Genotoxicity testing of Persicariae Rhizoma (*Persicaria tinctoria* H. Gross) aqueous extracts

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Received February 1, 2015; Accepted April 5, 2016

DOI: 10.3892/etm.2016.3273

Abstract. Persicariae Rhizoma (PR) has been used as an anti-inflammatory and detoxification agent in Korea, and contains the biologically active dyes purple indirubin and blue indigo. Despite synthetic indigo showing genotoxic potential, thorough studies have not been carried out on the genotoxicity of PR. The potential genotoxicity of an aqueous extract of PR containing indigo (0.043%) and indirubin (0.009%) was evaluated using a standard battery of tests for safety assessment. The PR extract did not induce any genotoxic effects under the conditions of this study. The results of a reverse mutation assay in four *Salmonella typhimurium* strains and one *Escherichia coli* strain indicated that PR extract did not increase the frequency of revertant colonies in any strain, regardless of whether S9 mix was present or not. The PR extract also did not increase chromosomal aberrations in the presence or absence of S9 mix. Although slight signs of diarrhea were restrictedly detected in the mice treated with 2,000 mg/kg PR extract, no noteworthy changes in the frequency of micronucleated polychromatic erythrocytes were observed at doses ≤2,000 mg/kg in a bone marrow micronucleus test. These results indicate the potential safety of the PR extract, particularly if it is consumed in small amounts compared with the quantities used in the genotoxicity tests.

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Key words: Persicariae Rhizoma, *Persicaria tinctoria* H. Gross, genotoxicity, reverse mutation assay, chromosomal aberration assay, mouse micronucleus assay

Introduction

Genotoxic substances are specific chemical or physical agents, including viruses and hormones, that can damage the genetic information within a cell, thereby inducing mutations or cancer, and genotoxicity refers to the capacity of a chemical or physical substance to damage genetic information. Although genotoxicity differs from mutagenicity, the terms are often used confusedly; all mutagens are genotoxic, however, not all genotoxic substances are mutagenic. Mutagens induce direct or indirect damage to DNA that leads to mutations, which are permanent and heritable changes that can affect somatic cells to be transmitted onto future generations (1). When DNA damage occurs, a DNA damage response, including cell cycle arrest, DNA repair and apoptosis, is induced in cells to suppress the induction of mutation; however, this DNA damage may not always be processed resulting in mutagenesis (2,3).

When evaluating genotoxic substances, scientists test for DNA damage in cells with exposure to the toxic agents. This DNA damage can be categorized as single- and double-strand breaks, loss of excision repair, cross-linking, the formation of alkali-labile sites, point mutations, and structural and numerical chromosomal aberrations (4). The loss of integrity of the genetic information can lead to cancer. To evaluate the potential of chemicals to cause DNA damage, various methods have been developed. Among these, the Ames assay, *in vitro* and *in vivo* toxicology tests and the Comet assay are widely used (5,6). The deleterious effect of the genotoxic substance is DNA damage induced by interaction with the DNA sequence and structure. On occasion, lesions within DNA under oxidative stress conditions can lead to mutation, and the accumulation of mutations can contribute to the development of cancer (7).

Natural materials have received special attention as a potential source of bioactive components in the pharmaceutical industry (8). Although materials such as herbs, medicinal plants and crude drug substances obtained from them are generally accepted as being safe (9), it is important to evaluate natural substances for mutagenicity. Evidence suggests that certain types of foods may cause toxic, genotoxic or carcinogenic hazards for humans (10). Contaminated foods are known to show harmful effects, and certain food additives may have mutagenic and/or carcinogenic potential (11). In addition, the cooking process may generate carcinogenic compounds (12),

and certain foods with natural constituents may show mutagenic and/or carcinogenic toxicity (13).

Persicariae Rhizoma (PR) is dried stem parts of *Persicaria tinctoria* H. Gross (Polygonaceae), and has been traditionally used as an anti-inflammatory and detoxification agent in Korea (14). PR contains two biologically active anti-inflammatory and antioxidative dyes, namely purple indirubin and blue indigo (15). Indirubin, a 3,2'-bisindole isomer of indigo, was first identified as the active ingredient of a traditional Chinese medicine preparation, Danggui Longhui Wan, which is used to treat various chronic diseases (16). Indirubin derivatives exhibit strong anti-inflammatory and anti-leukemic activities (17). A previous study showed that indirubin is a potent inhibitor of a wide range of kinases, but, in particular, it strongly suppresses the activation of cyclin-dependent kinases (18). Herbal extracts containing indigo or their derivatives have also exhibited potent antibacterial (19), antitumor (20), anti-inflammatory (21) and antioxidant (22) activities. In a previous study, it was revealed that natural indigo products showed no mutagenicity but all synthetic indigo products showed enzyme-mediated mutagenicity (23). It has also been demonstrated that the mutagenic effect of purified synthetic indigo and natural indigo may be attributed to one or more structurally related contaminants, and the metabolic activation of these promutagenic factors appears to involve glutathione (24). In addition, mutagenic effects of synthetic indigo of technical grade or 98% purity have been observed in the Ames test (25), and some synthetic colorants are harmful when ingested in large amounts (13). As mammalian intestinal microorganisms are able to cleave azo bonds and lead to the generation of degradation products such as aromatic amines that are potentially mutagenic and carcinogenic, the use of azo dyes has been limited (26). However, no preclinical studies of PR extract containing the natural dyes indigo and indirubin, have been performed, to the best of our knowledge, not even a basic rodent single-dose toxicity test. Moreover, the demand for PR extract has increased for use in foodstuffs and functional food materials, as well as for its nutritive value and as a natural dye. Therefore, the present study was conducted to test the genotoxicity of an aqueous extract of PR containing indigo and indirubin using a standard battery of tests, including the bacterial reverse mutation assay, the chromosomal aberration assay and the mouse micronucleus assay, as used to test other materials in our previous studies (27,28).

