Pharmacogn. Mag.
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ORIGINAL ARTICLE

In vitro Genotoxicity and *in vivo* Single-Dose Oral Toxicity of Polysaccharide Fraction from the Leaves of *Diospyros kaki* Thunb.

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Submitted: 18-Mar-2020 Revised: 22-Apr-2020 Accepted: 11-Aug-2020 Published: 11-Nov-2021

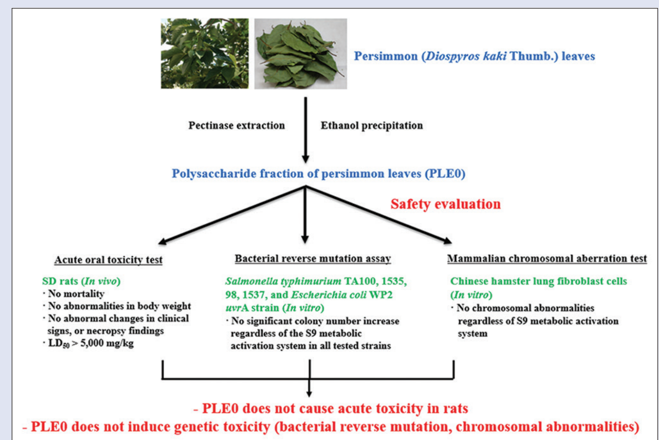
ABSTRACT

Background: Polysaccharides isolated from medicinal herbs are regarded as important bioactive materials due to their various physiological activities. In our previous study, the polysaccharide fraction (PLE0) was separated from pectinase-digested persimmon (*Diospyros kaki* Thunb.) leaves and showed immunostimulatory and anti-osteoporotic effects. However, there is currently no information regarding the toxicity of PLE0. **Materials and Methods:** As a toxicological evaluation, acute oral toxicity and *in vitro* genotoxicity assays (chromosomal aberration and bacterial reverse mutation tests) were performed. **Results:** In the single-dose acute oral toxicity test in female and male Sprague-Dawley (SD) rats, the median lethal dose of PLE0 was higher than 5000 mg/kg and adverse effects were not observed in terms of mortality and abnormal changes in clinical signs. Furthermore, chromosomal abnormality and bacterial reverse mutation tests showed that PLE0 displays no mutagenicity or clastogenicity. **Conclusion:** Based on these results, PLE0 was not mutagenic and did not induce chromosomal aberration *in vitro* under our experimental conditions and exhibited no acute oral toxicity at up to 5000 mg/kg in SD rats.

Key words: Acute oral toxicity, bacterial reverse mutation, chromosomal aberration, *Diospyros kaki* Thunb. leaves, polysaccharide

SUMMARY

- In this study, an acute oral toxicity test using Sprague-Dawley (SD) rats and *in vitro* genotoxicity tests were conducted as a preliminary evaluation of the possibility of the development of PLE0, a bioactive polysaccharide fraction isolated from pectinase-digested leaves of *Diospyros kaki*, as an herbal medicine or a functional food. Genotoxicity was assessed using *in vitro* chromosomal abnormality and bacterial reverse mutation assays. In the single-dose oral toxicity test, single-dose oral administration of PLE0 in SD rats (0, 1000, 2000, and 5000 mg/kg) resulted in no deaths or any unusual symptoms, such as significant weight changes. Therefore, the median lethal dose of PLE0 was determined to be higher than 5000 mg/kg. In the bacterial reverse mutation test, no significant colony number increases were found after PLE0 treatment, regardless of the S9 metabolic activation system. Furthermore, PLE0 did not induce chromosomal aberration in any of the treatment groups in the chromosomal abnormality test. Taken together, the above results suggest that PLE0 does not cause acute toxicity in rats and does not induce genetic toxicity under our experimental conditions (bacterial reverse mutation and chromosomal abnormality tests).



