ORIGINAL ARTICLE

Acute and 13-week subchronic toxicity studies of hot-water extract of *Cynanchi wilfordii* Radix in Sprague-Dawley rats

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Abstract

Cynanchi wilfordii Radix (CWR) is a herbal medicinal plant that is well-known and used in Asian countries as a health food. In this study, acute and 13-week subchronic oral toxicity studies of hot-water extract of CWR (CWR-WE) were performed in Sprague-Dawley rats. For the acute toxicity study, CWR-WE was administered once orally to five male and five female rats at doses of 800, 2000, and 5000 mg/kg. Mortality, clinical signs, and body weight changes were monitored over 14 days. There were no treatment-related changes in these parameters and the approximate lethal dose of CWR-WE in male and female rats was determined to be > 5000 mg/kg. For the subchronic toxicity study, CWR-WE was administered orally once daily to male and female rats for 13 consecutive weeks at doses of 0 (vehicle control), 500, 1000, and 2000 mg/kg/day (n = 10 rats/sex/group). There were no toxicologically significant changes with regard to clinical signs, body weight, food consumption, ophthalmology, urinalysis, hematology, clinical chemistry, organ weights, necropsy findings, and histopathological findings. These results suggest that the oral no observed adverse-effect level of CWR-WE is > 2000 mg/kg/day for both sexes, although target organs were not identified.

Keywords *Cynanchi Wilfordii* radix · Acute oral toxicity · Subchronic oral toxicity

Abbreviations

CWR	<i>Cynanchi wilfordii</i> radix
CWR-WE	Hot-water extract of CWR
GLP	Good laboratory practice
Glu	Glucose
KHP	Korean herbal pharmacopoeia
MFDS	Ministry of food and drug safety
NOAEL	No observed adverse-effect level

SD	Sprague-Dawley
WBC	White blood cell

Introduction

Although the use of plant extract-derived herbal medicines for the treatment of a wide variety of clinical diseases continues to increase, relatively little information is available regarding their modes of action. Their safety was established long ago through direct application to the human body and via experimental selection and usage. However, certain natural substances show potential toxicity and their safety is of constant concern [1–3]. Therefore, conducting systematic studies examining the safety of compounds is essential, even if the compound is a natural substance-derived medicine or health functional food.

Cynanchi wilfordii Radix (CWR) is defined as the root of *Cynanchum wilfordii* Hemsley (Asclepiadaceae) in the Korean herbal pharmacopoeia (KHP) [4]. It is well-known and widely used in Korean Oriental medicine [5] and the various pharmacological actions of CWR-containing products have been reported, including hypocholesterolemic

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effects [6], immune regulatory effects [7], hair protective effects [8], anti-inflammatory, antioxidant, and antimicrobial activities [9–11], neuroprotective effects [12], gastroprotective effects [13], melatonin inhibitory effects [14], anti-hyperlipidemia effects [15, 16], antihypertensive effects [17], vasodilatory effects [18], as well as preventive and therapeutic effects against estrogen carcinogenesis in menopausal women [19]. CWR contains biologically active compounds as well as β -sitosterol, wilfosiide C1N, wilfosiide C3N, wilfosiide K1N, methyleugenol, wilfosiide C1G, cynauriculosiide A, daucosterol, 2,4-dihydroxyacetophenone, cynanchone A, cynandione A, 2,5-dihydroxyacetophenone, acetovanillone, P-hydroxyacetophenone, sucrose, conduritol F, geniposiide, succinic acid, 3-(-D-ribofuranosyl)-2,3-dihydro-6H-1,3-oxazine-2,6-dione, bungeisiide A, and cynanon-esiide B [20–23].

Although numerous studies have examined the pharmacological actions of CWR and its preclinical effects, there are few studies evaluating its safety [24].

In this study, the single oral dose and 13-week subchronic oral dose toxicity studies of hot-water extract of CWR (CWR-WE) were conducted in Sprague-Dawley (SD) rats, followed by qualitative and quantitative examinations. The aim of these studies was to acquire safety data for the application of a natural substance-based medicine, including information about its no observed adverse-effect level (NOAEL) and target organs.

Materials and methods

Preparation of CWR-WE

CWR used in the study was cultivated in Yeongju City, Gyeongsangbuk-do (309-22, Kkotdongsan-ro 7beon-gil, Yeongju, Korea) in 2015. Pure CWR was isolated and tested via polymerase chain reaction analysis, which is the official testing method described in the herbal medicine standards of KHP. Heavy metal and pesticide contents in the samples were measured according to the specifications and test methods for residual contaminants in herbal medicines recommended by the Ministry of Food and Drug Safety (MFDS). Briefly, test solutions were prepared via acid digestion of CWR, and concentrations of lead (Pb), arsenic (As), and cadmium (Cd) were analyzed via inductively coupled plasma mass spectrometry (X-series II, Thermo Fisher Scientific, Waltham, MA, USA). Mercury (Hg) concentration was measured using a mercury analyzer (SP-3DS, NIC, Tokyo, Japan). In addition, the following contaminants were measured via organic solvent extraction and gas chromatography: total dichlorodiphenyltrichloroethane (DDT), dieldrin, total benzene hexachloride (BHC), aldrin, and endrin. Heavy metal concentrations were below acceptable reference values

(Pb, 5 ppm; As, 3 ppm; Cd, 0.3 ppm; Hg, 0.2 ppm), and no residual pesticide was detected.

