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Short communication

# Targeting cyst wall is an effective strategy in improving the efficacy of marketed contact lens disinfecting solutions against *Acanthamoeba castellanii* cysts

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ABSTRACT

*Acanthamoeba* cysts are highly resistant to contact lens disinfecting solutions. *Acanthamoeba* cyst wall is partially made of 1,4 β-glucan (i.e., cellulose) and other complex polysaccharides making it a hardy shell that protects the resident amoeba. Here, we hypothesize that targeting the cyst wall structure in addition to antiamoebic compound would improve the efficacy of marketed contact lens disinfecting solutions. Using chlorhexidine as an antiamoebic compound and cellulase enzyme to disrupt cyst wall structure, the findings revealed that combination of both agents abolished viability of *Acanthamoeba* castellanii cysts and trophozoites. When tested alone, none of the agents nor contact lens disinfecting solutions completely destroyed *A. castellanii* cysts and trophozoites. The absence of cyst wall-degrading enzymes in marketed contact lens disinfecting solutions render them ineffective against *Acanthamoeba* cysts. It is concluded that the addition of cyst wall degrading molecules in contact lens disinfecting solutions will enhance their efficacy in decreasing the incidence of *Acanthamoeba* effectively.


## 1. Introduction

*Acanthamoeba* keratitis is a serious human infection that can lead to blindness and often associated with inappropriate use of the contact lenses [1–3]. First reported in early 1970s [4], *Acanthamoeba* keratitis has remained a significant problem, despite our advances in antimicrobial chemotherapy and supportive care [5,6]. *Acanthamoeba* keratitis is characterized by blurred vision, sensitivity to light, conjunctivitis, eye lid swelling, and reddened eye with watery discharge, and severe pain [7]. Approximately 85–88% cases of *Acanthamoeba* keratitis are associated with the use of contact lens and hence contact lens wearers are at increased risk of this infection [8,9]. *Acanthamoeba* keratitis is often linked to contact lens disinfectants that fail to effectively decontaminate contact lens. A recent outbreak in the USA that reported to affect 138 people led to recall of contact lens disinfectants by the FDA and Health, Canada and has resulted in over 150 lawsuits against the manufacturer [10–12]. The available contact lens disinfecting solutions are often found ineffective or toxic to human cells, if not rinsed properly [13]. Recently, Lakhundi et al. [14] tested nine different contact lens disinfectants containing chlorhexidine or polyhexamethyl biguanide against *Acanthamoeba castellanii* and found none to be effective in destroying amoebae, albeit bacterial pathogens were killed. This is possibly due to low concentration of chlorhexidine or polyhexamethyl biguanide in contact lens disinfectants. A major challenge in eradicating *Acanthamoeba* is its ability to transform from an active trophozoite stage to a resistant cyst stage that remains dormant with little metabolic activity [15–17]. This may explain ineffectiveness of contact lens disinfectants containing chlorhexidine or polyhexamethyl biguanide against *A. castellanii* [14]. Recent work has shown that *Acanthamoeba* cyst wall is partially made of 1,4 β-glucan (i.e., cellulose) and other complex polysaccharides [16–18] making it a hardy shell that protects the resident amoeba. Here, we hypothesize that targeting the cyst wall structure together with antiamoebic compound, chlorhexidine, is an effective chemotherapeutic strategy to diminish viable amoeba. Using chlorhexidine as an antiamoebic compound and cellulase enzyme to disrupt cyst wall structure, we determined whether combination of both agents can enhance efficacy of marketed

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**Table 1**
List of contact lens disinfectants used in the present study, their ingredients, type of solution, minimum recommended disinfection time (MDRT) and manufacturer.

| Solution | Ingredients | Type | Minimum recommended disinfection time | Manufacturer |
|---|---|---|---|---|
| Ultimate plus | Polyhexamethylene biguanide, tromethamine, tyloxapol, hydroxy propyl methylcellulose (HPMC) and edetate disodium | Multipurpose solution | 4 h | ELKO Organization (Pvt.), Ltd. |
| Dura Plus | Edetate disodium, poloxamine, sodium chloride and aquahydrate™; preserved with OPTIMED (Polyaminopropyl Biguanide). Contains no chlorhexidine, no thimerosal and no sorbic acid | Multipurpose solution | 4 h | Sipic International Texas, USA |
| Opti-Free Express | Sodium chloride, sorbitol, edetate disodium, boric acid, aminomethyl propanol, citrate and tetronic® (polidronium chloride) 0.001% and ALDOX® (myristamidopropyl dimethylamine) 0.0005% | Multi-purpose disinfecting solution | 6 h | Alcon Laboratories, Inc., Fort Worth, TX |

contact lens disinfectants, including Opti-Free Express (Alcon Laboratories, Inc.), Ultimate Plus (ELKO Organization (Pvt.) Ltd.), Dura Plus (SIPIC International) against *A. castellanii* trophozoites and cysts, in vitro.

