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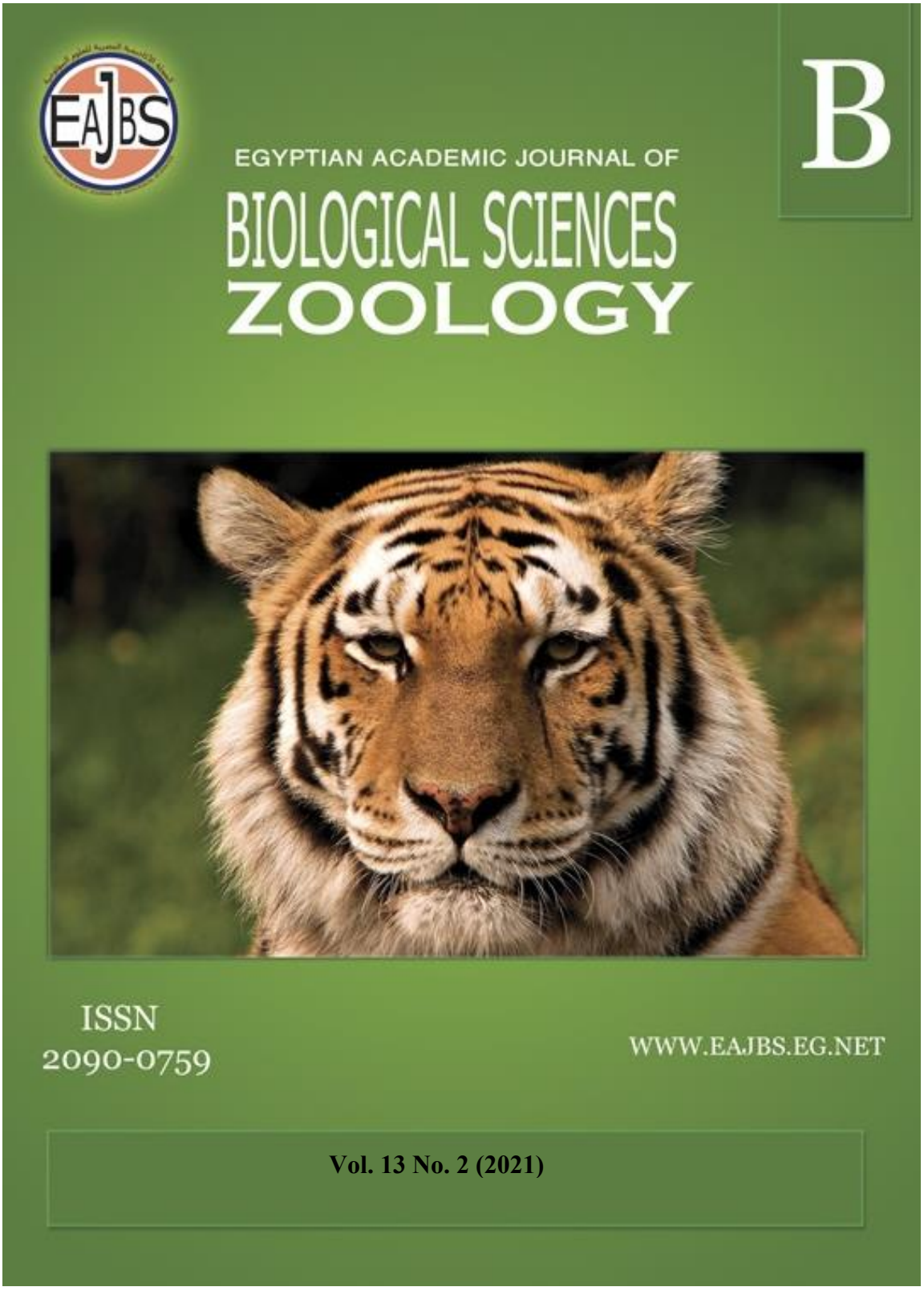

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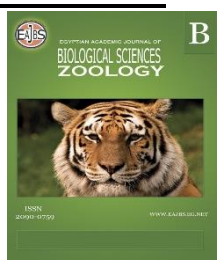

Bioactive Compounds of *Ziziphus spina-christi* Seeds Extract and Cellulase Enzyme Attenuates the Growth of *Acanthamoeba polyphaga* Isolated From Contact Lenses

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ABSTRACT

Background: Free-living *Acanthamoeba* spp. can cause sight-threatening amoebic keratitis and fatal granulomatous amoebic encephalitis. The difficulties in protecting against *Acanthamoeba* spp. frequently begin with a lack of diagnosis and continue with a lack of treatment. The current study aimed to evaluate the efficacy of ethanolic extract from *Ziziphus spina christi* (ZSC) seeds and cellulase enzyme as potential treatments *against* Acanthamoeba polyphaga compared to chlorhexidine (CHX) treatment. **Methodology:** *Acanthamoeba polyphaga* were isolated from contact lenses and contact lens solutions and were observed daily for 72–96 h and 3 weeks for trophozoites and cysts, respectively. Five groups, including ZSC, cellulase enzyme, the combination of ZSC and enzyme, CHX group, and control group were designed. **Results**: GC-MS analysis of the extract revealed ~ 85 bioactive compounds (primarily fatty acids and fatty acid derivatives). The antioxidant capacity of the extract at 800, 500, and 200 mg/ml was 1.972, 1.542, and 0.958 mg of ascorbic acid/g dry weight, respectively. Light and scanning electron microscopy observations revealed degeneration, decreasing in size, and distortion of the trophozoites and cysts. The viability of trophozoites and cysts was significantly reduced by different concentrations of the extract either alone or in combination with cellulase enzyme compared to 0.02% CHX. **Conclusion:** These results indicate that ethanolic extract from ZSC seeds (at the tested concentrations) and cellulase enzyme have anti-*Acanthamoeba* potential at various incubation periods.

INTRODUCTION

Acanthamoeba spp. are opportunistic free-living protozoans that feed on bacteria and yeast and can affect the eye (Lin *et al.*, 2019). Two different strains of these protozoans are pathogenic and nonpathogenic (Anger and Lally 2008). Given the ubiquitous distribution of *Acanthamoeba* spp. in the environment, humans have frequent contact with these amoeba (Siddiqui and Khan 2012). *Acanthamoeba* is a dimorphic organism, that has an active form, trophozoites, and a dormant form, cysts. Trophozoites can change into cysts by switching their phenotype under extreme and hostile environmental conditions, including a lack of food, high temperature, unsuitable osmolarity, and contact with antiseptic agents. *Acanthamoeba* strains have been isolated in air-conditioning units, contact lenses, and contact lens solutions (Siddiqui and Khan 2012). In particular, human contamination with pathogenic genotypes of *Acanthamoeba* is most likely to occur through infected contact lenses and contact lens solutions (Taher *et al.*, 2018).

