



Research Article

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Induction of apoptosis and anti HSV-1 activity of 3-(phenethylamino) demethyl(oxy)aaptamine from a Malaysian Aaptos aaptos

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ABSTRACT

The present study was investigated the cytotoxic activity of a new aaptaminoid of *A. aaptos* against human myeloid leukemia cell line HL-60 and murine myelomonocytic leukemia cell line WEHI-3B, as well as the mode of cells death and antiviral activity against Herpes simplex virus type 1 HSV-1, infected normal mammalian cells, *Cercopithecus aethiops* African green monkey kidney cell line VERO. Cell viability was evaluated by Methyl Thiazole Tetrazolium (MTT) assay, following the induction of apoptosis assessment by light microscopy and fluorescence microscopy (Acridine orange/ Propidium iodide (AO/PI) double staining analysis) while using hydrogen peroxide (H₂O₂) as a standard. The anti-HSV-1 activity was evaluated using neutral red uptake assay. A new alkaloid 3-(phenethylamino)demethyl(oxy)aaptamine(8-methoxy-2-(phenethylamino)-9H-benzo[d,e][1,6] naphthyridin-9-one) (**1**) and aaptamine (8,9-dimethoxy-1H-benzo[d,e][1,6]-naphthyridin) (**2**) strongly reduced HL-60 viability while also showing cytotoxicity against WEHI-3B, in which the mode of both cells death was through induction of apoptosis. In anti-herpetic investigation against HSV-1, only compound **2** showed strong anti-HSV-1 activity. The compounds however did not exert significant cytotoxic effect on VERO, confirming their tumour-selective cytotoxicity. Result concluded that compounds **1** and **2** produced a good cytotoxicity activity on HL-60 and WEHI-3B. Both compounds inhibited the cells via apoptotic cell death mechanism. In addition, compound **2** also exhibited a good antiviral activity on HSV-1.

Key words: *Aaptos aaptos*, aaptaminoids, cytotoxicity, apoptosis, HSV-1, AO/PI

INTRODUCTION

Marine sponges have been considered as a gold mine for the past 50 years due to the diversity of their secondary metabolites. Sponges produce a plethora of chemical compounds with widely varying carbon skeletons, which have been found to interfere with pathogenesis at many different points. The fact that a particular disease can be fought at different points increases the chance of developing selective drugs for specific targets. Chemical constituents of sponges have potential for many biological activities. Aaptaminoids are essential chemical markers for the genera *Aaptos* (sponge) as they have been identified to be present in samples collected from Indonesia, Vietnam, Taiwan and Okinawa [1]. Some studies reviewed the previously characterized aaptaminoids alkaloid compounds such as aaptamine, demethylaaptamine, iso-aaptamine, aaptosamine, aaptosine, demethyloxy-aaptamine [2], aaptosine [3], aaptosamine [4], 4-methylaaptamine [5], bisdemethylaaptamine and bisdemethylaaptamine-9-O-sulfate [6]. The

aaptaminoids alkaloid have been reported to possess many biological activities such as antineoplastic and α -adrenoceptor blocking [2], anti-HSV-1 [3], antioxidant [7], activator of p21 promoter [8], antifouling activity in the zebra mussel assay [9], cytotoxicity against cancer cell lines including murine lymphocytic leukemia P-338, human mouth epidermoid carcinoma KB16, human lung adenocarcinoma A54 and human colon adenocarcinoma HT-29 [10], DNA-binding [11] and antidepressant [12] activities.

Aaptos aaptos collected along water of Malaysia (particularly along the coast waters of Terengganu) [13] produced the new derivatives of aaptaminoid alkaloids which are 3-(phenethylamino)demethyl (oxy)aaptamine (**1**) and 3-(isopentylamino) demethyl(oxy) aaptamine (**3**) as well as aaptamine (**2**), 5 α -cholestan-3 β -ol and cholestanyl myristate from the Malaysian *A. aaptos* [14, 15]. Consequently, this paper reports the biological activities of **1** and **2** of *A. aaptos* mainly their cytotoxicity against cancer cells lines and modes of the cells death. It is generally accepted that cell death can either due to the consequence of a passive, degenerative process (necrosis), or the consequence of an active process (apoptosis). Apoptosis also known as programmed cell death is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own demise ('cellular suicide') [16]. Necrosis is a form of traumatic cell death that results from acute cellular injury ('cellular accident'). Apoptosis differentiates from necrosis as the processes enable the disposal of cellular debris through normal biological processes do not damage the organism in apoptosis and affecting only the cancerous cells instead of healthy cells. Apoptosis has been a preference because of the beneficial effects to the organism, rather than necrosis which almost always detrimental, and can be fatal [17, 18]. Therefore, in this study, we demonstrated the *in vitro* cytotoxic activity and mechanism of cell death tested by compounds **1** and **2** of *A. aaptos* on human myeloid leukemia (HL-60) and murine myelomonocytic leukemia (WEHI-3B). In addition, the effects of those compounds on *Herpes Simplex* virus type 1 were also investigated.

EXPERIMENTAL SECTION

2.1 Isolation of compounds 1 and 2

Samples of *A. aaptos* were collected from the coastal water of Bidong Island, Terengganu via SCUBA diving. Voucher specimens were deposited at the Biodiversity Museum, Institute of Oceanography, Universiti Malaysia Terengganu. The samples were cleaned, cut into small cubes (1 cm x 1 cm), dried in air-grafted oven (45°C), *prior* extraction with methanol. The extracts were filtered and dried under reduced pressure to yield the crude extract (CE). CE (50 g) was subjected to gravity column chromatography as described previously [14] to yield compounds **1** and **2**.

2.2 Cytotoxic activity against leukemic cell lines

2.2.1 Cell culture

Human myeloid leukemia (HL-60) and murine myelomonocytic leukemia (WEHI-3B) cancer cell lines used in this assay were obtained from American Type Culture Collection, ATCC. The cells were maintained in RPMI 1640 (Sigma-aldrich) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, sodium bicarbonate (0.2%), 1% penstrep and incubated at 37°C, 5% CO₂ and 90% humidity (Nuair CO₂ water-jacked incubator). After 72 h, cells were removed *prior* centrifugation (1200 rpm, 10 min) and later cell pellet was dispersed into single cell suspension by repeated pipetting (in 2 mL medium) while the supernatant was discarded. Accordingly, cell counting was done to obtain exponentially growing cells at concentration 2 x 10⁵ cells/mL to be used in MTT assay.

