Article

Melaleuca alternifolia Cheel (Tea Tree) and *Eucalyptus globulus* Labill. Essential Oils' Effectiveness Against an *Acanthamoeba polyphaga* Strain Responsible for Human Keratitis

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Abstract: Among free-living amoebae (FLA), *Acanthamoeba polyphaga* is an important causal agent of *Acanthamoeba* keratitis (AK), a severe and potentially sight-threatening condition. The present study evaluated the "in vitro" efficiency of *Melaleuca alternifolia* Cheel (tea tree) (tea tree oil—TTO) and *Eucalyptus globulus* Labill. (*Eucalyptus* essential oil—EEO) essential oils against an *Acanthamoeba* strain isolated from human keratitis. The Minimum Inhibitory Concentration (MIC) of the EOs and the Fractional Inhibitory Concentration (FIC) Index were used to evaluate the decrease in viable cells of *Acanthamoeba* over time and at different concentrations of EOs, used alone or in association. A relevant amoebicidal effect emerged during the first hours of exposure for both compounds, and TTO was the most effective. The TTO/EEO association clearly indicated a synergistic effect in all tests, and at 2 days post-treatment, no viable *A. polyphaga* cells were observed at all tested concentrations. In conclusion, the potential therapeutic use of EOs represents a promising therapeutic strategy for the treatment of AK.

Keywords: *Acanthamoeba polyphaga*; *Acanthamoeba* keratitis (AK); essential oils; *Melaleuca alternifolia* Cheel (tea tree); *Eucaliptus globulus* Labill

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1. Introduction

Acanthamoeba spp. are free-living amoebas (FLA), which are ubiquitous in both natural and anthropized environments. *Acanthamoeba* spp. are divided into 23 different genotypes (T1–T23), the most predominant of which is T4 [1,2]. FLA have been isolated from sewage and sewage-related environments [3], coastal marine waters [4], natural hot springs [5], drinking water [6], and air conditioning systems in hospital environments [7]. Swimming pools and recreational waters are among the environments where the greatest amount of human exposure to free-living amoebae occurs, and among the major genera of the medically important free-living amoebae, *Acanthamoeba* is the genus most involved in human infections [8].

Acanthamoeba spp. are responsible for keratitis (*Acanthamoeba* keratitis, AK), granulomatous amoebic encephalitis (GAE), lung infections, and skin infections in immunocompromised subjects. Epidemiological studies demonstrate an annual increase in *Acanthamoeba* infections, particularly AK infections [9].

The main disease caused by *Acanthamoeba*, AK, is a serious, painful eye infection that, if not diagnosed and treated early, can cause corneal perforation and fusion [10]. GAE is a rare but almost always fatal central nervous system infection affecting debilitated or immunocompromised individuals [11], although cases have been reported in apparently healthy patients. This rare central nervous system disease is highly fatal, with a mortality rate greater than 90%, leading to death within 1–2 months of symptom onset due to increased intracranial pressure. Between 1990 and 2020, 75 cases of *Acanthamoeba* spp. GAE have been described worldwide [12]. Other rare pathological conditions are cutaneous acanthamoebiasis (CA) [13] and *Acanthamoeba* pneumonia (AP), mainly observed in patients with AIDS and in patients with a low immune response, respectively. From 1990 to 2020, 19 cases of AP or similar lung infections were related to *Acanthamoeba* spp., mainly in the USA, but similar events have also been observed in Europe and Eastern countries [14].

