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Characterization and simultaneous quantification of seven triterpenoid saponins in different parts of *Xanthoceras sorbifolia* Bunge by HPLC-ESI-TOF

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In order to find new candidates against Alzheimer's disease, two new triterpenoid saponins, sorbifoside C (**1**) and D (**4**), and five known triterpenoid saponins (**2**, **3**, and **5–7**) were isolated and identified from the husks of *Xanthoceras sorbifolia* Bunge. Their structures were elucidated through 1D, 2D NMR and HR-MS. A fast, sensitive and reliable high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS) method has been developed and validated to simultaneously quantitate the content of seven triterpenoid saponins in different parts of *X. sorbifolia*. The quantification was accomplished on a triple quadrupole tandem mass spectrometer in the selected ion monitoring and positive ionization modes. Good chromatographic separation was achieved by gradient elution with the mobile phase consisting of acetonitrile and 0.05% formic acid in water during a total run time of 20 min. All of the seven compounds showed good linearity (r^2 > 0.998) in relatively wide concentration ranges. Satisfactory precisions (evaluated by intra- and inter-day tests) and recoveries/accuracies (98.0% to 102.0%) were obtained with RSD values less than 3.0%. The method was successfully adapted for simultaneous quantification of the seven analytes in different parts of *X. sorbifolia*. The results showed that triterpenoid saponin contents from different parts of the plant varied significantly and a reference for the choice of medicinal parts was accordingly provided.

Introduction

Xanthoceras sorbifolia Bunge (Sapindaceae) is an indigenous shrub distributed in Inner Mongolia, Liaoning, Hebei and Shanxi Provinces in China.[1] It has been used as a folk medicine for excellent treatment of rheumatism and enuresis in children.[2,3] The chemical constituents of *X. sorbifolia* are triterpenoid saponins, flavonoids, sterols and so on. Among them, triterpenoid saponins, rich in the husks of the plant, are its characteristic ingredients and responsible for its bioactivities, such as anti-inflammatory, anti-HIV, and antitumor activities, especially the function of improving intelligence.[4–6]

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disease characterized by memory loss and the decline of cognitive functions. It is among the most prevalent forms of dementia affecting the aging population, and pharmacological therapies to date have not been successful in preventing disease progression.[7,8] In order to find new candidates against AD, we have all long focused our interest on *X. sorbifolia*. Previous phytochemical studies revealed that the crude extract of the husks from *X. sorbifolia* could significantly ameliorate the impairment of learning and memory in several Alzheimer's disease animal models.[9,10] Moreover, Xanthoceraside and Xanifolia O54, triterpenoid saponin monomers isolated from the husks of the plant, exhibited neuroprotection of hippocampus nerve cells in both *in vitro* and *in vivo* animal experiments.[11,12] Taking previous findings into consideration, it is plausible that triterpenoid saponins from the husks play a key role in improving the cognitive performance of several Alzheimer's disease animal models. Therefore, the exploitation of the husks for pharmaceutical applications will no doubt widen the full use of the natural resources.

However, triterpenoid saponins have a high structural similarity and complexity, which result in comparable difficulties for their effective separation and complete identification. Thus information about triterpenoid saponin monomers against AD is still very limited with the exception of Xanthoceraside and Xanifolia O54, and there are only a few studies on the topic. With the aim of finding new candidates against AD, we isolated and identified seven angeloyl (Ang)-substituted triterpenoid saponins (**1–7**), two of which are new (**1** and **4**). The two new ones were named as sorbifoside C (**1**) and D (**4**), respectively. Then the structures of compounds **1–7** were identified by extensive spectroscopic analysis (1D and 2D

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NMR, and HR-MS). Comparing their structures with those of Xanthoceraside and Xanifolia O54 (Fig. 1), we found that they have similar triterpenoid skeletons, sugar moieties and functional groups. Since triterpenoid saponins are the major bioactive constituents of *X. sorbifolia*, the triterpenoid saponin monomers which we isolated from the husks have potential

