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Secondary metabolites from *Sambucus ebulus*

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Abstract: A new nonglycosidic iridoid, sambulin B (**1**), was isolated from the methanol extract of *Sambucus ebulus* L. leaves along with a recently reported new nonglycosidic iridoid, 10-*O*-acetylpatrinoside aglycone (sambulin A) (**2**); 2 flavonoids, isorhamnetin-3-*O*-β-D-glucopyranoside (**3**) and isorhamnetin-3-*O*-rutinoside (**4**); and a mixture of 2 flavonoids (**5**), quercetin-3-*O*-β-D-glucopyranoside and quercetin-3-*O*-β-D-galactopyranoside. Their structures were elucidated by 1-D and 2-D nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) experiments.

Key words: *Sambucus ebulus*, nonglycosidic iridoids, sambulin B, flavonoids

1. Introduction

The genus *Sambucus* (Adoxaceae) is represented by 2 species in the flora of Turkey.[1] Among these, *Sambucus ebulus* L. is a perennial herb known locally as mürver, sultanotu, and şahmehlemi.[1,2] In Anatolian folk medicine, *S. ebulus* is particularly used against inflammatory problems, i.e. rheumatic pain, edema, eczema, urticaria, burns, infectious wounds, and hemorrhoids as well as peptic ulcers.[3] Anti-inflammatory, antinociceptive, wound-healing, cytotoxic, antiulcer, and anti-*Helicobacter pylori* effects of *S. ebulus* were reported in several previous studies.[4–8] Simple phenols, flavonoids, anthocyanins, lignans, and iridoids have been shown to be the major secondary metabolites of the genus.[9–13]

We herein present the isolation and structure elucidation of a new nonglycosidic iridoid named sambulin B (**1**), along with an iridoid, 10-*O*-acetylpatrinoside aglycone (sambulin A), and 4 known flavonoids, isorhamnetin-3-*O*-β-D-glucopyranoside (**3**), isorhamnetin-3-*O*-rutinoside (**4**), and a mixture of 2 flavonoids, hyperoside (quercetin-3-*O*-β-galactoside) and isoquercitrin (quercetin-3-*O*-β-glucoside) (**5**). The chemical structures of the compounds are presented in the Figure.

2. Results and discussion

The leaves of *S. ebulus* were extracted with MeOH. The crude extract was dispersed in 90% MeOH, and then submitted to partition with organic solvents in increasing polarity. Two nonglycosidic iridoids (**1** and **2**) were isolated from the *n*-hexane and $CHCl_3$ subextracts, while 2 flavonol glycosides and a mixture of 2 flavonoid glycosides were obtained from the EtOAc subextract.

