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The mutagenic and antimutagenic effects of the traditional phytoestrogen-rich herbs, *Pueraria mirifica* and *Pueraria lobata*

W. Cherdshewasart[1], W. Sutjit[2], K. Pulcharoen[2] and M. Chulasiri[3]

[1]Department of Biology, [2]Biotechnology Program, Faculty of Science, Chulalongkorn University, Bangkok, Thailand
[3]Department of Microbiology, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand

Abstract

Pueraria mirifica is a Thai phytoestrogen-rich herb traditionally used for the treatment of menopausal symptoms. *Pueraria lobata* is also a phytoestrogen-rich herb traditionally used in Japan, Korea and China for the treatment of hypertension and alcoholism. We evaluated the mutagenic and antimutagenic activity of the two plant extracts using the Ames test preincubation method plus or minus the rat liver mixture S9 for metabolic activation using *Salmonella typhimurium* strains TA98 and TA100 as indicator strains. The cytotoxicity of the two extracts to the two *S. typhimurium* indicators was evaluated before the mutagenic and antimutagenic tests. Both extracts at a final concentration of 2.5, 5, 10, or 20 mg/plate exhibited only mild cytotoxic effects. The plant extracts at the concentrations of 2.5, 5 and 10 mg/plate in the presence and absence of the S9 mixture were negative in the mutagenic Ames test. In contrast, both extracts were positive in the antimutagenic Ames test towards either one or both of the tested mutagens 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide and benzo(*a*)pyrene. The absence of mutagenic and the presence of anti-mutagenic activities of the two plant extracts were confirmed in *rec*-assays and further supported by a micronucleus test where both plant extracts at doses up to 300 mg/kg body weight (equivalent to 16 g/kg body weight plant tuberous powder) failed to exhibit significant micronucleus formation in rats. The tests confirmed the non-mutagenic but reasonably antimutagenic activities of the two plant extracts, supporting their current use as safe dietary supplements and cosmetics.

Key words: Phytoestrogen; *Pueraria mirifica*; *Pueraria lobata*; Mutagenic test; Antimutagenic test; Micronucleus test

Introduction

Pueraria mirifica Airy Shaw et Suvatabandu is a perennial climbing vine native to Thailand, Myanmar, and Laos with the domestic Thai name of White Kwao Krua. The large-sized tubers of this plant have long been used in traditional Thai medicine for rejuvenating purposes and are found in at least 29 provinces of Thailand (1). The plant tubers are rich in phytoestrogens, especially miroestrol (2), deoxymiroestrol (3), and isoflavonoids (1), and have been shown to be effective as an alternative treatment of menopausal symptoms (4). Consumption of the tuberous powder generates a strong estrogenic activity as revealed by the induction of vaginal cornification in ovariectomized rats (5,6) and by the MCF-7 cell proliferation assay (7). Treatment of gonadectomized rats with plant tuber powder resulted in a reduction of LH and FSH levels (8), with dose-dependent estrogenic responses in female mice (9), female monkeys (10), and menopausal monkeys (11-13). The plant tuber powder also elicits dose-dependent physiological responses in male mice with a reduction in the weight of the epididymis and the seminal vesicles and reduced sperm motility and viability (14), promoting protection against osteoporosis in orchidectomized male (15) and female rats (16). *Pueraria lobata*, or Kudzu, is a tuberous plant found in China, Korea and Japan, which contains high amounts of isoflavonoids, especially puerarin and daidzein (1).

