Luteolin as an Anti-inflammatory and Anti-allergic Constituent of *Perilla frutescens*

Hiroshi Ueda, Chikako Yamazaki, and Masatoshi Yamazaki*

Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University; 1091–1 Suwarashi, Sagamiko-machi, Tsukui-gun, Kanagawa 199–0195, Japan.   Received April 15, 2002; accepted May 27, 2002

Oral administration of the perilla leaf extract (PLE) to mice inhibits inflammation, allergic response, and tumor necrosis factor-α production. We also found that PLE suppressed the tumor necrosis factor-α (TNF-α) production *in vitro*. Using the inhibitory activity of TNF-α production *in vitro* as the index for isolation, we searched the active constituents from PLE and isolated luteolin, rosmarinic acid and caffeic acid as active components. Among the isolated compounds, only luteolin showed *in vivo* activity: inhibition of serum tumor necrosis factor-α production, inhibition of arachidonic acid-induced ear edema, inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-induced ear edema and inhibition of oxazolone-induced allergic edema. These results suggest that luteolin is a genuinely active constituent which is accountable for the oral effects of perilla.

Key words   *Perilla frutescens*; luteolin; anti-inflammation; anti-allergy; tumor necrosis factor-α

The leaves of *Perilla frutescens* are used as a garnish with raw fish in Japan. It is believed that the aim of this use is not only as a flavor but also as an antidote to food poisoning.[1] Dried red perilla leaves are also used as 'soyou' in Chinese herbal medicine and it is one of the components of 'saiboku-to,' which is used to treat bronchial asthma. In a previous study, we reported that oral administration of a perilla leaf extract (PLE) to mice can inhibit the overproduction of tumor necrosis factor-α (TNF-α)[2] and shows anti-inflammatory and anti-allergic activity.[3] Perilla leaf extract has also been reported to suppress anti-DNP IgE production,[4] Th2-type cytokine production,[5] systemic allergic reaction induced by compound 48/80,[6] and IgA nephropathy.[7] Most of these clearly confirm our observations. However, these are parenteral effects and the active constituents were not well identified. Terpenoids, phenolics, flavonoids, cyanogenic glycosides, and anthocyanins have been reported as the chemical constituents of *Perilla frutescens*, but there has been no suggestion about the oral pharmacological effects of this plant. We investigated here the active constituents which have capability to inhibit TNF-α production, and inflammatory and allergic reactions when administered orally.

MATERIALS AND METHODS

Mice   Male ICR mice (4 or 6 weeks old) were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). The animals were given a standard laboratory diet and water *ad libitum*. The experiments were done under the control of the guidelines for Animal Experiments under the Law (No. 105) and Notification (No. 6) of the government.

Perilla Leaf Extract (PLE)   Dried leaves of a green type of perilla (*Perilla frutescens* (L.) Britton var. acuta Kudo forma viridis Makino; 5 g) were soaked in 5 ml of distilled water for 1 h and then homogenized for 10 min with Polytron equipment (Kinematika, Switzerland) at a power setting of 5. The homogenate was filtered through nylon mesh and centrifuged at 7000 *g* for 10 min at 4 °C. The resulting supernatant was passed through a membrane filter with a 0.45 μm pore size (Millipore, Tokyo, Japan) and 4.4 ml of PLE was acquired. This process was repeated many times to gain the sample for isolation.

Isolation of Active Constituents from PLE   5.92 g of exsiccated PLE, which was obtained from 253 ml of decoction, was dissolved with 5.92 ml of water and applied to reverse phase chromatography, MCI gel CHP 20P (28.5× 2.9 cm; Mitsubishi Chemical Co., Tokyo). The column was successively eluted with water (W), 50% methanol (WM), methanol (M), and 50% acetone (WA). These elutions were investigated for the dose response of the inhibitory activity of TNF-α production *in vitro* and their ED_{50} calculated.

The WM fraction was further extracted with 50% ethyl acetate (WM-EA) and fractionated by MCI gel column chromatography, which successively used 100 ml of 0, 15, 30, 45, and 60% of methanol to give Fr. A—E.

The M fraction was further extracted with ether (M-E) and subjected to silica gel column chromatography (benzene/acetone 97 : 3→49:51) to give Fr. 1—7.

