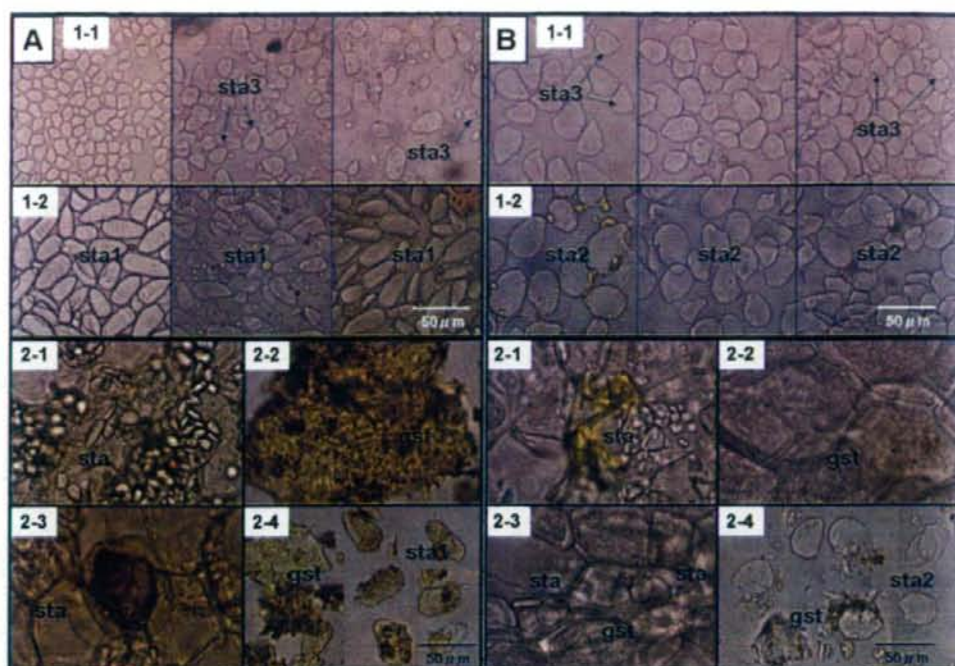
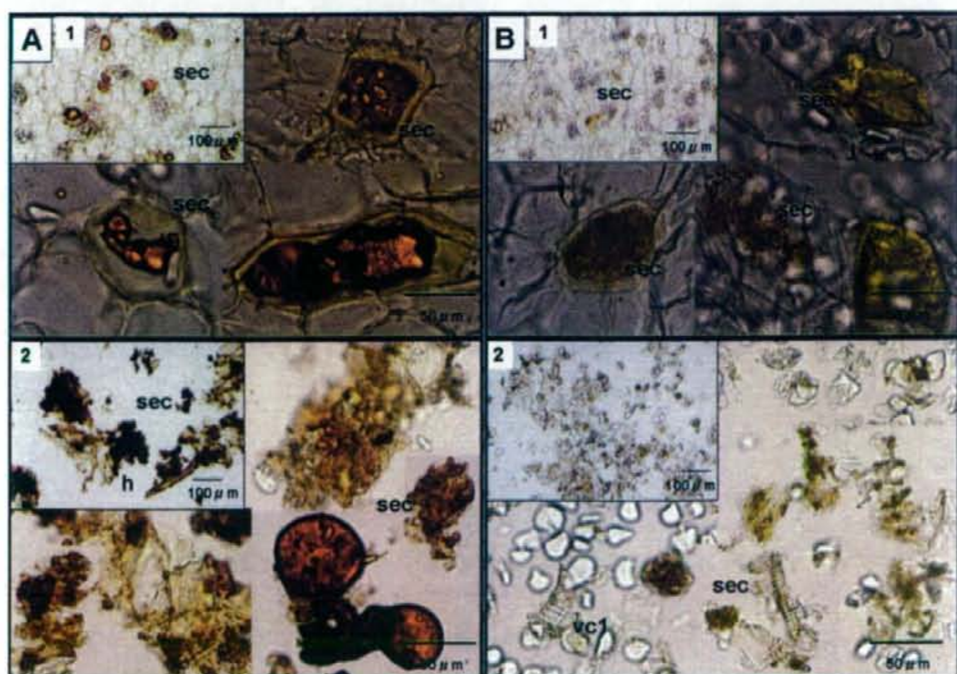


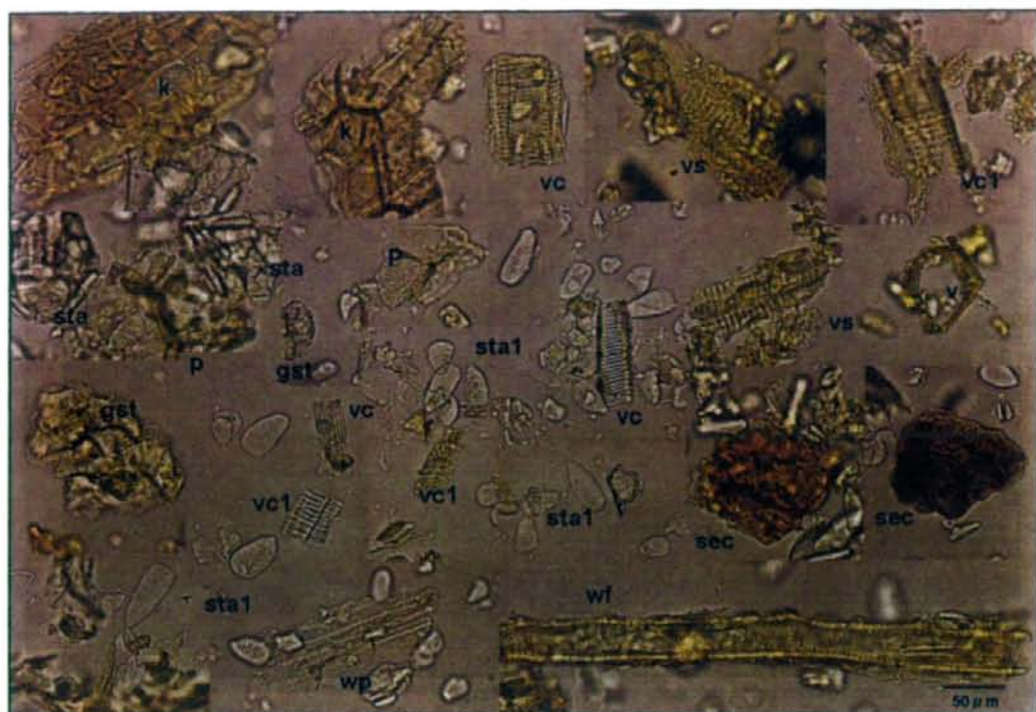
Pl.3 Shape of the starch grains and gelatinized starch of lateral rhizome

A: *C. longa* (Nos.1-3), B: *C. aromatica* (Nos.4-6). 1: shape of the starch grains in different collection time, 1-1: in September, 1-2: in February. 2: shape of the starch grains and gelatinized starch in different boiled time, 2-1: non boiled, 2-2: boiled for 1 minute, 2-3: boiled for 10 minutes, 2-4: starch grains in powdered lateral rhizome boiled more than 10 minutes.

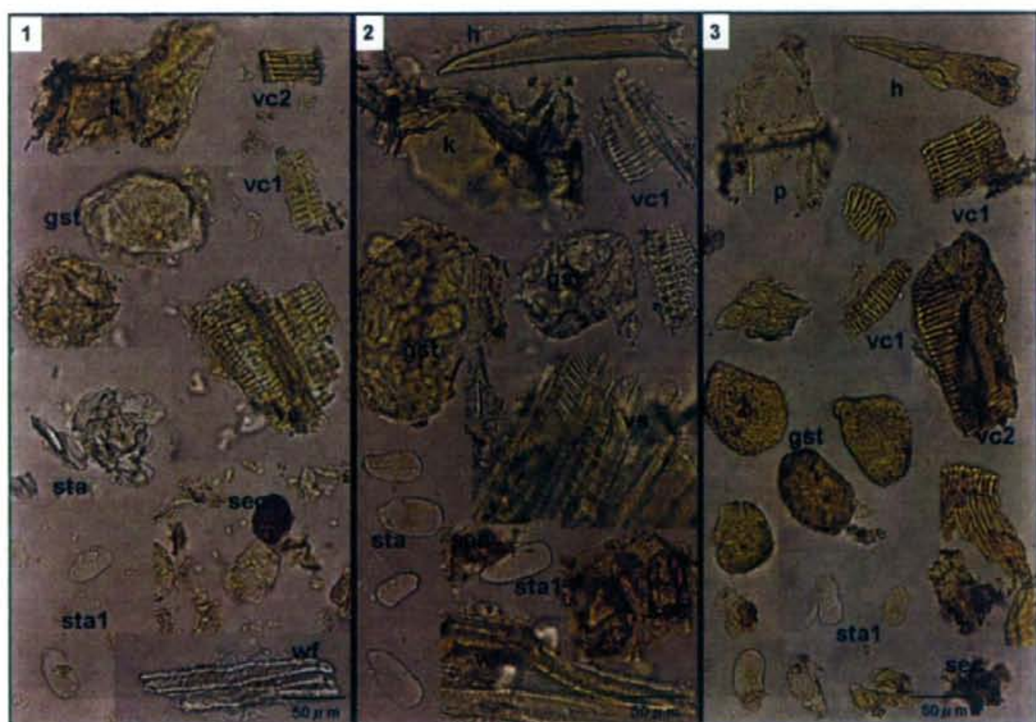


Pl.4 Appearance of inclusions

A: *C. longa*, B: *C. aromatica*. 1: secretions in a section mounted in mixture of glycerin and water, 2: secretions in powder mounted in water.