Amoeba can cause human infections such as amoebic keratitis (AK), a blinding infection of the cornea (Carnt and Stapleton 2016), and granulatous amoebic encephalitis (Marciano-Cabral and Cabral 2003), particularly in healthy and immunocompetent individuals. AK causes appear to be multifactorial, but most cases have been linked to wearing contact lenses and using their cleansing agents (Illingworth and Cook 1998).
Acanthamoeba treatment is usually problematic and not consistently effective because of a rigid, double-layer wall in *Acanthamoeba* cysts that can tolerate various physical and chemical conditions (Paknejad *et al.*, 2020).

However, effective topical treatments include aromatic diamines (Sun 2018), propamidine isethionate (Siddiqui and Khan 2012), hexamidine (Carnt and Stapleton 2016), polyhexamethylene biguanide, and CHX (Bouheraoua *et al.*, 2014). For example, CHX (0.02%) has recently treated infections from *Acanthamoeba* trophozoites and cysts (Dodangeh *et al.*, 2017), whereas, a combination of propamidine isethiobate and CHX can rapidly and effectively treat *Acanthamoeba* infection (Marciano-Cabral and Cabral 2003). However, most of the aformenthioned drugs are highly toxic to human corneal cells. They can cause the absence of epithelial cells, loss of keratocytes with apparent apoptosis, and loss of endothelial cells. Further, they are linked to corneal necrosis, iris atrophy, cataract formation, and ischemic ocular inflammation (Shi *et al.*, 2018). Also, *Acanthamoeba* trophozoites exhibit a higher level of resistance to common antiamoebic compounds. Therefore, applying a sufficient concentration of the drugs to the cornea remains an issue (Lorenzo-Morales *et al.*, 2015). Therefore, it is necessary to test the efficiency of safe, natural antiamoebic compounds against pathogenic *Acanthamoeba* isolates continuously.

Recently, researchers investigating novel therapeutic agents against *Acanthamoeba* infections focused on applying medicinal plants as sources of novel compounds with high antiamoebic activity and low toxicity that represent alternative drug treatments (El-Sayed *et al.*, 2012, Niyyati *et al.*, 2016). *Ziziphus spina christi* (ZSC) seeds are frequently used in traditional medicine in the Middle East and some Asian countries for many illnesses, including eye inflammations, and are potentially a good source of antimicrobial compounds (Hossain 2019). Moreover, *in vitro* studies have shown that the antioxidant activity of ZSC is partly attributable to the presence of phenolic compounds (Yahia *et al.*, 2020).

The cyst wall contains cellulose, which accounts for 10% of the total dry weight of the cyst (Khan 2006); as a result, it is resistant to chemotherapy, resulting in infection recurrence after treatment. Therefore, cellulose degradation by cellulase enzyme may

make amoeba more susceptible to available chemotherape utic agents. At a minimum effect, inhibiting the excystment process will impede infection recurrence (Lazuana *et al.* 2019). Therefore, in the present study, we hypothesised that ZSC seed extract applied alone or in combination with cellulase enzyme compared to CHX (as a reference drug) would be effective against the *Acanthamoeba* strain (*A. polyphaga*) isolated from cosmetic lenses and disinfectant solutions in Upper Egypt. Furthermore, we examined the tested compounds' role in preventing the conversion of trophozoites into cysts with sufficient treatment duration.

MATERIALS AND METHODS

Sample Collection and Culture:

One hundred samples of contact lenses and contact lens solutions were collected from contact lens wearers. The samples were placed onto the non-nutrient agar (1.5%) medium plates seeded with live *Escherichia coli.* The plates were incubated at 30°C for 4 days, after which they were examined after four days (Ithoi *et al.*, 2010, Mahmoud *et al.*, 2020).

Trophozoites and Cyst Preparation:

Trophozoites at the exponential growth stage 72 – 96 h and 3 weeks-old cysts, were harvested by flooding the agar surfaces with 5 mL of phosphate-buffered saline (PBS) and gently scraping them with an inoculating loop. Once harvested, samples were centrifuged at 600 × g for 10 min. The supernatant was aspirated, and the sediment was washed twice in PBS to exclude most of the bacteria. The trophozoites or cysts in the suspension were counted with a hemocytometer, and counts were adjusted to 25×10^4 amoebae/ mL, for the amoebicidal activity assays (Ithoi *et al.*, 2010).

Preparation of Ethanolic Extract from ZSC Seeds:

Seeds of ZSC were collected from natural growth areas in Assiut Governorate, Egypt. The plant was authenticated by a specialised taxonomist. Voucher specimens were recorded under herbarium reference number 1843 in the herbarium of the Department of Botany and microbiology, Faculty of Science, Assiut University, Egypt.
ZSC seeds were cleaned, dried, and ground into powder; 500 g of powder was soaked in 5 L of ethanol and allowed to stand for ~72 h in room temperature with frequent stirring for extraction purposes. After 72 h, the ethanol will separate the soluble components of the extract during this soaking period. Then, the solution was filtered using Whitman filter paper (guage 1). The filtrate was then dried using a rotary evaporator (Mann *et al.* 2008) and stored in – 4 °C until used.

GC/MS Analysis and Total Antioxidant Activity Of Ethanolic ZSC seed Extracts:

GC/MS analysis was conducted in Nawah Scientific**,** Almokattam, Cairo, Egypt, using a gas chromatography-mass spectrometry instrument (TRACE GC Ultra Gas Chromatographs; Thermo Scientific Corp., USA coupled with a thermo mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC/MS system had a TR-5 MS column (30 m × 0.32 mm i.d; 0.25 µM film thickness). Analyses were performed using helium as a carrier gas at a flow rate of 1.0 mL/min and a split ratio of 1:10 with the following temperature program: 60°C for 1 min; rising at 4.0°C/min to 240°C and the held for 1 min. The injector and detector were held at 210 °C. Diluted samples (1:10 hexane, v/v) of 1 µL of the mixtures were injected continuously. Mass spectra were obtained by electron ionization at 70 eV using a spectral range of m/z 40-450. The compounds were identified via a library search on a Wiley 275 L GC/MS database (Thermo Fisher Technology, Waltham, Massachusetts, USA) using AMDIS software (www.amdis.net), with identification acheived by retention indices (relative to

n-alkanes C8–C22 Wiley spectral library collection and NSIT library database). Curves were generated by running GC analysis of authentic representative compounds (Leary *et al.*, 2019).

