Antiallergic Effect of Flavonoid Glycosides Obtained from *Mentha piperita* L.

Toshio INOUE,^{a,b} Yukio SUGIMOTO,^a Hideki MASUDA,^b and Chiaki KAMEI^{*,a}

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Okayama University,^a 1-1-1 Tsushima-naka, Okayama 700-8530, Japan and Ogawa & Co., Ltd., Material R&D Laboratory,^b 15-7 Chidori, Urayasu, Chiba 279-0032, Japan. Received August 13, 2001; accepted October 31, 2001

Six flavonoid glycosides, eriocitrin (1), narirutin (2), hesperidin (3), luteolin-7-*O*-rutinoside (4), isorhoifolin (5), diosmin (6), rosmarinic acid (7) and 5,7-dihydroxycromone-7-*O*-rutinoside (8), were isolated from the aerial part of *Mentha piperita* L. Among these compounds, compound 4 showed a potent inhibitory effect on histamine release induced by compound 48/80 and antigen-antibody reaction. This compound was more effective than luteolin and luteolin-7-*O*-glucoside in inhibiting histamine release from rat peritoneal mast cells. Compound 4 also caused a dose-related inhibition of the antigen-induced nasal response and significant effects were observed at doses of 100 and 300 mg/kg. These results indicate that compound 4 may be clinically useful in alleviating the nasal symptoms of allergic rhinitis.

Key words *Mentha piperita* L.; flavonoid glycoside; antiallergic effect; rat peritoneal mast cell; histamine

Peppermint (*Mentha piperita* L.) belonging to the labiatae family is commercially cultivated in the temperate zones around the world, particularly in the United States, Canada and China, for its volatile oil, obtained by steam distillation from the plant's aerial parts.¹⁾ The volatile constituents (essential oil) obtained by steam distillation from the aerial parts have been widely used for a long time because its flavor, fragrance and carminative and stimulant properties. In addition, the volatile oil has been reported to show anti-inflammatory, antibacterial and antifungal properties.^{2–4)} As described above, the broad spectrum of bioactivity of this plant has usually been ascribed to the components of its essential oil. However, only a few studies have reported the quantitative and qualitative composition of the non-volatile constituents.^{5,6)}

We have reported that the 50% EtOH extract of peppermint inhibited histamine release from rat peritoneal mast cells induced by compound 48/80. A remarkable inhibition against nasal symptoms (sneezing and nasal rubbing) induced by antigen challenge was also recognized by the 50% EtOH extract of peppermint in actively sensitized rats.⁷⁾ However, the true characters of the effective component contained in this extract were not clarified. Therefore, the present study was undertaken to clarify the anti-allergic effects of certain compounds isolated from the extract of the peppermint.

MATERIALS AND METHODS

Apparatus The NMR spectra were measured using a Bruker AVANCE-400 NMR spectrometer. The spectral data are reported as ppm downfield from tetramethylsilane (TMS) ($\delta=0$). Preparative HPLC was performed using a Soken Hisep LC type C (Soken Chemical & Engineering Co., Ltd., Tokyo, Japan). TLC was performed on silica gel 60F₂₅₄ plates (Merck, Darmstadt, Germany). TLC spots were detected with 10% H₂SO₄ followed by heating. Column chromatography was carried out on Sephadex LH-20 (Amersham Pharmacia Biotech UK, Ltd., England) and DIAION HP-20 (Mitsubishi Chem. Ind., Co., Ltd., Tokyo, Japan).

Plant Material The dried leaves of Peppermint (*Mentha*

piperita L.) were purchased from TRI-WEST SPECIALTIES, L.L.C. (WA, U.S.A.).

