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ISOLATION AND CHARACTERIZATION OF COMPOUNDS FROM THE STEM BARK OF *UVARIA RUFA* (ANNONACEAE)

(Pemisahan dan Pencirian Sebatian dari Kulit Batang *Uvaria rufa* (Annonaceae))

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Abstract

Isolation of compounds from methanol extract of the stem bark of Uvaria rufa has been conducted by using radial chromatography. Their structures were elucidated by UV, IR, NMR and mass spectroscopy, and by comparison with the literature. Seven compounds were isolated namely benzyl benzoate (**1**), caryophyllene oxide (**2**), glutinol (**3**), 5-hydroxy-7-methoxyflavone (**4**), 5-hydroxy-6,7-dimethoxyflavone (**5**), 2,5-dihydroxy-7-methoxyflavanone (**6**) and 5,7-dihydroxyflavanone (**7**). Separation of the caryophyllene oxide (**2**), glutinol (**3**) and 5,7-dihydroxyflavanone (**7**) from Uvaria rufa has never been reported.

Keywords: *Annonaceae*, Uvaria rufa, *terpene, flavonoid*

Abstrak

Pengasingan sebatian dari ekstrak metanol kulit batang Uvaria rufa telah dilakukan dengan menggunakan kromatografi radial. Struktur semua sebatian ditentukan dengan spektroskopi UL, IM, RMN dan jisim, dan secara perbandingan dengan data kepustakaan. Tujuh sebatian telah diasingkan iaitu benzil benzoat (**1**), kariofilena oksida (**2**), glutinol (**3**), 5-hidroksi-7-metoksiflavon (**4**), 5-hidroksi-6,7-dimetoksiflavon (**5**), 2,5-dihidroksi-7-metoksiflavanon (**6**) dan 5,7-dihidroksiflavanon (**7**). Pemisahan kariofilena oksida (**2**), glutinol (**3**) dan 5,7-dihidroksiflavanon (**7**) dari Uvaria rufa belum pernah dilaporkan.

Kata Kunci: *Annonaceae*, Uvaria rufa, *terpena, flavonoid*

Introduction

Uvaria, a genus of Annonaceae family, consists of approximately 150 species that are distributed in several areas including tropical Africa, Madagascar, Indo-Malaya and Australia [30]. Most plants of the genus *Uvaria* are found to grow as vines on trees. Some are known as Larak (Malaysia), Kalak (Java) and Allagat or Hinlalaki (Luzon). *Uvaria rufa* or *U. ridleyi* is widely available in Peninsular Malaysia, and have been named by the locals as Larak or Pisang-pisang [16]. This plant was reported to be found in the north-west and southern part of Peninsular Malaysia, namely the River Tebrau in Johor and Pahang [4]. Traditionally, all parts of this plant can be used for specific purposes; the squeezed leaves afford a cinnamon bark-like smell and water decoctions can be consumed directly, while the fruits are believed to cure certain diseases like ulcers of the intestines [4]. It has also been mentioned that the water decoction of the roots are used to treat women after giving birth [11]. *Uvaria rufa* is a rich source of various new compounds as proven by the isolation of benzoylated derivatives [17], flavonoids [5] and flavonoid

glycosides [9], essential oils [3], oxygenated cyclohexanes [29] and polyoxygenated cyclohexenes [31]. In our recent study on methanol extract of the stem bark of *U. rufa*, we have successfully isolated seven compounds.

Materials and Methods

Plant material

The stem bark of *Uvaria rufa* was collected from the secondary forest in Parit, Perak, Malaysia. A herbarium specimen of BKP0001 was deposited at the Universiti Kebangsaan Malaysia Herbarium.

