ORIGINAL ARTICLE

Antioxidant and cytotoxic activities of Thai medicinal plants named Khaminkhruea: *Arcangelisia flava*, *Coscinium blumeanum* and *Fibraurea tinctoria*

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Abstract

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Antioxidant and cytotoxic activities of Thai medicinal plants named Khaminkhruea: *Arcangelisia flava*, *Coscinium blumeanum* and *Fibraurea tinctoria*
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The stems of *Arcangelisia flava, Coscinium blumeanum* and *Fibraurea tinctoria*, collectively known in southern Thailand as Khaminkhruea, were sequentially extracted with petroleum ether, chloroform, methanol, and boiling water to afford 12 crude extracts. All extracts have been assessed for antioxidant activity against DPPH (1,1-diphenyl-2-picrylhydrazyl) radical and cytotoxic activity against brine shrimp and human cancer cell line MCF-7 (breast adenocarcinoma). Methanol extract of *A. flava* (AFM), and methanol and chloroform extracts of *C. blumeanum* (CBM and CBC) exhibited moderate antioxidant activity with EC_{50} values of 25-55 μg/ml. Chloroform extracts of *A. flava* (AFC) and *F. tinctoria* (FTC), AFM, CBC and CBM showed pronounced cytotoxic activity against brine shrimp and MCF-7 cells with LC_{50} and IC_{50} values of 210-278 and 8-12 μg/ml, respectively. Bioassay-guided chemical investigation led to the isolation of berberine as a main compound of AFC, AFM, CBC and CBM. Palmatine and jatrorrhizine were found to be main alkaloids of FTC, and minor alkaloids of AFC, AFM, CBC and CBM. In addition, an ester triacontanyl caffeate was isolated for the first time from *C. blumeanum* (CBC). Chemical structures of the isolated

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compounds were determined by spectroscopic methods particularly NMR and mass spectrometry. Triacontanyl caffeate was mainly responsible for antioxidant activity of *C. blumeanum* with an EC_{50} value of 6.8 μg/ml. Jatrorrhizine possessed moderate antioxidant activity with an EC_{50} value of 98.0 μg/ml, whereas palmatine and berberine were found to be considerably less active (EC_{50} >100 μg/ml). The LC_{50} values of the four isolated compounds on brine shrimp were ranging from 37-206 μg/ml. The IC_{50} values of berberine, palmatine and jatrorrhizine against MCF-7 cells were in the range of 1-4 μg/ml. Triacontanyl caffeate was considerably less cytotoxic than the alkaloids with an IC_{50} value of 15.5 μg/ml.

Key words : *Arcangelisia flava, Coscinium blumeanum, Fibraurea tinctoria,* Menispermaceae, isoquinoline alkaloids, palmatine, jatrorrhizine, berberine, triacontanyl caffeate, antioxidant, cytotoxicity

บทคัดย่อ

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ฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ความเป็นพิษต่อเซลล์ของสมุนไพรไทยชื่อขมิ้นเครือ

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ได้นำสมุนไพรชื่อขมิ้นเครือซึ่งเป็นพืชท้องถิ่นในภาคใต้ของประเทศไทย 3 ชนิด คือ ***Arcangelisia flava, Coscinium blumeanum*** และ ***Fibraurea tinctoria*** มาศึกษาฤทธิ์ต้านอนุมูลอิสระ (เป็นกลไกหนึ่งของ antioxidant) และฤทธิ์ความเป็นพิษต่อเซลล์ พร้อมทั้งสกัดแยกและพิสูจน์เอกลักษณ์โครงสร้างทางเคมีของสารสำคัญในสมุนไพรเหล่านี้ โดยสกัดผงสมุนไพรแห้งซ้ำด้วยตัวทำละลาย 4 ชนิดตามลำดับความเป็นขั้วจากน้อยไปหามากคือ ปิโตรเลียมอีเทอร์ คลอโรฟอร์ม เมธานอล และน้ำต้มเดือด ได้สารสกัดรวม 12 ตัวอย่าง เมื่อนำมาทดสอบฤทธิ์ต้านอนุมูลอิสระด้วยวิธี DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay พบว่า สารสกัดเมธานอลของ ***A. flava*** สารสกัดเมธานอลและสารสกัดคลอโรฟอร์มของ ***C. blumeanum*** มีฤทธิ์ต้านอนุมูลอิสระที่ดี โดยมีค่าความเข้มข้นที่กำจัดอนุมูลอิสระได้ 50% (EC_{50}) อยู่ในช่วง 25-55 ไมโครกรัม/มล. ในขณะที่สารสกัดคลอโรฟอร์มของพืชทั้ง 3 ชนิด และสารสกัดเมธานอลของ ***A. flava*** และ ***C. blumeanum*** แสดงฤทธิ์ความเป็นพิษต่อไรน้ำเค็ม (brine shrimp) โดยมีค่า LC_{50} อยู่ในช่วง 210-278 ไมโครกรัม/มล. และมีฤทธิ์ต้านเซลล์มะเร็งเต้านม MCF-7 (breast adenocarcinoma) โดยมีค่า IC_{50} อยู่ในช่วง 8-12 ไมโครกรัม/มล. จากการศึกษาองค์ประกอบทางเคมีของสารสกัดที่แสดงฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ความเป็นพิษต่อเซลล์สามารถแยกได้สารบริสุทธิ์ 4 ชนิด ซึ่งเมื่อพิสูจน์เอกลักษณ์โครงสร้างทางเคมีโดยใช้เทคนิคทางสเปคโตรสโคปีพบว่าเป็นสารกลุ่มไอโซควิโนลีน แอลคาลอยด์ 3 ชนิด คือ palmatine, jatrorrhizine และ berberine ส่วนอีกหนึ่งชนิดเป็นสารกลุ่มเอสเทอร์ คือ triacontanyl caffeate ทั้งนี้ในสารสกัดคลอโรฟอร์มและสารสกัดเมธานอลของ ***A. flava*** และ ***C. blumeanum*** พบแอลคาลอยด์ทั้ง 3 ชนิด โดยมี berberine เป็นองค์ประกอบหลัก แต่ในสารสกัดคลอโรฟอร์มของ ***F. tinctoria*** มี palmatine และ jatrorrhizine เป็นองค์ประกอบหลัก (ไม่พบ berberine) ส่วน triacontanyl caffeate แยกได้จากสารสกัดคลอโรฟอร์มของ ***C. blumeanum*** ซึ่งเป็นครั้งแรกที่แยกสารนี้ได้จากต้น ***C. blumeanum*** ผลการทดสอบพบว่า triacontanyl caffeate เป็นสารสำคัญในการแสดงฤทธิ์ต้านอนุมูลอิสระของต้น ***C. blumeanum*** (EC_{50} เป็น 6.8 ไมโครกรัม/มล.) ส่วน jatrorrhizine มีค่า EC_{50} ในการต้านอนุมูลอิสระเป็น 98.0 ไมโครกรัม/มล. ในขณะที่ palmatine และ berberine มีฤทธิ์น้อยมาก (EC_{50} > 100 ไมโครกรัม/มล.) สำหรับฤทธิ์ความเป็นพิษต่อไรน้ำเค็มของสารทั้ง 4 ชนิดนั้น มีค่า LC_{50} อยู่ในช่วง 37-206 ไมโครกรัม/มล. ทั้งยังพบว่า palmatine, jatrorrhizine และ berberine แสดงความเป็นพิษต่อเซลล์มะเร็ง MCF-7 ได้ดี โดยมีค่า IC_{50} อยู่ในช่วง 1-4 ไมโครกรัม/มล. ในขณะที่ triacontanyl caffeate มีฤทธิ์ต่ำกว่าโดยมีค่า IC_{50} เป็น 15.5 ไมโครกรัม/มล.

