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Potent Antimutagenic and Their Anti-Lipid Peroxidative Effect of Kaikasaponin III and Tectorigenin from the Flower of *Pueraria thunbergiana*

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The MeOH extract of *Pueraria thunbergiana* (Leguminosae) flowers and its fractions were subjected to Ames test to test the antimutagenicity. EtOAc fraction (1 mg/plate) decreased the number of revertants of *Salmonella typhymurium* TA100 by 95% against aflatoxin B_1 (AFB_1). Phytochemical isolation of the EtOAc fraction afforded four isoflavonoids (tectorigenin, glycitein, tectoridin and glycitin) and one saponin (kaikasaponin III). Though the three isoflavonoids other than tectoridin showed significant antimutagenicity, the activity of kaikasaponin III was the most potent. Kaikasaponin III (1 mg/plate) decreased the number of revertants of *S. typhymurium* TA100 by 99% against AFB_1 but by 75% against N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). Tectorigenin (1 mg/plate) inhibited the AFB_1-induced mutagenicity by 90% and MNNG-induced one by 76%. Glycitein and glycitin were less active than tectorigenin and kaikasaponin III. This result suggested that kaikasponin III prevents the metabolic activation of AFB_1 and scavenge electrophilic intermediate capable of mutation. The two components with potent activities, tectorigenin and kaikasaonin III, significantly prevented the malondialdehyde formation caused by bromobenzene in the rat.

Key words: *Pueraria thunbergiana*, Leguminosae, Isoflavonoid, Kaikasponin III, Antimutagenic, anti-lipid peroxidative

INTRODUCTION

The roots of *Pueraria thunbergiana* have been widely used as antipyretics and analgesics to treat common cold in the prescription of Oriental medicine. Although the roots are much more widely used as an Oriental crude drug, the flower of the plant origin has been also used to treat *diabetes mellitus* and lingering intoxication (Kim, 1986). This drug belongs to anti-thirst drug in the traditional medicine of Korea. Our successive studies have suggested that these drugs contain antioxidant principles but no or minor prooxidant ones to manifest several biological effects in diseases associated with aging (Kim *et al.*, 1998; Lee *et al.*, 2000; Bae, *et al.*, 1999). Reactive oxygen species (ROS) including several of free radicals can provide oxidative damage on various cells and cause the diseases associated with oxidative stress. We are sure that the anti-thirst medicine in Oriental medicine could treat ROS-elicited diseases. There are several pharmacological reports on those actions of anti-thirst drugs (Park *et al.*, 1998; Kim *et al.*, 2001; Choi *et al.*, 2001).

The isolation of isoflavones (tectorigenin, glycitein) together with its glycosides (tectoridin, glycitin, 6″-O-xylosyltectoridin, and 6″-O-xylosylglycitin) from *P. thunbergiana* has been reported (Park *et al.*, 1999). Hypoglycemic and hypolipidemic effects in streptozotocin-induced diabetic rats were reported together with the inhibitory effect of NADPH-induced lipid peroxidation *in vitro* and the most active constituents were found as tectorigenin and kaikasaponin III (Lee *et al.*, 2000). Human intestinal bacteria hydrolyzed isoflavone glycosides isolated from *P. thunebergiana* to form isoflavone aglycones (Kim *et al.*, 1998). The active principles of antidiabetic herbal drugs are very often overlapped with antimutagenic substances indicating that the ROS shares the cause of

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diabetes mellitus and mutagenicity (Lee *et al.*, 2000; Lee *et al.*, 2000) in some aspects. Since a number of crude drugs contain many compounds with entirely different structures which can be seen in isoflavones and saponins, it is necessary to investigate as to whether the two different compounds with the same activities could be cooperated or not in anti-lipid peroxidative assays *in vivo*. The effect of mixed samples with several ratios of both active compounds was also investigated to search for the interference of the active compound to the other active one.

