Authentication and Chemical Study of Isodonis Herba and Isodonis Extracts

Takuro MARUYAMA,[a] Naoki SUGIMOTO,[b] Masanori KUROYANAGI,[c] Ik Hwi KIM,[a] Hiroyuki KAMAKURA,[a] Takeshi KAWASAKI,[d] Masao FUJITA,[d] Hiroshi SHIMADA,[e] Yutaka YAMAMOTO,[e] Atsuko TADA,[b] Takeshi YAMAZAKI,[b] and Yukihiro GODA*,[a]

[a] *Division of Pharmacognosy, Phytochemistry and Narcotics, National Institute of Health Sciences;* [b] *Division of Food additives, National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan:* [c] *School of Bioresources, Prefectural University of Hiroshima; 562 Nanatsuka, Shobara, Hiroshima 727–0023, Japan:* [d] *Uchida Wakan-yaku Co., Ltd.; 4–4–10 Higashinippori, Arakawa-ku, Tokyo 116–8571, Japan: and* [e] *Tochimoto Tenkaido Co., Ltd.; Oniya, Kaibara, Tamba, Hyogo 669–3315, Japan.*

Received July 30, 2007; accepted September 9, 2007; published online September 10, 2007

Isodonis Herba is used as a Japanese dietary supplement and folk medicine. The extract of the herb (Isodonis extract) is also used as a food additive whose major compound is enmein (1). Here we compared internal transcribed spacer sequences of nuclear ribosomal DNA from Isodonis Herba available on the Japanese and Chinese crude drug markets, and found that the former derived from *Isodon japonicus* and *Isodon trichocarpus*, while the latter derived from distinct species such as *Isodon eriocalyx*. The liquid chromatography/mass spectrometry profiles of Isodonis Herba were classified into four chemotypes (A to D) according to the ratio of the major constituents. Types B and C contained 1 and oridonin (2) as major components, respectively. An intermediate (or mixed) form of types B and C in various ratios was designed type A. Type D contained eriocalyxin B (3) as its major component. Japanese herba were types A—C, while Chinese herba were types C and D. The commercial Isodonis extract products tested were classified as type D, suggesting that they originated from Chinese Herba. Understanding the relationship between extract constituents and DNA profiles is important for the official specification of dietary supplements and food additives of plant origin.

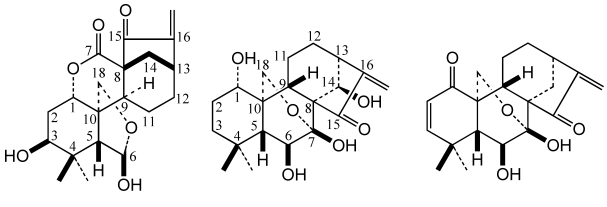
Key words Isodonis; LC/MS; internal transcribed spacer; folk medicine; food additive; dietary supplement

To ensure the quality of dietary supplements and food additives of plant origin, we have studied the main constituents of many products and identified the species from which they derive.[1—4] This work revealed a number of products that did not originate from the labeled material, including one-half of the commercial white kwao keur products purported to be made from the root of *Pueraria candollei* var. *mirifica*, two out of nine chondroitin sulfate products, and the commercial alkanet color, the major pigments of which were composed of shikonin and its derivatives rather than alkannin as labeled in Japan.

Isodon (previously *Rabdosia*) plants are widely distributed, and are the source of popular folk medicines in Japan and China. *Isodon japonicus* (Labiatae) is a perennial plant, which grows in Japan, Korea, eastern China, and far-eastern Russia.[5] In Japan, the aerial parts of *I. japonicus* and *Isodon trichocarpus* are used for the treatment of gastrointestinal disorders under the common name "enmei-so" (Isodonis Herba), which means "a grass for the prolongation of human life" in Japanese. "The Japanese Standard for Non-Pharmacopoeia Crude Drugs" defines these two species as the source plants of Isodonis Herba. In Japanese law system, Isodonis Herba are treated as "dietary supplements" and under the Japanese Food Sanitation Law when they are sold without the advertisement of their health effects. So, some dietary supplements utilizing the plants are sold in Japan, particularly in the form of herb tea. In China, other plants from this genus, such as *Isodon rubescens* and *Isodon eriocalyx*, are used as antibacterial and anti-inflammatory agents, and many studies have investigated the constituents and pharmacological activities of the former due to its anti-cancer activities.[6—10] As *Isodon* plants are rich in a wide range of diterpenoids, they are major targets of phytochemical studies.[11,12] The main constituents of *I. japonicus* include *ent*-6,7-seco kaurane, and *ent*-kaurane-type diterpenoids, such as enmein (**1**) and oridonin (**2**) (Fig. 1),[13—19] the antibacterial[20,21] and anti-inflammatory[22,23] activities of which have pharmacological effects against gastrointestinal disorders. In addition, some of the diterpenoids have an intensively bitter taste.[24]

