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ORIGINAL ARTICLE

The impact of vinegar on pathogenic *Acanthamoeba astronyxis* isolate

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Abstract *Acanthamoeba* keratitis (AK) is a severe corneal disease that was reported by WHO as the second most common infectious cause of blindness after trachoma; contact lens wear is considered one of the main risk factors in its transmission. Thus, the treatment of AK is crucial but, the inability of medical agents to completely eradicate the resistant cyst, together with their toxic effects, suggest that new agents are needed. Vinegar has been known long ago as a simple and available disinfectant with antimicrobial effects, so the present study aimed to test the effect of different concentrations of vinegar solution on *Acanthamoeba astronyxis* isolate, along the period of 1 h in comparison to parasite and chlorhexidine controls. Post hoc test analysis revealed a highly significant difference between the vinegar-treated parasites and both controls, as regards the viable and non-viable mean cysts count. Vinegar concentration of 5% exhibited the highest mean of non-viable cysts along the time intervals, while the lowest was shown with 0.04% where also, no viable cysts were detected at 60 min. All tested concentrations behaved in a time-dependent manner. There was a positive correlation with a significant outcome between the different concentrations and the mean of the non-viable parasites along time. Transmission electron microscopy of treated cysts revealed corrugated altered cell wall with loss of ridges and detachment and shrinkage of content. Treated trophozoites showed flattening of the acanthopodia with thinned out plasma membrane and degenerated cytoplasmic content. The study highlighted the potential use of vinegar as an adjuvant in the prevention and treatment of AK.

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Keywords *Acanthamoeba astronyxis* · Cysts · Trophozoites · Contact lenses · Vinegar · Disinfectant · Ultrastructure · Chlorohexidine

Introduction

Acanthamoeba species (sp.) are commonly found in a variety of habitats like, dust (Niyyati et al. 2009), air, soil (Sawyer 1989), fresh, sea, tap and bottled mineral water (Rivera et al. 1981; De Jonckheere 1991; Szenasi et al. 1998) or sewage (Schroeder et al. 2001). *Acanthamoeba* sp. can exist as motile trophozoites and double-walled cysts (Siddiqui and Khan 2012).

It is an opportunistic pathogen which can cause subacute granulomatous amoebic encephalitis, skin or sinus infections. The most common clinical presentation is *Acanthamoeba* keratitis (AK), which can result in corneal pain and blindness, in healthy individuals (Khan 2006).

The failure of medications to affect both forms of the parasite and their toxic effects on the cornea suggest that new agents are requested (Yildiz et al. 2018). AK has been documented with some commercially available multipurpose lens rinsing solutions (Zanetti et al. 1995; Hiti et al. 2002; Siddiqui and Khan 2012). The persistence of infection has been reported despite prolonged treatment with different drugs or even their combinations (Lorenzo-Morales et al. 2015). The prolonged treatment may lead to corneal abnormalities ending in drug resistance (Gooi et al. 2008).

The sanitizing characteristics of vinegar were proven among the natural products (Chang and Fang 2007). Investigations clearly demonstrated the antimicrobial criteria of vinegar, but mainly in the context of food safety (Sengun and Karapinar 2005). At the concentration of $\leq 0.0025\%$, acetic acid solutions were slightly effective at

inhibiting the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Rund 1996). Studies also proved the potency of diluted vinegar (2% acetic acid solution at pH2) in the treatment of ear infections; otitis externa, otitis media, and granular myringitis (Aminifarshidmehr 1996 and Jung et al. 2002). In the parasitological context, the lethal impact of vinegar on *Ascaris lumbricoides* eggs (Beyhan et al. 2016) and on *Giardia duodenalis* cysts has been described (Costa et al. 2009).

The present study aims to test the impact of vinegar solution in different concentrations on *Acanthamoebae astronyxis* along 1-h duration in comparison to parasite and chlorhexidine controls followed by an ultrastructure comparative evaluation, as an adjunctive in treatment or prevention of AK.

Materials and methods

Chemicals

Pasteurized natural cane vinegar 5% purchased from Int. Co. for the complementary and pharmaceutical industry, Egypt. Chlorhexidine digluconate and Roswell Park Memorial Institute Media (RPMI) 1640 media were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Acanthamoeba sp. isolation and identification

Corneal scrapings were collected from keratitis patients at the Research Institute of Ophthalmology, Egypt, and isolation was performed in the Diagnostic and Research Parasitology Unit, at Faculty of Medicine, Ain Shams University. The specimens were inoculated onto the surface of 1.5% non-nutrient agar (NNA) plates seeded with heat-inactivated *Escherichia coli* (*E. coli*) according to Init et al. (2010). The plates were examined for the presence of amoebic growth daily for 14 days with an inverted microscope (Olympus CKX41) using 40X objective. For positive samples, subcultures were done after 2 weeks on a new NNA-*E. coli* plate (Init et al. 2010).

