Note

Chemical Evidence for Potent Xanthine Oxidase Inhibitory Activity of *Glechoma hederacea* var. *grandis* Leaves (Kakidoushi-Cha)

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Summary  In this study, chemical evidence for the potent xanthine oxidase inhibitory activity of “kakidoushi-cha” (dry leaves of *Glechoma hederacea* var. *grandis*), a traditional folk tea consumed in Japan, was clarified on the basis of structure identification of the active constituents. Assay-guided fractionation and purification afforded 15 compounds from the most active chromatographic fraction of an extract of the tea. Two flavonoids, apigenin and luteolin, showed remarkable inhibitory activity against xanthine oxidase (XO). The contribution of these flavonoid constituents to the observed XO inhibitory activity of the methanol and boiling-water extracts of the tea was estimated to be ca. 35% and ca. 18%, respectively.

Key Words  xanthine oxidase inhibitor, identification, *Glechoma hederacea* var. *grandis*, traditional folk tea, kakidoushi-cha

Throughout Asia, people have utilized various indigenous plants for good health. Such plants, which are referred to as traditional folk teas, are now available in local farmers’ markets and can be easily obtained and used. The results of a survey of local traditional teas for health promotion conducted in the Shikoku area of Japan indicated that three types of the teas, “shiso-cha,” “seisou-cha,” and “kakidoushi-cha” prepared from the leaves of plants of the Labiatae family, had xanthine oxidase (XO) inhibitory activity (1). XO is a key enzyme that catalyzes the oxidative conversion of hypoxanthine and xanthine to uric acid in the purine catabolism pathway. Inhibition of XO activity can reduce the risk of hyperuricemia and gout, which have been recognized as lifestyle related diseases (2). It is well known that most Labiatae plants contain rosmarinic acid as a major phenolic (3, 4). Our previously reported high-performance liquid chromatography (HPLC) analysis of the three tea extracts revealed similar profiles; this study also reveals that rosmarinic acid is a common major constituent (1). Rosmarinic acid has various biological activities, including the inhibition of XO. However, the half maximal inhibitory concentration (IC₅₀) of rosmarinic acid for its XO inhibitory activity has varied from 2.06 to more than 200 μmol L⁻¹ (5–7). Our preliminary investigation of XO inhibition of rosmarinic acid revealed weak activity (below 30% inhibition at 300 μmol L⁻¹). Therefore, other inhibitors are expected to contribute to the XO inhibitory activity of these teas. Nakamishi et al. (8) have clarified that two powerful XO inhibitory phenolics, whose structures were similar to that of rosmarinic acid, existed in the leaves of *Perilla frutescens*. The strong XO inhibitory activity of “shiso-cha” and “seisou-cha” is attributed to the inhibitory properties of the compounds because the teas are prepared from the same plant species. However, the active XO inhibitory constituents of “kakidoushi-cha” have not been reported; therefore, we investigated the active constituents of “kakidoushi-cha” (dry leaves of *Glechoma hederacea* var. *grandis*) to uncover chemical evidence for the activity of the tea.

Materials and Methods

Chemicals and instruments. Xanthine oxidase (purified from buttermilk) and vanillic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Xanthine, uric acid, p-hydroxybenzoic acid, and protocatechuic acid were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Allopurinol, rosmarinic acid, and syringic acid were obtained from Sigma-Aldrich (St. Louis, MO). Caffeic acid, coumaric acid, ferulic acid, and luteolin were purchased from Tokyo Kasei (Tokyo, Japan). Rosmarinic acid methyl ester was synthesized from rosmarinic acid by Fisher esterification. Solvents for HPLC were purchased from Kishida Chemicals (Osake, Japan) as HPLC grade. All other solvents were obtained from Nacalai Tesque. Analytical HPLC was performed with a PU-2989plus low-pressure gradient system (JASCO, Tokyo, Japan) equipped with an SPD-M10AVP photodiode array detector (Shimadzu, Kyoto, Japan) and a U-620 column heater (Sugai, Wakayama, Japan). The obtained data were analyzed with Class-M10A software (Shimadzu). Preparative HPLC was performed with an LC-6AD pump (Shimadzu) equipped with a UV-8011 detector (Toosoh, Tokyo, Japan). ¹H-NMR spectra were measured with an ECS-400 spectrometer (JEOL, Tokyo, Japan). Mass spectra (MS) were obtained with a XEVO-
**XO Inhibitors from G. hederacea var. grandis**

**Plant materials.** Dry leaves of *Glechoma hederacea* var. *grandis* for a traditional folk tea were obtained at a local market in Kochi City, Japan. Dry leaves of *Glechoma hederacea* var. *grandis*, sold commercially as medicinal herbs, were also purchased from Uchida Wakanyaku Co. Ltd. (Tokyo, Japan) and Matsuura Yakugyo Co. Ltd. (Nagoya, Japan).

