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RESEARCH ARTICLE

Evaluation of the Genotoxicity of Bangpungtongsung-San, a Traditional Herbal Prescription

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ABSTRACT

Bangpungtongsung-san (BPS) is a traditional Korean herbal formula used as an anti-inflammatory, antipyretic, antiobesity and choleretic agent, which consists of 18 herbs. As part of a safety evaluation of BPS, the present study evaluated the potential genotoxicity of an aqueous BPS extract using a standard battery of tests, including, the Ames test, chromosomal aberration test and mouse micronucleus test. The BPS extract was not found to be genotoxic under the conditions of the Ames test. In micronucleus test, oral administration of BPS at doses up to 2,000 mg/kg did not increase the incidence of micronucleated polychromatic erythrocyte. The chromosomal aberration test showed that the BPS extract induced an increase in the number of structural and numerical chromosomal aberrations in the group treated with BPS at high dose levels (2,500 and 4,000 µg/mL) for 6 h, in the presence of the metabolic activation system (S-9 mix), compared with the vehicle control. In conclusion, these results indicate that BPS extract may act as a genotoxic agent.

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INTRODUCTION

Oriental traditional herbal medicines are used for therapy and prevention of disease. Traditional herbal medicines have few side effects, are highly effective, and the consumption of these medicines increased substantially in recent decades (Castro *et al*., 2009; Jung *et al*., 2011; Sarnthima and Khammuang, 2012). With increasing herbal medicine consumption, concerns were raised over the lack of quality control and scientific evidence of the efficacy and safety of herbal medicine (Ha *et al*., 2011; Shin *et al*., 2011a; Kim *et al*., 2012; Sindhu *et al*., 2012; Masood *et al*., 2013; Munir *et al*., 2013). However, few scientific studies explored the safety of herbal medicines.

Bangpungtongsung-san (BPS, Bofu-tsusho-san in Japanese) is a traditional Korean herbal formula, which is composed of 18 different herbs (Table 1). Currently, BPS is one of the most widely used herbal prescriptions in Korea and Japan. BPS was ranked fifth in production in Japan's herbal market (MHLW, 2008). BPS is generally used as an anti-inflammatory, an antipyretic, a choleretic, jaundice and diuretic agent for liver disorders (Ogawa *et al*., 2009; Weon *et al*., 2011). According to recent studies, BPS extracts are also effective in preventing obesity and various metabolic disorders (Nakayama *et al*., 2007; Shimada, 2008). BPS markedly reduced lipid plasma levels, the liver weight and number of fatty droplets in the liver cytoplasm and bodyweight gain (Nakayama *et al*., 2007).

Previously, we investigated the acute toxicity of BPS and the concentration of hazardous substances before and after a decoction to establish data on the safety of BPS (Seo *et al*., 2009; Shin *et al*., 2010). Thus, as part of the safety evaluation of BPS, an evaluation of the potential genotoxicity of an aqueous extract of BPS was conducted using a standard battery of tests including the Ames test, the chromosomal aberration assay and the mouse micronucleus assay recommended by the Korea Food and Drug Administration (KFDA).

MATERIALS AND METHODS

Preparation of BPS: BPS was prepared in the laboratory from a mixture of chopped crude herbs purchased from Omniherb (Yeongcheon, Korea) and HMAX (Jecheon, Korea). BPS was prepared as described in Table 1 and extracted in distilled water at 100°C for 2 h. The extract was evaporated to dryness and freeze-dried (yield = 17.7%).

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

Table 1: Composition of BPS

Scientific name	Amount (g)
Talcum	6.375
Glycyrrhiza uralensis	4.5
Gypsum	2.625
Scutellaria baicalensis	2.625
Platycodon grandiflorum	2.625
Ledebouriella seseloides	1.6875
Cnidium officinale	1.6875
Angelica gigas	1.6875
Paeonia lactiflora	1.6875
Rheum undulatum	1.6875
Ephedra sinica	1.6875
Mentha pulegium	1.6875
Forsythia koreana	1.6875
Erigeron Canadensis	1.6875
Schizonepeta tenuifolia	1.3125
Atractylodes japonica	1.31
Gardenia jasminoides	1.3
Zingiber officinale	6.25
Total amount	44.125