To prepare CWR-WE, dried CWRs were extracted three times with water at 100 °C for 4 h. The extracts were concentrated to 1/10 of the original volume and dried in a vacuum dryer at 50 °C for 24 h. The dried powder was ground in a mortar before passing through a 60-mesh sieve to obtain hot-water extract powder.

Preparation of dosing formulation

CWR-WE was prepared at appropriate concentrations by suspending in sterilized water for injection. Homogeneity and stability analyses for the subchronic toxicity study were conducted in a previous study. Dosing formulations were homogenous and stable for 4 h at room temperature and for 7 days under refrigeration. Following stability testing, dosing formulations were freshly prepared at least once a week and stored in refrigeration (3.9–5.4 °C).

The verification of the formulation concentrations via high-performance liquid chromatography (Prominence, Shimadzu Corp., Kyoto, Japan) using conduritol F as an analytic marker compound. Briefly, 5-mL sample aliquots were taken removed in triplicate from the middle layer of each batch prior to dosing and at week 13 of dosing period. Accuracy values for the 500, 1000, and 2000 mg/kg/day CWR-WE formulations were 87.92, 87.06, and 90.41%, respectively, prior to dosing, and 87.02, 86.94 and 88.12%, respectively, at week 13. These results were within an acceptable range of $\pm 20\%$ of nominal values.

Experimental animals and husbandry

Specific pathogen-free Crl:CD SD rats (5 weeks old) were obtained from Orient Bio Inc. (Seongnam, Korea) for use in acute and subchronic toxicity studies. During the study period, the animals were housed in a room maintained at a temperature of 22 ± 3 °C and a relative humidity of $50 \pm 20\%$, with artificial lighting from 07:00 to 19:00 at 150 to 300 Lux of luminous intensity and with 10–15 air changes/h. The animals were acclimatized to laboratory conditions for 7 days prior to the commencement of experiments. No more than three animals were housed together in stainless steel wire mesh cages (260 × 350 × 210 mm) during the quarantine and acclimation periods, and animal were housed individually during the treatment period. Each cage was labeled with the study number and animal identification number. Animals were provided ad libitum irradiation-sterilized pellet feed (Teklad certified irradiated global 18% protein rodent diet, 2918C, Harlan Co., Hayward, CA, USA) and groundwater disinfected by an ultraviolet sterilizer and ultrafiltration.

Study design overview

The present study was conducted at an institute that is approved by the MFDS for upholding good laboratory practice (GLP). The study was designed and conducted in keeping with the GLP and test guidelines of the Organization for Economic Cooperation and Development (OECD) and the MFDS. This study was also reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Biototech Co., Ltd (accredited by AAALAC International, 2008). IACUC approval numbers for the acute and repeated toxicity studies were 160086 and 160406, respectively.

Administration

The oral route was selected for the administration of CWR-WE, and it was administered directly into the stomach via gavage. In the acute toxicity study, animals were fasted overnight for approximately 16 h prior to dosing. Drinking water was provided ad libitum and food was provided approximately 4 h after administration. In the subchronic toxicity study, CWR-WE was administered orally once daily for 91 days without fasting. The administration volumes were 20 mL/kg/day for the acute toxicity study and 10 mL/kg/day for the repeated dose toxicity study based on the most recently measured body weights.

Acute oral dose toxicity study

A total of five male and five female rats (6 weeks old) per group were orally administered CWR-WE at doses of 0 (water injection, vehicle control), 800, 2000, and 5000 mg/kg. Body weights at beginning of the administration were 148.8–161.2 g for males and 122.3–136.2 g for females. All animals were observed for clinical signs (time of toxic symptom onset and recovery, etc.) and mortality at 30 min, 1, 2, 4, and 6 h after dosing and twice daily thereafter for the 14-day experimental period. Body weights were recorded once prior to dosing on the day of dosing and on Days 1, 3, 7 and 14 after dosing using an electronic balance (Sartorius, Göttingen, Germany). On day 14, all animals were euthanized with an overdose of CO₂ gas, exsanguinated via the abdominal aorta, and complete gross postmortem examinations were performed.

Subchronic oral toxicity study

Group designation The treatment doses for the 13-week subchronic toxicity study were selected based on the result of a preliminary 2-week repeated oral dose range finding toxicity study (Biototech study no.: R15020). Five male and five female rats (6 weeks old) per group were administered CWR-WE at doses of 0 (vehicle control), 500, 1000,

and 2000 mg/kg/day. No treatment-related changes were observed. Therefore, 2000 mg/kg/day was used as the high dose for the 13-week subchronic toxicity study, and the medium and low doses were set at 1000 and 500 mg/kg/day, respectively, by applying a geometric ratio of 1/2. Control animals received the vehicle only (water). A total of 10 male and 10 female rats (6 weeks old) per group were administered CWR-WE at doses of 0 (water, vehicle control), 500, 1000, and 2000 mg/kg/day. Body weights at the beginning of the administration were 197.3–217.5 g for males and 142.1–170.7 g for females.

