Safety evaluation of *Chlorella vulgaris* cultivated under heterotrophic conditions by single and repeated oral dose toxicity tests in animal models

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Safety evaluation of *Chlorella vulgaris* cultivated under heterotrophic conditions by single and repeated oral dose toxicity tests in animal models

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2014 년 12 월 면접보는 날부터 지금까지 만학도인 저를 입학동기라고 챙겨준 민호쌤과 화진쌤, 그리고 2020 년에 같이 졸업하자며 응원해준 졸업동기 지숙쌤에게도 감사한 마음을 전합니다. 본인의 경험을 후배에게 아낌없이 풀어주신 장혜윤 박사님, 행정업무에 도움을 주신 구정임 조교선생님과 정지수 조교선생님께도 감사인사를 드립니다.

입학할 때 그리고 졸업을 하는 지금도 많은 배려해주신 전진영 팀장님, 부족한 영어에 도움 주신 강성준 박사님, 든든한 응원군이신 한재갑 부장님을 비롯한 회사 동료분들께도 글로나마 감사의 말씀을 드립니다. 또한 이 연구에 참여하신 ㈜켐온 관계자들의 노고와 실험동물들의 희생을 감사히 생각하겠습니다.

언제나 우리딸 믿는다고 말씀하시며 제가 세상에 당당히 맞설 용기를 한 없이 불어넣어 주시는 부모님, 시도 때도 없이 논문 작업을 도와준 홍근이, 그리고 지난해 하늘로 가신 아버님과 홀로되신 어머님께도 그동안 못 전한 감사의 마음을 전달합니다. 저 보다 더 이 논문의 완성을 기다리며 집안일과 육아로 힘들었을 서영덕님과 공부하는 엄마 때문에 섭섭했을 우리집 보물 1 호 재인이에게도 고마움과 사랑을 듬뿍 드립니다.

마지막으로 이 논문을 끝이 아닌 또 다른 시작으로 여기며, 몸과 마음이 건강한 김병곤이 되라고 저에게 응원을 보냅니다.

2020 년 6 월

김 병 곤

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ABBREVIATIONS

ADI	Acceptable daily intake
A/G ratio	Albumin/globulin ratio
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APTT	Active partial thromboplastin time
AST	Aspartate aminotransferase
BUN	Blood urea nitrogen
Ca	Calcium
CHO	Total cholesterol
Cl	Chloride
CPK	Creatine phosphokinase
CRE	Creatinine
GLU	Glucose
HCT	Hematocrit
HGB	Hemoglobin concentration
IP	Inorganic phosphorus
K	Potassium

MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MLD	Minimum lethal dose
Na	Sodium
NOAEL	No observable adverse effect level
PLT	Platelet
PRO	Total protein
PT	Prothrombin time
RBC	Red blood cell
SPF	Specific pathogen free
T-BIL	Total bilirubin
TG	Triglyceride
WBC	White blood cell

ABSTRACT

Safety evaluation of *Chlorella vulgaris* cultivated under heterotrophic conditions by single and repeated oral dose toxicity tests in animal models

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Aims: Chlorella is a unicellular green algae that is mainly used as a dietary supplement or food. There are several species in the *Chlorella* genus, including *Chlorella vulgaris*. The study aimed to evaluate the safety of *C. vulgaris* cultivated under heterotrophic conditions as a food supplement.

Methods: The chlorella sample (*C. vulgaris*) used in this study was obtained from Daesang Corp. (Seoul, Korea). It was cultured under heterotrophic conditions with

glucose as a carbon source. A single oral dose toxicity test was conducted to evaluate the acute toxicity of *C. vulgaris* in rodents and non-rodents. The subacute toxicity was examined by repeated oral dose toxicity test in rodents for 13 weeks. In a single oral dose toxicity test in Sprague-Dawley (SD) male (n=15) and female (n=15) rats, *C. vulgaris* was administered orally at 0, 5,000, and 10,000 mg/kg and then mortality rate, general symptoms, changes in body weight, and autopsy observation were observed for 2 weeks after treatment. For the single oral dose toxicity test in male (n=6) and female (n=6) beagle dogs, *C. vulgaris* was administered orally at 0, 2,000, and 5,000 mg/kg. In the repeated oral doses toxicity test, SD male (n=40) and female (n=40) rats were treated with *C. vulgaris* at doses of 0, 300, 1,000, and 2,000 mg/kg/day; moreover, mortality, general symptoms, body weight, food and water intake, and organ weight were measured. Eye test, urinalysis, hematological test, blood coagulation time test, blood biochemical test, autopsy observation, and histopathological test were conducted.

Results: In a single oral dose toxicity test in SD rats, there were no animal deaths in all test groups. Although Polyuria was observed in all test groups and chlorella-colored feces was observed in the groups treated with 5,000 and 10,000 mg/kg of test substance. There were no significant changes in body weight and autopsy results. Thus, the minimum lethal dose (MLD) of *C. vulgaris* in rats was determined at more than 10,000 mg/kg. In beagle dogs, 5,000 mg/kg dose administration caused chlorella-colored feces and diarrhea. But there were no animal deaths and no abnormal observations in all test groups.

Therefore, the MLD of *C. vulgaris* in dogs was more than 5,000 mg/kg. In the repeated oral doses toxicity test, there were no animal deaths and no significant changes caused by *C. vulgaris*. Therefore, the no observed adverse effect level of *C. vulgaris* in rats was found to be more than 2,000 mg/kg/day, based on the highest dose.

Conclusion: The safety tests results showed that *C. vulgaris* led to no animal deaths and no significant toxic effects in our tested conditions. Therefore, *C. vulgaris* might be considered safe as a food and dietary supplement under the present dosage conditions. In addition, to estimate the acceptable daily intake, further studies are needed to subacute toxicity test for excess amount of chlorella and chronic toxicity test.

Keywords: Chlorella, *Chlorella vulgaris*, Acute toxicity, Subacute toxicity, Toxicity, Safety

I. INTRODUCTION

Microalgae or microscopic algae are usually found in freshwater and marine environments and have the ability to convert solar energy into chemical energy. Microalgae are widely consumed as health foods, carotenoid supplements, fatty acid supplements, and animal feed. Representative commercial products of microalgae as health foods or dietary supplements are chlorella, spirulina, and dunaliella. Commercial large-scale culture of chlorella was started in the early 1960's in Japan by "Nihon Chlorella" [1, 2].

Chlorella have been widely consumed as food [1, 2]. It contains essential amino acids, proteins, vitamins, minerals, and bioactive substances, such as chlorophyll, lutein, and β-carotene [2-5]. Chlorella has been shown to have health benefits including skin health-improving [6, 7], antioxidant [8-10], blood cholesterol-lowering [11-14], and immune-enhancing [15-18] effects.

Several species, including *Chlorella vulgaris*, *C. protothecoides*, *C. sorokiniana*, and *C. pyrenoidosa,* have been identified [19-21]. Among them, *C. protothecoides* strain S106 was generally recognized as safe (GRAS) by the US FDA 22]. There has been no report of chlorella's serious negative effects in humans [1, 10, 14, 15, 23, 24]. However, the components of chlorella may be different based on the culture conditions and strains [25, 26]. Safety evaluation studies have been performed on *C. sorokiniana* strain CK-22 [27], protein from *C. protothecoides* [28], high-lipid biomass from *C. protothecoides* [29],

carotenogenic *C. vulgaris* of orange color (which results from the carotenogenesis process) [30], They were reported that was safe under the tested conditions. But these studies were safety evaluation for chlorella cultured in different species or in different conditions from *C. vulgaris* conducted in this study. Neumann et al. [31] assessed the safety about histological parameter of *C. vulgaris* for 14-day repeated oral dose. There have been no studies on single and 13-week repeated oral dose toxicity test on biomass of green *C. vulgaris*, which is the most consumed chlorella supplement in Korea [32].

Therefore, this study investigated the safety of green *C. vulgaris* cultivated under heterotrophic conditions as a food and dietary supplement through single oral dose toxicity test in rodents and non-rodents as well as through repeated oral dose toxicity test in rodents.

II. BACKGROUND

1. Characteristics of chlorella

Chlorella is a eukaryotic microalgae that grows mainly in freshwaters, such as ponds and lakes. It has existed on the earth since the Precambrian period. It was discovered by a Dutch microbiologist, Martinus W. Beijerinck in 1890 [33]. It is spherical and approximately 2 to 10 μm in diameter. The reproductive rate of chlorella is faster than that of other industrial microalgae, capable of renewing into four new cells within 24 h [5]. The name chlorella was taken from “Chloros”, meaning green in Greek, and “'ella”, meaning small in Latin [34]. The characteristic green color and peculiar odor of chlorella are due to its high chlorophyll content. However, depending on the culture conditions, it may have a different colors, such as orange, rather than green [26, 30].

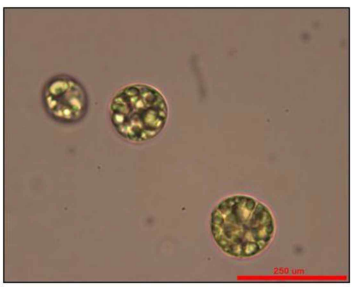

Figure 1. Optical micrograph of *C. vulgaris* cultivated under heterotrophic conditions (at 400×magnification)

Table 1. Taxonomic classification of *C. vulgaris*

Domain	Eukaryota
Phylum	Chlorophyta
Class	Trebouxiophyceae
Order	Chlorellales
Family	Chlorellaceae
Genus	*Chlorella*
Species	*Chlorella vulgaris*

Adapted from NCBI database [19] and Champenois et al. [20]

Chlorella belongs to the phylum Chlorophyta (Table 1), and several species, including *C. vulgaris*, *C. protothecoides*, *C. sorokiniana*, and *C. pyrenoidosa,* have been identified [19-21]. *C. vulgaris* can grow under autotrophic, heterotrophic, or mixotrophic condition [5, 31]. Autotrophic cultivation is a method of cultivation using light without a carbon source. It is generally cheaper than heterotrophic cultivation, which involves artificial supply of a carbon source, but has low productivity. Heterotrophic cultivation uses an organic carbon source, such as glucose. Thus, it is more productive, but costs more than autotrophic cultivation [25]. However, the economic efficiency of heterotrophic cultivation can be increased compared with that of autotrophic cultivation by developing strains and improving the culture conditions. Mixotrophic cultivation is a mixture of autotrophic and heterotrophic cultivations, with high productivity and reduced amount of the organic substrate used for biomass growth. However, it has not been used industrially owing to facility difficulties. There are differences in component between heterotrophic (indoor) and autotrophic (outdoor) cultivations (Table 2) [25, 35].

According to Yang et al. [36] energetics and carbon metabolism during the growth of microalgae vary depending on the culture conditions. Therefore, components such as pigment and lipid composition of chlorella are different depending on the culture conditions, even within the same species [26].

Table 2. Comparison of components between indoor (heterotrophic) and outdoor (autotrophic) chlorella

Component	Indoor Chlorella (mg/100 g)	Outdoor Chlorella (mg/100 g)
Chlorophyll	2,800 - 3,600	1,000 - 3,000
Carotene	70 - 120	5 - 50
Vitamin C	30 - 60	8 - 91
Calcium	80 - 1,200	80 - 170
Iron	40 - 50	70 - 200
Extract	17,000 - 21,000	14,000 - 26,000
Digestibility	82%	82%

Adapted from Jeon [35]

2. Nutrients of chlorella

Chlorella contains various nutrients, including proteins, essential amino acids, vitamins, and minerals, as well as many phytochemicals, such as chlorophyll [1, 2, 5]. Chlorella has a solid cell wall, which is mainly composed of cellulose, hemicellulose, proteins, lipids, and minerals. The sugar composition of the cell wall is a mixture of rhamnose, galactose, glucose, xylose, arabinose, and mannose [5]. The protein content of chlorella is 42%–61% of the dry cell weight [37-39]. It is a high-protein food, with higher protein content than that of chicken eggs (Table 3) [39]. Chlorella contains more amino acids than beef, except for cysteine (Table 4) [39]. It also contains lipids (14%–22% of the dry mass), carbohydrates (12%–17% dry weight) [40], and bioactive pigment substances, such as chlorophyll, lutein, astaxanthin, and β-carotene [5, 41, 42] (Table 5). Chlorella has high content of magnesium, calcium, and iron, which are needed for maintaining normal heart functions, blood formation, and circulation [5]. Chlorella is an excellent food that can improve the unbalanced diet of modern people, as it contains various nutrients.

Table 3. Comparison of biochemical properties and composition of chlorella, spinach, milk, and chicken egg (per 100g)

Component	Chlorella*	Spinach	Milk	Chicken egg
Protein	60.6 g	3.3 g	2.9 g	12.3 g
Carbohydrate	3.7 g	3.6 g	4.5 g	0.9 g
Fat	12.8 g	0.2 g	3.2 g	11.2 g
Ash	4.5 g	1.7 g	0.7 g	0.9 g
Fiber	13.0 g	3.5 g	-	-
Vitamin A	58,900 IU	2,900 IU	1,100 IU	640 IU
Vitamin B_1	1.29 mg	0.13 mg	0.03 mg	0.08 mg
Vitamin B_2	4.55 mg	0.23 mg	0.15 mg	0.48 mg
Niacin	32.1 mg	0.6 mg	0.1 mg	0.1 mg
Vitamin C	74 mg	65 mg	0 mg	0 mg
Vitamin E	22.8 mg	2.1 mg	0.1 mg	1.1 mg
Energy	372 kcal	25 kcal	59 kcal	162 kcal

* Chlorella made by pure culture using a fermenter Adapted from Kang et al. [39]

Table 4. Comparison of the amino acid composition of chlorella and beef (per 100g)

Amino acids	Chlorella* [A]	Beef (Fat-free) [B]	Relative Content (%) [A/B × 100]
Arginine	3.51 g	1.20 g	292.5
Lysine	4.88 g	1.70 g	287.1
Histidine	1.16 g	0.75 g	155.0
Phenylalanine	2.48 g	0.77 g	322.1
Tyrosine	1.64 g	0.63 g	260.3
Leucine	4.52 g	1.60 g	282.5
Isoleucine	1.04 g	0.88 g	118.2
Methionine	1.20 g	0.22 g	545.5
Valine	3.14 g	0.92 g	341.3
Alanine	4.38 g	1.10 g	398.2
Threonine	1.38 g	0.89 g	155.1
Tryptophan	1.01 g	0.21 g	481.0
Cysteine	0.71 g	0.76 g	93.4
Glutamic acid	6.60 g	2.90 g	227.6
Aspartic acid	4.86 g	1.80 g	270.0

* Chlorella made by pure culture using a fermenter Adapted from Kang et al. [39]

Table 5. Potential pigments content in *C. vulgaris* under different growth conditions

Pigments	μg/g (dw)
β-Carotene	7 - 12,000
Astaxanthin	555,000
Cantaxanthin	362,000
Lutein	52 - 3,830
Chlorophyll-a	250 - 9,630
Chlorophyll-b	72 - 5,770
Pheophytin-a	2,310 - 5,640
Violoxanthin	10 - 37

Adapted from Safi et al. [5]

3. Health benefits of chlorella

Because chlorella contains various nutrients, chlorella has been investigated as a future food to solve the food shortage problem caused by population growth after World War II [43]. Since various health benefits of chlorella have been studied, it is now consumed as a dietary supplement, and not as food [44-46].