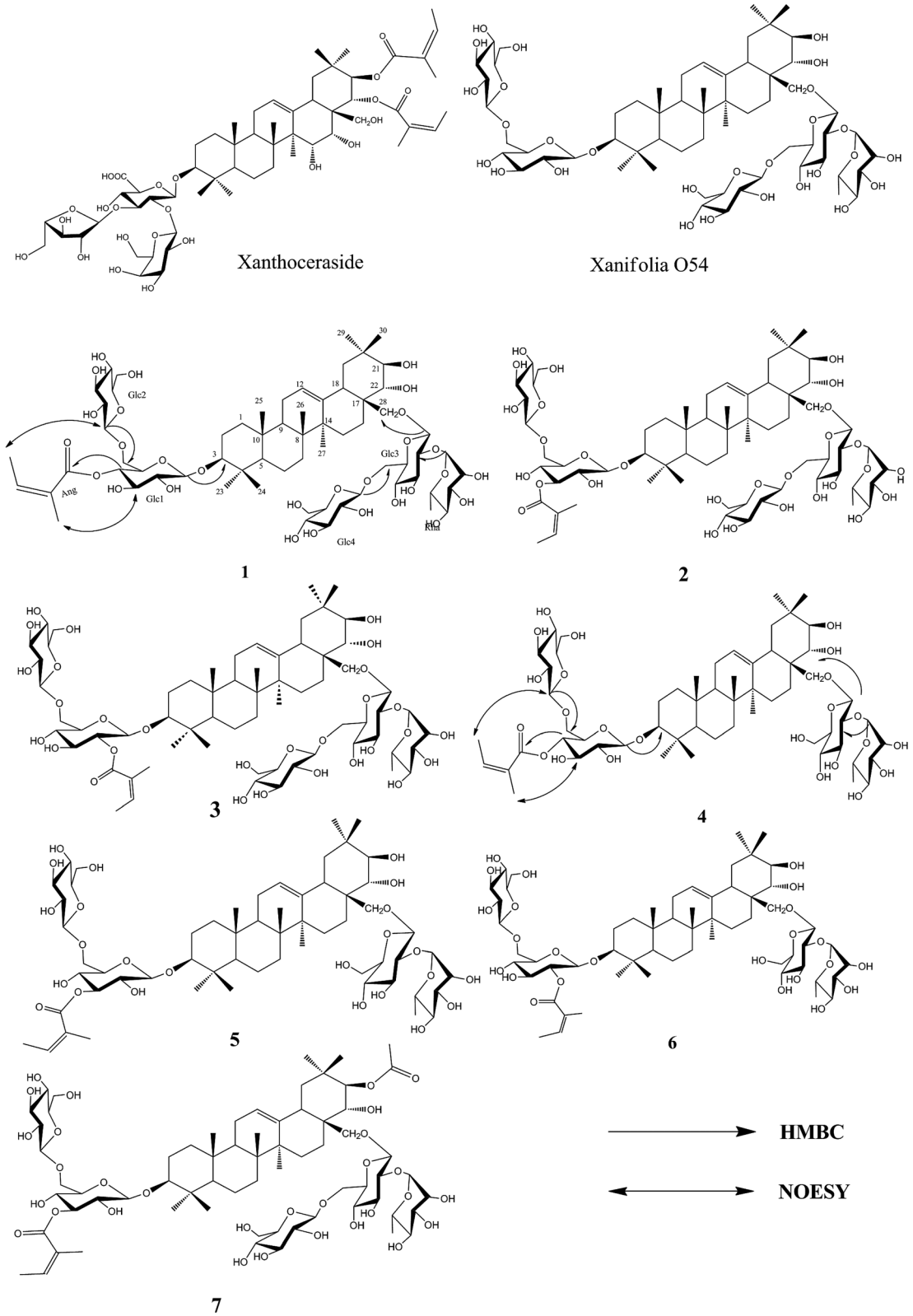

Fig. 1 Structures of compounds **1–7**, Xanthoceraside and Xanifolia O54 and key 2D NMR correlations of the new compounds **1** and **4**.

research value as new candidates for the prevention and treatment of AD.

Analytical methods for investigating triterpenoid saponins are mainly based on chromatographic separation using high-performance liquid chromatography (HPLC) and then detection using traditional UV, ELSD or mass spectrometry (MS).[13–16] Compared with the traditional HPLC method, an HPLC-ESI-MS method has advantages including high sensitivity, short analysis time and low consumption of samples. Due to its high sensitivity, the pre-treatment of the samples becomes very simple (no concentration or enrichment). Therefore, an HPLC-ESI-MS method was established and validated to simultaneously determine the contents of seven triterpenoid saponins in different parts of the plant. Although an HPLC-ESI-MS method had been established for the determination of triterpenoids in different parts of *X. sorbifolia* previously[17], the method we established was more time-saving and determined more compounds. The method was developed to explain the distribution of triterpenoid saponins in different parts of the plant and a reference for the choice of medicinal parts was thus provided.

Experimental

Chemicals

Methanol, acetonitrile and formic acid (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and deionized water was filtered through a 0.22 μm filter before use. Other reagents and solvents were of analytical grade from Shandong Yu Wang Chemical Reagent Factory (Shandong, China).

Plant materials and preparation of triterpenoid saponin analytes

Different parts of *X. sorbifolia* including husks, twig bark, twig xylem, seed coats, seed kernels, flowers and leaves for phytochemical studies were obtained from Chifeng City, Inner Mongolia, China, which were identified by Professor Ying Jia (Department of Traditional Chinese Medicine, Shenyang Pharmaceutical University, Shenyang, China).

Dried husks (7.5 kg) of *X. sorbifolia* were powdered and further extracted three times with 70% ethanol under reflux for 2 h. The ethanol extract was concentrated under a reduced pressure, and then loaded on a macroporous resin column for gradient elution with ethanol/water to give fraction 1 [water], fraction 2 [ethanol : water (3 : 7)], fraction 3 [ethanol : water (7 : 3)] and fraction 4 [ethanol : water (9.5 : 0.5)]. Fraction 3 (~100 g) was subjected to column chromatography on silica gel for gradient elution with dichloromethane/methanol repeatedly to give subfraction C [dichloromethane : methanol (100 : 20)] and D [dichloromethane : methanol (100 : 30)]. Then subfraction C was purified on a RP-HPLC using an ODS-A YMC column (10.0 × 250 mm, flow rate 4 mL min^{-1}) with acetonitrile/water (30 : 70) to afford compound **4** (15.6 mg, purities = 90.5%), compound **5** (22.4 mg, purities = 90.3%), compound **6** (17.6 mg, purities = 98.6%) and compound **7** (17.2 mg,

Table 1 NMR data assignments for compounds **1** and **4**[a]

