous acetic acid) and solvent B (acetonitrile with 1.0%, v/v, acetic acid). The three-dimensional HPLC chromatogram of GGT is shown in Figure 1. The retention times of the components were 17.0 min (liquiritin), 18.2 min (ferulic acid), 22.3 min (baicalin), 28.9 min (baicalein), 31.5 min (glycyrrhizin), and 33.9 min (wogonin). The linearity of the peak area (y) versus concentration (x, μg/ml) curve for liquiritin, ferulic acid, baicalin, baicalein, glycyrrhizin, and wogonin was used to calculate the content of the main components in GGT. Analytical results for each component are summarized in Table 2.

Cell viability

As shown in Figure 2, cell viability was not significantly reduced by GGT up to a concentration of 200 μg/ml in BEAS-2B and LNCaP cell lines. However, cytotoxicity for splenocytes was apparent at concentrations over 100 μg/ml

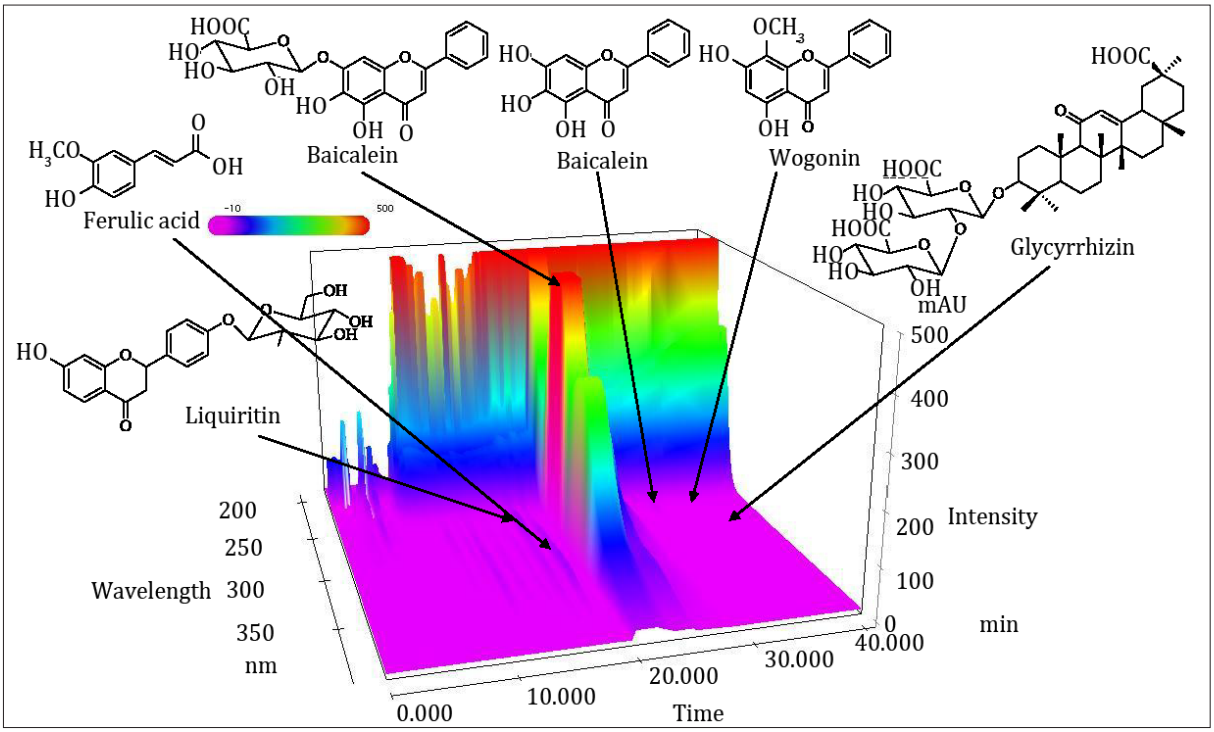

Figure 1: Three-dimensional HPLC chromatogram of GGT

and RBL-1 cells at concentrations over 200 μg/ml. Thus, at concentrations of 10–100 μg/ml, GGT itself did not cause cytotoxicity in RBL-1 cell lines.

Table 2: Contents of six components in the GGT by HPLC (n = 3)

Component	Mean (mg/g)	SD	RSD (%)
Liquiritin	4.00	0.02	0.60
Ferulic acid	0.69	0.0004	0.61
Baiclin	21.76	0.05	0.23
Baicalein	0.10	0.002	1.85
Glycyrrhizin	1.71	0.01	0.43
Wogonin	ND*	—	—

*ND mean was less than the limit of quantitation.