Recently, *P. mirifica* and *P. lobata* tuber powders have became highly popular as components of orally consumed traditional medicines and as a dietary supplement for the treatment of menopausal symptoms. In addition, a wide variety of newly formulated cosmetic products contain *P. mirifica* or *P. lobata* extracts and are popularly used as beauty aids by women. Thus, it is likely that consumers,

Correspondence: W. Cherdshewasart, Department of Biology, Faculty of Science, Chulalongkorn University, Phyathai Road, Bangkok 10330, Thailand. Fax: 662-218-5386. E-mail: cwichai@sc.chula.ac.th

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especially in Asia, are increasingly exposed (frequency and dose) to phytoestrogens from the two plants. Investigation of the mutagenic and/or antimutagenic potentials of herbal plants used in traditional medicine are generating great interest with the growing evidence of their safe consumption and/or low long-term genotoxic effects (17). A few studies using the Ames test and the micronucleus assay have been conducted on phytoestrogen-rich plants or products such as the equol-rich product (18) and genistein (19), showing that the test materials were safe. Therefore, given the increasing exposure to plant phytoestrogens, in the present study we evaluated the *P. mirifica* and *P. lobata* extracts in *in vitro* and *in vivo* studies using the Ames test for mutagenic and anti-mutagenic assays and the micronucleus test for genotoxicity assays to evaluate the genetic risk or safety of the two plant materials. The results of these studies should contribute directly to the development and/or consumption of products derived from the two plant powders and plant extracts.

Material and Methods

Chemicals

All chemicals were of analytical grade. Benzo(*a*)pyrene (B*(a)*P) was from Sigma (USA); dimethylsulfoxide (DMSO) and the Folin-Ciocalteu phenol reagent were from Merck (Germany); 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide (AF-2) was from Wako Pure Chemical Industrial, Ltd. (Japan). Ampicillin was purchased from General Drug House Co., Ltd. (Thailand). Tryptic soy broth and Bacto agar were from Difco Laboratories (USA), while nutrient broth No. 2 was from Oxoid, Ltd. (Basingstoke and Hants, England). Cyclophosphamide (CP) was purchased from Asta Medica (Germany).

AF-2 and B(*a*)P were used as mutagens. AF-2 was dissolved in DMSO at a concentration of 2 µg/mL for TA98 and 0.2 µg/mL for TA100 when tested in the absence of the S9 mixture. B(*a*)P was dissolved in DMSO at a concentration of 200 µg/mL for TA98 and 100 µg/mL for TA100 when tested in the presence of the S9 mixture. For the micronucleus assay, CP was dissolved in 0.7 mL 1% (v/v) EtOH and injected *ip* in rats at the dose of 80 mg/kg body weight.

Plant material

P. mirifica cultivar Wichai-III tuber roots were collected from Chiang Mai Province, Thailand. The plant was identified by one of us (W.C.) (voucher herbarium reference No. BCU 11045) in the Department of Botany, Faculty of Science, Chulalongkorn University (20). *P. lobata* tubers were collected from Ghangzhou Province, People's Republic of China, identified by Zhang Yam (Agro-Biotechnical Research Institute, Academy of Agricultural Science, Guangdong, People's Republic of China). The plant materials were cut with a blade into 2-3-mm thick pieces and oven dried at 70°C, ground into a powder of 100 mesh size and subsequently extracted with EtOH at room temperature. The EtOH solution was dried *in vacuo* (20). The weight/weight yield in terms of dry starting material was 3.70 and 3.48% for *P. mirifica* and *P. lobat*a, respectively. The plant extract was dissolved in DMSO, adjusted to the analyzed concentration, and filtered through a 0.45-µm sterile nitrocellulose membrane filter disc. For the micronucleus assay, the plant crude ethanolic extract was prepared in 0.7 mL 1% (v/v) EtOH.

The isoflavonoid contents of the tuber powder analyzed by RP-HPLC (1) were 35.55 mg/100 g puerarin, 27.39 mg/100 g daidzin, 58.00 mg/100 g genistin, 8.38 mg/100 g daidzein, and 1.99 mg/100 g genistein for *P. mirifica*, and 32.85 mg/100 g puerarin, 21.90 mg/100 g daidzin, 25.63 mg/100 g genistin, 10.34 mg/100 g daidzein, and 0.81 mg/100 g genistein for *P. lobata*.

