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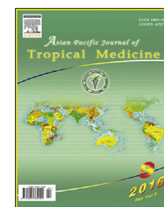

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# Anti-encystment and amoebicidal activity of *Lonicera japonica* Thunb. and its major constituent chlorogenic acid *in vitro*

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ABSTRACT

**Objective:** To examine the acanthamoebicidal effects of ethyl acetate, aqueous and butanol fractions of dried flower buds of *Lonicera japonica (L. japonica)* Thunb. (*Flos Lonicerae*) *in vitro*.

**Methods:** *Acanthamoeba triangularis* isolates were obtained from environmental water samples and identified by PCR. They were exposed to ethyl acetate, water and butanol fractions of *L. japonica* Thunb. at concentrations ranging from 0.5 mg/mL to 1.5 mg/mL. The extracts were evaluated for growth inhibition at 24, 48 and 72 h, respectively. Chlorogenic acid at a concentration of 1 mg/mL was examined for inhibition of encystment.

**Results:** Ethyl acetate fraction at a concentration of 1.5 mg/mL evoked a significant reduction of trophozoite viability by 48.9% after 24 h, 49.2% after 48 h and 33.7% after 72 h chlorogenic acid, the major active constituent of *L. japonica* Thunb. at the concentration of 1 mg/mL reduced the cysts/trophozoite ratio by 100% after 24 h, 84.0% after 48 h and 72.3% after 72 h. This phenolic compound at concentration of 1 mg/mL concurrent with 0.6% hydrogen peroxide inhibited hydrogen peroxide-induced encystment by 92.8% at 72 h.

**Conclusions:** Results obtained from this study show that ethyl acetate fraction at 1.5 mg/mL is the most potent fraction of *L. japonica* Thunb. and its major constituent chlorogenic acid showed the remarkable inhibition of encystment at a concentration of 1 mg/mL.

## 1. Introduction

*Acanthamoeba* is one of the most important related human pathogenic free living amoebas. Members of the genus *Acanthamoeba* Volkonsky, discovered in 1931, are protozoa living in soil, dust, water, sand, humidifiers, air conditioning units, contact lens and hospital facilities [1]. Some species in this genus are human pathogens [2], for instance *Acanthamoeba polyphaga* and *Acanthamoeba triangularis (A. triangularis)* which account for ophthalmic and cutaneous keratitis as well as granulomatous encephalitis [3]. Granulomatous amoebic encephalitis (GAE) is associated with immunosuppression and is usually fatal [4]. Treating acanthamoebiasis is difficult as there have been constant reports on the resistance to biocides, lack of nutrients or unfavorable temperature or osmolarity trigger within 24–48 h phenotype modification from vegetative trophozoites to cryptobiotic multiresistant cysts [5]. So far, there is no clear evidence of the complete molecular pathways commanding encystment but some recent studies suggest the participation of reactive oxygen species as well as a variation in redox state in the protozoa [6]. Evidence for the ability of medicinal plants

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to abrogate the survival of trophozoites and cysts *in vitro* has been accumulating. Among the pathogenic free living amoebae, *A. triangularis* shows typical morphological characteristics of group II in the genus *Acanthamoeba* that has been classified for the past 40 years [7]. *A. triangularis* has a double walled cyst which consists of thick wrinkled ectocyst and satellite or polygonal endocyst. The cyst diameter is between 12.0 and 22.5 μm and with (3–6) arms [8].

As part of an ongoing study to isolate novel chemopreventive or therapeutic compounds from Asian medicinal plants, we, therefore focused on the flower buds of *Lonicera japonica (L. japonica)* Thunb. or 'Jin Yin Hua' which are used externally in the form of water decoction for the treatment of acute conjunctivitis in traditional Chinese medicine [9]. Since conjunctivitis is a symptom of acanthamoebiasis, we prepared extracts in solvents of different polarity, from the dried flower buds of *L. japonica* Thunb. for their ability to inhibit the growth of trophozoites, cysts and encystment of *A. triangularis in vitro*. In the literature, studies demonstrate that extracts of flower buds of *L. japonica* Thunb. inhibit TNFα in lipopolysaccharide-stimulated RAW 264.7 macrophages [10] lower glycemia [11] and possess antioxidant properties on account of its major constituent chlorogenic acid [12]. Chlorogenic acid is one of the most abundant phenolic compounds in the flower buds for *L. japonica*. This conjugate of caffeic acid and quinic acid is produced by few plant species including *Coffea arabica*, *Artemisia iwayomogi* and *Castilla elastica*. Some evidence demonstrates that chlorogenic acid is antioxidant, antibacterial, anticarcinogenic, particularly hypoglycemic and hypolipidemic [13]. To date, no study on acanthamoebicidal activity on *L. japonica* Thunb. and its major constituent chlorogenic acid has been reported against pathogenic *Acanthamoeba* spp.