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Several plants belonging to the family Menispermaceae have been utilized for medicinal purposes in Thai traditional medicine. The family is well known as an important source of isoquinoline alkaloids, one of the largest groups of natural products which display interesting pharmacological activity (Shamma, 1972). A group of Menispermaceous plants known in Thai as Khaminkhruea, which are yellow-wood climbers derived from *Arcangelisia flava* (L.) Merr., *Coscinium blumeanum* Miers, *C. fenestratum* (Gaertn.) Colebr. and *Fibraurea tinctoria* Lour., have been used in traditional medicines of Thailand and some other Asian countries (Perry and Metzger, 1980). However, some other plants such as *Anamirta cocculus* Wight & Arn. (Menispermaceae) and *Combretum acuminatum* Roxb. (Combretaceae), which do not possess yellow wood, are also confusingly known in Thailand as Khaminkhruea (Smitinand, 1980; Forman, 1991). Three out of the four Khaminkhruea species, i.e. *A. flava, C. blumeanum,* and *F. tinctoria* are indigenous to southern Thailand, while *C. fenestratum* is the local plant of northeastern and eastern regions of Thailand. The botanical descriptions of these four species have been completely described in Flora of Thailand (Forman, 1991). The stems and roots of these three plants have been used in traditional medicine as bitter tonic and to treat a variety of diseases including jaundice and some infectious diseases such as diarrhea and skin abscess (Perry and Metzger, 1980). These suggest that they could be potential sources of biologically active compounds, which might be used as the lead molecules for development of new drugs. According to a review by Thornber (1970), the isoquinoline alkaloids palmatine and jatrorrhizine were commonly isolated from the three plants whereas the alkaloid berberine was reported from *A. flava* and *C. blumeanum*. Some minor alkaloids, for instance, columbamine, dehydrocorydalmine, homoaromoline and thalifendine were also isolated from the stem of *A. flava* (Verpoorte *et al*., 1982; Dechatiwongse Na Ayudhya *et al*., 1993). Furthermore, some furanoditerpenes were isolated from *A. flava* (Kunii *et al*., 1985) and *F. tinctoria* (Itokawa *et al*., 1986). It is notable that, among the three species, *C. blumeanum* has been the least studied one, of which there has been only one paper so far reporting about its alkaloid component (Tomita and Tani, 1941). So far no report concerning biological activity of *C. blumeanum* has been published. The extracts of *A. flava* and *F. tinctoria* showed antimicrobial activity (Pongpan *et al*., 1982; Avirutnant and Pongpan, 1983; Grosvenor *et al*., 1995). Ethanol extract of *A. flava* exhibited cardiotonic activity on turtle heart (perfusion) and hypotensive activity in dog (Estrada *et al*., 1963). On the other hand, no research work on antioxidant and cytotoxic activities of these three plants have been reported. Natural antioxidants have been known to be capable of inhibiting the formation of carcinogens from precursor substances, thus could provide preventive effect against cancer (Gordon, 1996). Upon our preliminary assay for antioxidant and cytotoxic activities of some medicinal plants used by folkloric medicine-men in southern Thailand, it was found that the methanol extracts of the stems of *A. flava, C. blumeanum*, and *F. tinctoria* showed appreciable effects. Therefore, further work on chemical investigation and biological evaluation of these three plants is of interest. In the present study we report antioxidant activity of the extracts of *A. flava, C. blumeanum* and *F. tinctoria* on DPPH radical, and their cytotoxic activity against brine shrimp and human cancer cell line MCF-7 (breast adenocarcinoma). In addition, the structure elucidation and the biological activities of the compounds isolated from the active extracts are also described.

Materials and Methods

DPPH radical scavenging assay (Antioxidant assay)

The following assay procedure was modified from those described by Blois (1958) and Yamasaki *et al*. (1994). Samples were dissolved in an appropriate vehicle, i.e. absolute ethanol, methanol or distilled water, to obtain the required concentrations and further diluted for at least 5 concentrations (two-fold dilutions). These vehicles

showed no effect on the assay. A portion of sample solution (500 µl) was mixed with an equal volume of $6x10^{-5}$ M DPPH (1,1-diphenyl-2-picrylhydrazyl; in absolute ethanol) and allowed to stand at room temperature for 30 min. The absorbance (A) of sample solution (in triplicate for each concentration) was measured at 520 nm, compared with that of control solution (maximum absorbance). The scavenging activity of samples corresponded to the intensity of quenching DPPH. The results were expressed as percentage inhibition, %inhibition = $[(A_{control} - A_{sample})/A_{control}] \times 100$. The effective concentration of sample required to scavenge DPPH radical by 50% (EC_{50} value) was obtained by linear regression analysis of dose-response curve plotting between %inhibition and concentrations.

