CYTOTOXIC ACTIVITY OF APORPHINE ALKALOIDS FROM ALSEODAPHNE PEDUNCULARIS (WALL. EX. NESS) MEISSN AGAINST HELA CELL LINE

Ulil Amna[1], Kartini Ahmad[1] and Mohd Azlan Nafiah[1]
[1]Department of Chemistry, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris, 35900 Tanjung Malim, Perak, Malaysia
*Corresponding author: ulil_amna@yahoo.com

Abstract:
Genus of Alseodaphne has been known as a source of apoprhine alkaloid that displayed potent cytotoxic activities against selected cell lines. Two aporphine alkaloids, Corydine 1 and norlirioferine 2 were isolated from leaves of Alseodaphne peduncularis (Wall. Ex. Ness) Meissn. (Lauraceae). 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. These compounds were then assayed for cytotoxicity against human uteric cervical tumor (heLa) and compare for safety with normal mouse fibroblast (NIH/3T3) cell lines by using the MTT assay. Compound 2 displayed potent cytotoxic activities against heLa cell, but compound 1 showed as inactive. Both compounds have no significant effect against NIH/3T3 cell lines indicated good for cancer agent treatment.
Keywords: *Lauraceae, Alseodaphne peduncularis, Aporphinoid and Cytotoxic*

INTRODUCTION

Plants are the most important sources of biologically active natural products that have differences in the structure and biological properties. Active compounds from plant resulted by secondary metabolite pathway. Plants have long history of use in the treatment of cancer. About 60% of anticancer agents used nowadays are obtained from natural sources. The present review presents that most of alkaloids isolated from large number of plant families showed specific emphases on their potential development as anticancer agents [1]. Plant secondary metabolites and their semi-synthetic derivatives have been used as anticancer drugs therapy, such as vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, etoposide, etoposide derived from epipodophyllotoxin and paclitaxel (taxol). Looking for new secondary metabolites is still necessary for the development of novel pharmaceuticals [1,2].

Alseodaphne peduncularis is a genus of Lauraceae, which is widely distributed in peninsular Malaysia and Sumatra. Most of this genus has been known as a source of aporphine alkaloids[3]. 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 [4,5]. This work aimed to identify the active compounds by assessing the cytotoxic activity of aporphinoids isolated from leaves of *Alseodaphne peduncularis (Wall. Ex. Ness) Meissn.* on HeLa 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 ECX (500 MHz) using $CDCl_3$ as a solvent. HRESIMS was obtained on Agilent 6530 Accurate-Mass Q-TOF LC/MS. UV spectra was obtained by using Perkin Elmer UV-Visible spectrophotometer with methanol as a solvent and the IR spectra was 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 were first defatted with hexane and filtered. After being dried, the samples residue was moisted 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 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 gram with 7.8% percentage of yields.

Isolation and purification

Isolation of alkaloids was performed by using common chromatographic techniques such as colomn chromatography (CC) and Preparative Thin Layer Chromatography (PTLC). The dichloromethane crude extract from *A. peduncularis* was subjected to CC over silica gel and eluted

with increasing polarity solvent system of hexane, dichloromethane and methanol. Fractions which have the same pattern shown in TLC were grouped into a series of fractions.

Isolation and purification (44 g) of sample yielded 14 fractions after grouped. Fraction 5 (100 mg) was then purified by PTLC to gave compound **1** (5 mg) and fraction 11 (2 g) gave **2** (100 mg) after purified by CC and 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 HeLa and NIH/3T3 cell lines. Both cells were recognized from the American Type Cell Collection (ATCC). Medium without compound was used as negative control and positive control was used vincristine sulfate as comparation. 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 sulphate 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 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). A dose response curve of the percentage of cell viable versus extract concentration was plotted.