Estimation of Total Antioxidant Activity For Extract Dilutions:

The phosphomolybdenum method is a quantitative method used for the determination of the antioxidant activity in terms of reduction of molybdate ions, based on the reduction of Mo (VI) to Mo (V) in the presence of the extracts to form a green coloured complex at acidic pH with maximum absorption of 695 nm (Aadesariya *et al.* 2017). In brief, a calibration curve was prepared by dissolving ascorbic acid in methanol, then different concentrations were prepared (100, 75, 50, 25, 10, 0 µg/mL). 10 µl of the extract and different concentrations of ascorbic acid were mixed with 3 mL of reagent solution in test tubes and incubated at 95 °C for 90 min. Then, samples were cooled to room temperature and the absorbance of the solution was measured at 695 nm using a UV-VIS spectrophotometer (UVmini-1240) against blank. 0.3 mL methanol was used as the blank. All the determinations were carried out in a triplicate and mean values were calculated. The total antioxidant activity in the extract was expressed as mg ascorbic acid equivalent (A.E) per gram dry weight (mg A.E/g DW).

Cellulase Production And Preparation:

Aspergillus flavus AUMC 10331 and *Aspergillus oryzae* AUMC 10329 were ultilized in solid-state fermentation (SSF) to synthesize cellulase and xylanase enzymes from rice husk (RH) (Moubasher *et al.*, 2019). Using microcrystalline cellulose, the cellulase enzyme had specific activity of 3200 IU/g enzyme at the standard assay conditions (pH 5.0 and 50 °C). The obtained cellulase enzyme was dissolved in citrate buffer (pH 4.5 – 5.5) and the activity was adjusted at 300 IU/ ml for this investigation.

Experimental Design:

Three serial dilutions, i.e., 200, 500, 800 mg/mL of the extract were prepared according to Dodangeh *et al*. (2017) to evaluate the amoebicidal activity. Five groups with three replicates were divided as follows:

Group 1: 100 µL of each serial dilution of ZSC extract was added to an equal volume of the calibrated trophozoite/cyst suspension (25×10^4/mL) in Eppendorf tubes.

Group 2: 100 µL of 0.02% CHX was added to an equal volume of the calibrated trophozoite/cyst suspension (25×10^4/mL) in Eppendorf tubes**.**

Group 3: Equal volumes (50 µL) of ZSC extract and cellulase enzyme were added to 100 µL of the calibrated cyst suspension (25×10^4/mL) in Eppendorf tubes.

Group 4: 300 U of cellulase was added to 100 µL of the calibrated cyst suspension (25×10^4/mL) in Eppendorf tubes.

Group 5: Equal volumes (100 µL) of the parasite and PBS were used as a control group. All tubes were mixed and incubated at 30ºC for 24, 48, and 72 h.

Efficacy of Ethanolic Extract Against Cultured Trophozoites and Cysts:

Following the incubation periods , cell viability was assessed using 0.4% trypan blue stain by adding 20 µL to an equal volume of treatment in tubes. These tubes were then vortexed and incubated for 3 min at room temperature. The number of viable trophozoites/cysts in each group was determined separately using a Thoma cell counting chamber. Nonviable cysts were transferred to non-nutrient agar medium plates enriched with *E. coli* and incubated at 26°C for three days to confirm the observed results.

Morphological Alterations of Trophozoites/Cysts:

After 72 h, in preparation for morphological examination by scanning electron microscopy, representative samples from each group were suspended in 0.1M sodium phosphate buffer at pH 7.4. Samples were then centrifuged at 500 × g for 2 min and washed three times with 0.1M sodium phosphate buffer at room temperature to remove

the remaining media. The pellets were fixed in 5% gluteraldehyde and then postfixed in 1% osmium tetroxide in 0.1M sodium phosphate buffer at 4°C for 2 h. Subsequently, the samples were washed three times and dehydrated using ethanol and propylene oxide, filtered using a millipore filter (diameter: 22 mm), dried for 24 h, and finally stained with contrast uranyl acetate and citrate. Morphological alterations were examined with a Zeiss Leo 435 VP scanning electron microscope (Leo Electron Microscopy Ltd Cooperation, Zeiss Leica, Cambridge, England) at 15 kV. A magnification of 1,500 – 2,500 k was used to capture images at the Electron Microscope Unit in Assiut University Egypt (Abdel-Zaher *et al.*, 2016)**.**

Statistical Analysis:

The percentage of viable cells was determined using Graphpad Prism 3.0, whereas, continuous variables were summarized with ranges, means, and standard deviations. Oneway ANOVA and Tukey's test were used to statistically compare group data. Results were considered statistically significant at $P \leq 0.05$.

RESULTS

Growth and Morphological Identification of *Acanthamoeba sp*. Stages:

Under a light microscope (Olympus, Japan), trophozoites were visible after four days and covered the entire agar surface after seven days. The morphological characteristics of the trophozoites and cysts that were used for the experiment were typical of *Acanthamoeba polyphaga* (Hassan *et al.*, 2021). Trophozoites were identified using the unique and characteristic presence of fine, tapering, thorn-like acanthopodia (**Fig. 1a**). The cysts were spherical or round, sometimes slightly deformed, and ranged in size from 10 to 26 µm. The ectocyst was wrinkled, whereas the inner cyst wall had a smooth and spherical shape (**Fig.1b**). The ectocyst was separated from the endocyst, except for the region of cyst pores (ostioles) where they met. In viable cysts, there was a single spherical nucleus with a central nucleolus. Granular cytoplasm appeared just under the cytoplasmic membrane.

GC/MS and Total Antioxidant Capacity of Ethanolic ZSC Seed Extract:

GC/MS analysis of the ethanolic extracts of ZSC seeds revealed about 85 bioactive compounds, including fatty acids, ketones, alkanes, phenols, saponins, glycosides, alkaloids, steroids, polysaccharides, and terpenoids. Chromatograms of the major 17 significant peaks and the components corresponding to the peaks were shown in Table 1 and Fig. 2.