Extraction and Isolation

Dried ground stem bark of *Uvaria rufa* (500 g) was macerated with methanol at room temperature. The filtrate was concentrated using rotary evaporator to yield a dark green extract (13 g, 2.65 %). A 3 g portion of the extract was fractionated by using radial chromatography (RC) with 4 mm thickness silica gel on a round glass plate eluted with increasing polarity of *n*-hexane-EtOAc. The eluents that showed the same profile on thin layer chromatography (TLC) chromatogram were combined to give three fractions (I-III). Purification of fraction I (600 mg) was carried out using RC with silica gel plate of 1 mm thickness eluted with 95:5 *n*-hexane-EtOAc in 5% polarity increment to yield compounds **1** (550 mg) and **2** (26 mg). Purification of fraction II (410 mg) was conducted utilizing another RC with silica gel plate of 1 mm thickness. Elution with 9:1 *n*-hexane-EtOAc produced compounds **3** (18 mg) and **4** (36 mg). Purification of fraction III (430 mg) was then performed using RC with silica gel plate of 1 mm. Elution with 7:2:1 *n*-hexane-EtOAc-$CHCl_3$ resulted in compounds **5** (28 mg), **6** (56 mg) and **7** (12 mg).

Compound Identification

Structures of pure compounds were determined based on the spectral data recorded on Shimadzu UV-260 spectrophotometer, Frontier Perkin-Elmer FTIR/NIR spectrophotometer and Bruker NMR 600 MHz Cryo-Probe instrument that included 1-D and 2-D NMR. Melting points were measured using Stuart SMP10 melting point apparatus. ESIMSs were recorded by using GC/MS Model GC5890 Series II/MSD-5970 HP. The isolation was then carried out by radial chromatography using round glass plates of the Merck Kieselgel 60 PF_{254} (art. no. 7749) and profile was analyzed using smaller pieces from aluminium sheets 20 x 20 cm of the Merck TLC silica gel 60 F_{254} of 0.25 mm thickness (art. no. 5554) and then detected under UV light (254 nm) or by $CeSO_4$ spraying reagent followed by heating.

Benzyl benzoate (**1**): Pale yellow oily liquid (550 mg), ESIMS $[M]^+$ at *m/z* 212, FT-IR (ATR, attenuated total reflectance) ν_{max} cm^{-1}: 3034-2953 (weak), 1715, 1601-1451, 1265 and 707-695. ^1H NMR ($CDCl_3$, 600 MHz) δ_H (ppm) and ^{13}C NMR ($CDCl_3$, 150 MHz) δ_C (ppm) are tabulated in Table 1.

Caryophyllene oxide (**2**): White amorphous (26 mg), 59-61 ^oC (60-61 ^oC, Chavan et al. 2010), ESIMS $[M]^+$ at *m/z* 220, FT-IR (ATR) ν_{max} cm^{-1}: 2927-2867, 1640, 970. ^1H NMR ($CDCl_3$, 600 MHz) δ_H (ppm) and ^{13}C NMR ($CDCl_3$, 150 MHz) δ_C (ppm) are tabulated in Table 2.

Glutinol (**3**): White amorphous (18 mg), m.p. 202-203°C, ElMS $[M]^+$ at *m/z* 426, FT-IR (ATR) ν_{max} cm^{-1}: 3391, 2927, 1726 and 1072 (weak). ^1H NMR ($CDCl_3$, 600 MHz): δ_H (ppm) 0.86, 0.96, 1.00, 1.01, 1.05, 1.10, 1.15, 1.17 (each 3H, *s*, $8CH_3$), 1.23-2.05 (23H, complex, $10CH_2$ + 3CH), 3.49 (lH, *d*, J = 13.8 Hz, H-3) and 5.64 (lH, *d*, *J* = 6.6 Hz, H-6). ^{13}C NMR ($CDCl_3$, 150 MHz) δ_C (ppm) data are tabulated in Table 3.

