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

Watcharapong Mitsuwana,b, Chea Sinse, Samell Keod, Suthinee Sangkanua, Maria de Lourdes Pereirae, Tajudeen O. Jimohf,g, Cristina C. Salibayh, Muhammad Nawaz, Roghayeh Norouzi, Abolghasem Siyadatpanahk, Christophe Wiartl, Polrat Wilairatanam, Polydor Ngoy Mutombon, Veeranoot Nissapatorna,*

School of Allied Health Sciences, Southeast Asia Water Team (SEA Water Team), World Union for Herbal Drug Discovery (WUHeDD), and Research Excellence Center for Innovation and Health Products (RECIHP), Walailak University, Nakhon Si Thammarat, Thailand

Akhhraratchakumari Veterinary College and Research Center of Excellence in Innovation of Essential Oil, Walailak University, Nakhon Si Thammarat, Thailand

Faculty of Health Sciences, University of Puthisastra, Phnom Penh, Cambodia

Academic Center for Education and Training (ACET), Phnom Penh, Cambodia

CICECO-Aveiro Institute of Materials and Department of Medical Sciences, University of Aveiro, Aveiro, Portugal

Akkhraratchakumari Veterinary College and Research Center of Excellence in Innovation of Essential Oil, Walailak University, Nakhon Si Thammarat, Thailand

Faculty of Health Sciences, University of Puthisastra, Phnom Penh, Cambodia

Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

Department of Biochemistry, Habib Medical School, Islamic University in Uganda, Kambala, Uganda

College of Science and Computer Studies, De La Salle University-Dasmariñas, Dasmariñas City, Cavite, Philippines

Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

Department of Nano-Medicine Research, Institute for Research and Medical Consultations (IRMC), Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia

Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

Ferdows School of Paramedical and Health, Birjand University of Medical Sciences, Birjand, Iran

School of Pharmacy, University of Nottingham Malaysia Campus, Selanger, Malaysia

Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Thailand

Independent Consultant, Neglected Tropical Diseases, Melbourne, Victoria, Australia

ARTICLE INFO

Keywords:
- \textit{Acanthamoeba triangularis}
- Adhesion
- \textit{Annona muricata}
- \textit{Combretum trifoliatum}
- Synergistic effects

ABSTRACT

Plants with medicinal properties have been used in the treatment of several infectious diseases, including \textit{Acanthamoeba} infections. The medicinal properties of Cambodian plant extracts; \textit{Annona muricata} and \textit{Combretum trifoliatum} were investigated against \textit{Acanthamoeba triangularis}. A total of 39 plant extracts were evaluated and, as a result, 22 extracts showed positive anti-\textit{Acanthamoeba} activity. Of the 22 extracts, 9 and 4 extracts showed anti-\textit{Acanthamoeba} activity against trophozoites and cysts of \textit{A. triangularis}, respectively. The minimum inhibitory concentration of \textit{A. muricata} and \textit{C. trifoliatum} extracts against trophozoites and cysts was 500 and 1,000 μg/mL, respectively. The combination of \textit{A. muricata} at 1/4×MIC with chlorhexidine at 1/8×C2MIC 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 \textit{A. muricata} and \textit{C. trifoliatum} extracts, respectively. A 90% of cysts adhered to the surface was decreased with pre-treatment of \textit{A. muricata} at 1/2×MIC (P < 0.05). A 75% of trophozoites and cysts from \textit{Acanthamoeba} adhered to the surface were removed after 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 \textit{A. muricata} and \textit{C. trifoliatum} as an option treatment against \textit{Acanthamoeba} infections.

* Corresponding author.
E-mail address: nissapat@gmail.com (V. Nissapatorn).

https://doi.org/10.1016/j.heliyon.2021.e06976
Received 2 November 2020; Received in revised form 17 December 2020; Accepted 27 April 2021
2405-8440/© 2021 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
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 (Jerice et al., 2019; Bunsuansa-kul 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 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 (Mohgahamtousi et al., 2015). It has been documented that the extract of *A. muricata* exhibited anti-microbial activity against *A. castellanii* (Mohgahamtousi 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...
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 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% DMSO and stored at 4 °C.

