CHEMICAL CONSTITUENTS FROM THE STEMS OF *Oxyceros bispinosus*

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Abstract: A study of the chemical constituents obtained from stems of *Oxyceros bispinosus* (Rubiaceae) led to the isolation and structure elucidation of four known compounds, 5(6)-gluten-3α-ol (**1**), quinovic acid 3-*O*-*β*-D-quinovopyranoside (**2**), daucosterol (**3**), and scopoletin (**4**). Its chemical structure was elucidated by IR, UV, HR-ESI-MS, NMR 1D and 2D experiments and compared with literatures. The present report is the first comprehensive study of this species.

Introduction: *Oxyceros bispinosus* (Griff.) Tirveng, a climbing shrub of the rubiaceae family, is mainly distributed in Phangnga, Krabi, Surat Thani, and Phuket provinces of southern Thailand and the tropical regions of Asia.[1] As a traditional Thai herbal medicine, the climbers of *O. bispinosus* are used as the diarrhea, dermatitis, and cure shingles. The plants of the family rubiaceae are rich in alkaloids,[2-4] saponin,[3] pyranoanthraquinones,[5] as the major constituents, some of them have demonstrated antifeedant,[6] antinociceptive,[7] and antidiarrhoeal activities.[8] As part of our ongoing search for new bioactive constituents from Thai medicinal plants, either wild or cultivated, our continued interest in discovering novel bioactive metabolites of *Oxyceros bispinosus* stems resulted in the isolation and identification of four known compounds (**1**–**4**) (Fig. 1). In this letter, the isolation and structure of the compounds are elucidated using spectroscopic methods, including UV, IR, 1D, 2D NMR and comparison with the literature values.

Methodology:

General Experimental Procedures

UV spectra were recorded in MeOH using SPECORD® 210 PLUS from analytic Jena spectrophotometer. IR spectra were obtained on a Shimadzu FTIR-8900 spectrophotometer. NMR spectra were acquired on a Bruker AVANCE400 spectrometer (at 400 MHz for ^{1}H and 100 MHz for ^{13}C) using $CDCl_3$ with TMS as the internal standard. Vacuum liquid chromatography (VLC) was carried out on silica gel 60H (Merck, 5-40 μm) and RP-18 (Merck, 15-25 μm). Fractions were monitored by TLC using Merck pre-coated silica gel $60F_{254}$ and RP-18 F_{254S} sheets and spots were visualized by using fluorescence (254 and 386 nm) and by heating silica gel plates sprayed with ceric sulphate solution.

Plant material

The stems of *O. bispinosus* were collected from Phangnga Province, Thailand, in January 2016. The identification of the plant material was authenticated by Dr. Piya Chalermglin, Thailand Institute of Scientific and Technological Research, Thailand. A voucher specimen (PKRU2016001) was deposited at the Laboratory of Natural Products Chemistry, Faculty of Science and Technology, Phuket Rajabhat University, Phuket, Thailand.

Extraction and Isolation

The fresh stems (8.1 kg) of *O. bispinosus* were extracted with EtOH (4 × 30.0 L) under heated (50 °C) for 4 × 2 h. Removal of the solvent in vacuo afforded a residue (254.18 g), which was suspended in H_2O (500 mL) followed by successive partition with EtOAc and n-

BuOH (each 5 × 2.0 L). The EtOAc extract (53.4 g) was performed on a vacuum liquid chromatography column (VLC) using silica gel 60H (1.27 kg, diameter × height: 18.0 × 10.0 cm) and eluted with a step gradient of using a EtOAc in hexane (1% EtOAc to 100% EtOAc) and 100% MeOH to yield 9 fractions (OS1-OS9). Fraction OS3 (1.48 g) was subjected to a silica gel 60H VLC column (19.2 g, diameter × height: 3.5 × 4 cm) eluted with increasing amount of EtOAc in hexane (1% EtOAc to 100% EtOAc) to afford eight subfractions (OS3.1–3.8). Subfraction OS3.4 was identified as **1** (white solid, 8.9 mg). Fraction OS7 (1.63 g) was separated over a silica gel 60H VLC column (39.2 g, diameter × height: 5.0 × 4 cm) eluted with increasing amount of acetone in hexane (2% acetone to 100% acetone) to gain ten subfractions (OS7.1-7.9). Subfraction OS7.3 (0.097 g) was further purified by over silica gel 60H VLC column (3.5 g, diameter × height: 1.5 × 4 cm) eluted with increasing amount of CH_2Cl_2 in hexane (80% CH_2Cl_2 to 100% CH_2Cl_2) to obtain ten subfractions (OS7.3.1–7.3.10). Subfraction OS7.3.3 was identified as **4** (yellow oil, 3.9 mg). Fraction OS9 (3.64 g) was chromatographed over a silica gel 60H VLC column (56.5 g, diameter × height: 6.0 × 4 cm) eluted with increasing amount of MeOH in CH_2Cl_2 (6% MeOH to 100% MeOH) to yield eight subfractions (OS9.1-9.8). Subfraction OS9.3 was identified as **2** (white solid, 1.28 g). Fraction OS9S (0.29 g) was chromatographed over a silica gel RP-18 VLC column with 100% MeOH to give four subfractions (OS9S.1–4). Subfraction OS9S.3 was identified as **3** (white solid, 26.6 mg).

