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Antiproliferative Effects of Alkaloids Isolated from the Tuber of *Stephania venosa via* the Induction of Cell Cycle Arrest in Mammalian Cancer Cell Lines

[1]Sirinapa Nantapap, [2]Chatchanok Loetchutinat, [3]Puttinan Meepowpan,
[4]Narong Nuntasaen and [1]Wilart Pompimon
[1]Laboratory of Natural Products, Center for Innovation in Chemistry, Department of Chemistry,
Faculty of Science, Lampang Rajabhat University, 52100 Lampang, Thailand
[2]Laboratory of Physical Chemistry, Molecular and Cellular Biology,
Center of Excellence for Molecular Imaging, Department of Radiologic Technology,
Faculty of Associated Medical Sciences, Chiang Mai University, 50200 Chiang Mai, Thailand
[3]Center for Innovation in Chemistry, Department of Chemistry,
Faculty of Science, Chiang Mai University, 50300 Chiang Mai, Thailand
[4]The Forest Herbarium, Department of National Park, Wildlife and Plant Conservation,
Ministry of Natural Resources and Environment, 10900 Bangkok, Thailand

Abstract: Problem statement: *S. venosa* (Menispermaceae) is used in traditional medicine. The constituents of *S. venosa* belonging to showed remarkable cytotoxic activity. According to previous research, *S. venosa* contains several alkaloids, such as protoberberine stephanine cyclanoline and N-methylstepholidine, kamaline, (+)-N-carboxamidostepharine, (-)-O-methylstepharinosine, (-)-stepharinosine, aporphine (-)-O-acethylsukhodiamine and oxostephanosine. The chemical and biological investigations of this plant are interesting to bioassay-guided fractionation, particularly Antiproliferative effects *via* the induction of cell cycle arrest in mammalian cancer cell lines. **Approach:** The research was carried out to extract, isolate, purify and elucidate structure of the active compound from the tuber *S. venosa*. Most of the solvent extracts and isolated compound were evaluated with kinds of mammalian cancer cell lines for investigation on antiproliferative effects. **Results:** Four alkaloids, tetrahydropalmatine (1), crebanine (2) O-methylbulbocapnine (3) and N-methyltetrahydropalmatine (4) were isolated from the tuber of *S. venosa*. Charaterization of the compounds were carried out by extensive NMR studies using COSY, HMQC, HMBC and DEPT in addition to other spectroscopic methods. These compounds (1, 2 and 3) were showed evidence of the anticancer activities for cell proliferation inhibition in K562, K562/Adr, GLC4 and GLC4/Adr cell lines due to G0/G1 obstruction by compound 2 and 3 and negligible S phase arrest by compound 1. **Conclusion:** The result showed slightly increase in S phase by the effect of compound 1, beside the G0/G1 phase was blocked by compound 2 and 3.

Key words: *Stephania venosa*, Menispermaceae, palmatine, aporphine, crebanine and bulbocapnine alkaloids, cell cycle arrest, cytotoxicity

INTRODUCTION

Stephania genus belong to the Menispermaceae family, comprises of about 45 species and they have been reported 15 species in Thailand (Pharadai *et al*., 1985). Several Stephania's species have been used in traditional medicine to treat a variety of disease (Likhitwitayawuid *et al*., 1993). This genus is also well known as an important source of alkaloids, one of the largest groups of natural products which reveal interesting pharmacological activity (Sugimoto *et al*., 1988). *S. venosa* has various local names. In the northeast, it is called "Kratomluad" and in the southwest called "Boraphet plungchang" or "Saboo luad". This plant is herbaceous perennial vines growing to around four meters tall. It has a large tuber (up to 20-

Corresponding Author: Wilart Pompimon, Laboratory of Natural Products, Center for Innovation in Chemistry, Department of Chemistry, Faculty of Science, Lampang Rajabhat University, 52100 Lampang, Thailand Tel/Fax: +665-4241052

40 cm in diameter) with bitter taste and red latex. The leaves are arranged spirally on the stem *Andare peltate*, with the leaf petiole attached near the centre of the leaf (Smitinand, 2001). According to previous research, *S. venosa* contains several alkaloids, such as protoberberine stephanine cyclanoline and N-methylstepholidine (Ingkaninan *et al*., 2006), kamaline (Banerji *et al*., 1994), (+)-N-carboxamidostepharine, (-)-O-methylstepharinosine and (-)-stepharinosine (Charles *et al*., 1987), aporphine (-)-O-acethylsukhodiamine and oxostephanosine (Pharadai *et al*., 1985). The constituents of *S. venosa* belonging to showed remarkable cytotoxic activity on human peripheral blood mononuclear cells (Sueblinvong *et al*., 2007), inhibition the treatment of Alzheimer's disease (Ingkaninan *et al*., 2006), cytotoxic activity against brine shrimp (Keawpradub *et al*., 2001) properties. Based on already known preliminary data from bioassay-guided fractionation we promote undertook the studies on cytotoxicity of four crude extracts e.g., hexane, ethyl acetate, acetone and methanol. We nearby the best results obtained by testing ethyl acetate and acetone extracts of *S. venosa* for examining with kinds of mammalian cancer cell lines, i.e., GLC4, GLC4/Adr, K562 and K562/Adr for antiproliferation assay and cell cycle arrest analysis. Furthermore, we herein report the isolation, purification and structure elucidation of these four compounds on the basis of extensive spectroscopic analysis (UV, IR, MS, ^{1}H NMR, ^{13}C NMR) as well as by comparison of their spectral data with previously reported values. Although these compounds are known natural products but this is the first report of the isolation from ethyl acetate and acetone extracts of this plant.

