CYTOTOXICITY EFFECT OF AAPTAMINE AND ITS DERIVATIVES ON *ACANTHAMOEBA CASTELLANII* (IMR ISOLATE)

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ABSTRACT: *Acanthamoeba is a free-living amoebae that is ubiquitously present in various natural environments. In this study, several of Aaptamine derivatives (**2-5**) were synthesized and evaluated for their cytotoxicity effect against Acanthamoeba castellanii (IMR isolate). The Acanthamoeba viability was determined using a range of concentration from 0 to 50 µg/mL for each compound. The treatment was done for 72 hours and Eosin staining was used to determine the cell viability. From the result obtained, Aaptamine (**1**) and its derivatives (**2-5**) have significant effect toward inhibition growth on Acanthamoeba with of 1,4-dibenzylaaptamine (**5**) was observed as the most potent compound as an anti-amoeba agent.*

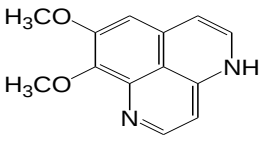
Keywords: Aaptamine derivatives, Acanthamoeba castellanii

1. INTRODUCTION

Acanthamoeba is one of the most widely distributed protozoa consisting of free-living and pathogenic microbes [1-2]. They can be found in soil, dust, water, seawater, domestic water tap, swimming pool, sewage and also bathroom tap water [3]. Pathogenic protozoa like *Acanthamoeba* can enter our body system through the respiratory tract, skin abrasions, contact lenses and surgical instruments [4]. In humans, *Acanthamoeba* is responsible for two common diseases such as fatal granulomatous amoebic encephalitis (GAE) which occur in human's central nervous system and eye keratitis [3, 5-6].

So far, no chemotherapeutic agent has been described as a single effective treatment for Acanthamoeba disease. Current treatment consists of a combination of topical antimicrobial agents, which can achieve high concentrations at the site of infection [7].

Aaptamine (**1**), also known as 8,9-dimethoxy-1H-benzo[de][1,6]naphtyridine, is a natural compound, initially isolated from the marine sponge *Aaptos aaptos* [8]. Aaptamine derivatives possess antimicrobial, antifungal, and antiretroviral as well as cancer-preventive activity [9,10].



Aaptamine **(1)**

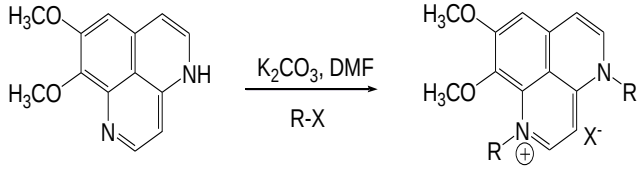
Figure 1: Structure of Aaptamine (1)

Previously we have reported on the synthesis and antibacterial activity of 1,4-dialkyl aaptamine derivatives [11,12]. In an effort to explore the possibilities offered by these derivatives as a potential anti amoebic agent, the aaptamine (**1**) and its derivatives (**2-5**) were examined for their anti-amoebic properties against *Acanthamoeba castellanii* (IMR isolate).

EXPERIMENTAL DETAILS

Preparation of Aaptamine Derivatives (compounds 2-5)

Aaptamine derivatives (compounds **2-5)** were synthesised by slightly modification of the published method [13] and characterized as previously described [11,12] (Scheme 1).



Aaptamine **(1)** **2-5**

2: R = CH_3, (80%)
3: R = CH_2CH_3, (68%)
4: R = $CH_2CH_2CH_3$, (61%)
5: R = Bn (73%)

Scheme 1: Synthetic route of the preparation of compounds 2-5

Amoeba Cultivation

In this study, *Acanthamoeba castellanii* (IMR isolate) was used. This isolate was cultivated in polypeptone-yeast-glucose (PYG). The amoeba culture was sub-cultured every three days and incubated at 30 °C. The process of sub-culturing is done by placing one drop of amoeba suspension from the original culture into a new tube containing the PYG medium. This process was conducted in an aseptic manner under the laminar flow.