As previously mentioned, the number of infections caused by *Acanthamoeba* is very high, resulting in an incidence of 23,561 annual cases, as reported in a recent study that collected data from 20 countries [15]. Risk factors for AK vary between developing and developed countries. In the latter, most AK cases (86%) are related to contact lens use (inadequate disinfection of the lens or insufficient efficacy of the solution), while in developing countries, trauma remains the main risk factor (27%). The current AK treatment protocol consists of topical agents (chlorhexidine, polyhexamethylene, and antifungal drugs); however, due to the ability to form cysts, the biological stage of the pathogen is particularly difficult to treat due to unfavourable environmental conditions and the emergence of drug-resistant parasites; alternative approaches with tolerable side effects are urgently needed. Despite the significant progress that has been made in identifying new therapeutic agents that are active against *Acanthamoeba* infections [16], to date, there are no highly effective and low-toxicity anti-amoebic drugs available. Recently, in fields including antiparasitics, the interest of researchers has focused on alternative therapeutics from herbal sources. Many natural compounds are widely studied for numerous biological activities, including the ability to antagonize pathogens of public health concern, including protozoan disease agents such as amoebae [17,18]. Of particular interest are various plant extracts, including essential oils (EOs) and mixtures of volatile organic compounds, which have attracted increasing interest over time due to their biological properties [19–23]. Notably, *Melaleuca alternifolia* and *Eucalyptus globulus* EOs are increasingly used in traditional medicine due to various medical implications such as antibacterial, anti-inflammatory, and antifungal effects. In our previous study, *M. alternifolia* Cheel (tea tree) (tea tree oil—TTO) and *E. globulus* Labill. (*Eucalyptus* essential oil—EEO) essential oils showed the ability to combat antibiotic-resistant bacteria even when organized in biofilms, which is a promising result for these two important public health problems [24].

To broaden the knowledge on the biological activity of these two essential oils, the aim of the present study was to evaluate "in vitro" the anti-acanthamoebic potential of natural extracts obtained from these two plants. In particular, the decrease in viable cells of an *Acanthamoeba* strain isolated from a person affected by AK was evaluated over time and with different concentrations of EOs used alone or in association.

2. Materials and Methods

2.1. Amoeba Strain

The amoeba strain used in this study was isolated in a clinical setting by corneal scrapes from a patient with keratitis. The strain was grown under axenic conditions as monolayers in 25 cm^2 tissue culture flasks (Sarstedt, Nümbrecht, Germany) in Peptone Yeast Extract Glucose (PYG) medium containing 0.75% (w/v) proteose peptone, 0.75% (w/v)

yeast extract, and 1.5% (*w*/*v*) glucose (Oxoid, Milan, Italy) at 30 °C, and monitored by examination under an inverted microscope.

For all experiments, five-day-old PYG cultures of protozoa, grown to confluence, were suspended by tapping the flask, washed three times in sterile phosphate-buffered saline solution (PBS), centrifuged at 2000 rpm, and resuspended in the same media.

2.2. Genotypic Characterization of Amoeba Strain

For the extraction of nuclear DNA, trophozoites were harvested by centrifugation at 2500 rpm for 5 min. After centrifugation, the supernatant was aspirated, and the pellet was resuspended twice in 25 mL of phosphate-buffered saline. Subsequently, the pellet was resuspended in lysis buffer (10 mM Tris HCl, pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS) and incubated at 60 °C for 5 min. Then, 2.5 µL of proteinase K and 5 mL of RNASE (1 mg/mL) were added and incubated at 60 °C for 1 h. After incubation, 250 µL of NaCl (5 M) was added and incubated on ice for 5 min then centrifuged at 2500 rpm for 15 min. The supernatant was transferred into a new tube and an equivalent volume of isopropanol was added. Following incubation at −20 °C for 60 min, the DNA pellet was washed with 500 µL of ethanol (70%). Ethanol was carefully removed using a micropipette, and after the pellet was dried, 200 µL tris–EDTA (TE) buffer (pH 8.0) was added (all reagents were purchased from Sigma-Aldrich, Milan, Italy).

DNA was subjected to PCR aiming for the specific recognition of 18S rDNA from amoebae of the genus *Acanthamoeba* using the primers JDP1 (5′-GGC CCA GAT CGT TTA CCG TGA A-3′) and JDP2 (5′-TCT CAC AAG CTG CTA GGG GAG TCA-3′).

