

# Synergistic Effect of *Garcinia Mangostana* Combined With Chlorhexidine on *Acanthamoeba Triangularis* Trophozoites and Cysts

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## Research

**Keywords:** *Garcinia mangostana* pericarp extract, Anti-Acanthamoeba activity, *Acanthamoeba triangularis*, Synergistic effect

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# Abstract

**Background:** Various parts of *Garcinia mangostana* Linn, including its pericarp have been traditionally used to treat different types of diseases. This study was carried out to determine the anti-*Acanthamoeba* activity of *G. mangostana* pericarp extract against *Acanthamoeba triangularis*.

**Methods:** The *G. mangostana* ethanolic pericarp extract was screened for the anti-*Acanthamoeba* activity and determined its minimal inhibition concentrations (MICs) by the microdilution method. Then, the time-kill kinetic assay of the extract was determined. The synergistic effect of *G. mangostana* extract and chlorhexidine was performed using the checkerboard method. Parasite morphology was detected by scanning electron microscopy (SEM).

**Results:** The MICs of extract were assessed on trophozoites and cysts with 250 and 4000 µg/mL, respectively. More so, at 2×MIC of extract exhibited inhibitory activity against trophozoites and cyst of *A. triangularis* for up to 7 days. Checkerboard assays showed synergistic activity of extract (500-1000 µg/mL) plus chlorhexidine (3.90-15.62 µg/mL) at a fractional inhibitory concentration index (FICI) of 0.18-0.37. The lowest FICI (0.18) displayed good synergism resulting in up to 16-fold reduction of drug MIC and reducing to 8-fold of extract MIC. The viability of cysts decreased to  $12.28 \pm 3.03$  cells/mL. FICI interpretation equal to 1 is considered an additive effect on *Acanthamoeba* trophozoites. The SEM results clearly showed that *Acanthamoeba* cells treated with a single drug of chlorhexidine and its combination with *G. mangostana* extract caused cell membrane damage and irregular cell shapes comparing with the control.

**Conclusion:** A good combinatorial relationship displayed by *G. mangostana* extract and chlorhexidine suggest a more reliable evidenced based therapeutic strategy. Therefore, this approach is promising and could be employed as an alternative treatment method for the management of *Acanthamoeba* infection.

## Introduction

*Acanthamoeba* is an opportunistic pathogen that is isolated from diverse natural environments [1]. This organism exists in two main forms: the trophozoite, which is the invasive stage; and the cysts, which is the highly resistant stage in a very harsh environment [2]. *Acanthamoeba* spp. have been divided into three groups (I, II, and III) based on characteristics of cyst such as size and shape. However, *Acanthamoeba* spp. were then classified into 20 different genotypes (T1–T20 Genotype) based on 18S rRNA gene sequencing. Genotype T4 is the most commonly isolated in both clinical and environmental samples, followed by genotype T3 and T5. In addition, the T4 genotype is the most virulent because it has a higher potential of binding to host cells than other genotypes [3].

*Acanthamoeba* spp. are the causative agents of amebic keratitis (AK) and granulomatous amebic encephalitis (GAE). AK can cause permanent vision loss or blindness [4]. The rate of infectious keratitis is becoming alarming in recent time which is an issue that could be linked to a sudden increase in the population of contact lens wearers [2]. Similarly, *Acanthamoeba* encysts sprawled deep within the corneal

stroma [5], as such, the cyst wall becomes impermeable to the existing drugs and this has reduced further research in the areas of drug formulations and designed for this organism.

*Garcinia mangostana* Linn is generally known as mangosteen and belongs to the family *Clusiaceae*. It is a tropical plant, widely grown in Southeast Asia [6]. The pericarps of this fruit have been used for traditional medicine in treating many diseases [7]. The xanthone group, enriched in *G. mangostana* extract exhibited anti-oxidant properties, anti-bacterial activity, anti-diabetic activity, anti-tumour, anti-inflammatory, anti-allergenic, anti-malarial activity, anti-bacteria, cytoprotective and anti-cancer properties [8]. In 2017, Cantos and Lee [9] reported the ovicidal activity of peel and seed extracts from *G. mangostana* against *Ascaris suum*. Next year, Canton and Jagunap [10] found that the extract from pericarp had better-inhibited ova fertilization than mebendazole.

Having searched through all available information on this plant, we discovered to our surprise that there is no single report on the anti-amoebic activity of *G. mangostana* extract against *Acanthamoeba* spp. Hence, our study sought to investigate the effective concentration of *G. mangostana* extract for inhibiting *Acanthamoeba* spp. and demonstrate synergistic effects of extract from *G. mangostana* pericarp and chlorhexidine on anti-amoebic activity against *A. triangularis*.

## Materials And Methods

### 1. Preparation of plant extracts

Dried mangosteen pericarp powder was extracted with ethanol. 50 g of powdered mangosteen pericarp was soaked in 200 mL of ethanol for 7 days. The extract was filtered through Whatman No. 1 using vacuum and pressure pump. The solution was evaporated to dryness under reduced pressure using a rotary evaporator to obtain a *G. mangostana* extract. The extract was dissolved in 100% DMSO and stored at -20 °C until use.

