Antifungal, Antioxidant and Antibiofilm Activities of Essential Oils of Cymbopogon spp.

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Abstract: Essential oils (EOs) of Cymbopogon citratus and Cymbopogon proximus are known as sources of monoterpenes and sesquiterpenoids, although their biological activities have not been well investigated. In this study, the compositions of C. citratus and C. proximus EOs of Egyptian origin and their antifungal and antibiofilm properties against Candida spp. and Malassezia furfur were investigated. Antioxidant activities were also evaluated. GC-MS showed the presence of nine and eight constituents in C. citratus and C. proximus EOs, respectively, with geranial and neral as the major compounds of C. citratus EO and piperitone and α-terpinolene as the major compounds of C. proximus EO. Both EOs showed antifungal (MIC values ranging from 1.25 to 20 µL/mL) and antibiofilm activities (% of reduction ranging from 27.65 ± 11.7 to 96.39 ± 2.8) against all yeast species. The antifungal and antibiofilm activities of C. citratus EO were significantly higher than those observed for C. proximus EO. M. furfur was more susceptible to both EOs than Candida spp. Both EOs exhibited the highest antioxidant activity. This study suggests that C. citratus and C. proximus EOs might be an excellent source of antifungal, antibiofilm and antioxidant drugs and might be useful for preventing Malassezia infections in both medical and veterinary medicine.

Keywords: antifungal; antioxidant; antibiofilm; Cymbopogon citratus; Cymbopogon proximus; essential oils

1. Introduction

Essential oils (EOs) are secondary metabolites and organic compounds with a low molecular weight produced by plants [1]. They play a role as regulators of cell metabolism under environmental stress and pathogenic attacks and are considered to be relevant therapeutic drugs for the treatment of animals and human infectious diseases [2,3]. In particular, EOs may represent sources of bioactive agents with a large spectrum of pharmacologic applications (i.e., antiphlogistic, spasmolytic, antinociceptive and antioxidant activities) [4]. In addition, their antimicrobial and antibiofilm activities stimulated the interest of the scientific community in proposing a solution for alarming multidrug resistance phenomena [5].

Several EOs displayed fungicidal and antibiofilm effects against different fungal pathogens, namely Aspergillus, Candida, Cryptococcus and Fusarium, which represent the major causes for human and animal diseases with high mortality, mainly in immunocompromised patients [6–8]. Recently, Malassezia yeasts have emerged as a threat to both human and veterinary medicine. These yeast species are known to cause skin disorders and
fungemia in immunocompromised patients [9]. Despite attempts to control such yeast infections with topical and systemic antifungals, recurrence of clinical signs of skin infections, as well as treatment failure in preventing or treating *Malassezia furfur* fungemia, have been reported, most likely due to the occurrence of resistant phenomena. Interestingly, essential oils are proposed as promising candidates to control or to prevent *Malassezia*-associated skin diseases both in humans (i.e., atopic dermatitis, dermatitis, pityriasis versicolor and *Malassezia folliculitis*) and in animals [9–12].

In particular, biofilm formation is one of the mechanisms related to multidrug resistance phenomena associated with the highest lethality of infected patients [13]. Several antifungal agents (e.g., amphotericin B, fluconazole, fluconazole, itraconazole and ketoconazole) fail to treat infections caused by yeasts forming biofilm [13], thus raising scientific efforts for the selection of new molecules. EOs have proven to be more effective against fungal biofilm than conventional drugs due to their high content of monoterpenes and sesquiterpenes [14]. Monoterpenes and sesquiterpenes have the potential to affect membrane integrity and suppress genes related to biofilm formation [15]. Earlier studies have shown a large variability in the monoterpenes and sesquiterpenoids composition of Mediterranean medicinal plants EOs (e.g., *Origanum vulgare* L. (oregano), *Salvia officinalis* L. (sage) and *Thymus vulgaris* L. (thyme) (*Lamiaceae*). Among Mediterranean plants, the genus *Cymbopogon* has been largely recommended for its high monoterpane and sesquiterpenoid content [16,17]. In particular, EOs of *Cymbopogon citratus* and *Cymbopogon proximus*, commonly named lemongrass and halfabar and largely diffused in Egypt, are used in traditional medicines as anti-diabetic, antihypertensive, antioxidant and anti-inflammatory drugs [18]. Although some investigations on the chemical and biological profiles of these species proved the presence of monoterpenes and sesquiterpenoids [19–21], there is a lack of scientific evidence regarding the chemical composition of Egyptian *C. citratus* and *C. proximus* EOs and their usefulness as antifungal, antibiofilm and antioxidant drugs. Thus, the present study was designed to characterize the composition of the EOs from *C. citratus* and *C. proximus* and to evaluate their antifungal, antibiofilm and antiradical properties.

