Metabolites of Orally Administered *Perilla frutescens* Extract in Rats and Humans

Takahiro NAKAZAWA and Keisuke OHSAWA*

Tohoku Pharmaceutical University, 4–4–1 Komatsushima, Aoba-ku, Sendai, Miyagi 981–8558, Japan.
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As a part of our search for bioactive substances from the leaves of *Perilla frutescens* BRITTON var. *acuta* KUDO (Perillae Herba, Labiatae), the aqueous extract was orally administered to rats and humans, and metabolites in the urine, plasma, and/or bile were analyzed by a high-performance liquid chromatograph (HPLC) equipped with a photodiode array detector. When the extract was administered to rats, 10 metabolites, *trans*-caffeic acid-4-*O*-sulfate (1), *trans*-*p*-coumaric acid-4-*O*-sulfate (2), *trans*-ferulic acid-4-*O*-sulfate (3), *trans*-*m*-coumaric acid-3-*O*-sulfate (4), *trans*-caffeic acid (5), *m*-hydroxyphenylpropionic acid (6), *trans*-*p*-coumaric acid (7), *trans*-*m*-coumaric acid (8), luteolin (9), and apigenin (10) were detected in the urine, whereas four metabolites, scutellarein-6, 7-di-*O*-β-glucuronide (11), apigenin-4′-*O*-sulfate-7-*O*-β-glucuronide (12), apigenin-7-*O*-β-glucuronide (13), and diosmetin-7-*O*-β-glucuronide (14) were found in the bile. Compounds 1—8 and 11—14 were also found in the plasma. When the extract was given to humans, however, two metabolites, 1-*O*-(2,4,5-trimethoxycinnamoyl)-β-glucuronic acid (15) and apigenin-4′-*O*-β-glucuronide (16), were found in the urine and plasma. Thus, a species difference in the metabolism of the extract constituents was observed between rats and humans. Structures 1—16 were identified based on their chemical and spectral data.

Key words *Perilla frutescens*; metabolite; apigenin; 2, 4, 5-trimethoxycinnamic acid; luteolin; diosmetin

The leaves of *Perilla frutescens* BRITTON var. *acuta* KUDO (Labiatae) are found in Hangekoubokuto, Saibokuto, and other traditional Chinese herbal medicines which are primarily used to treat inflammatory diseases, clinical depression, and anxiety-related disorders such as anxiety neurosis and anxiety hysteria.[1—7] One of the goals in our laboratory is to characterize the bioactive compounds of *P. frutescens*. Although many components such as essential oils,[8—10] flavones,[11—13] and phenylpropanoids and related compounds[14,15] have been identified in *P. frutescens*, to our knowledge, the bioactive compounds for the traditional use of the herbal medicine are still unidentified.

In most cases, traditional medicines are prepared by extraction with hot water and are orally administered so that the components present in the aqueous extract may be metabolized by gut flora before being absorbed into the body. Accordingly, to evaluate the bioactive compounds in the herbal medicines, investigation of the compounds actually absorbed into the body is necessary. Furthermore, when using an animal for an experiment, the reproducibility in humans will be required to the obtained result.

In our study on *P. frutescens*, we have preliminarily reported that rosmarinic acid, one of the major constituents in the herbal medicine which shows 5-lypoxygenase inhibitory[16] and anti-inflammatory activities,[17] was administered orally to rats and found to be metabolized into 6 phenylpropanoid derivatives.[18] Here, we describe the major metabolites in rat urine, bile, and plasma and human urine and plasma after the oral administration of an aqueous extract of *P. frutescens*.

MATERIALS AND METHODS

Apparatus Melting points were determined on a Yanagimoto micro melting point apparatus and are not corrected. IR spectra were measured with a Perkin Elmer FT-IR 1725X spectrometer. Optical rotations were in methanol using a Jasco DIP-360 digital polarimeter. UV spectra were taken on a Beckman DU-54 spectrometer. NMR spectra were recorded on a JEOL JNM-EX 400 (^{1}H: 400, ^{13}C: 100 MHz) spectrometer. Chemical shifts were given in δ value (ppm) downfield relative to tetramethylsilane. Electron impact (EI) and FABMS were measured with a JEOL JMS-DX 303 mass spectrometer. The HPLC system was comprised of a CCPM pump, CO-8010 column oven (Tosoh, Tokyo) and model MCPD-3600 photodiode array detector (Otsuka, Osaka).

Crude Drug The leaves of *Perilla frutescens* BRITTON var. *acuta* KUDO were commercially obtained from the Japanese market, Matuura Kampo Co., Ltd. in Nagoya.

Reagents Rosmarinic acid, scutellarein, scutellarein-7-*O*-β-glucuronide, luteolin-7-*O*-[β-glucuronosyl-(1→2)-β-glucuronide], and apigenin-7-*O*-[β-glucuronosyl-(1→2)-β-glucuronide] were isolated from *P. frutescens*.[13—15,19] *m*-Hydroxyphenyl-propionic acid was synthesized in the usual way. The identity of these compounds was confirmed by MS, ^{1}H, and ^{13}C-NMR spectroscopies before use.[13,14,19—22] *trans*-Caffeic acid-4-*O*-sulfate, *trans*-ferulic acid-4-*O*-sulfate, and *trans*-*m*-coumaric acid-3-*O*-sulfate were isolated from rat urine after the administration of rosmarinic acid in our laboratory. Details on isolation and identification were reported previously.[18] *trans*-Caffeic acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka), *trans*-*p*-coumaric and 2, 4, 5-trimethoxycinnamic acids were from Tokyo Chemical Industry Co., Ltd. (Tokyo), luteolin, apigenin, β-glucuronidase, and arylsulfatase from Sigma (St. Louis, MO, U.S.A.), and diosmetin from Funakoshi Industry Co., Ltd. (Tokyo). For column chromatography, Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) was used. All other reagents were of special grade.

