In vitro Susceptibility of Clinical Aspergillus Species to Some Antifungal Agents

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ABSTRACT
Aspergillus species are regularly involved in human broncho-pulmonary diseases, mainly in immunocompromised patients. The essential oils extracted from three different plants were tested for their inhibitory effect on the growth of five pathogenic Aspergilli including Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus versicolor and Aspergillus terreus which are pathogens with clinical importance. The results of the antimicrobial tests showed that the isolated essential oils inhibited the growth of Aspergillus species to varying degrees. The Ocimum basilicum oil completely inhibited the mycelial growth and spore germination of A. terreus and A. fumigatus at 200 and 250 ppm. In addition, this oil showed a very strong antifungal activity against the mycelial growth and spore germination of A. flavus and A. versicolor with 97.7, 97.5, 91.2 and 93.5%, respectively, when compared with control. The essential oil extracted from Eucalyptus globulus also exhibited significant antifungal activity against the Aspergillus species tested. This oil completely inhibited the mycelial growth and spore germination of A. fumigatus. Conversely, Nigella sativa oil exhibited moderate inhibitory activity against all the tested Aspergillus species. The present study supports the proposition that plant essential oils may have a role in both pharmaceutical and agricultural practices.

Key words: Nigella sativa, Aspergillus niger, essential oils, spore germination
potential of essential oils has been of considerable interest to the pharmaceutical industry. In addition, their use as alternative anti-infective agents has emerged from a growing trend to reduce the utilization of antibiotics against human pathogenic fungi (Abadio et al., 2011; Celikel and Kavas, 2008; Mohammadpour et al., 2012). The aim of this study was to evaluate the antifungal activity of some plant essential oils against five Aspergillus species. The median lethal dose (LD_{50}) values of the antifungal activity against these pathogens are available.

**MATERIALS AND METHODS**

**Tested microorganisms:** In this study, five clinical Aspergillus species responsible for human cutaneous infection and representative of medically relevant was provided by the Microbiology and Parasitology Department, Riyadh Military Hospital, Riyadh, Saudi Arabia. Isolates were cryopreserved in liquid nitrogen until the time of use. Before the experiments, the fungi were transferred to Potato dextrose agar media and incubated for 7 days in duplicate.

**Extracts and essential oils extraction:** The essential oils were extracted from Nigella sativa (seeds), Eucalyptus globulus (dried leaves) and Ocimum basilicum (dried leaves) using microwave-assisted hydrodistillation (30 min, 250 mL water) with a Clevenger-type distillation device and a Dean-Stark distillation trap in a domestic microwave oven (Stashenko et al., 2004). The extracts were obtained by soaking 50 g of the dried leaves or seeds in 200 mL of ethanol for 7 days at 28°C. The mixture was then filtered and concentrated using a rotary evaporator (Hei-VAP, Heidolph, Germany). Stock solutions of 40 and 20 mg mL^{-1} of the extracts and oils, respectively, were prepared in dimethyl sulfoxide for subsequent bioassays.

**Inhibitory effect of plant essential oils on the radial growth of fungal pathogens:** The antifungal activities of the essential oils against the test fungi were studied using a dual culture assay. Potato Dextrose Agar (PDA) was autoclaved and cooled to near 45°C. The plant essential oils were mixed with the sterile PDA to obtain final concentrations of 0, 100, 150, 200 and 250 ppm. Tween 80 (0.5%) was used as a surfactant to disperse the oil in the PDA which was poured in petri plates. Mycelial disks of 3 mm diameter cut out from the periphery of 7 days old cultures of the tested fungi, were aseptically inoculated upside down on the PDA. The plates were incubated at 27±2°C and there were four replicates per treatment. The growth of the tested fungi was recorded for 7 days and the percentage inhibition of the mycelial growth was computed by comparison with the control. The values were calculated using the following equation:

\[
\text{Inhibition} \%(\%) = \left(\frac{\text{Md} - \text{F}}{\text{Md}}\right) \times 100
\]

where, Md and F are the mean diameters of the mycelial growth of the control and the treatment groups, respectively. The median effective dose (ED_{50}) and ED_{95} were also determined.

