CHEMICAL CONSTITUENTS FROM THE LEAVES OF *Fissistigma rubiginosum*

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Abstract: An investigation on the chemical constituents in the leaves of *Fissistigma rubiginosum* was performed for the first time. Five known compounds including two flavonoids, (2*S*)-5,8-dihydroxy-7-methoxy-flavanone (**1**) and 6,7-dimethoxy-5-hydroxyflavone (**2**) one terpenoid, taraxerol (**3**), one sesquiterpenoid, selin-11-en-4α-ol (**4**), and one diterpenoid, phytol (**5**) were obtained. Their structures were elucidated on the basis of extensive spectroscopic analysis and compared with literatures.

Introduction: The genus *Fissistigma* (Annonaceae) comprises approximately 80 species, commonly found in India, Bangladesh, Myanmar, Thailand, Kampuchea, Malaysia, Indonesia.[1] Chemical investigation of various *Fissistigma* species have shown the present of alkaloids,[2-11] cyclopentenones,[12-13] flavonoids,[14-22] and sesquiterpenoids.[23] *Fissistigma rubiginosum* (A.DC.) Merr (synonym: *Uvaria rubiginosum*; *Melodorum rubiginosum*),) is an evergreen endemic climber of up to 10 m long, often with blackish bark and is widely distributed in the highland forest of the Southern Thailand. In Thailand, *F. rubiginosum* is locally known as 'Yan lueat'. In the present paper, we report the isolation and structural elucidation of two known flavonoids (**1**–**2**) and three known terpenoids (**3**–**5**) from the leaves of *F. rubiginosum*.

Methodology:

General Experimental Procedures

UV-Vis spectra were taken in MeOH solution on a SPECORD® 210 PLUS analytik Jena spectrophotometer. IR spectra were recorded with a Shimadzu FTIR-8900 IR spectrophotometer. NMR spectra were recorded in $CDCl_3$ or pyridine-d_5 with TMS as the internal reference on a Bruker AVANCE400 spectrometer (^{1}H at 400 MHz and ^{13}C at 100 MHz). Vacuum liquid chromatography (VLC) was carried out on silica gel 60H (Merck, 5-40 µm) and RP-18 (Merck, 15-25 µm). TLC was performed on precoated silica gel 60 F_{254} plates (Merck) and RP-18 F_{254S} plates (Merck). Fractions were monitored by TLC using Merck pre-coated silica gel 60F_{254} and RP-18 F_{254} sheets and spots were visualized by using fluorescence (254 and 386 nm) and by heating silica gel plates sprayed with 1% $Ce(SO_4)_2$ in 10% aq. H_2SO_4 solution.

Plant material

The leaves of *F. rubiginosum* were collected from Krabi Province, Thailand, in February 2016. The identification of the plant material was authenticated by Dr. Piya Chalermglin, Thailand Institute of Scientific and Technological Research, Thailand. A voucher specimen (PKRU2 0 1 60 01) was deposited at the Laboratory of Natural Products Chemistry, Faculty of Science and Technology, Phuket Rajabhat University, Phuket, Thailand.

Extraction and Isolation

Powdered leaves of *F. rubiginosum* (1.5 kg) were extracted with MeOH for three times (3 × 11 L, total amount 33 L) at room temperature, filtered residue, removed solvents under low pressure, obtained crude extract (157.41 g). Then, crude extract was dissolved with H_2O

and partitioned into EtOAc (35.7 g) and n-BuOH (43.03 g). The EtOAc extract was subjected to silica gel 60H (766.6 g, diameter × height: 12.5 × 5.0 cm) vacuum liquid column chromatography (VLC) and eluted with gradient solvent system of EtOAc in hexane (1% EtOAc to 100% EtOAc) and 100% MeOH to afford 12 fractions (FL1–FL12). FL3 (7.45 g) was recrystallize with MeOH to give **3** (white solid, 147.1 mg). FL4 (4.35 g) was chromatographed by VLC on silica gel 60H (49.06 g, 5.0 × 4.0 cm) and eluted with increasing amount of EtOAc in hexane (1% EtOAc to 100% EtOAc) to obtain 9 subfractions (FL4.1–4.9). FL4.4 (1.4 g) was applied on a silica gel 60H (16.8 g, 3.5 × 4.0 cm) VLC column and eluted with increasing amount of CH_2Cl_2 in hexane (30% CH_2Cl_2 to 100% CH_2Cl_2) to yield **4** (colorless oil, 15.5 mg). The same manner FL4 was applied to FL5 (4.56 g), eluted with increasing amount of EtOAc in hexane (100% hexane to 100% EtOAc) to give 5 subfractions (FL5.1–5.5). FL5.4 (2.57 g) was separated by silica gel 60H (70.6 g, 6.0 × 5.0 cm) VLC column, eluted with increasing amount of EtOAc in hexane (2% EtOAc to 100% EtOAc) to provide 7 subfractions (FL5.4.1–5.4.7). FL5.4.4 (0.45 g) was further purified by RP-18 (12.2 g, 2.5 × 4.0 cm) VLC column, eluted with 100% MeOH to give 4 subfractions (5.4.4.1–5.4.4.4). FL5.4.4.2 (0.282 g) was done as the same manner, further separated by VLC with increasing amount of CH_2Cl_2 in hexane (70% CH_2Cl_2 to 100% CH_2Cl_2) to affrord **2** (pale yellow solid, 5 mg). FL6 (2.64 g) was separated by silica gel 60H (49.4 g, 6.0 × 3.5 cm) VLC column, eluted with increasing amount of CH_2Cl_2 in hexane (50% CH_2Cl_2 to 100% CH_2Cl_2) to give 6 subfractions (FL6.1–6.6). FL6.2 (23.8 mg) was further recrystallize with MeOH to obtain **1** (yellow solid, 5.2 mg).