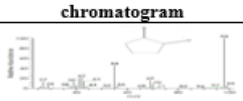
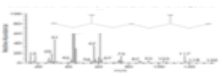
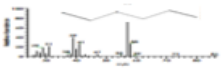
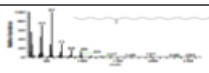
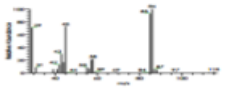
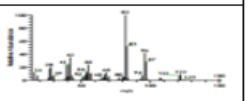
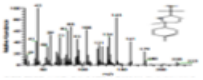
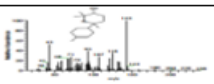
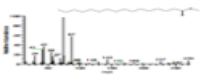
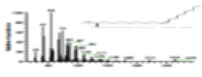
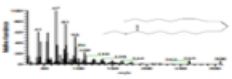
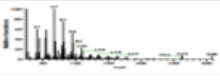
The total antioxidant capacity of the various concentrations of extracts was measured using the phosphomolybdenum method and expressed quantitatively in the plant's ascorbic acid equivalents/dry weight. The standard solution of (10–100 µg/mL) conformed to Beer's Law; the absorbance of the solution was 695 nm with a regression coefficient of 0.9979 (slope = 0.0079; intercept = 0.0214; Fig. 3). The standard curve equation was $y = 0.0079\ x + 0.0214$. The total antioxidant capacity primarily relates to thermodynamics with $x = y - 0.0214/0079$, where x and y are the concentration and bsorbance of the unknown samples, respectively. The total antioxidant capacity of 800 , 500 , and 200 mg/mL extracts was 1.972, 1.542, and 0.958 mg A.E/g, respectively.

Morphological Alterations of *Acanthamoeba* Trophozoites and Cysts:

Viable trophozoites and cysts were unstained, whereas dead cells were stained with trypan blue (Fig. 4). ZSC extract, cellulase enzyme, and CHX caused trophozoite accumulation, degeneration, and size reduction. Irregular and distorted shapes also occurred in both trophozoites and cysts (Fig. 4). The ultrastructure of *Acanthamoeba*

cysts and trophozoites after 72-h incubation revealed morphological changes and progressive destruction (Fig. 5).

Table 1: The chemical nature of the major 17 compounds of ethanolic extract of *Ziziphus spina christi* seeds.

No.	RT	Name of the compound	Molecular Formula	Area %	M. W	Nature of compound	Chemical extraction and chromatogram
1	4.52	1,2-Cyclopentanedione	C5H6O2	9.23	98	Cyclic diketone	
2	5.20	Diglycerol	C6H14O5	5.67	166	Fatty acid	
3	5.54	Formic acid, 2-propenyl ester	C4H6O2	19.79	86	Fatty acid	
4	6.66	Octadecane, 6-methyl	C19H40	1.40	268	Alkane	
5	7.67	Undecane	C11H24	5.93	156	Alkane	
6	7.86	Octadecane, 1(ethenyloxy)-	C20H40O	2.27	296	Stearic acid ester	
7	10.77	4-(1-Hydroxy-ethyl) ç butanolactone	C6H10O3	4.43	130	Terpenic derivatives	
8	13.43	8-Azabicyclo[3.2.1]octane-2-carbo xylic acid, 3-hydroxy-8-methyl-, methyl ester	C9H14N2	2.26	199	Atropane alkaloid	
9	15.73	Phenol, 2-methoxy-4-(2-propenyl)-	C10H12O2	4.90	164	phenol	
10	24.54	Bisabolol oxide B	C15H26O2	0.97	238	sesquiterpeineol essential oil	
11	27.00	2H-Pyran-3-ol, tetrahydro-2,2,6-trimethyl-6-(4-methyl-3-cycloh exen-1-yl)-, [3S-[3à,6à(R*)]]	C15H26O2	8.02	238	Sesquiterpenoids	
12	31.77	Hexadecanoic acid, methyl ester	C17H34O2	3.52	270	Fatty acid methyl esters	
13	33.44	Hexadecanoic acid, ethyl ester	C18H36O2	3.19	284	Palmitic acid ester	
14	35.72	7,10-Octadecadienoic acid, methyl ester	C19H34O2	2.26	294	Fatty acid	
15	35.89	9-Octadecenoic acid (Z)-, methyl ester	C19H36O2	4.06	296	Fatty acid	
16	37.32	Linoleic acid ethyl ester	C20H36O2	1.00		Fatty acid	
17	37.45	Ethyl Oleate	C20H38O2	4.64	310	Ethanolic fatty acid oleic	

Ethanolic Extract of ZSC Inhibits the Viability of *Acanthamoeba* Trophozoites and Cysts:

Treatment with the various extract concentrations significantly affected the cell viability of trophozoites and cysts relative to the viability of control cells. After incubation for 72 h with 200 , 500 , and 800 mg/mL plant extract, viability percentages were 20.00%, 18.00%, and 3.333% for trophozoites (Fig. 6a) and 41%, 23%, and 15% for cysts (Fig. 6b), respectively. Furthermore, all ZSC concentrations showed potent amoebicidial activity throughout different incubation periods. In the group of CHX, the viability of *A. polyphaga* trophozoites was zero after 24h and no growth was observed after incubation of the culture for 3 more days at 26ºC (Fig. 6a).

Cysticidal Effect Of Cellulase Enzyme Alone and In Combination With The Extract:

Treatment with the extract and cellulase produced a highly significant reduction in the viability of cysts compared to non-treated control group (Fig. 7a). The viability percentages of cysts treated with cellulase enzyme only were 43.6%, 18.3%, and 11.5% at 24, 48, and 72 h of incubation, respectively (Fig. 7b). In contrast, viable cells were not observed in samples treated with 0.02% CHX after 24 h of incubation (Fig. 7b).

