Original Article

Acute toxicity and genotoxicity of fermented traditional medicine oyaksungi-san

Hwayong Park, Youn-Hwan Huang, Jin Yeul Ma *

KM (Korean Medicine) Application Center, Korea Institute of Oriental Medicine, Daegu, Korea

ABSTRACT

Background: The traditional medicine oyaksungi-san (OY) has been prescribed in East Asia for hundreds of years for the treatment of stroke, paralysis, and ataxia. OY also has therapeutic effects on arthralgia, myalgia, and rheumatoid arthritis, and recent studies have shown its protective effects against apoptosis of hippocampal cells and its anti-inflammatory effects on the peripheral blood cells of patients with cerebral infarction. Many studies have explored the use of traditional medicine and herb materials in the development of safe, novel, and effective pharmaceuticals with fewer side effects. These efforts commonly adopt a bioconversion tool for fermentation with beneficial microbes. However, only pharmaceuticals with high levels of safety and low levels of toxicity can be used in the healthcare system.

Methods: OY water extract was fermented with Lactobacillus and assayed for acute toxicity and genotoxicity. Single dose acute toxicity, bacterial reverse mutation, chromosome aberrations, and micronucleus were observed and assayed in rats, histidine/tryptophan auxotrophic bacteria, Chinese hamster ovary fibroblast cells, and mouse bone marrow cells, respectively.

Results: All the experimental animals showed no abnormal behavior, clinical signs, body weight increases, or mortality. In the bacterial cultures, no revertant colonies were observed. Morphological and numerical chromosomal aberrations were not found in all metaphases examined. Frequency of induced micronuclei was not significantly increased in all doses applied.

Conclusion: As a whole, no acute toxicity or genotoxicity were observed in all the assays examined. Therefore, fermented OY is considered to be a safe material that can be used for development of complementary and alternative medicine using bioconversion.

© 2017 Korea Institute of Oriental Medicine. Published by Elsevier. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Oyaksungi-san (OY), also known as uuyaoshunqi-san in Chinese and uyakujyunki-san in Japanese, a traditional medicine formulated with 12 herbs, first appeared in the old medical book Tai-Ping-Hui-Min-He-Ji-Ju-Fang (Tai Ping Imperial Grace Formula) in the 11th century of Song Dynasty, China. Since then, OY prescriptions can be found in several...
other medical books, with minor differences in dosage of each constituent herbs. It has been widely prescribed in East Asia for hundreds of years for prevention and treatment of stroke, and consequent hemiplegia, paralysis, and ataxia. In addition, OY is known to have therapeutic effects on arthralgia, myalgia, and rheumatoid arthritis. Although scientific reports and evidence verifying efficacies of OY for these diseases cannot be found, research studies to determine any medicinal effects and activity mechanisms are actively being conducted. For example, recent studies have shown that OY has a protective effect against the H2O2-induced apoptosis of hippocampal cells\(^1\) and anti-inflammatory effects on the peripheral blood cells of patients with cerebral infarction.\(^2\) In addition, trigeminal neuralgia was significantly improved with a reduced dose of carbamazepine.\(^3\) Other studies have shown an inhibitory effect on colon cancer cell proliferation by inducing autophagy\(^4\) and inhibiting adipogenesis of adipocytes.\(^5\) For each of the 12 component herbs in OY, Angelicae Dahuricae Radix has an analgesic effect,\(^6,7\) whereas Ephedrae Herba and Zingiberis Rhizoma are associated with pulmonary vascular responses\(^8\) and peripheral circulation in autonomic disorders.\(^9\) Batryticatus Bombyx and Cnidii Rhizoma are neuroprotective\(^10,11\) and free radical scavenging agents.\(^12\) Citri Unshii Pericarpium and Aurantii Fructus Immaturus accelerate gastrointestinal motility.\(^12,13\) Zingiberis Rhizoma Crudus mitigates brain damage related to cerebral ischemia\(^14\) and shows anti-neuroinflammatory activity.\(^15\) Linderae Radix protects against postischemic myocardial dysfunction.\(^16\) Platycodi Radix and Citri Unshii Pericarpium show neuroprotective effects, including with regard to neurroblast cell proliferation.\(^17,18\)

Many studies have attempted to identify and understand the medicinal functions, therapeutic activities, and chemical compounds in traditional materials with novel pharmaceutical effects, and herbs are now being actively examined by many laboratories and companies. This scientific and industrial endeavor may be due to the safety of traditional medicine, which has fewer side effects because it relies on folk remedies that use natural materials. Moreover, bioconversion (e.g., fermentation or biological transformation, typically using microbes) is becoming an important tool for the development of compounds with new functions and effects. The beneficial microbe Lactobacillus is typically used for these purposes, and related products and fermented materials have protective and therapeutic effects on toxicity.\(^19\) inhibit cancer cell proliferation,\(^20\) and increase the quantities of effective compound.\(^21\) Compared to nonfermented OY, there is very little research on the efficacy increase or the improvement of medicinal properties by fermentation of OY. Moreover, medicinal and practical use in clinical application or bedside administration has not been carried out. Efficacy and activity improvement of fermented OY is still in the laboratorial research stage. Only one study on fermented OY has demonstrated the increased anti-inflammatory activity in mouse macrophage cells.\(^22\)

It is obvious that the achievement of safety in the absence of toxicity is the most important consideration when studying the activities and efficacy of potential pharmaceuticals to combat target diseases and ailments. For this reason, we conducted an acute single-dose toxicity and genotoxicity test, including bacterial reverse mutation, chromosomal aberration, bone marrow micronucleus, and chromatographic assays using fermented OY (OY744), prior to the efficacy and mechanism study. Overall, no toxicity was observed in OY744, which is suggestive of the safety and low toxicity of the fermented traditional medicine OY744 and may be a basis for research and development using bioconverted materials. As a whole, no toxicity was found in OY744 at all, and this result can be a predetermined evidence for safety and toxicity matters of fermented traditional medicine OY744, and preliminary informative reference in research and development using bioconverted materials.

