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Oxoaporphine alkaloids from the barks of *Platymitra siamensis* Craib (Annonaceae) and their cytotoxicity against MCF-7 cancer cell line

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ABSTRACT: Study on the chemical constituents of the dichloromethane (DCM) crude extract of *Platymitra siamensis* Craib has led to the isolation of four oxoaporphine alkaloids. The compounds were identified as liriodenine (**1**), O-methylmoschatoline (**2**), lysicamine (**3**) and cepharadione-A (**4**) which were isolated first time from this species. The structures of the isolated compounds were elucidated based on their spectral data (^{1}H, ^{13}C and LCMS) and reports in the literature. Here we observed that, only alkaloid **1** exhibited obvious cytotoxic effects against MCF-7 human breast cancer cells line with IC_{50} value of 31.26 µM. This work is the first attempt on phytochemical and bioactivity study on the genus of Platymitra.

KEYWORDS: *Platymitra siamensis* ; Annonaceae ; oxoaporphine alkaloids ; MCF-7 ; cytotoxic activity.

1. INTRODUCTION

Annonaceae is the largest flowering plant family in Magnoliales. There are 109 validly described and recognized genera and 2440 species in this family [1,2]. Annonaceae is also the most species-rich family in the order of Magnoliales [3]. However, the *Platymitra* is a very small genus that belongs to Annonaceae with only two species recognised. According to The Plant List, there are 3 species recorded. *P. arborea* is the only accepted name among the species in the genus *Platymitra* in Annonaceae family while the *P. siamensis* and *P. macrocarpa* are still in unresolved status [4]. On the other hand, botany wikispaces stated that *P. siamensis* (syn *P. macrocarpa*) is an abundant and important tree in Mainland Southeast Asia, and while *P. arborea* is often found abundantly in evergreen forests of the surrounding region of Thailand, Sumatra, Java and the Philippines [5].

The vernacular name of *P. siamensis* Craib in Malaysia is "mangitan" [6]. As stated by Whitmore, this species is rare and scattered in lowland forest, typically on hillsides [7]. In Malaysia, it can be found in several areas namely Langkawi, Kedah, Perak, Selangor, Negeri Sembilan and Johor. *P. siamensis* Craib can grow up to 12 m and 14 cm in diameter. The outer stem barks normally are grey or darkish grey in colour with irregular cracking but pale brown inner. The leaves are alternate simple, thinly coriaceous and 10-16 cm × 3.0-4.5 cm in size. Besides that, the leaves are shining dark green on the upside surface but paler for the downside.

In terms of its application, this species is an indigenous plant that has been reported to be widely used for interior construction, ship and boat building, furniture, agricultural implements and some ornaments [6-8]. Although Annonaceae family is well known to have numerous bioactive chemical compounds particularly alkaloids but, to date, there have been no reports on *P. siamensis* Craib chemistry and bioactivities [9]. Bioactive

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compounds from plants have been reported to exert anti-cancer activities with minimal side effects, as such search into these alternative natural resources including from genus *Platymitra* is important and timely.

This current study is the first attempt to isolate alkaloids from the barks of *P. siamensis* Craib and investigate its cytotoxicity activity against MCF-7 breast cancer cell lines. Breast cancer is the most common cancer in woman worldwide. According to the latest available data from GLOBOCAN, breast cancer is listed as the fifth cause of death from cancer overall (522,000 deaths; 25% of all cancers) with an estimated 1.67 million new breast cancer cases diagnosed in 2012 [10]. MCF-7 cells represent a very important candidate in research of breast cancer [11]. It is an ER-positive and progesterone receptor (PR)-positive [12], less aggressive and non-invasive cell line [13].

Liriodenine, was identified of having antimicrobial, antibiotic, antifungal, antitumor, cardiovascular effects and antiplatelet properties and actions [14]. In this study, four alkaloids were isolated and identified as liriodenine (**1**), *O*-methylmoschatoline (**2**), lysicamine (**3**) and cepharadione-A (**4**) (Figure 1). All compounds were isolated for the first time from *Platymitra* genus. The structures of the compounds were identified based on the spectroscopic data and comparison with literature.