Chemicals The chemicals used and their sources were as follows: Aluminum hydroxide (LSL Co., Tokyo, Japan), compound 48/80 (Sigma, St. Louis, MO, U.S.A.), gum arabic (Wako, Tokyo, Japan), *o*-phthalaldehyde (Sigma), luteolin (Funakoshi, Tokyo, Japan), luteolin-7-*O*-glucoside (Funakoshi), ovalbumin (Sigma), oxatomide (Kyowa Hakko Kogyo Co., Ltd., Shizuoka, Japan), L- α -phosphatidyl-L-serine (Sigma), tranilast (Kissei Pharmaceuticals Co., Ltd., Nagano, Japan).

Extraction and Isolation First, the essential oil was removed from the peppermint leaves and stems by steam distillation, and the residual leaves and stems were dried. The dried powdered leaves and stems (5 kg) were then extracted by refluxing in water (75 l, 1 h). The filtrate was evaporated under reduced pressure to yield a dark brown extract (1230 g). The extract was suspended in 50% EtOH and then defatted with *n*-hexane (1 l $\times$ 6). After removal of the solvent, the residue that dissolved in H₂O was subjected to chromatography on DIAION HP-20 (100 mm i.d., 3 l) and successively eluted with 7 l of H₂O, 50% EtOH and EtOH. A part of the 50% EtOH eluate was further purified by column chromatography on Sephadex LH-20 (30 mm i.d., 500 ml) and eluted with increasing amounts of methanol in H₂O (1:1–9:1, 5 l). Fractions of 100 ml each were collected and examined by silica gel TLC (CHCl₃:MeOH:AcOH:H₂O=60:20:15:5). Fractions with similar *R*_f (TLC) values were combined and concentrated to afford fractions A–E. Fraction C was rechromatographed by HPLC (50 mm i.d. column, 500 mm length, ODS-W 15–30 μ m, solvent 0 min: 45% MeOH, 22 min: 50% MeOH, flow: 100 ml/min) to yield 5 fractions, C-1, C-2, C-3, C-4 and C-5. A part of each fraction was further purified by repeated HPLC to give **1** (6570 mg), **2** (36 mg), **3** (230 mg), **4** (3250 mg), **5** (41 mg), **6** (93 mg) **7** (890 mg) and **8** (420 mg), respectively.

Animals Male Wistar strain rats (age: 7 weeks; body weight: 190–220 g) were obtained from Shimizu Laboratory Supplies (Kyoto, Japan). All of animals were maintained in an air-conditioned room at the controlled temperature of 24 $\pm$ 2 °C and humidity of 45 $\pm$ 15%. The animals were al-

* To whom correspondence should be addressed. e-mail: kamei@pheasant.pharm.okayama-u.ac.jp

lowed food and water *ad libitum*.

Compound 48/80-Induced Histamine Release from Isolated Rat Peritoneal Mast Cells Briefly, rat peritoneal mast cells were harvested from the abdominal cavity of the male rats and purified by Percoll density centrifugation. Physiological buffered saline (PBS; in mM: NaCl 154, KCl 2.7, CaCl₂ 5, N-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) 5; pH 7.4) was injected into the peritoneal cavity. The abdominal region was gently massaged for 90 s and peritoneal fluid was collected and centrifuged for 7 min at 100×*g*, 4 °C. After centrifugation, the cell pellets were pooled and washed twice in PBS(−) solution. The mast cell pellets purified by Percoll density centrifugation for 15 min at 200×*g*, 4 °C were washed twice in PBS(−) solution. Thereafter, equal numbers of mast cells (2.5×10⁶ cells/tube) were preincubated in 0.8 ml of 0.1% glucose containing physiological salt solution [PBS(+)] for 10 min at 37 °C. The test drugs dissolved in PBS(+) were added (0.1 ml) 10 min before compound 48/80 (final concentration: 0.5 μg/ml). The reaction was stopped 10 min later by cooling the tubes in ice water. The tubes were centrifuged for 15 min at 200×*g* and the histamine content was measured in the supernatant and precipitate using an autoanalyzer as previously reported.⁸⁾