The Isodonis extract from *I. japonicus* is also used as a natural food additive in Japan to give a bitter taste to processed foods, such as beverages, ice cream, and confectionery. "The List of Existing Food Additives" in Japan states that "Isodonis extract" is obtained from the stems or leaves of hiki-okoshi (*I. japonicus* HARA), and that the main bitter component is enmein (**1**).

On the other hand, some species of *Isodon* plants, collected and imported from various habitats and places are used as Isodonis Herba for a crude drug and a dietary supplement. In addition, it is possible to use these *Isodon* plants as the raw materials of the commercial Isodonis extracts. However, to our knowledge, there have been no previous investigations into the constituents and origins of Isodonis extract.



Enmein (**1**) Oridonin (**2**) Eriocalyxin B (**3**)

Fig. 1. Structures of *Isodon* Diterpenoids

* To whom correspondence should be addressed. e-mail: goda@nihs.go.jp

In the present study, the internal transcribed spacer (ITS) sequences of Isodonis Herba nuclear ribosomal DNA (rDNA) were analyzed to determine the original plant species from which they were derived. The major Herba diterpenoids were analyzed using liquid chromatography (LC)/mass spectrometry (MS). We then examined the relationship between the major constituents and the classification according to DNA analysis, and discussed the origin of Isodonis Herba and commercial Isodonis extract products.

Experimental

Materials and Reagents The commercial Isodonis extract product (Iso-E1) was obtained through the Japan Food Additives Association. Isodonis Herba samples (Iso-H1 to Iso-H8) were purchased from Japanese and Chinese local crude drug markets (Table 1). Authentic *I. japonicus* plants (Iso-1 to Iso-3) were provided by Japanese botanical gardens. *I. trichocarpus* (Iso-4 to Iso-6) and *I. shikokianus* var. *occidentalis* (Iso-7) plants were collected from the mountains in Ishikawa Prefecture and Toyama Prefecture, Japan (Table 2). Voucher specimens were deposited at our institute as herbarium specimens. For the LC/MS analysis, authentic **1** was purchased from Koshiro Company Ltd. (Osaka, Japan). All other chemical reagents were of research grade.

Instruments DNA sequencing analysis was performed using an ABI Prism 3100-Avant genetic analyzer (Applied Biosystems, Foster City, CA, U.S.A.) equipped with a 50-cm capillary array. The system was controlled by 3100/3100-Avant Data collection software, and the obtained electropherograms were analyzed using Applied Biosystems DNA sequencing analysis software version 5.1.

The LC/MS system (FractionLynx MS Autopurification System; Waters, Milford, MA, U.S.A.) consisted of a 2767 one-bed injection-collection sample manager, a 2525 binary high-pressure LC pump, a column/fluidic organizer (CFO), a 2996 photodiode array detector (PDA), and a ZQ single-quadropole mass spectrometer equipped with a Z-spray electrospray interface. The complete system was controlled by MassLynx software version 4.0. The electrospray sources ran at a 4.0-kV capillary voltage, 120 and 350 °C source and desolvation temperatures, respectively, and 350 and 50 l/h desolvation and cone gas flow rates, respectively. The cone voltage was 40 V. Full-scan acquisition between *m/z* 100 and 2000 was performed at a scan speed of 0.5 s/scan with a 0.1-s interscan delay. The solvent delivered to the electrospray interface was split in a 1 : 4 ratio, delivering to the interface at approximately 200 μl/min. The on-line PDA detector was monitored between 210 and 600 nm.

Nuclear magnetic resonance (NMR) spectra were recorded on the JEOL ECA-500 system (Jeol, Tokyo, Japan) in $CDCl_3$. The spectra were referenced internally to tetramethylsilane (TMS) in ^{1}H-NMR and to the solvent in ^{13}C-NMR. Assignment of the proton and carbon signals for all isolated compounds was confirmed by pulse-field gradient (PFG) ^{1}H–^{1}H correlation spectroscopy (COSY), PFG heteronuclear multiple quantum coherence (HMQC), and PFG heteronuclear multiple bond connectivity (HMBC) experiments.