5-Hydroxy-7-methoxyflavone (**4**): Yellow amorphous (36 mg), m.p. 164-165^oC, EIMS $[M]^+$ at *m/z* 268, UV [MeOH] λ_{max} 269 and 310 nm, [MeOH+NaOH] λ_{max} 283 and 377 nm, FT-IR (ATR), ν_{max} cm^{-1}: 3100-2800, 1652, 1600-1590, 1470-1430, 1350-1340 and 1270-1210. ^1H NMR ($CDCl_3$, 600 MHz) δ_H (ppm) and ^{13}C NMR ($CDCl_3$, 150 MHz) δ_C (ppm) are tabulated in Table 4.

5-Hydroxy-6,7-dimethoxyflavone (**5**): Yellow amorphous (28 mg), m.p. 158-159^oC, EIMS $[M]^+$ at *m/z* 298, UV [MeOH] λ_{max} 314 and 273 nm, [MeOH+NaOH] λ_{max} 314 and 384 nm, FT-IR (ATR) ν_{max} cm^{-1}: 2850-2950, 1620-1640, 1590-1600, 1430-1470, 1350-1340, and 1120-1150. ^1H NMR ($CDCl_3$, 600 MHz) δ_H (ppm) and ^{13}C NMR ($CDCl_3$, 150 MHz) δ_C (ppm) are tabulated in Table 4.

2,5-Dihydroxy-7-methoxyflavanone (**6**): Colourless crystals (56 mg), m.p. 174-176°C, EIMS $[M]^+$ at *m/z* 286, UV [MeOH] λ_{max} 289 nm, [MeOH+NaOH] λ_{max} 387 nm, FT-IR (ATR) ν_{max} cm^{-1}: 3409, 1640, 1575-1449, 1315, and 1156. ^1H NMR ($CDCl_3$, 600 MHz), δ_H (ppm) and ^{13}C-NMR ($CDCl_3$, 150 MHz) δ_C (ppm) are tabulated in Table 4.

5,7-Dihydroxyflavanone (**7**): Colourless amorphous (8 mg), m.p 285-286°C, EIMS $[M]^+$ at *m/z* 254, FT-IR (ATR) ν_{max} cm^{-1}: 3403, 1638, 1600-1590, 1447, 1317 and 1190-1156. ^1H NMR ($CDCl_3$, 600 MHz), δ_H (ppm) and ^{13}C-NMR ($CDCl_3$, 150 MHz) δ_C (ppm) are tabulated in Table 4.

Results and Discussion

Chromatographic separation of the methanol extract of the stem bark of *Uvaria rufa* on silica gel Merck $60F_{254}$ (art. no. 7749) resulted in the isolation of compounds **1-7** (Figure 1).

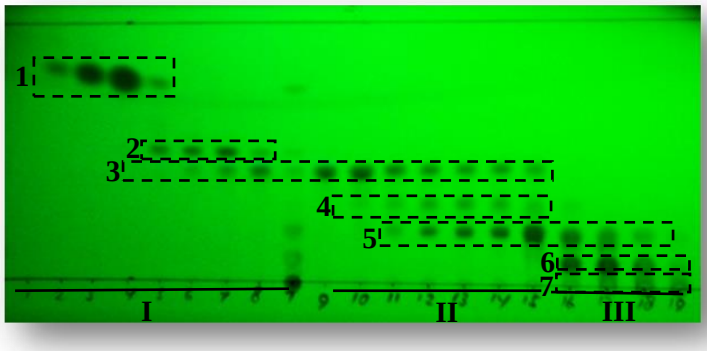
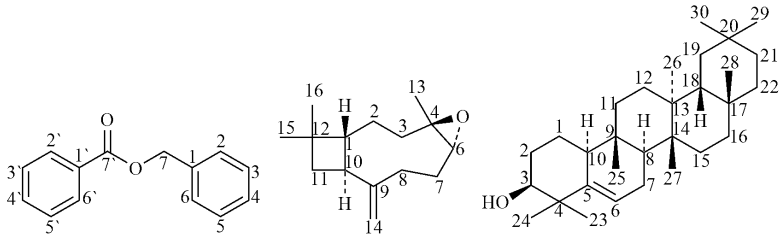
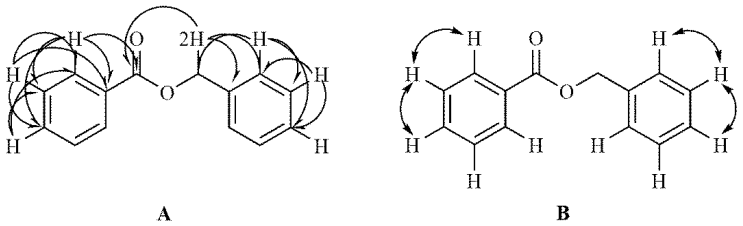