Acanthamoeba cysts and trophozoites were harvested from the 2-weeks old cultures (Sarhan et al. 2017). To increase the parasite yield, the sediment was transferred to the RPMI 1640 media, supplemented with 12% heat-inactivated calf serum (FCS), 0.5 mg/mL penicillin and

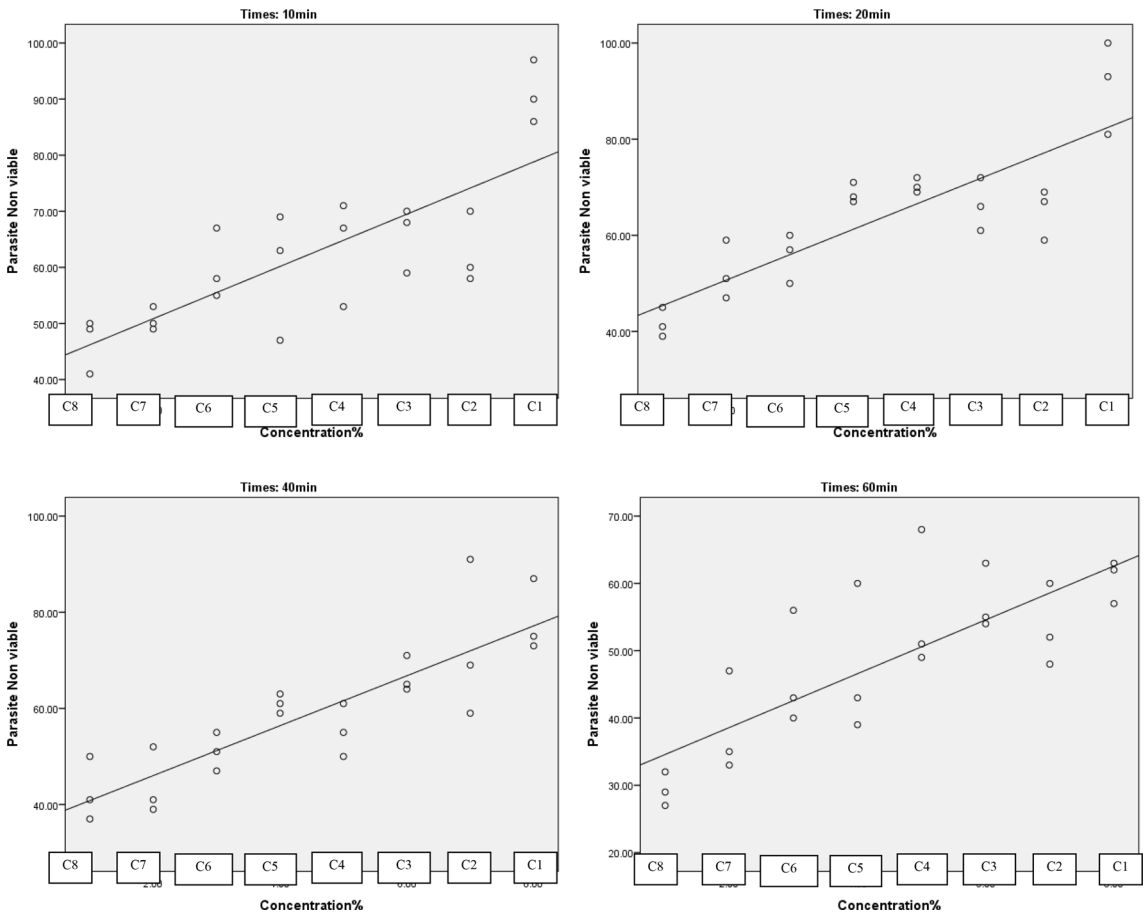

Fig. 1 Comparative scatter plot reflecting the relation between the serial dilutions of vinegar and the non-viable parasite mean at 10, 20, 40 and 60 min

0.5 mg/ml streptomycin incubated overnight at 30 °C (Sharief et al. 2008). Parasites in the resultant suspension were counted with a hemocytometer under light microscopy (Zeiss, primo star) using10X objective, and the suspension was standardized to 25 × 10^4/ml (Perrine et al. 1995). The culture was maintained in the laboratory. Molecular characterization, sequencing, genotyping and phylogenetic analysis were done by the authors to indentify the isolate which revealed *Acanthamoeba astronyxis* T7 genotype (Sarhan et al. 2017).