In case of anchorage-dependent WEHI-3B cells, trypsinization had been applied to dislodge the cells from the surface of the culture flask for cell counting as well as seeding in microwell plate. The medium was discarded from the cell culture flask and then added with 2 mL of accutase (Sigma-aldrich; 100x) to slowly cover all flat bottom flask surface. It was incubated (37°C, 5% CO₂, 90% humidity) until most of the cells rounded up and detached (5 min) *prior* adding the medium (2 mL) for cells resuspending, following cell counting to obtain exponentially growing cells at concentration 2 x 10⁵ cells/mL.

2.2.2 In vitro cytotoxicity analysis

The microculture cytotoxicity assay using Methyl Thiazole Tetrazolium (MTT) method was adopted from Mosmann [19] with slight modification. Exponentially growing cells (2 x 10⁵ cells/mL) (50 μ L) were seeded into 96-flat bottom microwell plate in the presence of 50 μ L samples at various concentrations (two-fold dilution topping from 30 μ g/mL to 0.46 μ g/mL). After 72 h of treatment, MTT solution (50 μ L) (5 mg/mL in PBS) was added into each well and then incubated for 3 h. Subsequently, the medium was replaced with 100 μ L of DMSO. The absorbance of MTT-formazan product was read at 570 nm and reference at 630 nm using microplate reader (TECAN, Infinite M200, USA). The concentration of compounds that reduce the treated-cell population by 50% compared to untreated control (cytotoxic dose at 50%; CD₅₀) was calculated. This value was determined by plotting the graph

percentage of cell viability against the concentration of sample tested. The CD_{50} values of compounds **1** and **2** calculated from MTT assay were used to treat HL-60 and WEHI-3B in mode of cell death assessment observation of their morphological changes protocols.

2.2.3 Morphological changes observation by light microscopy (LM)

Morphological changes of compounds **1** and **2**-treated HL-60 (at CD_{50} values; 1.1 and 5.2 $\mu\text{g/mL}$ respectively) as well as **1** and **2**-treated WEHI-3B (at CD_{50} values; 3.4 and 19.4 $\mu\text{g/mL}$ respectively) and untreated cells were observed under a Nikon Eclipse, TE2000-U (Japan) microscope using light microscopy (LM) every 24, 48 and 72 h. The photomicrographs of cells which underwent various morphological changes based on typical cell death features (apoptosis and necrosis) such as cell blebbing, swelling, shrinkage, fragmentation, etc. were captured and recorded using a Nikon Digital camera DXM1200F at 400X magnification with NIS-Element BR version 2.30 software.

2.2.4 Acridine orange/ Propidium iodide (AO/PI) staining analysis

Compounds **1** and **2**-treated HL-60 (at CD_{50} values; 1.1 and 5.2 $\mu\text{g/mL}$ respectively) as well as compounds (**1** and **2**)-treated WEHI-3B (at CD_{50} values; 3.4 and 19.4 $\mu\text{g/mL}$ respectively), untreated and hydrogen peroxide (H_2O_2) were harvested after 72 h and transferred to a microcentrifuge tube and centrifuged (1500 x g, 5 minutes) to collect both viable and dead cells. H_2O_2 was used as positive control.

Two DNA-intercalating nucleoprotein dyes, acridine orange (AO) and propidium iodide (PI) (Sigma) were individually prepared (10 $\mu\text{g/mL}$) in phosphate buffer saline (PBS). Then, AO and PI (50 μL each) mixture was added to the cells at 1:1 ratio. The stained-cells (20 μL) were transferred onto a slide, covered with a cover slip and immediately examined under a Nikon Eclipse, TE2000-U (Japan) fluorescence microscope. Images were captured by an attached Nikon Digital camera DXM1200F at 400X magnification with software NIS-Element BR version 2.30. The viable, apoptosis and necrosis cells were counted in every 100 cells (triplicate) and percentage of each were assessed.

2.3 In vitro antiviral analysis

Cercophithecus aethiops African green monkey kidney VERO cell line (ATCC[®] CCL-81TM) was cultured in Minimum Essential Medium (MEM) (Bio. Wittaker Europe, Belgium) and maintained (37°C, 5% CO_2) for the virus *Herpes Simplex* type 1 (HSV-1) stock propagation (75 cm^2 culture flasks seeded with 3.5×10^5 cells/mL). The cytotoxic and anti-HSV-1 activities by cell viability assays, following neutral red uptake assay were carried out as previously described by Rashid et al. [20].

2.4 Statistical analysis

All tests analyses were run in triplicate. The independent sample ANOVA test (SPSS version 11.5 for Windows) was used at 95% confident interval (CI) to compare the significant different between doses of samples treatment, while the independent sample Student *t*-test was used (at 95% CI) to compare the significant different between control (untreated) and doses of samples treatment. Results were expressed as the mean \pm standard error mean (S.E.M).

RESULTS AND DISCUSSION

3.1 Cytotoxicity effects of Aaptaminoids on leukemic cell lines

Research interests on aaptaminoids alkaloid from *Aaptos* species have begun since the firstly isolated aaptamine was first isolated by Nakamura et al. [2], followed by the discovery of other aaptaminoids which later found to possess many biological activities. Although the active aaptaminoid alkaloids structure-activity relationships have been extensively studied, mechanism of the bioactivity has received little attention [1]. Due to limited studies on the biological activities of aapaminoid compounds, we carried out a study on the characterization and determination of cytotoxicity mechanisms of new aaptaminoids.

The cytotoxicity effects of compound 3-(phenethylamino)demethyl(oxy)aaptamine (**1**) and aaptamine (**2**) (Figure 1) against HL-60 (Figure 2A) and WEHI-3B (Figure 2B) was investigated by using MTT assay. Compounds **1** and **2** exhibited strong cytotoxic activity against HL-60 with respective CD_{50} values were 5.2 ± 1.6 and 1.1 ± 0.1 $\mu\text{g/mL}$ (Table 1). As shown in Figure 1B, there were significant differences of cytotoxic percentage of high doses treated cells compared to either control (untreated) or lower doses treated suggesting that the compounds **1** and **2**-treated HL-60- induced cytotoxicity were in dose-dependent manner. In order to further investigate the potential of the aaptaminoid alkaloids as cytotoxic agent, both compounds were tested against murine myelomonocytic leukemia (WEHI-3B) (mouse leukemia cell line) as a biomimetic approach to the *in vivo* test. As a result, compound **2** displayed strong cytotoxicity against WEHI-3B with CD_{50} value was CD_{50} ; 3.4 ± 0.4 $\mu\text{g/mL}$, while **1** only showed moderate activity (CD_{50} ; 19.4 ± 3.8 $\mu\text{g/mL}$) (Table 1). The cytotoxicity activity of samples against cell lines

indicated by their cytotoxic index value. Sample which have cytotoxic index value (IC_{50} or CD_{50}) $< 30 \mu\text{g/mL}$, were considered have significant cytotoxic activity [21, 22].