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, 2 g proteose peptone, 2 g yeast extract, 0.98 g MgSO₄ × 7H₂O, 1 g sodium citrate dihydrate, 0.02 g Fe(NH₄)₂(SO₄)₂ × 6H₂O, 0.34 g KH₂PO₄, 0.355 g Na₂HPO₄×7H₂O, 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 described 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 × 10⁶ 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 (Bag et al., 2013). The relative percentage of cell viability was specified as: (mean of the treated parasite/mean of the control) × 100. Selection of the extracts was carried out when they showed >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 × 10⁴ 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 >90% the growth inhibition (mean ± 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 ≤ FICI <1.0 = partial synergy
- FICI = 1.0 = additive
- FICI >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 coverslip 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 cm²) 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 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 \( A570 \text{ nm of treated well/mean } A570 \text{ nm of control well} \) × 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 \( A570 \text{ nm of treated well/mean } A570 \text{ nm of control well} \) × 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 showed ≥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) |
|------|---------------------------------------|------------------------------|
|      | Tropozoites                           | Cysts                        |
| P001 | Buereria laevis Lindi                  | 60.00 ± 10.00                | 42.11 ± 5.26                |
| P002 | Asarada 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 | Xylosia xylocarpa                     | 56.67 ± 5.77                 | 45.17 ± 5.58                |
| P012 | Salacia chinensis                     | 86.67 ± 5.77                 | 83.88 ± 5.58                |
| P013 | Carapinopsis sappan                   | 60.00 ± 10.00                | 58.07 ± 5.58                |
| P018 | Ecora chinensis                       | 73.34 ± 5.77                 | 74.29 ± 5.58                |
| P019 | Anacardium occidentale                | 90.00 ± 0.00                 | 87.10 ± 5.58                |
| P060 | Althea lebeck                          | 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                 | 74.29 ± 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) | Tropozoites | 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       |       |
| *Althaea lebeck*       | 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* | |
| Tropozoites   | 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.
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 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).
3.9. 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...
negative controls for the trophozoites and cysts, respectively. The data was presented as mean 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 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 cell counting by trypan blue exclusion 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 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).

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., 2013). 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, D = 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.

*Combretum* 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-Bermego 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 in 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-derivate 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 Wilairatanata, Polydor Ngoy Mutombo: Contributed reagents, materials, analysis tools or data.

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

**Funding statement**

This work was supported by the project entitled “Medicinal under-exploited Thai native plant against *Acanthamoeba, Leishmania donovani,* and *Plasmodium falciparum* -Toward South East Asia collaboration initiative (Grant No. WUBG020-2564)” supported by The Royal Patronage of Her Royal Highness Princess Maha Chakri Sirindhorn, Walailak University, Thailand.

**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.
Acknowledgements

Authors are thankful to the Research Institute of Health Science, Walailak University for the laboratory facilities provided for these research activities. We would like to thank Research Center of Excellence in Innovation of Essential Oil, Walailak University and the project CICECO-Aveiro Institute of Materials, UIDB/50011/2020 and UIDP/50011/2020, national funds by FCT/MCTES.

References

Anato, M., Ketema, T., 2018. Anti-plasmodial activities of Combretum molle (Combretaceae) (Zooz) seed extract in Swiss albino mice. BMC Res. Notes 11, 312.

Baig, A.M., Iqbal, J., Khan, N.A., 2013. In vitro efficacies of clinically available drugs against growth and viability of an Acanthamoeba castellanii keratitis isolate belonging to the T4 genotype. Antimicrob. Agents Chemother. 57, 3561–3567.

Bunswansaksak, C., Mahboob, T., Hounkong, K., Laohaprapanon, S., Chitapornpan, S., Jawjit, S., Yasiri, A., Barurusux, S., Bunlesuek, K., Sawangjaroen, N., Salibay, C., Kaewjai, C., Pereira, M.L., Nissapatorn, V., 2019. Acanthamoeba in Southeast Asia: overview and challenges. Kor. J. Parasitol. 57, 341–357.