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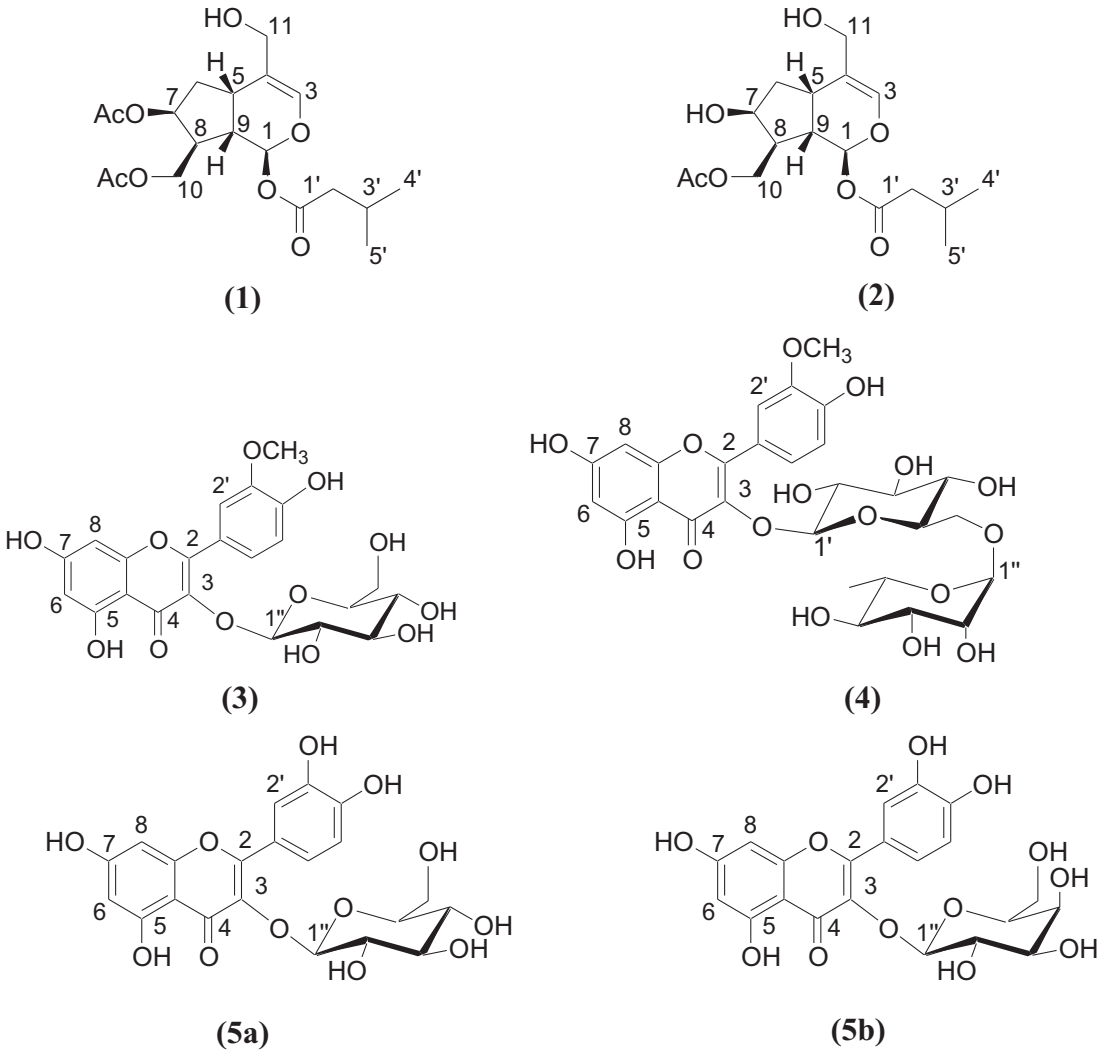

Figure. Structures of the isolated compounds.

Compound **1** was obtained as a colorless oily substance from the n-hexane subextract. The molecular formula, $C_{19}H_{28}O_8$, was deduced from the pseudomolecular ion peaks at m/z 407 $[M+Na]^+$ and 791 $[2M+Na]^+$ in the ESI-MS and by inspecting ^{13}C NMR data. It exhibited UV maxima at 241, 274, and 282 nm. The IR spectrum suggested the presence of hydroxyl group (3678 cm^{-1}), olefinic C–H (3100 cm^{-1}), and ester carbonyl (1738 cm^{-1}) functionalities. The ^1H NMR spectrum contained 1 olefinic (δ_H 6.34), 2 hydroxymethylene (δ_H 4.20 and 4.16, δ_H 4.09 and 4.00), 1 methylene (δ_H 2.15, 1.96), 1 oxymethyne (δ_H 5.30), 3 methyne (δ_H 3.01, 2.34, and 2.13), and 1 hemiacetal (δ_H 5.97) signals (Table 1). Moreover, the ^1H NMR spectrum displayed 2 equivalent secondary methyl resonances at δ 0.97 (d, J: 6.4 Hz). In the COSY spectrum, 1 methyne resonance and 1 methylene (δ_H 2.26) signal conjugated to a carbonyl function were observed in a spin system. These data were indicative of the presence of an isovaleryl moiety. The signals at 22.3 (2C), 25.6, 42.9, and 171.7 in the ^{13}C NMR spectrum supported this assumption.[14] Moreover, the presence of 2 acetoxy groups was evident from the signals at δ_H 2.05 (3H) and 2.04 (3H) and the corresponding carbon resonances at δ_C 21.0, 20.8, and 170.2 (2C). The ^{13}C NMR spectrum contained 19 signals; 5 of them were ascribed to an isovaleryl unit,

