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Acanthamoebicidal activity of periglaucine A and betulinic acid from *Pericampylus glaucus* (Lam.) Merr. *in vitro*

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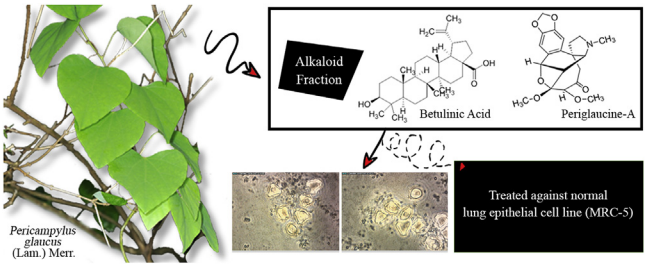
HIGHLIGHTS

- *Pericampylus glaucus* (L.) Merr., betulinic acid and periglaucine A contain effective anti-*Acanthamoeba* activity.
- To the best of our knowledge, this is the first report on the activity of alkaloid and triterpene against *Acanthamoeba*.
- This study confirms the ethnopharmacological potential of *Pericampylus glaucus* (L.) Merr. and constituents.

GRAPHICAL ABSTRACT



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ABSTRACT

Acanthamoeba species are pathogenic protozoa which account for amoebic keratitis, conjunctivitis and granulomatous amoebic encephalitis. These amoebae form cysts which resist drugs and more effective acanthamoebicidal agents are needed. Medicinal plants could be useful in improving the current treatment strategies for *Acanthamoeba* infections. In the present study, we examined the amoebicidal effects of *Pericampylus glaucus* (Lam.) Merr., a medicinal plant used for the treatment of conjunctivitis in Malaysia. Pathogenic *Acanthamoeba triangularis* were isolated from environmental water samples and treated with different concentrations of fractions obtained from *Pericampylus glaucus* (Lam.) Merr. as well as main constituents for 24–72 h. Chlorhexidine was used as a reference drug. Ethanol fraction of stem showed significant ($p < 0.05$) inhibition of trophozoites survival. Betulinic acid and periglaucine A from this plant at 100 μg/mL inhibited more than 70% survival of both cysts and trophozoites. The calculated therapeutic index for betulinic acid and periglaucine A was 170 and 1.5 for trophozoites stage and 3.75 and 8.5 for cysts stage. The observed amoebicidal efficacies indicate the beneficial aspects of this plant in the treatment of *Acanthamoeba* infection. Periglaucine A could also be of value for the treatment of *Acanthamoeba* infection.

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1. Introduction

Amoebae are protozoa living in air, drinking water, tap water, rivers, canals and dialysis units (Nakisah et al., 2012). These protozoa are responsible for potentially fatal disease such as granulomatous amoebic encephalitis, amoebic pneumonitis and amoebic keratitis (Jiang et al., 2015). These diseases are increasing gradually with time and number of reported amoebiasis cases are multiplying year after year (Nakisah et al., 2012; Lorenzo-Morales et al., 2015). Of clinical importance, there are several other dormant disseminating infections potentially affecting multiple organs such as lungs, skin, liver, trachea and adrenals (Khan, 2006; Khan et al., 2007; Kuźma et al., 2015). Members of the genus *Acanthamoeba* (Volkonsky, 1931) present 2 distinct stages in their life cycle: a feeding, vegetative "trophozoites" stage and a resistant "cyst" stage (Khan, 2006). *Acanthamoeba* species are classified on the basis of 18S RNA genetic groups from T1 to T 25. Among these genetic groups, T1, T4 and T5 are pathogenic to human (Jongwutiwes et al., 2000; Marciano-Cabral and Cabral, 2003). Species isolated in patients with keratitis are *Acanthamoeba castellani* (Douglas, 1930) *Acanthamoeba polyphaga* (Puschkarew, 1913), *Acanthamoeba rhysodes* (Bottone, 1993), *Acanthamoeba culberstoni* (Singh and Das, 1970), *Acanthamoeba griffini* (Sawyer, 1971) or *Acanthamoeba triangularis* (Pussard and Pons, 1977; Xuan et al., 2008). Whether cysts, trophozoites, or both are responsible for keratitis and blindness yet an unanswered (Ahearn and Gabriel, 1997). Drugs have been used to treat *Acanthamoeba* infection including chlorhexidine and a variety of antibiotics but treatment fails to overcome cysts with rigid double-layered walls (Polat et al., 2007). Cysts can survive after initial successful therapeutic treatment causing relapse of the disease (El-Sayed et al., 2012). Consequently, most of the patients require keratoplasty to efficiently treat amoebic keratitis (Illingworth and Cook, 1998; Khan, 2006).