DNA Sequencing Analysis of Isodonis Extract and Isodonis Herba A 20-mg sample of each product was frozen under liquid N_2 and crushed using a mixer mill, MM-300 (Qiagen, Hilden, Germany). Genomic DNA was extracted and purified from the powdered sample using a DNeasy Plant Mini Kit (Qiagen). The ITS region (small subunit rDNA-ITS1-5.8S rDNA-ITS2-large subunit rDNA) of the nuclear rDNA was PCR amplified using the obtained genomic DNA as a template. For the commercial samples except Iso-H4 to H6, nested PCR was used to compensate for low DNA yield. PCR was performed using a DNA engine PTC-200 (Bio-Rad, Hercules, CA, U.S.A.) with Gene TaqNT DNA polymerase (Nippon Gene, Tokyo, Japan) and the following program: 94 °C, 4 min; 40 cycles of 94 °C, 30 s, 50 °C, 30 s, and 72 °C, 45 s; then 72 °C, 4 min. The primers were designed based on the conserved sequence of the plant rDNA gene as follows: ITS-S1 5′-GGA-AGTAAAAGTCGTAACAAGG-3′ and ITS-AS1 5′-TTTTCCTCCGCT-TATTGATATGC-3′ for first-round PCR; and ITS-S2 5′-TCCGTAGGT-GAACCTGCGG-3′ and ITS-AS2 5′-GTAGTCCCGCCTGACCTG-3′ for second-round PCR. Excess primers and dNTPs were removed from the reaction mixture by Montage-PCR (Millipore, Billerica, MA, U.S.A.), and the amplicons were directly sequenced. Cycle sequencing was performed using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Subcloning of the amplicon into plasmid vectors was performed using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). The DNA sequences were aligned with a Clustal W program.[25] The genetic distances for all pairs of sequence were calculated using Kimura's two-parameter distance.[26] Neighbor-joining (NJ) tree[27] was constructed on the basis of their distances. The statistical support for the nodes of the tree was determined using bootstrap method[28] based on 10000 replicates.

Isolation of Oridonin (2) and Eriocalyxin B (3) MeOH extract (6.5 g) from 50 g Isodonis Herba (Iso-H2) was partitioned into hexane, $CHCl_3$, EtOAc, BuOH, and aqueous layers. The EtOAc portion (438 mg) was fractionated into six fractions by silica gel column chromatography with a gradient elution of hexane/acetone. The fourth fraction was subjected to reversed phase-high performance liquid chromatography (HPLC) equipped with YMC-Pack ODS-A (20×250 mm; YMC, Kyoto, Japan) with 45% MeOH isocratic elution, followed by treatment with activated charcoal and recrystallization from MeOH, affording 45 mg of **2**.

Oridonin (**2**): Colorless needles. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 1.14 (3H, s, Me-19), 1.30 (3H, s, Me-18), 1.36 (1H, m, H-3), 1.40 (1H, m, H-3), 1.48 (1H, d, J=6.9 Hz, H-5), 1.58 (1H, m, H-12), 1.84 (1H, m, H-2), 1.87 (1H, m, H-2), 1.93 (1H, m, H-9), 1.97 (1H, m, H-11), 2.43 (1H, m, H-12), 2.50 (1H, m, H-11), 3.21 (1H, d, J=9.2 Hz, H-13), 3.63 (1H, m, H-1), 4.28 (1H, dd, J=6.9, 10.3 Hz, H-6), 4.42 (1H, d, J=10.1 Hz, H-20), 4.80 (1H, d, J=10.1 Hz, H-20), 5.34 (1H, s, H-14), 5.50 (1H, s, H-17), 5.96 (1H, d, J=4.9 Hz, OH-1), 6.28 (1H, s, H-17), 6.94 (1H, d, J=10.3 Hz, OH-6). ^{13}C-NMR (125 MHz, pyridine-d_5) δ: 20.4 (C-11), 22.2 (C-19), 30.5 (C-2), 30.9

Table 1. Commercial Isodonis Extracts and Herba

Sample	Market	Collection distinct	Form
Iso-E1	Tokyo, Japan	Unknown	Extract[a]
Iso-H1	Osaka, Japan	China[b]	Herba
Iso-H2	Tokyo, Japan	Niigata Prefecture, Japan	Herba
Iso-H3	Osaka, Japan	Tokushima Prefecture, Japan	Herba
Iso-H4	Osaka, Japan	Niigata Prefecture, Japan	Herba
Iso-H5	Osaka, Japan	Niigata Prefecture, Japan	Herba
Iso-H6	China	Guizhou, China	Herba
Iso-H7	China	Henan, China	Herba
Iso-H8	China	Henan, China	Herba

a) Extract (Iso-E1) is a natural food additive used as a bittering agent in Japan. *b*) Herba was purchased from a Japanese market.