Antigen-Induced Histamine Release from Isolated Rat Peritoneal Mast Cells Equal numbers of mast cells (2.5×10⁶ cells/tube) were preincubated in 0.7 ml of 0.1% glucose containing physiological salt solution [PBS(+)] for 10 min at 37 °C. Thereafter, L- α -phosphatidyl-L-serine (final concentration: 1 μg/ml) suspended in PBS(+) was added (0.1 ml) and preincubated for 5 min. The test drugs dissolved in PBS(+) were added (0.1 ml) 10 min before ovalbumin (final concentration: 10 μg/ml). The reaction was stopped 10 min later by cooling the tubes in ice water. The following procedure is the same as that of the method already described.⁸⁾

Nasal Symptoms Induced by the Antigen Rats were actively sensitized by an injection of physiological saline containing 1 mg ovalbumin, 10¹⁰ cells of *B. pertussis* and 2 mg of aluminum hydroxide into the four foot pads on the first day. Five days later, they were boosted by a subcutaneous injection of 1 ml of physiological saline containing 0.5 mg ovalbumin into the rostral parts of the back. Fourteen days later, local sensitization was performed every day by dripping ovalbumin in saline (1 mg/ml, 10 μl/nostril) into the bilateral nasal cavities using a micropipette. The effects of the drugs on the nasal symptoms after antigen challenge were evaluated with rats from 28 to 42 d old after general sensitization. Before the experiment, the animals were placed in an observation cage (32×24×18 cm) for 10 min for acclimatization. After nasal instillation of 10 μl of antigen dissolved in saline (1 mg/ml) into the bilateral nasal cavities, the animals were placed in the observation cage (1 animal/cage), and sneezing and nasal rubbing were counted for 30 min. The drugs were orally administered 1 h before the antigen injection.

Nasal Symptoms Induced by Histamine Before the experiment, the animals were placed in an observation cage (32×24×18 cm) for 10 min for acclimatization. After nasal instillation of 10 μl (10 μmol/site) of histamine dissolved in saline into the bilateral nasal cavities, the animals were placed in the observation cage (1 animal/cage) and sneezing

and nasal rubbing were counted for 30 min. The drugs were orally administered 1 h before the histamine injection.

Statistical Analysis The data are presented as means±S.E.M. Statistical significance was tested by one-way analysis of variance using Dunnett's test. A probability value less than 0.05 was considered significant. The IC₅₀ values were calculated according to the probit method.

RESULTS AND DISCUSSION

Our previous paper revealed that the 50% EtOH extract of peppermint significantly inhibited histamine release from rat peritoneal mast cells. In addition, the 50% EtOH eluate separated by DIAION HP-20 column chromatography was more effective than the 50% EtOH extract of peppermint and showed a hydrophobic property. On the other hand, hexane and the other hydrophilic fractions had less effect.⁷⁾ Therefore, it seems likely that the active compounds contained in this fraction were quite soluble in water. In the present study, therefore, the extracting solvent was replaced by hot water instead of 50% EtOH. The hot water extract of *Mentha piperita* L. was further separated into water, 50% EtOH, EtOH and acetone eluates by DIAION HP-20 column chromatography. From the 50% EtOH eluate, 8 compounds, *i.e.*, eriocitrin (**1**), narirutin (**2**), hesperidin (**3**), luteolin-7-*O*-rutinoside (**4**), isorhoifolin (**5**), diosmin (**6**), rosmarinic acid (**7**) and 5,7-dihydroxycromone-7-*O*-rutinoside (**8**) were isolated (Fig. 1). The chemical structures of these compounds were elucidated by NMR including distortionless enhancement by polarization transfer (DEPT), ¹H-¹H-correlation spectroscopy (COSY), ¹H-detected heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC) experiments. These compounds **1**—**7** had already been identified by Didier and Bernard.¹⁾ If steam distillation was not used, compound **8** was not found out. Therefore, it was considered that compound **8** might be formed by the thermal decomposition of compound **1** during the steam distillation.⁹⁾

