Research Article

In Vitro Cytotoxic of Aporphine and Proaporphine Alkaloids from *Phoebe grandis* (Ness) Merr.

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ABSTRACT

Four known aporphinoids, *N*-nornuciferine **1**, caaverine **2**, sparsiflorine **3** and glaziovine **4** were isolated from the leaves of *Phoebe grandis* (Ness) Merr. (Lauraceae). All compounds were first isolated from this species. These compounds were assayed for cytotoxicity against human uteric cervical tumor (HeLa), human promyelocytic leukemia (HL-60) and normal mouse fibroblast (NIH/3T3) cell lines by using MTT assay. Compound **1** and **2** displayed low activity against HeLa cell, while **3** and **4** showed as good. Compound **4** also showed good cytotoxicity against HL-60 cell, while other displayed low to inactive. This phytochemical study involved extraction, separation and purification by using various chromatography methods and structural elucidation by using spectoscopic techniques such as UV, IR LC-MS and 1D and 2D NMR.

Keywords: Aporphine, Cytotoxic, Lauraceae, *Phoebe grandis* and Proaporphine

INTRODUCTION

Phoebe grandis is an evergreen tree belonging to the Lauraceae family which comprises a group of flowering plants which is widely distributed in tropics and subtropics, mainly in South-East Asia, South America and Brazil[1]. Most of this genus has been known as a source of isoquinoline alkaloids such as aporphine, oxoaporphine and proaaporphine[2,3]. Based on literature review, aporphine showed interesting biological activities, including antioxidant[4], anticancer[5], antimicrobial and antifungal[6]. More than 500 aporphine alkaloids, such proaporphines, oxoaporphines and aporphines have been isolated from various plant families and many of these compounds also displayed potent cytotoxic activities which may be exploited for the design of anticancer agents[7,8].

This work aimed to identify the active compounds by assessing the cytotoxic activity of aporphinoids isolated from leaves of *Phoebe grandis* (Ness) Merr. on selected cell lines and the structural evidence related to cytotoxicity is also discussed.

MATERIALS AND METHODS

General

Merck silica gel 60 (200-600 and 200–400 mesh) were used for column chromatography separations, aluminium support silica gel 60 F_{254} for Thin Layer Chromatography (TLC), and silica gel 60 F_{254} with gypsum for Preparative Thin Layer Chromatography (PTLC). NMR spectra were recorded on JEOL ECA (400 MHz) using $CDCl_3$ as a solvent. HRESIMS was obtained on Agilent 6530 Accurate-Mass Q-TOF LC/MS. UV spectra were obtained by using Perkin Elmer UV-Visible spectrophotometer with methanol as a solvent. IR spectra were obtained on Nicolet 6700 FTIR spectrophotometer with chloroform as a solvent. Rudolph autopol III polarimeter was used to obtained specific rotation with using methanol as a solvent. ELISA flourometer plate reader Infinite M200 was used to determined absorbance at 570 nm of cell viability.

Plant Materials

Leaves of *P. grandis* (1.4 kg) were collected from Bahau, Negeri Sembilan, Malaysia. The specimen was identified at Chemistry Herbarium, Faculty of Science, University of Malaya (KL 5540).

Extraction

P. grandis leaves extraction was carried out by exhaustive extraction using the Soxhlet extractor. Dried and grounded leaves of the plant were first defatted with hexane and filtered.

After being dried, the samples residue was moist with 28% of ammonia solution and left for two hour. This was to aggregate the nitrogen-containing compounds in *P. grandis* leaves. It was then re-extracted with dichloromethane to obtain dichloromethane crude extract. The crude extract was then dried using rotary evaporator. The yield of the dichloromethane crude extract obtained from leaves of *P. grandis* leaves was 31 gram with 2.2% percentage of yields.

Isolation and Purification

Isolation of alkaloids was performed by using common chromatographic techniques such as column chromatography (CC) and preparative thin layer chromatography (PTLC). The dichloromethane crude extract of *P. grandis* leaves was subjected to CC over silica gel and eluted with increasing polarity solvent system of hexane, dichloromethane and methanol. Isolation and

purification (20 g) of sample yielded 13 fractions. Fraction 6 gave compound **1** and **2** after purification by CC and PTLC. Fraction 11 gave compound **3** and fraction 5 gave compound **4** after purification by PTLC.

The structure of compounds were elucidated by using 1D-NMR (^{1}H, ^{13}C and DEPT) and 2D-NMR (COSY, HMQC and HMBC), LC-MS, UV and IR spectroscopic techniques and also compared to previous study.

