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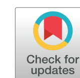
Research article

Potential anti-*Acanthamoeba* and anti-adhesion activities of *Annona muricata* and *Combretum trifoliatum* extracts and their synergistic effects in combination with chlorhexidine against *Acanthamoeba triangularis* trophozoites and cysts



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ABSTRACT

Plants with medicinal properties have been used in the treatment of several infectious diseases, including *Acanthamoeba* infections. The medicinal properties of Cambodian plant extracts; *Annona muricata* and *Combretum trifoliatum* were investigated against *Acanthamoeba triangularis*. A total of 39 plant extracts were evaluated and, as a result, 22 extracts showed positive anti-*Acanthamoeba* activity. Of the 22 extracts, 9 and 4 extracts showed anti-*Acanthamoeba* activity against trophozoites and cysts of *A. triangularis*, respectively. The minimum inhibitory concentration of *A. muricata* and *C. trifoliatum* extracts against trophozoites and cysts was 500 and 1,000 μg/mL, respectively. The combination of *A. muricata* at 1/4×MIC with chlorhexidine at 1/8×MIC demonstrated a synergistic effect against trophozoites, but partial synergy against cysts. A 40% reduction in trophozoites and 60% of cysts adhered to the plastic surface treated with both extracts at 1/2×MIC were noted comparing to the control ($P < 0.05$). Furthermore, a reduction of 80% and 90% of trophozoites adhered to the surface was observed after pre-treatment with *A. muricata* and *C. trifoliatum* extracts, respectively. A 90% of cysts adhered to the surface was decreased with pre-treatment of *A. muricata* at 1/2×MIC ($P < 0.05$). A 75% of trophozoites and cysts from *Acanthamoeba* adhered to the surface were removed after treatment with both extracts at 4×MIC ($P < 0.05$). In the model of contact lens, 1 log cells/mL of trophozoites and cysts was significantly decreased post-treatment with both extracts compared to the control. Trophozoites showed strong loss of acanthopodia and thorn-like projection pseudopodia, while cysts demonstrated retraction and folded appearance treated with both extracts when observed by SEM, which suggests the potential benefits of the medicinal plants *A. muricata* and *C. trifoliatum* as an option treatment against *Acanthamoeba* infections.

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1. Introduction

Infection caused by *Acanthamoeba* spp., free-living protozoa, has been concerned worldwide. *Acanthamoeba triangularis* (*A. triangularis*) is a causal agent of human disorders, such as granulomatous amebic encephalitis and *Acanthamoeba* keratitis (Jercic et al., 2019; Bunsuwansakul et al., 2019). Two stages of *Acanthamoeba* growth are well known and these include trophozoites and cysts. The trophozoites are vegetative moving amoebic form while cysts are a quiescent stage that persists in stress condition such as absence of nutrient. *Acanthamoeba* trophozoites exhibited adhesion ability for contact lens through acanthopodia (Lee et al., 2017). The occurrence of serious vision loss and total blindness induced by *Acanthamoeba* spp. is the major issue in contact lens users (Lee et al., 2017). The cyst contains double wall layers (ectocyst and endocyst walls) that are responsible for its antibiotic resistance (Hay et al., 1994) that leads to the difficulty in the treatment of *Acanthamoeba* infections.

To surmount infections induced by the pathogen, plant-derived compounds could be used as an alternative strategy for its treatment. A study has reported the inhibitory effect from the seed of *Trigonella foenum-graecum* extract on cysts from *A. castellanii* Kaya et al. (2019). Rhizome extract of *Curcuma longa* and its pure compound, Curcumin, showed anti-microbial activity against *A. triangularis* trophozoites and cysts (Mitsuwan et al., 2020a). Essentially, there is a recent interest in the combination therapy involving anti-microbial agents and other bio-active compounds from plant origin as a new method to treat infectious diseases. Synergistic effects of chlorhexidine in combination with cationic carbosilane dendrimers against both forms of *Acanthamoeba* spp., trophozoite and cyst have been reported (Heredero-Bermejo et al., 2016).

Annona muricata (*A. Muricata*), *Annonaceae* family, is a medicinal plant used in the treatment of many diseases (Moghadamtousi et al., 2015). It has been documented that the extract of *A. muricata* exhibited anti-microbial activity against *A. castellanii* (Moghadamtousi et al., 2015). *Combretum trifoliatum* (*C. trifoliatum*) belongs to *Combretaceae* family and it is a source of phytochemicals that has been usually used to treat several diseases (de Morais Lima et al., 2012). Anti-bacterial activity and synergistic effects of plants in the genus *Combretum* spp. in

Table 1. Cambodian medicinal plants used in this study.

No.	Family names	Local names spelt in English	Scientific names	Codes	Parts	Extracting Solvents	Extracting yield (%)	Extracted sample weight (mg)
1	Acantahceae	Mchul pich	*Barleria lupulina* Lindl.	P001	Whole plant	Methanol	2.20	93.33
2	Lauraceae	Choe sambol lveng	*Cinnamomum cassia* (L.) J. Presl	P005	Barks	Methanol	2.31	130.71
3	Acanthaceae	Smau bramat mnuhs	*Andrographis paniculata* (Burm. f.) Wall.	P003	Whole plant	Ethanol	2.27	136.18
4	Bixaceae	Chumpu chroluek	*Bixa orellana* L.	P006	Seeds	Methanol	0.83	276.22
5	Annonaceae	Tieb barang	*Annona muricata* L.	P004	Leaves	Ethanol	3.31	222.12
6	Fabaceae	So kram	*Xylia xylocarpa* Taub.	P011	Stems	Methanol	3.65	505.03
7	Celastraceae	Veay	*Salacia chinensis* L.	P012	Stems	Methanol	3.42	538.71
8	Fabaceae	Sbaeng	*Caesalpinia sappan* L.	P013	Wood	Methanol	4.25	639.26
9	Simaroubaceae	Doem bramat mnuhs	*Brucea javanica* (L.) Merr.	P007	Stems	Methanol	0.89	68.31
10	Bignoniaceae	Doem pika	*Oroxylum indicum* (L.) Kurz	P008	Stems	Ethanol	1.15	57.20
11	Meliaceae	Sdav	*Azadirachta indica* A. Juss.	P002	Barks	Ethanol	0.95	122.42
12	Zingiberaceae	Romiet	*Curcuma domestica* Valeton	P014	Rhizomes	Ethanol	2.10	61.31
13	Zingiberaceae	Ponlei	*Zingiber purpureum* Roscoe	P015	Rhizomes	Methanol	6.14	792.15
14	Lamiaceae	Trasiet	*Vitex negundo* L.	P016	Whole Plant	Ethanol	5.49	510.06
15	Moringaceae	Mrum	*Moringa oleifera* Lam.	P017	Seeds	Methanol	0.75	500.20
16	Rubiaceae	Kam rotehs	*Ixora chinensis* Lam.	P018	Stems	Methanol	1.21	400.15
17	Anacardiaceae	Svay chan ti	*Anacardium occidentale* L.	P019	Bark	Methanol	1.86	517.21
18	Arecaceae	Thnot	*Borassus flabellifer* L.	P020	Flowers	Methanol	1.32	201.56
19	Zingiberaceae	Khnhei	*Zingiber officinale* Roscoe	P021	Rhizomes	Methanol	0.81	247.55
20	Capparaceae	Doem rook kraham	*Capparis sepiaria* L.	P022	Leave	Methanol	1.54	309.01
21	Capparaceae	Thngan	*Crateva adansonii* DC.	P023	Stems	Methanol	0.73	488.82
22	Caricaceae	Lhong	*Carica papaya* L.	P024	Stems	Methanol	2.25	128.05
23	Costaceae	Trathok	*Costus speciosus* (J. Koenig) Sm.	P025	Rhizomes	Methanol	1.05	50.24
24	Myrtaceae	Puech thom	*Rhodomyrtus tomentosa* (Aiton) Hassk.	P051	Stems	Ethanol	5.29	105.12
25	Plantaginaceae	Eisei phsam srech	*Scoparia dulcis* L.	P052	Whole plant	Ethanol	5.4	124.22
26	Celastraceae	Ko muy sarsai muy roy	*Euonymus cochinchinensis* Pierre	P054	Barks	Ethanol	3.02	339.72
27	Araceae	Kdat haora	*Alocasia macrorrhiza* (L.) G. Don	PO55	Tubers	Methanol	3.12	253.19
28	Fabaceae	Srae muer	*Dicerma biarticulatum* (L.) DC.	P056	Arial parts	Methanol	2.58	116.54
39	Rubiaceae	Mroch sourt damrei	*Hydnophytum formicarum* Jack	P057	Tubers	Methanol	3.01	328.02
30	Phyllanthaceae	Kantuet prey	*Phyllanthus emblica* L.	P058	Fruits	Methanol	1.71	101.78
31	Fabaceae	Daem chre sar	*Albizia lebbeck* (L.) Benth.	P060	Bark	Methanol	3.12	77.02
32	Acoraceae	Kanteanghe phnom	*Acorus calamus* L.	P061	Bark	Methanol	4.89	75.03
33	Malvaceae	Tbalken	*Abutilon indicum* (L.) Sweet	P062	Bark	Methanol	2.87	84.05
34	Combretaceae	Voa tral	*Combretum trifoliatum* Vent.	P063	Roots	Methanol	5.04	55.02
35	Rutaceae	Daem reak sar	*Citrus medica* L.	P064	leaves	Methanol	3.13	72.03
36	Amaryllidaceae	Khtoem Sar	*Allium sativum* L.	P065	Bulbs	Methanol	1.52	76.02
37	Amaryllidaceae	Ka chay	*Allium tuberosum* Rottler ex Spreng.	P066	leaves	Methanol	25.66	55.03
38	Poaceae	Sloekakrei srok	*Cymbopogon nardus* (L.) Rendle	P067	leaves	Methanol	3.40	77.03
39	Asteraceae	Daem khmanh	*Eclipta erecta* L.	P068	Whole plant	Methanol	3.25	61.03

