

Original Article

Single, repeated dose toxicity and genotoxicity assessment of herb formula KIOM2012H



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ABSTRACT

Background: Traditional medicine and herbal prescriptions are becoming more popular, and they account for a large share of the world's healthcare research studies, developments, and market demands. Increasing scientific evidence of the substantive efficacies such as preventive health keeping pharmaceutical materials and dietary supplements can be found elsewhere. Above all, safety should be the critical premise for considering developmental materials such as pharmaceuticals without side effects and toxicity.

Methods: The authors formulated KIOM2012H (K2H) using four herbs that were reported to have medicinal effects—including anticancer, antiaging, antimicrobial, inflammation, and neuroprotective properties. In order to examine the toxicity, single and repeated dose toxicity, and genotoxicities of bacterial mutation, micronucleus, and chromosomal aberration assays were conducted.

Results: All experimental observations and results showed normal findings. Toxicities or abnormal signs were not observed in all experimental assays, including oral administration, animal behavior, clinical findings, and changes in body weight *in vivo*. *In vitro* bacterial cultures produced no revertant colonies, and no increased numbers of structural or numerical aberrant metaphases were found in the metaphase chromosomes examined. Moreover, no significant increased frequency of micronucleus was observed in any of the doses used. Overall, no acute toxicity or genotoxicity was found in all analysis parameters in all the assays conducted.

Conclusion: Reviewing the results as a whole, K2H extract was regarded as a safe material with no toxicity, and can be applied for the research and development of complementary and alternative medicines with improved efficacy in current therapeutic healthcare, based on traditional medicine and herb resources.

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1. Introduction

Asian countries and many other countries have their own traditional medicine that has been prescribed for a long time. Herbs and plants have also been used by the general public as folk remedies. This means that generally accepted and medicated herbal medicines have been empirically recognized as effective and safe, although their pharmacological safety and efficacies are not fully established and studies are still underway to understand them scientifically. Traditional medicine and herbs are becoming more common as practical complementary and alternative ways of modern preventive and therapeutic healthcare. It is reported that 70–95% of the population in many countries around the world use traditional medicine for primary healthcare.^{1,2} Natural product sales in the United States alone reached US\$14.8 billion in 2008, and global sales of Chinese medicine amounted to US\$83.1 billion in 2012 with exponential increase, which is expected to reach US\$115 billion by 2020 including all natural supplements and remedies.³ Indeed, current traditional medicines are sold as medicinal herbs and functional dietary supplements, and are usually supplied in the form of tea, beverages, tablets, and capsules that come from aqueous extracts or lyophilized formulations. Pharmaceutical companies are trying to find and develop new drugs from natural and herb resources. Actually, many drugs are based on these natural compounds, whereas chemically synthetic compounds and drugs are more common and more frequently adopted for drug development. Just as many plants and herbs have efficacies and many active natural compounds, the component herbs of K2H in this study are also known to have the following activities. The seeds and leaves of *Arctium lappa* were reported to have anticancer and antiulcer activities,^{4,5} antiaging,⁶ neuroprotective,⁷ and protective effect on atherosclerosis,⁸ and on cadmium toxicity in the liver.⁹ The root of *Glycyrrhiza uralensis* is known to have antiasthma,¹⁰ antimicrobial,^{11,12} antioxidant,¹³ hepatoprotective,¹⁴ neuronal cell protection,¹⁵ and tyrosinase inhibition¹⁶ effects, and has also been observed to stimulate osteoblast function¹⁷ and detoxification system via Nrf2 activation.¹⁸ The cortex of *Magnolia officinalis* has not only been shown to exhibit activities against inflammation,¹⁹ tumor,²⁰ and memory deficiency,²¹ but has also been observed to attenuate lipogenesis²² and hyperglycemia.²³ The root of *Zingiber officinale* was reported as effective against rheumatoid arthritis,²⁴ allergic asthma,²⁵ Alzheimer's disease,²⁶ cancer,²⁷ and diabetes-induced heart abnormality.²⁸ It was also shown to exert a hepatoprotective effect against hepatic injury, lipid accumulation,²⁹ and liver fibrosis.³⁰ Improvement of cognitive function by ginger extract was identified in the mouse hippocampus.³¹ As is well known, medicinal plants and herbs contain many natural compounds, which can be used for treatment of multiple diseases by combinatorial prescription, and development of multitarget drug, the so-called “multi-component” drug.^{32–34} In this respect, we have formulated K2H with four different herbs. K2H has already been identified earlier by the present authors for its lipid- and body weight-lowering activity in lipid- and high fat diet-induced cellular and animal model of nonalcoholic fatty liver disease.³⁵ Traditional medicine and folk medicines have been used for a

long time and are considered effective and safe, at least in the empirical sense. Nowadays, in the face of growing demand for natural and traditional medicines as drugs or functional foods, toxicological examination and secured safety with scientific evidence should be considered a first priority. In this study, single and repeated dose toxicity studies were conducted using rats, and genotoxicity studies by bacterial reverse mutation, micronucleus assay, and chromosomal aberration assay for toxicity and safety evaluation of K2H. Based on the results, no toxicities were observed in single and repeated dose toxicities, bacterial reverse mutation, and micronucleus assays, except for chromosomal aberrations.

2. Methods

2.1. Preparation of herb extract

To prepare K2H, four medicinal herb materials (Table 1) were purchased from a local distributor in Yeongcheon, Korea, and botanically identified by herb specialists and practitioners. Prior to extract preparation, all herb materials were immersed in distilled water for 1 hour at room temperature to enhance extraction yield, and boiled for 3 hours at 115 °C in the extractor (Cosmos 600; Kyungseo Machine, Korea). Extract was collected by filtering through a test sieve (106 µm; Retsch, Germany) to eliminate insoluble debris and residues, freeze-dried, and then stored at –20 °C until use. A voucher specimen (registration no. KIOM2012H) of the lyophilized extract was deposited in the institutional herbarium.

