Pharmacological Profile of Gastric Mucosal Protection by Marmin and Nobiletin from a Traditional Herbal Medicine, *Aurantii Fructus Immaturus*

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ABSTRACT—We studied the effects of marmin and nobiletin on the experimental acute gastric lesions, gastric transmucosal potential difference (PD) and gastric motor activity in rats and the contractions of isolated guinea pig ileum. Oral administration of marmin and nobiletin inhibited both the appearance of ethanol-induced gastric hemorrhagic lesions dose-dependently in a dose range of 10–50 mg/kg, with ED₅₀ values for marmin and nobiletin being 17.2 and 8.0 mg/kg, respectively. However, marmin and nobiletin had minimal effects on aspirin-induced gastric lesions at a dose of 50 mg/kg, respectively. Marmin and nobiletin had no significant influence on the basal PD. Intragastrical administration of marmin and nobiletin at a dose of 25 mg/kg significantly prevented the PD reduction induced by ethanol. Both marmin and nobiletin given intragastrically at 25 mg/kg significantly inhibited gastric motor activity measured as intraluminal pressure recordings. Marmin and nobiletin exhibited concentration-dependent relaxations of contractions induced by acetylcholine, transmural electrical stimulation and histamine in isolated guinea pig ileum, respectively. These findings suggest that the anti-ulcer effects of marmin and nobiletin are ascribed primarily to the maintenance of the mucosal barrier integrity and inhibition of gastric motor activity and secondarily due to the prevention of the effects of endogenous acetylcholine and histamine.

Keywords: Marmin, Nobiletin, Gastric mucosal protection, Gastric motor activity, Gastric transmucosal potential difference

The hot-water extract of *Aurantii fructus immaturus* has been used for a long time in traditional medical practice such as for the treatment of stomach disorders, and the anti-inflammatory and anti-allergy actions of the hot-water extract of *Aurantii fructus immaturus* have been previously reported (1, 2).

Recently, we have found that the hot-water extract of *Aurantii fructus immaturus* inhibited the formation of ethanol- or aspirin-induced gastric lesions (3). Furthermore, we have extracted, fractionated, isolated, purified and determined the chemical composition of the active ingredients from the hot-water extract of *Aurantii fructus immaturus* (3). The compounds extracted from the hot-water extract of *Aurantii fructus immaturus*, which inhibited ethanol-induced gastric mucosal damage, were identified as marmin (7-(6',7'-dihydroxygeranyl-oxy)coumarin, Fig.1) and nobiletin (5,6,7,8,3',4'-hexamethoxyflavone, Fig. 1) (3).

Marmin inhibited the contraction induced by BaCl₂ in

Fig. 1. Chemical structures of marmin and nobiletin.

isolated guinea pig ileum and acted as a kind of calcium antagonist on the calcium uptake of myocardial cells (4, 5). Nobiletin inhibited cAMP phosphodiesterase (6), had the inhibitory effect on rat passive cutaneous anaphylaxis (PCA) and had the inhibitory activity on histamine release increased (7). However, the anti-ulcer effects of marmin and nobiletin have not been studied.

To elucidate the anti-ulcer effects and pharmacological profiles of marmin and nobiletin, we examined the effects of marmin and nobiletin on ethanol- or aspirin-induced gastric lesions, basal transmucosal potential difference (PD) or ethanol-induced PD reduction and gastric motor activity in rats in vivo and the ileal contractions induced by acetylcholine, transmural electrical stimulation or histamine in guinea pig in vitro in comparison with the effects of pirenzepine.

MATERIALS AND METHODS

Procedure for isolation of marmin and nobiletin

Marmin and nobiletin were isolated from the plant, *Aurantii fructus immaturus* as described previously (3). Briefly, hot-water extracts of *Aurantii fructus immaturus* (2.6 kg) were subjected successively to silica gel column chromatography (developed with a mixture of methylene chloride and methanol) to yield marmin (0.58 g) and nobiletin (0.30 g). The structures of marmin and nobiletin were determined by spectroscopic methods (UV, MS, IR, ^1H NMR and ^{13}C NMR) and chemical transformation.