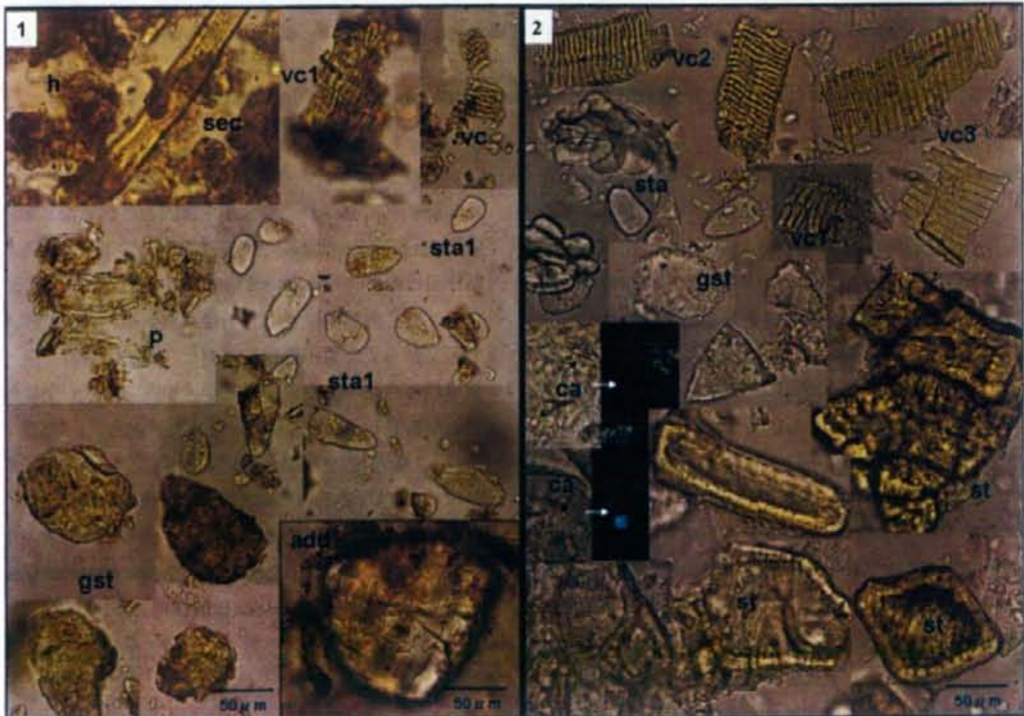


Pl.5 Elements of the powdered commercial turmeric No.7



Pl.6 Elements of the powdered commercial turmerics Nos.8, 10 and 13

Pl.7 Elements of the powdered commercial turmeric No.11



Pl.8 Elements of the powdered commercial turmerics Nos.9 and 12

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ORIGINAL PAPER

Comparison of the major flavonoid content of *S. baicalensis*, *S. lateriflora*, and their commercial products

Toshiaki Makino · Atsuyuki Hishida · Yukihiro Goda · Hajime Mizukami

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Abstract According to the notification for definition of pharmaceuticals from the Director-General of the Pharmaceutical and Food Safety Bureau, Ministry of Health Labour and Welfare of Japan, the roots of *Scutellaria baicalensis* (Chinese skullcap) and *S. lateriflora* (skullcap) are classified as "the raw materials exclusively used as pharmaceuticals", but their aerial parts are classified as "non-pharmaceuticals" so, in principle, there are no health claims for these materials and no descriptions of drug-like dosages or administration directions. Dried root of *S. baicalensis* is also registered in Japanese Pharmacopoeia XV as scutellaria root. Scutellaria root is considered to have the adverse drug reactions of interstitial pneumonia and drug-induced hepatopathy in kampo medicines (Japanese traditional herbal formulations), and baicalin, its major constituent, is considered to be the cause of the adverse reaction. This study was conducted to evaluate the validity of this borderline between pharmaceuticals and non-pharmaceuticals by analyzing the amounts of four flavonoids, including baicalin, in the roots, stems, and leaves of *S. baicalensis* and *S. lateriflora*, and in the commercial products herbal tea and dietary supplements prepared from *S. lateriflora*. These flavonoids were found in the root of *S. baicalensis*; its aerial parts, however, did not contain them. On the other hand, the amounts of those flavonoids in the aerial parts of *S. lateriflora* were larger than in the root. Herbal tea and dietary supplements of *S. lateriflora* obtained commercially also contained those flavonoids, and the dietary supplements contained amounts of them comparable with that in kampo medicine. These results suggest that classification that the aerial parts of *S. lateriflora* as non-pharmaceuticals in Japan needs reconsideration.

Keywords *Scutellaria baicalensis* · *Scutellaria lateriflora* · Baicalin · Wogonin · Kampo medicine · Dietary supplement

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Introduction

Scutellaria root is defined as the root of *Scutellaria baicalensis* (Labiatae) in the 15th edition of The Pharmacopoeia of Japan (JP XV) [1]. In Japanese traditional kampo medicine, scutellaria root is used for clearing away "heat" and drying "dampness", and is prescribed in formulae such as orengedokuto or shosaikoto [2]. Among the 210 OTC kampo formulae in Japan, scutellaria root is prescribed in 38, suggesting that this herb is one of the most frequently used herbal drugs in kampo medicine [2]. Recent pharmacological studies have revealed that scutellaria root has antibacterial [3], antioxidative [4], antiallergic [4], and antiulcer effects [5].

Since interstitial pneumonia caused by shosaikoto was reported in 1996, adverse drug reactions of kampo medicines such as interstitial pneumonia and drug-induced hepatopathy have accumulated, although their frequency is

not high [6]. Based on bibliographic survey, Yafune and Tsutani [7] predicted that the side effect of shosaikoto might be caused by scutellaria root, one of the herbal ingredients. Terada et al. [8] reported a pharmacoepidemiologic analysis indicating that 94 and 89% of the kampo herbal formulae applied in their hospital which caused interstitial pneumonia and drug-induced hepatopathy, respectively, contained scutellaria root as an ingredient. Nakazawa et al. [9] found that a herbal constituent common to several kampo formulae which caused drug-induced hepatic disorder in their hospital was scutellaria root. It is currently recognized that scutellaria root very occasionally causes the adverse drug reactions of interstitial pneumonia and drug-induced hepatopathy. However, the mechanism whereby scutellaria root causes these side effects is not fully understood. It has been predicted that baicalin, a major constituent in scutellaria root, would act as a hapten in patients with an allergic constitution [8, 9], but this hypothesis has not been proved by clinical or pharmacological studies.

The Japanese government has determined the borderline between pharmaceuticals and non-pharmaceuticals by listing the bulk ingredients (raw materials) exclusively used as pharmaceuticals [10]. Roots of *S. baicalensis* are classified as pharmaceuticals in the list. Considering the potent side effects of scutellaria roots, this classification seems adequate. On the other hand, the aerial parts of *S. baicalensis* are classified as non-pharmaceuticals in the list, which means the products can be sold freely without regulation by the Pharmaceutical Affairs Law as far as they do not present drug-like claims.

The aerial parts of *S. lateriflora* (skullcap) have been used as herbal tea and dietary supplements in European countries and the United States [11]. They are also imported into Japan. Although skullcap roots and aerial parts are classified as pharmaceuticals and non-pharmaceuticals, respectively, in the list, neither pharmacological nor chemical investigation of this species has yet been reported.

In this study we compared the chemical constituents of the roots, stems, and leaves of *S. baicalensis* and *S. lateriflora*, focusing on their major flavonoid compounds, baicalin, wogonin-7-*O*-glucuronide, baicalein, and wogonin. We also determined the amounts of these flavonoids in skullcap products obtained commercially.

Materials and methods

Materials

Baicalin, baicalein and wogonin were purchased from Wako Pure Chemicals (Osaka, Japan). Wogonin-7-*O*-glucuronide was kindly supplied by Tsumura (Tokyo, Japan). Other chemicals used were standard commercial products of analytical grade.

Scutellaria root (JP XV, cut-form) was purchased from Tsumura (Lot #23004351). *S. baicalensis* was harvested in the herbal garden of Nagoya City University (Nagoya, Japan; sample **#1**) and in a field of the Research Center for Medicinal Plant Resources, Tsukuba Division (Tsukuba, Japan; samples **#2–4**) in August 2006. *S. lateriflora* was harvested in a field of the Research Center for Medicinal Plant Resources, Tsukuba Division, in November 2006 (sample **#5**). Samples **#2** and **#3** were 1 and 7–8 years old, respectively; the ages of plant samples **#4** and **#5** were unknown. The voucher specimens of these samples were deposited in each research institute. These plants were used as samples after drying for two weeks under dark conditions.

Commercial products of skullcap herbal tea were purchased from Secret Garden (Shimanto, Kochi, Japan; sample **#6**), Harb Sache (New York, USA; sample **#7**), Ethnogens (Lawrence, KS, USA; sample **#8**), Herb-no-Kaori (Tokyo, Japan; sample **#9**), and E-tisane (Shizuoka, Japan; sample **#10**). Skullcap dietary supplement products were purchased from Nature's herb (American Fork, UT, USA; sample **#11**) and Nature's Answer (Hauppauge, NY, USA; sample **#12**). All the products were imported from the USA to Japan. Voucher specimens were deposited in the Department of Pharmacognosy, Graduate School of Nagoya City University, Nagoya, Japan.

Measurement of major flavonoid compounds characteristic of scutellaria

Analysis was conducted according to the protocol described in JP XV [1]. All samples except the dietary supplement products were finely pulverized and dried in a desiccator with silica gel for 24 h. Because sample **#11** (dietary supplement) was fine powder contained in capsules, the content was collected and used for analysis. Sample **#12** (dietary supplement) was a tincture, and was used as it is.

