Article

Synergistic Activity of New Diclofenac and Essential Oils Combinations against Different Candida spp.

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Abstract: According to recent studies, Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) have shown a good antimicrobial and antifungal activity. Their association with essential oils (EOs) could be useful for the treatment of infections caused by Candida spp. The aim of this study is to evaluate the synergistic antifungal activity of new combinations between Diclofenac Sodium Salt (DSS), a widely used NSAID, with EOs of Mentha × piperita, Pelargonium graveolens and Melaleuca alternifolia. The in-vitro antifungal activity was determined on different Candida strains. The determination of the chemical composition of EOs was carried out by gas chromatography-mass spectrometry (GC-MS). Susceptibility testing of planktonic cells was performed by using the broth microdilution assay and checkerboard methods. Minimum Inhibitory Concentrations (MIC) of DSS was in a range from 1.02 to 2.05 µg/mL reaching a MIC value of 0.05 µg/mL when combined with Pelargonium graveolens (FICI = 0.23–0.35) or Mentha × piperita (FICI = 0.22–0.30) EOs. These preliminary results show that the combination of the EOs with DSS improves the antifungal activity on all the tested Candida strains.

Keywords: synergism; Mentha × piperita; Pelargonium graveolens; Melaleuca alternifolia; Diclofenac Sodium Salt

1. Introduction

Fungal infections should not be underestimated, since their incidence in recent years has increased significantly, especially in immunocompromised patients [1]. Moreover, among all nosocomial fungal infections, those caused by Candida spp. are the most difficult to eradicate. Indeed, infections caused by Candida spp. can spread and colonize different tissue districts, causing considerable damage up to the compromise of organ functions. Candidiasis and candidemia show a wide spectrum of clinical symptoms of different entities depending on whether they are: superficial infections, affecting the skin and mucous membranes, or of deep and widespread severity [2,3].

Current pharmacological therapies are focused on the use of conventional antifungals such as Amphotericin B [4,5] and synthetic drugs belonging to the azoles class (e.g., Clotrimazole, Ketoconazole, Miconazole) that could also be prescribed in combination with each other depending on the severity of the infection [6]. Recently, the activity of different drugs belonging to other therapeutic classes are being evaluated in the drugs-repositioning strategy as antimicrobials [7]. Drugs such as Promazine (phenothiazine antipsychotic), Promethazine (antihistamine), Methyldopa (centrally acting antidepressant), Dobutamine (sympathomimetic) and Diclofenac (NSAIDs) have shown an interesting antimicrobial activity, and for this reason they have been defined as non-antibiotic drugs [8–12]. According
to these results, Diclofenac, also known as (2-[2-(2,6-dichloroanilino)phenyl] acetic acid), one of the more effective cyclooxygenase enzymes (COX) inhibitors, was selected for this research. Indeed, COX inhibition leads to blockage of prostaglandins (PGs) biosynthesis, contributing to a variety of physiological and pathological functions. Furthermore, current studies show that PGs may play a pivotal role in the regulation of eicosanoids pathway in Candida spp. and because of an impairment of their metabolism, the inhibition of PGs synthesis by Diclofenac should cause the fungus death [13–15]. Based on this evidence, DSS could be able to reduce the infection, acting as a COX inhibitory agent for the treatment of Candida infections.

Recently, research on EOs, whose antifungal activity in traditional medicine has been well documented, has aroused the interest of many researchers. Several recent studies confirmed the potential of these natural products as antifungal agents [16]. Therefore, it is not surprising that EOs are regarded as one of the most promising groups of natural products useful for the development of new broad-spectrum, cheaper, and safer drugs for the treatment of mycosis [17]. Although the precise mechanism of the antifungal action of EOs is not yet explained, the plasma membrane and the cell wall appear to be particularly affected [18,19]. Among EOs, it is already known that Mentha × piperita L. [20], Pelargonium graveolens L’Hér. [21], and Melaleuca alternifolia (Maiden & Betche) Cheel [22,23] have antifungal properties.

Starting from these results, the aim of these preliminary studies is to assess the synergistic effects of a new combination of DSS and EOs against planktonic cells of Candida spp., revealing new strategies for the repositioning of this anti-inflammatory drug.

2. Results

2.1. EOs Chemical Composition

EOs used in this study were analyzed using GC-MS. Their chemical composition is described in Table 1.