## 2. Materials and methods

### 2.1. Plant material and extraction

Dried flower buds of *L. japonica* Thunb. were purchased from a local Chinese medicinal store in Kuala Lumpur, Malaysia and were deposited in the herbarium of the University of Malaya with the voucher number KLU 47693. A total of 650 g of the powdered plant material was soaked with absolute ethanol at room temperature for 7 days. The tincture was filtered and evaporated to obtain a dark brown mass. The dried ethanol extract was resuspended in water and the aqueous phase was defatted with hexane and successively extracted with ethyl acetate and butanol. Upon evaporation, 0.5 g of ethyl acetate, 3 g of butanol and 11.3 g of aqueous fractions were obtained, kept in air tight containers and stored in a refrigerator until use.

### 2.2. Acanthamoeba cultivation on non-nutrient agar (NNA)

Positive environmental water samples were obtained from Department of Parasitology, Faculty of Medicine, University of Malaya. The water samples were concentrated. Parasites were cultured onto non-nutrient agar plates lawned with *Escherichia coli* and incubated at room temperature. The presence of *Acanthamoeba* was confirmed after 14 days of inoculation on the agar medium by observation of trophozoites and cysts using an inverted microscope. The morphological characteristics of both stages were observed as a trophozoite stage which comprises of a motile cell with prominent vacuole having acanthopodia with a diameter between 13 and 23 μm while a cyst stage consists of a triangular dormant double walled structure with a diameter between 12.0 and 22.5 μm. Axenic culture of *Acanthamoeba* was obtained. To yield the monogenous culture of *Acanthamoeba*, sub-cultures were routinely performed three to four times in the laboratory prior to DNA extraction of this pathogen.

### 2.3. Identification of Acanthamoeba using PCR

The feeding trophozoites were harvested using cold Page's Saline, (0.12 g NaCl, 0.004 g $MgSO_4 \cdot 7H_2O$, 0.004 g $CaCl_2 \cdot 2H_2O$, 0.142 g $Na_2HPO_4$, and 0.136 g $KH_2PO_4$ per liter of distilled water), and centrifuged at 3 500 rpm for 10 min, followed by DNA extraction using QIamp DNA blood mini kit (QIAGEN, Hiden, Germany). Polymerase chain reaction was performed in a 25 μL mixture of distilled water, 10X DNA polymerase buffer (Thermo Scientific, Lithuania, USA), 25 mM of magnesium chloride ($MgCl_2$) (Thermo Scientific, Lithuania, USA), 10 mM of deoxynucleotide triphosphate (dNTP) mix (Thermo Scientific, Lithuania, USA), 200 mol of each of *Acanthamoeba* genus-specific primer [14]: JDP1 (5′-GGCCCAGATCGTTACCG TGAA-3′) and JDP2 (5′-TCTCACAAGCTGCTAGGGAG TCA-3′), 1U of Taq DNA polymerase (Thermo Scientific, Lithuania, USA) and 5 μL of DNA template. The reaction was carried out at 94 °C for 5 min, followed by 40 cycles of annealing at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and an extension at 72 °C for 5 min. For negative control, the DNA template was replaced with the same volume of sterile distilled water.

### 2.4. Molecular analysis

PCR amplifications were carried out by targeting the 18S region and the amplicon at 450 bp was analyzed using 1.5% electrophoresis gel. The gel was stained with ethidium bromide and visualized under UV light. The positive samples were sent for sequencing and homology search was performed by BLAST software, obtained from National Centre for Biotechnology Information.