The following DNA thermal cycler was performed for 42 cycles: 95 °C for 7 min, then 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, followed by 40 cycles of 72 °C for 10 min, as described earlier [25].

The DNA extraction product was evaluated by electrophoresis on a 1% agarose gel, to determine the presence of DNA.

The QIAquick PCR Purification Kit (Qiagen, Milan, Italy) was employed to purify positive amplicons from PCR, to recover approximately 95% of clean DNA up to 10 kb (as per the manufacturer's instructions). After purification, the amplicons were sequenced by the ABI PRISM 3130XL Genetic Analyzer (Applied Biosystem, Milan, Italy) using amplification primers. The BLAST (version 1.30) program of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), available online, was used to perform sequence alignment and analysis.

A matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometer (TOF/MS) (bioMérieux, Craponne, France) was employed to obtain further confirmation of the strain's affiliation to the *Acanthamoeba* genus [26]. The MALDI-TOF MS compares spectra produced by intact cells against a database of unique, conserved peaks that are used to identify the species.

2.3. Essential Oils

M. alternifolia Cheel (tea tree) (TTO) and *E. globulus* Labill. (EEO) essential oils (EOs), isolated by hydro-distillation and characterized in a previous investigation, were chosen and employed in this study due to their strong biological activity, which is particularly evident when the two compounds are used in association [24].

2.4. EOs Minimum Inhibitory Concentration (MIC)

The MIC of each EO was determined by the broth microdilution method using 96-well microplates.

Each well was aliquoted with 95 µL of PYG and 5 µL of trophozoites up to a final inoculum concentration of 10^6 cells/mL. A volume of 100 µL of each EO serial dilution

was added to obtain concentrations ranging from 512 to 0.125 µg/mL. The plates were then incubated at 30 °C for 24 h. After incubation, cell viability was checked with a Bürker chamber under an inverted microscope using the trypan blue exclusion test.

The negative control wells consisted of amoeba cells in PYG without EOs [27].

2.5. Determination of the Fractional Inhibitory Concentration (FIC) Index

Using the Fractional Inhibitory Concentration (FIC) Index, the effect of EO/EO association on amoeba cells was tested. The potential synergistic amoebicidal activity of TTO and EEO was determined based on the FIC index method [28], and the results are expressed as follows: synergy (FIC $\leq$ 0.5), addition (0.5 $\leq$ FIC $\geq$ 1), indifference (1 $\leq$ FIC $\geq$ 4), and antagonism (FIC > 4).

2.6. Amoebicidal Activity of EOs

One hundred microliters of five-day-old PYG cultures of protozoa suspended in sterile PBS was seeded in each well of a microtiter plate (approximately 10^4 cells/well) [29] and incubated for 24 h at 30 °C. After incubation, the non-adherent cells were removed by washing the wells with 100 µL of Page's Amoeba Saline Solution (PAS) (Oxoid, Milan, Italy). TTO and EEO, used alone and in association, were added to the wells at the MIC and at the best synergistic concentration, as detected by the FIC Index assay. The amoeba cells were exposed to EOs and to EO/EO synergic association at 30 °C for 24 h. At predetermined times (1 h and 24 h), amoeba cells were counted using a Burker chamber, differentiating between dead and viable cells using trypan blue. At the end of each incubation period, an appropriate aliquot of trypan blue was added to each well to facilitate cell counting, resulting in a final concentration of 4%, and incubated for at least 10 min. Using an inverted microscope at 20X magnification, the cells were counted, distinguishing between dead cells, stained blue due to loss of membrane integrity and subsequent dye absorption, and viable cells, appearing white with intact membranes. Trophozoites not exposed to EO activity were used as a control.

2.7. Statistical Analysis

Statistical significance was determined by *t*-tests and ANOVA using the statistical program GraphPad Prism 9.2.0 (San Diego, CA, USA). Analysis was performed using Bonferroni's post hoc test. *p*-values were considered significant at $\leq$0.05. The experiment was performed in three replicates to check the reproducibility of the results.