**Inhibitory effect of plant essential oils on conidial germination of fungal pathogens:** The essential oils tested were dissolved in 0.1% Tween 80 in a 20 mL sterile glass tubes containing 5 mL potato dextrose broth to obtain the final concentrations (100, 150, 200 and 250) and 0.1% Tween 80 (v/v) without essential oil was used as the control (Elgorban et al., 2015). A total of 100 µL of conidia (1×10^6 spores mL^{-1}) fungal pathogens were added to each tube. Following a 24 h incubation at 27±2°C on a shaker (200 rpm), 100 conidia spores per replicate were observed microscopically to determine the germination rate. Four replicates were evaluated for every treatment and experiments were performed three times. The ED_{50} and ED_{95} were also determined.

**Statistical analysis:** Antifungal experiments were performed in triplicate and the data was analyzed using the statistics for the social sciences (SPSS) software and are presented as the Mean±Standard error of the mean (SEM). The ED_{50}, ED_{95} and slope of the activity of the essential oils against Aspergillus species were obtained using a probit analysis.

**RESULTS**

**Inhibitory effect of essential oils against A. terreus:** The data shown in Table 1 revealed that the O. basilicum oil completely inhibited the linear growth of A. terreus at concentrations of 200 and 250 ppm (ED_{50} 111.8 ppm L^{-1}, ED_{95} 232.3 ppmL^{-1} and slope 5.19±0.46). The oils of N. sativa and E. globulus significantly inhibited the radial growth of A. terreus. The N. sativa oil was more effective as an antifungal agent than the eucalyptus oil against the pathogen at a concentration of 250 ppm. The O. basilicum oil completely suppressed spore germination at concentrations of 150, 200 and 250 ppm (ED_{50} 86.5 ppm L^{-1}, ED_{95} 129.0 ppm L^{-1} and slope, 9.46±4.01). The efficacy of E. globulus and N. sativa followed with a 95.0 and 90.2% inhibition of spore germination, respectively, at 250 ppm.

**Inhibitory effect of essential oils against A. versicolor:** The oil of O. basilicum highly repressed the mycelial growth and spore germination of A. versicolor at 92.1% (ED_{50} 39.0 ppm L^{-1}, ED_{95} 455.8 ppm L^{-1} and slope, 2.38±0.21) and 93.5% (ED_{50} 110.6 ppm L^{-1}, ED_{95} 262.6 ppm L^{-1} and slope 4.38±0.26), respectively and this was significantly compared with the control. The oil extract of E. globulus significantly inhibited the mycelial growth of A. versicolor and showed a moderate effect on sporulation (76.7 and 74.8%, respectively), compared with the control (Table 2). Nigella sativa oil produced the lowest inhibition of the mycelial growth of A. versicolor and moderately reduced the spore germination with 59.9 and 67.8% inhibition, respectively compared with the control.
Inhibitory effects of the essential oils against *A. flavus*: The oil extracted from *O. basilicum* showed fungicidal activity against the mycotoxigenic *A. flavus* (Table 3). This oil completely suppressed the radial growth and spore germination of *A. flavus* at concentrations of 200 and 250 ppm (ED$_{50}$ 87.8 ppm L$^{-1}$, ED$_{95}$ 188.8 ppm L$^{-1}$ and slope 4.94±0.62). This efficacy was followed by that of *E. globulus* with 100% inhibition of mycelial growth (ED$_{50}$ 160.6 ppm L$^{-1}$, ED$_{95}$ 289.9 ppm L$^{-1}$ and slope 7.87±0.41) and spore germination of the pathogen at a concentration of 250 ppm compared to the control. The oil of *N. sativa* significantly reduced the mycelial growth of *A. flavus* (18.5%) and completely inhibited the spore germination of the fungus at a concentration of 250 ppm.