### 2. Cultivation of *A. triangularis*

*A. triangularis* WU19001, a strain from the recreational reservoir at Walailak University, Nakhon Si Thammarat Thailand was used in this study [11]. The parasite was grown in PYG medium (0.75% (w/v) proteose peptone, 0.75% (w/v) yeast extract and 1.5% (w/v) glucose). The trophozoites were observed after 48–72 hours of incubation at room temperature and were cultured in this medium for 1 week in order to obtain mature cysts. Trophozoites cells and cysts were centrifuged at 4000 rpm for 5 minutes and re-suspended in fresh PYG thereafter. The viability was investigated using 0.1% trypan blue assay and adjusted to  $2 \times 10^5$  cells/mL.

### 3. In vitro effect of plant extract against *A. triangularis*

One hundred microliters of trophozoites and cysts suspension ( $2 \times 10^5$  cells/mL) were added to 96-well microplates that contained 100  $\mu$ l of plant extract (final concentrations of 4000  $\mu$ g/mL). Chlorhexidine and 1% DMSO were used as positive and negative controls respectively. Viability was determined using 0.1% trypan blue that was obtained by manual count with inverted microscopy. The relative percentage of parasite viability was defined as (mean of the treated parasite/mean of the control)  $\times$  100.

## 4. Determination of minimal inhibitory concentration (MIC) of *G. mangostana* extract against *A. triangularis*

The minimum inhibitory concentration (MIC) for plant extract was determined using microtiter broth dilution method [11]. Plant extract was diluted to give a final concentration of 4000, 2000, 1000, 500, 250, 125, 62.5  $\mu$ g/mL into 96-well microplate. Then 100  $\mu$ l of  $2 \times 10^5$  cells/mL of trophozoites and cysts were inoculated in each well. Chlorhexidine and 1% DMSO were included as positive and negative control, respectively. The plates were incubated at room temperature for 24 hours. The MIC value of the extract was defined as the lowest concentration that inhibited  $> 90\%$  of growth, when compared with the negative control.

## 5. Time-kill Assay

Time-kill kinetics of the ethanolic extract of *G. mangostana* was carried out following the procedure as described [12] with modification. 100  $\mu$ L of  $2 \times 10^5$  cells/mL of trophozoites and cysts were inoculated into microcentrifuge tube containing PYG with extract at final concentration  $2 \times$  MIC, MIC,  $0.5 \times$  MIC, and  $0.25 \times$  MIC and incubated at room temperature for 1 week. The aliquots of *A. triangularis* were removed and counted at 24 hours intervals using hemocytometer. *A. triangularis* treated with 1% DMSO was used as a control.

## 6. Combination of *G. mangostana* extract with chlorhexidine in targeting trophozoites and cysts of *A. triangularis*

Checkerboard assay method was used to evaluate the interaction between *G. mangostana* extract and chlorhexidine against *A. triangularis*. The checkerboard method [13] used was the microdilution assay performed in a 96-well plate with final volume of 200  $\mu$ L. *G. mangostana* extract and chlorhexidine were diluted with PYG to obtain 4 times to its final concentrations of  $1/16$  MIC,  $1/8$  MIC,  $1/4$  MIC,  $1/2$  MIC and MIC. Fifty  $\mu$ l of extract and chlorhexidine were transferred to a microtiter plate containing 100  $\mu$ L of  $2 \times 10^5$  cells/mL of trophozoites and cysts. The plates were incubated at room temperature for 24 hours. The viability of the extract was defined as the lowest concentration that inhibited  $> 90\%$  of growth, when compared with the negative control. The assessment of results was defined as Fractional Inhibitory Concentration Index (FICI) that was calculated utilizing the following

FICI of combination = FIC A + FIC B

FIC A = MIC of chlorhexidine in combination / MIC of chlorhexidine alone

FIC B = MIC of extract in combination / MIC of extract alone.

The combination was considered synergistic for  $\Sigma\text{FIC} \leq 0.5$ , additive for  $0.5 < \Sigma\text{FIC} \leq 1$ , indifferent for  $1 < \Sigma\text{FIC} < 4$ , and antagonistic for  $\Sigma\text{FIC} \geq 4$ , according to EUCAST definition.

## 7. Scanning Electron Microscopic (sem) Study

*Acanthamoeba* trophozoites and cysts were treated with chlorhexidine and *G. mangostana* extract. After incubation, cells were collected by centrifugation at 4000 rpm for 5 minutes and re-suspended in phosphate buffer saline (PBS). Cells in DMSO (1%) were used as negative controls. Samples were fixed with 2.5% glutaraldehyde overnight. The sample was further dehydrated with graded alcohol series (20%, 40%, 60%, 80%, 90%, and 100% ethanol), mounted on aluminum stubs, and allowed to dry using a critical point dryer. The samples were then coated with gold particles and the morphology of *Acanthamoeba* trophozoites and cysts after treatment was subsequently examined under SEM (SEM-Zeiss, Munich, Germany) at the Center for Scientific and Technological Equipment, Walailak University, Nakhon Si Thammarat, Thailand.

## 8. Statistical Analysis

The experiments were performed in triplicate. All data were recorded and entered using the statistical package software (SPSS Inc. Chicago, IL, USA). The data were expressed as mean  $\pm$  SD. Statistical analysis was analyzed by the two-tailed unpaired Student's t-test. In all analyzes,  $P < 0.05$  was considered statistically significant.