Abbreviations used: B[a] P: Benzo[a] pyrene; CHL/IU: Chinese hamster lung fibroblast cells; 4NQO: 4-nitroquinoline-1-oxide; GLP: Good laboratory practice; HPAEC-PAD: High-performance anion-exchange chromatography with pulsed amperometric detection; HPSEC: High-performance size-exclusion chromatography; IACUC: Institutional Animal Care and Use Committee; ICR-191: Acridine mutagen ICR 191; KDO: Keto-3-deoxy-D-mannooctanoic acid; LD_{50}: The median lethal dose; NIFDS: National Institute of Food and Drug Safety Evaluation of Korea; OECD: Organization for Economic Cooperation and Development; PLE0: Polysaccharide fraction from pectinase-digested persimmon leaves; RI: Refractive index; RICC: Relatively increased cell count; SA: Sodium azide; SD: Sprague-Dawley; SPF: Specific-pathogen-free; 2-AA: 2-aminoanthracene; 2-NF: 2-nitrofluorene.

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DOI: 10.4103/pm.pm_94_20

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INTRODUCTION

Diospyros kaki Thunb., also known as persimmon or Oriental persimmon, is cultivated in several East Asian countries, including Korea.[1] The leaves of *D. kaki* have been traditionally used to treat hypertension, atherosclerosis, angina, internal hemorrhaging, ischemia stroke, and several infectious diseases in East Asia.[2] Various pharmacological functions of *D. kaki* leaves, such as antioxidant,[3] attenuation of neuroinflammation-mediated brain aging,[4]

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Cite this article as: Cho CW, Song YR, Hwang YH, Rhee YK, Lim WC, Choi JW, *et al*. *In vitro* genotoxicity and *in vivo* single-dose oral toxicity of polysaccharide fraction from the leaves of *Diospyros kaki* Thunb.. Phcog Mag 2021;17:406-12.

antitumor,[5] and anti-atherosclerosis effects,[6] have been recently reported. Most studies using *D. kaki* leaves have been performed with low-molecular-weight compounds such as polyphenols, flavonoids, and anthocyanins.[7] In addition to low-molecular-weight compounds, polysaccharides, a major type of macromolecule, from plant sources have a broad spectrum of biological effects including anticancer, antioxidant, and immune stimulation.[8] In line with these findings, we previously isolated the polysaccharide fraction (PLE0) from pectinase-digested *D. kaki* leaves and reported their immunostimulatory[9,10] and anti-osteoporotic[11] effects.

In general, medicinal herb extracts are considered to be safe because they have been used in traditional medicine and are perceived as natural products.[12] However, some medicinal herb extracts, including *Aristolochia manshuriensis* Kom. and *Acer tegmentosum*, were reported to display genotoxicity.[13,14] Therefore, it is important to evaluate the toxicity of newly developed extracts originating from traditional medicinal herbs to eliminate possible side effects. Furthermore, toxicity evaluation is a crucial step before human clinical trials.

To evaluate the toxicological profiles of PLE0, acute oral toxicity and genotoxicity were performed at good laboratory practice facility. The acute toxicity of PLE0 was analyzed using Sprague-Dawley (SD) rats after a single oral administration to determine the immediate toxic effects. In addition, the genotoxicity of PLE0 was assessed using *in vitro* mammalian chromosomal aberration and bacterial reverse mutation tests.

MATERIALS AND METHODS

Pilot-plant scale preparation of PLE0

In this study, PLE0 was prepared by enzymatic hydrolysis extraction at a pilot-plant scale, as described in our previous study.[10] *D. kaki* leaves were cultivated and collected from Yeongcheon-si (Gyeongsangbuk-do, Korea); this is one of the major *D. kaki* production regions in Korea. A commercial pectinase (Plantase MAX from *Aspergillus niger*; optimal pH, 3.0–5.0; optimal temperature, 10°C–55°C) was procured from Vision Corporation (Seongnam, Korea). Briefly, dried *D. kaki* leaves (10 kg) were crumbled and added into 200 L of tap water. The pH of blend was adjusted to 4.5–5.0, and enzyme digestion was implemented using pectinase at 50°C for 72 h in a circulated extraction tank, in which pectinase (1.5% v/w) was added twice at 0 and 36 h. Thereafter, the pectinase hydrolysate of *D. kaki* leaves was filtered through 0.5-μm depth cartridge filters to remove insoluble precipitates and concentrated under reduced pressure at 50°C to obtain the sugar degree of 5.0 degrees Brix. The polysaccharides from the aqueous extract were precipitated by adding 99% cold ethanol at a 1:3 ratio (v/v). The precipitate was then dissolved in purified water and dried with a spray dryer (Ein System, Seoul, Korea).

