In vitro activity of Melaleuca alternifolia (Tea tree) and Eucalyptus globulus essential oils on oral Candida biofilm formation on polymethylmethacrylate

Noumi Emira1*, Snoussi Mejdi2 and Mahjoub Aouni1

1Laboratoire des Maladies Transmissibles et des Substances Biologiquement Actives, Faculté de Pharmacie, Université de Monastir, Tunisie.

2Laboratoire de Traitement des Eaux Usées, Centre de Recherches et des Technologies des Eaux (CERTE), Technopole de Borj-Cédria, BP 273- Soliman 8020, Tunisie.

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Melaleuca alternifolia and Eucalyptus globulus essential oils are known for their antifungal activities and efficacy in the treatment of oral candidiasis. Candida biofilm increased resistance to antifungal agents that have activity against their planktonic cells. The aim of this study was to evaluate the potential role of M. alternifolia and E. globulus essential oils in the inhibition of Candida biofilm formation on polymethylmethacrylate (PMMA). The antifungal activity of M. alternifolia and E. globulus essential oils and adhesion and biofilm on PMMA inhibition capacity were tested on two oral Candida isolates and two reference type strains. The biofilm formation by Candida strains was quantified by colorimetric method based on the reduction of the 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl amino) carbonyl]-2H-tetrazolium hydroxide (XTT). M. alternifolia and E. globulus essential oils were active against clinical and reference Candida albicans and Candida glabrata strains in their planktonic and adherent phases. In fact, both minimum inhibition concentration (MIC) and 1/2 MIC values of these two plants essential oils can inhibit adhesion and biofilm formation of clinical and reference strains of Candida on PMMA. Also, E. globulus essential oil was more active on Candida biofilm formation on PMMA. M. alternifolia and E. globulus essential oils can inhibit Candida biofilm formation on PMMA. This may contribute to the use of these plants as alternative products for oral Candida biofilm prevention, control and treatment.

Key words: Candida, biofilm, polymethylmethacrylate, Melaleuca alternifolia, Eucalyptus globulus.

INTRODUCTION

Medicinal plants have been used in developing countries as alternative treatments to health problems. Many plant extracts and essential oils isolated from plants have been shown to exert biological activity in vitro and in vivo, which justified research on traditional medicine focused on the characterization of antimicrobial activity of these plants (Snoussi et al., 2008; Noumi et al., 2010a; Hajlaoui et al., 2010).

The therapy of deep fungal infections, particularly those caused by opportunistic pathogens, such as Candida albicans, remains a difficult medical problem (Bennet, 1992).

The infection of C. albicans will lead to the forming within the biofilm. Besides this, the action of antifungal may be limited by their penetration and chemical reaction into biofilm matrix, the extracellular polymeric material

*Corresponding author. E-mail: emira_noumi@yahoo.fr. Tel: +216 73 466244. Fax: +216 73 461830.
In this context, new agents from natural resources that can inhibit the growth of biofilm-associated microorganisms are greatly needed and would enhance the number of effective therapeutic alternatives (Alviano et al., 2005). Biofilm-associated or sessile, *C. albicans* organisms demonstrate increased resistance to traditional antifungal agents that have activity against their planktonic counterparts (Hawser and Douglas, 1995).

Since adhesion is an essential prerequisite in colonisation and infection, its role in the pathogenesis of several diseases by fungus is widely acknowledged. Several specific interactions between the genus *Candida* and other organisms, medical devices and host tissues have been described (Hawser and Douglas, 1995; Jain et al., 2007).

The essential oil of *Melaleuca alternifolia* contains ~100 components, which are mostly monoterpenes, sesquiterpenes and related alcohols. Tea tree oil (TTO) has been used medicinally in Australia, with uses relating primarily to its antimicrobial (Carson and Riley, 1993; Carson et al., 2002; Mondello et al., 2003), anti-inflammatory and antifungal especially antiancandidal properties (Hammer et al., 1998; Hammer et al., 2000; Noumi et al., 2010b). TTO efficiency was confirmed in the treatment of dandruff (Satchell et al., 2002) and oral candidiasis (Jandourek et al., 1998; Hammer et al., 2000). In fact, oral candidiasis is the most common mouth fungus infection in humans, especially caused by *C. albicans* (Pizzo et al., 2001, Ellepola and Samarayake, 1998). This yeast proliferates in resistant biofilm (Jain et al., 2007). Also, *Eucalyptus globulus* essential oil shows promise 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).

