Journal of Ethnopharmacology 133 (2011) 696–703

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Genotoxicity assessment of Pyungwi-san (PWS), a traditional herbal prescription

In Sik Shin[a,1], Chang Seob Seo[a,1], Hye Kyung Ha[a], Mee Young Lee[a], Dae Sun Huang[a], Jung Im Huh[b], Hyeun-Kyoo Shin[a,*]

[a] Herbal Medicine EBM Research Center, Korea Institute of Oriental Medicine, 483 Expo-ro, Yusung-gu, Daejeon 305-811, Republic of Korea
[b] Division of nonclinical studies, Korea Institute of Toxicology, P.O. Box 123, 19 Sinseongro, Yuseong-gu, Daejeon 305-343, Republic of Korea

ARTICLE INFO

Article history:
Received 15 June 2010
Received in revised form 8 October 2010
Accepted 23 October 2010
Available online 30 October 2010

Keywords:
Herbal medicine
Pyungwi-san
Bacterial reverse mutation test
Chromosomal aberration test
Micronucleus test
Gentotoxicity

ABSTRACT

Ethnopharmacological relevance: Pyungwi-san (PWS, Heii-san in Japanese) is a mixture of six herbs and is traditionally used in Northeast Asia (especially Korea and Japan) for the treatment of gastrointestinal disorder, such as dyspepsia and inappetance induced by gastric dilatation and gastrointestinal catarrh.
Aim of the study: Although PWS is a widely used herbal prescription in Korea and Japan, little information is available in the literature on the safety and toxicity of PWS. As part of a safety evaluation of PWS, the present study evaluated the potential genotoxicity of PWS using a standard battery of test.
Materials and methods: We prepared PWS using a water extraction method and simultaneously extracted three compounds from PWS using high performance liquid chromatography. The PWS extract that was obtained was assayed for genotoxicity using the standard three tests recommended by the Korea Food and Drug Administration. These tests included the bacterial reverse mutation test (Ames test), the chromosomal aberration test using China hamster lung cells, and the micronucleus test using ICR mice.
Results: The Ames test showed that the PWS extract did not induce an increase in the number of revertant colonies compared with vehicle control at any dose in all of tester strains. In the micronucleus test, no significant increase was observed in micronucleated polychromatic erythrocytes (MNPCEs) at any dose of PWS extract compared with vehicle control. Conversely, chromosomal aberration test showed that the PWS extract at a dosage of 4500 μg/mL induced an increase in the number of chromosomal aberrations in the 6 h group with metabolic activation compared with the vehicle control.
Conclusion: PWS extract exhibits genotoxicity, based on the results of the chromosomal aberration test. Thus, further detailed experiments will be needed to identify the ingredient responsible for inducing this genotoxicity and to determine its mechanism.

1. Introduction

Traditional oriental herbal prescriptions have become popular over the past decade; they are widely used for the treatment and prevention of various diseases due to their effectiveness (Jiang, 2005; Liu et al., 2008). In general, traditional oriental herbal prescriptions are a mixture of several herbs in a single preparation. These herbal prescriptions contain several active ingredients that exhibit various pharmacological activities. These pharmacological actions are referred to as "chemical combination effects" (Hiroaki et al., 2004). Accordingly, traditional oriental herbal formulas have been used as preventative measures against disease and to supplement other treatments to reduce the adverse effects of chemotherapy and surgical operations. Indeed, many physicians prescribe tradition herbal medicines in association with Western medicine (Neldner, 2000; Watanabe et al., 2001; Higuchi et al., 2002; Sasaki et al., 2002). As the use of herbal medicine increases, concerns have been raised over the lack of both quality control and scientific evidence of the efficacy and safety of herbal medicine (Rousseaux and Schachter, 2003; Firenzuoli and Gori, 2007). Currently, researchers using the protocols of evidence-based medicine have conducted extensive studies to establishing scientific evidence of efficacy of herbal medicines. However, few scientific studies have explored the safety and toxicity of herbal medicines.