DISCUSSIONS

Compound Characterization

Corydine **1** [7] was isolated from the leaves of *A. peduncularis* with a

Table 1 ^{1}H and ^{13}CNMR Data of Compound **1** and **2**

Position	^{1}H δ, $CDCl_3$ (*J*, Hz)		^{13}C (δ, $CDCl_3$)	
	1	**2**	**1**	**2**
1			142.3	145.1
1-OCH_3		3.65 (*s*)		60.3
1-OH	8.70 (*br*, *s*)			
1a			123.8	126.9
1b			127.8	127.3
2			149.2	156.3
2-OCH_3	3.88 (*s*)	3.88 (*s*)	56.0	55.9
3	6.67 (*s*)	6.59 (*s*)	111.3	110.8
3a			119.2	128.8
4	2.66 (*dd*, 2.85, 15.45) 3.16 (*m*)	2.69 (*m*) 3.03 (*m*)	28.8	28.8
5	2.53 (*dd*, 4.0, 23.5) 3.04 (*m*)	3.05 (*m*) 3.40 (*m*)	149.2	126.9
N-CH_3	2.54 (*s*)	3.84 ((*dd*, 13.2, 2.9)	43.8	
6a	2.96 (*d*, 13.1)	2.70 (*m*) 2.78(*dd*,13.8, 5.2)	62.7	53.7
7	3.02 (*d*, 3.4) 2.42 (*t*, 26.35)		35.4	36.3
7a			130.6	129.6
8	7.07 (*d*, 8.0)	6.78 (*s*)	124.4	114.1
9	6.86 (*d*, 8.0)		110.9	144.1
9-OCH_3		3.88 (*s*)		56.1
10			151.8	145.5
10-OCH_3	3.89 (*s*)		56.0	3.81 (*s*)
11		8.07 (*s*)	143.8	111.4
11-OCH_3	3.71 (*s*)		62.0	123.9
11a			126.4	53.7

molecular formula of $C_{20}H_{23}NO_4$ (m/z 342.1704 $[M+H]^+$). It was a 1,2,10,11-tetrasubstituted aporphine alkaloid suggested by UV spectrum that showed absorptions at 230, 265 and 305 nm [9]. The IR spectrum showed OH absorption band at 3278 cm^{-1}. Absorption peaks at 1425 and 1634 cm^{-1} indicated the presence of aromatic system [10].

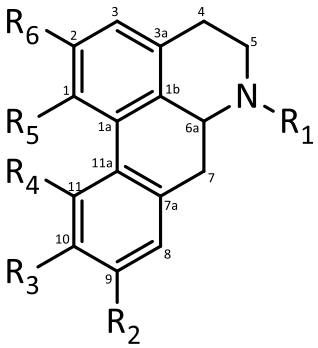
The specific ^{1}H NMR spectrum (Table 1) of 1, 2, 10, 11-tetrasubstituted aporphine alkaloid identified at a downfield region that showed proton peaks at δ 7.07 (*J*=8.0 Hz), 6.86 (*J*=8.0 Hz) and 6.67 (*s*) assignable to position H-8 and H-9 and H-3. A downfield chemical shift was also observed at δ 8.70 as a broad singlet and it was a typical of the hydroxyl group (OH)

that bonded at C-1. The *N*-methyl group appeared as singlet at δ 2.54. Three aromatic methoxyl groups observed at δ 3.71, 3.88 and 3.89, attached to C-11, C-2 and C-10, respectively.

The rest, aliphatic protons gave signals between δ 2.42 to 3.16 ppm attributed to H-4, H-5 and H-7. The assignments of all protons were made possible with the aid of COSY experiment that showed ^{1}H-^{1}H correlation of H-4/H-4, H-7/H-7 and H-8/H-9. The ^{13}C NMR spectrum (Table 1) gave a total of twenty carbon atoms, which consist of twelve aromatic carbons, four aliphatic carbons, three methoxyl groups and one of *N*-methyl group. Correlation between hydrogen and carbon also confirmed with HMQC and HMBC spectrums.

Compound **2** isolated from leaves of *A. peduncularis* known as aporphine alkaloids that substituted at position 1, 2, 9, 10. All five alkaloids showed UV absorption at range 280-284 and 303-310 nm which indicated this aporhine skeleton[6]. The IR spectrum showed absorption peak at 2800-3500 cm^{-1}, indicating the presence of a hydroxyl groups. An absorption of the aromatic system (C=C stretching) was observed at region 1600-1650 cm^{-1} [7].