Preparation of PYG Medium

PYG medium for amoeba cultivation was prepared by mixing the 3.75 g of protease, 3.75 g of yeast, 7.5 g of D+ glucose and 1000 ml of Page Amoeba Solution (PAS) solution. The mixture was then autoclaved at 121 °C for 15 minutes. The media was kept in the refrigerator for further use.

Preparation of Stock Solution

The stock solution of aaptamine (**1**) and its derivatives (**2-5**) were prepared by dissolving 0.001g of each compound in 20µL of dimethyl sulfoxide (DMSO). Then, 980µL of PYG media was added to prepare a 1000µL compounds. All the stock solution were prepared in separate vile and stored in the refrigerator at 4 °C for further use. Each compound (**1-5**) were prepared in a different concentration ranging from 1 mg/mL to 0.125 mg/mL by using two-fold dilution series for cytotoxicity assay.

Cytotoxicity Assay

IC_{50} Determination

The term IC_{50} can be defined as the molar concentration of an inhibitory agonist that reduces a response by 50% of the maximal inhibition that can be attained [14]. The *Acanthamoeba* were treated by aaptamine (**1**) and its derivatives (**2-5)** at different concentrations in 24-well plates. Hemocytometer was used to calculate the number of viable cells for treatment. PYG media with different concentration of compounds **1-5** were added into each well with four replicates. Two types of control that consist of positive and negative controls was used. Positive control consists of treatment of amoeba suspension with 4% Chlorhexidine while negative control involved the treatment of amoeba with media only.

Calculation for viable cell (hemocytometer):

Cell counting formula:

$$C = AV \times 2^{*} \times 10^{4}$$

Where C= Concentration of viable cells (cells/mL)

AV= Average number of viable cells counted in four corners

2*= Dilution factor

Determination of Amoeba Viability by Using Eosin Staining Method

PYG media in 24–well plates was sucked out before the plate is washed with 0.45% sodium chloride, NaCl. The plate was left to dry at room temperature before it fixed with methanol hydroxide, MeOH and was left to dry for 90 minutes. Then, 200 µL of 0.5% Eosin solution was added to each well to fix for 15 minutes. Next, the plate was washed with distilled water twice. 200 µL of 0.1 M sodium hydroxide, NaOH was added into each well to separate the cells. Lastly, the solution was taken to 96-well plate before read the absorbance value by using ELISA reader at 490 nm and the reference value of 630 nm.

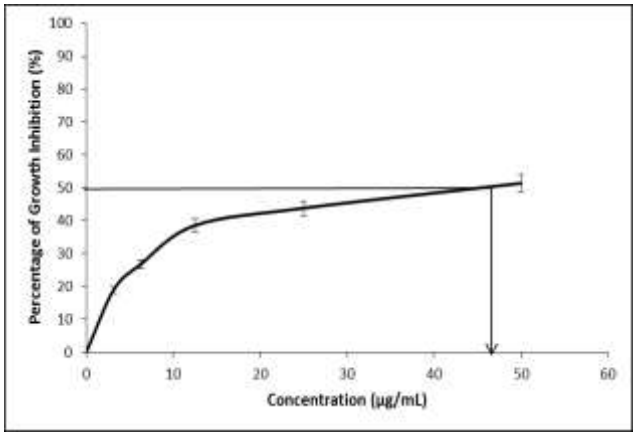
2. RESULTS AND DISCUSSION

Synthesis of Aaptamine Derivatives (2-5)

