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Hypoglycemic and Hypolipidemic Effects of Tectorigenin and Kaikasaponin III in the Streptozotocin-Induced Diabetic Rat and their Antioxidant Activity *In vitro*

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Tectorigenin and kaikasaponin III from the flowers of *Pueraria thunbergiana* showed potent hypoglycemic and hypolipidemic effects in the streptozotocin-induced diabetic rats. Intraperitoneal administration of these two compounds with 5 and 10 mg/kg, respectively, for seven days to streptozotocin-induced rats significantly reduced the blood glucose, total cholesterol, LDL- and VLDL-cholesterol and triglyceride levels when compared with those of control group. Glycitein in which 5-OH is unlinked and tectoridin (7-O-glycoside of tectorigenin) isolated from the flowers of *P. thunbergiana* did not improve hyperglycemia and hyperlipidemia. In addition, tectorigenin showed *in vitro* antioxidant effects on 1,1-diphenyl-2-pirylhydrazyl (DPPH) radical, xanthine-xanthine oxidase superoxide anion radical, and lipid peroxidation in rat microsomes induced by enzymatic and non-enzymatic methods. We further found that tectorigenin and kaikasaponin III protected the Vero cell line (normal monkey kidney) from injury by hydrogen peroxide. From these findings, it seems likely that the antioxidant action of tectorigenin and kaikasaponin III may alleviate the streptozotocin-induced toxicity and contribute to hypoglycemic and hypolipidemic effects.

Key words: *Pueraria thunbergiana*, Leguminosae, Tectorigenin, Hypoglycemic, Hypolipidemic, Antioxidant, Lipid peroxidation, Streptozotocin

INTRODUCTION

In the oriental herb medicine, the root of *Pueraria thunbergiana* (Leguminosae) has been very widely used as antypyretics and analgesics to treat common cold (Kim et al., 1986). Meanwhile, the flowers of *P. thunbergiana* have been used to treat diabetes mellitus and lingering intoxication. A number of constituents including kakkalide (irisolidone-7-O-β-D-xylopyranoside) together with irisolidone, genistein, daidzein have been isolated from the flowers of *P. thunbergiana* (Kurihara *et al.*, 1973, 1975, 1976). Kubo *et al.* (1975) reported the isolation of 6, 4'-dihydroxy-7-methoxyisoflavone as a new compound from the same plant. Recently, we have reported glycitein, tectoridin, glycitin, 6''-O-xylosyltectoridin and 6''-O-xylosylglycitin (Park *et al.*, 1999). As saponin constituents, Kinjo *et al.* (1988) have reported the isolation of 3-O-[α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranosyl(1→2)-β-Dglucopyranosyl] sophoradiol and kaikasaponin III from the flowers of *Pueraia lobata*. Miyao *et al.* (1988) have reported that kaikasaponin III exhibit antihepatotoxic activity more effective than soyasaponin I and glycyrrhizin though a former compound possesses cytotoxic activity at a high dose.

However, there have never been studies on the elucidation of active principles of *P. thunbergiana* flowers in a diabetic animal model. In this present study, we report the hypoglycemic and hypolipidemic principles of tectorigenin and kaikasaponin III in the streptozotocin (STZ)-induced diabetic rat.

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MATERIALS AND METHODS

Plant material and isolation

The flower of *Pueraria thunbergiana* was collected in September, on Chiak mountain, Kangwon province, Korea. A voucher specimen is deposited in the herbarium of Life Science and Natural Resources, Sangji University, Wonju, Korea. According to a previous report (Kinjo *et al.*, 1988),

the plant material was extracted and fractionated. From n-BuOH 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*-BuOH fraction was hydrolyzed by 1N-H_2SO_4 (MeOH-H_2O) solution under reflux for 3 h. 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 specimens, respectively and were shown to be present in the MeOH extract of *P. thunbergiana* on co-TLC.