Table 3: Clinical signs in rats treated orally with GGT for 4 weeks

Group	Salivation	Scab
Male		
V.CONTROL	0/5	0/5
500 mg/kg	0/5	0/5
1000 mg/kg	0/5	0/5
2000 mg/kg	5/5	0/5
Female		
V.CONTROL	0/5	0/5
500 mg/kg	0/5	0/5
1000 mg/kg	0/5	0/5
2000 mg/kg	4/5	0/5

Mortality and clinical signs

No mortality of either sex was observed in the 28 days repeated-dose oral toxicity testing in rats in GGT treatment. No GGT treatment-related clinical signs were detected in rats, regardless of gender, except for salivation in male and female rats treated with 2000 mg/kg GGT [Table 3].

Changes in body and organ weight

The development of animals during the experimental period corresponded to their species and age. Body weights were obtained once a week for both genders. Animals in all groups gained weight. No significant differences were observed among GGT treatment groups, compared with the V.CONTROL group, at any time point of the experimental period [Figure 3]. Changes in relative organ weight in the V.CONTROL and the GGT-treated groups are presented in Table 4. There were no significant differences between the V.CONTROL and GGT-treated groups in terms of the relative organ weights of male and female animals.

Changes in food and water consumptions

In male rats, treatment may partly be related to a slight decrease in food consumption when compared with the V.CONTROL group. Significant differences in male rat groups were observed following treatment with 500 mg/kg (days 16, 23, 28) and 2000 mg/kg (day 28) GGT [Figure 4]. In the female rats, no food consumption changes were observed in any group.

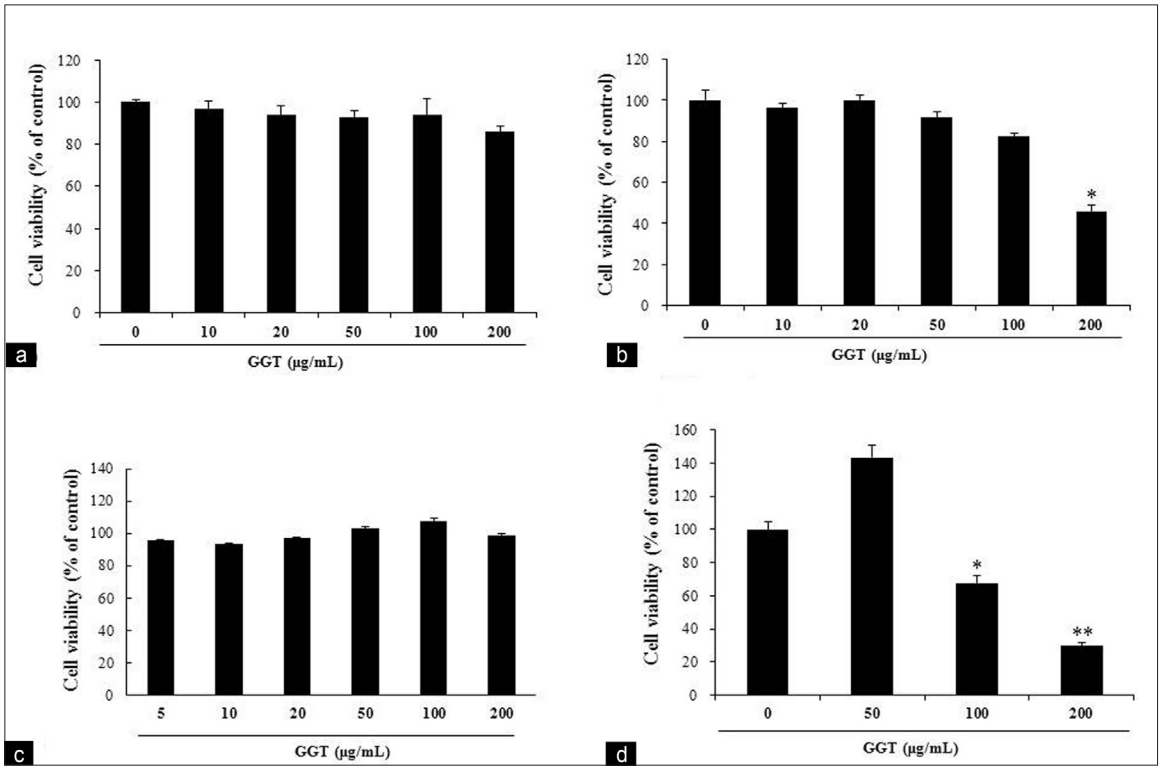

Figure 2: Cell viability after treatment with GGT. (a) BEAS-2B were incubated for 48 h with various concentrations of GGT (10–200 μg/ml). (b) RBL-1 were incubated for 24 h with various concentrations of GGT (10–200 μg/ml). (c) LNCap were incubated for 48 h with various concentrations of GGT (5–200 μg/ml). (d) Splenocytes were incubated for 48 h with various concentrations of GGT (50–200 μg/ml). Values are presented as mean ± SD of three experiments. *Significant differences from non-treatment (0) ($P < 0.05$); **significant differences from non-treatment (0) ($P < 0.01$)

