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Bioactive Flavonoids and Alkaloids from *Anomianthus dulcis* (Dunal) J. Sinclair Stem Bark

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Abstract

Three flavonoids **(1, 2, 3)** and two oxoaporphine alkaloids **(4, 5)** were isolated from *Anomianthus dulcis* stem barks. Structural elucidations of these compounds were established by spectroscopic data, as well as compared with literature data. Among them, liriodenine **(5)**, 9-methoxyliriodenine **(4)** and (2*S*)-5-hydroxy-6,7-dimethoxyflavanone **(1)** exhibited selectively potent cytotoxicity against human small cell lung cancer call line (NCI-H187) with IC_{50} values at 1.02, 1.28 and 1.73 µg/ml, respectively. (2*S*)-5-hydroxy-7,8-dimethoxyflavanone **(2)**, **(1)** and **(5)** were weakly active against human nasopharyngeal carcinoma cell line (KB) with IC_{50} values at 12.86, 15.45 and 13.45 µg/ml, respectively. While none of the isolated compounds showed cytotoxicity against breast cancer cell line (BC), **(5)** exhibited weakly active against BC with IC_{50} value at 14.57 µg/ml. Additionally, **(5)** showed strong anti-HSV-1 activity with IC_{50} value at 3.3 µg/ml. Compounds **(5)**, **(1)** and **(4)** exhibited antimycobacterial activity with minimum inhibitory concentrations (MICs) of 100, 200, and 200 µg/ml, respectively. However, the isolated compounds possessed neither antiplasmodial nor anti-HIV activities.

Key word: *Anomianthus dulcis***,** Flavonoids, Alkaloids

INTRODUCTION

Anomianthus dulcis (Dunal) J. Sinclair (Annonaceae), a shrub known as "Num Wua" in Thai, is widely distributed in Southern and Northeastern parts of Thailand[1]. This plant has been used in Thai folklore medicine for fever treatment[2]. Previous phytochemical investigation of its leaves revealed the presence of chrysin, *p*-coumaroyl-*b*-phenethylamine, pinocembrin, 3′-dihydroxy-4′,6′-dimethoxydihydrochalcone,5,7-dimethoxy-8-hydroxyflavanone, and 2′,3′-dihydroxy-4′, 6′-dimethoxychalcone[3]. However, there have been no reports about chemical study and biological activity of its stem bark. In this study, it is the first report of the isolation of flavonoids and oxoaporphine alkaloids from the stem barks together with their biological activities as anti-Herpes Simplex Virus type 1 (anti-HSV-1), cytotoxicity, anti-Human Immunodeficiency Virus (anti-HIV) and antiplasmodial activities.

MATERIALS AND METHODS

General experimental procedure

The UV spectra were obtained by

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using a Milton Roy Spectronic 3000 array. The IR spectra were measured on a Shimadzu IR-440 infrared spectrometer. EIMS were recorded on JEOL JMS D-300. HRMS were measured on Micro mass LCT. The ^{1}H and ^{13}C NMR (500 MHz and 400 MHz, using $CDCl_3$ as a solvent for measurement) spectra were obtained on a JEOL JNM-A500 NMR spectrometer and Varian mercury 400 spectrometer, respectively.

Plant materials

Plants were collected from Ubon-rachathani Province, Thailand. The plant samples used in this study were identified by Professor Wongsatit Chuakul, Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University. A voucher specimen (Collection No. Wong 2246) was deposited at Mahidol University Herbaria. The stem barks were chopped into small pieces, dried in an electric oven at 40°C and ground into powder.