**Extraction and HPLC analysis of the extracts.** The *G. hederacea* samples (2.0 g each) from the three different sources were extracted with 40 mL of boiling water for 1 h. After being cooled to room temperature, the extracts were filtered, and the filtered solutions were freeze-dried to prepare the water extracts (yield: 0.58 g (Kochi), 0.63 g (Matsuura), and 0.58 g (Uchida)) The leaves (1.98 kg) of *G. hederacea* from Uchida were also extracted with methanol (15 L) for 40 h at room temperature with stirring. The extract was evaporated under reduced pressure to give a methanol extract (176 g). HPLC analysis of the extracts was performed as follows. Five milligrams of each extract was dissolved in a mixture of methanol and H$_2$O [2 : 1 (v/v), 1.5 mL] and filtered with a membrane filter [Minisart RC4 (0.45 μm)]. Sartorius Stedim Japan, Tokyo, Japan], and 10 μL of the solution was subjected to HPLC under the following conditions: column, TSKgel ODS-80Ts (150 mm×4.6 mm i.d., Tosoh) with a guard column (TSK Guardgel ODS-80Ts, Tosoh); solvent system, solvent A, 1% acetic acid in H$_2$O; solvent B, CH$_3$OH, linear gradient from 20% (B) to 100% (B) for 50 min and then isocratic at 100% (B) for 10 min at a flow rate of 0.5 mL min$^{-1}$; detection, UV absorption at 230, 254, 280, 330, and 400 nm.

**XO inhibitory assay.** XO inhibitory assay was performed by following the method reported by Nagao et al. (9) with a slight modification. The reaction medium consisting of 110 μmol L$^{-1}$ xanthine aq (90 μL), a test sample in ethanol (10 μL), and 12.5 μmol L$^{-1}$ phosphate buffer (pH 7.8, 80 μL) were pre-incubated at 37°C for 10 min. Freshly prepared xanthine oxidase buffer solution (0.027 unit mL$^{-1}$, 20 μL) was added to the solution. After incubation at 37°C for 10 min, 3% HClO$_4$ aq (25 μL) was added to stop the reaction. Then, 20 μL of the solution was injected into the HPLC system to quantify the produced uric acid under the following conditions: column, Daisopak SP-120-5-ODS-BP (150 mm×4.6 mm i.d., Daiso, Osaka, Japan); solvent, CH$_3$OH–1% phosphoric acid aq=5 : 9 (v/v); flow rate, 0.5 mL min$^{-1}$; detection, 290 nm. Inhibition percent was calculated by the following equation:

$$\text{inhibition} \left(\%\right) = \left(\frac{\text{peak area of uric acid in control experiment}}{\text{peak area of uric acid in sample experiment}}\right) \times 100 / \text{peak area of uric acid in control experiment}.$$

**Fractionation of the methanol extract.** The methanol extract (165 g) of *G. hederacea* was subjected to silica gel column chromatography [880 g of silica gel 60 (230–400 mesh), Merck, Darmstadt, Germany] eluted with 3 L of methanol–CHCl$_3$–acetic acid mixture [10 : 90 : 1, 20 : 80 : 1, 30 : 70 : 1, 40 : 60 : 1, 60 : 40 : 1, 80 : 20 : 1, and 100 : 0 : 1 (v/v/v)]. The eluting solutions (1 L each) were combined on the basis of the results of silica gel thin layer chromatography analysis and separated into 10 fractions (yield after evaporation: fraction 1, 27.7 g; fraction 2, 1.2 g; fraction 3, 13.7 g; fraction 4, 1.5 g; fraction 5, 7.6 g; fraction 6, 11.4 g; fraction 7, 8.2 g; fraction 8, 30.2 g; fraction 9, 1.7 g; and fraction 10, 75.1 g).