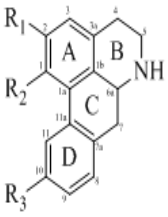
Cell Culture and MTT Cytotoxicity Assay

Cytotoxic activity in this study was treated against 3 types of cell, HL-60 (suspension cell), NIH/3T3 and HeLa (adherent cells). All cells were recognized from the American Type Cell Collection (ATCC). Medium without compound was used as negative control and positive control was used vincristine. The cells were cultured using RPMI 1640 culturing media and maintained at 37°C in 5% CO_2 atmosphere and counted using hemocytometer.

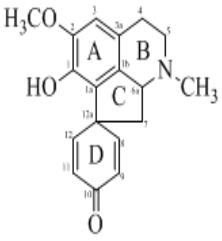
The MTT assay was carried out in the 96-wells plate. Briefly, a volume of 100.0 μL of complete growth medium was added into each well of 96-wells flat bottom microtiter plate (Nunclon, USA). The compounds or vincristine solution (95.0–105.0% purity by HPLC, Sigma, USA) at 60.0 μg/mL was aliquoted into wells in triplicate and serially diluted. A volume of 100.0 μL of 1x10^5 cells/mL HeLa or HL-60 or NIH/3T3 cells were seeded into 96-wells flat microtiter plates and incubated for 72 hours in CO_2 incubator. After 72 hours incubation, a volume of 20.0 μL of MTT solution (5.0 mg/mL) was added into each well and incubated for 4 hours. The culture medium was removed and 100.0 μL of 100% DMSO solution were added to each well to solubilise the formazan formed. The plates were read using the plate reader at 570nm wavelength (Infinite M200, Tecan, Switzerland).

RESULTS AND DISCUSSION

Compound Characterization



	R_1	R_2	R_3
1	OCH_3	OCH_3	H
2	OCH_3	OH	H
3	OCH_3	OH	OH



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N-nornuciferine **1**[9] was determined as $C_{18}H_{19}NO_2$ (m/z 282.1481 $[M+H]^+$). This compound obtained as a brown amorphous with $[\alpha]_D^{25}$ +149.89° (*c*= 0.02, CH_3OH). It was confirmed as 1, 2 disubstituted aporphine alkaloid by UV that showed peaks at 225, 245, 255 and 280 nm. The IR spectrum revealed the absorption at 1734 cm^{-1} which indicated as the presence of C=C stretching[10].

The ^{1}H NMR spectrum of **1** showed the presence of methoxyl groups at 3.66 (*s*) and 3.88 (*s*) that attached to C-1 and C-2, respectively. These positions also confirmed by HMQC experiment. Specifically, 1, 2 dioxygenated aporphine as showed by the presence of four aromatic protons at δ 7.23 (*m*), 7.21 (*m*), 7.30 (*m*) and 8.39 (*d, J*=7.45 Hz) assigned to four aromatic carbons of H-8, H-9, H-10 and H-11. Aromatic proton also gave peak at δ 6.65 as singlet that assigned to H-3. The other aliphatic protons appeared as multiplet and doublet of doublet at region δ 2.70-3.81 which in attributed to H-4, H-5, H-6a and H-7. The ^{13}C NMR spectrum showed the presence of eighteen carbon signals. There were four aliphatic carbons, two methoxy carbons and twelve aromatic carbons which consist of seven quaternary as supported by DEPT experiment.

Caaverine **2**[11] showed a brownish spot on TLC and its IR spectrum exhibited a broad absorption at 1760 and 1357 cm^{-1} were therefore assigned as aromatic rings and the absorption at 3017 cm^{-1} represented as the presence of hydroxyl group. This alkaloid showed specific rotation of $[\alpha]_D^{25}$ +94.93° (*c*= 0.02, CH_3OH). The molecular formula was determined as $C_{17}H_{17}NO_2$ (m/z 268.1334 $[M+H]^+$). The UV_{max} showed absorptions at 225, 245, 265 and 280 nm as the skeleton of 1, 2 disubstituted aporphine alkaloid. The pattern of ^{1}H and ^{13}C of **2** that shown in Table 1 and 2 were virtually the same as those for compound **1** suggested as the same skeleton.