For the past six decades, numerous pharmacological investigations have been carried out with various classes of natural products from plants specifically alkaloids, terpenes and phenols being evaluated (Butler, 2008). A wide range of potential biological activities against cancer, virus, parasites and bacteria have been exhibited by secondary metabolites (Patocka, 2003; Paduch et al., 2007). To date, there is comparatively a very few reports on natural products with specific toxicity for *Acanthamoeba*. A sesquiterpene and an abietane were reported to be active against *Acanthamoeba castellanii* (Martín-Navarro et al., 2010; Kuźma et al., 2015). Similarly, alkaloid fractions of *Pancratium maritimum* L. and *Peganum harmala*, have been reported to have weak activity against *Acanthamoeba castellanii* with MIC of 2 mg/mL (Shoaib et al., 2013). Carbazole alkaloids elicited strong activity with IC_{50} values of 3.04 ppm and 11.18 ppm respectively against *Acanthamoeba castellanii* (Sukari et al., 1998). In Southeast Asia, medicinal plants have been used traditionally for centuries to treat infectious diseases related to bacteria and parasites (Wiart, 2006).

In literature, there is a dearth of reports on the anti-acanthamoebic activity of Southeast Asian medicinal plants. *Pericampylus glaucus* (Lam.) Merr. (family Menispermaceae) is a common road-side climber which grows widely in Malaysia, Thailand and Philippine (Kifayatullah et al., 2015). In Philippines, the plant is called "*botang-botang*" and used to treat snake-bites. In Malaysia, this vine is used to treat fever, cough and asthma (Shipton et al., 2017). Stem juice has been used for conjunctivitis as eye drops in Malaysia (Ong, 2001). In this context, we examined the selective therapeutic toxicity of *Pericampylus glaucus* (Lam.) Merr. fractions and major constituents periglaucine A and betulinic acid towards *Acanthamoeba triangularis* (Liang et al., 1998; Yan et al., 2008; Shipton et al., 2017). The aims of the present investigation, therefore, were (i) to assess activities of fractions of *Pericampylus glaucus* (Lam.) Merr. and main constituents against *Acanthamoeba triangularis;* and (ii) to develop safe, effective, and inexpensive anti-*Acanthamoeba* formulation by using natural compounds to treat *Acanthoamoeba* infections.

2. Materials and methods

2.1. Plant material and extraction

2.1.1. Chemicals

Analytical grade n-hexane, chloroform, ethanol and dimethylsulphoxide (DMSO) were purchased from IDL Scientific Ltd. (Kuala Lumpur, Malaysia). Periglaucine A and betulinic acid were purchased from ChemFaces Ltd. (Hubei, China). RPMI 1640, quercetin, chlorhexidine and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma Aldrich Ltd. (St. Louis, USA). Fetal Bovine Serum (FBS) was purchased from Biosera Ltd. (Kansas, USA).

2.1.2. Collection and identification of Pericampylus glaucus (L.) Merr.

In this experiment, *Pericampylus glaucus* (L.) Merr. was selected based on the following criteria; i) It is used in Malaysia to treating conjunctivitis (Ong, 2001) ii) It has never been studied for anti-acanthamoebic activity and iii) It is a very common medicinal plant in Asia. Fresh *Pericampylus glaucus* (L.) Merr. was collected from the coordinates 2°56′53.4″N 101°53′51.7″E and 2°56′53.2″N 101°53′52.0″E in January 2013 from West Malaysia (Shipton et al., 2017). The plant was authenticated at the Forest Research Institute Malaysia. Voucher specimen no. PID 020115-02 was deposited in the School of Pharmacy, University of Nottingham Malaysia campus, Kuala Lumpur, Malaysia.

2.1.3. Plant processing

Stems and leaves of *Pericampylus glaucus* (L.) Merr. were separated and left to dry for a week in open air. The plant parts were cut into 2 cm pieces. After drying, the plant parts were ground into a fine powder using a blender (Philips, Shanghai, China) and weighed using a weighing balance (Sartorius AG, Germany).