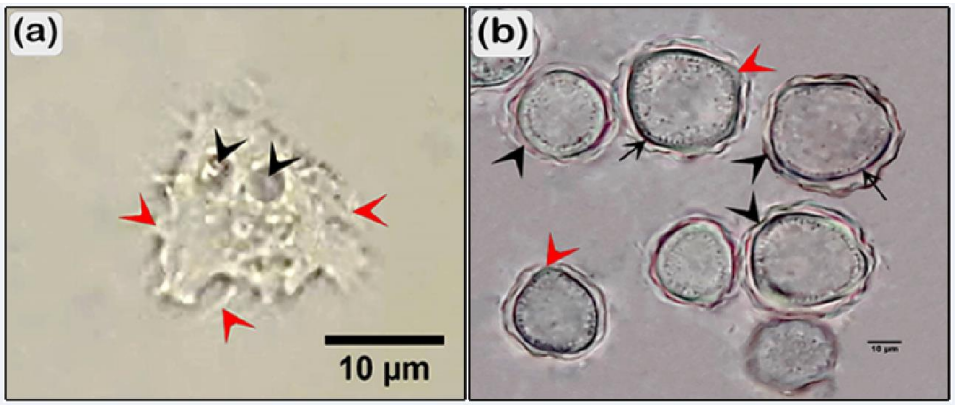


Fig. 1: Wet mount smears of *Acanthamoeba polyphaga* isolated from contact lens and contact lens solutions showing; (a) *A. polyphaga* trophozoite acanthopodia (red arrowheads) and contractile vacuoles (black arrowheads). (b) *A. polyphaga* cysts showing typical wrinkled ectocysts (black arrowheads), smooth endocyst (arrows), and ostioles (red arrowheads) (×400).

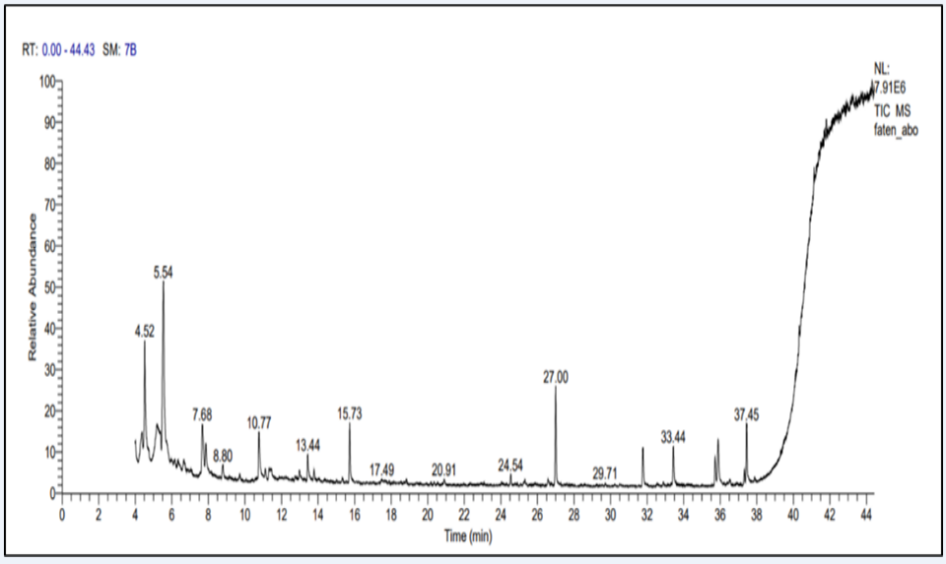


Fig. 2: Chromatogram (GC/MS) of ethanolic extract of ZSC seeds.

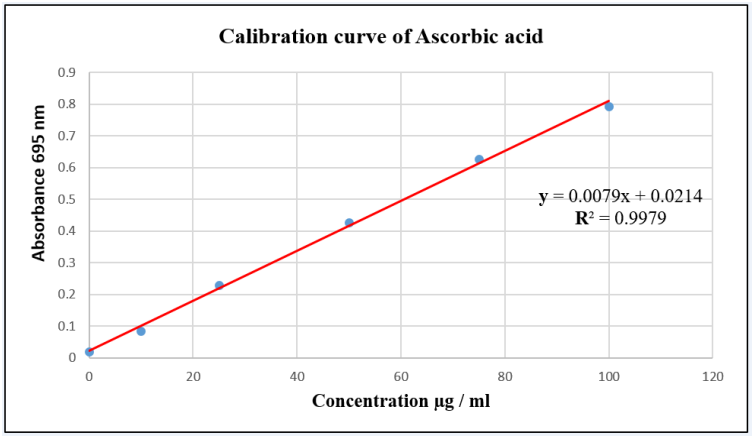


Fig. 3: Calibration curve of ascorbic acid for the total antioxidant capacity evaluation.

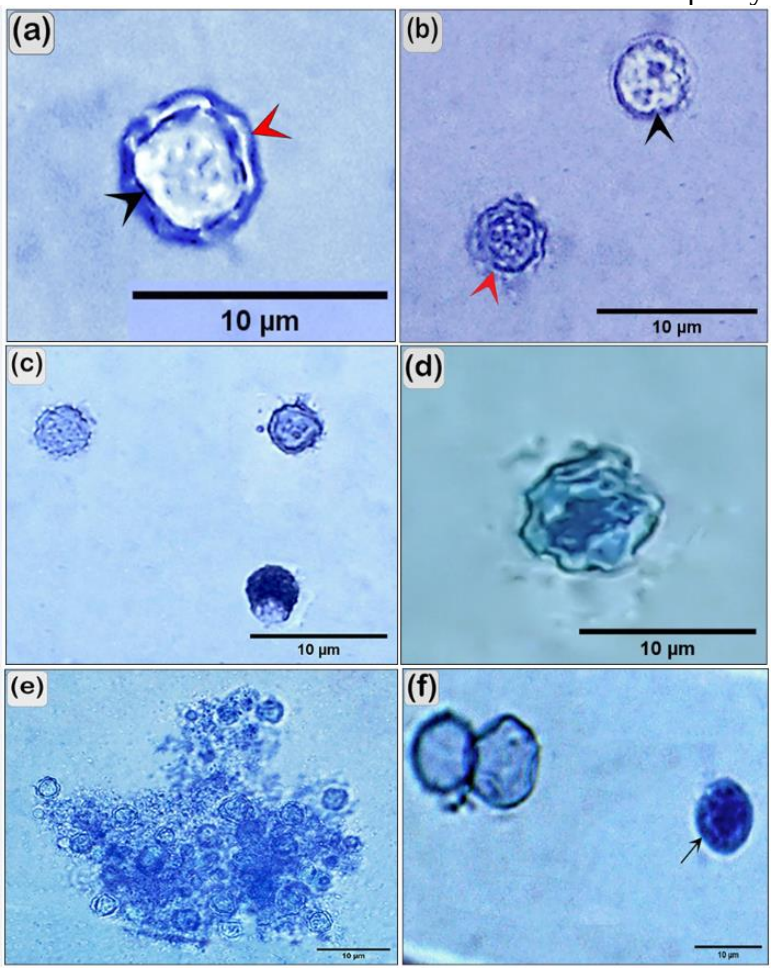


Fig. 4: Photomicrographs showing morphological alternations in *A. polyphaga* trophozoites and cysts in different treated groups. (a) Viable *A. polyphaga* cyst (un-stained) with wrinkled ectocyst (red arrowhead) and smooth endocyst (black arrowhead) in the control group; (b) Viable *A. polyphaga* trophozoite (black arrowhead) and stained non-viable cyst (red arrowhead); (c) Reduction in the size of non-viable trophozoites and deeply stained cyst; (d) Shrinkage of the non-viable cyst with the disintegration of outer surface architecture after incubation for 72 h; (e) Accumulation and degeneration of cysts after incubation for 72 h; (f) Interchanged shape of the cyst (arrow) and also stained trophozoites appeared with small size. All these changes can be noticed in different treated groups (×400).