MATERIALS AND METHODS

General experimental procedure: Melting point was measured on a BUchi 322 micro melting point apparatus and has to be uncorrected. Optical rotations were obtained using a JASCO DIP-1000 digital polarimeter. UV spectra were obtained on a Shimadzu UV-1601 spectrophotometer with MeOH as solvent. IR spectra in KBr disk were recorded on Shimadzu 8900 FTIR spectrophotometer. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were determined in $CDCl_3$ and CD_3OD solutions and were referenced to $CHCl_3$ as the internal standard (^{1}H = δ7.26; ^{13}C = δ77.0), using a DPX on a BrUker DPX 400 spectrometer for 1D and 2D determination. Low resolution mass spectra were recorded on a Thermo Finnegan Polaris Q mass spectrometer at 70 eV (probe) for EIMS, Mahidol University. Column chromatography was conducted on silica gel 60 (Merck 7734, 70-230 mesh). TLC was performed on aluminium backed pre-coated silica gel 60 PF_{254} sheets and detection with using UV detector.

Plant material: The *S. venosa* tuber part will be collected from Prachuapkhirikhan province of Thailand and identified by Forest Herbarium. A voucher specimen (BKF no. 140583) has been deposited at the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand.

Extraction and isolation: The air-dried powdered tuber of *S. venosa* (6.2 kg) were successively percolated with hexane (WP0554) (25 L×3 days×5 times) and then extracted with ethyl acetate, (WP0555) (25 L×3 days×7 times), acetone, (WP0556) (25 L×3 days×5 times) and methanol, (WP0557) (25 L×3 days×5 times) at room temperature, respectively and followed by filtration. The filtrates were combined and evaporated to dryness under reduced pressure to afford hexane, ethyl acetate, acetone and methanol extracts were 58.26, 126.00, 111.06 and 392.70 g, respectively. The extracts were submitted for bioassays. Preliminary biological investigation for cytotoxic of the four extracts (WP0554-0557) was carried out in the Laboratory of Natural Products Chemistry, Faculty of Science, Lampang Rajabhat University, Thailand. Effect of *S. venosa* extracts on cell viability was established that the ethyl acetate extract displayed strong activity in the K562, K562/Adr, GLC4 and GLC4/Adr assay with percentage inhibition to 50 (IC_{50}) at 11.9, 18.1, 20.9 and 19.7 μg mL^{-1}, respectively. Additionally, the acetone extract displayed activity in the K562, K562/Adr, GLC4 and GLC4/Adr assay with IC_{50}% at 16.7, 37.8, 40.5 and 42.3 μg mL^{-1}, respectively. From the bioassay-guided fractionation, the ethyl acetate and acetone fractions were separated by column chromatography, respectively. The ethyl acetate extract (126.00 g) was separated by column chromatography over silica gel 650 g, eluting with various proportions of ethyl acetate-n-hexane, followed by the increasing amount of methanol in ethyl acetate and finally with methanol. Fractions (1000 mL each) were collected and combined on the basis of TLC behavior. The solvents were evaporated to dryness to afford eight fractions (F_1-F_8). Fraction F_6 (7.92 g) which eluted by 30% ethyl acetate-n-hexane, was obtained as yellow solids mix fat. Further separation by column chromatography over silica gel 150 g. Gradient elution was started from pure n-hexane, gradually enriched with ethyl acetate in hexane up to 20% methanol-ethyl acetate. Fractions were collected and combined then solvent were

removed under reduced pressure to afford subfraction C_1 and C_2. Further, the subfraction C_1 (6.00 g) was separated by column chromatography over silica gel to yield yellow crystals subfraction G (4.85 g). The crystals were recrystallized with 95% ethanol to give purified yellow crystals 3.13 g which identified as tetrahydropalmatine (1). Fraction F_7 (43.49 g) which eluted by 30% ethyl acetate - n-hexane to 100% ethyl acetate, was obtained as light brown solids mix fat. Further separation by column chromatography over silica gel 600 g. Gradient elution was started from pure n-hexane, gradually enriched with ethyl acetate in hexane up to 20% methanol-ethyl acetate. Fractions (1000 mL each) were collected and combined on the basis of TLC behavior. The solvents were removed under reduced pressure to afford subfractions D_1-D_3. Further, the subfraction D_2 (19.84 g) was separated by column chromatography over silica gel to afford subfractions H_1-H_3. The subfraction H_2 (12.79 g) was separated by column chromatography over silica gel to yield light yellow crystals. Further, the crystals were recrystallized with 95% ethanol to give purified light yellow crystals 0.71 g which identified as crebanine (2).

The acetone extract (111.06 g) was separated by column chromatography over silica gel 500 g. Gradient elution was conducted initially with n-hexane, gradually enriched with ethyl acetate, followed by increasing amount of methanol in ethyl acetate and finally with methanol. Fractions (1000 mL each) were collected and combined on the basis of TLC behavior. The solvents were evaporated to dryness to afford four fractions (I_1-I_4). Fraction I_3 (25.07 g) which eluted by 20% methanol-ethyl acetate, was obtained as light brown crystals mix fat. Further, the crystals were recrystallized with pure methanol to give purified light brown crystals 1.72 g which established as O-methylbulbocapnine (3). Fraction I_4 (20.23 g) which eluted by 20% methanol-ethyl acetate, was obtained as light yellow crystals mix fat. Further, the crystals were recrystallized with pure methanol to give purified white crystals 0.73 g which identified as N-methyltetrahydropalmatine (4).