Preparation of Aqueous Extract *P. frutescens* (100 g) was added to 2 l of distilled water, and the entire mixture was boiled until the volume decreased to 1/2 of the original volume. The extract was then filtered through 5 layers of gauze to produce the filtrate, which was freeze-dried. Two hundred mg of the freeze-dried extract corresponded to 1 g of the

* To whom correspondence should be addressed.

herbal medicine. This freeze-dried extract (20 g) was stored at −20 °C until used.

Animals Male Sprague-Dawley rats (150—300 g) were purchased from Japan SLC, Inc. These animals were specifically pathogen-free and kept in environmentally controlled quarters (temperature: 22±2 °C, humidity: 55±10%, 12 h dark-light cycle) for at least 1 week before use. Normal food (Clea Japan Inc., Tokyo) and water were available at all times, except that it was withdrawn 18 h prior to experimentation.

Preparation of Rat Urine Samples Urine samples were collected for 24 h after oral administration of the extract (5 g/kg), luteolin-7-*O*-[β-glucuronosyl-(1→2)-β-glucuronide] (20 mg/kg), apigenin-7-*O*-[β-glucuronosyl-(1→2)-β-glucuronide] (20 mg/kg), and scutellarein-7-*O*-β-glucuronide (20 mg/kg) with a metabolic cage, and 1 ml of the individual collected urine samples was added to 5 ml of methanol. The solution (20 μl), filtered through a 0.45 μm filter membrane, was injected into the HPLC.

Preparation of Rat Bile Samples A polyethylene tube was inserted into the rat bile duct under pentobarbital sodium anesthesia. Bile samples were collected in methanol with cooling for 12 h after the oral administration of each drug. The solution (20 μl), filtered through a 0.45 μm filter membrane, was injected into the HPLC.

Preparation of Rat Plasma Samples After oral administration of the extract (5 g/kg), the animals were anesthetized with pentobarbital sodium and blood (5 ml from each animal) was collected 12 h after administration from the inferior *vena cava* with a heparinized tube and immediately centrifuged at 3000 rpm for 10 min at room temperature. Two milliliters of each individual plasma was added to 12 ml methanol and stirred well. The mixture was centrifuged at 3000 rpm for 10 min at room temperature and the supernatant was evaporated to dryness at 40 °C *in vacuo*. The residue was dissolved in 0.2 ml using water and 50 μl solution, filtered through a 0.45 μm filter membrane, then injected into the HPLC.

Preparation of Human Urine and Plasma Samples Three healthy male volunteers from this college took part in the testing program. The study was approved by the ethics committee of our department. Their ages were 25, 30 and 51, weight was 65, 70, and 65 kg, respectively, and each was given the aqueous extract (2 g) after informed consent to participate. They were given a restricted diet which consisted of white bread, egg, and water for 20 h before and after start of the experiment. Urine samples (0.6—0.8 l) were collected for 12 h after administration and then the urine was concentrated until the volume decreased to 1/10 of the original volume. Blood (10 ml) was collected at 5 h after the administration. These samples were treated in a similar manner as above.

HPLC Conditions For qualitative analysis of metabolites, HPLC conditions were as follows: column, TSK gel ODS-120T (Tosoh, Tokyo, 250 mm×4.6 mm i.d.); column temperature, 40 °C; flow rate, 1 ml/min; detection, by UV at 190—400 nm. The mobile phase was a gradient system with 0.1% trifluoroacetic acid (TFA) in H_2O (A) and CH_3CN (B). The gradient system was A/B=100/0 (0 min)→90/10 (60 min)→70/30 (100 min)→70/30 (120 min).

Preparative (prep.) HPLC conditions were as follows: column, TSK gel ODS-120T (Tosoh, Tokyo, 300 mm×7.6 mm i.d.); column temperature, room temperature; flow rate, 2 ml/min; detection, by UV at 220 or 280 nm. The mobile phase was a gradient system with H_2O (A) and CH_3CN (B). The gradient system was A/B=100/0 (0 min)→70/30 (30 min).

Isolation of Metabolites from Urine and Bile For metabolites isolation, rat urine (about 400 ml), rat bile (about 200 ml) and human urine (about 4000 ml) were collected as described above, after the extract administration. Rat urine was successively subjected to Sephadex LH-20 with H_2O and MeOH as eluents, the H_2O eluate fraction containing **2** was lyophilized and the MeOH fractions containing **7**, **9**, and **10** were evaporated to dryness under reduced pressure at 40 °C. Each fraction was further purified by a combination of column chromatography with Sephadex LH-20 (H_2O) and prep. HPLC, giving **2** (6 mg), **7** (6 mg), **9** (2 mg) and **10** (2 mg). Rat bile was chromatographed on Sephadex LH-20 (H_2O→MeOH), and the H_2O eluate fractions were further purified by prep. HPLC to give **11** (8 mg), **12** (10 mg), **13** (10 mg), and **14** (4 mg). Human urine was evaporated *in vacuo*, and the residue was suspended in 400 ml of H_2O and centrifuged at 3000 rpm for 10 min. The supernatant was chromatographed on Sephadex LH-20 (H_2O). Each fraction containing **15** or **16** was subjected to prep. HPLC to afford **15** (4 mg) and **16** (4 mg).

Enzymatic Hydrolysis Compounds **2** and **11**—**16** (each 1 mg) were incubated in 0.1 M citrate buffer (pH 5.2) with arylsulfatase (5 units) or β-glucuronidase for 2 h at 37 °C. The aglycons, *trans-p*-coumaric acid (t_R 63 min), scutellarein (t_R 94 min), luteolin (t_R 96 min), apigenin (t_R 103 min), diosmetin (t_R 105 min), and 2, 4, 5-trimethoxycinnamic acid (t_R 93 min), were identified by direct comparison with authentic samples.

Compound **2**: White powder, mp 260 °C (dec.). UV (MeOH) λ_{max} (log ε) 203 (4.05), 214 (4.03), 273 (4.10) nm. IR (KBr) ν_{max} 1670, 1624, 1507, 1054, 980 cm^{-1}. ^{1}H-NMR (CD_3OD, 400 MHz) δ: 6.51 (1H, d, J=15.8 Hz, H-8), 7.03 (2H, d, J=8.7 Hz, H-3, 5), 7.42 (1H, d, J=15.8 Hz, H-7), 7.51(2H, d, J=8.4 Hz, H-2, 6). ^{13}C-NMR (CD_3OD, 100 MHz) δ: 122.6 (C-3, 5), 124.9 (C-8), 129.5 (C-2, 6), 133.7 (C-1), 141.0 (C-7), 154.9 (C-4), 174.8 (C-9). FABMS *m/z*: 265 $[M-H+Na]^-$, 243 $[M-H]^-$, 163 $[M-H-SO_3]^-$.