Samples were weighed accurately, about 100 mg for the samples **#1–4** and **#6–11**, and 20 mg for sample **#5**, because the amount of sample **#5** was much less than other samples. Sample **#12** (250 μl) was collected accurately by micropipet, and used for analysis. The samples were extracted in 6 ml of a mixture of diluted phosphoric acid (1 in 146) and acetonitrile (18:7) by sonication for 30 min at room temperature. After centrifugation (1,200*g* for 10 min), the supernatant was transferred into a new tube and the residue was further extracted by the same procedure. The extraction was repeated three times and the

supernatants were combined. The volume of the extract was adjusted to 20 ml by addition of the extraction solvent. Sample preparation was conducted in triplicate.

For scutellaria root (JP XV) and samples **#6–8**, the extracts were also prepared as a decoction or herbal tea. Scutellaria root is usually prescribed with a daily dosage of 3 g in many kampo formulas such as shosaikoto. Therefore, 3 g scutellaria root was decocted in 100 ml H_2O for 20 min. For herbal tea, 300 ml hot water (98°C) was added to 1.5 g of each of samples **#6–8**, and the mixtures were left to stand for 10 min at room temperature. The resulting decoction and herbal tea were centrifuged at 1,200*g* for 10 min, and the supernatant was diluted to exactly 300 ml with water. The preparation was conducted in triplicate.

The extracts were diluted appropriately and analyzed by HPLC (Shimadzu LC-10A, Kyoto, Japan) under the following conditions: column, Inertosil ODS-2 (4.6 × 250 mm, GL Science, Tokyo); mobile phase, A: diluted phosphoric acid (1 in 146), B: acetonitrile, A:B = 8:2 (0 min) → 2:8 (18 min) → 2:8 (24 min) linear gradient; flow rate, 1 ml/min; column temperature, 50°C; detection, UV 277 nm. Retention times of baicalin, wogonin-7-*O*-glucuronide, baicalein and wogonin were 17.0, 18.8, 20.5 and 22.3 min, respectively. Calibration was achieved by linear regression analysis, using the least-squares method, of peak areas obtained from standard solutions ($r^2 = 0.999$; 0.4–800 μg/ml for baicalin and wogonin-7-*O*-glucuronide, and 0.5–10 μg/ml for baicalein and wogonin). Flavonoid content was expressed as % (*w*/*w*), mean ± SD ($n = 3$), except for sample **#12**, which was expressed as % (*w*/*v*). For the decoction, herbal tea, and dietary supplements, the flavonoid content was expressed as human daily dosage (mg) mean ± SD ($n = 3$).

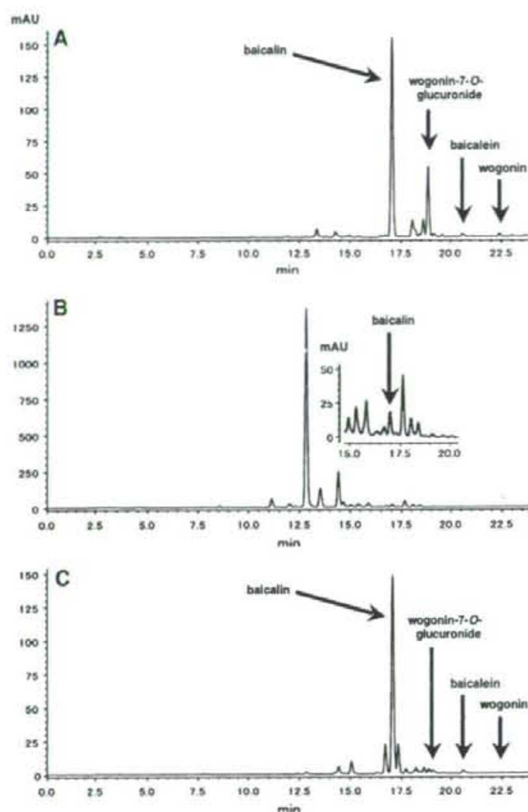

Fig. 1 Representative chromatograms of scutellaria root JP XV (**a**), leaf of *Scutellaria baicalensis* (sample **#2**, **b**), and skullcap herbal tea (sample **#8**, **c**). HPLC: column, Inertosil ODS-2 (4.6 × 250 mm), mobile phase, A: 0.68% phosphoric acid, B: methanol A:B = 8:2 (0 min) → 2:8 (18 min) → 2:8 (24 min) linear gradient, 1 ml/min, 50°C; detection, UV 277 nm; injection, 10 μl of the diluted sample solution (1:20 for scutellaria root, and 1:10 for sample **#8**. Sample **#2** was used without dilution)

Results

Figure 1 shows representative HPLC chromatogram of scutellaria root (JP XV), leaf of *S. baicalensis*, and skullcap herbal tea. Peak identification was carried out based on comparison of retention times and UV spectra with those of an authentic compounds. Table 1 indicates the amounts of baicalin, wogonin-7-*O*-glucuronide, baicalein, and wogonin in each sample. Scutellaria root (JP XV) and the roots of *S. baicalensis* contained these four flavonoids. The amounts of these flavonoids were much low in the aerial parts of the plant. On the other hand, *S. lateriflora* contained baicalin not only in the roots but in the aerial parts. The amount of wogonin-7-*O*-glucuronide in the roots of *S. lateriflora* was much less than that in the root of *S. baicalensis*.

Among the skullcap herbal tea products, these flavonoids were detected in samples **#6–9**, and the amounts of baicalein and wogonin were similar to that in scutellaria root (JP XV). Sample **#10** contained more wogonin-7-*O*-glucuronide and wogonin than samples **#6–9** whereas it contained only trace amounts of baicalin and baicalein. In dietary supplement products, sample **#11** (capsule-form) contained much more wogonin-7-*O*-glucuronide than baicalin and sample **#12** (tincture-form) also contained baicalin as a major flavonoid.

Next, in order to estimate human daily intake of the flavonoids, the samples were extracted in a manner similar to that in which they were used. Namely, scutellaria root was decocted as we prepare a kampo medicine and skullcap herbal tea was immersed in hot water. When scutellaria root (JP XV) was decocted, the baicalin content in the decoction was 101% that in the extract. Similarly, the baicalin content of skullcap herbal tea was

Table 1 Amounts of flavonoids in Scutellaria products

Sample	Baicalin (%)	Wogonin-7-*O*-glucuronide (%)	Baicalein (%)	Wogonin (%)
Scutellaria root (JP XV)	12.1 ± 0.4	3.71 ± 0.10	0.17 ± 0.008	0.104 ± 0.003
Root of *S. baicalensis* (**1**)	7.07 ± 1.05	3.16 ± 0.11	0.247 ± 0.049	0.061 ± 0.010
Root of *S. baicalensis* (**2**)	3.52 ± 0.14	1.03 ± 0.04	0.066 ± 0.009	0.016 ± 0.001
Root of *S. baicalensis* (**3**)	8.63 ± 1.55	3.46 ± 0.09	0.133 ± 0.045	0.051 ± 0.007
Root of *S. baicalensis* (**4**)	11.4 ± 2.7	5.07 ± 0.14	0.073 ± 0.020	0.029 ± 0.004
Leaf of *S. baicalensis* (**1**)	Trace	Trace	n.d.[a]	n.d.
Leaf of *S. baicalensis* (**2**)	0.057 ± 0.008	Trace	Trace	Trace
Leaf of *S. baicalensis* (**3**)	Trace	Trace	n.d.	n.d.
Stem of *S. baicalensis* (**1**)	Trace	n.d.	n.d.	n.d.
Root of *S. laterifolia* (**5**)	1.15 ± 0.04	0.161 ± 0.008	0.024 ± 0.002	0.027 ± 0.000
Stem of *S. laterifolia* (**5**)	0.82 ± 0.10	0.080 ± 0.006	0.022 ± 0.001	0.013 ± 0.004
Leaf of *S. laterifolia* (**5**)	2.83 ± 0.13	0.054 ± 0.004	0.041 ± 0.003	0.059 ± 0.009
Scullcap Tea Product (**6**)	5.22 ± 0.17	0.258 ± 0.011	0.888 ± 0.036	0.061 ± 0.005
Scullcap Tea Product (**7**)	1.54 ± 0.33	0.132 ± 0.013	0.112 ± 0.022	0.027 ± 0.004
Scullcap Tea Product (**8**)	4.98 ± 0.17	0.145 ± 0.000	0.107 ± 0.013	0.013 ± 0.002
Scullcap Tea Product (**9**)	2.47 ± 0.14	0.141 ± 0.015	0.132 ± 0.008	0.025 ± 0.002
Scullcap Tea Product (**10**)	Trace	0.749 ± 0.045	Trace	0.117 ± 0.021
Scullcap Dietary Supplement (**11**)	0.169 ± 0.006	0.533 ± 0.005	0.021 ± 0.005	0.109 ± 0.001
Scullcap Dietary Supplement (**12**)	1.40 ± 0.01	0.048 ± 0.006	0.084 ± 0.003	0.025 ± 0.000

Trace, less than 0.010%

[a] Not detected

96–104%. Daily doses of baicalin were then calculated on the basis of the ordinary dose of kampo medicines and the recommended amount of skullcap herbal tea and dietary supplement (Table 2). Daily intake of baicalin is about 319 mg from kampo medicines containing scutellaria root (JP XV), about 12–27 mg from 1.5 g herbal tea (about three spoons), and about 7–81 mg from the skullcap dietary supplement.

Discussion

Of various flavonoid compounds so far isolated from the roots of *S. baicalensis* and identified [12], we focused on four flavonoids, baicalin, baicalein, wogonin-7-*O*-glucuronide and wogonin in this investigation. The baicalin content of scutellaria root (JP XV) obtained commercially was 12.1%, which was in accordance with to the requirement of JP XV. The baicalin content of the roots of *S. baicalensis* cultivated in fields varied from 3.5% (sample **#2**) to 11.4% (sample **#4**), probably because of the age of a particular plant and/or environmental conditions. It was also found that aerial parts of *S. baicalensis* accumulated only trace amounts of these flavonoids. Because baicalin is regarded as the cause of the adverse effects of scutellaria root [8, 9], these results suggest that the Notification [10] that the borderline between pharmaceuticals and non-pharmaceuticals from *S. baicalensis*, i.e. between its root and its aerial parts, is adequate.