Chemical composition analysis of PLE0

The contents of neutral and uronic sugars in the PLE0 sample were analyzed by the phenol–sulfuric acid method[15] and the sulfuric acid–carbazole method,[16] respectively. The content of keto-3-deoxy-D-mannooctanoic acid (KDO)-like materials was determined according to the modified thiobarbituric acid method,[17] and protein content was determined by the Bradford assay. The sugar composition of the PLE0 sample was analyzed using high-performance anion-exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) in a high-performance ion chromatography system (ICS-5000; Dionex, Sunnyvale, CA, USA). For the analysis, the polysaccharide was hydrolyzed to monosaccharides by treatment with 2 M trifluoroacetic acid at 97°C for 5 h. A CarboPac PA1 column (4 mm × 250 mm, Dionex) at 30°C was used to separate the sugar components in the resulting hydrolysate. Neutral sugars were eluted under isocratic conditions with 18 mM NaOH, while uronic acids were separated with 100 mM NaOH and 100 mM NaOAc (flow rate: 1.0 mL/min). Each sugar component was identified and quantified based on the response curve of standard sugars (Sigma-Aldrich, St. Louis, MO, USA).

Molecular weight analysis of PLE0

The molecular weights of PLE0 were analyzed by a high-performance size-exclusion chromatography (HPSEC) system (PU-2089 Plus, JASCO, Tokyo, Japan) with a refractive index (RI) detector (RI-2031 Plus, JASCO). The combined columns of Asahipak GS-620 + GS-520 + GS-320 (0.76 × 30 cm each; Showa Denko Co., Tokyo, Japan) were used at 40°C. A solution of PLE0 (20 mg/mL, 20 μL) was injected and eluted with 50 mM ammonium formate (pH 5.5 with formic acid) at a flow rate of 0.4 mL/min. A pullulan standard set (Fluka, München, Germany) was used to calibrate the standard curve to estimate the molecular weights of PLE0.

Animals

For single-dose oral toxicity, 7-week-old specific-pathogen-free female and male SD rats were procured from Samtako Bio Korea Co., Ltd. (Osan, Korea). An irradiation-sterilized pellet diet for laboratory animals (2918C; Harlan Laboratories Inc., Madison, WI, USA) and groundwater (UV sterilized and filtered) were provided *ad libitum*. The rats were examined for appearance and individually weighed upon acquisition and acclimated under the laboratory conditions for 6 days before beginning experiments. All animal studies were approved (14-R507) by the Institutional Animal Care and Use Committee of the Chemon Non-clinical Research Institute (Suwon, Korea).

In vivo single-dose oral toxicity test

The acute single-dose oral toxicity assay was performed under the guideline (No. 2014-136) of the National Institute of Food and Drug Safety Evaluation (NIFDS) of Korea.[18] The single-dose oral toxicity of PLE0 in female and male SD rats (5/sex/group) was tested at 1000, 2000, and 5000 mg/kg. All SD rats were fasted overnight before administration, and PLE0 was directly administered by gavage with a feeding needle. The pellet diet was provided about 4 h after administration. PLE0 was dissolved in sterile distilled water (Daihan Pharmaceutical Co., Ltd., Seoul, Korea). During the observation periods, all rats were observed daily for 14 days for mortality and clinical signs. The day of the administration was designated as day 0. On day 14, rats were anesthetized by inhaling carbon dioxide and, after a laparotomy, were sacrificed by exsanguination.

