Full Length Research Paper

In vitro effect of *Melaleuca alternifolia* and *Eucalyptus globulus* essential oils on mycelia formation by oral *Candida albicans* strains

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Several mechanisms can inhibit mycelium formation which is an essential step for *Candida albicans* virulence. In this study, two essential oils of *Melaleuca alternifolia* and *Eucalyptus globulus* were tested for their ability to inhibit the mycelium formed on Lee medium by oral *C. albicans*. Lee medium was added with different concentrations of *M. alternifolia* essential oil ranging from 0.156 to 10 mg/ml and from 0.039 to 10 mg/ml for *E. globulus*. The results obtained showed that 5/17 of *C. albicans* strains were strongly mycelium producers, nine strains were moderately mycelium producers and three isolates were unable to form hyphae after 2 h of incubation in the same medium. *M. alternifolia* essential oil has an antimycelial activity against *C. albicans* isolates than *E. globulus* essential oil. In fact, only 1/2 minimum inhibitory concentration (MIC) (0.312 mg/ml) of *M. alternifolia* was able to inhibit total mycelium in *C. albicans* isolate while 2 MIC (0.312 mg/ml) of the second essential oil was necessary to inhibit germ tube formation in the same strain.

Key words: *Candida albicans*, *Melaleuca alternifolia*, *Eucalyptus globulus*, mycelium, lee medium.

INTRODUCTION

Oral candidiasis is an infection caused by abnormal growth of yeasts in the mucosa of the oral cavity. *Candida* species are opportunistic pathogens that occur as normal commensals in a large proportion of healthy individuals (Pei et al., 2007). *Candida albicans* is the most resistant strain to antifungal agents frequently isolated from oral candidiasis (Haberland-Carrodeguas et al., 2002). This species shows dimorphism, it reproduces itself by germination, creating budding cells called blastospores or blastoconidia, which are associated with normal colonization.

The conversion of yeasts to the filamentous stage (pathogenic) starts with production of germ tube that result in the formation of pseudohyphae or true hyphae (Chan et al., 1998; Villar et al., 2004). The formation of germ tubes and filaments by *C. albicans* has been observed in the tissues of mice after subcutaneous injection (Hill and Gebhardt, 1956). Their occurrence *in vitro* in the presence of various substances (Johnson, 1954) and serum (Reynolds and Braude, 1956) has been reported. This morphological transformation ability has been suggested to be an important factor for virulence (Hammer and Carson, 2000).

Plants are widely employed in folk medicine, mainly in communities with inadequate conditions of public health and sanitation. Several medicinal plants have been extensively studied in order to find more effective and less toxic compounds (Noumi et al., 2010a).

*Melaleuca alternifolia* (tea tree) and *Eucalyptus globulus* were usually used as a topical antifungal agent, with recent clinical data indicating efficacy in the treatment of oral candidiasis. The essential oil extracted from these plants has antimicrobial activity (Jandourek et al., 1998; D’Auria et al., 2001). The major component in tea tree essential oil is the erpinen-4-ol which is considered to be the most
active antimicrobial compound (Williams, 1998). In fact, tea tree oil shows some potentials of topical antifungal agent with recent clinical data indicating efficacy in the treatment of dandruff (Hammer 1998, 2003; Satchell et al., 2002) and oral candidiasis (Jandourek et al., 1998).

Thus, the objective of this study was to assess the possible in vitro antifungal activity of two plants essential oils (M. alternifolia and E. globulus) against C. albicans dimorphic commensal fungi, which can become a facultative pathogen under altered physiological situations for possible application as an antiseptic mouthwash.

