Identification of New Diterpenes as Putative Marker Compounds Distinguishing Agnus Castus Fruit (Chaste Tree) from Shrub Chaste Tree Fruit (Viticis Fructus)

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Key words
- *Vitex agnus-castus*
- *Vitex rotundifolia*
- *Vitex trifolia*
- Verbenaceae
- Lamiaceae
- Agnus Castus Fruit (ACF)
- Shrub Chaste Tree Fruit (SCTF)
- chastol
- marker compound

Abstract

Agnus Castus Fruit is defined in the European Pharmacopoeia as the dried ripe fruit of *Vitex agnus-castus*. In Europe it is used as a medicine targeting premenstrual syndrome and climacteric disorder. In Japan, Agnus Castus Fruit is becoming popular as a raw material for over-the-counter drugs and health food products, though its congenic species, *Vitex rotundifolia* and *Vitex trifolia*, have been used as Shrub Chaste Tree Fruit in traditional medicines. Therefore, it is important to discriminate these *Vitex* plants from the viewpoint of regulatory science. Here we tried to identify putative marker compounds that distinguish between Agnus Castus Fruit and Shrub Chaste Tree Fruit. We analyzed extracts of each crude drug by liquid chromatography–mass spectrometry, and performed differential analysis by comparison of each chromatogram to find one or more peaks characteristic of Agnus Castus Fruit. A peak was isolated and identified as an equilibrium mixture of new compounds named chastol (**1**) and epichastol (**1a**). The planar structures of **1** and **1a** were determined spectroscopically. Their relative configurations were revealed by nuclear Overhauser effect spectroscopy and differential nuclear Overhauser effect-NMR data. Since avoiding contamination from closely related species is needed for the quality control of natural pharmaceuticals, this information will be valuable to establish a method for the quality control of both, Agnus Castus Fruit and Shrub Chaste Tree Fruit products.

Supporting information available online at http://www.thieme-connect.de/products

Introduction

Vitex agnus-castus L. (Lamiaceae) is a deciduous shrub, native to the entire Mediterranean region and western Asia. Its fruit has been used for more than 2500 years to treat a variety of gynecologic conditions [1]. Various flavonoids [2,3], iridoids [4] and diterpenes [5] have been reported as secondary metabolites in the fruit of *V. agnus-castus*. Among them, 6*β*,7*β*-diacetoxy-13-hydroxy-labda-8,14-diene and rotundifuran have inhibitory effects on the dopamine-D_2 receptor [6].

Today, the dried ripe fruit of *V. agnus-castus* is defined under the names Agnus Castus Fruit (ACF) and Chaste Tree, respectively, in the European Pharmacopoeia [7] and the United States Pharmacopeia [8]. ACF products are distributed as over-the-counter (OTC) drugs to treat premenstrual syndromes (PMS) and climacteric disorders based on clinical evidence in Europe [9,10]. Although ACF has been generally distributed as a health food product in Japan, a direct OTC drug derived from ACF was recently approved by the Ministry of Health, Labour and Welfare. Western herbal medicine is currently one of the most attractive complementary medications that are useful for self-medication, and ACF extract is the second example of an active ingredient of a direct OTC drug derived from a Western medicinal herb available on the Japanese market; the first was an extract of the red leaves of *Vitis vinifera* L. (Vitaceae).

On the other hand, congenetic plants native to East Asia, *Vitex rotundifolia* L., *Vitex trifolia* L., and *Vitex negundo* L. are used as sources of crude drugs in traditional Oriental medicine. The fruits of *V. rotundifolia* and *V. trifolia* are prescribed as Shrub Chaste Tree Fruit (SCTF) with the Latin name Viticis Fructus in the Japanese standards for non-pharmacopoeial crude drugs 2012 (Non-

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received February 6, 2015
revised July 11, 2015
accepted August 10, 2015

Bibliography
DOI http://dx.doi.org/10.1055/s-0035-1558089
Planta Med 2016; 82: 147–153

ISSN 0032-0943

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JPS 2012), and are used as components of some traditional Japanese medicines (Kampo formulations) due to their pain relief, sedative and anti-inflammatory effects. Thus, fruits of several *Vitex* plants are properly considered herbal medications. However, the macroscopic appearance of *Vitex* fruits is very similar for ACF and its congener species. In fact, the 8th edition of the European Pharmacopoeia (EP8.0) requires a purity test by macroscopic analysis based on the size of the fruit to discriminate *V. agnus-castus* from other *Vitex* species. Considering the internationalization of the supply chain of herbal plants, this situation could lead to misidentification of the botanical origin of the crude drugs, resulting in unexpected adverse reactions as well as in a decline in their pharmaceutical efficacy. Therefore, an appropriate discrimination method for the drugs derived from *Vitex* fruits is necessary.