Figure.1. TLC profile of compounds present in the extract of stem bark of *Uvaria rufa* (Merck, art. no. 5554; n-hexane-EtOAc, 9:1). Combination of fractions 1-8, 9-15 and 16-19 gave respective fractions I, II and III. Similar horizontal spots designated as **1** (R_f 0.70), **2** (0.64), **3** (0.50), **4** (0.39), **5** (0.25), **6** (0.14) and **7** (0.06) represent respective benzyl benzoate (**1**), caryophyllene oxide (**2**), glutinol (**3**), 5-hydroxy-7-methoxyflavone (**4**), 5-hydroxy-6,7-dimethoxyflavone (**5**), 2,5-dihydroxy-7-methoxyflavanone (**6**) and 5,7-dihydroxyflavanone (7).



Benzyl benzoate (**1**) [1, 2; 18, 19; 23; 24] was obtained in the form of yellow pale oily liquid. The mass spectrum showed a molecular ion peak ESIMS $[M]^+$ at *m/z* 212 which was analyzed for the molecular formula $C_{14}H_{12}O_2$. FTIR (ATR) spectrum indicated that the compound contained a carbonyl group with stretching at 1715 cm^{-1}, ether at 1265 cm^{-1}, C=C aromatic at 1601-1451 cm^{-1} and *ortho* coupling in the benzene ring at 707-695 cm^{-1}. The presence of functional groups is supported by NMR analysis of 1-D (^1H, ^{13}C-NMR and DEPT) and 2-D (HSQC, HMBC and H-H COSY) data. Spectrum of ^{13}C-NMR showed 10 signals representing 14 carbons, which consisted of ten methine carbons for two phenyls at 133.1, 129.8, 128.7, 128.5, 128.3 and 128.2 ppm; a methylene carbon at 66.7 ppm; and three quaternary carbons including one carbonyl carbon at 166.4 ppm and two aromatic carbons at 136.2 and 130.3 ppm. Meanwhile, the ^1H NMR spectrum showed 7 signals representing 12 protons. Furthermore,

experiments of HMBC as shown in A and H-H COSY in **B** established the final structure of benzyl benzoate. 1-D and 2-D NMR data for benzyl benzoate (**1**) are tabulated in Table 1.



A **B**

Table 1. NMR data of benzyl benzoate (**1**)

No. of C	δ_C (ppm)	δ_H (ppm) (mult., *J* in Hz)	2-D NMR	
			HMBC	H-H COSY
1	136.2	-	-	-
2/6	128.2	7.53 (*d*, 8.1) (2H)	C-3/5, C-4, C-7	H-3/5
3/5	128.3	7.48 (*t*, 7.7) (2H)	C-2/6, C-4	H-2/6, H-4
4	128.5	7. 40 (*t*, 7.2) (1H)	C-2/6, C-3/5	H-3/5
7	66.7	5.44 (*s*) (2H)	C-7`, C-1, C-2/6	-
1`	130.3	-	-	-
2`/6`	129.8	8.17 (*d*, 8.1) (2H)	C-1`, C-3`/5`, C-4`, C-7`	H-3`/5`
3`/5`	128.7	7.45 (*t*, 7.5) (2H)	C-1`, C-2`/6`, C-4`	H-2`/6`, H-4`
4`	133.1	7.59 (*t* , 7.5) (1H)	C-2`/6`, C-3`/5`	H-3`/5`
7`	166.4	-	-	-