Clinical signs and mortality During the administration and observation periods, animals were observed daily and the type, date of occurrence, and severity of any clinical signs or mortality were recorded individually.

Body weights Body weights were recorded prior to dosing treatment administration on day 1, once a week during the treatment period, and on the day of necropsy. However, body weights recorded on the day of necropsy were not analyzed statistically as these data were obtained when animals were fasted.

Food consumption Food consumption was measured from the day of group assignment to the first day of treatment. During the treatment period, the mean daily food consumption was calculated using the total amount consumed in 7 days. However, food consumption over 6 days was used to calculate the mean daily food consumption in week 13.

Ophthalmological examinations Ophthalmological examinations were conducted on both eyes of all animals at week 13. A mydriatic agent (isopto atropine 1%, Alcon, Fort Worth, TX, USA) was instilled into the eyes of rats prior to examination. The anterior segment of the eye, transparent media, and ocular fundus were observed using an ophthalmoscope (All Pupil II, Keeler, Windsor, UK).

Urinalysis A urinalysis was conducted on all animals in week 13. Fresh urine (within 3 h after excretion) and cumulative urine (over a period of approximately 24 h) samples were collected. During fresh urine sample collection, animals were not administered any treatment or food, although drinking water was provided ad libitum. Parameters for the urinalysis including urine volume, color, turbidity, sediment, specific gravity, pH, and levels of protein, glucose (Glu), ketone bodies, occult blood, and bilirubin were investigated using a Combur¹⁰ Test[®]M stick (Roche, Basel, Switzerland) and via urine chemistry analysis (cobas u 411, Roche).

Hematology Prior to necropsy, all animals were fasted overnight for approximately 18 h. Animals were then anesthetized with isoflurane (Ifran liquid, Hana Pharm, Co., Ltd, Seoul, Korea) and blood samples (approximately 1 mL) were collected from the abdominal aorta. Blood samples were placed in evacuated tubes containing K₂-EDTA as an anticoagulant (BD[™] Vacutainer, Becton–Dickinson, Franklin Lakes, NJ, USA) and used for hematological analysis. The

following hematological parameters were evaluated using an autoanalyzer (Advia 2120i, Siemens, Munich, Germany): red blood cell (RBC) count, hemoglobin (HGB) concentration, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocytes, platelet count, white blood cell (WBC) count, and WBC differential count, including neutrophils, lymphocytes, monocytes, eosinophils, and basophils. Next, 2 mL blood was dispensed into microtubes containing 0.2 mL 3.2% sodium citrate, and plasma was obtained via centrifugation at 3000 rpm for 10 min (Union 32R plus, Hanil Science Industrial, Incheon, Korea). The plasma prothrombin time (PT) and activated partial thromboplastin time were calculated in seconds using the coagulation time method with a coagulation time analyzer (Coapresta 2000, Sekisui, Tokyo, Japan).

Serum biochemistry The remaining blood samples collected for hematological examination were centrifuged at 3000 rpm for 10 min (Union 32R plus, Hanil Science Industrial) to obtain serum. The following parameters were then analyzed using an automatic analyzer (7180, Hitachi, Tokyo, Japan) and electrolyte analyzer (EasyLyte, Medica Corporation, Bedford, MA, USA): alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), total bilirubin (TBil), glucose (Glu), total cholesterol (TChol), triglyceride (TG), total protein (TP), albumin (Alb), albumin/globulin (A/G) ratio, blood urea nitrogen (BUN), creatinine (Crea), inorganic phosphorus (IP), and calcium (Ca). The electrolytes including sodium ion (Na^+), potassium ion (K^+), and chloride ion (Cl^-) were also analyzed.

Gross findings Complete gross postmortem examinations of the external surface and internal organs were performed on all animals, and all grossly visible abnormalities were recorded.

Organ weights The following organs were weighed using an electronic balance (Sartorius), with paired organs weighed together: brain, heart, pituitary gland, lungs, spleen, liver, kidneys, adrenal glands, testes, prostate gland, uterus, and ovaries. Weights were converted to the relative organ weights based on the organ-to-fasting body weight ratio.

Histopathological examination The following organs and tissues were fixed in 10% neutral-buffered formalin, excepting the testes and eyes, which were fixed in Davidson's fixative: brain, pituitary, thyroid, parathyroid, thymus, lungs (including bronchi), trachea, heart, liver, spleen, kidneys, adrenal glands, salivary glands (submandibular, sublingual, and parotid glands), tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, pancreas, testes, epididymides, prostate gland, seminal vesicle, ovaries, uterus, vagina, urinary bladder, submandibular lymph node, mesenteric lymph node, eyes, optic nerve and Harderian gland, mammary glands (inguinal), skin (inguinal), sternum

(including bone marrow), femur (including bone marrow), and thoracic spinal cord.

