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Ocimum tenuiflorum L. and *Ocimum basilicum* L., two spices of Lamiaceae family with bioactive essential oils

Alessandra Piras[a], Maria Jose Gonçalves[b], Jorge Alves[c], Danilo Falconieri[d], Silvia Porcedda[a], Andrea Maxia[e,*], Ligia Salgueiro[b]

[a] Department of Chemical and Geological Sciences, University of Cagliari, Cittadella Universitaria, SP 8, Monserrato – Sestu km 0.700, 09042 Monserrato CA, Italy
[b] CIEPQPF and Faculty of Pharmacy, University of Coimbra, Azinhaga de Sta. Comba 3000-354 Coimbra, Portugal
[c] CNC.IBILI, Faculty of Medicine, University of Coimbra, Azinhaga de Sta. Comba, 3000-354 Coimbra, Portugal
[d] State Institute of Higher Education "Michele Giua", via Montecassino, 09134 Cagliari, Italy
[e] Department of Life and Environmental Sciences, Botany section, University of Cagliari, Viale Sant'Ignazio da Laconi 13, 09123 Cagliari, Italy

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ABSTRACT

The present study provides new insights to the antifungal mechanism of action of the essential oils of *Ocimum tenuiflorum* L. and *Ocimum basilicum* L., namely inhibition of germ tube formation, inhibition of biofilm formation and preformed biofilm disruption. The essential oils were characterized by GC and GC–MS. The major compounds were methyl eugenol (84.7%) and β-caryophyllene (7.4%) for *O. tenuiflorum* and linalool (35.1%), eugenol (20.7%) and 1,8-cineole (9.9%) for *O. basilicum*. The essential oil from *O. tenuiflorum* showed a more preeminent effect against *C. neoformans* (0.16 μL/mL) and dermatophytes (0.32 μL/mL). The effect on the germ tube formation of both essential oils was described here for the first time showing that *O. tenuiflorum* decreases germ tube formation by more than 50% at values four times lower than MIC (Minimal Inhibitory Concentration) while *O. basilicum* is able to decrease at values eight times lower than MIC. Furthermore, *O. basilicum* showed a more preeminent effect both in inhibition of *C. albicans* biofilm formation as well as in disruption of preformed biofilm. The activity of all major compounds was also determined, and their activity was in general similar to that of the essential oils thus suggesting that those are the main active compounds. Overall, this study highlights the antifungal activity of two widely used spices and complies with the antifungal uses described in folk medicine. In addition, it shows that both essential oils are able to inhibit virulence factors of *C. albicans* associated with resistance to treatment and relapse cases. Both species are of industrial interest as shown by their use on food and cosmetic industries which was reinforced by the results presented herein.

1. Introduction

Candidiasis is a fungal disease which affect several individuals. Although skin and mucous membrane are potential zones of infection, vagina and mouth are the most common zones of infection associated with *Candida* spp. (Pierce and Lopez-Ribot, 2013). Despite being a superficial infection, candidiasis can, in immunocompromised individuals, rapidly become systemic infections. Several *Candida* species are associated with candidiasis, such as, *C. krusei*, *C. parapsilosis* and *C. tropicalis* (Pinto et al., 2013), however *C. albicans* is the most common etiological agent (Cardoso et al., 2016). This fungus is highly opportunistic that colonize host tissues with ease (Raut et al., 2013) and rapidly form biofilms (Cardoso et al., 2016). These biofilms can be formed in the host tissues, however they are also very common on prosthetic apparatus, such as, catheters (Manoharan et al., 2017). This state is of major clinical relevance due to the increase resistance to antifungal agents associated with biofilms (Cardoso et al., 2016; Raut et al., 2013). Another fungus that is associated with life-threatening infections is *Cryptococcus neoformans* which is connected with cryptococcosis that affect the central nervous system and present a high mortality rate (Cardoso et al., 2016). In addition to the already mentioned fungal infections, dermatophytosis is a very prevalent fungal infection of hair, skin and nails. In fact, this type of infection are very common in most countries (Zeng et al., 2015). Dermatophytosis is mainly caused by dermatophytes of the *Trichophyton*, *Microsporus* and *Epidermophyton* genus. These fungi are able to infect keratinized regions of humans and animals (skin, hair) and cause lesions.