Caryophyllene oxide (**2**) [6; 8; 26] is a white amorphous. FT-IR (ATR) ν_{max} cm^{-1}: 1640 (epoxy), 2959-2858 (C-C cyclic aliphatic), 1541-1458 (weak, C=C), 1277 and 772 (epoxy), and ν_{max}1128-1073 (C=C olefinic). The ^{13}C-NMR data showed the presence of 15 carbon signals representing three quaternary carbons at δ_C of 151.8, 60.0 and 34.0 ppm; six methylene carbons at δ_C of 112.8, 39.8, 39.2, 30.2, 29.8 and 27.2 ppm; three methine carbons at δ_C of 63.8, 50.8 and 48.7 ppm; and three methyl carbons at δ_C of 29.9, 21.6 and 17.0 ppm. Based on the data, compound **2** can be assigned as a cyclic aliphatic sesquiterpene with an epoxy formed across C-4 and C-6, while a double bond is formed between C-9 and C-14. Meanwhile, 18 proton signals representing 24 protons appeared in the ^{1}H-NMR spectrum as shown in Table 2. Eighteen of the signals came from the three signals, one each from a methyl group at C-epoxy and the two non-equivalent gem-dimethyls at C-12; three signals from the three methine protons at C-1, C-6 and C-10; two respective signals of the saturated non-equivalent methylene protons C-2, C-3, C-7, C-8, C-11 and olefinic non-equivalent methylene protons C-14. The H-H COSY experiment indicated that protons at δ_H 4.89 and 5.00 ppm were related to each other and marked as olefinic methylene protons at C-14. Furthermore, the allylic methine proton at δ_H 2.65 ppm (C-10) was proven by an association between methine proton at C-1 (δ_H 1.77 ppm) and methylene protons at C-11 (δ_H 1.70 and 1.63 ppm). This assumption on compound **2** was highly supported by the similar parameters between ^{13}C- and ^{1}H-NMR data with those of caryophyllene oxide which was reported by Orihara et al. (1994) [20] (Table 2).

Table 2. Comparison of NMR data of caryophyllene oxide (**2)** with those of the literature*****

No. of C	δ_C (ppm)		No. of H	δ_H (ppm)
	2	*****		**2**
1	50.8	51.0	1	1.77 (*t*, 9.6)
2	27.2	27.6	2 α	1.45 (*m*)
			2 β	1.71 (*t*, 9.3)
3	39.2	39.7	3 α	2.13 (*m*)
			3 β	0.96 (*m*)
4	60.0	59.7	-	-
6	63.8	63.4	6	2.90 (*dd*)
7	30.2	30.2	7 α	1.26 (*m*)
			7 β	2.27 (*m*)
8	29.8	30.2	8 β	2.35 (*m*)
			8 α	2.11 (*m*)
9	151.8	152.4	-	-
10	48.7	49.0	10	2.65 (*m*)
11	39.8	40.0	11 β	1.70 (*dd*, 9.7, 3.6)
			11 α	1.63 (*s*)
12	34.0	34.1	-	-
13	17.0	17.4	13	1.23 (*s*)
14	112.8	113.0	14 α	4.89 (*d*, 6.6)
			14 β	5.00 (*d*, 6.6)
15	29.9	29.9	15	1.03 (*s*)
16	21.6	21.8	16	1.01 (*s*)