2. Methods

2.1. Chemicals and reagents

For high-performance liquid chromatography (HPLC) analysis, acetonitrile was purchased from J. T. Baker (Phillipsburg, NJ, USA). Trifluoroacetic acid, lithium, ferulic acid, naringin, and neohesperidin were obtained from Sigma–Aldrich (St. Louis, MO, USA). Glycyrrhizin were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Liquiritigenin and imperatorin were supplied by Faces Biochemical Co. (Wuhan, China). Hesperidin was from ICN Biomedicals (Santa Ana, CA, USA). Sodium azide (SA), 2-aminothracene (2-AA), 9-aminoacridine hydrochloride hydrate (9-AA), mitomycin C (MMC), cyclophosphamide (CPA), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), magnesium chloride (MgCl\(_2\)), potassium chloride (KCl), and glucose-6-phosphate (G6P) were obtained from Sigma–Aldrich. Lyophilized rat liver S9 fraction produced by inducer Aroclor 1254 was purchased from Molecular Toxicology Inc. (Boone, NC, USA).

2.2. Preparation of OY and OY744

The 12 herbs and medicinal materials in OY were purchased from local vendor Hyundai Herb (Youngcheon, Korea) and authenticated by herb specialist. OY was prepared according to the prescription described in Korean traditional medical book Fang-Yao-He-Bian (方藥合編), with slight modification (Table 1). Combined herb mixture was immersed in distilled water (DW) for 1 hour in an extractor (Cosmos-600, Kyungseo Machine, Incheon, Korea) at ambient temperature for high extraction yield and boiled for 3 hours at 115 °C. Solid insoluble residues were filtered out using a standard test sieve (106 μm, Retsch, Haan, Germany) and used for fermentation. OY extract was autoclaved and fermented by inoculating (1%, v/v) activated bacteria Bifidobacterium breve (1 × 10\(^6 – 5 \times 10^6\) colony-forming units/mL in MRS broth, obtained from Korea Food Research Institute, Sungnam, Korea) and incubating for 48 hours with aeration at 37 °C. OY744 was then lyophilized in a freeze dryer (Ishion, Dongducheon, Korea) and maintained at −20 °C until use. Part of OY744 was deposited in the institutional herbarium as a voucher specimen (Registration No. OY744).

2.3. Chromatographic analysis

To determine the phytochemical profiles and the contents of OY744, HPLC analysis was performed following a slightly mod-
Table 1 – Medicinal herbs composing oyaksungi-san.

| Scientific Name                          | Family Name | Herb Name                      | Source     | Weight (g) |
|------------------------------------------|-------------|--------------------------------|------------|------------|
| Angelica dahurica (Hoffm.) Benth. & Hook. | Apiaceae    | Angelica Dahuricae Radix       | Korea      | 200.0      |
| Bombax mori L.                           | Bombycidae  | Batryticasom Bombyx            | China      | 200.0      |
| Beauliera bassiana (Bals.) Vuill.        | Moniilaceae |                                |            |            |
| Citrus aurantium L.                      | Rutaceae    | Aurantii Fructus Immaturus      | China      | 200.0      |
| Citrus unshiu Yu-Tanaka ex Swingley Marcow. | Rutaceae    | Cirti Unshii Pericarpium        | Korea      | 300.0      |
| Cnidium officinale (Makino) Kitag.       | Umbelliferae | Cnidii Rhizoma                 | Korea      | 200.0      |
| Ephedra sinica Stapf.                    | Ephedraceae | Ephedrae Herba                 | China      | 300.0      |
| Glycyrrhiza uralensis Fisch.             | Leguminosae | Glycyrrhizae Radix et Rhizoma   | China      | 60.0       |
| Linderia styrchnofolia (Siebold & Zucc.) Fern.-Vill. | Lauraceae  | Linderae Radix                  | Korea      | 300.0      |
| Platycondan grandiflorus (Jacq.) A.D.C.  | Campanulaceae | Platycoyi Radix                | Korea      | 200.0      |
| Zingiber officinale Rosc.                | Zingiberaceae | Zingiberis Rhizoma             | Korea      | 100.0      |
| Zingiber officinalis Rosc.               | Zingiberaceae | Zingiberis Rhizoma Crudus      | Korea      | 75.4       |
| Ziziphus jujube Mill.                    | Rhamnaceae  | Zizyphi Fructus                 | Korea      | 100.0      |
| **Total**                                |             |                                |            | 2234.5     |

ified version of the method reported previously. Briefly, the HPLC-diode-array detection system (Lachrom Elite, Hitachi High-Technologies Co., Tokyo, Japan) was used. Chromatographic separation was achieved with Phenomenex Luna C18 column (4.6 mm x 250 mm, 5 μm). Gradient elution with 0.1% trifluoroacetic acid in deionized water (A) and acetonitrile (B) was performed as follows: 0–10 minutes with 20% B, 10–15 minutes with 20–35% B, 15–35 minutes with 35–45% B, 35–40 minutes with 45–50% B, 40–45 minutes with 50–60% B, and 45–60 minutes with 60–60% B. The flow rate and injection volume were 1 mL/min and 10 μL, respectively. An HPLC chromatogram was obtained at 190–400 nm UV. Standard (1.25–125 μg/mL) and sample (20 mg/mL) solutions were dissolved and diluted in methanol.