MATERIALS AND METHODS

Clinical strains, media and growth conditions

A total of 17 C. albicans strains including 16 clinical isolates and C. albicans ATCC 90028 type strain were used in the present study. Samples were collected from the oral cavity, the gingival sulci and the pharyngeal portion, by using a swabbing method. A sterile cotton swab (Nippon Menbo, Tokyo, Japan) was immediately cultured on Sabouraud chloramphenicol agar (Bio-rad, France) for 48 h at 35°C. All oral isolates were identified by standard microbiological methods: macroscopic test of culture on Sabouraud chloramphenicol agar and assimilation tests using the ID 32 C system (bio-Mérieux, Marcy l’Étoile, France) according to the manufacturer’s specification and the results were read using an automated microbiological mini-Api (bio-Mérieux). All strains were confirmed at the species level by amplification of a CaYST1 gene intron fragment (Noumi et al., 2010b).

Essential oils

M. alternifolia (Tea tree) essential oil (leaves) was purchased from Arkomédika (Laboratoires pharmaceutiques, BP 28-06511 Carros, France). E. globulus commercialized essential oil was kindly provided by the Laboratory of pharmacognosy from the faculty of Pharmacy (Monastir, Tunisia).

Micro dilution method of determination of the minimal inhibition concentration (MIC) and minimal fungidal concentration (MFC)

The minimal inhibition concentration (MIC) was defined as the lowest concentration of the compounds to inhibit the growth of the micro organisms. The minimal fungidal concentration (MFC) values were interpreted as the highest dilution (lowest concentration) of the sample, which showed clear fluid with no development of turbidity and without visible growth.

The (MIC) and the (MFC) values were determined for all Candida strains. The inoculums of the yeast strains were prepared from 12 h Sabouraud dextrose broth cultures and suspensions were adjusted to an optical density of 0.5 at 540 nm. The 96-well plates were prepared by dispensing into each well, 100 µl of nutrient broth and 5 µl of the inoculum. A 100 µl aliquot from the stock solutions of each plants extract was added into the first wells. Then, 100 µl from the serial dilutions were transferred into eleven consecutive wells. The last well containing 195 µl of nutrient broth without essential oil and 5 µl of the inoculum on each strip was used as the negative control. The final volume in each well was 200 µl. The plates were incubated at 37°C for 24 h. The plants essential oils tested in this study were screened two times against each strain. All tests were performed in triplicate.

Mycelation in Lee medium

The test consists of comparing the ability to form mycelium of different strains of C. albicans. For this, cells were grown in a small volume of Lee medium (15 ml), until they reach the end of the exponential phase. The cells were recovered by centrifugation at 2000 g for 5 min and washed 2 times with sterile water in order to remove the entire medium.

The pellet of cells was then resuspended in sterile water in order to obtain an O.D. = 0.6 at 600 nm and incubated 2 h at 28°C in order to stop the cell cycle of all the yeast. After this step, the cells were kept in the cold room at 4°C for 48 – 72 h in order to induce a metabolic starvation. The cells were recovered by centrifugation at 2000 g for 5 min and resuspended in a volume of prewarmed Lee necessary to obtain an OD 600nm = 0.3. The cells were then incubated at 28°C and the cells morphology was followed every hour by phase contrast microscopy.

Effect of essential oils on myceliation in Lee medium

Seven concentrations including (1/4 and 1/2 MIC; MIC; 2, 4 and 8 MIC; MFC) of M. alternifolia and nine concentrations including (1/4 and 1/2 MIC; MIC; 2, 4, 8, 16 and 32 MIC; MFC) of E. globulus essential oils were tested for their inhibitory potency against mycelium formation by one C. albicans strain. This strain was able to form high mycelium on Lee medium. The filament elongation was microscopically monitored.

RESULTS AND DISCUSSION

The activity of essential oils against bacteria has been studied for many years. As a result, several European, Chinese, African and Asian plant extracts have been evaluated for their antimicrobial and antifungal activities (Carson et al., 2002, 2006; Duarte et al., 2005). In fact, the current necessity of discovering new antifungal compounds that can inhibit the transition from non pathogenic form (yeast) to pathogenic form (filamentous) of C. albicans strains has stimulated research regarding the antifungal properties of plant compounds (Tampieri et al., 2005). In this interest, M. alternifolia and E. globulus essential oils have been tested for the first time in order to investigate their anti-Candida filament formation potency on the basis that they are commonly used in the world as additive in teeth brush. The main components of M. alternifolia were: terpinen-4-ol (40.44%), gamma terpinene (19.54%), alpha terpinene (7.69%) and 1,8-cineole (5.2%), while E. globulus essential oil was rich in 1,8-cineole (95.61%) identified using GC-MS system (unpublished data).

