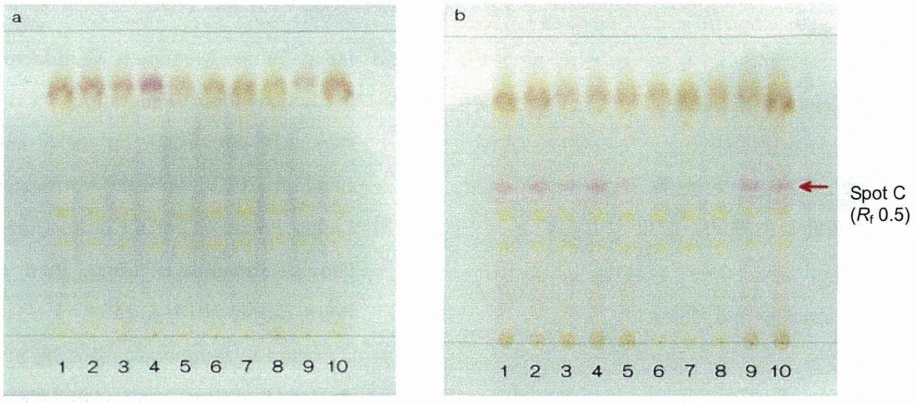

Fig. 3 TLC Comparison of Glycyrrhiza (a) and Prepared Glycyrrhiza (b) (Condition III)
Solvent system; ethyl acetate/methanol/water (20:3:2), Detection; Spraying 4-dimethylaminobenzaldehyde Test Solution.
Red arrow indicates the spot specific for Prepared Glycyrrhiza (Spot C, R_f 0.5).

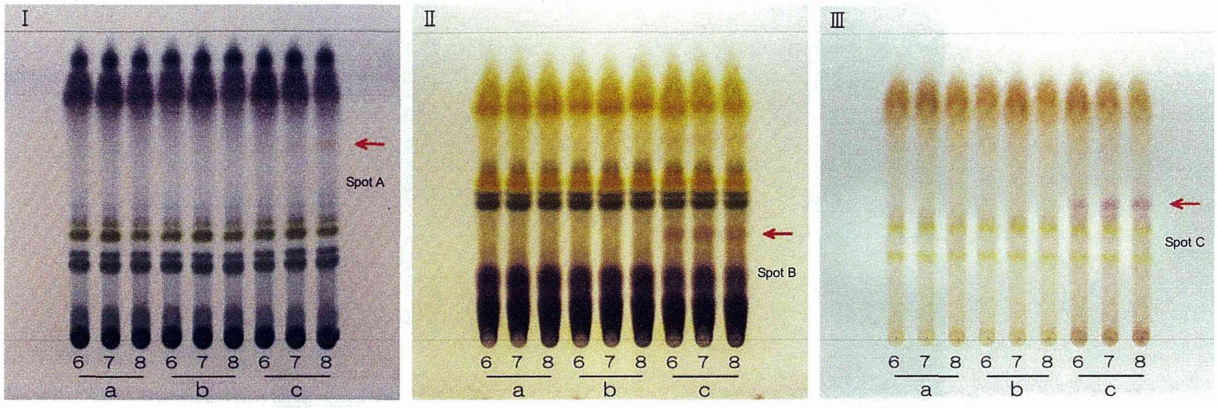

Fig. 4 TLC Comparison of Glycyrrhiza (a), Prepared Glycyrrhiza (b) and Re-heated Prepared Glycyrrhiza (c)
Solvent system and detection method in I, II and III are the same as in Fig. 1, Fig. 2 and Fig. 3, respectively.
Red arrows indicate Spot A (I), Spot B (II) and Spot C (III), respectively.

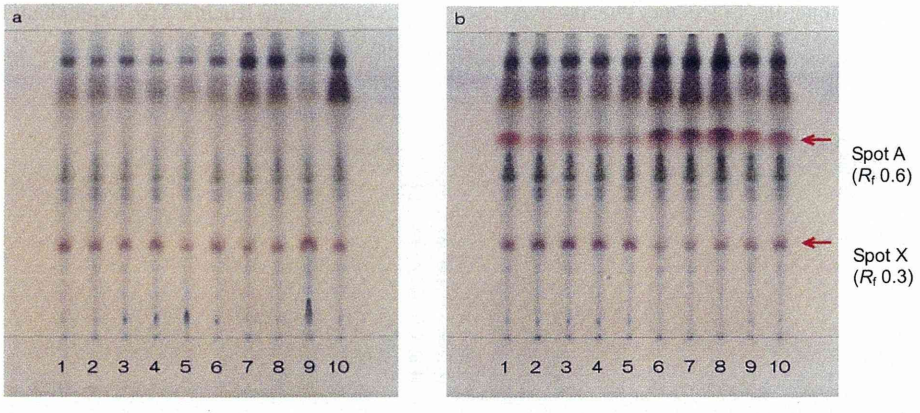

Fig. 5 Improved Sample Preparation Method for Comparison of Glycyrrhiza (a) and Prepared Glycyrrhiza (b) (Condition IV)
Solvent system; ethyl acetate/methanol/water (7:2:1), Detection; Spraying 4-methoxybenzaldehyde Test Solution then heated at 105℃.
Red arrows indicate Spot A (R_f 0.6) and Spot X (R_f 0.3).

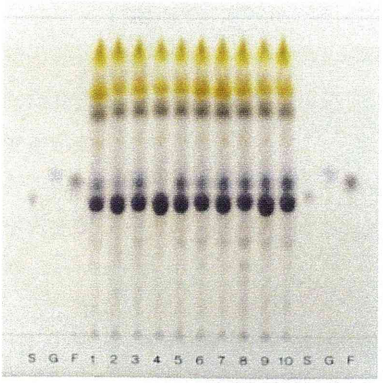

Fig. 6 TLC Detection of Sugars in Glycyrrhiza
Solvent system; 2-propanol/glacial acetic acid/diethylether/water (4:3:2:1), Detection; Spraying 1,3-naphthalenediol Test Solution then heated at 105℃.
S: sucrose; G: glucose; F: fructose; 1~10: Glycyrrhiza

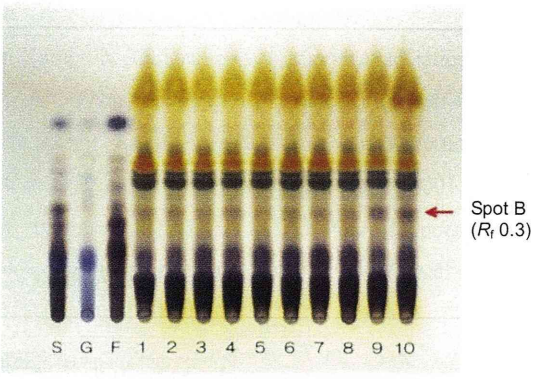

Fig. 7 TLC Comparison of Prepared Glycyrrhiza and Heat-treated (130℃, 30 min) Sugars (Condition II)
Solvent system; ethyl acetate/methanol/water (7:2:1), Detection; Spraying 1,3-naphthalenediol Test Solution then heated at 105℃. Red arrow indicates Spot B (R_f 0.3).
S: heat-treated sucrose; G: heat-treated glucose; F: heat-treated fructose; 1~10: Prepared Glycyrrhiza

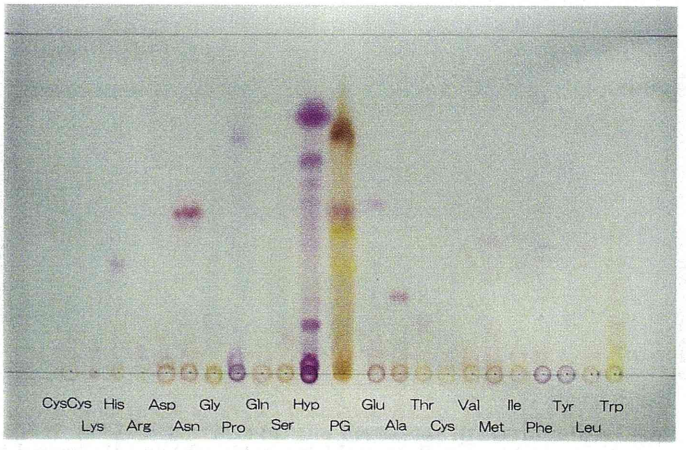

Fig. 8 TLC Comparison of Prepared Glycyrrhiza and Heat-treated (130℃, 30 min) Amino Acids with Fructose (Condition III)
Solvent system; ethyl acetate/methanol/water (20:3:2), Detection; Spraying dimethylaminobenzaldehyde Test Solution.
Cys-Cys: cystine; Lys: lysine; His: histidine; Arg: arginine; Asp: aspartic acid; Asn: asparagine; Gly: glycine; Pro: proline; Gln: glutamine; Ser: serine; Hyp: hydroxyprorline; Glu: glutamic acid; Ala: alanine; Thr: threonine; Cys: cysteine; Val: valine; Met: methionine; Ile: isoleucine; Phe: phenylalanine; Tyr: tyrosine; Leu: leucine; Trp: tryptophan; PG: Prepared Glycyrrhiza

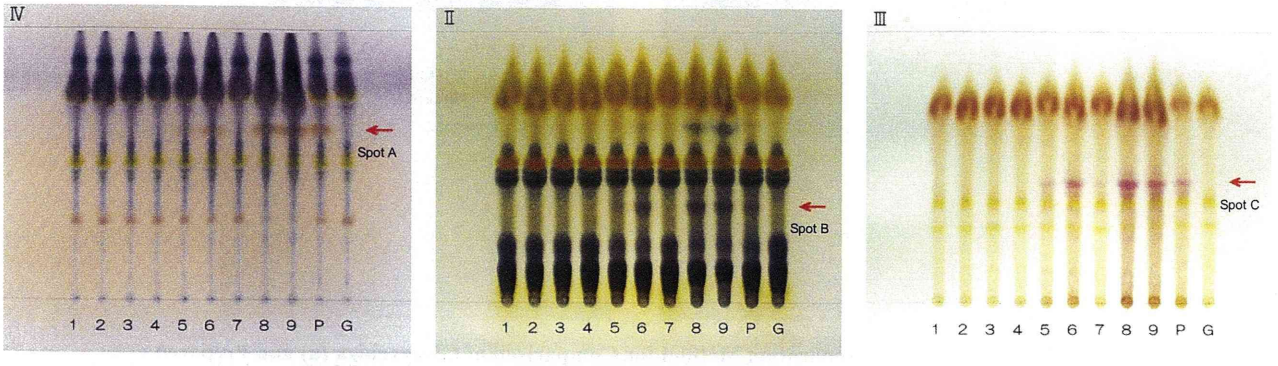

Fig. 9 Effect of Temperature and Time on TLC Pattern of Heat-treated Glycyrrhiza
Solvent system and detection method in IV, II and III are the same as in Fig. 5, Fig. 2 and Fig. 3, respectively.
Red arrows indicate Spot A (IV), Spot B (II) and Spot C (III), respectively.
1: 110℃, 10 min; 2: 110℃, 30 min; 3: 110℃, 60 min; 4: 130℃, 10 min; 5: 130℃, 30 min;
6: 130℃, 60 min; 7: 150℃, 10 min; 8: 150℃, 30 min; 9: 150℃, 60 min; P: Prepared Glycyrrhiza; G: Glycyrrhiza

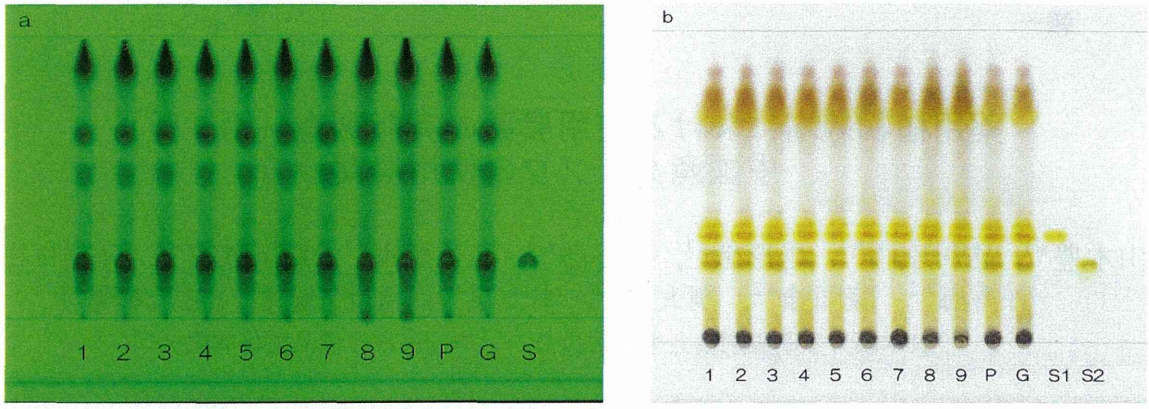

Fig. 10 Detection of Glycyrrhitic Acid (a) and Liquiritin (b) in Heat-treated Glycyrrhiza, Prepared Glycyrrhiza and Glycyrrhiza

a) Solvent system; 1-butanol/water/glacial acetic acid (7:2:1), Detection; UV 254 nm.

b) Solvent system; ethyl acetate/methanol /water (20:3:2), Detection; Spraying 10% sulfuric acid then heated at 105℃.