Bacterial strains

Salmonella typhimurium strain TA98 (*his D3052, rfa, uvrB, pKM101*) and strain TA100 (*his G46, rfa, uvrB, pKM101*) were provided by the Biochemistry and Chemical Carcinogenesis Section, Research Division, National Cancer Institute, Bangkok, Thailand. For all assays, a 20-µL inoculum of a thawed permanent culture was added to 20 mL nutrient broth No. 2. Recombination-proficient (*rec*$^+$) and recombination-deficient (*rec*$^-$) *Bacillus subtilis* strains H17 and M45, respectively, were stored frozen in the Department of Microbiology, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. For all assays, a 20-µL inoculum of a thawed permanent culture was added to 20 mL tryptic soy broth. The bacterial cultures were incubated overnight at 37°C with shaking at 200 rpm until an approximate concentration of 1.2×10^9 bacteria/mL was obtained as determined by spectrophotometry.

S9 mixture

Rat liver S9 fraction induced with sodium phenobarbital and 5,6-benzoflavone in male Sprague-Dawley rats was prepared as previously described (21).

Mutagenic and antimutagenic assays

Fifteen revertant colonies of *S. typhimurium* strain TA100 derived by induction with AF-2 were cultured in nutrient broth at 37°C in a shaking water bath at 200 rpm for 14 h. The turbidity was adjusted to be equivalent to McFarland 0.5 with phosphate buffer, pH 7.4, and 50 µL of the adjusted culture was mixed with 100 µL of the non-induced bacterial culture. The final culture was mixed with 500 µL phosphate buffer, pH 7.4, and 100 µL plant extract at a final concentration of 2.5, 5, 10, or 20 mg/plate. The plant extract was replaced with the same amount of 100 µL DMSO for a negative control. After overnight incubation, the numbers of individual colonies were counted. The dose that exhibits 50% lethality to the bacterial cells is defined as IC_{50}. The

plant extract was investigated in triplicate.

The Ames reversion assay was carried out on the sample solutions with and without the S9 mixture using the TA98 and TA100 indicator strains (22). The experiments were performed at a plant extract concentration of 0.25-10 mg/plate. The mixture, consisting of 0.1 mL sample solution, 0.5 mL of the S9 mixture, and 0.1 mL of the bacterial suspension, was pre-incubated for 20 min at 37°C with gentle shaking at 50 rpm. Molten soft agar (2.5 mL, containing 0.5 mM L-histidine and 0.5 mM D-biotin) was added, and the resulting mixture of 3.2 mL was layered onto minimal glucose agar plates. The tested plates were incubated for 2 days at 37°C and the numbers of revertant colonies formed were scored. Furylfuramide (AF-2) and B(*a*)P were used as positive controls. The antimutagenic activity of each plant extract was evaluated by comparison with the positive and negative controls. Antimutagenic assays consisted of 0.05 mL sample solution mixed with 0.05 mL reference mutagen (23). Puerarin, the most abundant isoflavonoid in *P. mirifica* and *P. lobata* (1), was also tested in parallel.

Rec-assay

The rec-assay was carried out using spores of *Bacillus subtilis* strains H17 and M45 as indicators. Spore-agar plates were prepared by mixing 10 mL tryptic soy agar with 0.1 mL spore suspension (2 x 10^7 spores/mL). A sterile cork borer 8 mm in diameter was used to pierce holes in the solidified agar with a total of 5 holes per plate. Each well received 0.02 mL DMSO and 0.02 mL sample solution at a concentration of 2.5, 5, or 10 mg/well. After overnight incubation, the diameters of the inhibition zones around each well were measured and the means for each paired treatment were compared between the H17 and M45 spore plates. AF-2 was used as a positive control (24). Each experiment was carried out in triplicate.