Compound **7**: White powder, mp 210—213 °C. High resolution EIMS (HREIMS) *m/z*: 164.0491 (calcd for $C_9H_8O_3$, 164.0473).

Compound **9**: Yellow powder, mp 328—330 °C. HREIMS *m/z*: 286.0452 (calcd for $C_{15}H_{10}O_6$, 286.0477).

Compound **10**: Yellow powder, mp 345—347 °C. HREIMS *m/z*: 270.0543 (calcd for $C_{15}H_{10}O_5$, 270.0520).

Compound **11**: Yellow powder, mp>350 °C. $[\alpha]_D^{28}$ −46.56° (c=0.1, MeOH). UV (MeOH) λ_{max} (log ε) 205 (3.98), 271 (3.62), 331 (3.75) nm. IR (KBr) ν_{max} 3415, 1654, 1609 cm^{-1}. ^{1}H-NMR (dimethyl sulfoxide (DMSO)-d_6, 400 MHz) δ: 3.12—3.65 (8H, m, H-2″—5″, H-2‴—H-5‴), 4.82 (1H, d, J=7.1 Hz, H-1″), 5.14 (1H, d, J=7.1 Hz, H-1‴), 6.82 (1H, s, H-3), 6.91 (2H, d, J=8.5 Hz, H-3′, 5′), 7.04 (1H, s, H-8), 7.90 (2H, d, J=8.5 Hz, H-2′, 6′). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 71.6 (C-4″, C-4‴), 72.9 (C-2‴), 73.7 (C-2″), 74.0 (C-5″ or C-5‴), 74.1 (C-5″ or C-5‴), 75.7 (C-3″), 75.9 (C-3‴), 95.1 (C-8), 100.9 (C-1‴), 102.5 (C-3), 103.8 (C-1″), 105.7 (C-10), 115.9 (C-3′, 5′), 120.6 (C-1′), 128.1 (C-2′, 6′), 129.2

(C-6), 152.0 (C-5), 152.3 (C-9), 156.0 (C-7), 161.4 (C-4′), 164.3 (C-2), 171.5 (C-6″ or C-6‴), 171.9 (C-6″ or C-6‴), 182.0 (C-4). Significant heteronuclear maltiple-bond correlation (HMBC) H-3→C-2, 4, 10, 1′, H-8→C-6, 7, 9, 10, H-2′, 6′→C-2, 1′–6′, H-3′, 5′→C-1′–6′, H-1″→C-6, H-1‴→C-7. FABMS *m/z*: 659 $[M-H+Na]^-$, 637 $[M-H]^-$, 461 [M–H–GlcUA]$^-$, 285 [M–H–2GlcUA]$^-$.

Compound **12**: Yellow powder, mp >350 °C (dec.). $[\alpha]_D^{28}$ −120.31° (*c*=0.2, MeOH). UV (MeOH) λ_{max} (log ε) 210 (4.69), 269 (4.59), 311 (4.44) nm. IR (KBr) ν_{max} 3415, 1655, 1609 cm^{-1}. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 3.13—3.66 (4H, m, H-2″—H-5″), 5.23 (1H, d, *J*=7.3 Hz, H-1″), 6.48 (1H, d, *J*=2.2 Hz, H-6), 6.90 (1H, d, *J*=2.2 Hz, H-8), 6.97 (1H, s, H-3), 7.37 (2H, d, *J*=8.8 Hz, H-3′, 5′), 8.04 (2H, d, *J*=8.8 Hz, H-2′, 6′), 12.91 (1H, s, 5-OH). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 71.8 (C-4″), 72.8 (C-2″), 74.8 (C-5″), 76.5 (C-3″), 94.5 (C-8), 99.2 (C-6), 99.5 (C-1″), 104.2 (C-3), 105.4 (C-10), 119.9 (C-3′, 5′), 124.5 (C-1′), 127.6 (C-2′, 5′), 156.8 (C-9), 157.0 (C-4′), 161.0 (C-5), 162.6 (C-7), 163.8 (C-2), 171.3 (C-6"), 182.0 (C-4). Significant HMBC H-3→C-2, 4, 10, 1′, H-6→C-5, 7, 8, 10, H-8→C-6, 7, 9, 10, H-2′,6′→C-2, 1′–6′, H-3′, 5′→C-1′–6′, H-1″→C-7. FABMS *m/z*: 547 $[M-H+Na]^-$, 525 $[M-H]^-$, 445 $[M-H-SO_3]^-$, 349 [M−H−GlcUA]$^-$, 269 [M−H−GlcUA-SO_3]$^-$.

Compound **13**: Yellow powder, mp 217—220 °C. $[\alpha]_D^{28}$ −116.33° (*c*=0.4, MeOH). UV (MeOH) λ_{max} (log ε) 203 (4.34), 267 (4.08), 331 (4.14) nm. IR (KBr) ν_{max} 3448, 1656, 1610 cm^{-1}. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 3.13—3.59 (4H, m, H-2″—H-5″), 5.05 (1H, d, *J*=7.1 Hz, H-1″), 6.41 (1H, d, *J*=1.4 Hz, H-6), 6.80 (1H, d, *J*=1.4 Hz, H-8), 6.81 (1H, s, H-3), 6.90 (2H, d, *J*=8.8 Hz, H-3′, 5′), 7.90 (2H, d, *J*=8.8 Hz, H-2′, 6′) 12.97 (1H, s, 5-OH). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 71.8 (C-4″), 72.8 (C-2″), 73.5 (C-5″), 76.4 (C-3″), 94.5 (C-8), 99.5 (C-1″), 99.5 (C-6), 102.7 (C-3), 105.1 (C-10), 115.9 (C-3′, 5′), 120.4 (C-1′), 128.4 (C-2′, 6′), 156.8 (C-9), 160.8 (C-5), 161.7 (C-4′), 163.0 (C-7), 164.2 (C-2), 171.8 (C-6″), 181.8 (C-4); Significant HMBC H-3→C-2, 4, 10, 1′, H-6→C-5, 7, 8, 10, H-8→C-6, 7, 9, 10, H-2′, 6′→C-2, 1′–6′, H-3′, 5′→C-1′–6′, H-1″→C-7. FABMS *m/z*: 467 $[M-H+Na]^-$, 445 $[M-H]^-$, 269 [M−H−GlcUA]$^-$.