Table 2: Contents of 4 compounds in the BPS by HPLC (n=3)

Compound	Mean (mg/g)	SD	RSD (%)
Geniposide	5.06	0.016	0.310
Liquiritin	7.33	0.015	0.200
Baiclin	28.44	0.110	0.388
Glycyrrhizin	7.81	0.016	0.198

High performance liquid chromatography (HPLC) analysis of BPS: HPLC analysis of BPS was conducted using a Shimadzu LC-20A HPLC system (Shimadzu Co., Kyoto, Japan). Lyophilized BPS 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, passed through a 0.2 μm membrane filter, and 10 μL aliquots of filtrate were injected into HPLC column. All calibration curves were obtained by assessment of peak areas from standard solutions in the concentration ranges: geniposide, 0.39-50.00 μg/mL; liquiritin, 0.39-50.00 μg/mL; baicalin, 1.56-200.00 μg/mL; glycyrrhizin, 0.79-100.00 μg/mL. The analysis was carried out at a flow rate of 1.0 mL/min with photodiode array detection at 254 and 280 nm. The injection volume was 10 μL.

The retention times of four compounds were 14.1, 17.3, 22.5 and 31.7 min for geniposide, liquiritin, baicalin, and glycyrrhizin, respectively. The linearity of peak area (y) versus concentration (x, μg/mL) curve for reference compounds was used to calculate the concentrations of the main components in BPS.

Ames test: The Ames test was conducted according to OECD guideline TG 471. *Salmonella typhimurium* strains TA98 and TA1537 (which detect frame-shift mutagens), strains TA100 and TA1535, and *Escherichia coli* WP2uvrA (which detect base-pair substitution mutagens) were used as tester strains, which were obtained from Molecular Toxicology Inc. (Boone, NC, USA). In this study, various concentrations of BPS (111.1−5,000 μg/plate) were incubated with tester strains in the presence or absences of metabolic activation (using S-9 mix) at 37°C for 48 h. Triplicate plates were run for each assay. The results were expressed as the mean number of revertant colonies with standard deviations.

Chromosome aberration assay: The chromosome aberration assay was performed according to OECD guideline TG 473. The chromosomal aberration test was performed using Chinese hamster lung (CHL) cells, which were obtained from the American Type Culture Collection (Manassas, VA, USA). The assay consisted of short-term (6 h) and continuous (24 h) treatments. Approximately 22 h after the start of the treatment, colchicine was added to each culture to make a final concentration of 1 μM. Slides of CHL cells were prepared by treating with hypotonic solution, methanol, and glacial acetic acid, followed by flame drying and Giemsa staining for metaphase plate analysis. Two hundred metaphases (100 metaphases from each duplicate culture) were selected and analyzed for each treatment group, using a light microscope at 1000x magnification. The results were expressed as the mean number of aberrant metaphases excluding gaps per 100 metaphases.

Micronucleus test: Six-week-old pathogen-free male ICR mice (25.3−28.3 g) were obtained from Orient Co., Ltd. (Sungnam, Korea) and used after one week of quarantine and acclimatization. This study was reviewed and assessed by the Institutional Animal Care and Use Committee of the Korea Institute of Toxicology. BPS was administered by gavage to male ICR mice once a day for two days at doses of 500, 1,000 and 2,000 mg/kg. Cyclophosphosphamide was administered by intraperitoneal injection at a dose of 70 mg/kg. At 24 h after the last treatment, mice were sacrificed and bone marrow cells were prepared as described by Shin *et al*. (2011b). Small round or oval bodies within erythrocytes with a size of about 1/5 to 1/20 of the diameter of a polychromatic erythrocyte (PCE) were regarded as micronuclei. A total of 2,000 PCEs were counted per animal by the same observer to determine the frequencies of micronucleated polychromatic erythrocytes (MNPCEs). The PCE/(PCE+NCE) ratio was calculated by counting 500 cells. The micronucleus test was conducted in accordance with OECD guideline TG 474.

Statistical analysis: The statistical analyses were based on the methods used in the published reports (Kim *et al*., 2010; Shin *et al*., 2012) using Statistical Analysis System (SAS) software (Ver. 9.1.3, SAS Institute Inc., NC, USA). Differences were regarded as statistically significant if $P<0.05$.