combination with antibiotic against bacteria have been reported (de Morais Lima et al., 2012).

Consequently, the aim of this study was to determine the anti-*Acanthamoeba* activity of Cambodian medicinal plants, including *A. muricata* and *C. trifoliatum* against the trophozoites and cysts of *A. triangularis*. Effects of *A. muricata* and *C. trifoliatum* extracts on the adhesion of the amoeba to plastic polystyrene was determined. Importantly, synergistic effects of the plant extract in combination with antibiotic was investigated.

2. Materials and methods

2.1. Preparation of plant extract and antimicrobial agents

Thirty-nine Cambodian medicinal plants were selected for this research (Table 1). Plants were dried and extraction of materials was done with 95% ethanol and methanol solutions. Then, the extracted solvents were vaporized under lowered pressure. The extracts were air dried and weighed. Percent yield of the extracted samples was determined. Chlorhexidine (Sigma-Aldrich, Missouri, USA) was used as positive control. The extracts and the antibiotics were dissolved in 100% dimethyl sulfoxide (DMSO) and stored at 4 °C till use.

2.2. Parasite culture

Acanthamoeba triangularis WU19001 was grown in culture flasks containing Peptone-Yeast Extract-Glucose (PYG) medium as reported (Niyyati et al., 2013; Mitsuwan et al., 2020b). The medium contained 18 g glucose, 20 g proteose peptone, 2 g yeast extract, 0.98 g $MgSO_4 \times 7H_2O$, 1 g sodium citrate dihydrate, 0.02 g $Fe(NH_4)_2(SO_4)_2 \times 6H_2O$, 0.34 g KH_2PO_4, 0.355 g $Na_2HPO_4 \times 7H_2O$, and 1,000 mL distil water. Cultured flasks were incubated at room temperature. Subsequently, trophozoites and cysts were harvested after 3 and 7 days, respectively.

2.3. Preliminary screening of antiparasitic activity of the extract against A. triangularis

Preliminary screening of anti-parasitic activity of Cambodian plant extracts against the trophozoite and cyst forms of the amoeba was evaluated by trypan blue exclusion assay as followed (Mitsuwan et al., 2020a). Briefly, trophozoites and cysts were cultivated as depicted above. Then, the microorganisms were rinsed twice by Page's saline solution (PAS). Subsequently, the samples were centrifuged at 4,000 rpm for 5 min. Viability of *A. triangularis* was evaluated by a trypan blue exclusion test. An aliquot of 100 μL of the cell suspension (2×10^5 trophozoites/mL) was dropped into 96 well plates, containing 100 μL of each extract at a concentration 1,000 μg/mL. The final concentration of DMSO presented in the extract was 1% DMSO. The samples were then incubated at room temperature for 24 h. One percent DMSO was used as negative control, while chlorhexidine was used as positive control. Inhibition of the parasite growth was investigated by trypan blue exclusion assay to determine the number of live (non-stained) and dead (stained) cells (Baig et al., 2013). The relative percentage of cell viability was specified as: (mean of the treated parasite/mean of the control) $\times 100$. Selection of the extracts was carried out when they showed $\geq$90% the growth inhibition of the trophozoites and cysts, compared with the negative control.

2.4. Minimal inhibitory concentration (MIC) determination

The determination of the MIC values of the extracts against the trophozoites and cysts was assessed by broth microdilution assay as reported in our previous study (Mitsuwan et al., 2020a). The trophozoites and cysts of *A. triangularis* were grown in PYG medium as mentioned above. One hundred microliters of the suspension (2×10^5 cells/mL) of each group of trophozoites and cysts were dropped into 96 well plates, containing 100 μL of successively diluted extracts at concentrations 125–1,000 μg/mL. One percent DMSO was used as negative control, while chlorhexidine was used as positive control. The sample was incubated at room temperature for 24 h. The MIC value was specified as the lowest concentration that caused $\geq$90% the growth inhibition (mean $\pm$ SD) of the trophozoites and cysts as measured by the exclusion assay, comparing to the negative control.

2.5. Combination of the plant extracts and antibiotic against A. triangularis

Checkerboard assay was used to examine synergistic effects of the plant extracts in combination with the antibiotic in comparison to their individual activities (Hwang et al., 2012). Briefly, the MIC of each extract and chlorhexidine alone was investigated as described above. The checkerboard with twofold dilutions of the plant extracts and chlorhexidine was carried out to explore the synergistic effects of the plant extract in combination with the antibiotics. The growth inhibition of the combinations and the agents alone was measured by trypan blue exclusion assay. Fractional inhibitory concentration (FIC index) was determined as follows:

FICI = (MIC of extract in combination/MIC of extract alone) + (MIC of chlorhexidine combination/MIC of chlorhexidine alone).

The index was interpreted as follows:

FICI <0.5 = synergism
$0.5 \leq$ FIC <1.0 = partial synergy
FIC $= 1.0$ = additive
FIC >2.0 = antagonism

2.6. Scanning electron microscopy

Effects of the extracts on morphology of the trophozoites and cysts was determined by Scanning electron microscopy (SEM) (Zeiss, Munich, Germany) as previously reported (Mitsuwan et al., 2020b) with slight changes. Parasite cells were given the extracts at concentration 4×MIC on a sterile glass covers lip in a 24-well plate. The sample was incubated at room temperature for 24 h. Subsequently, samples were rinsed thrice with phosphate buffer solution (PBS) and fixation was done with glutaraldehyde at concentration 2.5% in PBS for 24 h. The discs were then washed with PBS. Subsequently, samples were dehydrated in a series of graded ethanol (20–100%). The samples were then dried using a critical point dryer. The samples were then coated with gold particles. The morphology (size shape and structure) of *A. triangularis* post-treatment was observed under SEM.

