



Anti-Amoebic Activity of *Clitoria ternatea* L. Flower Extract against *Acanthamoeba culbertsoni* Genotype T4

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Abstract: Background: *Acanthamoeba culbertsoni* is an opportunistic protozoan capable of causing severe infections in humans, including *Acanthamoeba* keratitis (AK), which predominantly affects contact lens users. Current therapeutic options remain inadequate, as no available treatment can completely eradicate the infection, primarily due to the formation of resistant cysts. Natural plant-derived compounds such as those from *Clitoria ternatea* L. have gained attention for their potential antimicrobial properties. **Objectives:** Thus, the study aimed to evaluate the *in vitro* anti-amoebic activity of *C. ternatea* L. flower aqueous extract against *A. culbertsoni* genotype T4, as well as to assess its cytotoxic effects on human host cells. **Methods:** The *A. culbertsoni* strain (MH791017) isolated from contact lens paraphernalia was used throughout the study. Amoebicidal, encystation and excystation assays were performed to determine the extract's efficacy against trophozoite and cyst stages. Trophozoite viability was assessed using trypan blue exclusion with haemocytometer counting, while cytotoxicity was evaluated using the MTT assay on human keratinocyte (HaCaT) cells. Data were analysed using appropriate statistical tests, with significance set at $p < 0.05$ and IC_{50} values were calculated to determine dose-dependent effects. **Results:** The aqueous extract exhibited significant amoebicidal activity ($p = 0.001$), with 5 mg/mL markedly reducing trophozoite viability and complete inhibition observed at 30 mg/mL. Dose-dependent suppression of both encystation and excystation processes was observed with concentrations ≥ 20 mg/mL showing significant inhibition ($p < 0.05$). Cytotoxicity assays demonstrated a dose-dependent reduction in HaCaT cell viability, with substantial cytotoxic effects detected at concentrations ≥ 5 mg/mL. **Conclusion:** Overall, *C. ternatea* flower aqueous extract demonstrated potent *in vitro* activity against both trophozoite and cyst stages of *A. culbertsoni*, likely attributable to its anthocyanin and related phytochemical constituents. These findings support further investigation to optimise efficacy, minimise cytotoxicity and validate therapeutic potential in *in vivo* models.

Key Words: Amoebicidal, Anthocyanins, Cytotoxicity, Encystation, Excystation, HaCaT Cell

INTRODUCTION

Acanthamoeba spp. are opportunistic free-living amoebae capable of causing severe infections such as *Acanthamoeba* keratitis (AK) and granulomatous amoebic encephalitis (GAE) particularly among contact lens users and immunocompromised individuals [1]. The pathogenicity of *Acanthamoeba* is driven by its ability to transition between proliferative trophozoites and highly resistant cysts via encystation and excystation, processes that underlie therapeutic failure and recurrence [2]. Current treatment regimens, which include biguanides and diamidines remain

limited by cyst resilience and significant host cytotoxicity, highlighting an urgent need for safer and more effective dual-stage anti-amoebic agents.

Recent research has explored novel anti-*Acanthamoeba* agents beyond conventional antimicrobials. For example, *Mangifera indica* leaf extract demonstrated amoebicidal, anti-adhesive activity and low cytotoxicity in *Acanthamoeba* spp., underscoring the therapeutic potential of plant-derived compounds [3]. Likewise, *Camellia sinensis* extracts induced morphological and chemical changes in trophozoite and cyst forms of *Acanthamoeba castellanii*, indicating that

phytochemicals can affect both life stages [4]. Nano-formulations combining quercetin with zinc-copper nanoparticles exhibited significant activity against trophozoites, cysts, encystation and excystation, and reduced amoeba-mediated cytopathogenicity where a promising multi-targeted strategy [5]. Additionally, aqueous leaf extracts of *Rosa gallica* and *Picea orientalis* showed trophozoitocidal effects but limited cysticidal activity and significant cytotoxicity, illustrating persistent challenges in plant extract efficacy and safety [6]. These studies collectively demonstrate that while phytochemicals hold promise, a few have achieved both potent dual-stage activity and acceptable host safety profiles, and many have not been evaluated across trophozoite, encystation or excystation and cyst endpoints in a single comprehensive framework.

Clitoria ternatea L. (Fabaceae), commonly known as the Asian pigeonwings or *bunga telang*, is rich in anthocyanins, flavonoids and other secondary metabolites with established antioxidant, antimicrobial, anti-inflammatory and cytoprotective activities [7,8]. Although various parts of *C. ternatea* have been reported to possess antibacterial and antifungal activities [9,10], its anti-amoebic potential particularly against *Acanthamoeba* spp, which remains largely unexplored. Notably, *C. ternatea* extracts have not been systematically evaluated for their effects on amoeba encystation and excystation processes or assessed for cytotoxicity using relevant human cell models.

In the pursuit of novel therapeutic candidates against *A. culbertsoni*, this study represents the first (1) to evaluate the anti-amoebic potential of *C. ternatea* flower extract against both trophozoite and cyst stages of *A. culbertsoni* genotype T4, (2) to assess the effects of the extract on encystation and excystation processes, which were critical for parasite survival and recurrence as well as (3) to determine the cytotoxicity of the extract using human keratinocyte (HaCaT) cells to determine its safety profile toward host cells. This integrated approach enabled a comprehensive evaluation of both efficacy and biocompatibility, thereby providing novel insight into the dual-stage anti-amoebic potential of *C. ternatea* and supporting its development as a promising alternative therapeutic candidate for *Acanthamoeba* infections.