In sharp contrast with *S. baicalensis*, *S. lateriflora* contains these flavonoid compounds in the aerial parts in amounts comparable with those in the roots, although material from one plant only was subjected to analysis. We also analyzed five samples of the commercial product skullcap herbal tea, presumably derived from the aerial part of *S. lateriflora*. The baicalin content of samples **#6** and **#8** was approximately 5%, which was higher than that of samples **#7** and **#9**. From the fact that the former two samples were mixtures of mostly leaves with a small amount of stems while the latter two samples comprised mostly stems, it is predicted that the leaves of *S. lateriflora* might contain more baicalin than the stems. Sample **#10** contains wogonin-7-*O*-glucuronide as a major flavonoid and a trace amount of baicalin was present, suggesting that the original plant of sample **#10** might be a different chemotype compared with the other samples.

We also analyzed the amounts of the flavonoids in dietary supplement products from skullcap imported from the United States into Japan. Sample **#11** was a capsule form containing light green fine powder. The baicalin content of the powder was 0.17%, whereas the wogonin-7-*O*-glucuronide content was 0.53%. Considering the baicalin-to-wogonin-7-*O*-glucuronide ratio, the chemotype of sample **#11** could be the same as that of sample **#10**. Sample **#12** was a tincture contained in a glass bottle from which dark brown viscous suspension can be collected. This suspension contained 1.4% baicalin and 0.05% wogonin-7-*O*-glucuronide.

Table 2 Human daily intake of flavonoids in scutellaria products

Sample	Baicalin (mg)	Wogonin-7-*O*-glucuronide (mg)	Baicalein (mg)	Wogonin (mg)
Scutellaria root (JP XV)	319 ± 51	81.3 ± 13.7	3.76 ± 0.78	1.96 ± 0.360
Scullcap tea product (**6**)	27.1 ± 0.5	1.05 ± 0.05	2.82 ± 0.13	0.187 ± 0.002
Scullcap tea product (**7**)	25.9 ± 0.6	0.502 ± 0.102	0.074 ± 0.007	Trace
Scullcap tea product (**8**)	11.8 ± 1.5	0.450 ± 0.018	0.119 ± 0.013	0.056 ± 0.004
Scullcap dietary supplement (**11**)	6.69 ± 0.24	21.1 ± 0.2	0.829 ± 0.187	4.32 ± 0.057
Scullcap dietary supplement (**12**)	83.7 ± 0.59	2.88 ± 0.37	5.06 ± 0.17	1.50 ± 0.01

Scutellaria root JP XV (3 g) was boiled for 10 min. Each sample (1.5 g) of herbal tea (**6**–**8**) was extracted by immersion in hot water for 10 min. Dietary supplements (**11**, **12**) were recommended dairy intake written on labels. Trace, less than 0.010%

In kampo medicine, scutellaria root (3 g) is prescribed in orengedokuto or shosaikoto as a daily dose which corresponds to 319 mg baicalin. In contrast, when about three spoons of skullcap herbal tea was placed in hot water, daily intake of baicalin was calculated to be 12–27 mg, which corresponds to 4–8% of the daily dose of baicalin from the kampo medicines. The intake of wogonin-7-*O*-glucuronide from skullcap herbal tea was less than 2% of that from scutellaria root decoction. Furthermore, intake of baicalin was 6.69 mg from sample #**11** (capsule form) and 88.7 mg from #**12** (tincture form) when calculated from the daily intake written in the labels of the skullcap dietary supplements, the latter of which is more than one-third of the dose of kampo medicines. Daily intake of wogonin-7-*O*-glucuronide and wogonin from sample #**11** was 21.1 and 4.32 mg, respectively, which was comparable with that from kampo medicine (81.3 mg for wogonin-7-*O*-glucuronide and 1.96 mg for wogonin). When people take skullcap as herbal tea with the usual dosage, the flavonoid intake would be much lower than for the dosage of scutellaria root used for kampo medicines. However, the flavonoid intake from dietary supplement products containing *S. lateriflora* would be comparable with that from kampo medicine, suggesting that these dietary supplements could have the same adverse effects of interstitial pneumonia and drug-induced hepatopathy as kampo medicine, which contains scutellaria root and its flavonoid content.

Indeed, skullcap (leaves of *S. lateriflora*) has been reported to cause drug-induced hepatopathy [13]. However, skullcap is classified as the class 1, which is comparatively safe if the herb is used properly, because the hepatotoxicity was believed to be induced by germander contamination, which causes strong, acute hepatotoxicity [13]. It is predicted from the results of this study that the hepatotoxicity of skullcap described in Ref. [13] might be caused by baicalin and related compounds contained in skullcap itself.

In Japan, the roots of *S. baicalensis* and *S. lateriflora* are regulated as pharmaceuticals, while their aerial parts are non-pharmaceuticals. However, *S. lateriflora* contains baicalin and related compounds in whole plant materials, and the flavonoid content of the aerial part, especially the leaf, is more than in the root. Considering that these compounds have potent pharmacological activity and that scutellaria root, which contains these compounds, is suggested to be a possible source of the adverse reactions of kampo medicines, classification of the aerial parts of *S. lateriflora* as non-pharmaceuticals should be reconsidered for safety control of medicinal herbs. Further study is needed to determine experimental evidence of the adverse reactions related to scutellaria root or its components.

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NOTE

Chemical and biologically active constituents of *Pteris multifida*

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Abstract A new compound, 4-caffeoyl quinic acid 5-*O*-methyl ether (**2**), together with 12 known compounds—identified as (2*R*,3*R*)-pterosin L 3-*O*-*β*-D-glucopyrannoside (**3**), *β*-sitosterol *β*-D-glucopyranoside (**4**), apigenin 7-*O*-*β*-D-glucopyranoside (**5**), luteolin 7-*O*-*β*-D-glucopyranoside (**6**), sucrose (**7**), caffeic acid (**8**), pterosin C 3-*O*-*β*-D-glucopyranoside (**9**), pteroside C (**10**), 4,5-dicaffeoyl quinic acid (**11**), pteroside A (**12**), wallichoside (**13**) and (2*S*)-5,7,3′,5′-tetrahydroxyflavanone (**14**)—were isolated from *Pteris multifida*. The structure of the new compound was determined by means of physical, chemical and spectroscopic evidence. Compounds **5** and **6** were the main constituents of the plant, with yields of 0.19% and 0.16%, respectively. The cytotoxic activities of **2**, **3**, and **9–13** were evaluated against a human cell line (KB cells). Among the isolated compounds, pterosin C 3-*O*-*β*-D-glucopyrannoside (**9**) and 4,5-dicaffeoylquinic acid (**11**) showed a significant selective cytotoxicity (IC_{50} 2.35 and 5.38, respectively), while moderate activity was observed for compound **2** (IC_{50} 12.3). The chemosystematics of *Pteris* species is also discussed.

Keywords *Pteris multifida* · Pteridaceae · Quinic acid · Pterosin · Flavonoid · Chemosystematic · Cytotoxic activity

Introduction

Since the isolation and characterization of ptaquiloside (**1**) (Fig. 1), the carcinogenic illudane-type nor-sesquiterpenoid of bracken fern (*Pteridium aquilium*) [1–3], it has become important to investigate the content of putative carcinogens present in the Pteridaceae species used as food and/or as natural medicines. *Pteris multifida* Poir., known as spider brake fern, is one of the world's common plants, widely distributed in the temperate area of Japan. Decoction of the whole part of the plant is used in Chinese folk medicine to produce a solution that acts as an antipyretic, antidote, and stops bleeding [4]. Previous phytochemical investigation of the plant led to the isolation of kauranoids, C_{14} and C_{15} illudane-type sesquiterpenoids (known as pterosins), flavonoids, asperglauside and caffeoyl esters of quinic acid [5–9]. In the continuation of our systematic investigation aiming to isolate new and cytotoxic compounds from vascular plants, we have carried out a phytochemical investigation of *P. multifida* Poir., collected in Kure City, Hiroshima, Japan. This report deals with the isolation and structural determination of the chemical constituents of *P. multifida* and their cytotoxic activity.

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Results and discussion

The 1-butanol-soluble fraction of *P. multifida* was chromatographed repeatedly on silica gel column and ODS RP-18 medium pressure liquid column (ODS-MPLC) to afford a new compound, 4-caffeoylquinic acid (**2**), together with 12 known compounds, identified as (2*R*,3*R*)-pterosin L 3-*O*-*β*-D-glucopyrannoside (**3**) [10]; *β*-sitosterol *β*-D-glucopyrannoside (**4**); apigenin 7-*O*-*β*-D-glucopyrannoside (**5**) [7]; luteolin 7-*O*-*β*-D-glucopyrannoside (**6**) [7]; sucrose (**7**); caffeic acid (**8**); pterosin C, 3-*O*-*β*-D-glucopyrannoside (**9**) [7]; pteroside C (**10**) [11]; 4,5-dicaffeoylquinic acid (**11**) [12]; pteroside A (**12**) [12]; wallichoside (**13**) [13]; and (2*S*)-5,7,3′,5′-tetrahydroxyflavanone (**14**) [14]. The

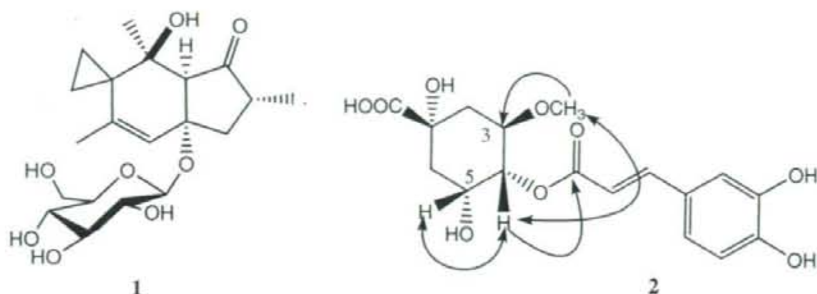

Fig. 1 Structures of compounds **1** and **2**. Important NOESY (*double arrow*) and HMBC (*arrow*) correlations observed

structures of the known compounds were elucidated through the interpretation of their physical and spectroscopic data, and by comparison with values reported in the literature.