Bacterial reverse mutation test

The bacterial reverse mutation assay was performed based on the Organization for Economic Cooperation and Development (OECD) guideline TG471[19] and guideline of NIFDS of Korea (No. 2016-141).[20] The histidine auxotroph *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 strains,[21] and the tryptophan auxotroph *Escherichia coli* WP2 *uvr*A strain,[22] were used. Preincubation was performed in the absence or presence of an S9 metabolic activation system. The metabolic activation S9 mix (5% S9, v/v) was made with Aroclor-induced rat liver S9 fraction (PN 11-01 L, Lot No: 3818; MOLTOX, Boone, NC, USA) and Cofactor-I (Wako, Osaka, Japan). Sodium azide (SA; 0.5 μg/plate) for *S. typhimurium* TA100 and TA1535, acridine mutagen ICR 191 (ICR-191; 0.5 μg/plate) for *S. typhimurium* TA1537, 2-nitrofluorene (2-NF; 2.0 μg/plate) for *S. typhimurium* TA98, and 4-nitroquinoline-1-oxide (4NQO; 0.5

µg/plate) for *E. coli* WP2 *uvr*A were used as positive controls for nonmetabolic activation. In samples subjected to metabolic activation, benzo[a] pyrene (B[a] P; 1.0 µg/plate) for *S. typhimurium* TA98 and 2-aminoanthracene (2-AA; 1.0-6.0 µg/plate) for *S. typhimurium* (TA100, TA1535, and TA1537) and *E. coli* WP2 *uvr*A were used as positive controls. All positive control reagents were purchased from Sigma-Aldrich. The result was judged positive if there was a dose–dependent increment of at least twofold over the range tested in the number of revertant colonies per plate (at least one strain in the presence or absence of the S9 metabolic activation).

In vitro mammalian chromosomal aberration assay

The chromosomal aberration assay was performed under the OECD guideline TG473[23] and guideline of NIFDS of Korea (No. 2016-141).[20] Chinese hamster lung fibroblast (CHL/IU; ATCC CRL-1935) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in minimal essential medium (Gibco BRL, Grand Island, NY, USA) with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA). The appropriate doses of PLE0 were determined in terms of solubility, osmolality, and cytotoxicity. Cytotoxicity was determined by calculating the relatively increased cell count (RICC) according to the following formula (1):

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

The inhibition of cell proliferation was observed with increasing PLE0 concentrations in all treatments. The highest dose required for the chromosomal abnormality assay was determined by calculating the concentration required to obtain a RICC value of 45 ± 5% in each treatment condition. B[a] *P* (20 µg/mL) with S9 (PN 11-01 L, Lot No: 3818; Moltox) metabolic activation and 4NQO (0.4 µg/mL) without S9 activation were used as positive controls. Isotonic $NaCl_2$ solution (0.9% w/v; Daihan Pharmaceutical Co., Ltd.) was used as a negative control. For short-term treatments, the cells were treated with PLE0 for 6 h without or with metabolic activation and then cultured for a further 18 h without metabolic activation according to the following procedure. In the 6-h treatment groups, cells were rinsed with phosphate-buffered saline (Gibco) after 6 h from the start of treatment. Thereafter, fresh medium was added and cultured continuously until the collection of metaphase cells. For continuous treatments, cells incubated without the S9 mix were cultured continuously for 24 h until the collection of metaphase cells without washing. At 22 h after the initial treatment, all flasks were treated with 50 µL colchicine solution (final concentration: 1 µM) and cells in metaphase cells were collected after a further 2 h. Medium containing the metaphase cells was centrifuged at 1,500 g for 5 min and stained with 5% Giemsa solution after fixation. Chromosomal abnormalities were identified and numbered based on the atlas of chromosome aberrations by chemicals.[24]

Statistical analysis

Animal body weight data were statistically analyzed by one-way analysis of variance. Fisher's exact test was applied for the *in vitro* chromosomal abnormality assay to compare data between the negative control group and the positive control group or the treatment groups. The significance level was set at $P < 0.05$. All statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA).

Table 1: Chemical properties of polysaccharides (PLE0) manufactured from pectinase-treated *Diospyros kaki* leaves

Details	PLE0[a]
Chemical composition (%)[b]	
Neutral sugar	54.9±0.5[c]
Uronic acid	35.8±1.0
KDO-like materials	3.0±0.2
Protein	6.2±1.3
Composition of sugar (mol%)[d]	
Arabinose	7.6±0.3
Fucose	2.6±0.3
Galactose	31.1±1.7
Glucose	14.5±3.8
Mannose	11.8±0.4
Rhamnose	8.3±0.8
Xylose	0.6±0.1
Galacturonic acid	17.9±3.7
Glucuronic acid	5.7±0.6