1~9: heat-treated Glycyrrhiza (1: 110℃, 10 min; 2: 110℃, 30 min; 3: 110℃, 60 min; 4: 130℃, 10 min; 5: 130℃, 30 min; 6: 130℃, 60 min; 7: 150℃, 10 min; 8: 150℃, 30 min; 9: 150℃, 60 min); P: Prepared Glycyrrhiza; G: Glycyrrhiza; S: glycyrrhitic acid; S1: liquiritin; S2: liquiritin apioside

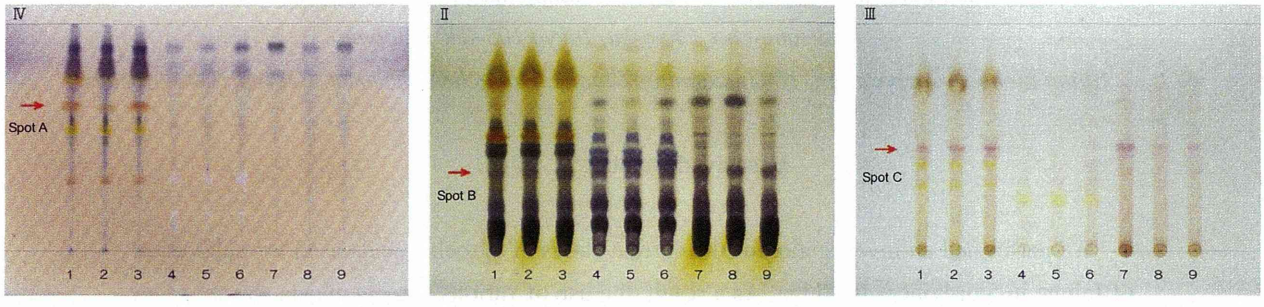

Fig. 11 TLC Comparison of Prepared Glycyrrhiza, Heat-treated Pueraria Root, and Heat-treated Astragalus Root

Solvent system and detection method in IV, II and III are the same as in Fig. 5, Fig. 2 and Fig. 3, respectively.

Red arrows indicate Spot A (IV), Spot B (II) and Spot C (III), respectively.

1~3: Prepared Glycyrrhiza; 4~6: heat-treated Pueraria Root; 7~9: heat-treated Astragalus Root

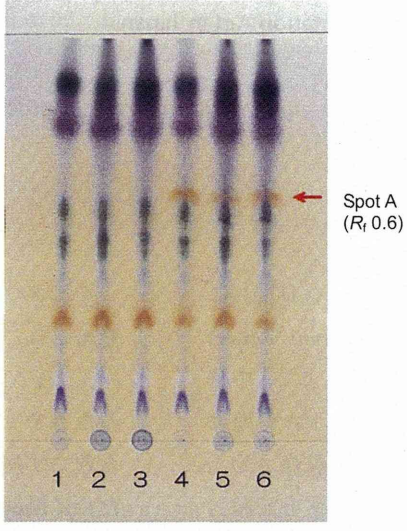

Fig. 12 TLC Comparison of Glycyrrhiza and Prepared Glycyrrhiza derived from *G. glabra*

Solvent system and detection method are the same as in Fig. 5 (Condition IV).

Red arrow indicates Spot A (R_f 0.6).

1~3: Glycyrrhiza; 4~6: Prepared Glycyrrhiza

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ORIGINAL PAPER

Identification of marker compounds for Japanese Pharmacopoeia non-conforming jujube seeds from Myanmar

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Abstract Jujube Seed is a crude drug defined as the seed of *Ziziphus jujuba* Miller var. *spinosa* Hu ex H.F. Chou (Rhamnaceae) in the Japanese Pharmacopoeia (JP). Most of the jujube seed in the Japanese markets is imported from China, with the rest obtained from other Asian countries. Here we confirmed the botanical origins of jujube seeds from both China and Myanmar by a DNA sequencing analysis. We found that the botanical origins of the crude drugs from China and Myanmar were *Z. jujuba* and *Z. mauritiana*, respectively. Although the jujube seed from China conforms to the JP, that from Myanmar does not. A method for discriminating jujube seeds from China and Myanmar using a chemical approach is thus desirable, and here we sought to identify a compound specific to *Z. jujuba*. Jujuboside A (**1**) was identified as a compound specific to *Z. jujuba*. To establish a purity test of Jujube Seed in the JP against *Z. mauritiana*, we fractionated the extract of *Z. mauritiana* seeds and identified frangufoline (**2**) and oleanolic acid (**4**) as the marker compounds specific to *Z. mauritiana*. Thin-layer chromatography (TLC) and gas chromatography-mass spectrometry analyses revealed that the latter compound was useful for testing by TLC analysis. The established TLC conditions were as follows: chromatographic support, silica gel; developing solvent, *n*-hexane:EtOAc:HCOOH = 10:5:1; developing length, 7 cm; visualization, diluted sulfuric acid; R_f value, 0.43 (oleanolic acid).

Keywords Jujube Seed · *Ziziphus jujuba* var. *spinosa* · *Ziziphus mauritiana* · Purity test · Oleanolic acid

Introduction

'Jujube Seed' (酸棗仁) is a crude drug defined as the seed of *Ziziphus jujuba* Miller var. *spinosa* Hu ex H.F. Chou (Rhamnaceae) in the Japanese Pharmacopoeia, 16th edition (JP16). It has been used for Kampo (i.e., Japanese traditional herbal medicine) formulae targeted at insomnia and psychoneurosis, based on its sedative ingredients such as saponins and flavonoids [1, 2]. Most Jujube Seeds in Japanese markets are imported from China, and the remaining stock is drawn from other Asian countries [3].

It has recently become clear that the botanical origin of 'jujube seeds' (in this manuscript, we refer to the seed of *Ziziphus* plants as "jujube seed" and that of *Z. jujuba* var. *spinosa* of JP grade as "Jujube Seed") from Myanmar are not *Z. jujuba* var. *spinosa*, but *Z. mauritiana* on the basis of genetic analyses [4], but the macroscopic distinction of the two species is difficult because of their similar appearance, especially in the cut and powder forms. A method for discriminating jujube seeds from China and those from Myanmar by an approach such as high-performance liquid chromatography (HPLC) or thin-layer chromatography

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(TLC) is desired to select jujube seeds conforming to the JP16.

In the present study, the botanical origins of jujube seeds from China and Myanmar were confirmed by a DNA sequencing analysis. We analyzed the extracts of the two types of jujube seeds by liquid chromatography-mass spectrometry (LC-MS) and TLC analyses, and we identified the specific compounds of each species of seed.

Materials and methods

Crude drugs and materials

The 11 samples of jujube seeds from China used in this study were collected from 1995 to 2012, and the five samples of jujube seeds from Myanmar were collected from 1993 to 2011. The details of the crude drug samples used are shown in Table 1. Authentic jujuboside A, oleanolic acid and spinosin were purchased from ChromaDex Co. (Boulder, CO, USA), Wako Co. (Osaka, Japan) and Apin Chemicals (Abingdon, Oxon, UK), respectively. Research-grade organic solvents were used for the LC–MS, TLC, and extraction and isolation.

General procedure

The LC-MS analysis was performed on an LC-20A HPLC system (pump, LC-20AD; degasser, DGU-20A_3; autosampler, SIL-20AC_{HT}; column oven, CTO-20A; diode array detector, SPD-M20A) equipped with an LCMS-2020 mass spectrometer (Shimadzu, Kyoto, Japan). The gas chromatography-mass spectrometry (GC–MS) analysis was performed on a GC-2010 system (Shimadzu) equipped with the GC-MS-QP2010 single quadrupole gas chromatograph-mass spectrometer (Shimadzu). ^{1}H- and ^{13}C-NMR spectra were recorded on a spectrometer (ECA-800, JEOL, Tokyo), and chemical shifts are expressed in δ (ppm) with tetramethylsilane (TMS). The high-resolution mass spectrum was measured on an LCMS-IT-TOF [a hybrid mass spectrometer coupling atmospheric pressure ionization with ion-trap (IT) and time-of-flight (TOF) technologies; Shimadzu] using glycyrrhizin as an external standard (*m/z* 821.3965). A ball mill (MM-300, Qiagen, Hilden, Germany), pulverizer (TI-200, Irie-Shokai, Tokyo), shaker (SR-2w, Taitec, Saitama, Japan) and centrifugation (6900, Kubota, Tokyo) were used to process the seeds.

Table 1 Date and area of collection of *Z. jujuba* and *Z. mauritiana*

No.	Date	Area
C-1	December 2012	Hebei, China
C-2	December 2011	Hebei, China
C-3	November 2010	Hebei, China
C-4	December 2009	Hebei, China
C-5	August 2009	Shaanxi, China
C-6	December 2008	Hebei, China
C-7	December 2007	Hebei, China
C-8	November 2006	Hebei, China
C-9	September 1999	Hebei, China
C-10	September 1997	Hebei, China
C-11	June 1995	Hebei, China
B-1	March 1993	Myanmar
B-2	January 2011	Myanmar
B-3	March 2011	Myanmar
B-4	January 2011	Myanmar
B-5	March 2011	Myanmar

DNA sequence analysis

A single seed (approximately 30–50 mg) of each sample was crushed with the ball mill in liquid N_2. Genomic DNA was extracted from the powdered sample using the Maxwell 16 Tissue DNA Purification Kit (Promega, Madison, WI). The internal transcribed spacer 2 (ITS2) region of nuclear rDNA was amplified by polymerase chain reaction (PCR) using the obtained genomic DNA as the template. The PCR was performed on a DNA engine PTC-200 (MJ Research, currently Bio-Rad, Hercules, CA) using BIOTAQ DNA polymerase (Bioline, London) with the following program: 95 °C for 10 min; 50 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s; 72 °C for 7 min. After the removal of excess primers and dNTPs from the reaction mixture using the MinElute PCR Purification Kit (Qiagen), the amplicon was directly sequenced on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems; currently Life Technologies, Carlsbad, CA). The cycle sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies). The DNA sequences were aligned using the Clustal W program [5].

LC-MS analysis

Sample preparation

Jujube seeds from China (C-1 to C-11) and from Myanmar (B-1 to B-4) were powdered by the ball mill at 20 Hz for 1 min. Thirty milliliters of MeOH was added to the powders (1 g) and then were shaken at 5 Hz for 20 min. After centrifugation at 8,000×*g* for 10 min, the supernatants were separated. The same procedure was applied to the residues, except that 15 mL of MeOH was used for the extraction. The total supernatants were combined and then adjusted to 50 mL by MeOH using a volumetric flask. These were used as the sample solutions.

LC-MS conditions

Analysis of a specific compound in jujube seed from China (condition A) An Inertsil ODS-3 column (5 μm particle size, 2.1 mm i.d. × 150 mm; GL Sciences, Tokyo) was used, and the mobile phase was 0.1 % formic acid aqueous solution-acetonitrile (65:35; v/v). One microliter of sample solution was analyzed at 203 nm with 0.2 mL/min at 40 °C. The MS analysis parameters were as follows: interface, ESI positive/negative mode; nebulizer gas flow, 1.5 L/min; drying gas flow, 10 L/min; CDL temperature, 250 °C; heat block temperature, 200 °C; detector voltage, 120 kV; interface voltage, 4.5 kV/−4.5 kV; interface current, 0.6 μA; scan range, *m/z* 100–1300.

Analysis of a specific compound in jujube seed from Myanmar (condition B) A specific compound in extracts of jujube seed from Myanmar was analyzed in the same way as in condition A, except for the following: the mobile phase, 0.1 % formic acid aqueous solution-acetonitrile (77:23, v/v); wavelength, 190 nm.