Micronucleus assay

Three independent *in vivo* micronucleus tests were performed in male Wistar rats, 6 rats per group weighing 160-200 g, supplied by the National Animal Production Center, Mahidol University, Nakohnpathom, Thailand. The rats were kept in a room with a 12-h photoperiod at a temperature of 25 ± 1°C with free access to food and tap water. The rats were sacrificed at 24, 48, and 72 h after the administration of *P. mirifica* or *P. lobata* extracts at a dose of 300 mg/kg body weight (equivalent to 16 g/kg body weight plant tuber powder) dissolved in 0.7 mL 1% EtOH (v/v); 80 mg/kg body weight CP (*ip*) and 0.7 mL 1% EtOH (v/v) were used as positive and negative controls, respectively (25). Air-dried slides with rat bone marrow micronuclei were stained with May-Grünwald and Giemsa. A total of 2000 polychromatic erythrocytes (PCEs) were scored per animal for the determination of micronucleated PCEs (MnPCEs). In the analysis of PCEs/non-chromatic erythrocytes (PCEs/NCEs), a total cell count of 1000 erythrocytes was scored per animal. The experimental protocol was approved by the Animal Ethics Committee in accordance with the guideline of Chulalongkorn University for the care and use of laboratory animals.

Statistical analysis

Data are reported as means ± SEM of three independent experiments. The unpaired Student *t*-test was used for analysis of the results and $P < 0.05$ was considered to be significant. The analyses were performed using the SPSS version 10.0 statistical software program.

Results

The results summarized in Table 1 reveal that the two plant extracts had a mild dose-dependent cytotoxicity (IC_{50} >1000 µg/mL), with the TA98 indicator strain being more sensitive than the TA100 strain, and a higher apparent toxicity for the *P. lobata* than *P. mirifica* extract.

The data summarized in Table 2 reveal that neither puerarin nor the *P. mirifica* and *P. lobata* extracts had detectable mutagenic activity towards *S. typhimurium* in the absence or presence of the S9 mixture. For both the TA98 and TA100 reporter isolates, the presence of the S9 mixture initiated a higher frequency of revertant colonies in the negative and

Table 1. Survival (cytotoxicity) test of the plant extracts on Ames test *Salmonella typhimurium* indicator strains TA98 and TA100.

Plant extract	Bacterial strain	Concentration (mg/plate)				IC_{50} (µg/mL)
		2.5	5.0	10	20	
Pueraria lobata	TA98	51.76 ± 7.64*	24.61 ± 0.98*	9.81 ± 0.84*	3.14 ± 0.12*	>1000
	TA100	79.75 ± 5.65*	46.36 ± 1.86*	42.13 ± 0.84*	20.38 ± 0.13*	>1000
Pueraria mirifica	TA98	74.41 ± 5.22*	41.81 ± 2.17*	36.67 ± 3.57*	35.36 ± 1.94*	>1000
	TA100	78.28 ± 2.69*	51.04 ± 1.73*	54.23 ± 3.03*	48.54 ± 3.04*	>1000

Data are reported as means ± SEM of three independent replicates. The test solution contained 2.5 mL top agar, 100 µL plant sample, 500 mL buffer and 100 mL bacterial culture (total volume of 3.2 mL). One milligram/plate plant extract is equivalent to 312.5 µg/mL. *$P < 0.05$ compared to the negative control (set as 100% survival; unpaired Student *t*-test).

positive controls as well as in the test samples including puerarin, *P. mirifica* and *P. lobata* extracts. Notice that a partial killing effect was observed at high doses (10 mg/plate) of the *P. mirifica* extract but not of the *P. lobata* extract. Nevertheless, compared to the negative control without the S9 mixture, only *P. mirifica* extracts at the concentration of

Table 2. Mutagenic and antimutagenic activity assays for *Pueraria mirifica* and *P. lobata* extracts and the isoflavonoid puerarin based on non-metabolic (without the S9 mixture) and metabolic activation (with the S9 mixture) using *Salmonella typhimurium* TA98 and TA100 strains.

