American Journal of Applied Sciences 10 (8): 787-792, 2013
ISSN: 1546-9239

doi:10.3844/ajassp.2013.787.792 Published Online 10 (8) 2013 (http://www.thescipub.com/ajas.toc)

Anticancer Activity of Isolated Chemical Constituents from *Miliusa smithiae*

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Received 2013-05-15, Revised 2013-05-27; Accepted 2013-07-08

ABSTRACT

Miliusa plants belonging to the family Annonaceae are found in Thailand and have been used as Thai traditional medicines. There have been a few previously reports on the chemical constituents of plants in this genus, describing the presence of aporphine alkaloids, terpenoids, flavonoids, phenylpropanoids, styrylpyrones, bis-styryls and homogentisic acid derivatives. *Miliusa smithiae*, a new species for Thailand and world, has not been studied chemical composition. The present study described phytochemical study of the leaves and twigs of *M. smithiae* together with their cytotoxicity. The *M. smithiae* was selected and percolated with hexane, ethyl acetate, acetone and methanol. The extracts were purified and elucidated chemical structures. The constituent of ethyl acetate extract of *M. smithiae* has been investigated. We isolated and identified two flavonoid derivatives, 5-hydroxy-3,7,4′-trimetoxyflavone (1) and 5,3′-dihydroxy-3,7,4′-trimetoxyflavone (2). The structures of these compounds were elucidated on the basis of spectroscopic evidence. Studies on ethyl acetate extract of *M. smithiae* has now resulted the isolation and structural characterization of two flavonoids. Their anticancer activities were evaluated using SRB assays. In this method, compound 2 showed potential activity in cell lines.

Keywords: *Miliusa smithiae*, Annonaceae, Flavonoid, Anticancer activity

1. INTRODUCTION

The genus *Miliusa* (Annonaceae family) consists about 40 species which grows in tropical rainforest of India, Thailand, South China and North Australia (Sawasdee *et al*., 2010). The plant is used in folk medicine for different symptom such as gastropathy and glomerulonephropathy (Kamperdick *et al*., 2002) in Chinese traditional medicine (Huong *et al*., 2008). The studies on phytochemical of genus *Miliusa* afforded aporphine alkaloids, terpenoids, flavonoids, phenylpropanoids, styrylpyrones, *bis*-styryls and homogentisic acid derivatives (Sawasdee *et al*., 2010). Several compounds from genus *Miliusa* showed antibacterial activity (Jumana *et al*., 2000), cytotoxic activity against human oral nasopharyngeal carcinoma (KB), human Hepatocellular carcinoma (Hep-G2 RD), human colon cancer (COl-2) human prostate adenocarcinoma (LNCaP), human lung cancer (Lu-1), human breast cancer (MCF-7) and human umbilical vein endothelial (HUVEC) cancer cell lines (Khumchompoo and Thongpukdee, 2007; Huong *et al*., 2005). *M. smithiae*, locally known as Rakungtai, grows widely in tropical rainforest in the south region of Thailand. It is a small tree, 2 to 6 m height, leaves 6 to 13 cm long, 2.5 to 4.5 cm wide and flower yellowish green. The plant is a new species in Thailand and world. There is no report about its chemical investigation for this species. Our preliminary screening tests for bioactivities of the crude extracts of *M. smithiae* revealed that the crude hexane extract exhibited cytotoxicity against MCF-7, murine lymphocytic leukemia (P-38), human oral

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nasopharyngeal carcinoma (KB), human colon cancer (Col-2), human lung cancer (Lu-1), rat glioma (ASK), noncancerous human embryonic kidney (Hek 239) and human urinary bladder (T24) cell lines with ED_{50} in the range of 1.16-13.31 μg mL^{-1}, while crude ethyl acetate showed cytotoxicity against those cell lines with ED_{50} in the range of 0.30-5.85 μg mL^{-1}. In the present study, we report the isolation and characterization of 5-hydroxy-3,7,4′-trimetoxyflavone (1) and 5,3′-dihydroxy-3,7,4′-trimetoxyflavone (2) or commonly called ayanin (Ali *et al*., 2006).