## 2. Methods

All chemicals were purchased from Sigma Laboratories (St. Louis, USA) or Oxoid (Hampshire, England) unless otherwise stated. Three Ultimate plus, Dura Plus, Opti-Free Express were purchased from local pharmacy in Karachi, Pakistan. *A. castellanii* belonging to the T4 genotype, isolated from a keratitis patient, was purchased from the American Type Culture Collection (ATCC 50492). *A. castellanii* was routinely cultured in PYG medium [proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v), and glucose 1.5% (w/v)] in T-75 tissue culture flasks at 37 °C without shaking [19]. The media were refreshed 15–20 h prior to experiments. *A. castellanii* adhering to flasks represented the trophozoite form and were collected by placing the flasks on ice for 30 min with gentle agitation and used as trophozoites.

To obtain cysts, amoebae trophozoites were inoculated on non-nutrient agar plates (without bacteria) at 30 °C and plates incubated for 14 days. Following this incubation, cysts were scraped off from the agar surface using 10 mL of sterile distilled water using a cell scraper. Next, cysts were centrifuged at 2000 × *g* for 10 min. The supernatant was aspirated and pellet resuspended in phosphate buffered saline and cysts enumerated using a haemocytometer.

Assays were performed on *A. castellanii* to evaluate amoebicidal effects of contact lens disinfectants in the presence and absence of antiamoebic drug and/or cellulase. The contact lens disinfection solutions used in this study along with their active ingredients and manufacturers' instructions are listed in Table 1. Briefly, $5 \times 10^5$ *A. castellanii* trophozoites or $5 \times 10^4$ *A. castellanii* cysts were incubated in contact lens disinfectants, together with various concentrations of chlorhexidine and/or various units of cellulase (final volume: 200 μL in Eppendorf tubes). The tubes were incubated at room temperature for recommended time, as per manufacturers' instructions. Following this incubation, the number of viable amoebae was determined by adding 0.1% Trypan blue exclusion staining (cells stained blue were considered nonviable while live cells were unstained). The numbers of amoebae were enumerated by haemocytometer counting. Additionally, viability of amoebae was determined using survival