2.1.4. Sequential solvent extraction of plant material

Powdered stem and leaves were successively soaked for 3 days at 60 °C with organic solvents of increasing polarity: hexane, chloroform and finally ethanol in a 1:3 ratio by volume for differential extraction of non-polar, mid-polar and polar constituents respectively (Harborne, 1998). The extracts were filtered using filter paper (Whatman International, Maidstone, England), concentrated to remove solvent using a rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland). Dry extracts were weighted and stored in glass scintillation vials (Kimble, MO, USA) at −20 °C until further use.

2.1.5. Extraction of alkaloid fraction

Alkaloids selectively were extracted by conventional method (Harborne, 1998). Powdered stems were defatted with hexane to remove non-polar products in an Erlenmeyer flask and macerated thrice in methanol overnight at 50 °C. The macerated methanol extract was then subjected to filtration and evaporation until complete dryness. Solid extract was then re-dissolved in 0.5% sulphuric acid. Ethyl acetate was used to remove the impurities by liquid-liquid separation. The remaining aqueous solution was adjusted to pH 9.0 with 25% ammonia and separated with chloroform. Finally, chloroform layer was evaporated using a rotary evaporator to yield alkaloid fraction. The calculated percent yield of alkaloid fraction was 0.07%.

2.1.6. Standardization of pure compounds

Qualitative HPLC analysis of pure compounds present in the potent fraction has been performed by standardization. An HPLC system (Agilent 110 Series) equipped with a quaternary pump, multi wavelength UV/visible detector, autosampler, fraction collector and 150 × 4.6 mm phenomenex biphenyl column (Phenomenex QC mix 870, Phenomenex, California, US) was used. Injection volume was 50 μL and the constant injection volume was 1.0 mL/min. Compounds were identified by using UV/Vis spectroscopy and by HPLC spectroscopy with authentic standards. The multiwave UV/VIS detector was set at 220 nm, 230 nm, 240 nm, 254 nm, 270 nm, 310 nm and 360 nm.

2.2. Acanthamoeba triangularis isolation

2.2.1. Acanthamoeba cultivation on non-nutrient agar (NNA)

Environmental water samples were kindly supplied by Department of Parasitology, Faculty of Medicine, University of Malaya. After concentration, water samples were cultured on non-nutrient agar plates (NNA) lawned with *Escherichia coli* and incubated at 26 °C. The presence of *Acanthamoeba triangularis* trophozoites was confirmed 48–72 h after inoculation on NNA medium by use of an inverted microscope. The morphological characteristics of trophozoites were observed as motile cells with prominent vacuole and acanthapodias. Cysts appeared as triangular (3–5 arms) dominant double-walled structures. The monogenous culture of *Acanthamoeba triangularis* was obtained by continuous sub-cultures prior to DNA extraction of the parasite (Mahboob et al., 2016).

2.2.2. Identification of Acanthamoeba using PCR

Page's Saline, PS (0.12 g NaCl, 0.004 $MgSO_4 \cdot 7H_2O$, 0.004 $CaCl_2 \cdot 2H_2O$, 0.142 Na_2HPO_4, and 0.136 g KH_2PO_4 per liter of distilled water) was used to harvest vegetative trophozoites. Suspensions were subjected to centrifugation at 3500 rpm for 10 min, followed by DNA extraction using QIamp DNA blood mini kit (QIAGEN, Hiden, Germany). Polymerase chain reaction was carried out in 25 μL mixture of distilled water, 10× DNA polymerase buffer (Thermo Scientific, Lithuania, USA), 25 mM of magnesium chloride ($MgCl_2$) (Thermo Scientific, Lithuana, USA), 10 mM of deoxynucleotide triphosphate (dNTP) mix (Thermo Scientific, Lithuana, USA), 200 mol of each primer: JDP1 (5′- GGCCCAGATCGTTACCG TGAA - 3′) and JDP2 (5′- TCTCACAAGCTGCTAGGGAGTCA - 3′), 1U of Taq DNA polymerase (Thermo Scientific, Lithuana, USA) with 5 μL of DNA template. The reaction was conducted at 94 °C for 5 min, followed by 40 cycles of annealing at 84 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and an extension at 72 °C for 5 min. DNA templates were retrieved with the same amount of distilled water as a negative control (Mahboob et al., 2016).