METHODS

Plant Materials

The blue flowers of *Clitoria ternatea* L. (Asian pigeon wings) used in this study were selected based on their documented medicinal relevance and high abundance of bioactive anthocyanins reported in floral tissues. Fresh, mature, and disease-free blue petals were collected from Jenderam Hulu Village, Dengkil, Selangor, Malaysia, during the flowering season in November 2022, a period associated with optimal phytochemical accumulation. The plant material was identified using standard morphological criteria and authenticated by Dr. Fadzureena Jamaludin, Research Officer at the Forest Research Institute Malaysia (FRIM). A voucher specimen was prepared and deposited in the FRIM

Herbarium, Selangor, Malaysia, under the accession number SBID: 023/22, ensuring traceability and taxonomic reliability. An aqueous extraction approach was employed in this study to reflect traditional medicinal practices and to prioritise biocompatibility for potential therapeutic application. Water was selected as the extraction solvent due to its safety, non-toxicity and suitability for isolating polar phytochemicals such as anthocyanins, flavonoids and phenolic compounds, which have been implicated in antimicrobial and anti-amoebic activities. Furthermore, the use of an aqueous extract minimizes solvent-related cytotoxicity and enhances translational relevance for future biomedical applications, particularly in ocular and topical formulations.

Extraction and Isolation

A total of 1 kg of *C. ternatea* flowers was collected and processed for extraction. The collected flowers were thoroughly cleaned and only healthy, undamaged blue petals were selected for subsequent freezing at -80°C . The frozen samples were then subjected to freeze-drying using a Scanvac Coolsafe system (Biopharmaceutic Laboratory, Atta-Ur-Rahman Institute for Natural Product Discovery, AuRIns) at -100°C for three days, following the procedure described by Muhammad *et al.* [11]. The dried samples were weighed to determine moisture loss before being pulverized using a Waring Commercial 8011G lab blender (Waring Commercial, HGB2NTGA model, USA) yielding 122.56 g of fine flower powder.

Extraction was carried out using the maceration technique with distilled water as the solvent at a ratio of 1:30 (w/v). The mixture was stirred continuously with a magnetic stirrer at room temperature for three days. The resulting extract was filtered through Whatman™ filter paper (110 mm) and the filtrate were concentrated under reduced pressure using a rotary evaporator (Heidolph Instruments, Germany) to obtain 10 g of dried extract, corresponding to an 8.16% yield. The solvent-free extract was transferred into universal bottles and stored at 4°C until further analysis.

Culture and Maintenance of *Acanthamoeba culbertsoni*

A. culbertsoni (genotype T4; MH791017), originally isolated from contact lens paraphernalia was employed in this study. Trophozoites were cultured monoxenically on 1.5% non-nutrient agar (NNA) plates (Sigma Aldrich, A7002, USA) overlaid with *Escherichia coli* and incubated at 30°C for three days. Cyst induction was performed using approximately 100 late-log-phase trophozoite cultures on 1.5% NNA medium, following the method described by Anwar *et al.* [12]. Briefly, trophozoite cultures were observed daily and harvested after three to four days, corresponding to the exponential growth phase. Two millilitres of Page's Amoebic Saline (PAS) were added to each plate, and trophozoites were gently scraped off using a sterile L-shaped spreader. The harvested cells were centrifuged at 3500 rpm for 10 minutes and fresh trophozoite suspensions were prepared prior to microscopic examination.

For cyst preparation, trophozoite cultures were maintained on NNA plates for up to two weeks at 30°C. Cultures were examined daily and harvested after 14 days, when approximately 95% of the cells had transformed into mature cysts. Cysts were collected as described above, the supernatants were discarded and the pellets were washed twice in PAS (3000 rpm for 5 minutes). The cyst suspensions were stored at 4°C and used for experiments within 14 days.

Both trophozoite and cyst populations were quantified using a haemocytometer (WITEG-Glasgeräte, Germany). Each sample was counted in triplicate and the mean value was used to determine the cell density per millilitre. Stock suspensions containing 5×10^5 cells/mL were prepared for subsequent anti-amoebic, encystation and excystation assays. Experimental assays were conducted using a final concentration of 1×10^5 trophozoites or cysts/mL, as previously described by Mohd Hussain *et al.* [13].

Anti-Amoebic Evaluation

The amoebicidal activity of *C. ternatea* flower aqueous extract against *A. culbertsoni* was evaluated to determine its effects on trophozoite viability. The assay was carried out following the procedure described by Siddiqui *et al.* [14], with minor modifications. Briefly, *A. culbertsoni* trophozoites were cultured in 96-well microplates (at a density of approximately 1×10^5 cells/mL in Page's Amoeba Saline (PAS)). A total of 100 µL of trophozoite suspension was dispensed into each well, followed by the addition of 100 µL of *C. ternatea* extract to achieve final concentrations of 5, 10, 15, 25 and 30 mg/mL. The total volume in each well was adjusted to 200 µL. PAS was used as the negative control, while 0.02% chlorhexidine (Sigma) served as the positive control due to its established anti-amoebic efficacy. Distilled water was included as the solvent control.

After incubation, the well contents were gently resuspended and 50 µL of the suspension was transferred to a new 96-well plate. Subsequently, an equal volume (50 µL) of 0.4% Trypan blue solution prepared in 1X phosphate-buffered saline (PBS) was added. Cell viability was determined using a haemocytometer, where viable trophozoites excluded the dye and remained unstained, whereas nonviable cells displayed blue staining due to compromised membranes. The assays were performed in triplicate and data were presented as the mean±standard error (SE) from at least three independent experiments conducted in duplicate. Inverted microscopic images were captured at 100X magnification using an inverted microscope.

