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In vitro evaluation of the amoebicidal activity of rosemary (*Rosmarinus officinalis* L.) and cloves (*Syzygium aromaticum* L. Merr. & Perry) essential oils against *Acanthamoeba polyphaga* trophozoites

Running heads:

Natural Product Research

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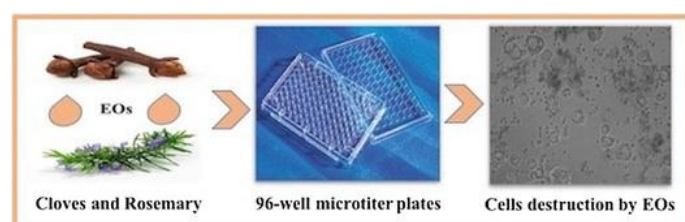
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Abstract

Several species of the genus *Acanthamoeba* cause human diseases. Treatment of infections involves various problems, emphasising the need to develop alternative antiprotozoal agents. We studied the anti-amoebic activity of Essential Oils (EOs), derived from rosemary (*Rosmarinus officinalis* L.) and cloves (*Syzygium aromaticum* L. Merr. & Perry), against *Acanthamoeba polyphaga* strain. The amoebicidal activity of cloves and rosemary EOs was preliminary demonstrated by the morphology change (modifications in the cell shape, the presence of precipitates in the cytoplasm, autophagic vesicles, membrane blends) of the treated trophozoites. The cell-counts, carried out after staining trophozoites with a Trypan blue solution, revealed that both EOs were active in a dose-dependent manner and in relation to the exposure time. This activity was evident after few hours, with encouraging results obtained in particular with cloves EO, able to act at the lower concentrations and after 1 h, probably for its high eugenol content (65.30%).



Keywords

Acanthamoeba polyphaga

essential oils

cloves

rosemary

1. Introduction

Acanthamoeba is a ubiquitous free-living protozoan that can exist as motile trophozoites and form cysts in response to adverse environmental conditions. Several species of *Acanthamoeba* are known to cause human disease, like keratitis that affects mainly the wearers of contact lenses, skin infections and other most insidious chronic diseases like systemic diseases or granulomatous amoebic encephalitis (GAE), especially in immunocompromised patients. *Acanthamoeba* can also serve as hosts for endosymbionts, representing a significant reservoir for environmental pathogen/opportunistic

serve as hosts for endosymbionts, representing a significant reservoir for environmental, pathogen/opportunistic microorganisms (*Legionella pneumophila*, *Aeromonas hydrophila*) and food-borne pathogens (*Listeria monocytogenes*, *Salmonella enterica serovar Enteritidis*, *Yersinia enterocolitica*), with important implications for human health (Snelling et al. 2006; Messi et al. 2011; Anacarso et al. 2012). The antiprotozoarian agents, used for the treatment of *Acanthamoeba* are often endowed with unpleasant side effects, irritating and toxic for the host, and chemical disinfectants used to prevent microbial growth in man-made aquatic systems are effective only in high concentrations, representing a health risk for human exposure to potentially hazardous by-products. Therefore, it is essential to develop alternative antiprotozoal agents with high activity, low toxicity and high efficacy. For these reasons, there is a growing interest in using natural antimicrobial compounds and, among these, Essential Oils (EOs), generally recognised as safe (GRAS) have already shown antimicrobial activity against bacteria of clinical origin, fungi, viruses (Cannas et al. 2016; Bouyahya et al. 2017) and, recently, special attention is also paid to their amoebicidal activity (Perez et al. 2012). There are several evidences of amoebicidal activity of essential oils obtained from *Euphorbiaceae*, *Asteraceae*, *Verbenaceae*, *Piperaceae* plants, *Peucedanum species*, *Allium sativum* extract (Polat et al. 2008; Rodio et al. 2008; Sauter et al. 2011, 2012; Malatyali et al. 2012; Vunda et al. 2012; Santos et al. 2016; Panatieri et al. 2017). The aim of the present investigation was to evaluate *in vitro* the amoebicidal effect against *Acanthamoeba polyphaga* strain of essential oils derived from two widely popular spices, rosemary (*Rosmarinus officinalis* L.) and cloves (*Syzygium aromaticum* L. Merr. & Perry).