Brine shrimp lethality assay

The assay was carried out according to the principle and protocol previously described by Meyer *et al*. (1982) and Solis *et al*. (1993) with slight modifications. Brine shrimp eggs (*Artemia salina*) were hatched in artificial sea water (40 g of sea salt per 1 L of distilled water) and supplemented with 6 mg/L dried yeast. After 36-48 hr incubation at room temperature (25-30ºC), the resulting nauplii (larvae) were attracted to one side of the vessel with a light source and collected with a measuring pipette. On 96-well microtiter plates, 100 µl of a suspension of nauplii containing 10-15 organisms was added to each well. Samples for testing were initially dissolved in an amount of DMSO and further diluted with artificial sea water to produce the required concentrations. The final concentration of DMSO in the test system was not greater than 2 % which has no effect on the growth of brine shrimp. Percent mortality of nauplii after exposure to test samples (serial dilutions) for 24 h was determined microscopically. A few drops of 10% formalin were added to each well and after 15 min the total numbers of nauplii in each well were counted compared with the non-treated control wells. Computer program for probit analysis (Finney, 1971) was used to determine the concentration at which lethality of brine shrimp represents 50% (LC_{50} value).

Assay for cytotoxic activity on human cancer cells

The human breast adenocarcinoma cell line MCF-7 was cultured in EMEM medium (with glutamine) supplemented with 10% heat-inactivated newborn calf serum, 50 IU/ml penicillin G sodium, 50 µg/ml streptomycin sulphate and 0.125 µg/ml amphotericin B. Cells were maintained at 37ºC in 5% CO_2 atmosphere with 95% humidity. According to growth profiles, the optimal plating density was determined to be 2000 cells/well to ensure the exponential growth throughout the experimental period and to ensure a linear relationship between absorbance and cell number when analyzed by SRB assay (Skehan *et al*., 1990). The cytotoxic activity was assessed according to the protocol described previously (Keawpradub *et al*., 1997). In brief, the tumour cells were seeded in 96-well plates and incubated to allow for cell attachment (18-24 hr). Cell viability (% survival) after exposure to test samples (serial dilutions) was determined colorimetrically (SRB assay) at 492 nm (Power Wave X plate reader: Bio-TEK Instruments, Inc.). Cell survival of the treated wells was measured as the percentage of absorbance compared to the control wells (non-treated cells, taken as 100 % survival). The final mixture used for treating the cells contained not more than 0.5% of the vehicle, the same as in vehicle-control wells, which showed no effect on cell growth. The IC_{50} value (effective concentration of sample required to inhibit cell growth by 50 %) was calculated from dose-response curves plotting between %inhibition and concentrations.

Instrumentation

Melting points were determined on a Buchi 520 melting point apparatus (uncorrected). Spectronic Genesys 5 UV spectrometer was used for measuring the absorbance in DPPH assay. IR spectra were recorded on a Jasco IR-810 spectrometer (KBr). UV spectrum was obtained from a Hewlett Packard 8452A diode array spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 500/125 MHz on a Varian Inova 500 NMR spectrometer. Chemical shifts (δ) were reported in ppm scale (*J*

in Hz), using either TMS or operating solvent as internal standards. A 90º-t_1-45º pulse sequence (COSY 45) was used for ^{1}H-^{1}H COSY experiments. Distortionless enhancement by polarization transfer (DEPT) experiments were carried out with the polarization pulse θ = 45º, 90º and 135º. EI-MS data were recorded on a Hewlett-Packard HP 5890 Series II Plus GC-HP 5972 Mass Selective Detector (EI mode with mass range of 20-700 amu) and a Finnigan MAT Incos 50 mass spectrometer. The TOF-ESI mass spectrometry was performed on a Mass spectrometer LCT (Micromass).

Chromatography

Analytical TLC was performed on precoated plates of silica gel 60 F_{254} (Merck, 0.2 mm thick). Developing solvent systems used were as follows: Toluene-MeOH-25% NH_3 (3:4:1), $CHCl_3$-MeOH (4:1), $CHCl_3$-MeOH (9:1), $CHCl_3$-MeOH (19:1), and EtOAc-MeOH (7:1). The zones were detected under UV at 254 nm and by spraying with Dragendorff's and anisaldehyde reagents. Preparative TLC was performed on silica gel 60 GF_{254} plates (Merck, 0.5 mm thick), activated at 105ºC for 30 min before use. The zones were detected under UV at 254 nm, scraped off, eluted with $CHCl_3$-MeOH (3:1) and evaporated to dryness. Silica gel 60 (Merck, 0.040-0.063 mm) was used for vacuum liquid chromatography (VLC) and column chromatography (CC). Sephadex LH-20 was purchased from Amersham Pharmacia Biotech AB (Sweden), suspended in methanol overnight before use.

Plant materials

The stems and leaves of *Arcangelisia flava* (L.) Merr., *Coscinium blumeanum* Miers and *Fibraurea tinctoria* Lour. (Menispermaceae) were collected at Songkhla Province and Surasthanee Province (southern Thailand) in March, 1999. Plant materials were identified by the author (NK). The herbarium vouchers have been deposited at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand, (accession numbers: MSPSU001-003 for *A. flava* (L.) Merr., *C. blumeanum* Miers and *F. tinctoria* Lour., respectively).