According to the stipulation for ACF in EP8.0 it must pass a content test, requiring a casticin content ≥ 0.08 % and an identification test confirming that both agnuside and aucubin are detectable by TLC. However, since these constituents have been isolated from SCTF [11–13], ACF products adulterated with SCTF could be overlooked under the current regulation. Therefore, for proper regulation, there is a pressing need to establish marker compounds for discriminating ACF from SCTF.

In the present study, we confirmed the source plants of commercially available ACF and SCTF by DNA sequence analysis. Subsequently, we analyzed extracts of the crude drugs by Orbitrap LC-MS to allow for an accurate mass analysis and a composition estimation, in order to identify possible characteristic compounds of ACF by comparing its chromatographic patterns in a differential analysis to SCTF. We isolated two novel diterpenes from ACF, which were not found in SCTF and elucidated their structures on the basis of spectroscopic data from NMR and MS.

Results

The botanical sources of crude drug samples designated as ACF and SCTF were determined by querying the GenBank nucleotide sequence database using the nucleotide sequences of the *mat*K and FLORICAULA/LEAFY (FLO/LFY) genes (**Table 1**). Partial sequences of the *mat*K gene from two ACF products, ACF-1 and 2, were identical to the sequence of *V. agnus-castus* (GenBank accession no. AB284182). Among the SCTF products, SCTF-1, 2 and 5 had sequences most similar to *V. trifolia* (AB284175), and SCTF-3 and 4 had sequences similar to *V. rotundifolia* (AB284177). Since the nucleotide sequences of *V. trifolia* and *V. rotundifolia* are too similar to identify these species based on only a single DNA region, the FLO/LFY region was also amplified to determine its sequence. The results based on the sequence of the FLO/LFY second intron agreed with a botanical origin of ACF-1 and 2 being *V. agnus-castus* L., whereas the nucleotide sequences obtained from SCTF-2, 3, 4 and 5 were more similar to these of *V. rotundifolia* L. (LC012527) than to those of *V. trifolia* L. (LC012528), and the sequence of SCTF-1 was not conclusive by direct sequencing. By subcloning a short fragment of the FLO/LFY second intron from SCTF-1, we found that SCTF-1 contained both *V. trifolia* and *V. rotundifolia*. Considering also the observation that the sequence similarities of SCTF samples to congeners other than *V. trifolia* L. and *V. rotundifolia* L. were not very high, we confirmed the usage of samples of the correct origin for the SCTF products.

To discover candidate marker compounds for ACF, an automated differential analysis of the metabolic profiles of ACF and SCTF crude drug samples was performed using SIEVE software. After alignment and peak detection, 2253 peaks were obtained, and these peaks were filtered to 23 on the basis of a ratio > 10.0 and a p-value < 0.15. Three of them were excluded from the candidates, because they had even mass numbers (*m/z* 350, 396 and 520 for $[M–H]^-$) and each calculated formula was not acceptable for natural products. Visual confirmation of the candidate peaks on chromatograms in each extracted mass range led to omitting 13 peaks, because their actual intensities from SCTF samples were too large to recognize them as the specific peaks for ACF samples. We finally obtained 7 peaks as putative marker compounds for ACF and selected ID 1552 (RT 19.02 min, *m/z* 335.2228, formula $C_{20}H_{31}O_4$ for $[M–H]^-$) as the first target to identify the chemical structure. The total ion chromatograms of the ACF and SCTF samples, the extracted ion chromatograms of ACF and SCTF samples at the range of *m/z* 335.22–335.23, the reconducted ion chromatogram of the Peak ID 1552 by SIEVE software, and the MS and MS/MS spectra of the Peak ID 1552 from ACF-2, are shown in **Fig. 1**.

The ACF health food product was furnished as a source material of Peak ID 1552 and repeated column chromatography and preparative TLC of a $CHCl_3$ extract of the ACF health food product afforded compound **1**/**1a** as a mixture. Compound **1**/**1a** was obtained as a white powder. The ^{1}H-NMR and ^{13}C-NMR spectroscopic data of **1**/**1a** (**Tables 2** and **3**) were very similar to those of known compounds leucasperols A and B [14], except for the chemical shift of the A-ring, especially at the C-3 position. The ^{13}C chemical shifts of C-3 of **1**/**1a** were δ_C 41.5/41.5, whereas those of leucasperols A and B were δ_C 78.2/78.4 (A/B). In addition to the high-field shift of C-3, DEPT signals of δ_C 41.5/41.5 indicated methylene groups. Moreover, the molecular formula of **1**/

Table 1 ACF and SCTF crude drugs used in this study and their botanical origin.