Susceptibility assay

Dilution of vinegar was carried out (v/v) in distilled water and 8 concentrations were prepared C1 (5%), C2 (2.5%), C3 (1.25%), C4 (0.625%), C5 (0.312%), C6 (0.156%), C7 (0.07%) and C8 (0.04%). The cysticidal effect of vinegar was determined in vitro. Using a microtiter plate, one hundred microliters (100 µl) of the calibrated parasite suspension in PBS was inoculated into each well of a 96-well plate, the plate was left for 30 min to avoid disturbance of adherence of amoebae onto the wells' surface. The PBS solution was discarded and 100 µl of each concentration of the vinegar solution were added into the wells.

The plate was examined after 10, 20, 40 and 60 min. In addition, control wells containing the parasite in PBS as a non-treated control and the parasite plus chlorhexidine digluconate (0.02%) (Prepared from a chlorhexidine solution 20% in H2O) as drug control, were submitted to the same procedure. Each experiment was performed in a triplicate.

After the incubation period, 100 µl from each well were mixed with 100 µl of 0.3% basic methylene blue, left for 10 min, then viable (unstained) and non-viable (stained) cysts were counted. For wells containing no viable cysts, the well content was re-inoculated onto NNA-*E.coli* plate, incubated at 30 °C for an additional 72 h, and examined to detect any viable cysts or trophozoites (Polat et al. 2008).

Transmission Electron Microscopy

Samples from *A. astronyxis* cysts and trophozoites treated with vinegar (C3) at 40 min were fixed in 2.5% glutaraldehyde plus 0.1 M cacodylate buffer then post-fixed with 1% (wt/vol) osmium tetroxide. Samples were dehydrated with ethanol and propylene oxide then embedded in epoxy resin. Thin sections (60 to 90 nm) were stained with uranyl acetate and lead citrate and examined under a Zeiss EM-910 by transmission electron microscope (TEM) (Carl Zeiss, Germany) (Debnath et al. 2012).

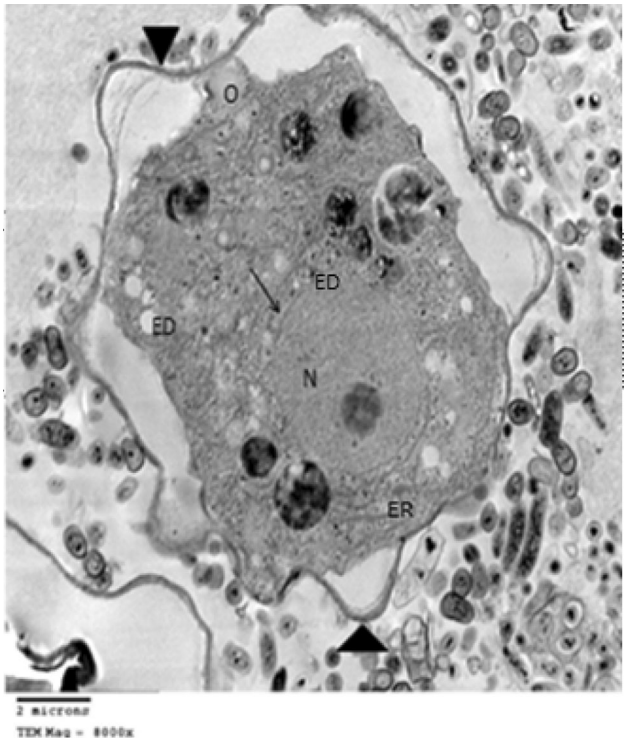

Fig. 2 TEM of control cyst showing thick connected ridges over their whole surface, with a pitted appearance and intact contour (arrow head). Ostioles (O) were recognized as distinct circular plugs. Intact endoplasm; nucleus (N) with a bilayer nuclear envelope (arrow) separating nucleus from the cytoplasm, endoplasmic reticulum (ER) and organelles, electron dense (ED) granules were seen in the cytoplasm and nuclear membrane

Statistical analysis

The statistical package for social sciences, version 20.0 (SPSS Inc., Chicago, Illinois, USA) was applied. Mean ± standard deviation (SD) expressed the quantitative data. Tests used included; A one-way analysis of variance (ANOVA), Post Hoc test with Least Significant Difference (LSD) and Spearman's rank correlation coefficient (rs). The confidence interval was set to 95% and the margin of accepted error was set to 5%. Probability (p value) ≤ 0.05 was considered significant, ≤ 0.001 was considered as highly significant and > 0.05 was considered insignificant.

Results

The results of the study are shown in Figs. 1, 2, 3, 4, 5 and Tables 1, 2.