The *Rf* values of TLC (Merck Kiesel gel F$_{254}$, *n*-butanol/ ethyl acetate/water 2 : 1 : 1) of each fraction were also checked step by step. The active fractions, which can be assumed to be fully purified, were analyzed by ^{1}H-, ^{13}C-NMR and IR data. PLE was applied to HPLC (Inertsil ODS-2, 250×4.6 mm i.d., GL Science, Tokyo; mobile phase, 1% ethyl acetate/acetonitrile 9 : 1→7 : 3, 0→30 min linear gradient; flow rate, 1 ml/1min; temperature, 25 °C; detection, UV absorption at 310 nm) for quantitative analysis.

Chemical Reagents   Romurtide was geneously provided by Daiichi Pharmaceutical (Tokyo) and was dissolved in water just before use. OK-432 (Picibanel) was supplied by Chugai Pharmaceutical (Tokyo) and was dissolved in saline just before use. The unit 1 KE, which was used to define the dose of OK-432, means that the reagent contained 100 μg of killed *Streptococcus pyogenes* Su.

12-*O*-Tetradecanoylphorbol-13-acetate (TPA), arachidonic acid (AA), and oxazolone (Ox) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetone and ethanol were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cells and Media   L929, a transformed murine fibroblast cell line, was grown in Eagle's minimum essential medium (Nissui Seiyaku Co., Tokyo) supplemented with 5% calf

serum (Hyclone Laboratories, Logan, Utah, U.S.A.) and passaged twice a week. RPMI1640 was also purchased from Nissui Seiyaku Co. and used for incubation of peritoneal cells.

Measurement of TNF-α Inhibitory Activity *in Vitro* Male ICR mice were intraperitoneally injected with 400 μl of 1% glycogen (Wako Pure Chemicals Industries) solution and peritoneal exudate cells were obtained after 18 h. The obtained peritoneal cells were cultured in 96-well plates at 2.0×10^5 cells in 200 μl of 1% FCS-RPMI/wells at 37°C in 5% CO_2 for 4 h. The wells were washed three times with phosphate buffered saline ($-$) and 200 μl of 5% FCS-RPMI containing test samples was added. One hour later, the wells were washed again with PBS ($-$) and were stimulated with 1 μg/ml of *Escherichia coli* O127:B8 lipopolysaccharide (LPS) (Difco, Michigan, U.S.A.) dissolved in 200 μl of 5% FCS-RPMI. Two hours later, TNF-α activity in the culture supernatants was evaluated by *in vitro* L929 cell cytotoxicity assay[8] using recombinant human TNF-α (National Biological Standards Board, Hertfordshire, U.K.) as an international standard.

Measurement of TNF-α Inhibitory Activity *in Vivo* The oral effect of perilla components on systemic TNF-α production was evaluated by the method previously reported.[2] Briefly, mice were orally administered romurtide (500 μg/200 μl/mouse) as a priming agent, and 3 h later were intravenously injected with OK-432 (3 KE/200 μl/mouse) as a triggering agent. Sample was orally administered with romurtide and the effect on TNF-α production was observed. Two hours after triggering, the animals were bled to obtain serum, and the serum was stored at -80°C. The TNF-α activity of the serum was evaluated by the *in vitro* L929 cell cytotoxicity with the method of Ruff and Gifford,[8] using international standard recombinant human TNF-α (National Biological Standards Board, Hertfordshire, U.K.).

Anti-inflammatory Test for AA-Induced Ear Edema AA-induced ear swelling in mice was caused by the method of Young *et al.*,[9] with modification. AA was dissolved in cold acetone at a concentration of 12.5 mg/ml and stored at -20°C until use.

Male ICR mice were orally administered test sample and 18 h later were again orally administered it. Three hours after the second administration, 20 μl of AA solution (12.5 mg/ml) was spread on both sides of the right ear of each mouse. Two hours later, ear swellings were determined by weighing the pieces of the ears obtained with a 5 mm diameter punch.

Anti-inflammatory Test for TPA-Induced Ear Edema TPA-induced ear swelling in mice was also performed by the method of Young *et al.*,[10] with modification. TPA was dissolved in cold acetone at a concentration of 400 μg/ml and stored at -20°C. TPA stock solution was diluted with additional cold acetone at 10 μg/ml just before use.