### 2.5. In vitro cultivation of trophozoite and cyst stages

Environmental isolates of *A. triangularis* were cultured on NNA plates coated with *E. coli* at 26 °C. Trophozoites at the exponential growth phase between 24 and 48 h were gently scrapped from the base of NNA culture plates with a sterile cell scraper. The trophozoites were washed twice with sterile Pages's Saline and were concentrated by centrifugation at 1 500*g* for 5 min [15]. The number of viable trophozoites was determined by 0.4% trypan blue exclusion and direct trophozoites count on hemocytometer [16,17]. The final concentration was adjusted to 100 000 trophozoites/mL and the trophozoites were used in the assay without delay. Two–three week old culture of *A. triangularis* was used in cysticidal assay. The cysts were harvested by washing in Page's Saline and were adjusted to a final concentration of 100 000 cysts/mL, as previously described. The viability of cysts was tested by 0.4% trypan blue method, and the inoculum with 100% viability was used

in the experiment to see the effects of *L. japonica* Thunb. extracts on cysts.

## 2.6. Amoebicidal activity testing

Two hundred microliters of calibrated trophozoites or cysts suspension and 200 µL of ethyl acetate, butanol or water fraction (0.5 mg/mL, 1.0 mg/mL and 1.5 mg/mL) of *L. japonica* Thunb. were mixed thoroughly in micro-centrifuge tubes and kept at 26 °C in the dark for 24, 48 and 72 h. Control tubes contain only sterile distilled water instead of plant extracts with 200 µL of trophozoites/cysts suspension. Chlorhexidine was used as a positive control at a concentration of 4 µg and 25 µg in 1 mL of sterile distilled water for trophozoites and cysts, respectively [18,19]. After 24, 48 and 72 h of incubation at 26 °C, 10 µL of trophozoites or cysts suspension was mixed with 10 µL of 0.4% trypan blue in a counting chamber. The specimens were incubated for 3 min at room temperature and the unstained (viable) and stained (nonviable) trophozoites were counted separately in hemocytometer [19].

## 2.7. Encystment assay

*A. triangularis* was divided into five experimental groups, each comprising 100 000 trophozoites/mL. The first group was treated with 100 µL of chlorogenic acid (Sigma Aldrich, St. Louis, USA) at a concentration of 1 mg/mL with 100 µL (10 000 trophozoites) of trophozoites suspension for 3 days. The second group was treated with 0.6% hydrogen peroxide for 3 days (10 µL of 6% $H_2O_2$ mixed with 90 µL of sterile distilled water) to 100 µL of trophozoites suspension. The third group was treated concomitantly with chlorogenic acid (1 mg/mL) and hydrogen peroxide (10 µL of 6% $H_2O_2$ mixed with 90 µL of sterile distilled water) to 100 µL of trophozoites suspension for 3 days. The fourth group was treated with chlorogenic acid (Sigma Aldrich, St. Louis, USA) at a concentration of 1 mg/mL for a day and then concomitantly hydrogen peroxide (10 µL of 6% $H_2O_2$ mixed with 90 µL of sterile distilled water) to 100 µL of trophozoites suspension for 2 days. The fifth group served as a control and treated with sterile distilled water and trophozoites suspension for 3 days. Each day, 10 µL of test solutions were pipetted out and mixed with 10 µL of 0.4% trypan blue and cells (trophozoites and cysts) were counted using hemocytometer.

## 2.8. Statistical analysis

The data analysis was performed by using SPSS software (Statistical Package for Social Sciences) version 21 (SPSS, Chicago, IL, USA). Data were presented as mean values with standard deviations and were analyzed by repeated measure of ANOVA followed by the Tukey test for the post hoc pairwise comparisons. $P < 0.05$ was regarded as statistical significance.

# 3. Results

## 3.1. Ethyl acetate, water and butanol fraction of L. japonica inhibit the growth of A. triangularis in vitro

*In vitro* amoebicidal effects were examined by evaluating the percentage of growth inhibition of ethyl acetate, water and butanol fraction of *L. japonica* in comparison with the untreated as a control. The positive standard chlorhexidine at a dose of 4 µg/mL reduced trophozoites availability by 57.9%, 71.4% and 71.5% in comparison with non-treated control after 24, 48 and 72 h, respectively. The ethyl acetate fraction at a concentration of 1.5 mg/mL evoked a significant reduction ($P < 0.05$) of trophozoites viability by 48.9% after 24 h, 49.2% after 48 h and 33.7% after 72 h as compared to the non-treated (control), as shown in Table 1.

Upon administration of the aqueous fraction at a concentration of 1.5 mg/mL, the survival of trophozoites showed a significant reduction ($P < 0.05$) by 25.0%, 12.8% and 44.7% in comparison with control after 24, 48 and 72 h, respectively, as demonstrated in Table 2.