### Table 1. Chemical composition of tested Essential Oils (Eos).

| N  | Components                              | LRI  | Al  | Pelargonium graveolens | Mentha × piperita | Melaleuca alternifolia |
|----|----------------------------------------|------|-----|------------------------|-------------------|-----------------------|
|    |                                        |      |     | AREA% ± SEM SI/MS      | AREA% ± SEM SI/MS | AREA% ± SEM SI/MS     |
| 1  | propanoic acid, ethylester             | 712  | 714 | 0.12 ± 0.012 86        | 0.11 ± 0.009 91   |                       |
| 2  | α-thujene                              | 924  | 926 | 0.04 ± 0.001 91        | 0.88 ± 0.020 91   |                       |
| 3  | α-pinene                               | 933  | 933 | 0.59 ± 0.050 97        | 1.40 ± 0.010 97   | 2.14 ± 0.120 96      |
| 4  | 1-methyl-3-(2-methyl-1-propenyl)-cyclopentane | 972  | 972 | 0.18 ± 0.050 80        |                   |                       |
| 5  | β-pinene                               | 975  | 975 | 1.43 ± 0.500 96        |                   |                       |
| 6  | trans-carene                           | 977  | 977 | 7.72 ± 2.110 91        |                   |                       |
| 7  | β-myrcene                              | 987  | 988 | 0.13 ± 0.100 91        |                   |                       |
| 8  | 2,6-dimethyl-2,6-octadiene             | 991  | 990 | 1.01 ± 0.090 96        |                   |                       |
| 9  | 3-octanol                              | 995  | 995 | 0.13 ± 0.150 90        |                   |                       |
| 10 | ν-cymene                               | 1021 | 1021| 0.10 ± 0.005 91        | 0.44 ± 0.050 95   |                       |
| 11 | p-cymene                               | 1025 | 1025| 2.21 ± 0.990 95        |                   |                       |
| 12 | (Z)–β-ocimene                         | 1027 | 1027| 0.10 ± 0.007 95        |                   |                       |
| 13 | 3-isopropenyl-5,5-dimethyl-cyclopentene| 1029 | 1028| 1.68 ± 0.030 81        | 1.68 ± 0.030 81   |                       |
| 14 | 1,8-cineole                            | 1031 | 1031| 9.07 ± 2.090 98        | 2.13 ± 0.700 98   |                       |
| 15 | limonene                               | 1033 | 1033| 0.22 ± 0.040 94        |                   |                       |
| 16 | β-phellandrene                         | 1035 | 1035| 0.53 ± 0.010 91        | 0.23 ± 0.005 91   |                       |
| 17 | γ-terpinene *                         | 1058 | 1060| 0.11 ± 0.002 96        | 17.18 ± 2.120 94  |                       |
| 18 | cis-linalool oxide                     | 1070 | 1074| 0.37 ± 0.001 90        |                   |                       |
| 19 | α-terpinolene                          | 1081 | 1082| 3.80 ± 0.020 96        |                   |                       |
| 20 | linalol                                | 1099 | 1098| 4.68 ± 0.850 95        |                   |                       |
| 21 | rose oxide                             | 1112 | 1112| 1.67 ± 0.050 90        |                   |                       |
| 22 | cis-p-menth-2-en-1-ol                  | 1119 | 1119| 0.33 ± 0.005 93        |                   |                       |
| 23 | p-menthone                             | 1154 | 1154| 2.19 ± 0.970 98        |                   |                       |
| 24 | iso-menthone *                         | 1164 | 1165| 4.61 ± 1.700 97        | 23.99 ± 2.490 97  |                       |
### Table 1. Chemical composition of tested Essential Oils (Eos).