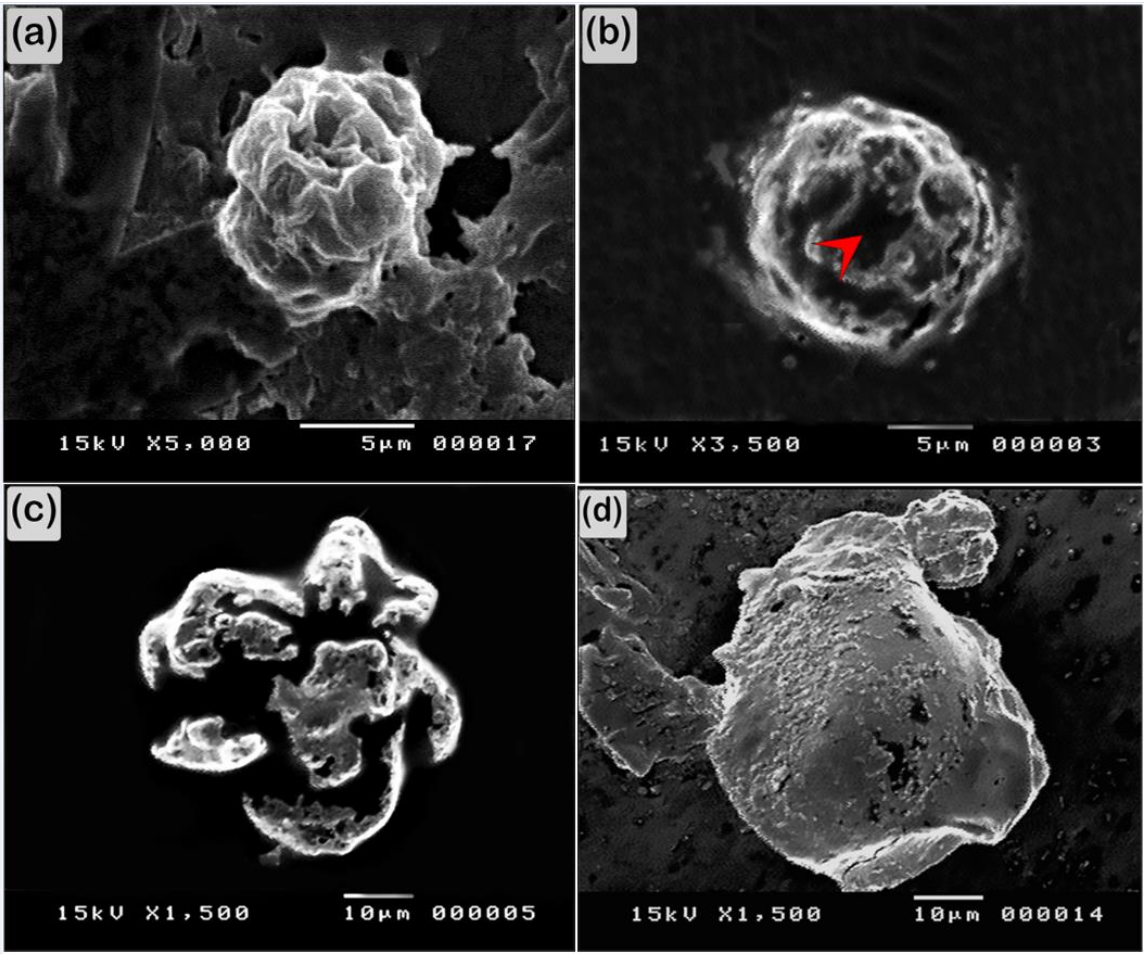


Fig. 5: Ultrastructure alterations of *A. polyphaga* showing; (**a**) Control cyst surface was appeared rough, thick wrinkles, and granulated; (**b**) Cysts treated with ZSC and cellulase enzyme after incubation for 72 h where the cyst surface was damaged, less wrinkled, and a collapsed areas (red arrowhead); (**c**) Cysts treated with high concentration of ZSC (800 mg/mL) after incubation for 72 h showing fully degenerated cyst; (**d**) Distorted trophozoites treated with high concentration of ZSC (800 mg/mL) after incubation for 72 h.

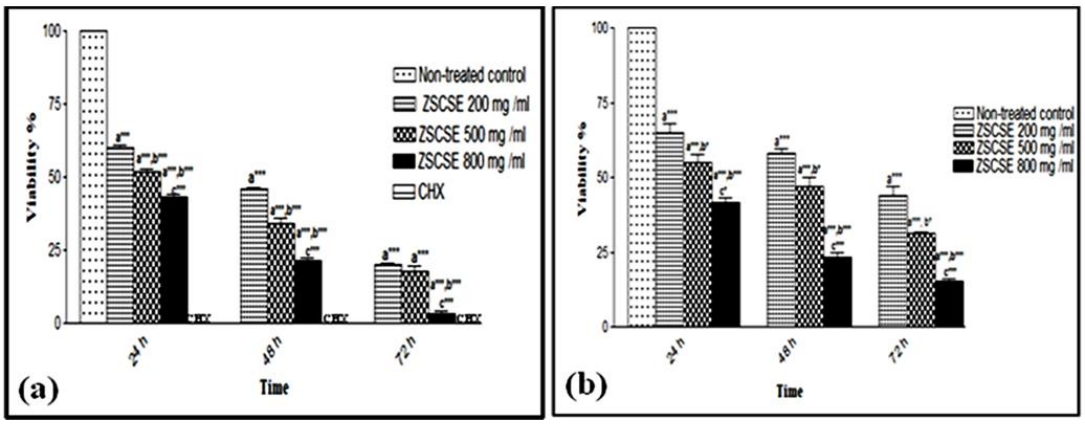


Fig. 6: Effect of extract only, CHX only on trophozoites (**a**) and cysts (**b**) compared to non-treated control. Data presented as mean ± S.E. where n= 3

*Significant at $P < 0.05$. ** Significant at $P < 0.01$. *** Significant at $P < 0.001$

[a] Comparison between control and different concentration.

