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Full Length Research Paper

Chemical composition and *in vitro* antifungal activity of the essential oil from *Cuminum cyminum* against various *Aspergillus* strains

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Antifungal activity of the essential oil obtained from *Cuminum cyminum* (*C. cyminum*) was investigated against different *Aspergillus* strains. Gas chromatography/mass spectrometry (GC/MS) analysis of the chemical composition of the oil was performed to determine their different components. The minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of *C. cyminum* essential oil and amphotericin B for seven strains of *Aspergillus* species, comprising *Aspergillus fumigatus* (*A. fumigatus*) PTCC 5009, *Aspergillus flavus* PTCC 5006, *Aspergillus niger* PTCC 5011, *Aspergillus terreus* PTCC 5267, *Aspergillus ochraceus* PTCC 5017, *Aspergillus nidulans* PTCC 5014 and *A. fumigatus* AIR13, were determined by a broth macro dilution method. *C. cyminum* oil contained α-pinene (30%), limonene (21%) and 1, 8-cineole (18.5%), as the major components. The MIC values of the oil were ranged from 0.3 to 74 mg/ml and *C. cyminum* oil demonstrated strong antifungal activity on *A. nidulans* (P<0.05). No isolate was detected with an MIC of amphotericin B > 2 µg/ml. Data showed that *C. cyminum* oil was more effective in comparison with amphotericin B. Furthermore, the study suggests that this essential oil can be used as an antifungal agent for the treatment of aspergillosis.

Key words: Fungitoxicity, *Aspergillus*, *Cuminum cyminum*, essential oil, amphotericin B.

INTRODUCTION

Invasive aspergillosis is a severe infectious disease marked by high morbidity and mortality frequently affecting patients with immune disorders such as prolonged neutropenia. For decades, amphotericin B has been an established treatment despite suboptimal responses and tolerability (Bodey and Vartivarian, (1989).

The exact reasons for such a dismal outcome remain unclear. It is widely believed that therapeutic failure is closely associated with nephrotoxicity, diagnostic delay, intrinsic resistance and poor immune status of the host (Herbrecht et al., 2002). Intriguingly, Lass-Flörl et al. (1998) proposed that resistance of the fungus to amphotericin B or poor pharmacokinetics of the drug might play a role in the dismal response. The potential for *Aspergillus fumigatus* (*A. fumigatus*) to develop resistance to amphotericin B was demonstrated by ultraviolet irradiation of germinating conidia enhanced the selection of amphotericin B-resistant mutants (Manavathu et al., 1998). Furthermore, Seo et al. (1999) reported the generation of polyene-resistant mutants following the repeated passage of *Aspergillus flavus* in graded concentrations of amphotericin B. These findings indicate that increased clinical use of amphotericin B may select for resistant *Aspergillus* isolates, possibly contributing to the poor outcome in aspergillosis.

Although new lipid formulations of amphotericin B have been developed which show a better safety profile, drug related renal impairment is still of concern and the acquisition cost of these therapies is high (Lass-Flörl et al., 1998). This situation forced the researchers to search for new antimicrobial substance from various sources including medicinal plants, because they have been reported to be safe and without side-effects. There are

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2600 plant species of which more than 700 are noted for their uses as medicinal herbs (Ali-Shtayeh and Abu, 1999). Previous studies indicated many essential oils which possess antifungal activity. The growth of *Aspergillus* species can be inhibited by essential oils as well. In this matter a number of essential oils such as *Zataria multiflora*, *Origanum majorana*, *Satureja hortensis*, *Eucalyptus globules*, *Cymbopogon martini,* and *Cinnamomum zylenicum* were studied (Khosravi et al., 2009; Salehi, 2006). *Cuminum cyminum* (Apiaceae) is an annual herbaceous plant (height: 15 to 50 cm) with fruits each one contains a 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 (Ali-Shtayeh and Abu, 1999). The aim of this study was to assess the antifungal activity of essential oil from *C. cyminum* against different *Aspergillus* strains.

