

## Research Article

**Cite this article:** Boonhok R *et al.* (2021). Amoebicidal activity of *Cassia angustifolia* extract and its effect on *Acanthamoeba triangularis* autophagy-related gene expression at the transcriptional level. *Parasitology* **148**, 1074–1082. <https://doi.org/10.1017/S0031182021000718>

Received: 16 December 2020

Revised: 7 April 2021

Accepted: 29 April 2021

First published online: 10 May 2021

**Keywords:**

*Acanthamoeba triangularis*; autophagy; *Cassia angustifolia*; encystation

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# Amoebicidal activity of *Cassia angustifolia* extract and its effect on *Acanthamoeba triangularis* autophagy-related gene expression at the transcriptional level

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**Abstract**

*Cassia angustifolia* Vahl. plant is used for many therapeutic purposes, for example, in people with constipation, skin diseases, including helminthic and parasitic infections. In our study, we demonstrated an amoebicidal activity of *C. angustifolia* extract against *Acanthamoeba triangularis* trophozoite at a micromolar level. Scanning electron microscopy (SEM) images displayed morphological changes in the *Acanthamoeba* trophozoite, which included the formation of pores in cell membrane and the membrane rupture. In addition to the amoebicidal activity, effects of the extract on surviving trophozoites were observed, which included cyst formation and vacuolization by a microscope and transcriptional expression of *Acanthamoeba* autophagy in response to the stress by quantitative polymerase chain reaction. Our data showed that the surviving trophozoites were not transformed into cysts and the trophozoite number with enlarged vacuole was not significantly different from that of untreated control. Molecular analysis data demonstrated that the mRNA expression of *AcATG* genes was slightly changed. Interestingly, *AcATG16* decreased significantly at 12 h post treatment, which may indicate a transcriptional regulation by the extract or a balance of intracellular signalling pathways in response to the stress, whereas *AcATG3* and *AcATG8b* remained unchanged. Altogether, these data reveal the anti-*Acanthamoeba* activity of *C. angustifolia* extract and the autophagic response in the surviving trophozoites under the plant extract pressure, along with data on the formation of cysts. These represent a promising plant for future drug development. However, further isolation and purification of an active compound and cytotoxicity against human cells are needed, including a study on the autophagic response at the protein level.

**Introduction**

*Acanthamoeba* species are free-living amoebae that commonly inhabit the environment, especially in soil and water. However, some species can be isolated from plants, animals and humans (Khan, 2006; Bunsuwansakul *et al.*, 2019). *Acanthamoeba* spp. are transmitted to humans by different routes, such as ocular, nasal and injured skin that still cause a mild to severe diseases (Neelam and Niederkorn, 2017; de Lacerda and Lira, 2021). In immunocompromised individuals, the clinical presentations may be more severe and may present cutaneous lesions (Morrison *et al.*, 2016), chronic sinusitis (Kim *et al.*, 2000) and granulomatous amoebic encephalitis (Matson *et al.*, 1988) depending on the site of infection. *Acanthamoeba* keratitis is the most frequent ocular disease caused by this protozoan parasite in healthy individuals and has recently been increased among contact lens wearers (Lorenzo-Morales *et al.*, 2015; Neelam and Niederkorn, 2017; Khan *et al.*, 2019). According

to genotypic diversity of *Acanthamoeba* species, the T4 genotype has a major impact on human health as it causes infection in several organs (Juarez *et al.*, 2018). *Acanthamoeba triangularis* is one of the T4 members widely distributed in the environment and has been shown to cause eye infection in humans (Xuan *et al.*, 2008; Hasni *et al.*, 2020). According to the life cycle of an amoeba, trophozoite is a metabolically active/infectious form that can be transmitted to humans. Upon infection, the trophozoite may expand further or become a cyst under stressful conditions. This dormant form is a major obstacle for *Acanthamoeba* treatment, as it is less sensitive to most of the available drugs. The preventing transmission of *Acanthamoeba* is as simple as learning and practicing healthy habits, especially for those who wear contact lenses. However, the available drug for *Acanthamoeba* treatment is quite limited. Chlorhexidine and polyhexamethylene biguanide (PHMB) are the two most common drugs used to treat *Acanthamoeba* spp. However, a recent report on the use of PHMB showed that *A. castellanii* from clinical isolates were resistant to this drug (Huang *et al.*, 2017).

Autophagy is an important intracellular mechanism for cell homeostasis. Damage or defective organelles, including large protein aggregates, are degraded by the autophagy mechanism (Yorimitsu and Klionsky, 2005; Feng *et al.*, 2014). The target proteins are enclosed in a double-membrane structure called autophagosome, which is further fused with lysosome for degradation. The biogenesis of the autophagosome is involved with several autophagy-related (Atg) proteins. In yeast and mammals, more than 30 Atg proteins have been reported (Feng *et al.*, 2014) whereas in the amoeba, a partial list of these Atg proteins, core machinery, is conserved (Picazarri *et al.*, 2008). Based on evidence so far, a role for *Acanthamoeba* autophagy has not been clearly demonstrated. Some Atg proteins have been partially characterized and reported to be associated with the *Acanthamoeba* encystation (Moon *et al.*, 2011, 2013; Song *et al.*, 2012; Kim *et al.*, 2015). Thus, further characterization of the autophagy pathway, including other cellular pathways related to encystation under stress conditions, are of interest. Altogether, evidence of drug resistance and knowledge of *Acanthamoeba* autophagy encourage researchers to pay more attention to drug development and the search for a natural compound or plant extract containing anti-*Acanthamoeba* activity (Elsheikha *et al.*, 2020) as to its ability to induce the amoeba encystation.

In this study, seven plant extracts from West Asia were screened for amoebicidal activity. The extract of *Cassia angustifolia* demonstrated a significant amoebicidal activity against *A. triangularis* trophozoite. Scanning electron microscopy (SEM) images also represented a morphological change in the cell membrane that can lead to cell death. The drug combination was also performed to explore if chlorhexidine could provide a synergistic effect with the plant extract. In addition to the amoebicidal activity, the encystation of surviving *Acanthamoeba* under a microscope and the autophagic response of *Acanthamoeba* at the transcriptional level were investigated. These data opened up another view of the plant extract apart from its cidal activity. Understanding the molecular mechanism in response to stress induced by the plant extract can provide an insight into the future drug development and treatment.