Materials and methods

Chemicals, culture medium and S9 activation system. Fetal bovine serum (FBS) was obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cyclophosphamide (CPA), ethylmethanesulfonate (EMS), sodium azide (SA), 4-nitroquinolone-1-oxide (4NQO), 9-aminoacridine (9-AA), 2-aminoanthracene (2-AA), indigo and indirubin standards, dimethylsulfoxide (DMSO), formic acid and acetonitrile were supplied by Sigma-Aldrich (St. Louis, MO, USA). Bacto agar was obtained from Difco (BD Biosciences, Franklin Lakes, NJ, USA). Cell culture-grade water used in the chromosomal aberration assay and the reverse mutation assay, culture medium, antibiotics and L-glutamine were purchased from Invitrogen (Thermo Fisher Scientific, Inc.).

The S9 mix was purchased from Molecular Toxicology, Inc. (Boone, NC, USA). For the reverse mutation assay, the S9 mix (per 1 ml) was composed of $MgCl_2.6H_2O$ (8 μmol), KCl (33 μmol), glucose 6-phosphate (5 μmol), nicotinamide adenine dinucleotide phosphate (NADPH; 4 μmol), nicotinamide adenine dinucleotide (NADH; 4 μmol), sodium phosphate buffer (100 μmol, pH 7.4) and S9 (50 μl). The S9 mix was used at 0.5 ml/plate, and the activities were determined using 2-AA. In the chromosomal aberration test, the S9 mix (per 1 ml) was composed of $MgCl_2.6H_2O$ (8 μmol), KCl (33 μmol), G-6-P (5 μmol), NADPH (4 μmol), NADH (4 μmol), sodium phosphate buffer (0.1 M, pH 7.4) and S9 (0.3 ml). The S9 mix was prepared just prior to use and maintained in an ice bath. The S9 mix was used at 0.5 ml/5 ml/T-25 flask, and the effectiveness of the S9 mix was identified by its ability to activate CPA to induce a mutagenic effect.

Test substance

Preparation of aqueous extract. Aqueous PR extract (yield, 12.00%) as a greenish brown powder was prepared using a rotary vacuum evaporator (Eyela N-1110; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and programmable freeze dryer (FDB-5503; Operon, Gimpo, South Korea). The extract was prepared from dried stem parts of *Persicaria tinctoria* H. Gross (Polygonaceae) collected from the South Korean province of Yeongcheon, which were purchased from Omniherb (Yeongcheon, South Korea) after checking the morphology under a microscope. Voucher specimens (code. CD2012Ku) were kept in the herbarium of the Medical Research Center for Globalization of Herbal Formulation, Daegu Haany University (Gyeongsan, South Korea). Next, 200 g herbs were boiled at 80˚C in 2 L distilled water for 3 h, then evaporated and lyophilized. As a result, 24 g extract was acquired (yield=12%). All test materials were stored in a refrigerator at -20˚C to protect them from light and degeneration until use.

Measurement of indigo and indirubin content in the PR extract. Standard solutions containing 1 μg/ml concentrations of indigo and indirubin in DMSO were prepared and diluted with 1:1 DMSO:acetonitrile mixtures to concentrations of 1, 5 and 10 ng/ml. Standard stock solutions and working solutions were stored at 4˚C. For preparation of the sample, samples of PR extract were weighed, and dissolved in 1:1 DMSO and acetonitrile mixtures. The samples were then sonicated for 1 h at room temperature. Prior to high performance liquid chromatographic system (HPLC) analysis, the sample preparations were filtered through a 0.45-μm membrane filter. A Waters Alliance HPLC system (Waters Corporation, Milford, MA, USA), equipped with a Waters 2489 UV/Visible detector was used for analysis. An Empower Data System and YMC-Pack Pro C-18 column (1.7 μm, 2.1x100 mm; both Waters Corporation) were used for recording the output signal of the detector and for separation, respectively. The mobile phase consisted of 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile. The flow rate in the gradient elution system was 1.0 ml/min, the injection volume was 10 μl, the UV detection wavelength was 540 nm and the column was maintained at room temperature.

Reverse mutation assay.

The bacterial reverse mutation assay was conducted according to standard procedures (29,30). *Salmonella typhimurium*

(TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2*uvr*A were used to determine whether reverse mutations were induced at histidine and tryptophan loci, respectively.

Materials. All strains were obtained from Molecular Toxicology Inc., and confirmed for retention of their characteristic phenotypic markers at the time of use, as previously described (31). Tester strains were inoculated into 25 ml 2.5% Oxoid nutrient broth no. 2 and incubated at 37˚C for 10 h in a 200-rpm shaking incubator (LabTech LSI-3016R; Daihan Labtech Co., Ltd., Namyangju, Korea). Minimal glucose agar plates were prepared with 1.5% Bacto agar, Vogel-Bonner medium E and 2% glucose/liter, and dispensed into 25-ml 100x15 mm Petri dishes. For the *E. coli* strain, 0.25 ml/l 0.1% tryptophan solution was added to the agar plates.

Treatment. Sterile distilled water was used to prepare PR extract solution and was also used as a negative control to compare with the PR extract. PR extract solution was diluted with sterile distilled water to prepare stock solutions for each treatment without (-S) or with (+S) the S9 mix. 2-AA, 9-AA and 4NQO solutions were prepared in DMSO. SA solutions were prepared in sterilized distilled water. Following autoclaving, 2 ml top agar was dispensed into 12x75-mm tubes at 45˚C. PR extract (0.1 ml), S9 mix (or sodium-phosphate buffer, pH 7.4; 0.5 ml) and bacterial culture (0.1 ml) were then added and mixed gently for 2-3 sec. This mixture was then poured onto a minimal glucose agar plate. In addition, the PR extract test solution and S9 mix, without bacterial culture, were plated to confirm sterility. When the top agar had hardened, the plates were turned upside down and incubated at 37˚C for ~48 h. Revertants were then counted and the formation of a background lawn and other abnormalities were observed. The PR extract was assayed in quadruplicate per concentration.