[b] Comparison between C200 with C500 and C800 at the same period.

[c] Comparison between C500 with C800 at the same period

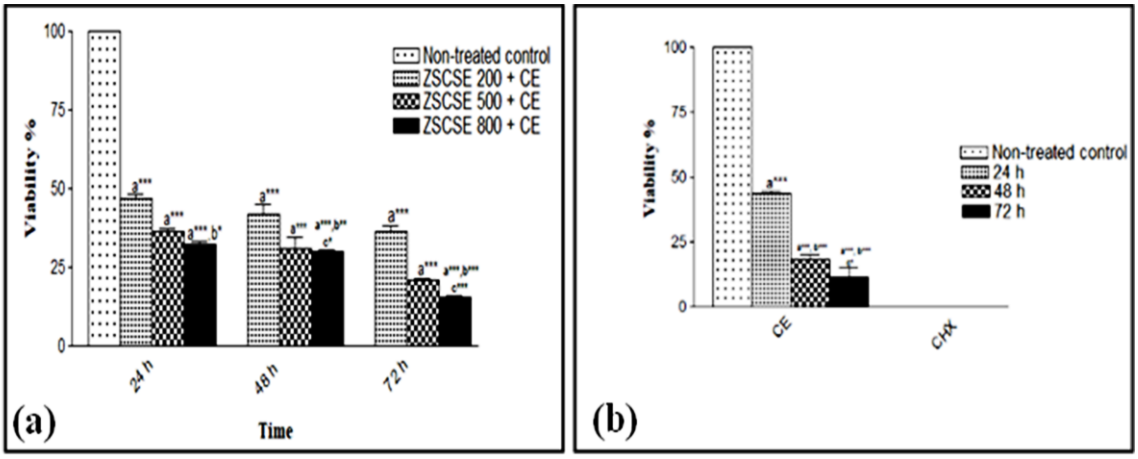


Fig. 7: (**a**) Effect of ZSCSE +CE on cysts compared to non- treated control.
[a] Comparison between control and different concentration.
[b]Comparison between C200 with C500 and C800 at the same period.
[c]Comparison between C500 and C800 at the same period.
(**b**): Effect of CE alone and CHX alone on cysts compared to non- treated control.
[a] Comparison between control and different periods compared to non-treated control.
[b] Comparison between 24 and 48h and 72.
[c] Comparison between 48h and 72h.
Significant at $P < 0.05$. ** Significant at $P < 0.01$. *** Significant at $P < 0.001$.
Data presented as mean ± S.E. where n= 3. ZSCSE: ZSC seeds extract; CE: cellulase enzyme.

DISCUSSION

Acanthamoeba is one of the most challenging infections in medical practice because of its wide range of clinical manifestations, symptoms, delayed diagnosis, and frequent lack of response to standard medical treatment (Lorenzo-Morales *et al.*, 2015)**.** In the present study, the isolated *Acanthamoeba* was identified morphologically as *Acanthamoeba polyphaga* per the previous descriptions of Azhar and Muslim (2017) and Hassan *et al*. (2021). *Acanthamoeba polyphaga* is recognized as Group II genotypes, which includes most pathogenic species regarding human keratitis (Niszl and Markus 1998).

To date, effective and safe treatment for this pathogen has yet to be developed (de Lacerda and Lira 2021). Treatment of pathogenic *Acanthmoeba* spp. with corticosteroids, antibacterial, antifungal, or antivial drugs may improve the condition and cause deterioration over time (Marciano-Cabral and Cabral 2003). Topical AK therapy must continue much longer than antibacterial therapy due to the amoebae encystment, which is difficult for the drugs to penetrate (Clarke *et al.*, 2012); however, prolonged treatment may lead to toxic effects on corneal tissue that can result in keratopathy (Lonnen *et al.*, 2014). Recently, a trend has arisen that involves shifting from the current chemical drugs to natural drugs (Dodangeh *et al.*, 2017). In this study, we evaluated the amoebicidal effects of alternative natural compounds to inhibit *Acanthamoeba* trophozoites and cysts viability by comparing these compounds' effects with those of CHX as a reference drug.

The medicinal plant ZSC contains biologically active ingredients that can potentially serve as antimicrobial, antioxidant, and anti-inflammatory agents (Asgarpanah and Haghighat 2012, Hossain 2019). Accordingly, we used chromatography analysis of ethanolic extract ZSC seeds and assessed their potential

effects alone or in combination with cellulase enzyme on *Acanthamoeba polyphage* isolated from contact lenses and disinfectant solutions. The ethanolic extracts of ZSC seeds alone exhibited statistically significant trophozoites and cysts viability inhibition in *Acanthamoeba* sp. cultures at 800, 500, and 200 mg/mL. These effects may be attributable to active compounds with different biological activities in ZSC seeds (Asgarpanah and Haghighat 2012)**.** Alfonso-Munoz and his colleagues (2018) reported that therapy should act on trophozoites, cysts, and inflammation via antiamoebic and anti-inflammatory agents.

Interestingly, chromatogram analysis of the ethanolic extract of ZSC seeds in this study revealed 17 compounds that might contribute to its medicinal properties. Antioxidant properties may be attributable to compounds such as phenols, 1, 2-cyclopentanedione, and octadecane 6-methyl (Krishnamoorthy and Subramaniam 2014, Rao and Naika 2018, Shyamala and Manikandan 2019)**.** The compound undecane plays key role in antimicrobial defence as a transducer for the immune sensor and its method of production (Krishnamoorthy and Subramaniam 2014). Moreover, other compounds, such as diglycerol, formic acid, and 2-propenyl ester fatty acid, have antimicrobial and antipyretic activities (Matsumura *et al.,* 1999, Jeeva and Krishnamoorthy 2018)**.** These active compounds may affect *Acathamoeba* cysts by binding to the mucopolysaccharides of the ostioles leading to penetration of the amoeba, cell membrane damage, cell lysis, and death (Lorenzo-Morales *et al.*, 2015)**.** In addition, *Acathamoeba* trophozoites are sensitive to many antifungals, antiseptics, and antiprotozoals (Carnt and Stapleton 2016, Garg *et al.*, 2017).