2.2.3. Molecular analysis

The 18S region was targeted for PCR amplification and 1.5% electrophoresis gel was used to analyze the amplicon at 450 bp. Ethidium bromide was used to the stain gel and was visualized under UV light. The positive samples were sent for sequencing and homology search was performed by BLAST software, obtained from National Centre for Biotechnology Information (NCBI). The *Acanthamoeba triangularis* was sequenced (KX232518) and found to form a clade with other pathogenic *Acanthamoeba* in T4 group (Majid et al., 2017).

2.2.4. In vitro cultivation of trophozoite and cyst stages

Cultivation of trophozoites and cysts was performed as previously reported by Mahboob et al. (2016). Environmental specimens were directly inoculated on NNA plates seeded with *Escherichia coli* bacterial suspension. Exponential growth of trophozoites was observed after 24–48 h incubation and the protozoa were then gently scraped from the base of NNA culture plates using Page's Saline solution with help of sterile cell scraper. Harvested trophozoites were washed twice with Page's saline solution and were concentrated by centrifugation at 1500*g* for 5 min. The viability of trophozoites was evaluated by microscopic trypan blue exclusion and direct trophozoites counts on hemocytometer. Trophozoites were then calibrated to a final concentration of 10^6 trophozoites/mL and used in assays without delay. Likewise, 2–3 weeks old culture of *Acanthamoeba triangularis* was used for cysts assays, and cysts were harvested with Page' s Saline solution and adjusted to a final concentration of 10^6 cysts/mL. The viability of cysts was determined by microscopic trypan blue exclusion method. Inoculums with 100% viability of cysts were subjected to experimental assays (Tepe et al., 2012).

2.3. Amoebicidal activity testing

In order to evaluate amoebicidal activity, 200 μL of calibrated trophozoite or cyst suspensions were mixed thoroughly with 200 μL of ethanol, chloroform or hexane fraction of *Pericampylus glaucus* (L.) Merr. stem or leaves at concentrations ranging from 0.5 mg/mL to 1.5 mg/mL, or alkaloid fraction, betulinic acid or periglaucine A dissolved in 1% of DMSO (at concentrations ranging from 25 μg/mL to 100 μg/mL) in micro-centrifuge tubes and left to incubate at room temperature for 1, 3, 6, 8, 24, 48 and 72 h. Negative control consisted of 200 μL of trophozoite/cyst suspensions with 200 μL of sterile distilled water. Positive control tubes contained 200 μL of 0.004% chlorhexidine with 200 μL of calibrated trophozoites or 0.025% chlorhexidine with 200 μL of calibrated cysts. Each experiment was performed 3 times. After 24, 48 and 72 h incubation, 10 μL of trophozoite/cyst suspensions were mixed with 10 μL of 0.5% trypan blue. The protozoa were incubated 3 min at room temperature and viable (unstained) and nonviable (stained) trophozoites/cysts were examined with a hemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) under an inverted microscope (Olympus BX51, Tokyo, Japan) (Mahboob et al., 2016).

2.4. Cytotoxicity tests

Apart from transmission via eyes and skin, one of route of transmission of *Acanthamoeba* into human is through nasal route. This route of transmission of *Acanthamoeba* includes lower respiratory tract results in colonization of amoeba in lungs (Marciano-Cabral and Cabral, 2003). Toxicity of fractions and pure compounds towards the mammalian cell line was tested using human lung epithelial cell line MRC-5. The cell lines were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS) using a humidified incubator with 5% CO_2. The stock solution of plant fractions or compounds was prepared by dissolving extracts or compounds in DMSO and RPMI 1640 with a final concentration of 0.1% DMSO. Further dilutions with concentrations ranging from 0.3125 to 200 μg/mL were prepared. The MTT assay was performed according to Mosmann (1983) with slight modifications. MRC-5 cells were grown in RPMI 1640 supplemented with 10% FBS. Trypsin-EDTA was used to detach cells and trypsinised cells at a concentration of 5000 cells per well/100 μL of medium were seeded onto a 96 well plate. The cells were allowed to attach for 24 h prior to removing the media and adding the plant fractions at various concentrations. Cells treated with quercetin were used as a positive control whilst untreated cells were used as negative control. After an incubation period of 72 h, the sample was removed and MTT (3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added. MTT was prepared at a final concentration of 5 mg/mL by

dissolving MTT salt in phosphate buffered saline (PBS). This stock solution was diluted 1: 10 into R0 RPMI. Hundred μL of diluted medium was added to each well and incubated at 37 °C. After incubating for 4 h, 75 μL of medium was removed from each well. Fifty μL of DMSO was then added to each well and mixed thoroughly with a pipette before incubating for 10 min. The absorbance was read at 570 nm using a microplate reader (Spectramax M3 microplate reader, Molecular devices, California, USA) (Mosmann, 1983; Shipton et al., 2017). Therapeutic index (TI), was calculated as follow:

$$\text{Therapeutic index (TI)} = \frac{CC_{50}\ human\ lung\ epithelial\ cell\ line}{IC_{50}\ of\ Acanthamoeba\ triangularis}$$