Cell lines and culture conditions: The bioactivity of crude extracts and pure compound were investigated on four cancer cell lines, that were adriamycin-sensitive erythroleukemia cell (K562), adriamycin-resistant erythroleukemia cell (K562/Adr) with the expressing of ABCB1/MDR1 P-glycoprotein, adriamycin-sensitive small cell lung cancer (GLC4) and adriamycin-resistant small cell lung cancer GLC4/Adr with the expressing of ABCC1/MRP1 (Perry and Metzger, 1980; Shamma, 1973). All cell lines were cultivated in RPMI-1640 medium supplemented with 1% fetal bovin serum 1% penicillin/streptomycin in a 37°C incubator under a 5% CO_2.

Anti-proliferation assay: The effect of crude extracts and pure compounds on cell proliferation were observed in those four cancer cell lines. The cellular viability of cell K562, K562/Adr, GLC4 and GLC4/Adr will be determined by using the conventional MTT-colorimetric method. Cells were incubated at cell culture condition with a various concentrations of pure compound up to 62.5 μg mL^{-1} and crude extracts up to 250 μg mL^{-1} for 72 h, the cells without crude extracts or pure compound of *S. venosa* were served as blank. At 72 h, 50 μL of MTT (2.5 mg mL^{-1}) was added to each well. The culture plates were gently shaken and incubated for 4 h. MTT in solution is converted to a blue formazan crystal by mitochondrial succinate dehydrogenase of living cells. The formazan crystals being formed within cells were solubilized with 50 μL DMSO and shaken well. The Optical Density (OD) of blue formazan chromophore was determined at 550 nm in an automated plate reader (Likhitwitayawuid *et al*., 1993). The cytotoxic parameter will be expressed as the concentration of compound in which the cellular proliferation was inhibited to 50% (IC_{50}%). Calculate the percentage of cell-growth inhibition (IC%) using the formula as the following equation:

$$IC\% = \frac{C_{72} - S_{72}}{C_{72} - C_0} \times 100$$

When:

C_0 = OD value represent the initial cell amount of non treated cell (control)

C_{72} = OD value represent the cell amount of control at 72 h

S_{72} = OD value represent the cell amount of treated cell at 72 h

Cell cycle distribution analysis: All four cell lines (5×10^5) were investigated the cell cycle analysis by cultured in completed RPMI-1640 medium in the present of compound 1, 2 or 3 at the concentration of IC_{50} value for 3, 6, 12, 24 and 48 h. Cells were harvested parallel with non treated cells and washed with Phosphate Buffer Solution pH 7.3 (PBS) twice before fixed with 70% ethanol overnight at 4°C. After fixation, cells were collected and resuspended in PBS and consequently added then mixed with final concentration of 1% triton-X100, 20 μg mL^{-1} RNase and 10 μg mL^{-1} propidium iodide. Then, cells were dark incubated at 37°C for 30 min. before cell cycle distribution analysis by using flow cytometer. The result was defined as the different between the %Cell of

treated cell (%$Cell_{treated}$) and %Cell of non treated cell (%$Cell_{control}$) in each cell phase.

RESULTS

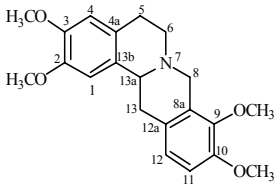
Chemical structure elucidation for this investigation, some of biological activity of *S. venosa* has been tested, by which; hexane, ethyl acetate, acetone and methanol extract were assayed for their anticancer activities. The active ethyl acetate and acetone extract were carried out for purification, separation, crystallization and structure explanation of the isolated tetrahydropalmatine and crebanine from ethyl acetate extract, O-methylbulbocapnine and N-methyltetrahydropalmatine from acetone extract. The structures have been established on the basis of spectral and physical evidence. It is worth nothing that the data from spectroscopic techniques, especially the ^{1}H, ^{13}C-1D and 2D NMR were performed on the accurate molecular structure in Table 1 and 2. The bioactivities of four pure compounds were investigated by studying the anti-proliferation and cell cycle analysis in four cancer cell lines are presented in Table 3 and 4.