## Materials and methods

### Plant collection and preparation of extracts

Dried medicinal plants were purchased from a perfume market in the eastern province of Azerbaijan, and the species was identified and authenticated in the botany section of the Agricultural Research Center in eastern Azerbaijan: *Artemisia absinthium* L.,

*Cuscuta epithimum*, *Achillea millefolium*, *Ferula assa-foetida*, *Teucrium polium*, *Melissa officinalis* and *C. angustifolia*. The whole dried plant was mechanically powdered using an electrical blender. The ethanolic extracts of plants were prepared by macerating 370 g of dry powdered plants in 70% aqueous ethanol for 3 days at room temperature (RT), and then filtered through filter paper (Whatman Ltd., Buckinghamshire, UK). The alcohol in the sample was removed by using a rotary vacuum evaporator. The extract was concentrated in an incubator at 37°C to obtain the dry powder extract.

### *Acanthamoeba* cultivation

*Acanthamoeba triangularis* strain WU19001 (Mitsuwan *et al.*, 2020) was cultured in 25 cm<sup>2</sup> cell culture flasks containing PYG medium [2% (w/v) proteose peptone, 0.1% (w/v) yeast extract, 400 µM CaCl<sub>2</sub>, 4 mM MgSO<sub>4</sub>, 2.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.5 mM KH<sub>2</sub>PO<sub>4</sub>, 50 µM (NH<sub>4</sub>)<sub>2</sub>Fe(SO<sub>4</sub>)<sub>2</sub>, 100 mM glucose] at 25°C in the dark without shaking (Taravaud *et al.*, 2017). The amoeba trophozoite was kept in PYG medium and replaced with fresh medium every 2 days until the harvest of *Acanthamoeba*. Regarding a cyst induction medium by Aqeel *et al.* (2013), a ready-to-use Page's saline (PAS) powder (HiMedia, Mumbai, India) that contained NaCl, MgSO<sub>4</sub>·7H<sub>2</sub>O, CaCl<sub>2</sub>·2H<sub>2</sub>O, Na<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub> and supplemented with 10% glucose for cyst induction in our laboratory and cultured for 5–7 days was performed. Then, the cysts were harvested by centrifugation.

### Plant extract preparation and determination of anti-*Acanthamoeba* activity

All plant extracts were dissolved in 100% dimethyl sulphoxide (DMSO) and prepared at a stock concentration of 100 mg mL<sup>-1</sup>. The screening of amoebicidal activity was performed on a 96-well black plate (SPL Life Sciences, Seoul, Korea). The plant extract was added at a final concentration of 1000 µg mL<sup>-1</sup>. *Acanthamoeba* trophozoites and cysts were harvested, washed twice with PAS buffer and counted using a haemocytometer. The trophozoite suspension was prepared in PYG medium whereas the cyst suspension was prepared in PAS containing 10% glucose. Amoebae trophozoites were added to the final cell number of 2 × 10<sup>4</sup> cells per well. Cells treated with 5 µg mL<sup>-1</sup> chlorhexidine, 1% DMSO and medium alone were included as controls. Edge wells were filled with PAS buffer to reduce the evaporation rate. After 24 h of treatment, the cells were stained with PrestoBlue<sup>®</sup> dye (Invitrogen, Waltham, USA), a new reagent based on resazurin to assess cell viability and cytotoxicity, in the final dilution of 1:20 for 30 min in an incubator at 37°C, followed by a fluorescence signal quantified by using a microplate reader (BioTek Synergy<sup>™</sup> MX microplate reader, VT, USA) at excitation/emission wavelength of 535/615 nm. The percentage viability of the parasite was then calculated using Prism 5 software (GraphPad Software, CA, USA). DMSO-treated condition was set to 100% cell viability. Then, the percentage of anti-*Acanthamoeba* activity was calculated using the formula: % amoebicidal activity = 100 – % cell viability. All experiments were conducted in triplicate with three independent experiments.

### Determination of minimal inhibitory concentration

The *C. angustifolia* extract which demonstrated the greatest amoebicidal activity was selected for further studies. The minimum inhibitory concentration (MIC) was identified using the microtiter broth dilution method (Mitsuwan *et al.*, 2020). The plant extract was prepared in a transparent 96-well plate (SPL Life Sciences, Seoul, Korea) with a 2-fold serial dilution. The extract

concentrations were 2048, 1024, 512, 256 and 128  $\mu\text{g mL}^{-1}$ , respectively. Then, 100  $\mu\text{L}$  of trophozoites and cysts of  $2 \times 10^5$  cells were added into each well. The plates were incubated at RT in the dark for 24, 48 and 72 h. Then, the cells were stained with Trypan Blue dye, and cell viability was quantified using a light inverted microscope, Eclipse TE2000-S (Nikon, Tokyo, Japan). The MIC value of the extract was defined as the lowest concentration with *Acanthamoeba* growth inhibition >90%.

### Scanning electron microscopy

To further investigate the effect of the *C. angustifolia* extract on the amoeba, the trophozoite and cyst forms were treated separately with 0.25 MIC concentration of 250  $\mu\text{g mL}^{-1}$  of *C. angustifolia* extract in a 24-well plate. Two pieces of sterile glass with high cell-binding affinity,  $3 \times 3 \text{ mm}^2$  in size, were added to the well, the cells were resuspended and allowed to stand for 24 h. The amoeba-coated glass was fixed with 2.5% glutaraldehyde overnight and dehydrated with a series of graded ethanol (20, 40, 60, 80, 90 and 100% ethanol). The samples were mounted on aluminium stubs, dried in a critical point dryer and coated with gold particles (Cressington 108 sputter coater, MA, USA). Then, the amoeba surface structure was observed under SEM (Gemini, Oberkochen, Germany).

### Analysis of cyst formation and vacuolization

The *Acanthamoeba* trophozoites were treated with a *C. angustifolia* extract of 250  $\mu\text{g mL}^{-1}$  and the surviving trophozoites were evaluated for cyst formation and vacuolization under stress induced by the plant extract 24 h after treatment. Cells were stained with Trypan blue and viable cells were analysed. At least 200 cells per condition were analysed for cyst formation and at least 100 surviving trophozoites per condition were examined for vacuole formation under the light microscope. The vacuole with a diameter  $\geq 5 \mu\text{m}$  was considered as an enlarged vacuole and the trophozoite containing at least one enlarged vacuole was counted as 1. Three independent experiments were performed.

### Drug combination

For the drug combination study, the experiment was set-up in the same manner as the MIC determination. The 2-fold serial dilution of the drug and plant extract was prepared and combined in a 96-well plate. The highest concentration was the MIC of drug/plant extract. Then,  $2 \times 10^5$  trophozoites were added and incubated at RT for 24 h in the dark. Then, the cells were stained with Trypan blue and analysed under an inverted microscope.