Judgment. Results are shown as the mean number of colonies± standard deviation (SD) of quadruplicate plates. After checking for contamination, the formation of a background lawn and other abnormalities were analyzed. If the ratio number of colonies on a treated plate/number of colonies on a negative control plate was <0.5, the test material was judged to have an antibacterial effect. A result was considered positive if the frequency of revertant colonies per plate in at least one strain was concentration-dependently increased over the range tested and/or there was a reproducible increase at one or more concentrations regardless of S9 activation.

Chromosomal aberration assay.

Assays were conducted using methods described previously (32) with slight modifications.

Materials. Chinese hamster lung (CHL) cells (33) were supplied by the Division of Genetic Toxicology, Department of Toxicology, Korea Food and Drug Administration (KFDA; Seoul, South Korea). Cells were grown in reconstituted minimum essential medium supplemented with sodium bicarbonate (2.2 g), L-glutamine (292 mg), streptomycin sulfate (100 μg/ml), sodium penicillin G (1x10^5 units) and FBS (10% v/v). Cells were maintained in incubator with a humidified atmosphere of 5% CO_2 in air at 37˚C. Rapidly growing cultures were separated with trypsin. Cells (4x10^4) were seeded in culture flasks (25-cm^2) in 5 ml medium and incubated for 3 days.

Treatment. In this assay, cells were directly treated with test (PR extract) and positive control materials (CPA and EMS). Cells were divided into series I, II and III groups. The PR extract was added following a 1 h incubation in 4.0 ml medium for series I (+S9) and 4.5 ml medium for series II and III (-S9). Cultures of series I and II were treated with PR extract for 6 h, the treatment mixture was then removed, and the cells were washed once with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) followed by further incubation in 5 ml fresh medium until harvesting. Cultures of series III were cultured with PR extract for 24 h and the washing procedure was omitted. At ~22 h after treatment, colchicine (1 μM) was treated to each culture, and the cultures were incubated for an additional 2 h. Mitotic cells were isolated by shaking gently. Following centrifugation at 150 x g for 5 min, the supernatant was removed and cell pellets were resuspended in 5 ml 0.075 M KCl solution. After 10 min at room temperature, fixative solution (methanol:glacial acetic acid, 3:1 v/v) was rapidly added and the suspension was kept at 4˚C for 20 min. The fixed cell suspension was dropped onto glass slides and then air-dried followed by staining with Giemsa (3% in Sorensen buffer, pH 6.8).

Judgment. The criteria for the identification of chromosomal aberrations were those of Evans (34). The structural aberrations were divided into two broad types: Chromatid and chromosome gaps, chromatid type deletions and exchanges; and chromosome type deletions and exchanges. Each slide was scanned systematically, and each set of metaphases was examined under x1,000 magnification. To identify aberrations, 100 metaphases on each slide that had a chromosome count of between 23 and 27 were examined. After recording each type of aberration, the number of aberrant metaphases (showing one or more aberrations, including/excluding gaps) and total aberrations (including/excluding gaps) were calculated. The results are shown as mean aberrant metaphases excluding gaps per 100 metaphases from quadruple flasks/dose.

In vivo mouse micronucleus assay

Animals and husbandry. A total of 62 male specific pathogen-free CrljOri:CD1 (ICR) mice (6 weeks old and body weight in the range of 31-34 g upon receipt; Orient Bio Inc., Seungnam, Korea) were obtained, and five groups of 10 mice each (total 50 mice) were selected based on the body weights at 9 days after acclimatization [mean ± standard deviation (SD), 36.16±1.98 g; range 33.6-40.7 g]. Animals were maintained at five per polycarbonate cage in a controlled room of 50-55% humidity and 20-25˚C temperature. The light:dark cycle was 12 h:12 h, and standard rodent chow (Samyang Feed, Seoul, Korea) and water were supplied *ad libitum*. All animals were maintained in accordance with international regulations for the usage and welfare of laboratory animals, and approved by the Institutional Animal Care and Use Committee of Daegu Haany University (Approval No. DHU2014-017).

Treatment. Mice were treated by oral gavage for 2 consecutive days with 500, 1,000 or 2,000 mg/kg/day PR extract in a volume of 10 ml/kg, dissolved in distilled water as vehicle or with an equal volume of vehicle (for vehicle control mice), with ~24 h between doses. As there are no available toxicological data for PR extract administered orally to female and male mice, the highest dosage used in the present study was selected