In Korea, four health claims of chlorella have been approved by the Ministry of Food and Drug Safety (MFDS): skin health-improving [6, 7], antioxidant [8-10], blood cholesterol-lowering [11-14], and immune-enhancing [15-18] effects. Chlorella has a skin-protective effect against UVB via production of MMP-1 and degradation of procollagen genes in human skin fibroblasts by [6], and protects against UVC-induced cytotoxicity [7]. Chlorella improves antioxidative capacity in rats with oxidative stress by reducing the production of radicals and promoting the capture and elimination of radicals [8, 9]. Ryu et al. [14] revealed that chlorella has beneficial health effects on the serum lipid profiles of mildly hypercholesterolemic subjects by improving serum carotenoid profiles in humans. Chlorella enhances the activity of NK cells and produces interferon-γ and interleukin-12 in humans [15]. Chlorella also increases the production of Th1-type cytokines, such as IFN-γ and IL-12, while not affecting Th2-type cytokines, such as IL-4, *in vivo* [18].

According to studies in rats, dietary chlorella has a protective effect on the toxicity of heavy metals, such as cadmium-induced liver damage and lead-induced oxidative

stress [47-49]. Moreover, it excretes dioxin-like PCB-138 and PCB-153 via urine [50]. In addition, it protects rats from heterocyclic amine-induced aberrant gene expression by increasing fecal excretion of unmetabolized PhIP [51].

Jung et al. [52] proved that chlorella could be a source of lutein, which helps maintain eye health; similar to lutein extracted from marigold flower, lutein from chlorella is absorbed from plasma.

III. MATERIALS AND METHODS

To evaluate the safety of substances for the human body, it is dangerous to test it directly on the human body; thus, a toxicity test is conducted on animals. General toxicity test (single- and repeated-dose) and genetic toxicity study are basics for safety assessment. Reproductive toxicity study, immunotoxicity study, and carcinogenicity study are conducted according to the characteristics of the raw materials [53]. In this study, acute toxicity was tested by a single dose toxicity study in rodents, the most widely used test animal in general toxicity studies. However, as the bioreaction to the test substance may be different depending on the type of animal, a single toxicity test was additionally performed in beagle dogs. Among non-rodents, beagle dog is easy to test, and many basic test data on beagle dog have accumulated; thus, these data can be used for interpreting and evaluating our test result. In addition, subacute toxicity test was performed through repeated 13-week administration in rodents to evaluate the toxicity that may occur during continuous medium-term intake.

The research was pre-approved by the animal care and use committee at Preclinical Research Center of ChemOn Corporation (06-RA-097, 06-DA-098, 06-RR-100) and was carried out in accordance with the “Toxicity Test Standards of Drug Medicine and the Like” of Korea’s MFDS Notification No. 2017-71 (August 30, 2017) and “Good Laboratory Practice” of Korea’s MFDS Notification No. 2018-93 (November 21, 2018).

1. Single oral dose toxicity test in Sprague-Dawley (SD) rats

1.1. Material and excipient

The chlorella sample (*C. vulgaris*) used in this study was obtained from Daesang Corp. (Seoul, Korea). *C. vulgaris* was isolated from freshwater samples collected in South Korea, and was cultured under heterotrophic conditions with glucose as a carbon source. After the culture was completed, the biomass was separated from the culture medium by centrifugation, and then prepared into a powder using a spray-dryer. It was green-colored due to chlorophyll. The nutritional components of chlorella used in this study are shown in Table 6. The content of the ingredient was measured according to the methods recorded in the Food Code [54] and the Health Functional Food Code [55], which were published by the MFDS of Korea.

Table 6. Composition of *C. vulgaris* used in this study

Ingredients	Content (per 100 g powder)
Protein	56.4 g
Ash	3.5 g
Fat	15.4 g
β-carotene	69.6 mg
Vitamin B_2	4.6 mg
Vitamin C	84.0 mg
Chlorophyll	2,550 mg
Letein	336.0 mg

Sterile water for injection (Daihan Pharm. Co., Ltd., Seoul, Korea) was used as a vehicle and kept refrigerated after opening. The chlorella sample used in this study was suspended in sterile water for injection according to the doses of each group assigned in the single dose toxicity studies before administration.

1.2. Animal husbandry and maintenance

In this study, specific pathogen-free (SPF) SD rats at 7 weeks of age, which were used for the single oral dose toxicity study, were provided by Koatech Co., Ltd., (Pyeongtaek, Korea). After 1 week of acclimation, the test substance was administered when the rats were 8 weeks of age.

Throughout the study period, the animals were housed in a room controlled at a temperature of 23 ± 3°C, relative humidity of 55% ± 15%, number of air changes of 10 to 20 times/h, lighting hour of 12 h (lights on: 8 a.m.; lights out: 8 p.m.), and illumination intensity of 150 to 300 Lux. During the test, the temperature and humidity in the animal room were measured every hour by using an auto-temperature and humidity measurer utilizing a computer system. The environmental conditions, such as the number of air changes and illumination intensity, were regularly measured.

The animals were acclimated in a stainless-steel net feeding cage (215 W × 355 L × 200 H mm) with a suitable amount of bedding materials. The animals were raised under a condition of five animals/feeding cage for the quarantine and adaptation periods, and under a condition of less than or equal to five animals/feeding cage for the administration

period. The feeding cages were distinguished by an individual identity card that had the test number and the animal number written.

The test animals were fed solid feeds sterilized by irradiation (Teklad global 18% protein rodent diet, 2918C; Harlan Co., Ltd., USA), supplied by Folas International. The animals were provided free access to the food.

The animals were also provided underground water sterilized by a UV sterilizer and a microfiltration system. Water was freely provided via a water bottle.

1.3. Administration

In a preliminary test, one animal each from the male and female groups was allocated as a high-dose group and administered 10,000 mg/kg; three other groups were administered the test substance at lower doses. No animal deaths nor specific symptoms were observed until 4 days after administration; thus, the group administered the maximum dose of 10,000 mg/kg was set as group 3, and one group receiving a lower dose was established as group 2. To observe the effect of liquid (sterile water for injection) overdose, an excipient-administered group was set as group 1 (Table 7).

Table 7. Test group organization of single oral doses toxicity test to SD rats

Group	Sex	Number of Animal	Liquid Dose (mL/kg)	Dose (mg/kg)
G1	Male	5	40	0
	Female	5	40	0
G2	Male	5	40	5,000
	Female	5	40	5,000
G3	Male	5	40	10,000
	Female	5	40	10,000

G1: Control material-administered group (Sterilized injection water-administered group),
G2, G3: Test sample-administered group

As the test sample was to be used for oral administration in humans, the test sample was orally administered to rats. During test sample administration, the animals were in a state of an empty stomach owing to one-night fasting. To administer the test sample, the abdominal skin of the animal was grasped with the hand, and then the test sample was orally administered into the stomach using a metal sonde. Half of the total administered volume was administered twice/day at hourly intervals on the day of treatment. The liquid dose was calculated based on the animal's weight, which was weighed just before sample administration. The liquid dose was calculated to be 40 mL/kg.

1.4. Observation of general symptoms and animal deaths

General symptoms were observed immediately after sample administration once a day over the whole administration period. They were observed every hour for 6 h later on the day of administration.

1.5. Weight measurement

The weight of all animals was measured before administration, and on the 1st, 3rd, 7th, and 14th days after administration.

1.6. Autopsy observation

Animals were anesthetized with ether on the 14th day after administration, and the postcavas and abdominal aorta were removed to exsanguinate the animals. Organs in the abdominal and thoracic cavities were then observed with the naked eye.

1.7. Statistical analysis

In the case of body weight increment, the mean and standard deviation were calculated. Next, the test sample-treated group was compared with the control group. *T*-test was used as a statistical method. The computer program SPSS v25 (IBM Corp, Armonk, New York, USA) was used for statistical analysis.

2. Single oral dose toxicity test in beagle dogs

2.1. Material and excipient

The chlorella sample (*C. vulgaris*) used in this study was obtained from Daesang Corp. Gelatin capsule (Torpac Inc., Fairfield, NJ, USA) was used as vehicle for oral administration.

2.2. Animal husbandry and maintenance

Beagle dogs are suitable and widely used experimental non-rodents for general toxicity testing. In this study, the beagle dogs, aged 5.5 months, for the single oral dose toxicity study were provided by Jung Ang Lab. Animal Inc. (Seoul, Korea). After 15 days of acclimation, the animals were administered the test substance at 6 months of age.

Throughout the study period, the animals were housed in a room with controlled conditions: temperature, 23 ± 3°C; relative humidity, 55% ± 15%; number of air changes, 10 to 20 times/h; lighting hour, 12 h (lights on, 8 a.m.; lights out, 8 p.m.); and illumination intensity, 150 to 300 Lux. During the test, the temperature and humidity in the animal room were measured every hour by using an auto-temperature and humidity measurer utilizing a computer system. The environmental conditions, such as the number of air changes and the illumination intensity, were regularly measured.

The animals were acclimated in a stainless-steel net feeding cage (895 W × 795 L × 765 H mm) during the adaptation and quarantine, administration, and observation periods. One animal each was raised for the quarantine and observation period after administration.

The feeding cages were distinguished by an individual identity card that had the test and animal numbers written.

The dogs were fed solid feeds for a pet dog (PuppyMac) supplied from BioMac Corp. (Suwon, Korea) at 300 g per day. Underground water sterilized by a UV sterilizer and a microfiltration system was provided freely to the dogs via an automatic waterer.

2.3. Administration

A preliminary test revealed that administration of the test sample at 1,000, 2,000, 5,000, and 10,000 mg/kg did not result in abnormal changes in male and female animals (one animal each). Accordingly, 2,000 and 5,000 mg/kg, which were generally used as a limit dose for a one-time oral administration [56], were set as the administration doses for the subsequent test. In addition, an excipient control group, which was administered an empty capsule, was established (Table 8).

Table 8. Test group organization of single oral dose toxicity test to beagle dogs

Group	Sex	Number of Animal	Dose (mg/kg)
G1	Male	2	0
	Female	2	0
G2	Male	2	2,000
	Female	2	2,000
G3	Male	2	5,000
	Female	2	5,000

G1: Control material-administered group (Empty gelatin capsule-administered group),
G2, G3: Test sample-administered group

As the test sample is to be used for oral administration in humans, the test sample was orally administered in this study. The animals were fasted overnight, and then administered the test sample. First, an animal was naturally positioned in the feeding cage, its mouth was opened, the capsule was put inside its inner tongue, and then its mouth was closed. After that, the pharyngolarynx of the animal was gently touched to ensure swallowing of the capsule. The test sample was administered once on the day of administration.

2.4. Observation of general symptoms and animal deaths

General symptoms were observed once a day in all animals over the whole administration period. However, on the day of administration, it was observed every hour from immediately after to 6 h after administration.

2.5. Weight measurement

The weight of all animals was measured before administration, and on the 1st, 3rd, 7th, and 14th days after administration.

2.6. Autopsy observation

All surviving animals were anesthetized by oral administration of pentobarbital (45 mg/kg; Entobar; Hanlim Pharm. Co., Ltd., Seoul, Korea) on the 14th day after administration, and then the animals were exsanguinated by cutting the armpit artery. All organs were then observed with the naked eye.

2.7. Statistical analysis

For body weight, the mean and standard deviation per group was calculated, and then the test sample-treated group was compared with the control group. *T*-test was used as a statistical method. The program SPSS v25 (IBM Corp.) was used for statistical analysis.

3. Thirteen-week repeated oral doses toxicity test in SD rats

3.1. Material and excipient

The chlorella sample (*C. vulgaris*) used in this study was obtained from Daesang Corp. Sterile water for injection (Daihan Pharm. Co., Ltd.) was used as the vehicle and was kept refrigerated after opening. The chlorella sample used in this study was suspended in sterile water for injection according to the doses of each group assigned in the repeated-dose toxicity study.

3.2. Animal husbandry and maintenance

In this study, SPF SD rats at 5 weeks of age were provided by Koatech Co., Ltd. After 1 week of acclimation, the animals were administered the test substance at 6 weeks of age.

Throughout the study period, the animals were housed in a room with controlled conditions: temperature, 23 ± 3°C; relative humidity, 55% ± 15%; number of air changes, 10 to 20 times/h; lighting hour, 12 h (lights on, 8 a.m.; lights out, 8 p.m.); and illumination intensity, 150 to 300 Lux. During the test, the temperature and humidity in the animal room were measured every hour by using an auto-temperature and humidity measurer utilizing a computer system. The environmental conditions, such as the number of air changes and the illumination intensity, were regularly measured.

The animals were acclimated in a polycarbonate feeding cage (235 W × 380 L × 175 H mm) with a suitable amount of bedding materials. The animals were raised under the

condition of less than or equal to five animals/feeding cage for the quarantine period and of two animals/feeding cage for the administration and observation periods. The feeding cages were distinguished by an individual identity card with the test and animal numbers written.

The animals were provided free access to solid feeds sterilized by irradiation (Teklad global 18% protein rodent diet, 2918C; Harlan Co., Ltd.), supplied by Folas International.

The animals were also provided underground water sterilized by a UV sterilizer and a microfiltration system. Water was provided *ad libitum* via a water bottle.

3.3. Administration

The preliminary test showed no remarkable toxicological changes, except that BUN was significantly increased in a male group administered with the test substance at 2,000 mg/kg/day. Accordingly, for this test, the high-dose was set to be 2,000 mg/kg/day, which was two-times higher than 1,000 mg/kg/day, the limit dose in the OECD Guidelines for toxicity testing of repeated oral administration [57], and the low-dose was set to be 300 mg/kg/day, which was two-times higher than the planned clinical dose (150 mg/kg/day). A medium-dose was set to be 1,000 mg/kg/day, i.e., half the high-dose. The control group was an excipient control group administered only sterile water (Table 9).

Table 9. Test group organization of repeated oral doses toxicity test to SD rats

Group	Sex	Number of Animal	Liquid Dose (mL/kg/day)	Dose (mg/kg/day)
G1	Male	10	10	0
	Female	10	10	0
G2	Male	10	10	300
	Female	10	10	300
G3	Male	10	10	1,000
	Female	10	10	1,000
G4	Male	10	10	2,000
	Female	10	10	2,000

G1: Control material-administered group (Sterilized injection water-administered group),
G2, G3, G4: Test sample-administered group

As the test sample is to be used for oral administration in humans, the test sample was orally administered in this study. For administration of the test sample, the abdominal skin of the animal was grasped with one hand, and then the test sample was orally administered into the stomach using a metal sonde. The test sample was generally administered in the morning on the administration day. The liquid dose was calculated based on the weight of the animal on the administration day, and was determined to be 10 mL/kg/day.