*(2S)-5,8-dihydroxy-7-methoxy-flavanone (**1**)*: yellow solid; $C_{16}H_{14}O_5$; UV (MeOH) λ_{max} nm (log ε) 232 (4.10), 280 (4.32), 325 (3.75) nm; IR ν_{max}^{ATR} 3420, 1680, 1590, 1210, 1200, 1145, 1032 cm^{-1}; ^1H and ^{13}C NMR, see Table 1.

*6,7-dimethoxy-5-hydroxyflavone (**2**)*: pale yellow solid; $C_{17}H_{14}O_5$; UV (MeOH) λ_{max} nm (log ε) 263 (4.50), 310 (4.10), 337 (4.01) nm; IR ν_{max}^{ATR} 3375, 1650, 1610, 1340, 1128, 1080, 1021 cm^{-1}; ^1H and ^{13}C NMR, see Table 1.

*taraxerol (**3**)*: white solid; $C_{30}H_{50}O$; m.p. 354-356 °C; IR ν_{max}^{ATR} 3483, 3010, 2916, 2850, 1461, 1442, 1415, 1380, 1033, 999, 813, 690 cm^{-1}; ^1H and ^{13}C NMR, see Table 2.

*selin-11-en-4α-ol (**4**)*: white solid; $C_{15}H_{26}O$; $[\alpha]_D^{25}$ **–** 69.2° (*c* 0.02, $CHCl_3$); m.p. 82-84 °C; UV (MeOH) λ_{max} nm (log ε) 278 (3.18) nm; IR ν_{max}^{KBr} 3314, 2926, 2853, 1641, 1454, 1377, 1169, 881 cm^{-1}; ^1H and ^{13}C NMR, see Table 3.

*phytol (**5**)*: colorless oil; $C_{20}H_{40}O$; UV (MeOH) λ_{max} nm (log ε) 239 (2.44) nm; ν_{max}^{ATR} 3336, 2868, 1672, 1461, 1377, 1365, 1168, 1002, 734, 410 cm^{-1}; ^1H and ^{13}C NMR, see text.

Results and Discussion: The CH_3OH-soluble extract of the leaves of *F. rubiginosum* was successively fractionated with EtOAc and *n*-BuOH. The EtOAc fraction was separated by a combination of silica gel 60H and RP-18 silica gel vacuum liquid column chromatography (VLC), to provide five compounds including two known flavonoids (**1**–**2**) and three known terpenoids (**3**–**5**) (Fig. 1).