Despite the existent antifungal therapies these infections still account for a high mortality rate, especially in immunocompromised individuals (Cardoso et al., 2016). In cases of dermatophyte infections,

* Corresponding author.
E-mail address: a.maxia@unica.it (A. Maxia).

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the relapse is unacceptably high (Pinto et al., 2013).Both the high mortality and the rate of relapse in fungal infections can be attributed to the poor arsenal, several side effects and the emergence of resistant strains (Cardoso et al., 2016). In fact, antifungal of the polyene class present severe toxicity to the host while azole-type antifungals are only fungistatic and very susceptible to resistance (Khan et al., 2010a,b), and amphotericin B is also highly toxic (Pozzatti et al., 2008). Furthermore, strains of *Candida* spp. resistant to azole-type antifungals, such as fluconazole, itraconazole, ravuconazole, ketoconazole and voriconazole have been described (Pozzatti et al., 2008).

Bearing this is mind, it is imperative that new, safer and more effective antifungal agents are discovered. Traditional medicine have been known in various parts of the world and about 80% of the world population still rely on traditional medicines as primary healthcare (Mandal et al., 2012). One of the most used agents in folk medicine are aromatic plants rich in essential oils (Cardoso et al., 2016; Pinto et al., 2013; Pozzatti et al., 2008). These mixture, in addition for the described antifungal effect, have a great advantage compared to synthetic antifungal that is the lower risk of resistance to the treatment (Zeng et al., 2015). Furthermore, several essential oils have been described as possessing anti-biofilm activity (Alves-Silva et al., 2016; Khan et al., 2014b; Manoharan et al., 2017) while the antifungal agents lack activity or require high concentrations to have any significant activity on biofilms (Manoharan et al., 2017).

The family Lamiaceae is widely distributed over the world and many plants of this family possess several purposes such as food flavouring, fragrances and medicinal properties (Sakkas and Papadopoulou, 2017). One of the most important genera in Lamiaceae is the *Ocimum* genus which is also considered to be the largest genera in this family (Chowdhury et al., 2017). Furthermore, the plants of this genus are called “king of herbs” due to the plethora of applications in folk medicine, perfumery and pharmaceutical and food industries (Simpson and Conner-Ogorzaly, 1986). Holy basil (*Ocimum tenuiflorum* L. syn. *O. sanctum* L.), also known as Tulsi in India, is native and widely spread in Asia (Saharkhiz et al., 2014). The medicinal properties of *O. tenuiflorum* have been described in the Ayurveda for thousands of years. In fact, this plant is regarded as a “elixir of life” by Ayurvedic medicine and is used to treat several ailments, such as common colds, headaches, stomach disorders, inflammation, heart disease, poisoning and malaria (Pattanayak et al., 2010) as well as psycho-physical discomfort, asthma and conjunctivitis (Khare, 2004).

Basil (*Ocimum basilicum* L.) is an annual herb that grow in several regions of the world (Hussain et al., 2008) which is frequently used as medicinal agent (Hossain et al., 2010). In truth, the leaves and flowering tops are alleged to possess carminative, galactagogue, stomachic and anti-spasmodic properties (Hussain et al., 2008). In addition, basil have been used for the treatment of several pathologies, such as, headaches, coughs, diarrhoea, constipation, warts, worms and kidney malfunctions (Araújo Silva et al., 2016; Simon and Morales, 1999).

Some studies have addressed the antifungal properties of *O. tenuiflorum* (Balakumar et al., 2011; Gopalkrishna et al., 2016; Joshi, 2013; Kalagatur et al., 2015; Khan et al., 2010a,b; Khan et al., 2014a,b; Rao et al., 2011; Zomorodian et al., 2015) and *O. basilicum* (Abou El-Soud et al., 2015; Avetisyan et al., 2017; Cardoso et al., 2016; Císarová et al., 2016; Fitsiou et al., 2016; Joshi, 2014; López et al., 2005; Nardoni et al., 2015; Pozzatti et al., 2008; Saxena et al., 2012; Shirazi et al., 2014; Soares et al., 2015). However, few discuss the effect on virulence factors with only scarce reports on inhibition of biofilm formation by *O. basilicum* (Cardoso et al., 2016). However, this genus is highly polymorphic (Maggio et al., 2016) and the composition of the essential oil is highly dependent on the location and growing conditions thus affecting the biological activities of the essential oil (Alves-Silva et al., 2013). Thus, this study aims to evaluate the antifungal activity of two species of the *Ocimum* genus, *O. basilicum* and *O. tenuiflorum* (syn. *O. sanctum*) and to assess their efficacy on virulence factors for *Candida albicans* being the first reported study on the inhibition of the germ tube formation, inhibition of the formation of biofilms and disruption of preformed biofilms. In addition, the chemical composition was also determined.