3. Results

3.1. Genotypic Characterization of Amoeba Strain

By BLAST analysis of the amplified gene products, >98% identity was obtained with the reference sequences of the *Acanthamoeba*18S rRNA gene. BLAST and MALDI-TOF MS analysis confirmed that the amoeba strain used in this study, isolated in a clinical context, belongs to the genus *Acanthamoeba*, and it was identified as *A. polyphaga*, belonging to the T4 genotype.

3.2. Minimum Inhibitory Concentration (MIC) and Fractional Inhibitory Concentration (FIC) Index of TTO and EEO

The Minimum Inhibitory Concentration (MIC) of each EO and the Fractional Inhibitory Concentration (FIC) Index are shown in Table 1.

TTO displays the best activity, with an MIC value of 16 µg/mL, while for EEO the MIC value was 32 µg/mL. The associated EO/EO also showed anti-amoebal and synergistic activity (FIC = 0.5), with a reduction in the concentrations of both active compounds of 75%.

Table 1. Minimum Inhibitory Concentration (MIC) of *M. alternifolia* Cheel (tea tree) and *E. globulus* Labill. (μg/mL) against *A. polyphaga* and Fractional Inhibitory (FIC) Index value.

EO	MIC EOs (μg/mL)	MIC EO/EO (μg/mL)	FIC Index
TTO	16	4	0.5
EEO	32	8	

3.3. Amoebicidal Activity of M. alternifolia (Tea Tree) (TTO)

As reported in Figure 1a, a powerful amoebicidal effect from the first hour of exposure emerged for TTO at all concentrations tested ($p < 0.0001$), especially for the concentrations 1xMIC and 2xMIC.

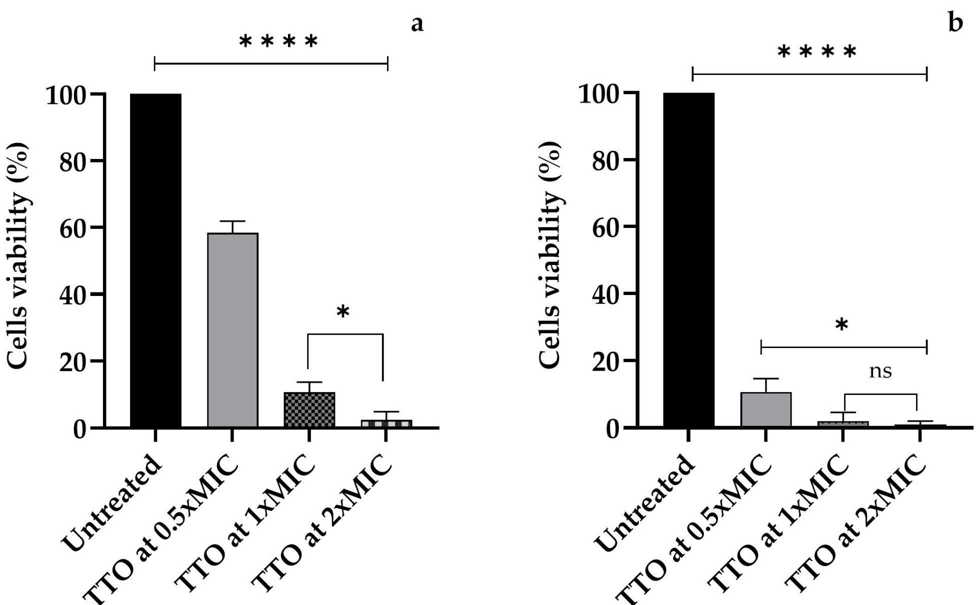


Figure 1. The cell viability of *A. polyphaga* trophozoites after (**a**) 1 h and (**b**) 24 h of exposure to *M. alternifolia* Cheel (tea tree) (TTO) essential oil at concentrations of 0.5xMIC, 1xMIC, and 2xMIC. Each bar represents the mean ± SD of the three determinations (error bar = S.D.; n = 3). $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) were considered significant during *t*-tests and ANOVA with the Bonferroni correction. ns stands for not statistically significant.

