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In vitro Cytotoxic Activities of Aporphine Alkaloids from the Leaves of *Alseodaphne peduncularis* (Wall. ex. Ness) Meissn

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Four known aporphine alkaloids namely laurotetanine **1**, *N*-methylaurotetanine **2**, norboldine **3** and boldine **4** were isolated from the leaves of *Alseodaphne peduncularis* (Wall. Ex. Ness) Meissn (Lauraceae). Phytochemical studies involved extraction, separation, and purification by using various chromatography methods and structural elucidation by using spectroscopic techniques such as UV, IR and 1D and 2D NMR. These alkaloids 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 the MTT assay. Alkaloids **1** and **2** showed cytotoxic activity effect against heLa cell line with CD_{50} value of 2 and 15 µg/ml, respectively, whereas alkaloids **3** and **4** showed very low activity with CD_{50} value of 42 and 46 µg/ml, respectively. All four alkaloids did not show cytotoxic activity against HL-60 and NIH/3T3 cell lines.

Key words: *Alseodaphne peduncularis*; aporphine alkaloids*; in vitro* cytotoxic; phytochemical studies

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Cancer is the most common and fatal disease that caused about 7.6 million deaths in 2008. During the last decade, research on natural compounds has been particularly successful in the field of anticancer drug research. About 60% of anticancer drugs used nowadays are obtained from natural sources. The present review presents that most of the alkaloids isolated from a large number of plant families including Annonaceae, Lauraceae, Monimiaceae, Menispermaceae, Hernandiaceae and Ranunculaceae showed specific emphases on their potential development as anticancer agents [1, 2].

Alseodaphne peduncularis belongs to the Lauraceae family have been recognized for a long time as a source of alkaloids included aporphine alkaloids [3]. Aporphinoids form an important group of plant secondary metabolites. More than 500 aporphine alkaloids, such proaporphines, oxoaporphines and aporphines have been isolated from various plant families, and many of these compounds displayed potent cytotoxic activities which may be exploited for the design of anticancer agents [2, 4]. Here, we diescribe the isolation, structural elucidation and compare the cytotoxicity of four known aporphine alkaloids isolated from the leaves of *A. peduncularis*:- laurotetanine **1**, N-methyllaurotetanine **2**, norboldine **3** and boldine **4**.

METHODOLOGY

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 ECX (500 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 and the IR spectra were obtained on Nicolet 6700 FTIR spectrophotometer with chloroform as a solvent.

Plant Materials

Leaves of A. peduncularis (3.5 kg) were collected from Mersing, Johor, Malaysia. The specimen was identified at Chemistry Herbarium, Faculty of Science, University of Malaya (KL 5165).

Extraction

A. peduncularis leaves extraction was carried out by exhaustive extraction using the Soxhlet extractor. Dried, grounded leaves of the plant (3.525 kg) were first defatted with hexane and filtered. After being dried, the samples residue was moistened with 28% of ammonia solution and left for two hours; this was to aggregate the nitrogen-containing compounds in *A. peduncularis* leaves. It was then re-extracted with dichloromethane to obtain dichloromethane (DCM) crude extract. The crude extract was then dried using rotary evaporator. The yield of the dichloromethane crude extract obtained from leaves of *A. peduncularis* was 275.0 g with 7.8% 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 (TLC). The DCM crude extract from *A. peduncularis* was subjected to CC over silica gel and eluted with increasing polarity solvent system of hexane, DCM, and methanol. Fractions which had the same pattern shown in TLC were grouped into a series of fractions.

Isolation and purification (44.0 g) of sample yielded 14 fractions after grouped. Fraction 5 (0.1g) was then purified by PTLC to give compound **2**. Fraction 7 (4.4 g) gave compound **1** (7.5 mg) and **4** (10 mg), while fraction 11 (2.0 g) gave compound **3** (9 mg) after purified with CC and TLC.

The structure of compounds was 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 the previous study.