2.2. Chemicals and reagents

The following chemicals—2-aminoanthracene (2-AA), benzo[a]pyrene (B[a]P), sodium azide (SA), 2-nitrofluorene (2-NF), 4-nitroquinoline-1-oxide (4NQO), acridine mutagen ICR 191 (ICR-191), cyclophosphamide monohydrate (CPA), acridine orange solution (AO), ethyl methanesulfonate (EMS), dimethylsulfoxide (DMSO), potassium chloride (KCl), magnesium sulfate, citric acid monohydrate, potassium phosphate dibasic anhydrous, sodium ammonium phosphate, glucose, sodium chloride, tryptophan, histidine, biotin, methyl alcohol, and glacial acetic acid—were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Oxoid Nutrient Broth No. 2 and Bacto agar were purchased from Thermo Scientific Inc. (Waltham, MA, USA) and BD (Franklin Lakes, NJ, USA), respectively. S9 mix (5% for bacterial reverse mutation assay and 30% for chromosomal aberration assay, v/v) was prepared using Aroclor 1254-induced rat liver S9 (Molecular Toxicology Inc., Boone, NC, USA) supplemented with cofactor-I (8 µM MgCl₂, 33 µM KCl, 5 µM glucose-6-phosphate, 4 µM nicotinamide adenine dinucleotide phosphate, 4 µM nicotinamide adenine dinucleotide, 100 µM sodium phosphate buffer, pH 7.4; Wako Pure Chem. Ind., Osaka, Japan). S9 mix was prepared fresh prior to use and kept on ice during the experiment. Information on chemicals and reagents not described here is supplied in the relevant sections in “Materials and methods” section.

Table 1 – Medicinal Herbs Used to Prepare K2H

Scientific name	Family name	Latin name	Origin	Amount (g)
<i>Arctium lappa</i> Linne	Compositae	Arctii semen	China	90.0
<i>Glycyrrhiza uralensis</i> Fischer	Leguminosae	Glycyrrhizae radix	China	75.0
<i>Magnolia officinalis</i> Rehder & Wilson	Magnoliaceae	Magnoliae cortex	China	60.0
<i>Zingiber officinale</i> Roscoe	Zingiberaceae	Zingiberis rhizoma	Korea	75.0
Total				300.0

K2H, KIOM2012H.

2.3. Single dose toxicity study

For single dose toxicity study, 7-week-old specific-pathogen-free (SPF) Sprague–Dawley (SD) rats (Samtako, Osan, Korea) were used after 7 days of acclimatization in the animal laboratory under the following conditions: temperature $23 \pm 3^\circ\text{C}$, humidity $55 \pm 15\%$, ventilation 10–20 times/h, and 12-hour lighting (8:00–20:00 hours, 150–300 lux). Diet (Teklad Global 18% Protein Rodent Diet 2918C; Harlan Laboratories Inc., Madison, WI, USA) and water were provided *ad libitum*. K2H dissolved in phosphate-buffered saline (PBS; 0, 2500, 5000, and 10,000 mg/kg) was given (20 mL/kg) once a day for 15 days via oral administration using sonde in five male and five female rats in each dose group. Body weight was measured on Days 1, 2, 4, 8, and 15. Every organ was examined for lesions by autopsy after CO₂ anesthesia and phlebotomy on Day 15. Statistical analysis was conducted using SPSS Statistics 22 for Medical Science (IBM, NY, USA), and a *p* value less than 0.05 was considered significant. Assuming the data normality, a parametric one-way analysis of variance (ANOVA) was applied. Lethal dose 50% (LD₅₀) was not calculated as no animal death was observed. The use of laboratory animals in this assay was approved by the Institutional Animal Care and Use Committee (Review No. 14-R581) and conducted according to the Good Laboratory Practice (No. 2014-67, Ministry of Food and Drug Safety, Republic of Korea), Standard for Toxicity Study of Pharmaceuticals (No. 2014-136, Ministry of Food and Drug Safety, Republic of Korea), and Organisation for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (1997) ENV/MC/CHEM(98)17.

2.4. Repeated dose toxicity study (2 weeks)

Two weeks repeated dose toxicity study was performed to determine the dose in 13 weeks repeated toxicity study. Laboratory animals (SPF SD rats), diet and water supply, and animal laboratory environment were the same as those described in the single dose toxicity study. Oral administrations were given (10 mL/kg/d) to five male and five female rats in each dose group (0, 625, 1250, 2500, and 5000 mg/kg) once a day for 2 weeks. Body weight, and diet and water consumptions were measured at Day 1 and once a week thereafter. On the day of sacrifice, fresh urine (0.3 mL) and blood samples (1.0 mL for hematology and 2.0 mL for blood biochemical analysis) were collected from postcaval vein after anesthesia using isoflurane inhalation. Multistix 10SG and CliniTek urinalysis system (Siemens, Munich, Germany) were used for urine analysis. For hematological analysis, blood was collected in K₂EDTA Vacutainer (BD) and analyzed using ADVIA 2120 hematology

system (Siemens, Munich, Germany). Blood samples collected in Insepack tube (Sekisui Medical Co., Tokyo, Japan) containing clot activator were coagulated (room temperature, 20 minutes), centrifuged (10 minutes, 3000 revolutions per minute; Combi-514R; Hanil Scientific Inc., Gimpo, Korea), and subjected to blood biochemistry analysis (AU680, Beckman Coulter, Brea, CA, USA). All organs were weighed and examined for lesions.

Statistical analysis was conducted using SPSS Statistics 22 for Medical Science, and a *p* value less than 0.05 was considered significant. Assuming data normality, parametric one-way ANOVA was applied for body and organ weights, hematological, and blood biochemical data analysis. The equality of variance was tested using Levene's test. Post hoc comparison was conducted using Duncan's multiple range test and Dunnett's T3 test, when there was significant and equal variance, and when there was unequal variance, respectively. Student *t* test was used for diet and water consumptions. The results of the urine analysis were compared with nonparametric Kruskal–Wallis *H* test using the ranked data. When the results were significant, significantly different groups compared with the vehicle control group were identified by Mann–Whitney *U* test. The use of laboratory animals in this assay was approved by the Institutional Animal Care and Use Committee (Review No. 15-R044) and conducted according to the Good Laboratory Practice (No. 2014-67, Ministry of Food and Drug Safety, Republic of Korea), Standard for Toxicity Study of Pharmaceuticals (No. 2014-136, Ministry of Food and Drug Safety, Republic of Korea), and OECD Principles of Good Laboratory Practice (1997) ENV/MC/CHEM(98)17.

