Positive Interaction of Thyme (Red) Essential Oil with Human Polymorphonuclear Granulocytes in Eradicating Intracellular *Candida albicans*

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Abstract

The essential oils have started to be recognized for their potential antimicrobial role only in recent years. Clinical experience showed that the efficacy of antimicrobial agents depends not only on their direct effect on a given microorganism but also on the functional activity of the host immune system. Since data on the effects of essential oils on the innate immune system are scanty and fragmentary, the aim of this study was to evaluate the influence of thyme (red) essential oil (EO), at subinhibitory/inhibitory concentrations, on intracellular killing activity by human polymorphonuclear granulocytes (PMNs) against *Candida albicans*. In order to provide a frame of reference for the activity of this EO, its *in vitro* killing activity in the absence of PMNs was also evaluated.

Results showed that EO at subminimal inhibitory (subMIC)/minimal inhibitory (MIC) concentrations significantly enhanced intracellular killing of *C. albicans* in comparison with EO-free controls and was comparable to the positive control (fluconazole). In *in vitro* killing assays without PMNs, we observed progressive growth of the yeast cells in the presence of EO subMIC/MIC concentrations. A positive antifungal interaction with phagocytes could explain why this EO, which appeared to be only fungistatic in time-kill assays, had efficacy in killing yeast cells once incubated with PMNs.

Key words

thyme (red) essential oil · *Candida albicans* · PMNs · intracellular killing · *in vitro* killing

The increasing recognition and importance of fungal infections, the difficulties encountered in their treatment, and the increase in resistance to antifungal agents have stimulated the search for new therapeutic alternatives [1]. The essential oils and products of plant secondary metabolism had a wide application in folk medicine, fragrance industries, as well as food flavoring and preservation, but only in recent years they have started to be recognized for their potential antimicrobial role [2–4]. The literature reports evidence suggesting that a larger number of plants and their constituents could show beneficial therapeutic effects, including antioxidant, anti-inflammatory, and immunomodulatory activity, which still need to be further investigated [5–9]. In particular, data on the effects of essential oils on the innate immune system are scanty and fragmentary. As PMNs play a pivotal role against invading microbial pathogens, enhanced PMN activity under the essential oils influence may contribute to their anti-infective properties [10,11].

The essential oil from *Thymus vulgaris* L. is widely used in folk medicine for the treatment of a variety of diseases since it possesses numerous biological properties including antibacterial, antifungal, and antioxidant activity [1,3,12–17].

In this paper, we report the interaction of thyme (red) EO with human PMNs, focusing on intracellular killing of *Candida albicans*. As a positive control, we used fluconazole, one of the most common antifungal drugs in candidiasis management, known to enhance the fungicidal activity of PMNs [10]. Moreover, in order to provide a frame of reference for the activity of this EO, its *in vitro* killing in the absence of PMNs was also evaluated.

EO MICs for *C. albicans* were 0.03 and 0.5% v/v, while fluconazole MICs were 0.5 and 8 µg/mL with inocula of 10^3 and 10^6 ufc/mL, respectively. In the absence of PMNs, the EO activity was only fungistatic at all concentrations tested, causing slight reductions (i.e., $\leq 3 \log_{10}$) in the starting inoculum, as shown in **Fig. 1**. At subMIC/MIC concentrations, killing activity was not sustained over time because yeast cell growth was seen at 24 h.

Clinical experience showed that the efficacy of antimicrobial agents depends not only on their direct effect on a given microorganism but also on the functional activity of the immune system [10,11,18]. Results on effects upon the PMNs intracellular killing showed that EO and fluconazole had similar candidacidal activity (**Table 1**). EO at 1 × MIC significantly increased the intracellular killing by phagocytes, with percentages that ranged from 50 to 73%, in comparison with controls (EO-free), ranging from 33 to 50% (**Table 1**; $p < 0.01$). In the presence of 1 × MIC fluconazole, intracellular yeasts were killed at 51–69–75% ($p < 0.01$).