A. culbertsoni exhibits a biphasic life cycle comprising trophozoite and cyst stages. Under favourable conditions, the organism exists as an active trophozoite. However, exposure to adverse environmental factors induces the transformation of trophozoites into double-walled dormant cysts, a process known as encystation. When environmental conditions become favourable, the trophozoites re-emerge from cysts through excystation, thereby completing the life cycle [15].

Encystation Assay

The encystation assay was performed to evaluate the inhibitory effect of the *C. ternatea* flower aqueous extract on the phenotypic transformation of *A. culbertsoni* trophozoites into cysts. The procedure was adapted from Anwar *et al.* [16], with minor modifications. Briefly, *A. culbertsoni* trophozoites (1×10^5 cells) were counted and exposed to varying concentrations of the *C. ternatea* extract (5, 10, 15, 25 and 30 mg/mL). Each concentration (100 µL) was added to 96-well microplates containing 100 µL of Page's Amoeba Saline (PAS), resulting in a total reaction volume of 200 µL per well.

The plates were incubated at 30°C for 72 h without agitation. Following incubation, the contents of each well were gently resuspended with sterile pipette tips and 50 µL of the suspension was transferred to new 96-well plates. An equal volume of 0.4% Trypan blue prepared in 1X PBS was added to each well. Trophozoites incubated solely in PAS served as the negative control, whereas those treated with water and 0.02% chlorhexidine (Sigma) were used as solvent and positive controls, respectively.

Mature, double-walled cysts were enumerated using a haemocytometer under a light inverted microscope, as described by Dudley *et al.* [17]. The assays were performed in triplicate and data were presented as the mean±standard error (SE) from at least three independent experiments conducted in duplicate. Representative micrographs were captured to illustrate the morphological effects of the extract on trophozoite-to-cyst transformation.

Excystation Assay

The effect of *C. ternatea* flower aqueous extract on the transformation of *A. culbertsoni* cysts into trophozoites was evaluated using an encystation assay adapted from Anwar *et al.* [16] with minor modifications. Briefly, *A. culbertsoni* cysts (1×10^5 cells) were counted and exposed to various concentrations of the extract (5, 10, 15, 25 and 30 mg/mL). The treatment was performed in 96-well microplates containing 100 µL of Page's Amoeba Saline (PAS) and 100 µL of the respective extract concentration, resulting in a total reaction volume of 200 µL per well.

For controls, cysts incubated with PAS alone served as the negative control, while those treated with sterile water and 0.02% chlorhexidine (Sigma) were used as solvent and positive controls, respectively. The plates were incubated at 30°C for 72 h without agitation. After incubation, the contents of each well were gently resuspended with sterile pipette tips and 50 µL of the suspension was transferred to new 96-well plates for observation. The assays were performed in triplicate and data were presented as the mean±standard error (SE) from at least three independent experiments conducted in duplicate.

Phenotypic transformation from cysts to trophozoites was examined under a light inverted microscope following the procedure described by Dudley *et al.* [17]. An equal volume of 0.4% Trypan blue solution prepared in 1X PBS was added to each well and viable trophozoites were counted

using a haemocytometer under a light inverted microscope. Representative images from each treatment were captured to document the morphological changes and the effects of the extract on cyst-to-trophozoite transformation.

Culture and Maintenance of Human Keratinocyte Cells (HaCaT)

The HaCaT human keratinocyte cell line (CVCL-0038, CLS 300493) was procured from Cell Line Service (CLS, Germany). As no primary human tissues or identifiable human data were involved, ethical approval was not required for this study in accordance with institutional and international guidelines for the use of established cell lines in *in vitro* research [Ethics Review Exemption: REC/06/2024 (PG/EX/28)]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (HiMedia Laboratories, India) supplemented with 10% fetal bovine serum (FBS) (Capricorn Scientific, Germany) and 1% penicillin–streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin) (HiMedia Laboratories, India) as described by Anwar *et al.* [12]. Cultures were maintained at 37°C in a humidified incubator with 5% CO₂ until a confluent monolayer was formed. Upon reaching confluence, the culture medium was aspirated and the cells were detached using 2 mL of trypsin solution. The cell suspension was centrifuged at 3500 × g for 5 min and the pellet was resuspended in 25 mL of fresh growth medium. Subsequently, 200 µL of the cell suspension was seeded into each well of a 96-well microplate and incubated for 24 h under standard culture conditions to allow uniform monolayer formation, which was confirmed microscopically. The established monolayer was then used for the cytotoxicity assay.

Cell Cytotoxicity Assay

The cytotoxic potential of the *C. ternatea* flower aqueous extract toward host cells was evaluated using the MTT assay, following the method described by Muniandy *et al.* [18] with minor modifications. Human keratinocyte (HaCaT) cells were employed as the model for host cell toxicity assessment. Briefly, HaCaT cells were seeded at a density of 1 × 10⁵ cells per well in 96-well microplates and incubated for 24 h to allow cell adherence. The confluent monolayers were subsequently exposed to 100 µL of *C. ternatea* extract at concentrations of 5, 10, 15, 25 and 30 mg/mL and incubated for an additional 24 h. After treatment, the culture medium was discarded and the cells were gently washed with phosphate-buffered saline (PBS) (Sigma Aldrich, USA) to remove residual extract. A fresh culture medium containing 0.5% (w/v) MTT reagent was then added to each well and incubated for 3 h to allow the formation of formazan crystals by viable cells. Untreated cells cultured in complete DMEM served as the negative control, while cells treated with 0.02% chlorhexidine served as the positive control and sterile distilled water was used as the solvent control. The MTT solution was carefully removed and the resulting crystals were solubilized in dimethyl sulfoxide

(DMSO) (Sigma Aldrich, USA). Absorbance was recorded at 570 nm using a Spectrostar Nano microplate reader (BMG Lab Tech, Germany) at 570 nm., where higher absorbance values corresponded to greater cell viability. The assays were performed in triplicate and data were presented as the mean ± standard error (SE) from at least three independent experiments conducted in duplicate. The percentage of cell survival was calculated using the following equation:

$$\text{Survival \%} = (\text{ABt} / \text{ABu}) \times 100$$

ABt and ABu denote the absorbance values of treated and untreated cells, respectively [19].