2.7. Effects of the plant extracts on adhesion of A. triangularis to polystyrene plastic surface

The activity of the plant extracts on adhesion of the amoeba was carried out using 96 well polystyrene plate (0.33 cm^2 of culture area, 0.075–0.2 mL of proposed working volume, VWR International, Missouri, USA). The experiment was performed as disclosed in our previous study (Mitsuwan et al., 2020b) with minimal changes. The parasite cultured in the culture medium were then grown in the medium supplemented with the extracts at sub-MICs. Sub-MICs of chlorhexidine were used as positive control, while 1% DMSO were used as negative control. The samples were then incubated at 25 °C for 48 h. Non-adhesive cells were eliminated by removing of the old medium. Later, the microplates were washed twice with PAS and air dried. Then, the samples were stained with 0.1% crystal violet assay for 30 min. Subsequently, the plates were washed twice by sterile distilled water. The samples were air dried overnight at room temperature. A total of 200 μL DMSO were added to dissolve the stained cells. The plates were measured at the optical density 570 nm. The activity of the extracts on the adhesion of *A. triangularis* adhesion was reported as relative percentage of the

adhesion. It was specified as: (mean A570 nm of treated well/mean A570 nm of control well) ×100.

2.8. Prevention of A. triangularis adhesion to polystyrene plastic plates by the extracts

Effects of the plant extracts to prevent the adhesion of *A. triangularis* to the plastic surface were performed in the microtiter plate as previously described (Mitsuwan et al., 2020b). The polystyrene wells were pre-treated with the extract at sub-MICs. The samples were incubated at 4 °C for 24 h. One percent DMSO was used as negative control, while chlorhexidine was used as positive control. The extracts were removed and substituted with PYG (100 μL). One hundred microliters of the parasites (3×10^5 cells/mL) were put into the polystyrene wells. The plates were incubated at room temperature for 24 h. Crystal violet assay was used to investigate the activity of the extracts to prevent *A. triangularis* adhesion to the surface as reported above.

2.9. Elimination of adhesive A. triangularis on polystyrene surface by A. muricata and C. trifoliatum extracts

Effects of the elimination of adhesive *A. triangularis* on the surface were done in 96 well plate as reported (Sudjana et al., 2012) with minor changes. In brief, an aliquot of 100 μL of the microorganism (3×10^5 cells/mL) was inoculated in the plate, incubated at room temperature for 24 h. After that, the pathogens were incubated in the medium containing the extracts at concentrations of the extracts at 2-4×MICs. The samples were incubated at room temperature for 24 h. It was noted that the final concentration of DMSO presented in the extract was 1% and 2% DMSO used as negative controls in trophozoite and cyst experiments, respectively. Also, chlorhexidine was included as a positive control. Plates were washed twice with PAS to remove non-adhesive cells. In order to investigate the elimination of the parasite, crystal violet assay was used to stain the adhesive cells on the plates. The plates were measured at the optical density 570 nm. Percentage of the survival cells was defined as: (mean A570 nm of treated well/mean A570 nm of control well)×100.

2.10. Activity of A. muricata and C. trifoliatum extracts on adherence of A. triangularis to contact lens

The activity of *A. muricata and C. trifoliatum* extracts to decrease the adhesion of the parasite on contact lens (Duna Plus, Singapore) was assessed as earlier reported (Mitsuwan et al., 2020b) with slight changes. Five hundred microliters of the microorganism (3×10^5 cells/mL) were dropped on contact lens in 24-well plate containing sub-MICs of the extracts. The samples were kept at room temperature for 24 h. One percent DMSO was used as negative control, while chlorhexidine was used as positive control. The samples were rinsed in PAS to remove non-adhesive cells. After that, the contact lens was solved in tubes holding 500 μL of PAS and mixed. The adhesive cells were stained using trypan blue. Then, the adhesive cells were detected under an inverted microscope (Nikon, Tokyo, Japan).

2.11. Statistical analysis

All the experiments were done in triplicate. The data were recorded and entered using the statistical package version 19 (SPSS Inc. Chicago, IL, USA) and the obtained results were presented as mean ± SD. Two-tailed unpaired Student's t-test was used to analyze the statistical analysis. It was reported that $P < 0.05$ was considered statistically significant difference.

3. Results

3.1. Plant extraction

A total of 39 Cambodian medicinal plants were collected and extracted using alcohol. Taxonomical data of specimens, parts collected, and common names are presented in Table 1. Percentage of the extracted yield values ranged from 0.73 to 25.66 (Table 1). *Allium tuberosum* leaf extract has highest extracted yield of 25.66%.

3.2. Preliminary screening of anti-Acanthamoeba activity of extracts against trophozoites and cysts of A. triangularis

Preliminary screening of antimicrobial activity of Cambodian plant extracts against the trophozoites and the cysts was determined at the concentration of 1000 μg/mL (the final concentration of DMSO was 1%). Of 39 evaluated plant extracts, 22 extracts had positively shown anti-*Acanthamoeba* activity (Table 2), whereas 17 extracts were yielded negative results of the activity (at the tested concentration). Consequently, the percent inhibition of the viability of the trophozoites treated with the extracts ranged from 0-90%, whereas against cysts, inhibition ranged from 0-90.33%. Selection of the extracts for further study was carried out when they showed ≥90% growth inhibition (mean ± SD) of trophozoites and cysts, comparing with the negative control.

3.3. Determination of MIC of selected plant extracts against A. triangularis trophozoites and cysts

Plant extracts that show ≥90% growth inhibition were chosen to determine the MIC values against *A. triangularis*. The extracts exhibited strong anti-parasitic activity against *A. triangularis* trophozoites and cysts. The results showed that the MIC values of the extracts ranging from 500–1,000 μg/mL, respectively (Table 3). *A. muricata* and *C. trifoliatum* revealed the strongest anti-*Acanthamoeba* activities against both trophozoites and cysts. The MIC values of both extracts against *A. triangularis* trophozoites and cysts were 500 and 1,000 μg/mL, respectively. Hence, both extracts were chosen for further studies. The MIC values of antibiotics against *A. triangularis* are presented in Table 3. The final concentration of DMSO was 1% presented in the extracts.

3.4. Synergistic effects of A. muricata and C. trifoliatum extracts in combination with chlorhexidine against A. triangularis

Due to the strong nature of the two layers of cyst walls, synergistic effects of *A. muricata* and *C. trifoliatum* extracts in combination with chlorhexidine against *A. triangularis* were determined by checker board assay. As shown in Table 4, combination of *A. muricata* at 1/4×MIC and chlorhexidine at 1/8×MIC demonstrated synergistic effects against *A. triangularis* trophozoites with FIC index as 0.375. In addition, partial synergy of 1/2×MIC *C. trifoliatum* plus 1/8×MIC chlorhexidine against the trophozoites was observed. Both *A. muricata* and *C. trifoliatum* extracts showed partial synergy in combination with chlorhexidine against *A. triangularis* cysts.