Table 4: Relative organ weights of rats (n = 5) treated orally with GGT (% body weight) for 4 weeks*

Items	Male				Female			
	V.CONTROL	500 mg/kg	1000 mg/kg	2000 mg/kg	V.CONTROL	500mg/kg	1000 mg/kg	2000 mg/kg
Mean body weights (g)	389.6 ± 33.2	378.8 ± 17.6	381.1 ± 13.3	395.6 ± 38.5	236.1 ± 16.3	246.5 ± 13.9	255.6 ± 13.8	241.9 ± 13.7
Brain	0.55 ± 0.03	0.53 ± 0.025	0.54 ± 0.05	0.52 ± 0.05	0.84 ± 0.06	0.83 ± 0.05	0.79 ± 0.05	0.82 ± 0.06
Pituitary gland	0.002 ± 0.001	0.002 ± 0.001	0.003 ± 0.001	0.003 ± 0.001	0.005 ± 0.002	0.005 ± 0.001	0.005 ± 0.001	0.005 ± 0.001
Lung	0.37 ± 0.02	0.37 ± 0.02	0.36 ± 0.02	0.42 ± 0.14	0.50 ± 0.03	0.48 ± 0.03	0.48 ± 0.03	0.52 ± 0.02
Liver	2.80 ± 0.15	3.01 ± 0.30	3.04 ± 0.18	3.00 ± 0.23	3.08 ± 0.22	3.09 ± 0.14	3.18 ± 0.38	3.31 ± 0.21
Spleen	0.18 ± 0.02	0.17 ± 0.01	0.19 ± 0.03	0.20 ± 0.02	0.23 ± 0.03	0.22 ± 0.02	0.22 ± 0.03	0.24 ± 0.02
Heart	0.32 ± 0.02	0.34 ± 0.02	0.35 ± 0.02	0.32 ± 0.02	0.37 ± 0.02	0.37 ± 0.02	0.37 ± 0.01	0.37 ± 0.02
Thymus	0.11 ± 0.01	0.13 ± 0.02	0.14 ± 0.03	0.14 ± 0.03	0.17 ± 0.04	0.20 ± 0.04	0.20 ± 0.06	0.17 ± 0.02
Kidneys	0.74 ± 0.05	0.76 ± 0.06	0.81 ± 0.06	0.77 ± 0.035	0.81 ± 0.03	0.84 ± 0.06	0.78 ± 0.08	0.81 ± 0.03
Adrenal glands	0.02 ± 0.002	0.02 ± 0.004	0.02 ± 0.002	0.02 ± 0.009	0.03 ± 0.006	0.03 ± 0.003	0.03 ± 0.005	0.03 ± 0.005

*Values are presented as mean ± SD.

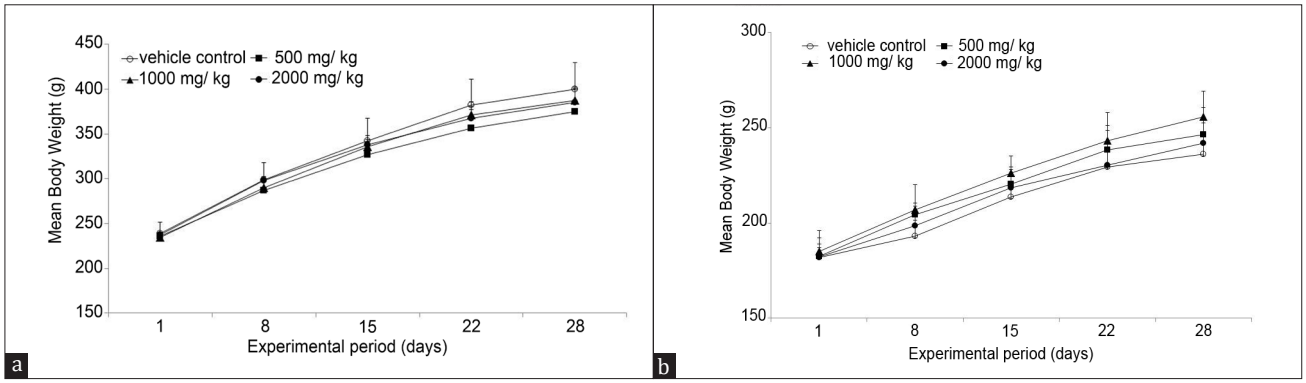

Figure 3: Mean body weight changes of male (a) and female (b) rats treated with GGT at dose levels of 0 (○), 500 (■), 1000 (▲), and 2000 (●) mg/kg/day for 4 weeks. Values are presented as mean ± SD.