The number and period of administration was once per day, 7 days per week, for 13 weeks.

3.4. Observation of general symptoms

General symptoms were observed immediately after the once-a-day administration over the whole administration period. The type, expression, and degree of the symptoms were recorded per individual animal as general symptoms.

3.5. Weight measurement

The weight of all animals was measured at the start of administration, once per week during the test period, and on the autopsy day.

3.6. Measurement of food intake

Food intake was measured at the administration day and once per week over the whole test period. The amounts of feed supplied and remaining were measured each day after weight measurement, and then calculated as an average intake amount per one animal (g/rat/day).

3.7. Measurement of water intake

Water intake was measured on the administration day. The amounts of water supplied and remaining were measured each day and then calculated as an average intake amount per one animal (g/rat/day).

3.8. Eye test

The eyes of all animals were observed upon their allocation into groups. The pupils were dilated using a mydriatic (Ocuhomapine, Lot 013118; Samil Pharm. Co., Ltd., Seoul, Korea), and then the eyeground parts of the eyes were observed and photographed using an eyeground camera (Genesis; Gowa Co., Ltd., Japan) at the final week of observation, and compared between the excipient control group and the high-dose group.

3.9. Urinalysis

At the final week of administration, each group of five animals were put in a metabolic cage, and 1.0 mL of new urine was collected for 3 to 4 h. Next, 0.3 mL urine sample was smeared on a urinalysis test paper (Multistix 10SG; Bayer, Elkhart, IN, USA) and a urine auto-analyzer (CliniTek 100; Bayer, Elkhart, IN, USA) to measure glucose, bilirubin, ketone body, specific gravity, occult blood, pH, protein, urobilinogen, nitrite, and leukocyte content in the urine. The color of 0.7 mL urine sample was observed both with the naked eye and by a urine auto-analyzer (CliniTek 100; Bayer). The same 0.7 mL sample was centrifuged for 5 min at 1,500 rpm in a centrifuge (MF300; Hanil Scientific Inc., Gimpo, Korea), and the sediment was then microscopically examined for the presence of red blood cells, leukocytes, epithelial cells, and cylinder epithelium. Urine amount was measured after collection for 24 h [59].

3.10. Blood test

Blood test was performed on the animals to be sacrificed by using a blood corpuscle auto-measuring instrument (Cell-Dyn 3700; Abbot, Illinois , USA) (Table 10). The animals were fasted overnight and anesthetized, and then blood was collected via the postcava for the blood test. EDTA-2K was used as an anticoagulant [58].

Table 10. Hematological test item and method

Item	Unit	Method
WBC (White blood cell count)*	$\times 10^3/mm^3$	Electrical resistance method
RBC (Red blood cell count)*	$\times 10^6/mm^3$	Electrical resistance method
HGB (Hemoglobin conc.)*	g/dL	Cyanmethemoglobin conversion method
HCT (Hematocrit)*	%	Calculation from RBC and MCV
MCV (Mean corpuscular volume)*	fL	Electrical resistance method
MCH (Mean corpuscular hemoglobin)*	pg	Calculation from RBC and HGB
MCHC (Mean corpuscular hemoglobin conc.)*	g/dL	Calculation from HGB and HCT
Platelet*	$\times 10^3/mm^3$	Electrical resistance method
Red blood cell percentage*	%	Light Scattering Detection Method
Reticulocyte**	ea/1,000	Smear method

* They were tested by using a blood corpuscle auto-measuring instrument (Cell dyn 3700; Abbot, Illinois , USA).

** It was measured by observing 1,000 red blood cells with an optical microscope.

3.11. Blood coagulation time test

Blood coagulation time test was performed on the same animals subjected to the blood test. Prothrombin time (PT) and active partial thromboplastin time (APTT) were measured by using a blood coagulation time analyzer (CA-50; Sysmex, Japan) on the autopsy day (Table 11). As an anticoagulant for a blood coagulation time test, 3.2% sodium citrate was used [58].

Table 11. Blood coagulation time test item and method

Item	Unit	Method
PT (Prothrombin time)*	second	Photometric scattered light detection method & Percentage test endpoint detection method
APTT (Active partial thromboplastin time)*	second	

*They were tested by using a blood coagulation time analyzer (CA-50; Sysmex, Japan).

3.12. Blood biochemical test

Blood biochemical test was performed on the same animals subjected to the blood test. Blood collected from the postcava was centrifuged at 3,000 rpm for 10 min to obtain serum for the blood biochemical test. They were measured by using an auto-analyzer (AU400; Olympus, Japan) and an electrolyte auto-analyzer (644 Na, K, Cl Analyzer; Ciba-Corning, USA) (Table 12) [58].

Table 12. Biochemical blood test item and method

Item	Unit	Method
AST (Aspartate aminotransferase)*	lU/L	IFCC method
ALT (Alanine aminotransferase)*	lU/L	IFCC method
ALP (Alkaline phosphatase)*	lU/L	P-NPP method
BUN (Blood urea nitrogen)*	mg/dL	Urease-UV method
CRE (Creatinine)*	mg/dL	Jaffe method
GLU (Glucose)*	mg/dL	UV method
CHO (Total cholesterol)*	mg/dL	Enzyme method
PRO (Total protein)*	g/dL	Biuret method
CPK (Creatine phosphokinase)*	lU/L	UV-Rate method
ALB (Albumin)*	g/dL	BCG method
BIL (Total bilirubin)*	mg/dL	Evelyn-Malloy method
A/G ratio*		Calculation from PRO and ALB
TG (Triglyceride)*	mg/dL	Enzyme method
IP (Inorganic phosphorus)*	mg/dL	Enzyme method
Ca (Calcium)*	mg/dL	O-CPC method
Cl (Chloride)**	mmol/L	Electrode method
Na (Sodium)**	mmol/L	Electrode method
K (Potassium)**	mmol/L	Electrode method

*They were measured by using an auto-analyzer (AU400; Olympus, Japan).

**They were measured by using an electrolyte auto-analyzer (644 Na, K, Cl Analyzer; Ciba-Corning, USA).

3.13. Autopsy

The postcavas of the same animals subjected to the blood test were cut to exsanguinate the animals. Next, all organs in the abdominal and thoracic cavities as well as the head were observed.

3.14. Organ weight measurement

After the autopsy, the following organs were removed and then weighed with an electronic scale. In the case of organs that are present on both sides of the body, both organs were measured. The organs included the brain, heart, lungs, liver, spleen, kidney, thymus, adrenal gland, thyroid gland, pituitary gland, prostate, testis, epididymis, uterus, and ovary.

3.15. Histopathological test

Microscopic examinations were performed on the organs and tissues collected from the excipient control group (0 mg/kg/day) and high-dose group (2,000 mg/kg/day). The organs of all the treated animals were fixed in 10% neutral formalin solution; the tissues were embedded in paraffin and sectioned at 5 μm thickness. Representative sections of each specified organ were stained with hematoxylin-eosin for microscopic examination.

3.16. Statistical analysis

The data obtained from the group administered with the test sample was compared with those from the excipient control group by using parametric and non-parametric multiple comparison tests. Regression analysis was used to confirm whether or not a dose correlation was significant concerning the test item of interest. The generation rate was expressed as a percentage. All statistical analyses were performed using SPSS v25 (IBM Corp.).

IV. RESULTS

1. Single oral dose toxicity test to SD rats

1.1. Mortality

There were no animal deaths during the whole test period in all test groups (Table 13).

Table 13. Mortality after a single oral dose in rats

Dose	Days after dosing									No. death
(mg/kg)	0	1	2	3	4	5	6	7	8~14	/No. dosed
Male										
CV 0	0*	0	0	0	0	0	0	0	0	0/5
CV 5,000	0	0	0	0	0	0	0	0	0	0/5
CV 10,000	0	0	0	0	0	0	0	0	0	0/5
Female										
CV 0	0	0	0	0	0	0	0	0	0	0/5
CV 5,000	0	0	0	0	0	0	0	0	0	0/5
CV 10,000	0	0	0	0	0	0	0	0	0	0/5

*Number of dead animals.
Day 0: The day of dosing
CV: *C. vulgaris* powder

1.2. General symptoms

Polyuria was observed between 1 and 3 h after administration in all test groups at the day of administration, and chlorella-colored feces was observed in both male and female rats in the groups treated with 5,000 and 10,000 mg/kg of test substance on the 1st day after administration (Table 14, 15).

Table 14. General symptoms after a single oral dose in male rats

Day	Signs observed	Dose (mg/kg)		
		CV 0	CV 5,000	CV 10,000
0	Appears normal	0/5*	0/5	0/5
	Polyuria	5/5	5/5	5/5
1	Appears normal	5/5	0/5	0/5
	Chlorella-colored feces	0/5	5/5	5/5
2	Appears normal	5/5	5/5	5/5
3	Appears normal	5/5	5/5	5/5
4	Appears normal	5/5	5/5	5/5
5	Appears normal	5/5	5/5	5/5
6	Appears normal	5/5	5/5	5/5
7	Appears normal	5/5	5/5	5/5
8	Appears normal	5/5	5/5	5/5
9	Appears normal	5/5	5/5	5/5
10	Appears normal	5/5	5/5	5/5
11	Appears normal	5/5	5/5	5/5
12	Appears normal	5/5	5/5	5/5
13	Appears normal	5/5	5/5	5/5
14	Appears normal	5/5	5/5	5/5

*Number of animals with the sign/Number of animals examined.

Day 0: The day of dosing

CV: *C. vulgaris* powder

Table 15. General symptoms after a single oral dose in female rats

Day	Signs observed	Dose (mg/kg)		
		CV 0	CV 5,000	CV 10,000
0	Appears normal	0/5*	0/5	0/5
	Polyuria	5/5	5/5	5/5
1	Appears normal	5/5	0/5	0/5
	Chlorella-colored feces	0/5	5/5	5/5
2	Appears normal	5/5	5/5	5/5
3	Appears normal	5/5	5/5	5/5
4	Appears normal	5/5	5/5	5/5
5	Appears normal	5/5	5/5	5/5
6	Appears normal	5/5	5/5	5/5
7	Appears normal	5/5	5/5	5/5
8	Appears normal	5/5	5/5	5/5
9	Appears normal	5/5	5/5	5/5
10	Appears normal	5/5	5/5	5/5
11	Appears normal	5/5	5/5	5/5
12	Appears normal	5/5	5/5	5/5
13	Appears normal	5/5	5/5	5/5
14	Appears normal	5/5	5/5	5/5

*Number of animals with the sign/Number of animals examined.

Day 0: The day of dosing

CV: *C. vulgaris* powder

1.3. Body weight

There were no significant changes in weight in female rats treated with the test sample (Figure 2). In contrast, the weight of male rats administered 10,000 mg/kg of the test sample significantly increased ($p < 0.05$) on the 1st day after administration, compared with that of the excipient control group counterpart.

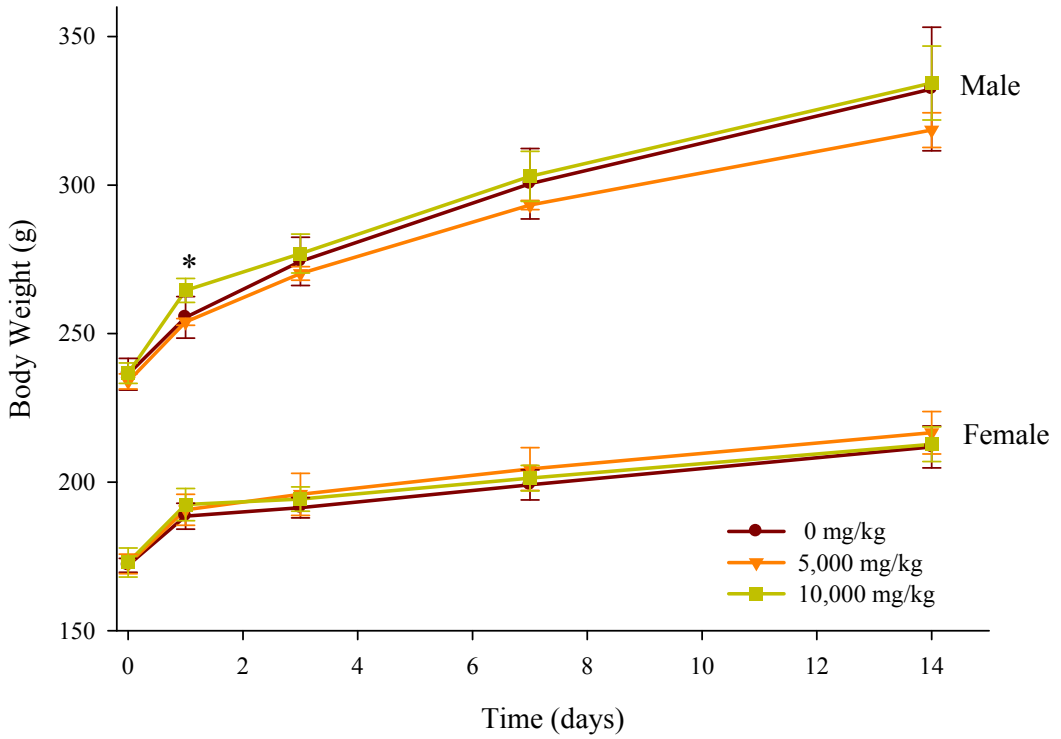

Figure 2. Body weight changes of rats given a single dose of *C. vulgaris* powder. Male and female rats were administered 0 (●), 5,000 (▼), 10,000 (■) mg/kg body weight of *C. vulgaris* powder. The values represent the means ± standard deviation (n=5).
*Significantly different from control value ($p<0.05$)

1.4. Autopsy observation

There were no specific changes in autopsy results between all male rats treated with the test sample (Table 16). On the contrary, female rats in the excipient control group showed pale yellow liquid and cyst formations in the oviducts. There was a dark red node around the ovaries in the group administered 5,000 mg/kg of the test substance (Table 17).

Table 16. Gross findings after a single oral dose in male rats

Dose (mg/kg)	Gross observation		Frequency	
	Organs	Gross findings	Death	Survivors
CV 0		No gross findings	0/0*	5/5
CV 5,000		No gross findings	0/0	5/5
CV 10,000		No gross findings	0/0	5/5

*Number of animals with the sign/Number of animals examined.
CV: *C. vulgaris* powder

Table 17. Gross findings after a single oral dose in female rats

Dose (mg/kg)	Gross observation		Frequency	
	Organs	Gross findings	Death	Survivors
CV 0		No gross findings	0/0*	4/5
	Oviduct	Cyst formation	0/0	1/5
		Pale yellow liquid content	0/0	1/5
CV 5,000		No gross findings	0/0	4/5
	Ovary round	Dark red node	0/0	1/5
CV 10,000		No gross findings	0/0	5/5

*Number of animals with the sign/Number of animals examined.
CV: *C. vulgaris* powder

2. Single oral dose toxicity test in beagle dogs

2.1. Mortality

There were no animal deaths during the whole test period in all the test groups (Table 18).

