Advanced Journal of Microbiology Research Vol. 2017
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

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

Emira Noumi[1]*, Mejdi Snoussi[1,2] and Amina Bakhrouf[1]

[1]Département de Microbiologie, Faculté de Pharmacie, Laboratoire d'Analyse, Traitement et Valorisation des Polluants de l'Environnement et des Produits, Monastir, Tunisie.
[2]Laboratoire de Traitement et de Recyclage des Eaux, Centre de Recherches et des Technologies des Eaux (CERTE), Technopole de Borj-Cédria, BP 273- Soliman 8020, Tunisie.

Accepted 15 April, 2016

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 specie 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

*Corresponding author. E-mail: emira_noumi@yahoo.fr. Tel: +216 73 466244. Fax: +216 73 461830.

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 (Laboratories 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 fungicidal 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 fungicidal 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, 95 l of nutrient broth and 5 l of the inoculum. A 100 l aliquot from the stock solutions of each plants extract was added into the first wells. Then, 100 l from the serial dilutions were transferred into eleven consecutive wells. The last well containing 195 l of nutrient broth without essential oil and 5 l of the inoculum on each strip was used as the negative control. The final volume in each well was 200 l. 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.

Myceliation 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 a 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 ½ 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 anticandidal activities 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

Table 1. MIC and MFC values of *M. alternifolia* and *E. globulus* essential oils against *C. albicans* strains.

Strains	MIC and MFC values (mg/ml)					
	M. alternifolia		*E. globulus*		AmB	
	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans						
ATCC 90028	0.312	>10	0.156	10	0.097	0.781
15_B	0.625	10	0.156	10	0.012	0.781

M. alternifolia, *Melaleuca alternifolia*; *E. Globulus, Eucalyptus globulus*; MIC, Minimal Inhibitory Concentration; MFC, Minimal Fungicidal Concentration; AmB, Amphotericin B.

Table 2. Origin of *C. albicans* isolates and their filamentation potency on Lee medium.

C. albicans strains	Body site	Filamentation potency
ATCC 90028		+
3^1	Oral cavity	+
4	Oral cavity	+
6	Oral cavity	+
7	Oral cavity	++
9	Oral cavity	-
10	Oral cavity	-
11	Oral cavity	+
13	Oral cavity	+
14	Oral cavity	++
15_B	Oral cavity	++
16	Oral cavity	++
17	Oral cavity	-
17_R	Oral cavity	++
18	Oral cavity	+
21	Oral cavity	+
65	Oral cavity	+

-: non filamentous strain; +: moderately filamentous strain; ++: strongly filamentous strain.

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* 15_B 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 lypophilic, 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).

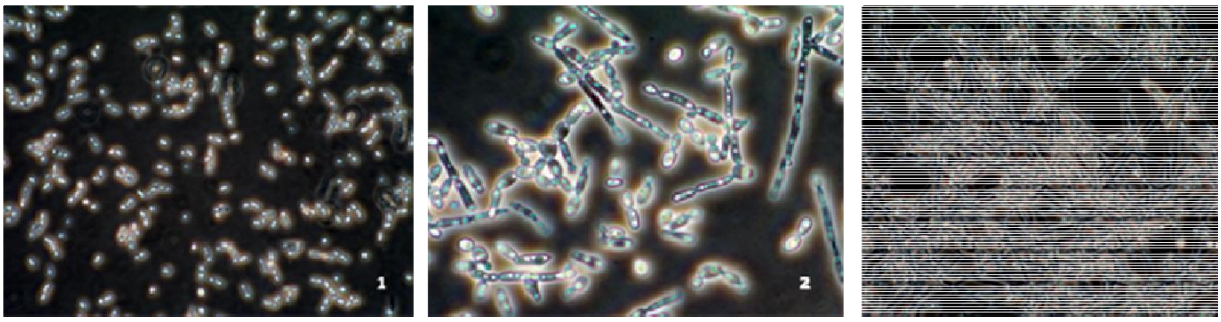

Figure 1. Microscopic observation of filamentaion range of *C. albicans* strains on Lee medium (×40). (1): *C. albicans* strain (non producer of filamentation) (strain 17); (2): *C. albicans* strain (moderately producer of filamentation) (type strain ATCC 90028) and (3): *C. albicans* strain (strongly filamentation producer) (strain 15_B).