Pyungwi-san (PWS, Heii-san in Japanese) is a traditional oriental herbal prescription that is used for the treatment of gastrointestinal disorders such as inappetance, abdominal distension, borborygmus and diarrhea induced by gastric atony, gastric dilatation and gastrointestinal catarrh. Currently, PWS is one of the most widely used herbal prescriptions in Korea and Japan (Lee, 2006; JKMA, 2006). In Korea, 56 traditional herbal prescriptions have been approved by

Abbreviations: 9-AA, 9-aminoacridine; 2-AA, 2-aminoanthracene; BP, benzo(a)pyrene; CPA, cyclophosphamide monohydrate; ER, endoreduplication; EMS, ethylmethanesulfonate; MNPCE, PCE with one or more micronuclei; 2-NF, 2-nitrofluorene; 4NQO, 4-nitroquinoline *N*-oxide; NCE, normochromatic erythrocyte; PCE, polychromatic erythrocyte; PP, polyploid; SA, sodium azide.

* Corresponding author. Tel.: +82 42 868 9464; fax: +82 42 864 2120.
E-mail address: hkshin@kiom.re.kr (H.-K. Shin).

[1] These two authors contributed equally to this work.

0378-8741/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jep.2010.10.050

Table 1
Composition of PWS.

Family name	Scientific name	Latin name	Amount (g)	Company of purchase	Source
Compositae	*Atractylodes japonica*	Atractylodis Rhizoma	7.5	HMAX	China
Rutaceae	*Citrus unshiu*	Citri Pericarpium	5.25	Omniherb	Jeju, Korea
Magnoliaceae	*Machilus thunbergii*	Magnoliae Cortex	3.75	HMAX	China
Monocotyledoneae	*Zingiber officinale*	Zingiberis Rhizoma Recens	3.75	Omniherb	Yeongcheon, Korea
Rhamnaceae	*Zizyphus jujuba*	Zizyphi Fructus	3.75	Omniherb	Yeongcheon, Korea
Leguminosae	*Glycyrrhiza uralensis*	Gycyrrhizae Radix	2.25	HMAX	China
Total amount			26.25		

the National Health Insurance Corporation (NHIC) since 1990 and PWS is most often used as a fifth in the 56 traditional herbal prescriptions (NHIC, 2009). PWS is composed of six different herbs (Table 1). In previous studies, PWS has been evaluated for various biological activities. Lee and Moon (2007) reported that PWS possesses an antioxidative activity from the results of different antioxidant tests including 1,1-diphenyl-2picryl-hydrazyl (DPPH) radical scavenging, superoxide anion radical scavenging, metal chelating hydrogen peroxide scavenging. Also, PWS was protected from gastrointestinal mucosal damage and may induce an increase in gastrointestinal motility (Riedlinger et al., 2001). However, little is known about the toxicity and safety of PWS, and standard genotoxicity studies have not been done until now.

Thus, as part of safety evaluations of PWS, an evaluation of the potential genotoxicity of PWS aqueous extract was conducted using the standard battery of tests recommended by the Korea Food and Drug Administration. The tests included the bacterial reverse mutation test (Ames test), the chromosomal aberrations test and the micronucleus test.

2. Materials and methods

2.1. Chemicals and reagents

A prescription of PWS was prepared in our laboratory from a mixture of chopped crude herbs purchased from Omniherb (Yeongcheon, Korea) and HMAX (Chungbuk, Korea). PWS was prepared as described in Table 1 and extracted in distilled water at 100 °C for 2 h. The extract was then evaporated to dryness and freeze-dried (yield; 23.4%).

For the high performance liquid chromatography (HPLC) analysis, liquiritin, glycyrrhizin (purity ≥ 98.0% for both) and hesperidin (purity ≥ 92.0%) were purchased from Wako (Osaka, Japan). The HPLC-grade reagents methanol, acetonitrile and water were obtained from J.T. Baker (Phillipsburg, NJ, USA). The glacial acetic acid was of analytical reagent grade and was procured from Merck KGaA (Darmstadt, Germany). Other chemicals were of analytical grade.