Table 2. Details of Authentic *Isodon* Plants Used in This Study

Sample	Species	Habitat	Voucher
Iso-1	*I. japonicus*	Tsukuba Division, Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation (NIBIO), Japan	0548-79TS
Iso-2	*I. japonicus*	Tanegashima Division, Research Center for Medicinal Plant Resources, NIBIO	0068-99TN
Iso-3	*I. japonicus*	The Botanical Garden for Medicinal Plant Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Japan	TM010
Iso-4	*I. trichocarpus*	Toyama city, Toyoma Prefecture, Japan[a]	TM011
Iso-5	*I. trichocarpus*	Kanazawa city, Ishikawa Prefecture, Japan[a]	TM007
Iso-6	*I. trichocarpus*	Kaga city, Ishikawa Prefecture, Japan[a]	TM008
Iso-7	*I. shikokianus* var. *occidentalis*	Kaga city, Ishikawa Prefecture, Japan[a]	TM009

a) Samples (Iso-4 to Iso-7) were wild plants, morphologically verified as authentic *Isodon* plants.

(C-12), 33.3 (C-18), 34.0 (C-4), 39.3 (C-3), 43.9 (C-13), 41.7 (C-10), 43.9 (C-13), 54.1 (C-9), 60.5 (C-5), 64.0 (C-20), 73.1 (C-1), 73.5 (C-14), 74.8 (C-6), 98.4 (C-7), 119.0 (C-17), 153.4 (C-16), 209.2 (C-15). ESI-MS (positive) *m/z* 387 $[M+Na\ (C_{20}H_{28}O_6Na)]^+$.

Commercial Isodonis extract (Iso-E1; 310 g) was partitioned into EtOAc, BuOH, and aqueous layers. The EtOAc portion (22 g) was fractionated into eight fractions using silica gel column chromatography with a gradient elution of $CHCl_3$/MeOH. The fourth fraction (4.2 g) was recrystallized from MeOH to give 1.8 g crystal composed of three compounds. The crystal (150 mg) was subjected to preparative thin-layer chromatography (TLC plate silica gel 60 F_{254}, 200×200×0.5 mm; Merck) with $CHCl_3$/MeOH (15/1), affording 80 mg of **3**.

Eriocalyxin B (**3**): Colorless powder. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.23 (3H, s, Me-19), 1.37 (3H, s, Me-18), 1.40 (1H, m, H-11), 1.50 (1H, m, H-12), 1.90 (1H, ddd, J=1.5, 4.9, 13.5 Hz, H-9), 2.04 (1H, d, J=8.8 Hz, H-5), 2.05 (1H, overlap, H-12), 2.14 (1H, ddd, J=1.0, 5.6, 12.6 Hz, H-14), 2.30 (1H, overlap, H-11), 2.33 (1H, br d, J=12.6 Hz, H-14), 3.46 (1H, br dd, J=4.6, 9.4 Hz, H-13), 3.95 (1H, dd, J=8.8, 12.0 Hz, H-6), 4.01 (1H, dd, J=1.7, 10.0 Hz, H-20), 4.29 (1H, dd, J=1.2, 10.0 Hz, H-20), 5.48 (1H, s, H-17), 5.81 (1H, d, J=12.0 Hz, OH-6), 5.85 (1H, d, J=10.0 Hz, H-2), 6.00 (1H, s, H-17), 6.77 (1H, d, J=10.0 Hz, H-3). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 19.2 (C-12), 24.7 (C-14, 19), 29.8 (C-11), 30.0 (C-18), 34.3 (C-13), 35.9 (C-4), 46.6 (C-10), 48.3 (C-9), 56.9 (C-5), 59.7 (C-8), 65.6 (C-20), 73.1 (C-6), 95.5 (C-7), 119.0 (C-17), 127.1 (C-2), 152.5 (C-16), 161.3 (C-3), 196.9 (C-1), 208.3 (C-15). ESI-MS (positive) *m/z* 367 $[M+Na\ (C_{20}H_{24}O_5Na)]^+$.