Cell Culture and MTT Cytotoxicity Assay

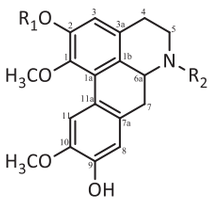
Cytotoxic activity in this study was treated with three types of cell which were developed by Mosmann [5], i.e. HL-60 (suspension cell), NIH/3T3 and heLa (adherent cells). All cells were recognized from the American Type Cell Collection (ATCC). Medium without compound was used as the negative control while vincristine was used as a positive control. 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 µl of complete growth medium was added to each well of 96-wells flat bottom microtiter plate (Nunclon, USA). For treatment cells, the compounds or vincristine solution (95 – 105% purity by HPLC, Sigma, USA) at 60.0 µg/ml was aliquoted into wells in triplicate and serially diluted and a volume of 100 µl of $1x10^5$ HL-60 (suspension cells) were seeded into 96-wells flat microtiter plates and incubated for 72 h in CO_2 incubator. In the case of adherent cells (heLa and NIH/3T3) seeding cells were done first about 24 h before treated with each compound and standard. After 72 h incubation, a volume of 20 µl of MTT solution (5.0 mg/ml) was added to each well and incubated for 4 h. The culture medium was removed, and 100 µl of 100% DMSO solution were added to each well to solubilise the formazan formed. The plates were read using the plate reader at 570 nm wavelength (Infinite M200, Tecan, Switzerland). A dose response curve of the percentage of cell viable versus extract concentration was plotted. The potency of cell growth inhibition for test agent was expressed as a CD_{50} value.

RESULTS AND DISCUSSION

Compound Characterization

Compound **1**, which was isolated as a major compound from the leaves of *A. peduncularis* is the a alkaloid with a molecular formula of $C_{19}H_{21}NO_4$ (m/z 328.1540 $[M+H]^+$). The UV spectra exhibited maximal absorption at 225, 280, 300 and 314 nm, indicating an aporphine substituted at positions 1, 2, 9, and 10 [6]. The IR spectrum showed absorption peak at 3280 cm^{-1}, indicating the presence of a hydroxyl group. An absorption of the aromatic system (C=C stretching) was observed at 1641 cm^{-1} [6].



	R_1	R_2
1	CH_3	H
2	CH_3	CH_3
3	H	H
4	H	CH_3

The ^{1}H NMR spectrum showed three aromatic protons resonated at δ 6.57 (H-3), 6.79 (H-8), and 7.99 (H-11) as a singlet. Three methoxyl signals which are attached to C-1, C-2, and C-10 showed as a singlet at δ 3.60, 3.87, and 3.81, respectively. The rest of the aliphatic protons appear as multiplets in the region between δ 2.80 and 3.50. The ^{1}H NMR data is summarized in Table 1. HMQC spectrum confirmed the direct correlation between hydrogen and carbon. The above observations were reinforced by COSY experiment which displayed correlations of H-4/H-5. The ^{13}C NMR spectrum of this compound showed the presence of nineteen carbons and DEPT experiment showed that there was three methoxyl carbons (1-OCH_3, 2-OCH_3 and 10-OCH_3); twelve aromatic carbons (C-1, C-1a, C-1b, C-2, C-3, C-3a, C-7a, C-8, C-9, C-10, C-11 and C-11a), one methine (C-6a) and three methylenes (C-4, C-5 and C-7), consistent with the structure proposed. The ^{13}C NMR data is also shown in Table 2. Structure assignment is also confirmed by HMBC experiment. On the basis of these spectral evidence and comparison with those in literature values [8, 9], compound **1** was identified as laurotetanine.

Compound **2**, obtained as a brown amorphous solid, showed an ion peak at m/z 342.1708 $[M+H]^+$ in its mass spectrum which was consistent with the molecular formula $C_{20}H_{23}NO_4$. This 1,2,9,10-tetraoxygenated aporphine moiety is indicated in the UV spectrum at 225, 280, 300 and 314 nm. Moreover, the IR spectrum exhibited the presence of a highly conjugated hydroxyl group at 3263 cm^{-1} and C=C stretching of the aromatic system at 1641 cm^{-1}.