Concentration or per plate	His$^+$ revertant colonies			
	TA98		TA100	
	- S9 mixture	+ S9 mixture	- S9 mixture	+ S9 mixture
Puerarin				
1.0 pM	27.10 ± 1.89	39.14 ± 3.74	82.38 ± 3.44	109.46 ± 7.76
100 pM	24.96 ± 1.01	30.23 ± 1.44	102.37 ± 9.24	96.53 ± 5.42
10 nM	27.23 ± 0.99	41.42 ± 2.35	103.41 ± 4.29	112.35 ± 3.68
1.0 µM	22.78 ± 2.25	30.33 ± 1.17	104.98 ± 6.43	109.11 ± 7.36
Puerarin plus AF-2 (0.1 µg/plate)				
1.0 pM	23.54 ± 1.61*	9.80 ± 1.55*	33.35 ± 2.43*	17.58 ± 0.63*
100 pM	16.79 ± 0.43*	1.52 ± 0.15*	39.62 ± 2.03*	6.78 ± 0.26*
10 nM	16.54 ± 1.16*	16.91 ± 1.62*	21.47 ± 1.87*	16.69 ± 1.18*
1.0 µM	7.42 ± 0.21*	28.60 ± 7.46*	63.24 ± 3.14*	49.52 ± 5.64*
P. lobota				
2.5 mg/plate	27.18 ± 2.73	46.37 ± 2.49	142.32 ± 1.46	151.46 ± 1.38
5 mg/plate	28.33 ± 1.42	51.38 ± 6.22	143.39 ± 1.87	160.17 ± 2.23
10 mg/plate	33.47 ± 1.38	51.26 ± 2.14	155.25 ± 4.34	163.58 ± 2.47
P. lobota plus AF-2 (0.1 µg/plate)				
2.5 mg/plate	6.83 ± 1.90*	27.03 ± 0.97*	7.97 ± 0.53*	18.50 ± 1.62*
5 mg/plate	16.15 ± 2.01*	28.52 ± 1.94*	16.02 ± 1.10*	26.81 ± 1.40*
10 mg/plate	29.74 ± 3.44	29.09 ± 2.26*	21.29 ± 1.12*	35.53 ± 2.01*
P. mirifica				
2.5 mg/plate	27.10 ± 0.87	47.18 ± 1.45	134.47 ± 1.35	137.29 ± 5.43
5 mg/plate	26.74 ± 2.96	48.93 ± 2.26	137.28 ± 3.26	142.37 ± 3.18
10 mg/plate	38.01 ± 1.03*	52.45 ± 1.46	PK	161.39 ± 9.32
P. mirifica plus AF-2 (0.1 µg/plate)				
2.5 mg/plate	8.21 ± 0.92*	0*	3.45 ± 0.21*	0*
5 mg/plate	18.32 ± 2.74*	0*	2.47 ± 0.80*	7.34 ± 0.82*
10 mg/plate	27.58 ± 1.91	0*	PK	42.09 ± 3.41*
DMSO				
100 µL/plate	27.31 ± 1.10	49.32 ± 3.70	136.02 ± 7.70	165.04 ± 18.00
AF-2				
0.01 µg/plate	505.66 ± 7.60*	NA	NA	NA
0.1 µg/plate	NA	NA	694.32 ± 8.80	NA
B(*a*)P				
5 µg/plate	NA	NA	NA	705.00 ± 29.11
10 µg/plate	NA	742.01 ± 22.51	NA	NA

Data are reported as means ± SEM of three independent replicates. DMSO = dimethylsulfoxide; AF-2 = 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide; B(*a*)P = benzo(*a*)pyrene; PK = partial killing effect; NA = not analyzed. If the number of revertant colonies is less than 2-fold that obtained in the negative control the sample is considered nonmutagenic. *P < 0.05 compared to the negative control (unpaired Student *t*-test).

10 mg/plate exhibited a significant increase in the number of bacterial revertant colonies.

All doses of puerarin, and *P. mirifica* and *P. lobata* extracts at the dose of 2.5 and 5 mg/plate but not at the dose of 10 mg/plate showed detectable antimutagenic effects towards the TA98 indicator isolate in the absence of the S9 mixture (Table 2) and the antimutagenic activities were stronger in the TA100 isolate than in the TA98 isolate. The presence of the S9 mixture led to a higher revertant colony frequency in both the negative and positive controls, but not in the plant extract samples, which showed a reduced revertant colony frequency in both the TA98 and TA100 indicator isolates. Only *P. mirifica* exhibited a dose-dependent antimutagenicity (Table 2) with no development of revertant colonies.