Sample ID	Product name	Distributor country	Lot Nos.	DNA identification		
				*mat*K region	LFY direct sequencing (1.3kp)	LFY subcloning (280 bp)
ACF-1	Agni casti fructus Ph. Eur	Germany	08015318	*V. agnus-castus*	*V. agnus-castus*	not sequenced
ACF-2	Agni casti fructus tot.	Germany	B116424	*V. agnus-castus*	*V. agnus-castus*	not sequenced
SCTF-1	Shrub Chaste Tree Fruit	Japan	024011001	*V. trifolia*	undetermined	*V. trifolia*, *V. rotundifolia*
SCTF-2	Shrub Chaste Tree Fruit	Japan	B2L0604	*V. trifolia*	*V. rotundifolia*	not sequenced
SCTF-3	Shrub Chaste Tree Fruit	Japan	6B22	*V. rotundifolia*	*V. rotundifolia*	not sequenced
SCTF-4	Shrub Chaste Tree Fruit	Japan	024007001	*V. trifolia*	*V. rotundifolia*	not sequenced
SCTF-5	Shrub Chaste Tree Fruit	Japan	313016	*V. trifolia*	*V. rotundifolia*	not sequenced

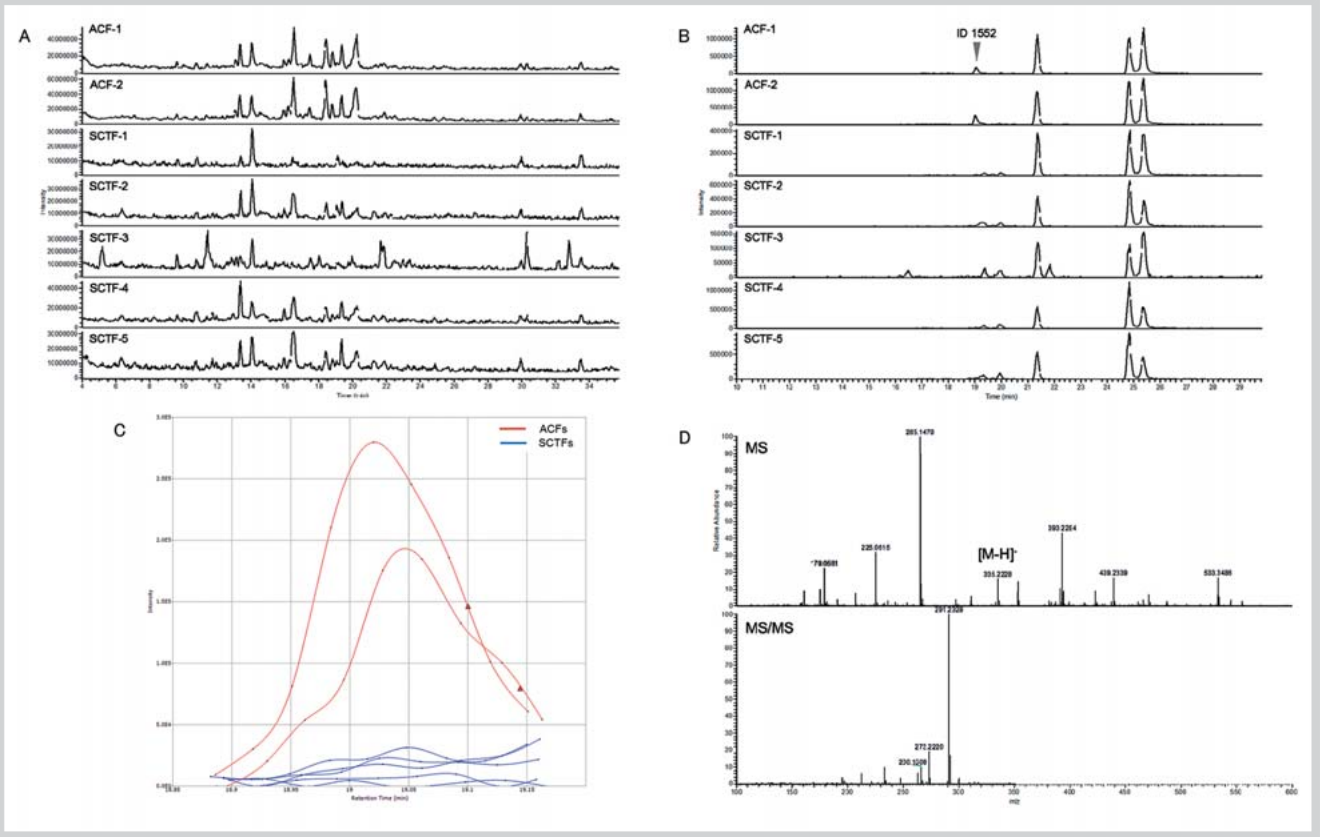

Fig. 1 **A** Total ion chromatograms of ACF and SCTF samples. **B** Extracted ion chromatograms of ACF and SCTF samples at *m/z* 335.22–335.23. **C** Re-conducted ion chromatogram of Peak ID 1552 by SIEVE software. **D** MS and MS/MS spectra of Peak ID 1552 from ACF-2. (Color figure available online only.)

Table 2 [1] H-NMR data.

Position	a/b*[1] (5:2 mixture)	1/1a (4:1 mixture)
	δ_H ($CDCl_3$, δ ppm, *J* in Hz)	δ_H ($CDCl_3$, δ ppm, *J* in Hz)
1	1.86 (1H, m) 1.44 (1H, dt, 12.5, 3.7)	1.56 (overlapping) 1.41 (1H, dd, 10.4, 4.8)