Extraction and Isolation

Dried ground stem barks (6 kg) were macerated several times with 80% ethanol to provide the ethanolic extract (F001, 1.48 kg, 24.66 % yield). The F001 was partitioned between CH_2Cl_2 and water (1 : 1), providing the water extract (F002, 820 g, 13.66 % yield) and the CH_2Cl_2 extract (F003, 118 g, 1.97 % yield). Then, F003 was further partitioned with n-hexane and 90% methanol (1 : 1) to provide the 90% methanol extract (F006, 72 g, 1.20 % yield) and the hexane extract (F007, 32 g, 0.53 % yield). The F002 was further partitioned with EtOAc to afford the EtOAc extract (F005, 42 g, 0.70 % yield) and the water extract (F004, 783 g, 13.05 % yield). Since F006 showed the strongest activity against brine shrimp lethality test (BST) and cytotoxicities (Table 3), F006 (30 g) was subjected to column chromatography on silica gel with gradient (n-hexane/CH_2Cl_2/MeOH) system to afford 9 fractions (A-I). The fraction D (1 g) was separated by Sephadex-LH 20 column chromatography (MeOH), silica gel column chromatography (n-hexane/CH_2Cl_2/EtOAc/MeOH gradient system) and then preparative thin-layer chromatography (toluene : n-hexane : acetone = 30 : 20 : 2.5) to give 3 compounds, **1** (24 mg), **2** (14 mg), and **3** (5 mg). The fraction E was purified by precipitating with EtOAc, silica gel column chromatography (n-hexane/CH_2Cl_2/EtOAc/MeOH gradient system) and then RP-18 column chromatography (20% water in methanol) to give two compounds, **4** (6 mg) and **5** (4 mg).

Bioassay

Brine shrimp lethality assay (BST): Samples were dissolved in DMSO, diluted to proper concentration (e.g., 1000, 100, 10 µg/ml) and dispensed (200 µl) in six replicates into wells of 96-well microplate. The negative control was DMSO in seawater. The 50 µl-portion of the suspension containing 4-8 shrimps was pipetted and added to each well. The covered microplate was incubated at room temperature for 24 hrs. After this period, the number of dead nauphii in each well was counted. The LD_{50} with 95% confident intervals was calculated using Finney statistical method of probit analysis[4].

Cytotoxicity assay: The cytotoxicity assay was tested against KB (oral human epidermo-carcinoma cell line), BC (breast carcinoma cell line), and NCI-H187 (human small cell lung cancer call line), employing the colorimetric technique[5-7]. Under our screening conditions, the reference compounds, ellipticine and doxorubicin, typically exhibited the activity with the IC_{50} values at the range of 0.2-0.9, and 0.1-0.2 µg/ml, respectively, and dimethyl sulfoxide (DMSO) was used as a negative control.

Anti-Herpes Simplex Virus type 1 assay: The bioassay was tested against HSV-1 strain ATCC VR 260, using colorimetric microtiter plate technique. Herpes simplex virus type 1 (HSV-1) was maintained in the Vero cell line (kidney fibroblast of an African green monkey), which was cultured in Eagle's minimum essential medium (MEM) with addition of heat-inactivated fetal bovine serum (FBS) (10%) and antibiotics. The test samples were put into wells of a microliter

plate at the final concentrations ranging from 20 to 50 µg/ml. The virus HSV-1 (30 PFU) was added into the 96-well plate, followed by plating of Vero cells ($1x10^5$ cells/ml); the final volume was 200 µl. After incubation at 37°C for 72 hrs, under 5% of CO_2 atmosphere, cells were fixed and stained, and the optical density was measured at 510 nm. Acyclovir, typically exhibited the antiviral HSV-1 with IC_{50} value at 1-2 µg/ml and DMSO were used as positive and negative control, respectively[5].

HIV Growth Inhibition assay: The T cell line, H9, was maintained in continuous culture with complete medium (RPMI 1640 with 10 % fetal calf serum [FCS] supplemented with L-glutamine) at 5% CO_2 and 37°C. Aliquots of this cell line were only used in the experiments when in log-phase of growth. Test samples were first dissolved in DMSO. The following were the final drug concentrations routinely used for screening: 100, 20, 4, and 0.8 mg/ml, but for active agents, additional dilutions were prepared for subsequent testing so that accurate EC_{50} value could be achieved. As the test samples were being prepared, an aliquot of the T cell line, H9, was infected with HIV-1 (IIIB isolate) while another aliquot was mock-infected with complete medium. The mock-infected was used for toxicity determinations (IC_{50}). The stock virus used for these studies typically had a $TCID_{50}$ value of 10^4 Infectious Units/ml. The appropriate amount of virus for a multiplicity of infection (moi) between 0.1 and 0.01 Infectious Units/cell was added to the first aliquot of H9 cells. The other aliquot of H9 cells only received culture medium and then was incubated under identical conditions as the HIV-infected H9 cells. After a 4 hour incubation at 37°C and 5% CO_2, both cell populations were washed three times with fresh medium and then added to the appropriate wells of a 24-well plate containing the various concentrations of the test drug or culture medium (positive infected control/negative drug control). In addition, AZT was also assayed during each experiment as a positive drug control. The plates were incubated at 37°C and 5% CO_2, for 4 days. Cell-free supernatants were collected on Day 4 for use in our in-house p24 antigen ELISA assay. P24 antigen is a core protein of HIV and therefore is an indirect measure of virus present in the supernatants. Toxicity was determined by performing cell counts by a Coulter Counter on the mock-infected H9 cells which had either received culture medium (no toxicity) or test sample or AZT.