Table 18. Mortality after a single oral dose in beagle dogs

Dose	Days after dosing									No. death
(mg/kg)	0	1	2	3	4	5	6	7	8~14	/No. dosed
Male										
CV 0	0*	0	0	0	0	0	0	0	0	0/2
CV 2,000	0	0	0	0	0	0	0	0	0	0/2
CV 5,000	0	0	0	0	0	0	0	0	0	0/2
Female										
CV 0	0	0	0	0	0	0	0	0	0	0/2
CV 2,000	0	0	0	0	0	0	0	0	0	0/2
CV 5,000	0	0	0	0	0	0	0	0	0	0/2

*Number of dead animals
Day 0: The day of dosing
CV: *C. vulgaris* powder

2.2. General symptoms

Chlorella-colored feces was observed on the 1st and 2nd day after administration in both male and female dogs administered 5,000 mg/kg of the test sample, and diarrhea was observed in male dogs of the 5,000 mg/kg group (Table 19, 20). In addition, sporadic symptoms of anorexia were observed in several groups, including the control group.

Table 19. General symptoms after a single oral dose in male dogs

Day	Signs observed	Dose (mg/kg)		
		CV 0	CV 2,000	CV 5,000
0	Appears normal	2/2*	2/2	2/2
1	Appears normal	2/2	2/2	0/2
	Diarrhea	0/2	0/2	1/2
	Chlorella-colored feces	0/2	0/2	2/2
2	Appears normal	2/2	2/2	0/2
	Diarrhea	0/2	0/2	1/2
	Chlorella-colored feces	0/2	0/2	2/2
3	Appears normal	2/2	2/2	2/2
4	Appears normal	2/2	2/2	2/2
5	Appears normal	2/2	2/2	2/2
6	Appears normal	2/2	2/2	2/2
7	Appears normal	2/2	2/2	2/2
8	Appears normal	2/2	2/2	2/2
9	Appears normal	2/2	2/2	2/2
10	Appears normal	2/2	2/2	2/2
11	Appears normal	1/2	1/2	2/2
	Anorexia	1/2	1/2	0/2
12	Appears normal	1/2	1/2	2/2
	Anorexia	1/2	1/2	0/2
13	Appears normal	2/2	2/2	2/2
14	Appears normal	2/2	2/2	2/2

*Number of animals with the sign/Number of animals examined

Day 0: The day of dosing

CV: *C. vulgaris* powder

Table 20. General symptoms after a single oral dose in female dogs

Day	Signs observed	Dose (mg/kg)		
		CV 0	CV 2,000	CV 5,000
0	Appears normal	2/2*	2/2	2/2
1	Appears normal	2/2	2/2	0/2
	Anorexia	0/2	0/2	1/2
	Chlorella-colored feces	0/2	0/2	2/2
2	Appears normal	2/2	2/2	0/2
	Anorexia	0/2	0/2	1/2
	Chlorella-colored feces	0/2	0/2	2/2
3	Appears normal	2/2	2/2	2/2
4	Appears normal	2/2	2/2	2/2
5	Appears normal	1/2	2/2	2/2
	Anorexia	1/2	0/2	0/2
6	Appears normal	1/2	2/2	1/2
	Anorexia	1/2	0/2	1/2
7	Appears normal	1/2	2/2	1/2
	Anorexia	1/2	0/2	1/2
8	Appears normal	2/2	2/2	2/2
9	Appears normal	2/2	2/2	2/2
10	Appears normal	2/2	2/2	2/2
11	Appears normal	1/2	2/2	1/2
	Anorexia	1/2	0/2	1/2
12	Appears normal	2/2	2/2	1/2
	Anorexia	0/2	0/2	1/2
13	Appears normal	2/2	2/2	2/2
14	Appears normal	2/2	2/2	2/2

*Number of animals with the sign/Number of animals examined

Day 0: The day of dosing, CV: *C. vulgaris* powder

2.3. Body weight

There were no significant changes in weight following administration of the test sample in both male and female dogs over the whole test period, compared with that in the control group (Figure 3, 4).

However, in female dogs of the control group, there was a slight decrease in weight on the autopsy day, compared with that on the day of administration; however, this decrease was not significant.

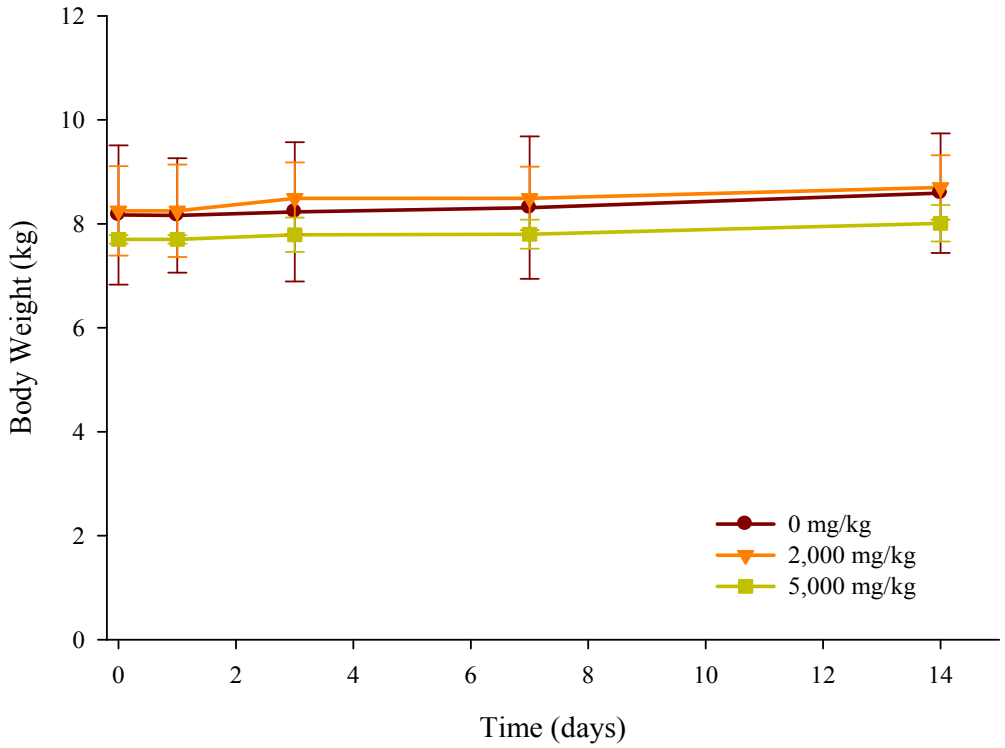

Figure 3. Body weight changes of male dogs given a single dose of *C. vulgaris* powder. Male dogs were administered 0 (●), 2,000 (▼), 5,000 (■) mg/kg body weight of *C. vulgaris* powder. The values represent the means ± standard deviation (n=2).

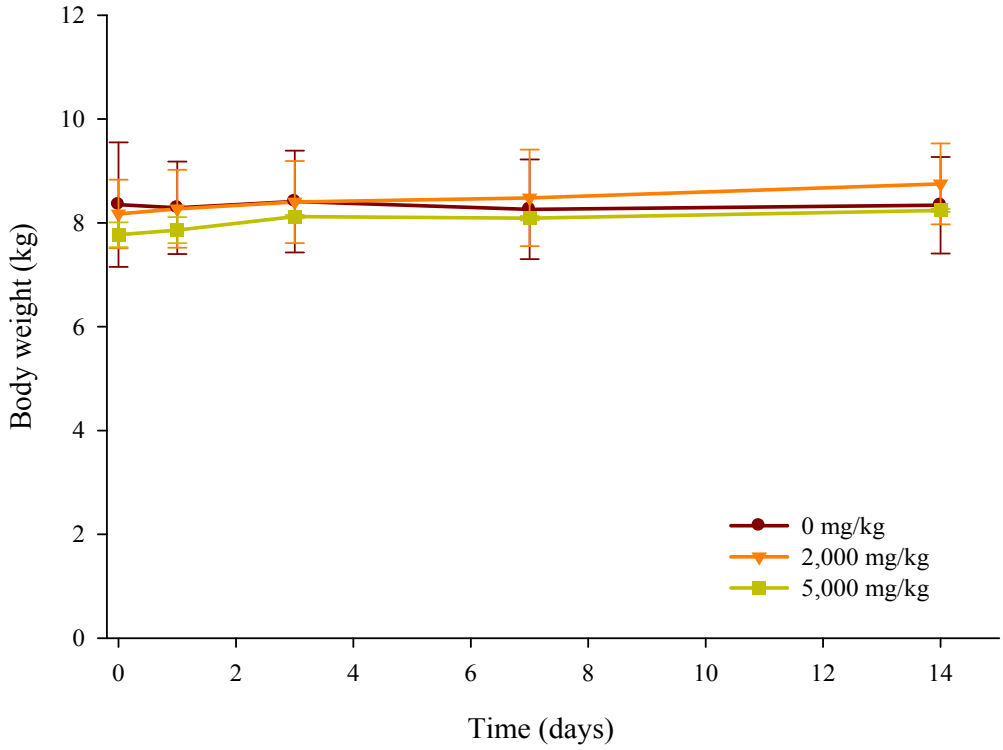

Figure 4. Body weight changes of female dogs given a single dose of *C. vulgaris* powder. Female dogs were administered 0 (●), 2,000 (▼), 5,000 (■) mg/kg body weight of *C. vulgaris* powder. The values represent the means ± standard deviation (n=2).

2.4. Autopsy observation

There were no abnormal autopsy results in all animals (Table 21, 22).

Table 21. Gross findings after a single oral dose in male dogs

Dose (mg/kg)	Gross observation		Frequency	
	Organs	Gross findings	Death	Survivors
CV 0		No gross findings	0/0*	2/2
CV 2,000		No gross findings	0/0	2/2
CV 5,000		No gross findings	0/0	2/2

*Number of animals with the sign/Number of animals examined
CV: *C. vulgaris* powder

Table 22. Gross findings after a single oral dose in female dogs

Dose (mg/kg)	Gross observation		Frequency	
	Organs	Gross findings	Death	Survivors
CV 0		No gross findings	0/0*	2/2
CV 2,000		No gross findings	0/0	2/2
CV 5,000		No gross findings	0/0	2/2

*Number of animals with the sign/Number of animals examined
CV: *C. vulgaris* powder

3. Thirteen-week repeated oral dose toxicity test in SD rats

3.1. Mortality

There were no animal deaths during the whole test period in all test groups (data not shown).

3.2. General symptoms

There was a temporary, partial loss of teeth in two animals from the 77th to the 81st day after administration in the male excipient control group (Table 23, 24).

3.3. Body weight

There were no significant changes in body weight caused by the test sample in both the male and female groups (Figure 5).

Table 23. General symptoms after repeated oral dose in male rats

Day	Signs observed	Dose (mg/kg)			
		CV 0	CV 300	CV 1,000	CV 2,000
0	Appears normal	10/10*	10/10	10/10	10/10
1~76	Appears normal	10/10	10/10	10/10	10/10
77	Appears normal	8/10	10/10	10/10	10/10
	Loss of teeth	2/10	0/10	0/10	0/10
78	Appears normal	8/10	10/10	10/10	10/10
	Loss of teeth	2/10	0/10	0/10	0/10
79	Appears normal	8/10	10/10	10/10	10/10
	Loss of teeth	2/10	0/10	0/10	0/10
80	Appears normal	8/10	10/10	10/10	10/10
	Loss of teeth	2/10	0/10	0/10	0/10
81	Appears normal	8/10	10/10	10/10	10/10
	Loss of teeth	2/10	0/10	0/10	0/10
82~91	Appears normal	10/10	10/10	10/10	10/10

*Number of animals with the sign/Number of animals examined
CV: *C. vulgaris* powder

Table 24. General symptoms after repeated oral dose in female rats

Day	Signs observed	Dose (mg/kg)			
		CV 0	CV 300	CV 1,000	CV 2,000
0	Appears normal	10/10*	10/10	10/10	10/10
1~91	Appears normal	10/10	10/10	10/10	10/10

*Number of animals with the sign/Number of animals examined
CV: *C. vulgaris* powder

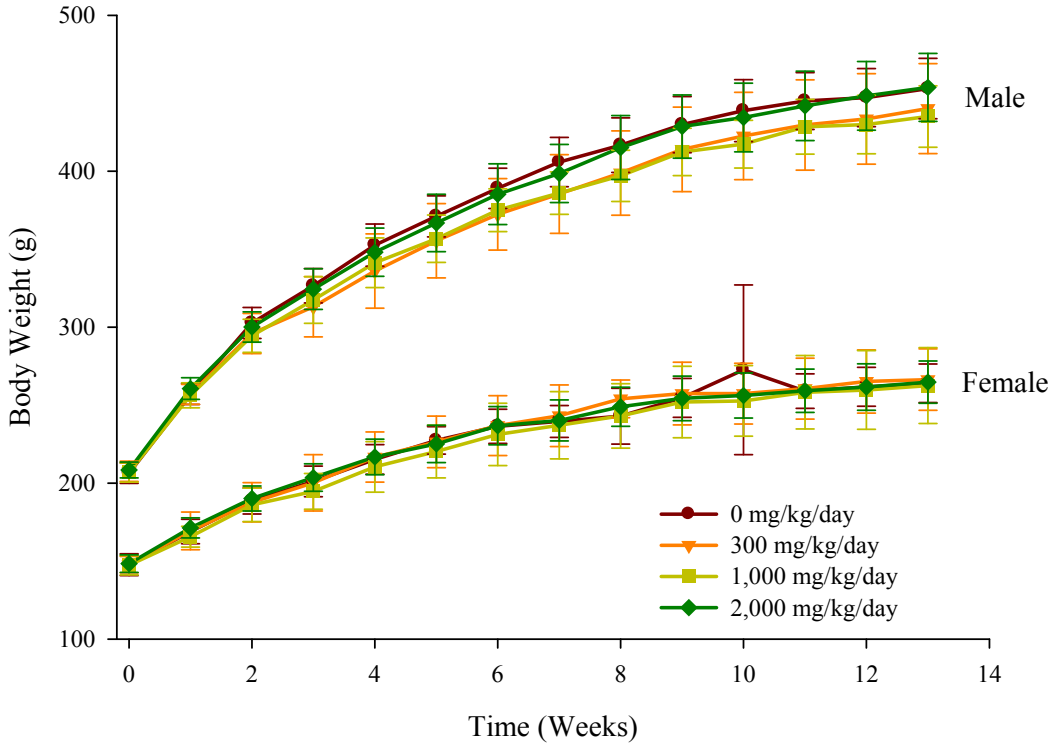

Figure 5. Body weight changes of rats administered *C. vulgaris* containing diet for 13-week. Male and female rats were administered 0 (●), 300 (▼), 1,000 (■), 2,000 (◆) mg/kg body weight/day of *C. vulgaris* powder. The values represent the means ± standard deviation (n=10).

3.4. Food and water consumption

There were no significant changes in food intake related to the test sample in both the male and female groups (Figure 6).

There was a significant increase ($p < 0.05$) in water intake during the 1st week after administration in male rats of the 2,000 mg/kg/day group (Figure 7). There were no significant changes in water intake in the female group (Figure 8).