In mutagenic test with H17 (*rec*$^+$), the *P. lobata* extracts exhibited a greater 0.28- and 0.23-fold clearance at the doses of 5 and 10 mg/well, with a significant difference in the corresponding M45/H17 ratios (Table 3). In contrast, the *P. mirifica* extracts showed an essentially neutral response. In the test with M45 (*rec*$^-$), the *P. mirifica* extracts exhibited no detectable mutagenic effects ($P < 0.05$) while the *P. lobata* extracts exhibited a greater clearance zone ($P < 0.05$), some 0.65-, 0.63-, and 0.65-fold than that of the negative control at 2.5, 5, and 10 mg/well, respectively, with a significant difference in the corresponding M45/H17 ratios.

In the antimutagenic assay with H17, the *P. mirifica* extracts did not differ significantly from the negative control. In contrast, the *P. lobata* extracts induced a significant increase in the frequency of revertant colonies, some 1.23- and 1.05-fold greater than that seen in the negative control at 2.5 and 5 mg/well, respectively, with a significant difference in the corresponding M45/H17 ratios. *P. mirifica* extracts exhibited a significant increase in revertant colonies, 1.32 and 1.13 times greater than that of the negative control, with a significant difference in the corresponding M45/H17 ratios. However, the increase in revertant colony frequency in the tested groups was lower than that observed in the positive control (Table 3).

P. mirifica and *P. lobata* extracts exhibited a lower MnPCEs/1000 PCE ratio compared to the positive CP control ($P < 0.05$), which was not significantly greater than that observed with the negative control (Table 4). Thus, the data showed no detectable genotoxicity of the plant extracts, a notion supported by the greater PCEs/NCEs ratio observed compared with the positive control ($P < 0.05$; Table 4), although not similar to the negative control.

Table 3. Mutagenic and antimutagenic activity assay of *Pueraria lobata* and *P. mirifica* extracts based on non-metabolic activation in a *rec* assay using *Bacillus subtilis* var. H17 (*rec*$^+$) and M45 (*rec*$^-$) as reporter strains.

Sample	Diameter of clear zone (mm)		M45/H17
	H17	M45	
DMSO			
20 µL/well	8.00 ± 0.00	8.00 ± 0.00	1.00 ± 0.00
AF-2			
0.1 µg/well	8.00 ± 0.00	21.36 ± 1.90*	2.67 ± 0.24*
P. lobata			
2.5 mg/well	9.66 ± 0.88	13.17 ± 1.04*	1.40 ± 0.40*
5 mg/well	10.23 ± 1.22*	13.00 ± 1.95*	1.35 ± 0.57*
10 mg/well	10.80 ± 1.40*	13.17 ± 0.52*	1.28 ± 0.24*
P. lobata plus AF-2 (0.1 µL/well)			
2.5 mg/well	8.00 ± 0.00	17.80 ± 1.33*	2.23 ± 0.21*
5 mg/well	8.00 ± 0.00	16.37 ± 1.37*	2.05 ± 0.22*
10 mg/well	8.00 ± 0.00	8.00 ± 0.00	1.00 ± 0.00
P. mirifica			
2.5 mg/well	8.00 ± 0.00	8.00 ± 0.00	1.00 ± 0.00
5 mg/well	8.00 ± 0.00	8.00 ± 0.00	1.00 ± 0.00
10 mg/well	8.00 ± 0.00	8.00 ± 0.00	1.00 ± 0.00
P. mirifica plus AF-2 (0.1 µL/well)			
2.5 mg/well	8.00 ± 0.00	18.57 ± 1.19*	2.32 ± 0.02*
5 mg/well	8.00 ± 0.00	17.07 ± 1.15*	2.13 ± 0.39*
10 mg/well	8.00 ± 0.00	8.00 ± 0.00*	1.00 ± 0.00

Data are reported as means ± SEM of three independent replicates. DMSO = dimethylsulfoxide; AF-2 = 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide. *$P < 0.05$ compared to the negative control (unpaired Student *t*-test).