Animals

Male Wistar rats (ST substrain from Japan SLC, Inc., Hamamatsu), weighing 180–280 g, were used for the in vivo studies. The rats were fasted overnight, but allowed free access to water up to the beginning of the experiment. For the in vitro studies, male guinea pigs (from Japan SLC, Inc.), weighing 250–550 g, were used.

Ethanol-induced gastric lesions

Ethanol-induced gastric mucosal lesions were produced according to the method of Robert et al. (8). Each drug or vehicle was given to rats orally 30 min prior to oral administration of 1 ml of 99.5% ethanol. One hour after ethanol treatment, the animals were killed. The stomach was removed and inflated by injecting 10 ml of 1% formalin to the gastric lumen for 10 min. Subsequently, the stomach was incised along the greater curvature and examined for lesions. The length (mm) of each lesion was measured under a dissecting microscope ($\times 10$) with a square grid, and the sum per stomach was used as the lesion index.

Aspirin-induced gastric lesions

Aspirin-induced gastric mucosal lesions were produced according to the method of Brodie and Chase (9). Each drug or vehicle was given to rats orally 30 min prior to oral administration of 200 mg/kg aspirin. Four hours after aspirin treatment, the animals were killed immediately after intravenous injection of 0.5 ml of 5% Evan's blue. The stomach was removed and inflated by injecting 10 ml of 1% formalin solution to the gastric lumen for 10 min. Subsequently, the stomach was incised along the greater curvature and examined for lesions. The length (mm) of each lesion was measured under a dissecting microscope ($\times 10$) with a square grid, and the sum per stomach was used as the lesion index.

Gastric potential difference (PD)

The experimental procedure was the same as that described by Nagashima et al. (10). After anesthesia with urethane (1.25 g/kg, i.p.), the trachea of the rat was cannulated. The abdomen was opened and the stomach exposed. The esophagus was ligated without disturbing the vagus nerve. One catheter filled with 3% agar in saturated KCl was inserted into the stomach through an incision in the duodenum and served as the intragastric electrode. A temporary gastric fistula was prepared by inserting a polyethylene tube into the forestomach. The fistula, which led to a three-way tap, was used for intragastric instillation and removal of gastric content. A second catheter filled with 3% agar and saturated KCl was inserted into the peritoneal cavity and served as the indifferent electrode. Both the intragastric and intraperitoneal electrodes were placed in separate beakers containing saturated KCl solution in which a balanced Ag-AgCl electrode was positioned. The whole interior of the stomach was gently rinsed with warm saline 3–4 times, and then 4 ml of saline was instilled into the stomach. The changes of PD were continuously monitored by a recorder connected to the millivoltmeter. PD recordings were taken at 5-min intervals during a 30-min period. The gastric PD measurements were done at the forestomach portion where the tip of the detecting electrode was placed. The influence of drugs on gastric PD was estimated by comparison with the values of PD in the corresponding control group after treatment with ethanol or drug alone. Each drug or vehicle was intragastrically administered through the fistula before treatment with 50% ethanol.

Gastric motor activity

Gastric motor activity was measured in conscious rats using a balloon positioned in the glandular part of the stomach, following the procedure described by Takeuchi and Nobuhara (11). Briefly, under ether anesthesia, a balloon (containing 0.8 ml of water) and support catheter

were placed in the glandular stomach through an incision of the forestomach. A temporary gastric cannula for administration of each test drug was placed into the forestomach. The animals were then placed in Bollman cages, and the support catheter was connected to a pressure transducer and polygraph device (Nihon Kohden, Tokyo). Gastric motor activity was continuously monitored on a recorder as intraluminal pressure for a 2-hr test period. Quantitative analysis of gastric motor activity was performed by counting the number of contractions with an amplitude of 10 cmH₂O or greater and by measuring the amplitude of each contraction (clear spike) over a 30-min period. Changes of the motor activity for each animal were expressed as a percentage of the frequency or the amplitude values to the corresponding values observed before administration of each drug. Data were presented as the mean $\pm$ S.E. for each time period from five to ten rats per group. Each drug or vehicle was given intragastrically through the cannula after the motor activity had been well stabilized.