while 4 of them were arising from 2 acetyl groups. The remaining 10 carbon atoms were in accordance with a C_{10}-iridoid core. The esterification sites of the acyl units were established by the long-range correlations in the HMBC. Thus, cross peaks between carbonyl carbon of isovaleryl (δ_C 171.7) with H-1 (δ_H 5.97) and carbonyl carbon of one of the acetyl units with H_2-10 (δ_H 4.20, 4.16) revealed the locations of acyl units to be at C-1 and C-10, respectively. The NMR data of **1** were very close to those of 10-*O*-acetylpatrinoside, a recently published molecule from the same species by another research group, except for the presence of an additional acetyl unit in compound **1**. When the ^{1}H NMR spectrum of **1** was carefully inspected, H-7 signal of this appeared to be shifted to downfield about 1 ppm (δ_H 5.30) compared to 10-*O*-acetylpatrinoside aglycone (**2**).[15] Moreover, the ^{13}C NMR spectrum of the compound showed that the C-7 signal shifted downfield 2.5 ppm, while the C-6 and C-8 signals shifted upfield around 3 ppm compared to 10-*O*-acetylpatrinoside aglycone (**2**). Therefore, the position of the second acetyl group was determined to be C-7(OH). To determine the relative stereochemistry of the chiral centers in **1**, a ROESY experiment was performed. ROe cross-peaks were observed between H-1α and H-8α, and H-7α and H-8α, showing that these protons lie on the same side (α) of the molecule. In contrast, correlations were observed between H-3 and H-11β, H-5 and H-9, H-5 and H-6β, and H-5 and H-11β, indicating that these protons were on the same side (β). Based on these findings the structure of compound **1** was elucidated as 7-*O*-acetyl derivative of 10-*O*-acetylpatrinoside aglycone (**2**). A literature survey revealed that compound **1** was a new nonglycosidic iridoid and was named sambulin B. On the other hand, for 10-*O*-acetylpatrinoside aglycone (**2**) we propose the trivial name sambulin A.

Table 1. ^{1}H (600 MHz, $CDCl_3$) and ^{13}C-NMR (150 MHz, $CDCl_3$) data and HMBC correlations of sambulin B (1) and 10-O-acetylpatrinoside (sambulin A (2)).