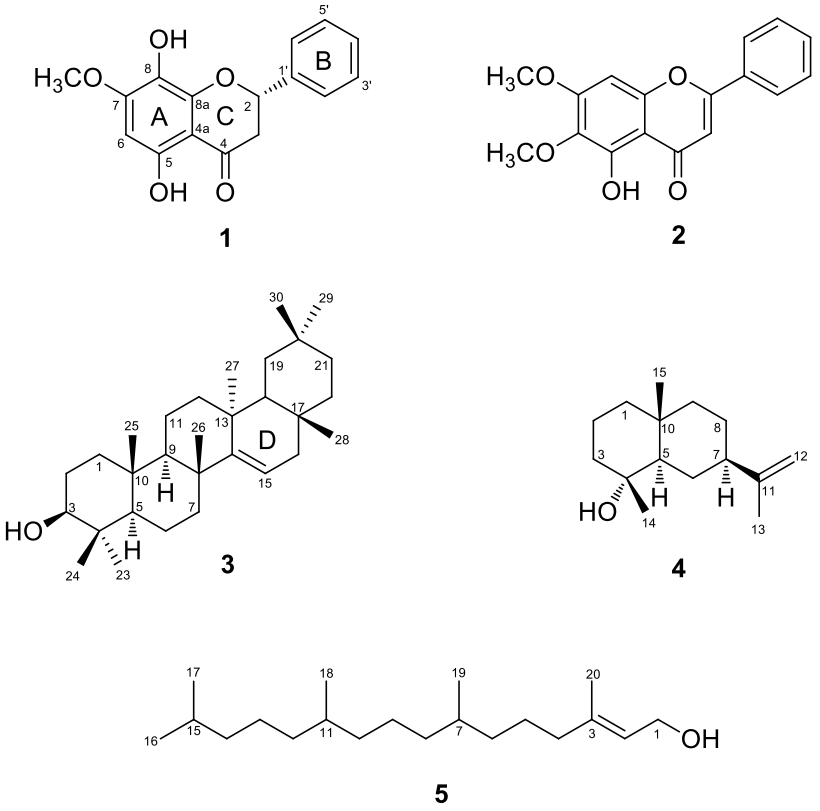

Figure 1. Structures of compounds **1**-**5**.

Compound **1** was obtained as a yellow solid with a molecular formula of $C_{16}H_{14}O_5$ based on ^{1}H and ^{13}C NMR spectroscopic data analysis, indicating 10 degrees of unsaturation. The IR spectrum of **1** showed absorption band at 3420, 1680 and 1590 cm^{-1} ascribable to hydroxyl, carbonyl and aromatic groups, respectively. The ^{1}H NMR spectrum (Table 1) of **1** show intramolecular hydrogen bonding signal at δ_H 12.39 (s) as well as display carbonyl signal in its ^{13}C NMR spectrum. The ^{1}H NMR showed a non-substituted phenyl moiety at δ 7.57 (2H, dd, *J* = 7.2, 2.0 Hz, H-2′, 6′) and 7.35 (3H, m, H-3′, 4′, 5′) related with the B-ring. A flavanone skeleton was evident from the coupling pattern of the C-ring protons δ 2.92 (1H, dd, *J* = 16.8, 2.8 Hz, H-3), 3.27 (1H, dd, *J* = 16.8, 12.4 Hz, H-3) and 5.61 (1H, dd, *J* = 12.4, 2.8 Hz, H-2). Analysis of the ^{13}C NMR spectrum and DEPT experiments (Table 1), confirmed a flavanone moiety with the presence of a typical ketone carbonyl signal at δ 196.7. The ^{1}H NMR spectrum of **1** also exhibited an aromatic methine proton as a singlet at δ 6.47 (1H, s, H-6), an aromatic methoxy group at δ 3.81 (3H, s, OCH_3-7). The positions of a methoxy group at C-7 and the phenolic hydroxyl group at C-8 were established from the connectivities indicated in the 2D HMBC experiments (Fig. 2). The methoxy proton at δ_H 3.81 showed the long-range HMBC correlation to the signal of C-7 (δ_C 157.8). The phenolic hydroxyl group at δ_H 12.39 was correlated with C-6 (δ_C 93.3), C-5 (δ_C 156.8) and C-4a (δ_C 103.3). The important long-rang correlations were also observed between H-6 (δ_H 6.47) and C-4a (δ_C 103.3), C-5 (δ_C 156.8), C-7 (δ_C 157.8) and C-8 (δ_C 128.5). The full assignment of ^{1}H and ^{13}C NMR (Table 1) resonances was confirmed by DEPT, ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY (Fig. 2). According to the evidence above, the structure of **1** was identified as (2*S*)-5,8-dihydroxy-7-methoxy-flavanone by comparing their spectroscopic data with literature values.[24]

Compound **2** was isolated as a pale yellow solid, had a molecular formula of $C_{17}H_{14}O_5$ as established by ^{1}H and ^{13}C NMR spectroscopic data analysis, suggesting 11 degrees of unsaturation. The UV and IR spectra of **2** showed very similar to those **1**. Seventeen ^{13}C NMR

signals, including twelve aromatic carbons, one unsaturated carbonyl carbon as well as two methoxy carbons indicated that **2** possessed flavone skeleton. The ^{1}H NMR spectrum (Table 1) of **2** showed the presence of five mutually coupling aromatic protons at δ 7.55 (3H, m, H-3′, 4′, 5′), 7.90 (2H, dd, *J* = 8.0, 1.6 Hz, H-2′, 6′), two singlet aromatic signals at δ 6.69 (1H, m, H-3) and δ 6.58 (1H, s, H-8) two methoxy groups at δ 3.93 (3H, s, OCH_3-6), 3.98 (3H, s, OCH_3-7), and a hydroxyl group at δ 12,69 (1H, s, OH-5). The full assignment of ^{1}H and ^{13}C NMR (Table 1) resonances was confirmed by DEPT, ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY (Fig. 2). According to the evidence above, the structure of **2** was elucidated as 6,7-dimethoxy-5-hydroxyflavone by comparing their spectroscopic data with literature values.[25]