Our previous study showed that the essential oils of *M. alternifolia* and *E. globulus* possessed a good *in vitro* antifungal activity against oral *Candida* isolates better than amphotericin B (Noumi et al., 2010b). Therefore, the aim of this study was to evaluate the potential role of *M. alternifolia* and *E. globulus* essential oils in the inhibition of *Candida* biofilm formation on polymethylmethacrylate (PMMA) in an attempt to contribute to the use of these plants as alternative products for oral *Candida* biofilm prevention, control and treatment.

**MATERIALS AND METHODS**

**Plant material and essential oil**

*M. alternifolia* (Tea tree) essential oil (leaves) was purchased from Arkomédica (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).

**Candida isolates**

Four *Candida* strains including two species (*Candida albicans* and *Candida glabrata*) were used in this study. Two clinical *Candida* strains: *C. albicans* (15a) and *C. glabrata* (15f) were isolated from the oral cavity of the same patient by using a swabbing method. All isolates were incubated at 30°C for 24 to 48 h on Sabouraud Chloramphenicol agar (Bio-rad, France) and yeast-like colonies were isolated and identified by the ID 32 C (bio-Mérieux, Marcy-l’Étoile, France) assimilation kit. The *C. albicans* ATCC 90028 and *C. glabrata* 90030 species were used as reference strains.

**Microdilution method of determination of the minimal inhibition concentration (MIC) and minimal fungicidal concentration (MFC)**

The MIC and the MFC values were determined for *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 95 µl of nutrient broth (1% NaCl) 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 (1% NaCl) without plants extract 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 18 to 24 h. The plants extract tested in this study was screened two times against each strain. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of the microorganisms. The 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. All tests were performed in triplicate.

**Biofilm formation on PMMA**

**Biofilm formation**

The acrylic resin strips of PMMA were prepared as described by our group in a previous study (Noumi et al., 2010c). The ability of *C. albicans* strains to form a biofilm was tested according to the protocol described by Chandra et al. (2001). For thus, cells were grown for 24 h at 37°C in yeast nitrogen base (YNB) containing 50 mM of glucose. Batches of medium were inoculated with overnight yeast cultures and incubated at 37°C in an orbital shaker operating at 150 rpm. Cells were harvested after 24 h (stationary growth phase), washed once with phosphate-buffered saline (PBS, pH 7.2), and standardized to a density of 1 × 10⁶ cells/ml.

A 80 µl quantity of the standardized *C. albicans* cells suspensions was applied to the surfaces of 1.5 cm² PMMA strips placed in a 12-well tissue culture plate. The cells were allowed to adhere for 90 min at 37°C (adhesion phase). Non-adherent cells were removed from the strips by being gently washed with 5 ml PBS. Strips were then submerged in 4 ml of YNB containing 50 mM of glucose. Strips to which no cells were added served as negative controls. Control and experimental strips were incubated at 37°C for 72 h (biofilm growth phase).

**Quantitative measurement of biofilm**

The biofilm formation by *Candida* strains was quantified by a colorimetric method which determines the mitochondrial dehydrogenase activity of the metabolic fungal cells. This colorimetric assay
investigates metabolic reduction of 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl amino) carbonyl]-2H-tetrazolium hydroxide (XTT) to a water-soluble brown formazan product.

Strips with biofilms were transferred to 12-well tissue culture plates containing 4 ml PBS/well. Fifty microliters of XTT (1 mg/ml in PBS) and 4 µl of menadione solution (1 mM in acetone) were added to each well. Plates were incubated at 37°C for 5 h. The entire contents of the well were transferred into a tube and centrifuged (5 min, 6000 g). XTT formazan in the supernatant was determined spectrophotometrically at 492 nm. Experiments were performed in triplicate.

