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Original Research Article

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

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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 *Ociumu 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.

Keywords: Essential oils, *Candida albicans*, *Ocimum* spp., bioactive compounds, spices.

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

61 fungi are able to infect keratinized regions of humans and animals (skin, hair) and cause
62 lesions.

63 Despite the existent antifungal therapies these infections still account for a high
64 mortality rate, especially in immunocompromised individuals (Cardoso et al., 2016). In
65 cases of dermatophyte infections, the relapse is unacceptably high (Pinto et al., 2013).
66 Both the high mortality and the rate of relapse in fungal infections can be attributed to
67 the poor arsenal, several side effects and the emergence of resistant strains (Cardoso et
68 al., 2016). In fact, antifungal of the polyene class present severe toxicity to the host
69 while azole-type antifungals are only fungistatic and very susceptible to resistance (A
70 Khan et al., 2010), and amphotericin B is also highly toxic (Pozzatti et al., 2008).
71 Furthermore, strains of *Candida* spp. resistant to azole-type antifungals, such as
72 fluconazole, itraconazole, ravuconazole, ketoconazole and voriconazole have been
73 described (Pozzatti et al., 2008).

74 Bearing this is mind, it is imperative that new, safer and more effective
75 antifungal agents are discovered. Traditional medicine have been known in various parts
76 of the world and about 80% of the world population still rely on traditional medicines as
77 primary healthcare (Mandal et al., 2012). One of the most used agents in folk medicine
78 are aromatic plants rich in essential oils (Cardoso et al., 2016; Pinto et al., 2013;
79 Pozzatti et al., 2008). These mixture, in addition for the described antifungal effect,
80 have a great advantage compared to synthetic antifungal that is the lower risk of
81 resistance to the treatment (Zeng et al., 2015). Furthermore, several essential oils have
82 been described as possessing anti-biofilm activity (Alves-Silva et al., 2016; Khan et al.,
83 2014b; Manoharan et al., 2017) while the antifungal agents lack activity or require high
84 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; H Gopalkrishna et al., 2016; Joshi, 2013; Kalagatur et al., 2015; A Khan et al., 2010; Amber Khan et al., 2010; Khan et al., 2014a, 2014b; Rao et al., 2011; Zomorodian et al., 2015) and *O. basilicum* (Abou El-Soud et al., 2015; Avetisyan et al.,

110 2017; Cardoso et al., 2016; Císarová et al., 2016; Fitsiou et al., 2016; Joshi, 2014;
111 López et al., 2005; Nardoni et al., 2015; Pozzatti et al., 2008; Saxena et al., 2012;
112 Shirazi et al., 2014; Soares et al., 2015). However, few discuss the effect on virulence
113 factors with only scarce reports on inhibition of biofilm formation by *O. basilicum*
114 (Cardoso et al., 2016). However, this genus is highly polymorphic (Maggio et al., 2016)
115 and the composition of the essential oil is highly dependent on the location and growing
116 conditions thus affecting the biological activities of the essential oil (Alves-Silva et al.,
117 2013). Thus, this study aims to evaluate the antifungal activity of two species of the
118 *Ocimum* genus, *O. basilicum* and *O. tenuiflorum* (syn. *O. sanctum*) and to assess their
119 efficacy on virulence factors for *Candida albicans* being the first reported study on the
120 inhibition of the germ tube formation, inhibition of the formation of biofilms and
121 disruption of preformed biofilms. In addition, the chemical composition was also
122 determined.

123

124 ## 2. Materials and Methods

125 ### *2.1. Plant material*

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

Commentato [JS1]: Reviewer 4: There is no information about preparation of plant material before hydrodistillation. The plant material was dried by forced ventilation. What's that mean? Which type of dryer the authors used? Also, the content of moisture in dried plant material is very important. Please give the information about characteristics of dry plant material.

137 ### *2.2. Essential oil isolation and analysis*

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

142 The samples were analysed by using a gas chromatograph equipped with a flame
143 ionization detector (GC-FID) to obtain the quantitative composition and by gas
144 chromatography coupled to mass spectrometry (GC-MS) for constituent identification.
145 Quantitative analyses of the extracts were performed using a gas chromatograph
146 (Agilent 7890A, Palo Alto, CA, USA), equipped with a 30 m × 0.25 mm i.d. with 0.25

147 μm stationary film thickness DB-5 capillary column (Agilent J&W) and a FID. The
148 following temperature program was used: from 60 ºC to 246 ºC at a rate of 3 ºC min^{-1}
149 and then held at 246 ºC for 20 min (total analysis time 82 min). Other operating
150 conditions were the following: carrier gas helium (purity ≥ 99.9999%, Air Liquide
151 Italy); flow rate, 1.0 mL min^{-1}; injector temperature, 250 ºC; detector temperature, 300
152 ºC. Injection of 1 μL of diluted sample (1:100 in hexane, w/w) was performed with 1:10
153 split ratio, using an autosampler (Agilent, Model 7683B).

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

170 *2.3. Minimum inhibitory and lethal concentration (MIC and MLC)*

171 Minimum inhibitory concentrations (MIC) and minimum lethal concentrations
172 (MLC) of the essential oils (EOs) and its main components were determined using
173 Clinical and Laboratory Standard Institute (CLSI) protocols, M27-A3 for yeasts (CLSI,
174 2008a) and M38-A2 for filamentous fungi (CLSI, 2008b) which were obtained from the
175 American Type Culture Collection (ATCC) or Spanish Type Culture Collection
176 (Colécion Española de Culturas Tipo – CECT) or clinical isolates. The MIC and MLC
177 were tested against yeasts, *Candida* spp. (*C. albicans* ATCC 10231, *C. guillermondii*
178 MAT23 *C. krusei* H9, *C. parapsilosis* ATCC 90018, *C. tropicalis* ATCC 13803) and
179 *Cryptococcus neoformans* CECT 1078 and filamentous fungi, both dermatophytes
180 (*Epidermophyton floccosum* FF9, *Microsporus canis* FF1, *Microsporum gypseum*
181 CECT 2908, *Trichophyton mentagrophytes* FF7, *T. mentagrophytes* var. *interdigitale*
182 CECT 2958, *T. rubrum* CECT 2794, and *T. verrucosum* CECT 2992) and *Aspergillus*
183 strains (*A. flavus* F44, *A. fumigatus* ATCC 46645 and *A. niger* ATCC 16404).