### Preparation of total RNA and cDNA synthesis

*Acanthamoeba* trophozoites of  $2 \times 10^5$  cells per 1 mL PYG medium per well were cultured in a 24-well plate in the presence or absence of the *C. angustifolia* extract at a concentration of 250  $\mu\text{g mL}^{-1}$ . The plate was incubated at RT for 24 h. The cells were harvested at 6, 12, 18 and 24 h after treatment. At each time point, untreated and *C. angustifolia* extract-treated cells, a total of two wells were collected into 1.5 mL Eppendorf tubes and kept on ice. The tubes were centrifuged and the culture medium was discarded. The cell pellet was vortexed and a 500  $\mu\text{L}$  TRI reagent (Molecular Research Center, Cincinnati, USA) was immediately added to the cells to preserve parasite RNA. The total RNA was extracted using a RNA extraction kit (Vivantis Technologies, Selangor, Malaysia). The total 100 ng mRNA was then converted to cDNA by the Viva cDNA synthesis kit (Vivantis Technologies,

Selangor, Malaysia) following the manufacturer's protocol. The cDNA was kept at  $-20^\circ\text{C}$  until use.

### Validation of polymerase chain reaction (PCR) primers

Specific primers targeting genes of interest were obtained from published research on *Acanthamoeba* spp. as shown in Table S1. Genes of interest were ATG3, ATG8b, ATG16, including the housekeeping gene, 18S rRNA. These primers were tested with DNA from the *A. triangularis* strain WU19001. The amplicon was purified, commercially sequenced (Apical Scientific Sdn. Bhd., Selangor, Malaysia) and blasted against *A. castellanii* NCBI databases before performing a real-time PCR.

### Analysis of gene expression by quantitative PCR

Real-time PCR was performed in duplicate with three independent experiments using an iTaq Universal SYBR Green Supermix kit (Bio-Rad, Hercules, USA). The qPCR reaction was conducted as follows. Ten microlitres of iTaq supermix, 2 $\times$  concentration, were added to the PCR tube followed by 100 ng cDNA, 1  $\mu\text{L}$  of 200 mM F+R primers and the total volume adjusted with DEPC water up to 20  $\mu\text{L}$ . The software setting for the thermal cycler, StepOnePlus Real-time PCR systems (Applied Biosystems, Waltham, USA), was as follows: holding stage 95 $^\circ\text{C}$  for 30 s, cycling stage for 40 cycles at 95 $^\circ\text{C}$  for 15 s, 60 $^\circ\text{C}$  for 60 s, and then, melting curve stage at 95 $^\circ\text{C}$  for 15 s, 60 $^\circ\text{C}$  for 60 s, 95 $^\circ\text{C}$  for 15 s with a temperature increase of 0.3 $^\circ\text{C}$ . The average  $\Delta\text{Ct}$ ,  $\Delta\Delta\text{Ct}$  and the relative expression of the mRNA were calculated. The 18S rDNA was used as an internal control.

### Statistical data analysis

All data from the extract testing assay were recorded, entered and edited using Microsoft Excel 2016 (Microsoft Corporation, Washington, USA) and data were obtained from three independent experiments with three technical replicates. The results were pooled to determine the mean  $\pm$  S.D. or mean  $\pm$  S.E.M. depending on the experiment and analysed using a two-tailed unpaired Student's *t*-test by Prism 5 (GraphPad Software, San Diego, USA). *P* values <0.05 were considered statistically significant.

## Results

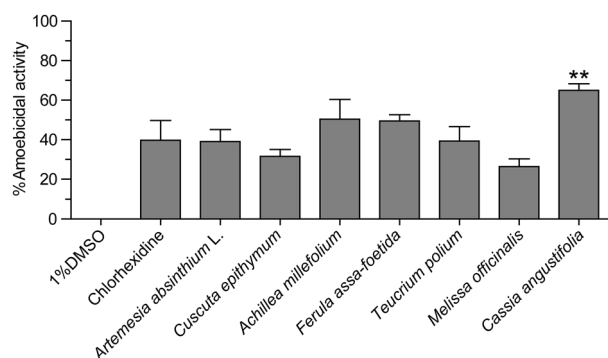
### Anti-Acanthamoeba activity of plant extracts

Seven plant extracts (Table 1) at the final concentration of 1000  $\mu\text{g mL}^{-1}$  were selected for an anti-*Acanthamoeba* activity against *A. triangularis* trophozoites. All plant extracts tested demonstrated amoebicidal activity to varying degrees. The percentage of cidal activity was in the range of 25–65% compared to the DMSO control. Among these plants, the extract of *C. angustifolia* demonstrated the highest cidal activity followed by *A. millefolium*, *F. assa-foetida*, *A. absinthium*, *T. polium*, respectively, whereas for the extracts of *C. epithymum* and *M. officinalis*, the percentage was below 35%. However, statistical analysis data revealed that the *C. angustifolia* extract was the only extract that significantly killed the *A. triangularis* trophozoite (Fig. 1).

*Cassia angustifolia* was further identified with its MIC against the *A. triangularis* trophozoite and cyst forms. In the 24 h treatment, the *C. angustifolia* extract had an inhibitory activity against the trophozoite with an MIC value of 1024  $\mu\text{g mL}^{-1}$ , whereas in the 48 and 72 h treatments, the MIC was 2048  $\mu\text{g mL}^{-1}$ . However, the inhibitory effect against cysts, their MICs at different time points were >2048  $\mu\text{g mL}^{-1}$  (Table S2).

**Table 1.** List of plants used in this study

Botanical name	Family	Part used
<i>Artemisia absinthium</i> L.	Asteraceae	Aerial parts (leaf and stem)
<i>Cuscuta epithymum</i>	Cuscutaceae	Aerial parts (leaf and stem)
<i>Achillea millefolium</i>	Asteraceae	Aerial parts (leaf and stem)
<i>Ferula assa-foetida</i>	Apiaceae	Aerial parts (leaf and stem)
<i>Teucrium polium</i>	Lamiaceae	Aerial parts (leaf and stem)
<i>Melissa officinalis</i>	Lamiaceae	Leaf
<i>Cassia angustifolia</i>	Caesalpinaceae	Leaf



**Fig. 1.** Screening of amoebicidal activity of the plant extract against *Acanthamoeba triangularis* trophozoites. *Acanthamoeba* trophozoites were treated with these extracts for 24 h at a final concentration of  $1000 \mu\text{g mL}^{-1}$ . Chlorhexidine of  $5 \mu\text{g mL}^{-1}$  and 1% DMSO were included as a positive and a negative control, respectively. The cells were stained with PrestoBlue dye and the fluorescence intensity was analysed. Cell viability in the DMSO control was set to 100% and the percentage of the extract-treated conditions was adjusted accordingly. Then, the percentage of amoebicidal activity was calculated using 100% cell viability. The experiment was performed in triplicate with three independent experiments. Bar graphs show mean  $\pm$  s.e.m. \*\**P* value < 0.01.