| N  | Components                        | Pelargonium graveolens | Mentha × piperita | Melaleuca alternifolia |
|----|-----------------------------------|------------------------|-------------------|------------------------|
|    |                                   | AREA% ± SEM            | SI/MS             | AREA% ± SEM            | SI/MS          | AREA% ± SEM            | SI/MS          |
| 25 | menthol *                         | 0.14 ± 0.003           | 91                | 35.60 ± 1.760          | 91             | 33.28 ± 2.750          | 83             |
| 26 | terpinen-4-ol *                   |                        |                   |                        |                |                        |                |
| 27 | isopulegone                        | 0.16 ± 0.002           | 96                |                        |                | 0.59 ± 0.010           | 87             |
| 28 | neo-iso-menthol                    | 9.33 ± 1.100           | 96                |                        |                | 3.84 ± 0.350           | 86             |
| 29 | α-terpineol                       | 0.45 ± 0.090           | 80                | 0.69 ± 0.100           | 86             | 1.21 ± 0.400           | 98             |
| 30 | citral                            | 0.70 ± 0.001           | 96                |                        |                |                        |                |
| 31 | piperitone                          |                        |                   |                        |                |                        |                |
| 32 | methyl acetate                     | 1.20 ± 0.030           | 96                |                        |                |                        |                |
| 34 | geraniol *                         | 11.70 ± 1.020          | 96                |                        |                |                        |                |
| 35 | citronellyl formate                | 6.85 ± 0.920           | 96                |                        |                |                        |                |
| 36 | geraniol formate                   | 2.69 ± 0.100           | 86                |                        |                |                        |                |
| 37 | 1,5,5-trimethyl-6-methylen-cyclohexene | 0.40 ± 0.005       | 91                |                        |                |                        |                |
| 38 | 1-H-indene-1-ethylidenedecata      | 0.33 ± 0.070           | 86                |                        |                |                        |                |
| 39 | citronellyl acetate                | 0.48 ± 0.050           | 94                |                        |                |                        |                |
| 40 | neryl acetate                      | 1.47 ± 0.250           | 86                |                        |                |                        |                |
| 41 | isolatedene                        | 1.07 ± 0.090           | 95                |                        |                |                        |                |
| 42 | β-bourbonene                       | 1.80 ± 0.140           | 95                |                        |                |                        |                |
| 43 | langifolene                        | 0.12 ± 0.009           | 90                |                        |                |                        |                |
| 44 | 1-H-indene-1-ethylidenedecata      | 0.64 ± 0.040           | 95                |                        |                |                        |                |
| 45 | α-guaiene                          | 0.39 ± 0.003           | 98                |                        |                |                        |                |
| 46 | (E)-caryophyllene                  | 1.63 ± 0.020           | 99                | 2.13 ± 0.950           | 99             | 1.09 ± 0.013           | 99             |
| 47 | β-copaene                          | 1.06 ± 0.015           | 99                |                        |                |                        |                |
| 48 | neryl propionate                   | 0.15 ± 0.023           | 80                |                        |                |                        |                |
| 49 | aromadendrene                      | 0.70 ± 0.090           | 99                | 4.41 ± 1.090           | 99             |                        |                |
| 50 | citronellyl propionate             | 1.06 ± 0.030           | 64                |                        |                |                        |                |
| 51 | humulene                           | 0.38 ± 0.003           | 97                | 0.12 ± 0.090           | 95             | 0.20 ± 0.001           | 97             |
| 52 | α-—amorphene                       | 0.87 ± 0.025           | 96                |                        |                | 0.32 ± 0.015           | 99             |
| 53 | (E)-β-farnesene                    | 0.10 ± 0.080           | 95                |                        |                |                        |                |
| 54 | γ—murolene                         | 0.73 ± 0.055           | 90                | 0.15 ± 0.090           | 83             |                        |                |
| 55 | epi-bicyclosesquiphellandrene      | 1.00 ± 0.078           | 87                |                        |                |                        |                |
| 56 | 4,11-selinadiene                   | 0.18 ± 0.074           | 92                |                        |                |                        |                |
| 57 | δ—selinene                         | 0.17 ± 0.007           | 97                |                        |                |                        |                |
| 58 | lideon                             | 3.93 ± 1.670           | 95                |                        |                |                        |                |
| 59 | δ—cadiene                          | 2.98 ± 0.430           | 95                |                        |                |                        |                |
| 60 | α-panasinsene                      | 0.16 ± 0.009           | 93                |                        |                |                        |                |
| 61 | α—calacorene                       | 0.11 ± 0.001           | 91                |                        |                |                        |                |
| 62 | geranyl butyrate                    | 1.49 ± 0.012           | 96                |                        |                |                        |                |
| 63 | neo-isolongifolene                 | 0.18 ± 0.004           | 83                |                        |                |                        |                |
| 64 | spathulenol                        | 0.35 ± 0.002           | 91                | 0.11 ± 0.008           | 99             |                        |                |
| 65 | phenethyl tiglate                  | 1.48 ± 0.015           | 90                |                        |                |                        |                |
| 66 | globulol                           | 0.54 ± 0.001           | 98                |                        |                |                        |                |
| 67 | caryophyllene oxyde                | 0.28 ± 0.070           | 95                |                        |                |                        |                |
| 68 | γ—eudesmol *                       | 7.02 ± 2.050           | 99                |                        |                |                        |                |
| 69 | (E)-citronellyl tiglate            | 0.38 ± 0.009           | 91                |                        |                |                        |                |
| 70 | geranyl tiglate                    | 1.57 ± 0.080           | 91                |                        |                |                        |                |