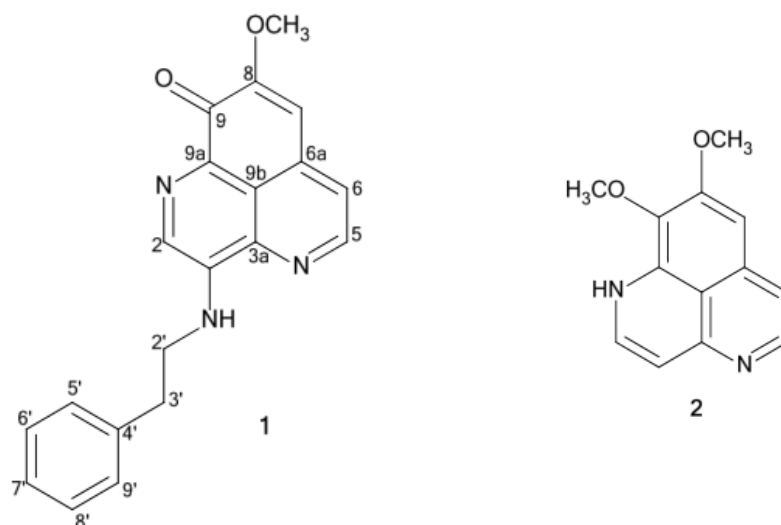


Figure 1: Aaptaminoid alkaloid compounds, 3-(phenethylamino)demethyl(oxy)aaptamine (or 8-methoxy-2-(phenethylamino)-9H-benzo[d,e][1,6]naphthyridin-9-one) (1) and aaptamine (or 8,9-dimethoxy-1H-benzo[d,e][1,6]naphthyridin) (2) [14]

Table 1: Cytotoxic activity of compounds 1 and 2 isolated from *A. aaptos* against HL-60 and WEHI-3B as well as their inactivation effect against HSV-1

Compounds	Cytotoxic Activity* ¹		Anti-HSV-1 Activity* ²		
	CD ₅₀ value ($\mu\text{g/mL}$)		EC ₅₀ ($\mu\text{g/mL}$)	% Protection	% Destruction
	HL60	WEHI-3B			
1	5.2 ± 1.6	19.4 ± 3.8	88.2 ± 0.4	55.5 ± 0.2^b	22.9 ± 0.02^b
2	1.1 ± 0.1	3.4 ± 0.4	7.0 ± 0.1	71.2 ± 0.4^a	0.9 ± 0.1^a

Note: 1; 3-(phenethylamino)demethyl(oxy)aaptamine and 2; aaptamine.

*¹; The exponentially growing cancer cells (2×10^5 cells/mL) were seeded after treatment for 72h. CD₅₀: cytotoxic dose at 50%, *²; Multiplicity of infection (MOI) used was 0.001 ID₅₀/cells ($2 \times 10^{8.5}$ ID₅₀/mL).

Vero cells were seeded for 1h before infected with HSV-1 and incubated with sample for 72h.

EC₅₀: Effective concentration of 50% cells protection. % Protection: Percentage of cells protection at optimal concentration, % Destruction: Percentage of cells destruction at concentration of maximum cells protection, ^aOptimal concentration=25 $\mu\text{g/mL}$, ^bOptimal concentration=100 $\mu\text{g/mL}$.

Importantly, in anticancer research, the assessment of mode of cell death is generated from the preference of compounds induced apoptosis rather than necrosis. Cell apoptosis is important to eliminate undesired cells during the development and homeostasis of multicellular organisms [23]. In contrast, the impact of necrosis was undesired due to the ultimate breakdown of the plasma membrane. This cell lyses releases the cytoplasmic contents including lysosomal enzymes and proteases are released into the extracellular fluid or culture supernatant, causing product degradation, and/or adsorption of the product. Further supplemented necrotic induced compound in *in vivo* study would exhibited the necrotic cell death associated with extensive tissue damage resulting in an intense inflammatory response to its surrounding tissue [16-18]. Hence, mode of cell death of the treated 1 and 2-HL-60 as well as 1 and 2-WEHI-3B were assessed through observation by LM and FM (AO/PI double staining analysis). To date, investigation of aaptaminoid compounds towards WEHI-3B and the modes of cell death were yet been discovered. Therefore, the present study would contribute the important data for marine natural product research community.