Antiplasmodial activity: The antiplasmodial activity was tested against *Plasmodium falciparum* (K1, multi drug resistant strain) and conducted following the microdilution radioisotope technique[8,9]. The standard sample was dihydroartemisinine (DHA) and DMSO was used as negative control.

Antimycobacterial activity: The test was evaluated against *Mycobacterium tuberculosis* $H_{37}Ra$, employing the Microplate Almar Blue Assay (MABA); the reference drugs were isoniazid, rifampicin and kanamycin sulfate, showing the MIC values of 0.1, 0.0023 and 2.5 µg/ml, respectively, and DMSO was used as negative control[10].

Spectroscopic data of isolated compounds

(2*S*)-5-hydroxy-6,7-dimethoxy-flavanone (1). Yellowish crystals; $[\alpha]_D$ –24.00°; UV (CH_2Cl_2), λ_{max} (log Є): 236 (4.19), 292 (4.28) and 346 (3.56) nm; IR (film) ν_{max}: 1955, 1641, 1574, 1455, 1297, 1112, 1020 cm^{-1}; HRMS: *m/z* 301.1080 $[M+H]^+$ ($C_{17}H_{17}O_5$, calcd. 301.1070); EIMS (70eV) *m/z* 300 [M^+] (100%): 299 (30), 285 (20), 233(25), 196 (50), 181 (85), 153 (40); ^{1}H- and ^{13}C-NMR ($CDCl_3$) were shown in Table 1.

(2*S*)-5-hydroxy-7, 8-dimethoxy-flavanone (2). Yellowish needles; $[\alpha]_D$ –28.00°; UV (CH_2Cl_2), λ_{max} (log Є): 229 (4.05), 292 (4.21) and 346 (3.66) nm; IR (film) ν_{max}: 1900, 1640, 1582, 1498, 1314, 1265, 1111, 1026 cm^{-1}; HRMS: *m/z* 301.1081 $[M+H]^+$ ($C_{17}H_{17}O_5$, calcd. 301.107); EIMS (70eV) *m/z* 300 [M^+] (100%): 299 (17), 285 (19), 223(25), 196 (62), 181 (85), 153 (47); ^{1}H- and ^{13}C- NMR ($CDCl_3$) were shown in Table 1.

5-hydroxy-7-methoxyflavone (3). White amorphous powder; UV (CH_2Cl_2), λ_{max} (log Є): 269 (4.09); IR (film) ν_{max}: 1650, 1616, 1451, 1381, 1158, 1116, 1032 cm^{-1}; HRMS: *m/z* 269.0816 $[M+H]^+$ ($C_{16}H_{14}O_4$, calcd. 269.0810); EIMS (70eV) *m/z* 268 $[M^+]$ (100%): 239 (51), 225 (12), 102 (8), 95 (13); ^{1}H- and ^{13}C-NMR ($CDCl_3$) were shown in Table 1.