The ^{1}H and ^{13}C NMR spectra of compound **2** were almost similar to compound **1**, which suggested a close structural relationship between these two compounds with the presence of *N*-methyl group at δ 2.54 and 43.9 in ^{1}H and ^{13}C NMR spectrum, respectively for compound **2**. The ^{1}H NMR spectrum of compound **2** exhibited three methoxyl singlets at δ 3.64, 3.87 and 3.88 positioned at C-1, C-10 and C-2, respectively, two singlets at δ 6.57 and 6.80 is assignable to H-3 and H-8 while the aliphatic protons gave a multiplet and doublet of doublet between δ 3.17–2.53 at position C-4, C-5, C-6a and C-7 that assigned based on COSY and HMQC spectrum. The $_{13}$C NMR and DEPT spectrum established the presence of twenty carbons, which consisted of four methyls which appeared as 1-OCH_3, 10-OCH_3, 2-OCH_3 and *N*-CH_3, three methylenes attributed to C-5, C-7 and C-4, four methines corresponding to C-8, C-11, C-3 and C-6a and nine quaternary carbon signals in the molecule at position C-1, C-1a, C-1b, C-2, C-3a, C-7a, C-9, C-10 and C-11a deshielded to downfield region due to diamagnetic anisotropy effects. Complete data of ^{1}H and ^{13}C NMR is tabulated in Tables 1 and 2, respectively. On comparison with literature values, the compound **2** was confirmed to be *N*-methyllaurotetanine [10].

Compound **3** was obtained as a dark brown amorphous solid. The UV maximum showed the wavelength at 219, 280 and 302 nm which indicated tetraoxygenated aporphine. The IR spectrum showed the presence of a hydroxyl group at 3300 cm^{-1}. The mass spectrum gave an $[M+H]^+$ ion peak at m/z 314.1387, consistent with a molecular formula of $C_{18}H_{19}NO_4$.

The ^{1}H NMR spectrum demonstrated the existence of two methoxyl groups as shown by two singlets at δ 3.61 and 3.91. These methoxyl groups were attached to C-1 and C-10 respectively, confirmed by HMQC spectrum. Three singlet signals represent three aromatic protons revealed at δ 6.65, 6.81 and 7.91 which could be assigned to H-3, H-8 and H-11, respectively. The remaining of aliphatic protons appeared as multiplets and doublets of doublets as H-4, H-5, H-6a and H-7. Data of ^{1}H NMR spectrum is summarized in Table 1. The cross-correlation deduced from COSY spectrum revealed the exact position of an aliphatic proton between H-4 and H-5. ^{13}C NMR established eighteen carbons were tabulated in Table 2. The signals are for three methoxyl carbons, twelve aromatic carbons, one methine and three methylenes. Four oxygenated aromatic quaternary carbon signals were observed at δ 148.1, 145.6, 145.1 and 141.9, indicating the presence of hydroxyl groups at C-2 and C-9, and methoxyl groups at C-1 and C-10, respectively. Extensive analysis of all spectroscopic data established the complete assignment of all the ^{1}H and ^{13}C signals of compound **3**, which eventually

confirmed the identification of the compound as norboldine similar with data from previous research [7, 8].

Compound **4** showed the same physical appearance as compound **3**. The mass spectrum gave a $[M+H]^+$ ion peak at m/z 328.1540, consistent with a molecular formula of $C_{19}H_{21}NO_4$. The UV experiments also showed the same spectrum as compound **3** that indicated the same skeleton between compounds **3** and **4**. The IR spectrum showed the presence of a hydroxyl group at 3265 cm^{-1}. Spectrum at 1625 cm^{-1} indicated the presence of conjugated carbonyl group [7].

The ^1H NMR and ^{13}C NMR spectra of compound **4** were almost similar to compound **3**. However, compound **4** exhibited the presence of *N* methyl signal in the ^1H NMR and ^{13}C NMR spectra at δ 2.53 and 43.9, respectively. The ^1H NMR spectrum showed the existence of two methoxyls by revealing two singlets attached to C-1 and C-10, and confirmed by HMQC spectrum. Three singlet signals representing three aromatic protons assigned to H-3, H-8 and H-11. The remaining aliphatic protons appeared as multiplets and doublet of doublets as H-4, H-5, H-6a and H-7. The cross-correlation deduced from COSY spectrum revealed the exact position of the aliphatic proton between H-4 and H-5.