*****Source: Orihara et al. (1994)

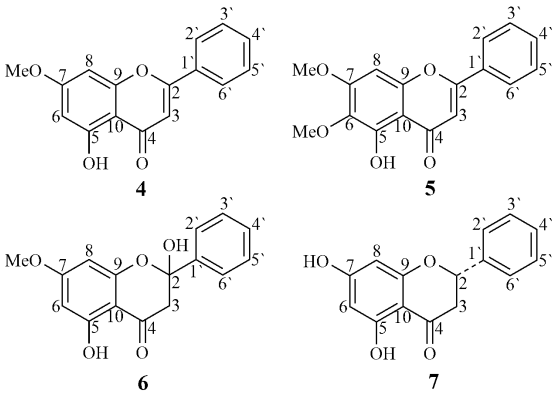
Glutinol (**3**) [13; 21; 27] is a white amorphous with m.p. 202-203°C. Its IR spectrum showed ν_{max} absorption peaks at 3525 (O-H); 2927, 2867 (C-H); 1385 (C-O) and 1453 (C=C). Compounds **3** gave positive response to Liebermann-Burchard spray reagent, which indicated the presence of a triterpenoid nucleus**.** This assumption was reinforced by the ^{13}C-NMR data that indicated the presence of thirty carbons as tabulated in Table 3 which included two olefinic carbon signals at δc 141.6 (C-5) and 122.1 (C- 6) and also one oxymethine at δc 76.3 (C-3). The ^{1}H-NMR spectrum of compound **3** displayed signals due to the eight methyls (δ_C 0.86-1.17), a carbinol proton H-3 at δ_H 3.5 and an olefinic proton H-5 at δ_H 5.6. A molecular ion peak $[M]^+$ at *m/z* 426 was exhibited in its mass spectrum data, which gave a possible molecular formula of $C_{30}H_{50}O$. The combined spectral data of ^{13}C NMR suggested that the triterpenoid was glutinol, which was further supported by comparison with the literature [28] as tabulated in Table 3.

Table 3. Comparison of ^{13}C-NMR data of glutinol (**3)** with those of the literature*****

No of C	3	**	No of C	3	**
1	18.2	18.2	16	36.0	36.0
2	27.8	27.8	17	30.1	30.1
3	76.4	76.3	18	43.1	43.1
4	40.8	40.8	19	35.1	35.1
5	141.6	141.6	20	28.3	28.2
6	122.1	122.1	21	32.1	33.1
7	23.6	23.7	22	39.0	39.0
8	47.4	47.5	23	25.5	25.5
9	34.9	34.9	24	29.0	29.0
10	49.7	49.7	25	16.2	16.2
11	34.6	34.6	26	19.6	19.6
12	30.4	30.4	27	18.5	18.4
13	39.3	39.3	28	32.0	32.0
14	37.8	37.8	29	34.5	34.5
15	33.1	32.1	30	32.4	32.4

**Source: Sule et al. 2011

The flavonoids were characterized as 5-hydroxy-7-methoxyflavone (**4**), 5-hydroxy-6,7-dimethoxyflavone (**5**) 2,5-dihydroxy-7-methoxyflavanone (**6**) and 5,7-dihydroxyflavanone (**7**) by spectroscopic methods as well as comparison of their physical and spectral data which was reported by [5], [7], [16] and [22]. The differences between compounds **4**, **5**, **6** and **7** lay on C-2, C-6 and C-7, where Compounds **4** and **6** had a methoxy bound at C-7, whereas in **5** two methoxys were bound to C-6, C-7 and also had a hydroxyl bound at C-2. For compound **7**, it had a hydroxyl at C-7 and two protons were bound to C-2 and C-6.



4 **5** **6** **7**

Table 4. NMR data of 5-hydroxy-7-methoxyflavone (**4**), 5-hydroxy-6,7-dimethoxyflavone (**5**), 2,5-dihydroxy7-methoxyflavanone (**6**) and 5,7-dihydroxyflavanone (**7**)