2.4. Experimental animals

Seven-week-old specific pathogen-free Sprague-Dawley rats and ICR mice were obtained from Orient Bio (Sungnam, Korea), and subjected to acute toxicity and micronucleus tests. Their exterior appearances were examined on acceptance, and they were housed and observed daily for general clinical signs during 1 week of quarantine and acclimation. Animals were then maintained under the following conditions: temperature: 22.7 ± 0.5 °C, humidity: 42.5 ± 1.5%, lighting: 12-hour (08:00–20:00, 290 lux), and ventilation (10–15 times/hour). Diet pellets (Harlan Laboratories, IN, USA) and filtered tap water were supplied ad libitum. This study using laboratory animals was approved by the Institutional Animal Care and Use Committee of Kore Institute of Oriental Medicine.

2.5. Acute toxicity

After 1 week of acclimation and quarantine, animals were divided into four experimental groups (5 males and 5 females in each group) according to body weight. Animals were fasted overnight and administered OY744 (dose volume of 10 mL/kg body weight dissolved in DW, 500 mg/kg, 1000 mg/kg, and 2000 mg/kg) using oral gavage. Diets and water were supplied again after 3 hours of administration. General behaviors, clinical signs, and mortality rates were observed once per day for 14 days, including once per hour during the 6 hours following administration. Body weights were recorded before and after administration on Days 1, 3, 7, and 14. Animals were euthanized by CO₂ asphyxia, and their organs were thoroughly examined for any gross alterations. Comparisons between each pair of group were made using one-way analysis of variance (ANOVA), and SPSS 12.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Acute toxicity assay was conducted according to the Korea Food and Drug Administration Notice #2009-183 (Good Laboratory Practice), and Notice #2009-116 (Guidelines for Toxicity Test of Drugs).

2.6. Bacterial reverse mutation assay

Bacterial reverse mutation assays were conducted using histidine auxotrophic Salmonella typhimurium (TA98, TA100, TA1535, and TA1537) and tryptophan auxotrophic Escherichia coli (WP2uvrA) purchased from Molecular Toxicology Inc. (Boone, NC, USA). Oxdox Nutrient Broth No. 2 (Thermo Scientific, Waltham, MA, USA) was used for preliminary cultivation in a shaking incubator for 10 hours at 180 rpm. Top agar for auxotrophic bacteria was composed of 5 g NaCl, 6 g Bacto agar, and 100 mL of 0.5 mM histidine/biotin in 1 L of DW. For tryptophan auxotrophic bacteria, 5 g NaCl, 6 g Bacto agar, and 100 mL of 0.5 mM tryptophan was used. Minimum glucose agar plates were prepared with 15 g agar, 20 g D-(+)-glucose minimum, and 100 mL of Vogel-Bonner medium E (10×). Vogel-Bonner medium E (10×) was prepared by dissolving 2 g MgSO₄·7H₂O, 20 g citric acid H₂O, 100 g K₂HPO₄, and 35.8 g NaNH₄PO₄·4H₂O in 1 L DW. Preliminary incubation was conducted according to the method reported previously. According to our preliminary study, OY744 was diluted in DW (313 μg/plate, 625 μg/plate, 1250 μg/plate, 2500 μg/plate, and 5000 μg/plate) in triplicate for the assays with and without S9 mix metabolic activation. The S9 mix was composed of 10% S9 tissue fraction, 8 mM MgCl₂, 33 mM KCl, 4 mM NADP, 4 mM NAD, and 5 mM glucose-6-phosphate in 100 mM phosphate-buffered saline (PBS, pH 7.4). Vehicle control was DMSO. The following positive controls (reference materials) were used in a metabolic non-activation system: AF-2 for TA98, TA100, and WP2uvrA; NaN₃ for TA1535; and 9-AA for TA1537. In the metabolic activation system, 2-AA was used as the positive control for all bacterial strains. A total of 0.1 mL of bacterial culture, 0.05 mL of test material OY744, 2.0 mL of top agar, and S9 mix (or 0.1 M PBS, pH 7.4, for the metabolic nonactivation system) were prepared on glucose agar plates and incubated at 37 °C for 48 hours. OY744 and S9 mix, without bacterial
culture, were also plated together to ensure sterility. Abnormalities and contaminations were thoroughly observed, and revertants were counted using a colony counter. A positive result was defined as a dose-dependent and/or increased number of revertant colonies per plate with and without S9 metabolic activation. This assay was conducted according to the Organization for Economic Cooperation and Development (OECD) guideline for the testing of chemicals No. 471 Bacterial Reverse Mutation Test. Bacterial reverse mutation assay was conducted according to the Korea Food and Drug Administration Notice #2009-183 (Good Laboratory Practice), Notice #2009-116 (Guidelines for Toxicity Test of Drugs), and OECD Guidelines for the Testing of Chemicals TG 471 “Bacterial Reverse Mutation Test.”