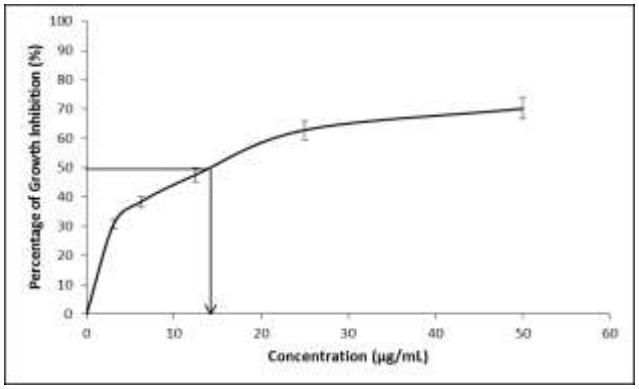
Compounds **2-4** were previously prepared by our research group, with yields ranging between 61% and 80% [11,12]. Compound **5** was synthesized according to the literature [13, 15] with slight modification to yield 73% of the desired molecule.

Cytotoxicity Test of Compounds 1-5 on *Acanthamoeba*

The cytotoxicity test of aaptamine and its derivatives (compound **1-5**) as well as the positive control, Chlorhexidine on *Acanthamoeba* was done by treating the amoebae at different concentration of the compounds in 24-well plate for 72 hours at 30 °C. Eosin dye assay was carried out to determine the number of viable cells and the IC_{50} value was obtained by plotting a graph concentration of compound against the percentage of viable cells using Microsoft Excel. Graph of percentage of cell inhibition against various concentrations of aaptamine (**1**) and its derivatives (**2-5**) are shown in Figure 2 (a-f).



(a)



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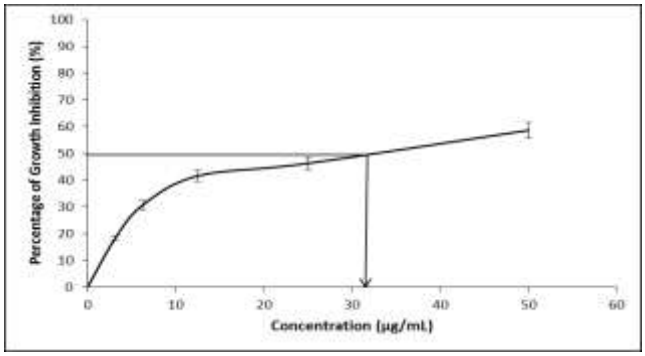

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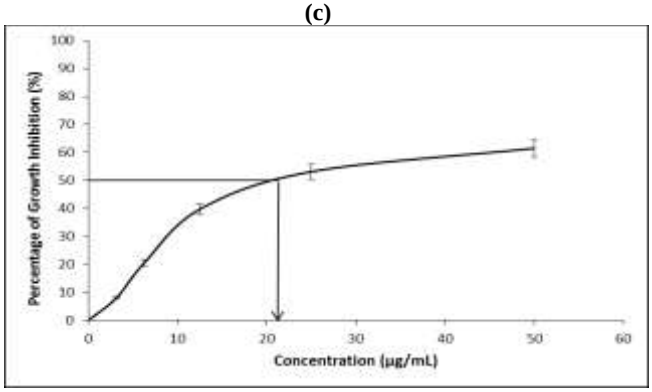

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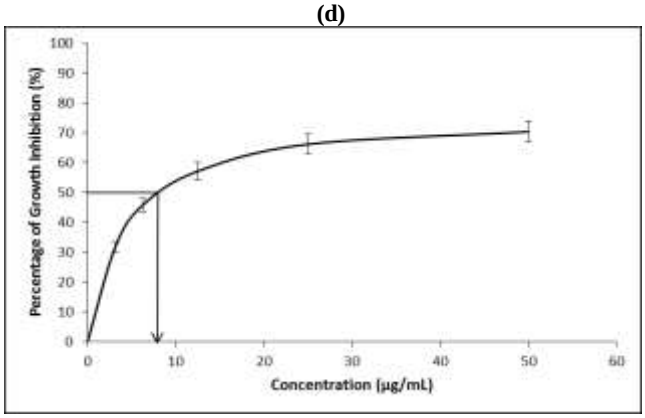