MATERIALS AND METHODS

Plant material

C. cyminum (Apiaceae; known as Ziree) seeds were collected from Tehran province, Iran in April 2011. The dried seeds were stored in a dark place until use. The sample was ground in a blender to produce a fine powder. Voucher specimen (no. 1172) has been deposited in the Herbarium of Pharmacognosy Department, School of Pharmacy, Shaheed Beheshti University of Medical sciences, Iran.

Preparation of *C. cyminum* essential oil

Essential oil was isolated by water distillation for 3 h from seeds, using a Clevenger type apparatus, according to the procedure described in the European Pharmacopoeia (Council of Europe, 1997). The oil was stored at -20°C sealed brown vial until use.

Analysis of chemical components of *C. cyminum* essential oil

GC/MS analysis was carried out using a Varian 3400 gas chromatograph coupled with a mass spectrometer (Saturn II; Varian, Sugarland, TX) under the following conditions: DB-1 MS column (60 m×0.25 mm, film thickness 0.25 µm); carrier gas, helium at a constant flow rate of 35 ml/min; ionization potential, 70 eV; and scan range, 40 to 300 amu. The oven temperature was programmed from 50 to 250°C at a rate of 4°C /min and the injector temperature was 240°C. Identification of the constituents of essential oil was made by comparison of their mass spectra and retention indices with those given in the literature and those of authentic samples (Adams, 2004).

Fungal strains and growth media

Six isolates of *Aspergillus* species, such as *A. fumigatus* PTCC 5009, *A. flavus* PTCC 5006, *Aspergillus niger* PTCC 5011, *Aspergillus terreus* PTCC 5267, *Aspergillus ochraceus* PTCC 5017 and *Aspergillus nidulans* PTCC 5014, were purchased from Persian Type Culture Collection (Tehran, Iran), and *A. fumigatus* AIR13 (originated from ostrich with severe disseminated aspergillosis) was obtained from Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Iran. Different *Aspergillus* strains were grown on sabouraud dextrose agar-SDA (Merck Co., Darmstadt, Germany) slants at 35°C until well sporulated (7 to 10 d). Conidia were harvested by adding sterilized Tween 80 solution (0,01%, v/v) (Klich, 2002).

Synthetic antifungal drug

Amphotericin B (Batch No. 20-914-29670, Squibb Institute for Medical Research, Princeton, NJ) was used as control drug in this study. It was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml and stored as 0.25 ml aliquots at -20°C. The frozen stock was thawed at room temperature and vortexed gently several times to ensure that any remaining crystals were completely dissolved before use. Since amphotericin B is light-sensitive, the stock solutions and the MIC tubes were covered with aluminum foil to prevent from light exposure. Drug concentrations ranging from 0.0625 to 128 µg/ml were used.

In vitro susceptibility test

Antifungal analysis and determination of the MIC and MFC of the oil and standard drug against different *Aspergillus* isolates were performed by a broth macrodilution technique in test tubes (National Committee for Clinical Laboratory Standards, 2002). Briefly, fresh conidia were collected from *Aspergillus* isolates and resuspended in sabouraud dextrose broth at a density (determined by haemocytometer count) of 2×10^4 conidia/ml. Twofold serial dilutions in DMSO ranging from 0.15 to 296 mg/ml were tested for essential oils. Two times the required concentrations of the drugs were prepared in the same medium (0.5 ml) by serial dilution in sterile 6-mlpolystyrene tubes (Falcon 2054, Becton Dickinson, Lincoln Park, NJ, USA) and inoculated with an equal volume (0.5 ml) of the conidial suspension.

The tubes were incubated at 35°C for 48 h and studied for visible growth after vortexing the tubes gently. The MICs were defined as the lowest concentrations of the oil which produced no visible growth (that is, 100% inhibition). The MICs of amphotericin B were defined as the first well with a significant growth reduction (90%) when compared to that of positive control. Endpoint for amphotericin B: isolates with MICs between 0.5 and 2 µg/ml had reduced dose-dependent susceptibility. Isolates with MIC ≤ 0.5 µg/ml were susceptible, whereas isolates with MIC ≥ 2 µg/ml were resistant (Hashemi et al., 2011). MFC values were determined as the highest dilutions (lowest concentrations) at which no growth occurred on the plates. The difference in the distributions of the essential oil and amphotericin B was determined with a chi-square (X^2) test. A *P* value less than 0.05 was statistically considered significant.