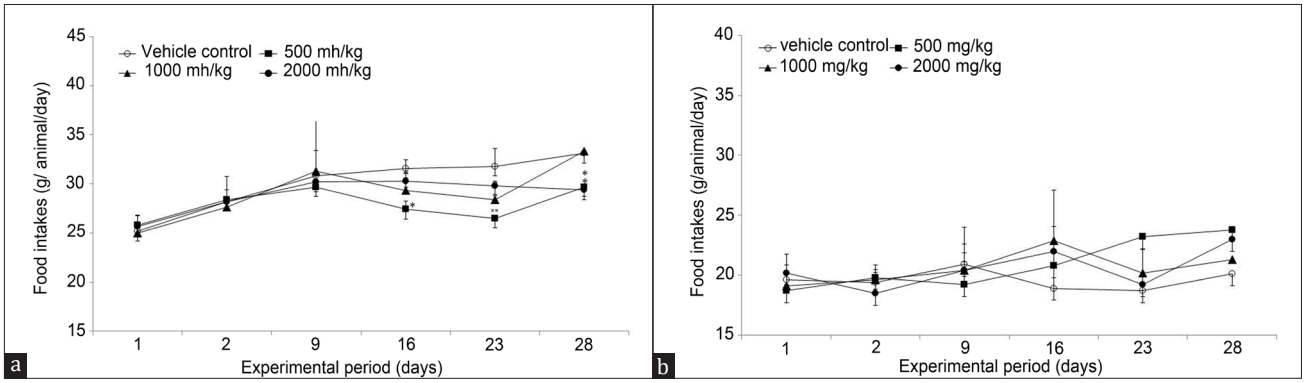

Figure 4: Food intakes in male (a) and female (b) rats treated with GGT at dose levels of 0 (○), 500 (■), 1000 (▲), and 2000 (●) mg/kg/day for 4 weeks. Values are presented as mean ± SD. *Significant differences from V.CONTROL group (P < 0.05); **significant differences from V.CONTROL group (P < 0.01)

Ophthalmological examination and urinalysis

There were no abnormal ophthalmologic findings among mice of each gender from the GGT-treated groups, compared with those of the V.CONTROL group (data not shown). There were no significant changes found in rats of either sex among any of the BPTS-treated groups for Vol, SG, pH, PRO, KET, GLU, BIL, NIT, URO, BIL, NIT, cast, EPI, RBC, WBC, and BLO when compared with V.CONTROL-group rats in this study (data not shown).

Hematology

Results of examination of the hematological parameters of the GGT-treated and V.CONTROL groups are shown in Table 5. In the male rat groups treated with 1000 and 2000 mg/kg GGT, PT was significantly higher than those of the V.CONTROL group. The hematological values of GGT-treated rats in the remaining groups differed slightly from those of the V.CONTROL groups, but were within the normal range for both sexes.

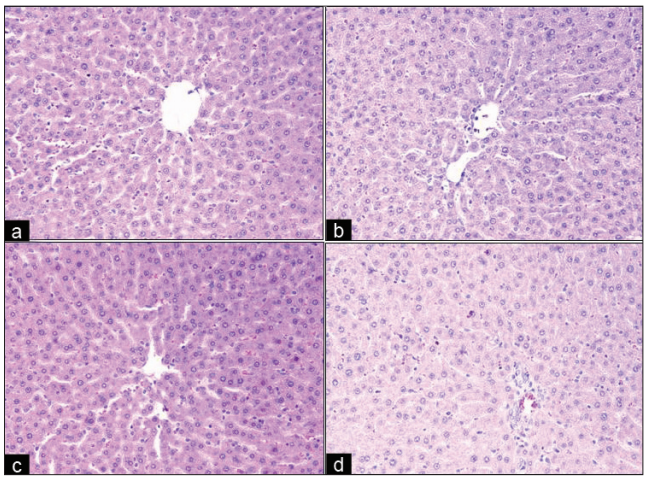

Figure 5: Histopathological findings in liver. (a) V.CONTROL male. (b) High dose male (2000 mg/kg GGT). (c) V.CONTROL female. (d) High-dose female (2000 mg/kg GGT)