DISCUSSION

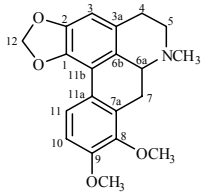
Tetrahydropalmatine (1): $C_{21}H_{25}NO_4$ (355, M^+) major alkaloid was separated from the active acetate extract of the tuber from *S. venosa*. It was obtained as yellow crystals, mp 143.8-145.0°C, $[\alpha]^{25}_{589}$ -74.90 (c 2.75, MeOH). The UV spectrum of the compound in methanol exhibited absorption maxima at 237 and 272 nm (log ε 0.87 and 0.75, respectively) which were characteristic of an isoquinoline alkaloid (Banerji *et al*., 1994). In addition, the IR spectrum revealed the presence of aromatic nucleus (1608, 1512 and 1458 cm^{-1}), an ether linkage (1280, 1230 cm^{-1}) and an o-disubstituted benzene moiety (780 cm^{-1}). The key fragmentation ions in the mass spectrum of compound 1 at m/z 355 [M^+], 340, 324, 190, 164 and 149 (base peak), were useful in obtaining the structure of 1 (Chen and Maclean, 1968). The ions at m/z 190 for $C_{11}H_{12}O_2N^+$, which loss of a single hydrogen from the neutral nitrogen containing fragment formed in retro Diels-Alder fragmentation and 164 for $C_{10}H_{12}O_2^+$ confirmed the structure of tetrahydroprotoberberine alkaloid derivatives. These two fragment ions resulted from a retro Diels-Alder opening of the ring C and ion 149 (base peak) for $C_9H_9O_2^+$ by misplace of CH_3 from ion $C_{10}H_{12}O_2^+$. In the 400 MHz ^{1}H NMR (Table 1) and ^{13}C NMR spectra of 1 ($CDCl_3$), signals for four protons on sp^2 carbons, four methoxyl groups and four CH_2 groups were discernible. The pair of doublets (AB pattern), δ 6.79 H-11 and 6.88 H-12 displayed on the aromatic region suggesting the presence of two ortho proton coupling with J = 8.38, together with two singlets (δ 6.73, H-1 and 6.62, H-4) reliable with two para related hydrogens in an aromatic of isoquinoline nucleus. The methylene proton H-8 displayed a pair doublets at δ 4.24 and 3.54 with the large between these signals ($^{1}J_{H\text{-}H}$ = 15.63 Hz) recommended geminal protons and the direct bond C-H correlation HMQC technique were established. So, the presence of deshielded, secluded methylene group is distinguishing of the bridge position (C-8) of protoberberine alkaloids (Joanne *et al*., 2003). The substantial down fielded shift of the ^{13}C signal of CH and CH_2 groups (δ 59.29 C-13a, 51.48 C-6, 53.96 C-8) indicated these carbons atom were adjacent to the nitrogen atom. The signal for the axial proton in the methylene group (δ 2.83 H-13b) appeared as a dd splitting pattern with $^{1}J_{H\text{-}H}$ = 15.63 Hz and $^{2}J_{H\text{-}H}$ = 11.49 Hz. This indicated that the dihedral angle between this proton and the adjacent CH proton must be very close to 180°. Correspondingly, the signal for the equatorial (geminal) proton (δ 3.27) was a dd pattern, with smaller vicinal coupling ($J_{H\text{-}H}$ = 3.65), suggesting a dihedral angle closer to 90° (Joanne *et al*., 2003). This was substantiation for the protoberberine system and these coupling constants necessitate an axially oriented H-13a. Furthermore, the aliphatic signals, resonated at δ 3.13, 2.66 (H-5a, b) and 3.21, 2.66 (H-6a, b) were multiplets arising from the CH_2-CH_2 spin system. In order to obtain more information about the location of substituents in the structure, a 2D NMR HMBC was carried out. From HMBC experiments showed the presence of two methoxyl groups as in the aromatic ring of the isoquinoline moiety. The HMBC was exhibited obvious correlation peak between the three carbons (C-2, 4a, 13b) and four aromatic carbons (C-1, 3, 5, 13b) with H-1 and H-4, respectively. Additionally, the two methoxyl groups located at the other aromatic ring, positioning 9 and 10 were confirmed by the HMBC correlation of proton 11 and 12. The structure of compound 1 was finally confirmed by direct comparison of the major chemical shift ^{1}H and ^{13}C NMR of the isolated compound with the value reported by Joanne *et al*. (2003).

Crebanine (2): The minor alkaloid was divided from the same extract of 1 by comprehensive column chromatography. It was obtained as light yellow crystals, mp 117.0-117.8°C, $[\alpha]^{25}_{589}$ -9.12 (c 1.88, MeOH), (339, M^+), corresponding to $C_{20}H_{21}NO_4$. The UV spectrum of the compound in methanol showed absorption bands maxima at 225 and 281 nm (log ε 1.33 and 1.32) which were characteristic of isoquinoline alkaloids (Banerji *et al*., 1994).

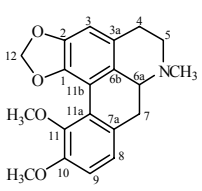
Table 1: ^{13}C-NMR, ^{1}H-NMR data for isolated alkaloids



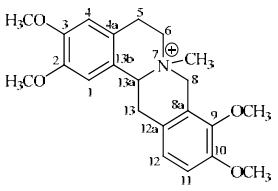
Tetrahydropalmatine (1)



Crebanine (2)



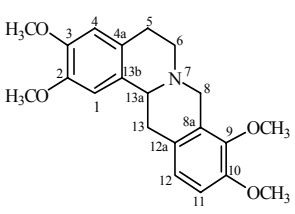
O-methylbulbocapnine (3)



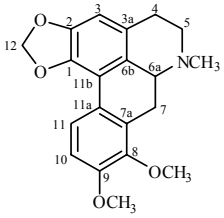
N-methyltetrahydropalmatine (4)