RESULTS AND DISCUSSION

Different studies have shown that despite the expanding number of antifungal agents, death rate caused by *Aspergillus* species has been increased during the recent decades due to drug-resistance occurrence, increased MIC and cross-resistance among the isolated species (Seo et al., 1999). Regarding the lack of effective response to conventional treatments and antifungal susceptibility patterns of the most common isolated *Aspergillus* species, this study was undertaken to

Table 1. The compositions of *C. cyminum* essential oil identified by 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%

Table 2. Antifungal susceptibility of *C. cyminum* essential oil and amphotericin B against different *Aspergillus* strains.

Fungal isolate	*C. cyminum* (mg/ml)		Amphotericin B (µg/ml)	
	MIC	MFC	MIC	MFC
Aspergillus fumigatus PTCC 5009	37	74	32	64
Aspergillus flavus PTCC 5006	4.7	4.7	32	64
Aspergillus niger PTCC 5011	37	74	32	64
Aspergillus terreus PTCC 5267	74	148	16	32
Aspergillus ochraceus PTCC 5017	18.5	37	32	64
Aspergillus nidulans PTCC 5014	0.3	0.6	4	8
Aspergillus fumigatus AIR13	18.5	37	32	64

investigate scientifically those plants which have been used in traditional medicines as potential sources of novel antimicrobial compounds. Previous publications have documented the antifungal activity of the essential oils including *Artemisia sieberi* (Khosravi et al., 2011), *Foeniculum vulgare* (Naeini et al., 2009), *Origanum vulgare* (Khosravi et al., 2011), *Rosmarinus officinalis* Centeno et al., (2010), *Zataria multiflora* (Shokri et al., 2011; Naeini et al., 2011), *Ziziphora clinopodioides* (Khosravi et al., 2011) and *Plargonium graveolens* (Naeini et al., 2011). The result obtained by GC/MS analyse of the essential oil obtained from *C. cyminum* was illustrated in Table 1. Seventeen components were identified in the oil. As a result of GC/MS analyse, *C. cyminum* contained α-pinene (30%), limonene (21%) and 1,8-cineole (18.5%), as the major compounds. The major components, such as α-pinene and 1,8-cineole, were also reported by Khosravi et al. (2011) and Gachkar et al. (2007) studies. Other components, such as sabinene, terpinen-4-ol, Terpinolene, γ-terpinene, ρ-cymene, α-thujene, myrcene and γ–terpineol, were also found in this oil at a total contribution of 2.68%. In this study all of the tested *Aspergillus* strains were susceptible to the essential oil of *C. cyminum* (Table 2). The MIC and MFC values of the oil were ranged from 0.3 to 74 mg/ml and 0.6 to 148 mg/ml, respectively. We also found that essential oil extracted from *C. cyminum* demonstrated strong antifungal activity on *A. nidulans*, followed by *A. flavus, A. ochraceus*, *A. fumigatus*, *A. niger* and *A. terreus*. There was significant difference between MICs for *A. nidulans* and other *Aspergillus* species ($P<0.05$). *A. nidulans* is a filamentous fungus characterized as an excellent model system for the study of mitotic crossing over and whose cells pass the great part of their cell cycle in G_2 phase. Since in this phase chromosomes are in duplicate, they significantly favor mitotic recombination