Norlirioferine **2** [8] was isolated as a brownish amorphous solid. The mass spectrum showed $[M+H]^{+}$ ion peak at 328.1534, corresponding with a molecular formula of $C_{19}H_{21}NO_4$. The ^{1}H NMR spectrum (Table 1) showed two singlet at δ 3.65 (3H) and 3.88 (6H) that overlapping attributed to the methoxyl group at C-9, C-2, and C-1, respectively.



	R_1	R_2	R_3	R_4	R_5	R_6
1	CH_3	H	OCH_3	OCH_3	OH	OCH_3
2	H	H	OCH_3	H	OCH_3	OCH_3

Table 2 *Cytotoxic Activity of Compound **1** and **2** against NIH/3T3 and HeLa Cell lines*

Compound Name	Cytotoxic Activities IC_{50} values (μg/mL)	
	NIH/3T3	heLa
Corydine **1**	> 60	> 60
Norlirioferine **2**	> 60	2
Vincristine sulfate	> 60	0.4

Three aromatic proton resonated at δ 6.59 (H-3), 6.78 (H-8), and 8.07 (H-11) showed as a singlet. Aliphatic protons appeared as H-4, H-5 and H-7. Multiplet signals at δ 3.40 and 3.03 corresponding to the C-5. Signals at δ 3.05 as multiplet and 2.68 as doublet with coupling constant of 6.8 Hz attributed to C-5. C-7 connected with signal at δ 2.70 (*m*) and 2.78 (J=*dd*, 8.6, 13.75 Hz). The above observations were reinforced by COSY experiment which displayed correlations of H-4/H-5 and HMQC which displayed direct correlation of ^{1}H-^{13}C. The ^{13}C NMR spectrum (Table 1) showed the presence of nineteen carbons, supported with DEPT experiment twelve were aromatic carbons revealed at the downfiled region, three methoxyl carbons appeared at δ 60.3, 56.1 and 55.9 attached at C-1, C-9 and C-2, respectively, one methines at δ 53.7 as position C-6a, and three methylenes observed at δ 43.0, 36.3 and 28.8 corresponding to C-5, C-7 and C-4, respectively.

***In- vitro* cytotoxic**

In this study, the compounds were evaluated for cytotoxicities against *NIH/3T3 and HeLa Cell lines*. The cytotoxicities were assayed at various concentrations under continuous exposure for 72 hours, are expressed in CD_{50} values (μg/mL), and were summarized in Table 2. 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 of less than

5.0μg/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 [9].

Positive control, vincristine sulfate, showed a very good anticancer agents where gave a strongly effect against heLa and HL-60 cell lines, but have no cytotoxic against a normal cell NIH/3T3. In case of cytotoxicity against heLa cell line, compound **2** showed very strong activity with CD_{50} values of 2 μg/mL while compound **1** displayed as inactive. Both compounds showed no significant cytotoxicity effect against NIH/3T3. This result indicated as good for anticancer agent that indicated as save for normal cell.

In addition, cytotoxicities were also enhanced by presence of methoxyl and *N*-methyl groups. Compound **2** which have no *N*-methyl group gave better cytotoxic activity against HeLa cell line. In case of compound **1**, cytotoxic activity also affected by substituent arrangement. Previous research reported that aporphine alkaloid with has 10-CH_3 and 11-OH substitution had no activity [7]. This type of structure made **1** being inactive in cytotoxic. On the other side, the low activity of this compound also caused of the presence of *N*-methyl group.

Conclusions

Isolation, identification and characterization using spectroscopic data of compounds isolated from the leaves of *Alseodaphne peduncularis* yielded two known aporphine alkaloids, corydine **1** and norlirioferine **5**. Compound **2** displayed potent cytotoxic activity against heLa cell, but compound showed as inactive. Both compounds have no significant effect against NIH/3T3 cell lines.

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