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The Protective Effect of a *Puerariae flos* Extract (Thomsonide) against Ethanol-Induced Gastric Lesions in Rats

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Abstract

Objectives: *Puerariae flos* has popularly been used to treat alcoholic disorders. However, the effect of *Puerariae flos* on alcoholic disorders in the gastrointestinal system has not been identified. We investigated the protective effect of an extract of *Puerariae flos* against the murine gastric mucosa. **Methods:** Thomsonide, the extracts containing large amounts of isoflavonoid and triterpenoid saponin, was obtained from *Puerriae flos* via Diaion HP-20 column chromatography using water and 99.5% ethanol. It was investigated whether thomsonide, as well as geranylgeranylacetone (teprenone), a popular anti-ulcer agent developed in Japan, had a cytoprotective effect that might be related to endogenous prostaglandins, which played an important role in preventing gastric mucosal lesions. **Results:** Thomsonide and teprenone inhibited ethanol-induced gastric lesions. Furthermore, thomsonide increased the production of PGE_2 and 6-keto$PGF_1\alpha$, a stable metabolite of PGI_2, in the gastric mucosa, and protective effects of thomsonide, as well as teprenone, against ethanol-induced gastric lesions were attenuated by pretreatment with indomethacin. **Conclusions:** These findings suggest that thomsonide, as well as teprenone, has the gastro protective effect which may be related to the cytoprotective activity of endogenous prostaglandins. The results of this study also suggest that the gastro protective effect of thomsonide may partially mitigate alcoholic disorders in the gastrointestinal tract, and support our pharmacological belief that *Puerariae flos* is useful for treatment of alcoholic disorders.

Keywords

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1. Introduction

Puerariae flos, the dried flower of *Pueraria thomsonii* Benth. (Leguminosae), is known to counteract the effects of orally consumed ethanol [1]. Kakkakaiseito is one of the well-known Chinese medicines prepared from *Puerariae flos* and several other herbs. Kakkakaiseito has traditionally been used to treat alcoholic disorders. Chemical studies have shown that *Puerariae flos* contains various isoflavonoids and triterpenoid saponins [2] [3]. Yamazaki *et al*. have reported that an extract of *Puerariae flos* promotes elimination of acetaldehyde from human blood [4], and improves learning and memory impairments, which are induced by ethanol or scopolamine [5]. However, despite the fact that ethanol is widely recognized to induce gastric mucosal lesions in mammals, only few extensive pharmacological studies have been conducted to evaluate the effect of *Puerariae flos* on alcoholic disorders in the gastrointestinal system. A large amount of alcohol consumption induces gastric mucosal lesions, while *Puerariae flos* has been used in traditional Chinese herbal medicine to treat alcoholic disorders, under the circumstances we investigated the possibility of *Puerariae flos* affording gastro protection against ethanol-induced gastric mucosal lesions. The present study was designed to investigate whether the extract of *Puerariae flos*, as well as geranylgeranylacetone, a popular anti-ulcer agent developed in Japan [6], had a cytoprotective effect that might be related to endogenous prostaglandins (PGs), which played an important role in preventing gastric mucosal lesions. In addition, since indomethacin, a cyclo-oxygenase (COX) inhibitor, has been used to determine the role of endogenous PGs, we evaluate the gastro protective effect of the *Puerariae flos* extract against ethanol-induced gastric mucosal lesions in indomethacin-pretreated rats.

2. Materials and Methods

2.1. Plant Materials

2.1.1. Extract Preparations

Puerariae flos, the air-dried flowers of *Pueraria thomsonii*, was purchased from Mikuni Co. (Osaka, Japan). A dried voucher specimen (PFT0013) was deposited in our laboratory for future reference. Preparations for extracts were made in the factory in Mikuni Co.; dry flowers of the plant (1.5 kg) were mixed with 30 L of boiling water, extracted for 2 h at 95°C, and filtered. The filtrate was concentrated and spray drying was carried out to obtain the aqueous extract (400 g). The aqueous extract was put into 2 L of boiling water, and was centrifuged. The supernatant obtained was subjected to Diaion HP-20 column chromatography using water and 99.5% ethanol to give ethanolic elution (6 L) containing large amounts of isoflavonoid and triterpenoid saponin. The ethanolic elution was then filtered, and the filtrate was further concentrated under reduced pressure to obtain 60 g of dried brownish powder. It had a characteristic odor and bitter taste, and was named as thomsonide (Lot 9,954,111). Thomsonide was used for the following experiments.