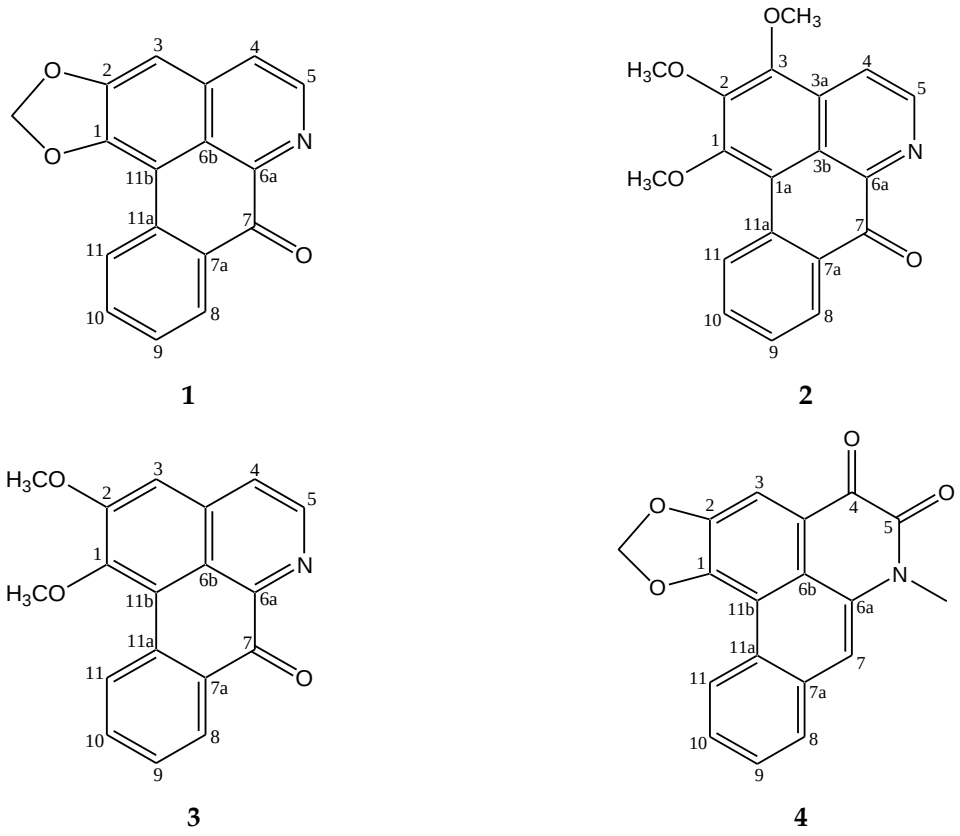

Figure 1. Chemical structures of **1 – 4** isolated from *P. siamensis* Craib.

2. RESULTS AND DISCUSSION

2.1. Structural Elucidation

Compound **1** was isolated for the first time from *Liriodendron tulipifera* L., and followed by other plant species [15]. Most are found in the families of Magnoliaceae, Annonaceae, Rutaceae, Monimiaceae, and Menispermaceae [14]. This compound was obtained as yellow needles. The MS analysis exhibited a molecular ion peak at *m/z* 275.1 $[M]^+$, suggesting the molecular formula $C_{17}H_9NO_3$. The ^1H NMR spectrum ($CDCl_3$, 500 MHz) of **1** showed two protons singlet at δ 6.36 corresponding to the presence of methylenedioxy group, OCH_2O. Meanwhile a proton singlet at δ 7.14 belonged to H-3. Two sets of one proton doublet at δ 7.74 and δ 8.86 represent H-4 and H-5, respectively. Two set of double of doublets at δ 8.55 and δ 8.59 were attributable to H-8 and H-11, respectively. In addition, two sets of one proton triplet of doublets at δ 7.55 and δ 7.72 were assigned to H-9 and H-10, respectively. The ^{13}C NMR spectrum of **1** exhibited seventeen signals. The spectrum indicated the presence of one methylene, seven methine and eight quartenary carbons and one carbonyl

carbon. Signal at δ 182.4 indicated the carbonyl carbon while signal δ 102.6 attributed to the methylenedioxy carbon. These spectroscopic properties were similar to those of the known compound liriodenine.