The ^{13}C NMR spectrum established the presence of nineteen carbons which belonged to two methoxyl carbons, one *N*-methyl carbon, twelve aromatic carbons and the rest aliphatic carbons as one methines and three methylenes confirmed by DEPT spectrum. Data were supported by HMQC and HMBC experiments. The complete ^1H and ^{13}C NMR data are summarized in Tables 1 and 2, respectively. Comparison with literature values [8] confirmed that the compound **4** is boldine.

Table 1. ^1H NMR [500 MHz, δ_H (J, Hz) of Compound **1**, **2**, **3** and **4** in $CDCl_3$.

Position	δ_H (*J*, Hz), ppm			
	1	2	3	4
1-OCH_3	3.60 (*s*)	3.64 (*s*)	3.61 (*s*)	3.59 (*s*)
2-OCH_3	3.87 (*s*)	3.88 (*s*)	–	–
3	6.57 (*s*)	6.57 (*s*)	6.65 (*s*)	6.61 (*s*)
4	2.80 (*m*) 3.17 (*m*)	2.67 (*dd,* 3.4, 16.6) 3.17 (*m*)	2.67 (*m*) 2.96 (*m*)	2.61 (*m*) 3.09 (*m*)
5	3.05 (*m*) 3.50 (*m*)	2.53 (*dd, 4*, 1) 3.07 (*d,* 5.1)	2.92 (*dd*, 5.1, 13.2) 3.32 (*m*)	2.49 (*dd*, 3.4, 11.4) 3.11 (*m*)
N-CH_3	–	2.54 (*s*)	–	2.53 (*s*)
6a	–	3.02 (*m*)	3.76 (*dd*, 5.1, 13.7)	3.02 (*m*)
7	2.90 (*dd*, 4.6, 13.7)	2.58 (*d*, 8.7*)* 2.96 (*dd*, 4, 13.7)	2.64 (*m*) 2.72 (*dd*, 5.1, 18.9)	2.58 (*m*) 2.95 (*dd*, 4, 13.7)
8	6.79 (*s*)	6.80 (*s*)	6.81 (*s*)	6.81 (*s*)
10-OCH_3	3.81 (*s*)	3.87 (*s*)	3.91 (*s*)	3.90 (*s*)
11	7.99 (*s*)	8.05 (*s*)	7.91 (*s*)	7.89 (*s*)

Table 2. ^{13}C NMR [125 MHz, δ_C] of Compound **1**, **2**, **3** and **4** in $CDCl_3$.

Position	δ_C, ppm			
	1	**2**	**3**	**4**
1	144.5	144.2	141.9	142.1
1-OCH_3	60.2	60.2	60.4	60.3
1a	127.0	126.9	125.6	126.0
1b	125.0	127.1	128.1	126.6
2	152.7	152.0	148.1	148.2
2-OCH_3	55.9	55.8	–	–
3	110.6	110.2	113.7	113.3
3a	127.8	128.8	130.3	129.8
4	27.6	29.1	29.1	28.8
5	42.4	53.3	43.3	53.4
N-CH_3	–	43.9	–	43.9
6a	53.4	62.6	53.8	62.6
7	35.1	34.1	36.8	34.0
7a	128.5	130.0	130.1	130.1
8	114.3	114.0	114.2	114.3
9	145.3	145.0	145.1	145.1
10	145.8	145.4	145.6	145.7
10-OCH_3	56.0	56.1	56.2	56.2
11	111.5	111.2	110.2	110.2
11a	123.6	123.9	123.8	123.6

In-vitro Cytotoxic

In this study, the compounds were evaluated for cytotoxicities activity against NIH/3T3, heLa and HL-60 cell lines. The cytotoxicity of compounds **1–4** was assayed at various concentrations under continuous exposure for 72 h, are expressed in CD_{50} values (µg/ml) and are summarized in Table 3. Results showed as CD_{50} 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 of 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 CD_{50} values of less than 5 µg/ml were considered very active, while compounds with the CD_{50} values between 5 and 10 µg/ml were classified as moderately active. Those compounds that have CD_{50} value of 10–25 µg/ml were considered to be weakly cytotoxicity [11].