[a]PLE0 was pilot-scale manufactured for industrial applications; [b]Percentage dry weight; [c]Values are shown as the mean±SD, *n*=3; [d]Mol% was calculated from the detected total sugar. KDO: 2-keto-3-deoxy-mannoctanoic acid; PLE0: Polysaccharide fraction from pectinase-digested persimmon leaves; SD: Standard deviation

RESULTS

Chemical and monosaccharide compositions and physico-chemical properties of PLE0

In this study, PLE0 from pectinase-treated *D. kaki* leaves was obtained at a yield of 4.08% in pilot-scale production. As shown in Table 1, PLE0 primarily contained neutral sugars (54.9%) and uronic acid (35.8%), with low concentrations of protein and KDO-like materials. Furthermore, the monosaccharide composition of PLE0 was analyzed by HPAEC-PAD. The results indicated that PLE0 contained mainly arabinose (7.6 mol%), galactose (31.1 mol%), glucose (14.5 mol%), rhamnose (8.3 mol%), and galacturonic acid (17.9 mol%); in addition, our results detected low proportions of fucose, xylose, and glucuronic acid. The high ratio of rhamnose, galactose, arabinose, and galacturonic acid indicated that PLE0 contains pectin-rich structures consisting of homogalacturonan, rhamnogalacturonan-I, and rhamnogalacturonan-II, as mentioned in our previous results.[10] The molecular weight distribution of PLE0 was confirmed by HPSEC-RI analysis [Figure 1]. PLE0 had a large major peak with the average molecular weight value of 19.1×10^3 g/mol and four smaller peaks with the average molecular weights of 88.5, 59.2, 1.6, and 1.0×10^3 g/mol. This result suggested that PLE0 consists of heterogeneous polysaccharide fractions. In our previous study, polysaccharides extracted from persimmon leaves at different maturity stages showed five distinct groups with average molecular weight values of 86.6, 21.6, 7.3, 2.2, and 1.2 kDa.[25]

In vivo single-dose oral toxicity test

In our dosage selection study, no animal death was found up to the highest concentration (5000 mg/kg). According to the dosage selection analysis, 5000 mg/kg was selected as the highest dose and lower dose groups (1000 and 2000 mg/kg) were added. A vehicle control group, treated with sterile distilled water, was also included. No unscheduled deaths or abnormal body weight changes were observed [Figure 2a]. In terms of body weight gain, one female group (1000 mg/kg) showed significantly reduced weight gain compared to the negative control group [Figure 2b]. In terms of necropsy, no significant gross changes in major organs (liver, kidneys, lungs, etc.) were detected in any of the

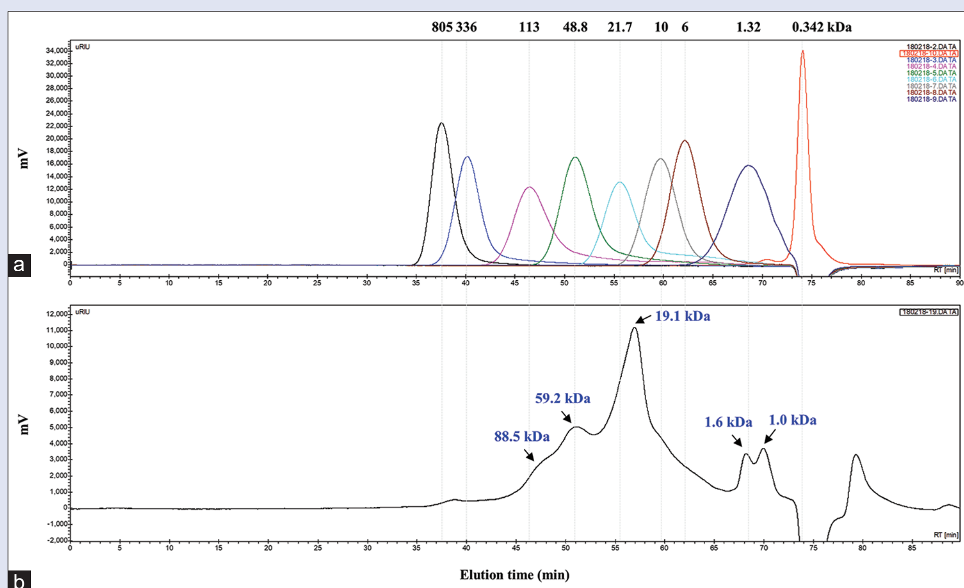

Figure 1: High-performance size-exclusion chromatography refractive index chromatograms of (a) pullulan standards and (b) PLE0