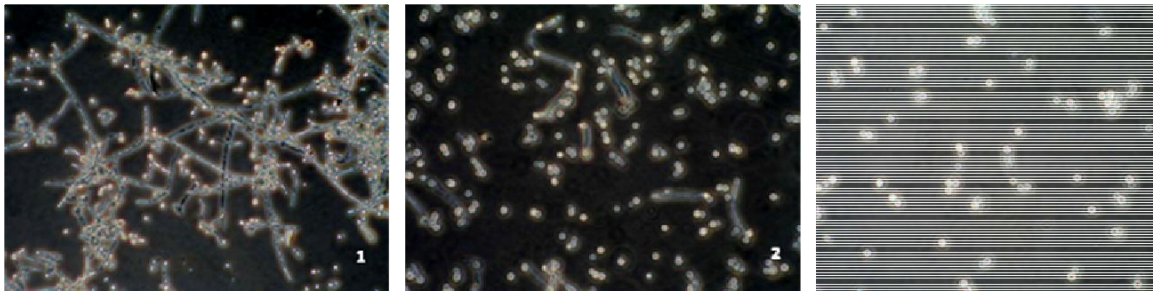

Figure 2. Microscopic observation showing the effect of *M. altenifolia* essential oil on the filamentation potency of *C. albicans* (strain 15_B; strongly filamentation producer) (×40). (1): stongly filamentation on Lee medium without essential oil (control); (2): Filamentation on Lee medium suplemented with 1/4 MIC (0.156 mg/ml) and (3): Total inhibition of germ tube formation on Lee medium suplemented with 1/2 MIC (0.312 mg/ml).

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 15_B) 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 15_B). 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.

ACKNOWLEDGMENT

The authors thank Pr. Eulogio Valentin (Departamento de Microbiologia y Ecología, Facultad de Farmacia, Universidad de Valencia, Burjassot, Valencia, Spain) for his help in photographs of myceliation.