2. Results

2.1. Chemical Composition of *C. citratus* and *C. proximus* Essential Oils

Extraction of *C. citratus* and *C. proximus* leaves by hydro-distillation produced EOs with yields of 3 and 3.75% (v/v), respectively. Their GC/MS profiles and chemical compositions (i.e., mass fragmentation and retention indexes) are presented in Figure 1 and Tables 1 and 2.

![Figure 1. Total ion current (TIC) chromatogram of the volatile oil of *Cymbopogon citratus* (a) and *Cymbopogon proximus* (b) (The peak numbers are described in Tables 1 and 2).](image-url)
Table 1. The main constituents of *Cymbopogon citratus* EO.

| Peak No. | RT    | Name                  | Formula | Classification              | MS (M/e)       | Area % |
|----------|-------|-----------------------|---------|-----------------------------|---------------|--------|
|          | m/z No | Scans                | Main Significant | Base Peak |                  |        |
|          | m/z    | Scans                | Fragments  | Peak        |                |        |
| 1        | 10.133 | 18                    | 69, 79, 93, 121 | 93         | 5.82             |
| 2        | 14.046 | 52                    | 55, 69, 79, 93, 107, 121, 136, 150 | 93         | 0.58             |
| 3        | 16.289 | 51                    | 55, 67, 91, 109, 134 | 91         | 1.01             |
| 4        | 16.93  | 51                    | 55, 67, 81, 91, 109, 119, 134, 152 | 67, 81     | 1.26             |
| 5        | 19.082 | 45                    | 69, 94, 134 | 69         | 37.49            |
| 6        | 19.568 | 40                    | 69, 79, 93, 121, 154 | 69         | 3.65             |
| 7        | 20.163 | 53                    | 69, 84, 109, 152 | 69         | 48.2             |
| 8        | 23.499 | 75                    | 55, 69, 79, 93, 121, 136, 154 | 69         | 1.91             |
| 9        | 24.993 | 20                    | 55, 69, 79, 93, 107, 119, 135, 161 | 93         | 0.07             |

Total Identification: 99.99
Total monoterpenes: 99.92
Total sesquiterpenes: 0.07

Monoterpenes were the most abundant compounds of both EOs representing 87.0 and 99.9% of the total oil composition. A total of nine compounds representing the whole bulk of *C. citratus* EO were identified, with geranial (48.2%) and neral (37.49%) being the major compounds (Figure 2). Eight compounds, representing 100% of *C. proximus* EO, were identified, with piperitone (66.99%) and α-terpinolene (15.7%) being the major compounds.
2.2. Antifungal Activity

The antifungal activities of *C. citratus* and *C. proximus* EOs obtained by the broth microdilution method are reported in Table 3. The minimal inhibitory concentration (MIC) and the minimal fungicidal concentration (MFC) values vary according to the EO’s origin. The MIC and MFC values of *C. citratus* EO were lower (MIC values from 1.25 to 5 µL/mL) than those registered for *C. proximus* EO (MIC values from 2.5 to 20 µL/mL). The *M. furfur* strains were the most sensitive species to both EOs. Among Candida spp., *C. catenulata* and *C. guilliermondii* were less sensitive to *C. citratus* EO.

Table 3. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *Cymbopogon citratus* and *Cymbopogon proximus* EOs and fluconazole (FLZ) against Candida spp. and Malassezia furfur.