% Characterized: 89.40, 96.22, 82.78

*Others*: 10.60, 3.78, 17.22

*: standard compounds. Linear retention index (LRI) on HP-5MS column was experimentally determined using a homologous series of C7–C40 alkanes standard mixture [24]. Arithmetic index (AI) was taken from Adams 4th Ed. (2007) [25] and/or the NIST 2017 Database [26]. Similarity index/mass spectrum (SI/MS) was compared with data reported on NIST 2017 Database and were determined as reported by Koo et al. [27], and Wan et al. [28]. Relative percentage values are means of three determinations with a structural equation modeling (SEM) in all cases below 10%.
About 45 compounds were identified in *P. graveolens* EO corresponding to 89.4% of the whole mixture. This EO was characterized by citronellol (26.5%), geraniol (11.7%), γ-eudesmol (7.02%), citronellyl formate (6.85%), linalol (4.68%) and iso-menthone (4.61%). Other compounds accounted for less than 2%. They were identified as β-bourbonene (1.8%), rose oxide (1.67%), (*E*)-caryophyllene (1.63%), geranyl formate and geranyl tiglate (1.57% both) and 2-phenylethyl tiglate (1.48%).

Pure *M. piperita* EO was characterized for 96% of its composition. Menthol (35.6%) and *neo*-menthol (9.33%) were the major components. Other compounds present in relevant amount were menthone (23.99%), 1,8-cineole (9.70%), *trans*-carene (7.72%) and (*E*)-caryophyllene (2.13%). Several compounds, such as α-pinene, β-pinene, piperitone and pulegone were present in an amount less than 2%, while others are in traces.

*M. alternifolia* EO was characterized for 82.78%. The major relevant compound was terpinen-4-ol (33.4%). γ-Terpinene accounted for 17.18% of the mixture, followed by aromadendrene (4.41%), ledene (3.93%), and α-terpinolene (3.80%). Several compounds such as α-pinene, *p*-cymene and δ-cadinene comprised about 2% of the mixture, while (*E*)-caryophyllene and isoledenne were about 1%.

### 2.2. Antifungal Activity

In this research, DSS was combined with different EOs to inhibit the fungal growth. The antifungal activity as MIC (minimal inhibitory concentration) of these combinations were reported in Tables 2–4. The FIC Index (FICI), a parameter that studies the synergism of two compounds, was also reported. Considering the combination between DSS and EOs, the lowest FICI values are 0.22 for *M. piperita* EO, 0.23 for *P. graveolens* and *M. alternifolia*. It is interesting to note that the concentration in µg/mL of DSS decreases from 2.05 to 0.06 when combined with *M. piperita* EO, to 0.05 in combination with *P. graveolens* EO and to 0.10 in association with *M. alternifolia* EO (Tables 2–4).

| Strains          | EO MIC ± SD | DSS MIC ± SD | DSS µg/mL | EO µg/mL | FICI  
|------------------|-------------|--------------|-----------|----------|-------
| *C. albicans* ATCC 10231 | 1.00 ± 0.480 | 1.02 ± 0.350 | 0.51  | 0.05  | 0.30  
| *C. albicans* ATCC 90028 | 1.00 ± 0.450 | 1.02 ± 0.370 | 0.51  | 0.05  | 0.30  
| *C. glabrata* ATCC 15126 | 1.00 ± 0.500 | 2.05 ± 0.790 | 0.10  | 0.51  | 0.30  
| *C. tropicalis* ATCC 750 | 1.00 ± 0.450 | 1.02 ± 0.350 | 0.20  | 0.06  | 0.22  
| *C. kefyr* ATCC 20493 | 0.25 ± 0.020 | 2.05 ± 0.800 | 0.20  | 0.13  | 0.30  
| *C. krusei* ATCC 6258 | 0.50 ± 0.030 | 1.02 ± 0.390 | 0.06  | 0.31  | 0.30  
| *C. albicans* A18 | 1.00 ± 0.080 | 2.05 ± 0.500 | 0.10  | 0.51  | 0.30  
| *C. albicans* 10A12 | 0.50 ± 0.030 | 1.02 ± 0.310 | 0.20  | 0.13  | 0.30  
| *C. albicans* 810 | 1.00 ± 0.20 | 1.02 ± 0.250 | 0.20  | 0.13  | 0.30  
| *C. krusei* 31A29 | 1.00 ± 0.310 | 2.05 ± 0.620 | 0.41  | 0.25  | 0.30  
| *C. parapsilosis* 11A13 | 1.00 ± 0.060 | 1.02 ± 0.200 | 0.05  | 0.51  | 0.30  
| *C. parapsilosis* 1A1 | 0.50 ± 0.020 | 2.05 ± 0.830 | 0.41  | 0.13  | 0.30  
| *C. parapsilosis* 911 | 0.25 ± 0.060 | 1.02 ± 0.270 | 0.10  | 0.06  | 0.22  
| *C. parapsilosis* 910 | 0.12 ± 0.040 | 1.02 ± 0.410 | 0.10  | 0.03  | 0.22  
| *C. tropicalis* 810 | 0.50 ± 0.020 | 1.02 ± 0.450 | 0.10  | 0.12  | 0.22  