After 24 h of exposure, a very high amount of amoebicidal activity was observed, up to the disappearance of viable cells upon treatment with a TTO concentration of 2xMIC, with a significant difference compared to the 0.5xMIC TTO concentration ($p = 0.016$) (Figure 1b).

3.4. Amoebicidal Activity of E. globulus Labill. (EEO)

A gradual increase in amoebicidal activity from the first hour of exposure emerged for all the EEO concentrations tested ($p = 0.0007$ for 0.5xMIC, $p < 0.0001$ for 1xMIC and 2xMIC) compared to the untreated sample. Cells treated with an EEO concentration of 2xMIC showed a decrease of 57.33% (compared to untreated cells) and a significant difference compared to the EEO concentrations of 0.5xMIC ($p < 0.0001$) and 1xMIC ($p = 0.0002$) (Figure 2a).

At 24 h post-treatment we observed a decrease in *A. polyphaga* cells compared to the untreated control of 57.3% for 0.5xMIC ($p = 0.0008$), 1xMIC ($p = 0.0003$), and 1xMIC ($p = 0.0001$) (Figure 2b).

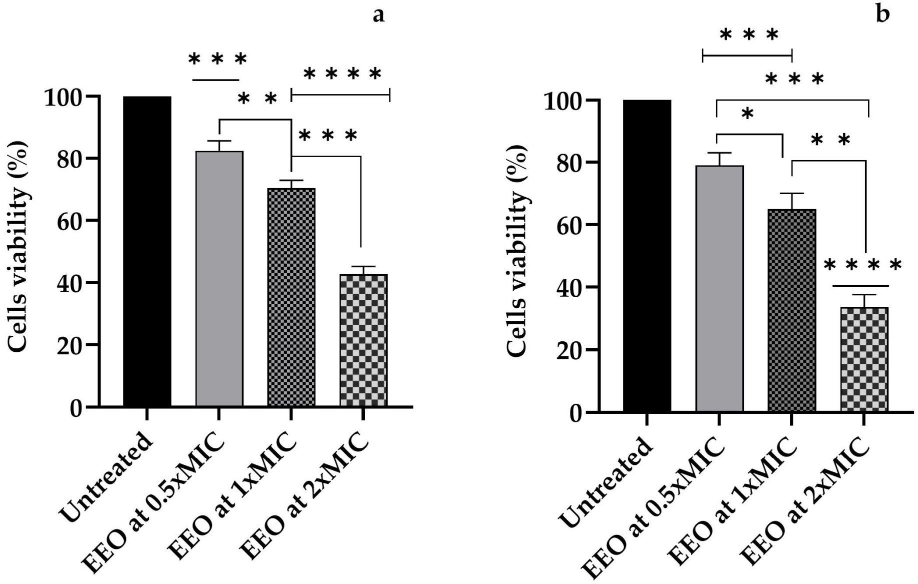


Figure 2. The cell viability of *A. polyphaga* trophozoites after (**a**) 1 h and (**b**) 24 h of exposure to *E. globulus* Labill. (EEO) essential oil at concentrations of 0.5xMIC, 1xMIC, and 2xMIC. Each bar represents the mean ± SD of the three determinations (error bar = S.D.; n = 3). $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) were considered significant during *t*-tests and ANOVA with the Bonferroni correction. ns stands for not statistically significant.