MATERIALS AND METHOD

Plant material and isolation

The flowers of *Pueraria thunbergiana* were collected at Mt Chiak, Kangwon Province, Korea, in September. A voucher specimen is deposited in the herbarium of Life Science and Natural Resources, Sangji University, Wonju, Korea. The plant material was extracted and fractionated as described previously (Kim *et al.*, 1998). From the ethyl acetate (EtOAc) fraction, glycitin, tectoridin and kaikasaponin III were isolated by chromatographic methods and identified by spectroscopic methods as our previous and Kinjo's study (Park *et al.*, 1999; Kinjo *et al.*, 1988). n-Butanol fraction also yielded kaikasaponin III by a similar phytochemical procedure. EtOAc fraction was hydrolyzed by 1N-H_2SO_4 (MeOH-H_2O) solution under reflux for 3 hours. After cooling, the reactant was fractionated with EtOAc and this fraction was washed with distilled water. This hydrolysate was chromatographed over silica gel with $CHCl_3$-MeOH-H_2O (7:3:1, lower phase). The major components, tectorigenin and glycitein, were purified by washing of corresponding subfraction with MeOH. These two compounds were identified by comparisons of mp, co-TLC and NMR spectral data with authentic samples, respectively and were shown to be present in the MeOH extract and $CHCl_3$- and EtOAc fraction of *P. thunbergiana* on co-TLC.

Mutagens

Aflatoxin B_1 (AFB_1) was purchased from the Sigma Chemical Co., St. Louis, MO. (USA) and dissolved in DMSO. N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) was obtained from the Aldrich Chemical Co., Milwuakee, WI (USA) and dissolved in distilled water.

Bacterial strains

The *Salmonalla typhimurium* TA100 bacterial strain, a histidine-requiring mutant, was provided by Dr. B. N. Ames, University of California, Berkeley, CA, USA, and maintained as described by Maron and Ames (Maron *et al.*, 1983). The genotypes of the test strains were checked routinely for their histidine requirement, deep rough (*rfa*) character, UV sensitivity (*uvr* B mutation) and presence of R factor.

S9 fraction and S9 mix

Male Sprague-Dawley rats were injected intraperitoneally with Aroclor 1254 dissolved in corn oil (500 mg/kg body weight) according to the method described by Maron and Ames (Maron *et al.*, 1983),. Five days after the injection, the rats were sacrificed, their livers were removed and rinced in 0.15 M KCl, and then homogenized with a Potter-Elvehjem apparatus. The homogenate was centrifuged at 9000 × g for 10 min in a refrigerated centrifuge, and the supernatant S9 fraction was distributed in 1.8-2.0 ml portions in plastic Nunc tubes, frozen quickly in a bed of crushed dry iced, and stored immediately at -80°C until use. The S9 required for the preparation of the S9 mix was thawed at room temperature and placed in a container of crushed ice. The S9 mix was prepared as soon as the S9 had thawed. The components of S9 mix were 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, 100 mM sodium phosphate, pH 7.4, and S9 at a concentration of 0.04 ml per ml of mix. The S9 mix was prepared freshly for each mutagenicity assay.

Antimutagenicity test

A modified plate incorporation procedure (Matsushima *et al.*, 1980) was employed to determine the effect of all isolates (tectorigenin, glycitein, tectoridin, glycitin, and kaikasaponin III) on AFB_1-induced mutagenicity. In brief, 0.5 ml of S9 mixture (0.5 ml of phosphate buffer for direct mutagen, *N-methyl-N′-nitro-N-nitrosoguanidine*(MNNG) was distributed in sterilized capped tubes in an ice bath, and then 0.1 ml of test bacterial suspension from an overnight culture (1-2 × 10^9 cells/ml) and 0.1 ml of test compounds (50 µl of mutagens and/or 50 µl of test compounds) were added. After vortexing gently and preincubating at 37°C for 30 min, 2 ml of the top agar supplemented with L-histidine and D-biotin kept at 45°C was added to each tube vortexed for 3 seconds. The resulting entire mixture was overlaid on the minimal agar plate. The plates were incubated at 37°C for 48 h and then the revertant bacterial colonies on each plate were counted. Toxicity tests for the different cells of the samples were also carried out, and the sample concentrations employed for the antimutagenic test did not show any toxicity on the test strain.