Extraction of plant materials

The ground-dried stem of each plant (1 kg each) was sequentially extracted with petroleum ether, chloroform, methanol, and boiling water. The extraction was repeated three times for each solvent. The combined extract of each solvent was evaporated under reduced pressure (except the water extracts which were dried by lyophilization) to give twelve crude extracts. The weights of each extract were as follows: petroleum ether extracts of *A. flava* (AFP), *C. blumeanum* (CBP) and *F. tinctoria* (FTP) were 0.95, 1.10 and 2.72 g, respectively; the chloroform extracts, i.e. AFC, CBC and FTC were 4.03, 14.80 and 44.84 g, respectively; the methanol extracts, i.e. AFM, CBM and FTM were 52.80, 59.50 and 31.50 g, respectively; and the water extracts, i.e. AFW, CBW and FTW were 2.85, 2.68 and 3.17 g, respectively.

Isolation of the active extracts

According to the results of preliminary activity assay (Table 1), the active extracts FTC, CBM, CBC, AFC, and AFM were subjected to chemical investigation as follows:

(1) Isolation of chloroform extract of *F. tinctoria* (FTC)

An aliquot of FTC (15.0 g) was fractionated by VLC using silica gel as an adsorbent. The system was sequentially eluted with $CHCl_3$ and MeOH in a polarity gradient fashion, by increasing polarity of MeOH from 0 to 100%, to obtain 36 fractions (200 ml each). Fractions 10-16 were purified by CC (silica gel) eluting with $CHCl_3$-MeOH (6:1, 2100 ml) to obtain compound **1** as yellow needles (2.967 g). Fractions 17-31 were subjected to CC (silica gel), sequentially eluted with $CHCl_3$-MeOH (4:1, 2000 ml and 3:1, 1200 ml). After crystallization in MeOH, compound **2** (730 mg) was obtained as orange plates. According to TLC analysis, **1** and **2** were major components of FTC.

Table 1. Antioxidant and cytotoxic activities of the extracts and isolated compounds from *A. flava, C. blumeanum* and *F. tinctoria*

Extract/Compound		Antioxidant against DPPH EC_{50} (µg/ml)	Brine shrimp lethality test LC_{50} (µg/ml)	Cytotoxic against MCF-7 cells IC_{50} (µg/ml)
A. flava:	AFP	>100±N=2)	>1000 (N=2)	>50 (N=2)
	AFC	>100±N=2)	266.7±22.3 (N=2)	8.6±1.0 (N=2)
	AFM	25.7±1.7 (N=2)	210.4±21.9 (N=2)	7.7±0.6 (N=2)
	AFW	68.3±0.8 (N=2)	>1000 (N=2)	>50 (N=2)
C. blumeanum:	CBP	>100 (N=2)	>1000 (N=2)	>50 (N=2)
	CBC	55.4±0.3 (N=2)	278.2±15.8 (N=2)	9.1±0.9 (N=2)
	CBM	50.1±0.6 (N=2)	221.6±27.8 (N=2)	10.9±1.1 (N=2)
	CBW	64.6±0.2 (N=2)	>1000 (N=2)	>50 (N=2)
F. tinctoria:	FTP	>100 (N=2)	>1000 (N=2)	>50 (N=2)
	FTC	78.8±1.6 (N=2)	252.7±28.4 (N=2)	11.2±1.2 (N=2)
	FTM	83.6±1.8 (N=2)	>1000 (N=2)	>50 (N=2)
	FTW	>100 (N=2)	>1000 (N=2)	>50 (N=2)
1 (Palmatine)		>100 (N=2)	135.4±6.1 (N=3) (384.7 µM)	3.0±0.5 (N=3) (8.5 µM)
2 (Jatrorrhizine)		98.0±1.7 (N=3) (289.9 µM)	206.6±11.7 (N=3) (611.2 µM)	3.9±0.5 (N=3) (11.5 µM)
3 (Berberine)		>100 (N=2)	37.1±5.9 (N=3) (110.4 µM)	1.0±0.2 (N=3) (3.0 µM)
4 (Triacontanyl caffeate)		6.8±0.4 (N=3) (11.3 µM)	65.2±5.6 (N=3) (108.7 µM)	15.5±1.9 (N=3) (25.8 µM)
Butylated hydroxytoluene*		18.8±1.5 (N=3) 85.4 µM	-	-
Caffeic acid*		0.9±0.1 (N=3) (5.0 µM)	-	-
			-	-
Strychnine hydrochloride*		-	175.7±6.7 (N=3) (474.9 µM)	-
Vinblastine sulphate*		-	-	1.2±0.1 nM (N=3)

- = Test not done
*** = positive standards**
N = Number of independent experiments

(2) Isolation of methanol extract of *C. blumeanum* (CBM)

An aliquot of CBM (15.0 g) was subjected to silica gel VLC, eluted with $CHCl_3$-MeOH (4:1, 3:1, 2:1, 600 ml each; 1:1, 1200 ml) and MeOH (1200 ml) to afford 21 fractions (1a-21a, 200 ml each). Fractions 1a-6a were subjected to CC (silica gel) eluting with $CHCl_3$-MeOH (6:1, 1400 ml and 3:1, 2000 ml) to obtain compound **1** (158 mg) and compound **3** (830 mg). Fractions 7a-16a were separated by CC (silica gel) eluting with $CHCl_3$-MeOH (9:1, 500 ml; 5:1, 600 ml; 3:1, 800 ml; 1:1, 800 ml) to afford 87 fractions (1b-87b, 30 ml each). Fractions 47b-76b were subjected to CC (silica gel), eluted with CHCl3-MeOH (4:1, 2500 ml) to yield compound **1** (90 mg) and compound

3 (237 mg). The polar fractions 17a-21a were pooled and separated by using Sephadex LH-20 as a stationary phase eluting with MeOH to obtain 37 fractions (1c-37c). Fractions 11c-18c were purified by CC (silica gel), eluted with $CHCl_3$-MeOH (3:1 and 1:1, 800 ml each) to afford compound **2** (225 mg).