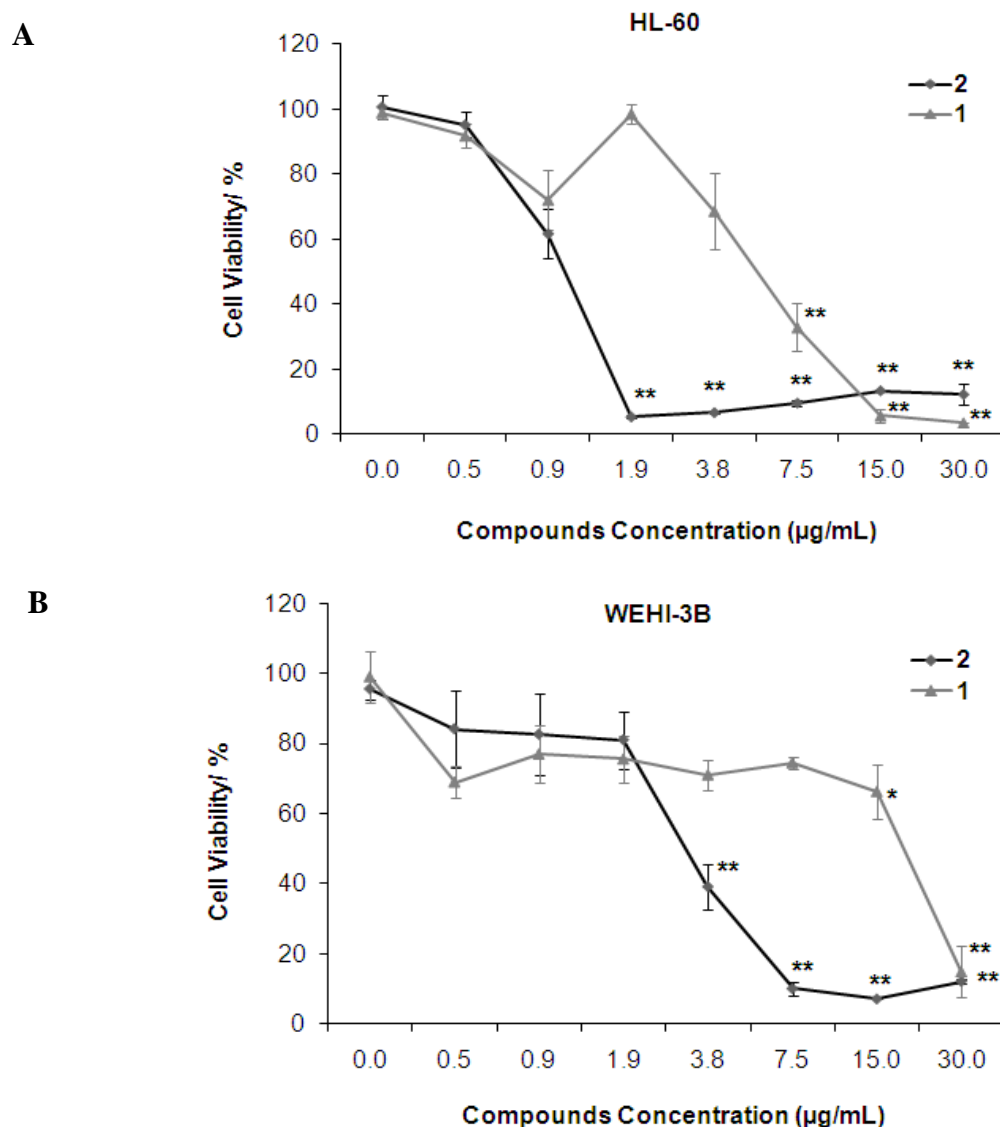


Figure 2: Cytotoxic activity of aaptaminoid alkaloids, of 3-(phenethylamino)demethyl (oxy)aaptamine (**1**) and aaptamine (**2**) against (A) HL-60 (B) WEHI-3B. Cells viability was determined by MTT assay

The CD_{50} values of **1** and **2** against HL-60 obtained were 5.2 and 1.1 µg/mL respectively as well as against WEHI-3B were CD_{50} were 19.4 and 3.4 µg/mL respectively. Results were expressed as the mean % \pm S.E.M of triplicate experiments. Asterisks indicate significant difference levels where $**p < 0.01$ between the experimental and control values using *t*-test.

3.1.1. Leukemic cell lines induction of apoptosis, observation by light microscopy (LM)

The untreated HL-60 (Figure 3A) and WEHI-3B (Figure 4A) cells appeared round and intact. It was found that in cultures, viable HL-60 cells were suspension, ovoid or round, while viable WEHI-3B cells appeared small single cells grew adherently and partly in suspension. After 24 h treatment with **1** and **2** at CD_{50} values, 5.2 and 1.1 µg/mL respectively, clear morphological changes were observed on HL-60 cells, of which, shrinking and blebbing, apoptotic cells and bodies were present. However, healthy cells were still evident (Figure 3B and 3E). After 48 h, HL-60 cells appeared with smaller size morphology indicating that the cells were shrunk, while the increased of apoptotic cells, bodies as well as cell debris showed the dramatic morphological changes (Figure 3C and 3F). After 72 h, numerous shrunken cells, apoptotic bodies and its debris were observed (Figure 3D and 3G).

However, the culture of WEHI-3B cells treated with **1** and **2** at CD_{50} values, 19.4 and 3.4 µg/mL respectively, showed obvious morphological changes only after 48 h (Figure 4C and 4F). After 24 h, large amount of floating cells appeared in the culture suggesting that the compounds caused the anchorage-dependent cells to dislodge from the surface of culture plate, resulting in cells floating in the media (Figure 4B and 4E). At 48 h treatment, cell blebbing was widely distributed in the culture. Round-shape normal cells were still present in both compounds cell-treated cultures. After 72 h treatment, surface membrane budding, cell shrinkage as well as cell fragmentation in membrane bound-vesicles (apoptotic bodies) were widely appeared in culture (Figure 4D and 4G).

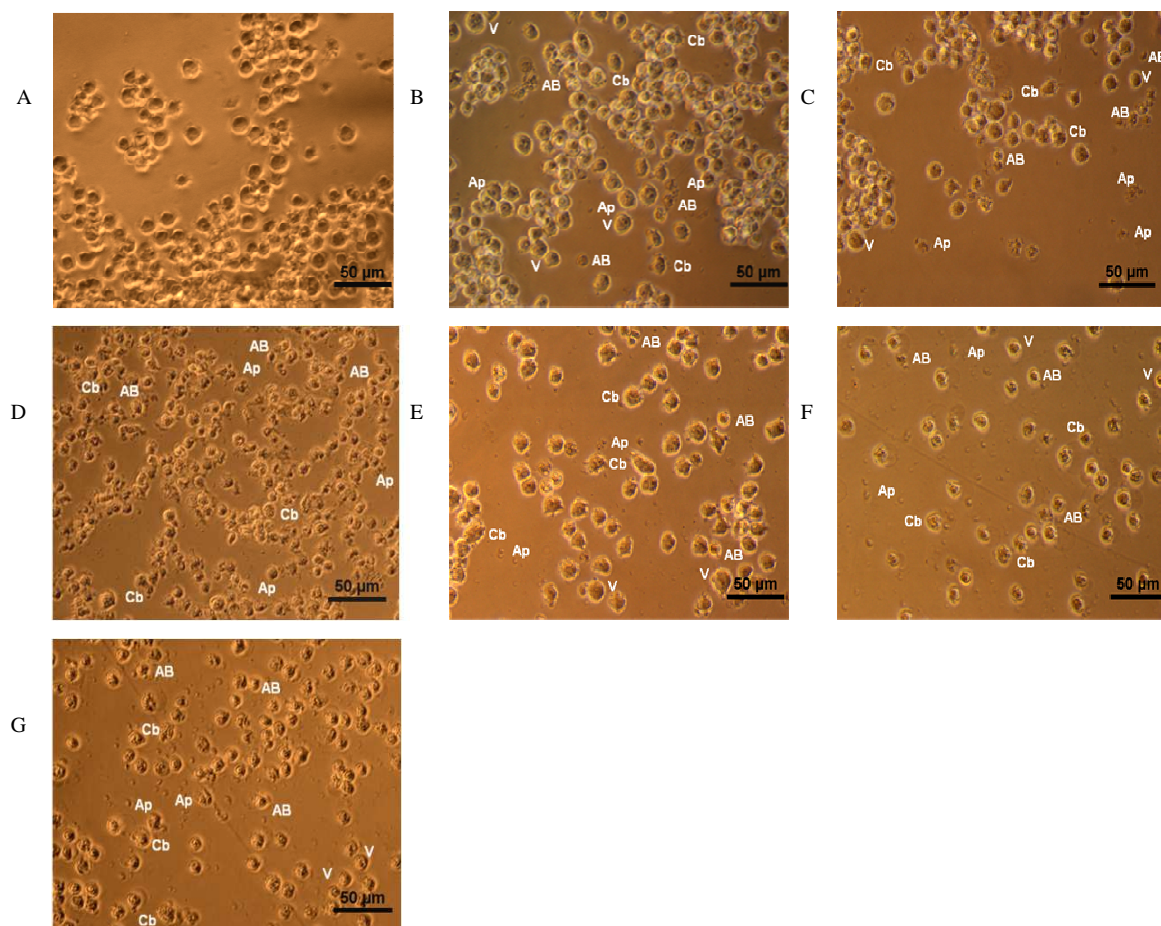


Figure 3: Morphology of HL-60 cells following (B) 24, (C) 48 and (D) 72 h individual treatment with compounds **1** at CD_{50} value 5.2 $\mu\text{g/mL}$ and (E) 24, (F) 48 and (G) 72 h with **2** at CD_{50} value 1.1 $\mu\text{g/mL}$

