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ORIGINAL ARTICLE/ARTICLE ORIGINAL

Analysis and in vitro anti-*Candida* antifungal activity of *Cuminum cyminum* and *Salvadora persica* herbs extracts against pathogenic *Candida* strains

Analyse et étude de l'activité antifongique in vitro d'extrait d'herbes de Cuminum cyminum *et de* Salvadora persica *sur des souches pathogènes de* Candida

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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.

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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.

MOTS CLÉS
Cuminum cyminum ;
Salvadora persica ;
Activité antifongique ;
Candida ;
Analyse GC/MS

Résumé
Objectif. — L'activité antifongique in vitro d'huile essentielle de *Cuminum cyminum* (*C. cyminum*) et d'extrait alcoolique de *Salvadora persica* (*S. persica*) a fait l'objet d'une étude afin d'évaluer leur efficacité contre *C. albicans* ATCC 14053, *C. dubliniensis* ATCC CD60, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258 et *C. parapsilosis* ATCC 22019.
Matériel et méthodes. — L'huile essentielle est obtenue par hydrodistillation des feuilles dans un appareil Clevenger et est analysée par chromatographie en phase gazeuse/spectrométrie de masse (GC/MS). La technique de diffusion avec des disques et de la macrodilution en milieu liquide ont été utilisées comme tests de sensibilité antifongique.
Résultats. — L'analyse GC/MS a permis d'analyser 17 composants ; les principaux constituants de l'huile essentielle de *C. cyminum* étaient α-pinène (30 %), limonène (21 %) et 1,8 -cineole (18,5 %). L'huile de *C. cyminum* avait un large spectre d'activité antifongique contre différents espèces pathogènes de *Candida*. Les zones d'inhibition variaient de 7 à 50 mm pour *C. cyminum* et de 0 à 10 mm pour *S. persica* contre les organismes testés. Les meilleures concentrations minimales inhibitrices (CMI) de l'huile de *C. cyminum* étaient associées à *C. albicans* et *C. dubliniensis* (289 mg/L) et les CMIs de l'extrait de *S. persica* étaient de 4,9 mg/mL et 20 mg/mL contre *C. albicans* et *C. dubliniensis*, respectivement.
Conclusion. — Les résultats suggèrent la possibilité de remplacer des produits chimiques antifongiques par de l'huile essentielle *C. cyminum* et par un extrait alcoolique naturel de *S. persica* comme inhibiteur de la croissance des plus importantes espèces pathogènes de *Candida* et comme thérapies alternatives pour la candidose.

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 sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases. World Health Organization (WHO) noted that majority of the world's population depends on traditional medicine for primary healthcare [41].

Essential oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties [8,9]. They are rich sources of biologically active compounds. There has been an increased interest in looking at antifungal properties of extracts from aromatic plants particularly essential oils. Therefore, it is reasonable to expect a variety of plant compounds in these oils with specific as well as general antifungal activity [24]. Essential oils such as aniseed, calamus, camphor, cedarwood, cinnamon, citronella, clove, eucalyptus, geranium, lavender, lemon, lemongrass, lime, mint, nutmeg, orange, palmarosa, rosemary, basil, vetiver and wintergreen have been traditionally used by people for various purposes in different parts of the world [31].

Cuminum cyminum (Apiaceae) is an annual herbaceous plant (height: 15—50 cm) with fruits, each one containing green seeds which has aromatic characteristics. It is applied in Iranian folk medicine since more than 200 years ago. It has been shown that its fruits have medicinal application in treatment of diarrhea, toothache and epilepsy [44]. *Salvadora persica* (*S. persica*) shrub has been used traditionally in folk medicine for different medical condition treatments. The habitual use of *S. persica* (chewing sticks or miswak) for dental hygiene is still wildly spread throughout parts of Asia, Africa and Middle East. It is one of the most important species with its reported strong antibacterial, antifungal and antiviral effects. Mechanical removal of dental plaque is regarded as an effective means of controlling progression of periodontal disease [37]. This study was undertaken with the intention of finding out the efficacy of the alcoholic extract from *S. persica* and the essential oil from *C. cyminum* as antifungal agents for therapeutic purposes and determination of chemical compositions of the oil by gas chromatography/mass spectroscopy (GC/MS) method.

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 N° 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. cyminum* essential oil

The study was carried out on essential oil sample obtained from *C. cyminum* 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 (N° 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% phenylmethylpolyxiloxane, 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].