Whereas, the butanol fraction at a concentration of 1.5 mg/mL, reduced the viability by 20.2% and 2.30% in comparison with control after 24 and 48 h, as shown in Table 3.

Overall, the results obtained from the *in vitro* assay on trophozoites demonstrate that the viability of trophozoites challenged with the ethyl acetate and aqueous fractions were significantly inhibited compared to control group. However, ethyl acetate, butanol, water fractions of *L. japonica* Thunb. and chlorhexidine did not show any significant growth inhibition against the resistant cystic stage (Figure 1).

Evaluation on the effect of chlorogenic acid and/or hydrogen peroxide against the encystment of *A. triangularis in vitro* was shown in Figure 2.

**Table 1**

Effect of *L. japonica* Thunb. ethyl acetate fraction on growth inhibition/stimulation of *Acanthamoeba triangularis*.

| Extract dose (mg/mL) | 24 h | | 48 h | | 72 h | |
|---|---|---|---|---|---|---|
| | Trophozoites | Cysts | Trophozoites | Cysts | Trophozoites | Cysts |
| Non-treated control group | 27.6 ± 2.5 (0) | 40.6 ± 1.5 (0) | 25.6 ± 1.1 (0) | 37.6 ± 4.5 (0) | 24.6 ± 0.57 (0) | 38.0 ± 3.4 (0) |
| [a]0.004%/[b]0.025% Chlorhexidine | 11.6 ± 1.5 (57.9⁻) | 36 ± 5.5 (11.4⁻) | 7.3 ± 3.7 (71.4⁻) | 27.6 ± 4.7 (26.5⁻) | 7.0 ± 2 (71.5⁻) | 31.6 ± 3.5 (16.6⁻) |
| *L. japonica* (1.5) | 19.6 ± 4.0*** (48.9⁻) | 44 ± 1.0 (8.2⁺) | 13.0 ± 1.0* (49.2⁻) | 28 ± 2.6 (25.6⁻) | 16.3 ± 5.1*** (33.7⁻) | 29.0 ± 1.0 (23.6⁻) |
| *L. japonica* (1.0) | 17.0 ± 1.0* (38.4⁻) | 32.0 ± 6.0 (21.2⁻) | 12.6 ± 2.3*(50.7⁻) | 31.0 ± 3.6 (17.6⁻) | 13 ± 1.7* (47.1⁻) | 29.0 ± 14.0 (23.6⁻) |
| *L. japonica* (0.5) | 27.3 ± 3.0** (1.0⁻) | 30.0 ± 1.0 (26.2⁻) | 18.0 ± 0***(29.6⁻) | 25.6 ± 8.5 (21.4⁻) | 11 ± 3.4* (55.2⁻) | 28.0 ± 4.3 (26.3⁻) |

[a]0.004% Chlorhexidine is for trophozoites. [b]0.025% Chlorhexidine is for cyst. (+) – stimulation, (−) – inhibition. *$P < 0.05$, statistically significant difference in comparison to non-treated control in the same time interval. **$P < 0.05$, statistically significant difference in comparison to drug control in the same time interval.

**Table 2**

Effect of *L. japonica* Thunb. water fraction on growth inhibition/stimulation of *Acanthamoeba triangularis*.

| Extract dose (mg/mL) | 24 h | | 48 h | | 72 h | |
|---|---|---|---|---|---|---|
| | Trophozoites | Cysts | Trophozoites | Cysts | Trophozoites | Cysts |
| Non-treated control | 27.6 ± 2.5 (0.0) | 40.6 ± 1.5 (0.0) | 25.6 ± 1.1 (0.0) | 37.6 ± 4.5 (0.0) | 24.6 ± 0.6 (0.0) | 38.0 ± 3.4 (0.0) |
| [a]0.004%/[b]0.025% Chlorhexidine | 11.6 ± 1.5 (57.9$^-$) | 36 ± 5.5 (11.4$^-$) | 7.3 ± 3.7 (71.4$^-$) | 27.6 ± 4.7 (26.5$^-$) | 7.0 ± 2.0 (71.5$^-$) | 31.6 ± 3.5 (16.6$^-$) |
| *L. japonica* (1.5) | 20.6 ± 3.0$^{**}$ (25.0$^-$) | 28.6 ± 6.6 (29.6$^-$) | 22.3 ± 0.5$^{***}$(12.8$^-$) | 34.0 ± 3.4 (9.7$^-$) | 13.6 ± 1.1*(44.7$^-$) | 32.6 ± 3.0 (14.2$^-$) |
| *L. japonica* (1.0) | 20.0 ± 4.3$^{**}$(27.5$^-$) | 36.6 ± 4.9 (9.9$^-$) | 22.0 ± 1.7$^{**}$ (14.0$^-$) | 35.6 ± 3.7 (5.4$^-$) | 13.6 ± 12.3*(44.7$^-$) | 34.0 ± 2.0 (10.5$^-$) |
| *L. japonica* (0.5) | 19.3 ± 3.0$^{*}$(30.0$^-$) | 35.6 ± 5 (12.4$^-$) | 19.0 ± 0$^{***}$ (25.7$^-$) | 29.3 ± 4.5 (22.0$^-$) | 21.0 ± 5.0**(14.6$^-$) | 30.6 ± 6.0 (19.4$^-$) |