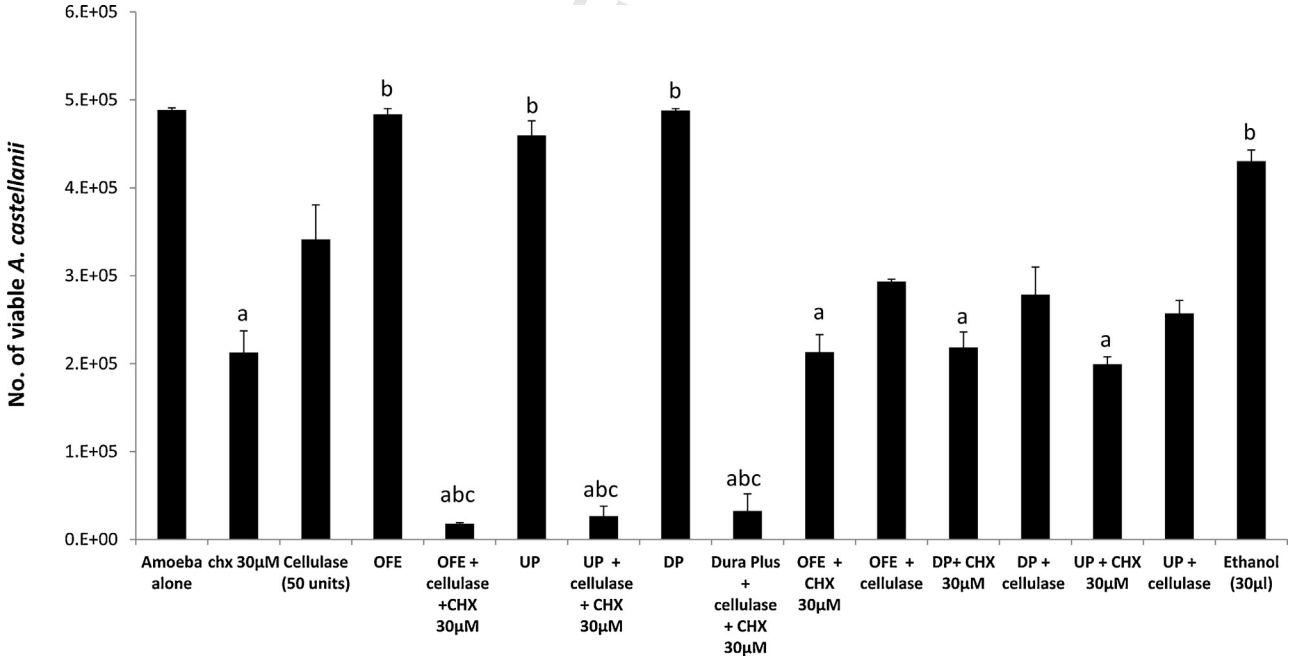


**Fig. 1.** The efficacy of CL disinfecting solution against keratitis isolate of *A. castellanii* belonging to T4 genotype. Chlorhexidine (CHX) and/or cellulase was added to three different contact lens disinfecting solutions, Opti-Free Express (OFE), Ultimate Plus (UP), Dura Plus (DP) to determine their effectiveness against *A. castellanii* trophozoites. Briefly *A. castellanii* ($5 \times 10^5$ trophozoites) were incubated with CL disinfecting solutions along with CHX and/or cellulase at room temperature for 6 h. Following this, number of viable amoebae were determined using Trypan blue exclusion assay as described in Methods. Note that CL solution plus CHX plus cellulase showed significant reduced the number of *A. castellanii* as compared to CL alone. The results represent the mean ± standard error of three independent experiments performed in duplicates. $^{a}P < 0.05$ versus *Acanthamoeba* alone, $^{b}P < 0.05$ versus chlorhexidine alone, and $^{c}P < 0.05$ versus cellulase alone.

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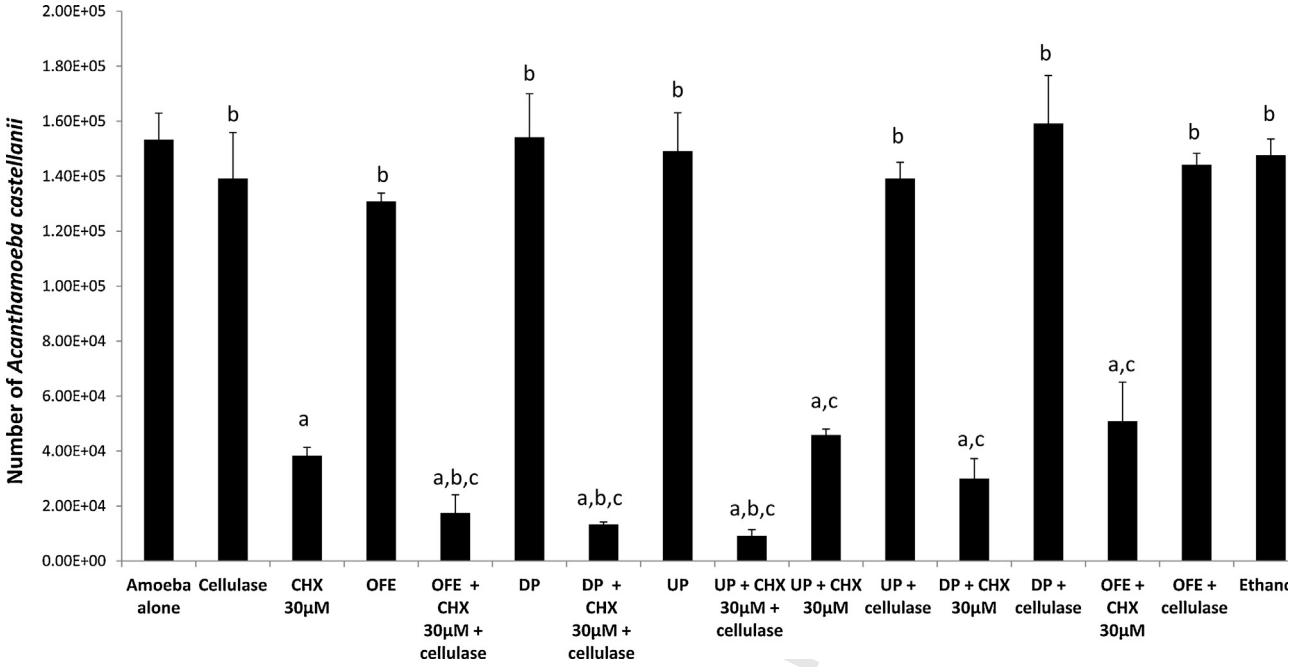