## Results

### 1. *G. mangostana* extract exhibited anti-*Acanthamoeba* activity against *A. triangularis*

Anti-*Acanthamoeba* activity was performed to observe the effects of the *G. mangostana* extract on viability of *A. triangularis*. The extract showed significant reduction in trophozoites viability as compared to the control ( $P < 0.05$ ) (Fig. 1a). At 250–2000  $\mu\text{g}/\text{mL}$  of extract reduced the trophozoites viability to 5.8–8.8%. Furthermore, the viability percentages of cysts decreased to 10.7–13.8% after incubated with 2000–4000  $\mu\text{g}/\text{mL}$  of extract ( $P < 0.05$ ) (Fig. 1b).

### 2. Minimum inhibitory concentration (MIC) of *G. mangostana* extract against *A. triangularis*

Extract of *G. mangostana* pericarp was determined for its anti-*Acanthamoeba* potential by MIC using microtiter dilution broth method. As shown in Table 1, the MICs of mangosteen ethanol extract against *A.*

*triangularis* trophozoites and cysts were 250 and 4000 µg/mL, respectively. The MIC values of chlorhexidine against trophozoites and cysts were 7.81 and 62.5 µg/mL, respectively.

Table 1  
Minimal inhibitory concentration (MIC) of *G. mangostana* extract and chlorhexidine against *A. triangularis* trophozoites and cysts.

Antimicrobial agents	MIC (µg/mL)	
	Trophozoites	Cysts
<i>G. mangostana</i> extract	250	4000
Chlorhexidine	7.81	62.5

### 3. Time-kill kinetic study of *G. mangostana* extract against *A. triangularis* trophozoites and cysts

The number of trophozoites reached from  $2.0 \times 10^5$  cells/mL to  $1.3 \times 10^6$  cells/mL within 7 days. Similarly, population of cysts exhibited an increase number from  $2.0 \times 10^5$  cells/mL to  $9.7 \times 10^6$  cells/mL (Fig. 2a and b). All concentrations of *G. mangostana* extract inhibited the growth of *Acanthamoeba* trophozoites and cysts when compared with the control. At  $2 \times$  MIC of the extract displayed strongest inhibitory activity in both forms of *Acanthamoeba*. After 7 days, the number of viable trophozoites were detected at  $2.1 \times 10^5$  cells/mL in the treatment with  $2 \times$  MIC extract (Fig. 2a), while the viable cysts was  $1.3 \times 10^5$  cells/mL (Fig. 2b).

### 4. Synergistic effects of *G. mangostana* extract in combination with chlorhexidine against *A. triangularis* by the checkerboard assay

Evaluation of synergistic effects between the extract and drug was determined in this study. Figure. 3 shows the viable trophozoites in combination test. The lowest FIC values (0.5) for each agent reduced viability of trophozoites to  $12.72 \times 10^5$  cells/mL. FICI interpretation equal to 1 is to be considered an additive effect (Table 2). *G. mangostana* extract showed synergistic (FICI < 0.5) activity with chlorhexidine against *A. triangularis* cysts. Set of combinations of extract showed FICI ranged from 0.18 to 0.37 (Table 2). The lowest FICI (0.18) displayed good synergy resulting in up to 16-fold reduction of drug MIC and a reduction to 8-fold of extract MIC. The viability of cysts decreased to  $12.28 \pm 3.03$  cells/mL (Fig. 4).

### 5. SEM analysis of *G. mangostana* extract effects on *A. triangularis*

The alterations due to the action of the extract was confirmed by SEM micrograph are shown in Figs. 5 and 6. Control trophozoites (Figs. 5a, e and i) treated with 1% DMSO exhibited normal cells with acanthopodia. Treated trophozoites with  $4 \times$  MIC of chlorhexidine showed flat cells and smooth surface which is a feature that could be related to a total destruction of acanthopodia (Figs. 5b, f, and j). In treatment with  $4 \times$  MIC of *G. mangostana* extract, the damage cells were observed as flat and smooth surface similar to  $4 \times$  MIC of chlorhexidine treated (Figs. 5c, g, and k). Trophozoites treated with the

combination of chlorhexidine/*G. mangostana* extract at FICI equal 1, SEM showed rounded morphology with pores on cell surface (Figs. 5d, h, and l). Also, the cysts micrographs of control cells showed intact cell with oval and smooth surface as showed in Figs. 6a, e and i. The oval cysts of *Acanthamoeba* were flat, irregularly shaped causing collapse of the ectocyst walls after treatment with 4 × MIC of chlorhexidine (Figs. 6b, f, and j), 4 × MIC of *G. mangostana* extract (Figs. 6c, g, and k) and the combination of chlorhexidine/*G. mangostana* extract at FICI equal 0.18 (Figs. 6d, h, and l).

**Table 2** Fractional inhibitory concentration index (FICI) of the combination test of *G. mangostana* extract with chlorhexidine in

targeting trophozoites and cysts of *A. triangularis*

<i>A. triangularis</i> infective stages	MIC chlorhexidine alone (µg/ml)	MIC extract alone (µg/ml)	MIC in combination (µg/ml)		FICI <sup>a</sup>	Effect
			chlorhexidine	extract		
Trophozoties	7.81	250	3.90	125	1	Additivity
Cysts	62.5	4000	3.90	500	0.18	Synergistic
			7.81	500	0.24	Synergistic
			15.62	500	0.36	Synergistic
			3.90	1000	0.31	Synergistic
			7.81	1000	0.37	Synergistic

<sup>a</sup> The combination was considered synergistic for  $\Sigma FIC \leq 0.5$ , additive for  $0.5 < \Sigma FIC \leq 1$ , indifferent for  $1 < \Sigma FIC < 4$ , and antagonistic for  $\Sigma FIC \geq 4$ .