2.5. Repeated dose toxicity study (13 weeks)

Laboratory animals (SPF SD rats), diet and water supply, and animal laboratory environment for 13 weeks repeated dose toxicity study were the same as those described above. Oral administrations were given (10 mL/kg/d) to 10 male and 10 female rats in each dose group (0 mg/kg, 1250 mg/kg, 2500 mg/kg, and 5000 mg/kg) once a day for 13 weeks. Clinical signs and general observations were recorded once a day, and diet and water consumptions and body weights were measured at Day 1 and once a week. Collected urine samples (0.3 mL) were subjected to the urinalysis system (CliniTek 100; Siemens, Munich, Germany) using Multistix (10SG; Siemens, Munich, Germany) and observed under the microscope for sediments. For whole blood analysis, blood samples collected in K₂EDTA Vacutainer (BD) were analyzed using ADVIA 2120 hematology system (Siemens, Munich, Germany). Blood clotting times were measured in ACL 100 Coagulation

Analyzer (Instrumentation Laboratories, Ramsey, MN, USA) using plasma separated from 1.8 mL blood collected in a microtube containing 0.2 mL sodium citrate (as an anticoagulant, 3.2%). Blood biochemical analyses were conducted using the same method described above. All organs were examined for lesions and their weight was recorded. Histopathological examination was conducted and graded using Pristima software (Xybion, Bensalem, PA, USA). Statistical analysis was conducted using SPSS Statistics 22 for Medical Science, and a *p* value less than 0.05 was considered significant. Assuming data normality, parametric one-way ANOVA was applied for body and organ weights, diet and water consumptions, urine, hematology, and blood biochemical data analysis. The equality of variance was assessed using Levene's test. Post hoc comparison was conducted using Duncan's multiple range test (with the same sample numbers) and Scheffe's multiple range test (with different sample numbers) in the case of equal variance by ANOVA. Dunnett's T3 test was applied for unequal variance. Urine analysis was conducted by nonparametric Kruskal-Wallis *H* test using ranked data, and significantly different groups were identified by Mann-Whitney *U* test. Histopathological data were subjected to nonparametric Fisher's exact two-tailed test. Use of laboratory animals in this assay was approved by the Institutional Animal Care and Use Committee (Review No. 15-R044) and conducted according to the Good Laboratory Practice (No. 2014-67, Ministry of Food and Drug Safety, Republic of Korea), Standard for Toxicity Study of Pharmaceuticals (No. 2014-136, Ministry of Food and Drug Safety, Republic of Korea), and OECD Principles of Good Laboratory Practice (1997) ENV/MC/CHEM(98)17.

2.6. Bacterial reverse mutation assay

Bacterial reverse mutation assay (also known as Ames test) was conducted using four strains of the histidine auxotrophic bacteria *Salmonella typhimurium* (TA100, TA1535, TA98, and TA1537) and tryptophan auxotrophic bacteria *Escherichia coli* WP2uvrA obtained from Molecular Toxicology Inc.^{36,37} These strains are listed in OECD guidelines as they are appropriate for this assay and have been proven for the sensitive detection of mutagenicity of diverse chemicals. With S9 metabolic activation, positive control material 2-AA was used in concentrations of 1.0, 2.0, 1.0, and 6.0 $\mu\text{g}/\text{plate}$ for strains TA100, TA1535, TA1537, and WP2uvrA, respectively. For bacterial strain TA98, B[a]P was used at a concentration of 1.0 $\mu\text{g}/\text{plate}$. Without S9 metabolic activation, positive control material SA (for TA100 and TA1535), 4NQO (for WP2uvrA), and ICR-191 (for TA1537) were all treated in the same concentration of 0.5 $\mu\text{g}/\text{plate}$. For TA98 strain, the positive control material 2-NF was used at a concentration of 2.0 $\mu\text{g}/\text{plate}$. K2H was prepared by dissolving in PBS and filtered through a 0.45- μm syringe filter. Oxoid Nutrient Broth No. 2 (2.5%) was used for cultivation, and a minimal medium composed of 1.5% Bacto agar, Vogel-Bonner medium E, and 2% glucose was prepared on a petri dish. For *E. coli*, 0.1% tryptophan was added to the minimal medium (0.25 mL/L). Top agars were prepared with 0.6% Bacto agar and 0.5% NaCl, and supplemented with 0.5 mM histidine-biotin (10 mL/100 mL) only for TA bacteria. Bacterial cultures (1 mL each) were mixed with 90 μL DMSO and kept frozen at -70°C . For master plates, bacteria were

cultured for 10 hours, plated on minimal media, and stored at 4°C for later use in bacterial trait identification and bacterial reverse mutation assay. Bacteria from master plates were inoculated in 20 mL of 2.5% Oxoid Nutrient Broth No. 2, then cultured for 10 hours in 37°C in a shaking incubator (120 revolutions per minute); bacteria were counted at 600 nm absorbance and kept at 4°C until use. Cytotoxicity such as sedimentation or increased colony number was not found in the dose-determining experiment (5–5000 $\mu\text{g}/\text{plate}$ treatment). Accordingly, dose groups were determined as 156, 313, 625, 1250, 2500, and 5000 $\mu\text{g}/\text{plate}$ with three plates for each concentration. Top agar (2 mL), S9 mix (0.5 mL; sodium phosphate buffer 0.5 mL for S9 mix negative), bacterial culture (0.1 mL), and test material (0.1 mL) were mixed together, and poured onto minimal glucose agar plate. Every plate was treated with viable cells of over 0.5×10^8 colony-forming units. Hardened plates were inverted and incubated at 37°C for 50 hours, and colonies were counted. It was classified as positive when the average colony number increased dose-dependently at least in one strain, regardless of S9 metabolic activation. Bacterial reverse mutation assay was conducted according to the OECD Principles of Good Laboratory Practice (1997) ENV/MC/CHEM(98)17, OECD Guideline for Testing of Chemicals TG471 (1997) Bacterial Reverse Mutation Test, Good Laboratory Practice (No. 2014-67, Ministry of Food and Drug Safety, Republic of Korea), and Standard for Toxicity Study of Pharmaceuticals (No. 2014-136, Ministry of Food and Drug Safety, Republic of Korea).