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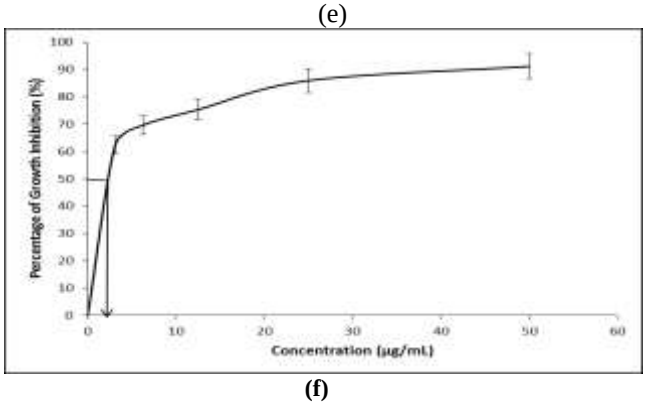

(f)

Figure 2: Percentage of inhibition of *Acanthamoeba castellanii* against concentration of (a) Aaptamine (1), (b) 1,4-dimethylaaptamine (2), (c) 1,4-diethylaaptamine (3), (d) 1,4-dipropilaaptamine (4), (e) 1,4-dibenzylaaptamine (5) and (f) Chlorhexidine (positive control)

the concentration of treatment versus percentage of viable cells (Figure 2). The number of viable cells of *Acanthamoeba* decreased as the concentration of the compound increased. The IC_{50} value obtained were 45 µg/mL for aaptamine (**1**), 15 µg/mL for 1,4-dimethylaaptamine (**2**), 32 µg/mL for 1,4-diethylaaptamine (**3**), 20 µg/mL for 1,4-dipropilaaptamine (**4**), 8 µg/mL for 1,4-dibenzylaaptamine (**5**) and 3 µg/mL for Chlorhexidine. The result of this study shows that 1,4-dibenzylaaptamine (**5**) has the greatest impact on *Acanthamoeba* growth compared to other compounds.

Morphological Changes in *Acanthamoeba* after IC_{50} Treatment of Aaptamine and Its Derivatives

The IC_{50} values obtained from the cytotoxicity test were used in the treatment to study the morphological changes of *Acanthamoeba*. There were two types of conditions given to the amoebae in this study. First, the amoebae were placed in a non-stress condition where no compounds were exposed to the amoebae which also known as a negative control in this study. Second, the amoebae were put in stress condition where the compounds, the aaptamine (**1**) and its derivatives (**2-5**) were exposed in the amoebae culture. The negative control of *Acanthamoeba* was act as a reference when the analysis was carried out to observe changes in the amoebae morphology when treated with the compounds.

In Figure 3 (a-g), the morphology of *Acanthamoeba* in control compared to the treated with aaptamine (**1**) and its derivatives (**2-5**) were greatly different in term of size, shape and the existence of the acanthapodia. This proved that all compounds caused the same effects to the amoebae. By using scanning electron microscopy, amoebae in non-stress condition were observed to display healthy and normal cell shape. In the other hand, amoebae in stress condition became reduced in size and highly damaged in term of the cell membrane and structures like acanthapodia.

The response of *Acanthamoeba* to Aaptamine (1) and Its Derivatives (2-5)

Based on the IC_{50} value used and the results of scanning electron microscopy, it can be confirmed that each compound has the ability as an anti-amoebic agent. These results also support the previous study [16]. In this study, 1,4-dibenzylaaptamine (**5**) has the lowest value of IC_{50} compared to other which was only 8 µg/mL to inhibit about 50% of amoebae growth. This indicates that this compound has the greatest anti-amoebic effect towards *Acanthamoeba* because in lower and small concentration, this compound was able to damage the amoebae structure as can be seen in Figure 3 (f).