Post hoc test analysis of data revealed a highly significant difference between vinegar-treated parasites and both parasite and drug controls, as regards the viable and non-viable mean count of cysts along the studied time intervals;

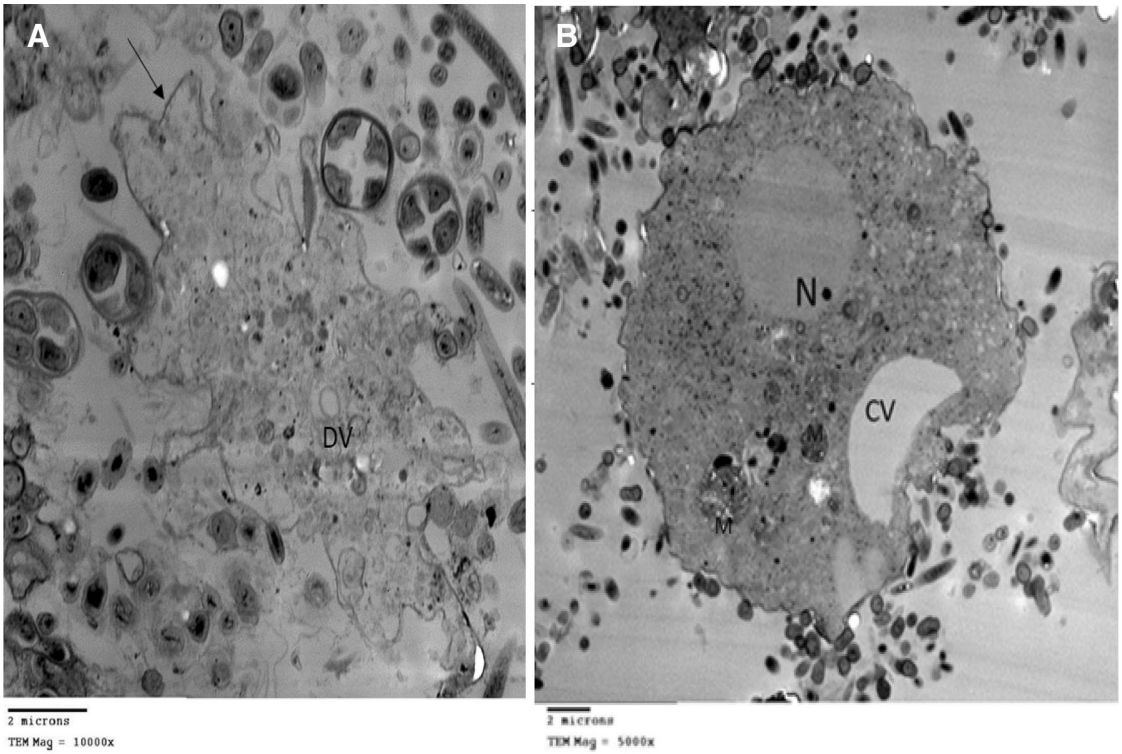

Fig. 3 TEMs of control trophozoites, **a** showing intact plasma membrane with acanthopodia (arrow) and compact content. **a**, **b** Showing well defined cytoplasmic organelles; nucleus (N), contractile vacuole (CV), digestive vacuoles (DV), and mitochondria (M)

10, 20, 40 and 60 min. Vinegar at C1 exhibited the highest non-viable count, while the lowest was shown with C8 (Table 1).

At 10 and 20 min, a highly significant difference was shown between the non-viable mean cysts count of C1 of vinegar and all the examined concentrations, as well as the parasite and drug controls. At 40 min, no significant outcome was revealed between C1 in relation to C2 and C3 and at 60 min, no significant difference was revealed between the non-viable mean cysts count of C1 in relation to C2, C3, and C4, denoting a proximate outcome between these concentrations (Table 1).

Concerning the mean of viable cyst count, at 10 min, the lowest mean was that of C1 and the highest was that of C8. At 20 min, no viable outcome was revealed from C1, C2, and C3 still, the highest mean was shown with C8. At 40 min, no viable outcome was revealed from all the concentrations except for C8. At 60 min no viable outcome was revealed from all the concentrations, reflecting a time-dependent action (Table 1).

Spearman's rank correlation coefficient revealed a positive correlation and a significant outcome between the different concentrations and the mean of the non-viable parasites along the time intervals (Table 2, Fig. 1).

Examination of the vinegar-treated cysts by TEM revealed corrugated altered cell wall with loss of ridges and retraction and shrinkage of content. While, vinegar-treated trophozoites showed flattening of the acanthopodia with thinned out plasma membrane and degenerated cytoplasmic content with structural damage (Figs. 2, 3, 4, 5).