2.7. Micronucleus assay

Positive control material CPA (Sigma-Aldrich) prepared in PBS was used as recommended in the OECD guideline TG474. Male Hsd:ICR (CD-1) SPF mice (Koatech, Pyeongtaek, Korea) were examined by their external appearance on arrival and acclimatized for 7 days. Animal laboratory conditions, diet, and water were the same as those described above in toxicity studies using rats. To determine the doses, K2H (10 mL/kg/d) was administered to three male and three female mice once a day for 2 days in the dose groups of 800 mg/kg/d, 2000 mg/kg/d, and 5000 mg/kg/d. Observations were conducted for 4 days including the treatment period. All animals showed compound-colored stool on Days 2 and 3, which reverted to normal by Day 4. Accordingly, K2H dose groups (0 mg/kg/d, 1250 mg/kg/d, 2500 mg/kg/d, and 5000 mg/kg/d) were administered once a day (i.e., 6 males in each group). Positive control CPA (70 mg/kg/d) was intraperitoneally administered once at Day 2. Two slide glasses of bone marrow specimens were prepared from each CO_2 -anesthetized mouse using 23G syringe and fetal bovine serum (HyClone; GE Healthcare Life Sciences, South Logan, UT, USA).³⁸ Cell suspensions were centrifuged, smeared, dried, and fixed in methanol for 5 minutes. Two slides were prepared for every animal. AO solution (0.05%) diluted in Sorensen buffer (1:4 v/v, pH 6.8) was applied on slide glass specimens and observed under the fluorescent microscope at $400\times$ magnification. Numbers of micronucleated polychromatic erythrocyte (MNPCE) were counted among 2000 polychromatic erythrocytes (PCEs) per animal, and MNPCE frequency was shown as the average number of MNPCEs per 2000 PCEs. Morphological examinations

were conducted according to the method described by Hayashi et al.³⁹ It is regarded as valid when the following conditions are satisfied. More than five animals should be alive at autopsy in every dose group. The average of PCE/red blood cell (RBC) ratios in control and dose groups should be more than 20% of the value in the negative group. Among 2000 PCEs, the average MNPCE frequency should be lower than 10.0 (0.5%) in the negative control and more than 50.0 (2.5%) in the positive control. Statistical analysis was performed using SPSS Statistics 22 for Medical Science, with a significance level of $p < 0.05$. Nonparametric Kruskal–Wallis H test using ranked data was performed for micronuclei frequency and showed no significant differences when comparing with the negative control. The significance rates of positive and negative control data were verified by Mann–Whitney U test. Parametric one-way ANOVA was applied for PCE/RBC ratio and body weight, assuming equal variance, which is verified by Levene's test. Significant differences were identified between the maximum dose group and the negative control group by ANOVA test and Duncan's multiple range test. It was regarded as cytotoxic when the PCE/RBC average ratio in dose groups is significantly reduced. It was regarded positive when the MNPCE frequency is statistically significant and increased dose-dependently in the treated groups, or when it shows reproducible increase in one or more dose groups. This assay was conducted according to the Good Laboratory Practice (No. 2014-67, Ministry of Food and Drug Safety, Republic of Korea), Standard for Toxicity Study of Pharmaceuticals (No. 2014-136, Ministry of Food and Drug Safety, Republic of Korea), OECD Guideline for Testing of Chemicals TG471 (1997) Mammalian Erythrocyte Micronucleus Test, and OECD Principles of Good Laboratory Practice (1997) ENV/MC/CHEM(98)17. The use of laboratory animals in this assay was approved by the Institutional Animal Care and Use Committee (Review No. 15-M094)

2.8. Chromosomal aberration assay

According to the OECD guideline TG473, B[a]P and EMS were used as positive control for the presence and absence of S9 mix, respectively. The Chinese hamster lung (CHL) cell line obtained from the ATCC (Manassas, VA, USA) was maintained at 37 °C humidified incubator (5% CO₂) in 1 L of medium composed of minimum essential medium (Gibco #41500-034; Gibco, Waltham, MA, USA), sodium bicarbonate (2200 mg), L-glutamine (292 mg), penicillin–streptomycin (Gibco #15140-122), and fetal bovine serum (100 mL). Actively proliferating 6×10^4 cells were seeded in 5 mL medium and treated after 3 days. The composition of the S9 mix is the same as that described in “Chemicals and reagents” section. To determine the dose concentrations in the main study, a preliminary study was conducted. In the preliminary study (5–5000 µg/mL), relative increase in cell count (RICC) for cytotoxicity index was calculated after 24 hours of treatment using the following formula:

$$\text{RICC}(\%) = \frac{\text{Increase in number of cells in treated cultures (final - starting)}}{\text{Increase in number of cells in control cultures (final - starting)}} \times 100$$

No sediments were observed in every dose group. However, suppressed cell proliferations were accompanied by dose increase. Accordingly, maximum doses were set at the con-

centrations where the estimated RICC value is $45 \pm 5\%$. Based on this, dose groups were organized as shown in Table 2, including negative and positive controls. Cells (6×10^4) were seeded and cultivated in 5 mL medium for 3 days with two culture flasks (bottom area, 25 cm²) for each dose. Prior to the treatment of K2H, the culture medium of each flask was removed, and 2.2 and 4.5 mL of the medium was added fresh to the S9+ group and S9– group, respectively, and treated with K2H in 1 hour. After 6 hours of treatment, the whole media solution was removed from treatment series 1 and 2, washed once with 5 mL Ca²⁺ and Mg²⁺ free Dulbecco's phosphate buffered saline (CMF D-PBS), fresh 5 mL culture medium was added, and culture was continued until harvest. Treatment series 3 consisted of kept culture without washing. After 22 hours of treatment, colchicine was treated to a final concentration of 1 µM for 2 hours, harvested, centrifuged, treated with hypotonic solution (75 mM KCl), fixed (methanol/acetic acid = 3:1, v/v), and dried, and metaphase chromosomes were prepared with 5% Giemsa staining. Chromosomal examination and analysis were performed according to the atlas of chromosome aberration by chemicals.⁴⁰ Structural (break and exchange) and numerical (diploid, polyploid, and endoreduplication) aberrations were analyzed. It is regarded as valid when the following conditions satisfied. Counting chromosomal aberrations should be possible in at least more than 100 metaphases. Metaphase chromosome frequency with structural aberrations (except gap) in the negative control should be lower than 5% and more than 10% in the positive control. Statistical analysis was performed using SPSS Statistics 22 for Medical Science, with significance level $p < 0.05$. For the structural aberrations, Fisher's exact test was conducted for comparing negative control and dose groups. When the aberration frequencies were significantly high, dose–response correlations were validated using the linear-by-linear association method in chi-square test. The same statistical analysis methods were also used for numerical aberrations. It is regarded as positive when the aberration frequencies of dose groups increased dose-dependently with statistical significance, or when reproducible positive responses were observed in at least one or more dose groups. Chromosomal aberration assay was conducted according to the OECD Principles of Good Laboratory Practice (1997) ENV/MC/CHEM(98)17, OECD Guideline for Testing of Chemicals TG473 (2014) *In Vitro* Mammalian Chromosomal Aberration Test, Standard for Toxicity Study of Pharmaceuticals (No. 2014-136, Ministry of Food and Drug Safety, Republic of Korea), and Good Laboratory Practice (No. 2014-67, Ministry of Food and Drug Safety, Republic of Korea).