[a]0.004% Chlorhexidine is for trophozoites. [b]0.025% Chlorhexidine is for cyst. (+) – stimulation, (–) – inhibition. $^{*}P < 0.05$, statistically significant difference in comparison to non-treated control in the same time interval. $^{**}P < 0.05$, statistically significant difference in comparison to drug control in the same time interval.

**Table 3**

Effect of *L. japonica* Thunb. butanol fraction on growth inhibition/stimulation of *Acanthamoeba triangularis*.

| Extract dose (mg/mL) | 24 h | | 48 h | | 72 h | |
|---|---|---|---|---|---|---|
| | Trophozoites | Cysts | Trophozoites | Cysts | Trophozoites | Cysts |
| Non-treated control | 27.6 ± 2.5 (0.0) | 40.6 ± 1.5 (0.0) | 25.6 ± 1.1 (0.0) | 37.6 ± 4.5 (0.0) | 24.6 ± 0.57 (0.0) | 38.0 ± 3.4 (0.0) |
| [a]0.004%/[b]0.025% Chlorhexidine | 11.6 ± 1.5 (57.9$^-$) | 36 ± 5.5 (11.4$^-$) | 7.3 ± 3.7 (71.4$^-$) | 27.6 ± 4.7 (26.5$^-$) | 7.0 ± 2.0 (71.5$^-$) | 31.6 ± 3.5 (16.6$^-$) |
| *L. japonica* (1.5) | 22.0 ± 3.0$^{**}$(20.2$^-$) | 36.0 ± 1.1 (11.4$^-$) | 25.0 ± 5.0$^{**}$ (2.3$^-$) | 36.0 ± 0 (4.4$^-$) | 27.6 ± 1.0$^{**}$ (12.1$^+$) | 38.0 ± 3.2 (0.0) |
| *L. japonica* (1.0) | 24.3 ± 2.0$^{**}$(11.9$^-$) | 41.6 ± 1.1 (2.3$^+$) | 22.6 ± 4.1$^{**}$ (11.7$^-$) | 35.6 ± 3.7 (5.4$^-$) | 22.0 ± 1.0$^{**}$ (10.5$^-$) | 36.3 ± 3.2$^{**}$ (4.4$^-$) |
| *L. japonica* (0.5) | 23.3 ± 4.1$^{**}$(15.5$^-$) | 37.6 ± 2.5 (7.3$^-$) | 17.3 ± 3.2 (32.3$^-$) | 37.6 ± 2.5 (0.0) | 18.0 ± 2.6 (26.8$^-$) | 33.3 ± 3.1 (12.3$^-$) |

[a]0.004% Chlorhexidine is for trophozoites. [b]0.025% Chlorhexidine is for cyst. (+) – stimulation, (–) – inhibition. $^{*}P < 0.05$, statistically significant difference in comparison to non-treated control in the same time interval. $^{**}P < 0.05$, statistically significant difference in comparison to drug control in the same time interval.