**Isolation of peak compounds from fraction 6.** Fraction 6, which showed the highest XO inhibitory activity, was purified on an ODS column (Cosmosil 140C18-OPN, 800 mL, Nacalai) eluted with 1 L of each of the following solvent mixtures: [CH$_3$OH–H$_2$O–acetic acid=10 : 90 : 1, 20 : 80 : 1, 30 : 70 : 1, 40 : 60 : 1, 80 : 20 : 1, and 100 : 0 : 1 (v/v/v)] to give seven fractions (yield: fraction 6-1, 3.43 g; fraction 6-2, 0.42 g; fraction 6-3, 0.50 g; fraction 6-4, 0.70 g; fraction 6-5, 0.83 g; fraction 6-6, 0.63 g; and fraction 6-7, 3.56 g). Compound 1 [2.0 mg; peak retention time (t$_R$) 34 min] was isolated from fraction 6-1 (100 mg) by preparative HPLC using an ODS column [TSKgel ODS-80Ts (250 mm×20 mm i.d.), Tosoh] with 20% CH$_3$OH–1% acetic acid in H$_2$O as an eluting solvent (flow rate, 8.0 mL min$^{-1}$). Compounds 2 (2.3 mg; t$_R$ 42 min), 3 (4.0 mg; t$_R$ 52 min), 4 (7.5 mg; t$_R$ 55 min), and 5 (2.3 mg; t$_R$ 59 min) were isolated from fraction 6-2 (100 mg) by the same HPLC system with 25% CH$_3$OH–1% acetic acid in H$_2$O as an eluting solvent. Compounds 6 (11.1 mg; t$_R$ 82 min) and 7 (5.2 mg; t$_R$ 90 min) were isolated from fraction 6-3 (100 mg) by the same HPLC system with 35% CH$_3$OH–1% acetic acid in H$_2$O as an eluting solvent. Compounds 8 (11.4 mg; t$_R$ 32 min) and 9 (6.3 mg; t$_R$ 39 min) were isolated from fraction 6-4 (100 mg) by the same HPLC system with 45% CH$_3$OH–1% acetic acid in H$_2$O as an eluting solvent. Compounds 10 (5.3 mg; t$_R$ 28 min), 11 (2.7 mg; t$_R$ 28 min), 12 (10.5 mg; t$_R$ 36 min), 13 (20.0 mg; t$_R$ 45 min), and 14 (20.0 mg; t$_R$ 65 min) were isolated from fraction 6-5 (100 mg) by the same HPLC system with 60% CH$_3$OH–1% acetic acid in H$_2$O as an eluting solvent. Compound 15 (2.1 mg; t$_R$ 42 min) was isolated from fraction 6-6 (0.6 g) by the same HPLC system with 70% CH$_3$OH–1% acetic acid in H$_2$O as an eluting solvent after removal of polymeric substances using Sephadex LH-20 (GE Healthcare Japan, Hino, Japan) column chromatography (CH$_3$OH).

**Identification of isolated compounds.** The chemical structure of each compound was identified primarily by examining the results of $^1$H NMR of both intact (solvent: CD$_3$OD or acetone-$d_6$) and corresponding acetylated compounds (solvent: CDCl$_3$), which were obtained from treatment with acetic anhydride and pyridine, and was confirmed by comparing its HPLC retention time and UV spectrum with those of a commercially available authentic sample.

**Quantitative analysis of luteolin and apigenin in the extracts.** Each calibration equation for luteolin and apigenin {peak area at 330 nm=9.46×10$^5$ [amount (nmol)]–0.85×10$^5$ (R$^2$=0.999) for luteolin; peak area at 330 nm=3.00×10$^5$ [amount (nmol)]–0.05×10$^6$ (R$^2$=0.999) for apigenin} was obtained by the absolute method.
calibration curve method using authentic luteolin and apigenin in HPLC. HPLC of the methanol and boiling-water extracts was performed with injections of 10 or 20 μL of the extract solutions [methanol extract solution: 20 mg in methanol (1 mL) and boiling-water extract solution: 20 mg in 30% methanol–H₂O (1 mL)] under the following conditions: column, TSKgel ODS-80Ts (150 mm × 4.6 mm i.d., Tosoh) with a guard column (TSK Guardgel ODS-80Ts, Tosoh); solvent system, solvent A, 1% acetic acid in H₂O; solvent B, CH₃OH, linear gradient from 20% (B) to 100% (B) for 50 min and then isocratic at 100% (B) for 10 min; flow rate, 0.5 mL min⁻¹; detection, UV absorption at 330 nm. The area values of luteolin (t_R 33.2 min) and apigenin peaks (t_R 36.7 min) thus obtained were converted to the corresponding concentrations (μmol L⁻¹) using the calibration equations described above.