Contractile response of smooth muscle strips of guinea pig ileum

Male guinea pigs were killed by stunning and exsanguination. The ileum was removed, dissected free of mesentery, and 2-cm segments of the mid-region ileum were set up in organ baths containing Tyrode solution (137.9 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl₂·6H₂O, 1.1 mM NaH₂PO₄·2H₂O, 1.8 mM CaCl₂·2H₂O, 11.9 mM NaHCO₃ and 5.6 mM glucose, in distilled water) gassed with 5% CO₂ and 95% O₂ and maintained at 37°C. Isometric tension was measured with a mechanical transducer. In the case of transmural electrical stimulated contractions, segments of ileum were set up as described by Paton (12) under 0.5 g resting tension, and transmural nerve stimulation to the muscle was applied through two parallel platinum rings separated by 10 mm. An electronic stimulator was used to deliver the rectangular pulses of supramaximal voltage and a 2-msec pulse width of 15 sec. When twitch responses to transmural stimulation had become constant, each drug was added to the bathing fluid in a cumulative-concentration schedule. The concentration of each drug required to reduce the twitch response by 50% was determined. In the case of acetylcholine- or histamine-induced contractions, segments of ileum were mounted on tissue holders under a resting tension of 0.5 g. Contraction with each test drug was obtained by the addition of acetylcholine (10⁻⁷ g/ml) or histamine (10⁻⁷ g/ml) 2 min after application of each test drug. The maximal contractile response obtained with the respective agonist was taken as the 100% response, and all subsequent contractions were calculated as a percentage of this response.

Drugs

Marmin, nobiletin and aspirin (Wako, Osaka) were suspended in 5% gum arabic solution in the in vivo study. Pirenzepine hydrochloride (Sigma, St. Louis, MO, USA), papaverine hydrochloride (Sigma), ethanol (Wako) and urethane (Wako) were dissolved in saline in the in vivo study. Marmin and nobiletin were dissolved in dimethylsulfoxide (DMSO), and pirenzepine hydrochloride, acetylcholine chloride (Sigma) and histamine dihydrochloride (Wako) were dissolved in distilled water in the in vitro study. The final concentration of DMSO in the bathing solution did not exceed 0.1%, and DMSO had no effect on the smooth muscle contraction at the final concentration.

Statistical analyses

All data are expressed as means $\pm$ S.E. The statistical significances were assessed by Dunnett's multiple comparison test following one-way analysis of variance (ANOVA) or by the *t*-test for paired results. *P* values less than 0.05 were considered statistically significant. The ED₅₀ values and IC₅₀ values (with 95% confidence limits) were calculated by the probit method.

RESULTS

Ethanol-induced gastric lesions

Severe band-like mucosal lesions were induced in the glandular stomach by 99.5% ethanol; the lesion index in the control group was 65.3 $\pm$ 14.3 mm (N=8). Marmin and nobiletin at doses ranging from 10 to 50 mg/kg, p.o. inhibited the formation of gastric lesions induced by

Fig. 2. Inhibitory effects of marmin, nobiletin, pirenzepine and papaverine on ethanol-induced gastric lesions in 24 hr-fasted rats. Gastric lesions were produced by ethanol (99.5%, 1 ml/rat, p.o.) in 24 hr-fasted rats. Each drug or vehicle was given orally 30 min before ethanol administration. Animals were killed 1 hr after ethanol treatment. Each value represents the mean $\pm$ S.E. of 5 to 8 rats per group. **P* < 0.05, significantly different from each control (vehicle-treated group) (Dunnett's *t*-test).

Fig. 3. Inhibitory effects of marmin, nobiletin and pirenzepine on aspirin-induced gastric lesions in rats. Gastric lesions were produced by aspirin (200 mg/kg, p.o.) in 24 hr-fasted rats. Each drug was given orally 30 min before aspirin administration. Animals were killed 4 hr after aspirin treatment. Each value represents the mean $\pm$ S.E. of 5 to 8 rats per group. * $P < 0.05$, significantly different from each control (vehicle-treated group) (Dunnett's *t*-test).

ethanol in a dose-dependent manner; the ED_{50} values for marmin and nobiletin (the doses that produced 50% inhibition of ethanol-induced gastric lesions) were 17.2 (95% confidence limit: 12.5–21.8) and 8.0 (3.1–11.8) mg/kg, respectively (Fig. 2). Pirenzepine at the dose of 10 mg/kg displayed only a 43% inhibition of the gastric lesions caused by ethanol (Fig. 2). On the other hand, papaverine at 100 mg/kg showed 69% inhibition of ethanol-induced gastric lesions (Fig. 2).