### Effect of plants oils on adhesion and biofilm formation on PMMA

#### Effect on adhesion

The ability of *Candida* strains to form a biofilm was tested according to the protocol described by Chandra et al. (2001). Thus, cells were grown for 24 h at 37°C in yeast nitrogen base (YNB) containing 50 mM of glucose. Batches of medium were inoculated with overnight yeast cultures and incubated at 37°C in an orbital shaker operating at 150 rpm. Cells were harvested after 24 h (stationary growth phase), washed once with phosphate-buffered saline (PBS, pH 7.2), and standardized to a density at 1 × 10^7 cells/ml.

A 80 µl quantity of the standardized *Candida* cells suspensions and 40 µl of the MIC or 1/2 of MIC was applied to the surfaces of 1.5 cm² PMMA strips placed in a 12-well tissue culture plate. The cells were allowed to adhere for 90 min at 37°C (adhesion phase). Non-adherent cells were removed from the strips by being gently washed with 5 ml PBS.

#### Effect on biofilm

Eighty microliters of the standardized *Candida* cells suspensions and 2 ml of the MIC or 1/2 of MIC was applied to the surfaces of 1.5 cm² PMMA strips placed in a 12-well tissue culture plate. The cells were allowed to adhere for 90 min at 37°C (adhesion phase). Non-adherent cells were removed from the strips by being gently washed with 5 ml PBS. Strips were then submerged in 4 ml of YNB containing 50 mM of glucose. Strips to which no cells were added served as negative controls. Control and experimental strips were incubated at 37°C for 72 h (biofilm growth phase).

#### Quantitative measurement of biofilm

Same colorimetric method protocol has been adopted as previously described by Chandra et al. (2001) to quantify the biofilm formation by *C. albicans* strains by determining mitochondrial dehydrogenase activity of the metabolic fungal cells.

### RESULTS

In this study, the anticandidal activities of *Melaleuca alternifolia* and *Eucalyptus globulus* essential oils against *Candida* strains were investigated. Antifungal effects were reported as inhibition zones using the disc diffusion method and in vitro activity as MIC and MFC values (Table 1).

The two plant essential oils showed significant antifungal activity against the four tested *Candida* strains. Overall, the best antifungal activity was against *C. albicans* ATCC 90028 for *Melaleuca alternifolia* (19.33 mm) and against *C. glabrata* ATCC 90030 for *Eucalyptus globulus* oil (22.33 mm). Essential oil of *Eucalyptus globulus* was more efficient and had the best antifungal effect for oral *C. albicans* strain (15_T) (IZ = 19.33 mm) as compared to the results obtained with amphotericin B (IZ = 11 mm) and also for *C. glabrata* ATCC 90030 strain (IZ = 22.33 mm) as compared to amphotericin B results (IZ = 14.33 mm).

Table 1 summarizes the MIC and MFC of the two plants essential oils. The lowest values of MIC were seen against two *C. albicans* isolates with *E. globulus* essential oil (strains 15_T and ATCC 90030; MIC: 0.078 mg/ml), followed by 0.156 mg/ml for *C. albicans* isolates (strains 15_T and ATCC 90028). The MFC values were similar for all *Candida* tested strains (10 mg/ml). The standard antifungal drug, amphotericin B, was more active against all oral and reference *Candida* strains (MIC range: 0.012 to 0.39 mg/ml; MFC range: 0.195 to 1.562 mg/ml) comparing the two essential oils.

The metabolic activity and total biomass of *C. albicans* and *C. glabrata* biofilms was determined by reduction of XTT dye. It was found out that the metabolic activity of *Candida* biofilm formed on PMMA did not differ between tested strains (Table 2).

The optical densities were 0.356 for *C. albicans* ATCC 90028 and 0.353 for the oral strain (15_T) of *C. albicans*.
Table 2. Quantitative estimation of *Candida* biofilm formation on polymethylmethacrylate biomaterial.