2. MATERIALS AND METHODS

2.1. General Experimental Procedure

Melting points were determined on a digital Electro thermal melting apparatus and uncorrected.IR spectra were recorded as KBr disks, using Shimadzu 8900 FTIR spectrophotometer and major bands (ν) were recorded in wave number (cm^{-1}). ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were determined in $CDCl_3$ and CD_3OD solution, the chemical shifts were recorded in δ values which were referenced to TMS as the internal standard in ppm down field from TMS (internal standard at δ 0.00). Low resolution mass spectra were recorded on a Thermo Finnegan Polaris Q mass spectrometer at 70 eV (probe) for EIMS. CC was carried out over silica gel (0.063-0.200 mm or less than 0.063 mm, MERCK). Fractions obtained from CC were monitored by TLC on silica gel 60 $F_{254,}$ aluminum sheets and the chromatograms were visualized at 254 and 366 nm and sprayed with anisaldehyde reagent and then heated until charred. Commercial grade solvents were distilled at their boiling point ranges prior to use for extraction and chromatographic separation (CC and preparative TLC), whereas AR solvents were used for crystallization.

2.2. Plant Material

The leaves and twigs of *M. smithiae* were collected from Kanchanaburi, a province of Thailand, in July, 2011 by Mr. Narong Nuntasaen. The plant was identified and the specimen has been deposited in the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of National Resources and Environment, Bangkok, Thailand.

2.3. Extraction and Isolation

Dried leaves and twigs from *M. smithiae* (1.4 kg) were successively defatted with hexane and then sequential extracted consecutively at room temperature with ethyl acetate, acetone and methanol for 5 times each (5×7 L). Removal of solvents from each extract under reduced pressure affords crude hexane (5.95 g) ethyl acetate (33.58 g), acetone (5.18 g) and crude methanol (53.17 g), respectively.

The crude ethyl acetate extract (33.58 g) was separated by column chromatography (CC) over silica gel 60 (Merck, 70-230 mesh) (66.62 g). Gradient elution was conducted initially with n-hexane, gradually enriched with ethyl acetate, followed by increasing amount of mixture between methanol and ethyl acetate and finally with methanol. Based on TLC patterns, overall 9 fractions were combined to give 8 fractions, A_1-A_8. Fraction A_3 (2.19 g), obtained from 10% ethyl acetate: hexane, was further purified by CC over silica gel, eluted with gradient systems of hexane: ethyl acetate and ethyl acetate: methanol follow by methanol. Fractions (200 mL, each) were collected and combined based on the basis of their TLC behavior to afford 4 subfractions, B_1-B_4. Subfraction B_1 (0.30 g), obtained from 10% ethyl acetate: hexane, was further purified by CC over silica gel, eluted with gradient systems of hexane: ethyl acetate. Fractions (25 mL, each) were collected and combined based on the basis of their TLC behavior to afford 2 subfractions, B_{1a}-B_{1b}. The precipitate in subfraction B_{1b} (0.10 g) was filtered out and then recrystallized from the combination ethanol to yield compound 1 (0.02 g). Fraction A_4 (3.0 g), obtained from 10% ethyl acetate: hexane, was further purified by CC over silica gel, eluted with gradient systems of hexane: ethyl acetate. Fractions (200 mL, each) were collected and combined based on the basis of their TLC behavior to afford 5 subfractions, C_1-C_5. The precipitate in subfraction C_2 (0.98 g) was filtered out and then recrystallized from the combination ethanol to yield compound 2 (0.16 g).

2.4. Evaluation of Cytotoxic Activity

The cytotoxic activities of the tested extracts and compounds from *M. smithiae* were carried out using the *in vitro* Sulforhodamine B (SRB) method (Vichai and Kirtikara, 2006) and ellipticine was used as a positive control. Test samples were dissolved in DMSO as a stock concentration at 4 mg mL^{-1} and were tested in triplicate with a final concentration of DMSO at 0.5%. The cancer cell lines were grown in a 96-well plate in the following media: P-388, in RPMI-1640 with 5% Fetal Bovine Serum (FBS). The P-388, KB, Col-2, MCF-7, Lu-l, ASK, Hek 293 and T24 cell lines were cultured in MEM (minimum essential medium with Earle's salt and L-glutamine) with 10% FBS, while Lu-1 was grown in MEM with 5% FBS. After drug exposure at 37°C for 72 h (48 h for P-388) with 5% CO_2 in air and 100%

relative humidity, cells were fixed with a final concentration of 10% trichloroacetic acid and stained with 0.4% sulforhodamine B in 1% acetic acid. The bound and dried stain was solubilized with 10 mM trizma base, after removal of the unbound dye by washing. The absorbance of wavelength at 510 nm was read on a Fluostar optima BMG plate reader. The cytotoxic activity is expressed as 50% effective dose (ED_{50}).