3. Results

3.1. Single dose toxicity study showed normal changes with no toxicity

In the single dose toxicity study, animal deaths or gross organ lesions were not observed, and body weight changes

Table 2 – Treatment Group Organizations and Summary of Chromosomal Aberrations in Cultured Cells

Treatment series	Dose (µg/mL)	S9 mix	Aberrations							PP + ER		No. aberrant metaphase		RICC (%)	
			Cs type		Ct type		Others	No. Gaps	No.	Decision	No. Gap+	No. Gap-	Decision		
			csd	cse	ctd	cte									
1	Vehicle	0	+	0.0	0.0	0.0	0.0	0.0	1.0	0.0	N	1.0	0.0	N	100
	K2H	875	+	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N	0.0	0.0	N	98
		1750	+	0.0	0.0	0.0	0.0	0.0	1.5	0.0	N	1.5	0.0	N	99
		3500	+	0.0	0.5	0.0	2.5	0.0	1.0	0.0	N	3.5	3.0*	P	46
2	B[a]P	20	+	0.0	2.0	2.0	34.0	0.0	2.0	0.0	N	24.5	24.0**	P	54
	Vehicle	0	-	0.0	0.0	0.0	0.0	0.0	0.5	0.5	N	0.5	0.0	N	100
	K2H	625	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N	0.0	0.0	N	101
		1250	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N	0.0	0.0	N	84
3		2500	-	0.0	0.0	0.5	9.0	0.0	0.0	1.5	N	4.5	4.5**	P	48
	EMS	800	-	0.5	2.0	0.0	22.0	0.0	1.0	0.0	N	20.0	19.5**	P	53
	Vehicle	0	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N	0.0	0.0	N	100
	K2H	200	-	0.0	0.0	0.0	0.0	0.0	0.5	0.0	N	0.5	0.0	N	83
		400	-	0.0	0.0	0.0	0.0	0.0	1.0	0.5	N	1.0	0.0	N	78
		800	-	0.0	0.0	0.0	0.0	0.0	1.0	0.0	N	1.0	0.0	N	49
	EMS	600	-	0.0	0.0	1.5	17.5	0.0	2.0	0.0	N	27.5	25.5**	P	62

Numbers of cells examined, aberrations, PP + ER, and metaphases are mean values.

Treatment series 1: Treatment time – Recovery time = 6–18 hours.

Treatment series 2: Treatment time – Recovery time = 6–18 hours.

Treatment series 3: Treatment time – Recovery time = 24–0 hours.

B[a]P, benzo[a]pyrene; Cs, chromosome; csd, chromosome type deletions; cse, chromosome type exchanges; Ct, chromatid; ctd, Chromatid type deletions; cte, chromatid type exchanges; EMS, ethyl methanesulfonate; ER, endoreduplication; Gaps: chromosome type + chromatid type gaps; K2H, KIOM2012H; N, negative; Others, metaphases with more than 10 aberrations (including gaps) or with chromosome fragmentation; P, positive; PP, polyploidy; RICC, relative increase in cell count.

* Significantly different at $p < 0.05$ (Fisher's exact test) as compared with negative control.

** Significantly different at $p < 0.01$ (Fisher's exact test) as compared with negative control.

showed normal increases. In male and female rats of over 2500 mg/kg dose groups, compound-colored stool, diarrhea, and soiled perineal region were observed on Day 2, which reverted to normal on Day 3. These observations did not affect body weight increase and were therefore considered temporary, as usually found in many other animal experiments. Statistically significant differences were not observed among dose groups. Lethal dose was not applied.

3.2. Repeated dose toxicity study showed no toxicity for 2 weeks of experiment

Compound-colored stools were observed during the dosing period (except Day 1) in all groups. The perineal region was found soiled on Day 2 in one female of the 5000 mg/kg/d group. Diet consumption was significantly low on Day 1 ($p < 0.05$) in 1250 mg/kg/d and 5000 mg/kg/d group females. In urine analysis, ketone body (KET), specific gravity (SG), and protein (PRO) values were significantly higher in males compared with the control group ($p < 0.05$, $p < 0.01$). The pH was significantly low in the male groups of ≥ 625 mg/kg/d ($p < 0.05$, $p < 0.01$), and leukocyte (LEU) was significantly high in males of the 625 and 1250 mg/kg/d dose groups ($p < 0.05$). Females in the 2500 mg/kg/d group showed significantly low pH and high PRO values ($p < 0.05$). However, all these changes were in the normal range with no dose–response correlation. Hematological results were also within the normal ranges. In blood biochemical analysis, the total bilirubin (TBIL) was significantly high in males of the 5000 mg/kg/d group ($p < 0.01$). Alkaline phosphatase (ALP) values were significantly high in females of 625 and 5000 mg/kg/d groups ($p < 0.05$). Absolute and relative weights in male liver and relative weight in female liver were significantly high in the 5000 mg/kg/d group ($p < 0.05$). Autopsy revealed a stomach full of test material in one male of the 625 mg/kg/d group. This is not attributable to fasting, because the fasting body weight and blood results were normal, and no other animals—even those kept in the same cage—showed a full stomach. Consequently, it is judged that these changes are irrelevant to the test material K2H with no dose–response correlation. All other observations, such as death, general clinical signs, body weight, water consumption, and hematological data, showed no changes due to the toxic effect.