Analysis of spinosin (condition C) Spinosin in crude extracts was analyzed in the same way as in condition A, except for the following: the mobile phase, 0.1 % formic acid aqueous solution-acetonitrile (85:15, v/v); wavelength, 254 nm.

Extraction and isolation

*Isolation of frangufoline (**2**)*

A jujube seed from Myanmar (B-5; 100 g) was crushed by the ball mill at 20 Hz for 1 min. The powder was extracted with MeOH (3 L) at room temperature for 24 h. After filtration, it was evaporated under reduced pressure to give the extract. The procedure was repeated twice to obtain the extract (Myn31, 9.7 g). It was suspended with EtOAc (300 mL) to separate the soluble fraction (Myn31-S, 3.7 g) and the insoluble fraction (Myn31-IS, 4.4 g) by filtration. A part of the soluble fraction (0.4 g) was dissolved in EtOAc (5 mL) and was subjected to a SNAP KP-Sil Samplet cartridge (3 g) (Biotage, Uppsala, Sweden). After being loaded on a SNAP Ultra column (25 g), the fraction was eluted with *n*-hexane-EtOAc on an Isolera Dalton (Biotage) to give four fractions [fr. S(1)-1, 314 mg; fr. S(1)-2, 25.6 mg; fr. S(1)-3, 7.5 mg; fr. S(1)-4, 5.2 mg]. Fr. S(1)-4 (5.2 mg) was suspended in *n*-hexane:EtOAc = 2:1 (3 mL) and then subjected to silica gel column chromatography (ϕ 1 × 4 cm) with *n*-hexane–EtOAc (2:1 → 0:1) to obtain frangufoline (**2**) (fr. S(1)-4-2, 0.4 mg), together with two fractions.

Frangufoline (**2**) [6, 7]: White amorphous powder. HRTOFMS *m/z* 535.3279 $[M + H]^+$ (calcd for $C_{31}H_{42}N_4O_4$, 535.3297) ^{1}H-NMR (800 MHz, $CDCl_3$): see Table 2. ^{13}C-NMR (200 MHz, $CDCl_3$): see Table 2.

*Isolation of oleanolic acid (**4**)*

A part of fr. Myn31-S (1.4 g) was dissolved in EtOAc (15 mL) and then subjected to a SNAP KP-SIL Samplet cartridge (10 g). After being loaded on a SNAP Ultra column (50 g), the fraction was eluted with *n*-hexane-EtOAc (2:1 → 0:1) by the Isolera Dalton to give two fractions [fr. S(2)-1, 1.3 g; fr. S(2)-2, 63.4 mg]. Fr. S(2)-2 (63.4 mg) was dissolved in EtOAc (3 mL) and then subjected to a KP-SIL Samplet cartridge (1 g). After being loaded on a SNAP Ultra column (10 g), the fraction was eluted with *n*-hexane–EtOAc (1:1 → 0:1) by the Isolera Dalton to give four fractions [fr. S(2)-2-1, 37.2 mg; fr. S(2)-2-2, 2.7 mg; fr. S(2)-2-3, 6.2 mg; fr. S(2)-2-4, 4.7 mg].

The remaining fraction (Myn31-S; 1.6 g) was dissolved in EtOAc (20 mL) and subjected to a KP-SIL samplet (10 g). After being loaded on a SNAP Ultra column (30 g), the fraction was eluted with *n*-hexane-EtOAc (2:1 → 0:1) by the Isolera Dalton to give three fractions [fr. S(3)-1, 1.4 mg; fr. S(3)-A, 12.6 mg; fr. S(3)-B, 34.6 mg].

Fr. S(2)-2-2 (2.7 mg) and fr. S(3)-A (12.6 mg) were combined on the basis of similar TLC patterns to obtain fr. C (15.3 mg). This fraction was dissolved in $CHCl_3$ (4 mL) and then subjected to silica gel column chromatography (ϕ 2.5 × 5 cm) and slowly eluted with $CHCl_3$ to obtain oleanolic acid (**4**) (fr. C-5, 2.5 mg), together with four fractions.

Oleanolic acid (**4**) [8]: White amorphous powder. ^{1}H-NMR (800 MHz, $CDCl_3$): δ_H 5.27 (1H, *dd*, $J = 4.0$, 3.2 Hz, H-12), 3.21 (1H, *dd*, $J = 11.2$, 4.0 Hz, H-3), 1.12, 1.07, 0.97, 0.92, 0.90, 0.76, 0.74 (3H, *s*, CH_3 × 7). ^{13}C-NMR (200 MHz, $CDCl_3$): d_C 182.9 (C-28), 143.7 (C-13), 122.7 (C-12), 79.1 (C-3), 55.3 (C-5), 47.7 (C-9), 46.6 (C-17), 45.9 (C-19), 41.7 (C-14), 41.1 (C-18), 39.3 (C-8), 38.8 (C-4), 38.5 (C-1), 37.1 (C-10), 33.9 (C-21), 33.2 (C-7), 32.7 (C-22), 32.5 (C-29), 30.8 (C-20), 28.2 (C-2), 27.8 (C-23), 27.3 (C-15), 26.0 (C-27), 23.7 (C-16), 23.5 (C-30), 23.0 (C-11), 18.4 (C-6), 17.2 (C-26), 15.6 (C-24), 15.4 (C-25).

TLC analysis

Sample preparation

Jujube seeds from China (C-1 to C-11) and from Myanmar (B-1 to B-5) were powdered by the ball mill at 20 Hz for 1 min. Five milliliters of MeOH was added to the powders (0.5 g), and then the mixture was shaken at 5 Hz for

Table 2 NMR data of compound **2** ($CDCl_3$)

Position	δ_H		δ_C	
	2 (800 MHz)	Ref. [7] (80 MHz)	**2** (200 MHz)	Ref. [7] (20 MHz)
1	6.35 (1H, *d*, J = 8.0 Hz)	6.35 (7.6)	115.6[f]	123.1
2	6.66 (1H, *dd-like*, J = 9.6, 8.0 Hz)	6.67 (10.2, 7.6)	125.7	125.9
3-NH	6.45 (1H, *d*, J = 9.6 Hz)	6.46 (10.2)	–	–
4	–	–	167.5	167.8
5	4.02 (1H, *ddd*, J = 7.2, 4.0, 3.2 Hz)	4.03 (7.6, 3.6)	52.7	52.7
6-NH	5.67 (1H, *d*, J = 7.2 Hz)	5.67 (7.6)	–	–
7	–	–	171.8	171.5
8	4.49 (1H, *dd*, J = 9.6, 6.4 Hz)	4.50 (10.3, 7.2)	55.3	55.4
9	5.00 (1H, *d*, J = 6.4 Hz)	5.01 (7.2, 1.7)	81.7	81.9
11	–	–	156.2	156.2
12[a]	7.19 (1H, *dd*, J = 8.0, 2.0 Hz)	7.06, 7.12	122.9	122.8
13[b]	7.06 (1H, *d*, J = 8.0 Hz)	7.06, 7.12	131.9[e]	131.8
14	–	–	132.0[e]	132
15[b]	7.03 (1H, *d*, J = 8.0 Hz)	7.06, 7.12	130.4	130.3
16[a]	7.12 (1H, *dd*, J = 8.0, 2.0 Hz)	7.06, 7.12	123.2[f]	115.5
17α	1.32–1.14 (overlapped)	1.20 (11.4, 3.6)	39.1	39.4
17β	1.68 (1H, *ddd*, J = 11.2, 4.0, 3.2 Hz)	1.69 (11.4)		
18	1.08 (1H, m)	1.11 (6.6)	24.5	24.5
19	0.64[c] (1H, *d*, J = 6.4 Hz)	0.61 (6.5)	23.3	23.1
20	0.59[c] (1H, *d*, J = 6.4 Hz)	0.65 (6.6)	20.5	20.6
21-NH	7.89 (1H, *d*, J = 9.6 Hz)	7.88 (10.3)	–	–
22	–	–	172.7	172.7
23	3.20–3.18 (overlapped)	3.2 (8.2)	70.5	70.6
24[α]	2.84 (1H, *d*, J = 16.0, 8.8 Hz)	2.85 (15.8, 8.2)	30.6	30.9
24[β]	3.20–3.18 (overlapped)	3.2 (15.8)		
25	–	–	140.5	140.4
26	7.27–7.23 (overlapped)	7.2	129.1	128.8
27	7.27–7.23 (overlapped)	7.2	128.6	129
28	7.17 (1H, *dd*, J = 7.2, 6.6 Hz)	7.2	126.3	126.2
29	7.27–7.23 (overlapped)	7.2	128.6	129
30	7.27–7.23 (overlapped)	7.2	129.1	128.8
32	2.24 (6H, *s*)	2.25	41.9	41.9
33	2.24 (6H, *s*)	2.25	41.9	41.9
34	1.92 (1H, *ddd*, J = 7.2, 6.4, 1.6 Hz)	1.9 (6.8, 6.7, 1.7)	29.4	29.4
35	1.00[d] (1H, *d*, J = 7.2 Hz)	1.02 (6.7)	20.5	20.4
36	1.28[d] (1H, *d*, J = 7.2 Hz)	1.29 (6.8)	15.1	15.1

[a–e] Interchangeable, [f]determined by 2D-NMR

20 min. After centrifugation at 8,000×*g* for 10 min, the supernatants were separated. These were used as the sample solutions.

TLC analysis

Sample solutions (10 μL) were applied on a TLC plate of silica gel and then developed with a mixture of *n*-hexane:EtOAc:HCOOH = 10:5:1 to a distance of 7 cm and air-dried. Diluted sulfuric acid was evenly sprayed on the plate, followed by heating at 105 °C for 10 min.

GC-MS analysis

The GC-MS analysis was carried out using a modification of Razborsek's method [9].

Preparation of TMS derivatives

A part of each sample solution (500 μL) prepared for the LC-MS analysis was evaporated, and then pyridine (50 μL) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA, 100 μL) were added to react for 30 min at 70 °C. The reaction mixture was adjusted to 1 mL with tetrahydrofuran (THF). This was used as the sample solution for GC-MS.

GC-MS analysis

A DB-5 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) was used. The carrier gas was helium at the flow rate of 0.9 mL/min, with a constant linear velocity of 35 cm/s. The oven temperature program was as follows: initial 105 °C (0.8 min), 12 °C/min to 200 °C (0.1 min), 7 °C/min to 290 °C (6 min) and 25 °C/min to 320 °C (10 min). Injector temperature was set at 290 °C. Injection volume was 2 μL. The transfer line temperature was held at 290 °C. MS was operated in EI ionization at 70 eV. The MS data were obtained in full scan mode (mass range *m/z* 40–700).

Results and discussion

Genomic DNA was extracted from each sample (B-1 to B-4 and C-1 to C-11 in Table 1) followed by the PCR amplification of the ITS2 region and direct sequencing to identify the botanical origins of the jujube seeds from China and Myanmar. We found that all 11 of the samples from China had the same 184-bp sequence except for two sites (aligned positions 88 and 94), which indicates nucleotide additivity [10]. A similarity survey using the blast search program revealed that the sequence is identical to that of *Z. jujuba* (GenBank accession nos.: GQ434736, DQ146572, JF421556, GQ434737, etc.) except for the above-mentioned two sites.

All five of the samples from Myanmar had the same 181-bp sequence except for three sites (aligned positions 69, 75 and 109), which indicates nucleotide additivity. A similarity survey indicated that the sequence is in agreement with that of *Z. mauritiana* (GenBank accession nos.: KC155274, JQ627047-50, DQ146589, etc.) except for the above-mentioned three sites. These results indicate that the botanical origins of jujube seeds from China and Myanmar are *Z. jujuba* and *Z. mauritiana*, respectively.

We next prepared the methanol extracts of 16 samples of jujube seeds (C-1 to C-11, B-1 to B-5 in Table 1) and analyzed them by LC-MS to identify each compound specific to *Z. jujuba* and *Z. mauritiana* (Fig. 1). When the extracts were analyzed in condition A, the extracts of *Z. jujuba* showed a peak at 8.5 min, but those of *Z. mauritiana* did not, indicating that this peak was specific to *Z. jujuba*. The analysis in condition B revealed a peak specific to *Z. mauritiana* at 11 min. Spinosin (**3**), a marker compound of Jujube Seed defined in the JP16, was detected in each extract (condition C). All samples in Table 1 showed reproducible results in each condition.