Animals and administration of isolated compounds

The animals (Sprague-Dawley male rat) were supplied by Daihan experimental animal center and adapted for a week at constant condition (temp. $22\pm1°C$, humidity: $55\pm2\%$, 12 h light/dark cycle). The rats with body weights of 190-210 g were used for this biological experiment. Hyperglycemic rats were induced by injecting STZ (50 mg/kg) dissolved in 0.01 M citrate buffer at tail vein. After one week, the rats showing the blood concentrations more than 300 mg/ml were considered as hyperglycemic rats. The samples were administered intraperitoneally to hyperglycemic rats with 5 mg/kg and 10 mg/kg, respectively for a week. The rats that final samples were administered were anesthetized with CO_2 gas and the bloods were collected from abdominal aorta. After the blood glucose levels were measured, the serums were isolated for determination of serum lipid concentration. The change of body weight was checked every 24 h from the initiating day.

Determination of blood glucose

The blood glucose levels in both normal and diabetic animals were determined using kit reagent (Exactech blood glucose strip) according to glucose oxidase method.

Determination of total serum cholesterol, triglyceride, HDL-, LDL- and VLDL- cholesterol

Kit reagent (AM 202-K, Asan) prepared by the enzyme method designed by Richmond *et al.* (1976) was used for the determination of total cholesterol. The kit reagent (AM 157S-K, Asan) prepared according to the method of McGown *et al.* (10) was used for determination of serum triglyceride. Kit reagent (AM 203-K, Asan) prepared according to enzyme method designed by Noma *et al.* (11,12) were used for the determination of HDL-cholesterol concentration. The concentrations of LDL-cholesterol and VLDL-cholesterol were determined as followings: LDL-cholesterol =[total-(HDL-cholesterol+triglyceride/5)]; VLDL-cholesterol =[total cholesterol-(HDL-cholesterol+LDL-cholesterol)]

DPPH radical scavenging effect

The scavenging effect corresponded to the intensity of quenching DPPH radical was determined as described by Xiong *et al.* (1996). The effects were expressed by the percent scavenging (%) of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical *vs.* that of control. The IC_{50} values were calculated from regression lines where the abscissa represented the concentration of tested compound and the ordinate the average percent reduction of DPPH radical from three separate tests. α-Tocopherol was used as a positive control.

Effects on superoxide anion radical generation

The production of superoxide anions in the xanthine/XOD system was determined as described by Xiong *et al.* (1996). The effects were expressed by the percent inhibition (%) of generation of superoxide anion radical *vs.* that of control. The IC_{50} values were calculated from regression lines where the abscissa represented the log concentration of tested compound and the ordinate *vs.* the mean percent inhibition of NBT reduction of three independent tests. Caffeic acid was used as a positive control.

Preparation of rat liver microsomal suspension

After animals had fasted for 24 h, the livers were perfused with an ice-cooled 0.9% NaCl solution *in situ*, then collected, homogenized and ultracentrifuged at 10,500 g for 60 min. The pellet obtained was resuspended in the same volume of 1.15% KCl solution. Protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Inhibitory effects on lipid peroxidation in rat liver microsomes

Lipid peroxidation in rat liver microsomes non-enzymatically induced by ascorbic acid/Fe^{2+} and enzymatically induced by ADP/NADPH/Fe^{+++} were measured by the method of Kiso *et al.* (1988). The percent inhibition of tested substances on the formation of lipid peroxides was calculated based on the amount of MDA (malondialdehyde) production of control after substracting that of normal. IC_{50} values were calculated from regression lines where the abscissa represented the log concentration of tested compounds and the ordinate *vs.* % inhibition of MDA reduction.

Cell viability assay

2×10^4 cells/well were inoculated in the medium in microtiter plates (96 wells per plate) for 24 h and then washed

with PBS in order to remove the medium containing the serum and to avoid direct reaction of H_2O_2 with compounds of the medium or the serum. Several concentrations of samples and H_2O_2 dissolved in PBS were incubated with the cells after the medium was removed for 45 min. After being rinsed twice with PBS to remove H_2O_2 and samples, the cells were incubated again with fresh medium containing serum for 24 h. After adding 50 μl of MTT solution (5 mg/ml in PBS), incubated at 37°C for 4 h, then the optical density was measured at 540 nm. For blank and control, PBS was used instead of sample solution. The effects were expressed by the percent preventing (%) of H_2O_2 damage vs. that of control. Catalase was used as positive control.

Statistics

All the data from the *in vivo* experiments were expressed as mean ± standard error and statistical significance was determined using Duncans new multiple range test. All the results obtained from antioxidant test *in vitro* were expressed the mean of three independent experiments.