**Inhibitory effect of the essential oils against *A. flavus*:** The *E. globulus* oil at concentrations of 200 and 250 ppm prevented the mycelial growth and spore germination of *A. flavus* that is particularly harmful to humans. The values for inhibition are given in Table 1 and slope 4.97±0.51 and ED$_{50}$ 85.2 ppm L$^{-1}$, ED$_{95}$ 178.5 ppm L$^{-1}$ and slope 5.11±0.69, respectively, than the control (Table 4). The oil extract of *O. basilicum* significantly inhibited the mycelial growth of *A. flavus* with 97.7 and 97.5% inhibition of the sporation of the fungus. These levels of efficacy were followed by the *N. sativa* oil with a 72.6 and 86.0% reduction in the mycelial growth and spore germination of *A. flavus*, respectively.

**Inhibitory effect of essential oils against *A. niger*:** The data shown in Table 5 revealed that *O. basilicum* oil exhibited strong antifungal properties against *A. niger* with a 73.3 and 89.0% inhibition of the mycelial growth (ED$_{50}$ 92.0 ppm L$^{-1}$, ED$_{95}$ 1243.6 ppm L$^{-1}$ and slope 1.45±0.18) and spore germination (ED$_{50}$ 136.8 ppm L$^{-1}$, ED$_{95}$ 277.0 ppm L$^{-1}$ and slope 5.11±0.69, respectively) compared to the control. This efficacy level was followed by that of the *E. globulus* oil with a 72.5% reduction of the radial growth and 84.5% inhibition of the spore germination of the fungus. The lowest mycelial growth (21.5 mm) was recorded for the *N. sativa* oil which was more effective against the germination of the fungal spores with 83.2% inhibition.

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**Table 1:** Inhibitory influence of plant essential oils against the mycelial growth and spore germination of *Aspergillus terreus*

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Cont. (100 ppm)</th>
<th>150 (ppm)</th>
<th>200 (ppm)</th>
<th>250 (ppm)</th>
<th>ED$_{50}$</th>
<th>ED$_{95}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. sativa</em></td>
<td>56.5±5.8</td>
<td>41.0±0.8</td>
<td>36.5±1.7</td>
<td>35.4</td>
<td>22.5±1.3</td>
<td>60.2</td>
</tr>
<tr>
<td><em>E. globulus</em></td>
<td>65.3±5.8</td>
<td>41.0±0.8</td>
<td>37.4</td>
<td>39.3±1.0</td>
<td>40.1</td>
<td>36.5±1.0</td>
</tr>
<tr>
<td><em>O. basilicum</em></td>
<td>56.5±5.8</td>
<td>29.3±0.5</td>
<td>25.0±0.8</td>
<td>55.8</td>
<td>0.0±0.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

**Table 2:** Inhibitory influence of plant essential oils against the mycelial growth and spore germination of *Aspergillus vesciolar*

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Cont. (100 ppm)</th>
<th>150 (ppm)</th>
<th>200 (ppm)</th>
<th>250 (ppm)</th>
<th>ED$_{50}$</th>
<th>ED$_{95}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. sativa</em></td>
<td>77.3±1.7</td>
<td>50.5±0.6</td>
<td>47.8±1.0</td>
<td>38.2</td>
<td>43.8±2.1</td>
<td>43.4</td>
</tr>
<tr>
<td><em>E. globulus</em></td>
<td>77.3±1.7</td>
<td>47.5±0.6</td>
<td>35.5±5.4</td>
<td>54.0</td>
<td>31.0±5.4</td>
<td>59.9</td>
</tr>
<tr>
<td><em>O. basilicum</em></td>
<td>77.3±1.7</td>
<td>31.8±1.2</td>
<td>30.3±0.5</td>
<td>60.8</td>
<td>20.0±1.8</td>
<td>74.1</td>
</tr>
</tbody>
</table>

**Table 3:** Inhibitory influence of plant essential oils against the mycelial growth and spore germination of *Aspergillus fumigatus*

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Cont. (100 ppm)</th>
<th>150 (ppm)</th>
<th>200 (ppm)</th>
<th>250 (ppm)</th>
<th>ED$_{50}$</th>
<th>ED$_{95}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. sativa</em></td>
<td>59.5±0.6</td>
<td>41.3±2.5</td>
<td>30.7</td>
<td>31.5±2.4</td>
<td>47.1</td>
<td>14.3±2.2</td>
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<tr>
<td><em>E. globulus</em></td>
<td>59.5±0.6</td>
<td>51.0±0.8</td>
<td>14.3</td>
<td>48.8±2.2</td>
<td>18.1</td>
<td>12.3±2.6</td>
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<tr>
<td><em>O. basilicum</em></td>
<td>59.5±0.6</td>
<td>21.0±8.8</td>
<td>64.7</td>
<td>12.0±2.9</td>
<td>79.8</td>
<td>0.0±0.0</td>
</tr>
</tbody>
</table>