Aspirin-induced gastric lesions

Multiple dotted lesions were produced in the glandular stomach by aspirin (200 mg/kg); the lesion index in the control group was 23.7 ± 3.4 mm ($N=8$). Marmin and nobiletin at 50 mg/kg, p.o. did not significantly inhibit

the aspirin-induced gastric lesions, but showed a 32% and 22% inhibition, respectively (Fig. 3). On the other hand, pirenzepine (0.1, 1.0 and 10 mg/kg) dose-dependently prevented the aspirin-induced gastric lesions; the ED_{50} value and 95% confidence limit for pirenzepine in the aspirin ulcer model was 0.21 (0.06–0.44) mg/kg (Fig. 3).

The basal PD

The normal stomach generated a PD of -30 to -35 mV (basal PD) (Table 1). Intragastrical administration of marmin and nobiletin had no effect on the basal PD. On the contrary, when the stomach was exposed to pirenzepine at 10 mg/kg, the PD was significantly increased.

Ethanol-induced PD reduction

As shown in Fig. 4, after intragastrical administration of 2 ml of 50% ethanol, the PD value declined from -30 to -35 mV to -15 to -20 mV. Intragastrical administration of marmin and nobiletin at 25 mg/kg significantly inhibited ethanol-induced PD reduction, but did not significantly inhibit it at 10 mg/kg (Fig. 4). On the other hand, pirenzepine at 10 mg/kg did not prevent the PD reduction induced by ethanol (Fig. 4).

Gastric motor activity

In the normal rat, the stomachs contracted at a frequency of 1–2/min with an amplitude of 32.3 ± 6.4 cmH₂O ($N=10$) as intraluminal pressure recordings. Intragastrically administered marmin (25 mg/kg) significantly reduced both the amplitude for 30 min and the frequency of contractions during 0–60 min and 90–120 min, and this effect was observed dose-dependently for marmin at 10 and 25 mg/kg (Figs. 5 and 6). In contrast, the intragastrical administration of 10 and 25 mg/kg of nobiletin significantly reduced the frequency of contractions for 30 min and for 90 min, respectively, but had no significant effect on the amplitude of contractions (Figs. 5 and 6).

Table 1. Effects of marmin, nobiletin and pirenzepine on basal transmucosal potential difference (PD) in anesthetized rats

Drug	Dose (mg/kg)	N	Transmucosal potential difference (–mV)		
			before drug	after drug	Δ PD
Control (5% gum arabic)	—	4	35.0 ± 1.4	37.3 ± 2.4	2.3 ± 0.4
Marmin	25	4	35.3 ± 2.5	35.5 ± 3.0	0.2 ± 0.7
Nobiletin	25	4	36.3 ± 0.8	39.3 ± 1.1	3.0 ± 1.3
Control (saline)	—	4	36.0 ± 1.1	36.0 ± 2.0	0.0 ± 1.7
Pirenzepine	10	6	34.2 ± 1.7	39.5 ± 3.4	$5.3 \pm 1.8^*$

Each drug was intragastrically instilled for 30 min. Each value represents the mean $\pm$ S.E. of the given number of experiments. * $P < 0.05$, significantly different from each control (paired *t*-test).

Fig. 4. Effects of marmin, nobiletin and pirenzepine on ethanol-induced transmucosal potential difference (PD) reduction in anesthetized rats. Each drug was intragastrically administered prior to treatment with 50% ethanol. Each point represents the mean $\pm$ S.E. of 5 to 10 rats per group. $\circ$: control (vehicle-treated group); A: marmin, 10 ($\bullet$) or 25 ($\blacksquare$) mg/kg, i.g.; B: nobiletin, 10 ($\bullet$) or 25 ($\blacksquare$) mg/kg, i.g.; C: pirenzepine, 10 mg/kg, i.g. ($\bullet$). * $P < 0.05$, significantly different from each control (Dunnett's t -test).