2	1.64–1.68 (1H, m) 1.59 (1H, dd, 9.1, 4.3)	1.57 (overlapping) 1.50 (1H, m)
3	3.11 (1H, dd, 11.6, 4.3)/3.07 (1H, dd, 11.3, 4.3) –	1.20 (overlapping) 0.97 (1H, ddd-like, 12.8, 6.3, 4.0)
5	2.63 (1H, s)/2.64 (1H, s)	2.51 (1H, s)/2.62 (1H, s)
7	2.41 (1H, td, 13.1, 0.9)/2.36 (1H, td, 13.1, 1.0) 2.06 (1H, dd, 13.1, 4.3)/2.01 (1H, overlapping)	2.43 (1H, m) 2.00 (1H td, 12.8, 4.8)
8	2.10–2.19 (1H, m)	2.09 (m)
11	2.14 (1H, dd, 9.4, 3.6) 1.83 (1H, m)	2.17 (1H, ddd, 13.0, 8.8, 3.2) 1.83 (1H, ddd, 13.0, 9.6, 4.8)
12	2.02 (1H, dd, 9.1, 3.6) 1.98 (1H, dd, 9.1, 4.3)	1.99 (1H, ddd, 12.8, 8.8, 4.8) 1.92 (1H, m)
14	2.43 (1H, dt, 11.9, 9.8) 1.92 (1H, ddd, 11.9, 7.3, 2.7)	2.43 (1H, ddd, 12.5, 9.6, 2.4) 1.94 (1H, ddd, 12.5, 7.2, 3.2)
15	4.11 (1H,ddd,9.8, 8.7, 2.7)/4.04 (1H, td, 8.5, 3.9) 3.79 (1H, td, 8.7, 7.3)/3.97 (1H, td, 8.5, 7.0)	4.13 (1H, ddd, 9.6, 8.8, 3.2)/4.06 (1H, m) 3.77 (1H, ddd, 8.8, 7.2, 2.4))/4.00 (1H, m)
16	4.81 (1H, s)/5.33 (1H, s)	4.78 (1H, s)/5.37 (1H, s)
17	0.95 (3H, d, 6.4)/0.96 (3H, d, 6.4)	0.93 (3H, d, 6.4)
18	1.08 (3H, s)/1.07 (3H, s)	0.91 (3H, s)/0.89 (3H, s)
19	1.16 (3H, s)/1.15 (3H, s)	1.14 (3H, s)/1.18 (3H, s)
20	0.88 (3H, s)/0.87 (3H, s)	0.81 (3H, s)/0.86 (3H, s)
16-OH		3.18 (1H, br s)*[2]

*[1]a: leucasperol A; b: leucasperol B; *[2] disappeared on treatment with D_2O; a pair of signals from tautomers is shown as "a/b"

Table 3 [13] C-NMR data.