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**Table 4: Inhibitory influence of plant essential oils against the mycelial growth and spore germination of *Aspergillus flavus***

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Cont. (ppm)</th>
<th>100 (ppm)</th>
<th>R.G.</th>
<th>Inh. (%)</th>
<th>R.G.</th>
<th>Inh. (%)</th>
<th>R.G.</th>
<th>Inh. (%)</th>
<th>R.G.</th>
<th>Inh. (%)</th>
<th>ED50 (ppm L−1)</th>
<th>ED95 (ppm L−1)</th>
<th>Slope±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. sativa</td>
<td>63.0±1.6</td>
<td>30.0±1.2</td>
<td>52.4</td>
<td>27.0±0.8</td>
<td>57.1</td>
<td>23.5±1.3</td>
<td>62.7</td>
<td>21.5±1.3</td>
<td>65.9</td>
<td>88.5</td>
<td>6122.3</td>
<td>0.89±0.18</td>
<td></td>
</tr>
<tr>
<td>E. globulus</td>
<td>63.0±1.6</td>
<td>37.5±2.1</td>
<td>40.5</td>
<td>35.5±0.6</td>
<td>43.7</td>
<td>32.0±2.2</td>
<td>49.2</td>
<td>17.3±1.7</td>
<td>72.5</td>
<td>157.6</td>
<td>1255.7</td>
<td>1.83±0.19</td>
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</tr>
<tr>
<td>O. basilicum</td>
<td>63.0±1.6</td>
<td>30.5±0.6</td>
<td>51.6</td>
<td>23.3±1.7</td>
<td>63.1</td>
<td>19.8±2.8</td>
<td>68.7</td>
<td>16.8±1.0</td>
<td>73.3</td>
<td>92.0</td>
<td>1243.6</td>
<td>1.45±0.18</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5: Inhibitory influence of plant essential oils against the mycelial growth and spore germination of *Aspergillus niger***

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Cont. (ppm)</th>
<th>100 (ppm)</th>
<th>R.G.</th>
<th>Inh. (%)</th>
<th>R.G.</th>
<th>Inh. (%)</th>
<th>R.G.</th>
<th>Inh. (%)</th>
<th>R.G.</th>
<th>Inh. (%)</th>
<th>ED50 (ppm L−1)</th>
<th>ED95 (ppm L−1)</th>
<th>Slope±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. sativa</td>
<td>63.0±1.6</td>
<td>47.0±1.4</td>
<td>52.9</td>
<td>40.3±1.3</td>
<td>59.6</td>
<td>20.8±1.0</td>
<td>79.2</td>
<td>16.8±1.3</td>
<td>83.2</td>
<td>100.1</td>
<td>494.0</td>
<td>2.37±0.20</td>
<td></td>
</tr>
<tr>
<td>E. globulus</td>
<td>63.0±1.6</td>
<td>59.8±2.6</td>
<td>40.1</td>
<td>37.8±1.0</td>
<td>62.2</td>
<td>22.8±1.3</td>
<td>77.2</td>
<td>15.5±1.3</td>
<td>84.5</td>
<td>121.0</td>
<td>419.7</td>
<td>3.05±0.20</td>
<td></td>
</tr>
<tr>
<td>O. basilicum</td>
<td>63.0±1.6</td>
<td>79.8±1.0</td>
<td>20.1</td>
<td>36.3±1.0</td>
<td>63.7</td>
<td>17.0±1.4</td>
<td>83.0</td>
<td>11.0±0.8</td>
<td>89.0</td>
<td>136.8</td>
<td>277.0</td>
<td>5.36±0.26</td>
<td></td>
</tr>
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</table>