2. Materials and methods

2.1. Plant material

Tulsi and Basil seedlings have been grown from seed in “Planta Medica” greenhouse in the Laboratory of Plant Biology and Pharmaceutical Botany of the University of Cagliari (UNICA). Tulsi seeds, from Indian origin, have been provided by Prof. S. B. Kasture (Pinnacle Biomedical Research Institute, Bhopal, India), while Basil seeds have been purchased from a specialist store in Cagliari, Italy. After 5 weeks, seedlings from *Ocimum tenuiflorum* was transplanted to “Planta Medica” greenhouse. While, *O. basilicum* seedlings were transplanted after 4 weeks. Two different sectors were maintained in accordance to the eco-physiological needs of each plant. After 2 months of growth, the plants were harvested and dried in a laboratory oven with forced-air ventilation at 40 °C for two days. Voucher specimens (CAG122/18A and CAG122/18B) were deposited in the *Herbarium Karalitanum* (CAG), Università di Cagliari, Viale S. Ignazio, 13 Cagliari, Italy.

2.2. Essential oil isolation and analysis

Isolation of essential oils by hydrodistillation were performed, from leaves and twigs, in a *Clevenger*-type apparatus for 3 h accordingly to the European Pharmacopoeia (Council of Europe, 2010). All essential oils were stored at 4 °C until use.

The samples were analysed by using a gas chromatograph equipped with a flame ionization detector (GC-FID) to obtain the quantitative composition and by gas chromatography coupled to mass spectrometry (GC–MS) for constituent identification. Quantitative analyses of the extracts were performed using a gas chromatograph (Agilent 7890A, Palo Alto, CA, USA), equipped with a 30 m × 0.25 mm i.d. with 0.25 μm stationary film thickness DB-5 capillary column (Agilent J&W) and a FID. The following temperature program was used: from 60 °C to 246 °C at a rate of 3 °C min^{-1} and then held at 246 °C for 20 min (total analysis time 82 min). Other operating conditions were the following: carrier gas helium (purity ≥99.9999%, Air Liquide Italy); flow rate, 1.0 mL min^{-1}; injector temperature, 250 °C; detector temperature, 300 °C. Injection of 1 μL of diluted sample (1:100 in hexane, w/w) was performed with 1:10 split ratio, using an autosampler (Agilent, Model 7683B).

GC–MS analyses were carried out using a gas chromatograph (Agilent 6890N) equipped with a 30 m 0.25 mm i.d. with 0.25 μm stationary film thickness HP–5 ms capillary column (Agilent J&W) coupled with a mass selective detector having an electron ionization device, EI, and a quadrupole analyser (Agilent 5973). The temperature program and the chromatographic operating conditions (except detector) were the same used for GC-FID. The MS conditions were as follows: MS transfer line temperature 240 °C; EI ion source temperature, 200 °C with ionization energy of 70 eV; quadrupole temperature 150 °C; scan rate, 3.2 scan s^{-1} at *m/z* scan range, (30–480). To handle and process chromatograms and mass spectra was used the software MSD ChemStation (Agilent, rev. E.01.00.237). Constituents of the samples were identified by comparing: mass spectra fragmentation patterns with those of a computer library (Adams, 2007; NIST/EPA/NIH, 2015), and linear retention indices (RI), based on a homologous series of C8–C26 *n*-alkanes compared with those of authentic products included in the laboratory database and/or literature data (Adams, 2007). Relative amounts of individual components were calculated based on GC peak areas without FID response factor correction.