## Discussion

Herb-drug interaction may offer potential health promoting effect by augmenting drug efficacy or diminishing potential side effects. As such, we have presented an effective treatment of *Acanthamoeba* infections with herb-drug based combination strategy in this study. Also, we have highlighted the potential of anti-*Acanthamoeba* activity of *G. mangostana* ethanol extract. It is a natural source that provides active components of *G. mangostana* also known as the “queen of tropical fruits”. The *G. mangostana* extracts have anti-oxidant, anti-tumoral, anti-allergic, anti-inflammatory, anti-bacterial and

anti-viral activities [8]. A few studies have reported the effects of *G. mangostana* extract on anti-parasitic activity but there was no report on its *Acanthamoebic* effects.

*A. triangularis* WU19001 was a selected strain parasite used in this study. *A. triangularis* was characterized in group II (type 4) typically a wrinkled ectocyst and an endocyst which could be stellate, polygonal, triangular, or oval [14]. *A. triangularis* was originally isolated from the human feces documented in France [15]. In 2008, the first case of keratitis caused by *A. triangularis* had been reported in Korea [16]. Also, six isolates of *A. triangularis* had also been reported from cases with contaminated contact lens in southeastern Korea. In support of those previous findings, our study revealed that *G. mangostana* extract from pericarp exhibits anti-*Acanthamoeba* activity against *A. triangularis*. The MIC values of *G. mangostana* extract were 250 and 4000 µg/ml against *Acanthamoeba* trophozoites and cysts, respectively (Table 1). Our finding is in agreement with earlier reported studies that found the compounds of *G. mangostana* extract with anti-parasitic activities. In literature, xanthenes have been identified as the main compound of *G. mangostana* extract. Of this, xanthenes have been isolated from pericarp, whole fruit, leaves and bark [3]. α- and γ-mangostin are the most abundant xanthenes found in the pericarp of mangosteen fruit. Other xanthenes in mangosteen pericarp include β-mangostin, gartanin, 8-deoxygartanin, garcinones A, B, C, D and E, mangostinone, 9-hydroxycalabaxanthone and isomangostin, among others [3, 17]. Interestingly, the α-mangostin compound of *G. mangostana* has been reported with IC<sub>50</sub> value of 17 µM against *Plasmodium falciparum* [18]. Another study showed that α-mangostin and δ-mangostin exhibited anti-malarial activity against *P. falciparum* chloroquine-resistant strain with IC<sub>50</sub> value 0.2 and 121.2 µM, respectively [19]. Furthermore, a previous study has reported the use of *G. mangostana* pericarp in the management of amoebic dysentery [8]. Xanthenes block the polymerization process, and accumulation of soluble heme-drug complexes increases the osmotic pressure in the vacuole, causing its lysis on trophozoite stage [20]. The synthetic Caged *Garcinia* xanthenes (CGXs) exhibit anti-malarial activity against *P. falciparum* with low toxicity. It was reported that CGXs treatment affect malaria parasites relating to morphological changes, significant reduction of parasitemia (the percentage of infected red blood cells), and aberrant mitochondrial fragmentation [21]. Benzophenone (isoxanthochymol) was isolated from the roots of *G. polyantha* which showed strong chemosuppressive activity of parasitic growth found in *P. falciparum* [22]. The benzophenones (guttiferone E, isoxanthochymol, and guttiferone H) isolated from *G. xanthochymus* exhibited antiplasmodial activity with IC<sub>50</sub> values in the range of 4.71–11.40 µM [23]. The tetraprenylated benzophenone 7-epiclusianone (7-epi) was isolated from the fruits of *G. brasiliensis* showed effective against *Schistosoma mansoni* [24]. In addition, biflavanones (GB-1a, GB-1, and GB-2), another active compounds, were isolated from *G. kola*. These three biflavanones displayed the potent inhibitory activity *in vitro* against *P. falciparum* proliferation and also antimalarial potency through oral administration in mice infected with *P. berghei* without signs of acute toxicity [25]. As kolaviron (KV), a biflavonoid fraction from *G. kola* seeds that has also been reported to exhibit anti-malarial activities in *P. berghei*-infected mice [26]. The purified bioflavonoid of the fruit pericarp of *G. brasiliensis*, showed significant activity as inhibitors of *Leishmania*'s proteases, with mean (± SD) IC<sub>50</sub> values of 15.0 ± 1.3 µg/mL [27]. Interestingly, the effect of xanthenes has also been increasingly reported on targeting the bacterial membrane as a

result of rapid bactericidal action led to reducing resistance process and mutational possibility [28]. Therefore, xanthenes, biflavanones, and/or benzophenones may play a major role on the effect against *A. triangularis* in this study, however, it further suggests for more comprehensive studies to evaluate the mechanism of action on these compounds against *A. triangularis*.