**DISCUSSION**

This present study assessed the inhibitory activity of three essential oils against the growth of five *Aspergillus* species *in vitro*. The essential oils of *O. basilicum* had a strong fungicidal effect against all the *Aspergillus* species. This oil completely inhibited the mycelial growth and spore germination of both *A. terreus* and *A. fumigatus* at concentrations of 200 and 250 ppm. In addition, this oil exhibited strong antifungal activity against the mycelial growth and spore germination of *A. flavus* (97.7 and 97.5% inhibition, respectively) and *A. niger* (91.2 and 93.5% inhibition, respectively). These results are in agreement with those of Singh et al. (2011) who reported that *O. basilicum* oil exhibited strong fungitoxicity against some aflatoxigenic fungi which contaminated food including *Fusarium oxysporum*, *A. flavus*, *Alternaria alternata*, *A. fumigatus*, *Curvularia lunata A. niger*, *Penicillium italicum* and *F. nivale*. *O. basilicum* oil showed great antimicrobial potential against *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus megaterium*, *Escherichia coli* and *Listeria monocytogenes* (Hossain et al., 2010). Al-Hajj et al. (2014) reported the antimicrobial activity of *O. basilicum* oil against *S. aureus*, *Streptococcus pneumonia*, *E. coli*, *Candida albicans* and *B. subtilis*. The previous results reported the excellent antimicrobial activity of *Ocimum* oil against the toxigenic strain of *A. flavus* (Kumar et al., 2011) and the multidrug-resistant bacterial strains of *Enterococcus, Pseudomonas* and *Staphylococcus* (Opalchenova and Obreshkova, 2003). The high antimicrobial activity of the *Ocimum* oil is probably attributable to methoxy eugenol which was found to be the main constituent followed by eugenol, 1,8-cineole and b-caryophyllene (Kumar et al., 2011).

Furthermore, Yavari et al. (2011) identified 21 ingredients in the oil extracted from the purple type of *O. basilicum*, including *trans*-ß-bergamotene, 1, 8-cineole and linalool as the main components which represent about 92.1% of the oil content. A number of other components that were present in significant amounts in the *Ocimum* oil included bicyclogermacrene, fenchone, ß-caryophyllene (E)-ß-farnesene and germacrene. All these components play an important role in the inhibition of microbial growth and spread shown by the oil. In addition, the essential oil of *Ocimum* contains some antioxidant components such as tocochromers, polyphenols and fatty acids (Moreira et al., 2010; Puupponen-Pimia, et al., 2001).

The *E. globulus* oil showed bioactivity against the *Aspergillus* species. This oil completely inhibited the mycelial growth and spore germination of *A. flavus* at concentrations of 200 and 250 ppm. In addition, it strongly inhibited the radial growth and spore germination of *A. fumigatus* by 100%. Similar results have been previously reported (Bakkali et al., 2008; Elgorban et al., 2015; Hatamleh et al., 2014; Tyagi and Malik, 2011). Vilela et al. (2009) reported that *E. globulus* oil and 1,8-cineole completely inhibited the growth of both *A. flavus* and *Aspergillus parasiticus* which also showed a reduction in aflatoxin B1 production. The results revealed extreme reduction in the growth of *A. flavus* and *A. fumigatus* by the methanolic extract and essential oil of *E. globulus* (Javed et al., 2012). In addition, *E. globulus* extract was highly effective against 16 isolates of *Pseudomonas aeruginosa* (Pereira et al., 2014).
E. globulus extract and oil has been due to the components such as 1,8-cineole, ρ-cymene, citronellol, eucomalol, citronella, citronellyl acetate, β-pinene, ρ-cymene and alloocimene (Tyagi and Malik, 2011; Nezhad et al., 2009), α-terpinol and aromadendrene (Bachir and Benali, 2012).

CONCLUSION

The present study suggests that O. basilicum and E. globulus oils could be sources of natural antifungal agents for use in the pharmaceutical and food manufacturing industries against pathogenic microbes. Additional research is required to obtain information on the practical efficiency of plant essential oils or extracts, in preventing the growth of food-borne and contaminating microbes, for specific applications and conditions.

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