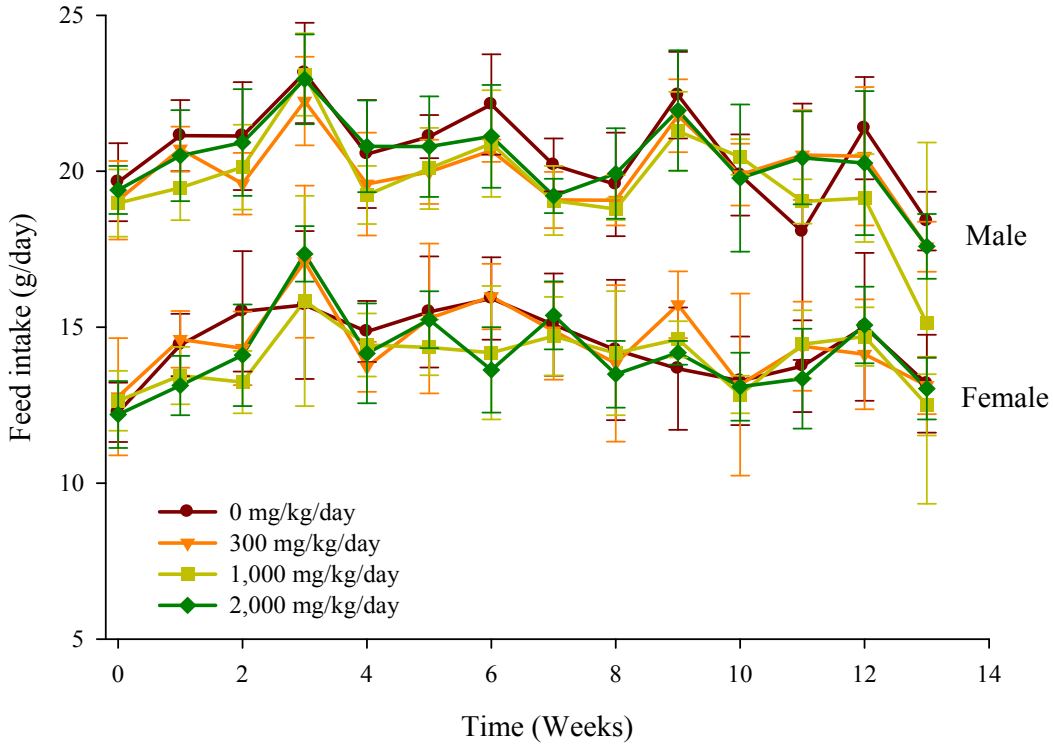

Figure 6. Food intake of rats administered *C. vulgaris* containing diet for 13-week. Male and female rats were administered 0 (●), 300 (▼), 1,000 (■), 2,000 (◆) mg/kg body weight/day of *C. vulgaris* powder. The values represent the means ± standard deviation (n=10).

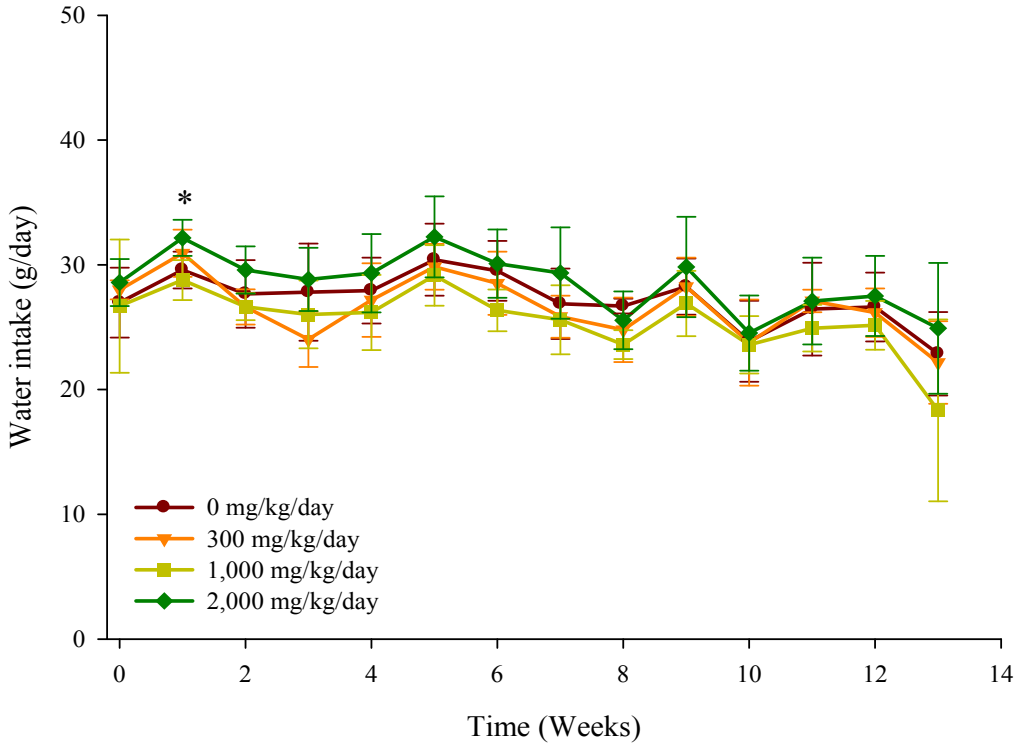

Figure 7. Water intake of male rats administered *C. vulgaris* containing diet for 13-week. Male rats were administered 0 (●), 300 (▼), 1,000 (■), 2,000 (◆) mg/kg body weight/day of *C. vulgaris* powder. The values represent the means ± standard deviation (n=10).
*Significantly different from control value ($p<0.05$)

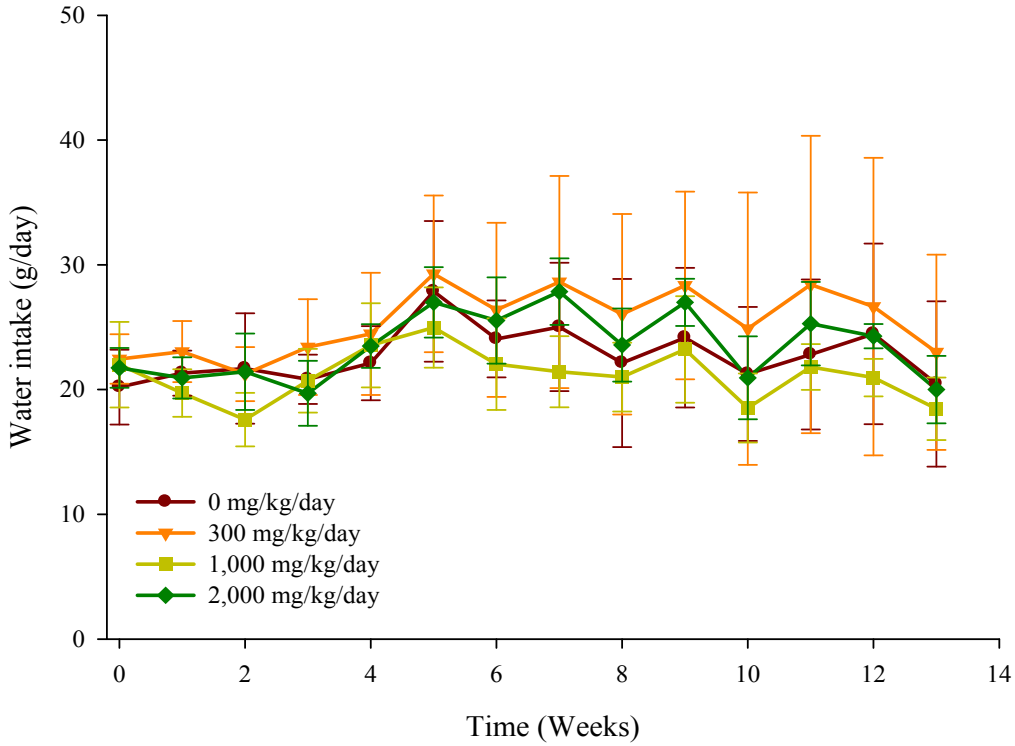

Figure 8. Water intake of female rats administered *C. vulgaris* containing diet for 13-week. Female rats were administered 0 (●), 300 (▼), 1,000 (■), 2,000 (◆) mg/kg body weight/day of *C. vulgaris* powder. The values represent the means ± standard deviation (n=10).

3.5. Eye test

There were no remarkable changes in eye test results related to the test sample (data not shown).

3.6. Urinalysis

There was a significant decrease in urine pH ($p<0.05$) in the male rats of the 2,000 mg/kg/day group (Table 25). There were no significant changes cause by the test sample in the female group.

Table 25. Urinalysis of male and female rats after repeated oral dose

Parameter	Result	Dose (mg/kg/day)							
		Male				Female			
		CV 0	CV 300	CV 1,000	CV 2,000	CV 0	CV 300	CV 1,000	CV 2,000
No. of animals examined		5	5	5	5	5	5	5	5
Specific gravity	≤1.005	0	0	0	0	0	1	0	0
	1.010	0	0	1	0	1	0	0	1
	1.015	3	1	1	0	0	1	0	1
	1.020	1	3	2	1	2	2	4	1
	1.025	0	1	0	3	2	0	1	2
	≥ 1.030	1	0	1	1	0	1	0	0
pH	6.5	0	0	1	0	0	0	0	0
	7.0	0	0	0	0	0	0	0	0
	7.5	0	0	0	0	0	0	1	0
	8.0	0	0	0	3*	0	0	0	0
	8.5	1	2	3	2	4	4	3	2
	≥ 9.0	4	3	1	0	1	1	1	3
Glucose	-	5	5	5	5	5	5	5	5
	+/-	0	0	0	0	0	0	0	0
Bilirubin	-	5	5	5	5	5	5	5	5
		0	0	0	0	0	0	0	0

*Significantly different from control value ($p<0.05$)

CV: *C. vulgaris* powder

Table 25. Urinalysis of male and female rats after repeated oral dose (continued)

Parameter	Result	Dose (mg/kg/day)							
		Male				Female			
		CV 0	CV 300	CV 1,000	CV 2,000	CV 0	CV 300	CV 1,000	CV 2,000
No. of animals examined		5	5	5	5	5	5	5	5
Ketone Body	-	1	2	1	1	5	5	5	5
	+/-	3	3	3	1	0	0	0	0
	+	1	0	1	3	0	0	0	0
Protein	-	0	0	0	0	2	2	1	2
	+/-	0	0	0	0	0	1	0	0
	1+	0	0	1	1	2	1	2	1
	2+	4	4	2	0	1	1	2	2
	3+	1	1	2	4	0	0	0	0
Urobilinogen	0.1	5	5	5	5	5	5	5	5
Nitrite	-	5	5	5	5	5	5	5	5
Occult Blood	-	2	3	5	5	4	5	5	5
	+/-	1	2	0	0	1	0	0	0
	1+	2	0	0	0	0	0	0	0
WBC	-	0	0	0	0	3	3	4	2
	+/-	1	0	2	0	2	1	0	3
	1+	2	3	2	3	0	1	1	0
	2+	2	2	1	2	0	0	0	0

CV: *C. vulgaris* powder

3.7. Hematological test

There were no significant changes in hematological test results related to the test sample in both the male and female groups (Table 26-28).

3.8. Blood coagulation time test

There were no significant changes in blood coagulation time related to the test sample in both the male and female groups (Table 29).

Table 26. Hematological values of male rats after repeated oral dose (mean ± SD, n=10)

Parameter	Dose (mg/kg/day)			
	CV 0	CV 300	CV 1,000	CV 2,000
WBC (× 10^3/μL)	9.98 ± 1.76	9.16 ± 1.34	9.97 ± 2.75	9.78 ± 1.62
RBC (×10^6/μL)	8.27 ± 0.23	8.19 ± 0.29	8.22 ± 0.23	8.41 ± 0.22
HGB (g/dL)	15.79 ± 0.36	15.68 ± 0.32	15.65 ± 0.56	15.90 ± 0.46
HCT (%)	41.99 ± 0.95	41.81 ± 0.76	41.63 ± 1.17	42.27 ± 1.32
MCV (fL)	50.79 ± 1.31	51.07 ± 1.78	50.78 ± 1.05	50.27 ± 0.89
MCH (pg)	19.08 ± 0.62	19.15 ± 0.49	19.07 ± 0.42	18.91 ± 0.39
MCHC (g/dL)	37.62 ± 0.66	37.49 ± 0.52	37.59 ± 0.62	37.65 ± 0.72
PLT (× 10^3/μL)	856.7 ± 33.3	822.2 ± 41.8	889.9 ± 75.0	881.6 ± 59.8
NEU (%)	18.24 ± 9.67	19.93 ± 6.53	21.57 ± 9.73	19.77 ± 11.23
LYM (%)	74.99 ± 9.87	73.09 ± 7.71	71.49 ± 9.78	72.38 ± 11.80
MONO (%)	3.20 ± 0.98	3.30 ± 2.30	3.07 ± 1.23	3.54 ± 1.40
EOS (%)	1.41 ± 0.59	1.51 ± 0.61	1.55 ± 0.55	1.83 ± 0.47
BASO (%)	2.18 ± 0.45	2.17 ± 1.10	2.31 ± 0.70	2.49 ± 0.54

CV: *C. vulgaris* powder

WBC: White blood cell count, RBC: Red blood cell count, HGB: Hemoglobin concentration, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: Platelet, NEU: Neutrophils, LYM: Lymphocytes, MONO: Monocytes, EOS: Eosinophils, BASO: Basophils

Table 27. Hematological values of female rats after repeated oral dose (mean ± SD, n=10)

Parameter	Dose (mg/kg/day)			
	CV 0	CV 300	CV 1,000	CV 2,000
WBC ($\times 10^3/\mu L$)	6.08 ± 1.35	6.66 ± 1.67	6.34 ± 1.26	6.78 ± 2.30
RBC ($\times 10^6/\mu L$)	7.21 ± 0.29	7.33 ± 0.30	7.21 ± 0.27	7.25 ± 0.15
HGB (g/dL)	14.71 ± 0.68	14.67 ± 0.45	14.33 ± 0.62	14.53 ± 0.59
HCT (%)	38.62 ± 1.53	38.95 ± 1.05	38.32 ± 1.63	38.76 ± 1.18
MCV (fL)	53.60 ± 1.07	53.22 ± 1.67	53.16 ± 1.18	53.44 ± 1.11
MCH (pg)	20.39 ± 0.69	20.06 ± 0.80	19.86 ± 0.60	20.03 ± 0.58
MCHC (g/dL)	38.05 ± 0.87	37.69 ± 0.62	37.38 ± 0.64	37.48 ± 0.66
PLT ($\times 10^3/\mu L$)	914.1 ± 59.0	911.6 ± 70.3	893.0 ± 60.2	930.4 ± 60.7
NEU (%)	11.32 ± 4.36	11.77 ± 4.54	9.17 ± 2.46	10.50 ± 2.35
LYM (%)	81.18 ± 6.55	81.47 ± 5.85	85.10 ± 3.57	84.38 ± 3.12
MONO (%)	3.08 ± 0.90	3.34 ± 1.16	2.45 ± 1.36	2.10 ± 1.13
EOS (%)	2.60 ± 2.78	1.58 ± 0.40	1.50 ± 0.30	1.57 ± 0.37
BASO (%)	1.81 ± 0.43	1.85 ± 0.51	1.80 ± 0.75	1.47 ± 0.62

CV: *C. vulgaris* powder

WBC: White blood cell count, RBC: Red blood cell count, HGB: Hemoglobin concentration, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: Platelet, NEU: Neutrophils, LYM: Lymphocytes, MONO: Monocytes, EOS: Eosinophils, BASO: Basophils

Table 28. Reticulocyte counts values of male and female rats after repeated oral dose (mean ± SD, n=10)

Sex	Dose (mg/kg/day)			
	CV 0	CV 300	CV 1,000	CV 2,000
Male	9.9 ± 1.4	10.6 ± 1.8	9.3 ± 3.4	9.8 ± 2.1
Female	10.9 ± 1.7	11.1 ± 1.9	11.0 ± 2.0	11.1 ± 1.9

The values represent a number of reticulocyte per 1,000ea of red blood cell.