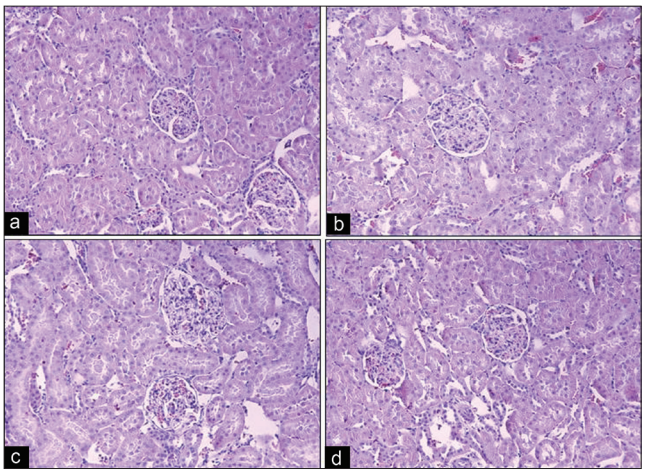

Figure 6: Histopathological findings in kidney. (a) V.CONTROL male. (b) High dose male (2000 mg/kg GGT). (c) V.CONTROL female. (d) High-dose female (2000 mg/kg GGT)

Serum biochemistry

Examination of the serum biochemistry values of the GGT-treated and V.CONTROL groups is shown in Table 6. In the male rat group (2000 mg/kg), Na values were significantly higher than those of the V.CONTROL group. The hematological values of GGT-treated rats in other groups differed slightly from those of the V.CONTROL group, but were within the normal range for both sexes.

Histopathological findings

Necropsy revealed no GGT treatment-related macroscopic changes in the liver [Figure 5] and kidney [Figure 6] of treated animals of both sexes.

DISCUSSION

The growing popularity of herbal medicines is attributable to their easy access, therapeutic efficacy, relatively low cost, and assumed absence of toxic side effects. The widespread public opinion is that herbal medicines are natural products, and therefore harmless with no adverse effects, and their consumption is not dangerous, even if the expected medical effect is not achieved. However, the safety of their use has recently been questioned, in view of reports of illness and fatalities.[8-10] In this study, we performed a 4-week repeated oral dose and cytotoxicity study of GGT. We obtained no evidence of adverse health effects in male or female Crl:CD (SD) rats following 4 weeks of dietary exposure to GGT. During the course of the investigation, no significant biological differences were observed in terms of body weight, body weight gain, feed consumption, and feed efficiency among both male and female rats. In addition, clinical observations, behavioral assessment, and relative organ weights were not changed in animals of both sexes in all treatment groups. No biologically significant differences in hematological profiles and biochemical parameters were observed.

Interestingly, minor changes in salivation were observed in male and female rats treated with 2000 mg/kg of GGT but not observed in 500 and 1000 mg/kg. In oriental medicine, GGT is known as one of the bitter herbal formulas. According to previous report, when rats lick a bitter taste solution, they secrete profuse amounts of saliva.[11] The salivation observed in this study was considered to be caused by bitter taste of GGT. In addition, the salivation induced by GGT treatment was not considered as toxic effects of that because it was not accompanied with pathological changes such as body weight changes and histopathological findings. In addition, other clinical signs are possibly not related to GGT treatment, because these findings occurred with a low incidence and did not exhibit a dose–response relationship. These findings are common in normal SD rats and may result from various factors, including stress caused by oral treatment and fighting among animals housed together.[12,13]

Oral administration of 500 mg/kg GGT significantly decreased the food intake in male rats at 16, 23, and 28 days, but did not affect body weight. Similarly, mean body weight was not altered in both sexes up to a dose of 2000 mg/kg. Therefore, any alterations in food consumption observed in these groups are regarded as incidental due to variations in measured values. Organ weight changes are generally accepted as a sensitive indicator of chemically induced changes to organs, and in toxicology experiments, comparison of organ weights between control and treated groups has conventionally been used to predict the toxic effects of specific compounds.[14,15]

In terms of relative organ weight, spleen weight was slightly increased in a dose-dependent manner, but not to a significant extent. Increased spleen weight without histopathological correlation, indicative of toxicity, is generally considered a physiological adaptation to high dietary levels of unsaturated fatty acids and not a manifestation of toxicity.[16,17] Oral administration of GGT up to 2000 mg/kg for 4 weeks