2.5. Statistical analysis

Data analysis was performed with SPSS software (Statistical Package for Social Sciences) version 21 (SPSS, Chicago, IL, USA). Data were presented as mean values with standard deviations and analyzed by repeated measure of ANOVA followed by Tukey's test for post hoc pairwise comparisons with positive and negative controls. A p value of <0.05 was regarded as statistically significant.

3. Results

3.1. Acanthamoebicidal activity

We examined the amoebicidal activity of hexane, chloroform and ethanol fractions sequentially extracted from the stems of *Pericampylus glaucus* (L.) Merr. towards *Acanthamoeba triangularis* by trypan blue exclusion method. The ethanol fraction of stems was significant by ($p < 0.005$) trophocidal compared to the positive control, chlorhexidine at 0.004% (Fig. 3). At a concentration of 0.5 mg/mL this polar fraction killed 98% trophozoites and this effect was significantly ($p < 0.05$) superior to the positive control, chlorhexidine at 4 μg/mL (Fig. 3). Since available literature suggests that alkaloids have the tendency to be toxic for *Acanthamoeba* we examined the activity of alkaloidal fraction of *Pericampylus glaucus* (L.) Merr. The alkaloid fraction inhibited cysts by 74.7%, 71.1% and 64.8% at concentrations of 100 μg/mL, 50 μg/mL and 25 μg/mL, respectively after 72 h as compared to chlorhexidine with 68.5% inhibition. At 100 μg/mL this fraction was more active than chlorhexidine at 25 μg/mL. In regard to trophocidal activity, the alkaloid fraction decreased number of viable trophozoites by 91.6%, 96.4% and 99% after 72 h at concentrations of 25 μg/mL, 50 μg/mL and 100 μg/mL, respectively (Table 1) (Fig. 2).

Betulinic acid, a triterpene and periglaucine A, an alkaloid are major constituents of *Pericampylus glaucus* (L.) Merr. (Shipton et al., 2017) (Fig. 1). We examined their amoebicidal effects at different intervals with concentrations ranging from 25 to 100 μg/mL. Betulinic acid, at 100 μg/mL demonstrated 76.3% cysticidal effect after 72 h. At concentrations ranging from 25 μg/mL to 100 μg/mL, this pentacyclic triterpene was dose and time-dependently cysticidal (Table 2). Betulinic acid decreased the number of viable trophozoites by 91.4% at 100 μg/mL, similarly to the positive control (Table 2). Periglaucine A at 100 μg/mL, inhibited 97.6% trophozoites survival and at 50 μg/mL was cysticidal by 77.9% after 72 h (Table 3).

3.2. Cytotoxicity tests

The toxicity of fractions and constituents towards human lungs epithelial cell line, MRC-5 was examined by classical MTT assay (Mosmann, 1983) with slight modifications to assess the degree of selectivity. Ethanolic extracts of stem *Pericamyplus glaucus* (L.) Merr. reduced cell proliferation with CC_{50} of 160 μg/mL against normal lung epithelial cell line and selective therapeutic index was below 1. It can be surmised that chemotherapeutics compounds present in the fraction ae mainly polar compounds. The CC_{50} of betulinic acid and periglaucine were 170 and 60 μg/mL respectively. Therapeutic index of periglaucine A, 170 and 8.5 against trophozoites and cysts appears as a therapeutic hint. The results of periglaucine A imply a possible hermetic response and showed the highest therapeutic index of 170 and 8.5 against trophozoites and cysts stage of *Acanthamoeba triangularis*. Proliferation was reduced in MRC-5 cell lines by alkaloid fraction of *Pericampylus glaucus* stem with CC_{50} of 18 μg/mL. Proliferation of cells increased significantly at lower concentrations but increased to a lesser extent at higher concentrations. However, betulinic acid also reduced proliferation in MRC-5 with CC_{50} of 60 μg/mL cell lines in a time dependent manner with therapeutic index of 1.5 and 3.7 for trophozoites and cysts respectively (Table 4).