RESULTS AND DISCUSSION

The isolated compounds from the flowers of *Pueraria thunbergiana* were identified to be glycitein (**1**), tectorigenin (**2**), glycitin (**3**), tectoridin (**4**) and kaikasaponin III (5) by comparison of spectral data with those of literatures and their chemical structures were shown in Fig. 1. The mean blood glucose levels of rats at 24 h after intraperitoneal administration of these compounds were shown in Table I. These levels were compared with those in normal and control rats administrated saline alone and STZ (50 mg/kg body), a known hyperglycemic agent, respectively. The treatments of tectorigenin significantly reduced elevated serum glucose by 53% in 5 mg/kg dose and 68% in 10 mg/kg, whereas tectoridin, glycitein and glycitin did not show any significant effect. Hypoglycemic effects of kaikasaponin III were found to be 39% in 10 mg/kg dose and 43% in 10 mg/kg dose. The body weight of rats after administration of **1**-**5** was shown in Table I. After 24 h, streptozotocin-treated animals showed considerable decrease of the body weight from 113.8 g of normal group to 54.8 g of only STZ-administered group. Tectoridin, glycitin and glycitein did not inhibit the decrease of body weight. In contrast, the treatment of **2** considerably prohibited the reduction of body weight, which inhibitory rates were shown by 61% in 5 mg/kg dose and 70% in 10 mg/kg, respectively. Kaikasaponin III administration also significantly inhibited body weight loss by 36% in 5 mg/kg dose and 48% in 10 mg/kg dose, respectively.

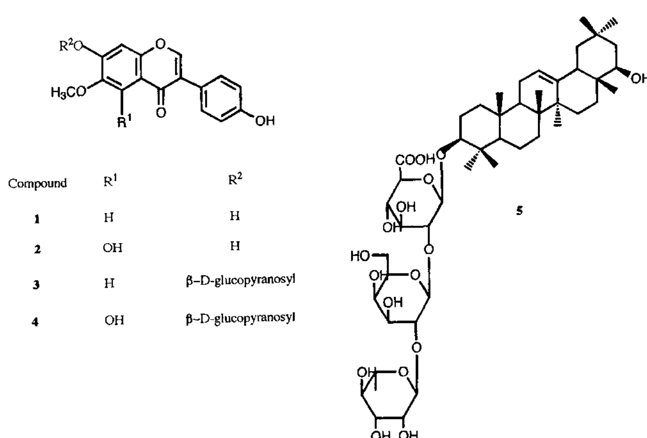

Fig. 1. Structures of isoflavonoids and a saponin isolated from Pueraria thunbergiana flowers.

Total serum cholesterol levels of the tectorigenin group were decreased by 61% in 5 mg/kg dose and 75% in 10 mg/kg dose, compared with those of control group which was elevated to 146.4 mg/dl from 71.2 mg/dl of the normal group by STZ administration (Table II). Kaikasaponin III treatment also significantly decreased the STZ-induced hypercholesterolemia, which inhibitory rates were shown by 20% in 5 mg/kg and 34.6% in 10 mg/kg dose. However, other isoflavonoids did not inhibit hypercholesterolemia. By STZ administration, lipoprotein cholesterols such as HDL-, LDL- and VLDL-cholesterol were considerably changed, respectively, when compared with those of normal rats. Potent inhibitory activities of tectorigenin were shown on lipoprotein value changes in Table II. However, glycitein, glycitin and tectoridin did not exhibit these effects.

From the above results, antidiabetic principles of the

Table I. Effects of the constituents of *P. thunbergiana* flowers on blood glucose concentration and body weight change in streptozotocin-induced hyperglycemic rats