Compound **2** was obtained as orange yellowish needles. The MS spectrum revealed a strong molecular ion peak at *m/z* 321.1 $[M]^+$, thus providing the possibility of the molecular formula to be $C_{19}H_{15}NO_4$. The ^1H NMR (500 MHz, $CDCl_3$) of compound **2** showed similarity to that of **1** indicating that **2** also is an oxoaporphine alkaloid. However, the spectrum of **2** did not contain signals arising from methylenedioxy group (O-CH_2-O), instead it displayed three methoxy signals at δ 4.08, δ 4.11 and δ 4.19. Therefore these three methoxyl carbons suggested to be located at C-1, C-2 and C-3, respectively. The ^{13}C NMR spectrum supported the structure of compound **2**. Three methoxyl carbons appeared at δ 61.1, δ 61.6 and δ 61.9 belong to OCH_3-1, OCH_3-2 and OCH_3-3, respectively. The carbonyl carbon signal appeared at δ 182.7 and no signal was found at δ 100.2, thus suggesting that the methylenedioxy carbon was replaced with two methoxyl carbons. These spectroscopic data were similar to those of the known compound *O*-methylmoschatoline; which has been reported to be isolated from two species of Annonaceae namely *Ellipeia cuneifolia* [16] and *Annona foetida* Mart [17].

Compound **3** was obtained as yellowish amorphous. The MS spectrum revealed a strong molecular ion peak at *m/z* 291.1 $[M]^+$, thus providing the possibility of the molecular formula to be $C_{18}H_{13}NO_3$. The ^1H NMR (500 MHz, $CDCl_3$) of compound **3** showed similar pattern with compound **2** due to the presence of two methoxyl signals at δ 4.02 and δ 4.11. There are one proton singlet H-3 at δ 7.24, two triplets of proton H-9 and H-10 (appearing at δ 7.58 and δ 7.79 respectively), two doublets of aromatic protons (H-4 and H-5 revealing separately at δ 7.81 and δ 8.89) and another two double of doublets signals of aromatic protons (assigned to H-8, at δ 8.60 and H-11 at δ 9.20). The ^{13}C-NMR spectrum showed eighteen carbon signals, including one carbonyl carbon resonated at δ 182.9 assigned to C-7. Two methoxyl carbons appeared at δ 56.3 and δ 60.8 were appointed to OCH_3-1 and OCH_3-2, respectively. In addition, seven methine aromatic carbons resonated at δ 106.5, δ 123.7, δ 145.2, δ 129.0, δ 128.9, δ 134.4 and δ 128.5 were designated to C-3, C-4, C-5, C-8, C-9, C-10 and C-11, respectively. Eight quaternary carbons assigned as C-1, C-1a, C-2, C-3a, C-3b, C-6a, C-7a and C-11a were identified at chemical shifts of δ 156.9, δ 120.0, δ 152.1, δ 135.6, δ 122.3, δ 145.5, δ 132.2 and δ 134.5, respectively. This compound has been reported to be previously isolated from leaves of *Phoebe grandis* (Nees) Merr. (Lauraceae) [18] and *Xylopia aethiopica* (Annonaceae) [19].