2.7. Chromosomal aberration assay

Chromosomal aberrations were observed according to the OECD guideline for the testing of chemicals No. 473 In Vitro Mammalian Chromosome Aberration Test using Chinese hamster ovary fibroblast cells (CHO-K1) obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), with minor modification of method reported previously. Briefly, cells were maintained in a 5% CO₂ incubator at 37 °C in F-12 nutrient mixture (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT, USA). Preliminary cytotoxicity was examined to observe any cytotoxic effect and to determine the concentration of OY744 (39.06 μg/mL, 78.13 μg/mL, 156.25 μg/mL, 312.5 μg/mL, 625 μg/mL, 1250 μg/mL, 2500 μg/mL, and 5000 μg/mL). Relative cell counts (RCCs) were determined by comparing cell counts in the treatment with those of the vehicle control as follows: RCC=(No. of treated cells/No. of control cells)×100 (%). Cells (2 × 10⁴–4 × 10⁴) were seeded in a 60-mm culture dish and incubated for 72 hours. After the medium was removed, 4.85 mL of a prewarmed fresh medium and 0.15 mL of OY744 (and 0.5 mL of S9 mix in a metabolic activation system) were added to a final volume of 5 mL and incubated for a further 6 hours (and 24 hours in the metabolic nonactivation system). The medium was removed again, and the cells were washed once with PBS and incubated for 18 hours with 5 mL of fresh prewarmed medium until metaphase cell harvest. After 22 hours of OY744 treatment, cells were incubated for another 2 hours with Colcemid (final conc. 0.2 μg/mL, Gibco, Carlsbad, CA, USA) and harvested by centrifugation (500 g, 5 minutes). After supernatant removal, cells were incubated in 75 mM KCI hypotonic solution for 20 minutes at 37 °C and fixed three times in Carnoy’s fixative (acetic acid: methanol=1:3). Chromosomes were stained with 5% Giemsa solution (Merck, Darmstadt, Germany) on glass slides. Chromosomal aberrations of 100 metaphase per slide (200 metaphases/dose) were examined by observing structural (gap, break, and exchange) and numerical (polyploidy and endoreduplication) anomalies, in accordance with the Atlas of chromosome aberration by chemicals. Statistical comparisons between the groups were conducted using the Chi-square test with SPSS 12.1; differences were considered significant when the p value was < 0.05. Chromosomal aberration assay was conducted according to the Korea Food and Drug Administration Notice #2009-183 (Good Laboratory Practice), Notice #2009-116 (Guidelines for Toxicity Test of Drugs), and OECD Guidelines for the Testing of Chemicals TG 473 “In Vitro Mammalian Chromosome Aberration Test.”

2.8. Bone marrow micronucleus assay

Carcinogenicity was evaluated using the micronucleus test with male ICR mice under the same condition used for the animal laboratory environment (see descriptions as described in the section on experimental animals). A preliminary study was conducted to determine the dose range and bone marrow cell collecting time of five groups (three mice/group): the vehicle control, positive control (mitomycin C 2.0 mg/kg), and the three treatment groups (1250 mg/kg, 2500 mg/kg, and 5000 mg/kg). After 3 hours of fasting, vehicle control and OY744 were administered orally using sonde, and the positive control was injected intraperitoneally. Two sets of these five groups were prepared for 24 hours and 48 hours treatment. Because the preliminary tests revealed no effects on mortality, clinical signs, and bone marrow cell proliferation, the dose range for the main study was determined as it was in the preliminary tests. In the main study, all conditions and groups were the same as those in the preliminary study, but six mice were included in each group. After 24 hours of administration, bone marrow cells were collected via FBS washing from the excised femurs of mice sacrificed by cervical dislocation. Three slides were prepared from each animal by smearing, drying, and fixation (methanol, 5 minutes). Cells were then stained with Giemsa (4%) for polychromatic erythrocytes (PCEs) and with acridine orange (40 μg/mL) for micronuclei in PCE. PCE with or without micronuclei and normochromatic erythrocytes (NCEs) were examined under light and fluorescent microscopes with the FITC filter. Cytotoxicity was evaluated by observing the PCE/NCE ratio in 200 erythrocytes. The frequency of micronucleated (MN) PCEs was observed in 2000 PCEs per animal. The micronuclei frequency and PCE/(PCE+NCE) ratio were statistically evaluated by one-way ANOVA. The results were considered reasonable when the PCE/(PCE+NCE) ratio was > 0.1, and they were considered positive when the micronuclei-inducing ratio (MN/PCE/2000 PCEs) was statistically significant and it dose-dependently increased or when it was positive at one or more doses. It was considered statistically significant when p<0.05; SPSS 12.0 was used for all analyses. Chromosomal aberration assay was conducted according to the Korea Food and Drug Administration Notice #2009-183 (Good Laboratory Practice), Notice #2009-116 (Guidelines for Toxicity Test of Drugs), and OECD Guidelines for the Testing of Chemicals TG 474 “Mammalian Erythrocyte Micronucleus Test.”