3.5. Amoebicidal Activity of Associated TTO/EEO

After 1 h of experimentation, the TTO/EEO association sample displayed a decrease in the cell viability of *A. polyphaga* ($p < 0.0001$) compared to the untreated sample. A very high amount of amoebicidal activity was observed for 1xMIC and 2xMIC ($p = 0.0001$ and $p < 0.0001$ compared to 0.5xMIC, respectively); cells treated with the highest concentration of TTO/EEO were no longer viable (Figure 3a).

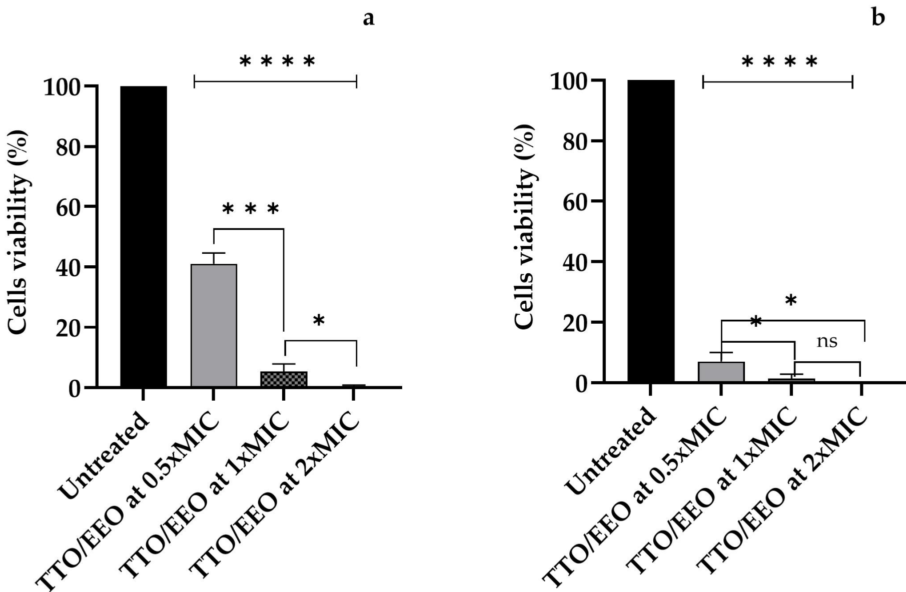
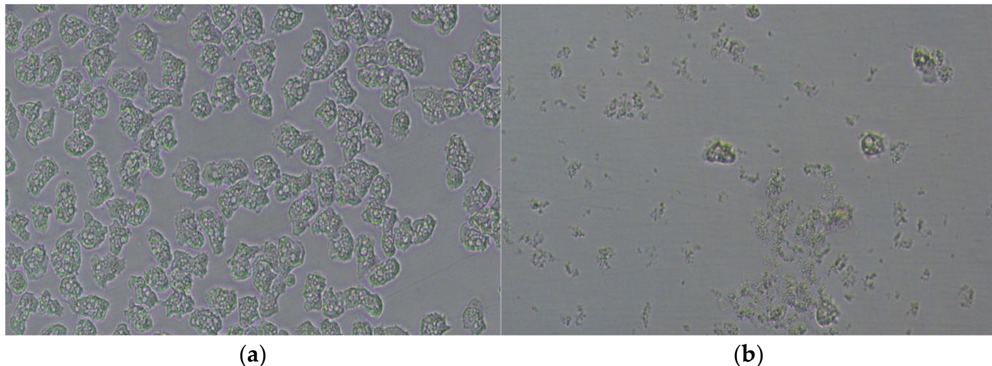


Figure 3. The cell viability of *A. polyphaga* trophozoites after (**a**) 1 h and (**b**) 24 h of exposure to the associated *M. alternifolia* Cheel (tea tree) (TTO)/*E. globulus* Labill. (EEO) essential oil at concentrations of 0.5xMIC, 1xMIC, and 2xMIC. Each bar represents the mean ± SD of the three determinations (error bar = S.D.; n = 3). $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) were considered significant during *t*-tests and ANOVA with the Bonferroni correction. ns stands for not statistically significant.