Animals

Male Sprague-Dawley rats (weighting 150-200 g) were

Table I. Effect of various fractions from the flower extract of *P. thunbergiana* on the mutagenicity induced by aflatoxin B_1 (0.5 μg/plate) and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG; 0.4 μg/plate) in *Salmonella typhymurium* TA100

Treatment	Revertants/plate (AFB_1)			Revertants/plate (MNNG)		
(mg/plate)	0.25	0.5	1.0	0.25	0.5	1.0
Mutagen+MeOH ext.	$859 \pm 25^{b1)}$ (12)[2)]	645 ± 27^{b} (38)	311 ± 14^{b} (78)	834 ± 18^{b} (16)	751 ± 14^{b} (26)	596 ± 18^{b} (43)
+$CHCl_3$ fr..	397 ± 7^{d} (68)	294 ± 6^{d} (80)	216 ± 12^{c} (90)	$721 \pm 1c^{c}$ (30)	613 ± 18^{d} (43)	505 ± 11^{c} (56)
+EtOAc fr..	274 ± 11^{e} (83)	227 ± 3^{e} (88)	173 ± 10^{d} (95)	639 ± 13^{d} (40)	467 ± 12^{e} (60)	365 ± 15^{d} (73)
+BuOH fr.	690 ± 11^{c} (32)	423 ± 22^{c} (65)	300 ± 15^{b} (80)	741 ± 18^{c} (27)	654 ± 16^{c} (38)	570 ± 25^{b} (48)
AFB_1 (Control)	957 ± 26^{a}	957 ± 26^{a}	957 ± 26^{a}	966 ± 19^{a}	966 ± 19^{a}	966 ± 19^{a}
Spontaneous	131 ± 9	131 ± 9	131 ± 9	139 ± 5	139 ± 5	139 ± 5

[1]Values represent mean ±S.D. based on three experiments.

[2]The values in parenthesis are percent inhibition.

[a-e]Means with the different letters on the same column are significantly different at the 0.05 level of significance as determined by Duncan's multiple range test.

Table II. Effect of the components from the *P. thunbergiana* on the mutagenicity induced by aflatoxin B_1 (0.5 mg/plate) and N-methyl-N¢-nitro-N-nitrosoguanidine (MNNG; 0.4 mg/plate) in *Salmonella typhymurium* TA100

Treatment (mg/plate)	Revertants/plate (AFB_1)			Revertants/plate (MNNG)		
	0.25	0.5	1.0	0.25	0.5	1.0
Mutagen+**1** (glycitein)	$1267 \pm 16^{b,c1)}$ (-)[2)]	287 ± 12^{c} (82)	219 ± 17^{d} (91)	694 ± 6^{c} (31)	612 ± 16^{d} (41)	463 ± 5^{d} (59)
+2 (tectorigenin)	859 ± 24^{b} (11)	288 ± 10^{c} (82)	226 ± 10^{d} (90)	441 ± 10^{e} (62)	375 ± 14^{f} (70)	321 ± 15^{e} (76)
+3 (glycitin)	837 ± 36^{c} (14)	305 ± 7^{c} (80)	283 ± 7^{c} (82)	831 ± 14^{b} (14)	751 ± 23^{b} (24)	648 ± 16^{b} (37)
+4 (tectoridin)	723 ± 20^{c} (28)	656 ± 31^{b} (35)	995 ± 21^{a} (-)	790 ± 18^{b} (19)	700 ± 30^{c} (30)	546 ± 20^{c} (49)
+5 (kaikasapon.)	741 ± 38^{d} (26)	166 ± 5^{d} (97)	154 ± 4^{e} (99)	538 ± 24^{d} (50)	431 ± 12^{e} (63)	329 ± 12^{e} (75)
AFB_1 (Control)	937 ± 7^{a}	953 ± 14^{a}	953 ± 14^{b}	950 ± 18^{a}	950 ± 18^{a}	950 ± 18^{a}
Spontaneous	144 ± 7	144 ± 7	144 ± 7	124 ± 6	124 ± 6	124 ± 6

[1]Values represent mean ±S.D. based on three experiments.

[2]The values in parenthesis are the inhibition rates (%).

[a-f]Means with the different letters are significantly different at the 0.05 level of significance as determined by Duncan's multipl e range test.

used for the study. Animals were fed with commercial standard rat diet and water *ad lib.*, and maintained at 20 ± 2°C with the illumination of a 12 hr light/dark cycle.