Discussion

AK is a severe infection of the cornea in contact lens users which may cause decreased vision from ulceration and scarring, or else may be complicated by the loss of vision. Thus, the contact lens hygiene with the proper selection of the disinfectants is crucial. The treatment of AK remains empirical and the in vitro activities of anti-*acanthamoeba* drugs varied according to the time of treatment or the selective response of different sp. (Ortilles et al. 2017).

Vinegar solution has always been known as a simple and available disinfectant. Previously, diluted vinegar proved to be beneficial as an adjuvant therapy with antimicrobials (Shamanna and Ganga 2018) and as an antiprotozoal agent (Sadjjadi et al. 2006; Costa et al. 2009).

Hoping to find an adjunctive agent for the treatment and prevention of AK and also providing a daily cheap and prompt contact lens cleansing measure which is selectively potent and fast acting, the present study tested the different

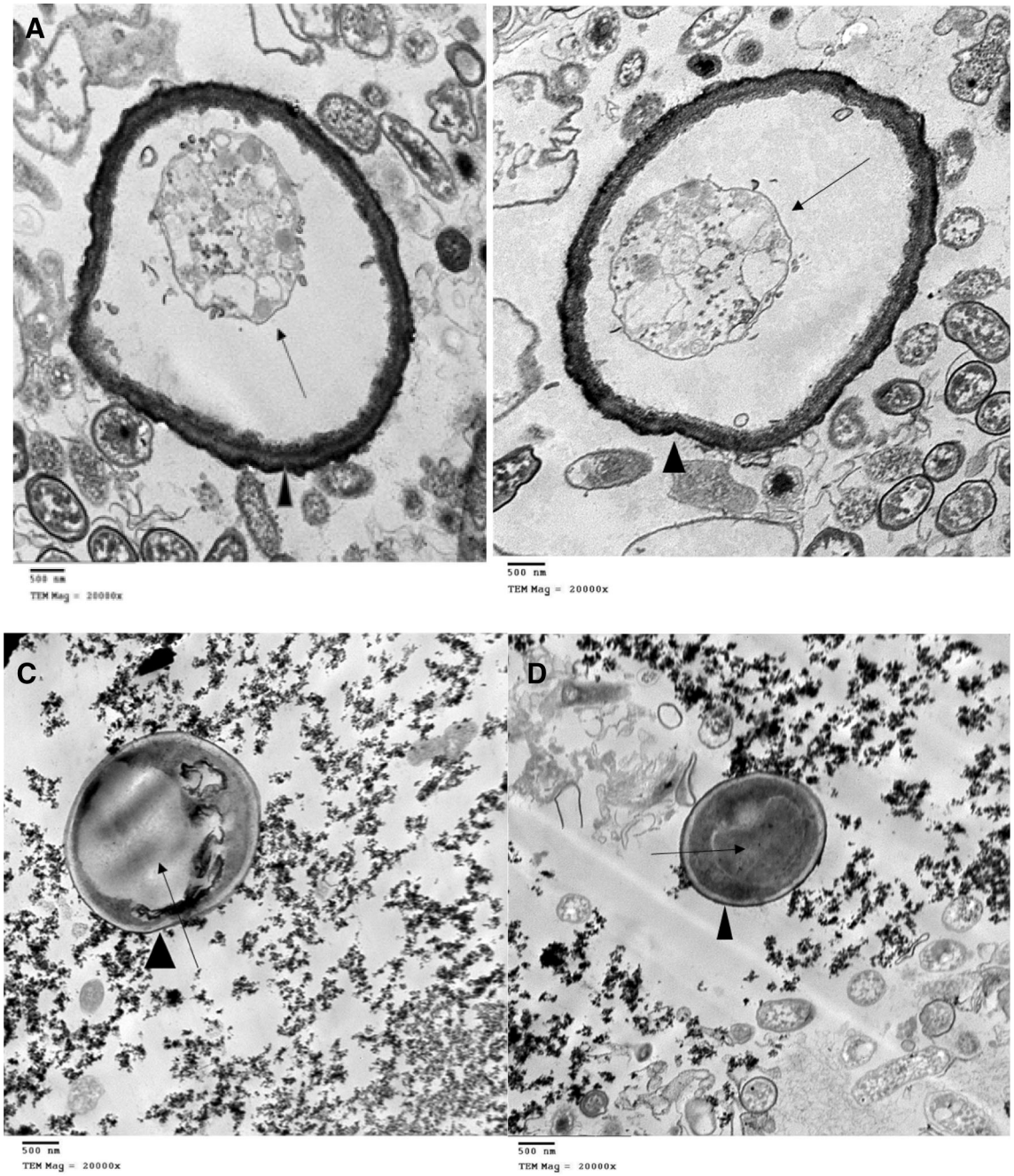

Fig. 4 TEMs of vinegar-treated cysts (C3) at 40 min showing corrugated thickened cell wall with loss of ridges and ostioles (arrow heads **a**, **b**), retraction and shrinkage of content from the cyst wall (collapsed, degenerated content) (arrows **a**, **b**). Evident thinning of the cell wall (arrow heads **c**, **d**) with disappearing of ridges and homogenization of intracellular content (hyaline changes) (arrows **c**, **d**)

concentrations of vinegar solution along 1 h in comparison to parasite and chlorhexidine controls.

