Regulatory Toxicology and Pharmacology 71 (2015) 365–370

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Genotoxicity studies on the root extract of *Polygala tenuifolia* Willdenow

Ki Young Shin [a,b,*], Beom Young Won [a], Hyun Jee Ha [a], Yeo Sang Yun [a], Hyung Gun Lee [a]

[a] Research & Development Center, Braintropia Co. Ltd., Anyang-si, Gyeonggi-do 431-716, Republic of Korea
[b] Department of Microbiology, College of Natural Science, Dankook University, Cheonan-si, Chungnam 330-714, Republic of Korea

ARTICLE INFO

Article history:
Received 5 December 2014
Available online 7 February 2015

Keywords:
Polygala tenuifolia Willdenow
Genotoxicity
Reverse mutation
Chromosomal aberration
Micronucleus test

ABSTRACT

The root of *Polygala tenuifolia* Willdenow has been used for the treatment against insomnia, amnesia, depression, palpitations with anxiety, and memory improvement. However, there is no sufficient background information on toxicological evaluation of the root to given an assurance of safety for developing dietary supplements and functional foods. As part of a safety evaluation, the potential genotoxicity of the root extract of *P. tenuifolia* was evaluated using a standard battery of tests (bacterial reverse mutation assay, chromosomal aberrations assay, and mouse micronucleus assay). In a reverse mutation assay using four *Salmonella typhimurium* strains and *Escherichia coli*, the extract did not increase the number of revertant colonies in any tester strain with or without metabolic activation by S9 mix, and did not cause chromosomal aberration in short-period test with the S9 mix or in the continuous (24 h) test. A bone marrow micronucleus test in ICR mice dosed by oral gavage at doses up to 2000 mg/kg/day showed no significant or dose dependent increase in the frequency of micronucleated polychromatic erythrocytes (PCE). These results indicate that ingesting the rot extract *P. tenuifolia* is not genotoxic at the proper dose.

1. Introduction

A number of medicinal plants have a long history of traditional use, and they have played many important roles for humans since the dawn of civilization (Pankaj et al., 2009), because medicinal herbs are used for the prevention and treatment of diseases (Firenzuoli and Gori, 2007; Wang et al., 2009). The use of herbal products as primary therapeutics or supplements for improving health-related conditions is popular worldwide (Seeff et al., 2001). Interestingly, medicinal plants have provided high opportunities for the development of herbal food products, dietary supplements, and functional foods (Chau and Wu, 2006). Recently, concerns have been raised over the lack of quality control and of scientific evidence for the efficacy and safety of these products (Firenzuoli and Gori, 2007; Rousseaux and Schachter, 2003). Especially, people become more interested in the food safety and well-being in recent years, so the demand for functional food from natural sources is increased (Lee et al., 2003), because medicinal plants have undesirable side effects (Chan and Cheung, 2000). In traditional oriental medicine, the root of *Polygala tenuifolia* Willdenow has been prescribed in Asia for thousands of years, because of its expectorant, tonic, tranquilizer, and antipsychotic properties (Spelman et al., 2006; Wang et al., 2007; Nagai and Suzuki, 2001; Klein et al., 2012; Jin et al., 2012). Previously, we reported that the root extract of *P. tenuifolia* can enhance memory and cognitive function in two animal models or two human models (Park et al., 2002; Lee et al., 2009; Shin et al., 2009a,b) and the acute or subchronic toxicity of the root extract was not toxic to dogs and rats (Shin et al., 2014). Several researchers have demonstrated the effects of *P. tenuifolia*, but information on its safety is lacking. Therefore, systematic evaluation of the safety of the root extract of this herb is necessary for development of new foods or drugs. In this study, we evaluated the potential genotoxicity of the dried root extract of *P. tenuifolia* was conducted using the standard battery of tests recommended by OECD and the Korea Food and Drug Administration (KFDA). The test included the bacterial reverse mutation test (Ames test), the chromosomal aberrations test and the micronucleus tests to assure its safe use in dietary supplements or functional ingredients.