	1		**4**	
Position	δ_H	δ_C	δ_H	δ_C
1	1.04/1.62 m	38.8	0.99/1.52 m	39.6
2	1.88/2.38 m	26.6	1.83/2.35 m	25.8
3	3.38 dd (11.3,4.6)	89.3	3.24 dd (11.6,4.8)	89.4
4	—	39.4	—	39.6
5	0.79 d (11.4)	55.7	0.68 d (11.6)	55.7
6	1.40/1.30 m	18.2	1.46/1.31 m	18.7
7	1.29 m	32.7	1.27 m	32.8
8	—	40.2	—	40.3
9	1.59 dd (11.2,7.0)	47.9	1.58 dd (11.4,6.6)	47.9
10	—	36.8	—	36.9
11	1.94/1.88 m	23.9	1.98/1.84 m	24.1
12	5.33 br.s	123.5	5.40 br.s	123.8
13	—	142.9	—	143.1
14	—	42.0	—	41.9
15	1.87/1.20 m	25.8	1.73/1.10 m	26.3
16	2.32/1.94 m	18.5	2.17/1.98 m	18.5
17	—	43.2	—	43.2
18	2.62 dd (13.8,3.1)	41.7	2.80 dd (13.2,2.9)	41.3
19	2.10/1.28 m	46.4	2.11/1.31 m	46.5
20	—	36.2	—	36.4
21	3.75 d (10.2)	76.8	3.74 d (10.2)	76.9
22	4.28 m	75.1	4.32 m	74.8
23	1.27 s	28.2	1.16 s	28.2
24	1.06 s	17.0	1.06 s	17.0
25	0.93 s	15.9	0.92 s	15.8
26	0.97 s	16.8	0.99 s	16.8
27	1.26 s	26.2	1.23 s	26.6
28	4.10 br.s	75.8	4.06 br.s	75.2
29	1.22 s	30.4	1.24 s	30.5
30	1.27 s	19.6	1.20 s	19.7
Glc1 1′	4.91 d (7.7)	106.6	4.91 d (8.4)	106.8
2′	3.99 m	75.1	4.28 m	75.2
3′	4.08 m	75.1	4.10 m	75.0
4′	5.57 (t, 9.6)	72.2	5.57 (t, 9.6)	72.4
5′	4.35 m	74.6	4.18 m	74.7
6′	4.81/4.35 m	69.4	4.88/4.15 m	69.6
Glc2 1″	5.02 d (8.2)	104.9	5.01 d (7.8)	105.0
2″	4.02 m	75.1	4.05 m	75.3
3″	4.19 m	78.2	4.22 m	78.5
4″	4.21 m	71.7	4.21 m	71.9
5″	3.91 m	78.3	3.94 m	78.5
6″	4.50/4.33 m	62.5	4.52/4.35 m	62.7
Glc3 1‴	4.71 d (7.5)	103.6	4.74 d (7.9)	103.7
2‴	4.23 m	75.7	4.33 m	75.9
3‴	4.22 m	79.9	4.35 m	80.3
4‴	4.22 m	71.5	4.20 m	71.6
5‴	3.95 d (9.6)	76.6	3.79 m	78.4
6‴	4.68/4.37 dd (13.2,3.0)	70.1	4.40 m	62.7
Rha 1⁗	6.52 br.s	100.8	6.61 br.s	100.6
2⁗	4.72 m	72.4	4.71 m	72.6
3⁗	4.69 m	72.4	4.67 m	72.4
4⁗	4.25 m	74.2	4.33 m	74.4
5⁗	4.81 m	69.2	4.45 m	69.1
6⁗	1.81 d (6.0)	18.9	1.85 d (6.0)	18.5
Glc4 1⁗′	5.01 d (7.8)	105.6	—	—
2⁗′	4.02 m	75.5	—	—
3⁗′	4.19 m	78.3	—	—
4⁗′	4.22 m	71.6	—	—
5⁗′	3.91 m	78.3	—	—
6⁗′	4.44/4.33 m	62.7	—	—
Ang 1	—	167.7	—	167.8

Table 1 (*Contd.*)

	1		**4**	
Position	δ_H	δ_C	δ_H	δ_C
2	—	128.3	—	128.4
3	5.87 dq (7.2,1.4)	137.8	6.00 dq (7.2,1.2)	137.9
4	1.99 dd (7.2,1.4)	15.9	2.01 dd (7.2,1.2)	16.0
5	1.87 d (1.2)	20.6	1.89 t (1.4)	20.7

[a] ^{1}H and ^{13}C NMR were measured in pyridine-*d*5 at 600 and 125 MHz, respectively. Multiplicities are indicated by usual symbols. Coupling constants (Hz) are in parentheses.

purities = 91.2%). In the same way, subfraction D was purified and eluted with acetonitrile/water (28 : 72) to afford compound **1** (13.3 mg, purities = 98.5%), compound **2** (30.8 mg, purities = 95.5%), and compound **3** (18.5 mg, purities = 96.5%) by RP-HPLC.

Structure elucidation

1D and 2D NMR spectra including ^{1}H-NMR, ^{13}C-NMR, HSQC, HMBC, ^{1}H–^{1}H COSY, NOESY and TOCSY were acquired on a Bruker Advance 600 NMR spectrometer. A Bruker LTQ-FTICR-MS spectrometer was used for high resolution in mass experiments. Thus, structure elucidation was based on interpretation of NMR and mass spectroscopic data.

HPLC-ESI-MS analyses

Liquid chromatography separation was performed on an XR LC-20AD Prominence™ HPLC system equipped with a binary pump, a degasser, an autosampler and a thermostatted column compartment (Shimadzu, Japan). A Phenomenex C18 column (250 mm × 4.6 mm, 5.0 μm) (Phenomenex, USA) protected by a high pressure column pre-filter (2 μm) (Shimadzu, Japan) was held at 30 °C. Chromatographic separation was achieved by gradient elution using a mobile phase consisting of 0.05% formic acid in water (A) and acetonitrile (B). The HPLC gradient program is as follows: 32% B → 33% B at 0.01–6.00 min; 33% B → 40% B at 6.01–15.00 min; 40% B → 32% B at 15.01–16.00 min; 32% B at 16.01–20.00 min. Efficient and symmetrical peaks were obtained at a flow rate of 1.0 mL min^{-1} with 30% of the eluent being split into the inlet of the mass spectrometer. All the samples were kept at 4 °C in an autosampler tray and the injection volume was 5 μL.