3.5. Inhibition of A. triangularis adhesion to polystyrene plastic surface by A. muricata and C. trifoliatum extracts

Effects of *A. muricata* and *C. trifoliatum* extracts at sub-MICs on adhesion of *A. triangularis* were determined in 96-well polystyrene plastic plates. As shown in Figure 1, both extracts substantially reduced the adhesion of *A. triangularis* trophozoites (Figures 1A and 2) and cysts (Figures 1B and 3) to the plastic surface ($P < 0.05$). Approximately 40% decrease in the trophozoites adhesion to the plastic surface was observed, while 60% inhibition was also detected in cysts treated with both extracts at 1/2×MIC. At the time point, non-encystation of the trophozoites was observed when the cells were challenged with the extracts as shown in Figure 2.

3.6. Prevention of A. triangularis adhesion to the plastic surface by A. muricata and C. trifoliatum extracts

Pre-treatment of the surface by *A. muricata* and *C. trifoliatum* extracts at sub-MICs was investigated in polystyrene 96-well plates. After that, the

Table 2. Percent inhibition of the viability of *Acanthamoeba triangularis* trophozoites and cysts treated with medicinal plant extracts, compared with the control.

Code	Plants	Percent Viability (Mean ± SD)	
		Trophozoites	Cysts
P001	*Barleria lupulina Lindl*	60.00 ± 10.00	42.11 ± 5.26
P002	*Azadirachta indica*	73.34 ± 5.77	42.11 ± 5.26
P003	*Andrographis paniculata*	73.34 ± 11.54	45.62 ± 8.03
P004	*Annona muricata*	90.00 ± 0.00	89.48 ± 5.26
P005	*Cinnamomum cassia*	86.67 ± 5.77	45.17 ± 5.58
P006	*Bixa orellana*	73.34 ± 5.77	52.63 ± 5.26
P007	*Brucea javanica*	90.00 ± 0.00	47.37 ± 11.17
P008	*Oroxylum indicum*	90.00 ± 0.00	54.84 ± 5.58
P011	*Xylia xylocarpa*	56.67 ± 5.77	45.17 ± 5.58
P012	*Salacia chinensis*	86.67 ± 5.77	83.88 ± 5.58
P013	*Caesalpinia sappan*	60.00 ± 10.00	58.07 ± 5.58
P018	*Ixora chinensis*	73.34 ± 5.77	74.20 ± 5.58
P019	*Anacardium occidentale*	90.00 ± 0.00	87.10 ± 5.58
P060	*Albizia lebbeck*	86.67 ± 5.77	56.15 ± 3.03
P061	*Acorus calamus*	90.00 ± 0.00	87.10 ± 5.58
P062	*Abutilon indicum*	86.67 ± 11.54	54.84 ± 5.58
P063	*Combretum trifoliatum*	90.00 ± 0.00	90.33 ± 0.00
P064	*Citrus medica*	16.66 ± 5.77	42.11 ± 5.26
P065	*Allium sativum*	83.34 ± 5.77	56.15 ± 6.07
P066	*Allium tuberosum*	76.67 ± 5.77	74.20 ± 5.58
P067	*Cymbopogon nardus*	76.67 ± 5.77	50.88 ± 8.03
P068	*Eclipta prostrata*	26.67 ± 5.77	31.58 ± 5.26

Table 3. Minimal inhibitory concentration (MIC) of medicinal plant extracts against *Acanthamoeba triangularis* trophozoites and cysts.

Antimicrobial agents	MIC (μg/mL)	
	Trophozoites	Cysts
Annona muricata	500	1,000
Cinnamomum cassia	1,000	>1,000
Brucea javanica	1,000	>1,000
Oroxylum indicum	1,000	>1,000
Salacia chinensis	500	>1,000
Anacardium occidentale	1,000	1,000
Albizia lebbeck	1,000	>1,000
Acorus calamus	1,000	1,000
Combretum trifoliatum	500	1,000
Chlorhexidine	16	64

trophozoites and cysts of *A. triangularis* were exposed to the wells. The findings demonstrated that *A. muricata* and *C. trifoliatum* extracts at sub-MICs significantly reduced the adhesion of the parasite to the surface ($P < 0.05$). Approximately 80% and 90% decrease in the trophozoite adhesion was detected in *A. muricata* and *C. trifoliatum* treatment, respectively (Figure 4A). Pre-treatment of the plastic surface with *A. muricata* at 1/2×MIC significantly decreased 90% of the cyst adhesion, compared with the control (Figure 4B) while 75% inhibition of cyst adhesion was detected after a treatment with *C. trifoliatum* extract comparing with the control.

3.7. Elimination of adhesive *A. triangularis* by *A. muricata* and *C. trifoliatum* extracts

Since *A. triangularis* trophozoites and cysts adhered to the surfaces of plastic and contact lens, we further investigated the inhibitory activity of the extracts to eliminate the parasite on the surface. Treatment of the plastic plates containing monolayer of trophozoites and/or cysts was performed to eliminate the parasite. Of more than 75% elimination in *Acanthamoeba* trophozoite and cyst adhesion to the surface was observed after the treatment with *A. muricata* and *C. trifoliatum* extract at 4×MIC (Figure 5). The final concentration of 2% DMSO presented in the extracts was used in cyst experiment. However, this concentration of DMSO did not affect the growth and morphology of *A. triangularis* as observed by trypan blue exclusion assay and inverted microscope (Fig. S1), respectively.

Table 4. Effects of *A. muricata* and *C. trifoliatum* extracts in combination with Chlorhexidine against *A. triangularis* trophozoites and cysts.

Growth stages	Concentrations of antimicrobial agents			FIC index	Description
	Chlorhexidine	Plants			
		A. muricata	*C. trifoliatum*		
Trophozoites	1/8 × MIC	1/4 × MIC	ND	0.375	Synergy
	1/8 × MIC	ND	1/2 × MIC	0.625	Partial synergy
Cysts	1/4 × MIC	1/2 × MIC	ND	0.750	Partial synergy
	1/2 × MIC	ND	1/4 × MIC	0.750	Partial synergy

MIC of *A. muricata*, *C. trifoliatum* and chlorhexidine against the trophozoites were 500, 500, and 16 μg/mL, respectively.
MIC of *A. muricata*, *C. trifoliatum* and chlorhexidine against the cysts were 1,000, 1,000, and 64 μg/mL, respectively.

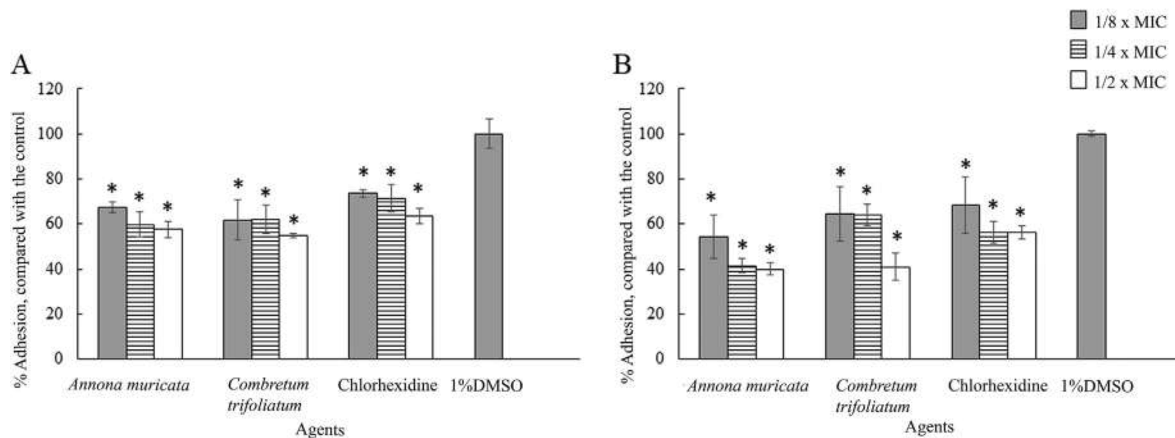

Figure 1. Effects of *A. muricata* and *C. trifoliatum* extracts on adhesion of *A. triangularis* WU19001 trophozoites (A) and cysts (B) at 24 h. The organism was treated with different sub-inhibitory concentrations of the agents, incubated at room temperature for 24 and 48 h. Inhibitory activity was carried out using crystal violet assay. Chlorhexidine and 1% DMSO were used as positive and negative controls, respectively. The relative percentage of the adherence was defined as: (mean of the treated cells/mean of the negative control) × 100, (*significant difference; $P < 0.05$).