Pirenzepine (10 mg/kg) significantly inhibited both the frequency during 0–120 min and the amplitude for 30 min, and papaverine (100 mg/kg) significantly suppressed the frequency for 60 min and the amplitude for 30 min

(Figs. 5 and 6).

Acetylcholine-, transmural electrical stimulation- or histamine-contracted ileum

Figure 7 shows the results for marmin, nobiletin and pirenzepine on the inhibition of acetylcholine (A)-, transmural electrical stimulation (B)- or histamine (C)-contracted ileum. In the acetylcholine-contracted ileum, marmin (10^{-7} – 3×10^{-4} g/ml), nobiletin (10^{-7} – 10^{-5} g/ml) and pirenzepine (10^{-8} – 10^{-6} g/ml) produced concentration-dependent relaxation of the contraction, the IC_{50} values being 2.56×10^{-4} , 3.73×10^{-6} and 2.05×10^{-7} g/ml, respectively. In the transmurally-stimulated ileum, marmin (10^{-7} – 3×10^{-4} g/ml), nobiletin (10^{-7} – 10^{-5} g/ml) and pirenzepine (10^{-8} – 10^{-6} g/ml) produced concentration-dependent inhibition of the twitch response, with the IC_{50} values of 8.12×10^{-4} , 4.05×10^{-6} and 4.52×10^{-8} g/ml, respectively. In the histamine-contracted ileum, marmin (10^{-7} – 10^{-4} g/ml) and nobiletin (10^{-7} – 10^{-4} g/ml) produced a concentration-dependent relaxation of the contraction, the IC_{50} values being 3.73×10^{-5} and 1.48×10^{-4} g/ml, respectively, but pirenzepine (10^{-8} – 10^{-5} g/ml) had little effect on the histamine-induced contractions. Table 2 summarizes the results.

DISCUSSION

Peptic ulcers are well accepted to be caused by an imbalance between the mucosal defensive factors and the aggressive factors of acid and pepsin (13). The mechanism by which ethanol induces gastric damage is thought to be due to the disruption of the defensive factors such as the gastric mucosal barrier, gastric mucus and mucosal circulation (14, 15). On the contrary, gastric acid plays a crucial role in the development of the aspirin-induced gastric lesions (16). The gastric lesion induced by ethanol was markedly prevented by drugs increasing the defensive factors, while the lesion induced by aspirin was prevented by drugs inhibiting the aggressive factors such as gastric acid (17, 18). Our previous studies have also shown that sucralate, 16,16-dimethyl prostaglandin E_2 and cetraxate, drugs increasing the defensive factors, effectively inhibited ethanol-induced gastric lesions (17, 19). Additionally, in the primary experiments, marmin and nobiletin displayed the inhibitory effects in acetylcholine-induced ileal contractions. Therefore, we studied the anti-ulcer effect and the pharmacological profile of marmin and nobiletin in comparison with that of pirenzepine.

In the present study, marmin and nobiletin markedly inhibited the formation of ethanol-induced gastric lesions, and the latter (ED_{50} value = 8.0 mg/kg) was about twice as potent as the former (ED_{50} value = 17.2 mg/kg) on the ethanol ulcer inhibition. However, the inhibitory effects

Fig. 5. Effects of marmin, nobiletin, pirenzepine and papaverine on the frequency of gastric contractions in rats measured as intraluminal pressure recordings. Each drug was given intragastrically after basal gastric motor activity had been well stabilized. Data are expressed as the percentage of the frequency values observed before administration of each drug and presented as the mean $\pm$ S.E. of 5 to 10 rats per group. $\circ$: control (vehicle-treated group); A: marmin, 10 ($\bullet$) or 25 ($\blacksquare$) mg/kg, i.g.; B: nobiletin, 10 ($\bullet$) or 25 ($\blacksquare$) mg/kg, i.g.; C: pirenzepine, 10 mg/kg, i.g. ($\bullet$); D: papaverine, 100 mg/kg, i.g. ($\bullet$). * $P < 0.05$, significantly different from each control (Dunnett's *t*-test).