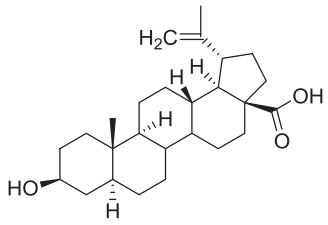
4. Discussion

Plants defend themselves from bacteria, parasites, insects and herbivores through the production of secondary metabolites which

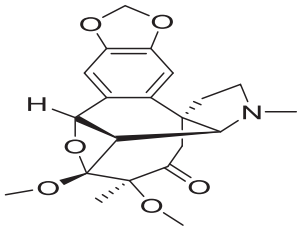
Table 1
Effect of Alkaloid extract of *Pericampylus glaucus* stem (Alk) on growth inhibition of *Acanthamoeba triangularis*.

Alk concentration (μg/mL)	Duration of assay (hours)					
	24 h		48 h		72 h	
	[a]Mean± SD	growth inhibition (%)	Mean ± SD	growth inhibition (%)	Mean ± SD	growth inhibition (%)
Trophozoites						
Non-treated control	238.6 ± 8.0	0	119.6 ± 5.0	0	140.3 ± 4.0	0
Chlorhexidine 4 μg/mL	2.6 ± 4.1	98.8	0.0 ± 0.0	100.0	6.3 ± 4.5	95.4
Alk, 100 μg/mL	8.3 ± 0.5*	96.5	18.6 ± 8.9*	79.8	31.3 ± 1.5*	99.0
Alk, 50 μg/mL	12.0 ± 5.5*	94.9	68.3 ± 1.4*	42.5	5.0 ± 1.0*	96.4
Alk, 25 μg/mL	25.3 ± 8.0*	89.3	31.6 ± 1.3*	73.8	17.6 ± 3.7*	91.6
Cysts						
Non-treated control	67.6 ± 7.2	0	65.6 ± 4.9	0	64.6 ± 5.5	0
Chlorhexidine 25 μg/mL	11.0 ± 3.0	87.0	8.33 ± 2.8	91.7	6.33 ± 4.5	68.5
Alk, 100 μg/mL	7.3 ± 5.8*	89.0	4.0 ± 2.6*	93.8	3.3 ± 2.3*	74.7
Alk, 50 μg/mL	13.0 ± 1.7*	80.5	7.0 ± 1.7*	89.2	5.6 ± 1.5*	71.1
Alk, 25 μg/mL	15.3 ± 3.0*	77.1	12.3 ± 2.5*	81.0	9.6 ± 2.0*	64.8

*$p < 0.05$, statistically significant difference in comparison to non-treated control in the same time interval.
[a] A mean value on the number of trophozoites/cysts.

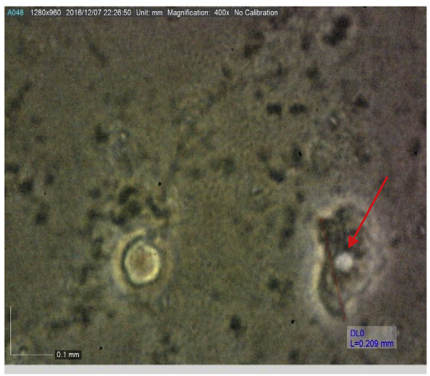


Chemical structure of betulinic acid

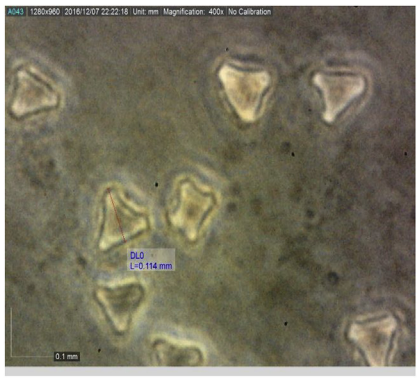


Chemical structure of periglaucine-A

Fig. 1. Chemical structures.



A- (LiM × 400) *Acanthamoeba triangularis* viable trophozoite showing typical contractile vacuole with no stain. LiM =light microscope



B- (LiM × 400) *Acanthamoeba triangularis* viable cysts showing smooth ectocyst and endocyst with no stain.

Fig. 2. Morphological observations of trophozoites and cysts.