Compound **4** was obtained as orange amorphous. The MS spectrum revealed a strong molecular ion peak at *m/z* 305.2 $[M]^+$, thus providing the possibility of the molecular formula to be $C_{18}H_{11}NO_4$. The ^1H NMR (500 MHz, $CDCl_3$) of compound **4** showed the presence of one *N*-methyl and one methylenedioxy group, OCH_2O with the singlets at δ 3.86 and δ 6.46, respectively. Two singlets appearing at δ 8.14 and δ 7.52 were assigned to shielded hydrogen atoms placed at C-3 and C-7. The aromatic protons revealed two double of doublets at δ 9.00 and δ 7.90 (assigned to H-11 and H-8, respectively) and two protons represented by multiplets at δ 7.69-7.71 (assigned to H-9 and H-10). The ^{13}C NMR spectrum of **4** exhibited eighteen signals. The spectrum indicated the presence of six methines at δ 109.0, δ 114.9, δ 128.4, δ 127.7, δ 126.8 and δ 128.4 which are assigned to C-3, C-7, C-8, C-9, C-10 and C-11, respectively. Signal at δ 174.6 and δ157.5 indicated the carbonyl carbons which are located at C-4 and C-5 while signal at δ 103.8 attributed to the methylenedioxy group. These spectroscopic properties were similar to those of the known compound cepharadione-A; previously isolated from roots of two species of Piperaceae; *Piper nigrum* [20] and *Piper betle* Linn [21]. The analysis of 1D NMR spectra data of compounds **1, 2, 3** and **4** were tabulated in Table 1 together with the sources of the references [22-24].

2.2. BIOACTIVITY

The anticancer activity of the crude extracts of dicholoromethane (DCM), hexane and methanol (MeOH); and four pure alkaloids from *P. siamensis* Craib were tested against MCF-7 human breast cancer cells. At the concentration tested (0-40 μM), crude extracts did not possess cytotoxic or anticancer effects against MCF-7 breast cancer cells. Meanwhile, pure compounds **1** exhibited IC_{50} value of 31.26 μM (Figure 2). However, compound **2, 3** and **4** did not show any inhibition on MCF-7 cells at concentration tested. Compound **1**, liriodenine has been reported to have anticancer effects against several types of cancer targeting at various pathways including apoptosis and cell cycle arrest without exerting cytotoxic effects to normal cells [25-27].

Table 1. ^{1}H-NMR and ^{13}C-NMR spectroscopic data ($CDCl_3$) of compound 1, 2, 3 and 4.

Position	Compound 1 [22,23]		Compound 2 [16]		Compound 3 [18]		Compound 4 [24]	
	δ^{13}C (ppm)	δ^{1}H (ppm) *J* (Hz)	δ^{13}C (ppm)	δ^{1}H (ppm) *J* (Hz)	δ^{13}C (ppm)	δ^{1}H (ppm) *J* (Hz)	δ^{13}C (ppm)	δ^{1}H (ppm) *J* (Hz)
O-CH_2-O	102.6	6.36 (*s*)	-	-	-	-	103.8	6.46 (*s*)
1	148.1	-	156.6	-	156.9	-	152.0	-
1a		-	115.7	-	120.0	-	-	-
2	151.9	-	147.4	-	152.1	-	148.3	-
3	103.3	7.14 (*s*)	148.5	-	106.5	7.24 (*s*)	109.0	8.14 (*s*)
3a	135.9	-	122.9	-	135.6	-	125.4	-
3b		-	131.2	-	122.3	-	-	-
4	124.4	7.74 (*d*) *J*=5.7	119.3	8.24 (*d*) *J*=5.2	123.7	7.81 (*d*) *J*=5.0	174.6	-
5	144.8	8.86 (*d*) *J*=5.5	144.5	8.98 (*d*) *J*=5.8	145.2	8.89 (*d*) *J*= 5.0	157.5	-
6a	145.2	-	145.5	-	145.5	-	131.8	-
6b	123.3	-	-	-	-	-	-	-
7	182.4	-	182.7	-	182.9	-	114.9	7.52 (*s*)
7a	131.2	-	131.5	-	132.2	-	129.1	-
8	128.8	8.55 (*dd*) *J*=8.0, 0.6	129.0	8.59 (*d*) *J*=8.3	129.0	8.60 (*dd*) *J*=7.6, 0.7	128.4	7.90 (*dd*) *J*=8.0, 0.6
9	128.7	7.55 (*td*) *J*=7.5, 0.6	128.2	7.55 (*dt*) *J*=6.9,7.5	128.9	7.58 (*t*)	127.7	7.69-7.71 (*m*)
10	134.0	7.72 (*td*) *J*=8.1, 0.6	134.5	7.76 (*dt*) *J*=9.2, 6.9	134.4	7.79 (*t*)	126.8	7.69-7.71 (*m*)
11	127.4	8.59 (*dd*) *J*=8.1, 0.6	127.7	9.13 (*d*) *J*=8.6	128.5	9.20 (*dd*) *J*= 7.6, 0.7	128.4	9.00 (*dd*) *J*=8.0, 0.6
11a	132.9	-	134.6	-	134.5	-	127.7	-
11b	108.1	-	-	-	-	-	116.0	-
OCH_3-1	-	-	61.1	4.08 (*s*)	56.3	4.02 (*s*)	-	-
OCH_3-2	-	-	61.6	4.11 (*s*)	60.7	4.11 (*s*)	-	-
OCH_3-3	-	-	61.9	4.19 (*s*)	-	-	-	-
N-CH_3	-	-	-	-	-	-	30.0	3.86 (*s*)