Position	a/b*	1/1a	1/1a
	δ_C ($CDCl_3$)	δ_C ($CDCl_3$)	DEPT
1	29.9/28.7	30.4/30.3	CH_2
2	26.5/26.5	17.2/17.3	CH_2
3	78.2/78.4	41.5/41.5	CH_2
4	37.4/37.4	31.2/30.9	C
5	58.5/58.1	58.2/57.6	CH
6	210.3/210.6	210.5/210.6	C
7	48.7/48.9	47.6/47.8	CH_2
8	38.6/37.8	37.4/36.6	CH
9	92.7/93.0	91.9/91.8	C
10	47.3/47.5	46.4/46.8	C
11	29.0/31.2	28.0/27.6	CH_2
12	36.6/37.2	35.4/35.4	CH_2
13	91.3/92.5	90.3/91.7	C
14	35.5/31.1	34.6/35.9	CH_2
15	64.8/66.3	64.0/65.5	CH_2
16	99.0/101.6	98.1/100.6	CH
17	16.9/17.6	16.0/16.7	CH_3
18	27.7/27.6	31.7/31.6	CH_3
19	14.9/15.0	20.8/21.7	CH_3
20	20.0/20.2	19.0/19.2	CH_3

* a: leucasperol A; b: leucasperol B; a pair of signals from tautomers is shown as "a/b"

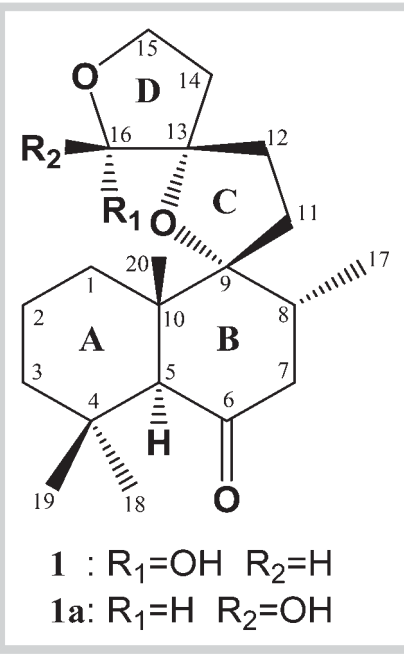

Fig. 2 Chemical structures of compounds **1** and **1a**.

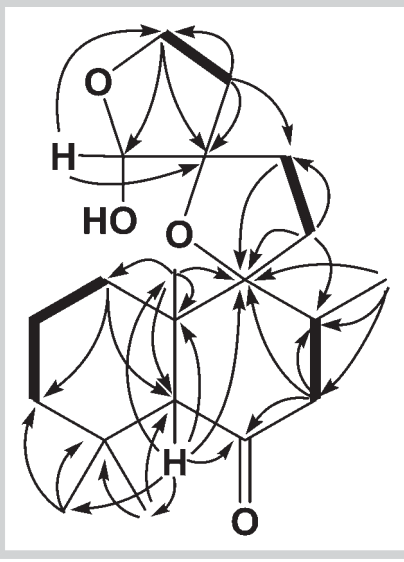

Fig. 3 COSY (bold) and HMBC (arrows) correlations of **1**.

1a was $C_{20}H_{32}O_4$, suggesting a dehydroxylate of leucasperols A and B ($C_{20}H_{32}O_5$). These data indicated that compound **1**/**1a** has a methylene group at the 3-position (**Fig. 2**). This structure was supported by the similar chemical shifts at C-3 (δ_C 44.1/44.1) of leucasperols A and B analogs bearing a hydroxyl group at C-6 and a methylene group at C-3 instead of a carbonyl group and a hydroxyl group, respectively [15]. The complete structures of **1**/**1a** were determined by an analysis of the heteronuclear multiple bond correlation (HMBC) and correlation spectroscopy (COSY) spectra, as shown in **Fig. 3**. In the nuclear Overhauser effect spectroscopy (NOESY) spectrum of compound **1** (**Fig. 4A**), correlations were observed between H-2 and H-20, H-11β and H-20, H-8 and H-11α, H-5 and H-18. In differential NOE experiments (**Fig. 4B**), irradiation of the H-16 proton resulted in NOE effects on H-1 (9.7%) and H-12 (3.3%). These data indicated that the relative configuration of compound **1** is as shown in **Fig. 4**. This configuration was supported by some reports on isolation of constituents having the same configuration from congener plants [16–19]. Since a compound bearing a hemiacetal group generally shows an equilibrium between two stereoisomers, the epimeric ratio varies depending on the solvent and on the temperature [20]. Compounds **1**/**1a** showed an equilibrium, and the epimeric ratios of **1** and **1a** were changed by the solvents used for NMR [**1**/**1a**; 4 : 1 mixture ($CDCl_3$), 5 : 4 mixture (DMSO-d_6); **Fig. 3S, Table 1S**, Supporting Information]. Consequently, the structures of **1** and **1a** were (2*R**,2″*R**,3*S**,4a″*S**,5′*R**,8a″*S**)-2-hydroxy-2″,5″,5″,8a″-tetramethyldodecahydro-2*H*,4″*H*-dispiro[furan-3,2′-furan-5′,1″-naphthalen]-4″-one and (2*S**,2″*R**,3 *S**,4a″*S**,5′*R**,8a″*S**)-2-hydroxy-2″,5″,5″,8a″-tetramethyldodecahydro-2*H*,4″*H*-dispiro[furan-3,2′-furan-5′,1″-naphthalen]-4″-one, respectively. We propose the trivial names chastol and epichastol for compounds **1** and **1a**.