Note; **1**: 3-(phenethylamino)demethyl(oxy)aaptamine and **2**: aaptamine. Cells were photographed at 400X magnification. Ap; Apoptotic cells, AB; Apoptotic bodies, Cb; Cell blebbing; V; Viable cell.

LM was employed to morphologically determine the mechanisms of cytotoxic effect of compounds **1** and **2** on HL-60 and WEHI-3B. Meanwhile, AO/PI staining showed clear comparison between viable, apoptotic and necrotic morphology induced by compounds **1** and **2**. According to Bank [24], AO/PI staining analysis enable to distinguish viable, early membrane-intact apoptotic and necrotic cell populations based on color emitted [25]. The AO/PI staining analysis was based on the selective permeability characteristic of cell components. Acridine orange (AO) is a cationic dye which develops a protonated positive charge after its passage across an intact cell membrane. Viable and healthy cell nuclear would appear homogeneously green showing intact and round-shaped nuclei under 488 nm emission filter. This is due to its ability to enter the cell and intercalate the DNA, permits electrostatic intercalation with DNA to produce a green fluorescence. It also accumulates in double stranded RNA and acidic organelles such as lysosomes giving it orange-red fluorescence instead of green [26]. Meanwhile, propidium iodide (PI), gives red fluorescence due to penetration of PI when intercalates into the leaked cytoplasmic membrane, nucleic acids or DNA belongs to cells that already lost their plasma membrane integrity (lysed membranes) of necrotic cells [18].

As reported previously, viable HL-60 cells would occasionally express pseudopods and are heterogeneous in size (9 to 25 μm in diameter), while viable WEHI-3B cells showed characteristics of myelomonocytic leukemia, specifically, a monomyelocytic leukemia cell line from the BALB/c mouse origin [27]. Meanwhile, AO/PI stained micrographic image of untreated HL-60 exhibited normal cell morphology as previously reviewed by Fleck *et al.* [28]. Morphology of HL-60 and WEHI-3B (LM and FM) individually treated with compounds **1** and **2** showed the characteristic features which attributed to apoptosis such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, surface membrane budding, blebbing, and chromatin (cytoplasmic) condensation as mentioned by Martin *et al.* [29], revealed that mode of cells death (HL-60 and WEHI-3B) induced by these aaptaminoids was through apoptosis.

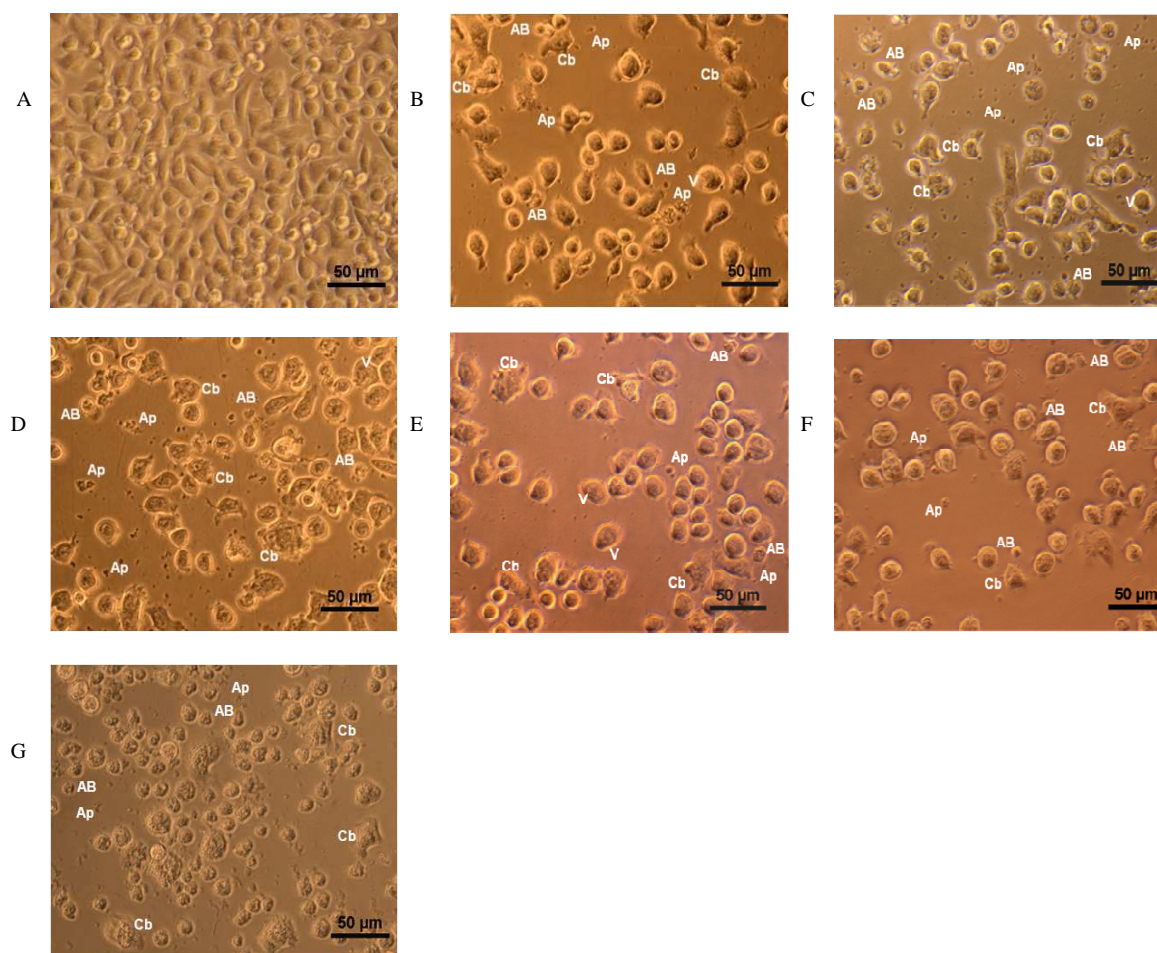


Figure 4: Morphology of WEHI-3B cells following (B) 24, (C) 48 and (D) 72 h individual treatment with compounds 1 at CD_{50} value 19.4 $\mu\text{g/mL}$ and (E) 24, (F) 48 and (G) 72 h with 2 at CD_{50} value 3.4 $\mu\text{g/mL}$

Note; 1: 3-(phenethylamino)demethyl(oxy)aaptamine and 2: aaptamine. Cells were photographed at 400X magnification. Ap; Apoptotic cells, AB; Apoptotic bodies, Cb; Cell blebbing; V; Viable cell.