Fig. 6. Effects of marmin, nobiletin, pirenzepine and papaverine on the amplitude of gastric contractions in rats measured as intraluminal pressure recordings. Each drug was given intragastrically after basal gastric motor activity had been well stabilized. Data are expressed as the percentage of the amplitude values observed before administration of each drug and presented as the mean $\pm$ S.E. of 5 to 10 rats per group. $\circ$: control (vehicle-treated group); A: marmin, 10 ($\bullet$) or 25 ($\blacksquare$) mg/kg, i.g.; B: nobiletin, 10 ($\bullet$) or 25 ($\blacksquare$) mg/kg, i.g.; C: pirenzepine, 10 mg/kg, i.g. ($\bullet$); D: papaverine, 100 mg/kg, i.g. ($\bullet$). * $P < 0.05$, significantly different from each control (Dunnett's *t*-test).

Fig. 7. Relaxatory effects of marmin, nobiletin and pirenzepine against contractions induced by acetylcholine (10^{-7} g/ml) (A), transmural electrical stimulation (ES: 100 V, 2 msec, 15 sec-width) (B) or histamine (10^{-7} g/ml) (C) in isolated guinea pig ileum. Each value shows the mean $\pm$ S.E. of 4 to 6 experiment. ●: marmin, ■: nobiletin, ▲: pirenzepine.

of these two drugs were smaller in the aspirin ulcer inhibition than in the ethanol ulcer inhibition. On the other hand, pirenzepine, a muscarine M_1 -receptor antagonist (20, 21), strongly prevented aspirin-induced gastric lesions at doses of more than 0.1 mg/kg, doses that inhibit carbachol-induced gastric acid output (data not shown), but showed only a 43% inhibition of ethanol-induced gastric lesions even at 10 mg/kg, a dose sufficient that inhibit gastric acid secretion. Therefore, it seems that the anti-ulcer effects of marmin and nobiletin are attributable to another mechanism that suppresses gastric acid secretion.

So-called mild irritants protect the gastric mucosa against strong irritants through the increased synthesis of mucosal prostaglandins (PGs) (22, 23). Mild irritants such as dilute ethanol, acetic acid and NaCl are known to produce a fall in the basal PD as a result of barrier disruption owing to damage in the gastric mucosa (24, 25). The cytoprotective properties of marmin and nobiletin seem to be different from those of such mild irritants, since marmin and nobiletin did not cause a fall in the basal PD.

The property of cytoprotection is shared by endogenous PGs, which protect the gastric mucosa against necrotizing agents such as absolute ethanol (8). The gastric PD, which represents the integrity of the gastric mucosal barrier, is considered to well reflect cytoprotective functions, since the PD values decline as a result of barrier disruption owing to damage in the gastric mucosa (26–30). We and other investigators have previously shown that sucralate and PGs derivatives, increasing the defensive factors, inhibit ethanol-induced PD reduction (10, 31, 32). In our present study, marmin and nobiletin significantly inhibited the PD reduction induced by ethanol at a dose that inhibited the formation of ethanol-induced lesions, thereby suggesting that marmin and nobiletin at least in part exert cytoprotective activities by maintaining the integrity of the gastric mucosal barrier. On the other hand, pirenzepine did not exhibit such a marked effect against ethanol-induced PD reduction.

The role of gastric motor activity in the pathogenesis of

Table 2. Inhibitory activities of marmin, nobiletin and pirenzepine against contractions induced by acetylcholine, transmural electrical stimulation (ES) or histamine in isolated guinea pig ileum

Agonist	IC ₅₀ values (g/ml)		
	marmin	nobiletin	pirenzepine
Acetylcholine	2.56×10^{-4} ($1.71 \sim 4.45 \times 10^{-5}$)	3.73×10^{-6} ($2.40 \sim 6.85 \times 10^{-6}$)	2.05×10^{-7} ($1.59 \sim 2.67 \times 10^{-7}$)
Electrical stimulation	8.12×10^{-4} ($2.91 \sim 54.0 \times 10^{-4}$)	4.05×10^{-6} ($2.72 \sim 6.72 \times 10^{-6}$)	4.52×10^{-8} ($3.14 \sim 6.19 \times 10^{-8}$)
Histamine	3.73×10^{-5} ($2.59 \sim 5.79 \times 10^{-5}$)	1.48×10^{-4} ($6.05 \sim 62.7 \times 10^{-5}$)	$> 10^{-5}$