Position	δ^1H (J Hz)	δ^{13}C (DEPT)	Position	δ^1H (J Hz)	δ^{13}C (DEPT)	Position	δ^1H (J Hz)	δ^{13}C (DEPT)	Position	δ^1H (J Hz)	δ^{13}C (DEPT)
1	6.73 (s)	108.59(CH)	1	-	142.00(C)	1	-	144.66(C)	1	6.79 (s)	109.73(CH)
2	-	147.48(C)	2	-	146.50(C)	2	-	150.06(C)	2	-	149.62(C)
3	-	147.42(C)	3	6.52(s)	106.78(CH)	3	6.70(s)	107.81(CH)	3	-	148.64(C)
4	6.62 (s)	111.33(CH)	3a	-	126.50(C)	3a	-	120.73(C)	4	6.69 (s)	111.50(CH)
4a	-	126.76(C)	4	(a)3.13(obsc)	29.14(CH_2)	4	(a)3.30(obsc)	26.93(CH_2)	4a	-	119.22(C)
5	(a)3.13(obsc)	29.06(CH_2)		(b)2.62(m)			(b)2.96(dd,17.39,		5	(a)3.30(obsc)	23.27(CH_2)
	(b)2.66(obsc)		5	(a)3.05(obsc)	53.59(CH_2)		3.53)			(b)3.16(dd,	
6	(a)3.21(obsc)	51.48(CH_2)		(b)2.51(t,d,		5	(a)3.80(obsc)	53.94(CH_2)		18.15, 6.86)	
	(b)2.66(obsc)			11.63, 3.69)			(b)3.54(obsc)		6	(a)3.82(obsc)	51.86(CH_2)
8	(a)4.24(d, 15.63)	53.96(CH_2)	6a	3.05(obsc)	61.85(CH)	6a	4.34(obsc)	69.11(CH)		(b)3.67(obsc)	
	(b)3.54(d,15.63)		6b	-	126.55(C)	6b	-	125.04(C)	8	4.94(s)	59.83(CH_2)
8a	-	127.70(C)	7	(a)3.67(dd,	26.89(CH_2)	7	(a)3.83(obsc)	25.41(CH_2)	8a	-	119.92(C)
9	-	145.06(C)		14.66, 4.3)			(b)2.60(m)		9	-	145.58(C)
10	-	150.24(C)		(b)2.29(m)		7a	-	126.77(C)	10	-	151.24(C)
11	6.79(d, 8.38)	110.94(CH)	7a	-	129.76(C)	8	7.87(d, 8.72)	124.81(CH)	11	6.87(d, 8.35)	113.35(CH)
12	6.88(d, 8.38)	123.82(CH)	8	-	151.98(C)	9	7.03(d, 8.72)	112.61(CH)	12	6.82(d, 8.35)	123.32(CH)
12a	-	128.61(C)	9	-	145.80(C)	10	-	153.94(C)	12a	-	121.16(C)
13	(a)3.27(dd,	36.28(CH_2)	10	6.87(d, 8.47)	110.20(CH)	11	-	147.03(C)	13	(a)3.45(d, 2.59)	34.00(CH_2)
	15.76, 3.65)		11	7.80(d, 8.47)	123.05(CH)	11a	-	124.44(C)		(b)3.00(dd,	
	(b)2.83(dd,		11a	-	124.59(C)	11b	-	117.60(C)		18.35, 10.12)	
	15.76, 11.49)		11b	-	116.46(C)	12	(a)6.15(d, 0.74)	102.86(CH_2)	13a	5.09(obsc)	64.98(CH)
13a	3.54(obsc)	59.29(CH)	12	(a)6.06(d,1.44)	100.56(CH_2)		(b)6.00(d, 0.74)		13b	-	123.93(C)
13b	-	129.66(C)		(b)5.91(d,1.44)		N-CH_3	3.21(s)	42.12(CH_3)	N-CH_3	3.40(s)	49.98(CH_3)
2-OCH_3	3.87(s)	56.05(CH_3)	N-CH_3	2.60(s)	43.95(CH_3)	10-OCH_3	3.90(s)	56.30(CH_3)	2-OCH_3	3.85(s)	56.03(CH_3)
3-OCH_3	3.89(s)	55.84(CH_3)	8-OCH_3	3.81(s)	60.65(CH_3)	11-OCH_3	3.86(s)	61.19(CH_3)	3-OCH_3	3.86(s)	56.18(CH_3)
9-OCH_3	3.85(s)	60.14(CH_3)	9-OCH_3	3.90(s)	55.71(CH_3)				9-OCH_3	3.84(s)	60.84(CH_3)
10-OCH_3	3.84(s)	55.81(CH_3)							10-OCH_3	3.82(s)	55.88(CH_3)

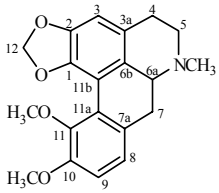
Table 2: ^{1}H-^{13}C, ^{1}H-^{1}H correlations for isolated alkaloids



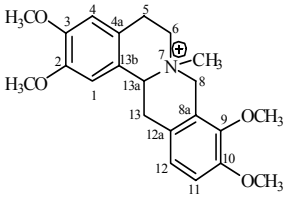
Tetrahydropalmatine (1)



Crebanine (2)



O-methylbulbocapnine (3)



N-methyltetrahydropalmatine (4)