5(6)-gluten-3α-ol **(1)**: violet solid; m.p. 201-203 °C; IR ν_{max}^{ATR} 3456, 2923, 2866, 1458, 1384, 1180, 1091, 1033, 968, 825, 659 cm^{-1}; ^1H and ^{13}C NMR see Table 1.

quinovic acid 3-O-β-D-quinovopyranoside (**2**): white solid; m.p. 245-250 °C; IR ν_{max}^{ATR} 3390, 2923, 2869, 1689, 1450, 1384, 1307, 1222, 1172, 1064, 1002, 975, 825, 655 cm^{-1}; ^1H and ^{13}C NMR see Table 1.

daucosterol **(3)**: white solid; UV (MeOH) λ_{max} (log ε) 277 (3.58) nm; IR ν_{max}^{ATR} 3375, 2958, 2931, 2866, 1460, 1365, 1164, 1103, 1070 cm^{-1}; ^1H and ^{13}C NMR see Table 2.

scopoletin **(4)**: Yellow oil; UV (MeOH) λ_{max}: 225, 240, 245 and 346 nm; IR (thin film) ν_{max}: 3450, 1725, 1630, 1590, 1450, 1180, 940, 820 cm^{-1}; ^1H and ^{13}C NMR see Table 3.

Results and Discussion:

The EtOH extract of the stems of *O. bispinosus* was fractionated with water, EtOAc and *n*-BuOH. From the EtOAc fraction, four known compounds as a glutinane triterpenoid (**1**), a ursane triterpenoid glucoside (**2**), a known steroid glucoside (**4**), and a known coumarin (**4**) (Fig.1) were obtained by a combination using of silica gel 60H and RP-18 vacuum liquid column chromatography (VLC). The structures of the known compounds were determined by interpretation of their spectroscopic data as well as by comparison with reported data.