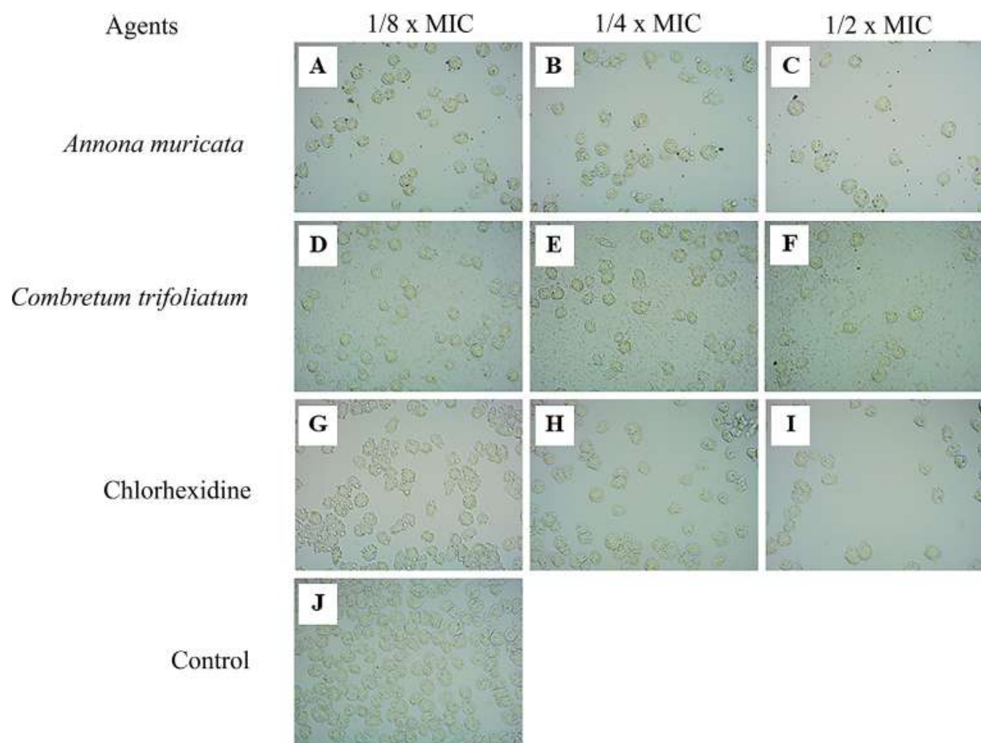

Figure 2. Effects of *A. muricata* and *C. trifoliatum* extracts on adhesion of *A. triangularis* WU19001 trophozoites at 24 h. The cells were grown in PYG medium, and treated with the agents at different concentrations, incubated for 24 h. Chlorhexidine and 1% DMSO was included as positive and negative control, respectively. Images of the adhesion were observed by inverted microscope (200X).

3.8. A. muricata and C. trifoliatum extracts reduced the adhesion of A. triangularis to contact lens

Both extracts of *A. muricata* and *C. trifoliatum* reduced the adhesion of *A. triangularis* trophozoites and cysts on the polystyrene surface. Therefore, we performed the effects of the extracts on the adhesion of *A. triangularis* to contact lenses to apply the potential extracts as the agent for cleaning of contact lens. It was found that the adhesion of *A. triangularis* was substantially inhibited by both the extracts at 1/2×MIC (Figure 6). Nearly, 1 log cells/mL of the trophozoites was decreased when the cells were treated with *A. muricata* and *C. trifoliatum* extracts at 1/2×MIC compared to the control. Additionally, the extracts at 1/2×MIC marginally inhibited the adhesion of the cysts on the lens surface.

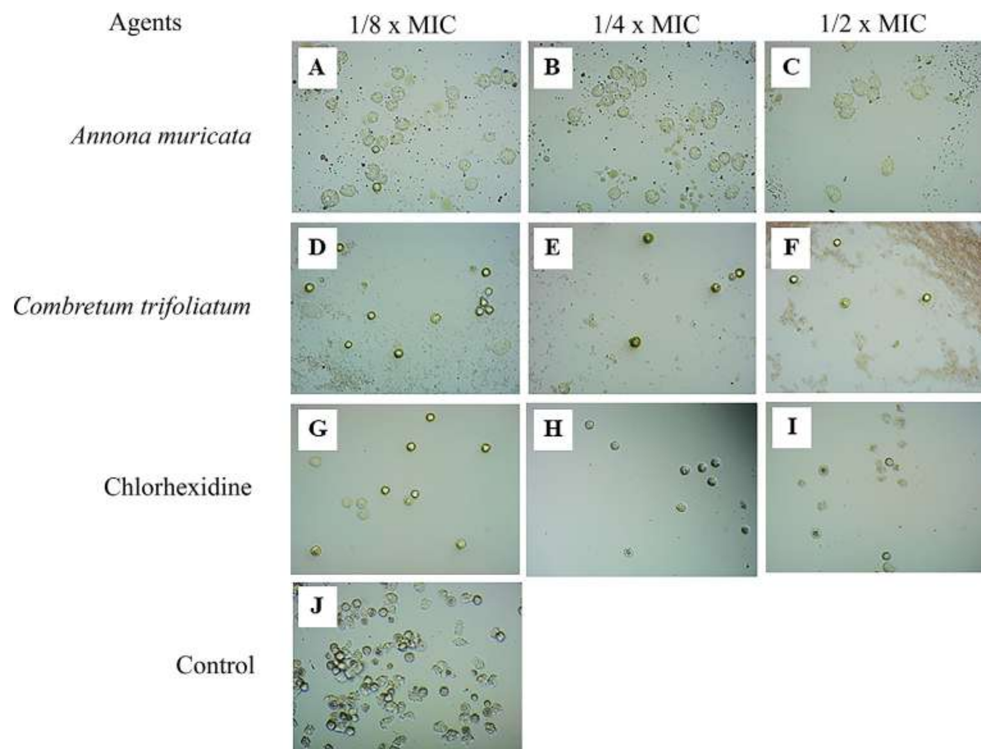

Figure 3. Effects of *A. muricata* and *C. trifoliatum* extracts on adhesion of *A. triangularis* WU19001 cysts at 24 h. The cells were grown in PYG medium, and treated with the agents at different concentrations, incubated for 24 h. Chlorhexidine and 1% DMSO was included as positive and negative control, respectively. Images of the adhesion were observed by inverted microscope (200X).