*a*: MIC minimal inhibitory concentration (%v/v for EO; µg/mL for DSS); *b*: concentration of DSS in the mixture; *c*: concentration of essential oil in the mixture; *d*: FICI: fractional inhibitory concentration index; DSS: Diclofenac Sodium Salt; EO: Essential Oil; SD: Standard Deviation.
Table 3. Antifungal activity of \textit{P. graveolens} Essential Oil (EO) and Diclofenac Sodium Salt (DSS) on different \textit{Candida} strains.

| Strains                  | EO MIC $^a$ ± SD | DSS MIC $^a$ ± SD | DSS µg/mL $^b$ | Synergism EO µg/mL $^c$ | Synergism FICI $^d$ |
|--------------------------|-----------------|------------------|----------------|-------------------------|-------------------|
| \textit{C. albicans} ATCC 10231 | 0.12 ± 0.021    | 1.02 ± 0.350     | 0.10           | 0.03                    | 0.23              |
| \textit{C. albicans} ATCC 90028  | 0.25 ± 0.017    | 1.02 ± 0.370     | 0.20           | 0.06                    | 0.30              |
| \textit{C. glabrata} ATCC 15126  | 0.25 ± 0.015    | 2.05 ± 0.790     | 0.20           | 0.06                    | 0.23              |
| \textit{C. tropicalis} ATCC 750    | 0.12 ± 0.013    | 1.02 ± 0.350     | 0.10           | 0.03                    | 0.23              |
| \textit{C. krusei} ATCC 204093  | 0.12 ± 0.014    | 2.05 ± 0.800     | 0.10           | 0.06                    | 0.30              |
| \textit{C. albicans} A18         | 0.25 ± 0.021    | 2.05 ± 0.500     | 0.41           | 0.06                    | 0.33              |
| \textit{C. albicans} 10A12       | 0.12 ± 0.012    | 1.02 ± 0.310     | 0.20           | 0.03                    | 0.30              |
| \textit{C. albicans} 810         | 0.12 ± 0.010    | 1.02 ± 0.250     | 0.10           | 0.03                    | 0.30              |
| \textit{C. krusei} 31A29         | 0.50 ± 0.084    | 2.05 ± 0.620     | 0.41           | 0.12                    | 0.30              |
| \textit{C. parapsilosis} 11A13   | 0.50 ± 0.082    | 1.02 ± 0.200     | 0.20           | 0.06                    | 0.30              |
| \textit{C. parapsilosis} 1A1     | 0.25 ± 0.070    | 2.05 ± 0.830     | 0.41           | 0.06                    | 0.26              |
| \textit{C. parapsilosis} 911     | 0.25 ± 0.072    | 1.02 ± 0.270     | 0.20           | 0.03                    | 0.30              |
| \textit{C. parapsilosis} 910     | 0.25 ± 0.079    | 1.02 ± 0.410     | 0.05           | 0.12                    | 0.30              |
| \textit{C. tropicalis} 810       | 0.25 ± 0.052    | 1.02 ± 0.450     | 0.10           | 0.12                    | 0.35              |

$^a$: MIC minimal inhibitory concentration (%v/v for EO; µg/mL for DSS); $^b$: concentration of DSS in the mixture; $^c$: concentration of essential oil in the mixture; $^d$: FICI: fractional inhibitory concentration index; DSS: Diclofenac Sodium Salt; EO: Essential Oil; SD: Standard Deviation.