In this study, the diluted vinegar solutions acted on *A. astronyxis* cysts in a time-dependent manner that was previously reported from other tested disinfectants as hydrogen peroxide (Hughes and Kilvington 2001). The highest efficacy of vinegar was revealed after 10 and 20 min of exposure to the concentrations of; 5, 2.5 and 1.25%, also the lower concentrations; 0.625 and 0.04%, reached their efficacious action at 40 and 60 min.

The cysticidal activity shown at 40 min from the vinegar concentrations; 0.625, 0.312, 0.15 and 0.07%, though most of the contact lens solutions potency was shown at 30–45 min (Zanetti et al. 1995; Greub and Raoult 2003), might give diluted vinegar the chance to be incorporated as a component of contact lens solutions to provide protection

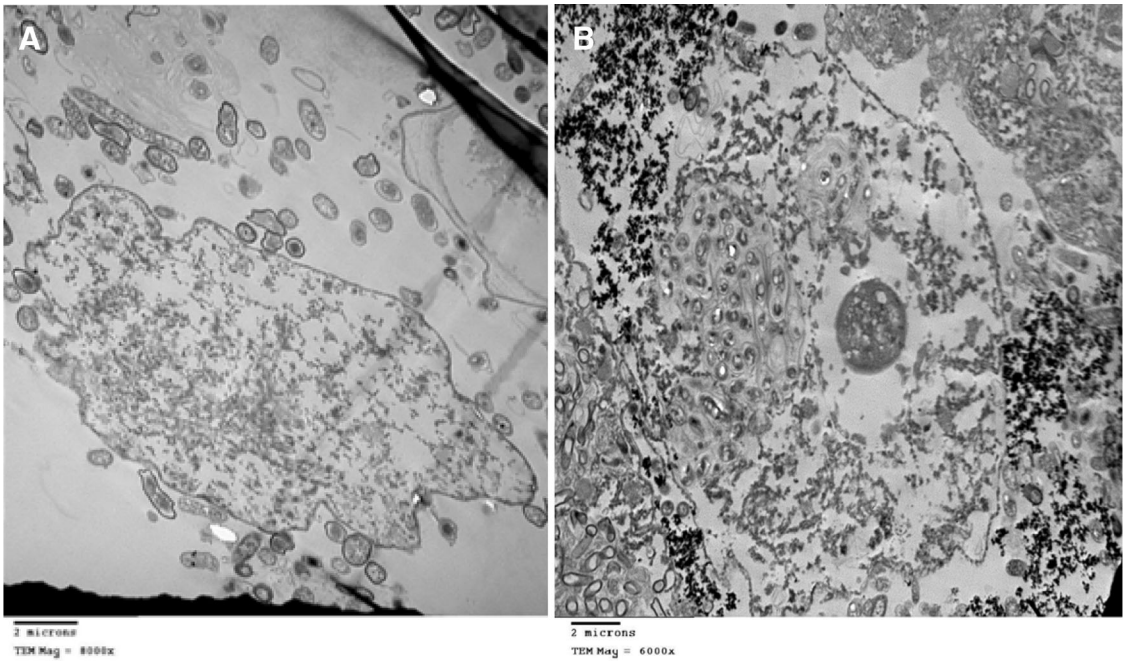

Fig. 5 TEMs of vinegar-treated trophozoites (C3) at 40 min showing; **a** shortened acanthopodia with thinned out plasma membrane and degenerated cytoplasmic content with structural damage. **b** Ghost like trophozoites with loss of acanthopodia and disrupted plasma membrane. The cytoplasmic contents were severely depleted with densely stained precipitates

against AK. This could be analogous to the use of boric acid, which has been used as a disinfectant in cases of conjunctivitis in the form of eyewash or eye ointment at different concentrations (Vastine et al. 1974), as well as one of the components of commercially available contact lens solutions that proved to be compatible with the ocular environment (Lehmann et al. 2010), similarly, hydrogen peroxide formulations have been commonly used for the disinfection of contact lenses with a trophocidal and cysticidal activity against *Acanthamoeba* sp. (Thomas 2013). Despite the inclusion of hydrogen peroxide (3%) in some commercial contact lens disinfectant, it was reported to be ineffective against *Acanthamoeba* sp. within the 30 min contact time recommended by the manufacturers (Ludwig et al. 1986; Zanetti et al. 1995), thus, studies were applied to enhance this activity (Hughes et al. 2003), which again stresses the urge of finding new adjunctive with a synergistic additives.