Compound	Dose (mg/kg)	Glucose concentration (mg/dl)	Body weight (g)
Normal		110.8 ± 12.9[a]	113.8 ± 10.4*[a]
STZ		352.7 ± 23.7[b]	54.8 ± 4.8[b]
Glycitein	5	325.3 ± 14.1[c,d]	47.5 ± 6.8[c]
	10	335.1 ± 36.6[b,c]	47.2 ± 5.2[c]
Tectorigenin III	5	223.5 ± 21.0[f]	90.8 ± 3.4[e]
	10	188.6 ± 17.6[g]	96.2 ± 5.3[e]
Glycitin	5	306.5 ± 16.3[c,d]	51.7 ± 3.9[b,c]
	10	297.1 ± 22.6[d]	54.8 ± 8.0[b]
Tectoridin	5	310.9 ± 20.6[c,d]	52.0 ± 4.5[b,c]
	10	300.5 ± 21.8[d]	56.6 ± 7.2[b]
Kaikasaponin III	5	257.7 ± 19.1[e]	76.1 ± 4.0[d]
	10	248.7 ± 27.9[e,f]	83.0 ± 7.5[d]
Glibenclamide	5	140.2 ± 13.6[a]	108.2 ± 9.7[a]

*Values are expressed mean ± S.D. for groups of six experiments. Values sharing the same superscript letter are not significantly different each other ($p<0.05$) By Duncans multiple range test.

Table II. Effect of the constituents of *Pueraria thunbergiana* flowers on the level of the serum HDL-, LDL-, VLDL-, total cholesterol and triglyceride concentration in streptozotocin-induced hyperglycemic rats.

Compound	Dose (mg/kg)	HDL-cholesterol (mg/dl)	LDL-cholesterol (mg/dl)	VLDL-cholesterol (mg/dl)	Total cholesterol (mg/dl)	Triglyceride (mg/dl)
Normal		38.0 ± 1.6*[a]	25.9 ± 5.2[a]	16.3 ± 5.2[a]	71.2 ± 1.0[a]	62.6 ± 2.8[a]
STZ		18.1 ± 0.9[b]	85.6 ± 1.8[b]	33.9 ± 1.2[b]	146.4 ± 4.2[b]	139.0 ± 6.2[b]
Glycitein	5	19.2 ± 0.6[b]	87.3 ± 1.2[b]	33.1 ± 1.3[b]	146.7 ± 3.1[b]	141.7 ± 5.6[b]
	10	19.1 ± 0.7[b]	87.3 ± 1.2[b]	33.7 ± 1.4[b]	139.8 ± 3.2[b,c]	136.1 ± 3.3[b,c]
Tectorigenin	5	30.3 ± 0.5[d]	57.7 ± 1.3[d]	24.6 ± 1.1[d]	100.9 ± 2.0[e]	94.5 ± 1.7[e]
	10	33.6 ± 1.4[e]	54.1 ± 2.3[d]	24.5 ± 0.9[d]	89.7 ± 0.8[f]	76.2 ± 2.2[f]
Glycitin	5	19.3 ± 0.5[b]	85.2 ± 1.4[b]	32.2 ± 1.2[b]	137.1 ± 2.0[c]	135.4 ± 1.8[c]
	10	19.8 ± 0.5[b]	86.4 ± 0.8[b]	33.6 ± 1.4[b]	138.4 ± 3.9[b,c]	129.7 ± 1.6[b,c]
Tectoridin	5	17.9 ± 0.4[b]	86.6 ± 1.0[b]	31.6 ± 1.0[b]	136.4 ± 3.3[c]	134.4 ± 1.9[c]
	10	18.0 ± 0.6[b]	86.9 ± 2.0[b]	33.2 ± 1.5[b]	138.7 ± 4.6[b,c]	128.7 ± 1.8[b,c]
Kaikasaponin III	5	22.3 ± 0.6[c]	72.3 ± 2.0[c]	27.9 ± 0.9[c]	131.5 ± 4.0[c]	116.1 ± 4.4[c]
	10	28.4 ± 0.4[d]	68.9 ± 1.4[d]	27.3 ± 0.9[c]	120.4 ± 3.2[d,f]	103.4 ± 4.0[d,f]
Glibenclamide	5	35.3 ± 3.2[a,d]	36.8 ± 2.3[d]	20.7 ± 2.6[a,d]	79.3 ± 5.1[a,d]	74.9 ± 6.3[a]

*Values are expressed mean ± S.D. for groups of six experiments. Values sharing the same superscript letter are not significantly different each other ($p<0.05$) by Duncans multiple range test.

Table III. IC_{50} values in antioxidant assay of the constituents of *Pueraria thunbergiana* flowers.