We then used high-resolution mass spectrometry to identify the peak specific to *Z. jujuba* to give *m/z* 1251.5957 as a formic acid adduct, indicating a molecular formula, $C_{58}H_{94}O_{26}$, which is identical to that of jujuboside A, a principal compound of *Z. jujuba*. The LC-MS analysis of an authentic sample of jujuboside A revealed the identical retention time and a mass spectrum identical to that of the specific peak (Fig. 2, Fig. S-1). Thus, the compound specific to *Z. jujuba* was identified as jujuboside A. Since spinosin was found to be present in both the seeds from China and those from Myanmar, jujuboside A is better as the marker compound to discriminate *Z. jujuba* from *Z. mauritiana*.

Next, to identify a marker compound that is specific to *Z. mauritiana* and that would be useful for the purity test of Jujube Seed in the JP against *Z. mauritiana*, we started to isolate a specific constituent in *Z. mauritiana* based on our comparison of the LC-MS data of *Z. jujuba* and those of *Z. mauritiana*.

Briefly, a seed of *Z. mauritiana* (100 g) was extracted with methanol at room temperature. Its extract's EtOAc-soluble fraction was repeatedly fractionated by silica gel column chromatography to obtain compound **2** with a pseudo-molecular ion peak, *m/z* 535 $[M + H]^+$. In high-resolution mass spectrometry, compound **2** showed *m/z* 535.3279 $[M + H]^+$, suggesting the molecular formula $C_{31}H_{42}N_4O_4$. As several cyclopeptide alkaloids have already been isolated from *Z. mauritiana* [11], we speculated that the structure of compound **2** was also similar to it. In a nuclear magnetic resonance (NMR) evaluation, compound **2** showed the same spectrum as the frangufoline isolated from Jujube Seed (Fig. 2) [7]. However, in the literature, the chemical shifts of C-1 and C-16 were described as δ_C 123.1 and δ_C 115.5 ppm, respectively (Table 2) [7]. In our present experiments, the ^1H-^1H COSY correlations between the signals at δ_H 6.35 (H-1) and δ_H 6.66 (H-2) and the signals at δ_H 7.12 (H-16) and δ_H 7.03 (H-15), and the HMBC correlations of the signals at δ_H 6.35 (H-1) to the signals at δ_C 131.9 (C-13) and δ_C 130.4 (C-15) were observed, and these data clearly suggested that the signals at 115.6 and 123.2 ppm should be assigned to C-1 and C-16, respectively. Since the other spectra were in accord with the structure of frangufoline, compound **2** was identified as frangufoline.

Generally, TLC analyses are used for the purity testing of crude drugs in the JP16, considering both efficiency and cost economy. Thus, in order to confirm whether compound **2** is suitable as a marker compound for the TLC purity test of *Z. jujuba* against *Z. mauritiana*, we sought to

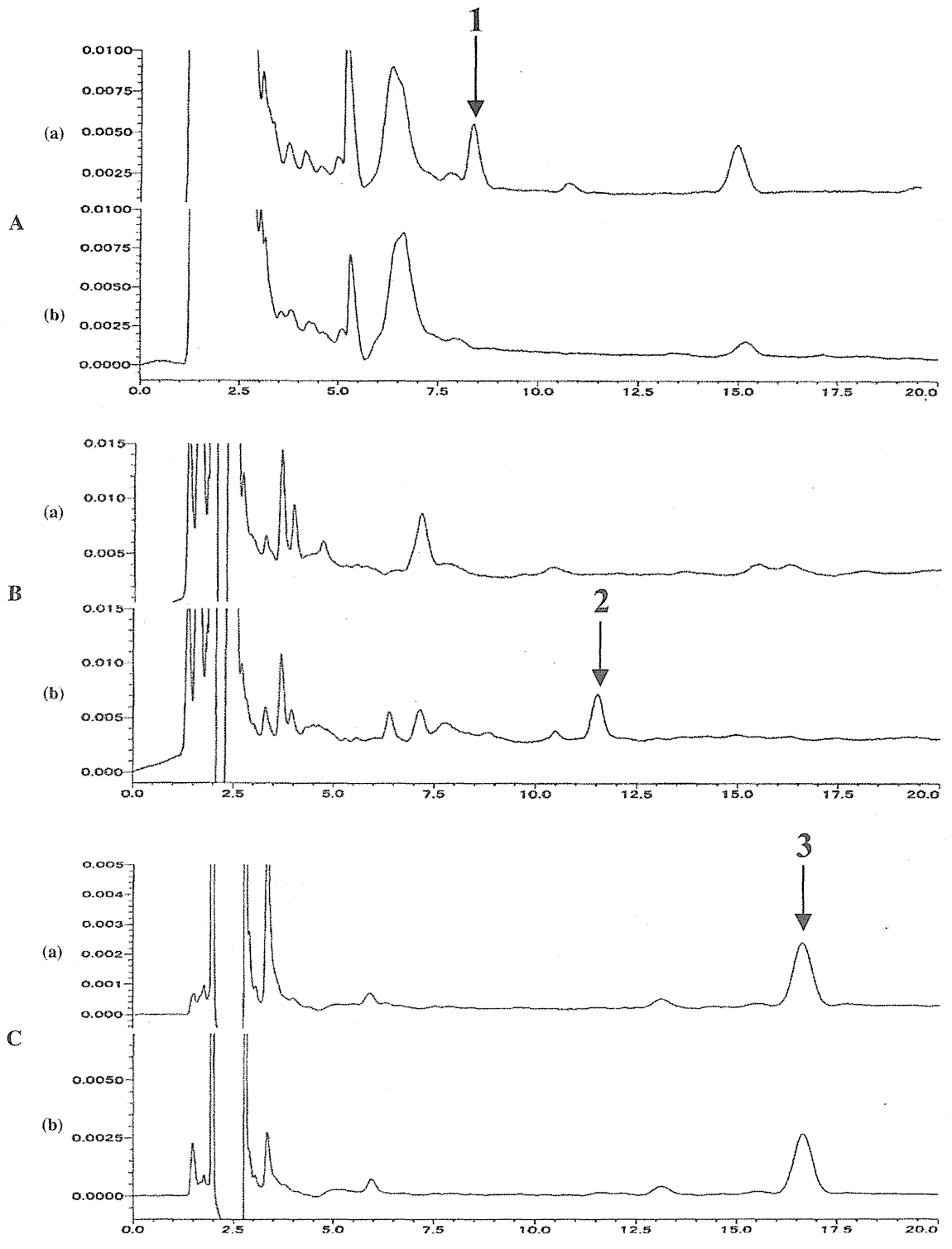

Fig. 1 The LC-PDA-MS chromatograms of jujube seeds from China (*a*) and from Myanmar (*b*) (**A** condition A, **B** condition B, **C** condition C)

determine whether **2** was detectable in the extracts of *Z. mauritiana* by TLC analysis. We found that it was difficult to detect **2** because of the low content of **2** and the lack of sensitive detection reagents. Therefore, we subsequently examined a characteristic spot from each extract on TLC.

When we analyzed each extract on normal-phase TLC using *n*-hexane:EtOAc:HCOOH = 10:5:1 as a developing solvent, a characteristic magenta spot turning to dark purple at R_f 0.43 was found by spraying with diluted sulfuric acid (Fig. 3). Thus, we isolated this compound by the same

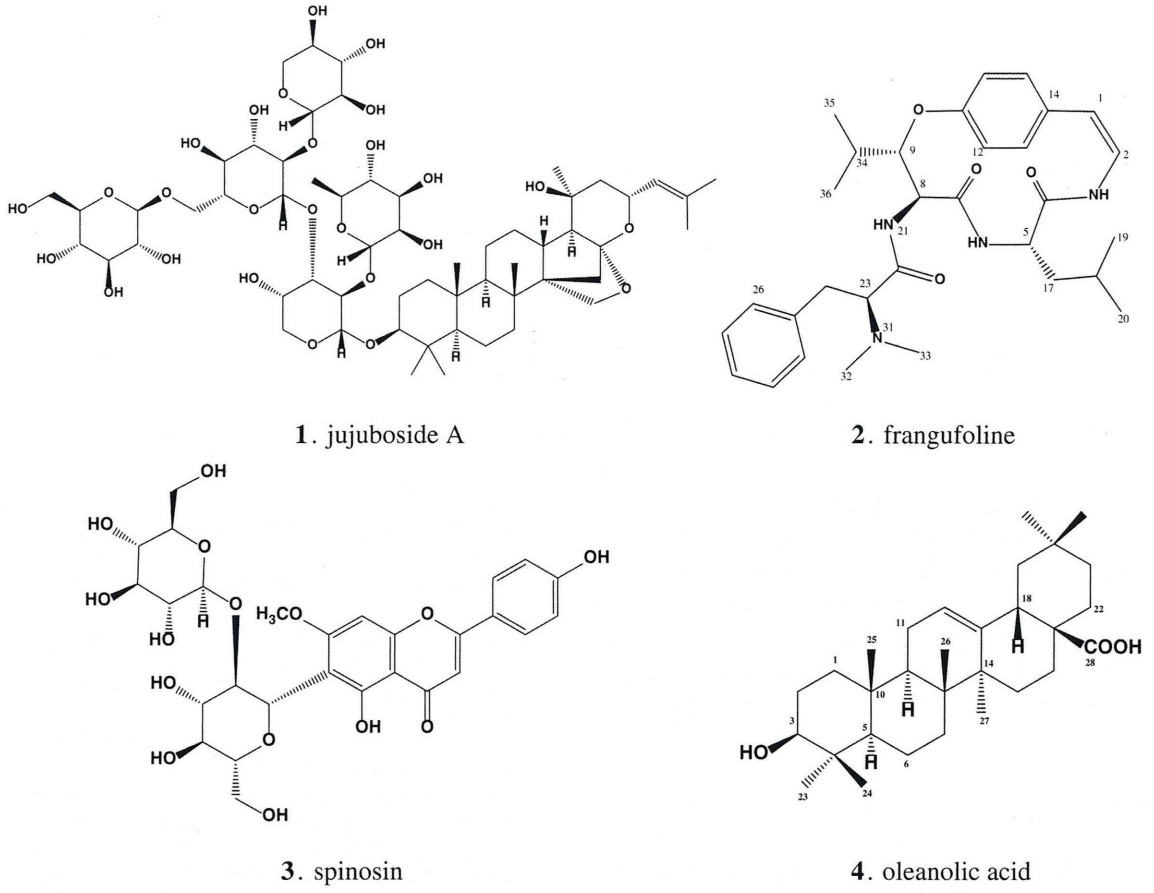

Fig. 2 Structures of compounds *1–4*

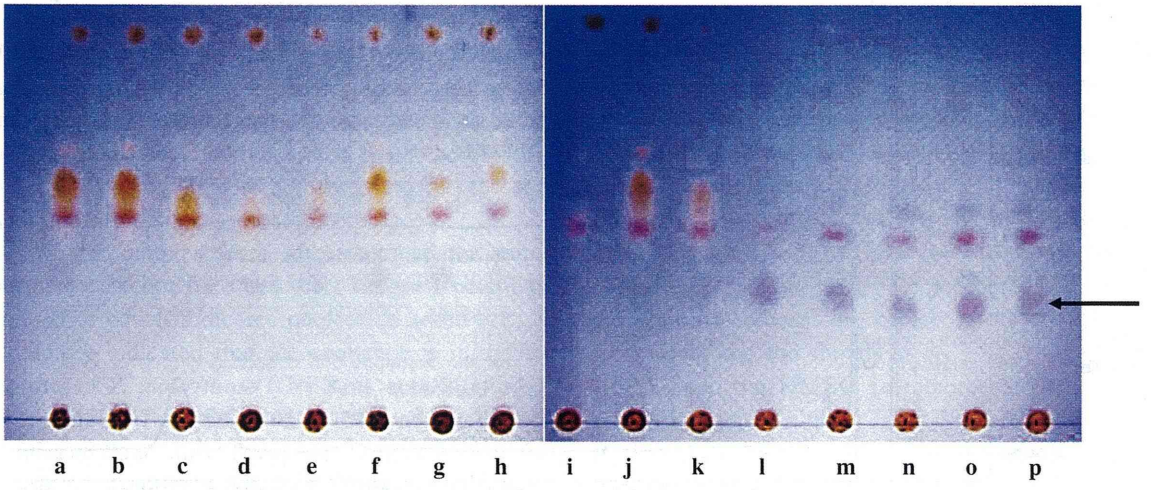

Fig. 3 The TLC analysis of *Z. jujuba* (*a–k*), *Z. mauritiana* (*l–p*). *Lanes a–p* are C-1 to C-11, B-1 to B-5, respectively. Developing solvent, *n*-hexane:EtOAc:HCOOH = 10:5:1; developing length, 7 cm; visualization, diluted sulfuric acid

procedure as that described above to obtain compound **4**. The ^{1}H-NMR spectrum of **4** showed olefin proton (δ_H 5.27 ppm), hydroxyl methine proton (δ_H 3.21 ppm) and seven methyl protons (δ_H 1.12, 1.07, 0.97, 0.92, 0.90, 0.76, 0.74 ppm). The ^{13}C-NMR spectrum displayed 30 carbon signals and carbonyl carbon of carboxylic acid (δ_C 182.9 ppm), suggesting oleanolic acid. The NMR data of **4** were in good agreement with those in the literature and an authentic standard. Therefore, compound **4** was identified as oleanolic acid [8] (Fig. 2).