	Sambulin B (1)		10-*O*-acetylpatrinoside aglycon (2)		HMBC (C→H)
Position	δ_C (ppm)	δ_H (ppm), *J* (Hz)	δ_C (ppm)	δ_H (ppm) *J* (Hz)	
1	91.1	5.97 *d* (4.7)	91.9	5.96 *d* (4.7)	H-3/H-8
3	138.4	6.34 *s*	138.0	6.39 *s*	H-1/H-5/H_2-11
4	116.9	-	118.0	-	H-3/H-5/H_2-6/H_2-11
5	32.0	3.01 *m*	32.5	3.07 *m*	H-1/H-3/H-7/H_2-11
6α	37.2	2.15 *m*	39.6	2.19 *m*	H-5
6β		1.96 *m*		1.70 *m*	
7	74.0	5.30 *m*	71.5	4.20 *m*	H-9/H_2-10
8	43.2	2.34 *m*	46.6	2.60 *m*	H_2-6
9	42.3	2.13 *m* †	41.1	2.16 *m*∗	H_2-6/H-8/H_2-10
10a	62.2	4.20 *dd* (11.2, 6.9)	62.8	4.49 *dd* (11.1,6.9)	H-8
10b		4.16 *dd* (11.2, 6.9)		4.14 *dd* (11.1, 6.9)	
11a	62.6	4.09 *d* (12.3)	62.5	4.10 *d* (12.3)	H-3
11b		4.00 *d* (12.3)		4.02 *d* (12.3)	
7-CO$\underline{CH}_3$	21.0	2.05 *s*			
7-$\underline{CO}CH_3$	170.2	-			
10-CO$\underline{CH}_3$	20.8	2.04 *s*	21.0	2.09 *s*	
10-$\underline{CO}CH_3$	170.2	-	171.8	-	H_2-10/CO$\underline{CH}_3$ (10)
1′	171.7	-	171.7	-	H-1/H_2-2′
2′	42.9	2.26 *m*	43.1	2.26 *m*	H_3-4′/H_3-5′
3′	25.6	2.30-2.22 *m* †	25.6	2.20-2.10 *m* †	H_2-2′/ H_3-4′/H_3-5′
4′	22.3	0.97 *d* (6.4)	22.4	0.98 *d* (6.4)	H_2-2′/H-3′
5′	22.3	0.97 *d* (6.4)	22.4	0.98 *d* (6.4)	H_2-2′/H-3′

*Unclear due to overlapping

In addition, 4 known flavonoids were isolated from the ethyl acetate subextract of *S. ebulus* methanol extract. The known compounds were identified by UV, IR, and 1-D and 2-D NMR techniques and by comparison with the previous published data. The structures of **3** and **4** were elucidated as isorhamnetin-3-*O*-β-D-glucopyranoside and isorhamnetin-3-*O*-rutinoside (Table 2). The NMR data of these compounds were found to be consistent with the published literature.[16] Inspection of the ^{1}H NMR spectra of **5** revealed that it was a mixture of 2 flavonoid glycosides. A careful inspection of the spectra led to the identification of quercetin-3-*O*-β-D-glucopyranoside and quercetin-3$-$*O*-β-D-galactopyranoside. Thus **5** was identified as an inseparable mixture of hyperoside (quercetin-3-*O*-β-galactoside) and isoquercitrin (quercetin-3-*O*-β-glucoside). The ^{13}C and ^{1}H NMR data of the hyperoside and isoquercitrin are given in Table 3; they were consistent with the previously published data.[17]

Table 2. ^{1}H (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) data of isorhamnetin-3-*O*-$\beta-$D-glucopyranoside (**3**) and isorhamnetin-3-*O*-rutinoside (**4**).

	Isorhamnetin-3-*O*-$\beta-$D-glucopyranoside (3)		Isorhamnetin-3-*O*-rutinoside (4)	
Position	δ_H, ppm (J, Hz)	δ_C, ppm	δ_H, ppm (J, Hz)	δ_C, ppm
2		157.2		159.2
3		130.9		132.9
4		178.0		179.9
5		160.5		164.0
6	6.21 *d* (2.1)	98.8	6.20 *d* (1.7)	100.7
7		165.1		163.5
8	6.41 *d* (2.1)	93.6	6.39 *d* (1.7)	95.6
9		157.0		159.0
10		104.1		104.9
1'		121.8		123.4
2'	7.91 *d* (2.1)	112.9	7.94 *d* (1.5)	116.4
3'		149.5		151.2
4'		147.0		148.9
5'	6.92 *d* (7.9)	114.7	6.90 *d* (8.5)	116.5
6'	7.58 *dd* (7.9, 2.1)	122.4	7.63 *dd* (8.5, 1.5)	124.5
1"	5.37 *d* (7.3)	100.2	5.22 *d* (7.6)	103.0
2"	3.24 *dd* (7.3, 5.2)	74.4	3.45-3.48 *m*	76.4
3"	3.46 *t* (7.3)	76.6	3.35-3.45 *m*	78.7
4"	3.30 *m*	69.9	3.35 *m*	72.1
5"	3.30 *m*	77.5	3.22 *m*	77.9
HA-6"	3.56 *dd* (12.0, 5.6)	61.2	3.55 *dd* (12.0, 5.1)	68.6
HB-6"	3.72 *dd* (12.0, 2.3)		3.72 *dd* (12.0, 2.5)	
OCH_3	3.94 *s*	55.4	3.94 *s*	57.2
1"			4.53*d* (1.7)	102.9
2"			3.20-3.80	72.4
3"			3.20-3.80	72.8
4"			3.20-3.80	74.4
5"			3.20-3.80	70.3
6"			1.1 *d* (6.5)	18.2