IC₅₀ values: The concentration that produces the 50% inhibition. The numbers in parentheses indicate the 95% confidence limits.

gastric lesions has been recently suggested (11, 33–35). For example, hypermotility might be a major factor in the genesis of gastric lesions induced by a necrotizing agent such as absolute ethanol (11, 33, 34), because there is a relationship between the grade of ethanol-induced gastric lesions and the increase of gastric motility. In the present study, marmin and nobiletin significantly inhibited gastric motor activity at a dose at which they inhibited ethanol-induced gastric lesions. Similar results were also obtained with papaverine, a non-specific smooth muscle relaxant. These findings suggest that the cytoprotective effects of marmin and nobiletin against gastric mucosal damage is attributed partially to the inhibition of gastric motor activity.

We have previously found that the hot-water extract of *Aurantii fructus immaturus* significantly inhibited both aspirin-induced and ethanol-induced gastric lesions (3). In this respect, there was a difference between the pharmacological mode of the hot-water extract of *Aurantii fructus immaturus* and those of marmin or nobiletin. Therefore, further experiments are required to examine which of the other ingredients contained in the hot-water extract of *Aurantii fructus immaturus* brought about this difference in the pharmacological mode.

Although marmin and nobiletin exhibited the mucosal protective effects, mechanisms of the anti-ulcer action of marmin and nobiletin remain unclear. Acetylcholine released from the cholinergic pathway of vagus nerve endings is considered to participate in modulating the gastric motor activity. Furthermore, there has been evidence showing that a significant amount of histamine is released from the gastric mucosa after damage with ethanol (36) and that mepyramine, a histamine H_1 -receptor antagonist, significantly suppressed ethanol-induced gastric hypermia (37). Therefore, it is conceivable that endogenous acetylcholine and histamine may play an important role in the development of gastric mucosal damage.

Nobiletin was approximately 15-fold more potent in preventing the ileal contraction induced by acetylcholine or electrical stimulation than by histamine, but was approximately 18-fold or 90-fold less potent in preventing acetylcholine- or electrical stimulation-contracted ileum than pirenzepine. Nobiletin has been reported to show inhibitory effects against cAMP phosphodiesterase and possess an inhibitory effect on histamine release from peritoneal mast cells (6, 7). In addition, in the case of smooth muscle, it has been proposed that the inhibition of cAMP phosphodiesterase is closely related to the relaxation of smooth muscle (38). Hence, the relaxatory effect of nobiletin on smooth muscle may be due to the inhibition of cAMP phosphodiesterase. Furthermore, although the inhibitory effect of nobiletin in the ileal contraction in-

duced by acetylcholine was weaker than that of pirenzepine, the relaxatory effect of nobiletin on smooth muscle may participate partly in the inhibitory effect of nobiletin against gastric mucosal damage as well as papaverine.

In contrast, marmin inhibited the ileal contraction induced by histamine approximately 7-fold and 22-fold more strongly than that induced by acetylcholine and electrical stimulation, respectively, and was approximately 1200-fold or 60-fold less potent in preventing acetylcholine-contracted ileum than pirenzepine or nobiletin. However, marmin (IC_{50} value = 3.73×10^{-5} g/ml) was approximately 1000 times weaker than diphenhydramine (IC_{50} value = 3.16×10^{-8} g/ml) in preventing histamine-induced ileal contraction. Therefore, the inhibitory effects of marmin on acetylcholine- or histamine-induced ileal contraction could not account for the inhibitory effect of marmin against gastric mucosal damage.

As described above, nobiletin and marmin, unlike pirenzepine, exhibited the protective effects on the mucosal barrier integrity in addition to the inhibition of gastric motor activity associated with the gastric mucosal protection. Additionally, the present findings revealed that nobiletin was more effective in preventing the ileal contraction induced by acetylcholine than by histamine, while marmin showed the reverse.

In summary, the present study revealed that the mucosal protective effects of marmin and nobiletin against ethanol injury were different from those of pirenzepine and mild irritants. Moreover, the present findings suggest that the protective effects of marmin and nobiletin are at least in part ascribed to the maintenance of the mucosal barrier integrity and inhibition of gastric motor activity.

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