In fact, the eradication of *Acanthamoeba* infection seems impossible due to highly resistance of the cysts to anti-amoebic drugs. Therefore, we also investigated the effective concentration of *G. mangostana* extract against *A. triangularis* trophozoite and cyst stages. Our results interestingly showed that *G. mangostana* extract can inhibit *A. triangularis* in a longer incubation period in PYG medium. It's worthy to note that the number of *Acanthamoeba* trophozoite and cysts were reduced significantly ( $P < 0.05$ ) in the presence of *G. mangostana* extract (MIC and  $2 \times$  MIC final concentrations). To our observation, the *G. mangostana* concentrations was found to have the highest growth-inhibitory for trophozoites at day 3 and cysts at day 2 (Figs. 2a and 2b) though there are few remaining *A. triangularis* survived due to diminishing the effect of *Garcinia* extract on the days after. To the best of our knowledge, we have shown the first potentially report of *G. mangostana* extract in its capacity to significantly inhibit *Acanthamoeba* cysts in an enrichment medium up to one week. This study have also shown the survival rate of *A. triangularis* ( $< 10\%$  in trophozoites and  $< 15\%$  in cysts) within the same period. Based on these results, it should therefore be better considered a 1-week than 3 days [29] as a general protocol for observation on the growth inhibitory effect of plant extract against *A. triangularis*. It is also very encouraging to explore the results shown that *G. mangostana* extract of  $2 \times$  MIC remarkably inhibited the growth of both trophozoites and cysts up to 7 days. Our finding is demonstrated similarly to a previous report on the growth inhibition of *A. culbertsoni* and *A. castellanii* incubated in the nonpolar extract of *Gastrochilus panduratum* from day 2 to day 7 [12]. Overall, this study therefore suggests that *G. mangostana* extract is a promising plant that shows the remarkable effect against *A. triangularis* infections.

Chlorhexidine is the mainstay drug of choice for *Acanthamoeba* keratitis and encephalitis due to it is active against both cysts and trophozoites [5]. In this study, a single drug chlorhexidine was therefore used against *A. triangularis* and successfully exhibited inhibitory activity on trophozoites and cysts with MIC values of 7.81 and 62.5  $\mu\text{g}/\text{mL}$  (Table 1), respectively. Our finding is in consistent with an earlier report on chlorhexidine used against *A. triangularis* exhibited amoebicidal and cysticidal properties at 200  $\mu\text{g}/\text{mL}$  (0.02%) [30]. While, a recent study [3] evaluated the efficacy of miltefosine, another orphan drug for the treatment of keratitis and encephalitis, against *Acanthamoeba* spp. cysts of genotypes T3, T4 and T5. Interestingly, the minimal cysticidal concentration (MCC) of 38.72 mM and 77.44 mM of miltefosine showed the effect against *Acanthamoeba* genotypes T4/T5 and T3, respectively. This suggests for further study to evaluate the effect of miltefosine against *A. triangularis*. In a matter of fact, a single drug used for treating infectious diseases includes this parasitic infection not only causes side effects, long-term use, costly, and drug resistance. Therefore, the combination approach is constantly introduced to encounter those pitfalls. Chlorhexidine has frequently been used in combination with aromatic diamidines [5], aminoglycosides, imidazoles and polyene [31], however these chemicals show side effects on keratocytes found in cases of human keratitis [32]. Recently, the finding of novel compounds with anti-*Acanthamoeba* activity of plant and herbs has been very encouraging to evaluate a

source of new molecules with anti-*Acanthamoeba* effects. At the same time, there is no data on the use of synergistic interaction between plant extract and synthetic drug against *Acanthamoeba* infection till date. To support this, our study also revealed an additive effect of a combination between *G. mangostana* extract and chlorhexidine against *Acanthamoeba* trophozoites (viable trophozoites < 90%) (Fig. 3). An additive effect occurs when substance added together enhances or improves the efficacies but not to the extent of synergic interaction [33]. Our better results were obtained when *G. mangostana* extract was combined with chlorhexidine to produce a synergistic effect against *A. triangularis* cysts. The FICI values demonstrated the synergy for concentration of 3.90 to 15.62 µg/mL of chlorhexidine and 500 to 1000 µg/mL of *G. mangostana* extract as shown the viability of less than 10% (Fig. 4). It is interestingly found that the concentration of chlorhexidine can be 4–16 times lower in the presence of *G. mangostana* extract but presenting the effects against cysts while reducing its toxicity. This finding is in line with a previous study demonstrated a synergistic effect against *A. polyphaga* from a combination of chlorhexidine digluconate (CLX) and carbosilane dendrimers containing ammonium or guanidine moieties [34]. From this study, it appears to be a promising combined agents to fight against the infection and especially resistance pattern of *Acanthamoeba* spp. in the future.

The mode of action considered in this study was confirmed by scanning electron microscopy (SEM) as shown in Figs. 5 and 6. Treated trophozoites showed similar flat cells and smooth surface as a result of total destruction of acanthopodia in the presence of *G. mangostana* extract and chlorhexidine. In combination, morphology of trophozoites were rounded and being observed of the presence of pores on its surface. Chlorhexidine is positively charged and ionic with the negatively charged plasma membrane of the parasite, resulting in membrane structure that gives rise to permeability modulation, ionic leakage, and cytoplasmic disruptions causing cellular damage and cell death [35–36]. The control cysts showed regular morphological characteristics. Overall, *A. triangularis* cysts were flat and morphological deformity (irregular in shape and size) as a result on destruction of the ectocyst walls after treatment given with *G. mangostana* extract, chlorhexidine and in combination of chlorhexidine/*G. mangostana* extract.

## Conclusion

In line with the data obtained from this study, *G. mangostana* ethanolic extract from pericarp possesses anti-*Acanthamoeba* activity against both trophozoites and cysts. Moreover, it is clear that we have presented for the first time, an anti-*Acanthamoeba* activity with emphasis on herb-drug combinatorial approach. This study therefore recommends further research in this area as it could provide an alternative treatment method for the management of *Acanthamoeba* infections.