Compound **14**: Yellow powder, mp 276—278 °C (dec.). $[\alpha]_D^{28}$ −58.02° (*c*=0.5, MeOH). UV (MeOH) λ_{max} (log ε) 203 (4.68), 252 (4.36), 267 (4.34), 340 (4.10) nm. IR (KBr) ν_{max} 3420, 1655, 1610 cm^{-1}. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 3.11—3.54 (4H, m, H-2″—H-5″), 3.86 (3H, s, –OC$\underline{H}_3$), 5.05 (1H, d, *J*=7.3 Hz, H-1″), 6.43 (1H, d, *J*=1.4 Hz, H-6), 6.81 (1H, d, *J*=1.4 Hz, H-8), 6.83 (1H, s, H-3), 7.10 (1H, d, *J*=8.5 Hz, H-5′), 7.48 (1H, d, *J*=2.0 Hz, H-2′), 7.57 (1H, dd, *J*=2.0, 8.5 Hz, H-6′), 12.93 (1H, s, 5-OH). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 55.7 (–O$\underline{C}$H$_3$), 71.8 (C-4″), 72.9 (C-2″), 73.5 (C-5″), 76.5 (C-3″), 94.5 (C-8), 99.6 (C-1″), 99.7 (C-6), 103.7 (C-3), 105.2 (C-10), 112.1 (C-5′), 112.9 (C-2′), 118.7 (C-6′), 122.7 (C-1′), 146.8 (C-3′), 151.2 (C-4′), 156.9 (C-9), 160.9 (C-5), 163.1 (C-7), 164.0 (C-2), 171.3 (C-6″), 181.8 (C-4). Significant HMBC H-3→C-2, 4, 10, 1′, H-6→C-5, 7, 8, 10, H-8→C-6, 7, 9, 10, H-2′→C-2, 1′, 3′, 4′, 6′, H-3′→C-1′, 2′, 4′, 5′, H-6′→C-1′, 2′, 4′, 5′, H-1″→C-7, OCH_3→C-4′. FABMS *m/z*: 497 $[M-H+Na]^-$, 475 $[M-H]^-$, 299 [M−H−GlcUA]$^-$.

Compound **15**: Yellow powder, mp 147—149 °C. $[\alpha]_D^{28}$ +76.72° (*c*=0.3, MeOH). UV (MeOH) λ_{max} (log ε) 202 (4.22), 238 (3.95), 289 (3.96), 351 (4.00) nm. IR (KBr) ν_{max} 1609, 1517 cm^{-1}: ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 3.05—3.51 (4H, m, H-2′–H-5′), 3.76 (3H, s, 5-OC$\underline{H}_3$), 3.86 (3H, s, 4-OC$\underline{H}_3$), 3.88 (3H, s, 2-OC$\underline{H}_3$), 5.42 (1H, d, *J*=7.8 Hz, H-1′), 6.56 (1H, d, *J*=16.1 Hz, H-8), 6.73 (1H, s, H-3), 7.30 (1H, s, H-6), 7.92 (1H, d, *J*=16.1 Hz, H-7). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 55.7 (5-O$\underline{C}$H$_3$), 56.0 (4-O$\underline{C}$H$_3$), 56.2 (2-O$\underline{C}$H$_3$), 71.9 (C-4′), 72.4 (C-2′), 74.2 (C-5′), 77.1 (C-3′), 94.0 (C-1′), 97.3 (C-3), 111.2 (C-6), 113.4 (C-1), 114.6 (C-8), 139.9 (C-7), 142.9 (C-5), 152.4 (C-4), 153.6 (C-2), 165.6 (C-9), 170.8 (C-6′). Significant HMBC H-3→C-1, 2, 4, 5, H-6→C-1, 2, 4, 5, H-7→C-1, 2, 6, 8, 9, H-8→C-1, 7, 9, H-1′→C-9, OCH_3 (δ 3.76)→C-5, OCH_3 (δ 3.86)→C-4, OCH_3 (δ 3.88)→C-2. FABMS *m/z*: 435 $[M-H+Na]^-$, 413 $[M-H]^-$, 237 [M−H−GlcUA]$^-$.

Compound **16**: Yellow powder, mp 216—218 °C (dec.). $[\alpha]_D^{28}$ −58.99° (*c*=0.4, MeOH). UV (MeOH) λ_{max} (log ε) 210 (4.31), 268 (4.14), 322 (4.07) nm. IR (KBr) ν_{max} 3049, 1656, 1610 cm^{-1}. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 3.17—3.57 (4H, m, H-2″—H-5″), 5.02 (1H, d, *J*=7.3 Hz, H-1″), 6.23 (1H, d, *J*=2.0 Hz, H-6), 6.52 (1H, d, *J*=2.0 Hz, H-8), 6.87 (1H, s, H-3), 7.18 (2H, d, *J*=9.0 Hz, H-3′, 5′), 8.02 (2H, d, *J*=9.0 Hz, H-2′, 6′), 12.89 (1H, s, 5-OH). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 71.8 (C-4″), 72.9 (C-2″), 73.6 (C-5″), 76.4 (C-3″), 94.0 (C-8), 98.8 (C-6), 99.6 (C-1″), 103.6 (C-3), 103.7 (C-10), 115.8 (C-3′, 5′), 123.7 (C-1′), 128.0 (C-2′, 6′), 157.2 (C-9), 160.3 (C-4′), 161.3 (C-5), 163.0 (C-2), 164.4 (C-7), 172.0 (C-6″), 181.7 (C-4). Significant HMBC H-3→C-2, 4, 10, 1′, H-6→C-5, 7, 8, 10, H-8→C-6, 7, 9, 10, H-2′, 6′→C-2, 1′–6′, H-3′, 5′→C-1′–6′, H-1″→C-4′. FABMS *m/z*: 467 $[M-H+Na]^-$, 445 $[M-H]^-$, 269 [M−H−GlcUA]$^-$.