| Yeast spp.                  | MIC Values | *C. citratus* EO | *C. proximus* EO | FLZ  |
|-----------------------------|------------|-----------------|-----------------|------|
|                             |            | MIC µL/mL       | MFC µL/mL       | MIC µL/mL | MFC µL/mL |
| *Candida tropicalis* (n = 7) | Range      | 2.5             | 2.5             | 20     | <20       | 4           | 4     |
|                             | MIC90      | 2.5             | 2.5             | 20     | <20       | 4           | 4     |
| *Candida catenulata* (n = 10)| Range     | 2.5–5           | 5               | 20     | <20       | 8           | 8     |
|                             | MIC90      | 5               | 5               | 20     | <20       | 8           | 8     |
| *Candida krusei guilliermondii* (n = 10) | Range | 2.5–5           | 5               | 20     | <20       | 8           | 8     |
| *Candida albicans* (n = 12)  | Range      | 2.5             | 2.5             | 20     | <20       | 4           | 4     |
|                             | MIC90      | 2.5             | 2.5             | 20     | <20       | 4           | 4     |
| *Malassezia furfur* (n = 9)  | Range      | 1.25            | 2.5             | 2.5    | >32       | >32         | >32   |
|                             | MIC90      | 1.25            | 2.5             | 2.5    | >32       | >32         | >32   |
| *Candida parapsilosis* (n = 8) | Range   | 2.5             | 2.5             | 20     | <20       | 4           | 4     |
|                             | MIC90      | 2.5             | 2.5             | 20     | <20       | 4           | 4     |
| ATCC 22019                   | Range      | 2.5             | 2.5             | 20     | <20       | 4           | 4     |
| *Candida krusei*             | Range      | 2.5             | 2.5             | 20     | <20       | >32         | >32   |
| ATCC 6258                    | MIC90      | 2.5             | 2.5             | 20     | <20       | >32         | >32   |

2.3. Inhibitory Effects of *Cymbopogon citratus* and *Cymbopogon proximus* EOs on Candida spp. and Malassezia furfur Biofilms

The XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetra-zolium-5-carboxanilide] reduction assay showed that all tested yeasts were able to form biofilm within 24 to 48 h. Among the tested yeast species, *C. tropicalis* strains were the highest biofilm producers, whereas *M. furfur* strains were the lowest. All Candida spp. strains were higher biofilm producers (*p < 0.05*) than *M. furfur* strains. A significant decrease in biofilm formation compared to the control was observed in the tested yeast strains when grown in the presence of *C. citratus* or *C. proximus* EOs (Figure 3), showing an inhibition percentage ranging from 27.65 ± 11.7 to 96.39 ± 2.8%. The antibiofilm properties of both *C. citratus* and *C. proximus* EOs were significantly higher than those registered with FLZ (percentage of biofilm inhibition ranging from 19.68 ± 13.1% to 57.22 ± 5.3%; Table 4).
The antibiofilm effects of both EOs were not related to their concentrations. The *C. citratus* EO exhibited significantly higher anti-biofilm activity than *C. proximus* EO (74.01 ± 11.5 to 96.39 ± 2.8% vs. 27.65 ± 11.7 to 96.39 ± 2.8%) against all tested yeast species except *C. parapsilosis*, *M. furfur* and *C. krusei* ATCC 6258 strains.

### 2.4. In Vitro Antioxidant Activity of *C. citratus* and *C. proximus* EOs

DPPH and ABTS radicals can accept an electron or hydrogen radical to become stable radicals. They lose absorption when accepting an electron or hydrogen radical [22], which results in a visually noticeable discoloration and indicates the ability of the EOs to act as free radical scavengers or hydrogen donors [22]. *C. citratus* and *C. proximus* EOs showed high radical scavenging abilities for DPPH and ABTS. The effective concentration at which 50%
of the DPPH or ABTS radicals were scavenged (EC_{50}) ranged from 28.73 to 42.18 µg/mL (Figure 4). No statistically significant differences were registered between C. citratus and C. proximus EOs in scavenging DPPH and ABTS. Trolox and vitamin C demonstrated higher scavenging activity for DPPH and ABTS than those registered for EOs.

![Figure 4. Radical scavenging activity against 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) and 2, 2′-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) of the C. citratus and C. proximus EOs.](image)

3. Discussion

The results of this study confirm that C. citratus and C. proximus are sources of terpenes and demonstrate that these EOs may represent excellent sources of antifungal, antioxidant and antibiofilm drugs. Interestingly, this study revealed for the first time the potent antifungal and antibiofilm activities against new and emerging yeast pathogens such as C. catenulata, C. guilliermondii and M. furfur. In particular, the chemical profiles of the EOs reveal the usefulness of both plants as sources of terpenes, as previously suggested [14]. In addition, since the yield of the EOs varies according to the plants, the results of this study suggest that C. citratus represents the better source for these compounds, thus confirming previous studies in which the yield of EOs of C. citratus and C. proximus of different origins (Burkina Faso, México, Algeria, and Egypt) were evaluated [23–26].