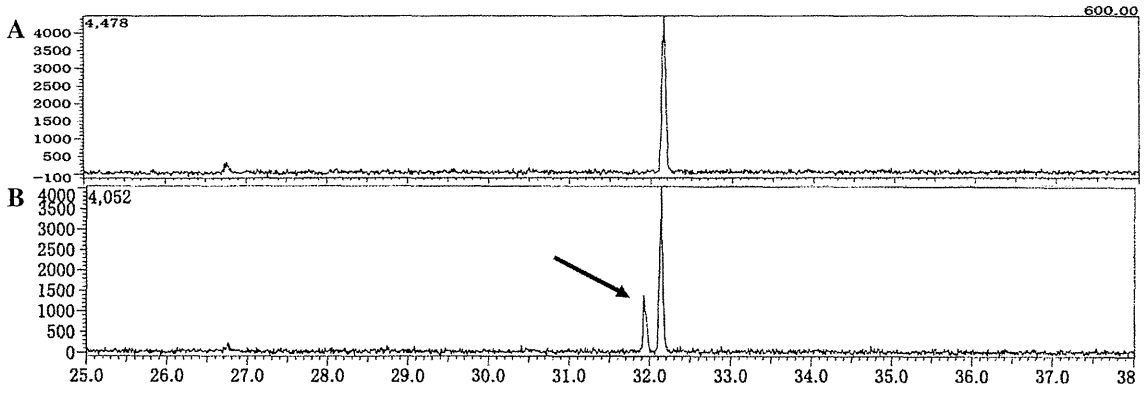

Fig. 4 Mass chromatograms at *m/z* 600 of methanol extracts from *Z. jujuba* (**A**) and *Z. mauritiana* (**B**) on GC-MS analysis

To confirm whether **4** characterizes *Z. mauritiana* against *Z. jujuba*, we analyzed each methanol extract by GC-MS (Fig. 4). A peak for **4** was detected only in the extracts of *Z. mauritiana*, and this peak was not detected in the extracts of *Z. jujuba* (LOD, 2.8 μg, Fig. S-2). It was thus revealed that **4** could be a marker compound in the TLC purity test of Jujube Seed against *Z. mauritiana*.

Conclusions

We searched for a marker compound to be used in the identification test for *Z. jujuba* var. *spinosa* and the purity test against *Z. mauritiana*. We identified jujuboside A (**1**) as a compound specific to *Z. jujuba*, and frangufoline (**2**) and oleanolic acid (**4**) as compounds specific to *Z. mauritiana*. Compound **4** is not a highly specific constituent, in contrast to compound **2**. However, compound **4** in extract showed a characteristic spot by TLC analysis and showed a peak that is specific to *Z. mauritiana* against *Z. jujuba* in the GC-MS chromatogram. Compound **4** can thus be used as a marker compound in the purity test of Jujube Seed against *Z. mauritiana*, although further validation studies are needed to establish the purity test as an official method.

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生薬「肉蓯蓉」と「和肉蓯蓉」の基原植物の成分比較

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Comparison of the Constituents of the Original Plants of Crude Drugs Cistanchis Herba and Boschniakiae Herba

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Cistanche salsa (C. A. Meyer) G. Beck, *Cistanche deserticola* Y. C. Ma and *Cistanche tubulosa* (Schrenk) Wight (*Orobanchaseae*), the sources of a crude drug called Cistanchis Herba (肉蓯蓉), and *Boschniakia rossica* (Cham. et Schltdl.) B. Fedtsch. ex Fedtsch. et Flerov, the source of a crude drug called Boschniakiae Herba (和肉蓯蓉) were analyzed by high performance liquid chromatography (HPLC) systems attached to a photodiode array (PDA) detector and a charged aerosol detector (CAD). Based on the HPLC chromatograms, *Boschniakia rossica* samples were easily distinguished from the *Cistanche* samples. For three *Cistanche* samples, principal component analysis (PCA) was carried out, based on the quantities of the 10 constituents (cistanoside F, echinacoside, cistanoside A, acteoside, tubuloside A, acteoside isomer, syringalide A 3'-α-L-rhamnopyranoside, cistanoside C, 2'-acetylacteoside and tubuloside B) which have pharmacological activities. The samples were not divided according to the plant species.

Keywords: *Cistanche salsa*; *Cistanche deserticola*; *Cistanche tubulosa*; Cistanchis Herba; *Boschniakia rossica*; Boschniakiae Herba; charged aerosol detection; principal component analysis

緒 言

生薬「肉蓯蓉」は, 神農本草経に上品として記載されており, 補腎陽, 益精血, 潤腸通便の効能が知られている[1]. 日本では, 日本漢方生薬製剤協会及び日本生薬連合会により 2007 年に作成された「公定規格に収載されていない生薬の規格及び試験方法」(以下「自主基準」)[2] において, 基原植物としてホンオニク *Cistanche salsa* (C. A. Meyer) G. Beck, *Cistanche deserticola* Y. C. Ma, *Cistanche tubulosa* (Schrenk) Wight 又はオニク *Boschniakia rossica* (Cham. et Schltdl.) B. Fedtsch. ex Fedtsch. et Flerov の 4 種が収載されてきたが, 2012 年に *B. rossica* が生薬「和肉蓯蓉」として日本薬局方外生薬規格 2012[3] に収載され, 同年「自主基準」[4] の肉蓯蓉から除外された. 中国では中華人民共和国薬典[1] において「肉蓯蓉」の基原植物として *C. deserticola* 及び *C. tubulosa* の 2 種が収載されており,

B. rossica は別の生薬「草蓯蓉」とされている[5].

上記の *Cistanche* 属植物 3 種からは，8-epiloganic acid, acteoside，acteoside isomer，2'-acetylacteoside，cistanin，cistanoside A, B, C, D, E, F, echinacoside, tubuloside A, B, C, D 及び syringalide A 3'-α-L-rhamnopyranoside などの成分が報告されており[6-17]，種間に共通の成分の存在がみられているが，含有量およびその組成比率が植物種により異なるとの報告もあり[16]，同一の生薬として扱って良いかを判断するには決め手に欠けるものであった．一方，*B. rossica* からは boschnaloside, rossicaside A, B, C, D, E, F 及び rossicasin A, B[18-21] など，*Cistanche* 属植物とは異なる成分が多く報告されているが，両者を同時に分析して比較した報告はない．

今回，これらを成分面から，より詳しく調査する目的で，多波長検出器に荷電化粒子検出器を追加した HPLC システムを用いて分析し，クロマトグラムの比較を行った．更に，*Cistanche* 属植物 3 種については，その成分面での類似性をより詳しく評価するために薬理活性作用が報告されている 10 成分 (Fig. 1) を定量して統計学的に解析した．これらのうち，acteoside, acteoside isomer, 2'-acetylacteoside, echinacoside, cistanoside A, cistanoside F, tubuloside A, tubuloside B 及び syringalide A 3'-α-L-rhamnopyranoside には抗酸化作用[22]，acteoside, cistanoside A 及び cistanoside C にはストレスによる学習行動や性行動の低下を防御する作用[23]，acteoside, acteoside isomer, 2'-acetylacteoside 及び tubuloside A には肝保護作用[24]，acteoside, acteoside isomer, 2'-acetylacteoside, echinacoside 及び syringalide A 3'-α-L-rhamnopyranoside には抗糖尿病作用[25] が報告されている．

材料及び方法

1. 供試試料及び標準物質

供試試料：*C. deserticola* 17 検体，*C. tubulosa* 7 検体，*C. salsa* 8 検体，*B. rossica* 4 検体の乾燥した茎を用いた (Table 1).

標準物質：定量に用いた標準物質は，養命酒製造株式会社中央研究所で単離，同定したものを用いた (Fig. 1). 同定は，UV, IR, ^{1}H-NMR および ^{13}C-NMR スペクトルにより行った．

2. 試料溶液の調製

18 号ふるいを通過した乾燥粉末試料 0.5 g に，薄めたメタノール (1→2) 50 mL を加え，15 分間振とう抽出した．ろ過後，残渣に対し同様の操作を 2 回行い，3 回分の抽出液を得た．この抽出液を減圧乾固した後，薄めたアセトニトリル (3→25) に溶解し，正確に 10 mL とした．これを，0.45 μm のメンブランフィルターでろ過し，HPLC 用の試料とした．

3. HPLC 装置および条件

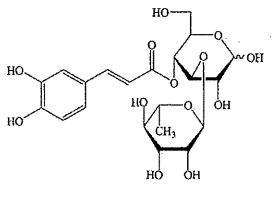
クロマトグラムの比較には，Agilent 1290 Infinity HPLC-CAD システム (Agilent Technologies, Inc.)；検出器：PDA (測定波長 190 ～ 400 nm) 及び CAD (荷電化粒子検出器) Corona ULTRA (Thermo Fisher Scientific, Inc.) を用いた．また，条件は以下に準じた．

定量には，以下の機器および条件を用いた．高速液体クロマトグラフ：SHIMADZU Prominence (Shimadzu Corporation)；ポンプ：LC20AD；検出器：SPD-M20A (PDA)；オートサンプラー：SIL-20AC；カラムオーブン：CTO-20AC；カラム：Inert Sustain ™ C18 / GL Science Inc. 4.6 mm×150 mm I.D., 3 μm；カラム温度：45℃；検出波長：UV 335 nm；流速：1 mL/min；注入量：10 μL；

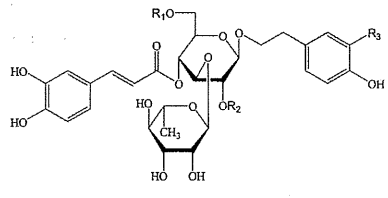
移動相：1 液 アセトニトリル，2 液 1.5%酢酸水溶液，リニアグラジエント法で 1 液の比率は，10 成分の純度測定：5% (0 分) →95% (60 分)，検量線作成および成分定量：5% (0 分) →6% (10 分) →13% (25 分) →14% (50 分) →18% (102 分)．