**Figure 1.** *Acanthamoeba triangularis* viable cysts.

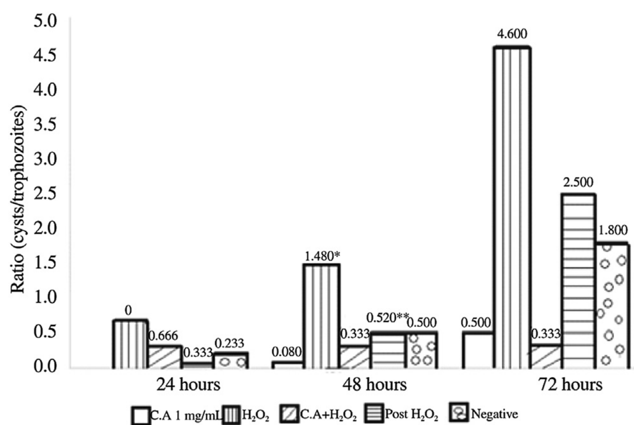


**Figure 2.** Effect of chlorogenic acid and/or hydrogen peroxide on encystment of *Acanthamoeba triangularis*. $^{*}P < 0.05$, statistically significant difference in comparison between chlorogenic acid and $H_2O_2$ in same interval. $^{**}P < 0.05$, statistically significant difference in comparison between chlorogenic acid and post $H_2O_2$ in same interval.

### *3.2. Inhibition of encystment by chlorogenic acid*

Chlorogenic acid, an ester of caffeic acid and quinic acid (Figure 2), at a concentration of 1.0 mg/mL, lowered the growth of trophozoites by 100%, 84.0% and 72.3% in comparison with control after 24, 48 and 72 h, respectively (Figure 3).

### *3.3. Encystment induced by hydrogen peroxide*

Following hydrogen peroxide addition to the culture medium, *A. triangularis* encysted with a ratio cysts/trophozoites increased by of 65.1%, 66. 3% and 60.9% after 24, 48 and 72 h, respectively as compared to control.

### *3.4. Effect of chlorogenic acid on encystment induced by hydrogen peroxide*

Concurrent administration of chlorogenic acid at a concentration of 1 mg/mL and 0.6% hydrogen peroxide sustained cysts/

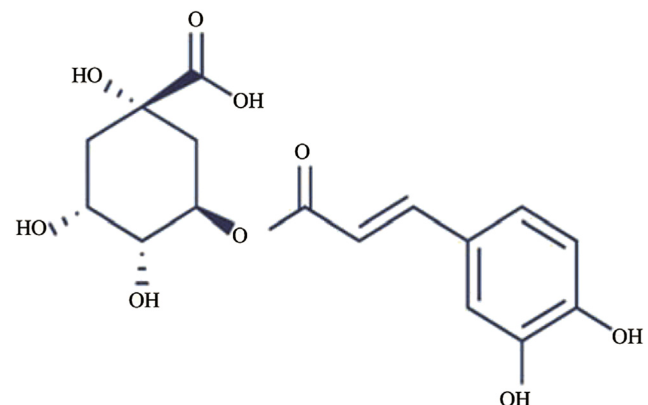


**Figure 3.** Chemical structure of chlorogenic acid.

trophozoites ratio as 0.333, 0.333, 0.333 after 24, 48 and 72 h, respectively. Initially cysts/trophozoites ratio increased by 30.1% after 24 h and a reduction by 33.4% and 81.5% after 48 and 72 h, respectively in comparison to the control.

### *3.5. Prophylactic effect of chlorogenic acid on encystment induced by hydrogen peroxide*

*A. triangularis* trophozoites were exposed to chlorogenic acid at a concentration of 1 mg/mL for 48 h and challenged with hydrogen peroxide. After 24 h, the ratio cysts/trophozoites were decreased by 34.3%. After exposure to hydrogen peroxide after 48 and 72 h, the ratio cysts/trophozoites were increased by 4.0% and 38.8%, respectively as compared to the control.

## 4. Discussion

*Acanthamoeba* encephalitis is a life threatening condition in immunocompromised patients for which current therapeutic agents have severe side effects. More often *Acanthamoeba* accounts for keratitis and/or conjunctivitis resulting in keratoconjunctivitis or blindness. Treatment of *Acanthamoeba* keratitis is difficult and requires topical application of drugs for several months, representing a major constrain for the patients because of lack of drugs to target both trophozoites and cysts.

As part of an ongoing study to isolate chemopreventive or therapeutic compounds from Asian medicinal plants, we became interested in the flower buds of *L. japonica* Thunb. or 'Jin Yin Hua' which are used for the treatment of acute conjunctivitis in traditional Chinese medicine [21]. The ethyl acetate fraction at concentrations ranging from 0.5 mg/mL to 1.5 mg/mL, after 72 h, dose dependently inhibited the growth of trophozoites and exhibited levels of potency superior to 0.004% chlorhexidine which is used for the treatment of *Acanthamoeba* keratitis [22]. In extreme temperature, desiccation, osmolarity and pH conditions, there is a phenotypic shift of *Acanthamoeba* from trophozoites into a round, doubled-walled cysts which allow the protozoa to remain alive for years in harsh environment. The duration of the viability of *Acanthamoeba* cysts is about 25 years in the natural environment with well-maintained invasive properties [23].

