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Flavones with Free Radical Scavenging Activity from *Goniothalamus tenuifolius*

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From the leaves of *Goniothalamus tenuifolius*, a new natural product namely 3'-hydroxy-3,5,7,4'-tetramethoxyflavone (**1**) was isolated, along with seven other known compounds (**2** - **8**). Each of these isolates was evaluated for free radical scavenging activity on the DPPH decoloration test. The data obtained in this study suggested that the *ortho* 3',4'-diphenolic structure was essential for the activity of these flavonol derivatives.

Key words: *Goniothalamus tenuifolius*, flavone, free radical scavenging activity, DPPH

INTRODUCTION

Goniothalamus tenuifolius King (Annonaceae), locally known as "Panan Kee Meaw," is a shrub or small tree growing in several parts of Thailand. A previous phytochemical study of the stem bark this plant revealed the presence of several antimalarial aristolactam alkaloids, including cepharanone B, taliscanine, aristolactam AII, velutinam and norcepharadione B (Likhitwitayawuid *et al.*, 1997). The leaves of this plant was found to be devoid of anti-plasmodial potential but showed free radical scavenging activity with DPPH in our TLC bioautographic assay. Our chemical investigation of the leaves of *G. tenuifolius* resulted in the isolation of a new natural product namely 3'-hydroxy-3,5,7,4'-tetramethoxyflavone (**1**), as well as seven other known compounds (**2-8**). Each of these isolates was then evaluated for free radical scavenging activity on the DPPH decoloration test.

MATERIALS AND METHODS

Plant material

The leaves of *Goniothalamus tenuifolius* were collected from Kaengkrachan, Phetchaburi province, Thailand, in March 1995. The plant was identified by comparison with herbarium specimens in the Department of Agriculture, Ministry of Agriculture and Co-operatives, Bangkok. A voucher specimen (VJ 04-2538) has been on deposit at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

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Instruments

UV spectra were obtained on a Milton Roy Spectronic 3000 Array spectrophotometer, and IR spectra on a JASCO FT-IR-300E spectrophotometer. ESITOF mass spectra were measured with a Micromass LCT mass spectrometer. ^{1}H-NMR (300 MHz), ^{13}C-NMR (75 MHz), NOESY, HMQC and HMBC spectra were obtained with a Bruker Avance DPX-300 FT-NMR spectrometer. Microtiter plate reading was performed on an Anthos HTL instrument.

Extraction and isolation

The dried leaves of *G. tenuifolius* (1.3 kg) were extracted with hexane, EtOAc and MeOH to give hexane (29 g), EtOAc (98 g) and MeOH (115 g) extracts. The EtOAc extract showed significant DPPH free radical scavenging activity on TLC, and was further investigated for its components. A portion of the EtOAc extract (45 g) was subjected to vacuum liquid column chromatography, eluted with pet. ether, EtOAc and MeOH in a polarity gradient

manner to give 10 fractions (A-J). Fraction B (460 mg) was separated by gel filtration (Sephadex LH-20; MeOH-$CHCl_3$ 3:2) to yield 5 fractions. Fraction 4 from this column was further separated on Sephadex LH-20 (MeOH-$CHCl_3$ 3:2) to give retusine or 5-hydroxy-3,7,3',4'-tetramethoxyflavone (**2**) (5 mg), whereas fraction 5 was chromatographed over Sephadex LH-20 (MeOH-EtOAc 1:1) to furnish *trans*-cinnamic acid (**3**) (10 mg). Fraction C (100 mg) was subjected to recrystallization ($CHCl_3$-MeOH) to give pachypodol or 5,4'-dihydroxy-3,7,3'-trimethoxy-flavone (**4**) (10 mg). Separation of fraction D (5 g) on Sephadex LH-20 (MeOH) yielded 5,7,3',4'-tetrahydroxy-3-methoxyflavone (**5**) (8 mg), and a fraction which was further separated on Sephadex LH-20 (MeOH) to give kumatakenin or 5,3',4'-trihydroxy-3,7-dimethoxyflavone (**6**) (86 mg). Fraction G (2.4 g) was fractionated on a silica column with gradient elution (pet. ether-EtOAc-acetone) to give 7 fractions. Fraction 3 from this column was further separated on silica gel ($CHCl_3$-acetone 9:1) to give 6 fractions. Evaporation of the solvent from fraction 2 gave 3,5,7,3',4'-pentamethoxyflavone (**7**) (220 mg). Repeated chromatography of fraction 3 on Sephadex LH-20 (acetone) yielded 4'-hydroxy-3,5,7,3'-tetramethoxyflavone (**8**) (5 mg). 3'-Hydroxy-3,5,7,4'-tetramethoxyflavone (**1**) (5 mg) was obtained fraction 4 after recrystallization from a mixture of EtOAc-$CHCl_3$.