Previously, peracetic acid was demonstrated to be effective against *Acanthamoeba* sp. and *Naegleria* trophozoites after exposure to 15 mg/l for 2 h, but the activity against *Acanthamoeba* cysts required longer incubation (18 h) and higher concentration (150 mg/l) (Greub and Raoult 2003), which may produce side effects from exposure. In contrast to our results, the effectiveness of tested vinegar concentrations against *A. astronyxis* cysts was within hour duration and with a lower scale of concentrations. This altered action could be attributed to the ingredient differences according to manufacturing or the combinations with other chemicals which could hinder the effect and delay the action.

The considerably shorter period of action in this study, adds to the potential of vinegar on Acanthamoebae. In contrast to chlorhexidine drug control (0.02%) that gave a significantly higher viable mean cyst count when compared to all the examined vinegar concentrations along the study duration. These chlorhexidine results matched those observed by Khunkitti et al. (1998), where the effect of chlorhexidine on *Acanthamoebae* sp. cysts was not reached until 24 h of exposure to doses higher than 100 µg/ml, moreover, most of the chlorhexidine exposed cysts retained their normal appearance and structure. Also, Lee et al. (2007) reported a lag in cysticidal effect of chlorhexidine till 8 h and full potential reached at 48 h, additionally, Padzik et al. (2018), reported the same observation, when examining chlorhexidine on environmental and corneal Acanthamoebae isolates where a lag of 24 h was shown and its full effectiveness was reached after 120 h. Together with the lengthy time needed for chlorhexidine in contact lens maintenance solutions to exert its cysticidal potency, it was incompetent against the potentially pathogenic strains of Acanthamoebae as confirmed by Martin-Navarro et al. (2008).

The TEM observations of the control trophozoites showed an intact plasma membrane with acanthopodia, compact content, well defined cytoplasmic organelles, this

Table 1 The viability of *Acanthmoeba astronyxis* (6 * 10^4) after treatment with serial dilutions of vinegar at different time intervals

Vinegar concentrations	10 min		20 min		40 min		60 min	
	Viable Mean ± SD	Non-viable Mean ± SD	Viable Mean ± SD	Non-viable Mean ± SD	Viable Mean ± SD	Non-viable Mean ± SD	Viable Mean ± SD	Non-viable Mean ± SD
C1 (5%)	0.67 ± 0.58	91.00 ± 5.57	0.00 ± 0.00	91.33 ± 9.61	0.00 ± 0.00	78.33 ± 7.57	0.00 ± 0.00	60.67 ± 3.21
C2 (2.5%)	1.00 ± 1.00	62.67 ± 6.43[a]	0.00 ± 0.00	65.00 ± 5.29[a]	0.00 ± 0.00	73.00 ± 16.37	0.00 ± 0.00	53.33 ± 6.11
C3 (1.25%)	1.00 ± 1.00	65.67 ± 5.86[a]	0.00 ± 0.00	66.33 ± 5.51[a]	0.00 ± 0.00	66.67 ± 3.79	0.00 ± 0.00	57.33 ± 4.93
C4 (0.625%)	1.33 ± 1.53	63.67 ± 9.45[a]	0.33 ± 0.58	70.33 ± 1.53[a]	0.00 ± 0.00	55.33 ± 5.51[a,b]	0.00 ± 0.00	56.00 ± 10.44
C5 (0.312%)	2.00 ± 1.00	59.67 ± 11.37[a]	1.00 ± 1.00	68.67 ± 2.08[a]	0.00 ± 0.00	61.00 ± 2.00[a,b]	0.00 ± 0.00	47.33 ± 11.15[a]
C6 (0.156%)	1.00 ± 1.00	60.00 ± 6.24[a]	1.33 ± 0.58	55.67 ± 5.13[a,b,c,d,e]	0.00 ± 0.00	51.00 ± 4.00[a,b,c]	0.00 ± 0.00	46.33 ± 8.50[a]
C7 (0.07%)	2.33 ± 1.53	50.67 ± 2.08[a,b,c,d]	2.00 ± 1.00	52.33 ± 6.11[a,b,c,d,e]	0.00 ± 0.00	44.00 ± 7.00[a,b,c,e]	0.00 ± 0.00	38.33 ± 7.57[a,b,c,d]
C8 (0.04%)	14.33 ± 4.16[a,b,c,d,e,f,g]	46.67 ± 4.93[a,b,c,d,e,f]	17.67 ± 2.08[a,b,c,d,e,f,g]	41.67 ± 3.06[a,b,c,d,e,f,g,h]	3.67 ± 1.53	42.67 ± 6.66[a,b,c,d,e]	0.00 ± 0.00	29.33 ± 2.52[a,b,c,d,e,f]
Parasite control	131.67 ± 3.06[a,b,c,d,e,f,g,h]	5.00 ± 1.00[a,b,c,d,e,f,g,h]	130.67 ± 3.21[a,b,c,d,e,f,g,h]	8.33 ± 2.08[a,b,c,d,e,f,g,h]	124.00 ± 7.94[a,b,c,d,e,f,g,h]	7.67 ± 2.08[a,b,c,d,e,f,g,h]	119.67 ± 8.33[a,b,c,d,e,f,g,h]	5.00 ± 2.00[a,b,c,d,e,f,g,h]
Chlorohexidine (Drug Control)	92.00 ± 7.00[a,b,c,d,e,f,g,h,i]	16.67 ± 3.51[a,b,c,d,e,f,g,h,i]	88.67 ± 2.52[a,b,c,d,e,f,g,h,i]	13.67 ± 3.21[a,b,c,d,e,f,g,h]	86.67 ± 9.71[a,b,c,d,e,f,g,h,i]	18.00 ± 3.00[a,b,c,d,e,f,g,h]	83.00 ± 7.00[a,b,c,d,e,f,g,h,i]	20.33 ± 6.11[a,b,c,d,e,f,g,i]
ANOVA	783.577	45.675	2734.405	81.826	382.185	31.311	481.739	20.469
p value	< 0.001**							