3.3. Repeated dose toxicity study showed no toxicity for 13 weeks of experiment

One female in the 1250 mg/kg/d group was found dead on Day 6. No general symptoms and changes related to the test material were observed until death. Autopsy findings showed soiled staining around the mouth and reddish discoloration, and a follicular foamy fluid in the lung. In all test groups, compound-colored stool was observed sporadically on Day 7 and after Day 10. Diarrhea was observed in all animals on Days 24–32, 34–62, and 65–92, and in four males on Days 63–64 in the 5000 mg/kg/d group. Hair loss was observed on Days 33–36 and 43–92 in one male of the 5000 mg/kg/d group, and salivation was observed in one male and one female of the 5000 mg/kg/d group at Day 8. Mean body weight in females was significantly high in the 5000 mg/kg/d group at Day 41; mean body weight gains were also significantly high (Fig. 1).

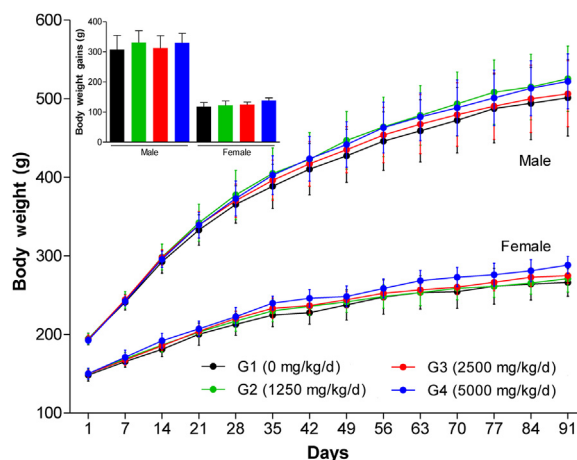


Fig. 1 – Body weight changes and gains in K2H administered male and female rats for 13 weeks of experimental period. K2H, KIOM2012H.

The average diet consumption was significantly high in males of the 5000 mg/kg/d group at Days 56 and 90 ($p < 0.01$), and significantly high in females of the ≥ 2500 mg/kg/d group at Day 77 ($p < 0.05$, $p < 0.01$). The average daily water consumption was significantly high during the entire experimental period in males and females of the 5000 mg/kg/d group ($p < 0.05$, $p < 0.01$). In males, KET was significantly high in the 5000 mg/kg/d group, and SG was high in the ≥ 2500 mg/kg/d group ($p < 0.05$). In 1250 and 5000 mg/kg/d group males, the PRO level was significantly high ($p < 0.05$, $p < 0.01$), but the change was not related to the test material owing to the lack of dose–response correlation. In addition, occult blood (BLO) levels were significantly low in males of ≥ 2500 mg/kg/d groups ($p < 0.05$), but the normal control values were negative and is accordingly judged to be changes not related to toxicity. Male reticulocyte (RET) values in the ≥ 2500 mg/kg/d group and male platelet (PLT) values in the 5000 mg/kg/d group were significantly high ($p < 0.05$). The eosinophil (EOS) percentile was significantly low in males of the ≥ 2500 mg/kg/d group ($p < 0.05$, $p < 0.01$), the activated partial thromboplastin time (APTT) was significantly shortened in the 2500 mg/kg/d male group ($p < 0.01$), and the PT was significantly prolonged in the 1250 mg/kg/d female group ($p < 0.05$), with no dose–response correlation. TBIL was significantly high ($p < 0.01$) in males of the 5000 mg/kg/d group and tended to high tendency in females. Male Cl^- concentration, female Na^+ and creatine phosphokinase (CPK) concentrations were significantly low in the 5000 mg/kg/d group ($p < 0.05$). Absolute and relative weights of the spleen, bilateral kidney, liver, and absolute weight of heart were significantly high in the 5000 mg/kg/d group ($p < 0.05$, $p < 0.01$). In addition, a significant relative increased liver weight was found in 2500 mg/kg/d group ($p < 0.05$). Absolute and relative weights of bilateral kidneys and liver were significantly high in females of the 5000 mg/kg/d group, and absolute weight of the bilateral ovary was high in females of the 5000 mg/kg/d group ($p < 0.05$). Relative weight of left kidney and heart were significantly low ($p < 0.05$, $p < 0.01$) in 1250 mg/kg/d group males, but this change is considered to be of no relation to the test

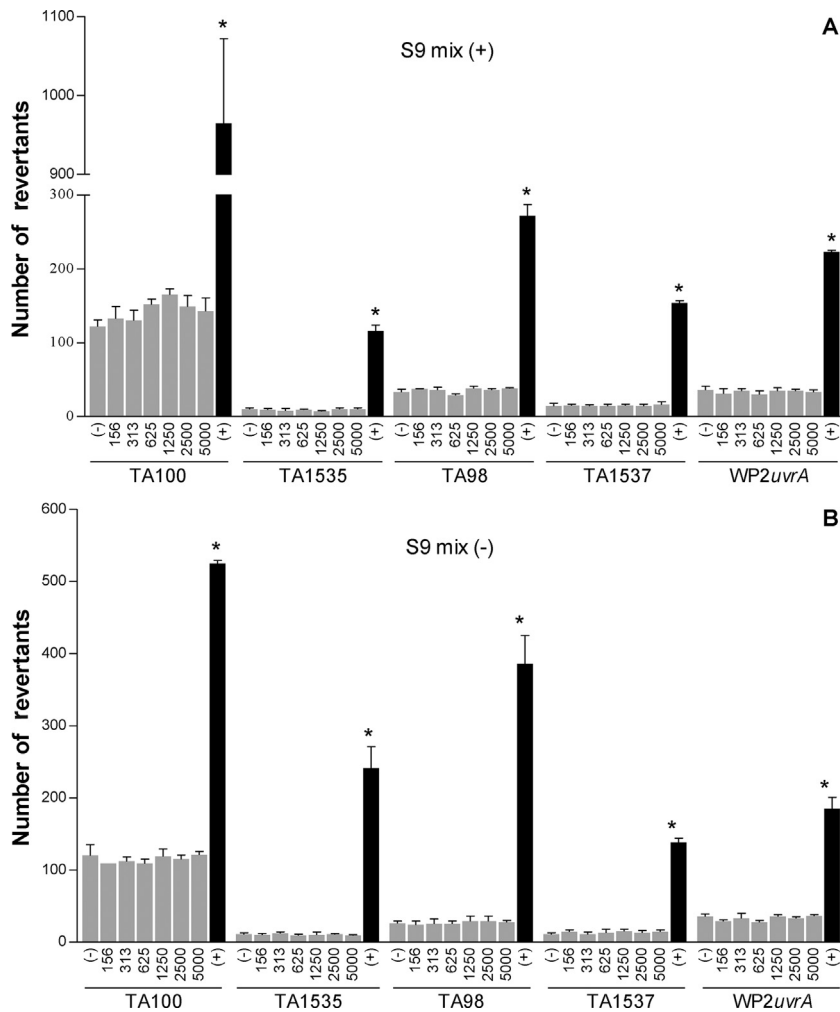


Fig. 2 – Effects of K2H on bacterial reverse mutation. (A) With S9 metabolic activation. (B) Without S9 metabolic activation. *Significantly different at $p < 0.05$ (Fisher's exact test) as compared with negative control. –, vehicle control; +, positive control; K2H, KIOM2012H.