(Unit: μg/ml)

Compound	DPPH[1)]	XOD[2)]	superoxide anion[3)]	lipid peroxidation	
				Fe^{2+}/ascorbate[4)]	Fe^{3+}/ADP/NADPH[5)]
Glycitein	71.0	>500	44.7	22.9	142.0
Tectorigenin	94.4	328.4	53.7	23.5	35.0
Genistein	>1,000	>500	292.2	30.9	44.7
Glycitin	61.0	>500	>500	66.8	>500
Tectoridin	357.5	180.8	>500	69.2	174.6
Kaikasaponin III	>1,000	>500	130	>500	>500
α-Tocopherol	3.6	-	-	-	-
Allopurinol	-	0.12	-	-	-
Caffeic acid	-	-	0.75	0.66	0.84

Each value is the mean of three independent experiments
[1)]Superscript means DPPH free radical scavenging assay
[2)]Superscript means xanthine oxidase inhibitory assay
[3)]Superscript means superoxide scavenging assay
[4)]Superscript means non-enzymatic (ascorbic acid)-lipid peroxidation inhibitory assay
[5)]Superscript means enzymatic (NADPH)-lipid peroxidation inhibitory assay

flowers of *P. thunbergiana* were revealed to be tectorigenin and kaikasaponin III in diabetic model rats. On consideration of bioactivity of kaikasaponin III derivatives in some literatures, the antidiabetic action of kaikasaponin III could be attributed to antioxidant action against various radicals occurred by STZ. It is well known that STZ generate free radicals and destroy β-cells residing in Langerhans islet. Pentacyclic triterpenoid saponins, such as soyasaponin I, soyasaponin βg, soyasaponin Ab and glycyrrhizin, prevent hydrogen peroxide damage against the mouse fibroblast cell, regardless of the presence of aromatic ring in their molecules. The effects by these saponins are considered as catalase mimic (Yoshikoshi *et al.*, 1996). Preventive effects of soyasaponin I and kudzusaponin SA isolated from the root of *P. lobata* have been studied by observing AST and ALT change in immunological injury of rat primary hepatocyte cultures (Arao *et al.*, 1997). Antihepatotoxic activity of kaikasaponin III and soyasaponin I have been studied by observing inhibitory effect on transaminase elevation by CCl_4 (Miyao *et al.*, 1988). By observing cell viabilities, we found that kaikasaponinm III protected lipid peroxidation in Vero and HepG2 cells from H_2O_2 damage (Table IV). Thus, these cytoprotective actions of kaikasaponin III against free radical generating agent were suggested to contribute to antidiabetic action *in vivo*.

Sato *et al.* (1992) have reported antioxidative mechnisms of pueraria glycosides and mangiferin. In this report, the

flavonoids possessing catechol moiety, such as pueraria glycoside-1 (PG-1) and mangiferin, showed antioxidant activity in DPPH assay system, which is being used to detect direct antioxidants from natural or synthetic substances. NADPH-dependent lipid peroxidation assay explains the secondary antioxidant effect owing to the inhibition of cytochrome P_{450} reductase. The former assay is used to find the inhibition of initiation phase of lipid peroxidation and the latter assay to find the inhibition of the terminating phase. Tectorigenin and glycitein in addition to their glycosides, tectoridin and glycitin, were shown to have antioxidatve potency in DPPH assay (Table III), while genistein did not show the activity up to 1 mg/ml. This result explains that 6-OMe in this 4',5,7-trihydroxyisoflavone system manifests the activity in DPPH assay, regardless of sugar linkage. This finding is in agreement with inactive result of PG-3 and daidzein both lacking 6-OMe (Sato *et al.*, 1992). It was suggested that meta-positioning of the two hydroxy in A-ring is the reason of inactive result of genistein in DPPH assay. Thus, the effects of active isoflavonoids in DPPH assay was attributed to the substitution of 6-OMe in 4',7-dihydroxyisoflavone skeleton. Isoflavonoids used showed variable positive results in non-enzymatic (ascorbic acid)-induced lipid peroxidation assay. In contrast, tectorigenin inhibited lipid peroxidation in microsomes initiated by the enzymatic (NADPH) system. This indicated that they could occur very different results between enzymatic and non-enzymatic lipid peroxidation. Thus, tectorigenin exhibited antioxidant ability to terminate free radical chain reaction during the lipid peroxidation in the enzymatic system of microsomes as well as in non-enzymatic system. In consequence, this isoflavone contributed to the inhibition of lipid peroxidation, possibly due to the suppression of cytochrome P_{450} reductase action. Other isoflavonoids, which exhibited inactive results *in vivo*, showed high IC_{50} values in NADPH-dependent lipid peroxidation assay. Tectorigenin and glycitein effectively scavenged superoxide anion radicals formed by xanthine oxidase, while other glycosides did not. The difference of some substituents in this isoflavone skeleton occurred the unexpected difference of activities. Interestingly, the activity of tectorigenin in NADPH-dependent-lipid peroxidation assay agrees with those of the present antidiabetic activity tests *in vivo*. The inhibitory effect on lipid peroxidation initiated by NADPH, for one of antioxidant activity tests, more reflected *in vivo* data than those of other antioxidant assays in the present study.