A similar picture was detected even at lower levels of EO (1/2 × MIC), where killing values (44–57–69%) were significantly higher than those of control systems (33–47–50%; $p < 0.05$) and overlapped with those observed in presence of 1/2 × MIC fluconazole (42–58–63%).

The mechanism of such enhancement is still unknown; despite the fact that this EO displayed only a fungistatic action in the absence of PMNs, it showed efficacy in killing yeasts once simultaneously incubated with PMNs, suggesting a positive antifungal interaction with phagocytes, as previously observed with other antifungal agents [10,19].

EO direct damage to the yeast cell may be, at least in part, responsible for changes that make the yeasts more susceptible to PMN lytic mechanisms. The EO used in this study is mainly composed of thymol, ρ-cymene, limonene, α-pinene, carvacrol, and γ-terpinene, at different percentages [3], but it is not clear which of the active ingredients accounted for the observed effect on yeast killing.

Recent literature data reported that thymol and carvacrol exhibit fungicidal activity in a dose-dependent fashion against yeasts, resulting from direct damage to cell membranes [20]. Since essential oils are phytocomplexes containing numerous molecules, their bioactivity could be the result of a synergism of all major and minor components [21,22]. In fact, antifungal susceptibility testing on thymol and carvacrol showed that these components exhibited MIC values higher (0.06% v/v) than those obtained with whole EO (0.03% v/v) against *C. albicans* (data not shown). Further investigations are needed to confirm these findings.

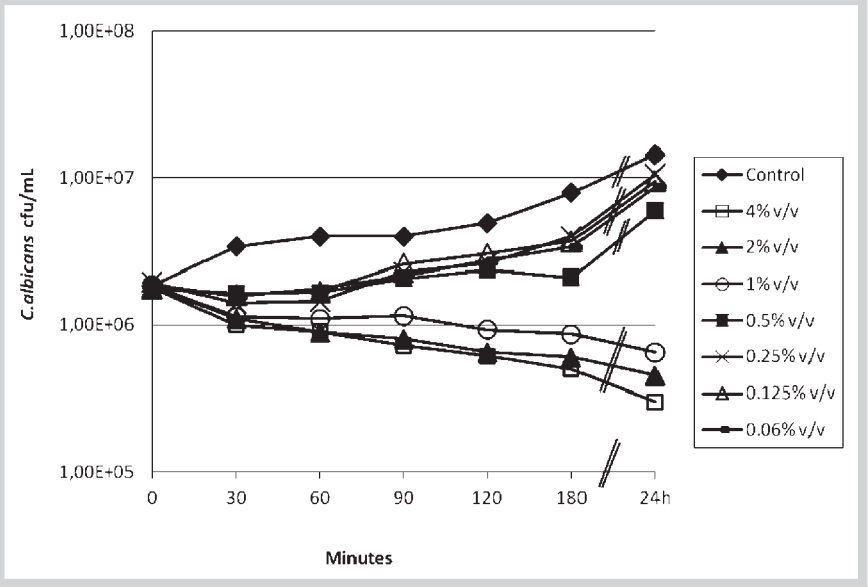

Fig. 1 Effect of thyme (red) essential oil on the *in vitro* killing of *Candida albicans*. *C. albicans* (10^6 cfu/mL) was incubated with the EO at 4% v/v (8 × MIC), 2% v/v (4 × MIC), 1% v/v (2 × MIC), 0.5% v/v (1 × MIC), 0.25% v/v (1/2 × MIC), 0.125% v/v (1/4 × MIC), and 0.06% v/v (1/8 × MIC).

Table 1 Effect of thyme (red) essential oil and fluconazole at 1/2 × MIC/1 × MIC on intracellular killing of *C. albicans* by human PMNs.