After 24 h of TTO/EEO exposure, the amount of viable cells of *A. polyphaga* in the sample treated with the 0.5xMIC concentration was only 7.0%, and the amount in the sample treated with the 1xMIC concentration was 1.3%. No viable cells were observed following treatment with the association at the 2xMIC concentration (Figure 3b).

Figure 4 shows an example of the ability of the TTO/EEO association to negatively affect the survival of amoebae, which leads to the rapid destruction of almost all *A. polyphaga* trophozoites and morphological changes in the residual cells.



(**a**) (**b**)

Figure 4. The rapid destruction of *A. polyphaga* trophozoites and the morphological changes in the residual cells observed by an inverted microscope (**a**) in the control and (**b**) after 1 h of contact with TTO/EEO at the 1xMIC concentration.

In the Supplementary Materials section, the morphological changes and reduction in viable count of *A. polyphaga* trophozoites at TTO/EEO concentrations of 0.5xMIC and 2xMIC are shown in Figures S1 and S2, respectively.

4. Discussion

Although AK is less common than other forms of infectious keratitis, its incidence has increased in recent decades, as reported in a study conducted between 2009 and 2015 on 224 patients diagnosed with *Acanthamoeba* keratitis in the Netherlands [30]. AK represents approximately 2% of all corneal infections worldwide [31], and contact lens use is one of the most common risk factors, particularly in developed countries, affecting 1–33 users per million each year [32]. The type of contact lens used also plays a role, with a more than three-fold increased risk for daily reusable lens users versus daily disposable lens users [33]. Daily reusable lens solutions can become contaminated at the point of use through contact with water or dirty hands [34], becoming a risk factor related to maintaining the sterility of the solution. Non-lens users can also develop this condition if exposed to environmental risks like contaminated tap water, swimming pools, hot tubs, and soil or dust [35,36].

As for the therapies currently used for the treatment of infections caused by *Acanthamoeba*, these still pose important challenges, such as toxicity to human cells and resistance to the drugs used. *A. polyphaga* is an opportunistic protozoan pathogen that is very difficult to eradicate. Therapy must be started as quickly as possible because if the parasite reaches the corneal stroma the therapeutic approach becomes more difficult, partly due to the ability of the protozoan to encyst. Cysts are in fact much more resistant to pharmacological treatment than trophozoites and require longer therapy cycles with sometimes uncertain outcomes. Therapy can, therefore, become long and demanding, and its management requires great experience because it is not always easy to evaluate the response to treatment, and complications can be very serious and difficult to manage. Resistance to therapy can develop during treatment, as well as drug-induced toxicity, with the latter leading to an initial worsening of both the inflammatory response and symptoms. New generations of drugs must be developed to help treat acanthamoebiasis, in order to

reduce both the recurrence of infection and the adverse reactions caused by the current therapies. Natural compounds like plant extracts and bacterial metabolites are very interesting and promising sources of future drugs [37–40]. Hadas et al. [41] showed that extracts from *Passiflora* spp. have amoebostatic and amoebicidal properties in concentrations from 4 to 12 mg/mL. A preliminary investigation into *Eryngium alpinum* L. extracts revealed remarkable amoebicidal action against trophozoites, which reached the highest antiamoebicidal effect after two days of treatment at the concentrations of 5 mg/mL, 2.5 mg/mL, and 0.5 mg/mL [42]. In recent years, various researchers have investigated the effectiveness of nanoparticle-conjugated drugs and/or naturally occurring plant compounds against *Acanthamoeba*. In addition to significant growth inhibition, these natural compounds and nanoconjugates do not exhibit in vitro cytotoxic effects against human cells [43]. The combined use of several natural compounds allows the reduction of their concentrations and, consequently, the decrease of toxic effects during therapy [44,45].

Other applied nanotechnologies have been shown to enhance the anti-*Acanthamoeba* activity in the encapsulated nanoparticles, opening the way for new therapeutic options [46,47].