Since the EO content and composition can be considerably affected by the geographical origin, in this study, the yield of EO of C. citratus was higher than those previously retrieved in the same plants of different origins with the geranial and neral as major compounds [19,23,24,26].

Both C. citratus and C. proximus EOs displayed growth inhibition activity against yeasts. This finding is in line with previous studies investigating different medicinal plants presenting a richness of terpenes (i.e., Origanum vulgare, Coriandrum sativum L., Juniperus communis L., Lavandula angustifolia Mill, Mentha arvensis L., Mentha pulegium L., Ocimum basilicum L.) [27].

Compared to C. proximus EO, the highest antifungal activity displayed by C. citratus EO could be related to its higher monoterpene content, including geraniol. Furthermore, the richness of geraniol in C. citratus EO might also cause destabilization of fungal cell membranes. In this sense, earlier studies have revealed the potent antifungal activity of geraniol at concentrations ranging from 30 to 130 µg/mL against Candida spp. due to its ability to disrupt cell membrane integrity by interfering with ergosterol biosynthesis and inhibiting the very crucial PM-ATPase [28,29]. Moreover, the high MIC of C. proximus EO herein observed is in accordance with the moderate activity of piperitone against Candida spp. [30]. These results confirm the studies previously performed on some yeast species using the same plants of different origins. Particularly, the results of this study
confirm previous findings about the inhibitory effect of *C. citratus* EOs from France and Brazil against some clinical *Candida* spp. (i.e., *C. albicans*, *C. krusei*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis*) [31] and extend the broad spectrum of antifungal activities to other rare opportunistic fungal pathogens, such as *C. catenulata*, *C. guilliermondii* and *M. furfur*. However, the MIC values herein registered for *C. citratus* EO against *Candida* spp. were slightly higher than those registered for *C. citratus* EO from Asia and lower than those for *C. citratus* EO from Brazil [32,33], suggesting that the chemo-geographical variation in *C. citratus* EO might also affect its antifungal activities [34,35]. On the contrary, the antifungal activity of *C. proximus* EO in this study is in contrast with previous studies in which only antibacterial activities were detected [19] and might be due to the low dose of EOs previously employed (i.e., 0.25 to 1 µL/mL vs. 2.5 to 20 µL/mL in our study).

Interestingly, *M. furfur* strains seem more susceptible than *Candida* spp. to both EOs and this might be due to the lipid capsule composition of *Malassezia* spp. that might favor EO solubilization, thus affecting their efficacy [36]. These findings propose that these EOs could be considered an effective alternative approach for the treatment of *M. furfur* skin infections, which are usually characterized by recurrences.

In particular, new guidelines for the treatment of these infections in animals propose the use of EOs as prophylactic procedures to decrease the risk of recalcitrant *Malassezia* spp. infection [37,38]. In addition, since these yeast species are considered emerging threats for immunocompromised patients (i.e., preterm infants), accurate hygiene of medical operators’ hands and incubators was usually required to prevent fungemia [39]. However, the chemical substances used for hygiene have very low efficacy against these yeast species; thus, EOs might be considered sources of active drugs for preventing strategies of *Malassezia* spp. systemic infections [40].

At present, this study demonstrated for the first time that *C. citratus* and *C. proximus* EOs are effective agents against biofilm formation. Anti-biofilm activities were also previously demonstrated for other EOs, including citronella, cinnamon, cascarilla bark and helichrysum [41], but the number of compounds with anti-biofilm effects are still scant and new molecules are requested. The excellent ability of *C. citratus* and *C. proximus* EOs to interfere with the mature biofilm of yeasts might be due to the hydrophobic interactions of monoterpenes with attachment forces such as Lifshitz-Van der Waals, Brownian, sedimentation and electrostatic interaction forces, which are useful for yeast attachment to different surface types [42].