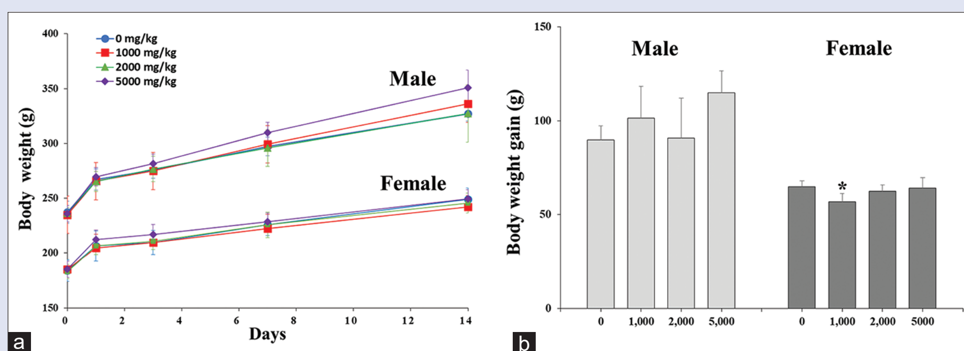

Figure 2: Body weight (a) and body weight gain (b) in single-dose oral toxicity test of PLE0. The day of administration was designated day 0. Male and female rats were randomly separated into four groups (5/sex/group). Data are expressed as the mean ± Sprague-Dawley. Body weight gain was calculated as body weight on day 14 – body weight on day 0. **P* < 0.05 compared to the vehicle control

PLE0-treated groups. Based on the above results, a single-dose oral administration of PLE0 in SD rats resulted in no mortality, abnormalities in body weight, abnormal changes in clinical signs, or necropsy findings.

Bacterial reverse mutation test

In our analysis to determine the optimal range of concentrations, no precipitations or other abnormalities were observed in any plates during colony counting. In all tested strains, no cytotoxicity was detected at PLE0 concentrations of up to 5000 μg/plate. The mean number of revertant colonies in the PLE0-treated groups was not increased in all tested strains, regardless of treatment with the S9 metabolic activation system [Figure 3]. On the other hand, positive results were obtained in all positive control groups for each strain.

In vitro chromosomal aberration analysis

Next, we evaluated whether PLE0 would induce structural or numerical chromosomal aberrations in CHL/IU cells. PLE0-induced chromosomal aberration results are summarized in Table 2. The frequency of structural abnormalities did not show a statistically significant increase at all concentrations of PLE0, and there was no concentration-dependent effect, regardless of the S9 metabolic activation system. On the other hand, the frequency of metaphase cells with structural abnormalities was significantly increased in the positive control groups. The frequency of numerical metaphase abnormalities was 0.0% in the all PLE0-treated and negative control groups, with no dose–response correlation observed.

DISCUSSION

The use of functional foods or medicines originating from medicinal herbs has increased not only in Asia but also worldwide.[26] In the United States, nearly 20% (1 in 5) of adults are known to use herbal products.[27] Herbal medicines have long been popular, and most users believe that herbal medicines are safe. In particular, from the viewpoint of safety, functional foods and medicines based on medicinal herbs are preferable over synthetic drugs.[12] However, there are still some safety issues surrounding medicinal herb extracts because of potential factors such as toxic materials within the medicinal herb itself, contamination by heavy metals and pesticides, and a variety of toxic materials that may be absorbed in the cultivation and extraction process.[27]

Therefore, this study focused on identifying the toxicological characteristics of PLE0, a bioactive polysaccharide fraction isolated