The ^{1}H NMR spectrum of **2** showed the presence of methoxyl groups attached at position C-2 as a singlet. Five aromatic protons appeared at the downfield region, three were multiplets at position H-8, H-9 and H-10, one doublet at position H-11 and one singlet peak which belongs to H-3. The rest, seven aliphatic proton were appeared as multiplets and doublet of doublets at position H-4, H-5, H-6a and H-7. The ^{13}C and DEPT NMR spectrums showed the presence of seventeen carbon signals, consisting of one methoxy carbon (1-OCH_3), three methylene carbons (C-4, C-5 and C-7), six methane carbons (C-3, C-6a, C-8, C-9, C-10 and C-11) and seven quaternary (C-1, C-1a, C-1b, C-2, C-3a, C-7a and C-11a).

Sparsiflorine **3**[12] was obtained as an amorphous solid with $[\alpha]_D^{25}$ +149.89° (*c*= 0.02, CH_3OH). The protonated molecular ion $[M+H]^+$ was observed at m/z 284.1280 (calcd for $C_{17}H_{17}NO_3$). The ^{1}H NMR spectrum indicated the presence of four aromatic protons at δ 6.57 (1H, *s*, H-3), 6.68 (1H, *dd*, *J*=5.75, 8.0Hz, H-9), 7.07 (1H, *d*, *J*=8.05 Hz, H-8) and 7.91 (1H, *d*, *J*=2.85 Hz, H-11), one methoxyl groups at δ 3.89 (3H, *s*, 2-OCH_3) and seven aliphatic protons represented at position H-4, H-5, H-6a and H-7.

The ^{13}C NMR showed the presence of seventeen carbon signals. The analysis of the DEPT spectrum with the aid of an HMQC experiment revealed the signals of one methoxyl group (δ 56.3) attached to position C-2, three methylenes at δ 29.1 (C-4), 43.3 (C-5) and 36.7 (C-7), five methines belongs to C-3, C-6a, C-8, C-9 and C-11 and

eight quaternary carbons (δ 119.0, 123.9, 127.8, 129.0, 133.4, 141.4, 145.8 and 154.7).

The aporphine skeleton confirmed with UV spectrum that showed absorption at 230, 260 and 280 nm (1, 2, 10 oxygenated aporphine).

Supported by IR experiment, hydroxyl groups showed of broad peaks at 3012 cm^{-1}. Peak at 1729 cm^{-1} displayed the presence of aromatic rings.

Glaziovine **4**[13], $[\alpha]_D^{25}$ +89.93° (*c*= 0.02, CH_3OH), was isolated as an amorphous material. The molecular formula of $C_{18}H_{19}NO_3$ was determined from ion peak at m/z 298.1441 $[M+H]^+$.

Its IR spectrum suggested the presence of a carbonyl (ν_{max} 1744 cm^{-1}) and hydroxyl (ν_{max} 3022 cm^{-1}) functionality. The proaporphine skeleton was showed by UV spectrums at 240, 265, 275 and 285 nm.

The ^{1}H NMR of **4** showed doublet of doublet signal of the vinyl protons at δ 6.37 (*dd*, *J*=1.7, 9.70 Hz) and 6.28 (*dd*, *J*=2.85, 9.70 Hz) for H-9 and H-11 and δ 6.85 (*dd*, *J*=2.85, 9.75 Hz) and 6.99 (*dd*, *J*=2.85, 9.70 Hz) for H-8 and H-12, respectively.

Protons at position 8 and 12 are more downfield because of their located at β to a carbonyl group, while the proton H-9 and H-11 are less shielded. A singlet at δ 6.57 is assigned to H-3. The presence of this signal showed that C-1 and C-2 are substituted, where singlet proton at δ 3.81 was assignable to C-2 as showed correlation in HMBC spectrum. As in the aporphine alkaloids, the aliphatic protons of H-4, H-5, H-6a and H-7 appeared at region δ 2.23-3.54 as multiplets and doublet of doublets with a complex pattern and the *N*-methyl signal resonated at δ 2.38 as a singlet with three protons.

Analysis of ^{13}C NMR and DEPT spectra revealed the presence of a carbonyl group (δ 186.0) at position C-10 that showed the basic skeleton of proaporhine, four aromatic carbons of vinyl ring (δ 153.4, 149.7, 128.7 and 127.6), six aromatic carbons revealed at ring A (δ 147.3, 140,9, 134.4, 124.4, 123.0 and 109.8), five aliphatic carbons consist of three methylenes (δ 55.1, 50,8 and 27.2), one methane (δ 65.8) and quaternary (δ 47.2) at position C-12a.

The complete data of ^{1}H and ^{13}C NMR for all the alkaloids were tabulated in Table 1 and 2, respectively.