RESULTS

HPLC analysis of BPS: HPLC analysis of BPS was applied for the simultaneous determination of four compounds in BPS, *i.e.,* geniposide, liquiritin, baicalin, and glycyrrhizin. Fig. 1 shows chromatograms of the reference compounds and the aqueous extract of BPS, with detection of eluents at 254 nm and at 280 nm. The linearity of the peak area (y) versus concentration (x, μg/mL) curve for each component was used to calculate the contents of the main components in the BPS. The correlation coefficients (R^2) of calibration curves for four constituents were greater than 0.9999. The concentrations of the four components in BPS ranged from 5.06 – 28.44 mg/g (Table 2).

Ames test: As shown in Table 3, there was no increase in the number of revertant colonies compared to vehicle

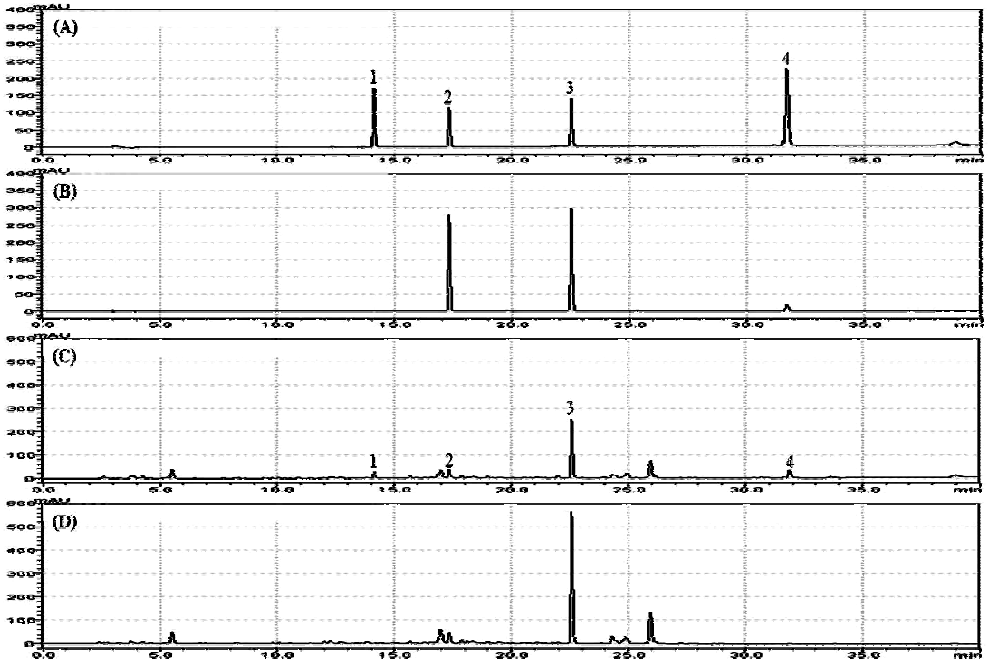

Fig 1: HPLC chromatogram of the standard mixture of four major compounds with detection at 254 nm (A) and 280 nm (B), BPS sample at 254 nm (C) and 280 nm (D). Geniposide (1), liquiritin (2), baicalin (3) and glycyrrhizin (4).