Position	HMBC Correlation	COSY correlation	Position	HMBC correlation	COSY correlation	Position	HMBC correlation	COSY correlation	Position	HMBC correlation	COSY correlation
1	C-2, C-4a, C-13b	-	1	-	-	1	-	-	1	C-2, C-3, C-4a, C-13b	-
2	-	-	2	-	-	2	-	-	2	-	-
3	-	-	3	C-1, C-2, C-3a,	-	3	C-1, C-2, C-3a, C-4	-	3	-	-
4	C-1, C-3, C-5, C-13b	-		C-4,C-11b		3a	-	-	4	C-2, C-3, C-4a, C-5	-
4a	-	-	3a	-	-	4	(a)C-5, C-6b	H-4b, H-5a	4a	-	-
5	(a)C-4a	H-6b, H-5b	4	(a)C-3a, C-5, C-6b	H-4b		(b)C-3, C-3a, C-6b	H-4a, H-5b	5	(a)C-4a	H-5b, H-6a
	(b)C-4a	H-5a, H-6a		(b)C-3, C-3a, C-6b	H-4a	5	(a)C-6b	H-4a, H-5b		(b)C-4a	H-5a, H-6b
6	(a)C-4a, C-5, C-8	H-6b	5	(a)C-3a, C-4, C-6a,	H-5b		(b)-	H-4b, H-5a	6	(a)-	H-5a, H-6b
	(b)C-4a, C-13a	H-6a, H-5a		N-CH_3		6a	-	H-7b		(b)-	H-5b, H-6a
8	(a)C-6, C-8a, C-9,	H-8b		(b)C-3a, C-4, C-6a,	-	6b	-	-	8	C-6, C-8a, C-9, C-12a,	-
	C-12a, C-13a			N-CH_3		7	(a)-	H-7b		C-13a, C-13b, N-CH_3	
	(b)C-6, C-13a	H-8a	6a	C-6b, N-CH_3	-		(b)C-7a	H-6a, H-7a	8a	-	-
8a	-	-	6b	-	-	7a	-	-	9	-	-
9	-	-	7	(a)C-6a, C-7a, C-9,	H-7b	8	C-7, C-7a, C-9,	H-9	10	-	-
10	-	-		C-11a			C-10, C-11, C-11b		11	C-9, C-10, C-12a,	H-12
11	C-8a, C-9, C-10, C-12	H-12		(b)C-6a, C-7a, C-8,	H-7a	9	C-10, C-11, C-11a	H-8	12	C-8a, C-9,C-10, C-13,	H-11
12	C-9, C-10, C-12a, C-13	H-11		C-9,C-10, C-11, C-11a		10	-	-		C-13a	
12a	-	-	7a	-	-	11	-	-	12a	-	-
13	(a)C-8a, C-12, C-13a	H-13b	8	-	-	11a	-	-	13	(a)C-12a	H-13b, H-13a
	(b)C-8a, C-12a, C-13a	H-13a, H-13a	9	-	-	11b	-	-		(b)C-12a, C-13a	H-13a, H-13a
13a	C-6, C-12a	H-13b	10	C-7a, C-8, C-9, C-11a	H-11	12	(a)C-1, C-2	-	13a	C-13b	H-13b, H-13a
13b	-	-	11	C-7a, C-8, C-9, C-10, C-11b	H-10		(b)C-1, C-2	-	13b	-	-
2-OCH_3	C-2, C-3	-		-		N-CH_3	-	-	N-CH_3	C-6, C-8	-
3-OCH_3	C-2, C-3	-	11a	-	-	10-OCH_3	C-9, C-10	-	2-OCH_3	C-2	-
9-OCH_3	C-9	-	11b	(a)C-1, C-2	-	11-OCH_3	C-11	-	3-OCH_3	C-3	-
10-OCH_3	C-10	-	12	(b)C-1, C-2	-				9-OCH_3	C-9	-
				C-4, C-5	-				10-OCH_3	C-10	-
			N-CH_3	C-8	-						
			8-OCH_3	C-9, C-10	-						
			9-OCH_3		-						

Table 3: The effect of isolated alkaloids on cell viability of K562, K562/Adr, GLC4 and GLC4/Adr

	IC_{50} (µg.mL^{-1}) (R value) n = 8			
Cell lines	Tetrahydropalmatine (1)	Crebanine (2)	O-methylbulbocapnine (3)	N-methyltetrahydropalmatine (4) (%)
K562	39.94±4.89 (0.7)	7.17±1.96 (1.1)	8.73±1.40 (1.2)	at 250 µg.mL^{-1} with IC 18.43
K562/Adr	27.07±5.27	8.24±1.83	10.13±2.51	at 250 µg.mL^{-1} with IC 20.90
GLC4	60.68±6.28 (0.3)	10.31±0.42 (1.0)	12.24±2.11 (1.2)	at 250 µg.mL^{-1} with IC 8.22
GLC4/Adr	20.21±4.89	10.14±3.83	14.18±4.03	at 250 µg.mL^{-1} with IC 31.08

Table 4: Cell cycle analysis; the effect of isolated alkaloids on cell cycle of K562, K562/Adr, GLC4 and GLC4/Adr

		$Cell_{treated}$(%) - $Cell_{control}$(%) (n = 4)								
		Tetrahydropalmatine (1)			Crebanine (2)			O-methylbulbocapnine (3)		
Cell lines	Time (h)	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M
K562	12	1.6	-2.6	1.0	2.3	-0.9	-1.3	1.2	-1.7	0.5
K562/Adr		-3.3	2.8	0.5	7.7	-7.0	-0.7	12.6	-21.6	9.0
GLC4		-1.8	2.7	-0.8	4.2	-4.7	0.5	7.3	-7.1	-0.2
GLC4/Adr		2.6	0.6	-3.2	-1.1	-0.1	1.2	-1.4	-2.2	3.6
K562	24	-3.4	1.7	1.7	-2.0	-10.7	12.7	18.4	-19.8	1.5
K562/Adr		-0.9	3.8	-2.9	1.2	0.0	-1.2	11.7	-10.7	-1.0
GLC4		-1.4	-3.6	5.0	1.2	1.5	-2.8	2.3	0.3	-2.7
GLC4/Adr		-1.0	1.9	-0.9	1.7	2.0	-3.7	4.5	1.0	-5.5