LC-MS Analysis of Isodonis Extract and Isodonis Herba Samples (1 g) were extracted with 50 ml EtOH at room temperature for 24 h. The extract was passed through 5C filter paper (Advantec, Ehime, Japan) and then the filtrate was concentrated *in vacuo*. The residue was dissolved with 5 ml EtOH and the solution was filtered through a 0.45 μm Milex-LH membrane filter (Millipore). The filtrate (5 μl) was injected into the LC/MS system under the following conditions: column, YMC-J'sphere-ODS-H80 (4.6×250 mm; YMC); mobile phase, 40% MeOH (0 min) to 100% MeOH (30 min); flow rate, 1.0 ml/min; detection, ultraviolet (UV) 230 nm; and electrospray ionization (ESI) positive scan mode. The retention times of the three authentic compounds under the abovementioned conditions were as follows: 5.9 min for **1**, 13.3 min for **2**, and 17.2 min for **3**.

Results and Discussion

In the current study, we initially investigated the genetic diversity among commercial Isodonis Herba products available in crude drug markets using DNA sequence analysis. Genomic DNA was prepared from each sample, and the ITS1 region of the nuclear rDNA was used for sequence alignment analysis, as the ITS2 region could not be analyzed by direct sequencing in some samples. Multiple sequences of Iso-H1 were detected, so the Iso-H1 amplicon was subcloned into plasmid vectors and three clones were sequenced.

Authentic *I. japonicus* (Iso-1 to Iso-3), *I. trichocarpus* (Iso-4 to Iso-6), and *I. shikokianus* var. *occidentalis* (Iso-7) all had specific sequences in the ITS1 region, and no intraspecific mutations were detected. These three Japanese species could thus be differentiated on the basis of the ITS1 sequences, which were registered in international nucleotide sequence databases (the DNA DataBank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank) as follows: *I. japonicus*, AB292804; *I. trichocarpus*, AB292805; and *I. shikokianus* var. *occidentalis*, AB292806. The phylogenetic tree constructed from the ITS1 sequences of commercial Isodonis herba is shown in Fig. 2. All of the Isodonis Herba samples collected from Japan (Iso-H2 to Iso-H5) were estimated to originate from *I. japonicus* or *I. trichocarpus*, based on their ITS1 sequences. Among them, Iso-H2, Iso-H4, and Iso-H5 from Niigata Prefecture, Japan, derived from *I. trichocarpus*. This result is consistent with the distribution area of the plant on the Japan Sea side.[29] By contrast, the ITS sequences of Chinese Isodonis Herba (Iso-H1, and Iso-H6 to Iso-H8) differed from both *I. japonicus* and *I. trichocarpus*, so they were classified into other clusters. Furthermore, Iso-H1 had multiple sequences in the ITS1 region, although a single population was used in the genomic DNA preparation. This suggests that the source plant is a hybrid derived from varieties or species of *Isodon* plants. Based on these results, we concluded that the Chinese Isodonis Herba were distinct from, but closely related to, the original species of Japanese Isodonis Herba.

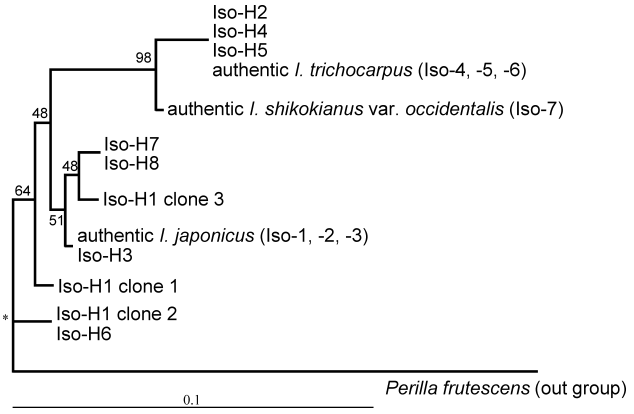


Fig. 2. Neighbor Joining Tree Constructed from ITS1 Sequences of Isodonis Herba

Bootstrap values in percent from 10000 replicates are indicated above the nodes. The tree is unrooted and branch lengths are proportional to the scale given in nucleotide substitution per site. *: trichotomy.