Table 3: Results of Ames test with BPS

Tester strain	Dose (μg/plate)	Revertant colonies/plate (Mean) [Factor][a)]	
		Without S-9 mix	With S-9 mix
TA100	0	107±4	96±1
	555.6	102±4 [1.0]	120±10 [1.3]
	1666.7	126±27[1.2]	149±3 [1.6]
	5000	152±35[1.4]	169±35 [1.8]
	SA(0.5)	284±33[2.7]	
	BP(2)		385±26 [4.0]
TA1535	0	10±3	12±1
	185.2	8±2 [0.8]	11±2 [0.9]
	555.6	12±3 [1.2]	11±2 [0.9]
	1666.7	9±3 [0.9]	13±1 [1.1]
	5000	10±3 [1.0]	15±4 [1.3]
	SA(0.5)	188±10[18.8]	
	2-AA(2)	9±2 [0.9]	71±11 [5.9]
TA98	0	26±2	33±7
	555.6	24±9 [0.9]	29±2 [0.9]
	1666.7	23±3 [0.9]	36±3 [1.1]
	5000	27±2 [1.0]	30±4 [0.9]
	2-NF (2)	336±39[12.9]	
	BP(2)	15±3 [0.6]	504±23 [15.3]
TA1537	0	7±1	22±5
	555.6	9±1 [1.3]	20±3 [0.9]
	1666.7	8±1 [1.1]	34±8 [1.5]
	5000	7±2 [1.0]	42±5 [1.9]
	9-AA (50)	189±49[27.0]	
	BP(2)		102±11[4.6]
E. coli	0	39±3	40±4
WP2uvrA	555.6	33±1 [0.8]	41±5 [1.0]
	1666.7	34±3 [0.9]	41±7 [1.0]
	4000	44±1 [1.1]	43±8 [1.1]
	5000	37±6 [0.9]	45±5 [1.1]
	4NQO (0.5)	154±36[3.9]	
	2-AA (4)		100±8 [2.5]
confirmatory test			
TA1537	0		22±4
	555.6		26±6 [1.2]
	1666.7		34±5 [1.5]
	5000		41±4 [1.9]
	BP(2)		131±6 [6.0]

[a)] No. of revertant colonies of treated plate/No. of revertant colonies of vehicle control plate; SA, Sodium azide; 2-NF, 2-Nitrofluorene; 9-AA, 9-Aminoacridine; 4NQO, 4-Nitroquinoline N-oxide; 2-AA, 2-Amino-anthracene; BP, Benzo(a)pyrene

control at any dose in TA100, TA1535, TA98 and WP2*uvrA* strains. However, in the TA1537 strain, the number of revertant colonies increased in a dose-dependent manner compared with the vehicle control in the presence of the S-9 mix. To confirm this result, the test was repeated. The number of revertant colonies increased in a dose-dependent manner similar to the first test. However, the numbers of revertant colonies did not increase by two-fold in both tests. The positive control treatments, 2-AA, 9-AA, BP, 2-NF, 4NQO and SA showed significant mutagenicity in all strains when compared with vehicle controls.

Chromosome aberration test: There was no increase in the frequency of metaphases with aberrant chromosomes for the 6 h and 22 h BPS treatment groups without the S-9 mix compared with the vehicle control. However, there was a statistically significant increase in the number of structural and numerical metaphase cells with structural aberrations at concentrations of 2,500 and 4,000 μg/mL after 6 h of treatment in the presence of the S-9 mix (Table 4).

Micronucleus test: Adverse effects, such as clinical signs or body weight change, were not observed for mice between the first and final administration in the vehicle control group, in the positive control group, or in the 500, 1,000 or 2,000 mg/kg/day BPS treatment groups (data not shown). There was no statistically significant increase in the number of MNPCEs at any BPS test dose compared to the vehicle control group (Table 5). There was also no significant cytotoxicity decrease for any group treated with BPS compared to the vehicle control, in terms of the mean value of the ratio of PCE/(PCE + NCE).

Table 4: Results of chromosome aberration assay and relative cell count with BPS

Nominal conc. Of test item (μg/mL)	S-9 mix	Times [a] (hours)	Mean Aberrant Metaphases	Mean Total Aberrations	Mean of PP + ER	Relative Cell Count (%)
6 h treatment (+ S-9 mix)						
0	+	6-18	1.0/1.0 [b]	1.5/1.5	0.5+0.0	100
1250	+	6-18	2.0/2.0	3.0/3.0	3.0+0.0	75
2500	+	6-18	8.5/7.5 [** c]	12.0/11.0	3.0+1.0 [* c]	60
4000	+	6-18	15.0/15.0 [** c]	24.5/24.5	2.0+2.5 [* c]	49
CPA 6	+	6-18	30.5/30.5 [** d]	57.0/56.5	0.5+0.0	65
6 h treatment (- S-9 mix)						
0	-	6-18	0.0/0.0	0.0/0.0	0.0+0.0	100
625	-	6-18		Not counted		101
1000	-	6-18	1.0/1.0	1.0/1.0	0.0+0.0	82
1250	-	6-18	2.5/2.5	3.5/3.5	0.5+0.0	50
EMS 800	-	6-18	19.0/19.0 [** d]	28.5/28.0	0.0+0.0	67
22h treatment (- S-9 mix)						
0	-	6-18	0.0/0.0	0.0/0.0	1.5+0.0	100
312.5	-	6-18		Not counted		102
625	-	6-18	1.0/1.0	1.5/1.5	1.5+0.0	86
1000	-	6-18	1.5/1.5	1.5/1.5	0.5+0.0	65
EMS 600	-	6-18	24.0/24.0 [** d]	36.5/36.0	2.0+0.0	66