184 Briefly, serial dilutions of EO and its main components (eugenol, methyl
185 eugenol and linalool) (10 µL/mL – 0.04 µL/mL) made in dimethylsulfoxide (DMSO)
186 was added to RPMI 1640 inoculated with cell suspensions adjusted to obtain a final
187 density of 1-3 x 10^3 cells/mL for yeasts or 1-3 x 10^4 cells/mL for filamentous fungi. The
188 tests tubes were then incubated at 35 ºC for 48h for *Candida* spp. and *Aspergillus* spp.,
189 or at 35ºC for 72h for *C. neoformans* or 30 ºC for 7 days for dermatophytes. At the end,
190 10 µL of each negative tube was taken and plated in Sabouraud dextrose agar (SDA)
191 and incubated at the same conditions as described before. MIC was the lowest
192 concentration were no growth was observed in the test tubes, while the MLC was the
193 lowest concentration were no growth was observed in SDA. Two reference compounds,
194 fluconazole (Pfizer) and amphotericin B (Fluka) were used in order to assess the

195 sensibility of tested strains. DMSO was used as positive control and the concentration
196 was never above 1%. All experiments were made in duplicate and in triplicate.

197 *2.4. Germ tube inhibition and disruption of preformed Candida albicans*
198 *biofilm*

199 *2.4.1. Germ tube inhibition*

200 In order to determine the effect of the oils and its main components on the yeast-
201 mycelium transition, cell suspensions of *C. albicans* ATCC 10231, from overnight
202 cultures on SDA, were prepared in NYP medium (N-acetylglucosamine [Sigma; 10^{-}
203 ^{3}mol/L], Yeast Nitrogen Base [Difco; 3.35 g/L], proline [Fluka; 10^{-3}mol/L], NaCl [4.5
204 g/L], and pH 6.7±0.1) (Marichal et al., 2009). The suspensions were adjusted to a
205 density of $1.0\pm0.2 \times 10^{6}$ CFU/mL and then distributed into glass test tubes (990 µL).
206 Dilutions of the EO, eugenol, methyl eugenol and linalool were added into the cell
207 suspension tubes, in 10 µL volumes, to achieve appropriate MIC and sub-inhibitory
208 concentrations. Following an incubation at 37ºC without agitation for 3 h, germ tube
209 formation was registered under a light microscope (40X). Germ tubes were considered
210 when the germinating protuberance was at least as long as the diameter of the
211 blastospore. DMSO in a maximum concentration of 1% (v/v) was used as control.
212 Results were presented as mean ± standard deviation (SD) of three independent
213 experiments made in duplicate.

214 *2.4.2. Inhibition of Candida albicans biofilm formation*

215 The capacity of the essential oils to inhibit the formation of *C. albicans* biofilm
216 was evaluated using the method described by Taweechaisupapong et al. (2010) slightly
217 modified. Briefly, 100 µL of serial dilutions of *O. tenuiflorum* and *O. basilicum*

218 essential oil made in RPMI-1640 was added to 96-well presterilized, polysterene and
219 flat-bottom microtiter plates. A cell suspension of *C. albicans* was made in RPMI-1640
220 from a 24h culture in YPD (Yeast Peptone Dextrose) broth. Of this cell suspension, 100
221 µL was added to the microtiter plate and incubated for 24 and 48h at 37ºC. At the end of
222 each time point the medium was removed and any nonadherent cells were removed by
223 thoroughly wash the wells with sterile PBS. The biofilm viability was determined using
224 the XTT/Menadione metabolic assay and the biofilm biomass was assessed using the
225 crystal violet method. A negative and positive controls comprising biofilm-free and a
226 compound-free wells, respectively, were also added.

227 *2.4.3. Disruption of preformed Candida albicans biofilm*

228 *C. albicans* biofilms were preformed as described by Taweechaisupapong et al.
229 (2012)slightly modified. Briefly, cells were cultured in Yeast Peptone Dextrose broth
230 (YPD) overnight at 37ºC, washed twice with PBS and resuspended in RPMI-1640
231 (1×10^6 cells/mL). Biofilms were formed on commercially available pre-sterilized
232 polystyrene flat-bottom 96-well microtiter plates. 100 µL of the cell suspension were
233 added to the wells and incubated at 37°C for 24 h. After biofilm formation, the medium
234 was discarded, and non-adherent cells were removed by thoroughly washing the
235 biofilms with sterile PBS. Residual PBS was removed by blotting with paper towels
236 before the addition of the essential oils. Then oils (0.08– 1.25 µL/mL for *Ocimum*
237 *tenuiflorum* and 0.16 – 2.5 µL/mL for *Ocimum basilicum*) were added to the biofilms
238 and incubated for 24 h at 37°C. Wells with DMSO in a maximum concentration of 1%
239 (v/v) was used as positive control, while negative control was biofilm-free wells. The
240 effect of the oils on biofilm viability was determined using the XTT/Menadione
241 metabolic assay.

2.4.4. 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carbox-anilide (XTT) metabolic assay

The biofilm viability was determined using the XTT/Menadione metabolic assay as described by Saharkhiz et al. (2012) with some modifications. In brief, to a XTT solution (1 mg/mL made in PBS), menadione (10 mM in acetone) was added to a final concentration of 4 µM. A volume of 100 µL of this solution was added to each well and incubated for 2h at 37ºC in the dark. After the absorbance was measured at 450 nm using a microtiter plate reader. The decrease in viability was determined as [(AbsEO/AbsC) x 100], where AbsEO was the absorbance in the different EO concentrations and AbsC the absorbance of compound-free wells. Results shown as mean ± SD of at least three independent assays made in duplicate.

2.4.5. Crystal violet assay

The effect of the essential oils on the biofilm biomass was quantified using the crystal violet assay as reported by Raut et al. (2013). Briefly, following medium removal, cells were fixed with methanol 99% for 15 min. The supernatant was removed, and the wells were air dried. Then, 100 µL of crystal violet solution (0.02%) was added to each well and left to stain the biofilm for 15 min. After CV removal, the wells were washed thrice with sterile water to remove any excessive reagent. Following, 150 µL of acetic acid 33% was added to release the stain from the cells and the supernatant was transferred to a new 96-well microtiter plate. The absorbance was then read at 620 nm using a microtiter plate reader. Biomass decrease was determined as following [(AbsEO/AbsC) x 100], where AbsEO is the absorbance of each well with essential oil and AbsC the absorbance in the control wells.

Results are shown as mean ± SD of three independent assays, made in duplicate.