Table 5: Hematological values of rats treated orally with GGT for 4 weeks*

Items	Male				Female			
	V.CONTROL	500 mg/kg	1000 mg/kg	2000 mg/kg	V.CONTROL	500mg/kg	1000 mg/kg	2000 mg/kg
WBC ($\times10^3$/μl)	9.9 ± 1.7	9.9 ± 1.7	14.6 ± 4.5	14.9 ± 5.2	10.6 ± 3.9	9.7 ± 2.5	8.7 ± 2.5	10.6 ± 2.2
RBC ($\times10^6$/μl)	8.2 ± 0.2	8.4 ± 0.2	8.5 ± 0.2	8.3 ± 0.4	8.4 ± 0.3	8.7 ± 0.3	8.2 ± 0.2	8.1 ± 0.5
HGB (g/dl)	15.7 ± 0.6	15.9 ± 0.3	15.9 ± 0.3	15.8 ± 0.7	15.9 ± 0.5	16.2 ± 0.4	15.3 ± 0.4	15.3 ± 0.9
HCT (%)	47.0 ± 1.9	48.1 ± 1.5	47.9 ± 1.4	47.2 ± 2.4	47.3 ± 1.6	48.4 ± 1.6	45.3 ± 1.5	45.6 ± 3.1
MCV (fl)	57.7 ± 2.0	57.0 ± 1.8	56.6 ± 2.1	57.0 ± 0.8	56.2 ± 0.3	55.9 ± 1.5	55.1 ± 1.3	56.4 ± 1.0
MCH (pg)	19.3 ± 0.6	18.8 ± 0.4	18.8 ± 0.6	19.1 ± 0.4	19.0 ± 0.3	18.7 ± 0.5	18.6 ± 0.3	18.9 ± 0.2
MCHC (g/dl)	33.4 ± 0.2	33.0 ± 0.5	33.3 ± 0.3	33.4 ± 0.4	33.7 ± 0.4	33.5 ± 0.4	33.8 ± 0.3	33.5 ± 0.5
PLT ($\times10^3$/μl)	1078 ± 130	1108 ± 143	1100 ± 54	1209 ± 232	1296 ± 181	1189 ± 139	1169 ± 154	1334 ± 224
Reticulocyte (%)	2.2 ± 0.3	2.2 ± 0.4	2.3 ± 0.2	2.4 ± 0.3	2.5 ± 0.2	2.1 ± 0.4	2.3 ± 0.6	2.6 ± 0.2
Neutrophils (%)	12.3 ± 3.2	11.2 ± 2.8	13.7 ± 3.5	13.1 ± 3.8	10.6 ± 2.5	10.3 ± 4.9	11.2 ± 2.2	9.8 ± 3.9
Lymphocytes (%)	82.6 ± 2.9	84.0 ± 3.1	81.3 ± 4.2	82.1 ± 4.2	84.7 ± 2.8	83.5 ± 5.7	83.8 ± 3.4	85.4 ± 4.3
Monocytes (%)	2.6 ± 0.7	2.6 ± 0.6	2.7 ± 0.6	2.5 ± 0.7	2.2 ± 0.7	2.7 ± 0.7	2.6 ± 1.0	2.7 ± 0.9
Eosinophils (%)	1.2 ± 0.3	0.8 ± 0.5	0.9 ± 0.2	0.7 ± 0.3	0.9 ± 0.3	1.0 ± 0.4	1.0 ± 0.2	0.9 ± 0.2
Basophils (%)	0.5 ± 0.1	0.5 ± 0.2	0.5 ± 0.2	0.6 ± 0.2	0.5 ± 0.2	0.6 ± 0.1	0.5 ± 0.2	0.4 ± 0.1
LUC (%)	0.8 ± 0.2	0.9 ± 0.2	0.8 ± 0.2	1.0 ± 0.3	1.0 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.8 ± 0.1
PT (sec)	13.7 ± 0.3	14.2 ± 0.6	14.5 ± 0.3	14.8 ± 0.5	14.4 ± 0.3	14.8 ± 0.5	14.7 ± 0.6	15.2 ± 0.8
APTT (s)	14.5 ± 0.8	14.2 ± 2.1	14.2 ± 1.6	13.7 ± 2.2	13.3 ± 2.9	13.2 ± 3.7	12.9 ± 0.8	12.7 ± 1.7

*Values are presented as mean ± SD.