3. CONCLUSION

In conclusion, four oxoaporphine alkaloids were isolated from DCM crude extract of *P. siamensis* Craib barks. Compound **1**, was found to be active at inhibiting MCF-7 breast cancer cell growth and this warrants further investigation. More new alkaloids are expected to be found in *P. siamensis* Craib since this species has not been studied before.

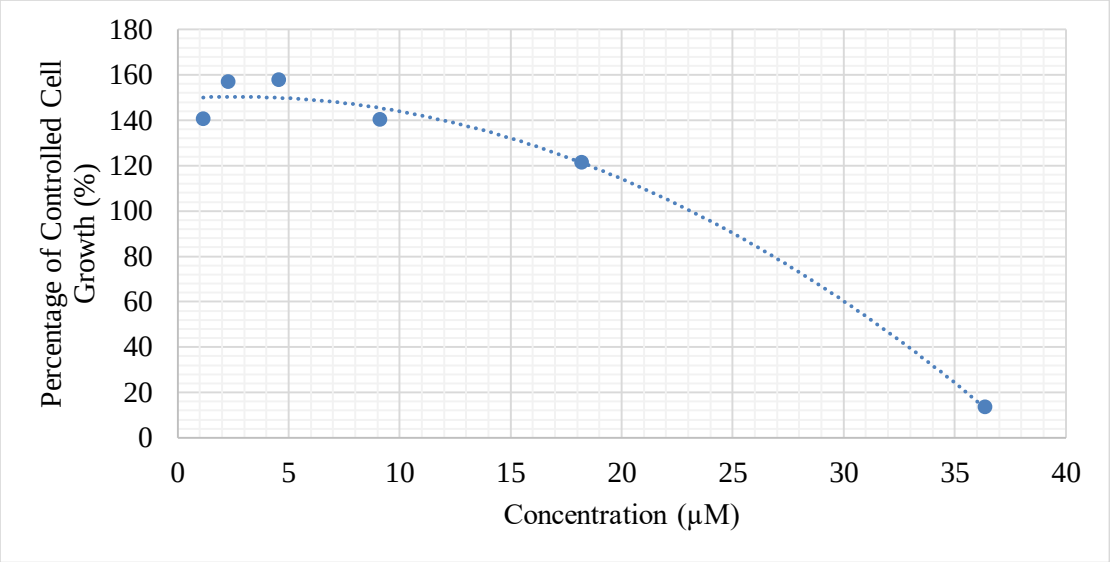

Figure 2. Percentage of Controlled Cell Growth (PCCG) *vs* concentration plot for pure compound **1.** IC_{50} value of 31.26 µM was obtained for compound **1**.