Evaluation of free radical scavenging activity

TLC Screening assay

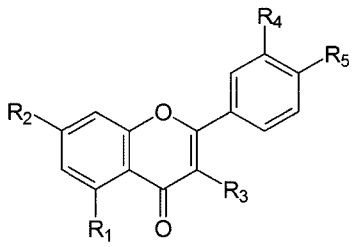
TLC Screening for free radical scavenging activity was done according to the method previously described (Takao *et al.*, 1994). In short, test samples were spotted on a TLC plate, and development was performed with a suitable solvent. After drying, the TLC plate was sprayed with 0.2% solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol and left 30 min before visual examination. Constituents possessing free radical scavenging activity appeared as yellow spots against purple background.

Determination of free radical scavenging activity

Assays of pure compounds (**1** - **8**) for free radical scavenging activity were conducted in duplicate using 96-well microtiter plates according to a protocol modified from the method of Braca *et al.* (2002). Test samples were initially prepared as solution in EtOH (400 μg/ml). Each compound was first tested at the concentration of 40 mg/ml. An IC_{50} value was determined if the compound showed more than 50% inhibition. For IC_{50} analysis, two-fold serial dilution was performed to give seven concentrations. The test was done by addition of the sample solution (20 μl) to the solution of 50 μM DPPH in EtOH (180 μl) in a 96-well microtiter plate. The reaction mixture was incubated at room temperature for 30 min, and then its absorbance at 510 nm was measured with a microplate reader. Quercetin was used as positive control.

RESULTS AND DISCUSSION

Compound **1** possessed the molecular $C_{19}H_{18}O_7$, as indicated from its $[M+H]^+$ ion at *m/z* 359.1129 ($C_{19}H_{19}O_7$, calcd. 359.1131) in the ESIMS. IR bands for OH, C=O and C=C functionalities were observed at 3421, 1631, and 1603 cm^{-1}. The UV spectrum of **1** exhibited absorptions at 250, 343 nm, indicative of a flavone skeleton (Markham, 1982). The ^1H NMR spectrum of **1** (Table I) showed typical signals for H-6 and H-8 of ring A at δ 6.31 (d, J = 2.1 Hz) and 6.48 (d, J = 2.1 Hz), respectively. In addition, four methoxyl proton signals were observed at d 3.85, 3.93, 3.87 and 3.95. This was supported by the ^{13}C NMR and DEPT spectra which displayed 19 carbon resonances, corresponding to four methoxyls, five methines and ten quaternary carbons (Table I). The methoxyl group at d 3.93 should be located at C-5, based on its NOESY interaction with H-6, whereas the methoxyl group at δ 3.87 should be at C-7, as shown by its NOESY interaction with H-6 and H-8. The third methoxyl group at d 3.85 should be placed at C-3 according to its NOESY cross peak with H-6' and H-2'. In the ^1H NMR spectrum, the B-ring aromatic protons appeared as an ABM splitting system at δ 7.65 (d, J = 2.1 Hz, H-2'), 7.69 (dd, J = 8.4,



	R_1	R_2	R_3	R_4	R_5
1	OCH_3	OCH_3	OCH_3	OH	OCH_3
2	OH	OCH_3	OCH_3	OCH_3	OCH_3
4	OH	OCH_3	OCH_3	OCH_3	OH
5	OH	OH	OCH_3	OH	OH
6	OH	OCH_3	OCH_3	OH	OH
7	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
8	OCH_3	OCH_3	OCH_3	OCH_3	OH
9	OH	OH	OH	OH	OH

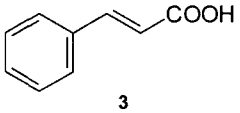


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Table I. ^{1}H and ^{13} NMR data of **1** and **8**