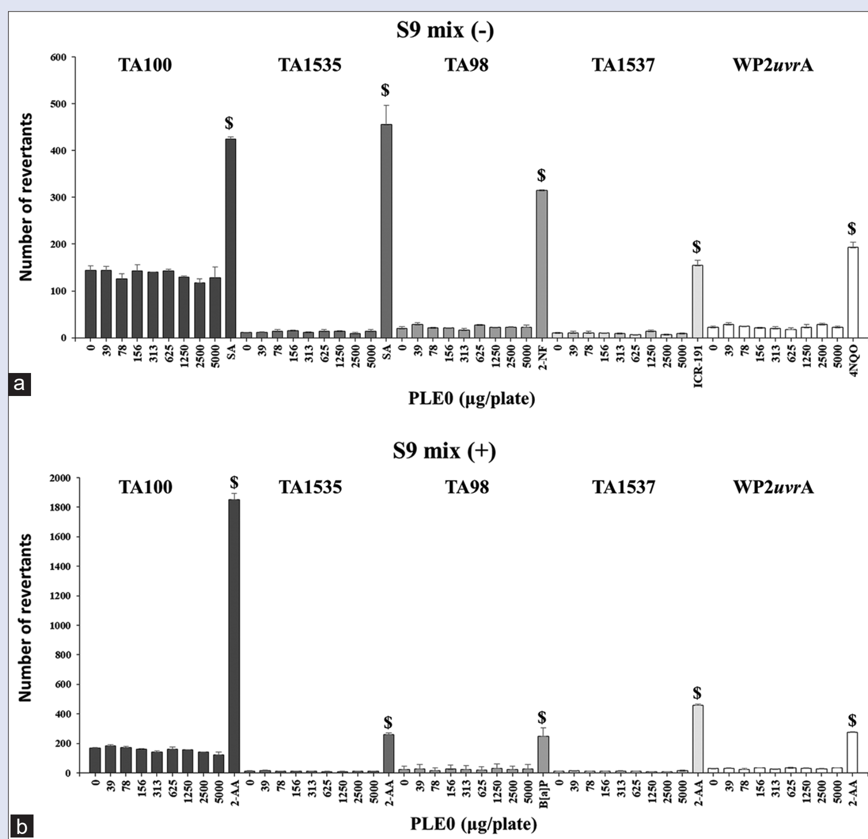

Figure 3: Effect of PLE0 on bacterial reverse mutation (a) without (–S9 mix) or (b) with (+S9 mix) the metabolic activation of *Salmonella typhimurium* (TA100, TA1535, TA98, and TA1537) and *Escherichia coli* (WP2 *uvr*A). $: Positive bacterial reverse mutation. SA: Sodium azide; 2-AA: 2-aminoanthracene; 2-NF: 2-nitrofluorene; ICR-191: Acridine mutagen ICR 191; 4NQO: 4-nitroquinoline-1-oxide; B[a] P: benzo[a] pyrene

Table 2: *In vitro* chromosome aberration test in Chinese hamster lung cells treated with polysaccharide fraction from pectinase-digested persimmon leaves

Trt-rec time (h)	S9 mix[a]	Dose (μg/mL)	Number aberrant metaphase[b] (%)	PP + ER (%)	RICC (%)
6-18	+	0	0 (0.00)	0 (0.00)	100
		400	0 (0.00)	0 (0.00)	76
		500	0 (0.00)	0 (0.00)	64
		550	0 (0.00)	0 (0.00)	66
		600	0 (0.00)	0 (0.00)	65
		700	0 (0.00)	0 (0.00)	66
		800	0 (0.00)	0 (0.00)	43
		B[a] *P* 20	47 (31.33)**	0 (0.00)	54
6-18	–	0	0 (0.00)	0 (0.00)	100
		200	0 (0.00)	0 (0.00)	70
		250	0 (0.00)	0 (0.00)	56
		300	0 (0.00)	0 (0.00)	50
		330	0 (0.00)	0 (0.00)	45
		350	0 (0.00)	0 (0.00)	49
		400	0 (0.00)	0 (0.00)	39
		4NQO 0.4	22 (14.67)**	0 (0.00)	65
24-0	–	0	1 (0.67)	0 (0.00)	100
		50	1 (0.67)	0 (0.00)	75
		80	0 (0.00)	0 (0.00)	64
		100	0 (0.00)	0 (0.00)	55
		120	0 (0.00)	0 (0.00)	48
		130	0 (0.00)	0 (0.00)	46
		150	0 (0.00)	0 (0.00)	39
		4NQO 0.4	18 (12.00)**	0 (0.00)	75