2.1.2. Analysis of Isoflavonoids and Triterpenoid Saponins in Thomsonide

Simultaneous analyses of isoflavonoids and triterpenoid saponins in thomsonide were performed by using HPLC coupled with an evaporative light scattering detector (ELSD) according to the method of Niiho *et al*. [7], as follows: 20 mg of thomsonide was dissolved in 6 mL volume of 80% ethanol, and then 10 μL of the ethanolic elution made by a membrane filter (Millipore Co., USA) was applied to HPLC-ELSD instrument system for analyses of isoflavonoids and triterpenoid saponins. HPLC-ELSD instrument system was equipped with a column heater (U-620, Sugai), a pump unit (CCPM-II, Tosoh), and ELSD (Model 300s, SofTA, USA). Analytical conditions for detecting isoflavonoids and triterpenoid saponin in thomsonide were set as follows: A C_{18} reversed-phase column (COSMOSIL 5C18-PAQ 250 mm × 3-mm i.d., Nacalai Tesque, Kyoto, Japan); a spray chamber at 40°C, and a drift tube at 60°C. The mobile phases were solvent A (trifluoroacetic acid (TFA):water = 0.05:100) and solvent B (acetonitrile: water:TFA = 150:100:0.05). The following solvent gradients were applied: from 100% A and 0% B to 17% A and 83% B within 30 min, and 0% A to 100% B within 45 min. The flow rate was 0.5 mL/min, and the injection volume was 10 μL. As shown in **Figure 1**, retention times of isoflavonoids were: 19.4 min in 6-hydroxygenistein 6, 7-di-O-glucoside, 20.2 min in glycitin, 21.4 min in tectorigenin 7-O-xylosyl-glucoside, 22.4 min in tectoridin, 26.5 min in glycitein, and 29.6 min in tectorigenin. Retention times of triterpenoid saponins were 35.4 min in soyasaponin I and 37.0 min in kaikasaponin III. According to the results of quantitative analysis by using a calibration curve of standard samples, thomsonide (100 mg) contained 3.99 mg 6-hydroxygenistein 6, 7-di-O-glucoside, 6.54 mg glycitin, 11.84 mg tectorigenin 7-O-xylosylglucoside, 5.82
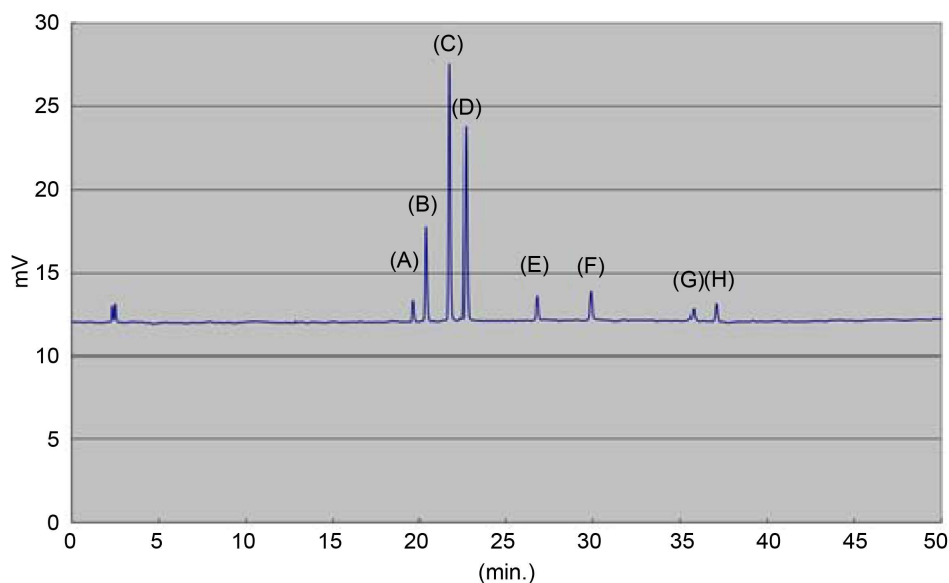
Figure 1. HPLC Chromatogram of Thomsonide. (A) 6-hydroxygenistein 6, 7-di-O-glucoside; (B) Glycitin; (C) Tectorigenin 7-O-xylosylglucoside; (D) Tectoridin; (E) Glycitein; (F) Tectorigenin; (G) Soyasaponin I; (H) Kaikasaponin III.

mg tectoridin, 2.39 mg glycitein, and 2.34 mg tectorigenin as isoflavonoids; 3.71 mg soyasaponin I and 3.21 mg kaikasaponin III as triterpenoid saponins.