Most of the chemicals used for the genotoxic evaluation of PWS, such as sodium azide (SA), 2-nitrofluorene (2-NF), 4-nitroquinoline *N*-oxide (4NQO), 2-aminoanthracene (2-AA), benzo(a)pyrene (BP), cyclophosphamide monohydrate (CPA), ethylmethanesulfonate (EMS) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Minimum essential medium (MEM) and fetal bovine serum (FBS) and penicillin–streptomycin were purchased from GIBCO-Invitrogen (Carlsbad, CA, USA).

The rat liver microsomal enzyme (S-9), which was prepared form male Sprague–Dawley rats induced with Aroclor-1254, was from Molecular Toxicology Inc. (Boone, NC, USA), and the cofactor for S-9 mix was from Wako Pure Chem. Ind., Ltd. (Japan). For the bacteria reverse mutation study, the S-9 mix 1 mL consisted of 8 μmol $MgCl_2 \cdot 6H_2O$, 33 μmol KCl, 5 μmol G-6-P, 4 μmol NADPH, 4 μmol NADH, 100 μmol sodium phosphate buffer (pH 7.4), and 50 μL S-9. S-9 mix was treated as 0.5 mL/plate and the activities were tested using 2-AA. For the chromosomal aberration study, the S-9 mix 1 mL consisted of 8 μmol $MgCl_2 \cdot 6H_2O$, 33 μmol KCl, 5 μmol G-6-P, 4 μmol NADPH, 4 μmol NADH, 100 μmol sodium phosphate buffer (pH 7.4), and 0.3 mL S-9. The S-9 mix was prepared just before use and kept in an ice bath. The S-9 mix was treated as 0.5 mL/5 mL/T-25 flask and the activity of S-9 mix in this study was confirmed by its ability to convert CP to induce a mutagenic effect.

2.2. HPLC analysis of PWS

HPLC, coupled with other techniques (particularly photodiode array (PDA) detection), is a convenient, widely used, and powerful approach for the rapid identification of constituents in botanical extracts and plants that is important in traditional Chinese medicine (Zhang et al., 2004). Therefore, we focused here on the quantitative determination of the main components of PWS and used HPLC-PDA-coupled methods to simultaneously detect the three main constituents (Fig. 1).

Lyophilized PWS extract was weighed (500 mg) into a 25 mL flask, and distilled water was added to the volumetric mark. The mixture was shaken for 10 min at room temperature. After extraction, the mixture was passed through a 0.2 μm membrane filter, and 10 μL aliquots of the filtrate were injected into the HPLC.

A methanol standard stock solution containing the compounds liquiritin, hesperidin and glycyrrhizin (all 1000 μg/mL) was prepared and diluted to the appropriate concentration range for the establishment of calibration curves.

A Shimadzu LC-20A HPLC system (Shimadzu Co., Kyoto, Japan) was used, consisting of a solvent delivery unit, an on-line degasser, a column oven, an autosampler, and a PDA detector. The data processor employed LC solution software (Version 1.24). The analytical column used was a Luna C18 (250 mm × 4.6 mm; particle size 5 μm, Waters Co., Milfore, MA, USA). The mobile phases consisted of solvent A (1.0%, v/v, aqueous acetic acid) and solvent B (acetonitrile with 1.0%, v/v, acetic acid). The gradient elution is described in Table 2. Column temperature was maintained at 40 °C. The analysis was carried out at a flow rate of 1.0 mL/min with PDA detection from 190 to 400 nm. The injection volume was 10 μL.

The retention times of the main compounds were 11.271, 13.720, and 31.970 min for liquiritin, hesperidin, and glycyrrhizin, respectively. The linearity of the peak area (y) vs. concentration (x, μg/mL) curve for liquiritin, hesperidin, and glycyrrhizin was used to calculate the contents of the main components in PWS.

Table 2
Gradient composition of the mobile phase for HPLC analysis PWS.

Time (min)	Flow (mL/min)	A (%)	B (%)
0	1.0	85	15
35	1.0	35	65
45	1.0	0	100
50	1.0	0	100
55	1.0	85	15
70	1.0	85	15

A, 1.0% acetic acid in H_2O; B, 1.0% acetic acid in acetonitrile.