9-methoxyliriodenine (4). Orange powder; UV (EtOH) λ_{max} (nm) (log Є), 245 (4.41), 271 (4.27), 315 (3.72) and 443 (3.56); IR (film) ν_{max} (cm^{-1}), 1604, 1571, 1489, 1358, 1050, 953 and 857; HRMS, *m/z* 306.0768 $[M+H]^+$ ($C_{18}H_{12}O_4N$, calcd. 306.0766); EIMS (70eV), *m/z* 305 $[M^+]$ (100%), 304 (60), 275 (18), 234 (18); ^{1}H- and ^{13}C-NMR ($CDCl_3$) were shown in Table 2.

Liriodenine (5). Yellow powder; UV (CH_2Cl_2) λ_{max} (nm) (log Є), 247 (4.21), 268 (4.01), 307 (3.62) and 415 (3.36); IR (film) ν_{max} (cm^{-1}), 1596, 1576, 1486, 1350, 1202, 1012, 963 and 820; HRMS, *m/z* 276.0670 $[M+H]^+$ ($C_{17}H_{10}O_3N$, calcd. 276.0660); EIMS (70eV), *m/z* 275 $[M^+]$ (50%), 246 (5), 188 (23), 162 (8); ^{1}H-NMR ($CDCl_3$) was shown in Table 2.

Table 1. ^{1}H NMR (500 MHz),^{13}C NMR (125 MHz) spectral data of compound **1**, **2** and **3** in $CDCl_3$

Position	(2S)-5-Hydroxy-6,7-dimethoxyflavanone (**1**)		(2S)-5-Hydroxy-7,8-dimethoxyflavanone (**2**)		5-Hydroxy-7-methoxyflavone (**3**)	
	δ_C (ppm)	δ_H (ppm) multiplicity (*J*, Hz)	δ_C (ppm)	δ_H (ppm) multiplicity (*J*, Hz)	δ_C (ppm)	δ_H (ppm) multiplicity (*J*, Hz)
2	79.1	5.39 (*dd*, 13.4, 3.1)	79.2	5.45 (*dd*, 12.2, 3.1)	164.0	-
3*eq*	43.4	2.80 (*dd*, 17.4, 3.1)	43.4	2.80 (*dd*, 17.1, 3.1)	105.9	6.65 (*s*)
3*ax*	43.4	3.06 (*dd*, 17.4, 13.4)	43.4	3.06 (*dd*, 17.1,12.2)		
4	196.3	-	196.0	-	182.5	-
5	155.0	-	159.9	-	162.2	-
6	130.6	-	93.1	-	98.2	6.37 (*d*, 2.4)
7	160.9	-	161.6	-	165.6	-
8	91.7	6.10 (*s*)	129.8	6.09 (*s*)	92.7	6.49 (*d*, 2.4)
9	158.6	-	153.5	-	157.8	-
10	103.2	-	102.9	-	105.7	-
1′	138.3	-	138.5	-	131.4	-
2′,6′	126.1	7.44 (*dd*, 8.9, 1.8)	126.0	7.46 (*dd*, 9.0, 1.8)	126.3	7.88 (*dd*, 7.9,2.1)
3′,5′	128.90	7.42 (*ddd*, 8.9, 7.6, 1.8)	128.8	7.41 (*ddd*, 9.0, 8.5, 1.8)	129.1	7.49 (*t*, 7.9)
4′	128.87	7.37 (*ddd*, 8.9, 7.6, 1.8)	128.7	7.36 (*ddd*, 9.0, 8.5, 1.8)	131.8	7.53 (*tt*, 7.9, 2.1)
5-OH	-	11.84 (*s*)	-	11.94 (*s*)	-	12.7 (*s*)
6-OCH_3	56.1	3.82 (*s*)	-	-	-	-
7-OCH_3	60.8	3.85 (*s*)	56.2	3.87 (*s*)	55.8	3.87 (*s*)
8-OCH_3			61.3	3.76 (*s*)	-	-

Table 2. ^{1}H NMR (400 MHz),^{13}C NMR (100 MHz) spectral data of compound **4** and **5** in $CDCl_3$