2.2. Animals

Male Sprague-Dawley and Wistar strain rats weighing 180 - 200 g were obtained from Japan SLC, Inc. (Shizuoka, Japan). All animals were placed in cages, which were kept in an air-conditioned room with illumination from 07:00 to 19:00 h. Room temperature (22°C ± 2°C) and humidity (55% ± 10%) were controlled automatically. The animals were given free access to laboratory chow (pellets) (Funabashi Farm Co., Ltd, Chiba, Japan) and water. All procedures were in accordance with the Guiding Principles for the Care and Use of Laboratory Animals as adopted by the Japanese Pharmacological Society. The study protocol was approved by the Animal Ethics Committee of Ohta's Isan Co., Ltd., Tokyo, Japan (20 January 2009; protocol No. 09-002).

2.3. Drug Preparations

Test compounds for oral administration were suspended in a vehicle containing Tween 80% and 1% sodium carboxymethylcellulose (CMC-Na) (1:19). The test compounds were administered by a feeding needle in a volume of 0.5 mL per 100 g body weight in the following concentrations: Thomsonide 100 - 400 mg/kg and geranylgeranylacetone (teprenone) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) 20 mg/kg. The concentration of each compound was chosen based on published literatures by Yamazaki *et al*. [5] and Watanabe *et al*. [8]. Thus, thomsonide was used at doses below 500 mg/kg, because even at 5g/kg, as a maximal practicable dose, it did not induce any marked change in behavior, except for the transient locomotor depression, nor lead to any deaths within 72 h.

2.4. Ethanol-Induced Gastric Mucosal Lesions in Intact Rats

Fifty male Sprague-Dawley strain rats were fasted for 24 h prior to the experiment, but allowed free access to water. In accordance with the method of Robert *et al*. [9], thomsonide was orally administered to rats at doses of 100, 200, and 400 mg/kg. Teprenone was orally administered to rats at a dose of 20 mg/kg as a positive control. The control group was treated with vehicles containing Tween 80% and 1% CMC-Na (1:19), instead of thomsonide. One hour later 1 mL of 99.5% ethanol was orally administered to the rats. One hour later, the rats were sacrificed under deep ether anesthesia, and the stomach was removed. After injecting 2% formalin into the sto-

mach through the glandular portion, the stomach was fixed in 2% formalin for 10 minutes. It was then cut along the greater curvature rinsed with tap water, and was spread out on a sheet of white paper for examination. The length (mm) of erosive lesions on the gastric mucosa was measured under a dissecting microscope, and the size of the gastric mucosal lesions was recorded as the sum of the lengths of the lesions.

2.5. Prostaglandin (PG) Content of the Gastric Mucosa

In the light of the method of Harada *et al.* [10], 24 male Wistar strain rats were fasted for 24 h prior to the experiment, but allowed free access to water. Thomsonide was orally administered to rats at doses of 200 and 400 mg/kg. Four hours later, the rats were sacrificed under deep ether anesthesia, and the stomach was removed immediately. The stomach was cut along the greater curvature and rinsed with ice-cold saline. In the light of the method of Powell [11], the corpus region was excised, weighed, and homogenized in 6 mL of 0.1 M phosphate-buffered saline containing 10^{-5} M indomethacin to prevent further formation of PGs, and then the homogenate was adjusted to pH 3 with 1.5 mL of 2 M hydrochloric acid. The homogenate was then centrifuged at $12,000 \times g$ for 20 min at 4°C, and the obtained supernatant was purified and was concentrated on Amprep C_2 (100 mg resin, Amersham Japan, Tokyo Japan) to extract in methyl formate, according to the Amersham manual. After drying the specimens obtained in a centrifugal evaporator, they were used to assay PGs, and their prostaglandin E_2 (PGE$_2$) and 6-keto prostaglandin $F_1\alpha$ (6-ketoPGF$_1\alpha$) contents were determined by ELISA with a commercial kit (Cayman Chemical Company, USA).