266

267 # 3. Results and discussion

268 ## *3.1. Essential oil composition*

269 Table 1 and Figure 1 resumes the chromatographic analysis of *O. tenuiflorum* and *O.*
270 *basilicum* essential oils which enabled the identification of 98.8% of the compounds for
271 *O. tenuiflorum* whereas for *O. basilicum* the identification was carried out in 97.6% of
272 compounds. The essential oil of *O. tenuiflorum* was predominantly constituted by
273 phenylpropanoids (84.8%), while *O. basilicum* presents high amounts of oxygenated
274 monoterpenes (47.7%), phenylpropanoids (21.1%) and hydrocarbon sesquiterpenes
275 (17.7%). Regarding the major compounds, *Ocimum tenuiflorum* essential oil was rich in
276 methyl eugenol (84.7%) with β-caryophyllene as the second major compound (7.4%).
277 The essential oil isolated from *Ocimum basilicum* was mainly constituted by linalool
278 (35.1%), eugenol (20.7%) and 1,8-cineole (9.9%). Previous studies have assessed the
279 chemical composition of essential oils of *O. tenuiflorum* and *O. basilicum* presenting a
280 high variability. Few studies have been conducted on Tulsi regarding the chemical
281 composition of the essential oil. The essential oil from Indian *O. tenuiflorum* was
282 characterized by high amounts of methyl chavicol and linalool (Amber et al., 2010;
283 Amber Khan et al., 2010). Some studies described oils from *O. tenuiflorum* rich in
284 methyl eugenol (Joshi, 2013; Rao et al., 2011), while others described that the major
285 compound was eugenol (Kalagatur et al., 2015; Kumar et al., 2010; Saharkhiz et al.,
286 2014). In other studies linalool was the major compounds (A Khan et al., 2010) whereas
287 some oils from *O. tenuiflorum* possess high amounts of 1,8-cineole (Zomorodian et al.,
288 2015). Several studies have described essential oils obtained from *O. basilicum* with
289 profile similar to the one described herein, having linalool (48.4 – 64.4%), 1,8-cineole

290 (10.3 – 12.3%) and eugenol (3.2 – 12.2%) (Abou El-Soud et al., 2015; Edris and Farrag,
291 2003; Rattanachaikunsopon and Phumkhachorn, 2010) as major compounds. Other
292 studies have described a different chemotype for *O. basilicum*, with linalool ranging
293 from 31.2 – 75.9% (Cardoso et al., 2016; Hussain et al., 2008; Nardoni et al., 2015;
294 Opalchenova and Obreshkova, 2003; Orhan et al., 2011; Pozzatti et al., 2008; Rao et al.,
295 2011; Snoussi et al., 2016; Soković et al., 2010; Zhang et al., 2009) and presenting
296 eugenol (Nardoni et al., 2015), 1,8-cineole (Orhan et al., 2011), methyl cinnamate
297 (Snoussi et al., 2016; Zhang et al., 2009) and epi-α-cadinol (Hussain et al., 2008),
298 geraniol (Cardoso et al., 2016) and methyl chavicol (Opalchenova and Obreshkova,
299 2003) also as major compounds. Previous studies also reported *O. basilicium* essential
300 oils with high amounts of methyl chavicol (45.8 – 86.4%) (Avetisyan et al., 2017; Bozin
301 et al., 2006; Císarová et al., 2016; Fitsiou et al., 2016; Hossain et al., 2010; López et al.,
302 2005; Rao et al., 2011; Shirazi et al., 2014; Sienkiewicz et al., 2013) and other ones
303 with methyl eugenol and methyl chavicol in similar proportions, 39.3% and 38.3%,
304 respectively (Joshi, 2014). Opposing, some authors have described essential oils from
305 *O. basilicum* with a chemical profile different from the others aforementioned, such as
306 one rich in 1,8-cineole (45%), α-pinene (14.3%) and camphor (7.3%) (Alexopoulos et
307 al., 2011), other enriched in α-terpineol (59.8%) and β-caryophyllene (10.5%) (Bayala
308 et al., 2014).

309 *3.2. Minimum Inhibitory and Lethal Concentrations (MIC and MLC)*

310 The antifungal activity of both essential oils was tested against yeasts, dermatophytes
311 and *Aspergillus* strains. Table 2 sum up the MIC and MLC of the essential oils against
312 all tested strains. Tulsi (*O. tenuiflorum*) shows a more preeminent antifungal activity
313 against all tested strains, especially against *C. neoformans* (MIC = 0.16 μL/mL) and
314 dermatophytes (0.32 μL/mL). However, this essential oil lacks fungicidal effect against

315 *Aspergillus* (MLC > 10 µL/mL) while the essential oil from *O. basilicum* shows both
316 fungistatic (MIC = 0.64 – 1.25 µL/mL) and fungicidal activity (MLC = 2.5 – 5 µL/mL).
317 Regarding the major compounds of basil essential oils reported herein, methyl
318 eugenol and eugenol have a very strong activity while linalool exerts a very weak
319 activity. In fact, eugenol shows a promising activity (MIC = 0.08 – 0.64 µL/mL) against
320 all tested strains. Considering this data, it is expected that the antifungal activity of the
321 essential oil isolated from *O. basilicum* might be due to the amount of eugenol since
322 linalool shows very weak activity. Regarding the major compound of *O. tenuiflorum*,
323 methyl eugenol has a MIC of 0.32 µL/mL for dermatophytes and 0.64 µL/mL for
324 yeasts. These values were very similar to those described for the essential oil of Tulsi,
325 therefore it is conceivable that the activity is due to methyl eugenol which is present is
326 very high amounts (84.7%).
327 Several authors have described the antifungal potential of *O. tenuiflorum* and *O.*
328 *basilicum* essential oils. Abou El-Soud et al. (2015) have described that *O. basilicum* is
329 able to reduce mycelial growth as well as aflatoxin B production in *Aspergillus flavus*.
330 Furthermore, it is also able to decrease the growth of *A. parasiticus* and the associated
331 mycotoxin production (Císarová et al., 2016). The *O. basilicum* var. *pilosum* is toxic
332 towards several phytopathogenic fungi (Edris and Farrag, 2003; Zhang et al., 2009),
333 while the var. Maria Bonita hinder the growth of both fluconazole-resistant and -
334 susceptible *C. albicans* and *Cryptococcus neoformans* (Cardoso et al., 2016). The
335 cultivars var. *purpureum* and var. *thyrsiflora* are able to inhibit the growth of
336 *Debariomyces hansenii* and *Candida albicans* (Avetisyan et al., 2017). The growth of
337 *Trichosporon ovoides* is inhibited by the presence of *O. basilicum* essential oil (Saxena
338 et al., 2012), in addition, the oil is also able to inhibit the growth of other dermatophytic
339 fungi (Bozin et al., 2006; Nardoni et al., 2015). Similarly, the essential oil of *O.*

340 *basilicum* inhibits the growth of *A. niger* (Fitsiou et al., 2016; Shirazi et al., 2014) and
341 *C. albicans* (López et al., 2005; Shirazi et al., 2014).