Statistical Analysis

All experimental results are presented as the mean ± standard error (SE) of at least three independent replicates. Statistical analyses were performed using Microsoft Excel (Microsoft Excel version 2021, Microsoft Corporation, USA). Differences among multiple treatment groups were assessed using IBM SPSS Statistics version 29.0.2.0 (IBM Corp., Armonk, NY, USA) such as Tukey's post hoc test, while pairwise comparisons were evaluated using a two-tailed unpaired Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant. The half-maximal inhibitory concentration (IC₅₀) values were determined by nonlinear regression analysis using Microsoft Excel (Microsoft Excel version 2021, Microsoft Corporation, USA), based on dose–response curves of treated versus control samples.

RESULTS

C. ternatea Extract Showed Anti-Amoebic Activity against *A. culbertsoni*

The anti-amoebic assay was performed to assess the effects of *C. ternatea* flower aqueous extract on viability of *A. culbertsoni*. The free-living amoeba was treated with various concentrations of extract ranging from 5 mg/mL to 30 mg/mL. Overall, the results revealed that tested extract showed a potent activity against *A. culbertsoni*. All tested concentrations significantly reduced viability dose-dependent. The reduction in trophozoite viability was statistically significant (*p* < 0.05, with Tukey's post hoc test while pairwise comparisons were evaluated using a two-tailed unpaired Student's *t*-test). As shown in (Figure 1A), the number of trophozoites was maintained at 1 × 10⁵ in the negative control. At 5 mg/mL, the concentration showed a significant (*p* = 0.001) activity against *A. culbertsoni* as compared to solvent control. However, at 25 mg/mL, the extract produced a 94% inhibitory effect on the viability of *A. culbertsoni* trophozoites and showed an IC₅₀ of 14.2 mg/mL. The most potent activity was recorded at 30 mg/mL, an upon treatment, 100% reduction in viability was recorded (Figure 1A). As shown in (Figure 1B), the images of individual wells from a 96-well plate recorded at 100X, showing the effects of various *C. ternatea* extract on the viability of *A. culbertsoni*.