CV: *C. vulgaris* powder

Table 29. Plasma coagulation values of male and female rats after repeated oral dose (mean ± SD)

Parameter	Dose (mg/kg/day)			
	CV 0	CV 300	CV 1,000	CV 2,000
Male				
PT (sec)	8.5 ± 0.2 (n=10)	8.7 ± 0.4 (n=10)	8.7 ± 0.4 (n=10)	8.7 ± 0.3 (n=9)
APTT (sec)	17.2 ± 0.8 (n=10)	17.7 ± 0.5 (n=10)	17.5 ± 0.7 (n=10)	17.4 ± 0.6 (n=10)
Female				
PT (sec)	8.1 ± 0.3 (n=10)	8.1 ± 0.2 (n=10)	8.0 ± 0.2 (n=10)	8.2 ± 0.3 (n=10)
APTT (sec)	15.6 ± 1.1 (n=10)	14.6 ± 1.4 (n=10)	15.3 ± 1.2 (n=10)	15.4 ± 1.1 (n=10)

CV: *C. vulgaris* powder

3.9. Blood biochemical parameters

There were no significant changes in blood biochemical parameters caused by the test sample in the male group (Table 30). However, there was a significant increase in ALT ($p < 0.05$) in female rats of the 300 mg/kg/day group, as well as significant increases in ALT and ALP ($p < 0.05$) in female rats of the 2,000 mg/kg/day group (Table 31).

Table 30. Serum biochemical values of male rats after repeated oral dose (mean ± SD, n=10)

Parameter	Dose (mg/kg/day)			
	CV 0	CV 300	CV 1,000	CV 2,000
TP (g/dL)	6.23 ± 0.21	6.23 ± 0.18	6.14 ± 0.15	6.14 ± 0.14
ALB (g/dL)	3.12 ± 0.05	3.12 ± 0.11	3.06 ± 0.08	3.05 ± 0.06
A/G (ratio)	1.01 ± 0.05	1.01 ± 0.03	1.00 ± 0.05	0.99 ± 0.03
AST (IU/L)	112.8 ± 25.6	125.9 ± 30.5	121.8 ± 25.0	109.5 ± 18.0
ALT (IU/L)	43.0 ± 8.0	46.3 ± 10.6	47.8 ± 21.2	44.9 ± 5.6
ALP (IU/L)	89.8 ± 14.3	94.3 ± 23.3	82.4 ± 17.1	87.1 ± 16.9
CPK (IU/L)	307.8 ± 129.5	358.0 ± 140.7	340.8 ± 136.2	277.7 ± 114.9
T-BIL (mg/dL)	0.23 ± 0.02	0.23 ± 0.01	0.22 ± 0.02	0.23 ± 0.02
CHO (mg/dL)	106.1 ± 13.1	99.5 ± 11.7	95.5 ± 12.1	101.3 ± 10.4
TG (mg/dL)	40.4 ± 7.1	41.8 ± 11.3	41.7 ± 6.6	47.5 ± 7.0
BUN (mg/dL)	15.2 ± 1.2	15.6 ± 1.2	14.6 ± 1.5	15.7 ± 1.7
CRE (mg/dL)	0.54 ± 0.04	0.53 ± 0.04	0.52 ± 0.06	0.51 ± 0.08
GLU (mg/dL)	121.4 ± 14.0	123.2 ± 6.5	121.4 ± 11.1	121.6 ± 9.4
IP (mg/dL)	10.03 ± 0.42	9.86 ± 0.30	8.78 ± 2.64	9.66 ± 0.27
Ca (mg/dL)	7.29 ± 1.12	7.07 ± 0.46	6.71 ± 0.33	6.66 ± 0.63
Na (mmol/L)	146.3 ± 1.6	147.0 ± 1.6	145.8 ± 1.9	145.6 ± 1.5
K (mmol/L)	4.87 ± 1.06	4.49 ± 0.18	4.57 ± 0.11	4.61 ± 0.19
Cl (mmol/L)	108.8 ± 1.9	109.9 ± 1.8	109.3 ± 1.4	107.8 ± 1.3

CV: *C. vulgaris* powder

TP: Total protein, ALB: Albumin, A/G ratio: Albumin/globulin ratio, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, CPK: Creatine phosphokinase, T-BIL: Total bilirubin, CHO: Total cholesterol, TG: Triglyceride, BUN: Blood urea nitrogen, CRE: Creatinine, GLU: Glucose, IP: Inorganic phosphorus, Ca: Calcium, Na: Sodium, K: Potassium, Cl: Chloride

Table 31. Serum biochemical values of female rats after repeated oral dose (mean ± SD, n=10)

Parameter	Dose (mg/kg/day)			
	CV 0	CV 300	CV 1,000	CV 2,000
TP (g/dL)	6.06 ± 0.26	6.09 ± 0.24	5.97 ± 0.23	6.05 ± 0.23
ALB (g/dL)	3.23 ± 0.13	3.20 ± 0.08	3.20 ± 0.08	3.23 ± 0.13
A/G (ratio)	1.14 ± 0.03	1.12 ± 0.08	1.15 ± 0.06	1.14 ± 0.05
AST (IU/L)	124.2 ± 15.0	125.8 ± 14.6	120.8 ± 16.0	130.8 ± 15.6
ALT (IU/L)	30.1 ± 4.2	35.2 ± 4.1*	30.6 ± 5.8	35.1 ± 4.7*
ALP (IU/L)	50.7 ± 9.9	56.5 ± 11.1	60.8 ± 13.2	65.5 ± 12.5*
CPK (IU/L)	434.0 ± 109.2	378.1 ± 73.8	360.2 ± 96.3	399.0 ± 85.2
T-BIL (mg/dL)	0.28 ± 0.03	0.26 ± 0.02	0.27 ± 0.03	0.27 ± 0.02
CHO (mg/dL)	117.2 ± 14.6	105.8 ± 16.8	103.9 ± 18.7	107.2 ± 13.6
TG (mg/dL)	42.4 ± 7.6	41.6 ± 8.1	37.5 ± 5.0	42.2 ± 8.4
BUN (mg/dL)	17.3 ± 1.5	18.2 ± 2.8	16.8 ± 1.9	16.1 ± 1.2
CRE (mg/dL)	0.59 ± 0.06	0.62 ± 0.08	0.61 ± 0.03	0.57 ± 0.04
GLU (mg/dL)	103.9 ± 9.0	103.2 ± 8.3	101.7 ± 7.0	104.2 ± 11.5
IP (mg/dL)	9.60 ± 0.25	9.62 ± 0.34	9.57 ± 0.47	9.35 ± 0.79
Ca (mg/dL)	6.57 ± 0.81	6.64 ± 1.25	6.27 ± 0.85	6.39 ± 0.86
Na (mmol/L)	145.0 ± 2.2	144.9 ± 1.7	144.9 ± 1.0	143.8 ± 2.5
K (mmol/L)	4.20 ± 0.28	4.49 ± 1.07	4.29 ± 0.31	4.23 ± 0.32
Cl (mmol/L)	108.1 ± 1.5	109.0 ± 1.4	109.1 ± 1.3	108.4 ± 1.9

CV: *C. vulgaris* powder

TP: Total protein, ALB: Albumin, A/G ratio: Albumin/globulin ratio, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, CPK: Creatine phosphokinase, T-BIL: Total bilirubin, CHO: Total cholesterol, TG: Triglyceride, BUN: Blood urea nitrogen, CRE: Creatinine, GLU: Glucose, IP: Inorganic phosphorus, Ca: Calcium, Na: Sodium, K: Potassium, Cl: Chloride

*Significantly different from control value ($p<0.05$)

3.10. Autopsy results

In the 300 mg/kg/day group, red discoloration was observed in the maxillary lymph nodes and in the thymus of one male rat each. Diffused red spots were observed in the thymus of one male rat of the 2,000 mg/kg/day group (Table 32). There was clear liquid in the uterus of one female rat each in the 1,000 and 2,000 mg/kg/day groups (Table 33).

Table 32. Gross findings after repeated oral dose in male rats

Organs	Gross findings	Dose (mg/kg/day)			
		CV 0	CV 300	CV 1,000	CV 2,000
Maxillary lymph node	No gross findings	10/10*	9/10	10/10	10/10
	Red discoloration	0/10	1/10	0/10	0/10
Thymus	No gross findings	10/10	9/10	10/10	9/10
	Red discoloration	0/10	1/10	0/10	0/10
	Diffused red spots	0/10	0/10	0/10	1/10

*Number of animals with the sign/Number of animals examined
CV: *C. vulgaris* powder

Table 33. Gross findings after repeated oral dose in female rats

Organs	Gross findings	Dose (mg/kg/day)			
		CV 0	CV 300	CV 1,000	CV 2,000
Uterus	No gross findings	10/10*	10/10	9/10	9/10
	Clear liquid contents	0/10	0/10	1/10	1/10

*Number of animals with the sign/Number of animals examined
CV: *C. vulgaris* powder

3.11. Organ weight

There was a significant decrease in heart weight ($p<0.05$) in male rats of the 1,000 mg/kg/day group (Table 34). There were also significant increases in the relative weight of the left ovary ($p < 0.05$) of female rats of the 300 and 2,000 mg/kg/day groups (Table 35).

Table 34. Absolute and relative organ weights of male rats after repeated oral dose (mean ± SD, n=10)

Parameter	Dose (mg/kg/day)			
	CV 0	CV 300	CV 1,000	CV 2,000
Body weight (g)	430.37 ± 18.51	417.90 ± 25.61	410.24 ± 21.34	431.12 ± 22.50
Brain (g)	2.1532 ± 0.4315	2.0411 ± 0.1086	1.9727 ± 0.0911	1.9902 ± 0.0898
Per body weight (%)	0.5032 ± 0.1190	0.4901 ± 0.0419	0.4818 ± 0.0307	0.4626 ± 0.0291
Heart (g)	1.3864 ± 0.0987	1.3702 ± 0.0715	1.2823 ± 0.0794*	1.3169 ± 0.0363
Per body weight (%)	0.3226 ± 0.0261	0.3288 ± 0.0240	0.3127 ± 0.0135	0.3060 ± 0.0136
Lung (g)	1.7252 ± 0.1407	1.7932 ± 0.3340	1.7024 ± 0.1756	1.7386 ± 0.1118
Per body weight (%)	0.4011 ± 0.0318	0.4323 ± 0.0997	0.4146 ± 0.0304	0.4044 ± 0.0355
Liver (g)	10.9963 ± 1.0541	10.6008 ± 0.5584	10.1025 ± 1.0880	10.7614 ± 0.8767
Per body weight (%)	2.5513 ± 0.1619	2.5400 ± 0.1106	2.4572 ± 0.1615	2.4947 ± 0.1281
Spleen (g)	0.7728 ± 0.0864	0.7578 ± 0.0919	0.7479 ± 0.0524	0.7947 ± 0.0587
Per body weight (%)	0.1798 ± 0.0199	0.1814 ± 0.0196	0.1825 ± 0.0126	0.1845 ± 0.0130
Kidney-left (g)	1.3147 ± 0.0935	1.3156 ± 0.1214	1.2415 ± 0.1168	1.3051 ± 0.1283
Per body weight (%)	0.3059 ± 0.0240	0.3156 ± 0.0327	0.3023 ± 0.0194	0.3027 ± 0.0252
Kidney-right (g)	1.3381 ± 0.0663	1.3219 ± 0.1162	1.2462 ± 0.1296	1.2787 ± 0.1165
Per body weight (%)	0.3112 ± 0.0151	0.3170 ± 0.0303	0.3033 ± 0.0213	0.2963 ± 0.0170
Thymus (g)	0.2948 ± 0.0333	0.2965 ± 0.0307	0.2721 ± 0.0444	0.2894 ± 0.0522
Per body weight (%)	0.0685 ± 0.0074	0.0713 ± 0.0099	0.0662 ± 0.0092	0.0671 ± 0.0114
Adrenal gland-left (g)	0.0482 ± 0.0658	0.0276 ± 0.0035	0.0278 ± 0.0040	0.0258 ± 0.0023
Per body weight (%)	0.0110 ± 0.0148	0.0066 ± 0.0009	0.0068 ± 0.0011	0.0060 ± 0.0004
Adrenal gland-right (g)	0.0501 ± 0.0742	0.0483 ± 0.0751	0.0280 ± 0.0107	0.0229 ± 0.0028
Per body weight (%)	0.0115 ± 0.0167	0.0121 ± 0.0198	0.0068 ± 0.0024	0.0053 ± 0.0006

CV: *C. vulgaris* powder
*Significantly different from control value ($p<0.05$)

Table 34. Absolute and relative organ weights of male rats after repeated oral dose (continued) (mean ± SD, n=10)