Microscopic examinations were performed on all organs and tissues from animals in the control and high-dose groups. Preserved organs and tissues were trimmed, dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin stain. All remaining organs and tissues were preserved in 10% neutral-buffered formalin. All gross lesions defined by the study pathologist were also included in this examination.

Statistical analysis

Statistical analyses of quantitative data including body weights, food consumption, urine volume, hematology, clinical chemistry, and organ weights were performed using the statistical analysis software (SAS) program (version 9.3, SAS Institute Inc., Cary, NC, USA). The results were expressed as mean $\pm$ standard deviation. In the acute toxicity and repeated oral dose toxicity studies, Bartlett's test was performed to assess the homogeneity of variance (significance level, 0.05). When the variances of all populations were assumed equal, a one-way analysis of variance (ANOVA) was conducted. Then, significance was further tested using Dunnett's test for multiple comparisons (significance levels, 0.05 and 0.01, two-tailed). When the variances were heterogeneous, a Kruskal–Wallis test was performed (significance level, 0.05).

Results

Acute oral toxicity study

No mortality was observed during the study period, and no treatment-related clinical signs were observed. Normal body weight gain was observed in both sexes in all dose groups during the study period. No abnormal gross findings were observed during necropsy in any of the animals (data not shown).

Subacute oral toxicity study

General findings No treatment-related deaths or abnormal clinical signs related to the administration of test article were observed. Furthermore, there were no treatment-related abnormal changes with regard to body weights, food consumption, and ophthalmoscopic examination in rats of either sex (data not shown).

Urinalysis No treatment-related changes were noted in the urinalysis findings (Table 1). All differences noted were thought to be a result of biological variations or were considered to be unrelated to the treatment because of their

Table 1 Urinalysis of male and female rats

Parameters	Result	Dose (mg/kg/day)							
		Male				Female			
		0	500	1000	2000	0	500	1000	2000
Glucose (mg/dL)	Negative	10	10	10	10	10	10	10	10
Bilirubin (mg/dL)	Negative	9	8	9	6	10	10	9	10
Ketone body (mg/dL)	1	1	2	1	4	0	0	1	0
	Negative	0	0	0	0	7	1	2	1
	5 (trace)	5	6	4	0	2	7	7	8
	15	5	4	6	8	1	0	1	1
Specific gravity	50	0	0	0	2	0	2	0	0
	1.011–1.020	0	0	0	0	2	0	0	0
	1.021–1.030	0	0	0	0	2	0	0	0
	1.031–1.040	0	0	1	0	2	2	1	1
	1.041–1.050	1	2	2	4	1	3	6	5
	1.051–1.060	3	5	3	1	3	2	2	2
pH	≥ 1.060	6	3	4	5	0	3	1	2
	6.0	0	0	1	2	1	3	3	4
	6.5	0	2	7	5	0	0	3	3
	7.0	5	3	2	3	0	1	2	0
	8.0	2	3	0	0	9	3	2	2
	9.0	3	2	0	0	0	3	0	1
Protein (mg/dL)	Negative	0	2	0	0	8	3	4	2
	25 (trace)	8	7	9	8	2	6	5	8
	75	2	1	1	2	0	1	1	0
Occult blood (Ery/uL)	Negative	7	8	7	7	9	10	10	10
	10	3	2	2	3	0	0	0	0
	25	0	0	0	0	1	0	0	0
	250	0	0	1	0	0	0	0	0
RBC (Ery/uL)	0	10	10	9	10	10	10	10	10
	> 100	0	0	1	0	0	0	0	0
WBC (Leko/uL)	0	10	10	10	10	10	10	10	10
Epithelial cell (mean/20 fields)	0	10	10	10	10	10	10	10	10
Casts (mean/field)	0	10	10	10	10	10	10	10	10
Urine color	Pale yellow	1	2	0	0	6	0	1	0
	Yellow	9	8	9	10	4	10	9	10
	Amber	0	0	1	0	0	0	0	0
Transparency	Clear	3	6	8	6	10	8	10	10
	Mild turbidity	7	4	1	3	0	1	0	0
	Turbidity	0	0	1	1	0	1	0	0
Urine volume* (mL/24 h)		7.5 ± 2.9	7.3 ± 3.4	8.8 ± 2.8	8.4 ± 4.3	9.7 ± 4.1	6.6 ± 3.1	7.7 ± 3.5	6.6 ± 2.4
No. of animals		10	10	10	10	10	10	10	10

*Values are mean $\pm$ SD

small magnitude and lack of relationship with the dose administered.

Hematology Results of the hematological parameters are shown in Table 2 and no treatment-related changes were noted.

Serum biochemistry Serum biochemical parameters are listed in Table 3 and no treatment-related changes were noted.