To induce irritant dermatitis, 20 μl of TPA solution (10 μg/ml) in acetone was spread on both sides of an ear of mice which had been orally administered a sample 3 and 21 h earlier. Four hours later, ear swelling was determined by weighing the pieces of the ears obtained with a 5 mm diameter punch.

Anti-allergic Test for Oxazolone-Induced Ear Edema Oxazolone-induced ear swelling in mice was also achieved by the method of Dietrich and Hess,[11] with modification.

Oxazolone was dissolved at a concentration of 5 mg/ml in ethanol for sensitization or in cold acetone for a challenge.

The abdominal region of mice was shaved with electric clippers. One day later, the mice were orally administered the sample, and three hours there after were sensitized by application of 100 μl of oxazolone ethanol solution to the abdominal region. After 7 d, the mice were challenged by spreading 20 μl of cold oxazolone acetone solution on either side of the ear. Twenty-four hours after the challenge, ear swellings were determined by weighing the pieces of the ears obtained with the 5 mm diameter punch.

Statistics The statistical difference from the control value was analyzed by Student's *t*-test or Dunnett test.

RESULTS

Identification of the Active Constituent(s) of Perilla Which Can Inhibit TNF-α Production *in Vitro* In our previous study we found that oral administration of PLE can inhibit systemic TNF-α production[2] and inflammatory and allergic ear edemas.[3] The purpose of this study was to determine the active constituent(s) which possess these activities.

We first investigated whether PLE can inhibit TNF-α production from LPS-stimulated peritoneal macrophage *in vitro* (Fig. 1). Glycogen-induced murine peritoneal macrophages were stimulated only by LPS and the TNF-α activity in culture supernatant was 5.5 U/ml. Pretreatment with PLE (2.5—20%) inhibited the TNF-α production in a dose dependent manner. Using the inhibitory activity of the TNF-α production *in vitro* as an index of purification of active components, we tried to isolate the active compounds.

The isolation procedure for active constituents of PLE is shown in Fig. 2. PLE (253 ml) was concentrated in vacuum with an evaporator and 5.92 g of exsiccated PLE was obtained. It was dissolved with 5.92 ml of water and applied to reverse phase chromatography with MCI gel CHP 20P (28.5×2.9 cm). The column was eluted successively with 890 ml of water (W), 400 ml of 50% methanol (WM), 400 ml of methanol (M), and 400 ml of 50% acetone (WA). Each
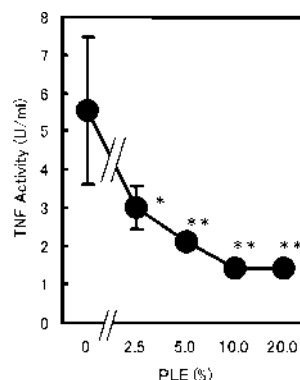

Fig. 1. Inhibition of TNF Production from Macrophage by Perilla

Male ICR mice were intraperitoneally injected with 400 μl of 1% glycogen solution and peritoneal exudate cells were obtained after 18 h. These cells were cultured in 96-well plates at 2.0×10^5 cells/200 μl/well at 37°C in 5% CO_2 for 4 h. The wells were washed three times with PBS ($-$) and 200 μl of 5% FCS-RPMI containing PLE (2.5—20.0%) was added. One hour later, the wells were washed again with PBS ($-$) and were stimulated with 1 μg/ml of *E. coli* O127:B8 lipopolysaccharide (LPS) dissolved in 200 μl of 5% FCS-RPMI. Two hours later, TNF-α activity in the culture supernatants was evaluated by *in vitro* L929 cell cytotoxicity assay as described in Materials and Methods. Each point indicates the mean±S.D. Significant differences from the control: *$p < 0.10$, **$p < 0.05$ by Dunnet test.
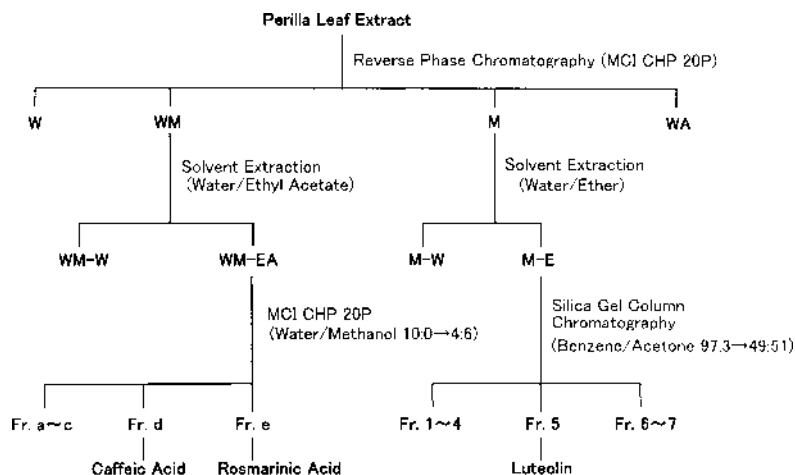
Fig. 2.   Isolation of the Active Constituents from Perilla Leaf Extract