342 Tulsi have been described as antifungal towards *C. albicans* and *C. tropicalis* (A
343 Khan et al., 2010). The essential oil is able to inhibit the growth of several food
344 spoilage-associated fungi, such as *Fusarium* spp. and *Aspergillus* spp. (Kumar et al.,
345 2010; Saharkhiz et al., 2014). This plant also decreases the growth of *Fusarium*
346 *graminearum* as well as the production of zearalenone by this food fungus (Kalagatur et
347 al., 2015). *O. tenuiflorum* is toxic towards several *Candida* spp. isolates, including
348 azole-resistant strains (Amber et al., 2010; Amber Khan et al., 2010). The activity of
349 this essential oil is thought to be due to plasma membrane lesion and inhibition of
350 ergosterol synthesis (Amber Khan et al., 2010) and by apoptosis induction (Khan et al.,
351 2014a). The essential oil of Tulsi is able to inhibit the growth of several yeasts
352 associated with oral infections (Zomorodian et al., 2015).

353 *3.3. Germ tube inhibition*

354 The effect of inhibitory and sub-inhibitory concentrations of *O. tenuiflorum* and *O.*
355 *basilicum* essential oils as well as major compounds are outlined on table 3. The
356 essential oil of Tulsi is able to inhibit the germ tube formation by more than 50% at
357 concentrations four times lower than the MIC, while *O. basilicum* is able to reduce at
358 concentrations eight times lower than the MIC. Although having a very high MIC ($\geq$5
359 µL/mL), linalool is able to decrease germ tube formation by more than 50% at
360 concentrations of 0.16 µL/mL (MIC/32) while eugenol shows an inhibition profile
361 similar to the one ascribed for the *O. basilicum* essential oil. Taking these results, it can
362 be expected that the observed activity of the essential oil is due to eugenol and linalool.
363 Regarding *O. tenuiflorum*, the major compound, methyl eugenol, is able to inhibit germ

364 tube formation at 0.16 µL/mL by 61.6%, therefore the activity of the whole oil might be
365 explained by synergistic effects between all compounds.
366 This study reports for the first time the capacity of these essential oils to inhibit the
367 germ tube formation thus highlighting the potential usage of these plants as treatment of
368 *C. albicans* infections by inhibiting some of the virulence factors of this pathogenic
369 yeast.

370 ### *3.4. Effect on Candida albicans biofilm*

371 The figures show the effect of on the formation of *C. albicans* biofilm for *O.*
372 *basilicum* and for *O. tenuiflorum*. It can be seen that although *O. basilicum* (Fig.2) have
373 a higher MIC (1.25 µL/mL) it shows a more promising effect on inhibiting the biofilm
374 formation both after 24 and 48h of biofilm formation, at values of MIC and 2xMIC, by
375 decreasing both biofilm biomass and viability. After 24h at concentrations two times
376 lower than the MIC (0.64 µL/mL) the essential oil is able to decrease biofilm viability.
377 The oil from *O. tenuiflorum* (Fig.3) although able to inhibit the biofilm formation it
378 does so in a less extent and fails to do so after 48h treatment. Furthermore, the essential
379 oil from sweet basil is able to decrease biofilm viability on preformed *C. albicans*
380 biofilms at values of 2xMIC (Fig. 4). These results demonstrate that both essential oils
381 decrease the biofilm formation whereas only sweet basil is able to disrupt preformed
382 biofilms thus highlighting the potential of using this essential oil on the treatment of
383 adherent candidiasis. For best knowledge of the authors only one study was conducted
384 on the capacity of these essentials oils to inhibit biofilms. Cardoso et al. (2016)
385 described that *O. basilicum* var. Maria Bonita is able to decrease the biofilm viability at
386 MIC and 2xMIC, although the authors do not assess the effect on preformed biofilm.

387 ## 3. Conclusions

388 Overall this study highlights the antifungal activity of two essential oils from the
389 *Ocimum* genus, namely *O. basilicum* and *O. tenuiflorum*. The first shows a strong effect
390 on the virulence factors of *C. albicans*, especially germ tube and biofilm formation as
391 well as disruption of mature biofilms. The latter also inhibits the virulence factors
392 however on a less extent. Furthermore, both plants present a very attractive essential oil
393 yield thus reinforce their industrial interest. Further studies are required so that the
394 absence of toxicity is demonstrated in pharmacologically relevant concentrations in
395 order to explore their pharmaceutical application.

396

397 **References**

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624
625 Figure captions

626 Figure 1 – GC-MS chromatograms of *Ocimum basilicum* (A) and *Ocimum tenuiflorum*
627 (B) essential oils.

628 Figure 2 – Effect of the essential oil from *Ocimum basilicum* on the formation of *C.*
629 *albicans* biofilms on biofilm biomass and viability after 24h (A and B,
630 respectively) and 48h (C and D, respectively). Results shown as mean ± SD of at
631 least three independent determinations made in duplicated. **** $p<0.0001$,
632 *** $p<0.001$ and ** $p<0.01$ after Dunnett's multiple comparison tests

633 Figure 3 – Effect of the essential oil from *Ocimum tenuiflorum* on the formation of *C.*
634 *albicans* biofilms on biofilm biomass and viability after 24h (A and B,
635 respectively) and 48h (C and D, respectively). Results shown as mean ± SD of at

636 least three independent determinations made in duplicated. *** $p<0.001$,

637 **$p<0.01$ and *$p<0.05$ after Dunnett's multiple comparison tests

638 Figure 4 – Effect of the essential oil from *Ocimum basilicum* on the preformed *C.*

639 *albicans* biofilms on biofilm viability. Results shown as mean ± SD of at least

640 three independent determinations made in duplicated. *$p<0.05$ after Dunnett's

641 multiple comparison tests

Table 1–Chemical composition of essential oil from *Ocimum sanctum* and *Ocimum basilicum*