Discussion

The *P. mirifica* and *P. lobata* extracts exhibited a dose-dependent cytotoxic effect towards *S. typhimurium* TA98

Table 4. Micronucleus assay in rat bone marrow at 24, 48, or 72 h after oral administration of *Pueraria mirifica* or *P. lobata* extracts at the dose of 16 g/kg body weight.

Sample	Time (h)	No. of MnPCEs/1000 PCEs (mean ± SEM)	PCEs/NCEs (mean ± SEM)
Pueraria lobata	24	0.33 ± 0.21*	0.63 ± 0.03*
	48	0.78 ± 0.27*	0.65 ± 0.03*
	72	1.13 ± 0.29*	0.70 ± 0.05*
Pueraria mirifica	24	1.74 ± 0.69*	0.59 ± 0.06*
	48	1.14 ± 0.29*	0.74 ± 0.05*
	72	0.98 ± 0.36*	0.63 ± 0.04*
1% EtOH (0.7 mL)	24	1.49 ± 0.22*	0.62 ± 0.03*
	48	1.97 ± 0.35*	0.65 ± 0.04*
	72	2.31 ± 0.33*	0.70 ± 0.03*
Cyclophosphamide (0.08 g/kg body weight, *ip*)	24	15.61 ± 0.89	0.39 ± 0.03
	48	16.12 ± 0.67	0.40 ± 0.05
	72	14.43 ± 1.16	0.34 ± 0.04

Data are reported as means ± SEM of 6 independent replicates. PCEs = polychromatic erythrocytes; MnPCEs = micronucleated PCEs; NCEs = non-chromatic erythrocytes. *$P < 0.05$ compared to the positive control (unpaired Student *t*-test).

and TA100 isolates. However, the IC_{50} values observed for both indicator strains were higher than 1000 µg/mL, demonstrating a mild cytotoxic effect. In the mutagenicity assay, some partial killing effect was observed at the higher dose of 10 mg/plate (3125 µg/mL) for *P. mirifica*. Thus, the possibility that the decrease in the number of revertant colonies might be partly due to the cytotoxic effects of the *P. mirific*a samples tested cannot be excluded. Oral administration of *P. mirifica* tuber powder may elicit adverse hematological effects in long-term chronic toxicity tests in rats receiving 250 mg/kg body weight for 6 months (26). The toxicity of *P. lobata* tuber powder has not been thoroughly addressed but no cytotoxicity to MCF-7 cells has been reported (20). In agreement, puerarin, the major isoflavonoid present in *P. mirifica* and *P. lobata* (2), had no detectable cytotoxic effect on MCF-7 at 1.0 pM to 1.0 µM (7). Cytotoxicity tests in MCF-7 with deoxymiroestrol and miroestrol isolated from *P. mirifica* revealed that, compared to genistein and daidzein, the two chemicals exhibited a higher estrogenic potency by promotion of cell growth and not a cytotoxic effect on $ERα^+$ cells (3,27). Thus, the cytotoxic effect of *P. mirifica* at 10 mg/plate observed here was probably due to other minor phytoestrogens or to potential synergistic interactions between components.

The results of the Ames tests revealed that the plant extracts had no detectable mutagenic activity towards the two indicator bacterial strains (TA98 and TA100) with or without prior metabolic activation by preincubation with the S9 mixture. Thus, the number of revertant colonies in each test sample was less than two times that seen with the negative control (spontaneous mutation). However, herbal consumption may elicit effects via the metabolite form of the phytochemicals. In tests on hepatocytes containing drug metabolizing enzymes, metabolic activation increased the estrogenic activity of *P. mirifica* extracts (28). This result was confirmed by the addition of liver metabolic enzymes to MCF-7 cells during the tests (29). Although there was a difference between non-metabolic and metabolic activation of the plant extracts with the S9 mixture, suggesting that metabolic activation of phytochemicals in both plant species might increase the mutagenic potential of the plant extracts, the metabolic dose required was still well outside the likely greatly exceeded the normal doses administered. Antimutagenic activity in the Ames test revealed that the *P. mirifica* extracts exhibited a higher antimutagenic than either the *P. lobata* extracts or puerarin, the key plant isoflavonoids. However, the antimutagenic potentials of these two plant extracts were still lower than that of another Thai herb, *Mucuna collettii* submitted to the same test (30). Thus, although not mutagenic, these two herbs are not likely to be preferred to others such as *M. collettii* for preventive medication against cancer, but are likely to continue to be used for the current purposes.