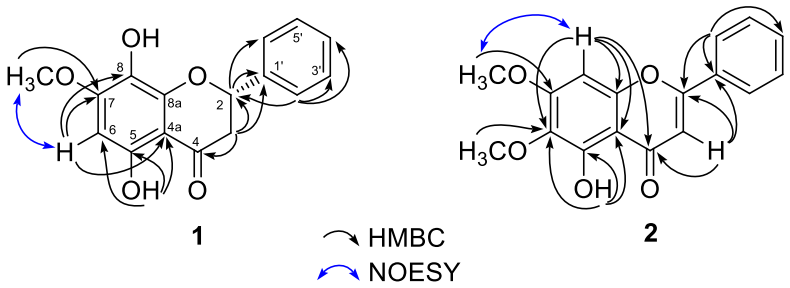

Figure 2. Selected HMBC and NOESY correlation of **1** and **2**.

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

	(2*S*)-5,8-dihydroxy-7-methoxy-flavanone (**1**)		6,7-dimethoxy-5-hydroxyflavone (**2**)	
Position	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
2	79.6, CH	5.61, dd (12.8, 2.8)	164.0, C	
3	43.5, CH_2	2.92, dd (16.8, 2.8)	105.7, CH	6.69, s
		3.27, dd (16.8, 12.4)		
4	196.7, C=O		182.3, C=O	
4a	103.3, C		106.1, C	
5	156.8, C		133.4, C	
6	93.3, CH	6.47, s	134.0, C	
7	157.8, C		159.0, C	
8	128.5, C		90.8, CH	6.58, s
8a	148.4, C		153.0, C	
1′	139.2, C		131.5, C	
2′,6′	126.9, CH	7.57, dd (7.2, 2.0)	126.3, CH	7.90, dd (8.0, 1.6)
3′,5′	128.8, CH	7.35, m	129.1, CH	7.55, m
4′	128.7, CH	7.35, m	131.8, CH	7.55, m
5-OH		12.39, s		12.69, s
6-OCH_3			61.1, CH_3	3.93, s
7-OCH_3	55.9, CH_3	3.81, s	56.6, CH_3	3.98, s

Compound **3** was obtained as a white solid. The IR spectrum indicated the presence of the hydroxyl group (3483 cm^{-1}) and a trisubstituted double bond (3010, 1461 and 813 cm^{-1}). The ^{1}H and ^{13}C NMR spectra of **3** showed signals for eight tertiary methyl groups at δ_H 0.80, 0.82, 0.90×2, 0.92, 0.95, 0.98, and 1.09 and δ_C 15.4×2, 21.3, 25.9, 28.0, 29.8, 29.9, 33.1 and 33.3, a trisubstituted double bond at δ_H 5.53 and δ_C 116.9, 158.1, and a hydroxymethine group at δ_H 3.20 and δ_C 79.1. These data were reminiscent of the presence of an taraxerane-type with a double bond located in D ring and a hydroxyl group at C-3. The full assignment of ^{1}H and ^{13}C NMR (Table 2) resonances was confirmed by DEPT, ^{1}H–^{1}H COSY, HSQC, and HMBC techniques. According to the evidence above, the structure of **3** was elucidated as taraxerol by comparing their spectroscopic data with literature values.[26,27]

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

Position	δ_C, type	δ_H (*J* in Hz)	Position	δ_C, type	δ_H (*J* in Hz)
1	37.7, CH_2		16	36.7, CH_2	1.92, dd (14.1, 2.4), 1.64 , m
2	27.1, CH_2		17	37.7, C	
3	79.1, CH	3.20, dd (10.2, 4.4 Hz)	18	48.7, CH	1.40, m
4	39.0, C		19	41.1, CH_2	
5	55.5, CH	0.78, dd (8.7, 2.0)	20	28.8, C	
6	18.8, CH_2		21	33.7, CH_2	
7	35.1, CH_2	2.03, tt (12.8, 3.2)	22	31.1, CH_2	
8	38.7, C		23	28.0, CH_3	0.98, s
9	49.3, CH	1.45, m	24	15.4, CH_3	0.80, s
10	38.0, C		25	15.4, CH_3	0.92, s
11	17.5, CH_2		26	29.8, CH_3	1.09, s
12	35.8, CH_2		27	25.9, CH_3	0.90, s
13	37.6, C		28	29.9, CH_3	0.82, s
14	158.1, C		29	33.3, CH_3	0.95, s
15	116.9, CH	5.53, dd (8.0, 3.2)	30	21.3, CH_3	0.90, s