Mass spectrometric detection was carried out on a QTRAP™ 4000 MS system from AB Sciex equipped with a TurboIonSpray source (Foster City, CA, USA). All the operations, the acquiring and analysis of data were controlled with Analyst (version 1.5.2, AB Sciex, USA). An ESI source was operated in the positive ionization mode, and spectra were acquired in the selected ion monitoring (SIM) mode. The ion spray voltage was set at 5500 V for positive mode; the curtain gas, gas 1 and gas 2 (using: nitrogen) were set at 20, 50 and 50 at a source temperature of 500 °C.

Target ions were monitored $[M + 2Na]^{2+}$ at *m*/*z* 698.60 for compounds **1–3**, $[M + 2Na]^{2+}$ at *m*/*z* 617.30 for compounds **4–6**, $[M + 2Na]^{2+}$ at *m*/*z* 719.30 for compound **7** and $[M + Na]^+$ at *m*/*z* 803.45 for compound IS (digoxin) using the selected ion monitoring (SIM) mode.

Calibration and quantification

The mixed standard stock solutions of compounds **1–7** were prepared in methanol at concentrations of 6.264, 90.9, 15.59, 14.36, 7.268, 6.454, and 5.884 μg mL^{-1}, respectively. Then the mixed standard stock solutions were further diluted with methanol to prepare a series of mixed working solutions for the establishment of calibration curves. IS (Digoxin) was diluted to a concentration of 0.5000 μg mL^{-1} with methanol as the working solution. All solutions were stored at 4 °C before use. Dried plant samples (husks, twig bark, twig xylem, seed coats, seed kernels, flowers, and leaves) were ground with a home blender, and then about 0.5 g of the powdered samples was extracted with 10 mL of methanol, respectively. The samples were put in an ultrasonic bath for 30 min (250 W, 40 kHz) at 30 °C and methanol was used to compensate the lost weight of extracted solutions. The extracted solution was filtered through a 0.22 μm filter before injection.

Results and discussion

Structure elucidation

All the known compounds (**2–3** and **5–7**) were identified by MS, 1D and/or 2D NMR experiments and compared with the published data.[18–23]

Table 2 Linear regression, LOD, LOQ, precision, repeatability, and stability of seven analytes

Analyte	Regression equation[a]	r^2	Linear range (μg mL^{-1})	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	Precision (RSD, %)		Repeatability (RSD, %)	Stability (RSD, %)
						Intra-day	Inter-day		
1	$y = 1.714x - 0.04490$	0.9982	0.03916–3.132	0.01291	0.03906	2.6	2.3	2.4	2.3
2	$y = 0.4366x + 0.6048$	0.9991	0.5684–45.47	0.01368	0.04165	2.8	2.4	2.0	3.0
3	$y = 0.804x + 0.1388$	0.9981	0.0975–7.797	0.01253	0.03879	2.8	2.2	3.0	2.8
4	$y = 0.7072x + 0.1392$	0.9980	0.0898–7.182	0.01404	0.04287	2.7	0.7	2.9	2.1
5	$y = 0.7965x + 0.02650$	0.9981	0.04542–3.634	0.01811	0.06140	1.1	1.5	1.6	2.6
6	$y = 1.924x - 0.02500$	0.9984	0.04034–3.227	0.01288	0.03909	1.8	2.9	2.4	1.7
7	$y = 0.3810x - 0.002300$	0.9992	0.03677–2.942	0.01204	0.03645	2.8	2.8	2.9	2.0

[a] y is the peak area ratios (analyte/IS) and x is the concentration injected.

Table 3 Recoveries of seven analytes

Analyte	Initial amount (μg)	Added amount (μg)	Detected amount (μg)	Recovery (%)	Average (%)	RSD (%)
1	1.421	0.6853	2.129	101.2	101.4	2.5
	1.404	0.6853	2.188	104.7		
	1.432	0.6853	2.086	98.5		
	1.426	1.371	2.786	99.6	99.0	0.5
	1.416	1.371	2.761	99.1		
	1.427	1.371	2.755	98.4		
	1.432	2.056	3.507	100.5	101.1	1.0
	1.404	2.056	3.469	100.3		
	1.399	2.056	3.546	102.6		
2	141.1	66.32	206.0	99.3	99.3	1.1
	141.1	66.32	203.2	98.0		
	142.2	66.32	209.9	100.7		
	139.9	132.6	268.0	98.3	99.4	0.8
	140.5	132.6	272.4	99.7		
	139.9	132.6	272.9	100.2		
	139.9	198.9	338.5	99.9	100.0	0.8
	139.9	198.9	342.5	101.1		
	141.1	198.9	336.9	99.1		
3	2.702	1.462	4.226	101.5	98.7	2.2
	2.691	1.462	4.089	98.4		
	2.691	1.462	3.996	96.2		
	2.702	2.924	5.601	99.6	100.7	2.9
	2.712	2.924	5.897	104.6		
	2.670	2.924	5.469	97.8		
	2.649	4.386	7.405	105.3	102.0	2.5
	2.649	4.386	7.139	101.5		
	2.670	4.386	6.994	99.1		
4	0.6563	0.2693	0.917	99.1	99.8	0.6
	0.6617	0.2693	0.930	99.9		
	0.6670	0.2693	0.942	100.6		
	0.6670	0.5387	1.203	99.7	101.3	2.0
	0.6670	0.5387	1.205	100.0		
	0.6723	0.5387	1.260	104.1		
	0.6723	0.808	1.446	97.7	99.9	2.1
	0.6670	0.808	1.465	99.3		
	0.6750	0.808	1.523	102.7		
5	24.18	11.35	35.13	98.9	101.2	2.7
	24.18	11.35	37.30	105.0		
	23.99	11.35	35.24	99.7		
	24.08	22.71	46.30	99.0	98.9	0.7
	23.89	22.71	46.52	99.8		
	23.89	22.71	45.68	98.0		
	23.80	34.07	57.29	99.0	99.8	0.7
	23.99	34.07	57.98	99.9		
	23.99	34.07	58.43	100.6		
6	0.2601	0.1240	0.3825	99.6	99.2	1.5
	0.2591	0.1240	0.3727	97.3		
	0.2591	0.1240	0.3862	100.8		
	0.2549	0.2480	0.5025	100.0	100.7	1.2
	0.2539	0.2480	0.5142	102.4		
	0.2549	0.2480	0.5017	99.8		
	0.2560	0.3720	0.6262	99.7	99.8	1.0
	0.2580	0.3720	0.6219	98.7		
	0.2580	0.3720	0.6371	101.1		
7	0.6218	0.2758	0.907	101.0	100.7	1.4
	0.6193	0.2758	0.884	98.8		
	0.6168	0.2758	0.912	102.2		
	0.6243	0.5516	1.143	97.2	100.2	2.5
	0.6193	0.5516	1.174	100.3		
	0.6267	0.5516	1.216	103.3		
	0.6292	0.827	1.416	97.2	98.0	0.8
	0.6292	0.827	1.422	97.6		
	0.6267	0.827	1.442	99.1		

