Analysis and in vitro anti-

Candida

antifungal activity of Cuminum cyminum and Salvadora persica herbs extracts against pathogenic Candida strains

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KEYWORDS
Cuminum cyminum; Salvadora persica; Antifungal activity; Candida species; GC/MS analysis

Summary
Objective. — The in vitro antifungal activities of essential oil from Cuminum cyminum (C. cyminum) and alcoholic extract from Salvadora persica (S. persica) were investigated in order to evaluate their efficacy against C. albicans ATCC 14053, C. dubliniensis ATCC CD60, C. glabrata ATCC 90030, C. krusei ATCC 6258 and C. parapsilosis ATCC 22019.

Methods. — The essential oil was obtained by hydrodistillation in a Clevenger apparatus and analyzed by gas chromatography/mass spectroscopy (GC/MS). The disc diffusion and broth macrodilution methods were used as antifungal susceptibility tests.

Results. — The GC/MS analysis allowed 17 components to be determined; the main constituents of C. cyminum essential oil were α-pinene (30%), limonene (21%) and 1,8-cineole (18.5%). C. cyminum oil had a broad-spectrum antifungal activity against different pathogenic Candida species. Inhibition zone values ranged from 7 to 50 mm for C. cyminum and 0 to 10 mm for S. persica against the organisms tested. The best minimal inhibitory concentration (MIC) of C. cyminum oil was associated with C. albicans and C. dubliniensis (289 mg/L) and the MICs of S. persica extract were 4.9 mg/mL and 20 mg/mL against C. albicans and C. dubliniensis, respectively.
Introduction

Fungal infections have increased over the last two decades, largely because of the increasing size of the population at risk, including patients who are immunocompromised, receiving parenteral hyperalimentation and/or broad-spectrum antibiotics and intravascular catheter users [6]. Although Candida albicans (C. albicans) is responsible for the majority of yeast infections in humans, several other emerging Candida species, including C. dubliniensis, C. glabrata, C. krusei, C. parapsilosis and C. tropicalis have also been associated with disease [21].

The resistance of pathogenic fungi, in particular C. albicans and non-C. albicans species isolated from patients, against antifungal agents has increased [10]. Based on the increasing side effects of polyenes and azoles, novel antifungal therapies with fewer side effects on humans are urgently required for effective management of candidiasis [36]. Down the ages essential oils and other extracts of plants have evoked interest as natural inhibitors to control the growth of the most important pathogenic Candida species and alternative therapies for candidiasis.

Conclusion. — The results suggested the potential substitution of the antifungal chemicals by C. cyminum essential oil and S. persica alcoholic extract as natural inhibitors to control the growth of the most important pathogenic Candida species and alternative therapies for candidiasis.

Materials and Methods

Preparation of S. persica extract

Miswak (S. persica) chewing sticks were purchased from Saudi Arabia. An ethanolic extract was prepared by grinding...
the chewing sticks to a fine powder and resuspending in 80% ethanol. The mixture was left for 3 days at room temperature and then filtered using Whatman No. 4 filter paper (Whatman Ltd., England). The ethanolic extract was then concentrated under vacuum, weighed and the residue was used in antifungal assay. The extract was stored at 4 °C until used [15].

Preparation of *C. cymun* essential oil

The study was carried out on essential oil sample obtained from *C. cymun* growing wild in Iran. The dried seeds were submitted to hydrodistillation for 3 h using Clevenger type apparatus, according to the European Pharmacopoeia [13]. One hundred grams of plant seeds was in turn fed into the Still and 120 mL of distilled water was added. Heating was at 100 °C at standard pressure. The volatile vapor that condensed at water temperature of 8 °C was collected in glass bottle, dried over anhydrous sodium sulphate and stored at 4 °C until used. Identification of the species was confirmed at the Herbarium of Pharmacognosy Department, School of Pharmacy, Shaheed Beheshti University of Medical sciences, Iran. A voucher specimen (No 1172) was preserved in scientific institute in Iran.