Subsequently, ethanol extracts prepared from authentic *Isodon* plants and Isodonis Herba were subjected to LC/MS analysis, in order to investigate the chemical diversity. The LC profiles at UV 230 nm differed according to the species and/or collection locations. Major chromatogram peaks were observed at retention time (RT) values of 5.9, 13.3, and 17.2 min, affording *m/z* 385 $[M+Na]^+$, 387 $[M+Na]^+$, and 367 $[M+Na]^+$ as the molecular related ions, respectively. The peak at RT 5.9 min was identified as **1** by comparison with the authentic sample. The peaks at RT 13.3 and 17.2 min were identified as **2** and **3**, respectively, after these constituents were isolated from Isodonis Herba and Isodonis extracts, and compared with reported ^{1}H- and ^{13}C-NMR spectra data.[30—33]

The profiles were classified into four chemotypes (A to D) based on the major constituents. Types B and C contained **1** and **2** as major components, respectively. The presence of an intermediate (or mixed) form of types B and C in various ratios was also observed, which was designated type A. Type D contained **3** as its major component, containing no traces of **1** or **2**. Typical LC profiles at 230 nm are shown in Fig. 3, and the results of the analysis of species and chemotype are summarized in Table 3. Among authentic *I. japonicus* (Iso-1 to Iso-3) and *I. trichocarpus* (Iso-4 to Iso-6), Iso-1 to Iso-5 contained both **1** and **2** while the predominant component varied according to the sample. Iso-6 mainly contained **2** and lacked **1**. Thus, authentic *I. japonicus* (Iso-1 to Iso-3) and *I. trichocarpus* (Iso-4 to Iso-6) were classified as type A, B, or C. By contrast, no peaks corresponded to any of the three compounds (**1**—**3**) in authentic *I. shikokianus* var. *occidentalis* (Iso-7), which showed an unidentified peak at an RT of 15.5 min as its main component.

Japanese Isodonis Herba (Iso-H2 to Iso-H5) contained

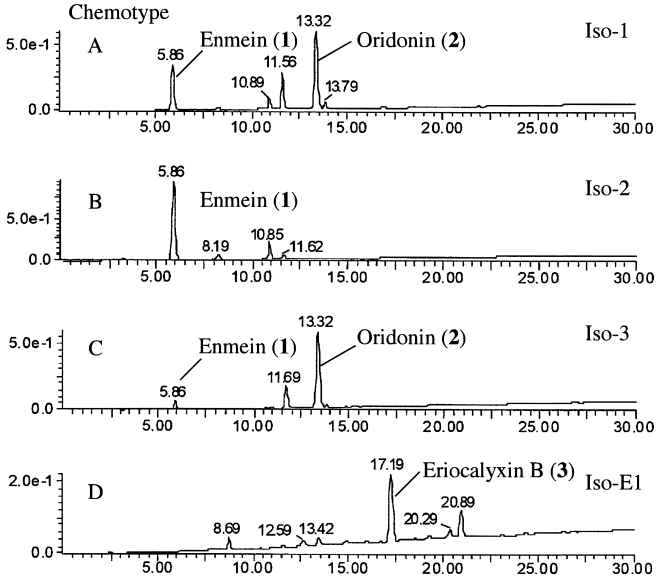


Fig. 3. Typical LC Profiles of Ethanol Extracts Prepared from Isodonis Extract and Isodonis Herba

The extracts were classified into chemotypes A, B, C and D by the major constituents: enmein (**1**) and oridonin (**2**) for A, **1** for B, **2** for C, eriocalyxin B (**3**) for D. The LC/PDA/MS conditions were described in experimental section.

Table 3. LC-PDA/MS Analysis of Authentic *Isodon* Plants and Isodonis Products

Sample	Species	Chemotype
Iso-1	***I. japonicus***	**A**
Iso-2	***I. japonicus***	**B**
Iso-3	***I. japonicus***	**A**
Iso-4	***I. trichocarpus***	**C**
Iso-5	***I. trichocarpus***	**B**
Iso-6	***I. trichocarpus***	**C**
Iso-7	***I. shikokianus* var. *occidentalis***	**Other**[a]
Iso-E1	Unknown	D
Iso-H1	Unknown	D
Iso-H2	Putative *I. trichocarpus*	C
Iso-H3	Putative *I. japonicus*	A
Iso-H4	Putative *I. trichocarpus*	C
Iso-H5	Putative *I. trichocarpus*	A
Iso-H6	Unknown	D
Iso-H7	Unknown	C
Iso-H8	Unknown	C

Authentic plants are indicated in the bold letter. *a)* No peaks corresponding to enmein (**1**), oridonin (**2**), or eriocalyxin B (**3**) were observed.

mainly **1** and/or **2**, and were classified as type A or C. Type B was not detected, even though it was predicted based on the results of authentic *I. japonicus* and *I. trichocarpus* in Japan. All Japanese Isodonis Herba contained **1**, while this was only weakly detected in Iso-H2 and Iso-H4. Chinese Isodonis Herba, Iso-H1, and Iso-H6 contained **3**, but lacked **1**, and were classified as type D. Iso-H7 and Iso-H8 mainly contained **2** and were type C. The commercial Isodonis extract product (Iso-E1) contained only **3**, and lacked both **1** and **2**, so was classified as the same type as Chinese Isodonis Herba Iso-H1 and Iso-H6 (type D).