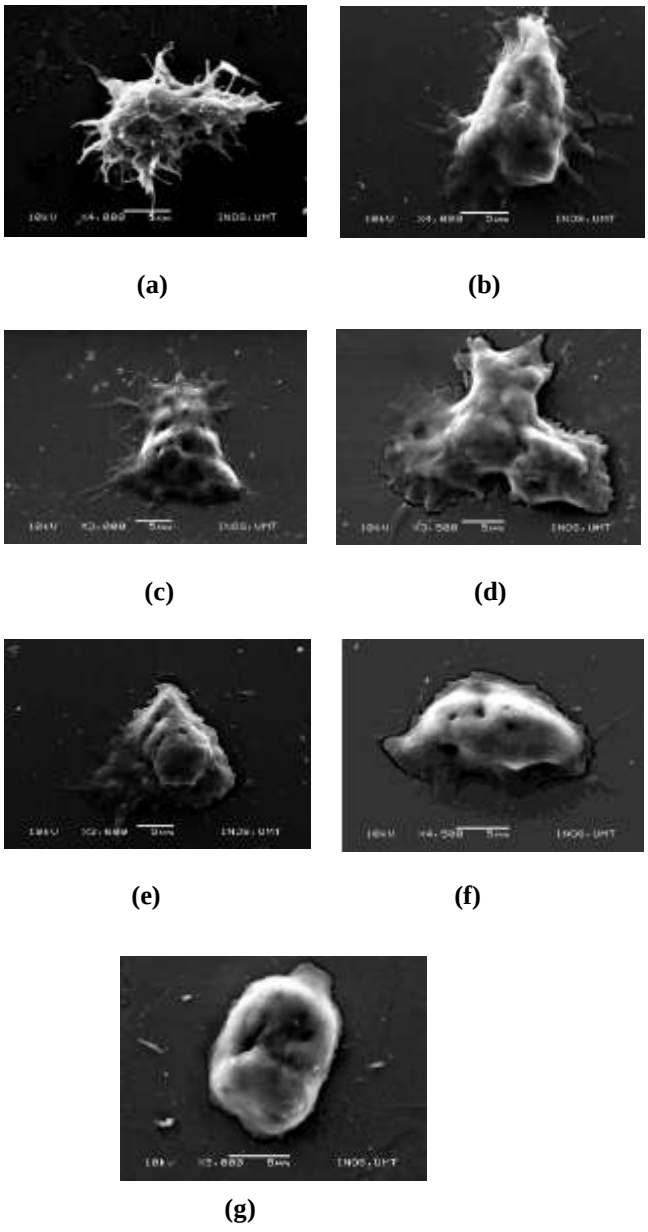

Figure 3: Trophozoite of *Acanthamoeba* under scanning electron microscopy (a) in control at 0 mg/mL concentration, (b) after treatment with 45 µg/mL aaptamine (1), (c) after treatment with 15 µg/mL 1,4-dimetylaaptamine (2), (d) after treatment with 32 µg/mL 1,4-diethylaaptamine (3), (e) after treatment with 20 µg/mL 1,4-dipropilaaptamine (4), (f) after treatment with 8 µg/mL 1,4-dibenzylaaptamine (5) and (g) after treatment with 3 µg/mL Chlorhexidine (positive control).

3. CONCLUSIONS

The result obtained from the cytotoxicity assay of aaptamine (**1**) and its derivatives (**2-5**) on *Acanthamoeba castellanii* (IMR isolate) shows that all compounds have growth inhibition ability at different concentration. Based on the IC_{50} value obtained, 1,4-dibenzylaaptamine (**5**) shows the strongest inhibitory effect compared to other compounds.

The effect of Acanthamoeba of the treatment of all compounds has been shown clearly on morphology images by scanning electron microscopy where the treated Acanthamoeba shows difference in morphology compared to control in terms of size, shape and particular structure. The images furthermore enhance the IC_{50} result by giving prove that can be seen clearly to show the anti-amoebic effect of aaptamine and its derivatives towards this pathogenic amoebae.

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