(Bergen and Morris, 1983). The exact reasons for high response of *A. nidulans* to the tested oil are unclear. Two mechanisms may explain the *A. nidulans*-toxicity induced by the essential oil: (a) the oil induces mitotic spindle disorganizations, stimulating the occurrence of non-disjunction events, or (b) the oil induces chromosome breaks, resulting in mitotic crossing-over or chromosome break-deletions (Ziogas and Kalamarakis, 2001). Zare et al. (2008) reported that the oil of *C. cyminum* was toxic to *Aspergillus* species. Similarly, Khosravi et al. (2011) reported high antifungal activity of *C. cyminum* against *A. fumigatus* and *A. flavus*. Rahman et al. (2000) reported that resistant A. *flavus* and *A. niger* isolates were moderately inhibited by *C. cyminum*. In a study conducted by Bansod and Rai (2008), the oil of *C. cyminum* showed low activity against *A. fumigatus* and *A. niger*. Karbin et al. (2009) also reported complete inhibition of *A. flavus* by *C. cyminum* oil. Along with the aforementioned report, our results are in agreement with those of Sing et al. (2002) who reported *C. cyminum* oil to be equally good or more effective when compared with standard antibiotics. In addition, *in vitro* antimicrobial activity of *C. cyminum* against human pathogenic fungi and commensally bacteria was studied by Chaumont et al. (2003) and Matan et al. (2006). The antimycotic activity of *C. cyminum* due to presence of a group of terpenes such as α -pinene and 1,8-cineole was well known (Davidson and Naidu, 2000; Hammer et al., 2003). Although, the antimicrobial activity of an essential oil attributed mainly to its major components, the synergistic or antagonistic effect of compounds in minor percentage in the mixture has to be considered (Souza et al., 2007). Besides the production of deoxyribonucleic acid (DNA)-breaks and inhibition of DNA synthesis caused by the oil, the lipophilic components of essential oil may react with the lipid parts of the cell membrane and influence their enzymes, carriers, ion channels and receptors. Owing to the critical role of the cytoplasmic membrane in cell wall synthesis and the presence of biosynthetic enzymes for the cell wall in this membrane, essential oil may affect membrane integrity and consequently lead to a loss of membrane fidelity and alter cell division (Ghfir et al., 1997).

Moreover, a marked depletion of cytoplasmic contents of hyphae could be accompanied by lysis and disruption of membranes of major organelles such as nuclei and mitochondria (Knobloch et al., 1986; Sikkema et al., 1995). Khosravi et al. (2011) observed that *C. cyminum* oil provided irreversible damage to cell wall (degenerative changes), cytoplasm membrane (irregular, dissociated from cell wall, invaginated) and nuclear membrane (folding) of *A. fumigatus* and *A. flavus*. However, most of the studies concerning *C. cyminum* showed that it has a moderate to high antifungal activity, which is consistent with the data of the present study. Amphotericin B has been used to treat fungal infections for over 40 years.

Despite of this long period of use, clinical resistance among *Aspergillus* species remains rare. It has been hypothesized that disruption of the complex interaction between amphotericin B and the plasma membrane requires multiple changes in the cell wall, making secondary resistance a challenge for the fungus and hence a rare event (Seo et al., 1999).

The results obtained from amphotericin B exhibited that all seven *Aspergillus* strains were resistance, representing MICs ranged from 4 to 32 µg/ml. Although essential oil values were high when compared with amphotericin B as reference antifungal agent, but these results were of interest as we were dealing with an essential oil and not a pure product. However, there was no agreement on the level of acceptance for plant when compared with standard antifungal drugs; therefore, some authors considered only activity comparable to antifungal drugs, while others considered even higher values. When comparing data obtained from different studies, most publications provide generalizations about whether or not plant oil possesses activity against fungi. However, not all provide details about the extent or spectrum of this activity. Some studies also show the relative activity of plant oils by comparing results from different oils tested against the same organism. Comparison of the data obtained in this study with previously published results is problematic. First, the composition of plant oil is known to vary according to local climatic and environmental conditions (Sivropoulou et al., 1995).

In view of this, many methods have been developed specifically for determining the antimicrobial activity of essential oils (Carson et al., 1995; Smith and Navilliat, 1997). The benefits of basing new methods on preexisting, conventional assays such as the NCCLS methods are that these assays tend to be more readily accepted by regulatory bodies (Carson et al., 1995). Also, these methods have been designed specifically for assessing the activity of antimicrobial compounds and factors affecting reproducibility have been sufficiently investigated. Although NCCLS methods have been developed for assessing conventional antimicrobial agents such as antibiotics, with minor modifications these methods can be made suitable for the testing of essential oils (Smith and Navilliat, 1997). The broad inhibition of fungal growth by *C. cyminum* essential oil, in addition to its availability as natural volatile product, justifies its possible rational use as an alternative antifungal compound to control the growth and dissemination of pathogenic *Aspergillus* species. Also, these preliminary findings need validation with a larger number of *Aspergillus* isolates from patients with prolonged exposure to amphotericin B.

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