Parameter	Dose (mg/kg/day)			
	CV 0	CV 300	CV 1,000	CV 2,000
Thyroid gland-left (g)	0.0083 ± 0.0023	0.0089 ± 0.0026	0.0096 ± 0.0017	0.0070 ± 0.0012
Per body weight (%)	0.0019 ± 0.0005	0.0021 ± 0.0006	0.0023 ± 0.0004	0.0016 ± 0.0003
Thyroid gland-right (g)	0.0086 ± 0.0017	0.0086 ± 0.0021	0.0102 ± 0.0035	0.0077 ± 0.0020
Per body weight (%)	0.0020 ± 0.0004	0.0020 ± 0.0005	0.0025 ± 0.0009	0.0018 ± 0.0005
Pituitary gland (g)	0.0112 ± 0.0008	0.0116 ± 0.0020	0.0109 ± 0.0019	0.0117 ± 0.0013
Per body weight (%)	0.0026 ± 0.0002	0.0028 ± 0.0005	0.0026 ± 0.0004	0.0027 ± 0.0003
Prostate (g)	0.5483 ± 0.1337	0.5386 ± 0.1462	0.5042 ± 0.1789	0.5368 ± 0.1041
Per body weight (%)	0.1268 ± 0.0274	0.1292 ± 0.0370	0.1232 ± 0.0432	0.1249 ± 0.0256
Testis-left (g)	1.9872 ± 0.1266	2.0231 ± 0.1055	2.0309 ± 0.1170	1.9855 ± 0.2147
Per body weight (%)	0.4623 ± 0.0313	0.4857 ± 0.0378	0.4955 ± 0.0241	0.4608 ± 0.0462
Testis-right (g)	2.0142 ± 0.1511	2.0227 ± 0.1133	2.0284 ± 0.1163	1.9863 ± 0.1734
Per body weight (%)	0.4686 ± 0.0376	0.4857 ± 0.0406	0.4951 ± 0.0294	0.4611 ± 0.0367
Epididymis-left (g)	0.6587 ± 0.0318	0.6775 ± 0.0403	0.6683 ± 0.0700	0.6730 ± 0.0595
Per body weight (%)	0.1532 ± 0.0083	0.1627 ± 0.0148	0.1628 ± 0.0143	0.1564 ± 0.0147
Epididymis-right (g)	0.6659 ± 0.0395	0.6876 ± 0.0501	0.6770 ± 0.0726	0.6825 ± 0.0452
Per body weight (%)	0.1550 ± 0.0111	0.1650 ± 0.0142	0.1648 ± 0.0127	0.1585 ± 0.0106

CV: *C. vulgaris* powder

Table 35. Absolute and relative organ weights of female rats after repeated oral dose (mean ± SD, n=10)

Parameter	Dose (mg/kg/day)			
	CV 0	CV 300	CV 1,000	CV 2,000
Body weight (g)	248.05 ± 12.55	248.63 ± 20.34	246.35 ± 22.91	250.71 ± 12.93
Brain (g)	1.8271 ± 0.0582	1.7950 ± 0.0961	1.8368 ± 0.0766	1.8451 ± 0.0838
Per body weight (%)	0.7388 ± 0.0523	0.7244 ± 0.0429	0.7492 ± 0.0483	0.7373 ± 0.0434
Heart (g)	0.8793 ± 0.0576	0.8860 ± 0.0560	0.8709 ± 0.0733	0.9061 ± 0.0643
Per body weight (%)	0.3547 ± 0.0189	0.3575 ± 0.0233	0.3541 ± 0.0154	0.3616 ± 0.0219
Lung (g)	1.3506 ± 0.1124	1.4076 ± 0.1369	1.3550 ± 0.0771	1.3774 ± 0.0816
Per body weight (%)	0.5445 ± 0.0358	0.5666 ± 0.0390	0.5523 ± 0.0351	0.5501 ± 0.0341
Liver (g)	6.1842 ± 0.5234	6.3390 ± 1.0115	5.7507 ± 0.6193	6.2858 ± 0.5936
Per body weight (%)	2.4918 ± 0.1445	2.5391 ± 0.2450	2.3341 ± 0.1146	2.5061 ± 0.1915
Spleen (g)	0.5960 ± 0.0637	0.6489 ± 0.0981	0.6025 ± 0.0826	0.6216 ± 0.0567
Per body weight (%)	0.2399 ± 0.0191	0.2614 ± 0.0362	0.2441 ± 0.0209	0.2481 ± 0.0207
Kidney-left (g)	0.7761 ± 0.0234	0.7570 ± 0.0562	0.7385 ± 0.0653	0.7741 ± 0.0555
Per body weight (%)	0.3134 ± 0.0149	0.3052 ± 0.0208	0.3002 ± 0.0147	0.3091 ± 0.0209
Kidney-right (g)	0.7700 ± 0.0412	0.7738 ± 0.0756	0.7500 ± 0.0570	0.7728 ± 0.0343
Per body weight (%)	0.3108 ± 0.0160	0.3114 ± 0.0234	0.3054 ± 0.0211	0.3086 ± 0.0144
Thymus (g)	0.2333 ± 0.0445	0.1980 ± 0.0541	0.2276 ± 0.0417	0.2199 ± 0.0277
Per body weight (%)	0.0939 ± 0.0167	0.0795 ± 0.0196	0.0923 ± 0.0146	0.0880 ± 0.0130
Adrenal gland-left (g)	0.0322 ± 0.0041	0.0298 ± 0.0078	0.0326 ± 0.0038	0.0311 ± 0.0024
Per body weight (%)	0.0130 ± 0.0014	0.0122 ± 0.0035	0.0133 ± 0.0013	0.0124 ± 0.0009
Adrenal gland-right (g)	0.0295 ± 0.0036	0.0315 ± 0.0041	0.0310 ± 0.0060	0.0292 ± 0.0037
Per body weight (%)	0.0119 ± 0.0014	0.0127 ± 0.0019	0.0125 ± 0.0017	0.0117 ± 0.0016

CV: *C. vulgaris* powder

Table 35. Absolute and relative organ weights of female rats after repeated oral dose (continued) (mean ± SD, n=10)

Parameter	Dose (mg/kg/day)			
	CV 0	CV 300	CV 1,000	CV 2,000
Thyroid gland-left (g)	0.0076 ± 0.0009	0.0085 ± 0.0033	0.0076 ± 0.0016	0.0079 ± 0.0024
Per body weight (%)	0.0031 ± 0.0003	0.0034 ± 0.0012	0.0031 ± 0.0006	0.0031 ± 0.0009
Thyroid gland-right (g)	0.0081 ± 0.0015	0.0084 ± 0.0024	0.0078 ± 0.0014	0.0076 ± 0.0019
Per body weight (%)	0.0033 ± 0.0006	0.0033 ± 0.0008	0.0032 ± 0.0006	0.0030 ± 0.0007
Pituitary gland (g)	0.0126 ± 0.0013	0.0125 ± 0.0017	0.0125 ± 0.0020	0.0126 ± 0.0017
Per body weight (%)	0.0051 ± 0.0004	0.0050 ± 0.0007	0.0051 ± 0.0007	0.0050 ± 0.0006
Uterus (g)	0.4953 ± 0.1122	0.6902 ± 0.3530	0.6969 ± 0.4569	0.6372 ± 0.2767
Per body weight (%)	0.1986 ± 0.0384	0.2748 ± 0.1290	0.2913 ± 0.2076	0.2534 ± 0.1039
Ovary-left (g)	0.0376 ± 0.0073	0.0463 ± 0.0086	0.0402 ± 0.0083	0.0460 ± 0.0080
Per body weight (%)	0.0152 ± 0.0029	0.0185 ± 0.0026*	0.0163 ± 0.0026	0.0183 ± 0.0028*
Ovary-right (g)	0.0431 ± 0.0093	0.0452 ± 0.0101	0.0407 ± 0.0065	0.0470 ± 0.0060
Per body weight (%)	0.0174 ± 0.0037	0.0181 ± 0.0035	0.0165 ± 0.0022	0.0188 ± 0.0027

CV: *C. vulgaris* powder

*Significantly different from control value ($p<0.05$)

3.12. Histopathological observation

Tissue samples from animals in the high-dose and excipient control groups were collected for microscopic examination. Unfortunately, histopathological examination could not be performed on 13 animals (3 in the male excipient control group, 3 in the male high-dose group, 3 in the female excipient control group, and 4 in the female high-dose group) because of a loss of fixed tissues.

Of the 7 rats in the male excipient control group, 4 showed hyaline casts in the kidneys, 1 showed prostatitis, and 1 showed basophilic kidney tubules (Table 36). In the 2,000 mg/kg/day group, 5 rats showed hyaline casts in the kidneys and 3 rats showed basophilic kidney tubules. Moreover, 2 rats had prostatitis, 2 had aspiration pneumonia, 1 had cyst formation in the adrenal glands, 1 had ultimobranchial cysts in the thyroid gland, and 1 had interstitial pneumonia in the lungs.

In the female excipient control group, 2 rats showed extramedullary hematopoiesis in the spleen, and 1 rat had ultimobranchial cysts in the thyroid gland (Table 37). Furthermore, in the 2,000 mg/kg/day group, 2 rats showed extramedullary hematopoiesis in the spleen, 2 showed ultimobranchial cysts in the thyroid gland, 1 showed endometrial fibrosis and atrophy, 1 showed hydrometra, 1 showed hyaline casts in the kidneys, 1 showed focal interstitial nephritis, and 1 showed aspiration pneumonia.

Table 36. Histopathological findings after repeated oral dose in male rats

Organs	Histopathological findings	Dose (mg/kg/day) CV 0		CV 2,000	
Number of animals		7		7	
		N	%	N	%
Skin (mammary glands)	No microscopic findings	7/7*	100	7/7	100
Pituitary gland	No microscopic findings	7/7	100	7/7	100
Adrenal gland	No microscopic findings	7/7	100	6/7	100
	Cyst formation	0/7	100	1/7	86
Testis	No microscopic findings	7/7	100	7/7	14
Epididymis	No microscopic findings	7/7	100	7/7	100
Prostate gland	No microscopic findings	6/7	86	5/7	71
	Prostatitis	1/7	14	2/7	29
Seminal vesicle	No microscopic findings	7/7	100	7/7	100
Urinary bladder	No microscopic findings	7/7	100	7/7	100
Kidney	No microscopic findings	3/7	43	2/7	29
	Hyaline cast	4/7	57	5/7	71
	Basophilic tubules	1/7	14	3/7	43
Aorta	No microscopic findings	7/7	100	7/7	100
Tongue	No microscopic findings	7/7	100	7/7	100
Eye	No microscopic findings	7/7	100	7/7	100
Harderian gland	No microscopic findings	7/7	100	7/7	100
Liver	No microscopic findings	7/7	100	7/7	100
Mesenteric lymph node	No microscopic findings	7/7	100	7/7	100
Spleen	No microscopic findings	7/7	100	7/7	100
Thyroid gland	No microscopic findings	7/7	100	6/7	86
	Ultimobranchial cyst	0/7	0	1/7	14
Trachea	No microscopic findings	7/7	100	7/7	100
Esophagus	No microscopic findings	7/7	100	7/7	100
Salivary gland	No microscopic findings	7/7	100	7/7	100
Maxillary lymph node	No microscopic findings	7/7	100	7/7	100
Heart	No microscopic findings	7/7	100	7/7	100
Thymus	No microscopic findings	7/7	100	7/7	100
Lung	No microscopic findings	7/7	100	4/7	58
	Aspiration pneumonia	0/7	0	2/7	28
	Interstitial pneumonia	0/7	0	1/7	14

*Number of animals with the sign/Number of animals examined

CV: *C. vulgaris* powder

Table 36. Histopathological findings after repeated oral dose in male rats (continued)

Organs	Histopathological findings	Dose (mg/kg/day) CV 0		CV 2,000	
Number of animals		7		7	
		N	%	N	%
Femoral muscle	No microscopic findings	7/7	100	7/7	100
Sciatic nerve	No microscopic findings	7/7*	100	7/7	100
Stomach	No microscopic findings	7/7	100	7/7	100
Duodenum	No microscopic findings	7/7	100	7/7	100
Pancreas	No microscopic findings	7/7	100	7/7	100
Jejunum	No microscopic findings	7/7	100	7/7	100
Ileum	No microscopic findings	7/7	100	7/7	100
Caecum	No microscopic findings	7/7	100	7/7	100
Colon	No microscopic findings	7/7	100	7/7	100
Rectum	No microscopic findings	7/7	100	7/7	100
Brain	No microscopic findings	7/7	100	7/7	100
Thoracic spinal cord	No microscopic findings	7/7	100	7/7	100
Sternum	No microscopic findings	7/7	100	7/7	100
Femur	No microscopic findings	7/7	100	7/7	100

*Number of animals with the sign/Number of animals examined

CV: *C. vulgaris* powder

Table 37. Histopathological findings after repeated oral dose in female rats

Organs	Histopathological findings	Dose (mg/kg/day) CV 0		CV 2,000	
Number of animals		7		6	
		N	%	N	%
Skin (mammary glands)	No microscopic findings	7/7*	100	6/6	100
Pituitary gland	No microscopic findings	7/7	100	6/6	100
Adrenal gland	No microscopic findings	7/7	100	6/6	100
Ovary	No microscopic findings	7/7	100	6/6	100
Uterus	No microscopic findings	7/7	100	4/6	67
	Endometrial fibrosis and atrophy	0/7	0	1/6	17
	Hydrometra	0/7	0	1/6	17
Vagina	No microscopic findings	7/7	100	6/6	100
Urinary bladder	No microscopic findings	7/7	100	6/6	100
Kidney	No microscopic findings	7/7	100	4/6	67
	Hyaline cast	0/7	0	1/6	17
	Focal interstitial nepharitis	0/7	0	1/6	17
Aorta	No microscopic findings	7/7	100	6/6	100
Tongue	No microscopic findings	7/7	100	6/6	100
Eye	No microscopic findings	7/7	100	6/6	100
Harderian gland	No microscopic findings	7/7	100	6/6	100
Liver	No microscopic findings	7/7	100	6/6	100
Mesenteric lymph node	No microscopic findings	7/7	100	6/6	100
Spleen	No microscopic findings	5/7	71	4/6	67
	Extramedullary hematopoiesis	2/7	29	2/6	33
Thyroid gland	No microscopic findings	6/7	86	4/6	67
	Ultimobranchial cyst	1/7	14	2/6	33
Trachea	No microscopic findings	7/7	100	6/6	100
Esophagus	No microscopic findings	7/7	100	6/6	100
Salivary gland	No microscopic findings	7/7	100	6/6	100
Maxillary lymph node	No microscopic findings	7/7	100	6/6	100
Heart	No microscopic findings	7/7	100	6/6	100
Thymus	No microscopic findings	7/7	100	6/6	100

*Number of animals with the sign/Number of animals examined

CV: *C. vulgaris* powder

Table 37. Histopathological findings after repeated oral dose in female rats (continued)

Organs	Histopathological findings	Dose (mg/kg/day) CV 0		CV 2,000	
Number of animals		7		6	
		N	%	N	%
Lung	No microscopic findings	7/7*	100	5/6	83
	Aspiration pneumonia	0/7	0	1/6	17
Femoral muscle	No microscopic findings	7/7	100	6/6	100
Sciatic nerve	No microscopic findings	7/7	100	6/6	100
Stomach	No microscopic findings	7/7	100	6/6	100
Duodenum	No microscopic findings	7/7	100	6/6	100
Pancreas	No microscopic findings	7/7	100	6/6	100
Jejunum	No microscopic findings	7/7	100	6/6	100
Ileum	No microscopic findings	7/7	100	6/6	100
Caecum	No microscopic findings	7/7	100	6/6	100
Colon	No microscopic findings	7/7	100	6/6	100
Rectum	No microscopic findings	7/7	100	6/6	100
Brain	No microscopic findings	7/7	100	6/6	100
Thoracic spinal cord	No microscopic findings	7/7	100	6/6	100
Sternum	No microscopic findings	7/7	100	6/6	100
Femur	No microscopic findings	7/7	100	6/6	100

*Number of animals with the sign/Number of animals examined

CV: *C. vulgaris* powder

V. DISCUSSION

1. Single oral dose toxicity test in SD rats

This test was performed to investigate the acute oral toxicity of *C. vulgaris* powder in SD rats. Male and female rats in the 5,000 and 10,000 mg/kg groups were compared with the excipient control group. Mortality rate, general symptoms, and body weight change were observed for 2 weeks before autopsy was performed.