Position	9-Methoxyliriodenine (**4**)		Liriodenine (**5**)
	d_C (ppm)	d_H (ppm) multiplicity (*J*, Hz)	d_H (ppm) multiplicity (*J*, Hz)
1	[a]	-	-
2	[a]	-	-
3	102.4	7.18 (*s*)	7.18 (*s*)
3a	135.8	-	-
4	[a]	7.81 (*d*, 6.5)	7.73 (*d*, 6.0)
5	144.8	8.93 (*d*, 6.5)	8.84 (*d*, 6.5)
6a	157.2	-	-
7	180.1	-	-
7a	151.8	-	-
8	124.3	8.06 (*d*, 3.0)	8.54 (*dd*, 10.5, 1.0)
9	159.7	-	7.54 (*ddd*, 10.5, 9.0, 1.0)
10	110.2	7.33 (*dd*, 10.5, 3.0)	7.70 (*ddd*, 10.0, 9.0, 1.0)
11	132.9	8.60 (*d*, 11.5)	8.62 (*ddd*, 10.0, 1.0)
11a	136.2	-	-
11b	129.1	-	-
11c	[a]	-	-
OCH_2O	102.3	6.39 (*s*)	6.39 (*s*)
9-OCH_3	55.8	4.03 (*s*)	-

[a] = Not detected

RESULTS AND DISCUSSION

The 80% ethanolic extract of *Anomianthus dulcis* stem barks (6 kg, dry weight) was partitioned following the standard procedure to give 6 extracts of F002, F003, F004, F005, F006 and F007. The bioassay (BST bioassay and HTCL cytotoxicity) guided fractionation using chromatographic technique to yield three flavonoids, (2*S*)-5-hydroxy-6,7-dimethoxyflavanone **(1)**, (2*S*)-5-hydroxy-7,8-dimethoxyflavanone **(2)** and 5-hydroxy-7-methoxyflavone **(3)** together with two oxoaporphine alkaloids, 9-methoxyliriodenine **(4)** and liriodenine **(5)** (Figure 1). Compounds **1-3** were isolated as yellowish crystals, yellowish needles and white amorphous powder, respectively. The structures of the compounds were elucidated by analysis of their spectroscopic data such as UV, IR, HRMS and NMR spectra (1D and 2D NMR) (Table 1) and comparison to the known compounds[11-15]. Compounds **4**, **5** were isolated as orange and yellow powder, respectively. The structures of the compounds were elucidated by analysis of their spectroscopic data such as UV, IR, HRMS and 1D NMR spectra (Table 2) and comparison to the known compounds[16-18].