RESULTS AND DISCUSSION

Using HPLC equipped with a photodiode array detector, 10 characteristic peaks, **1** (t_R 30 min), **2** (t_R 34 min), **3** (t_R 40 min), **4** (t_R 42 min), **5** (t_R 45 min), **6** (t_R 58 min), **7** (t_R 63 min), **8** (t_R 71 min), **9** (t_R 96 min), and **10** (t_R 103 min), were detected in the rat urine samples and 4 characteristic peaks, **11** (t_R 73 min), **12** (t_R 75 min), **13** (t_R 87 min), and **14** (t_R 89 min), were found in the rat bile samples after the oral administration of *P. frutescens* aqueous extract. These peaks except for **9** and **10** were also found in the rat plasma samples.

Compounds **1** and **3**—**10** were identified as *trans*-caffeic acid-4-*O*-sulfate (**1**), *trans*-ferulic acid-4-*O*-sulfate (**3**), *trans*-*m*-coumaric acid-3-*O*-sulfate (**4**), *trans*-caffeic acid (**5**), *m*-hydroxyphenylpropionic acid (**6**), *trans*-*p*-coumaric acid (**7**), *trans*-*m*-coumaric acid (**8**), luteolin (**9**), and apigenin (**10**) by direct comparison with authentic samples.

Enzymatic hydrolysis of **2** using arylsulfatase gave a product that was tentatively identified as *trans*-*p*-coumaric acid by comparing its t_R and UV spectrum during HPLC with those of an authentic sample. The IR spectrum of **2** showed absorption bands at 1670 and at 1624 and 980 cm^{-1} due to the conjugated carboxyl and trans double-bond functions, respectively. Intense absorption at 1054 cm^{-1} suggested a sulfate-conjugated structure for **2**. The negative FABMS of **2** showed a pseudomolecular ion peak at *m/z* 243 $(M-H)^-$ along with a fragment ion peak at *m/z* 163 $(M-H-SO_3)^-$

corresponding to coumaric acid mono-sulfate, indicating the presence of one sulfate group in **2**. Comparison of the ^{13}C-NMR spectrum of **2** with that of *trans-p*-coumaric acid indicated that C-4 signal of **2** shifted upfield by 5.7 ppm, accompanied by downfield shifts of C-3 (6.3 ppm) and C-5 (6.3 ppm), indicating the sulfate group is situated at C-4. Thus, the structure of **2** was concluded to be *trans-p*-coumaric acid-4-*O*-sulfate.

Compounds **11**—**14** exhibited absorption due to the conjugated carbonyl (1654—1656 cm^{-1}) in the IR spectra, and maximal absorption at 267—271 and 311—340 nm in the UV spectra. From these data, **11**—**14** were deduced to have a flavone skeleton. The presence of OH proton signals at low field positions (δ 12.07—δ 13.04 ppm) in the ^{1}H-NMR spectra suggested chelated C-5 hydroxyl group in **12**—**14**.

Enzymatic hydrolysis of **11** with β-glucuronidase gave the aglycone, which was assumed to be scutellarein from the HPLC behavior. In the negative FABMS of **11** showed a pseudomolecular ion peak at *m/z* 637 $(M-H)^-$ corresponding to scutellarein diglucuronide and fragment ion peaks at *m/z* 461 $(M-H-GlcUA)^-$ and 285 $(M-H-2GlcUA)^-$. The ^{1}H-NMR spectra of **11** showed signals due to a scutellarein skeleton [6.82 (1H, s, H-3), 6.91 (2H, d, J=8.5 Hz, H-3′, 5′), 7.04 (1H, s, H-8), 7.90 (2H, d, J=8.5 Hz, H-2′, 6′)], and two β-glucuronic acid moieties [3.12—3.65 (8H, m, H-2″—5″, H-2‴–H-5‴), 4.82 (1H, d, J=7.1 Hz, H-1″), 5.14 (1H, d, J=7.1 Hz, H-1‴)]. Furthermore, the ^{13}C-NMR data (see Materials and Methods) of **11** was also superimposable on that of scutellarein, except for the signals due to two β-glucuronic acids. In HMBC experiment, long-range correlations were observed between the following protons and carbons: H-3 and C-2, 4, 10, 1′; H-8 and C-6, 7, 9, 10; H-1″ and C-6; H-1‴ and C-7. Consequently, the structure of **11** was identified as scutellarein-6, 7-di-*O*-β-glucuronide.

Enzymatic hydrolysis of **13** with β-glucuronidase gave the aglycone apigenin by agreement with the HPLC behavior. In the negative FABMS, a pseudomolecular ion peak at *m/z* 445 $(M-H)^-$ corresponded to apigenin monoglucuronide. The ^{1}H-NMR spectrum showed signals due to an apigenin skeleton [6.41 (1H, d, J=1.4 Hz, H-6), 6.80 (1H, d, J=1.4 Hz, H-8), 6.81 (1H, s, H-3), 6.90 (2H, d, J=8.8 Hz, H-3′, 5′), 7.90 (2H, d, J=8.8 Hz, H-2′, 6′) 12.97 (1H, s, 5-OH)], and a β-glucuronic acid moiety [3.13—3.59 (4H, m, H-2″—H-5″), 5.05 (1H, d, J=7.1 Hz, H-1″)]. The ^{13}C-NMR data (see Materials and Methods) of **13** was superimposable on that of apigenin, except for the appearance of signals assignable to a β-glucuronic acid. In the HMBC experiment, long-range correlations were observed between the following protons and carbons: H-3 and C-2, 4, 10, 1′; H-6 and C-5, 7, 8, 10; H-8 and C-6, 7, 9, 10; H-1″ and C-7. Thus, **13** was identified as apigenin 7-*O*-β-glucuronide.