R_I[a]	R_I[b]	Compound	*O. sanctum*[c]	*O. basilicum*[c]	Identification
932	932	α-Pinene	0.1	0.6	MS, R_I, co-inj
947	946	Camphene	0.1	0.1	MS, R_I, co-inj
972	969	Sabinene	tr	0.5	MS, R_I, co-inj
976	974	β-Pinene	tr	1.0	MS, R_I, co-inj
990	988	Myrcene	-	1.0	MS, R_I, co-inj
1016	1014	α-Terpinene	-	0.1	MS, R_I, co-inj
1027	1024	Limonene	tr	tr	MS, R_I, co-inj
1030	1026	1,8-Cineole	tr	9.9	MS, R_I, co-inj
1046	1044	*trans*-Ocimene	-	1.6	MS, R_I
1057	1054	γ-Terpinene	-	0.1	MS, R_I, co-inj
1088	1086	Terpinolene	tr	0.2	MS, R_I, co-inj
1099	1095	Linalool	tr	35.1	MS, R_I, co-inj
1142	1141	Camphor	tr	0.3	MS, R_I, co-inj
1163	1165	Borneol	0.3	0.3	MS, R_I, co-inj
1176	1174	Terpinen-4-ol	-	0.2	MS, R_I, co-inj
1189	1186	α-Terpineol	-	1.1	MS, R_I, co-inj
1285	1287	Bornyl acetate	tr	0.8	MS, R_I, co-inj
1357	1356	Eugenol	0.1	20.7	MS, R_I
1374	1374	α-Copaene	0.4	0.7	MS, R_I, co-inj
1391	1389	β-Elemene	0.6	0.3	MS, R_I
1404	1403	Methyl eugenol	84.7	0.4	MS, R_I, co-inj
1418	1417	β-Caryophyllene	7.4	0.2	MS, R_I, co-inj
1435	1433	α-*trans*-Bergamotene	0.1	5.0	MS, R_I
1437	1437	α-Guaiene	tr	0.5	MS, R_I
1442	1440	*Z*-β-Farnesene	-	0.1	MS, R_I
1445	1448	Cis-Muurola-3,5-diene	-	0.3	MS, R_I
1451	1452	α-Humulene	0.5	0.5	MS, R_I, co-inj
1457	1454	*E*-β-Farnesene	-	0.3	MS, R_I
1461	1461	*Cis*-Cadina-1(6),4-diene	-	0.6	MS, R_I
1479	1484	Germacrene D	1.2	2.7	MS, R_I
1494	1500	Bicyclogermacrene	tr	1.2	MS, R_I
1503	1508	Germacrene A	2.6	1.8	MS, R_I
1512	1513	γ-Cadinene	0.1	2.4	MS, R_I
1522	1521	β-Sesquiphellandrene	0.1	0.5	MS, R_I
1530	1529	*E*-γ-Bisabolene	0.1	-	MS, R_I
1580	1582	Caryophyllene oxide	0.3	-	MS, R_I, co-inj
1612	1618	1,10-di-epi-Cubenol	tr	0.7	MS, R_I
1639	1638	epi-α-Cadinol	tr	5.6	MS, R_I
1652	1652	α-Cadinol	tr	0.3	MS, R_I
Total identified			98.9	97.6	
Hydrocarbon monoterpenes			0.2	5.0	
Oxygenated monoterpenes			0.4	47.7	
Hydrocarbonsesquiterpenes			13.2	17.0	
Oxygenated sesquiterpenes			0.3	6.7	
Oxygenated phenylpropanoids			84.8	21.1	

[a] Retention index determined on a HP-5 fused silica column relative to a series of n-alkanes (C8–C26);

[b] Retention index reported from the literature
[c]Composition of compounds are expressed as percentage of the whole oil (%); the chromatographic results, expressed as GC peak area percentages calculated without any response factor correction.
tr: trace, i.e., percentage lower than 0.1 %.

Strains	*Ocimum basilicum*		*Ocimum sanctum*		Eugenol		Methyl eugenol		Linalool	
	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC

Table 2 - Antimicrobial activity (MIC and MLC) of essential oil of *O. basilicum* and *O. sanctum* and major compounds against yeasts, dermatophytes and *Aspergillus* spp.

Candida albicans ATCC 10231	1.25	2.5	0.64	1.25	0.64	1.25	0.64	0.64	5	5
Candida tropicalis ATCC 13803	2.5-1.25	2.5	0.64	1.25	0.64	0.64	0.64	0.64	5	5
Candida krusei H9	1.25	2.5	0.64	2.5	0.64	1.25	N.D.	N.D.	10	10
Candida guillermondii MAT23	1.25	1.25	0.64	1.25	0.32	0.64	N.D.	N.D.	5	10
Candida parapsilosis ATCC 90018	1.25	2.5	0.64	2.5	0.64	1.25	032-0.64	0.64	10	10
Cryptococcus neoformans CECT 1078	0.16-0.32	0.64-0.32	0.16	0.64	0.16	0.64	0.32	0.32-0.64	5	5
T. mentagrophytes FF7	0.64	1.25	0.32	0.32	0.16	0.32	0.32	0.32	1.25	2.5
T. mentagrophytes var. *interdigitale* CECT 2958	0.64-0.32	1.25	0.32	0.64	0.16	0.32	0.32	0.64	2.5	2.5-5
Trichophyton rubrum CECT 2794	0.64	1.25	0.32	0.64	0.16	0.32	0.32	0.64	1.25	1.25-2.5
T. verrucosum CECT 2992	0.64	1.25	0.32	0.64	0.16	0.32	0.32	0.32	1.25-2.5	1.25-2.5
Microsporum canis FF1	0.64	1.25	0.32	0.64	0.16	0.32	0.32	0.32	2.5	2.5
M. gypseum CECT 2908	0.64	1.25	0.32	0.32-0.64	0.16	0.32	0.32	0.32-0.64	1.25-2.5	2.5
Epidermophyton floccosum FF9	0.64	0.64	0.32	0.32	0.16	0.16	0.32	0.32	1.25-2.5	2.5
Aspergillus niger ATCC16404	0.64	5	0.64	>10	0.32	1.25	0.64	>2.5	5	≥20
*A. fumigatus*ATCC 46645	1,25	5	0.64	>10	0.32	1.25	0.32	1.25	2.5	20
A. flavus F44	1.25	2.5	0.64	>10	0.32-0.64	1.25	0.64	>2.5	10	≥