Compound **4** was isolated as a colorless gum with $[\alpha]_D^{25} - 69.2°$ (*c* 0.02, $CHCl_3$). It molecular formula of $C_{15}H_{26}O$ as established by ^{1}H and ^{13}C NMR spectroscopic data analysis, suggesting 3 degrees of unsaturation. The IR absorption bands implied the presence of the hydroxy group (3314 cm^{-1}). The ^{1}H NMR spectrum (Table 3) of **4** displayed signals for an exomethylene at δ 4.70 (1H, s) and 4.71 (1H, br s) and three tertiary methyls at δ 1.75 (3H, s), 1.12 (3H, s), and 0.89 (3H, s). The ^{13}C NMR (Table 3) showed 15 carbon resonances, which were classified by DEPT and the HSQC spectra as three methyls, seven methylenes (one olefinic), two methines, and three quaternary carbons (one oxygenated and one olefinic). These spectral data suggested an eudesmane derivative. The full assignment of ^{1}H and ^{13}C NMR resonances was confirmed by DEPT, ^{1}H–^{1}H COSY, HSQC, and HMBC (Table 3) techniques. According to the evidence above, the structure of **4** was elucidated as selin-11-en-4α-ol by comparing their spectroscopic data with literature values.[28]

Table 3.
NMR (400 MHz, $CDCl_3$) data for selin-11-en-4α-ol (**4**)

Position	δ_C, type	δ_H (*J* in Hz)	HMBC
1	41.1, CH_2	0.86, m, 1.41, m	5
2	20.1, CH_2	1.57, m, 1.58, m	1, 3, 4, 10
3	43.4, CH_2	1.38, m, 1.81, m	14
4	72.3, C		
5	54.9, CH	1.27, m	6, 7, 10, 15
6	26.0, CH	1.85, m, 1.93, m	13
7	46.3, CH	1.95, m	13
8	26.9, CH_2	1.45, m, 1.46, m	10
9	44.7, CH_2	1.22, m, 1.46, m	5, 7, 10, 15
10	34.6, C		
11	150.7, C		
12	108.1, CH_2	4.70, br s 4.71, br s	7, 13
13	21.1, CH_3	1.75, s	7, 11, 12
14	22.7, CH_3	1.12, s	3, 4, 5
15	18.7, CH_3	0.89, s	1, 5, 9, 10

Compound **5** was obtained as a colorless oil. The IR spectrum revealed the presence of hydroxyl group (3336 cm^{-1}) and double bond (1672 cm^{-1}) in the molecule. Detailed analysis of the NMR data demonstrated that good agreement with phytol: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.40 (1H, t, *J* = 6.8 Hz, H-2), 4.14 (2H, d, *J* = 6.8 Hz, H-1), 1.98 (2H, t, *J* = 6.8 Hz, H-4), 1.66 (3H, s, H-20), 0.87 (6H, d, *J* = 6.4 Hz, H-16,17), 0.85 (3H, br d, H-19), 0.84 (3H, br d, H-18); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.2 (C-3), 123.1 (C-2), 59.4 (C-1), 39.9 (C-4), 39.4 (C-14), 37.4 (C-8), 37.3 (C-10), 37.2 (C-12), 36.7 (C-6), 32.8 (C-11), 32.7 (C-7), 28.0 (C-15), 25.1 (C-9), 24.8 (C-5), 24.5 (C-13), 22.7 (C-17), 22.6 (C-16), 19.7 (C-18,19), 16.2 (C-20). From all the evidence mentioned above, the structure of compound **5** was elucidated as phytol.[29]

Conclusion: The present report is the first study of *Fissistigma rubiginosum*. Five secondary metabolites (**1**–**5**) including two known flavonoids, (2*S*)-5,8-dihydroxy-7-methoxy-flavanone (**1**) and 6,7-dimethoxy-5-hydroxyflavone (**2**), one triterpenoid, taraxerol (**3**), one sesquiterpenoid, selin-11-en-4α-ol (**4**), and one diterpenoid, phytol (**5**) were isolated and characterised by spectrometric analysis (UV, IR, 1D and 2D NMR) as well as comparing with reported values.

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