Gross findings There were no abnormal findings related to the administration of test article. Spleen enlargement

Table 2 Hematological values of male and female rats

Items	Dose (mg/kg/day)			
	0	500	1000	2000
<i>Male</i>				
RBC ($10^6/\mu\text{L}$)	8.62±0.35	8.84±0.33	8.75±0.38	8.82±0.14
Hemoglobin (g/dL)	14.8±0.5	14.7±0.4	14.9±0.7	14.7±0.4
Hematocrit (%)	46.2±1.4	46.0±0.9	46.4±1.7	45.9±0.9
MCV (fL)	53.7±1.3	52.1±1.7	53.0±1.2	52.1±1.6
MCH (pg)	17.2±0.4	16.6±0.6	17.1±0.6	16.7±0.6
MCHC (g/ μL)	32.0±0.6	31.9±0.4	32.2±0.6	32.0±0.3
Platelet ($10^3/\mu\text{L}$)	939±94	967±70	885±97	982±126
Reticulocyte (%)	3.14±1.38	2.59±0.49	2.62±0.49	2.34±0.37
WBC ($10^3/\mu\text{L}$)	9.82±2.12	9.39±3.17	9.56±2.01	9.61±2.23
Neutrophils (%)	23.8±6.5	24.5±8.5	19.1±6.0	23.1±7.9
Lymphocytes (%)	71.6±6.8	70.8±9.2	75.9±6.2	71.6±8.3
Monocytes (%)	2.3±0.5	2.2±0.6	2.4±0.6	2.7±1.0
Eosinophils (%)	1.1±0.3	1.4±0.5	1.4±0.3	1.3±0.2
Basophils (%)	0.3±0.1	0.2±0.1	0.3±0.1	0.3±0.1
PT (second)	18.1±0.3	18.3±0.9	18.5±1.0	18.8±0.5
APTT (second)	16.8±1.5	17.1±0.6	17.1±1.3	16.9±1.3
No. of animals	10	10	10	10
<i>Female</i>				
RBC ($10^6/\mu\text{L}$)	8.10±0.36	7.95±0.41	8.04±0.35	8.10±0.32
Hemoglobin (g/dL)	14.4±0.5	14.3±0.8	14.6±0.6	14.5±0.4
Hematocrit (%)	44.2±1.4	43.6±2.2	44.3±2.1	43.8±1.2
MCV (fL)	54.6±1.8	54.8±1.1	55.1±1.6	54.1±1.6
MCH (pg)	17.8±0.7	18.0±0.4	18.2±0.6	17.9±0.6
MCHC (g/ μL)	32.7±0.4	32.8±0.5	33.0±0.6	33.1±0.4
Platelet ($10^3/\mu\text{L}$)	926±64	906±90	937±111	998±162
Reticulocyte (%)	2.31±0.37	2.18±0.32	2.58±1.07	2.59±0.86
WBC ($10^3/\mu\text{L}$)	4.36±1.49	4.61±0.71	5.21±1.24	6.32±2.28
Neutrophils (%)	18.2±4.2	17.7±5.5	22.2±12.4	22.1±10.6
Lymphocytes (%)	76.6±4.4	77.5±6.0	72.5±12.8	73.0±10.8
Monocytes (%)	2.2±0.6	2.0±0.5	2.5±0.8	2.0±0.7
Eosinophils (%)	1.9±0.5	1.8±0.8	1.5±0.4	1.8±0.9
Basophils (%)	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1
PT (second)	18.1±0.6	18.0±0.7	18.0±0.9	18.9±1.0
APTT (second)	14.2±0.7	13.6±1.4	13.7±2.3	14.6±1.5
No. of animals	10	10	10	10

Values are mean ± SD

RBC red blood cell count, MCV mean corpuscular volume, MCH mean corpuscular hemoglobin, MCHC mean corpuscular hemoglobin concentration, WBC white blood cell count, PT prothrombin time, APTT active partial thromboplastin time

was noted in one male treated with 500 mg/kg/day CWR-WE and small seminal vesicle was observed in one male in the 1000 mg/kg/day group. Red focus in glandular stomach or yellow nodule in uterus was observed in two female mice treated with 2000 mg/kg/day of CWR-WE in each.

All macroscopic findings at necropsy were observed in low incidence and were considered to be incidental and not dose-related.

Organ weights Organ weight measurements are shown in Table 4. A statistically significant increase in the absolute and relative spleen weights was evident in females in the 2000 mg/kg/day group when compared to those of control group females ($p < 0.05$).

Histopathological examination Histopathological findings showed various lesions (Table 5), including hypertrophy and vacuolation in the adrenal gland, retinal atrophy and dysplasia in the eyes, focal lymphocytic inflammatory infiltration in the Harderian gland, mononuclear infiltration in the heart, mineralization in the ileum, hyperplasia, dilation, tubular mineralization or basophilia in the kidney, extramedullary hematopoiesis, fatty change, mononuclear infiltration and hepatocyte necrosis in the liver, arterial wall mineralization and bronchiolo-alveolar hyperplasia in the lung including bronchi, luteal cyst in the ovary, concretions and lymphocytic infiltration in the prostate, lymphocytic infiltration and basophilic hypertrophic focus in the salivary gland, atrophy in the pancreas, atrophy in the seminal vesicle, congestion and extramedullary hematopoiesis in the spleen, regeneration of mucous neck cell in the stomach, fibrosis and ultimobranchial cyst in the thyroid, cyst of squamous cell in the uterus and submucosa inflammation in the trachea.