N.D. – Not determined

MIC and MLC are expressed as µL/mL

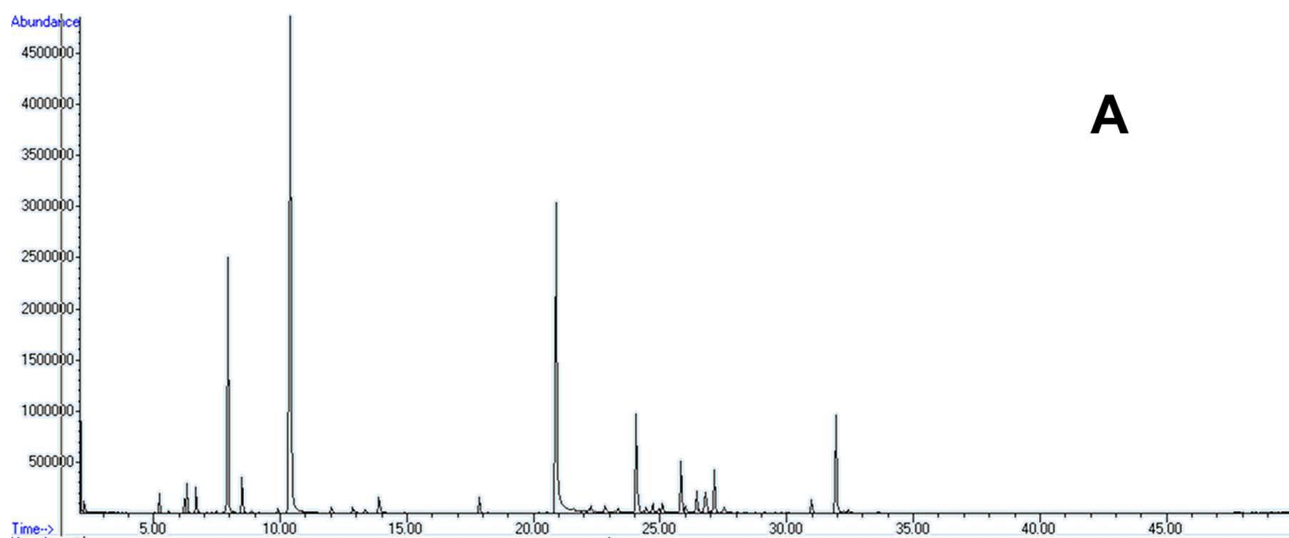
Table 3. Influence of sub-inhibitory concentrations of *Ocimum sanctum* and *O. basilicum* essential oils and major isolated compounds on germ tube formation of *Candida albicans* ATCC 10231.

	Ocimum sanctum	***Ocimum basilicum***	**Methyl eugenol**	**Eugenol**	**Linalool**
Control[(a)]	100	100	100	100	100
MIC/64 (Conc.[(b)])	-	-	-	-	83.3 (0.08)
MIC/32 (Conc.[(b)])	-	-	-	-	47.3 (0.16)
MIC/16 (Conc.[(b)])	-	89.4± 3.4 (0.08)	92.1 (0.04)	-	14.1 (0.32)
MIC/8 (Conc.[(b)])	71.5± 1.2 (0.08)	48.4±0.9 (0.16)	70.8 (0.08)	72.0 (0.08)	0.0 (0.64)
MIC/4 (Conc.[(b)])	44.7±1,4 (0.16)	12.2±3.6 (0.32)	61.6 (0.16)	21.2 (0.16)	0.0 (1.25)
MIC/2 (Conc.[(b)])	0±0 (0.32)	0±0 (0.64)	6.4 (0.32)	1.6 (0.32)	0.0 (2.5)
MIC (Conc.[(b)])	0±0 (0.64)	0±0 (1.25)	0.0 (0.64)	0.0 (0.64)	0.0 (5.0)

The results are expressed as mean ± standard deviation of a minimum of three independent experiments performed in duplicate

[a] Untreated samples including 1% DMSO are considered as control, with 100% filamentation.

[b] Absolute concentration in $\mu L.mL^{-1}$



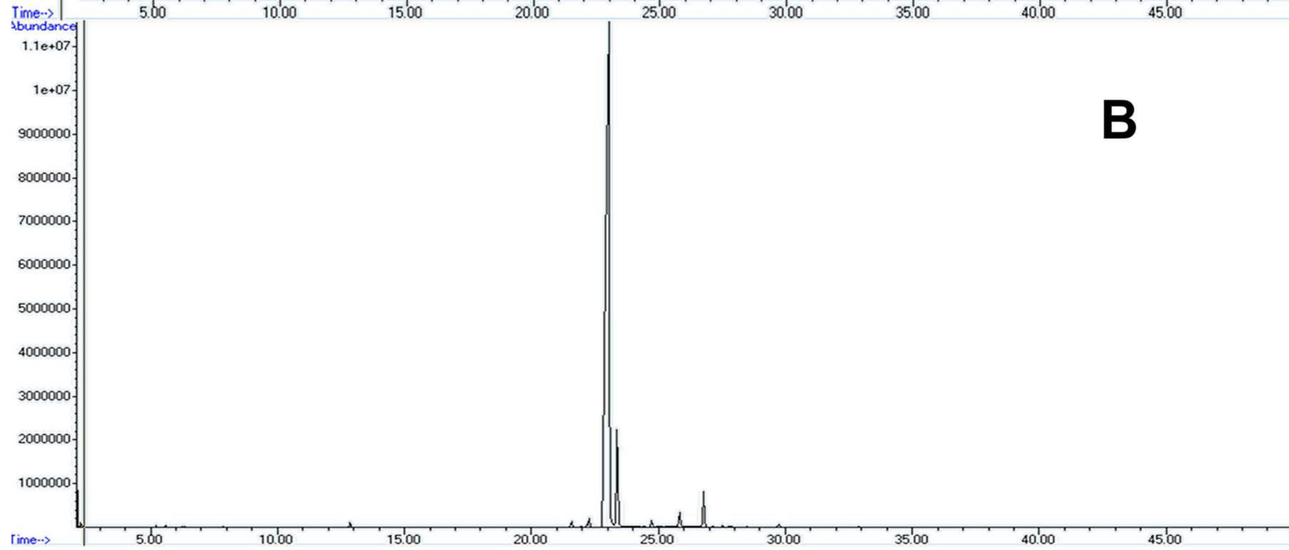
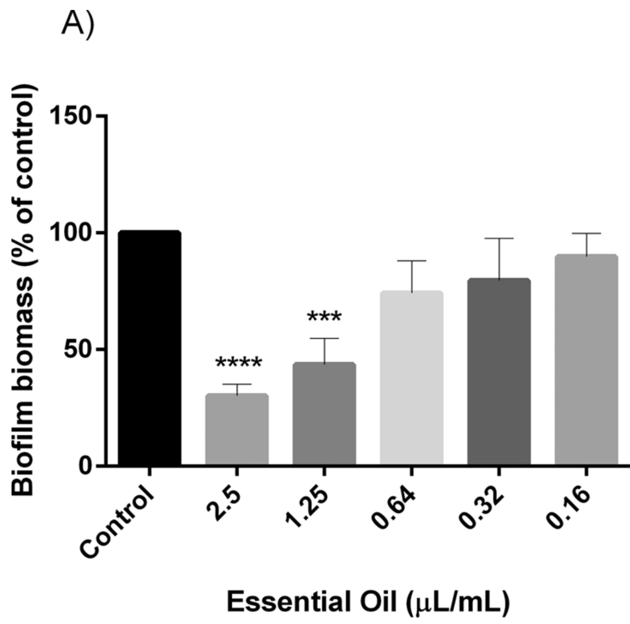
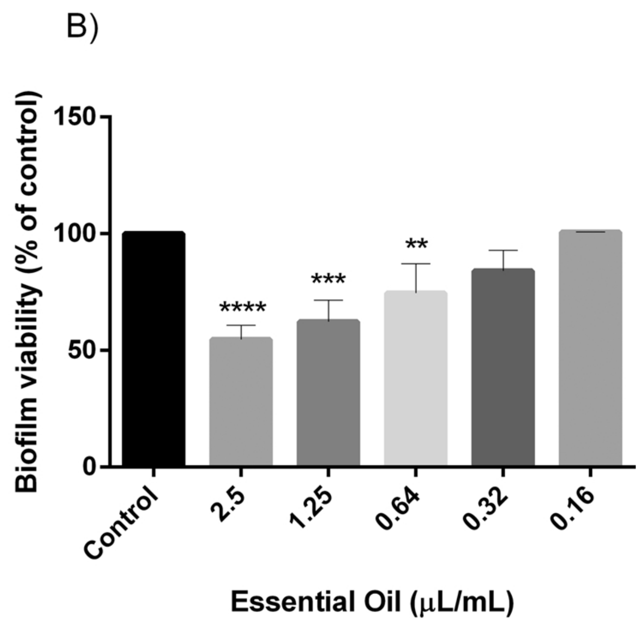
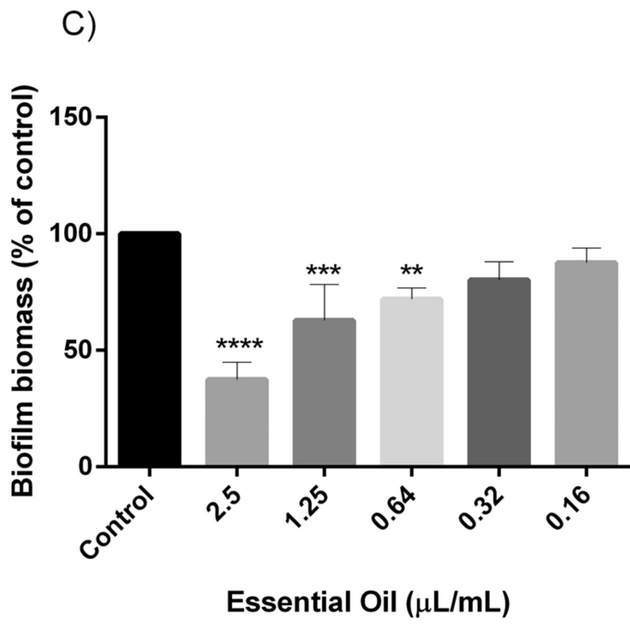