In the present study, we investigated in a first step, the antifungal activity of M. alternifolia and E. globulus essential oils. Table 1 shows the MIC and MFC values of M. alternifolia and E. globulus essential oils on C. albicans strains determined by microdilution method. The two plant essential oils showed significant antifungal activity against the tested C. albicans strains. Overall, the
best antifungal activity was against *C. albicans* ATCC 90028 for *M. alternifolia* (MIC: 0.312 mg/ml; MFC: >10 mg/ml). For *E. globulus* essential oil, the MIC value (0.156 mg/ml) and MFC value (10 mg/ml) were the same against the two tested strains (*C. albicans* 15B and *C. albicans* ATCC 90028). The standard antifungal drug, amphotericin B, was more active against *C. albicans* ATCC 90028 reference strain (MIC: 0.012 mg/ml; MFC: 0.781 mg/mg) when comparing the two essential oils (Table 1).

This high anticandidal activity of *M. alternifolia* essential oil supports the results found by Bard et al. (1988). In fact, tea tree oil has an antifungal potency, with recent clinical data indicating efficacy in the treatment of dandruff (Satchell et al., 2002) and oral candidiasis (Jandourek et al., 1998). Also, tea tree oil and components appear to affect membrane properties and integrity in a manner consistent with other lipophilic, membrane-active agents such as the terpenes, thymol (Shapiro and Guggenheim 1995) and geraniol (Hisajima et al., 2008). The major component of tea tree essential oil is the terpinen-4-ol, which is considered to be the most active antimicrobial compound (Williams, 1998).

For the ability of myceliation on Lee medium, our results showed that 5/17 of *C. albicans* strains were strongly mycelium producers, nine strains were moderately mycelium producers and three isolates were unable to form hyphae after 2 h of incubation in the same medium (Table 2 and Figure 1).

It is well known that the filamentous form is responsible for the pathogenesis of the fungal infection (Villar et al., 2004).
filamentous form could penetrate to epithelial layer to cause infection, while the yeast form remained on the surface. Therefore, inhibition of the filamentous form may prevent *C. albicans* infection such as oral and vaginal candidiasis.

Figure 2 illustrates the inhibition of the filamentous form of *C. albicans* by *M. alternifolia* essential oil using a light microscope. The control (Figure 2) shows only the filamentous form of *C. albicans*. When used at 1/4 MIC (0.156 mg/ml), tea tree oil showed moderate inhibition, whereas only 1/2 MIC (0.312 mg/ml) of *M. alternifolia* essential oil inhibited totally germ tube formation by oral *C. albicans* strain (strain 15a) showing the yeast form (Figure 2). When the filamentous form was deceased by the action of the two essential oils, the yeast form was increased as seen in Figure 2.

*M. alternifolia* and *E. globulus* essential oils both inhibit germ tube formation by *C. albicans* (Figure 2). Only 1/2 MIC (0.312 mg/ml) of *M. alternifolia* was able to inhibit total mycelium in a strongly mycelium producer *C. albicans* isolate (strain 15a). The inhibitory concentration required to inhibit the production of germ tube in the same strain was 2 MIC (0.312 mg/ml) for *E. globulus* essential oil. Our results showed also that these two essential oils affected filament elongation. Maruyama et al. (2008) demonstrated that vaginal Candida cells of mouse could change from hyphal form to the yeast form by washing with essential oils. Conversion of the filamentous form into the yeast form appeared to be the characteristic of components of essential oils and hydrosols.

**Conclusion**

*M. alternifolia* and *E. globulus* essential oils showed an anti-candidal activity and inhibited filamentation of these fungi. Such results apply the possibility of using these oils in mouth brush application.

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