### Ultrastructural effects of *C. angustifolia*

A morphological change of the amoeba upon exposure to a concentration of 0.25 MIC,  $250 \mu\text{g mL}^{-1}$ , of *C. angustifolia* extract was observed. The SEM was performed. The amoebae of both trophozoite and cyst forms were treated separately with the extract for 24 h, at RT in the dark. The amoeba was harvested and processed for SEM. Under the conditions treated with *C. angustifolia*, almost the entire surface of the trophozoites was covered by the plant extract. The cell membrane was ruptured and the formation of pores in the membrane was clearly seen (Fig. 2). This damage to the membrane can lead to the leakage of cytoplasmic content and finally result in the death of *A. triangularis* cells. In addition, acanthopodia of most of cells disappeared after 24 h of treatment, cells were much smaller compared to the untreated control, but did not shrink as expected. However, in part of cell membrane of some cells, the structure was still stabilized, which probably indicates the slow action of the extract.

In cysts treated with *C. angustifolia*, even the amoebicidal activity was not as potent as in trophozoites, SEM images revealed some morphological changes of the cyst (Fig. 3). Most of the *A. triangularis* cysts were slightly shrunk, pronounced circular or polygonal edges were obviously seen and a venation was no longer present in the membrane. Emphasizing on the membrane pore, its number was similar to that of the untreated control, but its size was slightly larger. However, due to the complexity of the double cyst wall, this limits the ability of most drugs/compounds to enter the cyst form and becomes the main barrier to *Acanthamoeba* treatment (Lorenzo-Morales *et al.*, 2013).

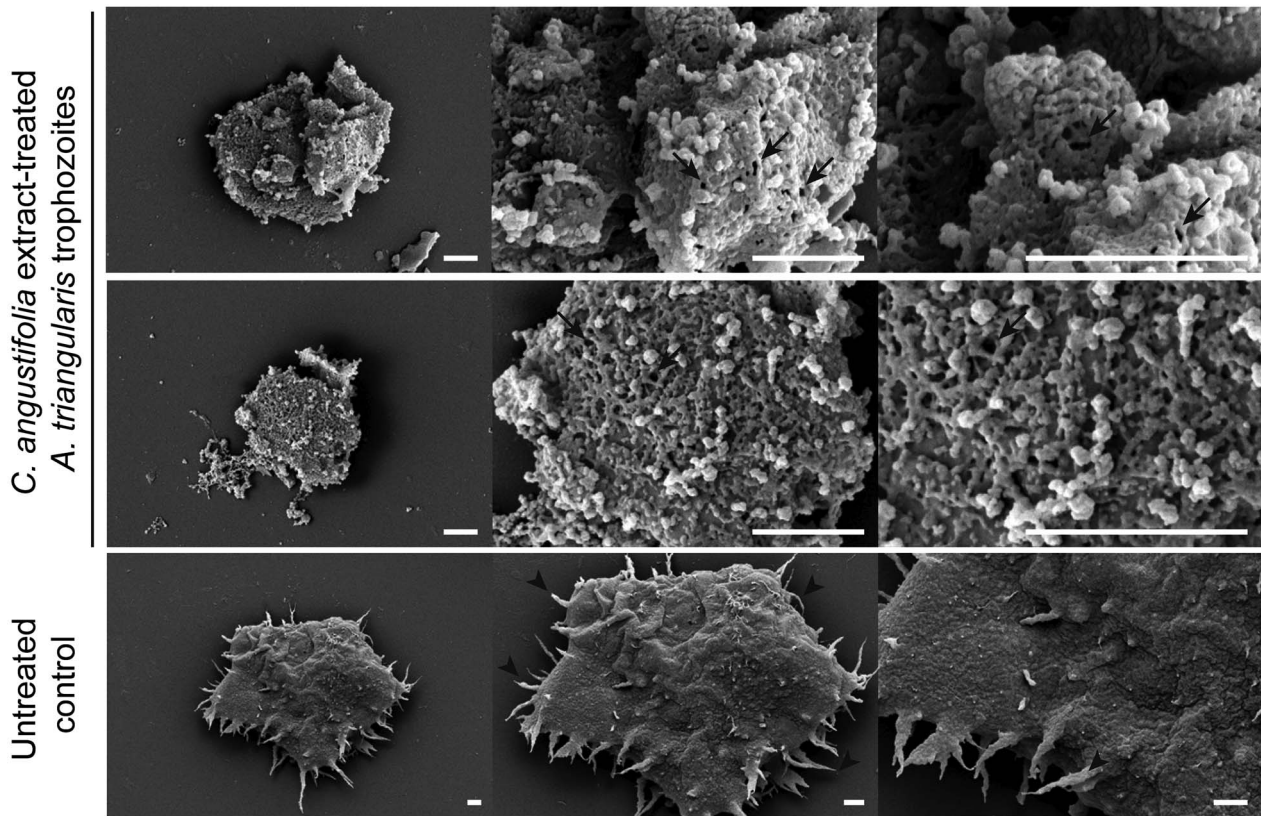
### Drug combination

Regarding a common anti-*Acanthamoeba* drug, chlorhexidine, we also combined the drug with the *C. angustifolia* extract against the trophozoite of *A. triangularis*. The concentrations of the drug/extract are shown in Table 2. At the maximum concentrations of the extract (MIC:  $1024 \mu\text{g mL}^{-1}$ ) and chlorhexidine (MIC:  $16 \mu\text{g mL}^{-1}$ ), the percent viability of the trophozoites fell into a range of 4–7%. Reduction of the concentration of chlorhexidine to  $8 \mu\text{g mL}^{-1}$  in combination with different concentrations of the extract, the percentage viability was markedly increased to 20–44% and their percentages were similar to those of chlorhexidine alone, 25%. At lower concentrations of chlorhexidine (4, 2, 1  $\mu\text{g mL}^{-1}$ ), a similar pattern of result was observed in which its percentage viability was at a level comparable to that of the drug alone. On the contrary, in the combination of fixed concentration of the extract,  $512 \mu\text{g mL}^{-1}$ , with varying concentrations of chlorhexidine, the percentage of viability was consistent in the range of 20–28% and close to the extract alone, 30%. When the concentration of the extract was reduced to 256, 128 and  $64 \mu\text{g mL}^{-1}$ , the percentage of viability was gradually increased to a level comparable to that of its own drug treated alone. Altogether, no synergistic, additive or antagonistic effects were observed in any combination of drugs that indicate their own inhibitory effects for *A. triangularis*.