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., Darmstdt, Germany) containing 5% chloramphenicol. The cultures were incubated at 37 °C and examined daily for one week.

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., Darmstdt, Germany). Essential oil from *C. cyminum* 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 mg/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. From 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. cyminum* 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

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).

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

reported by Khosravi et al. [23] and Gachkar et al. [17]. Li et al. [25] exhibited the five main chemical compositions including cuminal, safranal, 2-ethylidene-6-methyl-3,5-heptadienal, a-proyl-benzenemethanol and 1-methyl-4-(1-methylethyl)-1,4-cyclohexadiene of essential oil from *C. cyminum* seed. In another study performed by Derakhshan et al. [16], the main constituents of *C. cyminum* essential oil were found to be cumin aldehyde, ρ-mentha-1,3-dien-7-al, ρ-mentha-1,4-dien-7-al, γ-terpinene, ρ-cymene and ß-pinene. The changes in the essential oil compositions might have arisen from the climatical, seasonal, geographical and geological differences, as mentioned by Daferera et al. [14]. Other components, such as sabinene, terpinen-4-ol, Terpinolene, γ-terpinene, ρ-cymene, α-thujene, myrcene and γ–terpineol, were also found in tested oil at a total contribution of 2.68%. Although the anti-microbial activity of an essential oil attributed mainly to its major components, the synergistic or antagonistic effect of components in minor percentage in the mixture has to be considered [39].

Throughout the world, 182 species of plants have been used as chewing sticks; the most important is *S. persica*. One of the main constituents of the *S. persica* is Benzyl isothiocyanate (BITC) [5], which has virucidal activity against Herpes simplex virus, inhibits the growth and acid production of *Streptococcus mutans*, and is fungistatic to *C. albicans*. In vitro studies of miswak extract have also demonstrated some antibacterial activity against certain bacterial species implicated in periodontal disease and dental caries [1,2].

Contemporary data clearly showed that the broad-spectrum activity of *C. cyminum* includes antibacterial, antifungal, antiviral and antiprotozoal activities [26]. Of all these properties, antifungal activity has received the most attention. As shown in Table 2, antifungal effects were illustrated as inhibition zones using disc diffusion method. *C. cyminum* oil showed significant antifungal activity against all *Candida* strains tested except for *C. krusei* ATCC 6258, as compared to nystatin as positive control ($P < 0.05$). In the present study, inhibition zone values ranged from 7 to 50 mm against *Candida* strains. Essential oil of *C. cyminum* was more efficient and had the best antifungal effect for *C. dubliniensis* ATCC CD60 and *C. glabrata* ATCC 90030 (50 mm), followed by *C. albicans* ATCC 14053 (37 mm), *C. parapsilosis* ATCC 22019 (36 mm) and *C. krusei* ATCC 6258 (7 mm). In fact, our results are in agreement with previous works dealing with the high susceptibility of a wide range of yeasts [26,27,30,32,34], dermatophytes [33], *Aspergillus* species [22] and other filamentous fungi [8] against *C. cyminum* oil. It was demonstrated that the potential anti-microbial activity of *C. cyminum* is due to presence of a group of terpenes, such as α -pinene and 1,8-cineole [18]. Regarding to *S. persica* extract, the highest zone of growth inhibition was obtained for *C. albicans* ATCC 14053 strain, followed by *C. dubliniensis* ATCC 22019 and *C. glabrata* ATCC 90030 with a diameter of inhibition ranging from 10 mm to 7 mm, respectively, whereas *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were not susceptible (Table 2). Noumi et al. [29] studied the anti-candidal (*C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*,

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.

Isolate	*C. cyminum*			*S. persica*			Nystatin		
	MIC	MFC	DIZ	MIC	MFC	DIZ	MIC	MFC	DIZ
Candida albicans ATCC 14053	578	1156	37	4.9	10	9	0.25	0.25	25
Candida dubliniensis ATCC CD60	289	578	50	20	20	10	0.065	0.065	36
Candida glabrata ATCC 90030	578	1156	50	0	0	7	0.125	0.125	25
Candida krusei ATCC 6258	289	578	7	0	0	0	4	4	0
Candida parapsilosis ATCC 22019	578	1156	36	0	0	0	0.125	0.125	21

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 founded 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. cyminum* 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. cyminum* 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. cyminum* 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. cyminum* 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. cyminum* 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. cyminum* 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. ß-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. cyminum* 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. cyminum* 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. cyminum* 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 topic 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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