PLE was applied to reverse phase chromatography with MCI CHP 20P. The methanol fraction (M) was further fractionated by 4 step silica gel chromatography which used ether, benzene–ethyl acetate, chloroform–methanol and benzene–ethyl acetate again. The 50% methanol (W : M) fraction was also further extracted with 50% ethyl acetate and gel filtration chromatography with Sephadex LH-20. Each fraction was investigated for its *in vitro* TNF-α inhibitory activity. The final purified fractions were analyzed by ^{13}C-NMR and the components were identified.

elution was evaporated and 3.30 g (W), 0.72 g (WM), 0.22 g (M), 0.12 g (WA) of exsiccation was obtained. In each fraction the dose response of the inhibitory activity of TNF-α production *in vitro* was determined and the ED$_{50}$ calculated as the index for isolation. The ED$_{50}$ of TNF-α inhibitory activities was 1200 μg/ml (W), 100 μg/ml (WM), 130 μg/ml (M) and 350 μg/ml (WA), respectively. Those fractions were also checked by TLC (silica gel plates in *n*-butanol : acetic acid : water 2 : 1 : 1), but it was proved that some constituents were present in these fractions (data not shown). We therefore further fractionated the WM and M fractions which were suggested to contain the active constituent.

The WM fraction was further extracted with 50% of ethyl acetate; the activity was detected in this fraction (WM-EA; 115.2 mg) and it was further purified by MCI gel column chromatography, which used 100 ml of 0, 15, 30, 45, 60% of methanol, respectively. TLC analysis revealed that the fractions WM-EA-d (ED$_{50}$=33 μg/ml) and WM-EA-e (ED$_{50}$= 15 μg/ml) were sufficiently purified (data not shown). Further, ^{13}C-NMR analysis revealed that the main constituent is caffeic acid in the WM-EA-d fraction and rosmarinic acid in the WM-EA-e fraction by comparison with the literature data. HPLC analysis also gave rosmarinic acid (t_R=23 min; isolated amount, 14.9 mg) and caffeic acid (t_R=14 min; isolated amount, 3.2 mg) in comparison with the standard substance. Thus it was also suggested that caffeic acid and rosmarinic acid are candidates as the active constituents.

The M fractions were dissolved with water and further fractionated with ether. The activity was detected in the ether fraction (M-E) and it was further purified by silica gel column chromatography (benzene/acetone 97 : 3→49 : 51) resulting in finding of obtaining of the fraction M-E-5, which showed 39 μg/ml of ED$_{50}$. TLC analysis of the extract revealed the presence of flavonoids as its major constituents and ^{13}C-NMR analysis revealed luteolin as its only component in a comparison with the literature data. HPLC (MCI CHP 20P) separation of fraction M-E-5 also gave luteolin (t_R=8.3 min; isolated amount, 8.0 mg) as its major constituent in comparison with the standard substance. Thus it was suggested that luteolin is one of the active constituents.
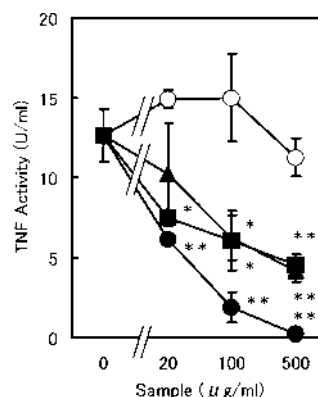