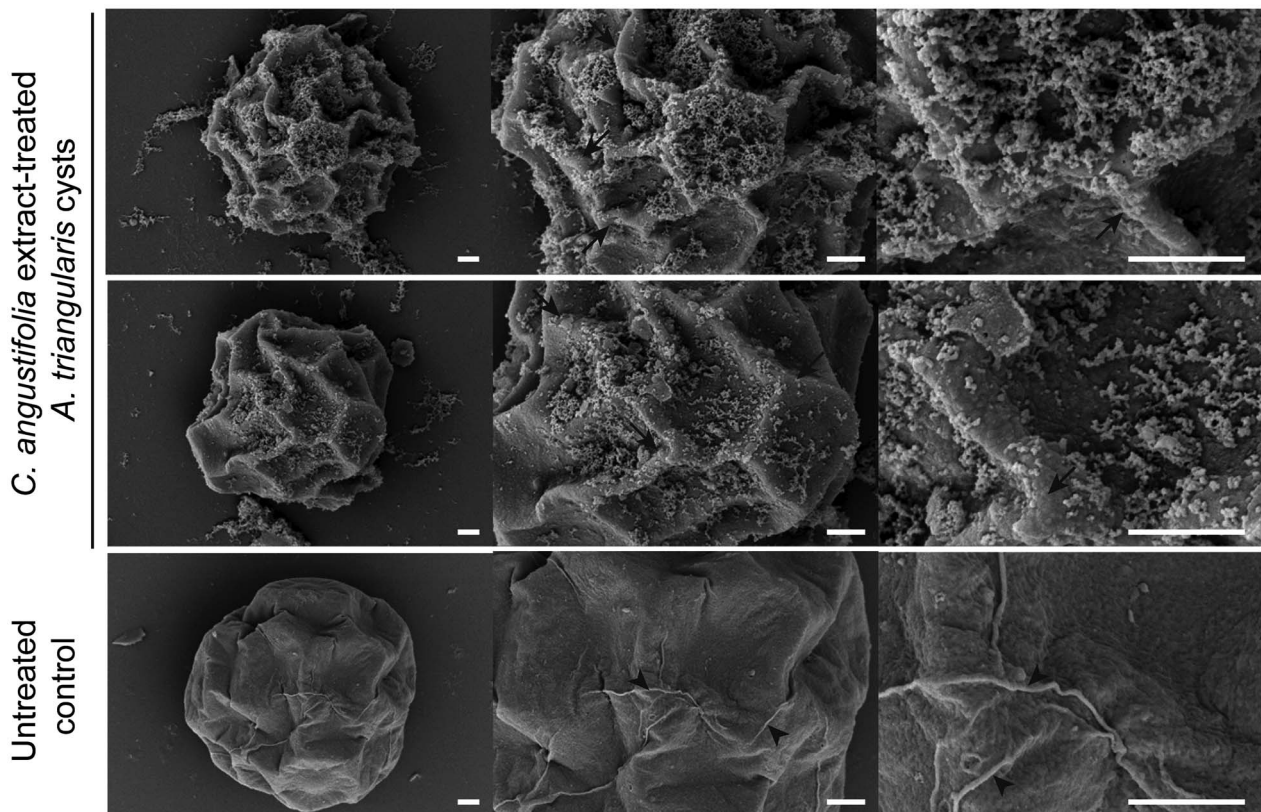
### Autophagic response to *C. angustifolia* extract

*Acanthamoeba* autophagy is not clearly identified. *Acanthamoeba* autophagy-related (Atg) proteins were partially characterized in *A. castellanii* and their functions have been reported to be associated with the amoeba encystation (Moon *et al.*, 2011, 2013; Song *et al.*, 2012). According to our screening result of the plant extract for the anti-*Acanthamoeba* activity, a stress-induced encystation was not observed upon 24 h treatment of *C. angustifolia*. The surviving *A. triangularis* remained in the trophozoite stage (Fig. 4A). Further quantification of stress-induced vacuolization was conducted on surviving trophozoites. The results showed that the percentage of trophozoites containing vacuoles was at a level comparable to the untreated control, 80–90% (Fig. 4B, left). We also considered the size of the vacuole to explore if it was enlarged (a diameter of  $\geq 5 \mu\text{m}$ ). However, the percentage of trophozoites with enlarged vacuole was not a significant difference between *C. angustifolia*-treated and untreated control (Fig. 4B, right). Regarding autophagy, a stress-sensing mechanism, we hypothesized that the extract may affect ATG gene expression which further impairs the encystation mechanism. Quantitative PCR was further performed. The validation of PCR primers targeting ATG3, ATG8b, ATG16, 18S rRNA genes in the *A. triangularis* genomic DNA was performed for the first time (Fig. S1). A single band from each PCR reaction was clearly seen on the agarose gel. These amplicons were subsequently sequenced to confirm a specific amplification, as shown in Table S3. Then, qPCR of *A. triangularis* treated with *C. angustifolia* was performed and the data are shown in Fig. 5.

Regarding Atg8b and Atg3 in *Acanthamoeba* spp., both were reported to be involved in the *Acanthamoeba* encystation (Moon *et al.*, 2011, 2013). Our qPCR results showed that after exposure to *C. angustifolia* extract, mRNA expression of ATG3 and ATG8b was consistent for the first 18 h compared to 0 h and increased significantly within 24 h post-treatment. However, Atg16, which is part of Atg12-Atg5 complex and required for an efficient Atg8 conjugation in autophagosome biogenesis (Yorimitsu and Klionsky, 2005; Fujita *et al.*, 2008), our qPCR data demonstrated that AcATG16 mRNA expression decreased over a period of 12 h after treatment and a significant reduction