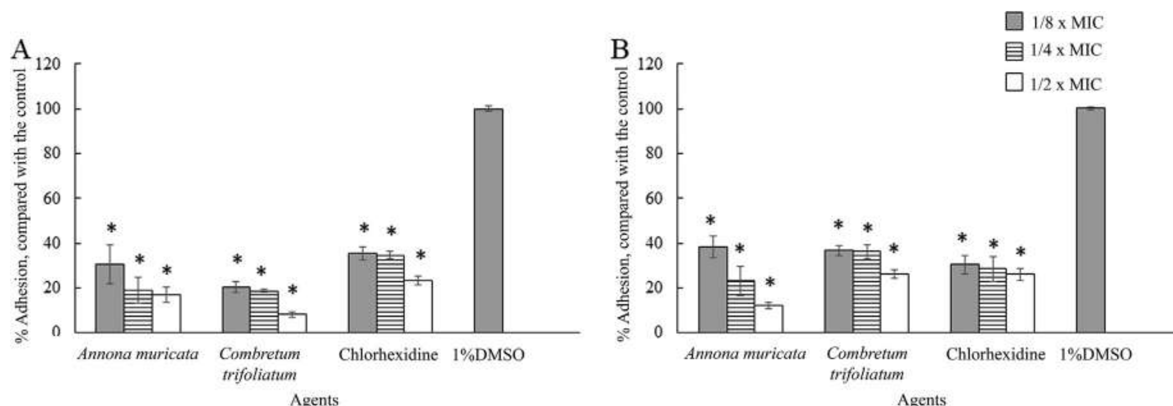

Figure 4. Prevention of adhesion of *A. triangularis* trophozoites (A) and cysts (B) to the plastic surface by *A. muricata* and *C. trifoliatum* extracts. The surface was treated with the extracts at different concentrations, kept at 4 °C for 24 h. Then, the parasitic cells were added, incubated at room temperature for 24 h. Inhibitory activity was carried out using crystal violet assay. Chlorhexidine and 1% DMSO were used as positive and negative controls, respectively. The relative percentage of the adherence was defined as: (mean of the treated cells/mean of the negative control) × 100, (*significant difference; $P < 0.05$).

3.9. Morphology of A. triangularis post-treatment with A. muricata and C. trifoliatum extracts

The morphology of trophozoites and cysts treated with both extracts was observed by SEM. Amoeboid cells with many envelop spikes of *A. triangularis* trophozoites were noted in the control group (Figures 7J-7L). It was found that the trophozoites contiguously adhered to the surface by several long acanthopodia (Figure 7K). While, it was observed that cells changed to abnormal shape when treated with *A. muricata* (Figures 7A-7C) and *C. trifoliatum* extracts (Figures 7D-7F). The treated cells showed lump shape like cystic form. Interestingly, the trophozoites cells had lost their mobility to each other and began to shrink after treatment with *A. muricata* and *C. trifoliatum* extracts. It has been highlighted that trophozoites treated *A. muricata* and *C. trifoliatum* extracts have lost robust acanthopodia (Figures 7A, 7B, 7D, and 7E). Dried shape of the cells and pore formation were detected following treatment with chlorhexidine (Figures 7G-7I).

The normal morphological characteristics such as triangular shape and soft surface of the cysts were observed in the control (Figures 8J-8L). It has been highlighted that the cysts treated with *A. muricata* (Figures 8A-8C) and *C. trifoliatum* (Figures 8D-8F) extracts demonstrated forms of retraction, compared with the control. Furthermore, folded cysts were observed when the cysts were treated with

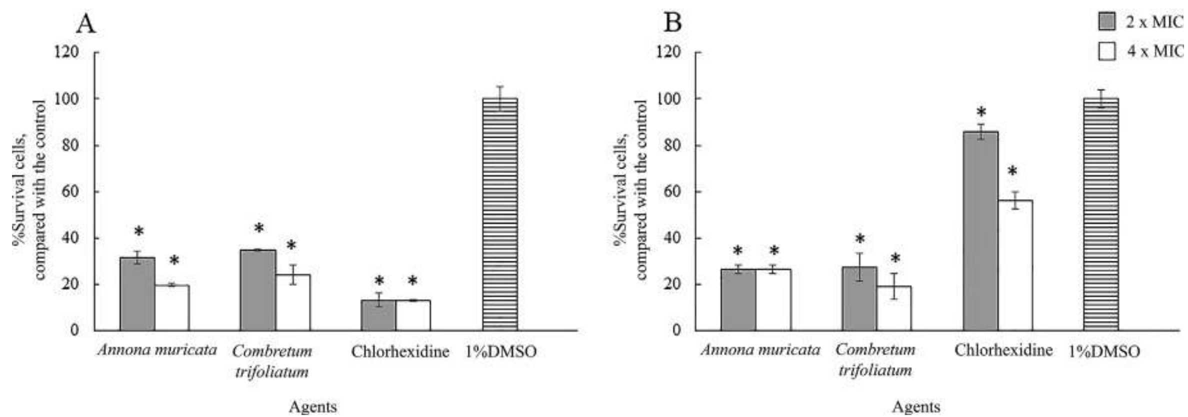

Figure 5. Effects of *A. muricata* and *C. trifoliatum* extracts on elimination of *A. triangularis* WU19001 trophozoites (A) cysts (B). The organism was cultured in PYG medium to form monolayer cells on 96 well plate at 24 h. The parasite was further treated with different concentrations of the agents, incubated at room temperature for 24 h. Inhibitory activity was carried out using crystal violet assay. Chlorhexidine was included as a positive control. While, 1% DMSO and 2% DMSO were used as negative controls for the trophozoites and cysts, respectively. The data was presented as mean ± SD (*significant difference; $P < 0.05$).

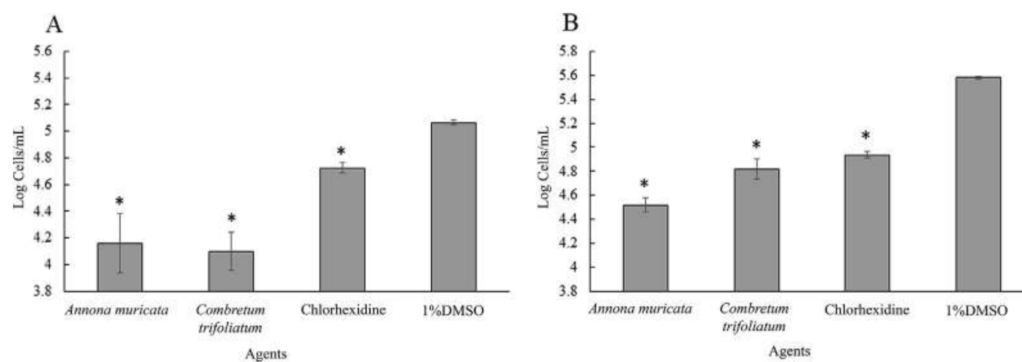

Figure 6. Effects of *A. muricata* and *C. trifoliatum* extracts on adhesion of *A. triangularis* WU19001 trophozoites (A) cysts (B) on contact lens. The organism was treated with 1/2×MIC of the agents, incubated at room temperature for 24 h. Inhibitory activity was carried out using cell counting by trypan blue exclusion assay. Chlorhexidine and 1% DMSO were used as positive and negative controls, respectively. The data was presented as mean ± SD (*significant difference; $P < 0.05$).

C. trifoliatum (Figures 8D-8F) and chlorhexidine (Figures 8G-8I). Additionally, the cell wall surface of shrink cysts treated with *C. trifoliatum* (Figure 8F) was vaguely perturbed when compared with the control and chlorhexidine treated cells. The final concentration of DMSO presented in the extracts at 4×MIC was 2%. However, this concentration of DMSO did not affect the growth and morphology of *A. triangularis* as observed by trypan blue exclusion assay and inverted microscope (Fig. S1), respectively.

4. Discussion

Acanthamoeba spp. is responsible for several infectious related diseases across the globe. As such, the resistance of its cystic stage has been a major factor to its potent strength against existing antibiotics. However, herbal-drug combination appears promising for the management of diseases caused by this parasite. In this study, we focused on plant-derived compounds that possess anti-*Acanthamoeba* activity as therapeutic strategy towards an efficient method for the management of this pathogenic parasite.