GC/MS method

The essential oil was chromatographed using a Hewlett Packard 5890 series II gas chromatograph (Hewlett Packard, Avondale, PA). It was fitted with a Mass Selective Detector HP 5971 A, an HP 7673 autosampler and a split-splitless injector, and connected to an MS ChemStation HP vs. C.00.07. A DB5MS fused silica column (5% phenylmethylpolysiloxane, 30 m × 0.25 mm i.d. film thickness 0.25 μm) (J & W Scientific Fisons, Folsom, CA) was employed. The injector and interface were operated at 200 °C and 280 °C, respectively. The oven temperature was programmed as follows: 60 °C raised to 180 °C (3 °C/min), and held for 15 min. Helium was the carrier gas at 0.9 mL/min; the sample (1 μL) was injected in the split mode (1:20). MS conditions were as follows: ionization voltage of 70 eV, scan rate 1.6 scan/sec, mass range 40–500, ion source temperature 180 °C. The essential oil components were identified by comparing their relative retention times and mass spectra with those of authentic samples (analytical standards from Aldrich, Acros and Fluka; purity > 97%). Sample solutions were prepared in n-hexane (GC grade, Merck) at 1.0% (w/w) [12].

Agar disc diffusion method

The antifungal assay was performed by the agar disc diffusion method [38]. In brief, fungal suspension containing 1 × 10^6 cell/mL of yeast was swabbed and spread on Sabouraud dextrose agar (Merck Co., Darmstadt, Germany). Essential oil from *C. cymun* and alcoholic extract of *S. persica* (40 μL) were applied on paper disc (6 mm in diameter) and placed on the inoculated agar. Nystatin (100 units/disc) was used as positive control standard to determine the sensitivity of fungus. The inoculated plates were incubated at 37 °C for 24 h. The antifungal activity was evaluated by measuring the diameter of the zone of inhibition against the test microorganisms in millimeters (mm). Each experiment was repeated in triplicate.

Broth macrodilution method

Minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined according to the reference documents M27-A for yeasts with modifications [28]. Twofold serial dilutions in DMSO ranging from 0.025 to 51.2 μg/mL were tested for herbs. Each tube was inoculated with 2.5 × 10^3 cell/mL of *Candida* suspensions. In addition, nystatin was used as standard antifungal drug. Twofold serial dilutions ranging from 0.016 to 16 μg/mL for nystatin were used. Each tube, 20 μL of culture was inoculated onto Sabouraud dextrose agar plate and incubated at 37 °C for 48 h. The plates were observed and the MFC was determined as the lowest concentration of plant oil and extract completely inhibiting the growth of *Candida* species. These experiments were performed in triplicate as well.

Statistical analysis

Student’s *t*-test was applied to determine the significance of difference between herbs and nystatin. Probabilities less than 0.05 were taken to be statistically significant.

Results and Discussion

*Candida* species are harmless commensal yeast-like fungi in healthy humans, which can cause mucosal as well as life threatening systemic infections under immune compromised situations [40]. These organisms can colonize or infect virtually all body sites because of its high adaptability to different host niches by the activation of appropriate sets of genes in response to complex environmental signals [20]. Early data on the susceptibility of fungi to different herbal essential oils were largely limited to *C. albicans*, which was a commonly chosen model test organism. Our study was to assess the possible inhibitory potential of the commonly used *C. cymun* essential oil and *S. persica* extract against several *Candida* species, which can become facultative pathogens under altered physiological situations.

The qualitative and quantitative compositions of the oil analyzed were shown in Table 1. Seventeen components representing 93.27% of the essential oil were identified. The oil was characterized by high amounts of *α*-pinene (30%), limonene (21%) and 1,8-cineole (18.5%), as the major compounds. Our findings are in agreement with the results

Test organisms

Standard *Candida* strains used in antifungal assay were: *C. albicans* ATCC 14053, *C. dubliniensis* ATCC CD60, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019. All organisms were obtained from Iranian Research Organization for Science and Technology (IROST). For fungal cultures, *Candida* species were inoculated on Sabouraud dextrose agar (Merck Co., Darmstadt, Germany) containing 5% chloramphenicol. The cultures were incubated at 37 °C and examined daily for one week.
Table 1  The compositions of Cuminum cyminum essential oil identified by gas chromatography/mass spectroscopy (GC/MS).