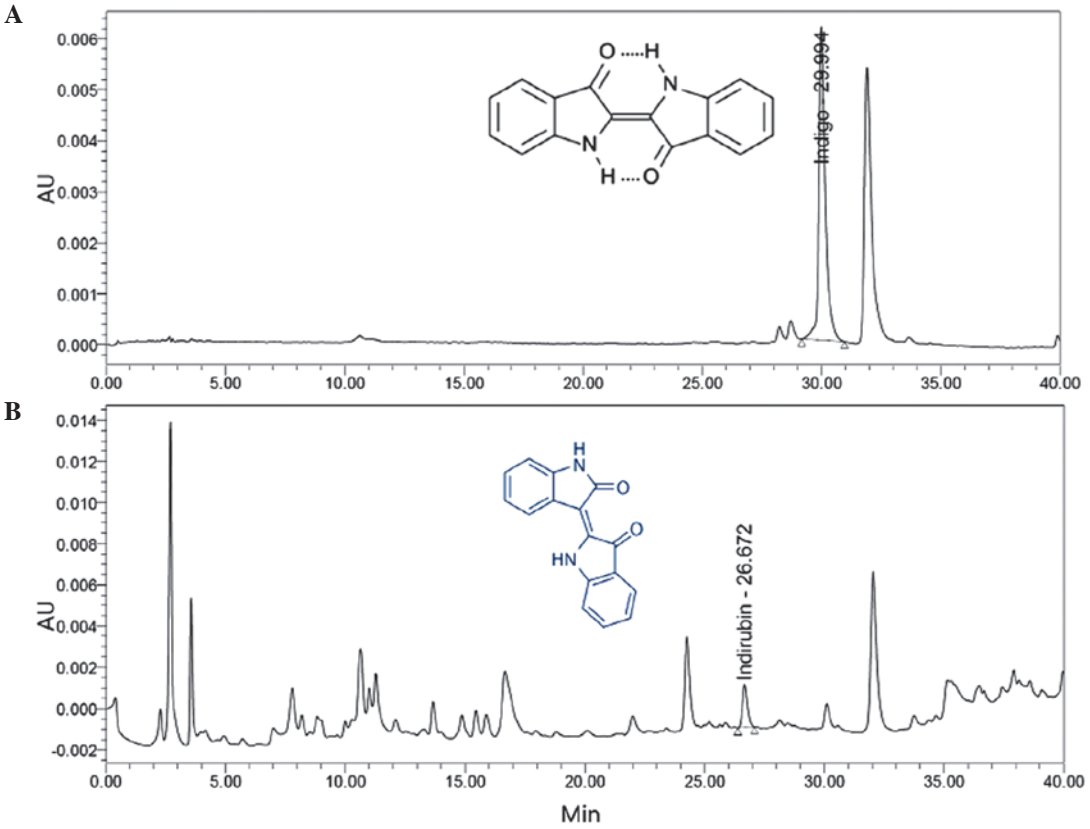

Figure 1. HPLC chromatograms of (A) indigo and (B) indirubin in PR extract. Determination of indigo and indirubin content in the PR extract was established by use of a HPLC system, and the results indicated that the lyophilized aqueous extract of PR contained 0.043% indigo and 0.009% indirubin. HPLC, high performance liquid chromatography; PR, Persicariae Rhizoma; AU, absorbance unit.

as 2,000 mg/kg, the limited dosage for rodents (35), and 1,000 and 500 mg/kg were selected for administration to the middle and lower dosage groups according to the recommendations of the KFDA. A similar group was treated once with the positive control (70 mg/kg of CPA) at ~24 h prior to sacrifice.

Observation of clinical signs. According to the functional observational battery test, all abnormalities were checked at least twice a day (36). If any abnormal clinical signs were detected, they were subdivided into three degrees according to the status of animals: 3+ severe, 2+ moderate and 1+ slight (37).

Changes of body weights. Body weights were measured at 1 day before administration, immediately prior to treatments and at sacrifice (24 h after the end of the second treatment).

Bone marrow preparation. Animals were asphyxiated with CO_2, and gross necropsy was performed in all surviving animals at 24 h after the end of the second treatment, and bilateral femurs were separated. Bone marrow preparations were made according to the method of Schmid (38). Bone marrow cells were collected from the femur in 3 ml inactivated FBS, centrifuged at 300 x g for 10 min in room temperature and smeared onto a slide. Preparations were then dried, and dipped into absolute methanol for 10-20 min. Fixed slides were serially stained using May-Grünwald stock (3 min; Sigma-Aldrich), May-Grünwald stock and tap water (1:1) diluted solution (2 min) and Giemsa and tap water (1:6) diluted solution (10 min), respectively.

Judgment. Slides were randomly observed through a microscope (Eclipse 80i, Nikon Corporation, Tokyo, Japan) at x400 magnification and examined blindly by two experts. Micronuclei were morphologically identified according to the criteria defined by Schmid (39). Micronuclei, which appeared as small round or oval-shaped bodies, ranging in size from ~1/5 to 1/20 of the diameter of a polychromatic erythrocyte (PCE) were counted and recorded. In total, 2,000 PCEs per animal were examined to determine the number of micronuclei. Cytotoxicity was evaluated by counting the frequency of PCEs and normochromatic erythrocytes (NCEs) in at least the first 500 erythrocytes from each animal. Furthermore, in cases where all of the PCE/[PCE + normochromatic erythrocyte (NCE)] ratios were >0.20, the experimental results were accepted (40).

Statistical analysis. Quantitative data are presented as means ± standard deviations. One-way analysis of variance (ANOVA) or the Kruskal-Wallis H test for multiple comparisons were conducted. When a significant difference was detected in a statistical hypothesis test, the Scheffe's test or Mann-Whitney U test with Bonferroni correction was conducted to determine the significantly different pairs of groups. Statistical analyses were performed using SPSS for Windows (version 14.0K; SPSS, Inc., Chicago, IL, USA), and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Indigo and indirubin content in the PR extract. Determination of the quantities of indigo and indirubin in the PR extract was established by use of HPLC. The indigo and indirubin contents

Table I. Results of preliminary range-finding tests in the bacterial reverse mutation assay.

Treatment	Dose (μg/plate)	Colonies/plate, mean (factor)[a]				
		TA98	TA100	TA1535	TA1537	WP2*uvr*A
Without S9						
Vehicle control[b]		29 (1.00)	127 (1.00)	14 (1.00)	11 (1.00)	21 (1.00)
PR extract	0.05	27 (0.93)	119 (0.94)	15 (1.07)	8 (0.73)	19 (0.90)
	0.5	26 (0.90)	132 (1.04)	12 (0.86)	9 (0.82)	20 (0.95)
	5	30 (1.03)	125 (0.98)	13 (0.93)	10 (0.91)	21 (1.00)
	50	29 (1.00)	106 (0.83)	12 (0.86)	11 (1.00)	17 (0.81)
	500	23 (0.79)	98 (0.77)	9 (0.64)	7 (0.64)	16 (0.76)
	1,000	10 (0.34)	51 (0.40)	5 (0.36)	5 (0.45)	9 (0.43)
	5,000	8 (0.28)	38 (0.30)	3 (0.21)	3 (0.27)	4 (0.19)
Sodium azide	0.5		398 (3.13)	483 (34.50)		
4-Nitroquinolone-1-oxide	0.5	282 (9.72)				132 (6.29)
9-Aminoacridine	50				295 (26.82)	
With S9						
Vehicle control[b]		38 (1.00)	119 (1.00)	9 (1.00)	17 (1.00)	17 (1.00)
PR extract	0.05	36 (0.95)	121 (1.02)	7 (0.78)	16 (0.94)	16 (0.94)
	0.5	40 (1.05)	117 (0.98)	9 (1.00)	18 (1.06)	14 (0.82)
	5	32 (1.84)	108 (0.91)	10 (1.11)	17 (1.00)	13 (0.76)
	50	35 (0.92)	105 (0.88)	8 (0.89)	15 (0.88)	18 (1.06)
	500	30 (0.79)	93 (0.78)	6 (0.67)	11 (0.65)	15 (0.88)
	1,000	15 (0.39)	42 (0.35)	3 (0.33)	6 (0.35)	7 (0.41)
	5,000	7 (0.18)	32 (0.27)	2 (0.22)	4 (0.24)	4 (0.24)
2-Aminoanthracene	0.4	406 (11.28)	252 (2.08)			
	2			327 (46.71)	351 (21.94)	
	4					223 (13.94)

[a]Factor is the number of colonies of the treated plate/number of colonies of the negative control plate. [b]Vehicle control, sterile distilled water. PR, Persicariae Rhizoma.

of the extract were calculated from the calibration curve of the standards. The results indicated that the lyophilized aqueous extract of PR contained 0.043% indigo and 0.009% indirubin (Fig. 1).