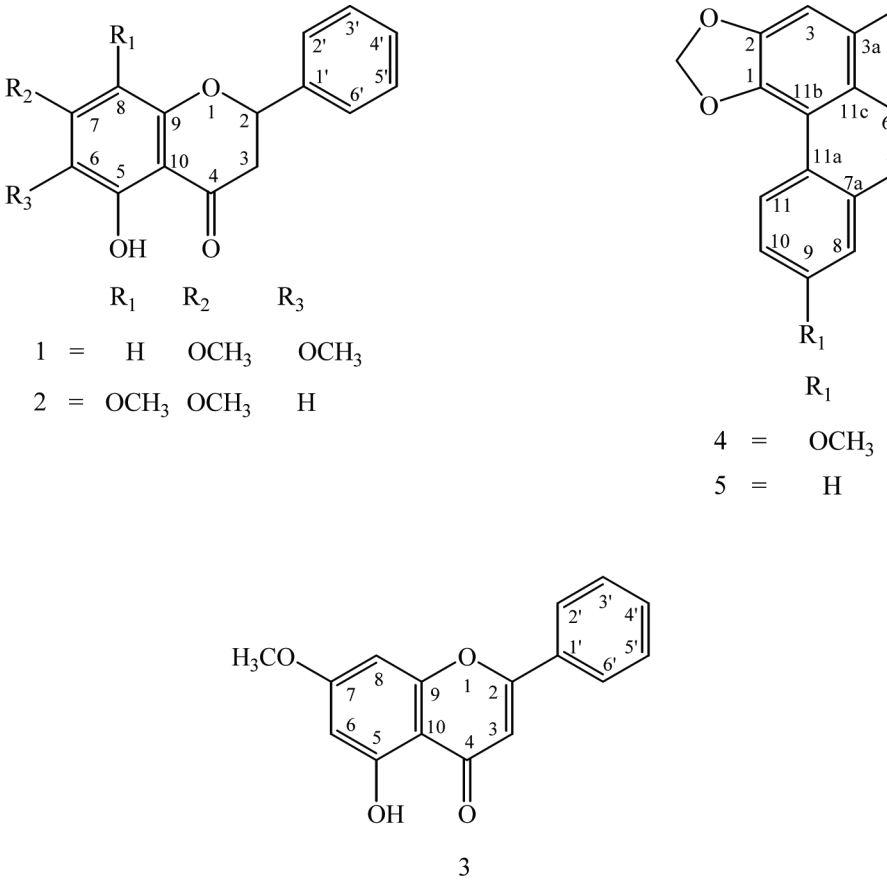

Figure 1. Structures of isolated compounds **(1-5)**

The ethanolic crude extract exhibited significant cytotoxic activity on brine shrimps with LD_{50} value at 4.0 µg/ml and against human tumor cell lines, NCI-H187, BC, and KB with IC_{50} values at the range of $7.8x10^{-2}$ - 0.31 µg/ml. The F006 was the most active fraction in all cell lines experiment and was about 10-100 times more potent than the positive controls, ellipticine and doxorubicine. Subfractions D, E, F and isolated pure compounds were found to be less active than F006. This may be the result of synergism between different compounds in the complex extracts or fractions. Among the isolated compounds, liriodenine **(5)** was found to be the most active compound. It exhibited the growth inhibition of NCI-H187, BC, and KB cell lines with IC_{50} values at 1.02, 13.45 and 14.57 µg/ml, respectively. Compound **4**, the liriodenine isomer, was selectively active against NCI-H187 with IC_{50} value at 1.28 µg/ml. Compounds **1** and **2** were flavanone isomers; however, **1** was found to be more active than **2** where the methoxy groups were substituted at C-6 and C-7 instead of C-7 and C-8. Compound **3**, a flavone, exhibited selective cytotoxicity against NCI-H187 with IC_{50} at 12.41 µg/ml. In conclusion, it was revealed that the alkaloid-based structure was likely to be more cytotoxic than the flavonoid-based structure (Table 3). Compounds **5**, **1** and **4** exhibited antimycobacterial activity with minimum inhibitory concentrations (MICs) of 100, 200, and 200 µg/ml, respectively. Only **5** showed strong activity as anti-HSV-1 with IC_{50} value at 3.3 µg/ml. Although the F006 and F007 extracts and some fractions were potential for anti HIV activity (Table 4), the isolated compounds showed no activity at the concentration of 25 µg/ml. It is possibly that the active compounds are not the same group of the isolated compounds and the anti-HIV activity of this plant is worthwhile to be further investigated. Additionally, none of the isolated compounds were active for

antiplasmodial activity at the concentration of 10 µg/ml (Table 5). Although all isolated compounds are known, it is the first report of the isolation from *Anomianthus dulcis* stem barks and the biological activities such as cytotoxicity, anti-HSV-1, anti-HIV and antiplasmodial activities. It was found that **1-4** were cytotoxic and **5** showed both cytotoxic and anti-HSV-1 activities. From the literature data, compounds **1-5** were previously reported as the followings. Compound **1** was isolated from *Onychium sikiculosum*[11]. Compound **2** was found in various plants such as *Andrographis paniculata*[12], *Miliusa balansae*[13] and *Andrographis lineata*[14]. Compound **3** was isolated from *Boesenbergia pandurata*[15]. Compound **4** was isolated from *Michelia lanuginose*[19]. *Xylopia lemurica*[20], and *Guatteria elata*[21]. Compound **5** was initially isolated from the heartwood of the yellow poplar, *Lirodendron tulipifera* (Magnoliaceae)[22], and was found in various annonaceous plants such as *Annona glabra*[22], *Annona montana*[16], *Artabotrys zeylanicus*[17], *Fissistigma glaucescens*[18], *Hornschuchia oblique*[23], and *Xylopia aethiopica*[24]. Compound **5** has been reported for the cytotoxicity against KB, A-549, P-388, and L-1210 cells with IC_{50} values at 1.00, 0.72, 0.70, 0.5, and 2.33 µg/ml, respectively[16].