Fig.1







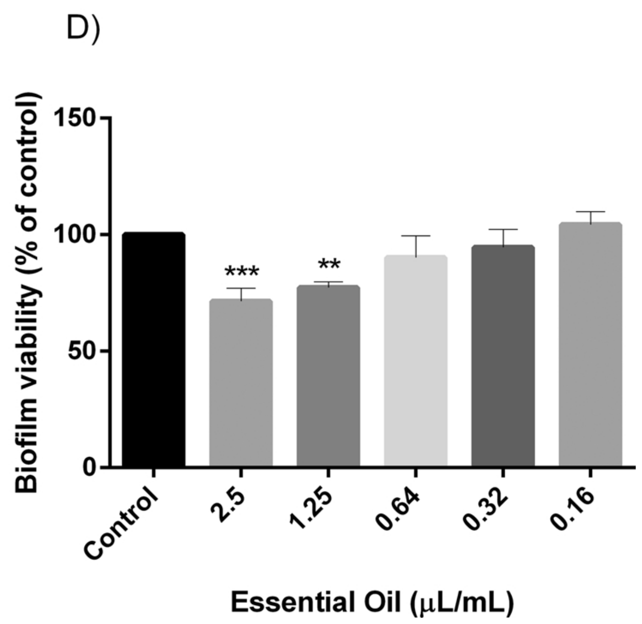
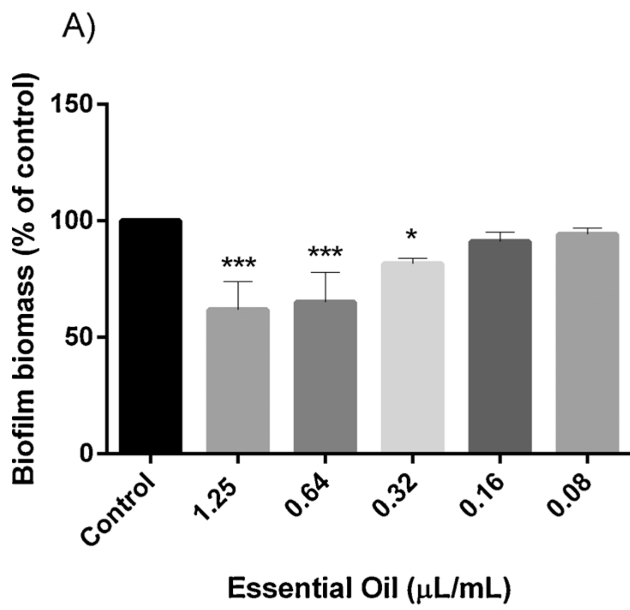
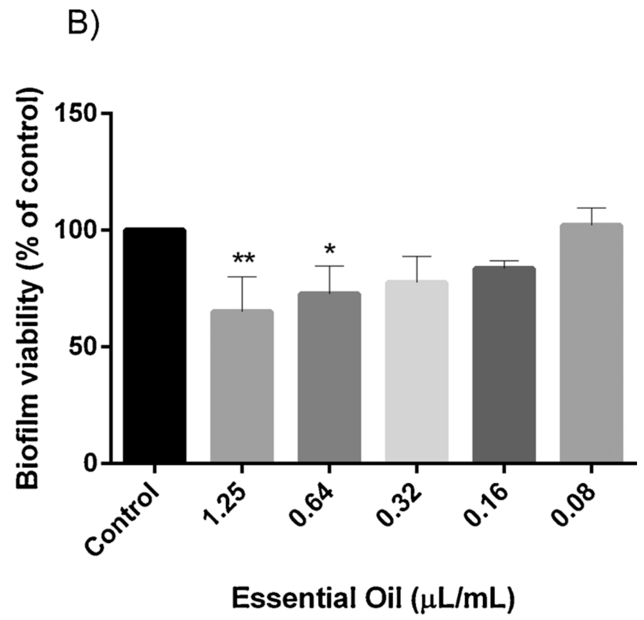
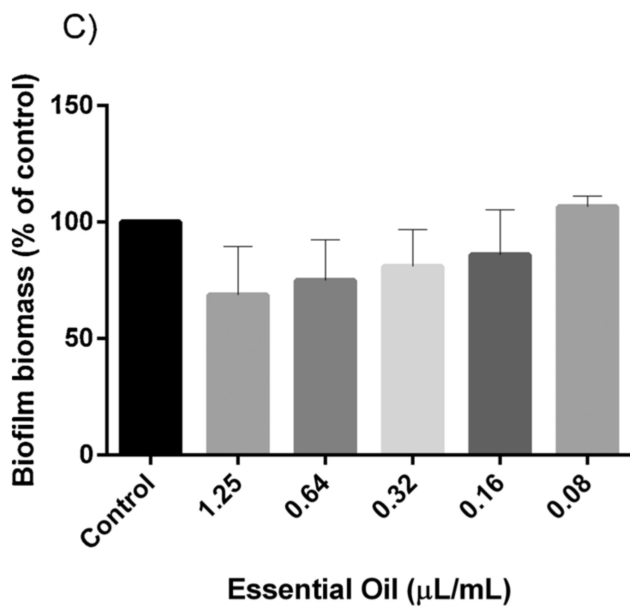