2.6. Ethanol-Induced Gastric Mucosal Lesions in Indomethacin-Pretreated Rats

Forty male Sprague-Dawley strain rats were fasted for 24 h prior to the experiment, but allowed free access to water. In order to clarify whether the gastro protective effects of thomsonide may be related to the local enzymatic conversion of arachidonic acid to PGs, indomethacin (Sigma-Aldrich, Inc., St. Louis, MO, USA) suspended in vehicle (5 mL/kg) containing Tween 80 and saline (1:19) was injected subcutaneously into the rats at a dose of 10 mg/kg, and thomsonide or teprenone was administered 30 min later. A volume of 1 mL at 99.5% ethanol was orally administered to the rats 1 h after administration of thomsonide or teprenone. One hour later, the rats were sacrificed under deep ether anesthesia, and the stomach was removed. The rest of the procedure was as described above.

2.7. Statistical Analysis

The data are expressed as means ± standard error of the mean (S.E.M). Data were evaluated for significant differences by the one-way analysis of variance (ANOVA) and Dunnet's multiple range test.

3. Results
3.1. Ethanol-Induced Gastric Mucosal Lesions in Intact Rats

One hour after administration of 1 mL of 99.5% ethanol, erosive mucosal lesions with congestion were observed in the stomach (**Figure 2(a)**). The lesions were markedly reduced by the pretreatment with thomsonide or teprenone (**Figure 2(b)** and **Figure 2(c)**). As shown in **Table 1**, the length of the lesions was 109.10 ± 8.29 mm in the vehicle-treated rats, *i.e.*, the control group. Pretreatment with thomsonide 60 min before ethanol administration inhibited dose-dependently the gastric lesions at doses ranging from 100 to 400 mg/kg. At doses of 200 mg/kg and above the effect was marked compared to the control group. In addition, pretreatment with teprenone at a dose of 20 mg/kg inhibited more significantly the gastric lesions compared to the control group.

Accordingly, thomsonide, as well as teprenone, had protective effects against ethanol-induced gastric lesions.

3.2. Prostaglandin (PG) Content of the Gastric Mucosa

As shown in **Table 2**, the levels of PGE$_2$ and 6-ketoPGF$_1\alpha$ in gastric mucosa were 50.23 ± 5.81 ng/g and 385.38 ± 112.08 ng/g, respectively, in the vehicle-treated group. Thomsonide increased the PGE$_2$ and 6-ketoPGF$_1\alpha$ contents of the gastric mucosa, and the increases at doses of 200 mg/kg and above were significantly compared to those in the vehicle-treated group. Thomsonide significantly increased the PGE$_2$ and 6-ketoPGF$_1\alpha$ contents of the gastric mucosa.
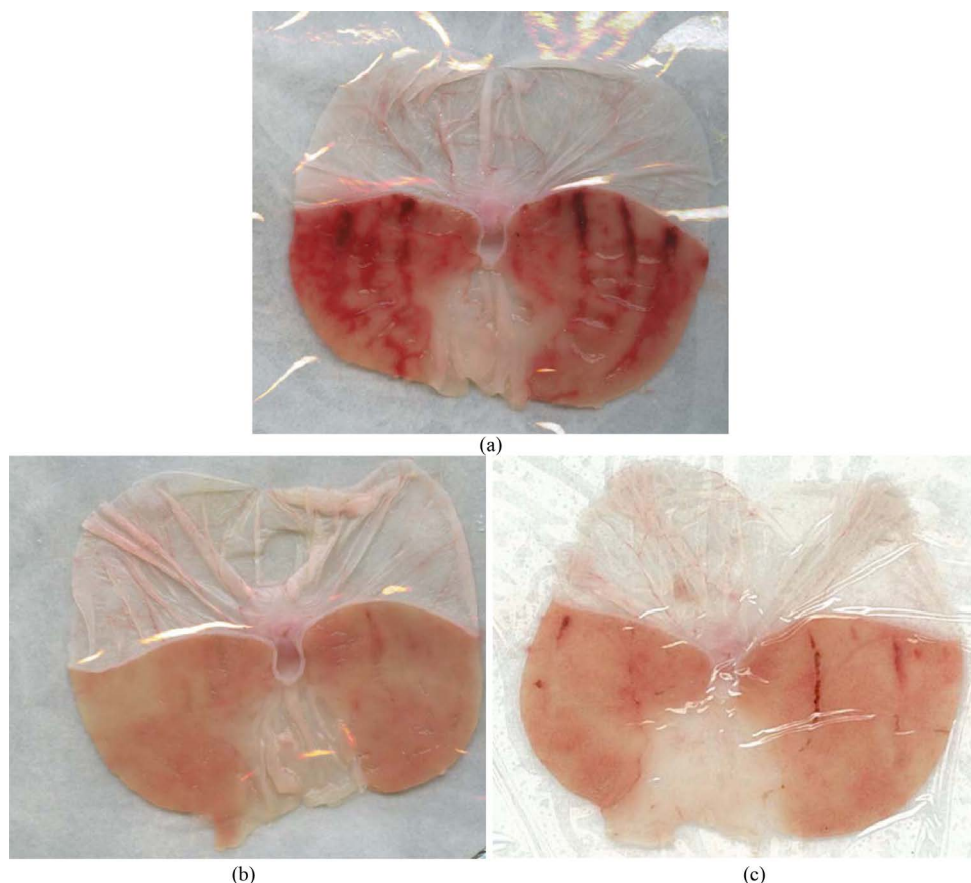
Figure 2. (a) Gross appearance of gastric mucosa 1 h after ethanol administration (Vehicle + ethanol). Erosive congestion was observed in the fundus. (b) Gross appearance of gastric mucosa 1 h after ethanol administration Pretreatment with Thomsonide at a dose of 400 mg/kg reduced the erosive congestion. (c) Gross appearance of gastric mucosa 1 h after ethanol administration Pretreatment with Tepreone at a dose of 20 mg/kg reduced the erosive congestion.