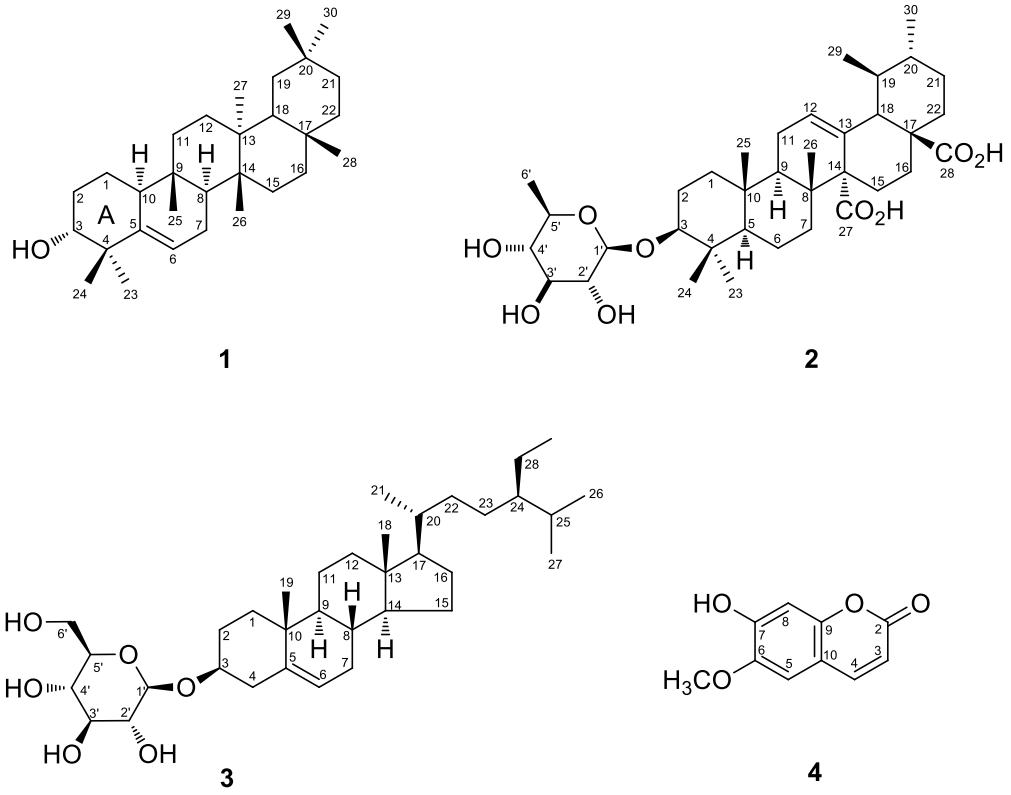

Figure 1. Structures of compounds **1**-**4**.

Compound **1** was obtained as a white solid. Its IR spectrum showed a hydroxyl band stretching at 3418 cm^{-1}. The ^{1}H NMR spectrum (Table 1) of **1** revealed the presence of a vinyl proton at δ 5.61 (br d, *J* = 6.0, H-6), one oxymethine proton at δ 3.45 (br s, H-3) and signals for eight methyls as singlets (δ 0.84, 0.94, 0.98, 1.00, 1.03, 1.08, 1.13, 1.15). Analysis of the ^{13}C NMR spectrum (Table 1), with the aid of DEPTs and HSQC experiments, revealed the presence of 30 carbon signals, involving eight methyls, one vinylic system at δ 122.0 (C-6) and 141.6 (C-5) and one oxygen-bearing carbon at δ 76.3 (C-3). These data suggested that **1** was a glutinane-type triterpenoid. Furthermore, a complete set of 2D NMR spectra (COSY, HSQC and HMBC) was acquired in order to corroborate the structure of **1**. The HMBC correlations observed from the signal at δ 3.45, attributed to the carbinol hydrogen at C-3, to the carbon resonances at δ 25.5 (C-23), 29.7 (C-24) and 141.6 (C-5) confirmed the cyclohexanol in A ring. The relative stereochemistry was established on the basis of the coupling constants and confirmed by a NOESY experiment. Thus, the observed cross peaks from H-3 to CH_3-23 and CH_3-24 indicated α-axial position for the hydroxyl group at C-3. Therefore, the structure of **1** was established as 5(6)-gluten-3α-ol, and comparing their spectroscopic data with literature values.[9]

Compound **2** was isolated as a white solid. The IR spectrum of 2 showed the bands at 3390 and 1689 cm^{-1} corresponding to hydroxyl and carbonyl group absorptions, respectively. The ^{1}H NMR spectrum of **2** (Table 1) showed signals readily recognized for four tertiary methyl singlet groups at δ 0.83, 0.89, 1.04 and 1.08, and for two secondary methyl doublet groups at δ 0.77 (d, *J* = 6.0 Hz) and 1.17 (d, *J* = 6.0 Hz), an olefinic proton at δ 5.95 (br s) and an oxygenated methine proton at δ 3.14 (dd, *J* =11.6, 3.9 Hz). Additionally, signals for an anomeric proton at δ 4.61 (d, *J* = 6.0 Hz) was also observed. The ^{13}C NMR spectrum (Table 1) showed 36 carbon signals, 30 for a triterpenoid aglycone and the remaining signals were for

the sugar moiety. The presence of two secondary methyl groups (δ 18.0 and 21.1), the chemical shifts of the olefinic carbons (δ 128.7 and 133.8) and two carboxyl groups (δ 177.8 and 179.8) suggested that **2** is an ursane-type triterpenoid with a double bond at C-12 and two carboxyl at C-27 and C-28. The further NMR analyses were performed with the aid of ^{1}H–^{1}H COSY, HSQC and HMBC spectroscopies. The sugar moiety of **2** was supposed to be *β*-rhamnopyranose based on the anomeric proton δ 4.61 (d, J = 6.0 Hz) and the ^{13}C NMR chemical shifts. Furthermore, the chemical shifts of the anomeric proton (δ 4.61) and carbon (δ 106.3) revealed that the rhamnopyranose was attached to the hydroxy group (C-3). This was confirmed by a long-range correlation between the anomeric proton and C-3 (δ 88.2) in the HMBC spectrum (Fig. 3). Thus, the structure of **2** was determined to be quinovic acid 3-O-β-D-quinovopyranoside, and comparing their spectroscopic data with literature values.[10]