Reverse mutation assay. The mutagenicity of the PR extract in bacteria was assessed up to a maximal dose of 1,000 μg/plate, because PR extract exhibited antibacterial activity at 5,000 and 2,500 μg/plate in all five test strains of the preliminary test (Table I). All strains showed normal growth, and the test material was freely soluble at all doses evaluated in the absence and presence of S9. The revertant frequencies of all PR extract doses in all tester strains were not changed, regardless of S9 activation, compared with those in the vehicle control cultures. All four positive control agents that were tested exhibited significant ($P<0.05$) increases in revertant frequencies of tested strains (Table II).

Chromosomal aberration assay. In the chromosomal aberration assay, the highest concentration of PR extract tested was 5,000 μg/ml since the calculated relative cell count for 5,000 μg/ml was > 89% in the preliminary experiments (Table III). There were no differences in structural and numerical chromosomal aberrations between all doses of PR extract in all three series of treatments (I, II and III) regardless of the presence or absence of the metabolic activator S9 when compared with those in the vehicle control. The two positive control agents tested in this study showed significant ($P<0.05$) increases in structural and numerical chromosomal aberrations (Table IV).

In vivo mouse micronucleus assay. No PR extract or CPA treatment-related unscheduled mortalities or changes in body weight were detected. All of the five experimental groups tested in the present study were subjected to bone marrow cell harvesting or blood sampling at 24 h after the end of the final treatment. In addition, no treatment-related abnormal clinical signs were observed in mice treated with any of the three different dosages of PR extract or CPA, with the exception of slight (1+) diarrhea signs, restrictedly and transiently detected

Table II. Results of the bacterial reverse mutation assay.

Treatment	Dose (μg/plate)	Colonies/plate				
		TA98	TA100	TA1535	TA1537	WP2*uvr*A
Without S9						
Vehicle control[a]		26.50±2.65	135.00±6.48	14.75±1.26	11.00±1.83	14.50±2.65
PR extract	0.01	25.25±2.50	131.75±8.34	15.25±2.63	10.75±1.26	14.75±3.59
	0.1	25.00±2.94	132.00±4.83	14.75±2.50	10.25±2.63	15.00±2.94
	1	26.75±3.59	133.25±5.97	14.50±1.73	10.25±1.71	14.25±2.22
	10	24.75±2.50	134.75±8.22	15.00±1.83	11.00±2.16	14.50±2.38
	100	25.50±2.08	137.25±5.74	14.25±1.71	9.75±2.50	12.75±1.71
	1,000	22.25±3.86	130.25±4.03	12.75±1.71	8.75±2.50	11.50±2.08
Sodium azide	0.5		456.50±69.63[b]	494.25±105.18[b]		
4-Nitroquinolone-1-oxide	0.5	346.50±73.58[b]				112.25±19.16[b]
9-Aminoacridine	50				273.75±19.45[b]	
With S9						
Vehicle control[a]		32.50±5.07	146.25±12.18	10.75±3.10	17.75±2.22	19.50±2.38
PR extract	0.01	30.75±7.54	145.50±10.91	11.00±2.16	18.00±2.94	18.25±2.75
	0.1	29.50±1.29	145.50±11.98	11.50±1.29	18.00±2.16	18.75±2.22
	1	32.50±6.19	146.75±9.67	9.50±1.29	17.25±1.71	18.25±3.50
	10	32.00±5.94	145.75±10.90	10.00±2.71	17.25±3.77	20.00±1.83
	100	29.50±6.76	139.75±10.40	9.75±1.26	16.50±2.65	17.50±2.08
	1,000	27.00±2.58	132.25±11.15	8.75±1.50	14.00±4.08	16.00±3.92
2-Aminoanthracene	0.4	311.50±27.33[b]	343.50±63.82[b]			
	2			231.75±47.13[b]	332.50±37.04[b]	298.50±11.45[b]
	4					

Values are expressed as mean ± standard deviation from quadruple plates/dose. [a]Vehicle control, sterile distilled water, [b]$P<0.05$ vs. vehicle control by Mann Whitney U test. PR, Persicariae Rhizoma.

Table III. Results of preliminary range-finding tests for the chromosomal aberration assay.

Treatment conditions	Dose (μg/ml)	Viable cells/flask	RCC[a] (%)
Treatment time 6 h, recovery time 18 h, with S9			
Vehicle control[b]		11,871.25±1,034.27	100.00
PR extract	0.05	11,327.00±941.60	95.42
	0.5	12,054.25±1,123.84	101.54
	5	11,482.50±871.57	96.73
	50	11,931.50±568.58	100.51
	500	12,085.25±77.74	101.80
	1,000	10,641.25±691.62	89.64
	5,000	11,292.75±975.90	95.13
Cyclophosphamide	6	7,694.00±822.28	64.81
Treatment time 6 h, recovery time 18 h, without S9			
Vehicle control[b]		11,954.00±2,032.45	100.00
PR extract	0.05	11,469.00±852.76	95.94
	0.5	11,859.50±1,540.14	99.21
	5	11,902.75±1,909.86	99.57
	50	12,344.00±1,063.40	103.26
	500	11,473.75±895.79	95.98
	1,000	12,118.00±1,116.35	101.37
	5,000	10,836.00±436.41	90.65
Ethylmethanesulfonate	800	7,609.75±465.86	63.66
Treatment time 24 h, recovery time 0 h, without S9			
Vehicle control[b]		11,220.75±978.78	100.00
PR extract	0.05	10,062.50±882.62	89.68
	0.5	11,643.25±1,526.13	103.77
	5	11,900.75±556.52	106.06
	50	11,304.50±2,231.19	100.75
	500	10,628.25±519.86	94.63
	1,000	11,380.00±2,133.52	101.42
	5,000	12,308.25±1,459.35	109.69
Ethylmethanesulfonate	600	7,019.75±1,146.26	62.56