Amoebicidal activity has been confirmed for the natural compounds used in the present investigation, which show remarkable effectiveness when combined. The TTO/EEO association clearly indicated a synergistic effect in all tests, and at 24 h post-treatment, no viable *A. polyphaga* cells were observed for the 2xMIC concentration. To further expand the knowledge about the well-known antagonistic potential of EOs [19], TTO and EEO were chosen for this preliminary study. In our previous investigation, *M. alternifolia* Cheel (tea tree) and *E. globulus* Labill. essential oils showed the ability to combat antibiotic-resistant strains even when organized in biofilms, with the results showing promise regarding these two important public health problems [24]. Lastly, the role of "trojan horse" played by this protozoan is well known. Water (*Legionella*, *Aeromonas hydrophila*) and food-borne (*Listeria monocytogenes*, *Salmonella enterica* serovar Enteritidis, *Yersinia enterocolitica*) pathogens are examples of this endosymbiotic relationship, which is capable of implementing environmental spread and resistance [48–50].

This endosymbiotic relationship is beneficial, allowing both partners to survive and take advantage of the utilization of nutrients (excreted catabolites, remains of dead bacteria, etc.), the development of new characteristics, and adaptation to new environments [51]. These host/parasite interactions indeed seem to be of considerable importance, increasing the parasites' potential virulence and their resistance to biocides and antibiotics [52–57].

Therefore, based on all these concerns and given the challenges associated with the current AK treatment protocols, new and alternative biocides must be investigated for future clinical and environmental purposes.

The data emerging from the present study suggest that both of the studied EOs are interesting natural compounds endowed with amoebicidal activity. TTO proved to be the most effective compound, with significant activity after only 1 h of exposure, especially for the concentrations 1xMIC and 2xMIC. EEO was less effective, and a reduction in viable amoeba cells of 62% was observed after 24 h for the highest concentration used (2xMIC). The synergistic interaction of TTO and EEO employed in the present investigation once again highlights the fact that the adequate association of these two EOs can improve the biological activity of the single compounds. This increased amoebicidal activity confirms that the biological activity of these EOs is not given exclusively by the action of their main compound, but is also due to the synergism established between all their different phytochemical active components. This is consistent with other studies confirming the activity of EOs against *A. castellani* [27,58] and *A. polyphaga* [59]. Other studies also agree that among the EOs with amoebicidal activity, tea tree EO is the most effective against

different strains of *Acanthamoeba*, both individually [60] and in synergistic combination with compounds like dimethyl sulfoxide, which is used as a disinfectant [61], and EO blends [44], similarly to what emerged in the present investigation.

5. Conclusions

All research data on the biological characteristics of natural substances encourage the scientific community to discover new therapeutics for possible pharmacological applications. The development of alternative therapies extracted from natural products represents an important step in trying to solve global health challenges, as stated by the results of this study. Therefore, the use of EOs, alone or in association with therapeutics, as alternative biocides in contact lens solution preservatives or for other potential applications in the prevention of AK infections may represent a potential future strategy. Although EOs have demonstrated amoebicidal activity, further studies are needed to evaluate their other properties of clinical interest, as seen in other studies. Their cytotoxicity, especially against human epithelial cells, will be explored, along with their other pharmacological properties, to better define the future impact of essential oils on human public health.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/10.3390/app15084198/s1, Figure S1. Rapid destruction of *A. polyphaga* trophozoites and morphological changes in residual cells observed by inverted microscope, after 1 h of contact with TTO/EEO at 0.5xMIC concentration (b) with respect to control (a). Scale bar is same for all images. Figure S2. Rapid destruction of *A. polyphaga* trophozoites and morphological changes in residual cells observed by inverted microscope, after 1 h of contact with TTO/EEO at 2xMIC concentration (b) with respect to control (a). Scale bar is same for all images.

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Conflicts of Interest: The authors declare no conflicts of interest.

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