Discussion

In the present study, the botanical origins of 2 ACF and 5 SCTF crude drug products were confirmed by DNA sequence analysis, and then extracts of each crude drug were analyzed by LC-MS. Subsequent differential analysis of ACF and SCTF by comparison of their chromatographic patterns revealed 7 peaks of *V. agnus-castus* that could distinguish it from both *V. rotundifolia* and *V. trifolia*. The calculated chemical formulas based on the accurate mass and MS fragmentation pattern of these peaks were collated with the phytochemical metabolite database, and 1 diterpene ($C_{20}H_{31}O_4$ for $[M-H]^-$), 5 iridoids [$C_{25}H_{29}O_{12}$ (2 peaks), $C_{25}H_{29}O_{13}$, $C_{26}H_{31}O_{14}$ and $C_{34}H_{35}O_{15}$ for $[M-H]^-$] and 1 unknown compound ($C_{13}H_{23}O_8$ for $[M-H]^-$) were predicted as the marker candidates. We isolated the diterpene candidate to identify new compounds, chastol (**1**) and epichastol (**1a**) as the first putative

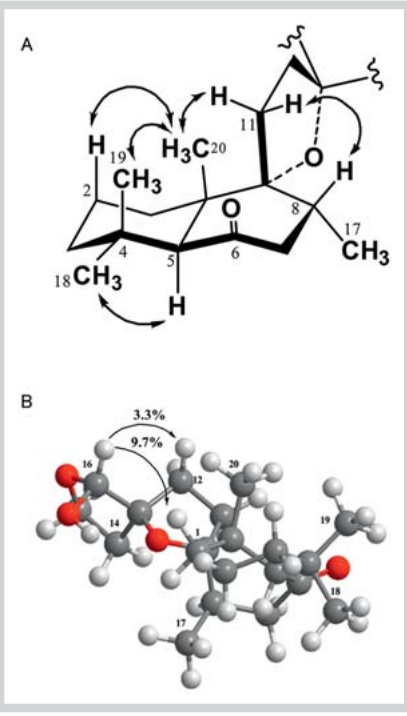

Fig. 4 **A** NOESY correlations of **1**, **B** Differential NOE data (%) of **1**. (Color figure available online only.)

marker compounds. Further study for the other candidates is ongoing.

Compounds **1** and **1a** are labdane-type diterpenes having two spirotetrahydrofuran rings, a carbonyl group at the 6-position and a methylene group at the 7-position. There have been no reports on the isolation of compounds bearing this structure, except for leucasperols A and B from *Leucas aspera* L. (Lamiaceae) [14]. The genus *Vitex* was earlier classified in the Verbenaceae family, based on morphological features (e.g., the modified Engler and Cronquist system), but later reclassified in the Lamiaceae family according to the APG system, which is based on DNA sequences [21]. Thus, the structural similarity of **1** and **1a** to leucasperols A and B bearing both a carbonyl group at the 6-position and a methylene group at the 7-position supports this reclassification from a chemotaxonomical viewpoint.

The distribution of standardized products in the market requires the correct recognition of the quality of the raw materials based on the composition and the content of chemical constituents, as well as the accurate botanical origin and the GMP-guiding manufacturing process. Though casticin, agnuside and aucubin are used as marker compounds for the standardization of ACF in EP8.0, some reports have shown [11–13] that they can be found in other *Vitex* species, and these compounds were indeed detected in SCTF crude drugs in our study (data not shown). Conversely, a much larger amount of compounds **1** and **1a** was detected from ACF than from SCTF, and the detection of these putative markers was also confirmed from commercial ACF medicines and health food products (**Table 4**, **Fig. 4 S**, Supporting Information). Therefore, the detection of the characteristic marker compounds for ACF, chastol and epichastol, should help preventing the use of adulterated materials in SCTF products, and would also be a useful means of identifying the origin of raw materials in ACF products.

In conclusion, we identified new diterpenes, chastol and epichastol, as putative marker compounds distinguishing Agnus Castus Fruit from Shrub Chaste Tree Fruit. Since avoiding contamination from closely related species is needed for the quality control of natural pharmaceuticals, this information should be valuable to establish a method for determining the quality of both ACF and SCTF products.