Interestingly, in this study, the antibiofilm activity should also be related to the antioxidant activities of *C. citratus* and *C. proximus* EOs. Indeed, both EOs, at very low concentrations, showed radical activities scavenging DPPH and ABTS in vitro (50%) comparable to those of synthetic antioxidants (i.e., butylated hydroxytoluene -BHT), possibly due to the high content of monoterpenes activities [43]. In particular, monoterpenes are able to absorb or neutralize free radicals due to their phenolic structure and redox properties [44,45]. In fungal cells, monoterpenes might act as pro-oxidants by disturbing the healthy redox cycle that might lead to an accumulation of reactive oxygen species (ROS) (i.e., hydrogen peroxide, superoxide and hydroxyl radicals) [46]. Usually, a healthy redox cycle promotes microbial attachment, thus favoring biofilm formation [39]. Inversely, in the presence of pro-oxidant compounds, a high level of ROS might favor a reduction of the extracellular polymeric substance (EPS) production, thus affecting the homogeneous structure, yeast numbers, and community composition of biofilm [46–48]. Recently, a strong association between oxidative stress and biofilm formation of bacteria and some yeast species has been demonstrated (*C. albicans*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*) [47,49]. In detail, in *C. albicans* cells, the polyphenols from plants (i.e., magnolol and honokiol) induce ROS accumulation, causing decreased expression levels of specific genes (i.e., Ras-like protein 1-RAS1, enhanced filamentous growth protein -EFG1, Ty-transcription activator-TEC1, and ATP pyrophosphate-lyase-CDC35) involved in adhesion, yeast hyphal transition and biofilm formation [50]. Similarly, compounds that could target oxidative stress regulators, including antioxidants, could potentially be exploited as novel strategies
for biofilm control [46]. However, the significantly higher antibiofilm activity of *C. citratus* EO compared to *C. proximus* EO might be attributable to the occurrence of specific components, mainly geranial and neral or to their synergetic activity, thus suggesting that the antibiofilm activities of EO might be due to different factors acting synergestically and/or additionally. In particular, it has been shown that geraniol is involved in the deterioration of the mature biofilm by affecting chitin and β-glucan synthesis, which are the major fungal cell wall components [51]. In addition, geranial and neral might act in synergy by decreasing intracellular adenosine triphosphate (ATP), pH and cell membrane integrity [52].

4. Materials and Methods

4.1. Plant Material and Essential Oil Isolation

*C. citratus* and *C. proximus* were collected from Siwa Oasis, governorate Nubian and Aswan governorate, Egypt, respectively, during September 2020. The plant species were identified by Dr. Monier Abd El-Ghani, Department of Taxonomy, Faculty of Science, Cairo University. Leaves of *C. citratus* and *C. proximus* were washed, dried in the shade, crushed into small pieces and 100 g were subjected to hydro distillation for 4 h. EO extraction was repeated 4 times. *C. citratus* and *C. proximus* EOs were extracted by steam distillation using a Karlsruhe apparatus. The resulting EOs were dried over anhydrous sodium sulfate and stored at −20 °C until their use. The EO concentrations tested for antifungal and antibiofilm activities ranged from 0.015 to 80 µL/mL being lower than those causing acute toxicity phenomena [24,53,54].

4.2. Identification of the Chemical Composition of EOs by Gas Chromatography–Mass Spectrometry Analysis (GC-MS)

The GC-MS system (Agilent Technologies) was equipped with a gas chromatograph (7890B) and mass spectrometer detector (5977A) at the Central Laboratories Network, National Research Centre (NRC), Cairo, Egypt. EOs were diluted with hexane (1:19, v/v). The GC-MS was equipped with an HP-5MS column (30 m × 0.25 mm internal diameter and 0.25 µm film thickness). Analyses were carried out using helium as the carrier gas at a flow rate of 1.0 mL/min at a split 1:30, injection volume of 1 µL at the following temperature program: 40 °C for 1 min; rising at 4 °C/min to 150 °C and held for 6 min; rising at 4 °C/min to 210 °C and held for 1 min. The injector and detector were held at 280 °C and 220 °C, respectively. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 50–900 and a solvent delay of 5 min. The identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in the Wiley and NIST Mass Spectral Library.