The IR spectrum of compound 2 showed four different C = C stretching bands. The bands at 1601, 1573, 1496, 1473 cm^{-1} indicated the presence of aromatic ring. The presence of the stretching bands at 1284 and 1234 cm^{-1} suggested the presence of a C-O-C linkage of ether and an o-disubstitued benzene moiety (821 cm^{-1}). The presence of the aporphine group in the sturcture was judged by the fragmentation pattern in the mass spectrum. The retro Diles-Alder fragmentation of the compound 2 yielded the ions at m/z 296 and 43, indicating the presence of two methoxy and methylenedioxy group in ring A, C and D whilst the fragment ion m/z 43 (methyl imine) confirmed the methyl group was on nitrogen of ring B. Peaks at m/z 324 and 308 (M-15 and M-31) peak are due to the loss of methyl and methoxyl from one of the aromatic methoxyl substituents (Ohashi *et al*., 1963). This is a typical behavior of aromatic methylether is observed. Stabilization of the two observed ions may be possible by initial fission of the benzylic bond and formation of a new ring. The N-methyl function of compound 2 exhibited an M-43 peak. Thus, the fragment lost must be methylene imine, expelled by cyclic process *via* retro Diles-Alder cycloaddition (Ohashi *et al*., 1963). Additionally, the base peak at m/z 149 came from the fragment ion at m/z 296. The 400 MHz ^{1}H NMR ($CDCl_3$) spectrum (Table 1) of 2 indicated that compound 2 was aporphine alkaloids crebanine (Joanne *et al*., 2003). This fact was evidenced by the presence of signals for three protons on aromatic carbons. Two of them appeared at a fairly downfield position as a doublet at δ 6.87 (J = 8.47) and 7.80 (J = 8.47) indicating the presence of an aporphine nucleus in the ring D. These signals were justifiably assigned to H-10 and H-11 (Banerji *et al*., 1994). In addition, the most up field proton appeared as a singlet at δ 6.52. The ^{1}H NMR spectrum exhibited two low field signals at δ 6.06 and 5.91 and the ^{13}C signal at δ 100.56 which has AB pattern with coupling constant, J = 1.4 Hz, displaying three-bond coupling to the ^{13}C signals at δ 142.00 (C-1) and 146.50 (C-2), respectively which consecutively showed long-range coupling the methylene dioxy protons in the HMBC experiment. The series of connectivities implied that the one aromatic ring present in the molecule bore the methylene dioxy group. The HMQC experiment revealed the C-4 - C-5 ethano group but C-7 was a methylene center. The H-7 signal is doublet of doublet (J = 14.66, 4.3) at δ 3.67 and 2.29 while the C-7 signal is shifted to higher field (δ 26.89). Additionally, the spectrum also indicated the presence of an N-methyl group with chemical shift at 2.60 s, which this signal exhibited HMBC correlation to carbon signals at δ 29.14 (C-4) and 53.59 (C-5). The two methoxyl groups as in the ring D were corroborate located at the C-8 (δ 151.98) and C-9 (δ 145.80) position by the HMBC correlation proton signals of H-7, 10 and 11. Based on above data, compound 2 was identified as crebanine. This compound has been isolated from *S. sasakii* and *S. capitata* (Joanne *et al*., 2003).

O-methylbulbocapnine (3): Isolated from the acetone extract was obtained as light brown crystals (mp 130.1-132.3°C) analyzed for $C_{20}H_{21}NO_4$ by means of HRAPCIMS measurement on the $[M+H]^+$ ion (m/z)

340.1540 calcd for 340.1543, $[\alpha]^{25}_{589}$ -9.27 (c 2.02, MeOH). The UV spectrum of the compound in methanol showed absorption bands maxima at 282 and 322 nm (log ε 1.37 and 0.92) which were characteristic of isoquinoline alkaloids (Banerji *et al*., 1994). This compound is isomeric with compound 2. The mass spectrum of compound 3 was suggested to have the bear a resemblance to structure as in 2, except the position of the two methoxyl are different from compound 2. The fragment ions in the mass spectrum at m/z 339, 296, 281, 266 and 149 (base peak) were in agreement with those observed in compound 2. In the mass spectrum of bulbocapnine, the M-15 peak is observed (Ohashi *et al*., 1963). Therefore this compound has four oxygen functions in the aromatic rings, so it is possible to stabilize the positive charge by distribution among several oxygen atom (Ohashi *et al*., 1963). The aromatic region of the ^{1}H NMR spectrum, which was run in CD_3OD (Table 1) showed the presence of three aromatic protons. Their multiplicity, two of them appeared at a fairly downfield position as a doublet at δ 7.87 (J = 8.72) and 7.03 (J = 8.72) and a ^{1}H singlet at δ 6.70, were suggestive of an aporphine nucleus in the ring D. Not only the vicinal coupling correlation observed H-8 and H-9 suggested the presence of ortho aromatic proton on the ring D but also H-H COSY crosspeaks between these two protons confirmed that they are vicinal methane proton. The HMQC and COSY experiments exposed that the C-4 - C-5 group but C-7 signal was a methylene center. The H-7b signal is multiplet because of long-range coupling with H-8, germinal, vicinal coupling with H-7a and H-6a, respectively. The HMBC sequence provided information on the long-range connectivities between various ^{1}H and ^{13}C atom. Its showed geminal, vicinal and long-range coupling interactions between H-8 (δ 7.87) and C-7a, C-9, C-7, C-10, C-11, 11b, between H-9 (δ 7.03) and C-10, C-11, C-11a, between H-3 (δ 6.70) and C-2, C-3a, C-1, C-4, between H-4a,b and C-3a, C-5, C-3, C-6b, between H-5a (δ 3.80) and C-6b. Additionally, the ^{1}H NMR spectrum exhibited two low field signals at δ 6.15 and 6.00 and the ^{13}C signal at δ102.86, similar to compound 2 which has AB pattern with coupling constant, J = 0.74 Hz, displaying three-bond coupling to the ^{13}C signal at δ 144.66 (C-1) and 150.06 (C-2), respectively which consecutively showed long-range coupling the methylene dioxy protons in the HMBC experiment. The two methoxyl groups as in the ring D were substantiate located at the C-10 (δ 153.94) and C-11 (δ 147.03) position by the HMBC correlation proton signals of H-8, H-9, 10-OCH_3 and 11-OCH_3. These data above were in agreement with the structure, O-methylbulbocapnine alkaloid previously reported for *L. megaphylla* Hemsl (Lauraceae) by Chou *et al*. (1994).