Table 4. Antifungal activity of \textit{M. alternifolia} Essential Oil (EO) and Diclofenac Sodium Salt (DSS) on different \textit{Candida} strains.

| Strains                  | EO MIC $^a$ ± SD | DSS MIC $^a$ ± SD | DSS µg/mL $^b$ | Synergism EO µg/mL $^c$ | Synergism FICI $^d$ |
|--------------------------|-----------------|------------------|----------------|-------------------------|-------------------|
| \textit{C. albicans} ATCC 10231 | 0.50 ± 0.021    | 1.02 ± 0.350     | 0.20           | 0.25                    | 0.45              |
| \textit{C. albicans} ATCC 90028  | 0.50 ± 0.020    | 1.02 ± 0.370     | 0.10           | 0.13                    | 0.23              |
| \textit{C. glabrata} ATCC 15126  | 0.50 ± 0.012    | 2.05 ± 0.790     | 0.20           | 0.13                    | 0.23              |
| \textit{C. tropicalis} ATCC 750    | 0.50 ± 0.015    | 1.02 ± 0.350     | 0.20           | 0.03                    | 0.23              |
| \textit{C. krusei} ATCC 204093  | 1.00 ± 0.112    | 2.05 ± 0.800     | 0.82           | 0.51                    | //                |
| \textit{C. krusei} ATCC 6258    | 0.50 ± 0.025    | 1.02 ± 0.390     | 0.40           | 0.25                    | //                |
| \textit{C. albicans} A18         | 0.25 ± 0.001    | 2.05 ± 0.500     | 0.82           | 0.15                    | 0.43              |
| \textit{C. albicans} 10A12       | 0.50 ± 0.025    | 1.02 ± 0.310     | 0.20           | 0.25                    | 0.45              |
| \textit{C. albicans} 810         | 0.50 ± 0.022    | 1.02 ± 0.250     | 0.40           | 0.06                    | 0.45              |
| \textit{C. krusei} 31A29         | 0.50 ± 0.027    | 2.05 ± 0.620     | 0.82           | 0.25                    | //                |
| \textit{C. parapsilosis} 11A13   | 0.50 ± 0.023    | 1.02 ± 0.200     | 0.05           | 0.25                    | 0.30              |
| \textit{C. parapsilosis} 1A1     | 0.50 ± 0.030    | 2.05 ± 0.830     | 0.20           | 0.25                    | 0.35              |
| \textit{C. parapsilosis} 911     | 0.50 ± 0.042    | 1.02 ± 0.270     | 0.05           | 0.25                    | 0.30              |
| \textit{C. parapsilosis} 910     | 0.50 ± 0.050    | 1.02 ± 0.410     | 0.40           | 0.03                    | 0.43              |
| \textit{C. tropicalis} 810       | 0.50 ± 0.045    | 1.02 ± 0.450     | 0.20           | 0.25                    | 0.45              |

$^a$: MIC minimal inhibitory concentration (%v/v for EO; µg/mL for DSS); $^b$: concentration of DSS in the mixture; $^c$: concentration of essential oil in the mixture; $^d$: FICI: fractional inhibitory concentration index; DSS: Diclofenac Sodium Salt; EO: Essential Oil; SD: Standard Deviation.

3. Discussion

The emergence and development of antifungal drug resistance in \textit{Candida} spp. constitute a serious concern. A successful combination of therapy for the treatment of fungal infectious diseases can achieve broader antifungal coverage and potentially reduce acquired resistance. The combination of repositioned drugs with EOs is also an interesting approach for the rapid identification of new therapies to treat acute infections. Several studies demonstrated that NSAIDs exhibited antifungal activity against \textit{Candida} species alone or in combination with antifungal agents [29,30]. The antifungal activity of NSAIDs is conceivably related to the inhibition of the COX leading to decrease the levels of prostaglandins that
are known to be produced by Candida spp. Among NSAIDs, DSS is an anti-inflammatory drug whose activity on eukaryotic fungal cells was likely determined by an impairment of PGs metabolism. In fact, DSS causes an inhibition of prostaglandin synthesis. Due to their potential therapeutic effects, EOs are widely used as alternative antimicrobial agents against various infections.

Our previous studies on EOs showed their synergy with some commercially available antibiotics and demonstrated the effectiveness of these associations by proposing the possibility of a new therapeutic use [31–35].