Table 1: ^{1}H [400 MHz, δ_H (*J*, Hz)] NMR Data of **1**, **2, 3** and **4** in $CDCl_3$

Position	^{1}H δ_H (*J*, Hz), ppm			
	1	**2**	**3**	**4**
1				
1-OCH_3	3.66 (*s*)			3.59 (*s*)
1a				
1b				
2				
2-OCH_3	3.88 (*s*)	3.91 (*s*)	3.89 (*s*)	3.81 (*s*)
3	6.65 (*s*)	6.59 (*s*)	6.57 (*s*)	6.57 (*s*)
3a				
4	2.70 (*m*)	2.67 (*m*)	2.66 (*m*)	2.77 (*dd*, 5.2, 16.6)
	3.00 (*m*)	3.00 (*m*)	3.00 (*m*)	2.95 (*m*)
5	2.99 (*m*)	2.96 (*dd*, 5.2, 12.0)	3.03 (*dd*, 2.9, 12.0)	2.50 (*m*)
	3.35 (*m*)	3.36 (*m*)	3.37 (*m*)	3.13 (*m*)
N-CH_3				2.38 (*s*)
6a	3.81 (*dd*, 4.6, 13.8)	3.86 (*dd*, 4.1, 13.8)	3.85 (*dd*, 2.3, 14.3)	3.45 (*m*)
7	2.75 (*m*)	2.73 (*t*, 8.0)	2.64 (*m*)	2.23 (*t*, 10.9)
	2.83 (*dd*, 4.6, 13.8)	2.84 (*dd*, 4.6, 14.3)	2.81 (*dd*, 4.6, 13.8)	2.35 (*m*)
7a				
8	7.23 (*m*)	7.22 (*m*)	7.07 (*d*, 8.05)	6.85 (*dd*, 2.9, 9.7)
9	7.21 (*m*)	7.21 (*m*)	6.68 (*dd*, 2.3, 8.0)	6.37 (*dd*, 1.7, 9.7)
10	7.30 (*m*)	7.31 (*m*)		
11	8.39 (*d*, 7.5)	8.39 (*d*, 8.1)	7.91 (*s*)	6.28 (*dd*, 2.3, 10.3)
11a				
12				6.99 (*dd*, 2.9, 9.7)
12a				

Table 2: ^{13}C [100 MHz, δ_C] NMR Data of **1**, **2, 3** and **4** in $CDCl_3$

Position	^{13}C δ_C, ppm			
	1	**2**	**3**	**4**
1	145.2	141.5	141.4	140.9
1-OCH_3	60.3			
1a	126.6	119.1	119.0	123.0
1b	129.0	129.1	129.2	124.0
2	152.1	145.9	145.8	147.3
2-OCH_3	56.0	56.2	56.3	56.6
3	111.8	110.0	110.1	109.8
3a	127.0	124.0	123.9	134.4
4	29.3	29.0	29.1	27.2
5	43.2	43.3	43.3	43.6
N-CH_3				47.2
6a	53.6	53.6	53.9	147.3
7	37.6	37.5	36.7	56.6
7a	136.2	135.8	128.7	
8	128.4	127.8	113.9	153.4
9	127.8	126.7	154.7	128.7
10	127.4	127.0	53.9	186.4
11	129.0	128.5	115.5	127.6
11a	132.2	132.5	127.8	
12				149.7
12a				50.8

Table 3: Cytotoxic Activity of Compound **1**, **2**, **3** and **4** against NIH/3T3, HeLa and HL-60 Cell lines

Compound Name	Cytotoxic Activities CD_{50} values (µg/mL)		
	NIH/3T3	**HeLa**	**HL-60**
N-nornuciferine **1**	17	15	37
Caaverine **2**	37	21	51
Sparsiflorine **3**	6	1	51
Glaziovine **4**	9	4	3.5
Vincristine (VCR)	> 60	0.4	1.5

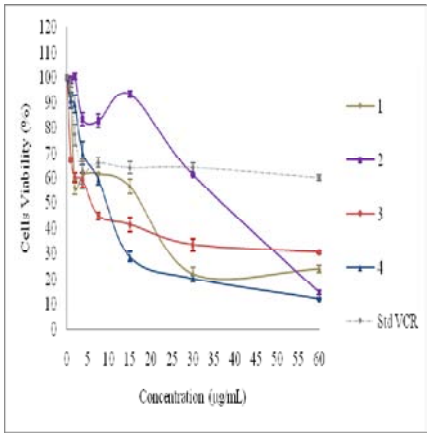

Figure 1: Effect of **1**, **2**, **3**, **4** and Standard Vincristine on the viability of NIH/3T3 Cell for 72 Hours Incubation