Determine ED_{50} value:

$$\% \text{ Survival} = \frac{OD(\text{test sample}) - OD(\text{Day 0})}{OD(0.5\% \text{ DMSO control}) - OD(\text{Day 0})} \times 100$$

2.5. Criteria of Activity

Extracts having an ED_{50}<20 µg mL^{-1} and pure compounds having an ED_{50}<4 µg mL^{-1} = Active; ED_{50}>20 µg mL^{-1} = No Response

3. RESULTS

The chromatographic procedure with the ethyl acetate extract of *M. smithiae* afforded two compounds. 5-hydroxy-3,7,4′-trimetoxyflavone (1) and 5,3′-dihydroxy-3,7,4′-trimetoxyflavone (2), were identified from the leaves and twigs extract. The structures of compounds were proposed by ^{1}H and ^{13}C NMR spectral data analysis and comparison with the literature data. The structures are shown in **Fig. 1**.

4. DISCUSSION

Compound 1 was obtained as yellow crystals, mp 205-206°C. It was determined as $C_{18}H_{16}O_6$ by its EIMS which showed the molecular ion peak at *m/z* 328 $[M]^+$. The UV spectrum of 1 showed three absorption bands at λ_{max}^{EtOH} 349 and 268 nm. The absorption band at λ_{max}^{EtOH} 349 is referred to Band I, which is considered to be associated with absorption due to the B-ring cinnamoyl system. The absorption bands at λ_{max}^{EtOH} 268 is typical for Band II involving the A-ring benzoyl system, which appear as two peaks depending on the B- ring oxidation pattern in flavonols. Thus, it was clearly identified as a flavonol derivatives. The IR spectrum of 1 exhibited the C = O stretching of a conjugated carbonyl group at 1658 cm^{-1} which slightly shifted to the longer wavelength due to the presence of an intramolecular hydrogen bonding between o-hydroxylaryl and keto group. The C = C stretching of the conjugated carbonyl was also observed at 1585 cm^{-1}. The compound was clearly proved to be phenolic by the presence of O-H stretching and C-O stretching bands of 3469 cm^{-1} and 1203 cm^{-1}, respectively. Information from the IR spectrum supported that one of the hydroxy group was at position-5 (the presence of *o*-hydroxyaryl ketone), which confirmed by a singlet at δ 12.65 of a chelated hydroxyl proton (5-OH) in the ^{1}H-NMR spectrum. Ring B of this compound was found to have a methoxy group at C-4′ since AA′BB′ pattern of H-2′, H-6′ and H-3′, H-5′ was observed at δ 8.08 and 7.02, respectively. ($J_{2',3'} = J_{3',2'} = J_{5',6'} = J_{6',5'} = 9.6$ Hz and $J_{2',6'} = J_{6',2'} = J_{3',5'} = J_{5',3'} = 2.2$ Hz). The remaining two aromatic protons of 1 also appeared as a pair of doublets [(δ 6.35 and 6.44 (J = 2.2 Hz)] corresponding to two *meta*-coupled protons, which resembled those of H-6 and H-8 of 1. ^{13}C NMR in $CDCl_3$ showed the presence of three methoxy carbons, six methine carbons, seven quarternary carbons and one carbonyl carbon (**Table 1**). Other connectivities in the structure were confirmed by the results from hetero-correlations (HMQC and HMBC) spectra (**Table 2**). The structure of compound 1 was finally confirmed by direct comparison of the value reported by Jang *et al*. (2004).