In the present study, we reported the effect of DSS in combination with EOs of M. piperita, P. graveolens and M. alternifolia on the growth of Candida spp. from ATCC collection and clinical isolation. As highlighted in our in-vitro assays, Candida spp. planktonic cells have shown their sensitivity to the compounds tested, both individually and in combination. Tables 2–4 show the antifungal activity against Candida spp. of DSS alone or in combination with EOs tested. The results obtained allow us to confirm the synergistic effect between DSS and the EOs under study. Indeed, the data clearly show a significant reduction in the active concentration of NSAID when used in association with EOs for all fungal strains tested. It is noteworthy that, when tested in association with M. piperita EO, the MIC value for DSS is reduced from 1.02 µg/mL to 0.05 µg/mL and from 1.02 µg/mL to 0.06 µg/mL for C. parapsilosis 11A13 and C. krusei ATCC 6258, respectively. With regard to the association with P. graveolens EO, it is particularly noteworthy that the MIC value of DSS is reduced from 1.02 µg/mL to 0.05 µg/mL for C. parapsilosis 910. Interestingly, the MIC value of DSS is reduced from 1.02 µg/mL to 0.05 µg/mL for C. parapsilosis 11A13 and C. parapsilosis 910, when tested in association with M. alternifolia EO. These promising results obtained allow us to confirm the synergistic effect between DSS and the EOs under study. This activity should be ascribed to the presence of fundamental active compounds in EOs such as terpene alcohols and hydrocarbons acting in association with DSS. The mechanism of action is conceivably multifactorial, deriving from the complex synergy of the components. As reported in several scientific works, the synergy of EO could be explained by their ability to disrupt the permeability barrier of the microbial plasma membrane [18,19]. This disruption could conceivably facilitate the entry of DSS into the microbial cell, thus interacting with the COX systems and ultimately causing its antifungal action.

4. Material and Methods

4.1. Material

The pure M. piperita EO (LOT F011023, 10/2023), the pure P. graveolens EO (LOT F810074, 07/2022) and the pure M. alternifolia EO (F911010, 04/2024) were provided by Puressentiel Italia (Milano, Italy) and were stored in a brown glass bottle at the temperature of 0–4°C until the testing analysis or microbiological assays. The DSS was purchased from Farmalabor (Canosa di Puglia—Bari, Italy). Solvents (analytical grade), n-alkanes standard mixture C7–C40 and all standard compounds (17, 24–26, 30, 34 and 68 listed in Table 1) used to compare GC-MS analyses were purchased from Supelco Sigma-Aldrich S.r.l. (Milano, Italy). Filters were supplied by Agilent Technologies Italia S.p.A (Milano, Italy). The culture media used were Sabouraud 2% dextrose broth (Oxoid, Italy) and Yeast Malt Broth (Oxoid, Italy). The antifungal activity was tested against many fungal strains and include different strains belonging to the American Type Culture Collection (ATCC, Rockville, MD, USA) or derived from clinical isolation. Strains from the ATCC were C. albicans (ATCC 10231), C. albicans (ATCC 90028), C. glabrata (ATCC 15126), C. tropicalis (ATCC 750), C. kefyr (ATCC 204093), C. krusei (ATCC 6258). All the isolates were from patients admitted to the intensive care unit of the Department of Biomedical Science and Human Oncology, University of Bari, Italy. The isolation and identification procedures were conducted in the Hygiene Section of the Department. Using conventional physiological and morphological methods (API systems), the strains were identified as C. albicans A18, C. albicans 10A12, C. albicans 810, C. krusei 31A29, C. parapsilosis 11A13, C. parapsilosis 1A1, C. parapsilosis 911, C. parapsilosis 910.
and C. tropicalis 810. All strains were grown and maintained on Sabouraud dextrose broth (Oxoid, Italy) at 37 °C.

4.2. Methods

4.2.1. Gas Chromatography and Mass Spectrometry Equipment

Gas chromatographic analysis of EOs were performed on an Agilent 6890 N gas chromatograph equipped with a 5973 N mass spectrometer, provided with a HP-5 MS (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 µm film thickness; J & W Scientific, Folsom) capillary column. The following temperature programmer was used: 5 min at 60 °C, then 4 °C/min to 220 °C, then 11 °C/min to 280 °C, held for 15 min, for a total run of 65 min. Injector and detector temperatures were 280 °C; the carrier gas was He; the flow rate was 1 mL/min; the split ratio was 1:50; the acquisition range was 29–400 m/z in electron-impact (EI) mode; and the ionization voltage was 70 eV.