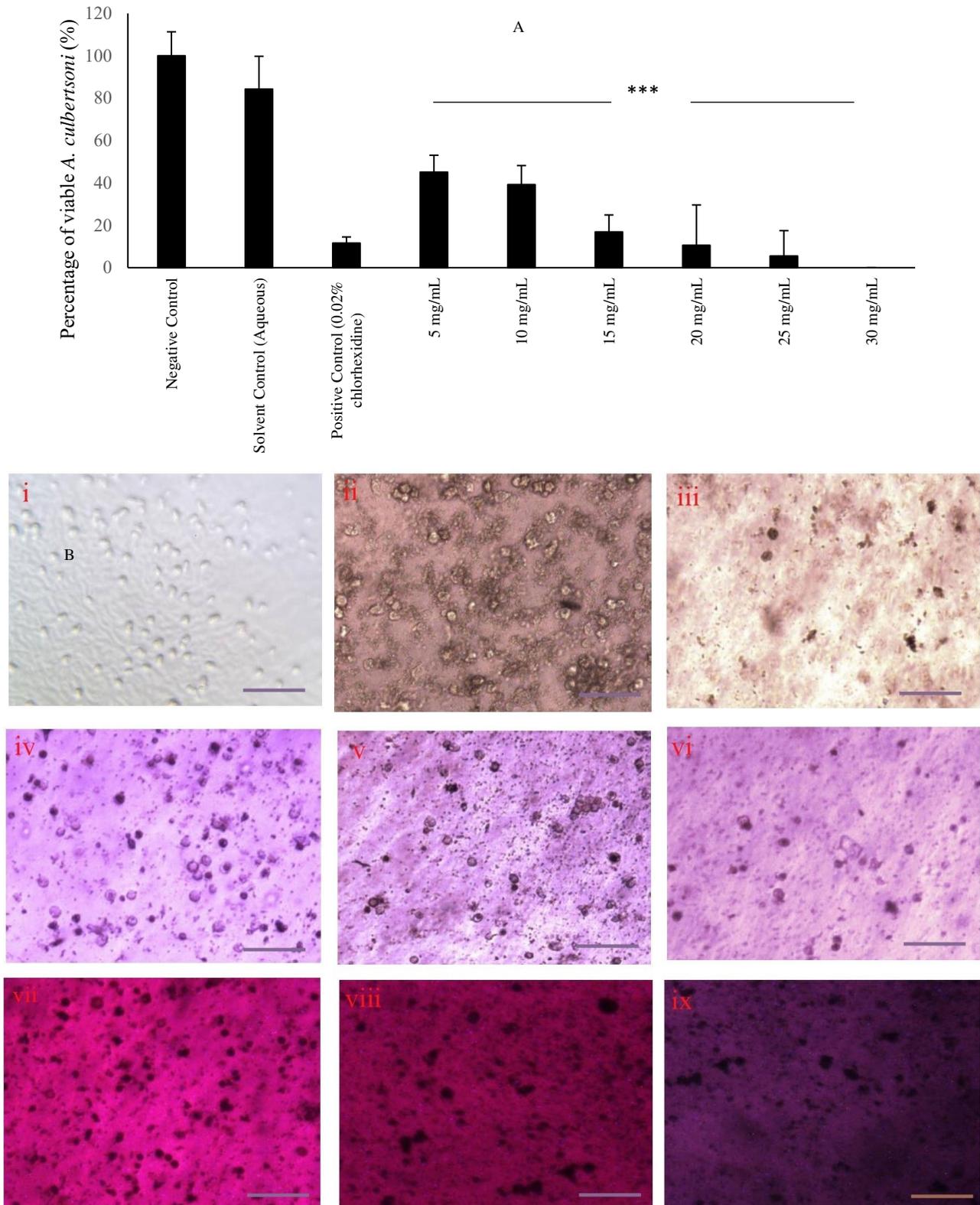


Figure 1: Anti-Amoebic Effect of *C. ternatea* Flower Aqueous Extract Against *A. culbertsoni* Trophozoites. (A) Percentage Viability of Trophozoites Following Treatment with 5–30 mg/mL of *C. ternatea* Extract. Data are Expressed as Mean \pm SE (N = 3 Independent Experiments). (***) $p < 0.05$ Compared To Solvent Control. (B) Representative Inverted Microscope Images Showing Trophozoite Morphology after Treatment. Scale Bar = 100 μ m. (I) Negative Control, (ii) Solvent Control (Aqueous), (iii) Positive Control (0.02% Chlorhexidine), (iv) 5 mg/mL Extract, (v) 10 mg/mL Extract, (vi) 15 mg/mL Extract, (vii) 20 mg/mL Extract, (viii) 25 mg/mL Extract And (ix) 30 mg/mL Extract