Table 1.
NMR (400 MHz) data for compounds **1** ($CDCl_3$) and **2** (pyridine-d_5)

Position	5(6)-gluten-3α-ol (**1**)			quinovic acid 3-O-*β*-D-quinovopyranoside (**2**)		
	δ_C,	type	δ_H (J in Hz)	δ_C,	type	δ_H (J in Hz)
1	18.3,	CH_2		38.8,	CH_2	1.60, m
2	27.8,	CH_2		26.5,	CH_2	
3	76.3,	CH	3.45, brs	88.2,	CH	3.14, dd (11.1, 3.9)
4	40.8,	C		39.2,	C	
5	141.6,	C		55.5,	CH	0.90, m
6	122.0,	CH	5.61, brd (6.0)	18.3,	CH_2	1.45, m
7	23.6,	CH_2		36.8,	CH_2	
8	47.4,	CH	1.50, m	39.7,	C	
9	34.8,	C		46.9,	CH	2.64, dd (10.8, 4.4)
10	49.7,	CH	2.01, m	36.7,	C	
11	34.6,	CH_2		23.1,	CH_2	1.82-1.92, m
12	30.3,	CH_2		128.7,	CH	5.95, brs
13	37.8,	C		133.8,	C	
14	39.8,	C		56.5,	C	
15	32.1,	CH_2		26.1,	CH_2	
16	38.9,	CH_2		25.2,	CH_2	
17	30.1,	C		48.4,	C	
18	43.0,	CH	1.55, m	54.7,	CH	2.74, d (11.2)
19	33.1,	CH_2		37.5,	CH	
20	28.2,	C		39.1,	CH	
21	35.1,	CH_2		30.3,	CH_2	
22	36.0,	CH_2		37.6,	CH_2	
23	25.5,	CH_3	1.13, s	27.7,	CH_3	0.89, s
24	29.7,	CH_3	1.03, s	16.8,	CH_3	1.08 s
25	16.2,	CH_3	0.84, s	16.3,	CH_3	0.83 s
26	18.4,	CH_3	1.08, s	18.6,	CH_3	1.04 s
27	19.6,	CH_3	1.15, s	177.8,	CO_2H	
28	32.0,	CH_3	1.00, s	179.8,	CO_2H	

29	34.5, CH_3	0.94, s	18.0,	CH_3	1.17, d (6.0)
30	32.4, CH_3	0.98, s	21.1,	CH_3	0.77, d (6.0)
1′			106.3,	CH	4.61, d (7.6)
2′			75.6,	CH	3.88, t (8.4)
3′			78.0,	CH	4.02, d (8.8)
4′			76.6,	CH	3.62, d (8.8)
5′			72.3,	CH	3.72, m
6′			18.6,	CH_3	1.59, d (6.0)

Compound **3** was obtained as a white solid. The IR spectrum showed absorptions at 3375 cm^{-1} corresponding to hydroxyl group absorption. The ^{1}H and ^{13}C NMR data of **3** (Table 3) were typical of a steroid glycoside. The ^{13}C NMR spectrum showed 35 signals, of which 29 were assigned to the stigmastane nucleus and six were in the glycosidic region corresponding to a hexose unit. These signals were sorted out by HSQC and DEPT 135° spectra as six methyl, ten methylene, fourteen methine and three quaternary carbon atoms. The steroidal nature of the aglycone moiety of **3** was indicated in the ^{1}H NMR spectrum by the angular methyl singlets at δ 0.66 (s, CH_3-18) and 0.92 (s, CH_3-19) and the characteristic oxymethine multiplet at δ 3.97 (m, H-3). The ^{1}H NMR spectrum showed the characteristic signal of an anomeric proton as a doublet at δ 5.04 (d, H-1′); the coupling constant J = 7.7 Hz indicated the β-configuration of the sugar residue. The anomeric carbon signal of the glucose moiety appeared at δ 102.6 (C-1′) and the downfield chemical shift value of C-3 of the aglycone at δ 78.4 showed the linkage of sugar unit at this carbon. This was further confirmed in the HMBC spectrum by the correlation between H-3 and C-1′. The full assignment of ^{1}H and ^{13}C NMR (Table 2) resonances was confirmed by DEPT, ^{1}H–^{1}H COSY, HSQC, HMBC, and NOESY (Fig. 2). According to the evidence above, the structure of **3** was determined as daucosterol by comparing their spectroscopic data with literature values.[11]