Values of viable cells/flask are expressed as mean ± standard deviation of two independent experiments. [a]RCC, relative cell count (%) = (cell count of treated flask/cell count of control flask x 100. [b]Vehicle control, sterile distilled water. PR, Persicariae Rhizoma.

in three (3/10; 30%) mice treated with 2,000 mg/kg PR extract following the first administration, but not after the second treatment (Table V).

Significant (P<0.01) increases in the numbers of micronucleated bone marrow polychromatic erythrocytes (MNPCEs) detected among 2,000 PCEs were observed in the CPA-treated group as compared with the vehicle control group, but no significant changes in MNPCE numbers were observed for the mice in the 2,000, 1,000 and 500 mg/kg PR extract-treated mice as compared with the vehicle control group. Although the numbers of PCEs in the 70 mg/kg CPA-treated mice were significantly (P<0.01) decreased as compared with those in the vehicle control group, individual PCE/(PCE + NCE) ratios were >0.25 in the present study. No significant or noteworthy changes in the PCE/(PCE + NCE) ratio were observed for the mice treated with 2,000, 1,000 and 500 mg/kg doses of PR extract when compared with corresponding ratio in the vehicle control (Table V and Fig. 2).

Discussion

Genotoxicity tests carried out using bacterial, yeast and mammalian cells are aimed to assess whether substances will damage genetic material (1,41). The primary use of *in vitro* testing is to evaluate whether chemical and physical agents, or environmental factors can damage genetic material. The information obtained from such tests can provide appropriate or sufficient control in the early development of vulnerable organisms to genotoxic substances (2,3). The bacterial reverse mutation assay (Ames assay) is used to detect for gene mutation (42) and various bacterial strains can be used to compare different changes in the genetic material. This assay detects

Table IV. Results of the chromosomal aberration assay.

Treatment conditions	Dose (μg/ml)	Aberrant metaphases		Total aberrations		PP	ER
		With gaps	Without gaps	With gaps	Without gaps		
Treatment time 6 h, recovery time 18 h, with S9							
Vehicle control[a]		0.50±0.58	0.00±0.00	1.25±0.96	0.75±0.96	0.50±0.58	0.25±0.50
PR extract	0.5	0.25±0.50	0.00±0.00	1.25±0.50	1.00±0.00	0.50±0.58	0.50±0.58
	5	0.75±0.50	0.00±0.00	1.00±0.82	0.25±0.50	0.25±0.50	0.00±0.00
	50	0.75±0.50	0.00±0.00	1.75±0.50	1.00±0.00	0.50±0.58	0.50±0.58
	500	0.25±0.50	0.00±0.00	0.50±0.58	0.25±0.50	0.25±0.50	0.00±0.00
	1,000	0.50±0.58	0.00±0.00	1.50±1.00	1.00±0.82	0.75±0.50	0.25±0.50
	5,000	0.50±0.58	0.00±0.00	1.00±0.00	0.50±0.58	0.25±0.50	0.25±0.50
Cyclophosphamide	6	26.75±3.30[b]	23.50±2.38[b]	28.75±3.50[b]	25.50±2.65[b]	0.75±0.50	1.25±0.96
Treatment time 6 h, recovery time 18 h, without S9							
Vehicle control[a]		1.00±0.82	0.25±0.50	2.00±1.41	1.25±0.96	0.50±0.58	0.50±0.58
PR extract	0.5	0.25±0.50	0.00±0.00	1.00±0.82	0.75±0.50	0.25±0.50	0.50±0.58
	5	0.50±0.58	0.00±0.00	1.50±0.58	1.00±0.82	0.50±0.58	0.50±0.58
	50	1.00±0.82	0.25±0.50	1.50±1.29	0.75±0.96	0.00±0.00	0.50±0.58
	500	0.25±0.50	0.00±0.00	0.75±0.50	0.50±0.58	0.25±0.50	0.25±0.50
	1,000	0.75±0.96	0.25±0.50	1.50±1.29	1.00±0.82	0.50±0.58	0.25±0.50
	5,000	0.75±0.96	0.25±0.50	1.25±0.96	0.75±0.50	0.25±0.50	0.25±0.50
Ethylmethanesulfonate	800	38.50±6.14[b]	32.00±4.97[b]	39.75±6.55[b]	33.25±5.38[b]	0.50±0.58	0.75±0.50
Treatment time 24 h, recovery time 0 h, without S9							
Vehicle control[a]		1.75±1.71	0.75±0.96	2.75±1.71	1.75±0.96	0.25±0.50	0.50±0.58
PR extract	0.5	2.25±1.26	0.75±0.96	3.25±2.22	1.75±1.71	0.00±0.00	0.50±0.58
	5	2.00±1.63	1.00±0.82	3.25±2.22	2.25±1.50	0.50±0.58	0.50±0.58
	50	2.00±1.83	0.75±0.96	3.25±0.96	2.00±0.82	0.50±0.58	0.25±0.50
	500	1.00±0.82	0.25±0.50	1.25±0.50	0.50±0.58	0.25±0.50	0.00±0.00
	1,000	1.25±1.89	0.50±1.00	3.00±2.83	2.25±2.22	0.50±1.00	0.75±0.96
	5,000	1.00±0.82	0.50±1.00	2.00±0.82	1.50±0.58	0.25±0.50	0.25±0.50
Ethylmethanesulfonate	600	56.25±11.41[b]	48.25±10.21[b]	59.25±13.57[b]	51.25±12.50[b]	0.75±0.50	1.00±0.82

Values are expressed as mean ± standard deviation of 100 metaphase cells from quadruple flasks/dose. PR, Persicariae Rhizoma; PP, polyploid; ER, endoreduplication. [a]Vehicle control, sterile distilled water. [b]$P<0.05$ vs. vehicle control by Mann Whitney U test.