[a]The presence (+) or absence (–) of S9 mix in the metabolic activation system; [b]Number of cells in metaphase with chromosome aberrations, One culture/dose was used. After gaps were excluded, 150 metaphase cells/culture were examined. ***P*<0.01 compared to the negative control (Fisher's exact test). Trt-rec time: Treatment time-recovery time; PP: Polyploidy; ER: Endoreduplication; RICC: Relative increase in cell count

from *D. kaki* leaves. These toxicological assessments are a fundamental requirement for future clinical studies aimed at using PLE0 in functional foods or pharmaceuticals. In addition, these toxicity assessments will provide helpful information in the evaluation of the initial safety dose and dose range for clinical trials and may assist in clinical monitoring to identify potential side effects.[28]

A single-dose oral administration of PLE0 in SD rats resulted in no mortality or abnormal changes in body weights, clinical signs, and necropsy findings. Therefore, the median lethal dose of PLE0 was predicted to be higher than 5000 mg/kg in both sexes. Body weight gain was significantly lower in the one female group (1000 mg/kg). However, this was thought to be unrelated to PLE0 because clinical signs related to the gastrointestinal system, such as diarrhea and soft stool, were not observed, and a dose–response correlation was not found. In the previous studies, polysaccharide fraction from *Curcuma longa* and *Pueraria lobata* extracts exhibited no toxicity in rats after the single-dose oral administration of 5000 mg/kg;[28,29] our results were consistent with these previous findings.

The bacterial reverse mutation test, which utilizes histidine- or tryptophan-requiring bacterial strains, is the most widely used test currently available for *in vitro* mutagen detection due to its rapidity and simplicity.[30] In this study, bacterial strains useful for the assessment of base-pair substitutions (*S. typhimurium* TA100 and TA1535 and *E. coli* WP2 *uvr*A) and frameshift mutations (*S. typhimurium* TA98 and TA1537) were used to detect the mutagenicity of PLE0. In this study, regardless of the metabolic activation system, the number of revertant colonies was not raised by PLE0 treatment at a dose range of 39–5000 μg/plate. The positive controls resulted in a significant increase of revertant colony numbers, confirming that the experiment was working properly. Thus, we made a conclusion that PLE0 did not induce point mutations to induce revertant colonies in the test strains.

The clastogenicity of PLE0 was also evaluated by a chromosomal aberration assay. For this assay, the CHL/IU cell line was used. This cell line has been widely used in genetic toxicity testing for a variety of chemicals and has proven to be suitable for the detection of chromosomal aberrations.[31,32] The proportion of cells in metaphase with structural and numerical chromosomal abnormalities in all PLE0-treated groups was not risen comparing with that in the negative control, regardless of the treatment method; thus, this result did not satisfy the positive judgment criteria. This result demonstrated that PLE0 does not induce chromosomal aberrations in CHL/IU cell line. Taken together, the current mutagenicity assessment data for PLE0 are in agreement with previous studies which show no genotoxic effects of the polysaccharide fraction from *C. longa*,[28] *Curcuma zedoaria*,[33] and *Astragalus membranaceus* aerial parts.[34]

Given the above results, PLE0 is considered to be no acute toxicity and displays no genotoxicity under the tested conditions. However, additional *in vivo* micronucleus assays are required to satisfy OECD guidelines, which recommend the inclusion of *in vivo* genetic toxicology tests to adequately investigate all possible genotoxic effects from the test material.[35]

CONCLUSION

Based on these results, PLE0 was not mutagenic and did not induce chromosomal aberration *in vitro* under our experimental conditions and exhibited no acute oral toxicity at up to 5,000 mg/kg in SD rats.

Financial support and sponsorship

This work was supported by the Main Research Program (E0201100) of the Korea Food Research Institute funded by the Ministry of Science and ICT.

Conflicts of interest

There are no conflicts of interest.

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