Those morphological changes during the apoptotic cell death as seen in Figure 4B, 4C, 5B and 5C, would occurred *via* a sequence of events which were formation of blebs on the intact external cell surface, followed by cell membrane broke up and enclosed various intact organelles [30]. The apoptosis characteristics such as cellular shrinkage attributed from internal water loss which showed by closer appearance of intracellular organelles in the reduced cell volume. The endoplasmic reticulum developed connections to the plasma membrane then channeled water out of the cell to give it a wrinkled appearance [30-31]. During this stage, the apoptotic bodies and the remaining cell fragments were undergone secondary necrotic which proved by the presence of intense small orange red cells fragment. These apoptotic bodies were a transition from an apoptotic to necrotic cells in order to swell and finally lysed from the culture medium. Among various ROS, H_2O_2 was chosen as standard apoptotic induced compound because its freely dispersion in and out of cells and tissues, aggravating cell proliferation and triggering cell death characteristics. H_2O_2 is one of the reactive oxygen species (ROS) which generated oxidative stress in the cell when the ROS overwhelms the defense systems and then alters the redox homeostasis of the cell. Oxidative stress could trigger the cell apoptosis provided that the ROS become putative mediator of apoptosis [32-34].

3.1.2 Acridine orange/ Propidium iodide staining analysis

The observation by fluorescence microscopy (FM) using AO/ PI staining was applied on the 1 and 2 HL-60 and WEHI-3B treated cell in order to assure the mode of cell death induced by compounds 1 and 2 (Figure 5 and 6). In the absence of compounds, the HL-60 (Figure 5A) and WEHI-3B (Figure 6A) displayed normal morphology. Both cells showed predominantly typical promyelocytes with large round nuclei, containing 2-4 nucleoli and dispersed nuclear chromatin. After 72 h, compounds 1 and 2-treated HL-60 as well as 1 and 2-treated WEHI-3B at their respective CD_{50} values showed the occurrence of apoptotic and necrotic cells.

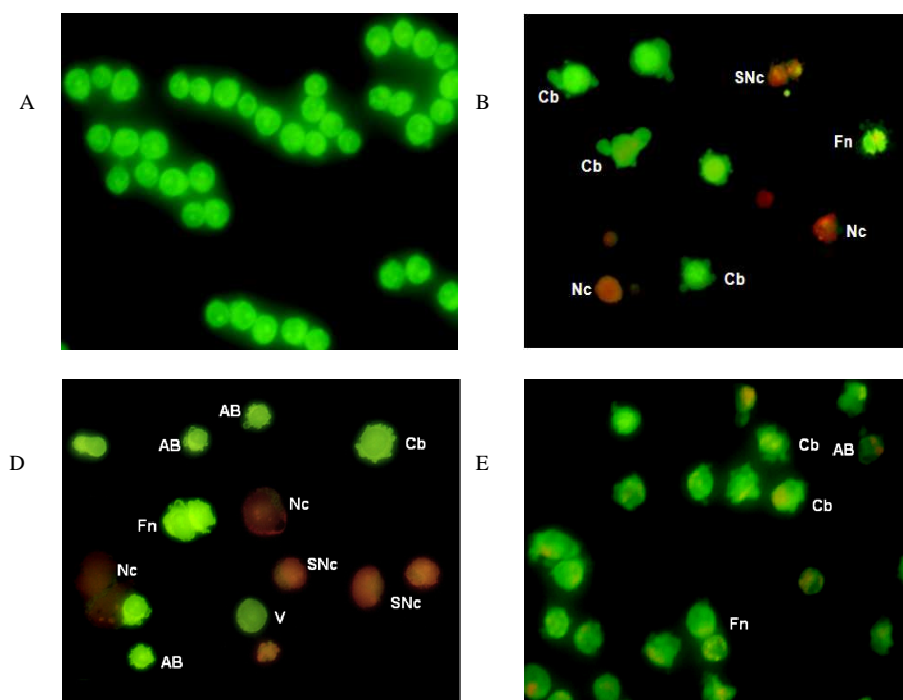


Figure 5: AO/PI stained micrographic images of HL-60 cells after 72 h treatment with; (A) Untreated, (B) 1, (C) 2 and (D) hydrogen peroxide (H_2O_2) at CD_{50} values (5.2 $\mu g/mL$, 1.1 $\mu g/mL$ and 25.0 μM respectively)

Note; 1: 3-(phenethylamino)demethyl(oxy)aaptamine and 2: aaptamine. Cells were photographed at 400X magnification. AB; Apoptotic bodies, Cb; Cell blebbing; Fn; Fragmented nuclei; Nc; Necrotic cell, SNc; Secondary necrotic cell, V; Viable cell.

Figure 5B, 5C, 6B and 6C showed the presence of tested cells that displayed green fluorescence which underwent membrane blebbing, chromatin aggregation, nuclear and cytoplasmic condensation and green fluorescence fragmented nuclei. Condensed chromatin material was observed as intense green patches. The partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) were also observed. Some small intense orange and red cells were also present in both cell populations. Which indicate moderate and low percentages of necrotic cells when tested with both compounds. H_2O_2 was used as positive control.