In this context, we examined the cystocidal effects of the 3 fractions obtained from the flower buds of *L. japonica* Thunb. The ethyl acetate fraction at 0.5 mg/mL evoked a non-significant decrease number of cysts by 26.3% after 72 h and this effect was superior to 0.025% chlorhexidine. Similarly, the aqueous fraction evoked a 29.6% reduction of cysts after 24 h at 1.5 mg/mL. The butanol at 1.5 mg/mL was comparatively least reactive and showed 11.6% of decrement of cysts viability after 24 h in comparison with control.

Emergence of resistance to biocides during differentiation of *Acanthamoeba castellanii* has been reported [24]. Cysts are difficult to eradicate, giving rise to the concept that agents inhibiting encystment of *Acanthamoeba* could facilitate the treatment of acanthamoebiasis. So far, we know that glycogen stores in *Acanthamoeba* serve as a source of glucose for the synthesis of cellulose during encystment. In fact, glycogen is the most rapidly degraded macromolecule during the early hours of encystment via increased enzymatic activity of glycogen phosphorylase [25]. In mammalian cells, glycogen phosphorylase is activated by adenosine monophosphate activated protein kinase which is allosterically activated by cellular starvation and reactive oxygen species [26]. In fact, evidence suggests that the generation of reactive oxygen species, coupled to redox cycling, driven by cytoplasmic and mitochondrial processes, is the core of observed rhythmicity and scale-free dynamics of *Acanthamoeba* [27]. In our study, none of the tested fractions was significantly active since the double walled structure of *Acanthamoeba* cysts made of protein and cellulose resists most chemotherapeutic agents. We further examined the effects of chlorogenic acid, against *A. triangularis* encystment induced by reactive oxygen species. Chlorogenic acid, the first isolated was from coffee beans [28] is the major active component of the flowers of *L. japonica* Thunb. [29]. Current encystment techniques employ axenic and xenic techniques which are based on bacterial deprivation and osmotic stress, respectively and hydrogen peroxide was used in this study as an oxidative cystogenic agent. Upon exposure of *A. triangularis* trophozoites to hydrogen peroxide in culture media, a significant increase in cysts/trophozoites ratio after 72 h occurred and co-administration of chlorogenic acid at a concentration of 1 mg/mL repressed the ratio by approximately 90% after 72 h. In addition, we examined the effect of chlorogenic acid pre-treatment on hydrogen peroxide encystment and found a reduction of cysts/trophozoites by about 60%. The precise mechanism underlying the anti-encystment effect of chlorogenic acid is, at this point, unknown. This phenolic compound is anti-inflammatory [30] and antioxidant [31] and accounts for most of the medicinal uses of the plant [32]. Resveratrol and demethoxycurcumin which are strong antioxidants have been recently reported to inhibit the growth of *Acanthamoeba in vitro* [33] and this is important because it raises the possibility that antioxidant principles may be able to inhibit encystment of *Acanthamoeba*. Thus, although highly speculative, since chlorogenic acid scavenges free radicals *in vitro* with an $IC_{50}$ equal to 10.2 μM [34], it is reasonable to speculate that this ester of caffeic acid and quinic acid inhibits encystment by scavenging reactive oxygen species in the cytoplasm of *Acanthamoeba* impeding thus downstream allosteric activation of adenosine monophosphate activated protein kinase and downstream glycogen phosphatase.

From the results obtained in this study, the flower buds of *L. japonica* being abundantly available in Asia may represent a source of lead for the treatment. Alternatively, chlorogenic acid shows as a promising candidate for the combined therapy for acanthamoebiasis with existing therapeutic agents. Therefore, this could be the major breakthrough not only for more future studies to explore more of its potential for not only *Acanthamoeba* but also other waterborne protozoan parasites. In addition, this could also serve as a potential alternative medicine for pathogenic *Acanthamoeba* causing keratitis in the future.

## Conflict of interest statement

We declare that we have no conflicts of interest.

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