Fig. 2







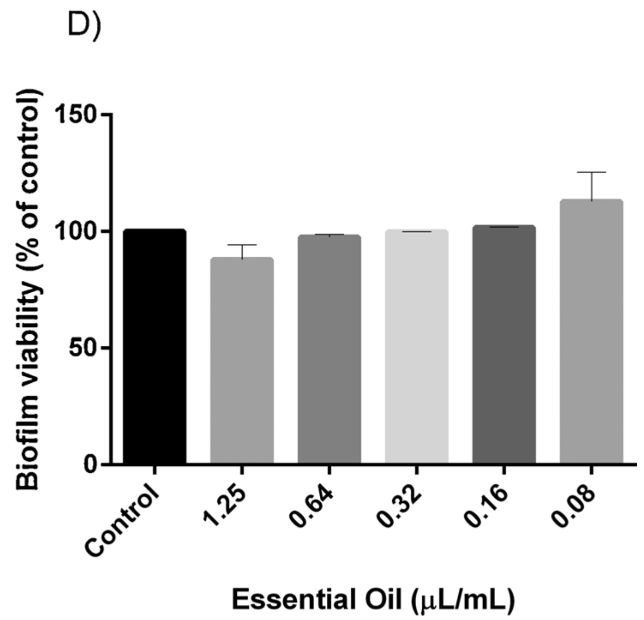


Fig. 3

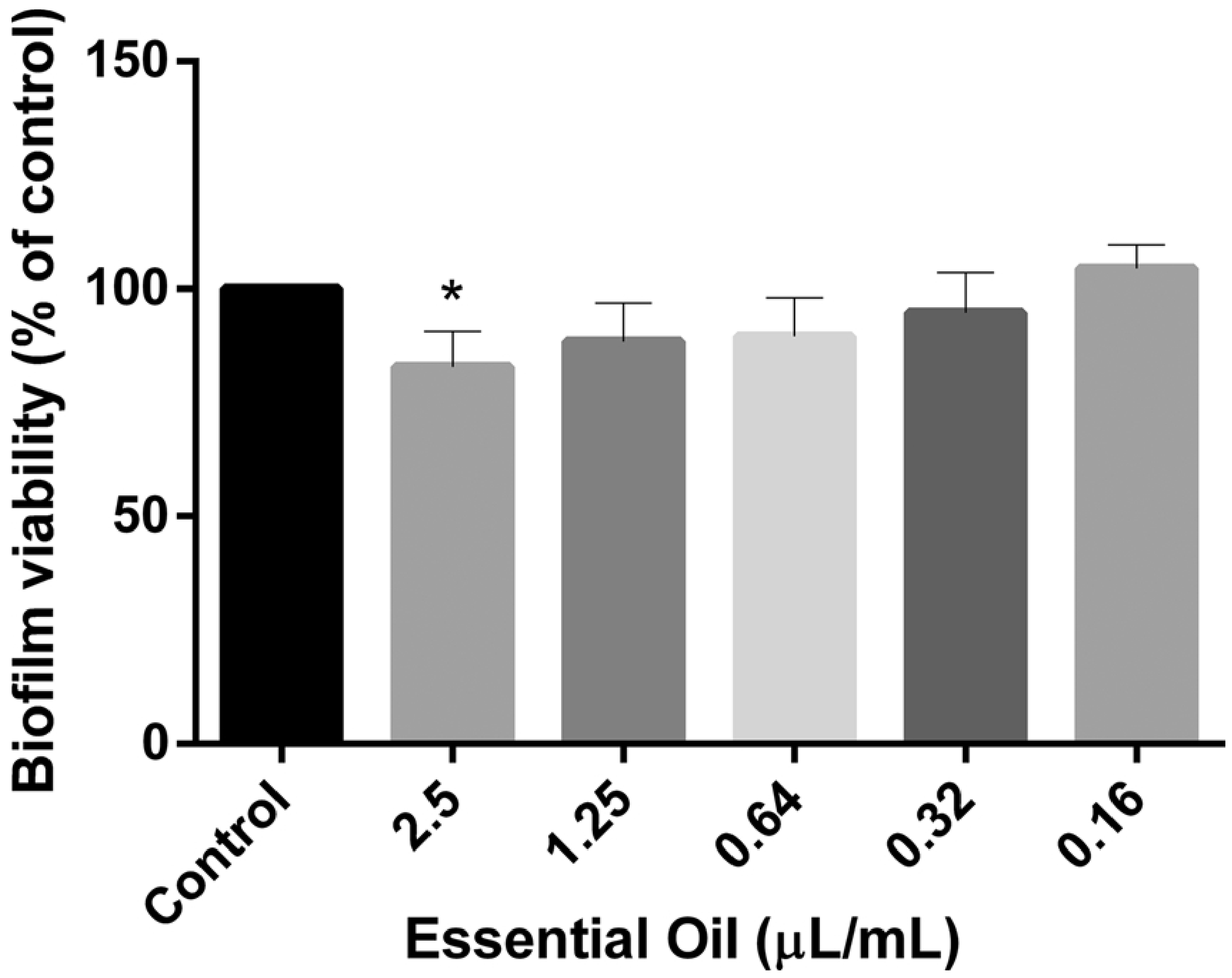


Fig. 4