2. Results and discussion

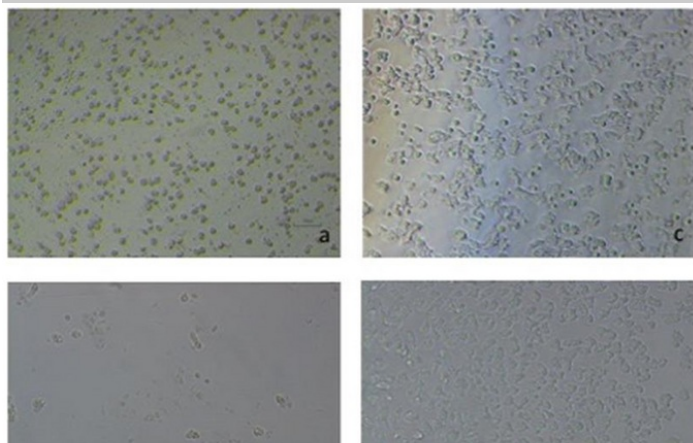
2.1. Distillation and qualitative-quantitative analysis of essential oils

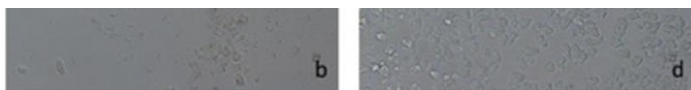
For each spice, the following total extract concentrations were obtained: 1,1 g for rosemary and 3,6 g for cloves. The yield of the distillation process was calculated by the formula: [(g final extracted compound/g initial compound)/100]. Yields of 1,8% and 12% were obtained for rosemary and cloves, respectively. With regard to the chemical composition, the main cloves oil constituents were eugenol (65.30%), β -caryophyllene (15.00%), 2-methoxy-4-[2-propenyl] phenol acetate (9.75%) and α -caryophyllene (2.85%). In rosemary EO, the main compositions were 1,8-cineole (25.23%), α -pinene (18.81%), camphor (15.26%), camphene (10.82%), borneol (2.87%), β -caryophyllene (1.91%) and bornyl acetate (1.51%).

2.2. Amoebicidal activity of essential oils

The amoebicidal activity against *A. polyphaga* ApUP of cloves and rosemary EOs, studied by microtitre plates method, is preliminary demonstrated by the morphology change of the treated trophozoites, compared to the control. Figure 1 (a, b, c, d) shows the most indicative changes in trophozoites morphology, after the last time of contact (144 h). The main morphological alterations were the modifications in the cell shape, the presence of precipitates in the cytoplasm, autophagic vesicles, membrane blends, observable at the highest and lowest concentration of 4 mg/mL and 0,1 mg/mL for rosemary EO and cloves EO, respectively. The cell count, carried out after staining trophozoites with a Trypan blue solution, revealed that both EOs were active in a dose-dependent manner and in relation to the exposure time. In particular, the rosemary EO (Figure 2) showed a good amoebicidal activity, but only at the higher concentrations (4 and 2 mg/mL); in both cases the total killing of cells was obtained at the end of the experiment (144 h), with a percentage reduction of 100%. At the concentrations of 1 and 0.1 mg/mL, the EO displayed a lower amoebicidal capacity, and gradual over time: after the first hour the adding of 1 mg/mL solution determined a 27% reduction, whereas a good killing percentage (86%) was observed after 144 h. At 0.1 mg/mL, a decrease in cell count of *Acanthamoeba* of about 50% was obtained, but only at the end of the experiment. The cloves EO showed a better amoebicidal activity than the rosemary EO (Figure 3). A total killing of *A. polyphaga* was obtained after 1 h using 1, 2, 4 mg/mL concentrations. With the concentration of 0.1 mg/mL a 43.37% reduction of amoeba was only observed but, even in this case, the total elimination of vital cells was obtained after 24 hours' incubation.

Figure 1. Effects of EOs of rosemary (a) and cloves (c) on *A. polyphaga* trophozoites vs. control (b and d, respectively). Trophozoites after exposure to the EOs appears as spheric and damaged forms or as few disaggregated forms and amorphous mass for rosemary and cloves, respectively.





Note: Inverted microscope (200× magnification).

Figure 2. Amoebicidal activity of rosemary EO at different concentrations (0.1, 1, 2, 4 mg/mL) presented as percentage of reduction of *A. polyphaga* trophozoites.