2. Material and methods

2.1. Extraction of Polygala tenuifolia

The dried root extract of *P. tenuifolia* (500 g) was refluxed with 75% ethanol for 4 h in a boiling water bath. This procedure was repeated twice and the ethanol solution was concentrated under

* Corresponding author at: Department of Microbiology, College of Natural Science, Dankook University, Cheonan-si, Chungnam 330-714, Republic of Korea. Fax: +82 031 425 3719.
E-mail address: newsky73@braintropia.com (K.Y. Shin).

http://dx.doi.org/10.1016/j.yrtph.2015.01.016
0273-2300/

vacuum. The concentrated ethanol fraction (125 g) of the plant root, obtained as described above, was used for this study (Park et al., 2002).

2.2. Bacterial mutation assay (Ames test)

The Ames test was carried out in according to the methods described in OECD (1997a) and the KFDA Notification No. 1999-61 (KFDA, 1999). Two strains of *Salmonella typhimurium*, TA 100 and TA 1535, and *Escherichia coli* WP2 *uvr*A were used for the detection of base-pair substitution mutations. Two additional *S. typhimurium* strains, TA 98 and TA 1537, were used for the detection of frameshift mutations. The tester strains were obtained from Molecular Toxicology, Inc. (111 Gibralter avenue, Annapolis, MD21401, USA) and Dr. M.H.L Green (University of Sussex, Falmer, Brighton, UK). The phenotypic properties of the *S. typhimurium* strains, including histidine requirement, presence of *uvr*B mutation, presence of *R*-factor, presence of *rfa* mutation, and number of spontaneous revertants were evaluated. For *E. coli*, the tryptophan requirement, presence of *uvr*A mutation, and the number of spontaneous revertants were checked according to the method of Maron and Ames (1983). These procedures were conducted at Preclinical Research Center (ChemOn, Inc., Gyeonggi-do, Korea) prior to the conduct of the study. The following chemicals were used as positive control: sodium azide (SA), 2-aminoanthracene (2-AA), 4-nitroquinoline-N-oxide (4-NQO), and 9-aminoacridine (9-AA) (all obtained from Sigma Aldrich Company). Sterile distilled water was included as a negative control and served as the solvent for sodium azide. All the other positive controls substances were dissolved in dimethylsulfoxide (Sigma–Aldrich Company). The metabolic activation system consisted of a commercial S9 fraction obtained from Molecular Toxicology, Inc. (Boone, NC, USA). The S9 fraction was prepared from the livers of male Sprague–Dawley rats pretreated with Aroclor 1254. Cofactor-1 ($MgCl{\cdot}6H_2O$, KCl, glucose-6-phosphate, NADPH, NADH, and sodium phosphate buffer) was supplied from Wako Pure Chemical, Ind., Ltd. (Japan). The S9 mix containing 50 μL of S9/mL solution was prepared according to standard methods. The study was conducted using the direct plate incorporation method. Briefly, this involved addition to a sterile tube of 2.0 mL top agar (45 °C), 0.1 mL test substance, 0.5 mL of the S9-mix or sodium phosphate buffer and 0.1 mL bacterial culture. Following vortexing the contents were poured onto 25 mL minimal glucose agar plates. Following this and the hardening of the top agar, plates were turned over and incubated at 37 °C for 48 h. At this time, the numbers of revertant colonies were counted. Two separate experiments were conducted, a dose-range finding test and a main test. The range-finding study was conducted in both the presence and absence of S9-mix and used 1 plate/dose level. The doses tested were 1.6, 8, 40, 200, 1000, 2500, 5000 μg/plate. Given the lack of growth inhibition of the colonies at any concentration, in the main study 5000 μg/plate was selected as the highest dose. Four lower doses of 62, 185, 556, 1667, and 5000 μg/plate were also included.