3. Results

3.1. Representative compounds were identified by chromatographic analysis

As shown in Fig. 1, seven phytochemicals in OY744 were identified through retention time and the UV spectrum in the HPLC-diode-array detection system. The quantities of liquiritin, ferulic acid, naringin, hesperidin, neohesperidin, liquiritigenin, and glycyrrhizin were 0.14 mg/g, 1.21 mg/g,
3.2. No toxicity and no abnormal findings were observed in acute toxicity assay using rats

Single-dose acute toxicity was evaluated in the Sprague–Dawley rats in the three experimental groups (500 mg/kg, 1000 mg/kg, and 2000 mg/kg) and the control group. During the 14 days of the total experimental period, no mortality or no abnormal clinical signs were observed. Behaviors and external appearances were normal, and no gross lesions of internal organs were observed. The body weights of both male and female rats showed normal increases without any significant changes (Fig. 2). According to these results, the lethal dose 50 (LD<sub>50</sub>) of OY744 might be over 2000 mg/kg.

3.3. Antibacterial effects were not found with no increase of revertant colony numbers

In the dose-determining preliminary study, no antibacterial or growth inhibitory effects were observed up to concentrations of 5000 μg/plate, regardless of metabolic activation. In the main study, no increases in the number of revertant colonies were observed in any bacterial strain at any concentration (Fig. 3).

3.4. Chromosomal observations showed normal numerical and structural metaphases

In the preliminary metabolic nonactivation study, the cell viabilities of the 24 hours treatment group were 53.45% and 72.41% at 1250 μg/mL and 625 μg/mL, respectively; in the 6 hours treatment/18 hours recovery group, they were 57.06% and 61.58% at 5000 μg/mL and 2500 μg/mL, respectively. Accordingly, the maximum concentrations of the 24 hours and 6 hours treatment/18 hours recovery groups were 1250 μg/mL and 5000 μg/mL, respectively. In the metabolic activation system, the 6 hours treatment group showed 58.65% and 67.67% cell viability at 5000 μg/mL and 2500 μg/mL concentrations, respectively; we used 5000 μg/mL as the maximum concentration for the main study. In the main study (metabolic nonactivation) for chromosomal aberration, the cell numbers of aberrant metaphase (gap–) were 0.0, 0.0, 0.0, and 0.5 in the negative control and 312.5 μg/mL, 625 μg/mL, and 1250 μg/mL in the 24 hours treatment group. In the 6 hours treatment/18 hours recovery group, the comparable figures were 0.5, 0.5, 1.0, and 1.5, respectively; in the negative control group, they were 1250 μg/mL, 2500 μg/mL, and 5000 μg/mL, respectively. Aberrant metaphase cell numbers in the metabolic activation system were 0.5, 0.0, 0.5, and 0.5 in the negative control group at 1250 μg/mL, 2500 μg/mL, and 5000 μg/mL, respectively. As we observed no statistically significant increases with regard to polyploidy and endoreduplication (Table 2), we concluded that OY744 had no effect on inducing chromosomal aberrations in CHO-K1 cells.

3.5. Micronucleus assay showed normal ranges in cytotoxic indicators

In the preliminary study, the MNPCEs per 2000 PCEs in the vehicle; the 1250 mg/kg, 2500 mg/kg, and 5000 mg/kg and the positive control MMC groups were 0.25, 0.23, 0.10, 0.15, and 11.52 and 0.23, 0.35, 0.22, 0.18, and 9.50 at sampling times of 24 hours and 48 hours, respectively (Fig. 4A). Induced MNPCE ratios of 2000 PCEs per animal in the vehicle; 1250 mg/kg, 2500 mg/kg, 5000 mg/kg; and the positive control groups were 0.30%, 0.23%, 0.27%, 0.24%, and 10.11%, respectively, in the main study (Fig. 4B). This reflected no statistically significant difference between vehicle and treatment groups. Instead, the positive control MMC group significantly differed from the vehicle group (p < 0.01). The cytotoxic indicators PCE/(PCE + NCE) in the preliminary study were 0.52, 0.43, 0.46, 0.42, and 0.43 at 24 hours sampling time and 0.43, 0.52, 0.46, 0.47, and 0.18 at 48 hours sampling time in the same control and treatment groups, respectively (Fig. 4C). The PCE/(PCE + NCE) results in the main study were 0.47, 0.47, 0.54,
Thus, Traditional herbal medicines, including those based on folk remedies, have been used for centuries to treat a wide array of ailments. These compounds, especially those used in folk medicine, are considered to be safe medicinal materials with few side effects. Nowadays, traditional medicine has increased in popularity and attracted attention worldwide as its significant therapeutic activities and efficacy as a dietary supplement have been shown with regard to improving health and overall condition. Traditional medicine is becoming more popular, and the sales of Chinese medicine accounted for US$3.1 billion in 2012, which represents an increase of >20% over the previous year. Expenditures for natural products in the US accounted for US$14.8 billion in 2008. Thus, many in-depth studies are being conducted to develop complementary and alternative pharmaceuticals. Herbal medicines are typically believed to be less toxic than synthetic chemical drugs. However, toxicity and safety assay are important for all pharmaceuticals. For this reason, we evaluated potential toxicities based on the single-dose acute toxicity and genotoxicities of OY744 using bacterial reverse mutation, chromosomal aberration, and micronuclei assays.