REFERENCES

Bard M, Albrecht MR, Gupta N, Guynn CJ, Stiliwell W (1988). Geraniol

interferes with membrane functions in strains of *Candida* and *Saccharomyces*. Lipids, 23: 534-538.
Carson CF, Hammer KA, Riley TV (2006). *Melaleuca alternifolia* (tea tree) oil: a review of antimicrobial and other medicinal properties. Clin. Microbiol. Rev., 19: 50-62.
Carson CF, Mee BJ, Riley TV (2002). Mechanism of Action of *Melaleuca alternifolia* (Tea Tree) Oil on *Staphylococcus aureus* Determined by Time-Kill, Lysis, Leakage, and Salt Tolerance Assays and Electron Microscopy. Antimicrobial Agents Chemother., 46:1914.
Chaffin WL, Lopez-Ribot JL, Casanova M, Gozalbo D, Martinez JP (1998). Cell Wall and Secreted Proteins of *Candida albicans* : Identification, Function, and Expression. Microbiol. Mol. Biol. Rev., 62: 130-180.
D'Auria FD, Laino L, Strippoli V, Tecca M, Salvatore G, Battinelli L, Mazzanti G (2001). *In vitro* activity of tea tree oil against *Candida albicans* mycelial conversion and other pathogenic fungi. J. Chemother., 13:377-383.
Duarte MCT, Figueira GM, Sartoratto A, Rehder VLG, Delarmelina C (2005). Anti-Candida activity *of Brazilian medicinal plants.* J. Ethnopharmacol. 97: 305-311.
Haberland-Carrodeguas C, Allen CM, Beck FM, Bueschiling WJ, Koletar SL, Sundstron P (2002). Prevalence of fluconazole-resistant strains of *C. albicans* in otherwise healthy outpatients. J. Oral Pathol. Med., 31: 99-105.
Hammer KA, Carson CF, Riley TV (2000). Melaleuca alternifolia (tea tree) oil inhibits germ tube formation by Candida albicans. Med. Mycol., 38: 354-361.
Hammer KA, Carson CF, Riley TV (1998). *In-vitro* activity of essential oils, in particular *Melaleuca alternifolia* (tea tree) oil and tea tree oil products, against *Candida* spp. J. Antimicrob. Chemother., 42: 591-595.
Hammer KA, Carson CF, Riley TV (2003). Antifungal activity of the components of *Melaleuca alternifolia* (tea tree) oil. J Appl Microbiol 95:853-60.
Hill DW, Gebhardt LP (1956) Morphological transformation of *Candida albicans* in tissues of mice. Proc. Soc. Exp. Biol. Med., 92: 640.
Hisajima T, Ishibashi H, Yamada T, Nishiyama Y, Yamaguchi H, Funakoshi K, Abe S (2008). Invasion process of *Candida albicans* to tongue surface in early stages of experimental murine oral candidiasis. Med. Mycol., 46: 697.
Jandourek A, Vaishampayan JK, Vazquez JA (1998). Efficacy of *Melaleuca* oral solution for the treatment of fluconazole refractory oral candidiasis in AIDS patients. AIDS, 12: 1033.
Johnson SA (1954). Candida albicans: Effect of Amino Acids, Glucose, pH, Chlortetrations on Its Growth. Amer Arch Derm Syph 70:49.
Maruyama N, Takizawa T, Ishibashi H, Hisajima T, Inouye S, Yamaguchi H, Abe S (2008). Protective activity of geranium oil and its component, geraniol, in combination with vaginal washing against vaginal candidiasis in mice. Biol. Pharm. Bull., 31: 1501.
Noumi E, Snoussi M, Hajlaoui H, Valentin E, Bakhrouf A (2010)a. Antifungal properties of *Salvadora persica* and *Juglans regia* 1 L. extracts against oral *Candida* strains. Eur. J. Clin. Microbiol. Infect. Dis., 29: 81-88.
Noumi E, Snoussi M, Hentati H, Mahdouani K, Del Castillo L, Valentin E, Sentandreu R, Bakhrouf A, 2010b. Adhesive properties and hydrolytic enzymes of oral *Candida albicans* strains. Mycopathologia, 169: 269-278.
Pei PC, Siti Rohani AH, Yee LL, Chin LP, Boon CT, Kee PN, Heng FS (2007). Genotyping and drug resistance profile of *Candida* spp. In recurrent and one-off vaginitis, and high association of non-*albicans* species with non-pregnant status. Infect. Gen. Evol., 7: 449-456.
Reynolds R, Braude A, 1956. The filament-inducing property of blood for *Candida albicans*; its nature and significance. Clin. Res. Proc., 4: 40.
Satchell AC, Saurajen A, Bell C, Barnetson RS, (2002). Treatment of dandruff with 5% tea tree oil shampoo. J Am Aca Dermato 47: 852–5.
Shapiro S, Guggenheim B (1995). The action of thymol on *oral* bacteria. Oral Microbiol. Immunol., 10: 241.
Tampieri MP, Galuppi R, Macchioni F, Carelle MS, Falcioni L, Cioni PL, Morelli I (2005). The inhibition of *Candida albicans* by selected essential oils and their major components. Mycopathologia, 159: 339-345.
Villar CC, Kashleva H, Dongari-Bagtzoglou A (2004). Role of *Candida albicans* polymorphism in interactions with oral epithelia cells. Oral Microbiol. Immunol., 19: 262.
Williams LR (1998). Clonal production of tea tree oil high in terpinen-4-ol for use in formulations for treatment of thrush. Complementary Therapies Nursing Midwifery, 4: 133.