Results and Discussion

XO inhibitory activity of the extracts from Glechoma hederacea

Sufficient amounts of G. hederacea leaves (“kakidoushi-cha”) were not able to be obtained for the identification of active constituents from the small local market. Therefore, we purchased two commercially available samples from medicinal herb companies and compared them with the sample purchased from the market. Although the HPLC analytical data for the extracts of the three samples were similar (data not shown). XO inhibitory activity was slightly different (Table 1). First, we selected the most potent sample and changed the employed extraction method from the boiling-water method to a methanol extraction method to obtain a sufficiently active extract. Methanol extraction has another advantage that the solvent is removed easily after extraction. The methanol extract was prepared in 176 g yield from 1.97 kg of dry G. hederacea leaves; the analysis results showed 62% inhibition against XO activity at a concentration of 150 μg mL⁻¹.

Identification of the constituents of the most active fraction

The methanol extract of G. hederacea was fractionated by silica gel column chromatography into 10 fractions, and the XO inhibitory activity of the fractions was evaluated. The data are presented in Table 1. Among the fractions, the activity of fraction 6 was the most potent (42% inhibition). From the HPLC analysis of fraction 6, rosmarinic acid was observed in very small amounts; however, it was observed in fractions 7 and 8 as a predominant peak. These results indicated that G. hederacea has powerful XO inhibitors other than rosmarinic acid. The most active fraction 6 was further purified by column chromatography using an ODS gel and subsequently by preparative HPLC to isolate 15 compounds, as shown in Fig. 1. The structures of the isolated compounds were deduced by ¹H NMR and MS analyses, and the results were confirmed by comparison with authentic samples. The structures of 13 compounds of the isolates have been successfully identified (Fig. 2). Although two compounds (9 and 12) could not be fully identified, the MS data for the compounds indicated that they were hexose-glycosides of apigenin. From the confirmed structures, the constituents of fraction 6 were

| Sample                                      | Concentration (μg mL⁻¹) | Inhibition (%)¹ | IC₅₀⁻² |
|---------------------------------------------|-------------------------|----------------|--------|
| Water extract of the tea from Kochi         | 150                     | 75.3±3.7       |        |
| Water extract of the tea from Matsuura      | 150                     | 32.9±5.0       |        |
| Water extract of the tea from Uchida        | 150                     | 41.5±2.6       | 235 μg mL⁻¹ |
| Methanol extract of the tea from Uchida     | 150                     | 62.0±4.3       | 104 μg mL⁻¹ |

1 Mean±SD (n=3) of inhibitory% of the extracts on activity of xanthine oxidase at the concentration of 150 μg mL⁻¹ or 50 μg mL⁻¹.

The amounts required for 50% inhibition on xanthine oxidase activity.
clarified to be various phenolic acids and several apigenin
derivatives including luteolin (13).

**XO inhibitory activity of the identified compounds**

The XO inhibitory activity of the isolated compounds was evaluated. The data are presented in Table 2. The assay was performed at relatively higher concentrations (90 µg mL⁻¹) to detect a lot of possibly active compounds as a first screening. Even at the high concentration, compounds 1 to 9 showed weak activity and compounds 10, 11, 12, and 15 showed moderate activity; however, compounds 13 and 14 showed remarkable activity. The isolation and structural analysis of the active compounds 13 and 14 clarified them to be luteolin and apigenin, respectively [13, 14]. ¹H NMR (CD₃OD, 400 MHz) δ 6.21 (1H, d, J = 2.1 Hz, H-6), 6.43 (1H, d, J = 2.1 Hz, H-8), 6.54 (1H, s, H-3), 6.89 (1H, m, H-5'), 7.38 (2H, m, H-2' and H-6'); ¹H NMR (acetone-d₆, 400 MHz) δ 6.24 (1H, s, H-3), 6.53 (1H, d,
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