**Fig. 2.** Chlorhexidine and/or cellulase was added to three different CL disinfecting solutions, Opti-Free Express (OFE), Ultimate Plus (UP) and Dura Plus (DP) to determine their effectiveness against *A. castellanii* cyst. Briefly *A. castellanii* cysts ($5 \times 10^4$) were incubated with CHX and/or cellulase at room temperature for 6 h. Next, drug-treated amoebae were centrifuged and pellets re-inoculated in fresh PYG at 30 °C for up to 72 h, followed by haemocytometer counting. Note that CL solution along with CHX and cellulase showed reduction in number of *A. castellanii* as compared to CL alone. The results represent the mean ± standard error of three different experiments performed in duplicates. $^{a}P < 0.05$ versus *Acanthamoeba* alone, $^{b}P < 0.05$ versus chlorhexidine alone, and $^{c}P < 0.05$ versus cellulase alone.

assays. Briefly, amoebae post-treatment, were resuspended in 1 mL of PBS and centrifuged at 1500 × *g* for 10 min. The supernatant was discarded and pellet resuspended in PBS. This process was repeated 3× to remove residual drugs, contact lens disinfectant, and cellulase. Finally, amoebae pellet was resuspended in 500 μL of growth medium, i.e., PYG and inoculated in 24-well plates for 72 h at 30 °C. *A. castellanii* in Roswell Park Memorial Institute-1640 (RPMI) medium alone served as negative control, while amoebae incubated with chlorhexidine alone served as positive control. All experiments were performed at least 3 times, in duplicate. The data are presented as mean ± standard error. For statistical comparisons, differences between groups were analyzed by a one-way analysis of variance (ANOVA), followed by Dennett's post-hoc test. A value of $P < 0.05$ was considered to be statistically significant.

## 3. Results

*A. castellanii* incubated with RPMI alone yielded $4.9 \times 10^5$ amoebae on average. The solvent alone had no effect on the viability of *A. castellanii* (Fig. 1). All three contact lens disinfectants, Ultimate plus, Dura Plus, and Opti-Free Express were ineffective in killing *A. castellanii* trophozoites as shown in Fig. 1. When contact lens disinfectants-treated amoebae were re-inoculated in fresh growth medium, PYG, healthy trophozoites emerged within 24 h. Contact lens disinfectants plus chlorhexidine (up to 30 μM) exhibited significant amoebicidal effects on the viability of *A. castellanii* trophozoites ($P < 0.05$), but when chlorhexidine-treated amoebae were re-inoculated in PYG, healthy trophozoites emerged at 72 h, albeit, in reduced numbers. In contrast, contact lens disinfectants in the presence of cellulase (50 units) and chlorhexidine (30 μM) abolished viability of *A. castellanii* trophozoites (Fig. 1). When incubated with growth medium, post-treatment, no viable amoebae were observed for up to 72 h.

Cysticidal assays were performed to determine the effectiveness of contact lens disinfecting solutions in the presence of chlorhexidine and cellulase. *A. castellanii* cysts incubated with the solvent alone remained viable (Fig. 2) and excysted as viable trophozoites upon inoculation in PYG (Fig. 3). Similarly, contact lens disinfectants had no cysticidal`` effects as demonstrated by Trypan blue staining and survival assay using PYG. Cellulase alone as well as in combination with contact lens disinfecting solution did not affect viability of cyst (Figs. 2 and 3). When chlorhexidine (30 μM) was included with contact lens disinfecting solution, it showed significant cysticidal effects ($P < 0.05$) (Fig. 2), however viable amoebae were observed upon inoculation in PYG. When both chlorhexidine (30 μM) and cellulase (30 μM) were added to contact lens disinfectants, the viability of *A. castellanii* cysts was abolished, as determined by Trypan blue exclusion assay and amoebae did not emerge as viable trophozoites in PYG (Figs. 2 and 3). These findings were consistent with all contact lens disinfectants tested in the present study. Similar results were observed when both chlorhexidine (30 μM) and cellulase (30 μM) were added to PBS, in the absence of contact lens disinfectants (data not shown). These findings are consistent with our previous findings [14], which showed that marketed contact lens disinfectants tested are ineffective against *A. castellanii* cysts.