4. HPLC クロマトグラム及び定量成分による比較

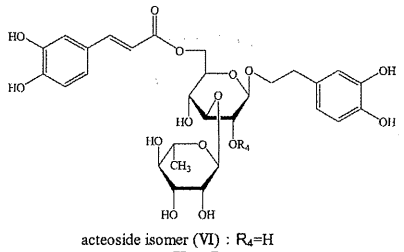
クロマトグラムの比較には *C. salsa*，*C. deserticola*，*C. tubulosa* 及び *B. rossica* の 4 基原種を用い，クロマトグラムのパターンを比較した．



cistanoside F (Ⅰ)



echinacoside (Ⅱ) : R_1=Glc, R_2= H, R_3=OH
cistanoside A (Ⅲ) : R_1=Glc, R_2= H, R_3=OCH_3
acteoside (Ⅳ) : R_1= R_2= H, R_3=OH
tubuloside A (Ⅴ) : R_1=Glc, R_2=Ac, R_3=OH
syringalide A 3'-α-L-rhamnopyranoside (Ⅶ) : R_1= R_2= H, R_3=H
cistanoside C (Ⅷ) : R_1= R_2= H, R_3=OCH_3
2'-acetylacteoside (Ⅸ) : R_1= H, R_2= Ac, R_3=OH



acteoside isomer (Ⅵ) : R_4=H
tubuloside B (Ⅹ) : R_4=Ac

Fig. 1 Chemical structures of 10 constituents

Table 1　Plant materials used for this study

Sample No.	species	place of collection	year collected
1	*Cistanche deserticola* Y. C. Ma	Alxa Left, Inner Mongolia, China	2010
2	*Cistanche deserticola* Y. C. Ma	Alxa Left, Inner Mongolia, China	2010
3	*Cistanche deserticola* Y. C. Ma	Alxa Left, Inner Mongolia, China	2010
4	*Cistanche deserticola* Y. C. Ma	Alxa Left, Inner Mongolia, China	2010
5	*Cistanche deserticola* Y. C. Ma	Alxa Left, Inner Mongolia, China	2010
6	*Cistanche deserticola* Y. C. Ma	Alxa Left, Inner Mongolia, China	2010
7	*Cistanche deserticola* Y. C. Ma	Alxa Right, Inner Mongolia, China	2010
8	*Cistanche deserticola* Y. C. Ma	Alxa Left, Inner Mongolia, China	2010
9	*Cistanche deserticola* Y. C. Ma	Alxa Left, Inner Mongolia, China	2001
10	*Cistanche deserticola* Y. C. Ma	Alxa Left, Inner Mongolia, China	1993
11	*Cistanche deserticola* Y. C. Ma	Ningxia, China	2006
12	*Cistanche deserticola* Y. C. Ma	Inner Mongolia, China	2007
13	*Cistanche deserticola* Y. C. Ma	Inner Mongolia, China	2009
14	*Cistanche deserticola* Y. C. Ma	Bayannur, Inner Mongolia, China	2011
15	*Cistanche deserticola* Y. C. Ma	Inner Mongolia, China	2007
16	*Cistanche deserticola* Y. C. Ma	Inner Mongolia, China	2008
17	*Cistanche deserticola* Y. C. Ma	Inner Mongolia, China	2008
18	*Cistanche tubulosa* R. Wight	Minfeng, Xinjiang Uyghur, China	2002
19	*Cistanche tubulosa* R. Wight	Hotan, Xinjiang Uyghur, China	2001
20	*Cistanche tubulosa* R. Wight	Hotan, Xinjiang Uyghur, China	2010
21	*Cistanche tubulosa* R. Wight	Hotan, Xinjiang Uyghur, China	2010
22	*Cistanche tubulosa* R. Wight	Hotan, Xinjiang Uyghur, China	2010
23	*Cistanche tubulosa* R. Wight	Hotan, Xinjiang Uyghur, China	2010
24	*Cistanche tubulosa* R. Wight	Hotan, Xinjiang Uyghur, China	2010
25	*Cistanche salsa* G. Beck	Ningxia, China	2011
26	*Cistanche salsa* G. Beck	Ningxia, China	2001
27	*Cistanche salsa* G. Beck	Ningxia, China	2011
28	*Cistanche salsa* G. Beck	Ningxia, China	2011
29	*Cistanche salsa* G. Beck	Ningxia, China	2011
30	*Cistanche salsa* G. Beck	Ningxia, China	2011
31	*Cistanche salsa* G. Beck	Ningxia, China	2011
32	*Cistanche salsa* G. Beck	Ningxia, China	2011
33	*Boschniakia rossica* B. Fedtschenko	Yamanashi, Japan	2012
34	*Boschniakia rossica* B. Fedtschenko	Yamanashi, Japan	2012
35	*Boschniakia rossica* B. Fedtschenko	Yamanashi, Japan	2012
36	*Boschniakia rossica* B. Fedtschenko	Yamanashi, Japan	2011

Samples were identified by Prof. Peng Fei Tu (No.1 to 10 and No.18 to 32) or Mr. Nobuyoshi Hanaoka (sample No.11 to 17 and No.33 to 36), respectively.

定量成分による比較には，*C. deserticola*, *C. tubulosa* 及び *C. salsa* の3基原種を用い，方法は多変量解析法の1つである主成分分析 (PCA:Principal Component Analysis) によって比較した．解析ソフトにはエクセル統計 2006 年版 for Windows (Microsoft Corporation) を用いた．

結果及び考察

1. HPLC クロマトグラムの比較

PDA 検出器を用いたクロマトグラムパターン (波長:190 ～ 400 nm)の比較 (Fig. 2) においては，*C. deserticola*, *C. tubulosa* 及び *C. salsa* の試料では，主なピークとして，保持時間28分付近及び38分付近に，同一の吸収スペクトルを示すピークが共通して認められ，*C. deserticola* の試料では，保持時間36分付近に，他の2種に認められないピークが認められた．*B. rossica* の試料では，28分付近及び74分付近に特徴的なピークが認められたが，28分付近のピークは，*C. deserticola*, *C. tubulosa* 及び *C. salsa* の試料から認められるピークとは異なる吸収スペクトルを示した．また，CAD 検出器を用いたクロマトグラムパターンの比較 (Fig. 3) においては，紫外吸収を持たない成分のピークも含め，PDA 検出器で検出される成分以外のピークを認めず，3 種の試料に共通に認められる2つのピーク成分の組成比率は，ほぼ同様のパターンを示した．

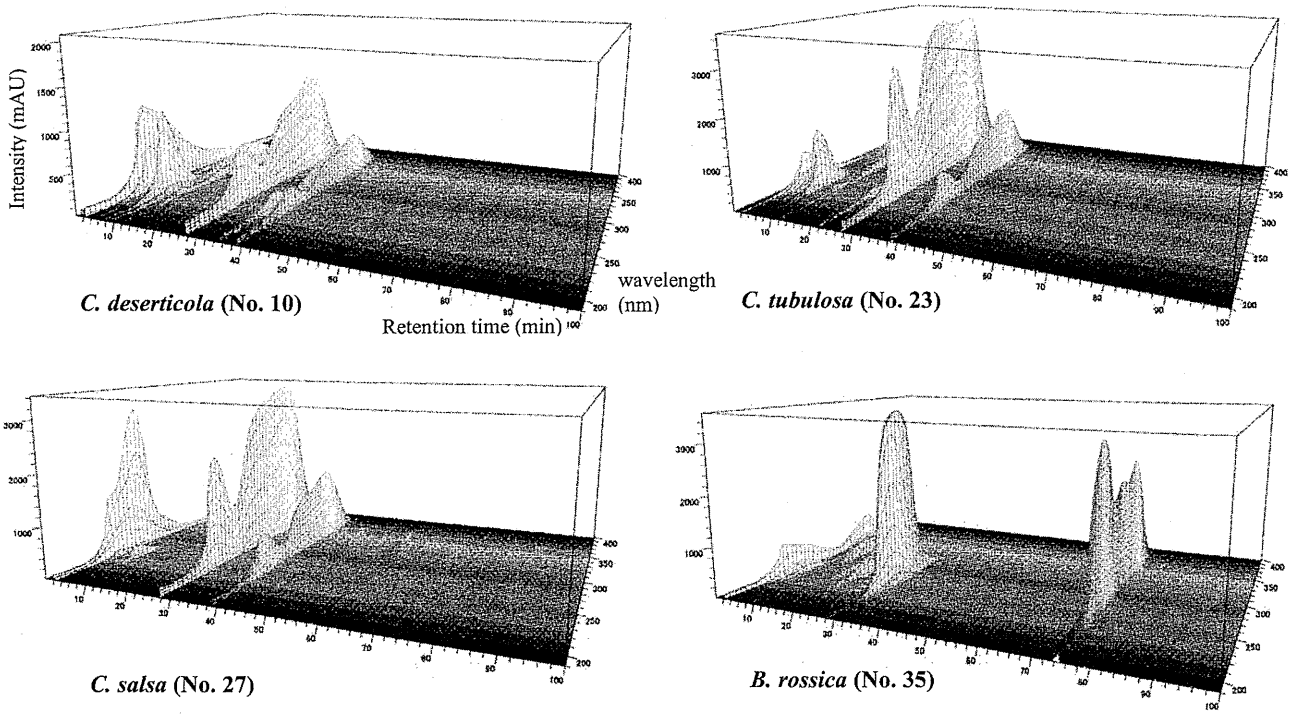


Fig. 2 HPLC-PDA chromatograms for typical *Cistanche deserticola*, *C. tubulosa*, *C. salsa* and *Boschniakia rossica* samples recorded at 190 nm and 400 nm

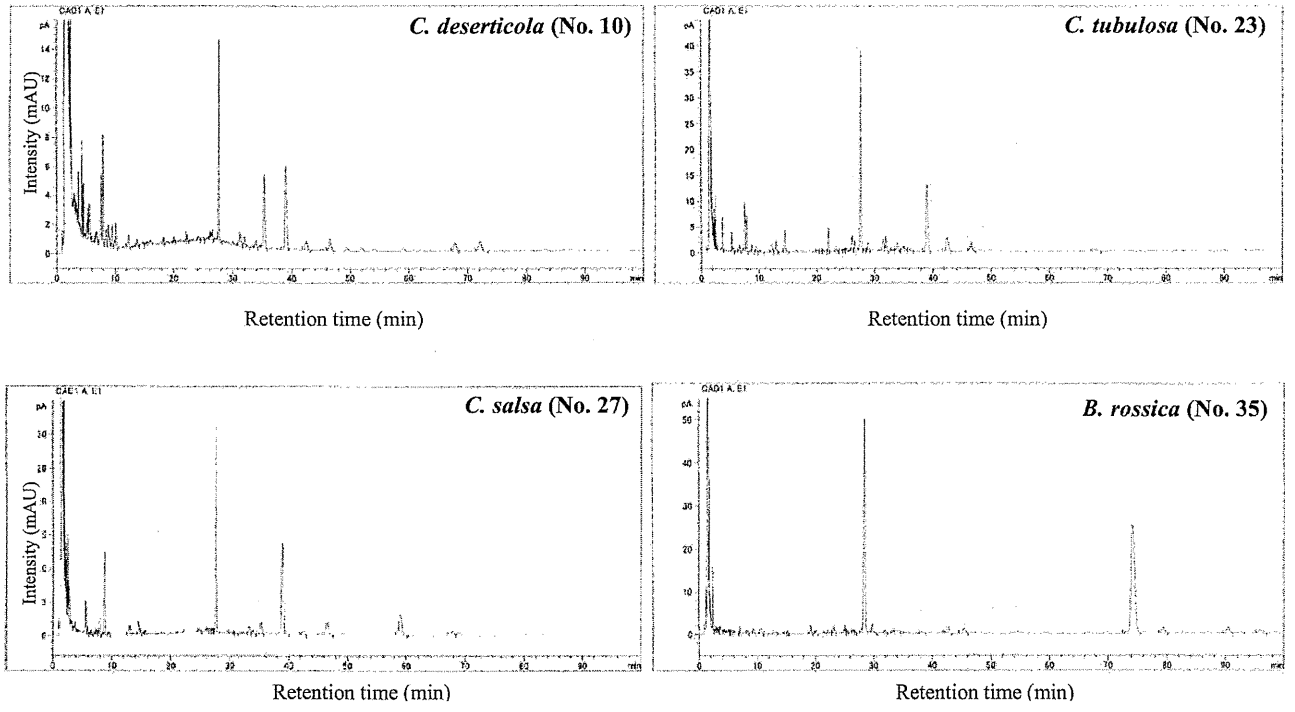


Fig. 3 HPLC-CAD chromatograms for typical *Cistanche deserticola*, *C. tubulosa*, *C. salsa* and *Boschniakia rossica* samples

Table 2 Pharmacologically active constituents contained in *Cistanche* samples

[n = 3 ; μg/g (%)]