## Declarations

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### **Ethics approval and consent to participate**

Not applicable

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

Data supporting the conclusions of this article are included within the article. The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

SS performed all the laboratory work, analyzed the data and wrote a draft of the paper; VN conceived the idea, designed the experiments related to natural products and parasitology, and overlooked at the laboratory work; WM assisted in the experiments; All authors read, reviewed, edited, agreed, and approved for the submission of the finalized manuscript.

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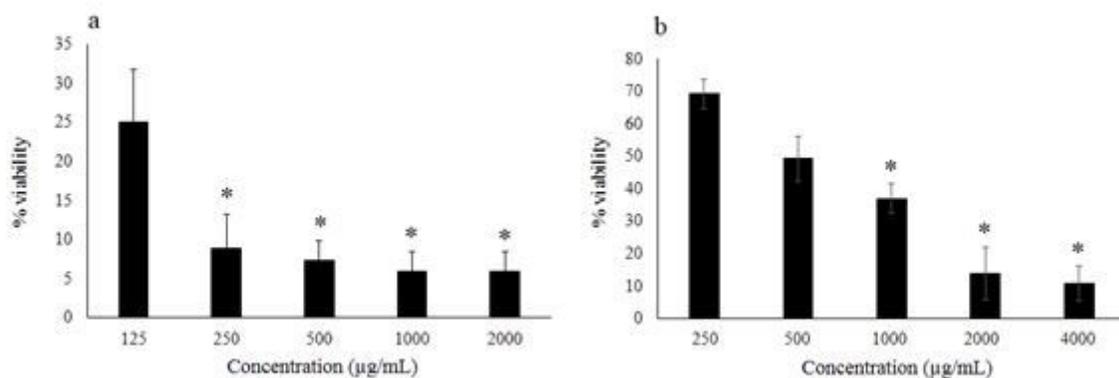
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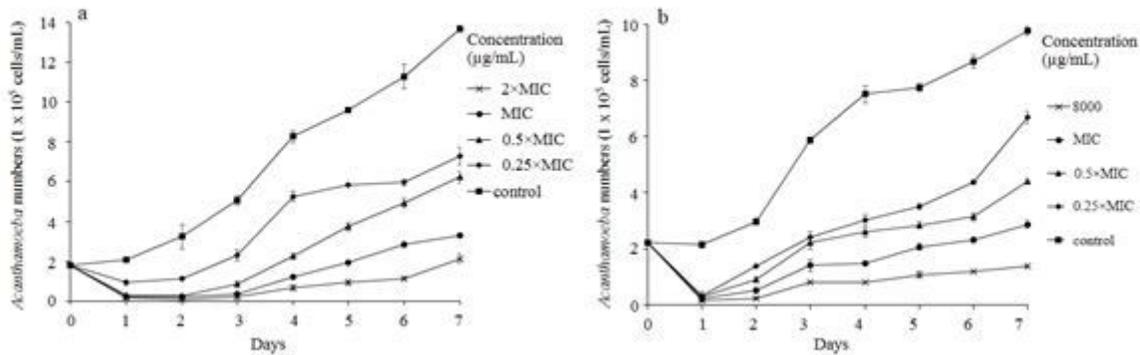
## Figures



**Figure 1**

Effect of *G. mangostana* extract on viability of *A. triangularis* trophozoites (a) and cysts (b). Parasite cells were treated with different concentrations of extract, incubated at room temperature for 24 hours.

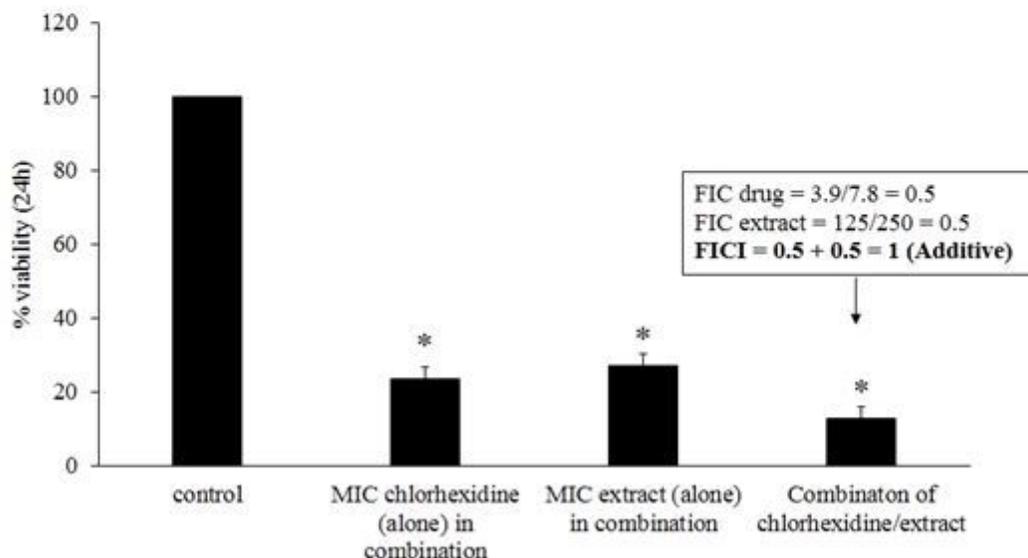
Inhibitory activity was carried out using trypan blue exclusion assay. 1% DMSO was used as negative control. The relative percentage of viability was defined as (mean of the treated /mean of the control)×100.