<table>
<thead>
<tr>
<th>Strain</th>
<th>XTT reduction (mean OD&lt;sub&gt;492nm&lt;/sub&gt; ± SD)</th>
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<tr>
<td><em>C. albicans</em></td>
<td></td>
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<tr>
<td>ATCC 90028</td>
<td>0.356 ± 0.006</td>
</tr>
<tr>
<td>15&lt;sub&gt;B&lt;/sub&gt;</td>
<td>0.353 ± 0.004</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td></td>
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<tr>
<td>ATCC 90030</td>
<td>0.325 ± 0.019</td>
</tr>
<tr>
<td>15&lt;sub&gt;T&lt;/sub&gt;</td>
<td>0.352 ± 0.01</td>
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Table 3. Effect of ½ MIC and MIC of *E. globulus* and *M. alternifolia* essential oils comparatively to amphotericin B on biofilm formation potency by *Candida* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>XTT reduction (OD&lt;sub&gt;492 nm&lt;/sub&gt; ± SD)</th>
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<tbody>
<tr>
<td></td>
<td><em>E. globulus</em></td>
</tr>
<tr>
<td></td>
<td>½ MIC</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td></td>
</tr>
<tr>
<td>ATCC 90028</td>
<td>0.242 ± 0.006</td>
</tr>
<tr>
<td>15&lt;sub&gt;B&lt;/sub&gt;</td>
<td>0.218 ± 0.003</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td></td>
</tr>
<tr>
<td>ATCC 90030</td>
<td>0.240 ± 0.004</td>
</tr>
<tr>
<td>15&lt;sub&gt;T&lt;/sub&gt;</td>
<td>0.210 ± 0.008</td>
</tr>
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</table>

MIC: Minimal inhibitory concentration.

The oral strain (15<sub>T</sub>) of *C. glabrata* was more adhesive (OD = 0.352) than the strain *C. glabrata* ATCC 90030 (OD = 0.325).

The results of this study showed that *M. alternifolia* and *E. globulus* essential oils were active against clinical and reference *C. albicans* and *C. glabrata* strains in their planktonic and adherent phases. In fact, for the effect on adhesion, the MIC value (0.156 mg/ml) of *E. globulus* essential oil can inhibit adhesion of *C. albicans* ATCC 90028 strain on PMMA (OD = 0.119); a similar result has been observed with amphotericin B. Also, 0.625 mg/ml of tea tree essential oil was active against adhesive oral strains of *C. albicans* (15<sub>B</sub>, OD = 0.133) and *C. glabrata* (15<sub>T</sub>, OD = 0.168) on PMMA. All these results are illustrated in Table 3.

Concerning the study of the effect of these two plant essential oils on biofilm formation by type and oral strains of *Candida* on PMMA, our results demonstrated that 1/2 MIC *E. globulus* (0.039 mg/ml) can inhibit biofilm formation by *C. glabrata* (15<sub>T</sub>) on PMMA (OD = 0.21) more than to amphotericin B (1/2 MIC = 0.195 mg/ml, OD = 0.175).

The reduction of the number of active *Candida* cells implicated on biofilm formation on PMMA is proportional to the reduction of the absorbance values of XTT. This result has been observed for the four *Candida* strains and with the two plants essential oils and with amphotericin B (Table 4).

Table 4 summarizes the variation in XTT reduction (biofilm formation) in function of MIC values of *M. alternifolia* and *E. globulus* essential oils. A MIC value of 0.625 mg/ml of *E. globulus* essential oils reduced the biofilm formation from (OD = 0.353) to (OD = 0.146) for the oral isolate of *C. albicans* and from (OD = 0.352) to (OD = 0.158) for the oral isolate of *C. glabrata*. The same concentration of *M. alternifolia* essential oil inhibited the biofilm formed by *C. albicans* (OD<sub>1</sub> = 0.353, OD<sub>2</sub> = 0.131) and also by *C. glabrata* strain (OD<sub>1</sub> = 0.352, OD<sub>2</sub> = 0.142). These results are approximatively similar to those obtained for inhibition of adhesion potency. This study demonstrated that *E. globulus* essential oil was more active on *Candida* biofilm formation on PMMA each times that MIC and 1/2 MIC values of this essential oil were lower than those of tea tree essential oil.