The present study showed the concentration of ZSC seed extract and exposure time was directly proportional to the viability of the cultured *Acanthamoeba*. These results are consistent with those of Niyyati *et al*. (2016), who reported that an aqueous total plant extract of *Ziziphus vulgaris* eliminated *Acanthamoeba* trophozoites and cysts at 200 mg/mL and 500 mg/mL, respectively, after 24 h of incubation. Furthermore, there was no cytotoxicity at the highest evaluated concentration. Similarly, Dodangeh *et al*. (2017) indicated that different concentrations of *Z. vulgaris* extract could eliminate the trophozoites and cysts of *Acanthamoeba in vitro***.** Clinically, treatment is usually applied hourly in the first 48–72 h (Garg *et al.,* 2017). The amoebicidal effect of ZSC may also be attributable to its free radical scavenging properties as an effective mechanism against both the trophozoite and cyst stages of *Acanthamoeba* spp. (Niyyati *et al.*, 2016).

Given the different genotypes, diverse stages, and different encystment capacities of *Acanthamoeba* trophozoites, combination therapy is typically more effective than monotherapy. Lindsay *et al*. (2007) reported that some therapeutic agents used against *Acanthamoeba* are only effective against trophozoites. However, our results showed that the combination of ZSC extract with cellulase enzyme achieved significant effects against cysts, particularly at the highest concentraction of the extract.

Acanthamoeba cysts walls have a chemical composition containing 33% cellulose (Weisman 1976). Indeed, cellulose (1,4-linked glucose) is the only target for the degradation of *Acanthamoeba* cysts. The use of enzymatic catalysts that can hydrolyze complex sugars can target cyst walls via the degradation of specific sugar linkages. Once cyst walls are degraded, it becomes easier to target *Acanthamoeba* (Anwar *et al.* 2018)**.** In the present study, the cellulase enzyme alone significantly reduced cyst viability relative to non treated control group. Furthermore, treatment with extract and cellulase combined caused morphological changes in cyst walls such as shrinkage and irregularly shaped cysts. This is consistent with Lazuana *et al*. (2019), who observed that a mixture of cellulase and disinfectant solution led to shrunken and irregular shaped cysts. In addition, a combination of CHX and cellulase can disrupt cyst wall structure and

enhance the efficacy of marketed contact lens disinfectants against *Acanthamoeba castellanii* trophozoites and cysts *in vitro* (Abjani *et al.*, 2017).

Conclusion:

In conclusion, the present study showed that ethanolic extract of ZSC seeds inhibited the viability and growth of *Acanthamoeba polyphaga* at various concentrations and under various incubation periods. This activity is also effective when combined with cellulase enzyme. Thus, further studies on this extract as a therapeutic agent, including additional *in vitro* and *in vivo* investigations, are recommended to determine the most appropriate dose and an incubation period for eliminating the highest percentage of cysts and trophozoites.

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Ethical Approval::

The National Ethics Committee of the Faculty of Science in Assiut University, Assiut, Egypt, approved this study. All methods were conducted per the relevant guidelines and regulations.

Conflict of interest: None declared.

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ARABIC SUMMARY

المركبات النشطة بيولوجياً من مستخلص بذور النبق (*Ziziphus spina-christi*) وإنزيم السليولاز يضعفا من نمو الأكانثاميبا (*Acanthamoeba polyphaga*) المعزولة من العدسات اللاصقة

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5- قسم الطفيليات الطبية, كلية الطب, جامعة أسيوط, أسيوط, مصر

مقدمة: الأكانثاميبا (*Acanthamoeba* spp.) يمكن أن تسبب التهاب القرنية الأميبي الذي يهدد البصر والتهاب الدماغ الأميبي الحبيبي. الصعوبات في الوقاية من الأكانثاميبا (*Acanthamoeba* spp.) تبدأ في كثير من الأحيان بنقص التشخيص والعلاج. تهدف الدراسة الحالية إلى تقييم فعالية المستخلص الإيثانولي من بذور نبات النبق (*Ziziphus spina christi* (ZSC)) وإنزيم السليولاز كعلاجات محتملة ضد الأكانثاميبا بوليفاجا (*Acanthamoeba polyphaga*) مقارنة بعقار الكلورهيكسيدين (CHX).

الطرق المستخدمة: تم عزل الأكانثاميبا بوليفاجا (*Acanthamoeba polyphaga*) من العدسات اللاصقة ومحاليل العدسات اللاصقة وتمت ملاحظتها يوميًا لمدة 72-96 ساعة و 3 أسابيع للتروفوزويتات والحويصلات، على التوالي. تم تصميم خمس مجموعات، تشمل المجموعة المعالجة بالمستخلص النباتى، المجموعة المعالجة بإنزيم السليلوز، المجموعة المعالجة بالمستخلص النباتى والإنزيم، المجموعة المعالجة بعقار الكلوروهيكسيدين، ومجموعة الكنترول. **النتائج**: كشف تحليل الكروماتوجراف الغازى (GC-MS) للمستخلص عن 85 مركبًا حيويًا (تشمل بشكل أساسي الأحماض الدهنية ومشتقات الأحماض الدهنية). كانت السعة المضادة للأكسدة للمستخلص عند 800 و 500 و 200 مجم / مل هى 1.972 و 1.542 و 0.958 مجم من حمض الأسكوربيك / جرام بالوزن الجاف, على التوالي. أظهر الفحص المجهري بأستخدام الميكروسكوب الضوئى والماسح الإلكتروني عن تفسخ وتناقص في الحجم وتشوه في التروفوزيتات والحويصلات. وكذلك أنخفاض نمو التروفوزويتات والحويصلات معنويا من خلال التركيزات المختلفة من المستخلص النباتى إما بمفرده أو بالاشتراك مع إنزيم السليلوز مقارنة بـعقار الكلوروهيكسيدين.

الخلاصة: تشير النتائج السابقة إلى أن المستخلص الإيثانولي من بذور النبق (عند جميع التركيزات المختبرة) وإنزيم السليلوز لهما تأثير مضاد للأكانثاميبا (*Acanthamoeba*) في فترات حضانة مختلفة.