4-Caffeoyl quinic acid 3-*O*-methyl ether (**2**) exhibited a molecular formula of $C_{17}H_{20}O_9$, as determined by the positive-ion HRESIMS (m/z 391.1008 $[M + Na]^+$). The IR absorption bands at υ_{max} 3368, 1723, and 1625 cm^{-1} suggested the presence of hydroxyl, carboxylic acid, and ester carbonyl groups. The ^{1}H-NMR spectrum showed signals of a caffeoyl moiety—δ_H 7.62 (1H, d, $J = 15.9$ Hz, H-β), 7.07 (1H, d, $J = 2.0$ Hz, H-2′), 6.95 (1H, dd, $J = 8.0$, 2.0 Hz, H-6′), 6.79 (1H, d, $J = 8.0$ Hz, H-5′), and 6.35 (1H, d, $J = 15.9$ Hz, H-α)—one methoxyl at δ_H 3.34 (3H, s), together with signals due to a quinic acid with H-3 appearing at δ_H 4.13 (1H, brs, see experimental). Morever, heteronuclear single quantum correlation (HSQC) and correlation spectroscopy (COSY) experiments allowed us to confirm the assignments of C-1 to C-6 of the quinic acid moiety. The allocations of the methoxyl group to be on the hydroxyl group at C-5 and the acyl moiety on that at C-4 were deduced as follows. HMBC correlations were observed between the methoxyl proton signal (δ_H 3.34) and C-3 (δ_C 67.7), and the proton signal at δ_H 4.92 (H-4) and δ_C 169.6 (O-C=O) (Fig. 1(**2**)). The NOE correlations observed between H-4 and H-5, and H-5 and the methoxyl group at C-3 confirmed their β-orientations (Fig. 1). Alkaline hydrolysis of **2** gave 5-*O*-methyl quinic acid methyl ester, the ^{1}H-NMR of which showed an upfield-shift of H-4 δ_H 3.95 (1H, dd, $J = 7.8$, 5.3 Hz) due to the presence of hydroxyl group at C-4. From the above data, the structure of **2** was deduced to be 4-caffeoylquinic acid 3-*O*-methyl ether.

Chemosystematics of the genus *Pteris*

The plants belonging to the genus *Pteris* have been shown to contain kauranoids, pterosins, pterosides, and flavonoids. Murakami and coworkers [7] investigated the chemical constituents of six species of *Pteris*: *P. tremula*, *P. dactylina*, *P. angustipinna*, *P. multifida*, *P. grevilleana*, and *P. cretica*. Pterosin B, the hydrolysis product of **1** has been found in all investigated species except the rhizome of *P. cretica*. Neither **1** nor pterosin B was detected during the present study. This is the second report of the presence of caffeoylquinic acid derivatives in the *Pteris* species, since the isolation of 5-*O*-caffeoyl, 3,5-di-*O*-caffeoyl, and 4,5-di-*O*-caffeoylquinic acids from the genus [9]. Compounds **5** and **6** (0.19% and 0.16%, respectively) were the main constituents of the title plant.

Table 1 Cytotoxicity (IC_{50} in μM) of compounds **2**, **3**, and **9**–**13** against human cancer cell line (KB cells) in vitro

Compound	IC_{50}
2	12.3
3	24.7
9	2.3
10	22.9
11	5.3
12	18.8
13	28.3

Cytotoxicity

The cytotoxic activity of compounds **2**, **3**, and **9**–**13** were tested against a human cancer cell line (KB cells); the results are presented in Table 1. Pterosin C 3-*O*-β-D-glucopyrannoside (**9**, IC_{50} 2.3) and 4,5-dicaffeoylquinic acid (**11**, IC_{50} 5.3) showed significant cytotoxicity. The location of the glucose moiety at C-3 of **9** seems to be important for the activity. These results were confirmed by the decrease (about tenfold) of the activity of **10**, in which the glucose attaches at C-14. Moreover, moderate activity was observed for compound **2** (IC_{50} 12.3).

Experimental

General experimental procedures

Optical rotations were measured on a JASCO P-1030 digital polarimeter. FT-IR spectra were recorded on a Horiba FT-710 spectrophotometer. UV spectra were measured with a

JASCO V-520 UV/VIS spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL α-400 spectrometer (400 and 100 MHz, respectively) with TMS as internal standard. HRESIMS were carried out on an Applied Biosystems QSTAR XL system mass spectrometer. Silica gel and ODS-MPLC were performed on silica gel 60 (Merck, 70–230 mesh) and Cosmosil $75C_{18}$-OPN (Nacalai Tesque Co, Ltd., Kyoto, Japan), respectively. Preparative HPLC was performed using an ODS-120T column (TSK gel, $\varphi = 7.8$ mm, $L = 30$ cm, Tosoh, Tokyo, Japan).

Plant material

The aerial parts of *P. multifida* were collected in Kure city, near Hiroshima, Japan, in May 2007, and a voucher specimen (07-PM-0504) was deposited in the Herbarium of the Department of Pharmacognosy, Graduate School Biomedical Sciences, Hiroshima University, Japan.

Extraction and isolation

Powdered aerial parts of *P. multifida* (800 g) were extracted with MeOH (18 l) two times at room temperature for 1 week. The extracts were filtered and concentrated in vacuo to yield a dark-green residue (133.6 g), which was then suspended in water (1 l) and partitioned with EtOAc (1 l) to afford 25.8 g of green residue after evaporation. Partition of the aqueous layer with 1-butanol (1 l) afforded 26.8 g of brown extract and silica gel column chromatography (ϕ = 5.3 cm, $L = 80$ cm) of the 1-butanol-soluble fraction using an isocratic solvent system (CH_2Cl_2-MeOH-H_2O:17:6:1) afforded 11 fractions. Compounds **4** (15 mg), **5** (1.52 g), and **6** (1.30 g) were purified from fractions 4, 7, and 8, respectively, by precipitation in MeOH. Fraction 6 was subjected to an ODS MPLC (10–100% MeOH) to give 10 fractions. Preparative ODS HPLC (solvent system: 17% aqueous CH_3CN) of sub-fractions 6–8 afforded compounds **12** (7.4 mg), **13** (2.2 mg), **3** (12 mg), **10** (6.1 mg), and **14** (18.4 mg). Compound **9** (2.2 mg) was obtained from preparative HPLC (solvent system: 17% aqueous CH_3CN) of the sub-fractions 6–10. Preparative ODS MPLC (10% aqueous MeOH to 100% MeOH) of fraction 11 yielded compounds **2** (116.8 mg), **7** (200.5 mg), **8** (25 mg), and **11** (13.4 mg).

4-Caffeoyl quinic acid 3-*O*-methyl ether (**2**)

Amorphous powder: [α]-43.1 (*c* 0.10, MeOH). IR υ_{max} (film): 3368, 2950, 1723, 1625, and 1029 cm^{-1}.

^{1}H-NMR (400 MHz, CD_3OD) δ: 7.62 (1H, d, $J = 15.9$ Hz, H-β), 7.07 (1H, d, $J = 2$ Hz, H-2'), 6.95 (1H, dd, $J = 8.0$, 2.0 Hz, H-6'), 6.79 (1H, d, $J = 8.0$ Hz, H-5'), 6.35 (1H, d, $J = 15.9$ Hz, H-α), 4.92 (1H, dd, $J = 7.4$, 3.3 Hz, H-4), 4.29 (1H, td, $J = 7.4$, 3.3 Hz, H-5), 4.13 (1H, brs, H-3), 3.34 (3H, s, OCH_3), 2.11 (2H, m, H-2a and 6a), and 1.95 (2H, m, H-2b and 6b).

^{13}C NMR (100 MHz, CD_3OD) δ: 182.2 (C-7), 169.6 (C = O of the caffeoyl moiety), 150.1 (C-4′), 147.6 (C-3′), 147.4 (C-β), 128.6 (C-1′), 123.5 (C-6′), 117.1 (C-5′), 116.2 (C-α), 115.8 (C-2'), 79.5 (C-4), 76.9 (C-1), 69.3 (C-5), 67.7 (C-3), 50.4 (OCH_3), 40.6x2 (C-2 and C-6); HRESIMS (positive-ion mode) *m/z* 391.1008 $[M + Na]^+$ ($C_{17}H_{20}O_9Na$, requires *m/z* 391.1005).

Alkaline hydrolysis of **2**

Compound **2** (26 mg) was dissolved in 2.7 ml MeOH, and 0.3 ml $NaOCH_3$ (1 M) was added. The reaction mixture after allowing to stand at room temperature for 4 h, was neutralized by passing through an amberlite IR-120 (H^+) ion-exchange resin to give brown precipitates (**2a**, 6.1 mg). The precipitates were identified as 5-*O*-methyl quinic acid methyl ester ([α]-43.1, c 0.10, MeOH); ^{1}H-NMR (400 MHz, CD_3OD) δ: 1.63 (1H, dd, $J = 13.8$, 5.8, H-2a), 1.70 (1H, dd, $J = 13.8$, 3.5, H-2e), 1.73 (1H, dd, $J = 13.8$, 7.8, H-6a:), 1.80 (1H, dd, $J = 13.8$, 4.0, H-6e), 4.10 (1H, brs, H-3), 3.95 (1H, dd, $J = 7.8$, 5.3, H-4), and 4.04 (1-H, td, $J = 7.8$, 4.0, H-5).