4.3. Antifungal Activities

4.3.1. Yeast Strains

A total of 68 strains isolated from the cloaca of domestic and wild animals or from the skin of hospitalized human patients with *M. furfur* fungemia were employed for antifungal testing (Table 5). The strains were identified biochemically and molecularly, as previously reported [55]. All strains were obtained from the fungal collection of the Department of Veterinary Medicine at the University of Bari Aldo Moro, Italy.
Table 5. Yeast strains used in this study.

| Yeast Species          | Collection Code | Origins                        |
|------------------------|-----------------|--------------------------------|
| Candida tropicalis (n = 7) | CD1693, CD1694, CD 1695, CD 1700, CD1701, CD1702, CD1703 | Lizards feces                  |
|                        | CD 1777, CD1778, CD1568, CD1569, CD1575, CD1577, CD1578, CD1579, CD1580, CD1581 |                                |
| Candida catenulata (n = 10) | CD 1631, CD 1635, CD1638, CD 1641, CD1642, CD 1645, CD1650, CD1651, CD 1659, CD 1661, CD 1662 | Lizards, Laying hens feces     |
| Candida krusei (n = 10) | CD 1606, CD 1644, CD 1653, CD1675, CD1676, CD 1733, CD1738, CD1740, CD 1741, CD1743 | Wild boars feces               |
| Candida guilliermondii (n = 10) | CD1601, CD1613, CD1616, CD1618, CD1620, CD1637, CD 1721, CD1729, CD1730, CD1755, CD1757, CD1760 | Lizards and wild boars feces   |
| Candida albicans (n = 12) | CD 1008, CD1009, CD1029, CD1042, CD1043, CD1058, CD1490, CD1492 | Lizards and wild boar feces    |
| Malassezia furfur (n = 9) | CD1042, CD1043, CD1058, CD1490, CD1492 | Human skin                     |
| Candida parapsilosis (n = 8) | CD1679, CD1681, CD 1682, CD1683, CD1684, CD 1691, CD1735, CD1736 | Lizards and wild boar feces    |
| Candida krusei | ATCC 6258 | American Type Culture Collection |
| Candida parapsilosis | ATCC 22019 | American Type Culture Collection |

4.3.2. Antifungal Activity

The minimal inhibitory concentration (MIC) and the minimal fungicidal concentration (MFC) of EOs were determined by broth microdilution methods according to the CLSI protocol for Candida and the CLSI modified protocol for Malassezia, as previously reported [56,57]. Stock inoculum suspensions of Candida spp. and M. furfur were adjusted to an optical density of 0.5–2.4 McFarland, respectively, equivalent to $5 \times 10^6$ colony forming units (CFU)/mL. Two serial dilutions of Candida spp. (1:10 v/v) and M. furfur (1:5 v/v) were performed in specific media (i.e., Roswell Park Memorial Institute-RPMI for Candida spp. and Sabouraud Dextrose broth—SAB + 1% Tween 80 for M. furfur). One hundred microliters of the final dilution were transferred into a 96-well microtiter plate. Serial 1:2 dilutions of EOs ranging from 0.015 to $20 \mu$L/mL were added to the wells of a 96-well plate (100 µL/well). The MIC end point was defined as the lowest concentration that produced a prominent decrease in turbidity (100%) relative to that of the drug-free control.

The MFC was measured by taking 100 µL of cell suspension from each well after 48 h (for Candida spp.) or 72 h (for M. furfur) of incubation at 32 °C, and then they were centrifuged, washed three times with fresh medium and vortexed for 10s. The solution was cultured on a specific medium (SDA for Candida spp. and SDA + 1% Tween 80 for M. furfur) at 32°C for 72 h. The MIC value was defined as the MIC values of drugs at which no visible growth was detected. The MIC and MFC values of fluconazole (FLZ) were also detected as positive controls.

The negative control was yeast in broth without any antifungal. The experiment was repeated in duplicate three times on different days. Data obtained were reported as MIC ranges and MIC$_{90}$ which indicate EO or drug concentration that inhibits the growth of 90% of the isolates.

4.4. Inhibitory Effects of Cymbopogon citratus and Cymbopogon proximus EOs on Candida spp. and Malassezia furfur Biofilms