Table V. Results of micronucleus test.

Treatment	Dose, mg/kg	Body weight (g)		No. of MNPCE[a]	PCE/(PCE + NCE)[b]	Slight diarrhea, n/total	Mortality, n/total
		First treatment	Sacrifice				
Vehicle control[c]		31.73±0.88	36.31±1.75	1.30±1.42	0.55±0.05	0/10	0/10
PR extract	2,000	31.88±1.03	36.35±1.63	0.90±0.88	0.55±0.05	3/10	0/10
	1,000	31.78±1.55	36.08±2.08	1.10±0.99	0.51±0.07	0/10	0/10
	500	31.60±1.17	36.30±1.08	0.40±0.52	0.51±0.05	0/10	0/10
Cyclophosphamide	70	31.92±0.86	36.08±0.97	41.70±16.89[e]	0.31±0.03[d]	0/10	0/10

Values are expressed as mean ± standard deviation of 10 mice. PR, Persicariae Rhizoma; MNPCE, micronucleated bone marrow polychromatic erythrocyte; PCE, polychromatic erythrocyte; NCE, normochromatic erythrocyte. Animals were treated for 2 consecutive days (vehicle and PR extract) or once (cyclophosphamide) and sacrificed 24 h after the last dose. [a]≥2,000 PCEs per animal were analyzed to determine the frequency of micronuclei. [b]Cytotoxicity was assessed by scoring the number of PCEs and NCEs in at least the first 500 erythrocytes for each animal. [c]Vehicle control, sterile distilled water. [d]P<0.01 vs. vehicle control by Scheffe's test. [e]P<0.01 vs. vehicle control by Mann Whitney U test.

specific genetic changes (mutations and cytogenetic abnormalities) and carcinogens; the types of mutations identified are frame shifts and base substitutions (29,30). The chromosome aberration assay is used to identify structural and numerical chromosomal aberrations in mammalian cells (32). Genotoxic substance-induced aberrations include chromatid and chromosome gaps, chromosome breaks, chromatid deletions, fragmentation, translocation and complex rearrangements (33). Thus, an increase in the frequency of structural or numerical aberrations indicates that a genotoxic substance is generating clastogenic or aneugenic effects (43). The micronucleus test is used to indicate the potential of genotoxic agents to influence chromosomal structure or destroy the mitotic spindle that controls chromosome number. An increase in the frequency of MNPCEs is regarded as a positive result of induced chromosomal damage (44). When DNA fragments or whole chromosomes are not incorporated into the main nuclei during mitosis, micronuclei are formed as small satellite structure nuclei (40).

Indigo and indirubin, which is a 3,2-bisindole isomer of indigo, are representative bioactive anti-inflammatory and antioxidative natural dyes (16,17), and herbal extracts containing indigo or their derivatives also have been shown to have potent antibacterial (19), antitumor (20), anti-inflammatory (21) and antioxidant (22) activities. However, certain synthetic colorants, if they enter into the human body in sufficient quantities, may be harmful (13). For example, mammalian intestinal microorganisms can generate degradation products such as aromatic amines from azo dyes under anerobic conditions. As these aromatic amines show potential mutagenic and carcinogenic effects, the use of azo dyes in foods has been limited (26). In addition, synthetic indigo of technical grade or 98% purity has exhibited mutagenic effects in the *Salmonella*/mammalian microsome assay (23) and also in the Ames test (25). PR is known to contain purple indirubin and blue indigo (15), and has been traditionally used as anti-inflammatory and detoxification agent in Korea (14). Until now, no preclinical studies of PR extracts containing indigo and indirubin, have been performed, to the best of our knowledge. Therefore, the genotoxicity of an aqueous extract of PR (yield=12.0%) containing indigo (0.043%) and indirubin (0.009%) was evaluated in the present study using a standard battery of various tests (27,28) as part of a safety testing process aiming to clarify its clinical safety. The PR extract was not found to be genotoxic under the conditions of the reverse mutation assay, chromosomal aberration assay or mouse micronucleus assay conducted in this study.

In the reverse mutation assay, with or without S9 mix as a metabolic activation system, no increase in revertant colony number was detected. In addition, the PR extract did not induce chromosomal aberration in a short-term exposure test with the S9 mix or in the continuous (24 h) test. Although slight signs of diarrhea were restrictedly and transiently detected in three (3/10; 30%) mice treated with 2,000 mg/kg PR extract after the first administration, there were no significant increases of the frequency of MNPCEs at doses ≤2,000 mg/kg.

Firstly, whether PR extract induces revertant colonies in four histidine auxotrophic strains of *S. typhimurium* and one tryptophan auxotrophic strain of E. coli was tested. There were no increases of the number of revertant colonies at any PR extract dose with or without the S9 mix metabolic activation system. These findings indicate a negative response to the PR extract in the bacterial reverse mutation assay. Secondly, to evaluate the mutagenicity of the PR extract, chromosome aberration testing was performed in cultured CHL cells. The number of aberrant metaphases did not increase in the PR extract-exposed groups at any dose or regardless of S9 mix, while a marked increase in the number of aberrant metaphases was detected in the positive controls. Thus, PR extract did not exhibit mutagenic potential in the chromosome aberration assay. Finally, to determine the mutagenic potential of the PR extract, a micronucleus assay was performed in male ICR mice. Although slight signs of diarrhea were restrictedly and transiently detected in three (3/10; 30%) mice following the first administration of 2,000 mg/kg PR extract, no significant increases in the incidence of MNPCEs or the ratio of PCEs to total erythrocytes, which is an indicator of cytotoxicity (40), were observed following treatment with ≥2,000 mg/kg PR extract. These results of the micronucleus test indicate that the PR extract was not mutagenic. *In vivo* micronucleus testing has been widely used to detect genotoxicity due to its simplicity and efficacy. Furthermore, if the micronucleus assay