2.3. Mouse micronucleus test

The micronucleus test was carried out in according to the methods described in OECD (1997b) and the KFDA Notification No. 1999-61 (KFDA, 1999). Total 30 healthy male ICR mice (8-weeks-of-age; Samtako, BIO KOREA) were used in the micronucleus assay to evaluate. The test substance was administered once a day for 2 days by gavage to male ICR mice at doses 0, 500, 1000 and 2000 mg/kg. The test substance was dissolved in DW and animals were orally administrated the test substance at the levels listed for two consecutive days. Cycliphosphamide (CPA) was administrated intraperitoneally at 70 mg/kg as the positive control. The clinical signs and body weight were assessed once daily. Bone marrow preparations were made according to Schmid (1975) and two slides of the cell suspension per animal were made. Following the sacrifice with fetal bovine serum using a disposable syringe with 23G needle, the cell suspension was centrifuged and the supernatant was decanted. After removing the supernatant, a small drop of the viscous suspension was smeared onto clean microscope slides. Preparations were air-dried, and fixed by submerging in absolute methanol for 5 min. Fixed slides were stained with May-Grunwald and Giamsa. Stained slides were rinsed with distilled water, dried, and mounted with mountant (Depex, Fluka). Slides were examined under 1000× magnification. Small round or oval shaped bodies, with a size of about 1/5–1/20 of the diameter of a polychromatic erythrocyte (PCE), were counted as micronuclei. A total of 2000 PCEs were scored per animal by the same observer for determining the frequencies of micronucleated polychromatic erythrocytes (MNPCEs). PCE/(PCE + NCE (normochromatic erythrocyte)) ratio was calculated by counting 500 cells.

2.4. Chromosome aberration in Chinese hamster lung (CHL) cells

The chromosome aberration study was carried out in accordance with the methods described in OECD (1997c) and the KFDA Notification NO. 1999-61 (KFDA, 1999). The experimental methods were based on the published reported by Ishidate et al. (1981) and Dean and Danford (1984). The Chinese hamster lung (CHL) fibroblast cell line used in the experiment was CHL/IU from American Type Culture Collection (ATCC #CRL-1935, CHL/IU). The cells were subcultured and maintained at Preclinical Research Center (ChemOn, Inc., Gyeonggi-do, Korea) prior to use in the study. The modal chromosome number of the CHL cells was 25 with a doubling time of approximately 15 h. CHL cell kept in liquid nitrogen was thawed and cultured more than 7 days. Microbial contamination was tested before starting experiment. Cell were cultured in reconstituted minimum essential medium (MEM, Gibco-BRL #41500-034) supplemented with 2200 mg of sodium bicarbonate, 292 mg of L-glutamine, antibiotics (Penicillin G and Streptomycin sulfate, Gibco-BRL 15140-122) and 10% (v/v) fetal bovine serum (FBS, Gibco-BRL #16000-044) per liter. CHL cell was seeded at 6×10^4 cells/mL using a plastic plate and incubated for 3 days at 37 °C with 95% air and 5% CO_2. Cells were subcultured every 2–3 days. Rapidly growing cultures were trypsinized, suspended in culture medium, and counted. Culture flasks (25 cm^2) were seeded with 6×10^4 cells, each in 5 mL medium, and incubated for 3 days. The test substance and the positive controls, cyclophosphamide (CPA) and ethylmethanesulfonate (EMS), were dissolved in DW. A range finding study was performed to determine the highest concentration for the main study. The highest concentration was set as 5000 μg/ml according to the OECD guideline (OECD, 1997c). The test substance and positive controls were treated in the presence (+S9) and absence (−S9) of the S-9 mix. A preliminary range-finding test (Study No. 04-VG-255P) was performed to select vehicle and dose levels of the present study. In the preliminary study cells were treated over at the doses of 0, 0.8, 2.3, 6.9, 20.6, 61.7, 185.2, 555.6, 1666.7 and 5000 μg/mL in the presence and absence of metabolic activation system, with the same method as present study. After 24 h from the start of treatment, the cells of each flask were dissociated and counted to calculate relative cell count (RCC) which was regarded as the index of cytotoxicity. Based on the results of the preliminary study the doses which induced inhibition of cell growth 50% or greater were calculated. The treatment plan of main study is as shown in the Supplementary 1. The presence of aberrant metaphases was evaluated in each treatment group at two concentrations that met the acceptance criteria for viability and analyzable metaphases. At the end of treatment, cells were washed with Ca^{2+} and Mg^{2+} free Dulbecco's phosphate buffered saline (CMFD-PBS), and fresh media was added for harvest.