Species	Sample No.	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅴ	Ⅵ	Ⅶ	Ⅷ	Ⅸ	Ⅹ	Total
C. deserticola	1	69.4	7080.3	5202.8	70.5	35.0	71.3	23.2	965.8	T	ND	13518.3
		(0.5)	(52.4)	(38.5)	(0.5)	(0.3)	(0.5)	(0.2)	(7.1)	(0.0)	(0.0)	
	2	21.0	3561.4	1608.7	219.6	35.5	58.9	ND	264.9	13.7	ND	5783.7
		(0.4)	(61.6)	(27.8)	(3.8)	(0.6)	(1.0)	(0.0)	(4.6)	(0.2)	(0.0)	
	3	75.8	4333.4	3797.4	219.9	150.1	108.5	33.8	294.9	15.3	T	9029.2
		(0.8)	(48.0)	(42.1)	(2.4)	(1.7)	(1.2)	(0.4)	(3.3)	(0.2)	(0.0)	
	4	111.2	112.6	158.8	64.3	ND	6.7	13.0	31.0	T	ND	497.7
		(22.3)	(22.6)	(31.9)	(12.9)	(0.0)	(1.3)	(2.6)	(6.2)	(0.0)	(0.0)	
	5	87.9	2521.8	496.4	383.8	16.1	79.3	20.2	182.1	19.2	ND	3806.7
		(2.3)	(66.2)	(13.0)	(10.1)	(0.4)	(2.1)	(0.5)	(4.8)	(0.5)	(0.0)	
	6	15.0	858.8	2443.8	ND	ND	21.3	T	109.4	ND	ND	3448.2
		(0.4)	(24.9)	(70.9)	(0.0)	(0.0)	(0.6)	(0.0)	(3.2)	(0.0)	(0.0)	
	7	487.1	458.4	96.5	1628.3	98.9	476.8	408.8	169.3	761.6	313.5	4899.2
		(9.9)	(9.4)	(2.0)	(33.2)	(2.0)	(9.7)	(8.3)	(3.5)	(15.5)	(6.4)	
	8	1041.1	7207.9	5495.3	7976.8	1004.0	1073.4	191.2	842.0	13854.0	2194.7	40880.5
		(2.5)	(17.6)	(13.4)	(19.5)	(2.5)	(2.6)	(0.5)	(2.1)	(33.9)	(5.4)	
	9	218.6	86.3	37.4	472.2	6.2	82.0	15.5	68.1	217.7	30.6	1234.5
		(17.7)	(7.0)	(3.0)	(38.3)	(0.5)	(6.6)	(1.3)	(5.5)	(17.6)	(2.5)	
	10	209.2	3621.9	1959.0	2773.3	163.9	454.6	161.5	74.9	348.3	ND	9766.5
		(2.1)	(37.1)	(20.1)	(28.4)	(1.7)	(4.7)	(1.7)	(0.8)	(3.6)	(0.0)	
	11	730.4	14776.9	5337.4	8079.1	752.3	1051.4	405.0	2021.2	2402.0	492.6	36048.3
		(2.0)	(41.0)	(14.8)	(22.4)	(2.1)	(2.9)	(1.1)	(5.6)	(6.7)	(1.4)	
	12	571.6	1526.1	374.3	16881.0	482.3	4185.7	1735.0	236.1	15331.8	5740.2	47064.0
		(1.2)	(3.2)	(0.8)	(35.9)	(1.0)	(8.9)	(3.7)	(0.5)	(32.6)	(12.2)	
	13	462.5	11345.7	6933.8	4154.0	149.1	384.6	112.2	932.2	227.9	38.3	24740.4
		(1.9)	(45.9)	(28.0)	(16.8)	(0.6)	(1.6)	(0.5)	(3.8)	(0.9)	(0.2)	
	14	248.3	6145.8	6324.0	2421.0	121.0	281.6	221.1	914.3	602.2	74.9	17354.1
		(1.4)	(35.4)	(36.4)	(14.0)	(0.7)	(1.6)	(1.3)	(5.3)	(3.5)	(0.4)	
	15	103.6	10237.8	5334.7	4097.0	976.1	478.3	41.4	630.6	4337.3	501.9	26738.7
		(0.4)	(38.3)	(20.0)	(15.3)	(3.7)	(1.8)	(0.2)	(2.4)	(16.2)	(1.9)	
	16	656.5	915.9	298.4	4191.4	111.0	394.3	338.5	1162.5	5087.8	413.7	13570.1
		(4.8)	(6.7)	(2.2)	(30.9)	(0.8)	(2.9)	(2.5)	(8.6)	(37.5)	(3.0)	
	17	533.6	2488.9	986.7	7857.9	354.1	951.8	928.5	788.4	9296.8	1423.7	25610.4
		(2.1)	(9.7)	(3.9)	(30.7)	(1.4)	(3.7)	(3.6)	(3.1)	(36.3)	(5.6)	
	Mean	331.9	4545.9	2757.9	3617.1	262.1	597.7	273.5	569.9	3089.2	660.2	16705.3
		(4.3)	(31.0)	(21.7)	(18.5)	(1.2)	(3.2)	(1.7)	(4.1)	(12.1)	(2.3)	
C. tubulosa	18	505.8	8305.3	24.8	2177.1	367.4	550.8	168.9	T	4.8	36.1	12141.0
		(4.2)	(68.4)	(0.2)	(17.9)	(3.0)	(4.5)	(1.4)	(0.0)	(0.0)	(0.3)	
	19	179.0	8219.1	41.0	4835.4	244.3	282.5	210.6	ND	71.0	12.7	14095.5
		(1.3)	(58.3)	(0.3)	(34.3)	(1.7)	(2.0)	(1.5)	(0.0)	(0.5)	(0.1)	
	20	464.1	41076.0	286.9	9285.0	ND	1643.5	443.8	159.3	22.7	ND	53381.3
		(0.9)	(76.9)	(0.5)	(17.4)	(0.0)	(3.1)	(0.8)	(0.3)	(0.0)	(0.0)	
	21	548.6	14406.9	74.9	3593.1	730.2	578.6	279.6	T	137.9	62.4	20412.2
		(2.7)	(70.6)	(0.4)	(17.6)	(3.6)	(2.8)	(1.4)	(0.0)	(0.7)	(0.3)	
	22	408.0	8338.4	34.8	6699.4	635.2	1007.8	327.3	ND	265.6	19.0	17735.5
		(2.3)	(47.0)	(0.2)	(37.8)	(3.6)	(5.7)	(1.8)	(0.0)	(1.5)	(0.1)	
	23	133.7	12539.4	70.2	6067.4	982.7	721.7	109.1	T	254.9	63.1	20942.3
		(0.6)	(59.9)	(0.3)	(29.0)	(4.7)	(3.4)	(0.5)	(0.0)	(1.2)	(0.3)	
	24	167.1	3785.1	26.2	2325.3	546.6	235.2	63.3	ND	110.7	7.8	7267.3
		(2.3)	(52.1)	(0.4)	(32.0)	(7.5)	(3.2)	(0.9)	(0.0)	(1.5)	(0.1)	
	Mean	343.8	13810.0	79.9	4997.5	500.9	717.2	228.9	22.8	123.9	28.7	20853.6
		(2.0)	(61.9)	(0.3)	(26.6)	(3.4)	(3.5)	(1.2)	(0.0)	(0.8)	(0.2)	
C. salsa	25	24.5	1451.9	9.7	1312.6	45.6	330.0	ND	ND	121.8	24.3	3320.5
		(0.7)	(43.7)	(0.3)	(39.5)	(1.4)	(9.9)	(0.0)	(0.0)	(3.7)	(0.7)	
	26	119.1	18589.9	751.5	43604.4	271.4	6297.6	409.0	1773.4	667.3	144.9	72628.6
		(0.2)	(25.6)	(1.0)	(60.0)	(0.4)	(8.7)	(0.6)	(2.4)	(0.9)	(0.2)	
	27	64.0	8720.6	591.9	6574.9	214.9	772.4	27.7	1806.0	326.8	23.4	19122.5
		(0.3)	(45.6)	(3.1)	(34.4)	(1.1)	(4.0)	(0.1)	(9.4)	(1.7)	(0.1)	
	28	75.1	2049.8	107.4	3886.8	499.5	318.3	17.2	793.9	6089.1	511.5	14348.6
		(0.5)	(14.3)	(0.7)	(27.1)	(3.5)	(2.2)	(0.1)	(5.5)	(42.4)	(3.6)	
	29	71.0	293.6	8.9	548.6	ND	76.2	ND	141.9	18.7	ND	1158.9
		(6.1)	(25.3)	(0.8)	(47.3)	(0.0)	(6.6)	(0.0)	(12.2)	(1.6)	(0.0)	
	30	14.9	17631.9	1064.2	13460.9	188.9	1716.5	22.3	2977.2	121.6	ND	37198.3
		(0.0)	(47.4)	(2.9)	(36.2)	(0.5)	(4.6)	(0.1)	(8.0)	(0.3)	(0.0)	
	31	26.3	10037.5	141.4	2042.3	709.6	464.9	ND	97.0	182.2	38.2	13739.4
		(0.2)	(73.1)	(1.0)	(14.9)	(5.2)	(3.4)	(0.0)	(0.7)	(1.3)	(0.3)	
	32	206.0	829.4	38.6	3974.7	477.5	386.1	42.4	116.2	13802.4	1752.0	21625.4
		(1.0)	(3.8)	(0.2)	(18.4)	(2.2)	(1.8)	(0.2)	(0.5)	(63.8)	(8.1)	
	Mean	75.1	7450.6	339.2	9425.6	300.9	1295.3	64.8	963.2	2666.2	311.8	22892.8
		(1.1)	(34.9)	(1.3)	(34.7)	(1.8)	(5.2)	(0.1)	(4.9)	(14.5)	(1.6)	

Ⅰ : cistanoside F, Ⅱ : echinacoside, Ⅲ: cistanoside A, Ⅳ: acteoside, Ⅴ: tubuloside A, Ⅵ: acteoside isomer, Ⅶ: syringalide A 3'- α -L-rhamnopyranoside, Ⅷ: cistanoside C, Ⅷ: cistanoside C, Ⅸ: 2'-acetylacteoside, Ⅹ: tubuloside B.

ND: not detected, T: trace, (%): Each 10 constituents content / Toral 10 constitutents content × 100.

これらのことから，*C. deserticola*, *C. tubulosa* 及び *C. salsa* は，主な 2 成分の組成比率も含め，成分的に大きく異なるものではないと判断された．また，*B. rossica* は，これら *Cistanche* 属植物 3 種とは異なり，成分的に大きく異なるものであった．

2. 成分の定量

Cistanche 属植物試料において，成分の含有量及び組成比率は個体差が認められたが，既報 [16]とほぼ同様の傾向が認められた(Table 2)．すなわち，echinacoside (Ⅱ) は *Cistanche* 属植物 3 種に共通して多く，*C. tubulosa* に極めて多い．cistanoside A (Ⅲ)は *C. deserticola* に特徴的に多く，*C. tubulosa* 及び *C. salsa* に少ない．acteoside (Ⅳ)は 3 種に共通して多い．2'-acetylacteoside (Ⅸ)は *C. deserticola* 及び *C. salsa* に多く，*C. tubulosa* に少ない傾向が認められた．また，cistanoside F (Ⅰ) 及び syringalide A 3'-α-L-rhamnopyranoside (Ⅶ) は他の 2 種と比較すると *C. salsa* に少なく，cistanoside C (Ⅷ)及び tubuloside B (Ⅹ)は *C. tubulosa* に少ない傾向が認められた．なお，*Boschniakia* 植物の *B. rossica* からはいずれの成分も検出されなかった (Fig. 4)．

各植物種の echinacoside (Ⅱ)及び acteoside (Ⅳ) の含有量は，3000 μg/g を超える試料が多く存在し，その成分の組成比率は，共通して他の成分より高い傾向にあった．また，cistanoside F (Ⅰ)，tubuloside A (Ⅴ) 及び syringalide A 3'-α-L-rhamnopyranoside (Ⅶ) は，含有量が 1000 μg/g を超える試料はほとんどなく，これらの成分の組成比率は低い傾向にあった．acteoside isomer (Ⅵ)，cistanoside C (Ⅷ)及び tubuloside B (Ⅹ)は，いくつかの試料で，含有量が 1000 μg/g を超えるものが存在したが，成分の組成比率は低い傾向にあった．