N-methyltetrahydropalmatine (4): the EIMS analysis gave a molecular formular $C_{22}H_{28}NO_4$ and a molecular ion peak m/z 371$[M+H]^+$. The mass spectrum of compound 4 was suggested to have the resemble structure as in 1, except the methyl group on the nitrogen atom. The fragment ions in the mass spectrum at m/z 371, 355, 339, 206, 164, 151, 149 and 55 (base peak) were in accordance with those observed in compound 1. The ion at m/z 55 (1-methyl-1H-azinine) resulted from a rearrangement involving the isoquinoline moiety in the structure. It was obtained as white crystals, mp 168.8-171.4°C, $[\alpha]^{25}_{589}$ -34.79 (c 1.44, MeOH). The UV absorption maxima at 227 (log ε1.2), 284 (log ε 0.74) and IR absorption bands (1612, 1519, 1500 and 1461 cm^{-1}), an ether linkage (1272, 1230 cm^{-1}) and an o-disubstitued benzene moiety (786 cm^{-1}) of 1 and 4 were similar. On the other hand, the 400 MHz ^{1}H NMR spectrum of 4 (Table 1) demonstrated the presence of four aromatic protons, twelve alkyl protons for four methoxyl groups, eight alkyl protons for four methylene groups, one methine proton and more higher field shift of N-CH_3 proton among the methyl protons. The five alkyl protons resonated in the downfield region when comparison with 1 at δ 3.82 (H-6a), 3.67 (H-6b), 4.94 (H-8) and 5.09 (H-13a), indicating that they are in the region of the ammonium cation. In addition, the pair of doublets (AB pattern), δ 6.87 H-11 and 6.82 H-12 displayed on the aromatic region suggesting the presence of two ortho protons coupling with J = 8.35 Hz, together with two singlets (δ 6.79, H-1 and 6.69, H-4) consistent with two para related hydrogens in an aromatic of isoquinoline nucleus. Form the low field shift of the H-8 proton, resonated at δ 4.94 is distinguishing of the bridge position (C-8) of protoberberine alkaloids (Joanne *et al*., 2003). The ^{13}C NMR spectrum of closely resembled that of 1. On the basis of systematic ^{13}C NMR spectroscopic analysis of protoberberine systematical structure or palmatine alkaloid stated that the resonance for saturated carbon C-5, C-6, C-8 and C-13 were found at δ 23.27, 51.86, 59.83 and 34.0, respectively. The four methoxyl protons at δ_H 3.85, 3.86, 3.84 and 3.82 exhibited HMBC correlations to their aromatic carbons δ_C 149.62 (C-2), 148.64 (C-3), 145.58 (C-9) and 151.24 (C-10), correspondingly. Additionally, the HMBC spectrum promote confirmed the involvement of C-2, C-3 as in the ring A and C-9, C-10 on the ring D by the correlation with H-1, 4 and

H-11, 12, respectively. Further analysis of HMBC spectrum confirmed the location of methyl group on heteroatom nitrogen which was clearly shown as the protons correlation of position H-8 whiles the N-CH_3 proton was also showed HMBC correlation with C-6 and C-8. This structure was agreement with the related structure, N-methylstepholidine which reported by Ingkaninan *et al*. (2006).

Antiproliferative effects: Compound 1, 2 and 3 exhibited the anti-proliferation activity on four cancer cell lines with the %IC_{50} value of microgram per milliliter ranging. Among those compounds, crebanine (2) presented the most proliferation inhibition whereas dislocation of dimethoxy on ring D from C-8 and C-9-C-10 and C11 of compound 3 showed faintly lesser cytotoxic than its isomer. The bioactivity of two protoberberine alkaloids (i.e., compound 1 and 4) was clearly revealed the antiproliferation in compound 1 while at the concentration of 250 μg mL^{-1}, compound 4 can inhibit cell proliferation at about 18, 20, 8 and 31% in K562, K562/Adr, GLC4 and GLC4/Adr, respectively. This can be suggested that the N-CH_3 proton may perturb the intercalation of compound in between base pair of DNA. The Resistance factor (R) can be presented and calculated by the IC_{50}% for resistance cells divided by its corresponding sensitive cells. These results indicated that the R values of compound 2 and 3 are about 1-1.2 and 0.3-0.7 for compound 1. These signify that those 3 compounds are not recognized either by P-glycoprotein or MRP1 protein, especially compound 1 which is 3 times more toxic in GLC4/Adr cell compare with its sensitive cell. According to DNA is the drug target of these compounds (Bhadra *et al*., 2008) cell cycle analysis was performed by incubate four cell lines with each compound at the IC_{50} concentration. The result showed slightly increase in S phase by the effect of compound 1, beside the G0/G1 phase was blocked by compound 2 and 3 (Table 4).

CONCLUSION

The investigation focused on the phytochemical of Thai medicinal plant together with biochemical evaluation. The results presented herein reveal the four alkaloids derivative from the only species, Venosa of the genus Stephania which has been potent on antiproliferative effect. Moreover, these three compounds can play an important role for solving the multidrug resistance in 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.

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