Materials and Methods

Materials

ACF crude drugs of EP8.0 grade and medicines were purchased from pharmacies in Europe. SCTF of non-JP crude drug standards grade and ACF health food products were purchased from manufacturers in Japan and from internet stores, respectively. Details are shown in **Tables 1** and **4**.

General procedure

Optical rotation was measured with a DIP-370 digital polarimeter (Jasco). IR spectra were recorded on an FT/IR 6100 Fourier transform infrared spectrometer (Jasco). ^{1}H-, ^{13}C-, and 2D-NMR spectra were recorded on an ECA-800 or ECA-600 spectrometer (JEOL), and the chemical shifts were referenced to TMS as the internal standard.

DNA sequence analysis

Total DNA was extracted from 10–50 mg of powdered samples using a Maxwell 16 Tissue DNA Purification Kit (Promega). A partial sequence of the chloroplast mat*K* gene (1165 bp) was amplified via PCR using AmpDirect Plus PCR buffer (Shimadzu) and EX Taq DNA polymerase (Takara) with specific primers 3F_KIMf (5′-CGT ACA GTA CTT TTG TGT TTA CGA G-3′) and 1R_KIMr (5′-ACC CAG TCC ATC TGG AAA TCT TGG TTC-3′) under the following conditions: 94 °C for 2 min, 30 cycles of 94 °C for 10 sec, 55 °C for 30 sec, and 72 °C for 1 min, then 72 °C for 4 min. Fragments of the nuclear FLORICAULA/LEAFY (FLO/LFY) second intron were also amplified with the specific primer set Vitex-LFint2F1 (5′-AAT GCC GCG AGT TCT TGA TA-3′) and Vitex-LFint2R1 (5′-GCA TAC CTG AAC ACT TGG TTT G-3′) for full-length fragments and the set Vitex-LFint5F (5′-CTC TTT TGG TGT TGG GGG TAG-3′) and Vitex-LFint5R (5′-GTG AAA CAC CCA TGA ATT GTT AG-3′) for a 377 bp fragment. The resulting PCR products were detected by an MCE-202 MultiNA microchip electrophoresis system (Shimadzu) and purified using a MinElute PCR purification kit (QIAGEN). The

Table 4 ACF medicines and health food products containing chasteberry.

Sample ID	Product	Distributor country	Form	Contents	Lot Nos.
ACF-11	Medicine	Germany	tablet	dry extract 5 mg	0000049883
ACF-12	Medicine	Germany	capsule	dry extract 5 mg	93323
ACF-13	Medicine	Switzerland	tablet	dry extract 20 mg	100056
ACF-21	Health food product	USA	capsule	dry extract 225 mg	109344
ACF-22	Health food product	USA	capsule	powdered fruit 400 mg	110723

377 bp fragment of the FLO/LFY second intron from SCTF1 was subcloned into the PCR2.1-TOPO vector (Invitrogen) for sequencing. Sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and each nucleotide sequence was determined by an ABI PRISM 3130 genetic analyzer (Applied Biosystems).

Liquid chromatography-tandem mass spectrometry analysis

An aliquot (200 mg) of each powdered crude drug sample was extracted with 10 mL of 75% MeOH by sonication for 3 h at room temperature and centrifuged at 12 000 *g* for 10 min to obtain a supernatant. The supernatant was diluted 20-fold to a final concentration of 1 mg/mL and filtered through a 0.45 µm Ultrafree-MC centrifugal filter unit (Millipore) before LC/MS. A 10 µL aliquot of the filtrate was injected and LC/MS/MS analyses were carried out using a Prominence UFLC (Shimadzu) equipped with a Hypersil GOLD column (2.1 × 100 mm, 1.9 µm; Thermo Scientific) and coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific). The mobile phase, consisted of water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B), was delivered at a flow rate of 0.2 mL/min. The gradient started at 20% solvent B and increased linearly to 90% in 35 min, then to 100% in 5 min. The column temperature was 40 °C. The mass spectra were obtained in electrospray ionization-negative mode using the following parameters: needle voltage: 3000 V; capillary temperature: 300 °C; target mass resolution: 30 000; *m/z* range: 100–2000. The most intense ions in the full-scan mass spectrum were isolated with a 3.5 Da window and fragmented by collision-induced dissociation with a collision energy of 35 V. Mass calibration was accomplished by using polythyrosine as an external standard according to the manufacturer's instructions.