Sorbifoside C (**1**) was obtained as a white powder from methanol, the charged $[M + Na]^+$ ion at *m/z* 1373.67049 (calcd for $C_{65}H_{106}O_{29}Na$, 1373.67120) by HR-ESI-MS established the molecular formula as $C_{65}H_{106}O_{29}$. Comparing the NMR data of sorbifoside C (**1**) with those of compound **2**, it can be concluded that their basic structures were similar, but the position of the angeloyl group was different. In the ^1H NMR spectrum, the proton signal ascribed to H-4′ was shifted downfield, suggesting the connection of the angeloyl group. This presumption was corroborated by the HMBC (Fig. 1) long-range correlation of δ 5.57 (H-4′) and δ 167.7 (Ang-1). Furthermore, C-4′ was shifted downfield, whereas C-3′ and C-5′ were shifted upfield clearly. In addition, in the NOESY (Fig. 1) spectrum, the correlations between δ 1.99 (Ang-4) and δ 5.02 (H-1″) and δ 1.87 (Ang-5) and δ 4.08 (H-3′) were observed to certify the connection of the angeloyl group. The linkage sequence of the sugar units at C-3 was established by the HMBC (Fig. 1) correlations from δ 4.91 (H-1′) to δ 89.3 (C-3) and δ 5.02 (H-1″) to δ 69.4 (C-6′). Similarly, the sugar chain at C-28 was deduced from HMBC correlations from δ 4.71 (H-1‴) to δ 75.8 (C-28), δ 6.52 (H-1⁗) to δ 75.7 (C-2‴), and δ 5.01 (H-1⁗) to δ 70.1 (C-6‴). The same result with regard to the sugar sequence could also be drawn from the NOESY experiment. The ^1H and ^{13}C NMR data were fully assigned by the ^1H–^1H COSY, HSQC, HMBC, NOESY and TOCSY spectra (Table 1). The structure of sorbifoside C is shown in Fig. 1.

Sorbifoside D (**4**) obtained as a white powder from methanol and has a molecular formula of $C_{59}H_{96}O_{24}$ determined by the HR-ESI-MS ion at *m/z* 1211.62230 $[M + Na]^+$ (calcd for $C_{59}H_{96}O_{24}Na$, 1211.61838). The NMR (Table 1) spectroscopic features suggested that sorbifoside D shared the same triterpenoid skeleton and sugar moieties with compound **5** and the same position of the angeloyl group with sorbifoside C. Four anomeric proton signals at δ 4.91 (H-1′), 5.01 (H-1″), 4.74 (H-1‴) and δ 6.61 (H-1⁗) correlated with carbon signals at δ 89.4 (C-3), 69.6 (C-6′), 75.2 (C-28), and 75.9 (C-2‴), respectively, in the HMBC spectrum, suggesting the linkage sequence of the sugar units at C-3 and C-28. The proton signal ascribed to H-4′ was shifted downfield in the ^1H NMR spectrum and the key correlation between δ 5.57 (H-4′) and δ 167.8 (Ang-1) was observed in the subsequent HMBC (Fig. 1) experiment. The connection of the angeloyl group was thus corroborated. Moreover, the correlations between δ 2.01 (Ang-4) and δ 5.01 (H-1″) and δ 1.89 (Ang-5) and δ 4.10 (H-3′) were observed in the NOESY (Fig. 1) spectrum, which also certify the connection of the angeloyl group. The ^1H and ^{13}C NMR chemical shift assignments were accomplished by a combination of ^1H–^1H COSY, HSQC, HMBC, NOESY and TOCSY experiments. The structure of sorbifoside D is shown in Fig. 1.

Acid hydrolysis of sorbifoside C and D was also conducted according to the method which had been published. D-Glucose and L-rhamnose present in the sugar fraction were determined by comparing their retention times and optical rotation with those of the standard samples.