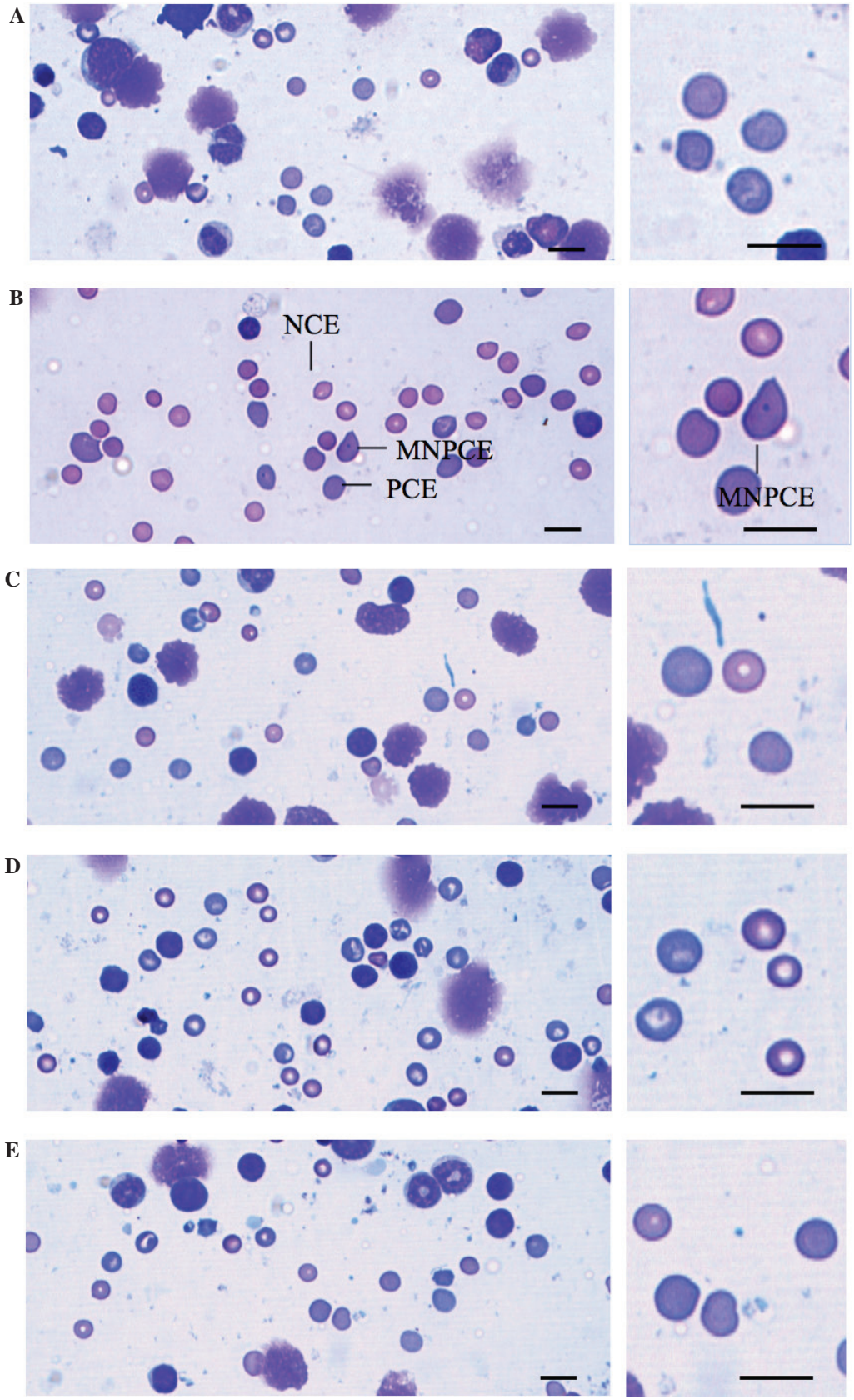

Figure 2. Representative cytology of bone marrow cell smears of mice treated with (A) vehicle, (B) CPA, (C) 2,000 mg/kg PR extract, (D) 1,000 mg/kg PRextract and (E) 500 mg/kg PR extract. In the prepared bone marrow cell smears, PCEs, NCEs and MNPCEs were counted on the basis of morphology. NCEs containing nuclei were not calculated. Significant ($P<0.01$) increases of the numbers of MNPCEs among 2,000 PCEs were detected in the CPA group as compared with the vehicle control, but no notable changes in MNPCE numbers were observed for mice treated with any of the three doses of PR extract when compared with the vehicle control. Although PCE numbers in the 70 mg/kg CPA-treated mice were significantly ($P<0.01$) decreased as compared with the vehicle control, individual PCE/(PCE + NCE) ratios were >0.25. No significant changes on the PCE/(PCE + NCE) ratio were observed for mice treated with any of the three PR extract doses as compared with the vehicle control. CPA, cyclophosphamide; PR, Persicariae Rhizoma; PCE, polychromatic erythrocyte; NCE, normochromatic erythrocyte; MNPCE, micronucleated bone marrow polychromatic erythrocytes. Scale bar, 5 μm.

is conducted in combination with other cytogenetic assays, this assay has greater relevance in the evaluation of the mutagenicity and carcinogenicity of test materials (40). All mice used in this study, including CPA-treated mice, were found to have normal body weights and weight gains throughout the experimental testing period, in comparison with age-matched normal reference mice (45), and no mortalities were recorded.

In summary, the results of the present study indicate that the aqueous PR extract should be safe to use, particularly if it is consumed in small amounts compared with the doses used in the genotoxicity tests. Furthermore, these results suggest that PR extract may be a useful bioactive agent following further toxicity evaluation.

Acknowledgements

This study was supported by the National Research Foundation of Korea (NRF) funded by the Korean government (Ministry of Science, ICT and Future Planning; grant no. 2011-0030124) and as a Basic Science Research Program by the Ministry of Education, Science and Technology (grant no. NRF-2012R1A1A2043886).

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