Several iridoids have been reported from *Sambucus* species.[12,18,19] Recently 6 new 'Valeriana-type' iridoid glycosides were isolated from *S. ebulus* leaves. However, sambulin B is a new nonglycosidic ester iridoid, isolated from the leaves of *S. ebulus*. Since iridoids are accepted as significant chemotaxonomic markers, the occurrence of

such rare 'Valeriana-type' nonglycosidic iridoids might contribute to the chemotaxonomy of the genus *Sambucus* (formerly Caprifoliacae) within its new family (Adoxaceae).

Table 3. ^{1}H (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) data of quercetin-3-*O*-β-D-glucopyranoside (**5a**) and quercetin-3-*O*-β-D-galactopyranoside (**5b**).

	Quercetin-3-*O*-β-glucopyranoside (5a)		Quercetin-3-*O*-β-D-galactopyranoside (5b)	
Position	δ_H, ppm (J, Hz)	δ_C, ppm	δ_H,ppm (J, Hz)	δ_C, ppm
2		158.8		158.5
3		136.0		136.0
4		179.6		179.5
5		163.1		163.1
6	6.20 *d* (1.7)	99.9	6.20 *d* (2.1)	99.9
7		166.7		164.1
8	6.39 *d* (1.7)	94.8	6.39 *d* (2.1)	94.8
9		158.6		156.3
10		105.5		103.9
1'		123.1		123.1
2'	7.66 *d* (2.2)	116.1	7.84 *d* (2.3)	116.0
3'		145.9		145.9
4'		150.0		149.9
5'	6.84 *d* (8.5)	117.6	6.86 *d* (8.8)	117.8
6'	7.63 *dd* (8.5, 2.2)	123.2	7.59 *dd* (8.8, 2.3)	121.8
1"	5.25 *d* (7.6)	104.5	5.15 *d* (8.2)	102.3
2"	3.42 *dd* (7.6, 9.0)	75.8	3.53 *dd* (8.2, 9.3)	73.2
3"	3.48 *m*	78.4	3.22–3.34 *m*	75.1
4"	3.35 *m*	71.3	3.61 *d* (3.2)	70.1
5"	3.22 *m*	78.2	3.22-3.34 *m*	77.2
6"	3.58 *dd* (12.0, 5.5)	62.6	3.60*	61.9
	3.71 *dd* (12.0, 2.5)		3.71 *dd* (12, 2.5)	

*Unclear due to overlapping

3. Experimental

3.1. General

UV spectra were recorded on an HP Aglient 8453 (USA). IR spectra were recorded on a PerkinElmer-2000 FT-IR spectrometer (USA). NMR spectra were recorded on a Varian Mercury-Mx spectrometer (USA) at 600 MHz for ^{1}H NMR and at 150 MHz for ^{13}C NMR, with $CDCl_3$ and CD_3OD as solvents. Optical rotations were determined on an Opt. Act. Ltd. AA-5 polarimeter. Kieselgel 60 (0.063–0.200 mm; Merck, Darmstadt, Germany), Sephadex LH-20 (Lipophilic Sephadex, 25–100 μm, Sigma-Aldrich, USA), and Polyamide (Fluka, USA) were used for column chromatography (CC), and precoated Kieselgel 60 F_{254} (Merck) plates were used for thin layer chromatography. For medium-pressure liquid chromatography (MPLC) a CombiFlash Companion (Teledyne Isco, USA) apparatus equipped with RediSep columns (C18, 130 and 43 g; Teledyne Isco, USA) was used.