Table 6: Serum biochemical values of rats treated orally with GGT for 13 weeks*

Items	Male				Female			
	V.CONTROL	500 mg/kg	1000 mg/kg	2000 mg/kg	V.CONTROL	500 mg/kg	1000 mg/kg	2000 mg/kg
GLU (mg/dl)	123 ± 23.6	134 ± 21.1	120 ± 36.2	129 ± 16.7	96 ± 20.1	98 ± 29.1	120 ± 22.0	120 ± 13.2
BUN (mg/dl)	13.0 ± 1.3	13.3 ± 2.0	14.9 ± 1.6	13.6 ± 2.4	15.5 ± 1.3	17.9 ± 3.4	14.6 ± 1.3	17.2 ± 4.7
CREA (mg/dl)	0.55 ± 0.02	0.56 ± 0.05	0.51 ± 0.04	0.54 ± 0.06	0.61 ± 0.07	0.64 ± 0.06	0.64 ± 0.03	0.70 ± 0.09
TP (g/dl)	6.2 ± 0.23	6.4 ± 0.31	6.4 ± 0.15	6.4 ± 0.29	7.0 ± 0.13	7.1 ± 0.26	6.7 ± 0.32	6.9 ± 0.22
ALB (g/dl)	3.9 ± 0.12	4.0 ± 0.15	4.1 ± 0.09	4.0 ± 0.05	4.4 ± 0.13	4.5 ± 0.10	4.3 ± 0.09	4.5 ± 0.16
A/G (ratio)	1.8 ± 0.07	1.8 ± 0.10	1.7 ± 0.06	1.7 ± 0.17	1.8 ± 0.16	1.8 ± 0.11	1.8 ± 0.15	1.9 ± 0.10
TCHO (mg/dl)	63 ± 6.6	62 ± 10.1	50 ± 12.1	58 ± 15.0	69 ± 4.2	69 ± 17.1	67 ± 18.5	64 ± 10.7
TG (mg/dl)	40 ± 19.6	47 ± 13.0	35 ± 9.1	36 ± 12.4	34 ± 8.2	35 ± 4.2	31 ± 11.4	32 ± 9.8
PL (mg/dl)	95 ± 7.6	100 ± 10.3	84 ± 15.2	91 ± 17.0	129 ± 5.6	125 ± 21.5	121 ± 29.4	120 ± 20.8
AST (IU/l)	125 ± 33.6	127 ± 24.5	115 ± 16.7	107 ± 7.8	127 ± 21.5	120 ± 20.4	131 ± 20.5	125 ± 24.4
ALT (IU/l)	33 ± 1.7	33 ± 3.8	36 ± 3.0	35 ± 6.0	30 ± 3.0	35 ± 12.0	29 ± 5.0	29 ± 4.5
TBIL (mg/dl)	0.11 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.11 ± 0.01	0.18 ± 0.05	0.16 ± 0.02	0.16 ± 0.04	0.15 ± 0.02
ALP (IU/l)	441 ± 90.1	544 ± 92.7	453 ± 62.9	473 ± 65.1	330 ± 44.9	343 ± 74.6	361 ± 56.9	294 ± 61.4
CK (IU/l)	810 ± 307.4	837 ± 298.4	675 ± 200.9	534 ± 147.0	760 ± 204.8	545 ± 214.4	810 ± 242.3	605 ± 178.1
Ca (mg/dl)	11.5 ± 0.5	11.9 ± 0.4	11.9 ± 0.38	11.9 ± 0.5	12.1 ± 0.18	12.4 ± 0.3	11.9 ± 0.5	12.1 ± 0.9
IP (mg/dl)	9.0 ± 1.3	9.9 ± 1.5	9.7 ± 0.7	9.5 ± 1.7	9.6 ± 0.5	9.7 ± 0.5	9.4 ± 1.2	9.4 ± 1.0
Na (mmol/l)	144 ± 2.1	144 ± 0.8	142 ± 1.8	144 ± 1.1	143 ± 2.3	143 ± 0.7	144 ± 0.4	144 ± 1.3
K (mmol/l)	7.0 ± 1.3	7.2 ± 1.9	7.6 ± 1.0	6.7 ± 1.6	7.7 ± 0.6	7.9 ± 0.8	6.8 ± 1.2	7.4 ± 0.9
Cl (mmol/l)	102 ± 1.1	102 ± 2.0	100 ± 2.3	102 ± 0.8	103 ± 1.7	103 ± 1.3	103 ± 2.5	103 ± 0.8
GGTE (IU/l)	0.18 ± 0.25	0.10 ± 0.23	0.07 ± 0.16	0.08 ± 0.19	0.39 ± 0.28	0.24 ± 0.34	0.29 ± 0.28	0.21 ± 0.48

*Values are presented as mean ± SD.

did not have a significant adverse effect, evaluated on the basis of several biochemical and histological parameters. Biochemical parameters of liver function, such as hepatocyte integrity (AST and ALT), bile duct alterations (ALP), and liver function (bilirubin), did not reveal hepatic damage following administration of GGT. Moreover, GGT did not induce statistically significant alterations in the hematological profiles of rats, and no treatment-related gross pathologies and microscopic findings were observed. The absence of biologically significant differences in these toxicological endpoints supports the safety of GGT usage. In addition, evaluation of *in vitro* cytotoxicity of GGT against the BEAS-

2B, RBL-1, LNCaP cell lines, and splenocytes disclosed no cytotoxic effects up to concentrations of 200, 100, 2000, and 50 μg/ml, respectively. Accordingly, we suggest that GGT may have beneficial biological activities. Our findings collectively indicate that GGT does not exert toxic effects in male and female rats up to a concentration of 2000 mg/kg/day (the highest dose tested) upon a 4-week repeated oral administration. However, further investigations, including genotoxicity and subchronic toxicity studies, are required before reaching a definitive conclusion about the oral safety dose of GGT. Experiments should additionally be performed in a suitable non-rodent model before extrapolating the results in humans.