Enzymatic hydrolysis of **12** with β-glucuronidase (containing arylsulfatase activity) and also arylsulfatase gave aglycone apigenin based on agreement with the HPLC behavior. The negative FABMS showed a pseudomolecular ion peak at *m/z* 525 $(M-H)^-$ and ion peaks corresponding to $(M-H-SO_3)^-$, $(M-H-GlcUA)^-$, and $(M-H-GlcUA-SO_3)^-$ at 445, 349 and 269, respectively. Furthermore, the intense absorption at 1051 cm^{-1} in the IR spectrum suggested a sulfate-conjugated structure for **12**. The ^{1}H- and ^{13}C-NMR spectra of **12** was similar to **13**, except for B-ring signals. **12** would thus appear to contain one sulfate-conjugate of **13**. Comparison of the ^{13}C-NMR spectrum of B-ring of **12** with that of **13** showed that C-4′ signal of **12** shifted upfield by 4.7 ppm, along with downfield shifts of C-3′ (4.0 ppm) and C-5′ (4.0 ppm). These shifts suggested a sulfate group at C-4′. Thus, **12** was identified as apigenin-4′-*O*-sulfate-7-*O*-β-glucuronide.

Enzymatic hydrolysis of **14** with β-glucuronidase gave the aglycone diosmetin by agreement with the HPLC behavior. In the negative FABMS, a pseudomolecular ion peak at *m/z* 475 $(M-H)^-$ corresponded to diosmetin monoglucuronide. The ^{1}H-NMR spectrum showed signals assignable to a diosmetin skeleton [3.86 (3H, s, –OC$\underline{H}_3$), 5.05 (1H, d, J=7.3 Hz), H-1″), 6.43 (1H, d, J=1.4 Hz, H-6), 6.81 (1H, d, J=1.4 Hz, H-8), 6.83 (1H, s, H-3), 7.10 (1H, d, J=8.5 Hz, H-5′), 7.48 (1H, d, J=2.0 Hz, H-2′), 7.57 (1H, dd, J=2.0, 8.5 Hz, H-6′), 12.93 (1H, s, 5-OH)], along with those of glucuronic acid moiety [5.05 (1H, d, J=7.3 Hz), 3.11—3.54 (4H, m, H-2″–H-5″)]. The ^{13}C-NMR data (see Materials and Methods) of **14** was superimposable on that of diosmetin, except for the signals due to a β-glucuronic acid. The HMBC experiment of **14** showed long-range correlations between the following protons and carbons: H-3 and C-2, 4, 10, 1′; H-6 and C-5, 7, 8, 10; H-8 and C-6, 7, 9, 10; H-1″ and C-7. Thus, **14** was identified as diosmetin-7-*O*-β-glucuronide. Structures of these metabolites are shown in Chart 1.

In the aqueous extract of *P. frutescens*, we detected four major constituents, which were identified as luteolin-7-*O*-[β-glucuronosyl-(1→2)-β-glucuronide] (t_R 73 min), apigenin-7-*O*-[β-glucuronosyl-(1→2)-β-glucuronide] (t_R 78 min), scutellarein-7-*O*-β-glucuronide (t_R 80 min), and rosmarinic acid (t_R 88 min) by comparison with authentic samples; **1**—**14** except for **5**, however, were not detected in the extract. On the other hand, the above flavones and rosmarinic acid present in the extract were difficult to detect as unchanged form in the urine and plasma samples when the extract was given to rats. We previously reported that when rosmarinic acid was orally administered to rats, it was metabolized to **1**, **3**—**6**, and **8** with cleavage of the ester bond, selective *para*-dehydroxylation, methylation, and sulfate-conjugation and finally excretion into the urine.[18] Griffith,[23] Abe *et al.*,[24] and Hattori *et al.*[25] reported that many flavonoid glycosides were absorbed as aglycone after hydrolysis with gut flora and excreted into urine or bile mainly as conjugated forms. These findings suggest that **1**—**14** must be metabolites produced in the body from the corresponding phenylpropanoids or flavonoids *via* some biotransformation mechanisms such as hydrolysis in the gut flora and methylation and conjugation in the liver.

After scutellarein-7-*O*-β-glucuronide was fed to rats, the aglycone scutellarein was contained in the urine and **11** was found in the bile. Apigenin-7-*O*-[β-glucuronosyl-(1→2)-β-glucuronide] was also given to rats, and aglycone apigenin (**10**) was detected in the urine and **12** and **13** in the bile. On the other hand, when luteolin-7-*O*-[β-glucuronosyl-(1→2)-β-glucuronide] was administered to rats, in addition to the aglycone luteolin (**9**), diosmetin was also detected in the urine and **14** was found in the bile. Thus, we concluded that rosmarinic acid, scutellarein-7-*O*-β-glucuronide, apigenin-7-*O*-[β-glucuronosyl-(1→2)-β-glucuronide], and luteolin-7-*O*-[β-glucuronosyl-(1→2)-β-glucuronide] were the most possible precursors of **1**, **3**—**6**, and **8**, **11**, **12** and **13**, and **14**, re-