3.2. Plant material

The leaves of *S. ebulus* were collected from Uludağ, Bursa (Turkey) in June 2009. The plants were identified by Prof Dr Erdem Yeşilada (Department of Pharmacognosy, Faculty of Pharmacy, Yeditepe University, İstanbul, Turkey). A voucher specimen (YEF 09017) was deposited at the Herbarium of Yeditepe University.

3.3. Extraction and isolation

The air dried and powdered leaves of *S. ebulus* (330 g) were extracted with methanol (MeOH) (2.3 l) over 24 h with intermittent stirring in a water bath (40 °C). The extract was filtered through filter paper and evaporated to dryness under reduced pressure to give crude MeOH extract (60.5 g, yield: 18.3%). The MeOH extract was then redissolved in 200 mL of MeOH in H_2O (10%) and extracted with *n*-hexane (9 × 200 mL). Combined *n*-hexane extract was evaporated under reduced pressure to yield *n*-hexane subextract (8 g, yield: 13.8%). Then MeOH was removed from the remaining extract and diluted with distilled H_2O and fractionated by successive solvent extractions with chloroform ($CHCl_3$) (4 × 200 mL), ethyl acetate (EtOAc) (4 × 200 mL), and *n*-butanol saturated with H_2O (*n*-BuOH) (4 × 100 mL). Each extract after solvent extraction was evaporated to dryness under reduced pressure to yield $CHCl_3$ subextract (22.5 g, yield: 39.2%), EtOAc subextract (2.5 g, yield: 4.3%), and *n*-BuOH subextract (9 g yield: 15.5%), and remaining water (11.5 g, yield: 19.8%).

3.3.1. Isolation of the components from EtOAc subextract

The EtOAc subextract (2 g) was further subjected to column chromatography (CC) over polyamide (25 g) and eluted with H_2O/MeOH mixtures in different ratios (0%–100%) to yield 4 fractions: SE_{EtOAc} I Fr. 1–4 (746 mg), SE_{EtOAc} I Fr. 5–10 (400 mg), SE_{EtOAc} I Fr. 12–14 (415 mg), and SE_{EtOAc} I Fr. 15–20 (227 mg). SE_{EtOAc} I Fr. 12–14 was subjected to MPLC over a RediSep Rf Reversed-phase C18 column (130 g) and eluted with mixtures of MeOH/H_2O (15%–100%) to yield 2 fractions: SE_{EtOAc} II Fr. 26–34 and SE_{EtOAc} II Fr. 35–49. Further CC of SE_{EtOAc} II Fr. 26–34 over SiO_2 (12 g) yielded a mixture (**5**) of hyperoside (quercetin-3-*O*-β-galactoside) and isoquercitrin (quercetin-3-*O*-β-glucoside) by eluting with mobile systems of MeOH/$CHCl_3$ (from 10% to 15%). The other fraction SE_{EtOAc} II Fr. 35–49 yielded isorhamnetin-3-*O*-β-D-glucopyranoside (**3**) by CC over SiO_2 (15 g) and eluting with mobile systems of MeOH/$CHCl_3$ (from 2% to 20%).

Furthermore, fractions of polyamide CC were further subjected to chromatographic separation by MPLC. SE_{EtOAc} I Fr. 15–20 was applied to MPLC over a RediSep flash column (12 g) and eluted with a MeOH/$CHCl_3$ solvent system (0%–80%). SE_{EtOAc} I Fr. 5–10 was applied to a MPLC RediSep 13 g reverse phase C18 column and eluted with MeOH/H_2O (5%–70%) to yield isorhamnetin-3-*O*-rutinoside (**4**).