Discussion

In this study, qualitative and quantitative examinations were performed after single-dose and 13-week repeated oral administrations of CWR-WE in SD rats. Evaluation of gross toxicity, NOAEL, and target organs confirmed the safety data of CWR-WE as a functional food.

In the acute oral toxicity study, no test article-related deaths or abnormalities in clinical signs, body weights, and necropsy finding were observed. In the 13-week subchronic oral toxicity study, no test article-related deaths or abnormalities with regard to clinical signs were observed. In addition, no significant changes in body weight, food consumption, ophthalmoscopic examination, urinalysis, hematological and serum biochemical examinations, necropsy findings and histopathological examination were observed in relation to the administration of the test article.

A statistically significant increase was noticed in the absolute and relative spleen weights in females in the 2000 mg/kg/day treatment group, although this change was not considered to be of toxicological significance as no changes in spleen-associated parameters were observed in the hematological and serum biochemical examinations

Table 3 Serum biochemical values of male and female rats

Items	Dose (mg/kg/day)			
	0	500	1000	2000
<i>Male</i>				
ALT (IU/L)	32.9 ± 10.5	29.1 ± 11.1	28.7 ± 4.1	36.4 ± 27.7
AST (IU/L)	97.4 ± 18.9	103.1 ± 37.4	97.6 ± 21.4	116.7 ± 57.4
ALP (IU/L)	302.9 ± 77.2	269.7 ± 74.2	288.8 ± 54.5	246.2 ± 42.5
GGT (IU/L)	0.33 ± 0.11	0.39 ± 0.21	0.33 ± 0.10	0.79 ± 1.39
Glucose (mg/dL)	129 ± 9	132 ± 8	130 ± 12	132 ± 10
BUN (mg/dL)	12.3 ± 1.4	12.7 ± 1.7	12.7 ± 1.3	12.6 ± 1.5
Creatinine (mg/dL)	0.45 ± 0.04	0.47 ± 0.03	0.47 ± 0.03	0.46 ± 0.03
TBil (mg/dL)	0.06 ± 0.02	0.05 ± 0.02	0.03 ± 0.02	0.06 ± 0.05
TCho (mg/dL)	74 ± 18	75 ± 19	70 ± 12	82 ± 19
Triglyceride (mg/dL)	60 ± 26	59 ± 29	59 ± 27	55 ± 23
TP (g/dL)	5.9 ± 0.1	6.1 ± 0.3	6.0 ± 0.2	6.0 ± 0.3
Albumin (g/dL)	2.4 ± 0.1	2.4 ± 0.1	2.4 ± 0.1	2.4 ± 0.1
A/G (ratio)	0.68 ± 0.03	0.67 ± 0.05	0.69 ± 0.04	0.68 ± 0.06
IP (mg/dL)	5.70 ± 0.49	5.82 ± 0.43	6.03 ± 0.62	5.81 ± 0.56
Ca (mg/dL)	10.0 ± 0.2	10.1 ± 0.2	10.0 ± 0.3	10.0 ± 0.3
Na ⁺ (mmol/L)	138.4 ± 1.6	138.5 ± 1.1	138.3 ± 0.7	137.9 ± 1.4
K ⁺ (mmol/L)	4.22 ± 0.15	4.28 ± 0.23	4.23 ± 0.23	4.27 ± 0.16
Cl ⁻ (mmol/L)	106.4 ± 1.6	106.8 ± 0.9	106.3 ± 1.1	106.2 ± 1.6
No. of animals	10	10	10	10
<i>Female</i>				
ALT (IU/L)	27.3 ± 7.3	27.4 ± 5.9	31.3 ± 24.7	30.0 ± 21.7
AST (IU/L)	88.3 ± 23.8	97.6 ± 19.2	119.0 ± 51.6	103.7 ± 48.1
ALP (IU/L)	133.2 ± 49.8	149.9 ± 46.5	152.5 ± 26.4	143.2 ± 35.1
GGT (IU/L)	0.59 ± 0.15	0.73 ± 0.34	0.64 ± 0.30	0.67 ± 0.14
Glucose (mg/dL)	129 ± 9	133 ± 9	134 ± 16	129 ± 8
BUN (mg/dL)	14.6 ± 1.7	14.2 ± 2.1	14.1 ± 1.4	14.1 ± 1.3
Creatinine (mg/dL)	0.51 ± 0.03	0.51 ± 0.04	0.51 ± 0.04	0.51 ± 0.04
TBil (mg/dL)	0.09 ± 0.04	0.07 ± 0.01	0.08 ± 0.05	0.08 ± 0.04
TCho (mg/dL)	82 ± 15	91 ± 22	91 ± 25	97 ± 23
Triglyceride (mg/dL)	22 ± 9	30 ± 12	30 ± 14	40 ± 33
TP (g/dL)	6.4 ± 0.4	6.5 ± 0.3	6.5 ± 0.5	6.4 ± 0.4
Albumin (g/dL)	2.9 ± 0.2	2.9 ± 0.3	3.0 ± 0.3	2.8 ± 0.3
A/G (ratio)	0.82 ± 0.04	0.82 ± 0.09	0.83 ± 0.07	0.80 ± 0.10
IP (mg/dL)	4.34 ± 0.82	4.55 ± 0.44	4.70 ± 0.56	4.70 ± 0.57
Ca (mg/dL)	10.0 ± 0.4	10.0 ± 0.4	10.0 ± 0.4	10.0 ± 0.3
Na ⁺ (mmol/L)	137.7 ± 1.0	137.6 ± 1.0	137.4 ± 0.9	136.9 ± 1.6
K ⁺ (mmol/L)	4.04 ± 0.24	3.95 ± 0.19	4.03 ± 0.27	3.91 ± 0.37
Cl ⁻ (mmol/L)	107.8 ± 1.4	107.5 ± 1.4	107.0 ± 1.3	106.7 ± 1.3
No. of animals	10	10	10	10