The absence of detectable mutagenic activity in these two plant extracts observed in the Ames test was supported by the results of the *rec* assays, which revealed no mutagenic effects for either extract. The antimutagenicity tests by *rec* assays also supported the notion that *P. mirifica* and *P. lobata* had a closely similar antimutagenic potential. Even though this study did not involve cells harboring estrogen receptors, previous work using the $ERα^+$ cell line, MCF-7, showed an anti-proliferation effect only at high doses of both plants extracts (20). Even though *P. lobata* extracts had a lower estrogenic effect than *P. mirifica* regarding vaginal cornification (8) and uterotrophy (31) and a lower cytotoxicity than *P. mirifica* in MCF-7 (20), in the present study there was not much difference in the non-mutagenic and anti-mutagenic activities of the two plants.

An additional genotoxicity study was performed using the micronucleus assay, where an increase in the frequency of MnPCEs in the plant extract-treated animals is an indication of induced chromosome damage. The *P. mirifica* and *P. lobata* extracts caused a delayed and rapid response, respectively, with the maximum micronucleus formation being observed at 72 and 24 h after treatment, respectively (Table 4). However, the amount of micronucleus formation in both cases was not significantly greater than the negative control in contrast to the positive control, supporting a weak genotoxic effect of the two plant extracts.

Both the *P. mirifica* and *P. lobata* plant extracts used in the present study are known to be relatively rich in isoflavonoids such as puerarin, daidzin, genistein, and daidzein (1,32). Puerarin had an antiproliferative effect on colon cancer cells (33). Daidzein expressed anti-genotoxic activity while genistein enhanced DNA damage (34). Daidzein and genistein exhibited negative results in the Ames tests for mutagenicity assay (19,35). In addition, the isoflavonoid levels within the two plants were related to the antioxidant activity of the plant (36).

Taking all of these data into account, we conclude that the non-mutagenic and anti-mutagenic properties of *P. mirifica* and *P. lobata* are derived partly from their major isoflavonoid constituents. The two plant products are thus likely to be safe for human consumption because the normal human consumption doses are significantly lower than the highest dose used in these assays. Recently, the global demand for phytoestrogen products has been steadily increasing, since these products can be used instead of estrogen replacement therapy for menopausal women. The raw materials derived from the two plants have thus rapidly diversified into a wide variety of cosmetics, dietary supplements and food ingredients. However, a precautionary concern is that the extracts from plants harvested from various sources or at different ages or during different seasons and tested at different doses may all elicit different test results (37). Thus, for example, crude plant extracts prepared from *P. mirifica* tuber also collected from Chiang Mai Province, Thailand, potentially acted as mutagenic agent at the doses of 600 and 800 mg/kg body weight by inducing higher frequencies of micronucleus formation in male rats, although no sup-

porting data from Ames tests or *rec* assays were provided (38). In the present study, only the 300 mg/kg body weight plant extract was tested, which is equivalent to a single consumption of 16 g/kg body weight.

The present results strongly indicate that *P. mirifica* and *P. lobata* extracts are mostly non-mutagenic and anti-mutagenic. In addition, data from previous work with human mammary adenocarcinoma cells (MCF-7) (20) and mammary tumor induction in female rats (39) have especially supported the anti-breast cancer potential of *P. mirifica*. Most importantly, we have shown that the traditional knowledge regarding the preparation and consumption of the tuber products of these two plants is soundly based on, or at least does not contradict, currently recognized safety criteria. The present study is the first mutagenic/antimutagenic investigation conducted on these phytoestrogen-rich plants whose consumption has recently become popular. The data reported here demonstrate that the phytoestrogens or products derived from these plants may be consumed safely.

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