MTT cytotoxic assay

The assay was performed using human epidermoid carcinoma KB cells and the viability estimated by means of colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay [15]. Dulbecco's modified Eagle medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/ml of kanamycin and 0.5 μg/ml of amphotericin B, was used as the cell culture medium. KB cells (5×10^3 cells/well in 96-well plates, 100 μl/well) were cultured in a CO_2 incubator at 37 C for 24 h. The test compounds, dissolved in DMSO (final concentration below 0.1%) were added to each well at 1% as the final concentration. After 72 h incubation, MTT solution was added to each well, and the plates ware incubated for another 1.5 h. The viability was compared with that of control cells incubated in the same medium without the test compounds. The cytotoxic activity was evaluated as the IC_{50}.

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ORIGINAL PAPER

Chemical constituents of imported Rosae fructus

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Abstract Rosae fructus is a traditional Chinese crude drug, used for purgative purposes. It is included in the Japanese Pharmacopoeia XV, in which its origin is stated to be *Rosa multiflora* Thunberg. These days, some imported Rosae fructus are on the market and the pharmacological activity of the imported product is in question. The chemical constituents of Japanese Pharmacopoeial Rosae fructus, imported from the People's Republic of China and whose plant origin is expected to be *Rosa* aff. *multiflora*, were investigated to give 2-hydroxynaringin 5-*O*-*β*-D-glucopyranoside and L(*S*)-pyroglutaminic acid derivative as new compounds. However, although we made every effort, the major flavonoids in *R. multiflora*, multiflorins A and B, and multinoside A acetate, could not be isolated.

Keywords Rosae fructus · *Rosa* aff. *multiflora* · Rosaceae · Flavonoid

Introduction

In Rosae fructus, some flavonol glycosides, i.e., multiflorins A and B and multinoside A and its acetate, are major constituents and play an important role in its purgative effect [1, 2]. Although the market for Rosae fructus is not very large, there are some rumors that imported Rosae fructus does not possess sufficient pharmacological activity. In this study, the chemical constituents of Japanese Pharmacopoeial Rosae fructus, imported from the People's Republic of China, are investigated.

Results and discussion

From Rosae fructus, imported from the People's Republic of China, two new compounds (**1** and **2**) (Fig. 1) along with a methyl ester of **2** (**3**), which must be formed during isolation, quercetin (**4**) [3], seven flavonoid glycosides, multinoside A (**5**) [3], isoquercitrin (**6**) [3], quercetin 3-*O*-*β*-D-glucuronide methyl ester (**7**) [4], hyperin (**8**) [3], isorhamnetin 3-*O*-*β*-D-glucoside (**9**) [5, 6], taxifolin 3-*O*-*β*-D-xylopyranoside (**10**) [7], heliocioside A (**11**) [8], syringin (**12**) [9, 10], (6*S*,9*R*)-roseoside (**13**) [11], and 9′-tetrahydroxy-3-methoxy-8-*O*-4′-neolignane 3′-*O*-*β*-D-glucopyranoside (**14**) [12] were isolated by means of various chromatographic techniques. The known compounds were identified by spectroscopical comparison with data reported in the literature.

2-Hydroxynaringin 5-*O*-*β*-D-glucopyranoside (**1**), $[\alpha]_D$ −61.7, was isolated as an amorphous powder and its elemental composition was determined to be $C_{21}H_{22}O_{11}$ on negative-ion HR-FAB-MS. In the IR spectrum, absorption bands for hydroxyl groups (3,344 cm^{-1}), a conjugated carbonyl group (1,689 cm^{-1}), and aromatic rings (1,618 and 1,514 cm^{-1}) were observed. The UV absorption maxima indicated the phenolic feature of **1**. In the ^{13}C-NMR spectrum, some signals appeared as dual peaks in the ratio of nearly 1:1 (Table 1). However, essentially 21 signals were used to establish the structure. Six signals

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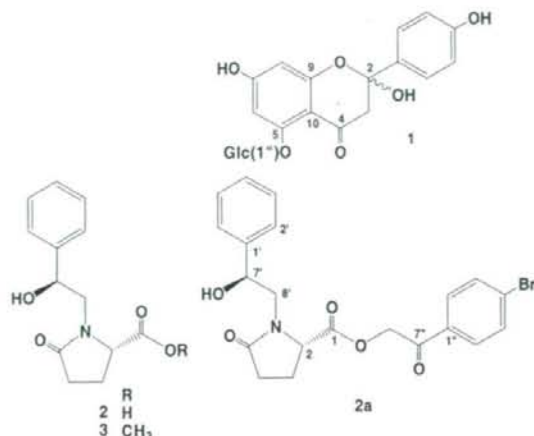

Fig. 1 Structures of new compounds, 1, 2 and 3, and *p*-bromophenacyl ester of 2 (**2a**)

were assigned for β-glucopyranose, ten sp^2 signals, two of which exhibited double strength, for two benzene rings, one highly deshielded signal [δ_C 196.91 (196.82)] for a carbonyl carbon, and δ_C 42.12 (41.96) and 107.62 (107.57) signals for methylene and ketal carbons, respectively. In the ^{1}H-NMR spectrum, essentially two aromatic proton signals coupled in an AA′BB′ system and two *meta* coupled aromatic protons were also observed. Based on this evidence, the fundamental structure of **1** was expected to have a flavanone skeleton, and position 2 possessed a hydroxyl group to form a hemiketal functional group. Key HMBC correlations from δ_H 3.10 and 3.07 to δ_C 107.62 (107.57) and δ_C 196.91 (196.82) supported the structure (Fig. 2). The position of the sugar linkage was established to be on the hydroxyl group at C-5, based on the HMBC correlation from the anomeric proton [δ_H 4.87 (4.85)] to C-5 [δ_C 158.41 (158.29)]. This was further confirmed by the differential NOE experiment, in which irradiation of the anomeric proton significantly enhanced the peak area of the H-6 signal [δ_H 6.06 (6.05)]. The mode of the sugar linkage was determined to be β from the coupling constants (8 Hz) of the anomeric proton, and the absolute configuration of glucose was established to be of the D-series from the results of chirality analysis of the hydrolyzate of **1**. Therefore, the structure of **1** was elucidated to be 2-hydroxynaringin 5-*O*-β-D-glucopyranoside, as shown in Fig. 1. 2-Hydroxynaringin exists as an interconvertible epimeric mixture of hemiketal and 1,3-diketone forms [13–15]. However, based on the mass spectral and NMR data, the hemiketal form was dominant.

Compound **2**, $[\alpha]_D^{21}$ +30.3, was isolated as an amorphous powder, but its elemental composition could not be determined since no molecular ion peak was observed in the high-resolution electrospray time-of-flight mass spectrum (HR-ESI-TOF-MS) or on HR-FAB-MS. The IR spectrum showed strong absorption bands at 3,395 cm^{-1} for a hydroxyl group, which tailed to around 2,500 cm^{-1}, at 1,651 cm^{-1} for an amide carbonyl group, and at 1,590 cm^{-1} for a carboxylate carbonyl group. From this evidence, compound **2** was implied to be present as a zwitterion. In the ^{13}C-NMR spectrum together with the ^{1}H-NMR spectrum, carbon signals for two carbonyl carbons, one monosubstituted benzene ring, three methylenes, and two methines with electronegative substituents were observed. The methylene signal at δ_C 51.5 suggested that compound **2** contained a

Table 1 NMR spectroscopic data for **1** (100 MHz for C and 400 MHz for H, CD_3OD)

	1			
.	C		H	
2	107.62	107.57	–	
3	42.12	41.96	3.10 1H, d, 16	
			3.07 1H, d, 16	
4	196.91	196.82	–	
5	158.41	158.29	–	
6	97.74	97.40	6.06 ½H, d, 2	6.05 ½H, d, 2
7	171.46		–	
8	93.38	93.28	5.94 1H, d, 2	
9	174.50		–	
10	103.69	103.50	–	
1′	125.51	125.60	–	
2′, 6′	132.51		6.99 2H, d, 8	
3′, 5′	115.83	115.80	6.58 1H, d, 8	5.57 1H, d, 8
4′	157.20	157.17	–	
1″	101.73		4.87 ½H, d, 8	4.85 ½H, d, 8
2″	74.07	74.03	[a]	
3″	78.34	78.27	[a]	
4″	71.20		[a]	
5″	77.38	77.32	[a]	
6″	62.39		3.86 1H, dd, 12, 5	
			3.67 ½H, dd, 12, 2	3.68 ½H dd, 12, 2

[a] Overlapped between 3.40 and 3.57

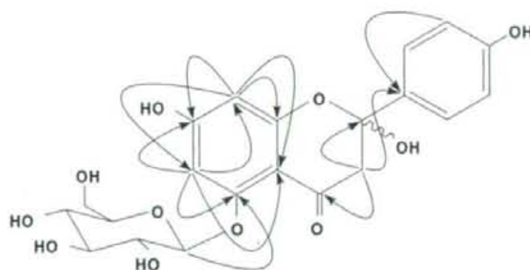

Fig. 2 Diagnostic HMBC correlations of **1**. *Arrowheads* denote carbons and *arrowtails* protons

nitrogen atom, and the H-H COSY spectrum showed two distinct connectivities, H-2 to H_2-3 and then to H_2-4, and H-7′ to H-8′. Based on the HMBC spectral data, although several correlations substantiated partial structures (Fig. 3), the planar structure could not be elucidated. Thus, compound **2** was reacted with *p*-bromophenacyl bromide to yield the *p*-bromophenacyl ester of **2** (**2a**), which gave a suitable crystal for X-ray crystallographic analysis. The structure of compound **2a** was confirmed to be *N*-7-hydroxyphenylethynyl L(*S*)-pyroglutamic acid, as shown by the ORTEP drawing in Fig. 4. Therefore, the structure of **2** was elucidated to be a free acid form of **2a**.