Optimization of the extraction procedure

Different extraction methods (ultrasonic and refluxing), extraction solvents (0, 30%, 50%, 70%, 100% ethanol, methanol and acetonitrile, respectively), and extraction times (15, 30, 45 and 60 min) were assessed based on single factor experiments in order to obtain satisfactory extraction efficiency for all the analytes. First we investigated ultrasonic and refluxing methods and found that there was no significant difference between ultrasonic and refluxing methods; the former was finally chosen for its higher convenience and time-saving ability. In terms of extraction solvent, 100% methanol was regarded as the preferred choice in the present study since a variety of compounds can be extracted effectively. Then the volume of the solvents (*i.e.* 100% methanol) with material to solvent ratios (w/v) as 1/10, 1/20 and 1/50 was investigated, since there was no significant difference between the latter two groups, 100% methanol with the w/v value as 1/20 was selected. After that, the extraction time was also investigated; 30 min was chosen as the appropriate condition for extraction because the content of analytes was almost unchanged even when the extraction time was prolonged. Taken together, the best extraction efficiency was obtained by ultrasonic extraction with 100% methanol (1/20, w/v) for 30 min.

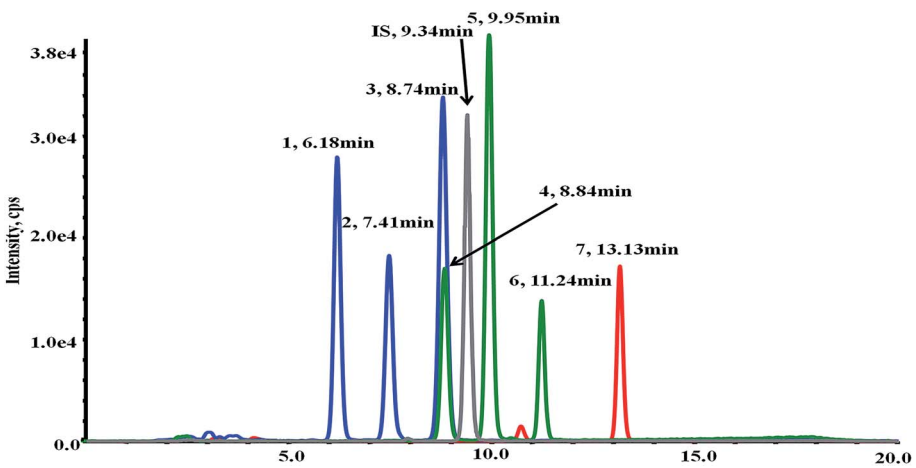

Fig. 2 Full chromatogram containing all seven compounds (standard mixture) and IS.

Calibration curves and limits of detection and quantification

The calibration curves of the seven triterpenoid saponins were assayed at six appropriate concentrations for each. A linear relationship was obtained while plotting peak area ratios (analyte/IS) against the analyte concentration. The mean values of the regression parameters and the linearity range of the different analytes are listed in Table 2. All the analytes showed good linearity ($r^2 \geq 0.998$) over relatively wide concentration ranges. The data of the LOD and LOQ were experimentally verified by injecting the seven analytes at LOD (signal-to-noise = 3) and LOQ (signal-to-noise = 10) concentrations. The results are shown in Table 2.

Precision, repeatability and stability

The precision of the method was determined by analyzing six repeated injections of the seven analytes and IS during a single day and by duplicating the experiments on three successive days. The repeatability of the developed assay was determined by analyzing 6 different sample solutions prepared from the same sample. The stability of sample solutions was analyzed at 0, 2, 4, 8 and 12 h within one day at room temperature, and the results indicated that seven analytes were all stable in samples kept for 12 h at room temperature. As shown in Table 2, the relative standard deviation (RSD) was used to express precision, repeatability and stability.