The sequence of Iso-H1 clone 2 was identical to that of Iso-H6 (Fig. 2), and both contained **3** as their main component. This compound was first isolated from *I. eriocalyx*,[32] which was distributed in Yunnan, Guizhou, and Sichuan provinces in China, and was used as an antibacterial and anti-inflammatory agent in Yunnan province under the name "Yanshukang". Taken together, these facts and our results indicate that the Iso-H6 purchased from the crude drug market in Guizhou province was derived from *I. eriocalyx*.

Our results showed that the Japanese Isodonis Herba products (Iso-H2 to Iso-H5) originated from *I. japonicus* and *I. trichocarpus*, while the Chinese Isodonis Herba products (Iso-H1, and Iso-H6 to Iso-H8) originated from distinct species, such as *I. eriocalyx*. Furthermore, one (Iso-H1) of the Isodonis Herba products purchased in a Japanese market was made from other Chinese *Isodon* plants rather than *I. japonicus* and *I. trichocarpus*, which are defined as the source plants in "the Japanese Standard for Non-Pharmacopoeia Crude Drugs". We deduced that the Isodonis extract product (Iso-E1), which can be processed from Isodonis Herba, was also not made from the stated source plant with **1** as its major component, but was from the incorrect species with **3** as its major one.

A qualitative theory on the relationship between bitterness and the chemical structures of bitter *Isodon* diterpenoids has previously been proposed.[24] According to this theory, it is necessary for a bitter compound to contain at least one "unit" of bitter taste, which consists of a proton-donor group (PD) and a proton-acceptor group (PA) that must be within a distance of about 1.5 Å, thereby making it possible to form an intramolecular hydrogen bond. In the paper,[24] **1** and **2** were regarded as bitter compounds based on a qualitative test. Another paper[33] reported **3** as a bitter agent under the name, rabdosianone I. Furthermore, the structures of **1**, **2**, and **3** found in Isodonis Herba from Japan and China match the above criteria. Namely, the 6-aldehyde and 18-hydroxyl groups equilibrated with the acetal ring in **1** serve as PA and PD, respectively. The 6-hydroxyl and 15-carbonyl groups in **2** and **3** work as PD and PA, respectively. These facts indicate that the compounds are bitter. Therefore, the extract made from Chinese *Isodon* plants could also be used as a bitter agent.

In recent years, it has been reported that the bitter taste perception is involved with the G protein-coupled receptors (GPCRs) called T2Rs or TRBs.[34] Whether the response of the GPCRs against the *Isodon* diterpenoids relates to the above theory is of current interest.

Based on the results of the present study, we suggest that the definition of Isodonis extract in "the List of Existing Food Additives" in Japan should not be restricted to *I. japonicus* and that it should be expanded to *Isodon* plants. In addition, we recommend that the description of its main bitter components should also be changed to *ent*-6,7-seco-kaurane or *ent*-kaurane-type diterpenoids from enmein.

Acknowledgements We thank G. Honda (currently, Faculty of Pharmaceutical Sciences, Himeji Dokkyo University) and M. Ito from the Graduate School of Pharmaceutical Sciences, Kyoto University, Japan; F. Kiuchi, S. Katsuki, and A. Hishida from the Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation, Osaka, Japan, and C. Mouri from the Graduate School of Natural Science and Technology, Kanazawa University, Japan, for kindly supplying authentic *I. japonica*, *I. trichocarpa*, and *I. shikokianus* var. *occidentalis*. We are grateful to the Japan Food Additives Association for their kind supply of commercial Isodonis extract. This research was supported by grants from the Ministry of Health, Labor and Welfare, and the Japan Health Sciences Foundation.