## 4. Discussion

The treatment of *Acanthamoeba* keratitis is challenging and chances of recurrent infection are high [7]. Moreover, if it is not treated promptly and aggressively, it may lead to blindness. Contact lens wearers are at an increased risk because of ineffective lens hygiene, use of homemade contact lens solution, limescale, hard water, and use of expired contact lens solutions as potential risk factors [9,20–22]. The cascade of events triggering *Acanthamoeba* keratitis need to be understood to target treatment regimens at specific molecules or mechanisms, to explore disease-modifying strategies.

Given the nature of the disease and its devastating consequences, it is important to increase public awareness and



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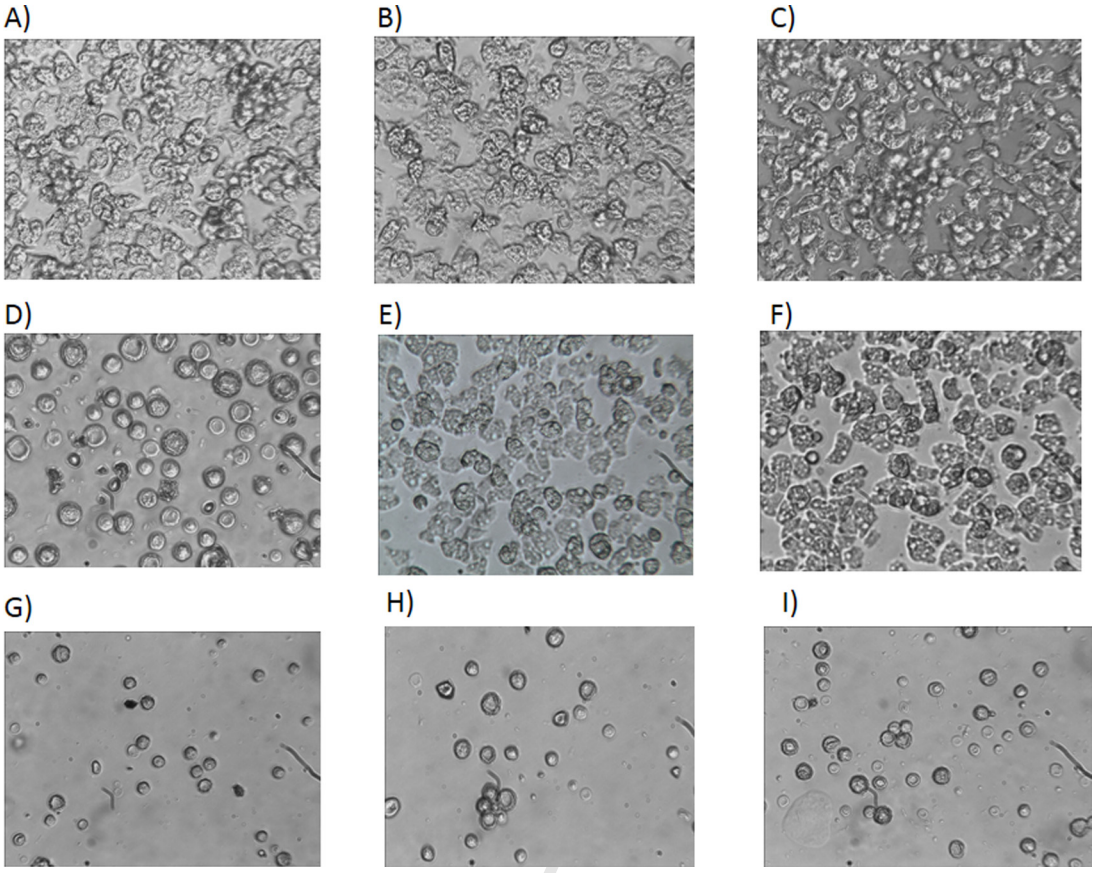