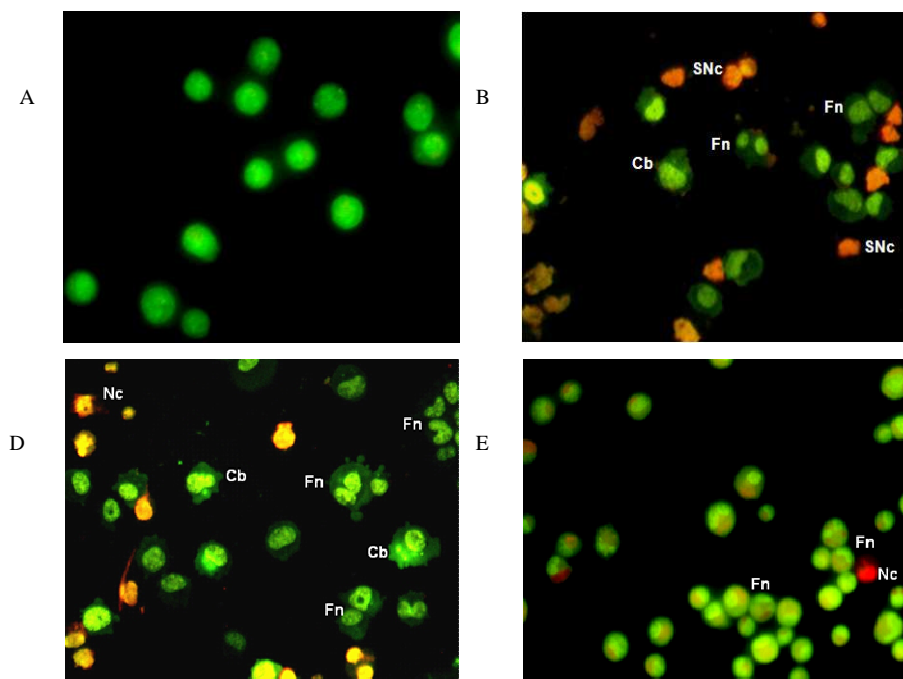


Figure 6: AO/PI stained micrographic images of WEHI-3B cells after 72 h treatment with; (A) Untreated, (B) 1, (C) 2 and (D) hydrogen peroxide (H_2O_2) at CD_{50} values (3.4 $\mu g/mL$, 19.4 $\mu g/mL$ and 85.0 μM respectively)

Note; 1: 3-(phenethylamino)demethyl(oxy)aaptamine and 2: aaptamine. Cells were photographed at 400X magnification. AB; Apoptotic bodies, Cb; Cell blebbing; Fn; Fragmented nuclei; Nc; Necrotic cell, SNc; Secondary necrotic cell, V; Viable cell.

The present of visible, apoptotic and necrotic were observed based on AO/PI staining. As showed in Figure 7, high percentages of apoptotic cells were obtained from **1** and **2**-treated-HL60 (**1**; 71% and **2**; 55%) as well as **1** and **2**-treated-WEHI-3B (**1**; 57% and **2**; 61%) cells. However, percentages of necrotic HL-60 cells after treatment with **1** and **2** were much lower with 12 and 31% respectively, and as for WEHI-3B cells were 37 and 34% respectively. Meanwhile, more than 80% of cells were apoptotic after treatment with hydrogen peroxide (H₂O₂). Majority of H₂O₂ treated cells showed apoptosis with apoptotic percentage of H₂O₂ treated-HL-60 and WEHI-3B were 84 and 95% respectively.

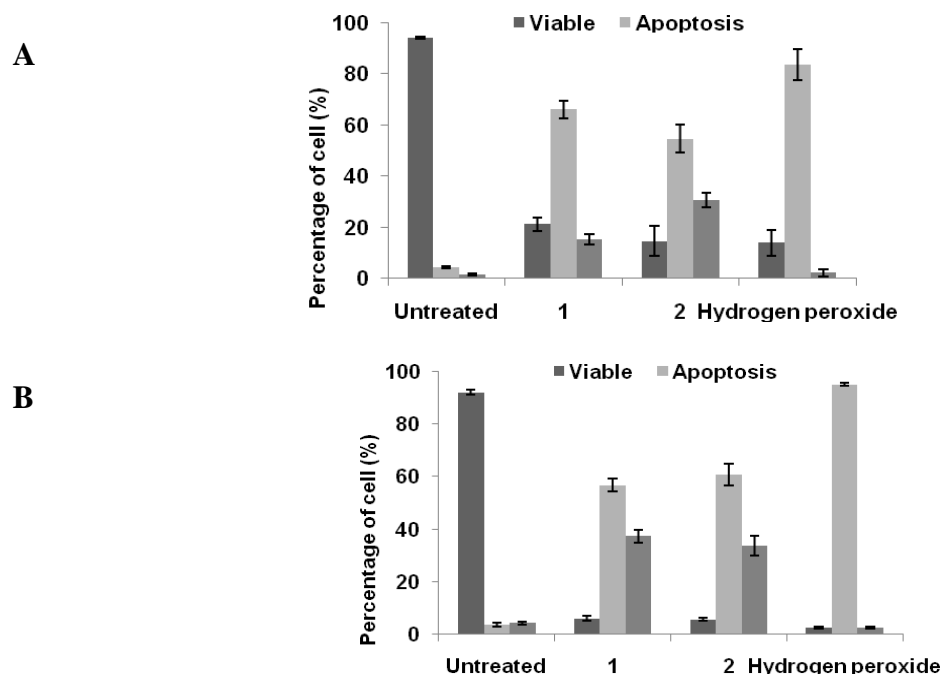


Figure 7: Mode of cell death following 72 h individual treatment of (A) HL-60 with compounds **1**, **2** and hydrogen peroxide at CD₅₀ values (5.2 µg/mL, 1.1 µg/mL and 25.0 µM respectively), and (B) WEHI-3B with compounds **1**, **2** and hydrogen peroxide at CD₅₀ values (3.4 µg/mL, 19.4 µg/mL and 85.0 µM respectively) using AO/PI double staining method. Result were expressed as the means \pm S.E.M of three independent experiments (n=3, ANOVA).

3.2 Anti-HSV-1 activity

In this study, compound **2** showed potential anti-HSV-1 activity in which $71.2 \pm 0.4\%$ protection was achieved at 25 µg/mL in which the EC₅₀ value was 7.0 ± 0.1 µg/mL, added with its low cytotoxic effect (0.9% destruction). However, compound **1** showed lower inhibition of HSV-1 with only 55.5% protection at 75 µg/mL and EC₅₀ value 88.2 µg/mL while showed cytotoxicity against normal Vero cells, revealing that it was not a safe anti-HSV-1 drug (Table 1). However, Figure 8 showed that at concentration less than 25 µg/mL, the cytotoxic effect of both compounds against normal Vero cells were still very low and did not achieve cytotoxic concentration of 50% cells destruction (CC₅₀).