**Figure 2**

*G. mangostana* extract inhibits *A. triangularis* growth in vitro. To determine the effect of extract on trophozoites (a) and cysts (b), assays were performed by inoculating  $2 \times 10^5$  cells/mL in PYG medium in the presence of extract at 2×MIC, MIC, 0.5×MIC and 0.25×MIC final concentrations. Inhibitory activity was carried out using trypan blue exclusion assay up to a week. 1% DMSO was used as negative control.

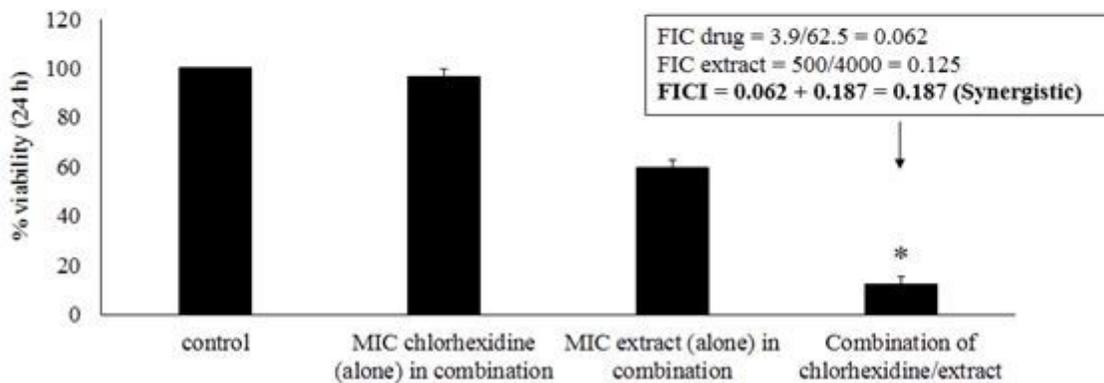
7.81	9.09 ± 3.14	9.09 ± 3.14	5.45 ± 0	5.45 ± 0	5.45 ± 0	0
3.90	23.63 ± 3.14	40.00 ± 3.14	36.36 ± 3.14	27.27 ± 0	<b>12.72 ± 3.14</b>	0
1.95	47.27 ± 3.14	52.72 ± 3.14	45.45 ± 3.14	34.54 ± 3.14	23.63 ± 3.14	0
0.97	54.54 ± 5.45	54.54 ± 0	45.45 ± 3.14	40.00 ± 3.14	25.45 ± 3.14	5.45 ± 0
0.48	78.18 ± 3.14	74.54 ± 3.14	60.00 ± 5.45	47.27 ± 3.14	29.09 ± 3.14	5.45 ± 0
0	100 ± 0	72.72 ± 6.29	58.18 ± 3.14	47.27 ± 3.14	27.27 ± 0	12.72 ± 3.14
	0	15.62	31.25	62.5	125	250



**Figure 3**

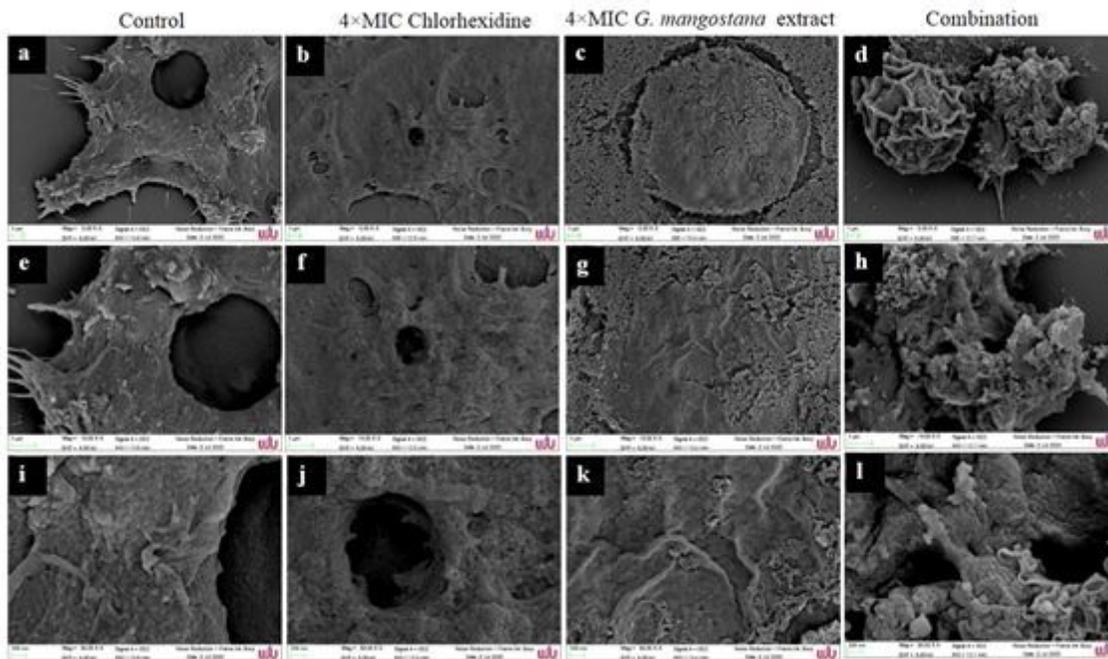
Combination of *G. mangostana* extract and chlorhexidine for anti-amoebic effects on trophozoites of *A. triangularis*. Parasites were grown in PYG medium in the presence of extract alone and in combination with chlorhexidine for 24 hours. Inhibitory activity was carried out using trypan blue exclusion assay. 1% DMSO was used as negative control. The relative percentage of viability was defined as (mean of the treated / mean of the control) × 100.