**Fig. 3.** Representative effects of contact lens disinfecting solutions against *A. castellanii* cysts. Briefly, *A. castellanii* cysts ($5 \times 10^4$) were incubated with CHX and/or cellulase and treated amoebae were incubated in PYG for 72 h as described in Fig. 2. (A) amoeba alone; (B) amoeba + contact lens disinfecting solution (similar results were observed for all disinfectants tested); (C) amoeba + contact lens disinfecting solution + cellulase (similar results were observed for all disinfectants tested); (D) amoeba + contact lens disinfecting solution + chlorhexidine (30 μM) (similar results were observed for all disinfectants tested); (E) amoeba + contact lens disinfecting solution; (F) amoeba + contact lens disinfecting solution + ethanol; (G) amoeba + Ultimate plus + chlorhexidine (30 μM) + cellulase; (H) amoeba + Dura Plus + chlorhexidine (30 μM) + cellulase; and (I) amoeba + Opti-Free Express + chlorhexidine (30 μM) + cellulase. X250. Results are representative of three independent experiments.

improve preventative strategies, especially among contact lens users who are at increased risk. Of concern, recent studies demonstrated inefficacy of marketed contact lens disinfectants in destroying *A. castellanii*, in particular against the cyst stage [14]. Cysts are partially made of cellulose, hence we proposed that adding cellulase and chlorhexidine will be a promising strategy in targeting *A. castellanii* trophozoites and cysts.

Cellulase used in the present study was isolated from *Trichoderma reesei,* a non-pathogenic fungal strain that serves as a major producer of biomass degrading enzymes. Cellulases and most hemicellulases belong to a group of enzymes known as glycoside hydrolases. In most cases, cellulases have a small independently folded carbohydrate-binding module which is connected to the catalytic domain by a flexible linker. The carbohydrate binding module increases the enzyme activity by binding to the crystalline cellulose. Cellulases follow two different catalytic mechanisms; the retaining and the inverting mechanisms. In both mechanisms, two catalytic carboxylate residues are involved and catalyze the reaction by acid–base catalysis [23]. Chlorhexidine is a commonly used disinfectant. It is a positively charged molecule that interacts effectively with the negatively charged membranes of different species of *Acanthamoeba* disrupting the cell membranes and interfering with osmosis, resulting in leakage of cytoplasmic contents and cell death [24,25]. It is on the list of the most important medication needed in a basic health system determined by World Health Organization [26] that is widely used as an antiseptic.

When tested alone, none of the contact lens disinfecting solutions completely destroyed *A. castellanii* trophozoites and cysts. The addition to chlorhexidine destroyed trophozoites but did not completely destroyed cysts, as viable trophozoites emerged when treated cysts were inoculated in fresh growth medium, PYG. In contrast, chlorhexidine plus cellulase-treated cysts were abolished and they were unable to revive in the growth medium, PYG. A likely explanation for these findings is that cellulase destroyed cyst walls, allowing chlorhexidine to target cell membranes of resident trophozoite resulting its destruction. Overall, *Acanthamoeba* trophozoites are highly sensitive to chlorhexidine and a combination of cellulase plus chlorhexidine proved lethal against both the cyst form and the trophozoite form and exhibited 100% kill rate. Thus the absence of cyst walls degrading enzymes in contact lens disinfecting solutions render them ineffective against *Acanthamoeba* cysts. Although toxic effects of new formulations on host cells needs to be determined, but these findings suggest that the addition of cyst walls degrading molecules in contact lens disinfecting solutions will enhance their efficacy in eradicating *Acanthamoeba*. Additionally, the addition of cyst walls degrading molecules in drug formulation in the treatment of *Acanthamoeba* keratitis. Future studies will unravel the precise biochemistry of cyst walls of *Acanthamoeba* to identify additional targets for the development of effective contact lens disinfecting solutions as well as chemotherapeutic approaches.

## Authors' contribution

RS and FA conceived the study. NAK and RS designed the experiments. All experiments were performed by FA under the supervision of FAY, NAK and RS. FAY and FA performed analyses and interpretations. FA wrote the first draft of the manuscript. FAY, NAK, and RS corrected the manuscript. All authors approved the manuscript.

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