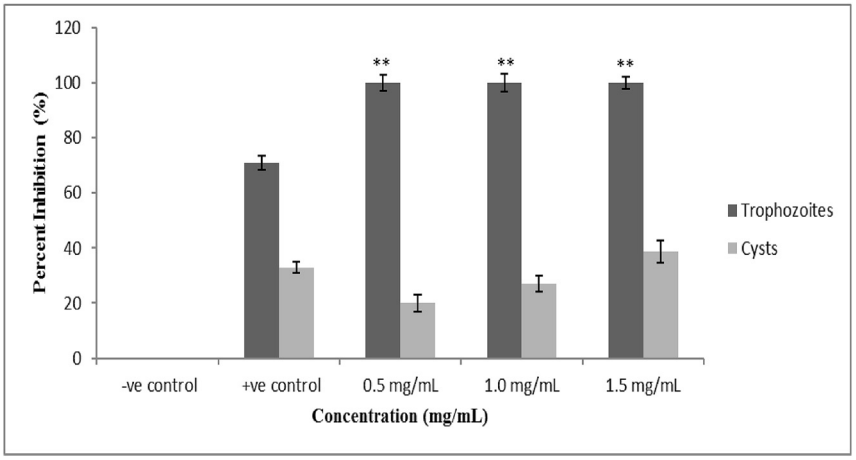


Fig. 3. Effect of *Pericampylus glaucus* (Lam.) Merr. ethanol stem on trophozoites and cysts of *A. triangularis*.
$^{**}p < 0.05$, statistically significant difference in comparison to drug control in the same time interval.

are pharmacologically active (Derda et al., 2009). There is a critical need to develop new drugs for *Acanthamoeba* infections and medicinal plants offer a vast untapped reservoir of potential leads (Khan, 2006; Malatyali et al., 2012). To date, no drugs have ever been licensed to treat *Acanthamoeba* keratitis (Kuźma et al., 2015). In this context, we examined the toxins of *Pericampylus glaucus* (L.) Merr., used to treat conjunctivitis in Malaysia against *Acanthamoeba triangularis in vitro* (Ong, 2001). The highest cysticidal activity was demonstrated by betulinic acid, followed by periglaucine A and lastly the alkaloid, fraction. The therapeutic selectivity index provide insight into specifically toxic to this pathogenic parasite. Periglaucine A with therapeutic index 170 could be considered as a promising agent for a future chemotherapeutic against amoebiasis. The mechanisms of the anti-*Acanthamoeba* properties of terpenes are closely linked to their lipophilic character (Trombetta et al., 2005). Betulinic acid has been reported to induce

Table 2
Effect of Betulinic acid (BA) on growth inhibition of *Acanthamoeba triangularis*.

BA concentration (μg/mL)	Duration of assay (hours)					
	24 h		48 h		72 h	
	[a]Mean± SD	growth inhibition (%)	Mean ± SD	growth inhibition (%)	Mean ± SD	growth inhibition (%)
Trophozoites						
Non-treated control	238.6 ± 8.0	0	119.6 ± 5.0	0	140.3 ± 4.0	0
Chlorhexidine 4 μg/mL	2.6 ± 4.1	98.8	0.0 ± 0.0	100.0	6.3 ± 4.5	95.4
BA, 100 μg/mL	57.3 ± 4.8*	75.1	6.3 ± 3.5*	94.6	12.0 ± 5.1*	91.4
BA, 50 μg/mL	88.6 ± 4.6*	62.7	70.0 ± 2.1*	41.4	40.0 ± 1.7*	71.4
BA, 25 μg/mL	60.0 ± 2.0*	74.4	140.0 ± 3.5*	4.6	144.6 ± 6.5*	0.47
Cysts						
Non-treated control	67.6 ± 7.2	0	65.6 ± 4.9	0	64.6 ± 5.5	0
Chlorhexidine 25 μg/mL	11.0 ± 3.0	47.0	8.33 ± 2.8	71.7	6.33 ± 4.5	68.5
BA, 100 μg/mL	10.3 ± 3.2*	61.0	6.66 ± 0.5*	69.1	2.3 ± 1.5*	76.3
BA, 50 μg/mL	26.0 ± 7.2*	41.0	19.3 ± 5.6*	50.2	10.3 ± 2.0*	63.8
BA, 25 μg/mL	18.6 ± 2.5*	51.4	2.6 ± 9.5*	42.0	14.6 ± 5.0*	57.0

*$p < 0.05$, statistically significant difference in comparison to non-treated control in the same time interval.
[a] A mean value on the number of trophozoites/cysts.