(3) Isolation of chloroform extract of *C. blumeanum* (CBC)

An aliquot of CBC (6.0 g) was subjected to CC (silica gel), eluted with EtOAc-MeOH (4:1, 1500 ml) and $CHCl_3$-MeOH (3:1, 1200 ml; 2:1, 600 ml; 1:1, 300 ml) to afford 72 fractions (50 ml each). Fractions 9-16 were separated by Sephadix LH-20 (eluted with MeOH), followed with preparative TLC using $CHCl_3$-MeOH (19:1) as developing solvent to yield 56 mg of compound **4**. Fractions 42-66 were subjected to CC (silica gel), eluted with $CHCl_3$-MeOH (4:1, 2000 ml) to obtain compound **1** (145 mg) and compound **3** (640 mg). Trace amount of compound **2** was detected (TLC analysis) in fractions 67-72.

(4) Isolation of chloroform extract of *A. flava* (AFC)

An aliquot of AFC (3.0 g) was subjected to CC (silica gel), eluted with *n*-Hexane-$CHCl_3$ (1:1, 500 ml) and $CHCl_3$-MeOH (19:1, 9:1, 4:1, 2:1, 500 ml each) to obtain 50 fractions. Fractions 35-43 were separated by preparative TLC using Toluene-EtOH-25% NH_3 (3:4:1) as developing solvent to yield compound **1** (23 mg) and compound **3** (388 mg). Trace amount of compound **2** was detected (TLC analysis) in fractions 46-50.

(5) Isolation of methanol extract of *A. flava* (AFM)

An aliquot of AFM (15.0 g) was subjected to VLC (silica gel), eluted with $CHCl_3$-MeOH (9:1, 1000 ml; 4:1, 1200 ml; 2:1, 1000 ml) and MeOH (1000 ml) to obtain 4 fractions (F1-F4), respectively. Fraction F2 was subjected to CC (silica gel), eluted with $CHCl_3$-MeOH (6:1, 1400 ml and 3:1, 1600 ml) to obtain 60 fractions (1a-60a). Fractions 27a-42a were subjected to CC (silica gel), eluted with $CHCl_3$-MeOH (6:1, 1400 ml and 3:1, 1200 ml) to obtain compound **1** (78 mg) and compound **3** (940 mg). Fraction F3 was subjected to CC (silica gel), eluted with $CHCl_3$-MeOH (4:1, 2000 ml) to obtain 40 fractions (1b-40b). Fractions 18b-26b, in which a major alkaloid was detected, were pooled and crystallized with MeOH to yield 370 mg of compound **3**. Fractions 32b-40b were purified by preparative TLC using Toluene-MeOH-25% NH_3 (3:4:1) as developing solvent to yield 88 mg of compound **2**.

Physical properties and spectral data

Palmatine (1): yellow needles ($CHCl_3$/MeOH), m.p. 195-198ºC (decomposed). UV λ_{max} (MeOH) nm: 228, 268, 344, 430. IR ν_{max} (KBr) cm^{-1}: 2950, 1605, 1510, 1360, 1270, 1110, 1020, 800. EI-MS *m/z* (rel. int. %): 352 $[M]^+$ (5): $C_{21}H_{22}NO_4^+$, 337 (26), 323 (22), 321 $[M\text{-}OCH_3]^+$ (6), 115 (26), 55 (53), 43 (100). ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.88 (1H, s, H-8), 9.07 (1H, s, H-13), 8.20 (1H, d, J = 9.0 Hz, H-11), 8.03 (1H, d, J = 9.0 Hz, H-12), 7.72 (1H, s, H-1), 7.08 (1H, s, H-4), 4.95 (2H, br-t, J = 5.8 Hz, H-6), 4.09 (3H, s, 9-OCH_3), 4.06 (3H, s, 10-OCH_3), 3.93 (3H, s, 2-OCH_3), 3.86 (3H, s, 3-OCH_3), 3.22 (2H, br-t, J = 5.8 Hz, H-5). ^{13}C NMR (125 MHz, DMSO-d_6): δ 151.7 (C-3), 150.4 (C-10), 148.9 (C-2), 145.6 (C-8), 143.8 (C-9), 137.9 (C-13a), 133.3 (C-12a), 128.8 (C-4a), 127.0 (C-11), 123.6 (C-12), 121.5 (C-8a), 120.1 (C-13), 119.1 (C-13b), 111.5 (C-4), 109.2 (C-1), 62.1 (9-OCH_3), 57.2 (10-OCH_3), 56.4 (2-OCH_3), 56.0 (3-OCH_3), 55.5 (C-6), 26.1 (C-5).

Jatrorrhizine (2): orange plates (MeOH), m.p. 194-196ºC (decomposed). UV λ_{max} (MeOH) nm: 226, 266, 346, 432. IR ν_{max} (KBr) cm^{-1}: 3400 (broad), 2945, 1600, 1540, 1440, 1360, 1330, 1270, 1240, 1110, 1070, 1020, 908, 875. EI-MS *m/z* (rel. int.%): 339 $[M+H]^+$ (87), 338 $[M]^+$ (88): $C_{20}H_{20}NO_4^+$, 337(100), 336 (76), 324 (56), 323 (76), 322 (65), 294 (23), 161 (31), 50 (40), 36 (59). ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.85 (1H, s, H-8), 9.01 (1H, s, H-13), 8.19 (1H, d, J = 9.0 Hz, H-11), 8.02 (1H, d, J = 9.0 Hz, H-12), 7.73 (1H, s, H-1), 6.91 (1H, s, H-4), 4.93 (2H, br-t, J = 6.0 Hz, H-6), 4.09 (3H, s, 9-OCH_3), 4.06 (3H, s, 10-OCH_3), 3.93 (3H, s, 2-OCH_3), 3.15 (2H, br-t, J = 6.0 Hz, H-5). ^{13}C NMR (125 MHz, DMSO-d_6): δ150.7 (C-3), 150.4 (C-10), 148.3 (C-2), 145.5 (C-8), 143.9 (C-

9), 138.5 (C-13a), 133.6 (C-12a), 129.1 (C-4a), 127.1 (C-11), 123.5 (C-12), 121.5 (C-8a), 119.7 (C-13), 117.8 (C-13b), 115.2 (C-4), 109.8 (C-1), 61.8 (9-OCH_3), 57.0 (10-OCH_3), 56.2 (2-OCH_3), 55.3 (C-6), 25.5 (C-5).