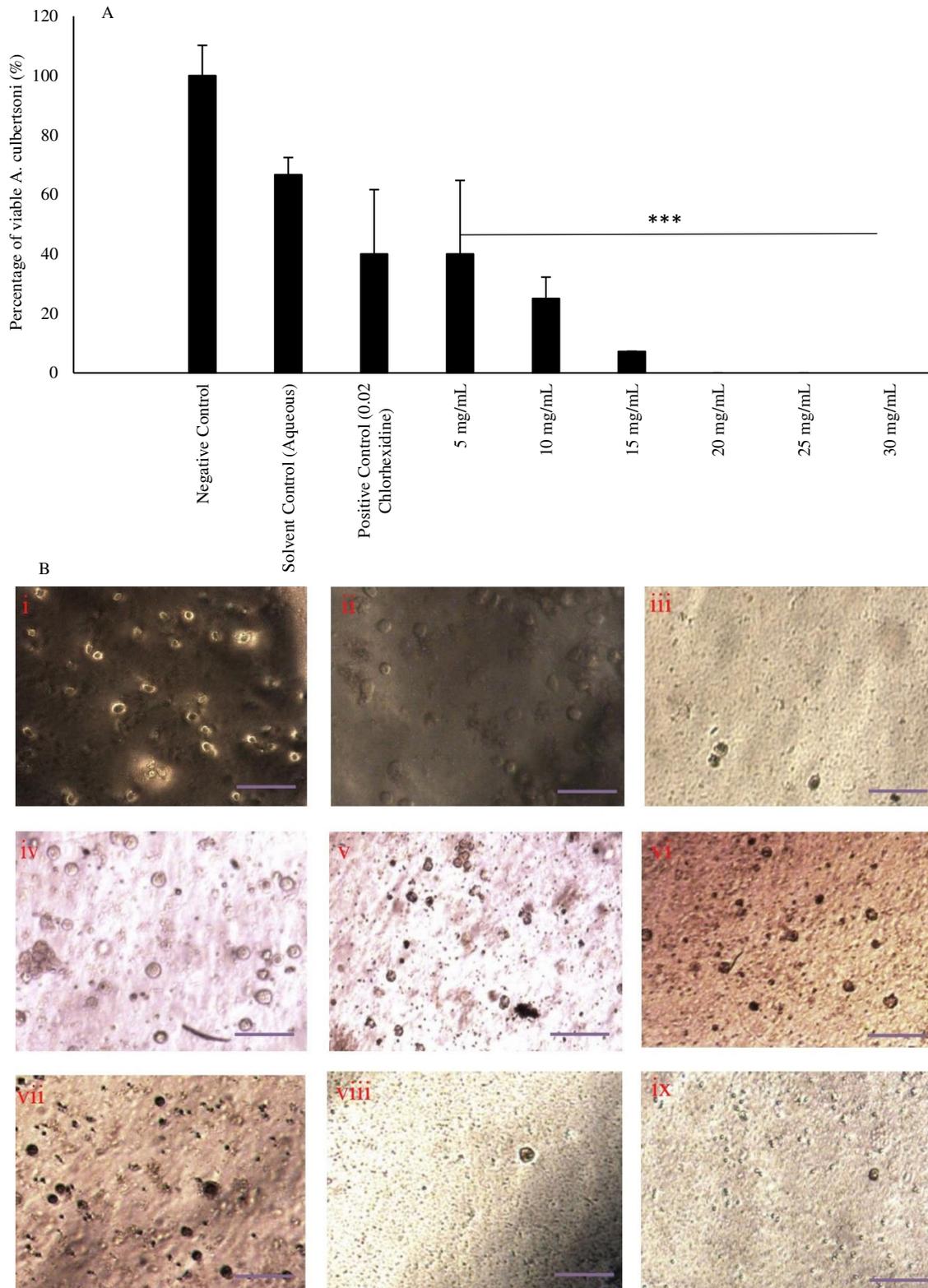


Figure 2: Encystation Assay. (A) Percentage Viability of Phenotypic Transformation of Trophozoites to Cysts Following Treatment With 5–30 mg/mL of *C. ternatea* Extract. Data are Expressed as Mean \pm SE (N = 3 Independent Experiments). (***) $p < 0.05$ Compared to Solvent Control. (B) Representative Inverted Microscope Images Showing Trophozoite Morphology after Treatment. Scale Bar = 100 μ m. Trophozoites were Treated With 5–30 mg/mL of *C. ternatea* Extract (I) Negative Control, (Ii) Solvent Control (Aqueous), (Iii) Positive Control (0.02% Chlorhexidine), (Iv) 5 mg/mL Extract, (V) 10 mg/mL Extract, (Vi) 15 mg/mL Extract, (Vii) 20 mg/mL Extract, (Viii) 25 mg/mL Extract and (Ix) 30 mg/mL Extract

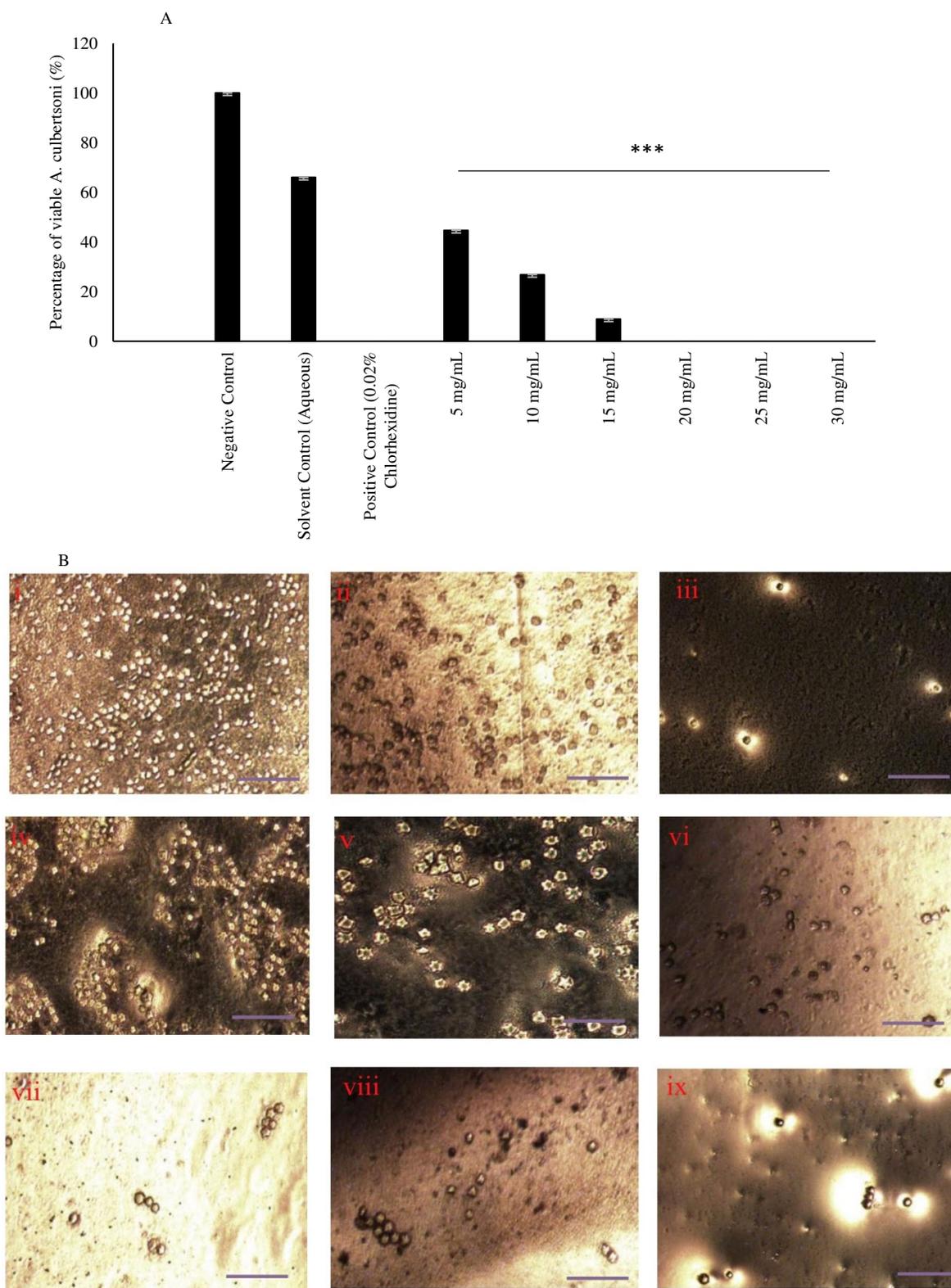


Figure 3: Excystation Assay. (A) Percentage Viability of Phenotypic Transformation of Cysts to Trophozoites Following Treatment with 5–30 mg/mL of *C. ternatea* Extract. Data are Expressed as Mean \pm SE (N = 3 Independent Experiments). (***) $p < 0.05$ Compared to Solvent Control. (B) Representative Inverted Microscope (100x) Images Showing Cyst Morphology after Treatment. Scale Bar = 100 μ m. Cysts were Treated with 5–30 mg/mL of *C. ternatea* Extract (I) Negative Control, (Ii) Solvent Control (Aqueous), (Iii) Positive Control (0.02% Chlorhexidine), (Iv) 5 mg/mL Extract, (V) 10 mg/mL Extract, (Vi) 15 mg/mL Extract, (Vii) 20 mg/mL Extract, (Viii) 25 mg/mL Extracta and (Ix) 30 mg/mL Extract

The life cycle of *A. culbertsoni* is composed of two stages: an active trophozoite and a dormant cyst stage. The tested *C. ternatea* extract was also assessed for its possible effects on the phenotypic alteration of *A. culbertsoni*.