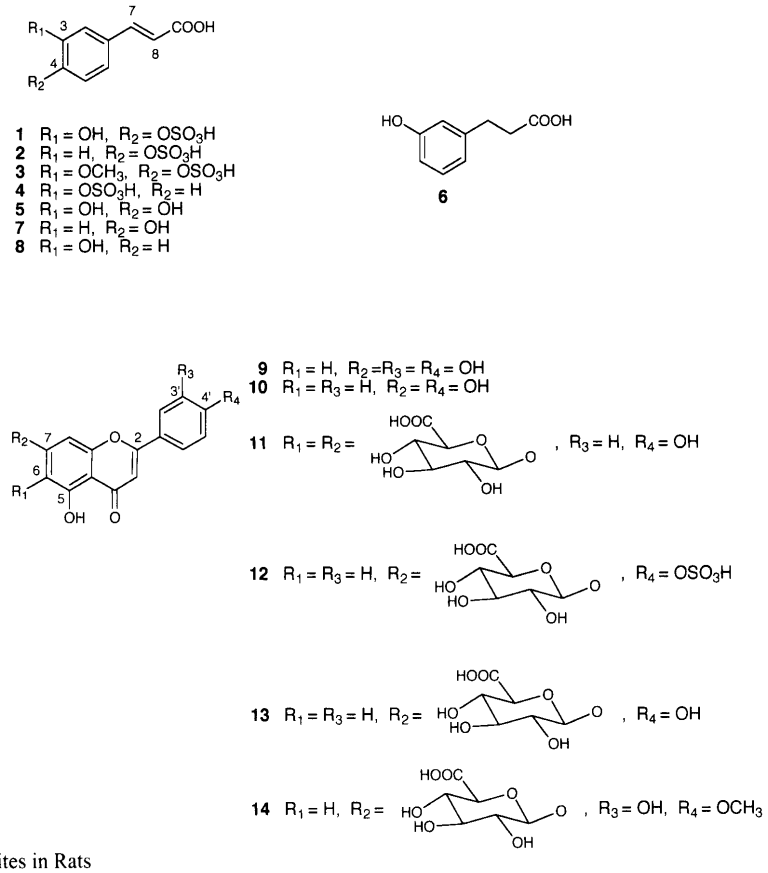


Chart 1. Structures of Metabolites in Rats

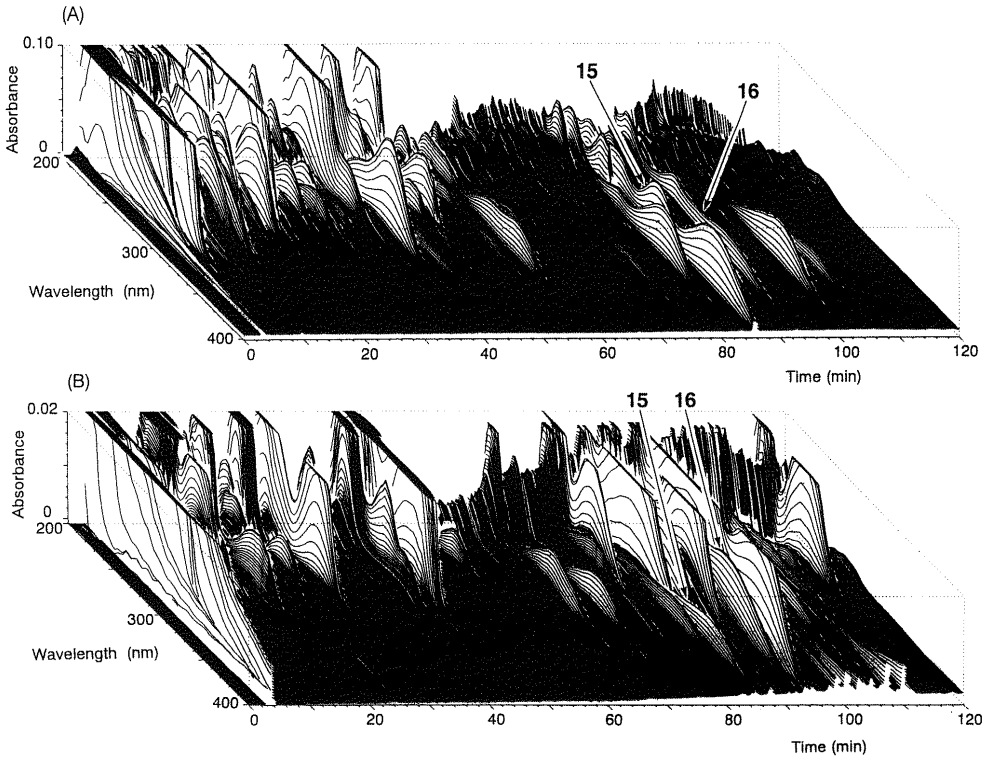
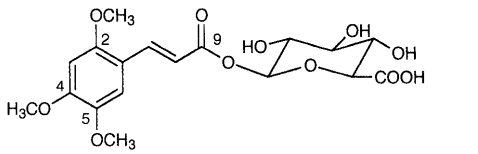


Fig. 1. HPLC Profiles of (A) Urine Samples (B) Plasma Samples after the Oral Administration of *Perilla frutescens* Aqueous Extract to Humans

spectively. In our present study, however, the putative origins of *trans-p*-coumaric acid (**7**) and the sulfate (**2**) were not identified. These two compounds may be derived from malonylshisonin and shisonin, which are the major pigments of *P. frutescens* and which contain the *p*-coumaryl group.[12]

These results, obtained when the extract was administered to rats, prompted us to perform similar experiments with humans. When humans received the extract, luteolin-7-*O*-[β-glu-



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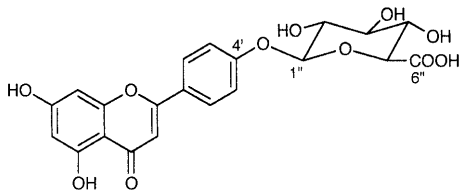

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Chart 2. Structures of Metabolites in Humans

curonosyl-(1→2)-β-glucuronide], apigenin-7-*O*-[β-glucuronosyl-(1→2)-β-glucuronide], scutellarein-7-*O*-β-glucuronide, and rosmarinic acid *per se* were not detected in the urine or plasma samples as well as in rat given the extract, although two characteristic peaks, **15** (t_R 85 min) and **16** (t_R 90 min), which were not contained in non-treated samples, were detected (Fig. 1). Compounds **1**—**14** were not present. Thus, a species difference was observed between the rat and human when the extract was ingested.