Our study assessed the anti-*Acanthamoeba* activity of Cambodian medicinal plants, including *A. muricata* and *C. trifoliatum* extracts against trophozoites and cysts of *A. triangularis*. To support our findings, amebic metabolic activity of *A. castellanii* was reduced following its treatment with multipurpose solutions containing *A. muricata* extract. Moreover, the solution suppressed pseudocyst formation in the organism (Ramírez et al., 2018). *A. muricata* aqueous leaf extract inhibited the growth of *Plasmodium berghei* infected mice with no toxicity (Somsak et al., 2016). It has also been described that ethyl acetate extract of *A. muricata* leaves exhibited anti-microbial activity against *Leishmania* spp. and *Trypanosoma cruzi* (Osorio et al., 2007). Besides, anthelmintic effects of the aqueous leaf extract against eggs, infective larvae and adult forms of *Haemonchus contortus* isolated from sheep have been reported (Ferreira et al., 2013).

The plant species contained several phytochemicals including alkaloids, megastigmanes, flavonol triglycosides, phenolics, cyclopeptides, and essential oils (Moghadamtousi et al., 2015). It has been reported that acetogenins are the most prevalent bioactive compounds of Annonaceae family, including *A. muricata*. Acetogenins is a unique group of derivatives of long chain fatty acids (Sun et al., 2016). Acetogenins has been documented to have anti-proliferation activity on human prostate cancer cell PC-3 (Sun et al., 2016). So far, there is dearth of information of these plant activities against *Acanthamoeba* spp.

Combretum species are widely used in traditional medicine against many infectious diseases including malaria (de Morais Lima et al., 2012). *Combretum mole* extract had anti-plasmodial activity against *P. berghei* in Swiss albino mice (Anato and Ketema, 2018). In addition, *C. fragrans* and *C. padoides* extracts revealed marked inhibition against Gram-positive bacteria such as *Staphylococcus aureus*, *S. epidermidis* as well as *Enterobacter aerogenes*, a Gram-negative bacterium (Fyhrquist et al., 2002). Moreover, there is no report on the activity of *Combretum* species including *C. trifoliatum* extracts against free-living amoeba including *Acanthamoeba* spp. Therefore, this study has revealed the anti-*Acanthamoeba* activity of *C. trifoliatum* extracts against both trophozoites and cysts of *A. triangularis*. To our knowledge, for the first time, the

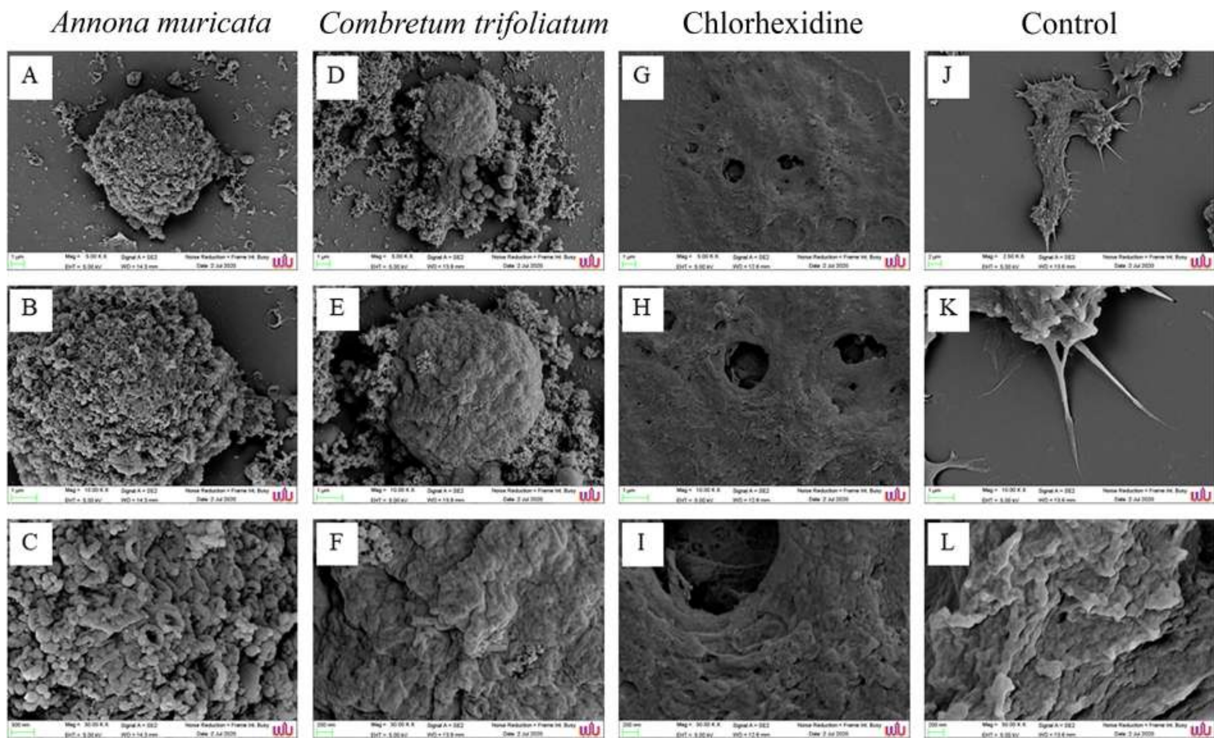

Figure 7. Morphology of *A. triangularis* trophozoites after treatment with *A. muricata* (A–C) and *C. trifoliatum* (D–F) extract observed by SEM. The cells were treated with the extracts at 4×MIC. Chlorhexidine (G–I) and 1% DMSO (J–L) were used as positive and negative control, respectively. Magnifications were revealed as: J = 2,500X; A, B, E, D, G, H, K = 10,000X; C, F, I, L = 30,000X.