The positive control, vincristine, demonstrated excellent anticancer which gave a strong effect against heLa and HL-60 cell lines, but are not cytotoxic against a normal cell NIH/3T3. Compound 1 showed very potent activity against heLa cell line with CD_{50} value of 2 µg/ml, compound **2** displayed low activity with CD_{50} value of 15 µg/ml, but compound **3** and **4** showed no activity. Most of the isolated compounds showed no significant cytotoxicity effect against NIH/3T3 and HL-60. It was a good indicator that all four compounds were not cytotoxic against the normal

Table 3. Cytotoxic Activities of Compound **1**, **2**, **3** and **4** against NIH/3T3, heLa and HL-60 Cell lines.

Compound Name	Cytotoxic Activities IC_{50} values (µg/ml)		
	NIH/3T3	heLa	HL-60
Laurotetanine **1**	> 60	2	> 60
N- methyllaurotetanine **2**	> 60	15	> 60
Norboldine **3**	> 60	42	> 60
Boldine **4**	> 60	46	> 60
Vincristine (positive control)	> 60	0.4	1.5

cell, NIH/3T3. But, it was not surprising too that all compounds also gave negative results against HL-60 cell line, as in general, suspension cell lines were spread more quickly and widely than adherent ones.

Comparison of all four known aporphine alkaloids from isolated of *A. peduncularis*, the result was consistent with the finding in the literature that cytotoxicities were enhanced by the presence of methoxyl group [1]. The results showed that compounds **1** and **2** which had **3** methoxyl groups gave better cytotoxicities than compounds **3** and **4** with have fewer methoxyl groups. The weak cytotoxic activities displayed by compound **2** compared with compound **1** and compound **4** compared with compound **3** were also due to the presence of the *N*-methyl group [1]. Cytotoxic activities of all four compounds against NIH/3T3, heLa and HL-60 cell lines compared with positive control showed in Figures 1, 2 and 3, respectively.

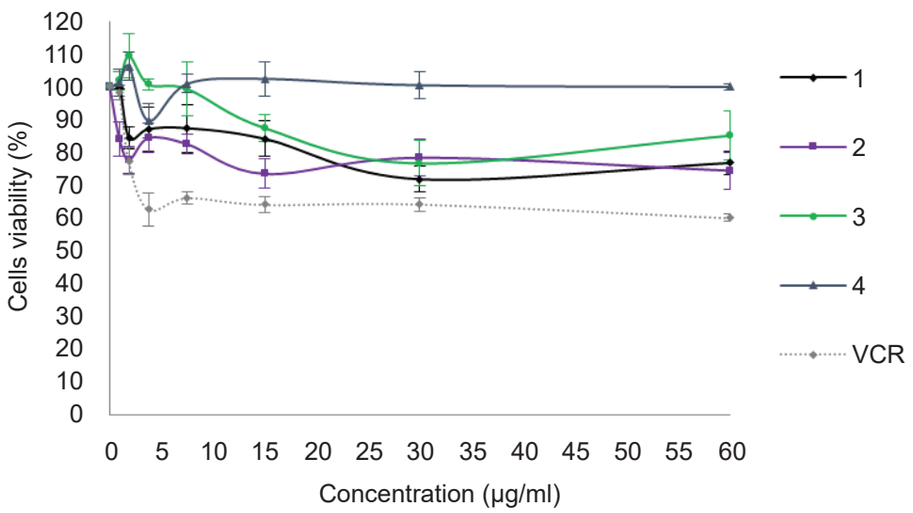


Figure 1. Effect of Compound **1**, **2**, **3**, **4** and Vincristine Standard on the viability of NIH/3T3 cell for 72 h incubation.