Administration of samples and induction of malondialdehyde (MDA)

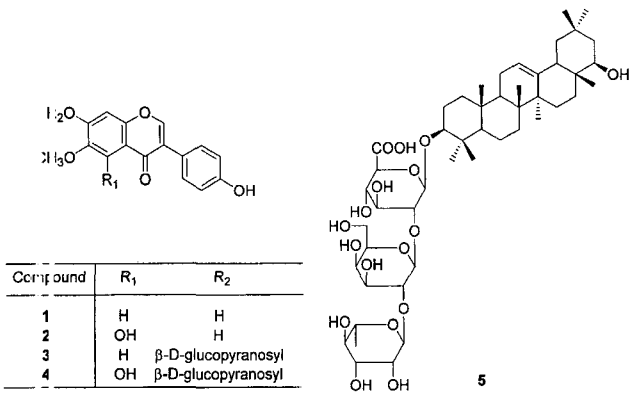
Animals were intraperitoneally injected daily with each dose of 10 mg/kg tectorigenin and 10 and 20 mg/kg kaikasaponin III and the mixture (6, 10, 9, 15 mg/kg) of tectorigenin-kaikasaponin III with the proportion (1:1, w/w) and (1:2, w/w), respectively, for one week. Then, bromobenzene (480 mg/kg) was intraperitoneally injected twice a day for two days. The animals were decapitated 24 h after the final treatment of bromobenzene.

Measurement of MDA production

The animals were sacrificed by exsanguination from the abdominal aorta under slightly anesthesia with CO_2 gas. The liver was exhaustively perfused with ice-cold normal saline through the portal vein until uniformly pale and weighed. The thiobarbituric acid (TBA) reactive substance in the liver was measured as a marker of lipid peroxidation by the method of Ohkawa *et al.* (1979). An aliquot (0.4 ml) of 10% liver homogenate in 0.9% NaCl was added to 1.5 ml of 8.1% SDS, 1.5 ml of 20% acetate buffer (pH 3.5) and 1.5 ml of 0.8% TBA solution. The mixture was heated at 95°C for 1 h. After cooling, 5.0 ml of n-butanol:pyridine (15:1) was added for extraction, and the absorbance of the n-butanol:pyridine layer at 532 nm was measured for the determination of TBA reactive substance.

RESULTS AND DISCUSSION

We isolated four isoflavonoids and one saponin and conducted Ames test using *Salmonella typhymurium* TA100. The total extract (MeOH extract) exhibited more potent antimutagenic activity against AFB_1 than against MNNG, suggesting that the extract contributed to prevent metabolic activation of AFB_1 or scavenge the electrophilic intermediate capable of inducing mutation. AFB_1, an indirect mutagen, requires metabolic activation for the mutagenicity and therefore uses S9 mix whereas MNNG, a direct mutagen, do not that requirement. Overall estimation on Table I and II showed that the active



Compound	R_1	R_2
1	H	H
2	OH	H
3	H	β-D-glucopyranosyl
4	OH	β-D-glucopyranosyl

Fig. 1 Structures of isoflavonoids and a saponin isolated from *Pueraria thunbergiana* flowers

constituents were mainly distributed to EtOAc fraction. Isolation on EtOAc fraction yielded the five compounds as shown in Fig. 1. When 1 mg/plate of kaikasaponin III and AFB_1 was added to the assay system, the number of the revertants per plate decreased to 99% compared to the control. Although glycitein, tectorigenin and glycitin also showed significant antimutagenic activity, kaikasaponin III was the most potent leading to the spontaneous group. However, the antimutagenicity of tectoridin did not show the dose-dependent fashion. Although the herb drugs with antimutagenicity shows exactly dose-dependent action in Ames test based on our previous and present research, the solates from the drug very often show dose-independent activity indicating that it could be prooxidant in certain concentration. The Ames test, which is one of precise assays for antimutagenicity, exhibited very similar activities between the effects of tectorigenin and glycitein at higher doses (0.5 and 1.0 mg/plate) when tested in AFB_1 treatment but showed the significant activity discrepancy at 0.25 mg/plate.