Table 1. Effects of thomsonide and teprenone on ethanol-induced gastric lesions in intact rats.

		N	Gastric lesions (mm)	Inhibition (%)
Vehicle		10	109.10 ± 8.29	
	100 mg/kg	10	90.60 ± 14.73	16.96
Thomsonide	200 mg/kg	10	$64.70 \pm 7.93^{**}$	40.70
	400 mg/kg	10	$24.90 \pm 6.91^{**}$	77.18
Teprenone	20 mg/kg	10	$42.80 \pm 10.57^{**}$	60.77

$^{**}p < 0.01$ (ANOVA and Dunnet's multiple range test) significantly different from control.

Table 2. Effects of thomsonide on PGE_2 and 6-ketoPGF$_1\alpha$ content of gastric mucosa in rats.

|		N	PGE_2 (ng/g)	6-ketoPGF$_1\alpha$ (ng/g)
Vehicle		8	50.23 ± 5.81	385.38 ± 112.08
Thomsonide	200 mg/kg	8	$90.92 \pm 9.25^{**}$	$847.16 \pm 74.41^{**}$
	400 mg/kg	8	$90.82 \pm 6.30^{**}$	$980.08 \pm 69.39^{**}$

$^{**}p < 0.01$ (ANOVA and Dunnet's multiple range test) significantly different from Vehicle treated group.

3.3. Ethanol-Induced Gastric Mucosal Lesions in Indomethacin-Pretreated Rats

Gastric mucosal lesions were also observed in rats, which were treated with ethanol following indomethacin pre-