Compound **3**, $[\alpha]_D^{29}$ +17.8, was isolated as an amorphous powder and its elemental composition was determined to be

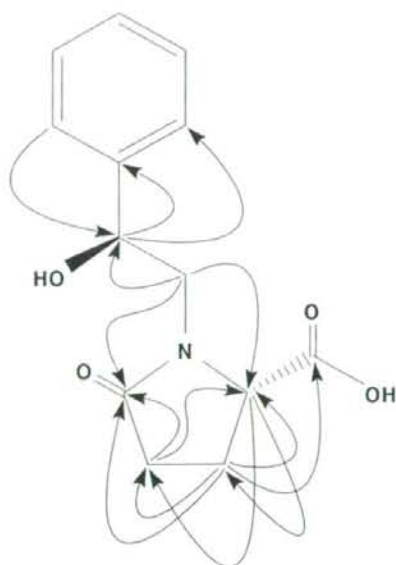

Fig. 3 Supportive HMBC correlations of **2**. *Arrowheads* denote carbons and *arrowtails* protons

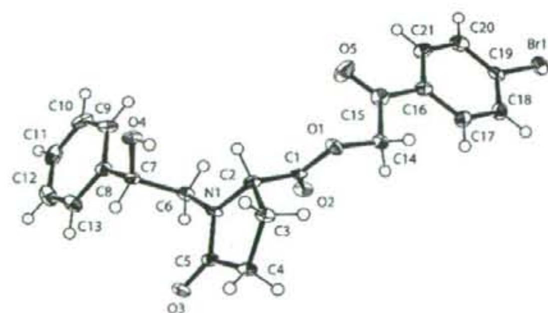

Fig. 4 ORTEP drawing of **2a**. The structure has X-ray crystallographic numbering

$C_{14}H_{17}O_4N$ on HR-ESI-TOF-MS. The ^{1}H- and ^{13}C-NMR spectra showed that compound **3** was an analogous compound to **2**, except for the presence of a methoxy signal at δ_H 3.76 on δ_C 53.0. Thus, the structure of **2** was elucidated to be the methyl ester of **2**. This compound may be formed during extraction and isolation due to exposure to methanolic conditions.

Although the isolation work was extensive, only multinoside A (**5**) (0.01%) was isolated, and multiflorins A and B and multinoside A acetate have so far not been isolated. Until Japanese Pharmacopoeia XIV, the origin of Rosae fructus was stated to be the fruit of *Rosa mutiflora* Thun. or a related species, *R. wichuraiana* Crépin. However, in the current Japanese Pharmacopoeia XV, *R. wichuraiana* is excluded, following the research finding by Seto et al. that no pharmacologically active principles are included in *R. wichuraiana* [3]. Thus, the imported Rosae fructus used in this study, although it was available as a Japanese Pharmacopoeially compatible material, cannot be *R. multiflora*, which is the only species in the current Japanese Pharmacopoeia. The results of qualitative analysis of the flavonoid glycoside contents of Rosae fructus, obtained from several sources, and genetic investigation will appear elsewhere.

Experimental

General experimental procedures

Mps were measured with a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1030 polarimeter. IR and UV spectra were measured on Horiba FT-710 and JASCO V-520 UV/VIS spectrophotometers, respectively. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM α-400 spectrometer at 400 and 100 MHz, respectively, with tetramethylsilane as the internal standard. Positive-ion HR-FAB-MS was performed with a JEOL SX-102 spectrometer and an Applied Biosystem QSTAR XL system ESI (Nano Spray)-TOF mass spectrometer.

A highly porous synthetic resin (Diaion HP-20) was purchased from Mitsubishi Kagaku (Tokyo, Japan). Silica gel column chromatography (CC) and reversed-phase [octadecyl silica gel (ODS)] open CC were performed on silica gel 60 (Merck, Darmstadt, Germany) and Cosmosil $75C_{18}$-OPN (Nacalai Tesque, Kyoto, Japan) [$\Phi = 50$ mm, $L = 25$ cm, linear gradient: MeOH–H_2O (1:9, 1 l) → (1:1, 1 l), fractions of 10 g being collected], respectively. The droplet counter-current chromatograph (DCCC) (Tokyo Rikakikai, Tokyo, Japan) was equipped with 500 glass columns ($\Phi = 2$ mm, $L = 40$ cm), and the lower and upper layers of a solvent mixture of $CHCl_3$–MeOH–H_2O–*n*-PrOH (9:12:8:2) were used as the stationary and mobile

phases, respectively. Five-gram fractions were collected and numbered according to their order of elution with the mobile phase. HPLC was performed on an ODS-3 column (Inertsil; GL Science, Tokyo, Japan; $\Phi = 6$ mm, $L = 25$ cm), and the eluate was monitored with a UV detector at 254 nm and a refractive index monitor.

Plant material

Rosae fructus, purchased from a crude drug store in Hiroshima, was imported from the People's Republic of China by Kinokuniya Kan-yakkyoku (Tokyo, Japan).

Extraction and isolation

Rosae fructus (5.0 k) was crushed and extracted three times with MeOH (15 l) at 25°C for 1 week and then concentrated to 3 l in vacuo. The extract was washed with *n*-hexane (3 l, 35.4 g) and then the MeOH layer was concentrated to a gummy mass. The latter was suspended in water (3 l) and then extracted with EtOAc (3 l) to give 24.7 g of an EtOAc-soluble fraction. The aqueous layer was extracted with 1-BuOH (3 l) to give a 1-BuOH-soluble fraction (106 g), and the remaining water-layer was concentrated to furnish 475 g of a water-soluble fraction.

The 1-BuOH-soluble fraction was applied to a Diaion HP-20 column ($\Phi = 90$ mm, $L = 40$ cm) using H_2O–MeOH (4:1, 6 l), (2:3, 6 l), (3:2, 6 l), and (1:4, 6 l), and MeOH (6 l), 1 l fractions being collected. The residue (13.7 g in fractions 10–16) of the 40–60% MeOH eluent was subjected to silica gel (450 g) CC, with elution with $CHCl_3$ (3 l) and $CHCl_3$–MeOH [(99:1, 3 l), (97:3, 3 l), (19:1, 3 l), (37:3, 3 l), (9:1, 3 l), (7:1, 3 l), (17:3 3 l), (33:7, 3 l), (4:1, 3 l), (3:1, 3 l), and (7:3, 3 l)], 250-ml fractions being collected. An aliquot (57.1 mg) of fractions of 24–31 (135 mg) of the 1% MeOH eluate was separated by HPLC with H_2O–MeOH (7:3) to give 28.9 mg of **3** from the peak at 58 min. The residue (607 mg) of the 7.5% MeOH eluate in fractions 62–73 was separated by ODS open CC to afford 10.1 mg of crude **4**, which was then crystallized from MeOH to give 3.6 mg of **4** as a crystalline state. The residue (2.00 g) of the 10% MeOH eluate in fractions 78–83 was applied to ODS open CC to give five fractions. From the first fraction (40.7 mg in fractions 80–88), 5.8 mg of **12** was obtained by DCCC in fractions 32–36. From the second fraction, 160 mg of **13** was isolated. From the third fraction (85.7 mg in fractions 119–130), 8.6 mg of **10** was obtained by DCCC in fractions 20–26. The residue (111 mg in fractions 174–183) of the fourth fraction was crystallized from MeOH to give 51.9 mg of **7**. From the fifth fraction (75.1 mg in fractions 184–191), 6.2 mg of **9** was obtained by DCCC in fractions 61–64. From the residue (607 mg in fractions 62–73) of the 12.5% MeOH eluate, 104 mg of **8** was isolated as a precipitate and the residue of the mother liquid was subjected to ODS open CC to give three fractions. From the first fraction (159 mg in fractions 105–116), 51.6 mg of **1** was isolated by DCCC in fractions 15–19. From the second fraction (77.3 mg in fractions 117–127), 3.1 mg of **11** was isolated by DCCC in fractions 20–21. The residue (114 mg in fractions 176–180) of the third fraction was compound **7**. The residue (1.76 g) of the 20–25% MeOH eluate in fractions 109–126 was subjected to ODS open CC to give 104 mg of **2** in fractions 40–43, 12.9 mg of **14** in fractions 104–107 and 563 mg of **5** in fractions 160–186.

2-Hydroxynaringin 5-O-β-D-glucopyranoside (1)

Amorphous powder, $[\alpha]_D^{21}$ −61.7 (*c* 0.24, MeOH). IR ν_{max} (film) cm^{-1}: 3,344, 2,924, 1,689, 1,618, 1,514, 1,447, 1,354, 1,100, 1,074, 832. UV λ_{max} nm (log ε): 290 (4.17), 225 (4.04). ^1H- and ^{13}C-NMR (400 and 100 MHz, respectively, CD_3OD): see Table 1. HR-FAB-MS (negative-ion mode): *m/z* 449.1084 $[M - H]^-$ (calcd for $C_{21}H_{21}O_{11}$: 449.1071).

Compound 2

Amorphous powder, Amorphous powder, $[\alpha]_D^{29}$ +30.3 (*c* 0.61, MeOH). IR ν_{max} (film) cm^{-1}: 3,395, 1,651, 1,590, 1,457, 1,418, 1,230, 1,060. UV λ_{max} (MeOH) nm (log ε):280 sh (1.97), 259 (2.34), 229 (2.42). ^1H-NMR (400 MHz, CD_3OD) δ:7.37 (2H, d, $J = 7$ Hz, H-2′ and 6′), 7.30 (2H, d, $J = 7$ Hz, H-3′ and 5′), 7.23 (1H, t, $J = 7$ Hz, H-4′), 4.86 (1H, dd, $J = 9, 3$ Hz, H-7′), 4.30 (1H, m, H-2), 3.71 (1H, dd, $J = 14, 3$ Hz, H-8′a), 3.28 (1H, dd, $J = 14, 9$ Hz, H-8′b), 2.39 (1H, m, H-4a), 2.24 (2H, m, H-3a and 4b), 2.01 (1H, m, H-3b). ^{13}C-NMR (100 MHz, CD_3OD) δ: 180.4 (C-1), 178.8 (C-5), 143.5 (C-1′), 129.3 (C-3′ and C-5′), 128.6 (C-4′), 126.9 (C-2′ and C-6′), 73.3 (C-7′), 65.3 (C-2), 51.5 (C-8′), 31.0 (C-4), 24.9 (C-3).