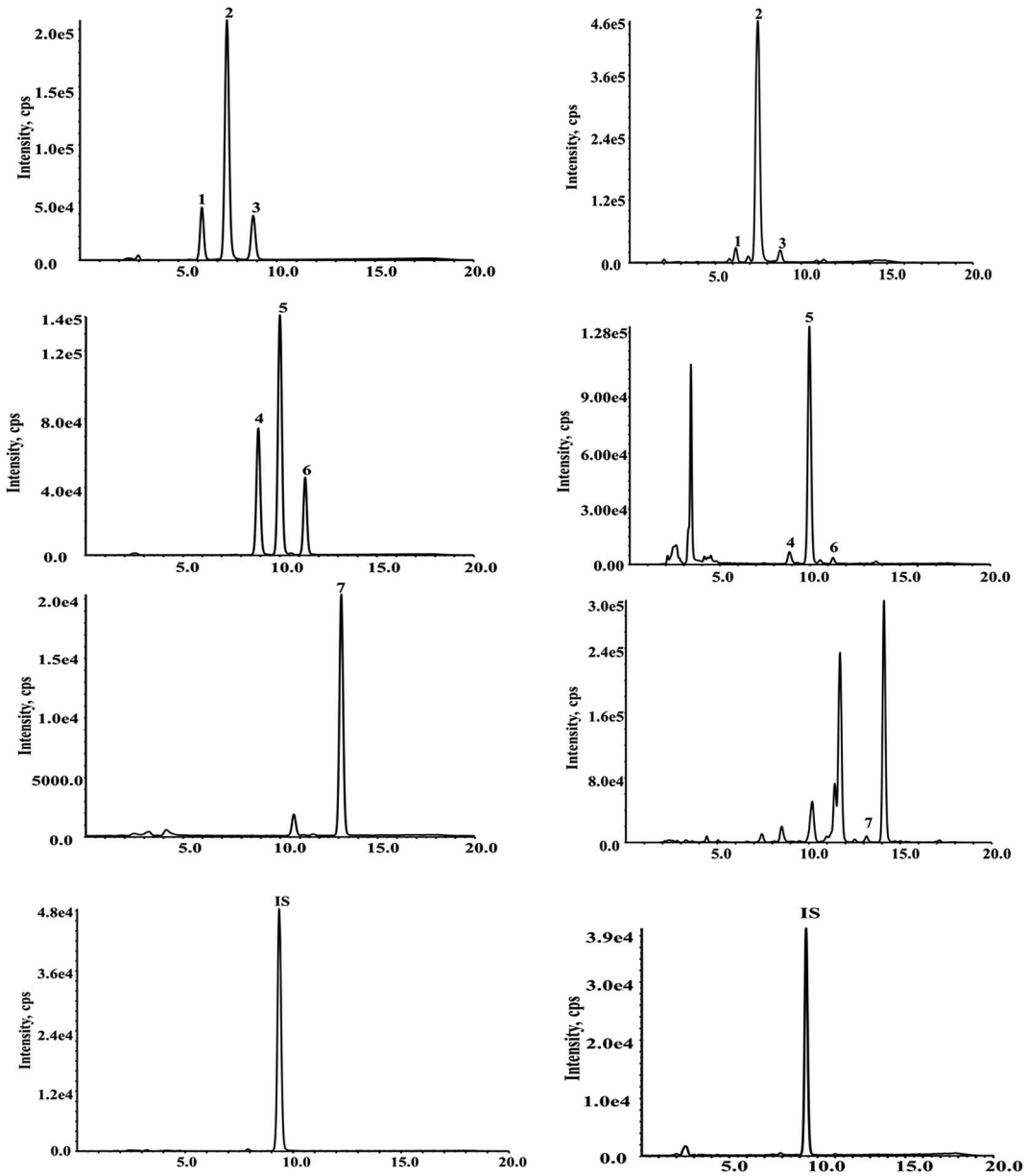

Fig. 3 Representative SIM chromatograms left: mixed standards, right: husks of *X. sorbifolia*. Selected ions corresponding to compounds **1–7** were monitored at *m/z* 698.60 (**1–3** $[M + 2Na]^{2+}$), *m/z* 617.30 (**4–6** $[M + 2Na]^{2+}$), *m/z* 719.30 (**7** $[M + 2Na]^{2+}$), and *m/z* 803.40 (IS $[M + Na]^{+}$), respectively.

Accuracy

The accuracy of the method was determined by recovery tests. The tests were carried out by adding three concentration levels of mixed standard solutions to approximately 0.25 g of the husk powder of *X. sorbifolia* samples. The results of recovery tests are shown in Table 3. The data indicated that recoveries of all analytes were consistent, precise and reproducible at different concentrations.

Sample analysis

The validated method was successfully applied to simultaneously determine the seven triterpenoid saponins in different parts of *X. sorbifolia* (husks, twig bark, twig xylem, seed coats, seed kernels, flowers and leaves). Representative chromatograms are shown in Fig. 2 and 3. The content of the seven compounds in different parts of *X. sorbifolia* was quantified and the mean contents in each sample are shown in Table 4 by three replicate injections.

Previously, different parts of *X. sorbifolia*, such as twigs, seeds, husks, leaves and even flowers, have been used as folk medicines in China.[24] With in-depth study of phytochemistry, triterpenoid saponins were considered as the major bioactive constituents, which exhibit a variety of biological activities, especially the function of improving intelligence. For example, the crude extract of the husks from *X. sorbifolia* could significantly rescue learning and memory deficits in several Alzheimer's disease animal models.[25] Because of the structure complexity, the synthesis of triterpenoid saponin monomers is very difficult. Therefore, the isolation and identification of triterpenoid saponin monomers from the plant is a prerequisite for further pharmacokinetics and pharmacodynamics study against AD. However, there is limited information on the distributions of triterpenoid saponins in different parts of the plant. To solve the problem, an HPLC-MS method was established for simultaneously determining the contents of seven triterpenoid saponins in different parts of the plant in this study. Our data in Table 4 showed that the content of seven triterpenoid saponins varied significantly in different parts of the plant, even in the case of isomers (compounds **1–3** and compounds **4–6**). Compound **2** was the most abundant triterpenoid saponin in all the plant parts with the exception of twig bark, whereas new compounds **1** and **4** were found as minor triterpenoid saponins only in some parts of the plant. Husks contained all the seven kinds of triterpenoid saponins, four of which (**2–3**, **5** and **7**) were in their highest concentrations. Other three kinds of triterpenoid saponins (**1**, **4** and **6**) were in the highest concentration in twig bark. Only one kind of triterpenoid saponin was provided by twig xylem and seed coats at very low concentrations. Based on the above data, husks were undoubtedly the largest reservoir of the seven triterpenoid saponins. Moreover, obtaining triterpenoid saponins from husks of ripe fruits will do less harm to the growth of the plant than obtaining from other parts of the plant. So it is obvious that the husks of *X. sorbifolia* are the best choices for people as a sustainable medicinal source to obtain triterpenoid saponin monomers.

Table 4 Contents (μg g^{-1}) of seven analytes in different parts of *X. sorbifolia*[a]

	Analyte[b]						
Plant part	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Husks	5.662	564.2	13.50	2.668	95.9	1.028	2.497
Twig bark	7.485	78.76	12.08	26.98	23.67	6.604	—
Twig xylem	0.942	—	—	—	—	—	—
Seed coats	—	35.37	—	—	—	—	—
Seed kernels	—	15.60	—	—	1.627	—	—
Flowers		188.6	—	—	2.251	—	1.086
Leaves	—	100.4	—	—	2.970	—	—

[a] "—": below the LOD. [b] The notation for analyte refers to Fig. 1.