Fig. 3.   Inhibition of TNF Production from Macrophage by Perilla Constituents

Male ICR mice were intraperitoneally injected with 400 μl of 1% glycogen solution and peritoneal exudate cells were obtained after 18 h. These cells were cultured in 96-well plates at 2.0×10^5 cells/200 μl/well at 37 °C in 5% CO$_2$ for 4 h. The wells were washed three times with PBS ($-$) and were added with 200 μl of 5% FCS-RPMI containing 20—500 μg/ml of test samples (●, luteolin; ○, luteolin 7-*O*-β-glucuronide; ▲, caffeic acid; ■, rosmarinic acid). One hour later, the wells were washed again with PBS ($-$) and were stimulated with 1 μg/ml of *E. coli* O127 : B8 Lipopolysaccharide (LPS) dissolved in 200 μl of 5% FCS-RPMI. Two hours later, TNF-α activity in the culture supernatants was evaluated by the *in vitro* L929 cell cytotoxicity. Each data point indicates the mean±S.D. Significant differences from the control: $*$ $p<0.10$, $**$ $p<0.05$ by Dunnet test.

We did not investigate only the purified samples but also commercially pure substances for their *in vitro* TNF-α inhibitory activity (Fig. 3). When peritoneal macrophages were pre-treated with caffeic acid, rosmarinic acid, or luteolin at 20—500 μg/ml for 1 h, these components inhibited the LPS-stimulated TNF-α production in a dose dependent manner. Further HPLC analysis of the WM-W fraction revealed the existence of luteolin 7-*O*-β-glucuronide. Our investigation of the isolated glucoside, found that it has no activity.

Inhibitory Activity of the Isolated Compound on TNF-α Production *in Vivo*   When mice were primed with an oral administration of romurtide (500 μg/mouse) and then triggered with an intravenous injection of OK-432 (3 KE/mouse), TNF-α was induced in the serum.[2] When mice were administered luteolin (1—1000 μg/mouse) with romurtide, TNF-α production was significantly inhibited in a dose-de-

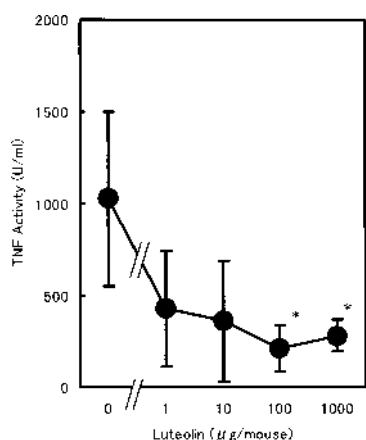Fig. 4. Inhibition of Serum TNF Production by Oral Administration of Luteolin

Male ICR mice ($n=3$) were orally administered romurtide (500 μg/400 μl/mouse) and luteolin (1—1000 μg/400 μl/mouse). Three hours later, the mice were injected intravenously with OK-432 (3 KE/200 μl/mouse). Two hours later, the animals were bled and the serum TNF-α activity was measured by an L929 cell cytotoxicity assay. Experiments were done three times with comparable results. The statistical difference was analyzed by Dunnet test. Significant differences from the control: * $p<0.05$.

Fig. 6. Inhibition of TPA-Induced Ear Edema by Orally Administered Perilla Constituents

Male ICR mice ($n=10$) were orally administered samples (closed bar; 1 mg/400 μl) or distilled water (open bar) and 18 h later the oral administrations were repeated. Three hours later, 200 ng of TPA was spread on both sides of the right ear of both groups of mice. The control group was not treated (hatched bar). Four hours later, ear swelling was determined by weighing the pieces of the ears obtained with a 5 mm diameter punch. Data are the mean±S.D. Statistical difference was analyzed by Student's t-test. Significant differences from the control: * $p<0.02$.
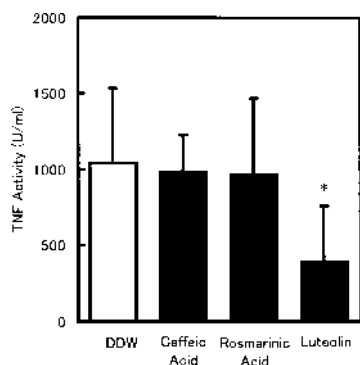