References

1) Sakai S., Maruyama T., Kawahara N., Goda Y., *Jpn. J. Food Chem.*, **12**, 10—15 (2005).
2) Maruyama T., Sakai S., Kawahara N., Goda Y., *Jpn. J. Food Chem.*, **12**, 76—79 (2005).
3) Sakai S., Otake E., Toida T., Goda Y., *Chem. Pharm. Bull.*, **55**, 299—303 (2007).
4) Goda Y., *Farumashia*, **42**, 905—907 (2006).
5) Li H.-W., *J. Arn. Arb.*, **69**, 289—400 (1988).
6) Ikezoe T., Chen S. S., Tong X.-J., Heber D., Taguchi H., Koeffler H. P., *Int. J. Oncol.*, **23**, 1187—1193 (2003).
7) Liu J. J., Huang R. W., Lin D. J., Peng J., Wu X. Y., Pan X. L., Li M. Q., Lin Q., *J. Int. Med. Res.*, **32**, 617—625 (2004).
8) Sartippour M. R., Seeram N. P., Herber D., Hardy M., Norris A., Lu Q., Zhang L., Lu M., Rao J. Y., Brooks M. N., *Int. J. Oncol.*, **26**, 121—127 (2005).
9) Chen S., Cao J., Halicka H. D., Huang X., Traganos F., Darzynkiewicz Z., *Int. J. Oncol.*, **26**, 579—588 (2005).
10) Ikezoe T., Yang Y., Bandobashi K., Saito T., Takemoto S., Machida H., Togitani K., Koeffler H. P., Taguchi H., *Mol. Cancer Ther.*, **4**, 578—586 (2006).
11) Fujita E., Node M., *Fortschritte der Chemie Organischer Naturstoffe*, **46**, 77—157 (1984).
12) Sun H.-D., Huang S.-X., Han Q.-B., *Nat. Prod. Rep.*, **23**, 673—698 (2006).
13) Fujita E., Fujita T., Shibuya M., *Tetrahedron Lett.*, **7**, 3153—3162 (1966).
14) Li J., Liu C., An X., Wang M., Zhao T., Yu S., Zhao G., Chen R., *Yaoxue Xuebao*, **17**, 682—687 (1982) [*Chem. Abstr.*, **98**, 59801p (1983)].
15) Liu C., Li J., An X., Cheng R., Shen F., Xu Y., Wang D., *Yaoxue Xuebao*, **17**, 750—754 (1982) [*Chem. Abstr.*, **98**, 77989k (1983)].
16) Fujita E., Fujita T., Katayama H., Shibuya M., *Chem. Commun*, **1967**, 252—254 (1967).
17) Fujita E., Fujita T., Katayama H., Shibuya M., Shingu T., *J. Chem. Soc*. (C), **1970**, 1674—1681 (1970).
18) Fujita E., Taoka M., Shibuya M., Fujita T., Shingu T., *J. Chem. Soc.*, *Perkin Trans. I*, **1973**, 2277—2281 (1973).
19) Fujita E., Taoka M., Nagao Y., Fujita T., *J. Chem. Soc.*, *Perkin Trans. I*, **1973**, 1760—1765 (1973).
20) Kubo I., Taniguchi M., Satomura Y., Kubota T., *Agr. Biol. Chem.*, **38**, 1261—1262 (1974).
21) Fujita E., Nagao Y., Kaneko K., Nakazawa S., Kuroda H., *Chem. Pharm. Bull.*, **24**, 2118—2127 (1976).
22) Hwang B. Y., Lee J.-H., Koo T. H., Kim H. S., Hong Y. S., Ro J. S., Lee K. S., Lee J. J., *Planta Med.*, **67**, 406—410 (2001).
23) Lee J.-H., Koo T. H., Hwang B. Y., Lee J. J., *J. Biol. Chem.*, **277**, 18411—18420 (2002).
24) Kubota T., Kubo I., *Nature* (London), **223**, 97—98 (1969).
25) Thompson J. D., Higgins D. G., Gibson T. J., *Nucl. Acids Res.*, **22**, 4673—4680 (1994).
26) Kimura M., *J. Mol. Evol.*, **16**, 111—120 (1980).
27) Saitou N., Nei M., *Mol. Biol. Evol.*, **4**, 406—425 (1987).
28) Felsenstein J., *Evolution*, **39**, 783—791 (1985).
29) Hara H., *J. Jpn. Bot.*, **47**, 193—203 (1972).
30) Meng X., Wang Q., Chen Y., *Phytochemistry*, **28**, 1163—1165 (1989).
31) Xian-Rong W., Hong-Ping W., Hui-Ping H., Han-Dong S., Su-Qing W., Ueda S., Kuroda Y., Fujita T., *Phytochemistry*, **38**, 921—926 (1995).
32) Wang Z., Xu Y., *Yunnan Zhiwu Yanjiu*, **4**, 407—411 (1982) [*Chem. Abstr.*, **98**, 104285m (1983)].
33) Yamada Y., Sako N., Ando E., Yamada M., Kikuzaki H., Yamamoto T., *Biosci. Biotechnol. Biochem.*, **63**, 524—529 (1999).
34) Margolskee R. F., *J. Biol. Chem.*, **277**, 1—4 (2002).