Table 2.
NMR (400 MHz, $CDCl_3$) data for daucosterol (**3**).

Position	δ_C, type	δ_H (J in Hz)	Position	δ_C, type	δ_H (J in Hz)
1	37.5, CH_2	1.00-1.75, m	19	19.4, CH_3	0.92, s
2	30.2, CH_2	1.76, m -2.15, brd	20	36.4, CH	1.39, m
3	78.4, CH	3.97, m	21	19.0, CH_3	0.99, d (6.4)
4	39.3, CH_2	2.48-2.73, ddd	22	34.2, CH_2	1.10-1.40, m
5	141.0, C		23	26.4, CH_2	1.26, m
6	121.9, CH	5.35, brt (2.5)	24	46.0, CH	1.00, m
7	32.2, CH_2	1.55-1.92, m	25	29.5, CH	1.68, m
8	32.0, CH	1.40, m	26	19.2, CH_3	0.85, d (7.0)
9	50.3, CH	0.86, m	27	20.0, CH_3	0.87, d (7.0)
10	36.1, C		28	23.4, CH_2	1.31, m
11	21.3, CH_2	1.45, m	29	12.1, CH_3	0.89, t (7.6)
12	40.0, CH_2		1′	102.6, CH	5.04, d (7.7)
13	42.5, C		2′	75.3, CH	4.04, t (8.2)
14	56.8, CH	0.96, m	3′	78.5, CH	4.31, t (9.0)
15	24.5, CH_2	1.05-1.57, m	4′	71.7, CH	4.21, t (7.5)
16	28.5, CH_2	1.25-1.85, m	5′	78.1, CH	3.95, m

17	56.3, CH	1.10, m	6′	62.8, CH_2	4.35, dd (11.8, 2.4)
18	12.0, CH_3	0.66, s			4.53, dd (11.8, 5.4)

Compound **4** was isolated as a yellow oil. The IR spectrum showed absorption bands at 3450, 1725 and 1630 cm^{-1}, indicating presence of a hydroxyl group, a carbonyl group and an aromatic ring system, respectively. The UV spectrum displayed absorption bands at 225, 240, 245 and 346 nm, indicating presence of a 7-oxygenated coumarin moiety.[12] The ^{1}H NMR spectrum (Table 3) of **4** showed two proton doublets at δ 7.60 and 6.28 (J = 9.4 Hz, H-3 and H-4, respectively). The presence of further two proton singlets at δ 6.88 and 6.86 in the ^{1}H NMR spectrum, and resonance of carbons bearing two oxygen moieties at δ 150.4 and144.8 indicated a 6,7-disubstituted coumarin. According to the evidence above, the structure of **4** was determined as scopoletin by comparing their spectroscopic data with literature values.[13]

Table 3.
NMR (400 MHz, $CDCl_3$) data for scopoletin (**4**).

Position	δ_C, type	δ_H (J in Hz)
1		
2	162.1, CO	
3	112.5, CH	6.28, d (9.4)
4	143.8, CH	7.60, d (9.4)
5	107.9, CH	6.86, s
6	144.8, C	
7	150.4, C	
8	103.2, CH	6.88, s
9	150.1, C	
10	111.2, C	
6-OCH_3	56.3	3.93, s

Conclusion: In this paper, four known secondary metabolites, 5(6)-gluten-3α-ol (**1**), quinovic acid 3-*O*-*β*-D-quinovopyranoside (**2**), daucosterol (**3**), and scopoletin (**4**) were isolated from the stems of *Oxyceros bispinosus*. Their structures were elucidated on the basis of extensive NMR spectroscopic analyses and chemical method.

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