[*,**] Significant different from the control at P<0.05 and 0.01; [a] Treatment time-recovery time; [b] Gaps included/excluded, means of duplicate cultures; 100 metaphases were examined per culture; [c] χ^2-test and Fisher' exact test; [d] Fisher's exact test; PP, Polyploid; ER, Endoreduplication; CPA, Cyclophosphamide monohydrate; EMS, Ethylmethanesulfonate

Table 5: Results of Micronucleus test with BPS

Chemical treated	Dose (mg/kg)	MNPCE/2000 PCEs (Mean±SD)	PCE/(PCE+NCE) (Mean±SD)
Vehicle	0	1.67±1.15	0.52±0.02
BPS	500	0.33±0.58	0.56±0.04
	1000	2.00±1.00	0.52±0.05
	2000	1.33±1.53	0.49±0.10
CPA	70	63.33±7.02 [*a]	0.37±0.13

* Significantly different from the control at $P < 0.05$; a) Mann-Whitney's U-test; MNPCE, PCE with on or more micronuclei; PCE, Polychromatic erythrocyte; NCE, Normochromatic erythrocyte; CPA, Cyclophosphamide monohydrate

DISCUSSION

As part of a safety evaluation of BPS, this study evaluated the potential genotoxicity of BPS using the three standard tests recommended by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and by the KFDA: the Ames test, the chromosomal aberration test, and the micronucleus test using ICR. A search of the published literature indicates that this is the first study to describe the genotoxicity of BPS.

In the Ames test, no positive mutagenic response was observed in any of the tester strains compared to the concurrent vehicle control groups regardless of S-9 mix application. However, in the TA1537 stain, the number of revertant colonies increased in a dose-dependent manner compared with the vehicle control in the presence of the S-9 mix in both first test and confirmation test. However, we do not think the BPS extract induced a mutagenic effect because the numbers of revertant colonies did not increase by two-fold, which would indicate genotoxicity, compared with the vehicle control. Therefore, under the conditions used in the present study, the Ames test demonstrated that BPS is not mutagenic to the bacterial strains TA98, TA100, TA1335, TA1537 and WP2uvrA.

In the chromosomal aberration test using CHL cells, there was a statistically significant increase in the number of structural and numerical metaphase cells with structural aberrations at concentrations of 2,500 and 4,000 μg/mL after 6 h of treatment in the presence of the S-9 mix (Table 4). Positive controls showed significant increase in the frequency of metaphases with aberrant chromosomes. The number of metaphases with structural aberrations in the vehicle and positive control groups was within the range established in our historical data. These findings confirm that the methodologies used in this study were valid. Therefore, these results indicated that the mutagenic potential of BPS is caused by both BPS and its metabolism.

The micronucleus test is a popular and useful in vivo procedure for the detection of chemically-induced chromosome damage because of its simplicity and efficacy. The number of reports using micronucleus testing increased dramatically in the scientific literature over the past decade (Naya *et al*., 2011) and the value of this test for examining the mutagenicity and carcinogenicity of chemicals has been emphasized, particularly when it is used in combination with other cytogenetic assays (Song *et al*., 2012). In present test, no abnormal changes in general appearance or body weight were observed for mice between the first and final administration in all groups (data not shown). There was no statistically significant increase in the number of MNPCEs at any BPS test dose compared to the vehicle control group. There was also no significant cytotoxicity decrease for any group treated with BPS compared to the vehicle control, in terms of the mean value of the ratio of PCE/(PCE + NCE). From these results, we conclude that BPS did not induce mutagenesis under the conditions of this study.

Conclusion: The study was evaluated the genotoxicity of BPS using a standard battery of tests to establish a genotoxicity profile for BPS. The results showed that BPS might exhibit genotoxic effects in chromosomal aberration test. Thus, we consider that BPS extract may act as a genotoxic agent.

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