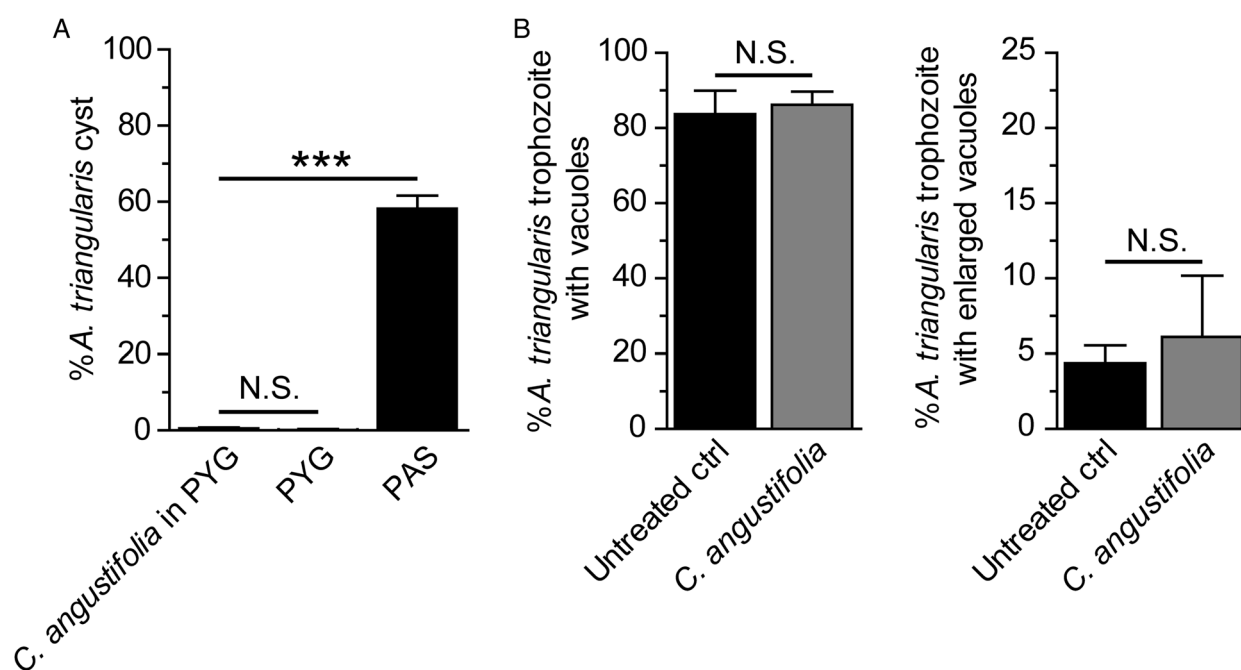
**Fig. 2.** SEM imaging of *A. triangularis* trophozoites treated with *Cassia angustifolia* extract. Cells were treated with the compound for 24 h in a 24-well plate. Cells were then fixed and processed for SEM. Arrows indicate porous membrane, whereas arrow heads indicate acanthopodia. Scale bars = 1  $\mu$ m.



**Fig. 3.** SEM imaging of *A. triangularis* cysts treated with *C. angustifolia* extract. Cells were treated with the compound for 24 h in a 24-well plate, and then fixed and processed for SEM. Arrows indicate a pronounced edge, whereas arrow heads indicate venation. Scale bars = 1  $\mu$ m.

**Table 2.** Activity of *C. angustifolia* in combination with chlorhexidine against *Acanthamoeba triangularis*

<i>C. angustifolia</i> ( $\mu\text{g mL}^{-1}$ )		Chlorhexidine ( $\mu\text{g mL}^{-1}$ )					
	0	1	2	4	8	16	
1024	4.0 $\pm$ 0	4.0 $\pm$ 0	4.0 $\pm$ 4.0	4.0 $\pm$ 0	5.3 $\pm$ 2.3	5.3 $\pm$ 2.3	
512	30.6 $\pm$ 4.6	280. $\pm$ 0	28.0 $\pm$ 0	25.3 $\pm$ 4.6	20.0 $\pm$ 0	4.0 $\pm$ 4.0	
256	58.6 $\pm$ 2.3	60.0 $\pm$ 0	46.6 $\pm$ 4.6	34.6 $\pm$ 2.3	30.6 $\pm$ 2.3	4.0 $\pm$ 0	
128	80.0 $\pm$ 4.0	77.3 $\pm$ 2.3	70.6 $\pm$ 2.3	44.0 $\pm$ 0	32.0 $\pm$ 0	4.0 $\pm$ 4.0	
64	97.3 $\pm$ 2.3	90.6 $\pm$ 2.3	78.6 $\pm$ 2.3	58.6 $\pm$ 6.1	44.0 $\pm$ 4.0	6.6 $\pm$ 4.6	
0	100.0 $\pm$ 0	76.0 $\pm$ 0	65.3 $\pm$ 9.2	48.0 $\pm$ 6.9	25.3 $\pm$ 4.6	4.0 $\pm$ 0	



**Fig. 4.** Cyst formation and vacuolization in surviving trophozoites. *Acanthamoeba triangularis* trophozoites were cultured in PYG medium and treated with the 250  $\mu\text{g mL}^{-1}$  *C. angustifolia* extract for 24 h. Trophozoites cultured in PYG and PAS media alone were included as negative and positive controls for encystation, respectively. (A) At least 200 cells per condition were analysed for cyst formation. (B) Surviving trophozoites of at least 100 cells per condition were examined for vacuole formation. Percentages of trophozoites containing vacuoles (left) and trophozoites with enlarged vacuole (diameter  $\geq 5 \mu\text{m}$ ) (right) were analysed. The data were obtained from three independent experiments. Bar graphs show mean  $\pm$  s.d. N.S., not significant. \*\*\**P* value  $< 0.001$ .

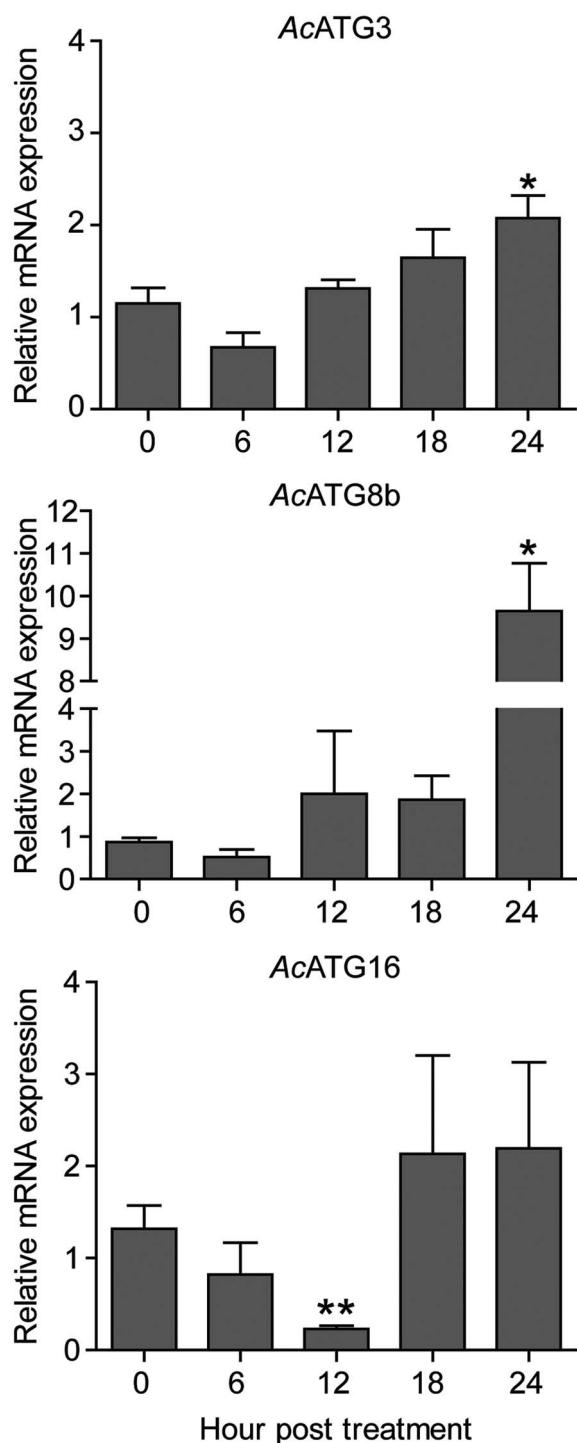
was observed at 12 h. However, its expression was recovered 18 h post-treatment. This indicates the expression profile of selected ATG genes upon treatment with the *C. angustifolia* extract.