To determine the antiallergic properties of these compounds, the study of histamine release from rat peritoneal mast cells induced by compound 48/80 in rats was used. As shown in Fig. 2, the histamine release in the control group induced by compound 48/80 (0.5 μg/ml) was 36.8±4.4% (*n*=6) of the total content. Compound **4** dose-dependently inhibited the histamine release and significant differences

Fig. 1. The Chemical Structures of Compounds **1**—**8**

Fig. 2. Effects of Compounds 1–6 on Histamine Release from Rat Peritoneal Mast Cells Induced by Compound 48/80 (0.5 µg/ml)

Each column and vertical bar represents the mean $\pm$ S.E.M. for 6 animals. **, $p < 0.01$ compared with the control group (Dunnett's test).

Fig. 3. Effect of Compound 4 on Histamine Release from Rat Peritoneal Mast Cells Induced by Antigen

Each column and vertical bar represents the mean $\pm$ S.E.M. for 8 animals. *, $p < 0.05$ and **, $p < 0.01$ compared with the control group (Dunnett's test).

were observed at concentrations of 30 µM or more. However, the other compounds had no effect on the histamine release even at a dose of 100 µM. Moreover, compound 4 showed the dose-dependent inhibition on histamine release induced by antigen and significant differences were observed at 30 µM or more (Fig. 3). Kimata *et al.* reported that luteolin, which is the aglycon of compound 4, is a potent inhibitor of human mast cell activation induced by stimulation of A23187 and antihuman IgE.¹⁰ As shown in rat present study, compound 4 also showed an inhibition of histamine release induced by compound 48/80 and antigen from peritoneal mast cells. In addition, Amellal *et al.* have reported that the catechol structure in the B ring and the C2–C3 double bond in the C ring is essential for the inhibition of the histamine release.¹¹ Therefore, it seems reasonable that compound 4 had a potent inhibitory effect on the histamine release from rat peritoneal mast cells. However, the role of the sugar moiety at the C-7 position is unclear. The effects of the sugar moiety attached to the C-7 position in the A ring were then studied by using aglycon (luteolin) and the compounds having a sugar at the C-7 position of luteolin (luteolin-7-*O*-glucoside and com-

Table 1. IC₅₀ Values of Different Flavonoids for the Histamine Release from Rat Peritoneal Mast Cells Induced by Compound 48/80 (0.5 µg/ml)

Compounds	IC ₅₀ values (95 % confidence limits) (µM)
Luteolin	54.4 (54.2–54.6)
Luteolin-7- <i>O</i> -glucoside	41.6 (32.2–51.0)
Compound 4 (luteolin-7- <i>O</i> -rutinoside)	21.9 (19.9–24.1)

Fig. 4. Effects of Compound 4 on Sneezing (A) and Nasal Rubbing (B) Induced by Antigen in Rats

Each column and vertical bar represents the mean $\pm$ S.E.M. for 9 animals. *, $p < 0.05$ and **, $p < 0.01$ compared with the control group (Dunnett's test).

pound 4). As a result, luteolin, luteolin-7-*O*-glucoside and compound 4 caused a dose-dependent inhibition of histamine release. The IC₅₀ value for the luteolin was 54.4 (54.2–54.6) µM. Compound 4 was more effective than luteolin and luteolin-7-*O*-glucoside in inhibiting histamine release (Table 1). From these results, it is considered that the presence of the sugar moiety at the C-7 position in the A ring might enhance the inhibition of histamine release.