The signals in the NMR spectra of **15** were similar to those of 2, 4, 5-trimethoxycinnamic acid. However, the ^{1}H-NMR spectrum showed one β-anomeric proton at δ 5.42 (d), and in the negative FABMS, a pseudomolecular ion peak at *m/z* 413 $(M-H)^-$ corresponded to trimethoxycinnamic acid monoglucuronide and fragment ion peaks at *m/z* 237 $(M-H-GlcUA)^-$. Furthermore, enzymatic hydrolysis of **15** with β-glucuronidase gave a product identified as 2, 4, 5-trimethoxycinnamic acid by direct comparison with authentic samples. The location of the glucuronic acid moiety was found by HMBC between the anomeric proton at δ 5.42 and C-9 at δ 165.6 for 2, 4, 5-trimethoxycinnamic acid moiety. Thus, **15** was identified as 1-*O*-(2, 4, 5-trimethoxycinnamoyl)-β-glucuronic acid.

Compound **16** was recognized as an isomer of **13** from the negative FABMS. The enzymatic hydrolysis of **16** with β-glucuronidase gave the aglycone apigenin by agreement with the HPLC behavior. The ^{1}H- and ^{13}C-NMR spectra were analogous to those of **13**, but in the HMBC experiment, long-range correlations were observed between the following protons and carbons: H-3 and C-2, 4, 10, 1′; H-2′, 6′ and C-2, 1′–6′; H-3′, 5′ and C-1–6′; H-1″ and C-4′. **16** was thus identified as apigenin-4′-*O*-β-glucuronide. Structures of these metabolites are shown in Chart 2.

Rosmarinic acid, which is the major constituent of *P. frutescens*, has been shown to inhibit 5-lipoxygenase[16] and to have anti-inflammatory activity,[17] however, it was not detected in human urine or plasma after oral administration of the extract. We infer that rosmarinic acid in the extract may be predominantly metabolized to **15** in the case of humans through hydrolytic cleavage in the gastrointestinal tract and hydroxylation, methylation, and glucuronidation in the liver, whereas it is metabolized to **1**, **3**—**6**, and **8** in rats. Compound **16**, found in human urine and plasma, may be derived from apigenin-7-*O*-[β-glucuronosyl-(1→2)-β-glucuronide], which is metabolized to **12** and **13**, and to its aglycone apigenin (**10**) in rats, through glucuronidation in the liver following hydrolysis with gut flora. Thus, the preferred route of apigenin metabolism will be 4′-*O*-β-glucuronidation in humans, against 7-*O*-β-glucuronidation and/or 4′-*O*-sulfation in rats. These considerations led us to believe that the action of metabolites **15** and **16** detected in human urine and plasma and its aglycones rather than the constituents *per se* present in *P. frutescens* would be important for the efficacy of *P. frutescens*. Although, to the best of our knowledge, the pharmacological activity of **15** has not yet been studied, Viola *et al.* reported that apigenin, the aglycone of **16**, had a clear anxiolytic activity in mice in the elevated plus-maze without evidencing sedation or muscle relaxant effects at doses similar to those used for classical benzodiazepines.[26] We therefore believe that it play an important role in the efficacy of *P. frutescens* for treating anxiety neurosis. Further studies are now being done on the biological activity of these metabolites.

REFERENCES

1) Kinebuti A., *Chiryo*, **71**, 1745—1750 (1989).
2) Narita H., *Chiryo*, **71**, 1798—1799 (1989).
3) Narita H., *Jpn. J. Neuropsychopharmacol.*, **12**, 165—172 (1990).
4) Khoriyama E., *Nihon Toyo Igaku Zasshi*, **43**, 115 (1992).
5) Ozaki T., Shimomura T., *Komposinryo*, **12**, 21—25 (1993).
6) Cyong J.-C. *Kampoigaku*, **19**, 397—401 (1995).
7) Yamagiwa M., *Kampoigaku*, **22**, 19—22 (1998).
8) Ito H., *Yakugaku Zasshi*, **84**, 1123—1125 (1964).
9) Ito H., *Yakugaku Zasshi*, **90**, 883—892 (1970).
10) Koezuka Y., Honda G., Tabata M., *Phytochemistry*, **25**, 859—863 (1986).
11) Ishikura N., *Agric. Biol. Chem.*, **45**, 1855—1860 (1981).
12) Yoshida K., Kondo T., Kameda K., Goto T., *Agric. Biol. Chem.*, **54**, 1745—1751 (1990).
13) Yoshida K., Kameda K., Kondo T., *Phytochemistry*, **33**, 917—919 (1993).
14) Aritomi M., Kumori T., Kawasaki T., *Phytochemistry*, **24**, 2438—439 (1985).
15) Okuda T., Hatano T., Nishibe S., *Yakugaku Zasshi*, **106**, 1108—1111 (1986).
16) Kimura Y., Okuda H., Okuda T., Kubo M., *J. Trad. Med.*, **12**, 180—186 (1995).
17) Gracza L., Koch H., Löffle E., *Arch. Pharm.*, **318**, 1090—1095 (1985).
18) Nakazawa T., Ohsawa K., *J. Nat. Prod.*, **61**, 993—996 (1998).
19) Yung-Qi W., Matuzaki K., Takahashi K., Okuyama T., Shibata S., *Chem. Pharm. Bull.*, **36**, 3206—3209 (1988).
20) Kelley C. J., Mahajan J. R., Books L. C., Neubert L. A., Breneman W. R., Carmack M., *J. Org. Chem.*, **40**, 1804—1815 (1975).
21) Kelley C. J., Harruff R. C., Carmack M., *J. Org. Chem.*, **41**, 449—455 (1976).
22) Meselhy M. R., Nakamura N., Hattori M., *Chem. Pharm. Bull.*, **45**, 888—893 (1997).
23) Griffith L. A., "The Flavonoids: Advances in Research," ed. by London, 1982, pp. 681—715.
24) Abe K., Inoue O., Yumioka E., *Chem. Pharm. Bull.*, **33**, 208—211 (1990).
25) Hattori M., Shu Y. G., El-Sedamy A., Nanba T., Kobashi K., Tomimori T., *J. Nat. Prod.*, **51**, 874—878 (1988).
26) Viola H., Wasowski C., Levin de Stein, M. Wolfman C., Silveira R., Dajas F., Media J. H., Paladini A. C., *Planta Med.*, **61**, 213—216 (1995).