treatment (**Figure 3(a)**). As shown in **Table 3**, in rats treated with indomethacin, the ethanol-induced gastric lesions measured 115.90 ± 14.05 mm in the vehicle-treated group, as opposed to 107.30 ± 17.41 and 78.00 ± 13.70 mm in the thomsonide groups given the 200 mg/kg dose and 400 mg/kg dose, respectively. The ethanol-induced gastric lesions in the indomethacin-pretreated rats measured 79.60 ± 9.45 mm in the teprenone group given the 20 mg/kg dose. The differences in gastric lesions between the thomsonide group or teprenone group and the vehicle-treated group were not significant, although these lesions were barely reduced by thomsonide or teprenone pretreatment (**Figure 3(b)** and **Figure 3(c)**). Pretreatment with indomethacin attenuated the protective effect of thomsonide, as well as teprenone, against the ethanol-induced gastric lesions.
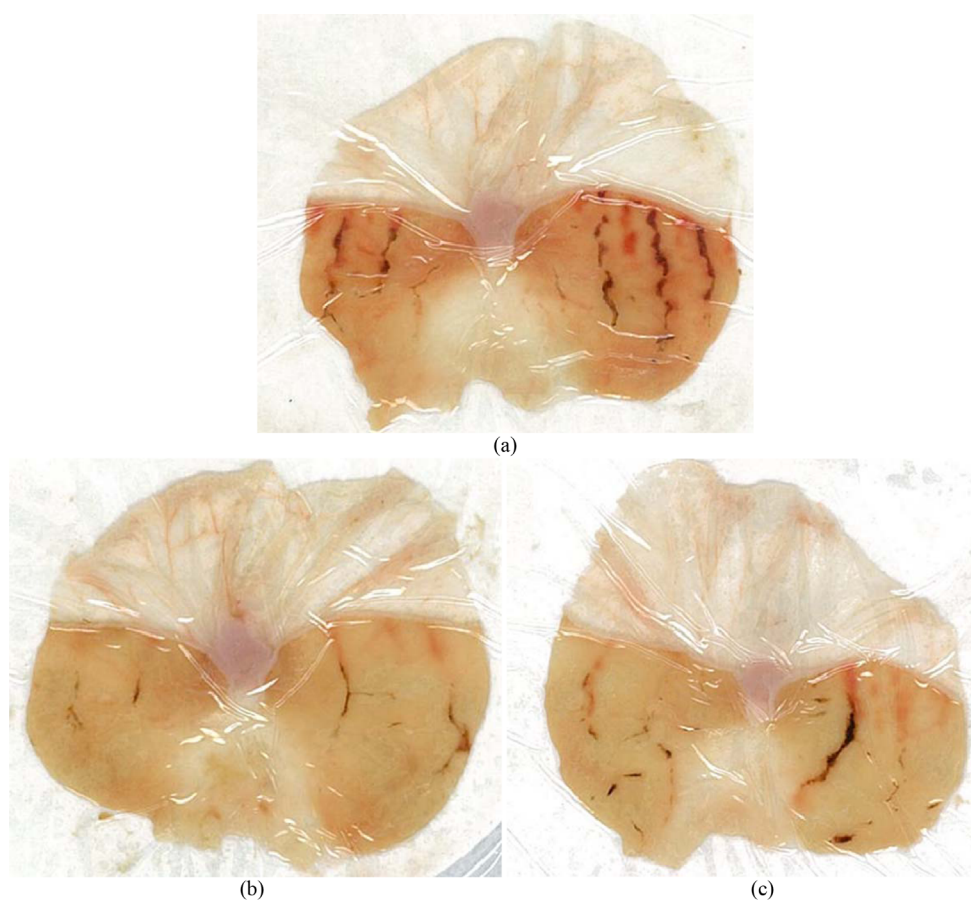
Figure 3. (a) Gross appearance of gastric mucosa 1 h after ethanol administration in rat pretreated with indomethacin. Hemorrhage and erosion were observed in the fundus of the vehicle-treated rats administered with ethanol following indomethacin pretreatment. (b) Gross appearance of gastric mucosa 1 h after ethanol administration in rat pretreated with indomethacin. Protective effects of thomsonide at a dose of 400 mg/kg against ethanol-induced gastric lesions were markedly diminished by indomethacin pretreatment. (c) Gross appearance of gastric mucosa 1 h after ethanol administration in rat pretreated with indomethacin Protective effects of teprenone at a dose of 20 mg/kg against ethanol-induced gastric lesions were markedly diminished by indomethacin pretreatment.

Table 3. Effects of thomsonide and teprenone on ethanol-induced gastric lesions in indomethacin-pretreated rats.

		N	Gastric lesions (mm)	Inhibition (%)
Vehicle		10	115.90 ± 14.05	
Thomsonide	200 mg/kg	10	107.30 ± 17.41	7.42
	400 mg/kg	10	78.00 ± 13.70	32.70
Teprenone	20 mg/kg	10	79.60 ± 9.45	31.32

Not significantly different from Vehicle.