No. of C	δ_C (ppm)				No. of H	δ_H (ΣH, mult., *J* in Hz			
	4	**5**	6	7		4	5	6	7
-	-	-	-	-	-	-	-	-	-
2	164.0	164.0	105.7	79.3		-	-	-	5.44 (*dd*, 3, 13.2)
3	105.9	104.9	56.4	43.4	3	6.67 (*s*)	6.69 (*s*)	3.06 (*s*)	3.11 (*d*, 12.6)
								3.22 (*s*)	2.08 (*d*, 13.2)
4	182.5	182.8	183.0	195.8	-	-	-	-	-
5	162.2	157.6	159.0	164.4		-	-	-	-
6	98.2	132.0	90.7	95.5	6	6.39 (*s*)	-	6.13 (*s*)	5.86 (*br-s*)
7	165.6	158.8	163.0	164.6		-	-	-	-
8	92.7	95.9	90.7	96.8	8	6.51 (*s*)	6.45 (*s*)	6.12 (*s*)	6.02 (*s)*
9	157.8	149.5	153.4	163.2		-	-	-	-
10	105.7	105.4	105.6	102.8		-	-	-	-
1`	131.9	131.4	132.7	138.3		-	-	-	-
2`/6`	126.3	126.4	126.3	126.2	2`/6`	7.89 (*d*,7.8)	7.96 (*d*, 7.8)	7.69 (*d*, 7.8)	7.46 (*m*)
3`/5`	129.1	129.2	131.4	129.0	3`/5`	7.54 (*m*)	7.56 (*m*)	7.54 (*m*)	7.45 (*m*)
4`	126.3	126.4	129.1	126.2	4`	7.52 (*m*)	7.54 (*m*)	7.54 (*m*)	7.46 (*m*)
6OMe	-	61.7	-	-	6OMe	-	3.97 (*s*)	-	-
7OMe	55.9	56.4	55.7	-	7OMe	3.89 (*s*)	3.96 (*s*)	3.84 (*s*)	-
-	-	-	-	-	-OH	12.73 (*s*)	12.57 (*s*)	11.93 (*s*)	12.05 (*s*)

Chemotaxonomic Significance

Benzyl benzoate (**1**) and caryophyllene oxide (**2**) are the main components of essential oils that have been frequently isolated from the family Annonaceae. Compound **1** is the main precursor in the biogenesis of the highly oxidized cyclohexenoid reported earlier from the leaves of *Uvaria rufa* [29]. It has been found in most of *Uvaria* species such as *U. purpurea*, *U. versicolor*, *U. klaineana*, *U. ferruginea*, *U. pauci-ovulata*, *U. narum* and *U. chamae* [1, 2; 3; 14, 15]. Meanwhile, the sesquiterpene **2** is frequently isolated from many species of *Uvaria*, *Cyathostemma*, *Mitrephora* and *Cananga* [3]. Glutinol (**3**) is a triterpene, which was reported in *Uvaria narum* and *U. hookeri* [12; 21]. This compound was also found in the family Olacaceae, especially *Olax* oliv. group [28] and in *Acer mandshuricum* (Aceraceae) [10]. Flavonoids are molecules with antioxidant properties that are widely occurring in the plant kingdom [25]. The presence of flavonoid group is very common in *Uvaria* species. Previous studies have reported the isolation of six flavonoids from the bark and roots of *Uvaria rufa* including 5-hydroxy-7-methoxyflavone (**4**), 5-hydroxy-6,7-dimethoxyflavone (**5**), 2,5-dihydroxy-7-methoxyflavanone (**6**), 2,5-dihydroxy-6,7-dimethoxyflavanone, 5-hydroxy-7-methoxyflavanone and 7-O-methylwogonine [9]. To the best of our knowledge, this is the first time 5,7-dihydroxyflavanone (**7**) has been found from *Uvaria rufa*., which contributes to the database of flavonoids from *Uvaria* species.

Conclusion

Benzyl benzoate (**1**), caryophyllene oxide (**2**), glutinol (**3**), 5-hydroxy-7-methoxyflavone (**4**), 5-hydroxy-6,7-dimethoxyflavone (**5**), 2,5-dihydroxy-7-methoxyflavanone (**6**) and 5,7-dihydroxyflavanone (**7**) were successfully isolated from the methanol extract of *Uvaria rufa.* To the best of our knowledge, this is the first report of caryopyllene oxide, glutinol and 5,7-dihydroxyflavanone from the stem bark of *Uvaria rufa.*

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