Thus, tectorigenin may inhibit free radical chain reaction due to its antioxidant ability in the mechanistic action different from those of tested isoflavonoids. For one of the elucidation of this fact, the cytoprotective activity tests against monkey kidney Vero and human hepatoblastoma HepG2 cells damaged by H_2O_2 were carried out. At 100 μg/ml concentration of tectorigenin, this compound showed nearly perfect cell viabilities by 100% in HepG2 cells and 95.2% in Vero cells (Table IV). The cytoprotective action of kaikasaponin III was also found, as shown in Table IV. Glycitein, tectoridin and tectorigenin showed significantly lower cell viabilities than tectorigenin and kaikasaponin III. Sakurai *et al.* (1989) have reported that addition of superoxide dismutase together with catalase almost completely prevent the inhibition of insulin release by removing reactive oxygens, $\cdot O_2^-$ and H_2O_2. Thus, the protection of β-cells in Langerhans islet from reactive oxygen radicals should contribute to insulin secretion. Miura *et al.* (1982) have reported that singlet oxygen ($^1O_2$) produced from $\cdot O_2^-$ generated by xanthine oxidase system cause lipid peroxidation of erythrocyte. Scavenging effect of tectorigenin on reactive oxygen radicals may possibly protect β-cells in Langerhans islet from injury due to several kinds of radical species, so that these biological mechanisms may contribute to improving diabetic symptoms as already described above. However, it is hard to exclude that **2** and **5** can be also involved in other mechanistic actions than described in this report, because these two compounds have comparatively high IC_{50} in lipid peroxidation assay compared with a positive control.

Conclusively, it was suggested that the two compounds, tectorigenin and kaikasaponin III, show antidiabetic activity through each antioxidant action via catalase mimic action, respectively. The catalase mimic action of tectorigenin was found to be the inhibitory action on NADPH-induced lipid peroxidation, whereas that of kaikasaponin III may have other inhibitory actions on lipid peroxidation,

Table IV. Cell viability incubated compounds isolated from *P. thubergiana* with H_2O_2 in HepG2 and Vero cells

Sample	Concentration (μg/ml)	Cell viability (%)	
		HepG2	Vero
Genistein	100	82.7	79.8
	50	76.4	84.5
	25	63.0	73.8
Glycitein	100	63.8	63.1
	50	63.0	58.3
	25	63.8	69.0
Tectorigein	100	100.0	95.2
	50	88.2	82.1
	25	74.8	79.8
Kaikasaponin III	100	81.6	85.2
	50	76.1	79.0
	25	73.5	72.4
Catalase	12.5	100.0	92.9
	2.5	96.8	86.9
	0.5	82.7	83.3

Each value is the mean of three independent experiments
Cells were treated with 5.0 mg/ml

maybe like the action on lipoxigenase. In addition, it was suggested that tectorigenin has unique antidiabetic action *in vivo* and antioxidant action *in vitro*, which was largely different from the actions of isoflavones tested. Since both compounds, an isoflavone (**2**) and a triterpenoid saponin (**5**), co-exist in the flowers of *P. thunbergiana*, studies on the synergistic action of lipid peroxidation inhibition by simultaneous administration are under investigation, *in vivo* and *in vitro*.

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