4.2.2. Compound Identification

For chemical characterization, EOs were diluted 1:100 in ethyl acetate and after filtration, 1 µL of each EO solution was injected into the GC-MS. Identification of the EOs’ components was done by comparison with authentic standards available in the authors’ laboratory. Qualitative analyses were carried out comparing the calculated Linear Retention Indices (LRIs) and Similarity Index Mass Spectra (SI/MS) for the obtained peaks with the analogous data from NIST 2017 and Adams 4th ed. (2007) databases. LRI of each compound was obtained by temperature programming analysis and was calculated in relation to a homologous series of n-alkanes (C7–C40) under the same operating conditions. LRI was calculated following the Van den Dool and Kratz equation [22] and compared with the Arithmetic Index (AI) from NIST 2017 database [26] and Adams, 4th ed. (Adams 2007) [25]. SI/MS were determined as reported by Koo et al. [27]. Component relative percentages were calculated based on GC peak areas without using correction factors.

4.2.3. Preparation of The Test Solution

The EOs are solubilized in ethanol in 1:5 proportions and then diluted in Sabouraud added with tween 80. DSS should be solubilized in DMSO and subsequently in culture medium.

4.2.4. Antifungal and Susceptibility Tests

The antifungal activity of DSS was evaluated using a microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI, M27-A3) [36]. Four double serial dilutions of the EOs were prepared following the same method used to evaluate the MIC described in our previous works [31,32]. Minimum inhibition concentration (MIC) determinations were made in triplicate. Two-fold serial dilutions of the NSAID were made with Yeast Malt Broth (YMB) to give concentrations ranging from 2.05 µg/mL to 0.03 µg/mL. MICs indicating the bacteriostatic effect of the DSS were obtained following incubation at 37 °C for 48 h. MICs were recorded as the lowest concentration of tested compound that completely inhibited fungal growth.

4.2.5. Checkerboard Test

The checkerboard method was utilized to determine the synergistic, additive, or antagonistic effects of the combination of DSS and EOs. The tested dilutions were based on the MIC of the two substances. The combination of two compounds was synergistic when the FICI was ≤0.5, additive when the FICI was >0.5 and <1, and antagonistic when the FICI was >1. The test was performed using sterile 96-well microtiter plates containing DSS and EOs in two-fold serial concentrations. MICs were obtained following incubation at 37 °C for 48 h.
Each test was performed in triplicate. A synergistic effect (FICI ≤ 0.5) between the two compounds is indicated as a concave curve, additive (FICI >0.5 and <1) interactions are represented by a straight line, and a convex curve indicates antagonism (FICI ≥ 1). This procedure allowed to evaluate with accurately the effect of synergism on the fungal growth.

4.3. Statistical Analysis

Every experiment for GC-MS has been replicated three times across three different days. The microbiological assays were performed for five times in five different days, giving an amount of 25 replicates.

Statistical analysis for microbiological assay (standard deviation, SD) and for chemical determination of structural equation modeling (SEM) was performed using Microsoft Excel.

5. Conclusions

The synergistic associations of drugs represent a valid approach in the antimicrobial therapies that have provided positive results in recent years. The rediscovery of natural products and their use in medical practice is quite recent and derives above all from the need to overcome the undesirable effects induced by conventional antimicrobials. The success of therapies based on natural products of plant origin has been scientifically evaluated with irrefutable research protocols in laboratory settings as well as in clinical practice. Our previous studies on EOs, based on the synergy with antibiotics, demonstrated the effectiveness of these associations by proposing the possibility of their possible therapeutic use. The data reported in this study underline that EOs, commonly sold and distributed, possess in vitro a decisive and strong action towards fungal *Candida* cells, belonging to different species in association with DSS, an NSAID whose activity against *Candida* spp. has been successfully confirmed. Results obtained indicate that small quantities of DSS and EO in association possess an excellent inhibitory capacity towards different strains of *Candida* spp. The effectiveness is conceivably the result of a multifactorial action, which escapes any resistance mechanisms that are now widespread and increasingly worrying. The in-vitro assays of these associations validate a sure efficacy against *Candida* infection, hither to never treated in scientifically proven research works. Further studies in the sector of EOs in association with NSAIDs are necessary to give us a better understanding of these phenomena related to fungal antibiosis from combinations of drugs and natural products. In this context our results may represent an interesting starting point for an alternative route to new synergistic antifungal therapies against fungal infections, overcoming the high cost of new drugs and the potential risk of antagonistic interactions. We are confident that these finding could represent a valid alternative to protect human health from infectious diseases.

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Abbreviations

DSS  Diclofenac Sodium Salt
Eos  Essential Oils
GC  Gas Chromatography
MS  Mass Spectrometer
SEM  Structural Equation Modeling
LRI  Linear Retention Indices
AI  Arithmetic Index
SI/MS  Similarity Index/Mass Spectra
MIC  Minimal Inhibitory Concentration
FICI  fractional inhibitory concentration

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