There were no animal deaths in all test groups in this experiment. The polyuria observed on the day of administration was considered a general symptom caused by the large volume of treatment. Moreover, the chlorella-colored feces observed on the 1st day after administration was considered to result from undigested test sample, which was also caused by the large amount of test sample administered. Accordingly, these phenomena were not considered the toxic effects of *C. vulgaris*. The increase in body weight in male rats of the 10,000 mg/kg group on the 1st day after administration was not considered a serious change due to toxicity. Changes in the oviducts of female rats in the excipient control group, as shown in autopsy, are often observed in female rats of the same age and linage. Therefore, these changes might be accidental. Moreover, the changes around the ovaries in the middle-dose group were regarded as accidental.

Based on the above results, the minimum lethal dose (MLD) of *C. vulgaris* powder in rats under the present test conditions was established to be more than 10,000 mg/kg.

2. Single oral dose toxicity test in beagle dogs

This test was performed to investigate the acute oral toxicity of *C. vulgaris* powder in beagle dogs. Male and female dogs were allocated to the 0, 2,000, and 5,000 mg/kg groups. Next, mortality rate, general symptoms, and body weight change were measured for 2 weeks after administration, and autopsy was performed.

In this experiment, no animals died due to the test sample in all test groups. Nevertheless, chlorella-colored feces was observed in the 5,000 mg/kg group on the 1st and 2nd day after administration. The chlorella-colored feces might be caused by the administration of the test sample in a large amount. Diarrhea was observed in one male dog of the 5,000 mg/kg group, and this might occur due to the sensitivity of this particular dog to the excessive amount of test sample. Although sporadic symptoms of anorexia were observed, anorexia was observed in a healthy normal dog, regardless of the dose; thus, this symptom was not considered a toxic effect of the test sample. Other than the above results, there were no abnormal findings in the other parameters.

Accordingly, it was determined that the test sample caused no toxicity following one-time administration under the present test conditions. Based on this result, the MLD of *C. vulgaris* powder under the present test conditions was more than 5,000 mg/kg in both male and female beagle dogs.

3. Thirteen-week repeated oral doses toxicity test in SD rats

This test was conducted to investigate the 13-week subacute oral toxicity of *C. vulgaris* powder. Male and female rats were allocated to the 300, 1,000, and 2,000 mg/kg/day groups, and then compared with the excipient control group. Mortality rate, general symptoms, body weight change, and food and water intake were measured, and eye test, urinalysis, hematological and blood biochemical tests, autopsy, and histopathological tests were performed after 13 weeks of repeated oral administration of the test sample.

In this experiment, there were no animal deaths and no general symptom changes caused by *C. vulgaris*. There were no significant changes in body weight and food intake caused by *C. vulgaris*. There were no significant changes in water intake caused by the test sample, but there was a significant, temporary increase in water intake on the 1st week in male rats of the 2,000 mg/kg/day group, but there were no changes in the female rats. In contrast, the other parameters showed no significant changes. Therefore, the observed changes were not considered the toxic effects of *C. vulgaris*.

There were no significant changes in eye test and urinalysis results caused by *C. vulgaris*. However, there was a significant decrease in urine pH in male rats of the 2,000 mg/kg/day group. As there were no significant changes in kidney-related biochemical parameters and histopathological findings, the observed changes were not considered the toxic effects of the test sample.

There were no significant changes in hematological test result and blood coagulation time caused by the test sample. Blood biochemical test results showed significant increases in ALT in the female 300 and 2,000 mg/kg/day groups. However, the changes were within the normal range and lacking a dose correlation [60, 51]. Moreover, there were no significant changes in histopathological findings. Therefore, those changes were not considered the toxic effects of the test sample. Furthermore, the significant increase in ALP in the female 2,000 mg/kg/day group was also within the normal range [60, 61]. Thus, we concluded that the changes were not due to the toxicity of *C. vulgaris*.

Autopsy results showed no significant changes caused by the test sample. However, clear liquid was observed in the uterus of several female animals with normal physiological function, and thus this finding was not considered the effect of the sample. In addition, red discoloration in the lymph nodes of the lower jaw and thymus is a common change found during the autopsy process after ether anesthesia. Therefore, these changes might not be caused by *C. vulgaris*.

In addition, there was a significant decrease in heart weight in the male 1,000 mg/kg/day group, and a significant increases in the relative weight of the left ovary of the female 300 and 2,000 mg/kg/day groups. Nevertheless, the changes were within the normal range and lacking a dose correlation [61]. Moreover, there were no significant changes in histopathological findings, the changes were not considered the toxic effects of the test sample.

There were no significant changes in histopathological findings caused by *C. vulgaris*. However, hyaline cast in the kidneys is a common change observed in male rats; moreover, prostatitis and basophilic kidney tubules are commonly found in experimental rats. Hence, the changes were not considered the toxic effects of the test sample. The ultimobranchial cysts observed in the thyroid gland of male rat is an embryo residue from an initial developmental process of the thyroid gland. The frequency of extramedullary hematopoiesis observed in the spleen of female rats is similar to that in the excipient control group. In addition, the frequencies of cyst formation in the adrenal glands, endometrial fibrosis and atrophy, hydrometra, and focal interstitial nephritis were low, and these changes occasionally occurred in normal rats as well [62, 63]. Based on these results, it was concluded that the changes were not due to the toxicity of *C. vulgaris*. The changes observed in the lungs were similar to those reported previously [64, 65], whereas the frequency of unknown pneumonitis was high in 2- to 5-month-old SD rats in an SPF feeding condition.

The above results showed no specific changes in the test results after 13 weeks of repeated oral administration of *C. vulgaris* powder. Therefore, we concluded that the no observed adverse effect level (NOAEL) was more than 2,000 mg/kg/day in both male and female rats.

Szabo et al. [28] did not evaluate the safety of biomass, but the safety of isolated protein from *C. protothecoides*, and concluded that the proteins are safe. Day et al. [29] determined that the NOAEL of high-lipid biomass (fat: 47.8%) from *C. protothecoides*

was 7557 and 8068 mg/kg body weight/day for male and female rats, respectively. De Mello-Sampayo et al. [30] assessed the safety of *C. vulgaris* with a low chlorophyll content following cultivation under special conditions and found that they could safely administer a dosage of 427.5 mg/kg/day to rats. But theirs was a carotenogenic biomass different from the one obtained with normal green chlorella and the dose was lower than that used in our study. Neumann et al. [31] assessed the safety of *C. vulgaris* after cell disruption, and they proved that 14 days of *C. vulgaris* diet showed no adverse effects at concentrations of up to 25% in mice. However, it was a short-term study, and the parameters, such as histological parameters, were only partially evaluated. Moreover, there was no information on food intake. In a study conducted by Himuro et al.[27], the NOAEL of *C. sorokiniana* strain CK-22 was estimated to be 5.94 and 6.41 g/kg body weight/day for male and female rats, respectively. As safety may vary depending on the intake, species, and culture methods, additional experiments will be needed to determine the safety of chlorella intake regardless of the species.

VI. CONCLUSION

The present study was conducted to evaluate the safety of *C. vulgaris* by a single oral dose toxicity test in rodents and non-rodents, as well as by a repeated oral dose toxicity test in rodents.

Single oral dose toxicity test of *C. vulgaris* at concentrations of 5,000 and 10,000 mg/kg in rats resulted in no animal deaths in all test groups. Although polyuria and chlorella-colored feces were observed on the day of administration and on the 1st day after administration, respectively, those results were considered not to be caused by *C. vulgaris*, but by the large amount of test sample administered. Moreover, changes in body weight and autopsy results were not caused by the toxicity of *C. vulgaris*. Thus, the MLD of *C. vulgaris* in rats was established to be more than 10,000 mg/kg.

Single oral dose toxicity test of *C. vulgaris* at concentrations of 2,000 and 5,000 mg/kg in dogs resulted in no animal deaths in all test groups. As in a single oral dose toxicity test in rats, chlorella-colored feces was caused by the large dose of the administered sample. Although diarrhea was observed in one dog after oral administration, there were no abnormal observations in the other parameters. Therefore, the MLD of *C. vulgaris* in dogs was determined to be more than 5,000 mg/kg under the present test conditions.

Thirteen-week repeated oral dose toxicity test of *C. vulgaris* at concentrations of 300, 1,000, and 2,000 mg/kg/day in SD rats resulted in no animal deaths and no significant

changes caused by *C. vulgaris*. Therefore, the NOAEL of *C. vulgaris* in rats was more than 2,000 mg/kg/day, which was the highest dose tested.

This study provided information on the acute and subacute toxicity of *C. vulgaris* cultivated under heterotrophic conditions, which has not been reported in previous studies; thus, the findings of this study are meaningful. However, this study had limitations to be considered. First, this study was conducted in animals, not human participants. As humans and experimental animals are different from each other, their bioreactions are also different; thus, caution must be paid when evaluating the safety of *C. vulgaris*. Second, owing to ethical considerations, the toxicity of *C. vulgaris* could not be examined at higher concentrations. Although the NOAEL of *C. vulgaris* in rats was determined to be 2,000 mg/kg/day or more, it is difficult to calculate the acceptable daily intake (ADI) from the NOAEL. Generally, the ADI is calculated by applying a safety factor of 100 to the NOAEL value [66]. However, there has been no research to evaluate the safety at an intake amount higher than the dose administered in this study; therefore, the ADI could be miscalculated using the NOAEL value in this study. Hence, to estimate the ADI, it would be necessary to obtain the NOAEL value through additional experiments using higher doses. In addition, foods are continuously consumed throughout life, unlike medicine, and thus the effect of their long-term intake should be evaluated. However, as this study only evaluated the subacute, a chronic toxicity test is required as a follow-up study. Moreover, because chlorella is a food with a long history of consumption, its safety

can also be determined by undertaking case studies of the side effects of its consumption, instead of conducting animal safety assessments.

In conclusion, *C. vulgaris* led to no animal deaths and no significant toxic effects in our tested conditions. Therefore, *C. vulgaris* might be considered safe as a food and dietary supplement under the present dosage conditions. In addition, to estimate ADI, further studies are needed to subacute toxicity test for excess amount of chlorella and chronic toxicity test.

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국문초록

동물모델에서 단회 및 반복 경구투여 독성평가를 통한 암배양 *Chlorella vulgaris* 의 안전성 평가

목적: 클로렐라는 2~10 μm 크기의 단세포 녹조류로 *Chlorella vulgaris* 를 포함한 여러가지 종이 일반식품이나 식이보충제로 섭취되고 있다. 클로렐라는 녹색을 나타내는 엽록소뿐만 아니라 단백질, 비타민 등 영양성분을 고루 함유하고 있으며, 면역증진, 콜레스테롤 개선, 피부건강 및 항산화 기능 등 다양한 건강기능성을 나타내는 식품원료이다. 클로렐라는 광배양과 암배양이 가능하며 배양방법이나 종에 따라서 영양성분의 변화가 나타날 수 있다. 따라서 본 연구에서는 암배양으로 배양된 *C. vulgaris* 가 식품으로서 안전한지 평가하고자 한다.

방법: 본 연구에 사용된 클로렐라는 대상㈜에서 공급받은 것으로, 포도당을 탄소원으로 공급한 암배양 조건에서 배양된 *C. vulgaris* 건조분말이다. *C. vulgaris* 에 대한 급성 독성연구로 설치류 및 비설치류에 대한 단회 경구투여 독성평가를 수행하였고, 아급성 독성연구로 설치류에 대한 반복 경구투여 독성평가를 실시하였다. Sprague-Dawley (SD) 랫트를 이용한 설치류 단회

경구투여 독성평가는 각 군별로 암수 각 5 마리에 대해 *C. vulgaris* 분말을 0, 5,000 그리고 10,000 mg/kg 씩 투여하고 사망률, 일반증상, 체중변화 및 부검소견을 관찰하였다. 비글 견을 이용한 비설치류 단회 경구투여 독성평가에서는 *C. vulgaris* 분말을 군별로 암수 각 2 마리에 0, 2,000 및 5,000 mg/kg 용량으로 투여하고 사망률, 일반증상, 체중변화와 부검소견을 관찰하였다. 반복 경구투여 독성평가에서는 SD 랫트에 13 주동안 *C. vulgaris* 를 0, 300, 1,000 및 2,000 mg/kg/day 농도로 투여한 뒤, 사망률, 일반증상, 체중변화, 사료 및 물 섭취량, 안검사, 요검사, 혈액학 및 핼액생화학 검사, 장기중량, 부검 및 조직병리학적 소견을 관찰하였다.

결과: SD 랫트를 이용한 단회 경구투여 독성평가 결과, 단회에 과량의 시험물질이 투여되어 다뇨와 클로렐라 색 변이 관찰되기는 하였지만 *C. vulgaris* 의 독성에 의한 것은 아닌 것으로 판단되었으며, 모든 평가군에서 시험동물의 사망도 없었다. 또한 체중변화 및 부검에 있어서도 심각한 변화는 나타나지 않았다. 랫트에 대한 *C. vulgaris* 의 최소 치사량 (minimum lethal dose, MLD)은 10,000 mg/kg 이상으로 판단된다. 비글 견을 이용한 단회 경구투여 독성평가 결과, 단회에 다량의 클로렐라를 투여함으로 인해 나타난 클로렐라색 변과 민감한 한 개체의 비글 견에서 관찰된 설사가 나타나기는 했지만 모든 평가군에서 일반증상, 체중변화 및 부검에 있어 비정상적인 증상은 나타나지 않으며, 시험동물의 사망도 없었다. 이번 연구 조건에서 비글 견에 대한 *C. vulgaris* 의 최소 치사량 (MLD)은 5,000 mg/kg 이상이다. 반복

경구투여 독성평가 결과, *C. vulgaris* 에 의한 사망이나 관찰한 모든 지표에 있어 유의적인 변화가 없었다. 이 결과로 볼 때 무독성량 (no observed adverse effect level , NOAEL)은 투여량 중에서 가장 많은 양인 2,000 mg/kg/day 로 판단된다.

결론: 랫트와 비글 견을 이용한 단회 및 반복 경구투여 독성평가에서 *C. vulgaris* 투여로 인한 사망이나 유의적인 독성 변화를 보이는 이상소견이 없었다. 따라서 *C. vulgaris* 는 본 연구조건의 농도에서는 섭취하기에 안전한 원료로 판단되나, 일일섭취허용량 (acceptable daily intake, ADI)을 산출하기 위해서는 본 연구조건 보다 많은 투여량에 대한 아급성 독성평가 및 만성독성 연구가 필요하다.

핵심어: 클로렐라, *Chlorella vulgaris,* 급성 독성, 아급성 독성, 독성, 안전성