Position	$^{1}H^{a}$		^{13}C	
	1	**8**	**1**	**8**
2	-	-	152.3	152.4
3	-	-	141.3	140.8
4	-	-	174.0	173.7
5	-	-	161.0	160.8
6	6.31 (d, 2.1)	6.32 (d, 2.1)	95.7	95.7
7	-	-	163.8	163.6
8	6.48 (d, 2.1)	6.48 (d, 2.1)	92.4	92.3
9	-	-	158.8	158.8
10	-	-	109.5	109.4
1'	-	-	124.1	122.7
2'	7.65 (d, 2.1)	7.72 (d, 1.8)	114.1	110.8
3'	-	-	145.4	146.1
4'	-	-	148.2	147.5
5'	6.93 (d, 8.4)	7.00 (d, 8.4)	110.3	114.3
6'	7.69 (dd, 8.4, 2.1)	7.61 (dd, 8.4, 1.8)	121.2	121.9
MeO-3	3.85 (s)	3.84 (s)	59.8	59.9
MeO-5	3.93 (s)	3.94 (s)	56.3	56.1
MeO-7	3.87 (s)	3.88 (s)	55.7	55.7
MeO-3'	-	3.94 (s)	-	56.6
MeO-4'	3.95 (s)	-	56.0	-
OH-3'	5.67 (s)	-	-	-
OH-4'	-	5.97 (br s)	-	-

[a]Multiplicities and coupling constants (Hz) are in parentheses.

2.1 Hz, H-6') and 6.93 (d, J = 8.4 Hz, H-5'). This suggested that ring B was 3',4'-disubtituted with a phenolic and a methoxyl (d 3.95) group. The NOESY interaction of the methoxyl protons with H-5' placed this methoxyl at C-4', and hence the phenolic was situated at C-3'. Thus, compound **1** was identified as 3'-hydroxy-3,5,7,4'-tetramethoxyflavone. Although this compound has been earlier prepared (Bouktaib, *et al.*, 2002, Beutler *et al.*, 1998, Parmar *et al.*, 1996, Parmar *et al.*, 1994, Wang et al., 1989), this is the first time it has been found as a naturally occurring compound. In addition, this study has provided the first ^{13}C NMR report of **1** through HMQC and HMBC experiments.

Identification of the known compounds was done by comparison of ^{1}H and ^{13}C-NMR, and MS data with reported values for **2** (Ohashi *et al.*, 1999; Dong *et al.*, 1999), **3** (Hanai *et al.*, 2001), **4** (Itokawa *et al.*, 1981), **5** (Urbatsch *et al.*, 1975; Wang et al., 1989), **6** (Urbatsch *et al.*, 1976) and **7** (Dong *et al.*, 1999), except for **8** which was identified only from its ^{1}H and MS data (Higa *et al.*, 1987). Its first ^{13}C NMR data (Table I) were obtained in this study through analysis of the HMQC and HMBC spectra. It

Table II. Free radical scavenging activity of compounds **1** - **8**

Compounds	Percent scavenging activity (40 μg/mL)	IC_{50} (μM)
1	11.49	nd
2	4.27	nd
3	6.20	nd
4	13.69	nd
5	80.31	6.4
6	82.15	5.8
7	0.61	nd
8	19.07	nd
Quercetin (**9**)	80.19	6.6

nd = not determined.

should be noted that **1** and **8** are structural isomers, differing only in the locations of the OH and OMe groups at C-3' and C-4'. When the ^{1}H NMR properties of the three aromatic protons on ring B of the two compounds were carefully examined, it was found that H-2' was obviously affected by the substituent at C-3', and its position could be used as a diagnostic signal for differentiating the two isomers. In **1**, H-2' resonated at a position between H-5' and H-6', appearing at d 7.65 (d, J = 2.1 Hz), whereas in **8**, H-2' was the most deshielded signal, showing at d 7.72 (d, J = 1.8 Hz). HMQC correlations of H-2' and H-5' indicated reverse ^{13}C NMR assignments of C-2' and C-5' for the two compounds, which were further confirmed by HMBC connectivites.

Table II shows the results of the tests for free radical scavenging activity of the isolates (**1** - **8**), with quercetin (**9**) as positive control. It can be seen that for these flavonol derivatives, the presence of OH is required for the activity. For example, compounds **7,** due to its lack of OH, had no ability to scavenge DPPH radicals. Flavones with one OH group, such as **1**, **2**, and **8** showed recognizable activity. Compound **2** demonstrated lower percent inhibition than that of **1** and **8**, probably because of the low reactivity of its OH-5 caused by the H-bonding with the keto group. This phenomenon was also seen in **4**, in which low activity was observed despite the existence of two OH groups. The most potent compounds are **5** and **6**, being as strong as the positive control quercetin (**9**). The fact that the flavonol derivatives **5, 6**, and **9** shared the 3',4'-dihydroxy functionality as their common attribute suggested that this *ortho*-diphenolic structure is essential for their free radical scavenging activity.

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