Fig. 5. Orally Administered Luteolin, But Not Caffeic Acid or Rosmarinic Acid Inhibits Systemic TNF Production

Male ICR mice ($n=3$) were orally administered romurtide (500 μg/400 μl/mouse) and test samples (closed bar; 1 mg/400 μl/mouse) or DDW (open bar). Three hours later, the mice were injected intravenously with OK-432 (3 KE/200 μl/mouse). Two hours later, the animals were bled and the serum TNF-α activity was measured by L929 cell cytotoxicity assay. Data are the mean±S.D. Experiments were done three times with comparable results. The statistical difference was analyzed by Dunnet test. Significant differences from the control: * $p<0.10$.

Fig. 7. Inhibition of AA-Induced Ear Edema by Orally Administered Luteolin

Male ICR mice ($n=10$) were orally administered luteolin (closed bar; 1 mg/400 μl) or distilled water (open bar) and 18 h later the oral administrations were repeated. Three hours later, both sides of the right ear of both groups of mice were spread with 250 μg of AA. The control group was not treated (hatched bar). Two hours later, the cut pieces of the ears were weighed. Data are the mean±S.D. Statistical difference was analyzed by Student's t-test. Significant differences from the control: * $p<0.02$.

pendent manner (Fig. 4). Caffeic acid and rosmarinic acid (1 mg/mouse), however, did not inhibit TNF-α production *in vivo* despite their positive action *in vitro* (Fig. 5).

Inhibitory Activity of the Isolated Compounds on Ear Edema We have also shown that oral administration of PLE can inhibit inflammatory and allergic ear edemas. Therefore, we were interested in whether oral administration of these substances could also inhibit inflammatory and allergic ear edema. We selected AA-induced ear edema and TPA-induced ear edema as experimental inflammatory models. These models are highly sensitive to non-steroidal anti-inflammatory drugs, and have long been accepted as useful phlogistic tools for investigating new anti-inflammatory drugs.

Figure 6 shows the preventive effect of orally administered luteolin on the murine ear inflammation which was provoked by topical application of TPA. In our experimental protocol, oral administration of luteolin was done as pretreatment
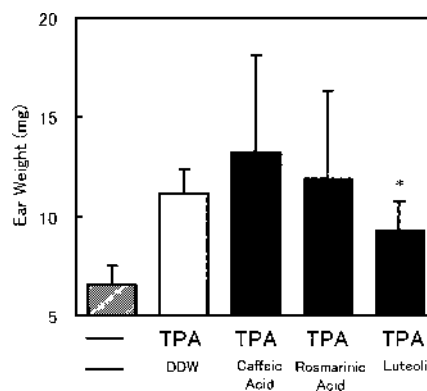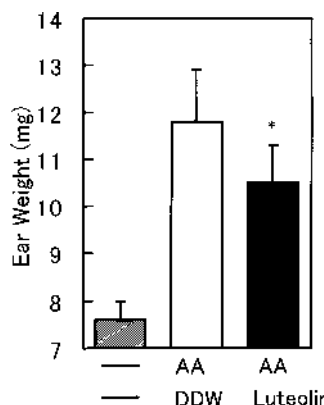
against any inflammatory stimulant. This protocol is based on the results of PLE.[2,3] Our previous study revealed that only 3 h pretreatment of PLE or luteolin was effective and that combined use of 3 and 21 h pretreatment further augmented the effectiveness.

The weight of a cut piece of untreated murine ear was 6.6±0.9 mg and it increased to 11.2±1.2 mg 4 h after topical application of TPA on the ear. When luteolin was orally administered to mice 3 h before topical application, weight of the piece of the ear significantly decreased to 9.3±1.5 mg ($p<0.02$).

Oral administration of luteolin also inhibited AA-induced ear edema (Fig. 7). Weight of the cut piece of the ear was 11.8±1.1 mg 2 h after topical application of AA. When luteolin was orally administered to mice 3 h before topical application, the weight significantly decreased to 10.5±0.8 mg ($p<0.02$).