material with no dose–response correlation. Autopsy findings showed ragged, glandular, and whitish discolored stomach in two females of the 5000 mg/kg/d group. In one male of the 1250 mg/kg/d group, the right kidney was enlarged, and the renal pelvis was rough, swallowed, bulged, and hardened. Hair loss was found in one male of the 5000 mg/kg/d group. Clear fluid retention in the uterus was observed in four, three, two, and four cases in all dose groups. Chronic progressive nephropathy and tubular basophilia in male kidneys and tubular basophilia in female kidney were found. Vacuolation in zona fasciculata was observed in male adrenal cortex (≥ 1250 mg/kg/d groups), and retrograde nephropathy was observed in one male of the 1250 mg/kg/d group. Renal tubule dilation and degeneration, infiltration of lymphocytes, neutrophils, plasma cells, and pelvis dilations were found in both medulla and cortex. Clear fluid retention in the uterus was identified as dilation of lumen. It is considered that this does not indicate a dose–response correlation, nor is it a spontaneous change associated with the sexual cycle, and thus is not an effect of the test material.

3.4. Antibacterial toxic effects were not found in bacterial reverse mutation assay

No increased colony numbers and toxicity were found in all bacterial strains at all concentrations used, regardless of S9 mix. All positive controls yielded obvious positive results as expected, and colonies due to microbial contamination were not found, which confirms the sterility of the test material K2H and S9 mix. Results are summarized in Fig. 2.

3.5. Micronucleus assay revealed cytotoxic indicators in normal range

The MNPCE frequencies observed in 2000 PCEs per animal were 1.50, 1.17, 1.33, and 2.00 for the negative control, 1250, 2500, and 5000 mg/kg/d groups, respectively. There was no statistically significant increase in MNPCE frequencies in the test groups compared to the negative control group. By contrast, in the positive control group, the MNPCE frequency was 57.17, which was significantly increased ($p < 0.01$) compared to the negative control group. Ratios of PCE/RBC, known as cytotoxicity indicator, were 0.57, 0.54, 0.56, and 0.58, for the

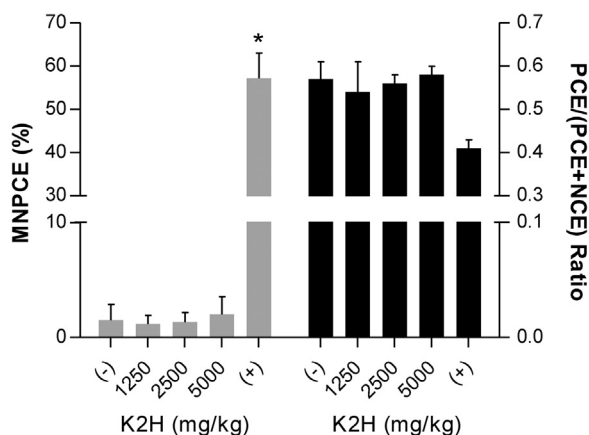


Fig. 3 – Effect of K2H on micronucleated polychromatic erythrocyte (MNPCE, left Y axis) and PCE/(PCE + NCE) (polychromatic/normochromatic erythrocyte) ratio (right Y axis).

*Significantly different at $p < 0.05$ (Fisher's exact test) as compared with negative control.

–, vehicle control; +, positive control; K2H, KIOM2012H; NCE, normochromatic erythrocyte; PCE, polychromatic erythrocyte.

negative control, 1250, 2500, and 5000 mg/kg/d groups, respectively, which were not statistically significant. Meanwhile, the PCE/RBC ratio was 0.41 in the positive control and significantly decreased ($p < 0.01$) compared to the negative control. Results of micronucleus assay are shown in Fig. 3.

3.6. Metaphases were normal in numerical and structural chromosome aberration assay

In 6 hours of treatment with metabolic activation, the frequencies of structural aberrations were 0.0, 0.0, 0.0, and 3.0 for the vehicle, 875, 1750, and 3500 $\mu\text{g}/\text{mL}$, respectively, and a significant increase was observed in 3500 $\mu\text{g}/\text{mL}$ group with RICC 46% ($p < 0.05$). In order to assess dose–response correlations, a linear-by-linear association method of the chi-square test was applied, and a significant result was obtained ($p < 0.01$). The frequencies of numerical aberrations were 0.0 in the negative control group and all dose groups. There was a significant increase in the frequency of structural aberrations in the positive control group (24.0, $p < 0.01$). In 6 hours of treatment without metabolic activation, the frequencies of structural aberrations were 0.0, 0.0, 0.0, and 4.5 in the vehicle, 625 $\mu\text{g}/\text{mL}$, 1250 $\mu\text{g}/\text{mL}$, and 2500 $\mu\text{g}/\text{mL}$, respectively, and a significant increase was observed in the 2500 $\mu\text{g}/\text{mL}$ group with RICC 48% ($p < 0.01$). Linear-by-linear association of chi-square test revealed significant dose–response correlation ($p < 0.01$). The frequencies of numerical aberrations were less than 1.5 in the negative control group and all dose groups, and no significant increase was observed in all groups compared to the negative control group. As a matter of course, the frequency of structural aberration was significantly high in the positive group (19.5, $p < 0.01$). In 24 hours of treatment without metabolic activation, structural aberration was not observed in the vehicle and all treated groups (200, 400, and 800 $\mu\text{g}/\text{mL}$).

The frequencies of numerical aberrations were ≤ 0.5 in the negative control group and all dose groups, with no significant increase. As a matter of course, there was a significant increase in the frequency of structural aberration in the positive control (25.5, $p < 0.01$). Consequently, the frequency of structural chromosome aberrations were increased in groups treated for 6 hours with or without metabolic activation, but there was no increase in numerical aberration. All results of chromosomal aberrations are summarized in Table 2.