The biofilm reduction of Candida spp. and M. furfur by C. citratus and C. proximus EOs was evaluated according to a previously reported method [58]. Candida spp. and M. furfur biofilms were performed in microtiter plates by adding 100 µL of cell suspension (1 × 106 cells/mL) suspended in RPMI 1640 medium (Candida spp.) and in SAB supplemented with 1% Tween 80 (M. furfur) and incubated at 37 °C for 24 h for Candida spp.
and 48 h for M. furfur. The wells were then washed twice with sterile phosphate buffered saline (PBS) and 100 µL of RPMI or and SAB tween 1% containing C. citratus (10 and 20 µL/mL) or C. proximus EOs (80 and 40 µL/mL) or FLZ (16 and 8 µg/mL) were added. A medium (100 µL) without EOs was used as a negative control for biofilm growth. Microtiter plates were incubated at 37 °C for an additional 24 h for Candida spp. and 48 h for M. furfur. Then, the medium was removed, and the wells were washed twice with sterile PBS (200 mL per well). Semi-quantification of the fungal cell viability in wells of microtiter plates was calculated using a colorimetric XTT[2,3-bis-(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide reduction assay. XTT (Sigma-Aldrich, Milan, Italy) was prepared in a saturated solution at 0.5 g/L in PBS. The solution was filter sterilized with a filter with a pore size of 0.22 mm, aliquoted and stored at −80 °C. Prior to each assay, an aliquot of stock XTT was supplemented with menadione (10 mM stock to a final concentration of 1 µM; Sigma-Aldrich, Milan, Italy). 100 µL of XTT-menadione solution was added to each pre-washed biofilm and control well. The microtiter plates were incubated in the dark at 37 °C for 3 h. Following incubation, 80 µL of the resulting-colored supernatant was transferred to a new microtiter plate and the colorimetric change from XTT reduction was read in at 490 nm using a microtiter plate reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA). The results were reported as a percentage of biofilm inhibition using the following formula: % inhibition = [(control OD 490 nm − Test OD 490 nm)/control OD 490 nm] × 100.

4.5. In Vitro Antioxidant Activity of C. citratus and C. proximus EOs

The radical scavenging activity of the EOs against 2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) was determined as previously reported[59]. Briefly, 1 mL of EO solution in methanol (Sigma-Aldrich, Milan, Italy) ranging from 0.5 to 70 µg/mL was combined with 2 mL of methanol DPPH solution (0.1 mM). The obtained samples were mixed vigorously and kept in the dark for 60 min. The absorbance was measured at 517 nm using a double beam UV-VIS spectrophotometer (Shimadzu UV-1601, Kyoto, Japan). Methanol was used as a negative control. Ascorbic acid and Trolox were used as positive controls. The percentage inhibition of the DPPH radical was calculated according to the following formula: % Inhibition = [(control OD − sample OD)/control OD] × 100, where A is absorbance at 517 nm. The results were expressed as of EC50 (µg EO /mL), which is the concentration necessary to obtain a 50% reduction of DPPH• radical.

The 2,2′-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) radical scavenging activity of EOs was determined as previously reported[60]. ABTS•+ was generated by the reaction of a 7 mM aqueous solution of ABTS with 2.45 mM aqueous solution of K2S2O8 which was conducted in the dark at room temperature for 16 h before use. The ABTS•+ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. About 0.15mL of different concentrations of EOs was mixed with 2.85 mL of ethanolic solution of ABTS•+, and the absorbance was read at 734 nm using a spectrophotometer after 15 min. Ethanol was used as a negative control. Ascorbic acid and Trolox were used as positive controls. The ABTS•+ inhibition radical was calculated according to the following formula: % inhibition = [(control OD − sample OD)/control OD] × 100, where A is the absorbance at 734 nm. The results were expressed in terms of EC50 (µg EO /mL), which is the concentration necessary for 50% reduction of ABTS•+ radical. EC50 was calculated from the graph plotting the percentage of radical scavenging activity (DPPH or ABTS) against EO concentration (1.5, 2.5, 5, 10, 20, 30, 50 and 70 µg/mL).

4.6. Statistical Analysis

The statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows program (version 13.0, SPSS Inc., San Rafael, CA, USA). One-way analysis of variance (one-way ANOVA) with post-hoc Tukey HSD (Honestly Significant Difference) was used to compare the differences among the MIC, EC50, biofilm optical densities of different yeast species, and the percentage of biofilm inhibition of...
C. citratus and C. proximus EOs. Differences were considered statistically significant when p < 0.05.

5. Conclusions

Conclusively, this study suggests that C. citratus and C. proximus EOs could be considered an excellent source of pharmacology ingredients to treat aging-associated diseases caused by free radicals for their antioxidant activities and to treat or prevent fungal infections and in particular might be considered as a drug source for preventing long treating Malassezia spp. skin infections in both medical and veterinary medicine. This study confirms the potential benefits of the use of natural antioxidants as antibiofilm compounds. Further investigations on the mechanism of action of antioxidant agents in treating, preventing and eradicating fungal biofilm are required.

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