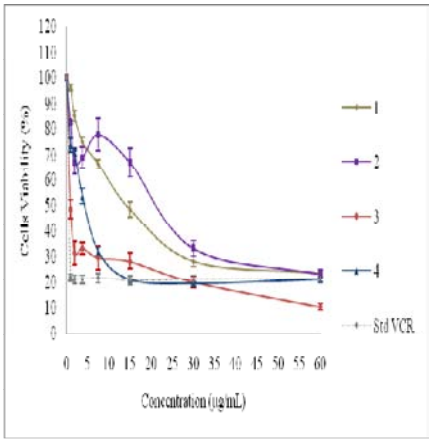

Figure 2: Effect of **1**, **2**, **3**, **4** and Standard Vincristine on the viability of HeLa Cell for 72 Hours Incubation

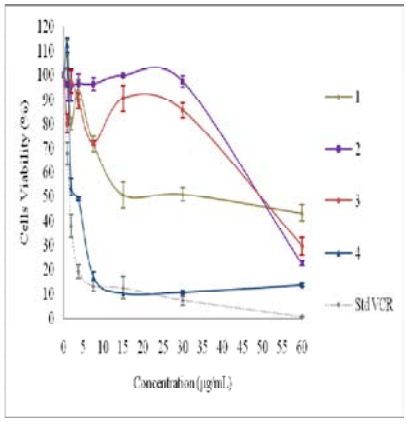

Figure 3: Effect of **1**, **2**, **3**, **4** and Standard Vincristine on the viability of HL-60 Cell for 72 Hours Incubation

In-vitro Cytotoxic

In this study, the compounds were evaluated for cytotoxicities against NIH/3T3, HeLa and HL-60 cell lines. The cytotoxicity of **1–4** was assayed at various concentrations under continuous exposure for 72 hours, are expressed in CD_{50} values (µg/mL), and were summarized in Table 3.

Result expressed as CD_{50} that represent the compound concentration doses that reduced the mean absorbance at 570 nm to 50% of those in the untreated control wells. The CD_{50} value was obtained from the plot between the concentrations of compound versus percent of cell viability.

The value was used to describe the degree of cytotoxicity of the compounds towards cell lines. Compounds which demonstrated the CD_{50} value less than 5.0 µg/mL were considered very active, while compounds with the CD_{50} value between 5.0 and 10.0 µg/mL were classified as moderately active. Those compounds that have CD_{50} value of 10–25 µg/mL were considered to be weak in cytotoxicity[14].

Isolated alkaloids from *P. grandis* were classified as 1, 2 substituted aporphine, 1, 2, 10 substituted aporphine and proaporphine.

Aporphine type of 1, 2 substituted gave a less activities compared to another aporhine type. In this case, **1** and **2** showed weak activities against HeLa cell and no activities against HL-60.

The presence of methoxyl group also effect the value of cytotoxicity, where **1** that has 2 methoxyls gave a higher value of 15 µg/mL than **2** which has one methoxyl and showed a value of 21 µg/mL. The 1, 2, 10 substituted aporphine showed a better cytotoxic activity than **1** and **2**. This **3** gave a very strong activity against HeLa cell with the value of 1 µg/mL.

A very good activity was also showed from proaporhine **4** that gave strong cytotoxicities against HeLa and HL-60 cell lines with the values of 4 and 3.5 µg/mL, respectively. Proaporhine has been known as a good cytotoxic due to the presence of carbonil group at C-10[2].

Beside all good activities against cancer cell line, compound **3** and **4** also displayed significant activities against normal cell NIH/3T3.

This result showed that these alkaloids were not safe for using as a drug. Cytotoxic activity of all four compounds from *P. grandis* against NIH/3T3, HeLa and HL-60 cell lines compared with positive control showed in Figure 1, 2 and 3, respectively.

CONCLUSION

Isolation, identification and characterization using spectroscopic data of compounds isolated from the leaves of *Phoebe grandis* yielded six known aporphine alkaloids, *N*-nornuciferine **1**, caaverine **2**, sparsiflorine **3** and glaziovine **4**. Compound **3** and **4** displayed strong cytotoxic activities against HeLa cell and only **3** showed as active against HL-60. Besides of all good activities against cancer cell line, compound **3** and **4** also displayed significant activities against normal cell NIH/3T3. This result showed that these alkaloids were not safe for using as drugs.

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