Conclusion

In this study, we isolated and identified seven triterpenoid saponins, two of which are new (**1** and **4**). Then an HPLC-MS method was established to explain the distribution of triterpenoid saponins in different parts of *X. sorbifolia*. In comparison to the conventional HPLC method, the method has several advantages including accurate quantification, short analysis time and good reproducibility. This paper is a fundamental study for providing a reference for the finding of candidates against AD.

Abbreviations

IS	Internal standard
HPLC-MS	High-performance liquid chromatography-tandem mass spectrometry
SIM	Selected ion monitoring mode
NMR	Nuclear magnetic resonance spectrometry
HR-MS	High resolution-mass spectrometry
COSY	Homonuclear chemical shift correlation spectrometry
HMBC	^{1}H detected heteronuclear multiple bond connectivity
HSQC	^{1}H detected heteronuclear multiple-quantum coherence
TOSCY	Total correlation spectrometry
NOESY	Two dimensional nuclear Overhauser effect spectrometry

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References

1 X. F. Li, S. L. Hou, M. Su, M. F. Yang, S. H. Shen, G. M. Jiang, D. M. Qi, S. Y. Chen and G. S. Liu, *Environ. Manage.*, 2010, **46**, 579.

2 Y. J. Chen, T. Takeda and Y. Ogihara, *Jpn. J. Pharmacogn.*, 1984, **38**, 203.
3 Y. J. Chen, T. Takeda and Y. Ogihara, *Chem. Pharm. Bull.*, 1985, **33**, 1043.
4 C. B. Cui, X. S. Yao and Y. J. Chen, *J. Shenyang Coll. Pharm.*, 1991, **8**, 36.
5 P. K. Chen, *Biochem. Pharmacol.*, 2007, **73**, 341.
6 C. Ma, N. Nakamura, M. Hattori, H. Kakuda, J. Qiao and H. Yu, *J. Nat. Prod.*, 2000, **63**, 238.
7 A. Morinaga, K. Ono, J. Takasaki, T. Ikeda, M. Hirohata and M. Yamada, *Exp. Neurol.*, 2011, **228**, 298.
8 T. Vazin, K. A. Ball, H. Lu, H. Park, Y. Ataeijannati, T. H. Gordon, M. Poo and V. D. Schaffer, *Neurobiol. Dis.*, 2014, **62**, 62.
9 X. X. Liu, X. F. Ji, L. L. Lu, B. Z. Yang, L. H. Wang and L. B. Zou, *Chin. Tradit. Herb. Drugs*, 2007, **38**, 1859.
10 X. X. Liu, X. Yang, C. Qu, Z. Wu, B. Z. Yang, L. H. Wang and L. B. Zou, *Tradit. Chin. Drug Res. Clin. Pharmacol.*, 2007, **18**, 23.
11 P. Liu, L. B. Zou, Q. Jiao, T. Y. Chi, X. F. Ji, Y. Qi, Q. Xu and L. H. Wang, *Neurosci. Lett.*, 2013, **543**, 115.
12 G. Jin, L. H. Wang, X. F. Ji, T. Y. Chi, Y. Qi, Q. Jiao, Q. Xu, X. Y. Zhou, R. Zhang and L. B. Zou, *Neurosci. Lett.*, 2014, **573**, 58.
13 X. Li, N. Li, X. Li, D. L. Meng and S. L. Shi, *J. Shenyang Pharm. Univ.*, 2008, **25**, 211.
14 J. B. Xie, Z. M. Bi and P. Li, *Acta Pharmacol. Sin.*, 2003, **38**, 534.
15 X. J. Hu, X. B. Liu, M. H. Gong, M. Luan, Y. L. Zheng, G. L. Wu, J. Z. Shentu and L. Zhang, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2014, **967**, 156.
16 S. Ji, Q. Wang, X. Qiao, H. C. Guo, Y. F. Yang, T. Bo, C. Xiang, D. A. Guo and M. Ye, *J. Pharm. Biomed. Anal.*, 2014, **90**, 15.
17 J. H. Ling, L. L. Liu, Y. X. Wang, Z. Y. Li, R. Liu, Q. Li, Y. Wang, B. Z. Yang, X. H. Chen and K. S. Bi, *J. Pharm. Biomed. Anal.*, 2011, **55**, 259.
18 W. Li, X. Li, D. L. Meng, P. Zhang and Z. L. Li, *J. Asian Nat. Prod. Res.*, 2007, **9**, 7.
19 H. W. Fu, Y. Guo, W. Li, D. Q. Dou, T. G. Kang and K. Z. Koike, *J. Nat. Med.*, 2010, **64**, 80.
20 L. L. Yu, X. L. Tang, L. X. Chen, M. M. Wang, J. F. Jian, S. J. Cao, X. B. Wang, N. Kang and F. Qiu, *Fitoterapia*, 2012, **83**, 1636.
21 L. L. Yu, X. B. Wang, X. C. Wei, M. M. Wang, L. X. Chen, S. J. Cao, N. Kang and F. Qiu, *Bioorg. Med. Chem. Lett.*, 2012, **22**, 5232.
22 W. Xiao, Y. Wang, P. Zhang, N. Li, S. Jiang, J. H. Wang, J. Huang and X. Li, *Eur. J. Med. Chem.*, 2013, **60**, 263.
23 H. Cui, H. Xiao, X. K. Ran, Y. Y. Li, D. Q. Dou and T. G. Kang, *J. Asian Nat. Prod. Res.*, 2012, **14**, 216.
24 G. Ao, A. H. Ju and Z. B. Xiao, *China J. Tradit. Chin. Med. Pharm.*, 2009, **24**, 769.
25 X. F. Ji, X. X. Liu, Z. Wu, B. Z. Yang, L. H. Wang and L. B. Zou, *J. Shenyang Pharm. Univ.*, 2007, **24**, 232.