Les constituants de l’huile essentielle de Cuminum cyminum identifiés par chromatographie en phase gazeuse/spectrométrie de masse (GC/MS).

<table>
<thead>
<tr>
<th>No.</th>
<th>Cuminum cyminum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-pinene 30</td>
</tr>
<tr>
<td>2</td>
<td>Limonene 21</td>
</tr>
<tr>
<td>3</td>
<td>1,8-cineole 18.5</td>
</tr>
<tr>
<td>4</td>
<td>Linalool 10</td>
</tr>
<tr>
<td>5</td>
<td>Linalyl acetate 4</td>
</tr>
<tr>
<td>6</td>
<td>α-terpineol 3</td>
</tr>
<tr>
<td>7</td>
<td>α-terpineol acetate 1.5</td>
</tr>
<tr>
<td>8</td>
<td>Geraniol 1.5</td>
</tr>
<tr>
<td>9</td>
<td>Methyl eugenol 1.2</td>
</tr>
<tr>
<td>10</td>
<td>Sabinene 0.5</td>
</tr>
<tr>
<td>11</td>
<td>Terpinen-4-ol 0.5</td>
</tr>
<tr>
<td>12</td>
<td>Terpinolene 0.5</td>
</tr>
<tr>
<td>13</td>
<td>γ-terpinene 0.5</td>
</tr>
<tr>
<td>14</td>
<td>ρ-cymene 0.3</td>
</tr>
<tr>
<td>15</td>
<td>α-thujene 0.2</td>
</tr>
<tr>
<td>16</td>
<td>Myrcene 0.1</td>
</tr>
<tr>
<td>17</td>
<td>γ-terpineol 0.08</td>
</tr>
<tr>
<td>Total</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>93.27</td>
</tr>
</tbody>
</table>

Table 2  Antifungal susceptibility of Cuminum cyminum essential oil, Salvadora persica extract and nystatin against various Candida strains.

Sensibilité aux antifongiques de l’huile essentielle de Cuminum cyminum, de l’extrait de Salvadora persica et à la nystatine de diverses souches de Candida.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>C. cyminum</th>
<th>S. persica</th>
<th>Nystatin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (mg/L)</td>
<td>MFC (mg/L)</td>
<td>DIZ (mm)</td>
</tr>
<tr>
<td>Candida albicans ATCC 14053</td>
<td>578</td>
<td>1156</td>
<td>37</td>
</tr>
<tr>
<td>Candida dubliniensis ATCC CD60</td>
<td>289</td>
<td>578</td>
<td>50</td>
</tr>
<tr>
<td>Candida glabrata ATCC 90030</td>
<td>578</td>
<td>1156</td>
<td>50</td>
</tr>
<tr>
<td>Candida krusei ATCC 6258</td>
<td>289</td>
<td>578</td>
<td>7</td>
</tr>
<tr>
<td>Candida parapsilosis ATCC 22019</td>
<td>578</td>
<td>1156</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>MIC (mg/L)</td>
<td>MFC (mg/L)</td>
<td>DIZ (mm)</td>
</tr>
<tr>
<td>Candida albicans ATCC 14053</td>
<td>4.9</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Candida dubliniensis ATCC CD60</td>
<td>20</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Candida glabrata ATCC 90030</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Candida krusei ATCC 6258</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Candida parapsilosis ATCC 22019</td>
<td>0.125</td>
<td>0.125</td>
<td>25</td>
</tr>
</tbody>
</table>

MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration; DIZ: diameter inhibition zone (mm); Cuminum cyminum (mg/L); Salvadora persica (mg/mL); Nystatin (mg/L). Candida species were defined as susceptible and resistant species to nystatin in values ≥15 mm and none zone in agar disc diffusion method, respectively.
C. famata, C. kefyr, C. sake, C. holmii, C. lusitaniae, C. intermedia, C. atlantica and C. maritima) potency of S. persica. They found that S. persica extract exhibited antifungal activity against the tested species, representing C. albicans as the most susceptible isolate.