4. Discussion

No reports have discussed the gastro protective effect of *Puerariae flos*, although preparations of this plant have been used in the traditional Chinese herbal medicine to counteract the effects of alcohol consumption. In the present study we found that thomsonide, an extract of *Puerariae flos*, as well as teprenone, had a protective effect against ethanol-induced gastric mucosal lesions. The results of our present study also showed that thomsonide significantly increased PGE_2 and 6-ketoPGF$_1\alpha$ contents of the gastric mucosa, and that pretreatment with indomethacin attenuated the protective effect of thomsonide against ethanol-induced gastric mucosal lesions. Furthermore, our present results showed that the protective effect of teprenone against ethanol-induced gastric mucosal lesions, was attenuated by pretreatment with indomethacin. Some reports have already shown that teprenone has a protective effect against ethanol-induced gastric mcosal lesions [8] and increases the PGE_2 content of the gastric mucosa through induction of COX-2 [12]. These findings are generally consistent with our observations. A variety of PGs have been shown to protect the gastrointestinal mucosa against various noxious agents. Robert *et al*. [9] have described the protective property of PGs, which is independent of their acid-inhibiting property, as "adaptive cytoprotection". PGE_2 and PGI_2 are well known to be involved in the regulation of a variety of gastrointestinal functions. Araki *et al*. [13] have reported that endogenous PGE_2 protects the gastric mucosa through the EP_1 receptor. The above findings suggest that the gastro protective effects of thomsonide may be related to the cytoprotective activity of endogenous PGE_2 through the EP_1 receptor. In addition, thomsonide increased the gastric mucosal content of 6-ketoPGF$_1\alpha$, a stable metabolite of PGI_2, indicating that thomsonide has a significant effect on the production of PGI_2. Konturek *et al*. [14] have reported that PGI_2 increases the postprandial serum gastrin level and mucosal blood flow in the resting mucosa in dogs with Heidenhain pouches; Gaskill *et al*. [15] have reported that PGI_2 increases gastric mucosal blood flow by the mechanism, which is dependent on cyclic AMP. In light of these findings, thomsonide may protect ethanol-induced gastric mucosal lesions by maintaining gastric blood flow through the increase in the gastric mucosal content of PGI_2, a precursor of 6-ketoPGF$_1\alpha$, as well as the cytoprotective activity of endogenous PGE_2.

Puerariae flos is known to be rich in isoflavonoids and triterpenoid saponins. Kinjo *et al*. [16] have reported that *Puerariae flos* contains a variety of triterpenoid saponins including Kakkasaponin I. Matsuda *et al*. [17] have reported that oleanene-type triterpenoid saponins have a protective effect against ethanol-induced gastric mucosal lesions. Martinez *et al*. [18] have also reported that oleanolic acid can be regarded as a bioactive molecule, which may induce PGs release in the COX-2-dependent manner. In view of the above evidence, it is plausible that thomsonide, which is rich in oleanene-type triterpenoid saponins, has a protective effect against the formation of gastric mucosal lesions. It is supposed that Kakkasaponin, a component of thomsonide, may protect the gastric mucosa from ethanol-induced lesions by increasing the production of endogenous PGs through overexpression of COX-2 in the gastric mucosa. The results of the present study prove our supposition that thomsonide has a gastro protective effect, and that the protective effect of thomsonide may be due to the local enzymatic conversion of arachidonic acid to PGs, which are known to have cytoprotective properties. The results also suggest that the gastro protective effect of thomsonide may partially mitigate alcoholic disorders in the gastrointestinal tract. Furthermore, the results support our pharmacological belief that *Puerariae flos* is useful for the treatment of alcoholic disorders.

5. Conclusion

Thomsonide, an extract of *Puerariae flos*, has been investigated for its effect on ethanol-induced gastric mucosal lesions. Thomsonide, as well as teprenone, inhibited dose-dependently the formation of ethanol-induced gastric mucosal lesions. At doses of 200 mg/kg and above, thomsonide produced the significant effect, and increased significantly the PGE_2 and 6-ketoPGF$_1\alpha$ contents of the gastric mucosa. Furthermore, indomethacin attenuated the protective effect of thomsonide, as well as teprenone, against the formation of ethanol-induced gastric mucosal lesions. These results elucidate that thomsonide has a gastro protective effect and that its protective effect may be produced by increasing endogenous PGs in gastric mucosa.

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