Differential liquid chromatography-mass spectrometry data analysis

Differential expression analysis for LC/MS-based metabolomics was performed using the SIEVE 2.0 software (Thermo Scientific) with the Base Peak Framing algorithm for chromatographic alignment, framing, and differential analyses. MS chromatograms were unaligned by setting the alignment bypass to "true". For the frame creation, *m/z* width was set to 10 ppm (± 5 ppm), retention time width to 0.3 min, and peak intensity threshold to 275 000. Two sample differential analyses were carried out between ACF and SCTF to obtain the ratio of peak intensity and the p value, indicating the level of statistical significance. Chemical formulas of the candidate compounds were extracted from the accurate mass with a tolerance < 4 ppm via the elemental composition tool in Xcalibur (Thermo Scientific).

Liquid chromatography-mass spectrometry for isolation

ACF, SCTF, and health food products containing chasteberry extract were extracted with $CHCl_3$ to a final concentration of 2 mg/mL and filtered through filter paper to remove muddy substances. After filtration through a 0.45 µm Ultrafree-MC centrifugal filter unit, 10 µL of the filtrate was injected and LC-MS/MS carried out using a Prominence UFLC, equipped with an Inertsil ODS-4 column (4.6 × 250 mm, 5 µm; GL Sciences), coupled to an LTQ Orbitrap XL mass spectometer. The mobile phase, consisted of water (solvent A) and MeOH (solvent B), was delivered at a flow rate of 1.0 mL/min. The gradient started at 50% solvent B for 10 min and increased linearly to 80% in 5 min, then to 100% in 15 min. The column temperature was 40 °C.

Extraction and isolation

The content of capsules (180 g or 580 capsules) of a commercial health food product derived from ACF extract (ACF-21) was extracted ultrasonically with $CHCl_3$ (7.2 L) for 3 h. After filtering the extract, it was evaporated under reduced pressure to give a crude dry extract (3.41 g), which was dissolved in $CHCl_3$ (8 mL) and applied to silica gel (10 g). This silica gel was added to the top of a silica gel column for chromatography (Ø 4.4 × 21 cm), eluted with *n*-hexane–EtOAc (1 : 0 → 1 : 4) to give six fractions [fr. A-1, *n*-hexane (600 mL) eluate; fr. A-2, *n*-hexane : EtOAc = 4 : 1 (600 mL) eluate; fr. A-3, *n*-hexane : EtOAc = 7 : 3 (600 mL) eluate; fr. A-4, 150 mg, *n*-hexane : EtOAc = 3 : 2 (600 mL) eluate; fr. A-5, *n*-hexane : EtOAc = 1 : 1 (600 mL) eluate; fr. A-6, *n*-hexane : EtOAc = 1 : 4 (600 mL) eluate]. Fr. A-4 (150 mg) was dissolved in $CHCl_3$ (1 mL) and chromatographed on a silica gel column (Ø 2 × 25 cm) with $CHCl_3$:MeOH (1 : 0 → 1 : 1) to give six fractions [fr. B-1, $CHCl_3$ eluate (100 mL); fr. B-2, 10 mg, $CHCl_3$:MeOH = 99 : 1 (100 mL) eluate; fr. B-3, 40 mg, $CHCl_3$:MeOH = 19 : 1 (100 mL) eluate; fr. B-4, 30 mg, $CHCl_3$:MeOH = 9 : 1 (100 mL) eluate; fr. B-5, 10 mg, $CHCl_3$:MeOH = 4 : 1 (100 mL) eluate; fr. B-6, $CHCl_3$:MeOH = 1 : 1 (100 mL) eluate]. Fr. B-4 (30 mg) was repeatedly separated by preparative TLC (*n*-hexane : EtOAc, $CHCl_3$:MeOH) to obtain compound **1**/**1a** [1.7 mg, R_f 0.50 (yellow spot; *n*-hexane : EtOAc 1 : 1; *p*-anisaldehyde–sulfuric acid)].

Chastol/epichastol (**1**/**1a**): white powder; $[\alpha]_D^{25}$ +16.7 (*c* 0.3, $CHCl_3$), +3.3 (*c* 0.3, DMSO); IR ($CHCl_3$) ν_{max} 3749, 2917, 1703, 1540, 1124, 940 cm^{-1}; ^{1}H-NMR, see ◉ **Table 2**; ^{13}C-NMR, see ◉ **Table 3**; HRTOFMS *m/z* 335.2223 $[M-H]^-$ (calcd. for $C_{20}H_{31}O_4$, 335.2222).

Supporting information

^{1}H-NMR and ^{13}C-NMR spectra of compound **1** are available as Supporting Information.

Conflict of Interest

▼

All authors declare no conflict of interest associated with this manuscript.

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