Table 2 – Effect of fermented oyaksungi-san (OY744) on chromosomal aberration in Chinese hamster ovary (CHO)-K1 cells.

| Treatment (μg/mL) | S9 mix | Timea (h) | No. of total chromosomal aberrations (mean) | No. of cells with chromosomal aberrations (mean) | FP + ER (mean) | RCCb (%) |
|------------------|--------|-----------|---------------------------------------------|-------------------------------------------------|---------------|---------|
|                  |        |           | Gap (−) Gap (+)                              | Gap (−) Gap (+)                                  |               |         |
| Vehicle 0.00     | –      | 24–0      | 0.0 0.5                                     | 0.0 0.5                                         | 0.0           | 100.00  |
| OY744 312.50     | –      | 24–0      | 0.0 0.0                                     | 0.0 0.0                                         | 0.0           | 79.31   |
| OY744 625.00     | –      | 24–0      | 0.0 0.5                                     | 0.0 0.5                                         | 0.0           | 72.41   |
| OY744 1250.00    | –      | 24–0      | 0.5 0.5                                     | 0.5 0.5                                         | 0.0           | 53.45   |
| MMC 0.04         | –      | 24–0      | 29.0 29.0                                   | 29.0 29.0                                       | 0.0           | –       |
| Vehicle 0.00     | –      | 6–18      | 0.5 1.0                                     | 0.5 1.0                                         | 0.0           | 100.00  |
| OY744 1250.00    | –      | 6–18      | 0.5 0.5                                     | 0.5 0.5                                         | 0.0           | 64.97   |
| OY744 2500.00    | –      | 6–18      | 1.0 1.0                                     | 1.0 1.0                                         | 0.0           | 61.58   |
| OY744 5000.00    | –      | 6–18      | 1.5 1.5                                     | 1.5 1.5                                         | 0.0           | 57.06   |
| MMC 0.04         | –      | 6–18      | 19.0 20.0                                   | 19.0 19.5                                       | 0.0           | –       |
| Vehicle 0.00     | +      | 6–18      | 0.5 0.5                                     | 0.5 0.5                                         | 0.0           | 100.00  |
| OY744 1250.00    | +      | 6–18      | 0.0 0.5                                     | 0.0 0.5                                         | 0.0           | 56.39   |
| OY744 2500.00    | +      | 6–18      | 0.5 0.5                                     | 0.5 0.5                                         | 0.0           | 67.67   |
| OY744 5000.00    | +      | 6–18      | 0.5 1.0                                     | 0.5 1.0                                         | 0.0           | 58.65   |
| CPA              | 10.00  | +         | 24.5 26.0                                   | 24.5 26.0                                       | 0.0           | –       |

CPA, cyclophosphamide; ER, endoreduplication; MMC, mitomycin C; PP, polyploidy; RCC, relative cell count.
a Treatment time–recovery time.
b RCC (%) = (number of treated cells/number of control cells) × 100.

0.51, and 0.35, respectively, which reflected no significant inhibition of bone marrow cell proliferation (Fig. 4D). There were no significant body weight changes in animals.

4. Discussion

For hundreds of years, numerous herbs and medicinal materials have been used for the treatment of many diseases and ailments. These compounds, especially those used in folk medicine, are considered to be safe medicinal materials with few side effects. Nowadays, traditional medicine has increased in popularity and attracted attention worldwide as its significant therapeutic activities and efficacy as a dietary supplement have been shown with regard to improving health and overall condition. Traditional medicine is becoming more popular, and the sales of Chinese medicine accounted for US$3.1 billion in 2012, which represents an increase of >20% over the previous year. Expenditures for natural products in the US accounted for US$14.8 billion in 2008. Thus, many in-depth studies are being conducted to develop complementary and alternative pharmaceuticals. Herbal medicines are typically believed to be less toxic than synthetic chemical drugs. However, toxicity and safety assay are important for all pharmaceuticals. For this reason, we evaluated potential toxicities based on the single-dose acute toxicity and genotoxicities of OY744 using bacterial reverse mutation, chromosomal aberration, and micronuclei assays.
The single-dose acute toxicity of OY744 (up to 2000 mg/kg) was observed during 14 days of animal experiments. No toxic clinical signs or parameters and no abnormal behaviors or local lesions of internal organs were observed throughout the experiments. Body weights also showed only normal increases; therefore, the LD_{50} of OY744 may be over 2000 mg/kg. In the bacterial reverse mutation assay, mutations, such as frame-shift or base pair substitution and the number of revertant colonies were not affected at concentrations up to 5000 mg/kg. Based on this result, OY744 can be considered safe with no mutagenicity in the bacterial strains at any concentration. Observation of chromosomal aberrations involving structural and numerical abnormalities is one of the integral components of genotoxic assays. The mammalian cell line CHO-K1 is commonly used for the evaluation of potential clastogens, as they are well-known mutagen-sensitive and informative cells. Chromosomal aberrations such as gap, break, exchange, aneuploidy, and polyploidy were examined thoroughly in metaphase, and no mutagenic aberrations were observed at any concentration of OY744 with or without S9 mix. This result suggests that OY744 is not a clastogen in this assay system. The mutagenic toxicity of OY744 was also evaluated with bone marrow cells from ICR mice administered single doses of 1250 mg/kg, 2500 mg/kg, and 5000 mg/kg in both the preliminary (with sampling times of 24 hours and 48 hours) and main studies. In the preliminary
Fig. 4 – Effect of fermented oyaksungi-san (OY744) on the incidence (%) of (A, B) micronucleated polychromatic erythrocyte (MNPCE) and (C, D) polychromatic erythrocyte (PCE)/normochromatic erythrocyte (NCE) ratio in the preliminary and main studies, after oral administration (0, 1250, 2500, and 5000 mg/kg). A and C, preliminary study; B and D, main study; (−), 0 mg/kg; (+), MMC 2.0 mg/kg.