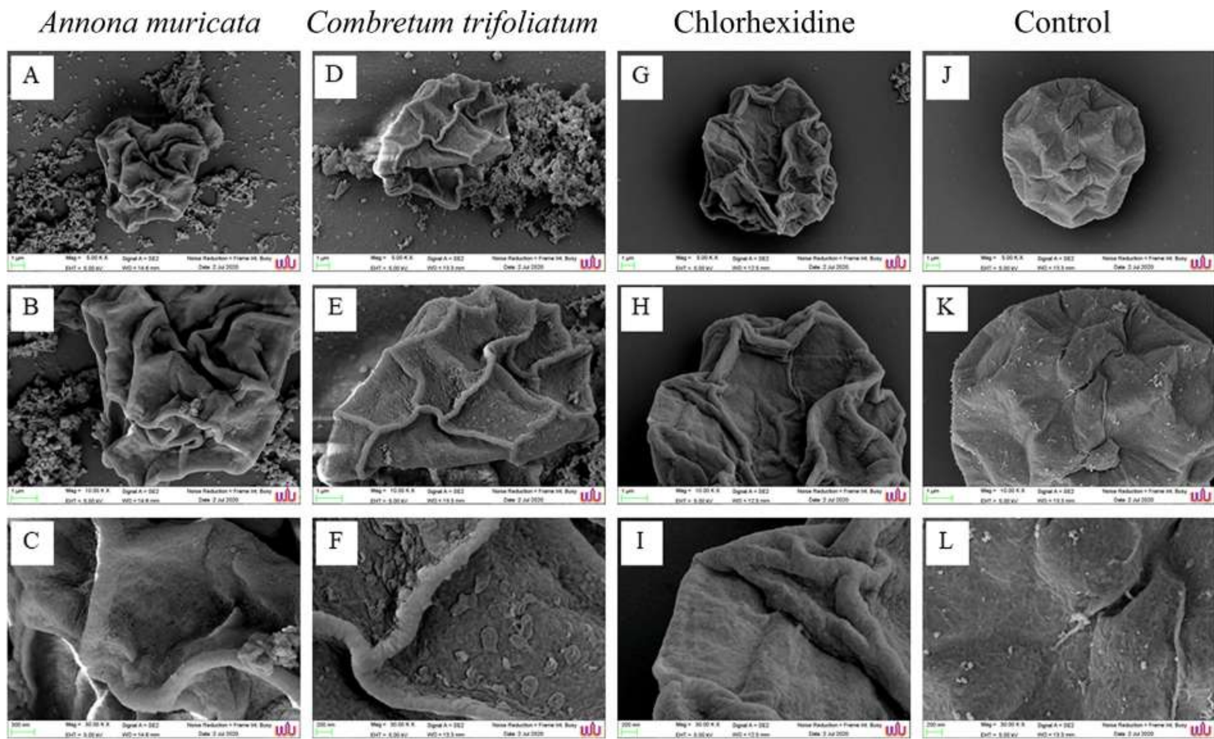

Figure 8. Morphology of *A. triangularis* cysts after treatment with *A. muricata* (A–C) and *C. trifoliatum* (D–F) extract observed by SEM. The cells were treated with the extracts at 4 × MIC. Chlorhexidine (G–I) and 1% DMSO (J–L) were used as positive and negative control, respectively. Magnifications were revealed as: A, D, G, J = 5,000X; B, E, H, K = 10,000X; C, F, I, L = 30,000X.

evidence-based report on *A. triangularis* inhibition with this plant species is described.

C. trifoliatum extract combined with chlorhexidine revealed a partial synergy against *A. triangularis* of both trophozoites and cysts. Similarly, synergistic effects of the plant in the genus *Combretum* spp. in combination with antibiotics against bacteria have been reported (Chukwujekwu and van Staden, 2016). It has been highlighted that the combination of *A. muricata* and chlorhexidine at sub-MICs demonstrated synergy against the trophozoites. Combination therapy of antibiotics plus antibiotics or other bio-active compounds to treat infectious diseases such as tuberculosis is gradually becoming a subject of interest and applied to others. Currently, treatment of *Acanthamoeba* infections comprises of drug combination therapy of biguanides, amidines, and azoles (Sifaoui et al., 2020). Also, synergistic effects of chlorhexidine plus cationic carbosilane dendrimers against *A. polyphaga* trophozoites and cysts have been documented (Heredero-Bermejo et al., 2016). Essentially, herbal-based combinations could reduce drugs cytotoxicity, cost effect, and the requirement for long-term treatment (Sifaoui et al., 2020).

Our study has demonstrated that *A. muricata* and *C. trifoliatum* extracts significantly exhibited anti-*A. triangularis* adhesion to the plastic surface and contact lens. This result is in agreement with the previous study reported on *A. muricata* inhibited the adhesion of *Streptococcus mutans* on hydroxyapatite discs, resulting in plaque forming inhibition (Rahman et al.,2018). It was also observed that flat and adjacent trophozoites adhered to the surface via several acanthopodia in the control, while the treated trophozoites demonstrated shrunken cells. Moreover, the trophozoites treated with *A. muricata* and *C. trifoliatum* extracts displayed a lump shape like cystic form. In addition, small pores formation was also noted when the cells were treated with *A. muricata* extract. It has been emphasized that the trophozoites treated with both *A. muricata* and *C. trifoliatum* extracts have lost strong acanthopodia. Similarly, *A. lugdunensis* L3a trophozoites treated with contact lens care multiuse solutions demonstrated a shrunk-like cystic shape (Lee et al., 2017). Clearly, *A. triangularis* cysts treated with *A. muricata* and *C. trifoliatum* extracts demonstrated deformities of retraction and shrink cells, compared with the smooth surface control.

Acanthopodia have been considered as the main adhesion structure of the organism to attach to the surfaces such as contact lenses (Lee et al., 2017). A high number of the acanthopodia was detected from the pathogenic *Acanthamoeba* while the non-pathogenic parasites possessed less numbers of acanthopodia (Siddiqui and Khan, 2012). Furthermore, the adhesion of the pathogenic trophozoites to corneal cells was mediated by the acanthopodium spikes (spike-like pseudopodium) Khan (2001). It has been reported that absence of acanthopodia in *Acanthamoeba* trophozoites could not adhere to the corneal epithelial cells (Khan, 2004). In general, a mannose-binding protein participating in the adhesion of *Acanthamoeba* spp. to the host cells is expressed and located at acanthopodia (Garate et al., 2005). In addition, it has been documented that the suppression of mannose-binding protein reduced the binding of the organism to the corneal cells (Garate et al., 2006). Hence, the loss of acanthopodia after treatment with *A. muricata* and *C. trifoliatum* extracts could inhibit *Acanthamoeba* adhesion to the surface. We hypothesized that the loss of acanthopodia and the presence of shrunk cells after treatment with the extracts could reduce adhesion of parasites to the surface.

Overall, the results demonstrated that *A. muricata and C. trifoliatum extracts* showed anti-*Acanthamoeba* and anti-adhesion activities against *A. triangularis*. Though, isolation of bio-active compounds presented in the plant species could not be possible due to the limitation of related facilities such as HPLC. Therefore, the combination of the extracts and available drug, chlorhexidine, has been used as an alternative approach for amoebicidal activities against *Acanthamoeba*. To support this, nanoparticle synthesis using plant extracts or plant-derive compounds has been reported as one option to enhance the efficiency of these compounds against the pathogens. Recently, there has been a report on the synthesis of nanoparticles using gallic acid, a component of *Leea indica* loaded in poly-D, L-lactide-co-glycolide nanoparticles inhibited the growth of trophozoites and cysts of *A. triangularis* (Mahboob et al., 2020). In light of our promising results, future study is strongly recommended to investigate the mechanism of the pure compounds in terms of nano-synthesis, metabolomics or docking simulation that will further enhance the discovery on the drug target of *Acanthamoeba* infection.

5. Conclusion

In summary, this research demonstrated that *A. muricata* and *C. trifoliatum* extracts substantially inhibited the growth of *A. triangularis* trophozoites and cysts. Synergistic effect of *A. muricata* extract combined with chlorhexidine against *A. triangularis* trophozoites was observed. Furthermore, both *A. muricata* and *C. trifoliatum* extracts showed partial synergy in combination with chlorhexidine against *A. triangularis* cysts. Both extracts considerably inhibited the adhesion of *A. triangularis* trophozoites and cysts to the plastic surface. Also, pre-treatment of the plastic surface with *A. muricata* at 1/2×MIC significantly diminished 90% of the cyst adhesion, compared with the control. It has been highlighted that the trophozoites treated with *A. muricata* and *C. trifoliatum* extracts have lost strong acanthopodia.

Declarations

Author contribution statement

Watcharapong Mitsuwan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Chea Sin, Samell Keo: Performed the experiments; Analyzed and interpreted the data.

Suthinee Sangkanu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Maria de Lourdes Pereira, Tajudeen O. Jimoh: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Christina C. Salibay, Muhammad Nawaz, Roghayeh Norouzi, Abolghasem Siyadatpanah, Christophe Wiart, Polrat Wilairatana, Polydor Ngoy Mutombo: Contributed reagents, materials, analysis tools or data.

Veeranoot Nissapatorn: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

No data was used for the research described in the article.

Competing interest statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2021.e06976.

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