## Discussion

The incidence of *Acanthamoeba* infection has increased annually, especially in contact lens wearers (Khan, 2006; Ibrahim *et al.*, 2009; Nielsen *et al.*, 2020; de Lacerda and Lira, 2021). Along with the reports on drug resistance (Iovieno *et al.*, 2010; Huang *et al.*, 2017) and the limited anti-*Acanthamoeba* drugs available (Lorenzo-Morales *et al.*, 2015; Szentmáry *et al.*, 2019), these attract the researcher's attention to focus on the identification of new medicinal plants for therapeutic purposes of *Acanthamoeba* infection and a compound with greater inhibitory activity and less toxicity is urgently needed (Niyati *et al.*, 2016; Mahboob *et al.*, 2018; Sanguan *et al.*, 2018; Kolören *et al.*, 2019; Elsheikha *et al.*, 2020). Besides, a treatment strategy of multidrug combination therapy may be needed for drug resistance cases (Tyers and Wright, 2019). Several phytochemicals extracted

from many plants have been evaluated as a source of amoebicidal agent, for example *Allium sativum*, *Origanum* spp., *Arachis hypogaea*, *Curcuma longa* L., *Pancreatum maritimum* L., *Pterocaulon polystachyum*, *Croton isabelli*, *Artemisia argyi*, *Piper* spp., *Citrus* spp., *Murraya paniculata*, *Amomum uliginosum*, *Kaempferia pandurata*, etc. (Sanguan *et al.*, 2018; Kolören *et al.*, 2019; Anwar *et al.*, 2020).

*Cassia angustifolia* is well known in West Asia, in particular in Yemen, a region in Southern Arabia, and is called Arabia Senna. The medicinal properties of Senna leaf are commonly used to treat constipation, loss of appetite, hepatomegaly, splenomegaly, indigestion, malaria, jaundice and anaemia (Tripathi, 1999), anti-diabetic, antioxidant and hepatoprotective activity of leaves of *C. angustifolia* has recently been reported (Bellassoued *et al.*, 2019; Jani and Goswami, 2020). The anthelmintic activity of *C. angustifolia* leaves has also been reported against *Heterakis gallinarum*, *Raillietina tetragona* and *Catatropis* sp. which resulted in helminth paralysis and death after exposure to the plant extract (Kundu *et al.*, 2014). A study on rat tapeworm, *Hymenolepis diminuta* reported that the anthelmintic activity of the *C.*



**Fig. 5.** Transcriptional expression of autophagy genes. *Acanthamoeba triangularis* trophozoites were cultured in PYG medium in the presence or absence of the extract and incubated for 24 h. The cells were harvested every 6 h and the relative changes in the mRNA levels of the ATG3, ATG8b and ATG16 genes were measured using quantitative PCR. The level of indicated transcripts from each time point was expressed as a relative mRNA expression. 18S rRNA was used as an internal normalization gene. The expression level was compared to that of time zero. The data were obtained from three independent experiments. Bar graphs show mean  $\pm$  s.e.m. \* $P < 0.05$ ; \*\* $P < 0.01$ .

*angustifolia* plant targeted the mitochondrial membrane and led to the loss of its architecture (Ukil *et al.*, 2018). In our study, the amoebicidal activity of *C. angustifolia* leaves extract was demonstrated against trophozoites and cysts of *A. triangularis*. The effect of *C. angustifolia* extract on trophozoites and cysts was observed by SEM. SEM images exhibited some morphological changes. Acanthopodia, a crucial component of the trophozoites

for cell adhesion, movement and capture of food particles, has disappeared. A loss of membrane integrity was observed in the trophozoites membrane, including an obvious porous formation. These changes can lead to a leakage in the cytoplasmic content and death of the amoeba. This fact is in agreement with Heredero-Bermejo *et al.*'s (2020) finding that the membrane alteration led to the *Acanthamoeba* cell death. In addition, the presence of pores in the membrane after *C. angustifolia* treatment may indicate the amoebic cell death by necrosis (Zhang *et al.*, 2018). However, further investigation to rule out apoptotic cell death is needed. Different degrees of susceptibility to the plant extract, including anti-*Acanthamoeba* drugs available for trophozoites and cysts, are due to a different membrane architecture of the cells, resulting in different degrees of cell permeability. Although the MIC against the cyst form was higher, which means less potency compared to the trophozoites stage, but morphological changes were still presented, that is, cell shrinkage, much smaller cell compared to the untreated control and more pronounced edges of the cyst wall.