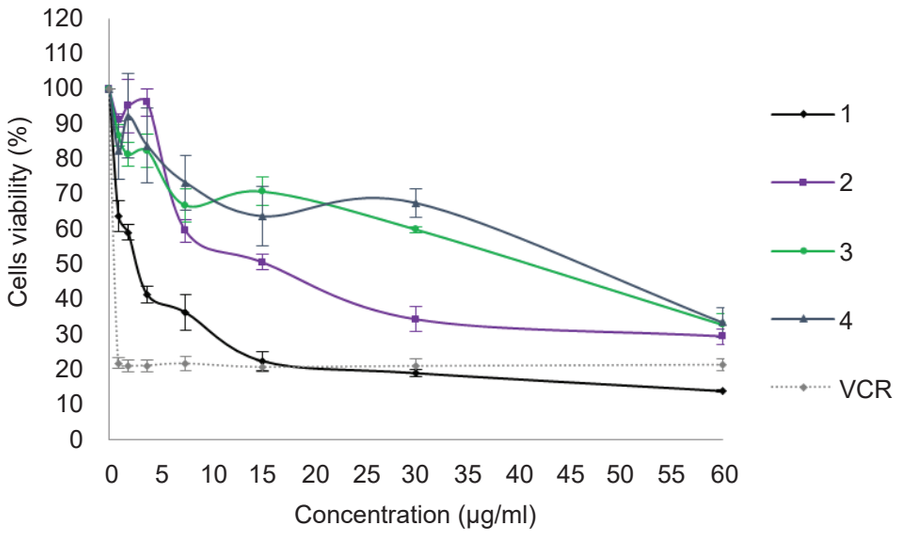


Figure 2. Effect of Compound **1**, **2**, **3**, **4** and Vincristine Standard on the viability of heLa cell for 72 h incubation.

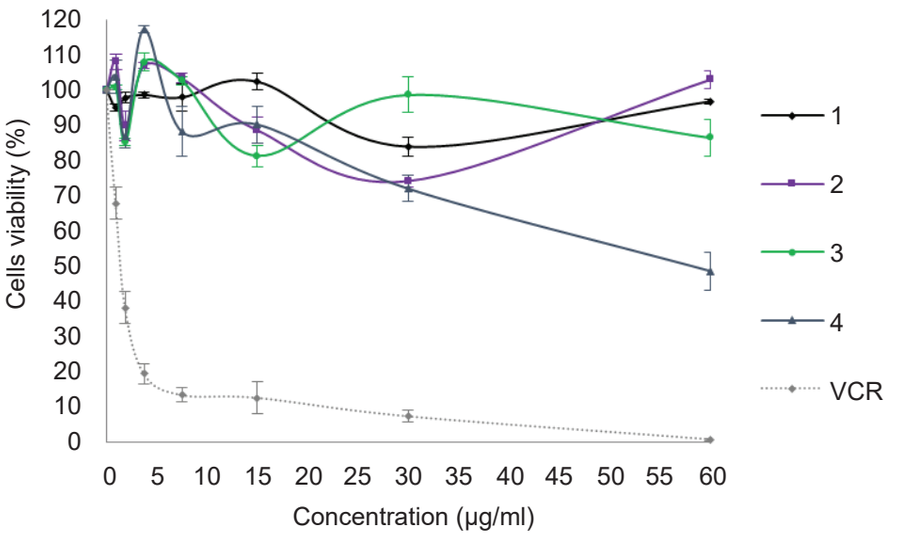


Figure 3. Effect of Compound **1**, **2**, **3**, **4** and Vincristine Standard on the viability of HL-60 cell for 72 h incubation.

CONCLUSIONS

Isolation, identification, and characterization using spectroscopic data of compounds isolated from the leaves of *A. peduncularis* yielded four known aporphine alkaloids, laurotetanine **1**, *N*-methyllaurotetanine **2**, norboldine **3** and boldine **4**. Compound **1** displayed potent cytotoxic activities against heLa cell, but compounds **2**, **3** and **4** showed as low to inactive. All four compounds had no effect against HL-60 and NIH/3T3 cell lines.

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