Table 3
Effect of Periglaucine A (PGA) on growth inhibition of *Acanthamoeba triangularis*.

PGA concentration (μg/mL)	Duration of assay (hours)					
	24 h		48 h		72 h	
	[a]Mean±SD	growth inhibition (%)	Mean ± SD	growth inhibition (%)	Mean ± SD	growth inhibition (%)
Trophozoites						
Non-treated control	238.6 ± 8.0	0	119.6 ± 5.0	0	140.3 ± 4.0	0
Chlorhexidine 4 μg/mL	2.6 ± 4.1	98.8	0.0 ± 0.0	100.0	6.3 ± 4.5	95.4
PGA, 100 μg/mL	2.0 ± 2.6*	99.1	19.0 ± 5.5*	84.0	3.3 ± 0.5*	97.6
PGA, 50 μg/mL	12.0 ± 2.6*	94.9	49.0 ± 2.6*	58.8	25.0 2*	89.8
PGA, 25 μg/mL	25.6 ± 7.5*	89.2	102.6 ± 6.2*	14.2	13.0 ± 3.0*	86.9
Cysts						
Non-treated control	67.6 ± 7.2	0	65.6 ± 4.9	0	64.6 ± 5.5	0
Chlorhexidine 25 μg/mL	11.0 ± 3.0	67.0	8.33 ± 2.8	71.7	6.3 ± 4.5	68.5
PGA, 100 μg/mL	4.3 ± 0.5*	73.5	5.0 ± 2.0*	72.3	2.6 ± 1.5*	75.8
PGA, 50 μg/mL	3.6 ± 2.0*	74.5	2.0 ± 1.0*	76.9	1.3 ± 0.5*	77.9
PGA, 25 μg/mL	14.6 ± 2.6*	59.1	7.6 ± 3.0*	68.2	4.0 ± 1.0*	73.7

*$p < 0.05$, statistically significant difference in comparison to non-treated control in the same time interval.
[a] A mean value on the number of trophozoites/cysts.

Table 4
Cytotoxicity tests. CC_{50} and IC_{50} of Fractions/Compounds.

Compound/Fraction	IC_{50} (μg/mL)[a]		CC_{50} (μg/mL)[b]	TI[c]	
	Trophozoites	Cysts		Trophozoites	Cysts
P. glaucus Stem ethanol	200	1800	160	0.8	0.08
Alkaloid Fraction	2	**16**	18	9	1.12
Periglaucine A	1	20	**170**	**170**	**8.5**
Betulinic acid	40	**16**	60	1.5	3.75

IC50 ug/mL-Bold shows the least IC50 against cyst stage, CC50ug/mL-Bold shows the highest CC50 value that proliferated MRC-5 cells by 50%, TI-Bold shows highest therapeutic index against trophozoites and cysts.
[a] Concentration at which growth of trophozoites/cysts inhibited by 50%.
[b] Concentration that proliferated MRC-5 cell lines by 50%.
[c] Therapeutic index, ratio of CC_{50} to IC_{50}.

apoptosis in several tumor cells by alterating in mitochondrial function while remain nontoxic for normal human cell line at concentration of 10 μg/mL (Fulda et al., 1998). Inhibition of lipoisomerase has also been reported to be induced by betulinic acid, which catalyses changes in DNA topology and consequently lead to apoptosis (Chowdhury et al., 2002). Alkaloids, such as berberine, cryptolepene and carboline alkaloids, induced cell death by induction of apoptosis and inhibition of vascular contraction by L-type Ca^{2+} channels and mitochondrial insults (Hazra et al., 2012; El-Sayed et al., 2012). Here we provide evidence that *Pericampylus glaucus* (L.) Merr., betulinic acid and periglaucine A are amoebicidal with potencies superior to chlorhexidine. The actual mechanisms of action of betulinic acid and periglaucine A towards *Acanthamoeba triangularis* are unknown and requires further investigation.

5. Conclusion

In conclusion, this study demonstrates that betulinic acid and

periglaucine A from *Preicampylus glaucus* (L.) Merr. have excellent anti-*Acanthamoeba* activity. This Malaysian medicinal plant has the potential to be used for medical purpose and to be utilized as anti-*Acanthamoeba* agent. These findings provide a basis for the use of this plant as an alternative therapy and to constitute an effective chemotherapeutic agent with less toxicity. Our promising results also justify the traditional use of *Pericampylus glaucus* for treating conjunctivitis.

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