C. ternatea* Extract Showed Significant Effects on Encystation of *A. culbertsoni

The encystation assay was performed to assess the inhibition of the phenotypic transformation of *A. culbertsoni* trophozoites into cysts. As shown in (Figure 2A), the *C. ternatea* L. flower aqueous extract significantly reduced the encystation at all tested concentrations (5 mg/mL to 30 mg/mL) in a dose-dependent manner. The reduction in cyst viability was statistically significant ($p < 0.05$, with Tukey's post hoc test while pairwise comparisons were evaluated using a two-tailed unpaired Student's *t*-test). Preliminary results showed that *C. ternatea* extract significantly inhibited the phenotypic transformation of *A. culbertsoni*. The result demonstrated a statistically significant ($p = 0.001$) inhibition of trophozoite to cyst transformation at concentration 5 mg/mL as compared to solvent control, indicating that the extract effectively disrupts encystation under *in vitro* conditions. At 15 mg/mL, the extract inhibited 98% of the trophozoites from undergoing encystation and showed an IC_{50} of 11.7 mg/mL. While no viability was recorded at 20, 25 and 30 mg/mL, suggesting that the extract interferes with the physiological or molecular mechanisms required for encystation. The typical images of a 96-well plate showing the viability of cysts inhibited by *C. ternatea* extract are shown in (Figure 2B).

C. ternatea* Extract Showed Significant Effects on Excystation of *A. culbertsoni

The excystation was performed to see if *C. ternatea* L. flower aqueous extract had any inhibitory effects on the conversion

of *A. culbertsoni* cysts to trophozoites. As shown in (Figure 3A), it illustrates that *C. ternatea* extract showed potent inhibitory effects on the excystation. The reduction in trophozoite viability was statistically significant ($p < 0.05$, with Tukey's post hoc test while pairwise comparisons were evaluated using a two-tailed unpaired Student's *t*-test). The maximum activity was recorded for the concentration 20 mg/mL and above, which inhibited 100% of newly emerged trophozoites. This effect was highly significant suggesting a robust suppression of excystation activity. While *C. ternatea* extract at 15 mg/mL reduced the viability of emerged trophozoites to 95% and showed an IC_{50} of 4.5 mg/mL. The affected reduction in viable trophozoites emerging from cysts implies that the extract interferes with essential metabolic or structural processes required for successful excystation. (Figure 3B) shows the illustrative images of a 96-well plate showing the effect of *C. ternatea* extract on the phenotypic alteration of cysts in trophozoites.

***C. ternatea* Extract Exhibited High Cytotoxicity toward HaCaT Cells**

MTT assay was performed in order to determine the toxicity of *C. ternatea* flower aqueous extract against the HaCaT cell line. As shown in (Figure 4), it revealed the effects of 5 mg/mL to 30 mg/mL of *C. ternatea* extract on the viability of HaCaT cells after 24 h of treatment. A significant dose-dependent reduction in cell viability was observed at concentrations ≥ 5 mg/mL, dropping below 50%, indicating the onset of cytotoxicity. At the lowest concentration (5 mg/mL), cell viability was reduced to $35.51 \pm 3.26\%$. Further increases in concentration progressively decreased viability toward HaCaT cells, reaching $18.31 \pm 2.55\%$ at 30 mg/mL. Furthermore, statistical analysis revealed a significant ($p =$

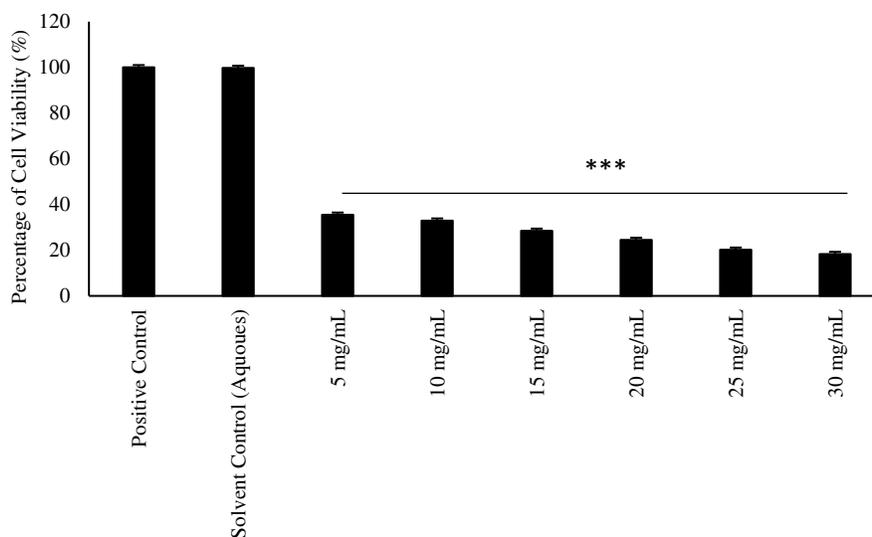


Figure 4: *In Vitro* Cytotoxicity Induced by *C. ternatea* Flower Aqueous Extract Against the HaCaT Cell Line. Briefly, the HaCaT Cells were Treated with different Concentrations (5–30 mg/mL) of Samples and Incubated at 37°C. *C. ternatea* Extract Demonstrated Higher Toxicity at Lower Tested Concentrations. Aqueous Was Used as a Solvent Control, while Untreated Hacat Cells were used as a Positive Control. Data are Expressed as Mean±SE (N = 3 Independent Experiments). (***) $p < 0.05$ Compared to Solvent Control. Data are Expressed as Mean±SE of Triplicate Experiments