Table 3 Cytotoxicity of the fractions and isolated compounds

Sample	BST	Cytotoxicity in HTCL test (IC_{50} µg/ml)		
	(LD_{50} µg/ml)	NCI-H187	KB	BC
F001	4.0	$7.8x10^{-2}$	0.19	0.31
F003	0.3	$2.9x10^{-4}$	$7.8x10^{-7}$	$2.0x10^{-2}$
F006	0.2	$1.8x10^{-4}$	$1.0x10^{-2}$	$5.0x10^{-2}$
F007	5.4	$4.9x10^{-4}$	$2.4x10^{-4}$	$2.7x10^{-4}$
Fr.D	6.4	$4.4x10^{-2}$	$9.0x10^{-2}$	0.15
Fr.E	ND	0.30	1.25	0.91
Fr.F	0.2	$6.0x10^{-3}$	$1.0x10^{-3}$	$5.0x10^{-3}$
(2*S*)-5-Hydroxy-6,7-dimethoxyflavanone (**1**)	ND	1.73	15.45	Inactive
(2*S*)-5-Hydroxy-7,8-dimethoxyflavanone (**2**)	ND	Inactive	12.86	Inactive
5-Hydroxy-7-methoxy-flavone (**3**)	ND	12.41	Inactive	Inactive
9-Methoxyliriodenine (**4**)	ND	1.28	Inactive	Inactive
Liriodenine (**5**)	ND	1.02	13.45	14.57
Ellipticine	ND	0.35	0.83-0.92	0.83-0.92
Doxorubicine	ND	ND	0.13	0.15

BST = Brine shrimp lethality test
NCI-H187 = Human small cell lung cancer
KB = Human nasopharyngeal carcinoma
BC = Human breast cancer
ND = Not determined

Table 4 Anti-HIV activity of the extracts (F006-F007) and the isolated fractions of F006 (Fr.A-Fr.G)

Sample	Anti-HIV in H9 lymphocytes		
	IC_{50} (µg/ml)	EC_{50} (µg/ml)	TI (IC_{50}/EC_{50})
F006	22.97	No suppression	No suppression
F007	>25	No suppression	No suppression
Fr.A	nt*	nt	nt
Fr.B	>25	19.47	1.28
Fr.C	14.12	No suppression	No suppression
Fr.D	11.94	No suppression	No suppression
Fr.E	nt	nt	nt
Fr.F	8.63	No suppression	No suppression
Fr.G	12.77	No suppression	No suppression
1	>25	No suppression	No suppression
2	>25	No suppression	No suppression
AZT	500	0.00824	60712

* nt = not tested due to the insufficient amount of sample

Table 5 Biological activities of isolated compounds

Compound	Anti-HSV-1 ($IC_{50,}$ µg/ml)	Cytotoxicity against VERO ($IC_{50,}$ µg/ml)	Antiplasmodial ($IC_{50,}$ µg/ml)	Anti-HIV (TI = IC_{50}/EC_{50})	Anti-TB (MIC, µg/ml)
1	Inactive	>50	Inactive	Inactive	200
2	Inactive	>50	Inactive	Inactive	Inactive
3	Inactive	>50	Inactive	nt*	Inactive
4	Inactive	>50	Inactive	nt	200
5	3.3	20.5	Inactive	nt	100
Acyclovir	1.7 ± 0.5				
Ellipticine		0.4 ± 0.1			
Dihydro-artemisinine			4.2		
AZT				60712	
Rifampicin					0.0023
Kanamycin					0.5
Isoniazid					0.1