The cellular reaction may be involved in antiinflammation or anti-lipid peroxidative mechanisms. Miyao *et al.* (1988) reported kaikasaponin III and soyasaponin, major triterpene saponins, prevented hepatotoxicity from CCl_4-induced injury. It was also reported that *in vitro* hepatoprotective effects of saponins from immunological injury in primary cultured rat hepatocyte (Arao *et al.*, 1997). We have also reported the cytoprotective action of kaikasaponin III against H_2O_2-induced cytotoxicity (Lee *et al.*, 1999) though it has cytotoxicity at high doses (Miyao *et al.*, 1988). We assume that the signal transduction responsible for the weak cytotoxicity may influence on such antimutagenic responses. Therefore, kaikasaponin III could effectively scavenge ROS and show antimutagenicity based on the reported evidences.

We have previously reported the differentiation and apoptosis effects of tectorigenin on HL-60 cell based on signal transduction (Lee *et al.*, 2002). Additionally, the cytoprotective *in vitro* and hypoglycemic effect *in vivo* of tectorigenin was attributed to NADPH-induced lipid peroxidation (Lee *et al.*, 1999). Kim et al. (Kim *et al.*, 1999) reported the inhibitory effect of tectorigenin on nitric oxide production indicating that the activity may be associated with anti-lipid peroxidation and antiinflammation. The inhibitory effects of tectorigenin on cyclooxigenase, lipoxygenase (You *et al.*, 1999), PGE_2 formation (Shin *et al.*, 1999) has been also reported. These reported evidences might reflect the anti-inflammatory properties. Moreover, the apoptotic phenomenon caused by at a relatively high dose of tectorigenin could be triggered by the inhibition of *bcl*-2 and autophosphorylation of epidermal growth factor (EGF) receptor (Lee *et al.*, 2002).

Table III. Effect of tectorigenin, kaikasaponin III and their mixtures on the hepatic lipid peroxidation content in bromobenzene (BB)-treated rats *in vivo*.

Group	Dose (mg/kg)	MDA (nmol/g tissue)	Inhibition (%)
Non-treated control	0	18.9 ± 0.72[e]	100
BB	480	49.3 ± 1.98[a]	0
Tectorigenin	10	31.7 ± 2.00[d]	58
Tect-Kaik (5:5)[a]	10	34.6 ± 1.49[d]	48
Tect-Kaik (3:3)	6	39.6 ± 2.11[c]	32
Tect-Kaik (5:10)	15	32.7 ± 1.68[d]	55
Tect-Kaik (3:6)	9	38.7 ± 2.34[c]	35
Kaikasaponin III	10	43.7 ± 2.28[d]	18
Kaikasaponin III	20	38.6 ± 1.90[c]	35
Ascorbic acid	100	29.1 ± 2.04[e]	66

[a]The ratio in mixed sample was shown as weight.
Rats were intraperitoneally administered in various concentrations of samples daily for a weak and then bromobenzene (BB, 480 mg/kg) was intraperinoneally injected twice at 12 hr intervals for two days. Rats were decapidated 24 hr after the injection of BB. Values are mean ±S.D. for five animals. Values followed by the same letter are not significantly different from the control ($p < 0.05$).

Treatment of bromobenzene, one of carcinogens, with 480 mg/kg dose significantly increased MDA indicating the induction of lipid peroxide. The pretreatment of all the test samples significantly inhibited the lipid peroxide compared with the control ($p < 0.05$). Tectorigenin and kaikasaponin III also significantly inhibited the lipid peroxidation (Table III). On the other hand, the mixture composed of tectorigenin and kaikasaponin III showed also effectively inhibited the lipid peroxidation from the control.

Since bromobenzene could be metabolized to the epoxide intermediate, bromobenzene 3,4-oxide, with a similar fashion of AFB_1 metabolism in liver, we used bromobenzene as a lipid-peroxidative agent. Tectorigenin and kaikasaponin III significantly inhibited the lipid peroxidation in bromobenzene-induced rats though the activity of the former compound was more potent than that of the latter. The potency of the two compounds was

not consistent between the two assay tests, but provided both positive results in anti-lipid peroxidative effect *in vivo*. The results of mixed sample treatments with different proportions indicated that one anti-lipid peroxidative component in plant do not interfere with the other one in anti-lipid peroxidative test. Based on the antimutagenicity and known bioactivity mechanism, it was suggested that the *P. thunbergiana* belonging to so-called anti-thirst drugs used in traditional medicine in Korea should be more investigated for understanding of the intervention in ROS-mediated disease.

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