Berberine (3): yellow needles (MeOH), m.p. 203-205ºC (decomposed). UV λ_{max} (MeOH) nm: 230, 266, 348, 428. IR ν_{max} (KBr)cm^{-1}: 2950, 1600, 1505, 1390, 1360, 1335, 1280, 1235, 1100, 1040, 815. TOF-ESI-MS *m/z* (rel. int. %): 337 $[M+H]^+$ (8), 336 $[M]^+$ (100): $C_{20}H_{18}NO_4^+$. ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.88 (^{1}H, s, H-8), 8.93 (1H, s, H-13), 8.19 (1H, d, *J* = 9.0 Hz, H-11), 8.00 (1H, d, *J* = 9.0 Hz, H-12), 7.79 (1H, s, H-1), 7.08 (1H, s, H-4), 6.16 (2H, s, O-CH_2-O), 4.92 (2H, br-t, *J*= 6.4 Hz, H-6), 4.09 (3H, s, 9-OCH_3), 4.06 (3H, s, 10-OCH_3), 3.20 (2H, br-t, *J* = 6.4 Hz, H-5). ^{13}C NMR (125 MHz, DMSO-d_6): δ150.6 (C-10), 150.0 (C-3), 147.9 (C-2), 145.6 (C-8), 143.9 (C-9), 137.7 (C-13a), 133.2 (C-12a), 130.9 (C-4a), 127.0 (C-11), 123.7 (C-12), 121.6 (C-8a), 120.6 (C-13b), 120.4 (C-13), 108.6 (C-4), 105.6 (C-1), 102.2 (O-CH_2-O), 62.1 (9-OCH_3), 57.3 (10-OCH_3), 55.4 (C-6), 26.5 (C-5).

Triacontanyl caffeate (4): yellowish brown solid. UV λ_{max} (MeOH) nm: 218, 300, 328. IR ν_{max} (KBr) cm^{-1}: 3480 (OH), 3315, 2910, 2845, 1710 (C=O), 1680 (C=C), 1605 (aromatic C=C), 1280 (C-O). TOF-ESI-MS *m/z* (rel. int. %): 600 $[M]^+$ (1): $C_{39}H_{68}O_4$, 495 (11), 454 (20), 434 (100), 181 (80), 163 [caffeoyl]$^+$ (72), 149 (33). ^{1}H NMR (500 MHz, CD_3OD): δ7.54 (1H, d, *J* = 16.0 Hz, H-7), 7.04 (1H, d, *J* = 2.1 Hz, H-2), 6.94 (1H, dd, *J* = 8.2, 2.1 Hz, H-6), 6.78 (1H, d, *J* = 8.2 Hz, H-5), 6.25 (1H, d, *J* = 16.0 Hz, H-8), 4.16 (2H, t, *J* = 6.6 Hz, -O-CH_2-), 1.69-1.26 (*circa* 56 H, m), 0.88 (3H, t, *J* = 6.6 Hz: terminal CH_3). ^{13}C NMR (125 MHz, CD_3OD): δ169.4 (C=O), 149.5 (C-4), 146.8 (C-3), 146.7 (C-7), 127.7 (C-1), 122.9 (C-6), 116.5 (C-5), 115.2 (C-8), 115.1 (C-2), 65.6 (-O-CH_2-), 33.1-23.7 (DEPT: 28 x CH_2), 14.4 (CH_3).

Alkali hydrolysis of compound 4: A solution of **4** (10 mg) in 4 ml of 10% KOH and 10 ml of $CHCl_3$ was refluxed on a boiling water bath for 1 hr. The mixture was extracted with $CHCl_3$, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The obtained residue was purified by preparative TLC [silica gel, $CHCl_3$: MeOH (19:1)] to yield 4.2 mg of triacontanyl alcohol (1-triacontanol; $C_{30}H_{62}O$), which gave purple colour with anisaldehyde spray reagent: TOF-ESI-MS *m/z* (rel. int.%): 437 $[M-H]^+$ (18), 411 (22), 319 (60), 317 (40), 279 (56), 192 (100), 149 (68).

Results, Discussion and Conclusion

Activity of the extracts and characterization of the isolated compounds

The results of antioxidant activity against DPPH radical and cytotoxic activity of the extracts from *A. flava, C. blumeanum* and *F. tinctoria* are depicted in Table 1. Methanol extract of *A. flava* (AFM), and methanol and chloroform extracts of *C. blumeanum* (CBM and CBC) showed moderate antioxidant activity with EC_{50} values of 25.7, 50.1 and 55.4 µg/ml, respectively. Pronounced cytotoxic activity was exhibited by the chloroform extracts of the three plants (AFC, CBC and FTC), and the methanol extracts of *A. flava* (AFM) and *C. blumeanum* (CBM) with LC_{50} values in the range of 210-278 µg/ml against brine shrimp, and IC_{50} values ranging from 8-12 µg/ml against MCF-7. AFM was the most active extract on both activities tested. In contrast, the petroleum ether extracts lacked both antioxidant and cytotoxic activities. This suggested that the active constituents should be polar compounds. In addition, the water extracts AFW and CBW showed some degree of antioxidant activity with EC_{50} values of 68.3 and 64.6 µg/ml, respectively, but lacked cytotoxic activity. In general, *A. flava* and *C. blumeanum* were found to possess stronger antioxidant activity than *F. tinctoria*, but the three species showed comparable cytotoxic activity.