Recently, we developed a new animal model for allergic rhinitis; sneezing and nasal rubbing in this model are useful for evaluating the effects of antiallergic drugs on allergic rhinitis.¹² Therefore, effects of the compound 4 on the antigen-induced nasal symptoms in rats were studied, and the results are shown in Fig. 4. The sneezing frequency in the control induced by the antigen was 39.9 $\pm$ 5.2 times/30 min. Compound 4 caused a dose-related inhibition of this response and significant effects were observed at doses of 100 and 300 mg/kg. Tranilast inhibited the antigen-induced sneezing at a dose of 300 mg/kg. Nasal rubbing was also significantly inhibited by compound 4 at doses of 100 mg/kg or more. Tranilast significantly inhibited this response at a dose of 300 mg/kg. In summary, compound 4 caused an inhibition not only histamine release but also nasal symptoms induced by antigen. Therefore, it seems likely that antiallergic effects of compound 4 are attributable to antigen-antibody reaction.

Saito *et al.* proposed that chemical mediators, including histamine, released from the mast cells caused sneezing and

Fig. 5. Effects of Compound 4 on Sneezing (A) and Nasal Rubbing (B) Induced by Histamine (10 μ mol/Site)

Each column and vertical bar represents the mean $\pm$ S.E.M. for 10 animals. **; $p < 0.01$ compared with the control group (Dunnett's test).

nasal rubbing.¹³⁾ Therefore, whether or not compound 4 showed an inhibitory effect on sneezing and nasal rubbing induced by histamine in rats was studied. As shown in Fig. 5, compound 4 showed no significant effect on sneezing or nasal rubbing induced by histamine even at a dose of 300 mg/kg. Oxatomide, which showed both anti-histaminergic activity and an inhibition of histamine release,¹⁴⁾ inhibited the histamine-induced sneezing at a dose of 30 mg/kg.

However, compound 4 only showed an inhibited histamine release from rat peritoneal mast cells. Therefore, the inhibitory effects of compound 4 on sneezing and nasal rubbing induced by antigen challenge may be responsible for the inhibition of histamine release from mast cells in the nasal mucosa.

These results indicated that compound 4 is an active substance of *Mentha piperita* L. and may be effective in alleviating the nasal symptoms of allergic rhinitis in clinical trials.

REFERENCES

- 1) Didier J. G., Bernard P. P., *J. Agric. Food Chem.*, **42**, 679—684 (1994).
- 2) Juergens U. R., Stober M., Vetter H., *Eur. J. Med. Res.*, **3**, 539—545 (1998).
- 3) Tassou C. C., Drosinos E. H., Nychas G. J., *J. Appl. Bacteriol.*, **78**, 593—600 (1995).
- 4) Sarbhoy A. K., Varshney J. L., Maheshwari M. L., Saxena D. B., *Zbl. Bakt. II*, **133**, 723—725 (1978).
- 5) Hoffmann B. G., Lunder L. T., *Planta Med.*, **50**, 361 (1984).
- 6) Duband F., Carnat A. P., Carnat A., Petitjean-Freytet C., Clair G., Lamaison J. L., *Ann. Pharm. Fr.*, **50**, 146—155 (1992).
- 7) Inoue T., Sugimoto Y., Masuda H., Kamei C., *Biol. Pharm. Bull.*, **24**, 92—95 (2001).
- 8) Zhao Q. E., Mihara T., Sugimoto Y., Kamei C., *Biol. Pharm. Bull.*, **19**, 237—240 (1996).
- 9) Stoker M., Pohl R., *Phytochemistry*, **15**, 571—572 (1976).
- 10) Kimata M., Shichijo M., Miura T., Serizawa I., Inagaki N., Nagai H., *Clin. Exp. Allergy*, **30**, 501—508 (2000).
- 11) Amellal M., Bronner C., Briancon F., Haag M., Anton R., Landry Y., *Planta Med.*, **1**, 16—20 (1985).
- 12) Sugimoto Y., Kawamoto E., Chen Z., Kamei C., *Immunopharmacology*, **48**, 1—7 (2000).
- 13) Saito H., Asakura K., Kataura A., *Arerugi*, **43**, 579—584 (1994).
- 14) Patella V., de Crescenzo G., Marino O., Spadaro G., Genovese A., Marone G., *Int. Arch. Allergy Immunol.*, **111**, 23—29 (1996).