3.3.2. Isolation of the components from $CHCl_3$ subextract

The $CHCl_3$ (10 g) subextract was applied to CC over SiO_2 (200 g) and eluted with first a solvent system composed of EtOAc/*n*-hexane in different rates (20%–100%) and then with MeOH/$CHCl_3$ mixtures (10%–50%) to give 4 fractions: SE_{CHCl3} I Fr. 2–7 (332 mg), SE_{CHCl3} I Fr. 8–13 (794 mg), SE_{CHCl3} I Fr. 14–16 (6.4 g), and SE_{CHCl3} I Fr. 17–23 (806 mg). SE_{CHCl3} I Fr. 14–16 was fractionated by CC over SiO_2 (150 g). Elution was started with $CHCl_3$ and then mixtures of MeOH/$CHCl_3$ (2.5%–60%) were used. Four subfractions were obtained: SE_{CHCl3} II Fr. 59–63 (1.289 g), SE_{CHCl3} II Fr. 71–72 (273 mg), SE_{CHCl3} II Fr. 73–74 (180 mg), and SE_{CHCl3} I Fr. 78–86 (615 mg). SE_{CHCl3} II Fr. 59–63 was applied to MPLC over a RediSep silica flash column (40 g) eluting with acetone/$CHCl_3$ (15%–90%) and 10-*O*-acetylpatrinoside aglycone (**2**) was obtained.

3.3.3. Isolation of the components from hexane subextract

The hexane subextract was applied to CC over Sephadex (16 g) and eluted with $CHCl_3$/MeOH (50%) to yield 2 fractions: SE_{Hexane} I Fr. 9–12 (38 mg) and SE_{Hexane} I Fr. 13–15 (75 mg). The MPLC (RediSep Silica Flash Column (4 g)) of SE_{Hexane} I Fr. 9–12 with EtOAc/n-hexane (15%–80%) as eluent system yielded sambulin B (**1**).

3.4. Spectroscopic characteristics of the isolated compounds

Sambulin B (**1**): Colorless oily substance; $C_{19}H_{28}O_8$, $[a]_D^{20}$ = +39.1 (c = 0.41, $CHCl_3$); UV ($CHCl_3$): λ max 241, 274, 282, IR (KBr) υ_{max} cm^{-1}: 3678, 3100, 1738, 1235, ESI-MS: m/z = 407 $[M+Na]^+$, 791 $[2M+Na]^+$, ^1H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) Table 1.

10-*O*-acetylpatrinoside aglycone (sambulin A) (**2**): Colorless oily substance; $C_{17}H_{26}O_7$, $[a]_D^{20}$ = –4.6 (c = 0.33, $CHCl_3$); UV ($CHCl_3$): λ max 241, 274, 282; IR (KBr) υ_{max} cm^{-1}: 3418, 1736, 1372, 1235; ESI-MS: m/z = 343 $[M+H]^+$, ^1H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$): Table 1.

Isorhamnetin-3-*O*-β-D-glucopyranoside (**3**)**:** Yellow amorphous powder; $C_{22}H_{22}O_{12}$, UV (MeOH): λ_{max} 264, 354 nm, IR (KBr) υ_{max} cm^{-1}: 3409, 1653, 1607, HR-ESI-MS: m/z =501.1003 $[M+Na]^+$, ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) Table 2.

Isorhamnetin-3-*O*-rutinoside (**4**)**:** Yellow amorphous powder; $C_{28}H_{32}O_{16}$, UV (MeOH): λ_{max} 266, 354 nm, IR (KBr) υ_{max} cm^{-1}: 3452, 2934, 1665, 1608, HR-ESI-MS: m/z =647.1583 $[M+Na]^+$, ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD): Table 2.

Mixture of isoquercitrin (**5a**) **and hyperoside** (**5b**) (2/3) **(5):** Yellow amorphous powder; UV (MeOH): λ_{max} 266, 352 nm, IR (KBr) υ_{max} cm^{-1}: 3415, 1655, 1605, HR-ESI-MS: m/z = 487.0847 $[M+Na]^+$, ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD): Table 3.

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