Phytochemical investigation of the active extracts FTC, CBM, CBC, AFC and AFM was carried out in order to isolate compounds responsible for the observed activities. Although AFW and CBW showed moderate antioxidant activity, no pure compounds were isolated. This is due to the complexity and extremely high polarity of their chemical compositions. However, according

to TLC analysis, some very polar phenolic compounds and traces of alkaloids were detected. It is possible that the phenolic compounds could account for one of the active compounds responsible for the observed antioxidant activity. Compounds **1** and **2** were isolated as main constituents of FTC, and as minor components of AFC, AFM, CBC and CBM. Compound **3** was isolated as a main constituent of AFC, AFM, CBC and CBM, and was not detected in FTC and the other extracts of *F. tinctoria*. These three compounds gave positive results with Dragendorff's reagent suggesting that all of which could be alkaloids. The mass spectra of **1, 2** and **3** exhibited molecular ion peaks at *m*/*z* 352, 338, and 336, respectively, consisting with the molecular formulae $C_{21}H_{22}NO_4^+$ (D.B.E.=11.5), $C_{20}H_{20}NO_4^+$ (D.B.E.=11.5), and $C_{20}H_{18}NO_4^+$ (D.B.E. =12.5), respectively. These non-integer values of the index of hydrogen deficiency suggested that **1, 2** and **3** could be quaternary compounds or ammonium salts. This was strongly supported by the presence of one-proton singlet at δ9.88, 9.85 and 9.88 in ^{1}H-NMR spectra of **1, 2** and **3**, respectively. The extremely downfield shifts of these protons indicated that the corresponding carbon (C-8) of each compound was directly attached to the quaternary nitrogen. Comparison the details of ^{1}H and ^{13}C NMR data of **1-3** with the published assignments led to characterization of **1, 2** and **3** to be palmatine, jatrorrhizine (Hussain *et al*., 1989) and berberine (Janssen *et al*., 1989), respectively (Figure 1). The unambiguous NMR assignments of the alkaloids **1-3** in the present work were achieved through 2D-NMR, particularly HMQC and HMBC experiments. Alkaloid profiles of the three plants obtained from the present work are in good agreement with the previous reports (Thornber, 1970; Verpoorte *et al*., 1982; Boonyaprakarn *et al*., 1983). It is of interest to note that the obtained alkaloid pattern of *C. blumeanum*, which contains **3** as a major alkaloid, is in accordance with that of the related species *C.*

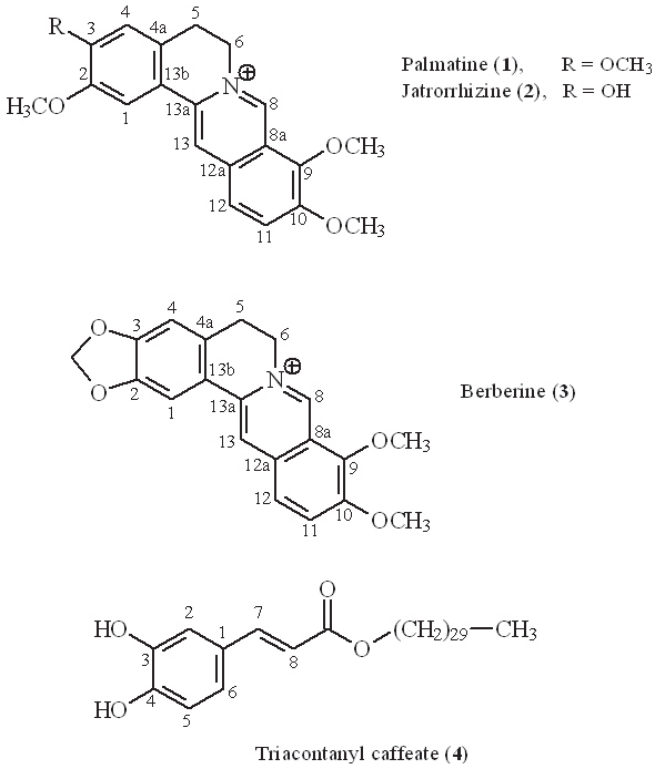

Figure 1. Chemical structures of the isolated compounds.

fenestratum (Siwon *et al.*, 1980; Pinho *et al.*, 1992).

The time of flight electrospray ionization (TOF-ESI) mass spectrum of **4** (isolated from CBC) showed weak molecular ion peak at *m/z* 600, consistent with the molecular formula $C_{39}H_{68}O_4$ (D.B.E. = 6). ^{1}H and ^{13}C resonances in the NMR spectra of **4** revealed that it is composed of two parts, the aromatic moiety and *n*-alkanol. The presence of a carbonyl function at δ169.4, which showed 3-bond correlation with the oxygen-bearing methylene protons at δ4.16 in the HMBC spectrum, suggested the existence of an ester function. This was further supported by the presence of absorption bands of carbonyl and double bond functions belonging to α,β-unsaturated ester (1710 and 1680 cm^{-1}) in the IR spectrum. Furthermore, the characteristic signals of two olefinic protons of *trans*-Ar-CH=CH-(C=O)- fragment were observed as two doublets (1H each) at δ7.54 and 6.25 (*J*=16.0 Hz) with their corresponding carbon signals at δ146.7 and 115.2, respectively. A cross peak of 3-bond correlation between the carbonyl function (δ169.4) and the olefinic proton at δ7.54 (H-7) was clearly observed in the HMBC spectrum. Having allocated the chemical shifts of H-7 (δ 7.54) and H-8 (δ6.25), the assignments of aromatic protons and carbons were achieved by analysis of 3-bond cross peaks of C-1/H-8, C-2/H-7, C-3/H-5, C-4/H-2, C-4/H-6 and C-6/H-7 in the HMBC spectrum. The obtained chemical shifts and multiplicities of three aromatic protons at δ7.04, 6.94 and 6.78, and the resonances of the six aromatic carbons were in accordance with those of caffeic acid (Fukuoka, 1982). This was supported by the presence of an intense caffeoyl ion peak at *m/z* 163 (72%) in the mass spectrum. The DEPT spectra of **4** gave details of the *n*-alkanol portion, which was characterized by an oxygen-bearing methylene carbon at δ65.6 (δ_H 4.16), the intense and severely overlapping long chain methylene functions in the range of δ33.1-23.7 (28 x CH_2), and the terminal methyl function at δ14.4 (δ_H 0.88). It was notable that NMR signals for neither methoxyl nor aliphatic methine functions were observed. Upon alkali hydrolysis of **4**, the aliphatic alcohol was obtained as main product and characterized by mass spectrometry to be triacontanyl alcohol (*m/z* 438). It is obvious that esterification