Concentration drug ( $\mu\text{g/mL}$ )	62.50	7.01 $\pm$ 3.03	5.26 $\pm$ 0	5.26 $\pm$ 0	5.26 $\pm$ 0	0	0
	31.25	29.82 $\pm$ 3.03	22.80 $\pm$ 3.03	7.01 $\pm$ 3.03	7.01 $\pm$ 3.03	7.01 $\pm$ 3.03	0
	15.62	57.89 $\pm$ 0	17.54 $\pm$ 3.03	7.01 $\pm$ 3.03	7.01 $\pm$ 3.03	7.01 $\pm$ 3.03	3.50 $\pm$ 3.03
	7.81	87.71 $\pm$ 0.67	45.61 $\pm$ 3.03	7.01 $\pm$ 3.03	7.01 $\pm$ 3.03	7.01 $\pm$ 3.03	7.01 $\pm$ 3.03
	3.9	96.49 $\pm$ 3.03	49.12 $\pm$ 3.03	12.28 $\pm$ 3.03	14.03 $\pm$ 3.03	10.52 $\pm$ 0	10.52 $\pm$ 0
	0	100 $\pm$ 0	59.64 $\pm$ 3.03	59.64 $\pm$ 3.03	24.56 $\pm$ 3.03	7.01 $\pm$ 3.03	8.77 $\pm$ 3.03
		0	250	500	1000	2000	4000
			Concentration extract ( $\mu\text{g/ml}$ )				



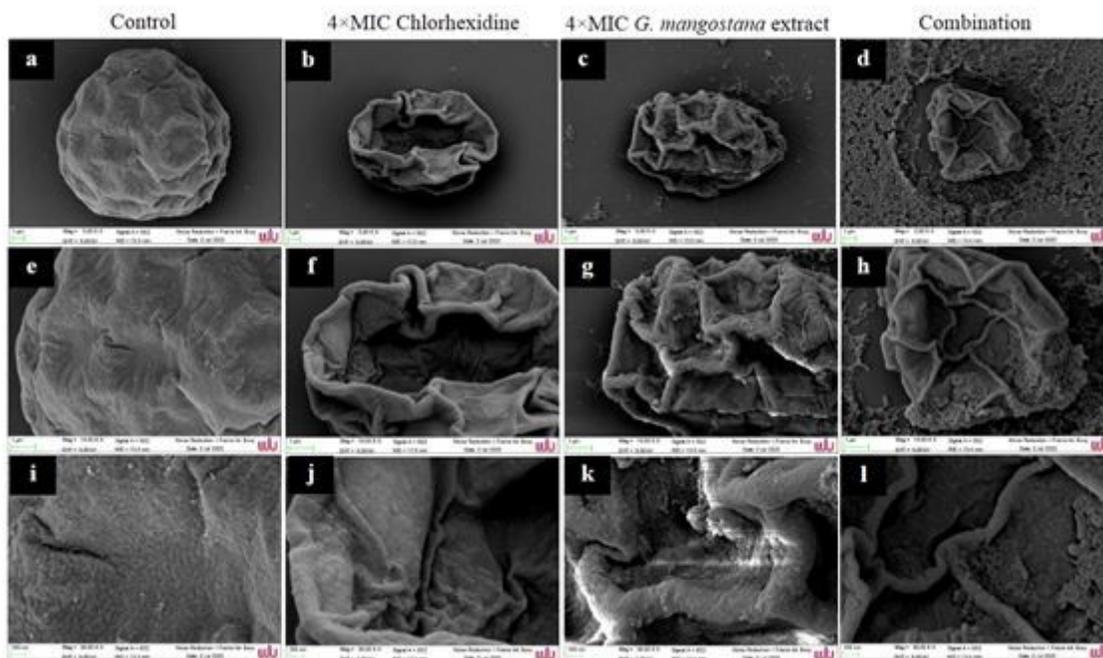
**Figure 4**

Combination of *G. mangostana* extract and chlorhexidine for anti-amoebic effect on cysts of *A. triangularis*. Parasites were grown in PYG medium in the presence of extract alone and in combination with chlorhexidine for 24 hours. Inhibitory activity was carried out using trypan blue exclusion assay. 1% DMSO was used as negative control. The relative percentage of viability was defined as (mean of the treated / mean of the control)  $\times$  100.



**Figure 5**

Scanning micrographs of *A. triangularis* trophozoites after treatment with *G. mangostana* extract, chlorhexidine and in combination at 24 hours. Control trophozoites (a,e,i); trophozoites were treated with 4xMIC chlorhexidine (b,f,j); 4xMIC *G. mangostana* extract (c,g,k) and combination (d,h,l), respectively. Magnification: a-d = 5000X; e-h = 10000X; i-l = 30000X.



**Figure 6**

Scanning micrographs of *A. triangularis* cysts after treatment with *G. mangostana* extract, chlorhexidine and in combination at 24 hours. Control cysts (a,e,i); cysts were treated with 4×MIC chlorhexidine (b,f,j); 4×MIC *G. mangostana* extract (c,g,k) and combination (d,h,l), respectively. Magnification: a-d = 5000X; e-h = 10000X; i-l = 30000X.