Values are mean ± SD

ALT alanine aminotransferase, AST aspartate aminotransferase, ALP alkaline phosphatase, GGT gamma glutamyl transpeptidase, BUN blood urea nitrogen, TBil total bilirubin, TCho total cholesterol, TP total protein, A/G albumin/globulin, IP inorganic phosphorus

and no accompanying morphological changes were revealed via histopathological analysis. Histopathological lesions observed in the treatment and control groups

may be due to spontaneous generation in 20-week-old SD rats, and these nonspecific changes were considered to be unrelated to the test article [25–27].

Table 4 Absolute and relative organ weights of male and female rats

Items	Dose (mg/kg/day)			
	0	500	1000	2000
<i>Male</i>				
Body weight ^a (g)	535.8 ± 63.9	539.3 ± 50.8	532.2 ± 48.4	547.1 ± 48.6
Brain (g)	2.18 ± 0.10	2.16 ± 0.11	2.13 ± 0.09	2.15 ± 0.07
% to body weight	0.41 ± 0.05	0.40 ± 0.03	0.40 ± 0.04	0.40 ± 0.03
Pituitary (g)	0.0117 ± 0.0019	0.0122 ± 0.0018	0.0120 ± 0.0013	0.0122 ± 0.0014
% to body weight	0.0022 ± 0.0003	0.0023 ± 0.0002	0.0023 ± 0.0002	0.0022 ± 0.0002
Heart (g)	1.55 ± 0.16	1.57 ± 0.12	1.55 ± 0.08	1.59 ± 0.15
% to body weight	0.29 ± 0.02	0.29 ± 0.02	0.29 ± 0.02	0.29 ± 0.02
Lungs (g)	1.62 ± 0.15	1.72 ± 0.07	1.67 ± 0.17	1.64 ± 0.19
% to body weight	0.30 ± 0.03	0.32 ± 0.03	0.32 ± 0.03	0.30 ± 0.03
Liver (g)	13.36 ± 2.36	14.19 ± 2.09	13.33 ± 1.52	14.29 ± 2.18
% to body weight	2.49 ± 0.22	2.62 ± 0.23	2.50 ± 0.13	2.60 ± 0.19
Spleen (g)	0.96 ± 0.18	1.02 ± 0.36	0.93 ± 0.10	0.91 ± 0.11
% to body weight	0.18 ± 0.03	0.19 ± 0.06	0.18 ± 0.02	0.17 ± 0.02
Kidneys (g)	3.24 ± 0.37	3.22 ± 0.33	3.19 ± 0.21	3.38 ± 0.43
% to body weight	0.61 ± 0.07	0.60 ± 0.04	0.60 ± 0.05	0.62 ± 0.04
Adrenals (g)	0.0622 ± 0.0094	0.0601 ± 0.0081	0.0572 ± 0.0097	0.0618 ± 0.0093
% to body weight	0.0116 ± 0.0015	0.0112 ± 0.0013	0.0109 ± 0.0021	0.0113 ± 0.0017
Testes (g)	3.59 ± 0.2	3.60 ± 0.23	3.60 ± 0.34	3.43 ± 0.32
% to body weight	0.68 ± 0.12	0.67 ± 0.04	0.68 ± 0.08	0.63 ± 0.08
Prostate gland (g)	0.59 ± 0.20	0.55 ± 0.17	0.60 ± 0.16	0.61 ± 0.13
% to body weight	0.11 ± 0.04	0.10 ± 0.03	0.11 ± 0.04	0.11 ± 0.03
No. of animals	10	10	10	10
<i>Female</i>				
Body weight ^a (g)	285.5 ± 27.4	296.5 ± 24.3	297.2 ± 28.3	289.0 ± 30.1
Brain (g)	1.96 ± 0.10	2.03 ± 0.11	1.98 ± 0.10	2.03 ± 0.08
% to body weight	0.69 ± 0.08	0.69 ± 0.07	0.67 ± 0.07	0.71 ± 0.07
Pituitary (g)	0.0162 ± 0.0028	0.0180 ± 0.0046	0.0171 ± 0.0024	0.0175 ± 0.0021
% to body weight	0.0057 ± 0.0010	0.0061 ± 0.0014	0.0058 ± 0.0007	0.0061 ± 0.0009
Heart (g)	0.95 ± 0.07	0.96 ± 0.09	0.98 ± 0.07	0.98 ± 0.12
% to body weight	0.33 ± 0.02	0.32 ± 0.02	0.33 ± 0.02	0.34 ± 0.02
Lungs (g)	1.16 ± 0.14	1.19 ± 0.19	1.16 ± 0.08	1.22 ± 0.06
% to body weight	0.41 ± 0.02	0.40 ± 0.05	0.39 ± 0.03	0.43 ± 0.04
Liver (g)	7.02 ± 0.71	7.60 ± 0.82	7.66 ± 0.84	7.67 ± 1.02
% to body weight	2.47 ± 0.21	2.57 ± 0.24	2.58 ± 0.23	2.65 ± 0.19
Spleen (g)	0.54 ± 0.07	0.56 ± 0.09	0.61 ± 0.08	0.63 ± 0.05*
% to body weight	0.19 ± 0.02	0.19 ± 0.03	0.20 ± 0.02	0.22 ± 0.03*
Kidneys (g)	1.81 ± 0.15	1.94 ± 0.20	1.89 ± 0.13	1.93 ± 0.17
% to body weight	0.64 ± 0.05	0.66 ± 0.07	0.64 ± 0.06	0.67 ± 0.03
Adrenals (g)	0.0672 ± 0.0066	0.0720 ± 0.0163	0.0727 ± 0.0082	0.0697 ± 0.0111
% to body weight	0.0236 ± 0.0021	0.0244 ± 0.0052	0.0247 ± 0.0035	0.0242 ± 0.0034
Ovary (g)	0.0823 ± 0.0153	0.0856 ± 0.0180	0.0858 ± 0.0102	0.0857 ± 0.0221
% to body weight	0.0288 ± 0.0042	0.0290 ± 0.0060	0.0291 ± 0.0048	0.0298 ± 0.0074
Uterus (g)	0.65 ± 0.24	0.72 ± 0.26	0.81 ± 0.30	0.73 ± 0.21
% to body weight	0.23 ± 0.09	0.24 ± 0.09	0.27 ± 0.10	0.26 ± 0.08
No. of animals	10	10	10	10