The oral effect of luteolin on sensitization was of interest, because our previous kinetic study had shown that oral ad-
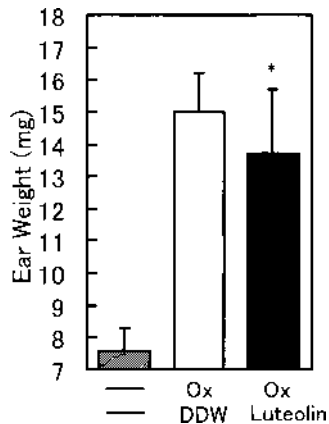
Fig. 8. Inhibition of Oxazolone-Induced Ear Edema by Orally Administered Luteolin

Male ICR mice ($n=10$) were sensitized by application with oxazolone (500 μg) in the abdominal region and after 7 d were challenged by spreading oxazolone (100 μg) on the ear. Luteolin (closed bars; 1 mg/mouse) or DDW (open bar) was orally administered 3 h before the sensitization. Twenty-four hours after the challenge, ear swelling was determined by weighing the cut pieces of the ears. No treatment was made to the control group (hatched bar). Data are the mean±S.D. Statistical difference was analyzed by Student's t-test. Significant differences from the control: * $p<0.05$.

ministration of PLE inhibits sensitization but not challenge. When luteolin was orally administered to mice 3 h before sensitization, the ear edema was decreased (Fig. 8). Luteolin inhibited oxazolone-induced ear edema which consisted of type IV allergy.

The flare and itching was also inhibited by oral administration of luteolin on both inflammatory and allergic models. Luteolin possessed anti-inflammatory and anti-allergic actions, but caffeic acid and rosmarinic acid could not inhibit these ear edemas (data not shown).

DISCUSSION

We found here that PLE suppressed the TNF-α production *in vitro* and searched the active constituents of PLE to use this activity as an index for isolation. We isolated luteolin, rosmarinic acid and caffeic acid as constituents which can inhibit TNF-α production *in vitro*. Among the isolated compounds, only luteolin showed *in vivo* TNF-α inhibitory activity and anti-inflammatory and anti-allergic activity when orally administered to mouse. We therefore believe that luteolin is the genuinely active constituent which is consistently accountable for the oral effects of perilla.

Luteolin content was 9—13 μg/ml in PLE (data not shown) and generally consistent with the dose response of PLE. There is little doubt that luteolin is the main constituent responsible for the oral effect of perilla, but other constituents which have only slight activity alone may also support some combined effects, because the activity of luteolin is slightly weaker than PLE.

We isolated luteolin-7-*O*-β-glucuronide and luteolin-7-*O*-[β-glucuronosyl-(1→2)-β-glucuronide] in addition to luteolin aglycone from PLE. These glycosides were present in an amount approximately 100 times higher than aglycone in PLE (data not shown), but had no activity. Apigenin, scutellarein, and their glycosides were also obtained from Perilla.[12—14] We investigated the effects of various types of flavonoids which have a structure similar to luteolin, *e.g.*

quercetin and apigenin, but these had no activity (manuscript in preparation). These results suggest that the structure of luteolin (3′,4′,5,7-tetrahydroxyflavone) is necessary to possess these effects.

Luteolin is also reported to inhibit NO production,[15] active oxygen species,[16] TNF-α induced ICAM-1 expression,[17] and metallopeptidases.[18] Those findings may be related to the anti-inflammatory and anti-allergic actions shown here, however, most of them are *in vitro* findings. We suggest that the oral effects of these activities must be investigated because the *in vitro* activity may be different with *in vivo* oral activity as shown here. Luteolin administered orally may also affect these activities because it may have some oral bioactivity as described here, and it has been shown to be absorbed from intestine and to be present in human serum;[19] luteolin from perilla leaf extract can also be metabolized in rat.[20]

Luteolin is reported to antagonize an adenosine A$_1$ receptor[21] and to posses a significant estrogenicity like soy isoflavones.[22] We therefore considered the possibility that the antagonization of adenosine A$_1$ receptor may trigger these activities and investigated the effect of estradiol on TNF-α production. The estradiol had no effect on our examination protocol, however, so we speculate another mechanism exists which induces cytokine inhibition and anti-inflammation. Luteolin is also reported to inhibit protein kinase C and tyrosine kinase.[23,24] Inhibition of these signal transductions results in the cytokine inhibition, and therefore our next study will be on the relationship between these current results and signal transduction.

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