	Mean SI ± SEM (% of initial fungal population killed by PMNs in absence or presence of fluconazole or EO)				
Time (min)	Controls	1/2 × MIC fluconazole (4 µg/mL)	1 × MIC fluconazole (8 µg/mL)	1/2 × MIC thyme (red) EO (0.25% v/v)	1 × MIC thyme (red) EO (0.5% v/v)
30	1.67 ± 0.03 (33%)	1.58[b] ± 0.08 (42%)	1.49[a] ± 0.07 (51%)	1.56[b] ± 0.07 (44%)	1.50[a] ± 0.01 (50%)
60	1.53 ± 0.02 (47%)	1.42[b] ± 0.08 (58%)	1.31[a] ± 0.06 (69%)	1.43[b] ± 0.11 (57%)	1.36[a] ± 0.04 (64%)
90	1.50 ± 0.03 (50%)	1.37[b] ± 0.17 (63%)	1.25[a] ± 0.06 (75%)	1.31[b] ± 0.07 (69%)	1.27[a] ± 0.02 (73%)

[a] Significantly different from the controls ($p < 0.01$); [b] significantly different from the controls ($p < 0.05$)

Materials and Methods

A clinical *C. albicans* strain was isolated from blood, identified by conventional methods and subcultured on Sabouraud dextrose agar (SAB). Yeasts cultures consisted entirely of blastoconidia and had a slight tendency to differentiate into pseudohyphae during the course of the experiments [10].

The thyme (red) EO commercially obtained from Azienda Agricola Aboca was the same batch used and characterized by GC-FID analyses in a previous study [3]. Its major constituents were thymol (26.5%), *ρ*-cymene (16.2%), limonene (13.2%), α-pinene (13.2%), carvacrol (7.8%), and γ-terpinene (4%).

Antifungal susceptibility testing was based on the CLSI M27-A3 [23] method, with some modifications; the final EO concentrations ranging from 1 to 0.0019% (v/v). EO and fluconazole (Sigma-Aldrich; purity ≥ 98% by HPLC) MIC values for *C. albicans* were determined with an inoculum of 10^3 cfu/mL and an inoculum of 10^6 cfu/mL to perform tests with and without phagocytes. *In vitro* killing was performed by using a 10^6 cfu/mL starting yeast inoculum and EO at 4% v/v (8 × MIC), 2% v/v (4 × MIC), 1% v/v (2 × MIC), 0.5% v/v (1 × MIC), 0.25% v/v (1/2 × MIC), 0.125% v/v (1/4 × MIC), and 0.06% v/v (1/8 × MIC). EO-free controls were included. 500 µL aliquots were removed at 0, 30, 60, 90, 120, 180 min, and 24 h, serially tenfold diluted and plated onto SAB agar. After 24–48 h at 37 °C, results were reported as log cfu/mL. Fungicidal activity was defined as a 99.9% (≥ 3 $\log_{10}$) reduction in viable cell counts as compared with the starting inoculum [11].

Human PMNs were separated from lithium heparinized venous blood using Ficoll–Paque (Pharmacia S. p. A.) and adjusted to 10^6 cells/mL in RPMI1640 [11]. Viability, determined by trypan blue exclusion, was greater than 95%.

The EO effect on the intracellular killing of *C. albicans* by PMNs was investigated by incubating blastoconidia (10^6 cfu/mL) and PMNs (10^6 cells/mL) for 30 min to allow phagocytosis to proceed. The PMN-yeast cell mixtures were centrifuged at 200 *g* for 5 min to remove extracellular blastoconidia. An aliquot of PMNs was lysed by adding sterile water, and intracellular viable yeast counting was performed (time zero). PMNs were incubated further with 0.25% v/v and/or 0.5% v/v (1/2 MIC and 1 × MIC, respectively) of EO and at time *X* (30, 60, 90 min), the viable counts were measured in the same way. EO-free controls were included. As positive controls, 1/2 × MIC (4 µg/mL) and 1 × MIC (8 µg/mL) fluconazole were included. Killing values were expressed as a survival index (SI), which was calculated by adding the number of surviving blastoconidia at time zero to the number of survivors at time *X* and dividing by the number of survivors at time zero. According to this formula, if fungal killing was 100% effective, the SI would be 1 [10, 11].

Results are expressed as the mean ± standard error of the mean (SEM) of 10 separate experiments, each performed in quadruplicate. Statistical evaluation of the differences between test and control results was performed by Tukey's test. *In vitro* killing was compared using Student's unpaired t-test.

Conflict of Interest

The authors report no conflicts of interest.

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