[a]significant difference between Vinegar C1, [b]significant difference between Vinegar C2, [c]significant difference between Vinegar C3, [d]significant difference between Vinegar C4, [e]significant difference between Vinegar C5, [f]significant difference between Vinegar C6, [g]significant difference between Vinegar C7, [h]significant difference between Vinegar C8, [i]significant difference between parasite control

Table 2 The correlation between the serial dilutions of vinegar and the non-viable parasites along the different time intervals

Parasite	Time (10 min)		Time (20 min)		Time (40 min)		Time (60 min)	
	R	*p* value	R	*p* value	R	*p* value	R	*p* value
Viable	− 0.338	0.107	− 0.354	0.089	− 0.264	0.212	–	–
Non viable	0.782	< 0.001	0.839	< 0.001	0.862	< 0.001	0.790	< 0.001

R Spearman's rank correlation coefficient

was in accordance with the description of Hashim and Amin (2013). The TEM of the control cysts showed prominent double-layered cell wall, with intact contour and ostioles, intact endoplasm showing well-defined organelles and a number of oval mitochondria, these were consistent with the observations reported by Behera and Satpathy (2017). Electron-dense granules were seen in the cytoplasm and the nuclear membrane of the untreated cysts, it was suggested, that they might be corresponding to the interphase stage of the dividing cells (Hashim and Amin 2013), these have been reported in *A. palestinensis*, *A. castellanii* (Neff strain) (Chomicz et al. 2005; Martin-Gonzalez et al. 2006) and *Paradermamoeba levis* (Smirnov and Goodkov 2004).

The vinegar-treated cysts showed ultrastructure deformities, in the form of loss of ridges, thinning of the cell wall with retraction and shrinkage of the content, additionally, vinegar-treated trophozoites showed shortened acanthopodia, degenerated cytoplasmic content with structural damage. Similar effects were reported after lengthy exposure to chlorhexidine (Perrine et al. 1995). These deformities can be attributed to the passive diffusion of vinegar into the cell with the subsequent leakage and release of the intracellular constituents through the plasma membrane eventually resulting in cell death (Khunkitti et al. 1998).

Conclusions and recommendations

This is the first study on diluted cane vinegar 5% as an acanthamoebicidal. Although the outcomes did not define the exact mechanism of action, they highlighted its impact. The evidence provided suggests that a major target site was the plasma membrane and the organelles and that the intracellular damage contributes to the vinegar lethal effect. As a preliminary study a range of dilutions was chosen and applied to provide an initial point of start. Yet, new studies will be continued on a wider scale applying the cytotoxicity test to know the safest dose that can be used as contact lens disinfectant.

Author's contribution All authors participated in the *Acanthamoeba* culture and re-culture after drug application. Dr. Amira Elsaady was responsible for the sample collection, Dr. Rania Sarhan visualized the *Acanthamoeba* over the electron microscopy and Dr. Hayam Ezz Eldin performed the statistical analysis of the study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was taken from patients; the study was approved by the Research Ethics Committee, Faculty of Medicine, Ain Shams University, Egypt under Registration Number FWA 00006444.

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