Chukwujezokwu, J.C., van Staden, J., 2016. In vitro antibacterial activity of Combretum edwardii, Combretum kraussii, and Maytenus nemerosa and their synergistic effects in combination with antibiotics. Front. Pharmacol. 7, 208.

de Morais Lima, G.R., De Sales, I.R.P., Caldas Filho, M.R.B., De Jesus, N.Z.T., de Sousa Fulcao, H., Barbosa-Filho, J.M., Cabral, A.G.S., Souso, A.L., Tavares, J.F., Baista, L.M., 2012. Bioactivities of the genus Combretum (Combretaceae): a review. Molecules 17, 9142–9206.

Ferreira, L., Castro, P., Chagas, A., França, S., Beleboni, R., 2013. In vitro anthelmintic activity of aqueous leaf extract of Annona muricata L. (Annonaceae) against Haemonchus contortus from sheep. Exp. Parasitol. 134, 327–332.

Fyhruquist, P., Mwaisukuli, L., Haggstrom, C.A., Vuorela, H., Hiltunen, R., Vuorela, P., 2002. Ethnobotanical and antimicrobial investigation on some species of Terminalia and Combretum (Combretaceae) growing in Tanzania. J. Ethnopharmacol. 79, 169–177.

Garate, M., Cubillos, I., Marchant, J., Panjwani, N., 2005. Biochemical characterization and functional studies of Acanthamoeba mannose-binding protein. Infect. Immun. 73, 5775–5781.

Garate, M., Marchant, J., Cubillos, I., Cao, Z., Khan, N.A., Panjwani, N., 2006. In vitro pathogenicity of Acanthamoeba is associated with the expression of the mannose-binding protein. Invest. Ophthalmol. Vis. Sci. 47, 1056–1062.

Hay, J., Kirkness, C.M., Seal, D.V., Wright, P., 1994. Drug resistance and Acanthamoeba keratitis: the quest for alternative antiprotosomal chemotherapy. Eye 8, 555–563.

Herederoberrejio, I., Sanchez-Nieves, J., Solivert, J., Gomez, R., De la Mata, F., Capanino, J., Perez-Serrazano, J., 2016. In vitro anti-Acanthamoeba synergistic effect of chlorhexidine and cationic carbosilane dendrimers against both trophozoite and cyst forms. Int. J. Pharm. 509, 1–7.

Hwang, I.S., Hwang, J.H., Choi, H., Kim, K.I., Lee, D.G., 2012. Synergistic effects between silver nanoparticles and antibiotics and the mechanisms involved in. J. Med. Microbiol. 61, 1719–1726.

Jercic, M.I., Aguayo, C., Saladriaga-Córdoba, M., Muino, L., Chenet, S.M., Lagos, J., Osuna, A., Fernández, J., 2019. Genotypic diversity of Acanthamoeba strains isolated from Chilean patients with Acanthamoeba keratitis. Parasites Vectors 12, 58.

Kaya, Y., Baldemir, A., Karaman, Ü., Ilbaz, N., Arici, Y.K., Kaçmaz, G., Koleren, Z., Konca, Y., 2019. Amebicidal effects of fenugreek (Trigonella foenum-graecum) against Acanthamoeba cysts. Food Sci. Nutr. 7, 563–571.

Khankhan, N.A., 2001. Pathogenicity, morphology, and differentiation of Acanthamoeba. Curr. Microbiol. 43, 391–395.

Khankhan, N.A., 2004. The pathogenesis of Acanthamoeba infections: current status and future implications. Eivetext. Monograph. 1–19.

Lee, S.M., Lee, J.E., Lee, D.I., Yu, H.S., 2017. Adhesion of Acanthamoeba on cosmetic contact lenses. J. Kor. Med. Sci. 33, e26.

Mahboob, T., Nawar, M., Pereira, M.L., Tam-Chye, T., Samadi, C., Jekaran, S.D., Wiatr, C., Nissapatorn, V., 2020. PLGA nanoparticles loaded with Gallic acid: a constituent of Leea indica against Acanthamoeba triangularis. Sci. Rep. 10, 8954.

Mitsuwan, W., Bunsuwanwansuk, C., Leonard, T.E., Laohaprapanon, S., Hounkong, K., Bunlesuek, K., Kaewjai, C., Mahboob, T., Sumudi Raja, D., Dhibi, M., et al., 2020a. Curcuma longa ethanol extract and Curcumin inhibit the growth of Acanthamoeba triangularis trophozoites and cysts isolated from water reservoirs at Walailak University, Thailand. Pathog. Glob. Health 114, 194–204.