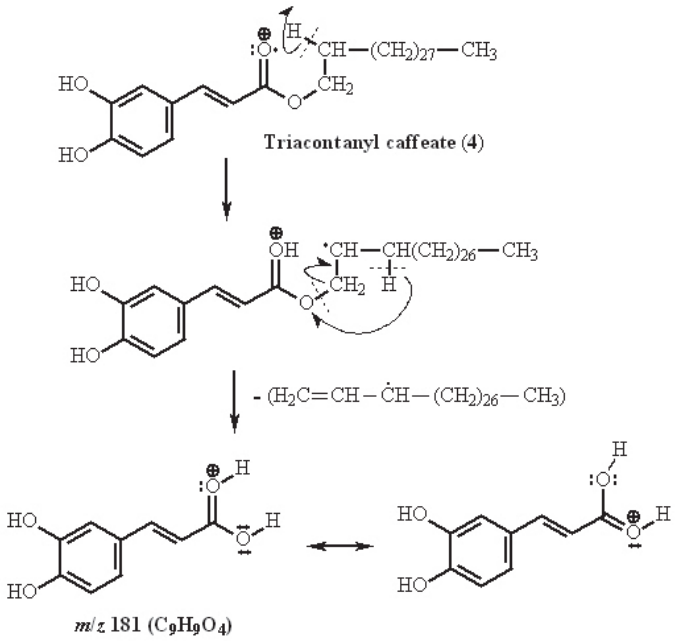

Figure 2. Rearrangement of the two hydrogen atoms of compound 4 with elimination of an allylic radical.

between caffeic acid and triacontanyl alcohol gave rise to compound **4**. Rearrangement of the two aliphatic hydrogen atoms adjacent to an ester function, as shown in Figure 2, resulted in elimination of an allylic radical and giving the intense ion peak at m/z 181 (80%) in mass spectrum of **4**. The structure of **4** was, therefore, concluded to be triacontanyl caffeate, which was previously isolated from the stem bark of *Pongamia glabra* (Saha *et al*., 1991). However, this compound has not previously been reported from *C. blumeanum, A. flava* and *F. tinctoria*.

Activity of the isolated compounds

The antioxidant activity against DPPH radical and cytotoxic activity against brine shrimp and human cancer cell line MCF-7 (breast adenocarcinoma) of compounds **1-4** are depicted in Table 1. The antineoplastic agent vinblastine sulphate, the cytotoxic alkaloid strychnine hydrochloride, and the two well-known antioxidants BHT (butylated hydroxytoluene) and caffeic acid were taken as positive standards for comparison with the tested compounds accordingly. Of the four compounds, **4** was found to be the most active one against DPPH radical with an EC_{50} value of 6.8 µg/ml (11.3 µM), which was more active than BHT but less active than caffeic acid. The strong antioxidative effect of **4** was due to the 3,4-dihydroxy-substituted aromatic fragment of its caffeoyl moiety, which was able to scavenge DPPH radical in the molecular ratio of one to two as those of caffeic acid and protocatechuic acid (Keawpradub *et al*., 2001). Among the three alkaloids, **2** showed moderate antioxidant activity whereas **1** and **3** almost lacked antioxidant activity (EC_{50} >100 µg/ml). The percent scavenging at 100 µg/ml of **1** and **3** were 18.3% and 5.5%, respectively (data not shown). The phenolic moiety in the structure of **2** should be responsible for the observed scavenging activity against DPPH radical by acting as an electron donator. Upon giving an electron to DPPH radical, the double bond conjugation system of **2** was rearranged to become quinonoid form with the molecular ratio of one to one, hence less active than **4**. It could be concluded that **2** was at least, in part, responsible for antioxidant activity of the three plants, while **4** plays a key role for free radical scavenging effect of *C. blumeanum*. However, since the scavenging activity of **2** was considerably less active than those of the active extracts of the three plants, it is likely that some other active compounds remain to be determined.

The three alkaloids (**1-3**) exhibited pronounced cytotoxic activity against brine shrimp and MCF-7 cells with LC_{50} and IC_{50} values ranging from 37-206 µg/ml (110-611 µM) and 1-4 µg/ml (3.0-11.5 µM), respectively. The cytotoxic activity of **4** against brine shrimp was comparable with those of the alkaloids, but considerably less active against MCF-7. This, however, was substantially less potent than vinblastine sulphate tested in the same system. Berberine (**3**) was found to be the most cytotoxic compound against brine shrimp and MCF-7 cells with LC_{50} and IC_{50} values of 37.1 (110.4 µM) and 1.0 µg/ml (3.0 µM), respectively. The pronounced activity of **3** observed in the present work provides additional evidence to support the assumption that the 1,2-methylenedioxy moiety of isoquinoline alkaloids is essential for cytotoxic activity (Likhitwitayawuid *et al*., 1993). It is important to note that IC_{50} values of the three alkaloids (**1-3**) against MCF-7 cells were less than 4 µg/ml, which is considered to possess significant cytotoxic effect according to the criterion of cytotoxic activity for pure compound on cancer cells recommended by the U.S. National Cancer Institute (Suffness and Pezzuto, 1991). It is obvious that the three alkaloids were mainly responsible for the observed cytotoxicity, which could be used as markers for such activity of the stems of the three species of Khaminkhruea investigated. The present work gives additional evidence that these three plants are potential sources of biologically active compounds. Further investigation of the active extracts and isolated compounds in animal models for the therapeutic efficacy and toxicity would provide evidence to determine whether these medicinal plants could be beneficial for drug discovery.

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