Compound 3

Amorphous powder, $[\alpha]_D^{29}$ +17.8 (*c* 0.54, MeOH). IR ν_{max} (film) cm^{-1}: 3,418, 1,736, 1,674, 1,454, 1,417, 1,229, 1,180. UV λ_{max} (MeOH) nm (log ε):278 sh (2.11), 247 (2.62). ^1H-NMR (400 MHz, CD_3OD) δ:7.36 (4H, m, H-2′, 3′ 5′ and 6′), 7.26 (1H, tt, $J = 7, 2$ Hz, H-4′), 4.85 (1H, dd, $J = 9, 4$ Hz, H-7′), 4.61 (1H, m, H-2), 3.85 (1H, dd, $J = 14, 4$ Hz, H-8′a), 3.76 (3H, s, −OMe), 3.04 (1H, dd, $J = 14, 9$ Hz, H-8′b), 2.45 (1H, m, H-4a), 2.39 (1H, m, H-3a), 2.34 (1H, m, H-4b), 2.07 (1H, m, H-3b). ^{13}C-NMR (100 MHz, CD_3OD) δ:178.4 (C-5), 174.1 (C-1), 143.7 (C-1′), 129.5 (C-3′ and C-5′), 128.7 (C-4′), 126.9 (C-2′ and C-6′), 73.8 (C-7′), 63.0 (C-2), 53.0 (−OMe), 51.0 (C-8′), 30.4 (C-4), 24.0 (C-3). HR-ESI-TOF-MS (positive-ion

mode) m/z: 286.1063 $[M + Na]^+$ (Cacld for $C_{14}H_{17}O_4NNa$: 286.1049).

*Acid hydrolysis of **1***

Compound **1** (520 μg) was hydrolyzed with 1 N HCl (0.1 ml) at 100°C for 2 h. The reaction mixture was partitioned with an equal amount of EtOAc (0.1 ml), and the water layer was analyzed with a chiral detector (JASCO OR-2090plus) on an amino column [Asahipak NH2P-50 4E, CH_3CN–H_2O (4:1), 1 ml/min]. The hydrolyzate of **1** gave a peak for D-glucose at the retention time of 15.0 min (positive optical rotation sign). The peak was identified by co-chromatography with authentic D-glucose.

*Preparation of p-bromophenacyl ester (**2a**)*

Compound **2** (18.3 mg) was dissolved in 1.0 ml of EtOH and the solution was made slight basic by means of 0.1 N NaOH with a few drops of phenol phthalein solution as an indicator. After the addition of a small amount (2.0 mg) of **2**, *p*-bromophenacyl bromide (25.3 mg) in 1.0 ml of EtOH was added, followed by refluxing for 9 h. After cooling, the solvent was evaporated off and the residue was purified by silica gel CC ($\Phi = 1.0$ cm, $L = 40$ cm) with $CHCl_3$ (50 ml) and then $CHCl_3$–MeOH (49:1, 150 ml) (1-ml fractions being collected) to give crude crystals in fractions 138–175, which were then recrystallized from MeOH to give 10.3 mg of colorless plates (**2a**). *p*-Bromophenacyl ester (**2a**): colorless plates (MeOH), mp. 161–163°C, $[\alpha]_D^{24}$ +6.8 (c 0.31, $CHCl_3$). IR ν_{max} (KBr) cm^{-1}: 3,314, 2,937, 1,751, 1,704, 1,664, 1,585, 1,455, 1,402, 1,175, 1,066, 970, 705. UV λ_{max} (EtOH) nm (log ε):257 (3.07), 210 (3.09). ^{1}H-NMR (400 MHz, $CDCl_3$) δ:7.75 (2H, d, $J = 7$ Hz, H-3″ and 5″), 7.65 (2H, d, $J = 7$ Hz, H-2″ and 6″), 7.41 (2H, dd, $J = 7, 2$ Hz, H-2′ and 6′), 7.35 (2H, td, $J = 7, 2$ Hz, H-3′ and 5′), 7.29 (1H, tt, $J = 7, 2$ Hz, H-4′), 5.41 (1H, d, $J = 16$ Hz, H-8″a), 5.33 (1H, d, J = 16 Hz, H-8″b), 5.02 (1H, dd, $J = 8, 2$ Hz, H-7′), 4.28 (1H, dd, $J = 9, 4$ Hz, H-2), 3.90 (1H, dd, $J = 14, 2$ Hz, H-8′a), 3.39 (1H, dd, $J = 14, 8$ Hz, H-8′b), 2.59 (1H, m, H-4a), 2.42 (2H, m, H-3a and 4b), 2.34 (1H, m, H-3b). ^{13}C-NMR (100 MHz, $CDCl_3$) δ:190.4 (C-7″), 177.4 (C-5), 171.8 (C-1), 141.9 (C-1′), 132.5 (C-2″ and 6″), 129.6 (C-1″), 129.2 (C-3″ and 5″), 128.5 (C-3′ and 5′), 127.5 (C-4′), 125.7 (C-2′, 6′ and 4″), 73.8 (C-7′), 66.4 (C-8″), 62.0 (C-2), 51.3 (C-8′), 29.4 (C-4), 23.7 (C-3). HR-ESI-TOF-MS (positive-ion mode) m/z: 468.0402 and 470.0402 $[M + Na]^+$ (Calcd for $C_{21}H_{20}O_5N^{79}BrNa$ and $C_{21}H_{20}O_5N^{81}BrNa$: 468.0417 and 470.0392, respectively).

*Single-crystal X-ray structure analysis of **2a***

A suitable crystal (0.43 × 0.30 × 0.05 mm) was used for analysis. The data were measured using a Bruker SMART 1000 CCD diffractometer, using Mo $K\alpha$ graphite-monochromated radiation ($\lambda = 0.71073$ Å). The structure was solved by a direct method using the program SHELXTL-97 [16]. The refinement and all further calculations were carried out using SHELXTL-97 [16]. The absorption correction was carried out utilizing the SADABS routine [17]. The H atoms were included at calculated positions and treated as riding atoms using the SHELXTL default parameters. The non-H atoms were refined anisotropically, using weighted full-matrix least-squares on F^2. Figure 4 was drawn with ORTEP32 [18]. Crystal Data: $C_{21}H_{20}BrNO_5$, $M = 446.29$ g mol^{-1}, monoclinic, $P2_1$, $a = 6.3415(14)$ Å, $b = 9.367(2)$ Å, $c = 16.499(4)$ Å, $\beta = 90.214(3)°$, $V = 980.1(4)$ Å^3, $T = 90$ K, $Z = 2$, $D_c = 1.512$ g cm^{-3}, μ(Mo Kα) = 2.130 mm^{-1}, $F(000) = 456$; 3,296 reflections were measured, 2,805 were unique ($R_{int} = 0.0468$) and used in all calculations. Final goodness-of-fit = 1.017, $R_1 = 0.0502$, $wR_2 = 0.1175$ based on $I > 2\sigma(I)$. The absolute parameter was 0.014(13) [19]. CCDC deposit contains the supplementary crystallographic data. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK; fax: +44-1223-336033; or e-mail: deposit@ccdc.cam.ac.uk.

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ORIGINAL PAPER

Botanical origin of Indian celery seed (fruit)

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Author Proof

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Abstract In the course of our study on the traditional medicines and foodstuffs used in Pakistan, we investigated the origin of Indian celery by using the analysis of the internal transcribed spacer (ITS) sequence of nuclear rDNA and a phytochemical approach. We found that the source plant of the Indian celery containing coumarin derivatives such as seselin (**1**), bergapten (**2**) and isopimpinellin (**3**) was not common celery, *Apium graveolens*. Our results suggest the source plant is *Seseli diffusum* even though Indian workers reported that *A. graveolens* seeds contain the aforementioned compounds. In addition, a market survey of the Indian celery in Pakistan and related countries revealed that the Indian celery seeds in Pakistani markets are mainly composed of three species which have been confused in rural markets.

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Introduction

Indian celery is used in Pakistan to treat amenorrhea, urinary discharges, fever with cough, rheumatism, chest pains and inflammation. Indian celery is prescribed as an antispasmodic for use in treating bronchitis, asthma and, to some extent, liver obstructions, intestinal debility and spleen disorders [1].

In the course of bioassay screenings of traditional medicines and foodstuffs used in Pakistan, we observed that the 80% methanol/water extract of the Indian celery seeds (botanically fruit), available as "ajmod" in a local market in Karachi, showed several interesting biological activities: one was the cytotoxicity to yeast strains ascribed to hexane and chloroform extracts; the others were antioxidant and spasmolytic activities of water-soluble fractions[1]. The phytochemical study of the constituents of the Indian celery guided by the activities revealed that nine coumarin derivatives (**1**–**9**; Fig. 1) and a phenylpropanoid (**10**; Fig. 1) were responsible for its biological activities (see footnote 1).

In the late 1970s to early 1980s, Indian authors reported the presence of angular and linear furocoumarin derivatives (**1**–**6**, **8**, **9**, isoimperatorin, osthenol, apiumetin etc.) from the seeds which they perceived to be of *Apium graveolens* [2–10]. Judging from the similarity to the chemical composition reported by the Indian researchers, it was noticeable that they treated the same species as our material. On the other hand, the seeds of common celery,

[1] Details will be reported in our successive papers.

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