* nt = not tested due to the insufficient amount of sample

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References

1. Smitinand T. Thai Plant Name. Revised edition. Bangkok: The Forest Herbarium, Royal Forest Department, 2001.
2. วงศ์สถิตย์ ฉั่วกุล และ ฉวีวรรณ ใจแก้ว. สมุนไพรพื้นบ้านจังหวัดอุบลราชธานี (3). วารสารสมุนไพร 2545; 5:28-56.
3. Sinz A, Matusch R, Elsacker F, Santisuk T, Chaichana S, Reutrakul V. Phenolic compounds from *Anomianthus dulcis*. *Phytochemistry* 1999; 50:1069-72.
4. Solis PN, Wright CW, Anderson MM, Gupta MP, Phillipson JD. A microwell cytotoxic assay using *Artemia salina* (brine shrimp). *Planta Medica* 1993; 59:250-2.
5. Skehan P, Storreng R, Scudiero D, Monks A, McMahon J, Vistica D, *et al*. New colorimetric cytotoxicity assay for anticancer drug screening. *J Natl Cancer Inst* 1990; 82:1107-12.
6. Chung HS, Woo WS. A quinolone alkaloid with antioxidant activity from the aleurone layer of anthocyanin-pigmented rice. *J Nat Prod* 2001; 64: 1579-80.
7. Plumb JA, Milroy R, Kaye SB. Effect of the pH dependence of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazoliumbased assay. *Cancer Res* 1989; 49: 4435- 40.
8. Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science* 1976; 193(4254):673-5.
9. Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrob Agents Ch* 1979; 16:710-8.
10. Collins LA, Franzblau SG. Microplate Alamar Blue Assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrob Agents Ch* 1997; 41(5):1004-9.
11. Wu TS, Kuoh CS, Ho ST, Yang MS, Lee KK. Flavanone and other constituents from *Onychium siliculosum*. *Phytochemistry* 1981; 20:527-9.
12. Gupta KK, Taneja SC, Dhar KL, Atal CK. Flavonoids of *Andrographis paniculata*. *Phytochemistry* 1983; 22(1): 314-5.
13. Kamperdick C, Van NH, Sung TV. Constituents from *Miliusa balansae* (Annonaceae). *Phytochemistry* 2002; 61:991-4.
14. Kishore PH, Reddy MVB, Reddy MK, Gunasekar D, Caux C, Bodo B. Flavonoids from *Andrographis lineata*. *Phytochemistry* 2003; 63:457-61.
15. Jaipetch T, Reutrakul V, Tuntiwachwuttikul P, Santisuk T. Flavonoids in the black rhizomes of *Boesenbergia pandurata*. *Phytochemistry* 1983; 22(2):625-6.
16. Wu YC, Chang GY, Duh CY, Wang SK. Cytotoxic alkaloid of *Annona montana*. *Phytochemistry* 1993; 33(2):497-500.
17. Wijeratne EMK, Hatanaka Y, Kikuchi T, Tezuka Y, Gunatilaka AAL. A dioxoaporphine and other alkaloids of two annonaceous plants of Sri Lanka. *Phytochemistry* 1996; 42(6):1703-6.
18. Lo WL, Chang FR, Wu YC. Alkaloids from the leaves of *Fissistigma glaucescens*. *J Chinese Chem Soc* 2000; 47: 1251-6.
19. Talapatra SK, Patra A, Talapatra B. Alkaloids of *Michelia lanuginosa* Wall. *Tetrahedron* 1975; 31:1105-7.

20. Nieto M, Leboeuf M, Cave A. Isolement de la lanuginosine a partir d'une annonacee malgache, *Xylopia lemurica*. *Phytochemistry* 1975; 14:2508-9.
21. Hsu CC, Dobberstein RH, Cordell GA, Farnsworth NR. Isolation of lauterine and oxoputerine, two new oxoaporphine alkaloids from *Guatteria elata. Lloydia* 1977; 40(2):152-6.
22. Warthen D, Gooden EL, Jacobson M. Tumor inhibitors: Liriodenine, a cytotoxic alkaloid from *Annona glabra*. *J Pharm Sci* 1969; 58(5):637-8.
23. Fechine IM, Tavares JF, Silva MSD, Barbosa- Filho JM, Agra MDF, da-Cunha EVL. Two new alkaloids from *Hornschuchia obliqua. Fitoterapia* 2003; 74:29-33.
24. Harrigan GG, Gunatilaka AAL, Kingston DGI. Isolation of bioactive and other oxoaporphine alkaloids from two annonaceous plants, *Xylopia aethiopica* and *Miliusa* cf. *banacea. J Nat Prod* 1994; 57(1):68-73.