Significantly different from vehicle control: * $p < 0.05$. Values are mean ± SD^aThe body weights were measured right before necropsy after overnight fasting

Table 5 Histopathological changes of male and female rats

Organs	Lesions	Male Dose (mg/kg/ day)		Female Dose (mg/ kg/day)	
		0	2000	0	2000
Adrenal gland	Hypertrophy, zona glomerulosa, multifocal	1	1	0	0
	Vacuolation, cortical, diffuse	2	0	0	0
Eye	Atrophy, retinal, focal	1	0	1	1
	Dysplasia, retinal, focal	1	1	1	0
Harderian gland	Infiltrate, lymphocytic inflammatory	0	1	1	0
Heart	Infiltrate, mononuclear, myocardium	1	1	0	1
Kidneys	Hyperplasia, oncocytic, focal	1	0	1	1
	Tubular basophilia, focal	2	1	1	2
	Dilation, tubule, focal	0	0	1	0
	Mineralization, tubular	0	0	0	1
Liver	Extramedullary hematopoiesis	1	0	0	0
	Fatty change, periportal	0	1	0	1
	Infiltration, mononuclear, multifocal	2	3	2	3
	Necrosis, hepatocyte, multifocal	0	1	0	0
Lung including bronchi	Mineralization, arterial wall, focal	2	0	1	0
	Hyperplasia, bronchiolo-alveolar, focal	0	0	0	1
Prostate	Concretions	1	0	–	–
	Infiltration, lymphocytic, interstitial	0	1	–	–
Salivary gland	Basophilic hypertrophic focus	0	1	0	0
	Infiltrate, lymphocytic, focal	0	0	1	0
Spleen	Extramedullary hematopoiesis	3	1	1	1
	Congestion	1	0	0	1
Thyroid	Fibrosis, unilateral	0	1	0	0
	Ultimobranchial cyst	0	0	0	1
Trachea	Inflammation, acute, submucosa	1	0	0	0
Ileum	Mineralization, Peyer's patch, focal	0	0	0	1
Pancreas	Atrophy, acinar, focal	0	0	0	1
Stomach	Regeneration, mucous neck cell, focal	0	0	0	1
Uterus	Cyst, squamous, cervix	–	–	–	1
Ovary	Cyst, luteal	–	–	0	2
	Decreased number/absent corpora lutea	–	–	0	1
No. of animals		10	10	10	10

–Not applicable

In conclusion, no deaths occurred in the acute oral toxicity study. Therefore, the approximate lethal dose of CWR-WE was determined to be higher than 5000 mg/kg in male and female rats. In the subchronic toxicity study, no toxicologically significant changes were observed in any CWR-WE-treated animal. These results suggest that the NOAEL of CWR-WE is greater than 2000 mg/kg/day in both sexes, and the target organs were not identified.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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