Previously, aaptaminoid derivatives such as aaptamine, isoaptamine and demethyl(oxy)aaptamine have demonstrated to be significantly active against cancerous cell lines KB16, A549, HT-29, and P388 [10] as well as HSV-1 [5]. Demethyl(oxy)aaptamine and 4-methylaaptamine (concentration of both were 2 µg/mL) were found to inactivate 78 and 80% of HSV-1 replication in Vero cells respectively and not cytotoxic at concentration below 20 mM. They proved to be more active than acyclovir (EC₅₀; 8.6 mM), and more efficient than other previously tested alkaloids. Larghi *et al.* [1] concluded that the aaptamines target was the immediate-early protein ICP 27, which would regulates splicing, termination, and nuclear export events of viral transcripts, resulting in impairment of all other steps of HSV-1 replication. Besides that, the mechanism also affects additional targets such as viral penetration which differed aaptaminoids mechanism of action from acyclovir (standard). However, among aaptaminoid compounds, demethyl(oxy)aaptamine latter cytotoxicity against normal Vero cell lines was high in comparison to the control, while also gave cytotoxic effect towards human peripheral blood mononuclear, PBM and T-lymphoblastoid, CEM cells [10, 35].

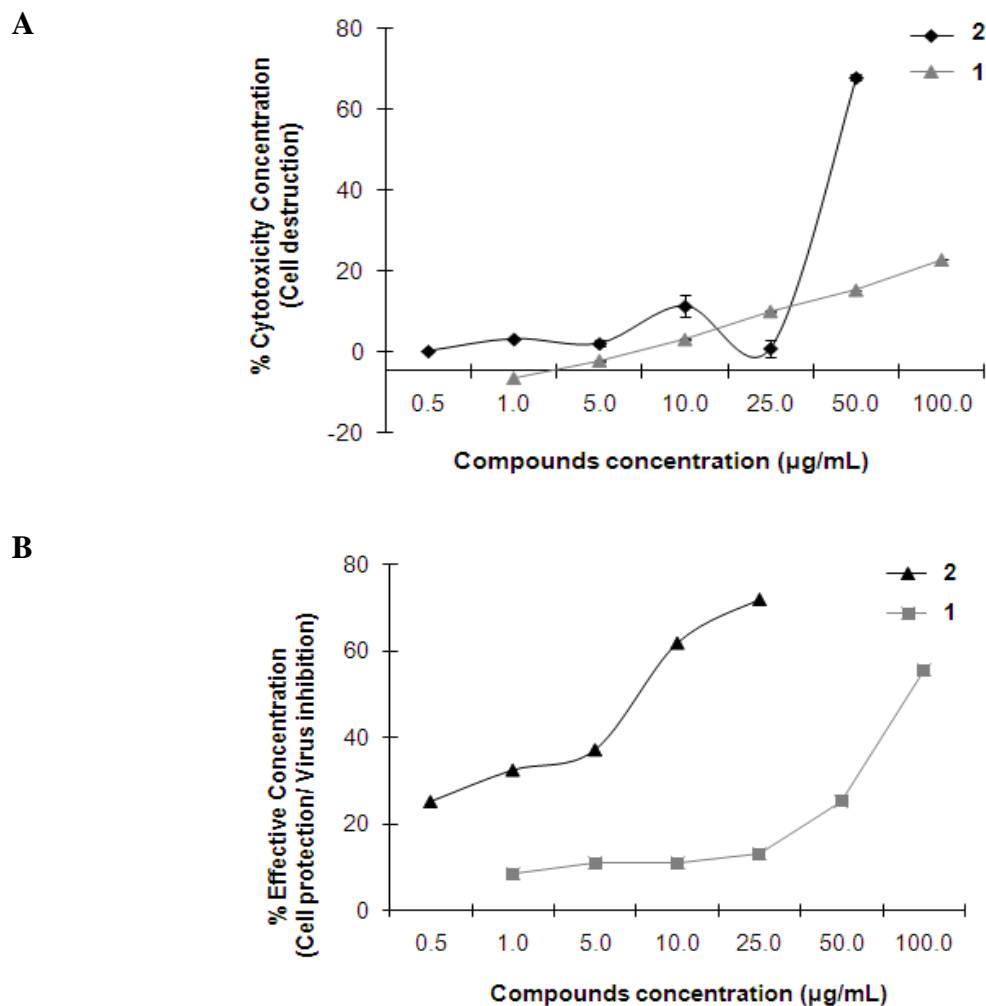


Figure 8: (A) Cytotoxicity against normal Vero cells and (B) anti-HSV-1 activity of 3-(phenethylamino)demethyl(oxy)aaptamine (1) and aaptamine (2) assayed by neutral red uptake method. Multiplicity of infection (MOI) used was 0.001 ID₅₀/cells ($2 \times 10^{8.5}$ ID₅₀/mL). Results were expressed as the mean % \pm S.D of triplicate experiments. Vero cells were seeded for 1 h before infected with HSV-1 and incubated with sample for 72h.

Larghi *et al.* [1] reviewed many studies that showed the introduction of an acyl side chain at C-9 or N-4 would decreased the activity indicating that their cytotoxicity against cancer cell lines was somehow related to the number of carbon atoms at these side chains. There were reported importance in the presence of a free hydroxyl group (-OH) at C-9 such as isoaptamine such as compound demethyl(oxy)aaptamine would resulted in the increase of activity more than aaptamine skeleton. Meanwhile, the additional methylene groups side chain at N-4 and free carbonyl function at C-9 revealed the slightly increasing anti-HSV-1 activity. Hence, the studies showed that other additional analogs of aaptamine and isoaptamine other functional group at C-9 or N-4, bearing shorter or longer the side chains would reveal less potent activity. Our results are consistent with these previous findings, adding that we found out the introduction of a phenethylamino side chain at C-3 of demethyl(oxy)aaptamine (forming compound **1**) have resulted in decreasing of cytotoxic activity against leukemic cell lines and anti-HSV-1 activity compared to aaptamine (**2**). Therefore, the results revealed that anti-leukemic and anti-HSV-1 activities of aaptaminoids were somehow also related to the functional group at C-3 chains besides C-9 and N-4. Besides that, we have revealed that HL-60 and WEHI-3B modes of cell death were through induction of apoptosis.

CONCLUSION

The cytotoxic activity of a new alkaloid, 3-(phenethylamino)demethyl(oxy)aaptamine(8-methoxy-2-(phenethylamino)-9H-benzo[*d,e*] [1,6]naphthyridin-9-one) (**1**) and aaptamine (8,9-dimethoxy-1H-benzo[*d,e*][1,6]-naphthyridin) (**2**) against HL-60 and WEHI-3B by induction of apoptosis was revealed through observation by LM and FM, AO/PI double staining analysis, which would promote further evaluation experiment such as flow cytometric analysis, caspase analysis etc. The cytotoxicity against WEHI-3B findings also enabled to provide

positive hypotheses of cytotoxic activity of compounds **1** and **2** in mice leukimic *in vivo* study. In addition, compound **2** also exhibited a good antiviral activity on HSV-1. Finally, the aptaminoids are believed to be the cytotoxic principles of the genus *Aaptos* sponge extract.

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