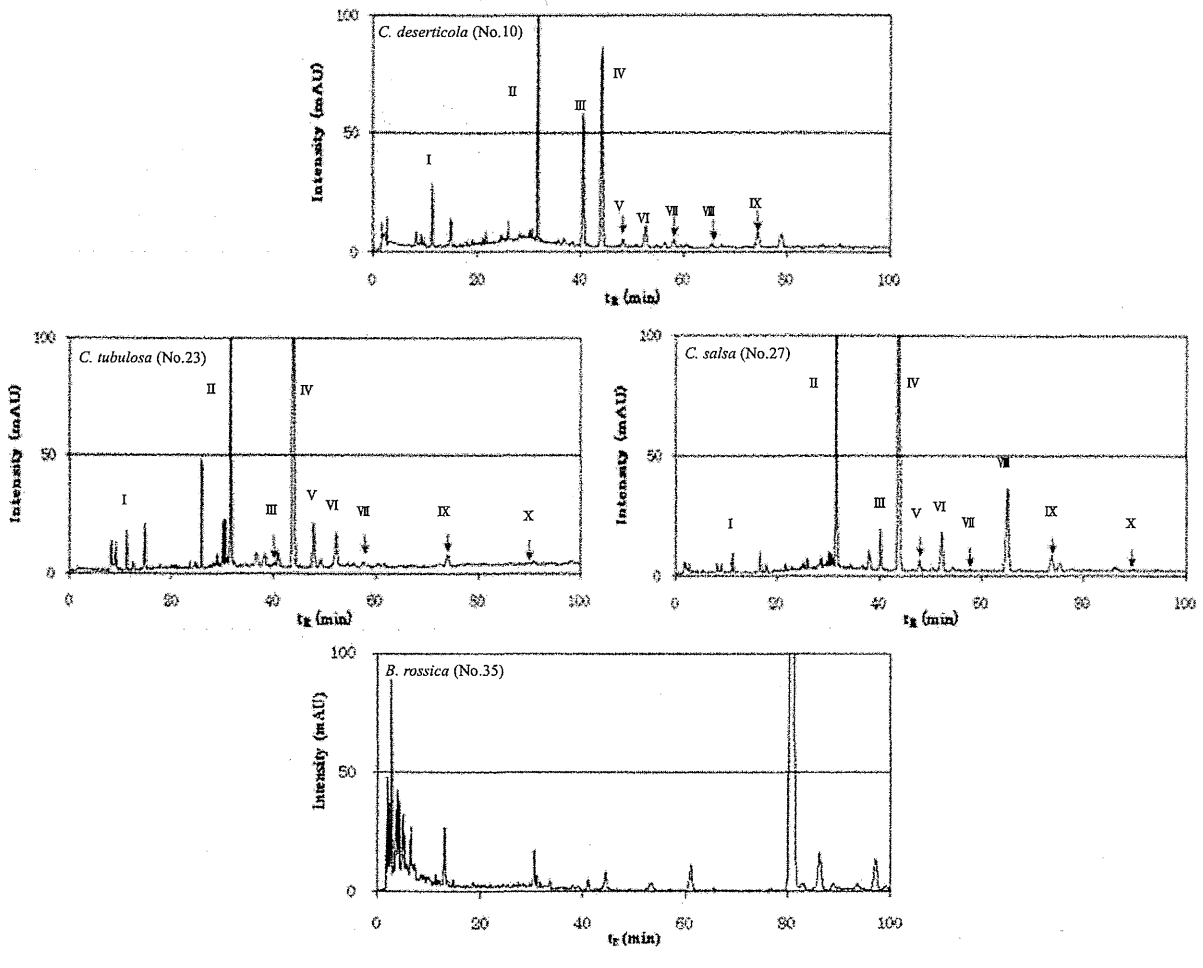


Fig. 4 HPLC chromatograms for typical *Cistanche deserticola*, *C. tubulosa*, *C. salsa* and *Boschniakia rossica* samples recorded at 335 nm

Table 3 Eigenvectors and eigenvalues from the data of HPLC analyses for *Cistanche* samples

Variable	Z1	Z2	Z3	Z4	Z5	Z6
cistanoside F (Ⅰ)	0.315	-0.186	0.345	0.231	-0.493	0.459
echinacoside (Ⅱ)	0.112	0.476	0.162	0.492	-0.322	-0.123
cistanoside A (Ⅲ)	0.031	0.052	0.693	-0.358	-0.121	-0.589
acteoside (Ⅳ)	0.362	0.429	-0.188	-0.034	0.179	-0.062
tubuloside A (Ⅴ)	0.223	-0.109	0.353	0.560	0.610	-0.048
acteoside isomer (Ⅵ)	0.405	0.341	-0.261	-0.039	0.075	-0.204
syringalide A 3'-α-L-rhamnopyranoside (Ⅶ)	0.433	-0.133	-0.201	-0.037	-0.381	-0.128
cistanoside C (Ⅷ)	0.156	0.421	0.302	-0.458	0.175	0.560
2'-acetylacteoside (Ⅸ)	0.384	-0.357	0.073	-0.142	0.231	0.129
tubuloside B (Ⅹ)	0.428	-0.320	-0.097	-0.168	0.030	-0.183
Eigenvalues	3.675	2.103	1.442	0.972	0.740	0.449
Cumulative contribution ratio	36.7%	57.8%	72.2%	81.9%	89.3%	93.8%

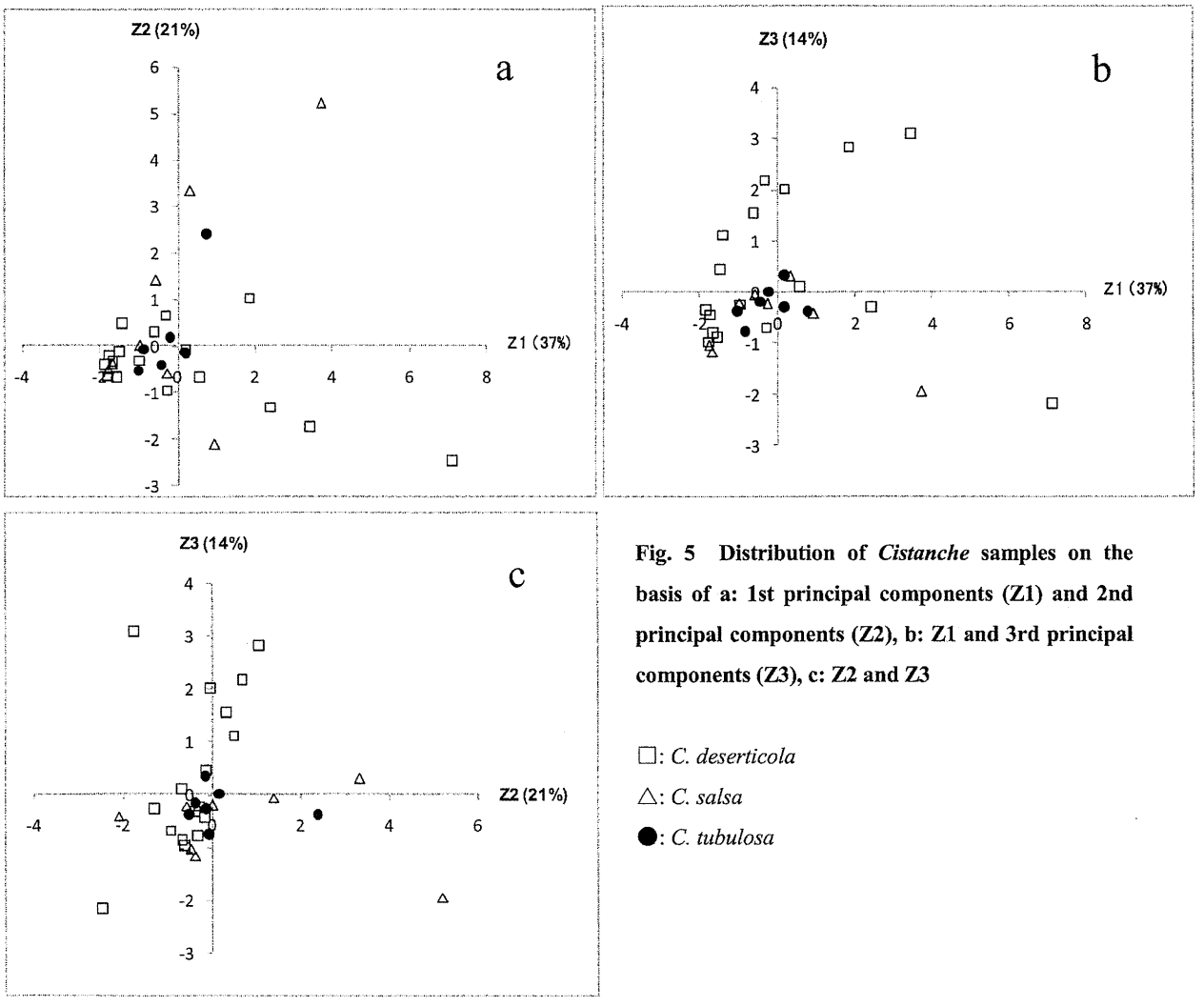


Fig. 5 Distribution of *Cistanche* samples on the basis of a: 1st principal components (Z1) and 2nd principal components (Z2), b: Z1 and 3rd principal components (Z3), c: Z2 and Z3

□: *C. deserticola*

△: *C. salsa*

●: *C. tubulosa*

3. 主成分分析(PCA)

PCAの第1主成分 (Z1) から第6主成分 (Z6) までの固有ベクトル, 固有値および累積寄与率をTable 3に示した.

固有値1以上の主成分はZ1からZ3までであり, Z3までの累積寄与率からZ1～Z3で全体の72.2%が説明できると判断した. Z1～Z3の主成分得点に基づく散布図をFig. 5に示した. 試料は植物種別に不連続なクラスターを作ることなく分布しており, 今回測定した10成分では基原種を識別することはできないと考えられる.

種別にプロットの分布を比較すると, *C. tubulosa* は狭い範囲に集まり, *C. deserticola* 及び *C. salsa* は広い範囲に分布していた. 主成分別にみると, Z1軸からなる散布図 (Fig. 5a及びb) では, No.8, 12 (いずれも *C. deserticola*), 及び26 (*C. salsa*)が正の方向に離れて分布する形となった. これは, Z1の固有ベクトルが全て正の値であるため全ての成分含有量と正の相関があり, このうち syringalide A 3'-α-L-rhamnopyranoside (Ⅶ), tubuloside B (Ⅹ) 及びacteoside isomer (Ⅵ) の影響が大きいことを反映したものである. Z2軸からなる散布図 (Fig. 5a及び5c)では, No.26, 30 (いずれも *C. salsa*) 及び20 (*C. tubulosa*) が正の方向に離れて分布する形となった. これは, Z2の固有ベクトルが正の方向に大きく影響している echinacoside (Ⅱ), acteoside (Ⅳ), 及びcistanoside C (Ⅷ) と, 負の方向に大きく影響している2'-acetylacteoside (Ⅸ) とtubuloside B (Ⅹ) のバランスを反映したものである. すなわち, No.26は正の方向に影響の大きい3成分を多く含有し, No.30はこれらの3成分を多く含有しつつtubuloside B (Ⅹ) 及びtubuloside F (Ⅰ) が少なく, No.20 は tubuloside A (Ⅴ), 2'-acetylacteoside (Ⅸ)及びtubuloside B (Ⅹ)が少なかったためである. Z3軸からなる散布図 (Fig. 5b及びc) では, No.8, 11, 13, 14及び15 (全て *C. deserticola*) が正の方向に分布し, 逆にNo.12 (*C. deserticola*) 及び26 (*C. salsa*) は負の方向に離れて分布する形となった. これは, Z3の固有ベクトルからcistanoside A (Ⅲ)の影響が正の方向に大きく, 負の値である acteoside (Ⅳ), acteoside isomer (Ⅵ), syringalide A 3'-α-L-rhamnopyranoside (Ⅶ) およびtubuloside B (Ⅹ) の影響(絶対値)が小さいためであり, *C. deserticola* がcistanoside A (Ⅲ) を多く含有するという特徴を反映したものである. ただし, No.12 はcistanoside A (Ⅲ)が少なく acteoside (Ⅳ), acteoside isomer (Ⅵ), syringalide A 3'-α-L-rhamnopyranoside (Ⅶ)及びtubuloside B (Ⅹ) を多く含有していたため, 例外的な存在となった.

結論

今回の多波長検出器 (190 ～ 400 nm) 及び荷電化粒子検出器によるHPLCクロマトグラムは, *Cistanche* 属植物3種 (*C. deserticola*, *C. salsa* 及び *C. tubulosa*) は類似しており, *B. rossica* はこれらと異なっていた. また, 本試験において定量した *Cistanche* 属植物から報告されている薬理活性成分も, *B. rossica* からは検出されなかった. よって, *B. rossica* は肉蓯蓉とは別の生薬として扱うのが妥当と考える.

一方, 今回調査した *Cistanche* 属植物3種の試料からは, 守屋らの報告[16, 26]と同様に含有量及びその比率に種特有のパターンは認められるものの, 3種のすべてから10成分の生理活性成分が検出され, これに基づくPCAにおいても, 種間の境界は認められなかった. したがって, 同じ生薬として扱って問題ないと考えられる.

なお, *C. deserticola* 及び *C. salsa* の成分含有量及びその比率が *C. tubulosa* より変異が大きかったが, これは守屋らの報告[26]にあるように, 今回調査した試料においても *C. deserticola* 及び *C. salsa* は, 栽培管理が進んでいる *C. tubulosa* に比べて乾燥処理が様々で, 乾燥方法により, 硬いものや弾力性に富むものがあり, この2基原種では, 成分の変化による影響が, より大きく反映したのではないかと推察される.

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