Apart from the cidal activity of the *C. angustifolia*, encystation of the surviving trophozoites left by the treatment is a secondary concern. Our data showed that there was no induction of cyst formation. The percentage of surviving trophozoites containing vacuole and the cell with enlarged vacuole were not significantly different from the untreated control. This may indicate a specific stress-induced encystation in *Acanthamoeba* spp. Cyst form is metabolically inactive and more resistant to environmental stress. Under the condition of depleted nutrients, the amoeba immediately undergoes encystation (Chagla and Griffiths, 1974). The *Acanthamoeba* cyst consists of a double wall called ectocyst and endocyst (Anwar *et al.*, 2018). Several pathways are involved with the amoeba encystation (Picazarri *et al.*, 2008; Bouyer *et al.*, 2009; Moon *et al.*, 2012). Autophagy is one of the mechanisms that support this transformation and some autophagy proteins have been reported (Picazarri *et al.*, 2008; Moon *et al.*, 2009; Song *et al.*, 2012; Moon *et al.*, 2015). Atg8 was first identified in *A. castellanii* and this protein was further characterized at the molecular level. Their data demonstrated that *A. castellanii* Atg8 (*AcAtg8*) was highly expressed during encystment and the fluorescence analysis data showed that *AcAtg8* diffused into a cytosol in the trophozoites stage, whereas in the cyst, it formed as puncta structures. The Atg8 positive membrane was further identified as an autophagosome, as it showed a co-localization with LysoTracker (Moon *et al.*, 2009). Another isoform was identified later, *AcAtg8b*. This protein was slightly longer than the *AcAtg8* protein. However, the *AcAtg8b* was highly expressed during encystation and its RNA interference data revealed that decreased expression of *AcAtg8b* mRNA significantly reduced *Acanthamoeba* encystation (Moon *et al.*, 2013). *AcAtg3*, a member of the Atg8 conjugation system, was further characterized in *A. castellanii*. Its molecular data showed that its mRNA expression level was not changed during the encystation. However, microscopic data revealed that the formation of mature cyst was significantly reduced in Atg3-depleted cells (Moon *et al.*, 2011). It was later reported that *AcAtg16* was highly expressed during the *A. castellanii* encystation. The immunofluorescence analysis showed that the *AcAtg16* protein was partially located in the autophagosome of the *Acanthamoeba* cyst. The reduction of this protein inhibited the formation of autophagosomes and further affected the efficiency of cyst formation (Song *et al.*, 2012). Our quantitative PCR data revealed that *Acanthamoeba* autophagy responded quickly to the *C. angustifolia* extract, which is similar to the autophagy mechanism in other organisms that respond immediately to cellular stress (Yorimitsu and Klionsky, 2005; Gatica *et al.*, 2018). The transcriptional expression of the autophagy genes was also affected. *AcAtg16* was significantly downregulated 12 h after treatment,

whereas transcripts of *AcATG3* and *AcATG8b* were consistent during the first 18 h after treatment. However, at 24 h, *Atg16* was recovered to a basal level, whereas transcripts of *AcATG3* and *AcATG8b* were slightly increased. Regarding our data on cyst formation, there was no cyst induction after treatment with *C. angustifolia*, the reduction in *ATG16*, as well as a constant level of *ATG8b* can attenuate the induction signal for *Acanthamoeba* encystation. In agreement with data previously published by Moon, *et al.* (2013), that the increase in *Atg8b* expression was associated with the amoeba encystation (Moon *et al.*, 2013), our data also confirmed a previous study by Moon *et al.* (2011) that the level of *ATG3* mRNA was consistent and expressed independently of encystment induction. However, the increase in *AcATG3* and *AcATG8b* in 24 h was of interest. It is possible that one of the active compounds within the extract may be active at this time point, which initiates an encystation signal or the autophagy may be delayed in coping with the stress induced by the *C. angustifolia* extract. The latter, the autophagy mechanism, may work independently form the amoeba encystment. For those *Atg*, *Atg3*, *Atg8* and *Atg16* proteins, they normally work together on basal autophagy and autophagosome biogenesis upon autophagic induction. The latter requires an increased expression of all *Atg* proteins. According to our data, *AcAtg16* was the only one affected. It is possible that *Atg16* protein has an additional role in response to the stress and is probably to be specific or non-specific to this plant in which stress may enhance protein translation, function, recycling and/or degradation. Another possibility is that its transcription may be disrupted by any of biomolecules in the extract. Thus, the isolation and purification of bioactive molecules are needed to characterize a specific response related to a certain *ATG* transcript. Certainly, a study at the protein level provides more information on how each *Atg* protein works in *Acanthamoeba* spp. including its correlation with *ATG* mRNA. Further investigations of the *ATG* expression profile including *ATG16* in response to other plant extracts are of interest. This is the first time that autophagy in *A. triangularis*, another important member of T4 genotype, was evaluated. The data provide insight into the autophagic mechanism of the *Acanthamoeba* in response to stress and an understanding of this mechanism may be useful for the development of drugs for *Acanthamoeba* infection.

As mentioned earlier on the limitation of commercially available drugs for the treatment of *Acanthamoeba*, monotherapy with chlorhexidine or PHMB has been used. As evidence of other infectious diseases in long-term treatment, combination therapy is usually superior to monotherapy for infections, especially in drug-resistant cases (Martínez-Sagasti *et al.*, 2016; Tepekule *et al.*, 2017) and synergistic effect of drug combinations is a promising approach to fight chronic diseases. This leads to our drug combination study, *C. angustifolia* extract and chlorhexidine. However, our results did not show any synergistic effect in any combination below its MIC, which indicates its own cidal activity against *Acanthamoeba* trophozoites. The investigation of the drug combination with an active compound purified from the plant extract is also needed.

Altogether, our study presents an extra property, amoebicidal activity, of the leave extract of *C. angustifolia*. The autophagy of *Acanthamoeba* was explored at the transcriptional level, indicating the expression profile of genes related to autophagy in response to the stress induced by this plant (Fig. S2). However, the autophagic response to other plants, as well as the response of other pathways related to the *Acanthamoeba* encystation are of interest and need further study. Moreover, investigations on active compounds, cytotoxicity against human cells, drug combination studies are essential for drug development and therapeutic purposes of protozoan infection, including free-living amoebae.

**Supplementary material.** The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182021000718>

**Author contributions.** R.B. and V.N. conceptualized the research; R.B. performed *Acanthamoeba* culture; R.B. performed compound treatment assay and quantitative PCR; S.S. performed drug combination study; W.M. and S.S. prepared *Acanthamoeba* sample for SEM; A.S. prepared plant extracts and provided information related to the plants; S.S., W.M., N.C., S.W., F.M., M.P., P.W., M.R., C.W., H.A.T. and K.G.D. provided critical suggestions; R.B. performed overall data analysis; R.B. wrote the manuscript and collated comments from all authors; V.N., P.W. and R.N. provided critical suggestions, comments on the manuscript, and financial support.

**Financial support.** We highly appreciate the support of The Royal Patronage of Her Royal Highness Princess Maha Chakri Sirindhorn – Botanical Garden of Walailak University, Nakhon Si Thammarat under the project entitled: Medicinal under-exploited Thai native plant against *Acanthamoeba* spp., *Leishmania donovani* and *Plasmodium falciparum* – Toward South East Asia collaboration initiative (WUBG020-2564), Thailand, Walailak University grant No. WU-IRG-63-073, Wasinee Poonsawat, the Research Institute of Health Science (RIHS) staff for the laboratory facilities and Benjaporn Somjit at the Center for Scientific and Technological Equipment for SEM imaging, Walailak University. We would also like to acknowledge the project CICECO-Aveiro Institute of Materials, UIDB/50011/2020 and UIDP/50011/2020, national funds by FCT/MCTES.

**Conflict of interest.** None.

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