Compound 2 was obtained as yellow crystals, mp 227-228°C. The mass spectrum showed $[M]^+$ at *m/z* 344 corresponding to the molecular formula of $C_{18}H_{16}O_7$. The UV absorption bands typical for flavonol were observed at λ_{max}^{EtOH} 357 and 256 nm. The conjugated carbonyl absorption band at 1650 cm^{-1} together with a broad O-H stretching band at 3421 cm^{-1} in the IR spectrum indicated the possibility of having a conjugated carbonyl group chelated to a phenolic OH group, while the band at 1558 cm^{-1} was referred to C=C stretching of the conjugated carbonyl system. The ^{1}H-NMR spectrum (**Table 1**) revealed the presence of three hydroxy protons at δ 12.63 (chelated OH) and 5.80. Beside these hydroxyl protons, three methoxy protons were observed as singlets at δ 3.87, 3.88 and 3.99. An ABX pattern at δ 7.70 (d, 2H, H-2′ and H-6′) and 7.73 (d, 1H, $J_{5',6'} = J_{6',5'} = 8.5$ Hz) were assigned to H-6′, H-2′ and H-5′ of ring B, respectively. The outstanding two aromatic protons of 2 also appeared as a pair of doublets [(δ 6.35 and 6.44 (J = 2.2 Hz)] corresponding to two *meta*-coupled protons, which resembled those of H-6 and H-8 of 2. ^{13}C NMR in $CDCl_3$ showed the presence of three methoxy carbons, five methine carbons, eight quarternary carbons and one carbonyl carbon (**Table 1**). ^{1}H-^{13}C correlations observed in the structure were confirmed by the results from hetero-correlations (HMQC and HMBC) spectra (**Table 2**). The

structure of compound 2 was finally confirmed by direct comparison of the value reported by Lima *et al*. (2010).

Compound 2 obtained in the present investigation was evaluated against a panel of mammalian cancer cell lines and the noncancerous human embryonic kidney cell Hek 293 (**Table 3**). The compound showed cytotoxicity to P-388, Col-2, MCF-7, ASK and Hek 293 cell lines. The flavonoid showed high selectivity toward cancer cells, thus making the compound as attractive anticancer agent.

Table 1. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz) data for isolated flavonoids in $CDCl_3$

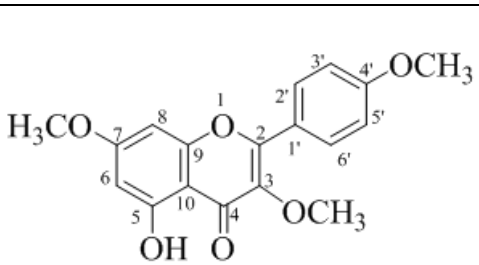
	5-hydroxy-3,7,4′-trimetoxyflavone (**1**)			5,3′-dihydroxy-3,7,4′-trimetoxyflavone (**2**)	
Position C	*δ ^{1}H (J Hz)	δ ^{13}C (DEPT)	Position C	*δ ^{1}H (J Hz)	δ ^{13}C (DEPT)
2	-	156.94 (C)	2	-	156.63 (C)
3	-	138.96 (C)	3	-	138.25 (C)
4	-	178.76 (C)	4	-	178.83 (C)
5	-	161.98 (C)	5	-	161.99 (C)
6	6.35 d (2.2)	97.79 (CH)	6	6.35 d (2.2)	97.88 (CH)
7	-	165.32 (C)	7	-	165.44 (C)
8	6.44 d (2.2)	92.12 (CH)	8	6.44 d (2.2)	92.13 (CH)
9	-	156.72 (C)	9	-	156.76 (C)
10	-	105.9 (C)	10	-	106.08 (C)
1′	-	122.17 (C)	1′	-	123.65 (C)
2′	8.08 d (9.6)	130.14 (CH)	2′	7.70 d (2.1)	114.41 (CH)
3′	7.02 d (9.6)	114.18 (CH)	3′	-	145.97 (C)
4′	-	161.60 (C)	4′	-	148.76 (C)
5′	7.02 d (9.6)	114.18 (CH)	5′	6.95 dd (8.5, 2.1)	110.38 (CH)
6′	8.08 d (9.6)	130.14 (CH)	6′	7.73 d (8.5)	121.59 (CH)
3-OCH_3	3.86 s	60.11 (CH_3)	3-OCH_3	3.87 s	60.16 (CH_3)
7-OCH_3	3.87 s	56.03 (CH_3)	7-OCH_3	3.88 s	55.79 (CH_3)
4′-OCH_3	3.90 s	55.33 (CH_3)	4′-OCH_3	3.99 s	56.04 (CH_3)
5-OH	12.65 s	-	5-OH	12.63 s	-
			3′-OH	5.80 s	-

*δ in ppm from TMS (coupling constants (J) in Hz are given in parentheses)