4. Discussion

The present study was conducted to evaluate the toxicity of the traditional medicine formula K2H. No animal death, abnormal body weight changes, or gross lesions were found in the single dose toxicity assay using rats. Therefore, the approximate lethal dose is considered to be more than 10,000 mg/kg in both males and females. Two weeks repeated dose toxicity study was conducted to determine the doses of 13 weeks repeated toxicity study. Observed increases in KET, SG, and PRO in males in 625 mg/kg/d and more dose groups, and increase in TBIL and liver weight in males and females in the 5000 mg/kg/d group were not accompanied by any corresponding changes in blood chemistry and organ weight. However, these increases are considered to be changes in dose–response correlation within normal variation.

In the assay of 13 weeks repeated dose toxicity, only one female death was observed in the 1250 mg/kg/d dose group, and is not related with dose–response correlation. In addition, the presence of a foamy fluid in lung bronchus indicated physical injury, which is considered an accidental change by the test material. Colored stools observed in all test groups are the excretion of the test material or its metabolite, and it is considered to be part of the changes induced by the test material itself. Diarrhea or soft stool observed in the 5000 mg/kg/d dose group was consistently observed throughout the experimental period with dose–response correlation and was judged to be attributable to the test material. However, it is not considered a toxicological change because it is not accompanied by other related changes such as weight loss. According to the research report on glycyrrhizin, which is one of the components of K2H, administered to rats with a dose of 2000 mg/kg/d for 14 days,⁴¹ and another report on body weight observed during the 7 days of administration to male mice at 500 mg/kg/d,⁴² the body weight increase seems to be one of the changes that can be attributed to the test material, but it is not considered a toxicological change due to a small variation. Hematological tests showed that the increase of RET in males in over 2500 mg/kg/d groups and the increase of PLT in males of the 5000 mg/kg/d group were related to the absolute and relative weight increase in the male spleen in the 5000 mg/kg/d group; histopathological examinations does not show concordance with any changes, and it is not considered to be toxic. Based on the results of the blood biochemical tests, the increase of TBIL concentration in males and females of the 5000 mg/kg/d group seems to be one of the changes with dose–response correlation induced by the test material. However, histopathologic change was not observed in the liver, and is not considered to be a toxicologically deleterious change. In addition, the

decrease in CPK observed in the 5000 mg/kg/d group appears to be a dose–response correlated change attributable to the test material. However, CPK is present in the skeletal muscle, myocardium, brain, and smooth muscles, and is not a toxic change because CPK concentration in the blood is rather increased when those organs were damaged. The relative and absolute liver weight increases in male and female rats of the 2500 mg/kg/d group were in dose–response correlation, and according to reports glycyrrhizin and glycyrrhetic acid significantly increased the levels of CYP3A, CYP2B1, and CYP1A2 activity in the liver⁴³; these changes seem to be due to the test material. However, these changes are not considered toxicologically deleterious because of the lack of corresponding changes in blood biochemical markers and histopathological examinations associated with liver. Increase of daily water consumptions in 2500 mg/kg/d females and 5000 mg/kg/d males and females, increase of urine KET in 5000 mg/kg/d males, increase of SG in ≥ 2500 mg/kg/d males, decrease of Cl^- and Na^+ in males and females of the 5000 mg/kg/d group, as well as relative and absolute kidney weight increases in 2500 and 5000 mg/kg/d groups were dose–response correlated, and seem to be attributable to the corresponding changes in chronic progressive nephropathy and increased tubular basophilia.

However, chronic progressive nephropathy can be observed in SD rats and also in the vehicle control, and differences in incidence are so small. In addition, increase of tubular basophilia was found in only a small number of animals, and those lesions were minimal and are therefore believed to be a nontoxicological effect. Vacuolations in zona fasciculata were found in the adrenal cortex of 2500 mg/kg/d group males, which seems to be attributable to the test material. However, these changes are known to occur naturally as an effect of the test material or stress^{44,45} and are also not considered toxic changes.

The reverse mutation test satisfied all the validity criteria. Mean colony numbers were not increased in all test strains, regardless of metabolic activation. This finding did not satisfy the criteria for a positive result. Therefore, K2H is not considered to induce reverse mutation under the present assay conditions.

In the micronucleus test, result of MNPCE for 2000 PCEs per animal showed no significant and dose-dependent increase in all treated groups compared to the negative control, or no reproducible increase in one or more dose groups, which does not satisfy the criteria for a positive result. The increase in body weight in high dose groups on the day of the autopsy was statistically significant but was considered to have no significant implication. Therefore, K2H was not considered to induce micronuclei of mice bone marrow cells under the present test conditions.

In the analysis of chromosomal aberration, frequencies of structural aberration in groups treated for 6 hours (with or without metabolic activation) were increased compared to the negative control. These results satisfied all criteria for a positive decision. Accordingly, K2H can be considered to induce chromosomal aberrations in cultured CHL cells under the present experimental conditions. However, according to the OECD Guideline for Testing of Chemicals TG473 (2014) *In Vitro* Mammalian Chromosomal Aberration Test, it

is described that “if no precipitate or limiting cytotoxicity is observed, the highest test concentration should correspond to 10 mM, 2 mg/mL or 2 $\mu\text{L}/\text{mL}$, whichever is the lowest.” In the present study, the K2H doses that induced structural aberration are 3500 $\mu\text{g}/\text{mL}$ (with metabolic activation) and 2500 $\mu\text{g}/\text{mL}$ (without metabolic activation), which exceed the doses recommended in the OECD guideline. Under this criterion, K2H can be considered to have no genotoxicity. However, further in-depth genotoxic studies should be conducted, especially for the development of pharmaceuticals and functional food or additives, in the medicinal sense of healthcare material. In addition, as mentioned in the Introduction section, K2H was previously noted to have a lipid-lowering and body weight-reducing effect in mice fed with high-fat diet.³⁵ The body weight of high-fat diet mice was reduced according to the increasing doses of K2H, whereas normal increases were observed in the present study. Therefore, this can be useful to further investigate in more detail the mechanism related to lipid, fat, and obesity, with the safety-assured material, K2H.

Conflict of interest

The authors declare that there are no conflict of interests.

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