*Differed significantly from the control group at p < 0.05 (one-way ANOVA).
†Differed significantly from the control group at p < 0.01 (one-way ANOVA).

study, no specific or significant differences in the appearance of the treatment and vehicle control groups were observed. Based on the results of the preliminary study, the main study was conducted with the highest concentration, 5000 mg/kg, and a sampling time of 24 hours, which produced no significant inhibition of bone marrow cell proliferation (i.e., ratio of PCE/NCE). No significant differences were observed regarding MNPCEs compared with the vehicle control, whereas the positive control showed a significant increase in this regard. Based on these results, OY744 is not inducible for micronuclei in bone marrow cells under these experimental conditions.

There are several studies on the pharmaceutical efficacy and biological activities of OY, as described in the Introduction. Based on further studies, concerns about safety issues have increased as new materials and new uses for extant materials have been developed. However, despite the need for research on safety, studies regarding toxicity have not been reported. Furthermore, traditional medicines are typically composed of natural materials and herbs and are processed either physically or biologically. Traditional medicine and herbs are heat or naturally dried, boiled, and steamed in the preparation of the formula, prescription, and decoction. Moreover, in biological processes, microbes are used for the fermentation of the herb extract and to prepare probiotics. The achievement of safety in the absence of toxicity is fundamental in the development of pharmaceuticals and in research using herbs. The present research involved a single-dose acute and genotoxicity study on the fermented traditional medicine OY744. Fermented OY has not been used in clinical practice and also not been prescribed as a medicine in healthcare system. It is still under research in this laboratory for later development of pharmaceuticals and medical applications. There are many research reports and comments on beneficially improved and strengthened efficacies, and biological and medical usefulness is recognized elsewhere, with increasing scientific evidence regarding targeted therapeutic activities in many diverse diseases and ailments. However, these research reports should be the solid basis for development of medicines with further studies and clinical trials. The importance of toxicity testing prior to the development of medicines is undoubtedly important. Taken together, none of the observations or findings revealed toxicity or mutagenicity. This result can be used as basis for the development of traditional pharmaceuticals and the performance of related researches of complementary and alternative medicines.
Conflicts of interest

The authors have no conflict of interest.

Acknowledgments

This work was supported by the grant (No. K15280) awarded to Korea Institute of Oriental Medicine (KIOM) from the Ministry of Science, ICT, and Future Planning (MSIP), Republic of Korea.