The MICs and MFCs of the oil and extract from plants tested were given in Table 2. The most susceptible yeasts were C. dubliniensis ATCC 22019 and C. krusei ATCC 6258 (289 mg/L) against C. cymnum oil, and C. albicans ATCC 14053 (4.9 mg/mL) and C. dubliniensis ATCC 22019 (20 mg/mL) against S. persica extract. There are no validated criteria for the MIC end points for in vitro testing of plant extracts, however, Aligiannis et al. [3] proposed classification for plant materials, based on MIC results as follows: strong inhibitors-MIC up to 0.5 mg/mL; moderate inhibitors-MIC between 0.6 and 1.5 mg/mL and weak inhibitors-MIC above 1.6 mg/mL. In our study, we tested up to maximum concentration of 40 mg/mL. Based on the above classification, C. cymnum and S. persica showed strong to moderate activity to one or more tested microorganisms. As shown in Table 2, S. persica extract was significantly 1000 times less efficient than C. cymnum oil (P < 0.05).

The therapeutic arsenal available for the treatment of fungal infections is limited to some antifungal agents [4]. For the polyene drugs, nystatin has been used for several decades as one of the principal treatments for mucosal candidiasis caused by Candida species in the world [11]. This history may partly explain the low cure rates with this drug for the non-albicans species, which may be developing resistance, although this did not occur for C. albicans [43]. However, the high frequency of mucosal Candida isolates with dose-dependent susceptibility to this drug, as observed in some studies, has caused concern, since the recommended dose may be insufficient to achieve the desired therapeutic efficacy. It is also necessary to mention that there is no resistance to polyenes, such as nystatin and amphotericin B among Candida species except some C. lusitaniae strains.

Nystatin (4 μg/mL) completely inhibited the growth of all Candida species tested. Interestingly, in the present study, nystatin did not affect C. krusei strain, while inhibition zone of C. cymnum essential oil for this organism was 7 mm. As per the literature reports, one of the predominant reasons for drug resistance of C. krusei appears to be restricted penetration of drugs inside the exopolymeric matrix, which can bind or restrict the diffusion of the antifungals [7]. C. cymnum oil can overcome this barrier and act as a much better antifungal agent as compared to nystatin.

Nystatin binds to ergosterol, a major component of the fungal cell membrane. When present in sufficient concentrations, it forms pores in the membrane that lead to K⁺ leakage and death of the fungus. Interestingly, the novel inhibitory effect of nystatin in our study was of inferior efficacy as compared to herbs against all reference Candida strains. Previous study clearly demonstrated that the C. cymnum oil can act as a potent antifungal agent against Candida species, and can function similar to antifungal antibiotics such as nystatin. Essential oil components have the capability to alter cell permeability by entering between the fatty acyl chains making up membrane lipid bilayers and disrupt the lipid packing. Due to this, the membrane properties like membrane fluidity/permeability and functions may get changed [19]. This may also affect the regulation and function of the membrane bound enzymes that alter the synthesis of many cell wall polysaccharide components (i.e. β-glucan, chitin, and mannan) and alter the cell growth and morphogenesis [35]. Moreover, some essential oils can cause extensive cellular damage at much lower concentrations, probably due to better penetration and contact. The major components of C. cymnum are terpenes, which have the capability to inhibit the respiration of Candida, and may have adverse effects on mitochondria. It may be the cause of cell death and other morphological changes [42]. Khosravi et al. [23] also indicated that the major anti-microbial components (terpenes) are enriched in C. cymnum essential oil and cause the destruction and lysis of memberanous organelles, including nuclei and mitochondria and disorganization of cytoplasmic contents.

In conclusion, the results of the present study showed that C. cymnum essential oil and S. persica alcoholic extract had strong to moderate activity against different pathogenic Candida species. In fact, the herbs could be alternative substances for fungi control, in particular C. krusei and strains that have acquired resistance to conventional antifungal agents. It is necessary to mention that there is a great difference between topical and systemic antifungals. However, further studies are needed to purify the major components of these plants and to assess their appreciable antifungal actions against Candida species in vivo.

Disclosure of interest

The authors have not supplied their declaration of conflict of interest.

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References