4. MATERIALS AND METHODS

4.1. General experimental procedure

Analytical grade reagents and chemicals were used unless otherwise stated. DCM and MeOH were obtained from Systerm®. Hexane was purchased from HmbG® Chemicals and $CDCl_3$ were obtained from ACROS® Organics. MCF-7 human breast cancer cells were purchased from American Type Culture Collection (ATCC® HTB-22™). The IR spectra were carried out on the Thermo Nicolet FTIR model 6700 spectrophotometer. Meanwhile, the mass spectra were recorded on Agilent Technologies GCMS-5975C VL MSD Spectrometer (USA). The UV-Vis spectra were carried out using UV-Vis spectrophotometer model Agilent Cary 60 and the NMR spectra were performed using JEOL JNM-ECX (500 MHz). Silica gel 60, 200 – 400 mesh was used for column chromatography (CC). The TLC plates were pre-coated with aluminium supported silica gel 60 F_{254} from Merck, observed under UV light (254 nm and 365 nm) model UVGL-58 and followed by spraying with Dragendorff's reagent to determine the presence of alkaloids.

4.2. Plant material

Barks of *P. siamensis* Craib with herbarium number KL 5753 was identified and collected by phytochemical group of Chemistry Department, University of Malaya, Kuala Lumpur from Hutan Simpan Sungai Badak, Jitra, Kedah in Malaysia on 7th October 2010. The voucher specimen was deposited at Chemistry Department, University of Malaya, Kuala Lumpur.

4.3. Extraction and Isolation

Cold maceration technique was used in this work. The dried and powdered bark (1800 g) of *P. siamensis* Craib was defatted with hexane for 72 h at room temperature to give hexane extract (7.8 g, 0.0043%). After drying for 24 h, the sample was moistened with 40% of ammonia (NH_3) solution for 3 h before being submitted for another extraction using DCM for 72 h. The sample was then filtrated and concentrated to yield DCM extract (8.8 g, 0.0049%). About 1.2 g of DCM extract was kept for bioactivity and the remaining (about 7.6 g) was chromatographed on silica gel CC using DCM/MeOH (100:0 to 0:100, v/v) to yield 229 fractions with 50 mL each. These fractions were evaluated and pooled according to TLC analysis yielding 57 fractions (P1-P57). Fractions P17-P22 were subjected to CC on silica gel using solvent system of DCM/MeOH (100:0 to 99:1) and gave 15 fractions. The sub-fractions 3-6 gave liriodenine (**1**) (7.5 mg, 0.00042%)**.** The sub-fractions 10-13 gave cepharadione-A (**4**) (2.9 mg, 0.00016%). Fractions P30-P35 were subjected to CC on silica gel using solvent system of DCM/MeOH (100:0 to 98:2) and gave 27 fractions. The sub-fractions 8-11 gave *O*-methylmoschatoline (**2**) (2.7 mg, 0.00015%) while the sub-fractions 17-22 were subjected to microcolumn on silica gel using solvent system of DCM/MeOH (99.5:0.5 to 98:2) and produced 11 fractions. The fractions 2-6 produced lysicamine (**3**) (1.9 mg, 0.00011%).

4.4. Cell line and culture medium

MCF-7 human breast cancer cells were thawed from liquid nitrogen storage and cultured in growth medium (Dulbecco's modified eagle's medium, DMEM) with 10% (v/v) fetal bovine serum (FBS). For cell viability assay, cells were detached, counted using trypan blue dye exclusion method, and seeded into 96-well flat bottom tissue culture plate. Seeding concentration was set at 1 x 10^5 cells/mL and cells were allowed to propagate for 24 h before introduction of samples to the growth medium. After 24 h of growth, 10 µL of SAS was pipetted into each designated well and the plate was incubated for 48 h. Then, 10 µL of CCK-8/WST-8/Cell Counting Kit-8 reagent was added and the plate was further incubated for 1 h. After 1 h, optical density (OD) reading was measured at 450 nm wavelength. The assay was carried out in three independent experiments. IC_{50} value (concentration of compound that yields 50% less cells compared to the control) was derived from curve-fitting methods. OD data was used to plot the dose-response between the compound concentration and growth inhibition percentage. The percentage of the controlled cell growth (PCCG) is determined as follows:

$$PCCG = \left(\frac{OD_{sample} - OD_{blank}}{OD_{negative\ control} - OD_{blank}}\right) \times 100\ \%$$

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Conflict of interest statement: The authors declare that there is no conflict of interest in this research.

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