Table 2. ^{1}H-^{13}C, ^{1}H-^{1}H correlations for isolated flavonoids

	5-hydroxy-3,7,4′-trimetoxyflavone (**1**)			5,3′-dihydroxy-3,7,4′-trimetoxyflavone (**2**)	
Position H	HMBC Correlation	COSY Correlation	Position H	HMBC Correlation	COSY Correlation
2	-	-	2	-	-
3	-	-	3	-	-
4	-	-	4	-	-
5	-	-	5	-	-
6	C-5, C-7, C-8, C-10	-	6	C-5, C-7, C-8, C-10	-
7	-	-	7	-	-
8	C-6, C-7, C-9, C-10	-	8	C-6, C-7, C-9, C-10	-
9	-	-	9	-	-
10	-	-	10	-	-
1′	-	-	1′	-	-
2′	C-2, C-3′	H-3′	2′	C-2, C-3′, C-4′	-
3′	C-1′	H-2′	3′	-	-
4′	-	-	4′	-	-
5′	C-2′, C-1′, C-6′	H-6′	5′	C-1′, C-2′, C-4′, C-6′	H-6′
6′	C-3′, C-4′, C-5′	H-5′	6′	C-4′	H-5′
3-OCH_3	-	-	3-OCH_3	C-3	-
7-OCH_3	-	-	7-OCH_3	C-7	-
4′-OCH_3	-	-	4′-OCH_3	C-4′	-

5-OH	-	-	5-OH	-	-
			3′-OH	-	-



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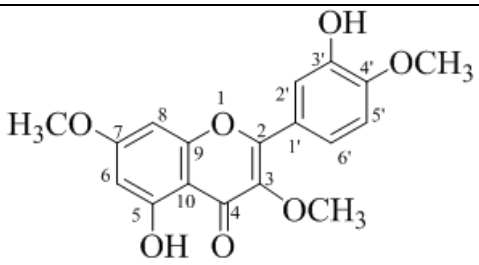

(2)

Fig. 1. Structures of isolated compounds

Table 3. Cytotoxicity of crude extracts and pure compounds from *M. smithiae*

Crude extracts/ Pure compounds	Cytotoxicity (ED_{50}, μg mL^{-1}) Cancer cells						Normal cells	
	P-388	KB	Col-2	MCF-7	Lu-1	T24	ASK	Hek293
Hexane	9.07	12	8.53	1.16	11.98	13.31	11.6	6.74
Ethyl acetate	2.07	5.45	1.98	0.3	5.85	3.29	3.83	<4.00
Acetone	NR	NR	NR	NR	NR	NR	NR	NR
Methanol	NR	NR	NR	NR	NR	NR	NR	NR
5,3′-dihydroxy-3,7,4′-trimetoxyflavone (**2**)	3.6	NR	0.76	0.68	NR	NR	16.08	2.81
Ellipticine (Positive control)	0.4	0.48	0.51	0.37	0.23	0.58	0.23	0.58

Cytotoxic assay: ED_{50} less than 20 μg mL^{-1} were considered active for extracts and less than 4 μg mL^{-1} for pure compounds. P388: murine lymphocytic leukemia, KB: human oral nasopharyngal carcinoma, Col-2: human colon cancer, MCF-7: human breast cancer, Lu-1: human lung cancer, human colon cancer, T24: human urinary bladder cancer cell, ASK: rat glioma cell, Hek293: noncancerous human embryonic kidney cell, NR: no response (ED_{50}>20 μg mL^{-1})

5. CONCLUSION

Phytochemical investigation of the crude ethyl acetate extract from *M. smithiae* had led to the isolation of two flavonoid derivatives, 5-hydroxy- 3,7,4′-trimetoxyflavone (1) and 5,3′-dihydroxy-3,7,4′-trimetoxyflavone (2). Compound **2** was showed potential anticancer activities. Moreover, the compound can play an important role for solving the cancer therapy. It is noted that the worthy finding of this study could be considered as a valuable economic medicinal natural products which helpful the cancer rehabilitation to human health.

6. ACKNOWLEDGEMENT

The researchers are grateful to Center for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education for financial support. We are grateful to Dr. Puttinan Meepowpan for recording the NMR spectra. We would also like to thank Mr. Narong Nantasaen for collecting the plants sample.

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