0.001) reduction in viability at concentrations ≥ 15 mg/mL compared to the positive control (untreated HaCaT cells). Based on the MTT assay, *C. ternatea* flower aqueous extract exhibited marked cytotoxicity toward HaCaT cells at concentrations ≥ 5 mg/mL. Therefore, concentrations below 5 mg/mL may represent a sub-cytotoxic range and warrant further evaluation for selective anti-amoebic activity with minimal host cell toxicity.

DISCUSSION

Acanthamoeba spp. are opportunistic pathogens capable of causing severe and potentially fatal infections, particularly among contact lens users and immunocompromised individuals. Despite advances in chemotherapeutic development, effective treatment remains challenging due to the remarkable resistance of the cyst stage and the absence of licensed, *Acanthamoeba*-specific therapeutics [1,14]. Current treatment regimens rely primarily on biguanides and diamidines, such as chlorhexidine and polyhexamethylene biguanide, which exhibit activity against both trophozoites and cysts but are limited by significant host-cell cytotoxicity and prolonged treatment duration [20,21]. These constraints highlight the need for safer, multi-targeted anti-amoebic agents.

In this context, the present study provides the first evidence that *C. ternatea* flower aqueous extract exhibits dual-stage anti-amoebic activity against *A. culbertsoni* genotype T4. Unlike many phytochemical studies that focus exclusively on trophozoite killing, this work demonstrates inhibitory effects on trophozoite viability as well as suppression of encystation and excystation which processes that are critical for disease persistence and recurrence. This broader activity profile distinguishes *C. ternatea* from several previously reported plant extracts that display limited or stage-specific efficacy.

Comparatively, plant-derived compounds such as tea tree oil (*Melaleuca alternifolia*), *Nigella sativa*, *Allium sativum* and *Artemisia* spp. have shown anti-*Acanthamoeba* activity primarily against trophozoites, with inconsistent or weak effects on cysts [22]. Similarly, flavonoid-rich extracts from *Thymus vulgaris* and *Origanum* species demonstrated amoebicidal effects but were associated with notable cytotoxicity at effective concentrations [23]. In contrast, the ability of *C. ternatea* extract to interfere with both encystation and excystation suggests a more comprehensive disruption of the *Acanthamoeba* life cycle, which is essential for long-term therapeutic success.

The observed anti-amoebic activity may be attributed to the phytochemical composition of *C. ternatea*, particularly its high anthocyanin, flavonoid and phenolic content. Anthocyanins are known to compromise microbial membrane integrity, induce oxidative stress and inhibit key metabolic enzymes [24,25]. In *Acanthamoeba*, encystation involves glucosidase-mediated mobilization of glycogen reserves to generate glucose units required for cellulose synthesis in the cyst wall [1]. Given that *C. ternatea* petals possess documented α -glucosidase inhibitory activity [24],

it was plausible that the extract disrupts cyst wall biogenesis by interfering with carbohydrate metabolism. This metabolic interference may explain the reduced encystation and impaired excystation observed in the present study, providing a mechanistic basis for its dual-stage efficacy.

Cytotoxicity assessment using HaCaT keratinocytes revealed a concentration-dependent response, with higher extract concentrations inducing significant toxicity, while lower concentrations exhibited minimal or no cytotoxic effects. This pattern was consistent with reports on other plant-derived anti-amoebic agents, where crude extracts often display limited selectivity at elevated doses [23]. Notably, compared with chlorhexidine in which well documented to cause corneal epithelial damage even at therapeutic concentrations the sub-cytotoxic activity range of *C. ternatea* extract suggests a potentially safer profile for topical or adjunctive applications [26]. Variations in cytotoxicity reported across different studies likely reflect differences in extraction solvents, phytochemical composition and target cell lines [27,28].

Despite these promising findings, several limitations should be acknowledged. The present study was conducted entirely *in vitro* and the observed anti-amoebic and cytotoxic effects may not fully reflect *in vivo* pharmacodynamics, tissue penetration or host immune interactions. Furthermore, the use of a crude aqueous extract precludes precise identification of the bioactive compounds responsible for the observed effects. Future studies should therefore focus on phytochemical fractionation, molecular mechanism validation and *in vivo* evaluation to establish the therapeutic relevance and safety of *C. ternatea*-derived compounds.

CONCLUSIONS

In summary, *C. ternatea* flower aqueous extract exhibited dual-stage anti-amoebic activity against *A. culbertsoni* genotype T4, effectively inhibiting trophozoite viability as well as encystation and excystation processes. The extract showed minimal cytotoxicity toward HaCaT cells at concentrations ≤ 2.5 mg/mL, indicating a favourable preliminary safety profile. These findings support the potential of *C. ternatea* as a promising plant-derived candidate for the development of alternative or adjunctive anti-*Acanthamoeba* therapies, particularly for topical applications. Further *in vivo* validation and mechanistic studies are required to confirm its efficacy, safety and clinical relevance.

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Conflict of Interest

The authors certify that this study has no conflicts of interest. Financial, professional or personal ties had no bearing on how the research was conducted, interpreted or reported.

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