REFERENCES

1. Je JI, Shin HT, Chung SH, Lee JS, Kim SS, Shin HD, et al. Protective effects of wuyaoqunisan against H2O2-induced apoptosis on hippocampal cell line HiBS. Am J Chin Med 2002;30:561–70.
2. Kim Y, So HS, Kim JK, Park C, Lee JH, Woo WH, et al. Anti-inflammatory effect of oyaksungisan in peripheral blood mononuclear cells from cerebral infarction patients. Biol Pharm Bull 2007;30:1037–41.
3. Kogure T, Sato H, Kishi D, Tatsumi T. The effect of traditional herbal medicines; uyakujunkisan on trigeminal neuralgia in an elderly patient - a case report and literature review. Pain Pract 2008;8:408–11.
4. Yim NH, Jung YP, Kim A, Ma CJ, Cho WK, Ma JY. Oyaksungisan, a traditional herbal formula, inhibits cell proliferation by induction of autophagy via JNK activation in human colon cancer cells. Evid Based Complement Alternat Med 2013;2013:231874.
5. Yoo SR, Seo CS, Shin HK, Jeong SJ. Traditional herbal formula oyaksung-san inhibits adipogenesis in 3T3-L1 adipocytes. Evid Based Complement Alternat Med 2015;2015:949461.
6. Sarker SD, Nahar L. Natural medicine: the genus Angelica. Curr Med Chem 2004;11:1479–500.
7. Liao ZG, Liang XL, Zhu JY, Zhao GW, Yang M, Wang GF, et al. Correlation between synergistic action of Radix Angelica dahurica extracts on analgesic effects of Corydalis alkaloid and plasma concentration of dl-THP. J Ethnopharmacol 2010;129:115–20.
8. Fields AM, Kaye AD, Richards TA, Ibrahim IN, Heavner JE, Dewitt BJ, et al. Pulmonary vascular response to ma huang extract. J Altern Complement Med 2003;9:727–33.
9. Kanai S, Okano H, Abe H. Efficacy of toki-shiyakukaku-gosyuuyu-syokyo-to (danggui-sini-jia-wuzhuuyu-shengjiang-tang) on peripheral circulation in autonomic disorders. Am J Chin Med 1997;25:69–78.
10. Koo BS, An HG, Moon SK, Lee YC, Kim HM, Ko JH, et al. Bombycis corpus extract (BCE) protects hippocampal neurons against excitatory amino acid-induced neurotoxicity. Immunopharmacol Immunotoxicol 2003;25:191–201.
11. Ramalingam M, Yong-Ki P. Free radical scavenging activities of Cnidium officinale Makino and Ligusticum chuanxiong Hort. methanolic extracts. Pharmacog Mag 2010;6:323–30.
12. Lyu JH, Lee HT. Effects of dried Citrus unshiu peels on gastrointestinal motility in rodents. Arch Pharm Res 2013;36:641–8.
13. Fang YS, Shan DM, Liu JW, Xu W, Li CL, Wu HZ, et al. Effect of constituents from Fructus Aurantii Immaturus and Radix Paeoniae Alba on gastrointestinal movement. Planta Med 2009;75:24–31.
14. Wattanathorn J, Jittiwat J, Tongtun T, Muchimapura S, Ingkaninan K. Zingiber officinale mitigates brain damage and improves memory impairment in focal cerebral ischemic rat. Evid Based Complement Alternat Med 2011;2011:429505.
15. Park C, Kim HG, Ju MS, Ha SK, Park Y, Kim SY, et al. 6-Shopol, an active compound of ginger, protects dopaminergic neurons in Parkinson’s disease models via anti-neuroinflammation. Acta Pharmacol Sin 2013;34:1311–9.
16. Wang N, Minatoguchi S, Arai M, Uno Y, Hashimoto K, Xue-Hai C, et al. Linderia strychnifolia is protective against post-ischemic myocardial dysfunction through scavenging hydroxyl radicals and opening the mitochondrial KATP channels in isolated rat hearts. Am J Chin Med 2004;32:587–98.
17. Son JH, Park YH, Lee SI, Yang HD, Moon HI. Neurprotective activity of triterpenoid saponins from Platycodi radix against glutamate-induced toxicity in primary cultured rat cortical cells. Molecules 2007;12:1147–52.
18. Seo KH, Lee DY, Lee DS, Kang HC, Kim HS, Kim YC, et al. Hepatoprotective and neuroprotective tocopherol analogues isolated from the peels of Citrus unshiuMarcovich. Nat Prod Res 2015;29:571–3.
19. Baek MW, Seok SH, Lee HY, Kim DJ, Lee BH, Ahn YT, et al. Protective and therapeutic effects of an extract mixture of alder tree, labiate herb, milk thistle green bean-rice bran fermentation, and turnip against ethanol-induced toxicity in the rat. J Vet Sci 2008;9:31–7.
20. Oh J, Jeon SB, Lee Y, Lee H, Kim J, Kwon BR, et al. Fermented red ginseng extract inhibits cancer cell proliferation and viability. J Med Food 2015;18:421–8.
21. Xu C, Ji GE. Bioconversion of flavones during fermentation in milk containing Scutellaria baicalensis extract by Lactobacillus brevis. J Microbiol Biotechnol 2013;23:1422–7.
22. Oh YC, Cho WK, Oh JH, Im GY, Jeong YH, Yang MC, et al. Fermentation by Lactobacillus enhances anti-inflammatory effect of oyaksungisan on LPS-stimulated RAW 264.7 mouse macrophage cells. BMC Complement Altern Med 2012;12:17.
23. Woen JB, Yang HJ, Ma JY, Ma CJ. Simultaneous determinations of six active components in traditional herbal medicine oyaksungisan by HPLC-DAD. J Nat Med 2012;66:510–5.
24. Maron DM, Ames BN. Revised methods for the Salmonella mutagenicity test. Mutat Res 1983;113:173–215.
25. Ishidate Jr M, Yoshikawa K. Chromosome aberration tests with Chinese hamster cells in vitro with and without metabolic activation – a comparative study on mutagens and carcinogens. Arch Toxicol 1980;44:1–4.
26. Dean BJ, Danford N. Assays for the detection of chemically-induced chromosome damage in cultured mammalian cells. In: Venitt J, Parry JM, editors. Mutagenicity testing – a practical approach. Oxford: IRL Press; 1984:187–232.
27. Japanese Environmental Mutagen Society-Mammalian Mutagenicity Study Group. Atlas of chromosome aberration by chemicals. Tokyo, Japan: Japanese Environmental Mutagen Society-Mammalian Mutagenicity Study Group; 1988.
28. Cheung F. TCM: made in China. Nature 2011;480:S82–3.
29. World Health Organization. WHO traditional medicine strategy 2014–2013. Geneva, Switzerland: World Health Organization; 2013.
30. Wang J. Evidence-based medicine in China. Lancet 2010;375:532–3.
31. Kroes BH, van den Berg AJ, Abeyesekera AM, de Silva KTD, Labadie RP. Fermentation in traditional medicine: the impact of woodfordia fruticose flowers on the immunomodulatory activity, and the alcohol and sugar contents of Nimba arishita. J Ethnopharmacol 1993;40:117–25.
32. Lee JH, Lee JH, JIn JS. Fermentation of traditional medicine: present and future. Orient Pharm Exp Med 2012;12:163–5.