**DISCUSSION**

Antifungal agents may enter the oral cavity in concentrations, which are equal to or over MIC (Pizzo, 2001). TTO is employed for its antimicrobial property and is incorporated as the active ingredient in many tropical...
formulations used to treat cutaneous infections. In fact, our results agree with previous works dealing about the high susceptibility of a wide range of yeasts, dermatophytes, and other filamentous fungi (Carson et al., 2006). In this context, TTO has been clinically evaluated for the treatment of several fungal infections including oral candidiasis (Jandourek et al., 1998).

Hammer et al. (1998) tested in vitro the antifungal activity of 24 essential oils against 14 Candida species isolates. They founded that E. globulus essential oil inhibit the growth of C. albicans ATCC 10231 at MIC = 1% (v, v) and from 0.12 to 0.5% for all Candida spp. tested.

MIC assays showed that M. alternifolium and E. globulus essential oils have a fungicidal effect on Candida tested strains. This result is similar to that obtained by our group researchers who verified the decrease in the growth of Candida strains cultured in medium supplemented with different concentrations of each plant essential oil. Also, TTO and E. globulus essential oils inhibit germ tube formation and mycelia conversion in C. albicans. In fact, only 1/2 MIC (0.312 mg/ml) of M. alternifolium was able to inhibit totally 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 (Noumi et al., 2010b).

Tampieri et al. (2005) found that 1, 8-cineole (81.4%) and limonene (7.01%) were the main components of E. globulus essential oil and that these two components have the same fungistatic activity at >1000 and 1000 ppm, respectively. Devkatte et al. (2005) studied the in vitro efficacies of 38 plant essential oils against four isolates of C. albicans. The E. globulus oil caused 6 to 10 mm zone inhibition (ZI) and tea tree caused 11 to 24 mm ZI. TTO caused fungicidal effect at 0.25% concentration comparatively to 1.5 to 2.5% for E. globulus essential oil.

The adhesion process of Candida is a complex issue and involves different factors and this virulence factor of Candida seems to influence its infection and colonization potential (King et al., 1980). The in vitro activity of plant extracts or essential oils decreases as the biofilm phenotype develops, as noted previously with traditional antifungal drugs (Alviano et al., 2005).

From this study, it appears that plants essential oils significantly prevent the formation of biofilm at low concentrations (0.039 mg/ml). Treated Candida cells with tea tree and E. globulus essential oils demonstrated reduction in adhering cells and biofilm development. In fact, biofilm-associated or sessile C. albicans organisms demonstrate increased resistance to traditional antifungal agents that have activity against their planktonic counterparts (Hawser and Douglas, 1995). Also, this suggests that essential oils may be exerting a metabolic interference in Candida biofilm. As biofilm formation and development involves a series of steps, cell responses and interactions, and any intervention on these steps may possibly inhibit Candida biofilm formation (Sangetha et al., 2009). This finding suggests that the potential bioactive compound(s) in tea tree and E. globulus essential oils have distinct influence on Candida cell growth, function and biofilm formation by interfering with any of the steps involved in biofilm development.

Braga et al. (2007) demonstrated that thymol interfered with the starting phases of biofilm formation by reducing the amount of metabolically active yeast as well as the dimorphic switching from yeasts to filamentous forms. Also, the effects of fresh garlic extract against planktonic Candida spp. have been demonstrated in vitro and have been attributed to the action of allicin (Ghannoum, 1988). The results of this study suggest that M. alternifolium and E. globulus essential oils have a potential anti-adhesive effect of Candida strains on PMMA. Generally, the beneficial effects attributed to the plants may be due to one or more photochemicals, including antioxidants, flavans, and other substances present in the extract (Balasenthil et al., 1999). Therefore, the results of this study reinforce the possible effect of the plants essential oils in the prevention of diseases of the buccal cavity.

Conclusion

Conclusively, the preliminary evidence for the antifungal activity of M. alternifolium and E. globulus essential oils against oral Candida were presented. Its action against this yeast includes inhibition of adhesion and biofilm formation on prosthetic biomaterial (PMMA) and this raises the possibility that these plants may have therapeutic use for oral candidiasis and possibly other oral infections.

REFERENCES