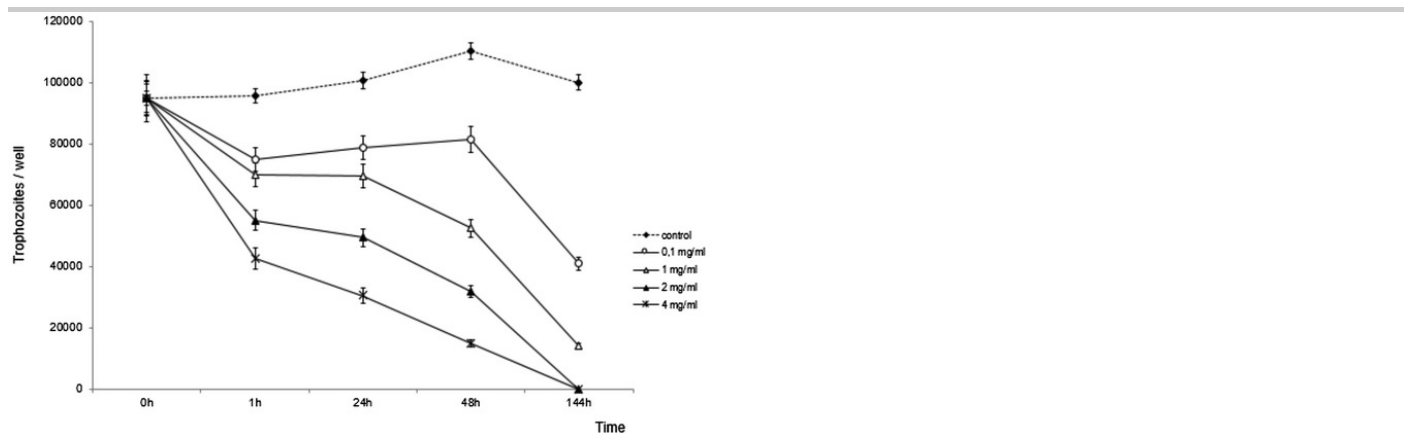
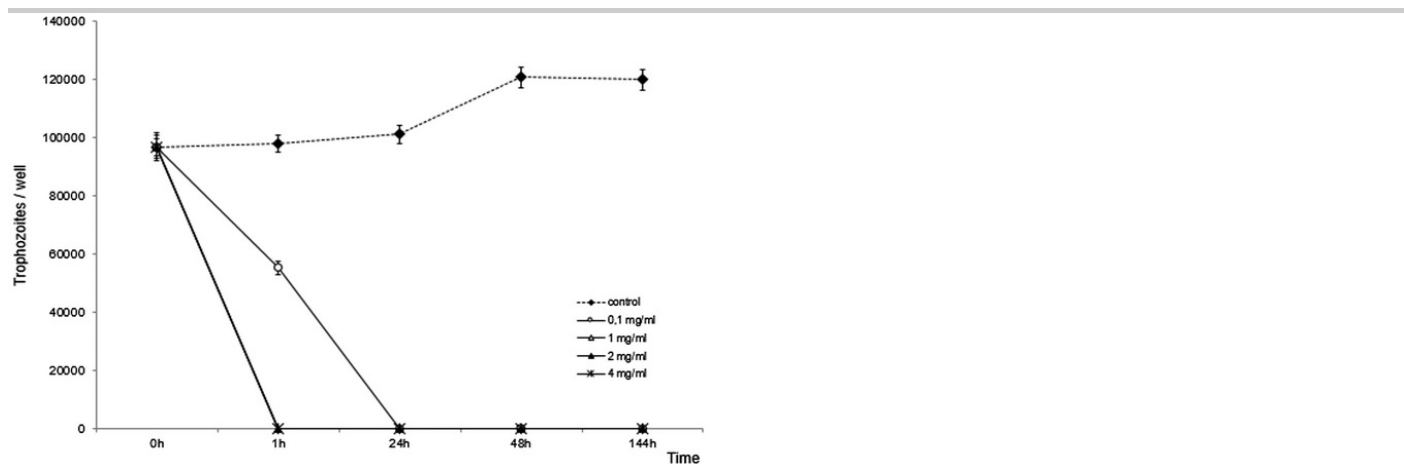


Figure 3. Amoebicidal activity of cloves EO at different concentrations (0.1, 1, 2, 4 mg/mL) presented as percentage of reduction of *A. polyphaga* trophozoites.



3. Conclusions

Recently, the antiprotozoal activity of many essential oils has been reported. Laboratory tests have been carried out to determine the effect of various essential oils against *A. polyphaga*. In the present investigation, we report the amoebicidal activity of two widely used natural substances after few hours of contact, with encouraging results obtained in particular for the cloves EO. In fact, a total killing of *A. polyphaga* trophozoites was obtained after 1 h using concentrations of 1, 2, 4 mg/mL. Both EOs were also able to prevent cysts formation. This capability is very important because trophozoites can turn into cyst forms during the therapy, causing difficulties and treatment failures. Other factors, as the emerging resistance to pharmacotherapy (Turner et al. 2000 ; Johnston et al. 2009) can also lead to delayed suitable therapy. Even in this case, as already emerged for the problem of antibiotic resistance in pathogenic bacteria (Yap et al. 2014), the combination of essential oils with antiprotozoal agents could be also useful in the fight against drug-resistant *Acanthamoeba*. Actually, in many fields there is a «return to the natural», and the results here reported fall in this philosophy. The essential oils from rosemary (*R. officinalis* L.) and clove (*Syzygium aromaticum* L. Merr. & Perry), in particular, are obtained from natural popular spices that could represent a promising alternative to treat infectious diseases or to employ as food preservative (Budri et al. 2015 ; Vital et al. 2016 ; Jardak et al. 2017 ; Ugalde et al. 2017). Further studies will be necessary to determine the toxicity, the molecular mechanisms involved in the amoebicidal activity, the pharmacokinetic and pharmacodynamics parameters and the molecular targets of these two spices.

Supplemental data

Supplemental data for this article can be accessed at <https://doi.org/10.1080/14786419.2017.1399390>.

Disclosure statement

AQ2 No potential conflict of interest was reported by the authors.

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