Mitsuwan, W., Sangkanu, S., Romyasamit, C., Kaewjai, C., Simoh, T.O., Pereira, M.L., Siyadatpanah, A., Kayesth, S., Nawar, M., Rahamatulailah, M., Butler, M.S., Wilsairatana, P., Wiatr, C., Nissapatorn, V., 2020b. Curcuma longa rhizome extract and Curcumin reduce the adhesion of Acanthamoeba triangularis trophozoites and cysts in polystyrene plastic surface and contact lens. Int. J. Parasitol. Drugs Drug. Resist. 14, 218–229.

Moghaddamtousi, S.Z., Fadaeinainab, M., Nikzad, S., Mohan, G., Ali, H.M., Kadir, H.A., 2015. Annona muricata (Annonaceae): a review of its traditional use, isolated acetogenins and biological activities. Int. J. Mol. Sci. 16, 15625–15658.

Moghadamtousi, S.Z., Fadaeinasab, M., Nikzad, S., Mohan, G., Ali, H.M., Kadir, H.A., 2015. Annona muricata (Annonaceae): a review of its traditional use, isolated acetogenins and biological activities. Int. J. Mol. Sci. 16, 15625–15658.

Neff, O., Barrios-Torres, R.L., Piñero, J.E., 2018. Combined amoebicidal effect of atorvastatin and commercial eye drops against Acanthamoeba triangularis keratitis isolate belonging to the T4 genotype. Antimicrob. Agents Chemother. 52, 3561–3567.

Neff, O., Barrios-Torres, R.L., Piñero, J.E., 2018. Combined amoebicidal effect of atorvastatin and commercial eye drops against Acanthamoeba triangularis keratitis isolate belonging to the T4 genotype. Antimicrob. Agents Chemother. 52, 3561–3567.

Osuna, A., Fernandez-Alvarado, J., Robledo, M., 2007. Antiprotozoal and cytotoxic activities in vitro and in vivo of Colombian Annonaceae. J. Ethnopharmacol. 111, 630–635.

Rahman, F.A., Hanafi, T., Uami, T.W., 2018. The effect of ethanol extract of sour sop (Annona muricata L.) on adhesion of Streptococcus mutans ATCC 25668 to hydroxyapatite discs. Majalah. Kedokteran. Gigi. Indonesia. 4, 22–26.

Ramírez, P.Y.P., Villavicencio, L.L.F., Ortega, A.F.R., Ramos, S.S., Domínguez, J.P.C., Castro, J.C.V., 2018. Actividad antiparasitaria de Annona muricata contra Acanthamoeba castellanii. Jovenes. In. La Ciencia. 3, 215–219.

Siddiqui, R., Khan, N.A., 2012. Biology and pathogenesis of Acanthamoeba. Parasites Vectors 5, 6.

Sifanou, I., Capote Yanes, E., Reyes-Batlle, M., Rodriguez-Exposito, R.L., Pizero, J.E., Lorenzo-Morales, J., 2020. Combined amoebicidal effect of atorvastatin and commercial eye drops against Acanthamoeba castellanii Neff: In vitro assay based on mixture design. Phathogens 9, 219.

Somah, V., Polwong, N., Chachiyon, S., 2016. In vivo antimalarial activity of Annona muricata leaf extract in mice infected with Plasmodium berghei. J. Pathog. 2016, 3264070.

Sudjana, A.N., Carson, C.F., Carson, K.C., Riley, T.V., Hammer, K.A., 2012. Candida albicans adhesion to human epithelial cells and polystyrene and formation of biofilm is reduced by sub-inhibitory Melaleuca alternifolia (tea tree) essential oil. Med. Mycol. 50, 863–870.

Sun, S., Liu, J., Zhou, N., Zhu, W., Dou, Q.P., Zhou, K., 2016. Isolation of three new Annonaceous acetogenins from Graviola fruit (Annona muricata) and their anti-proliferation on human prostate cancer cell PC-3. Biorg. Med. Chem. Lett 26, 4382–4385.