ACKNOWLEDGEMENT

This research was part of a project (The Evidence Based Medicine for Herbal Formula) funded by the Herbal Medicine EBM Research Center of the Korea Institute of Oriental Medicine.

REFERENCES

1. Watanabe S, Imanishi J, Satoh M, Ozasa K. Unique place of Kampo (Japanese traditional medicine) in complementary and alternative medicine: a survey of doctors belonging to the regional medical association in Japan. Tohoku J Exp Med 2001;194:55-63.
2. Castro LS, Perazzo FF, Maistro EL. Genotoxicity testing of *Ambelania occidentalis* (Apocynaceae) leaf extract in vivo. Genet Mol Res 2009;8:440-7.
3. Rousseaux CG, Schachter H. Regulatory issues concerning the safety, efficacy and quality of herbal remedies. Birth Defects Res B Dev Reprod Toxicol 2003;68:505-10.
4. Firenzuoli F, Gori L. Herbal medicine today: clinical and research issues. Evid Based Complement Alternat Med 2007;4:37-40.
5. Tang JL, Liu BY, Ma KW. Traditional Chinese medicine. Lancet 2008;372:1938-40.
6. Lee JT. Research on intake of Chinese medicine by Korea. Korean: Korea Food and Drug Administration; 2007.
7. Kim SJ, Jeong HJ, Moon PD, Lee KM, Lee HB, Jung HJ, *et al*. Anti-inflammatory activity of Gumiganghwaltang through the inhibition of nuclear factor-kappa B activation in peritoneal macrophages. Biol Pharm Bull 2005;28:233-7
8. Stewart MJ, Moar JJ, Steenkamp P, Kokot M. Findings in fatal cases of poisoning attributed to traditional remedies in South Africa. Forensic Sci Int 1999;101:177-83.
9. Ernst E. Toxic heavy metals and undeclared drugs in Asian herbal medicines. Trends Pharmacol Sci 2002;23:136-9.
10. Veiga-Junior VF, Pinto AC, Maciel MA. Medicinal plants: safe cure? Quim Nova 2005;28:519-28.
11. Matsuo R, Yamauch Y, Kobashi M, Funahashi M, Adachi A. Role of parabrachial nucleus in submandibular salivary secretion induced by bitter taste stimulation in rats. Auton Neurosci 2001;88:61-73.
12. Kim JC, Kang BH, Shin CC, Kim YB, Lee HS, Kim CY, *et al*. Subchronic toxicity of plant sterol esters administered by gavage to Sprague–Dawley rat. Food Chem Toxicol 2002;40:1569-80.
13. Burns RA, Wibert GJ, Diersen-Schade DA, Kelly CM. Evaluation of single cell sources of docosahexaenoic acid and arachidoinc acid: 3 month rat oral safety study with an in utero phase. Food Chem Toxicol 1999;**37:**23-36.
14. Peters JM, Boyd EM. Organ weights and water levels of the rat following reduced food intake. J Nutr 1966;90:354-60.
15. Pfeiffer CJ. A mathematical evaluation of the thymic weight parameter. Toxicol Appl Pharmacol 1968;13:220-7.
16. Lina BA, Wolterbeek AP, Suwa Y, Fujikawa S, Ishikura Y, Tsuda S, *et al*. Sub chronic (13 week) oral toxicity study preceded by an in utero exposure phase with arachidonate enriched triglyceride oil (SUNTGA40S) in rats. Food Chem Toxicol 2006;44:326-35.
17. Burn CC, Peters A, Day MJ, Mason GJ. Long-term effects of cage-cleaning frequency and bedding type on laboratory rat health, welfare, and handleability: a cross-laboratory study. Lab Anim 2006;40:353-70.

How to cite this article: Lee M, Shin I, Seo C, Kim J, Ha H, Huh J, Shin H. A 4-week repeated dose oral toxicity and cytotoxicity study of gumiganghwaltang in Crl:CD (SD) rats. Toxicol Int 2011;18:146-54.
Source of Support: Herbal Medicine EBM Research Center of the Korea Institute of Oriental Medicine. **Conflict of Interest:** None declared.