Antifungal activity of the *Trachyspermum ammi* essential oil on some of the most common fungal pathogens in animals

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Abstract:

BACKGROUND: The increasing resistance to antifungal drugs and the reduced number of available drugs led to the search for therapeutic alternatives among aromatic plants and their essential oils, empirically used by antifungal effects. **OBJECTIVES:** The purpose of the current study was to evaluate the antifungal activity of *Trachyspermum ammi* essential oil (EO) against the most frequent pathogenic fungi including *Candida*, *Aspergillus*, *Chrysosporium* and *Trichophyton* species. **METHODS:** EO from the seeds of the plant was obtained by hydrodistillation. Susceptibility tests were expressed as growth inhibition zone (diameter) using disk diffusion method and minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) using broth microdilution method. **RESULTS:** Results of susceptibility tests showed that *T. ammi* EO was effective against all the tested strains. The diameters of growth inhibition zone of the EO were between 11 mm and 60 mm. The EO was also the most active, with MIC and MFC values ranging from 0.3 to 2.5 mg/ml and 0.6 to 5 mg/ml, respectively. The EO of *T. ammi* showed a significant degree of antifungal activity against different *Candida* species in comparison with other fungi ($p<0.05$). **CONCLUSIONS:** The present study indicated that *T. ammi* EO has considerable antifungal activity, deserving further investigations for its clinical application for treatment of fungal infections.

Introduction

Fungal agents are widespread and can be isolated from a wide range of animals, from the soil and the environment. This makes fungal diseases as a group of transmissible infections in which animals can represent important reservoirs and asymptomatic carriers for people in close contact with them. The important role of farm and pet animals as carriers and spreaders is well known. Fungal infections in animals and immunocompetent individuals are commonly associated with asymptomatic infections or mild and transient local skin or mucosal lesions (Khosravi et al., 2006), but they can represent important risk factors in immunocompromised subjects, due to the impairment of their immune systems. Since the 1960s, when the use of antibiotic therapies has been established, a drastic increase of fungal infections was observed (Vandeputte et al., 2012). Now, emerging fungal pathogens

have been described causing severe infections, which include yeasts as azole-resistant *Candida* and non-*Candida* species, and filamentous fungi such as species of the genus *Trichophyton* and *Aspergillus* and some representatives of the hyaline moulds. Therefore, fungal infections are seen as an important threat to global health that needs to receive proper attention (Gauthier and Keller, 2013). Currently, the limited number of antifungals available, the increased multiresistance and the adverse effects are the major obstacles for fungal infection therapy. Additionally, the recent emergence of opportunistic fungal infections reinforced the necessity for discovering novel antifungal agents. For this purpose, molecules with a new mechanism of action and able to escape from the current fungi mechanism of resistance against antifungals are of interest (Vandeputte et al., 2012).

Medicinal plants are usually used in traditional medicine as antimicrobial agents (Hammer et al., 2002). These plants have continued to be used not only for primary health care of the poor in the developing countries, but also in countries where conventional medicine is predominant in the national health care system. According to the World Health Organization (WHO), herbal medicines serve the health needs of about 80% of the world's population, especially for millions of people in the vast rural areas of developing countries (WHO, 2001). Many researchers have shown that secondary plant metabolites, such as essential oil (EO), can exhibit important antifungal activity against yeasts, dermatophyte and *Aspergillus* strains (Pina-Vaz et al., 2004; Salgueiro et al., 2004), and have therapeutic potential, mainly in fungal diseases involving mucosal, cutaneous and respiratory tract infections (Pinto et al., 2003). Major constituents of the EO are phenolic compounds (terpenoids and phenylpropanoids) like thymol, carvacrol or eugenol, of which antimicrobial activity is well documented (Cavaleiro et al., 2006). The limited occurrence of these phenols in nature is one of the reasons why *Trachyspermum ammi* (*T. ammi*) EO containing thymol has been of great interest for some time (Gandomi et al., 2014). *T. ammi*, known as ajowan, is an annual herbaceous plant belonging to the highly valued medicinally important family, Apiaceae (Gersbach and Reddy, 2002). Ajowan is widely distributed and cultivated in various regions such as Iran, Pakistan, Afghanistan and India (Shojaaddini et al., 2008). Similar to most species of the family Apiaceae, ajowan is famous for its brownish EO. Usually, thymol is the main ajowan EO constituent and may yield 35% to 60% (Ishikawa et al., 2001). The non-thymol fraction (thymene) contains para-cymene, gamma-terpinene, alpha-pinene, beta-pinene, α-terpinene, styrene, delta-3-carene, beta-phyllanderene, terpinene-4-ol and carvacrol (Ranjan et al., 2012). The purpose of this study was to assess the antifungal effect of *T. ammi* EO against important pathogenic fungi for its potential use as a natural antifungal drug.

Material and Methods

Fungal organisms: A total of 16 pathogenic fungi including *Candida albicans* (no. 5), *C. glabrata* (no. 3), *C. tropicalis* (no. 2), *C. krusei* (no. 1), *C. parapsilosis* (no. 1), *Aspergillus* niger (no. 1), *A. fumigatus* (no. 1), *Trichophyton rubrum* (no. 1) and *Chrysosporium farinicola* (no. 1) were used as test microorganisms. Identification of different *Candida* species was performed by germ tube test, CHROM agar, β-glucosidase, chlamydospore production and API20C system (BioMérieux, Paris, France) for sugar assimilation. The macro- and microscopic identifications of filamentous fungi were determined according to the criteria of Klich (2002) and Larone (2002). All fungal isolates were stored in Sabouraud dextrose broth (Merck Co., Darmstadt, Germany) with glycerol at -70°C until used.

Herbal EO: Seeds of *T. ammi* were collect-

ed in July 2014 from Isfahan, Iran. Plants were taxonomically identified at the Pharmacognosy Department, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. The vouch¬er herbarium number was 15125. The seeds were thoroughly washed and dried in the shade at room temperature for 24 h. The dried *T. ammi* seeds were submitted to hydrodistillation in a Clevenger-type apparatus at 100oC for 3 h, according to the procedure described in the European Pharmacopoeia (Council of Europe, 1997). The EO was isolated and dried over anhydrous sodium sulfate and then stored in a dark glass bottle at 4oC until needed.

Preparation of fungal inoculum: Filamentous fungi and yeasts were grown on Sabouraud dextrose agar (Merck Co., Darmstadt, Germany) slants at 30oC for 7-21 days and 35oC for 2 days, respectively. Fungal cells were harvested by adding 10 ml of sterile distilled water containing 0.05% Tween 20 (Merck Co., Darmstadt, Germany) and scraping the surface of the culture to free the cells. The fungal suspensions were counted with a hemocytometer and further diluted to give a concentration of (1-5) × 106 cell/ml (Naeini et al., 2009; Naeini and Shokri, 2012).

Antifungal susceptibility assay: A) Disk diffusion method): Tests to assess the antifungal activity were performed using disk diffusion based on the M44-A method for yeasts (CLSI, 2004) and the M51-P method for filamentous fungi (CLSI, 2008). *T. ammi* EO (10, 20 and 30 µl) was inoculated to 6-mm-diameter disks and placed on Sabouraud dextrose agar (Merck Co., Darmstadt, Germany) plates inoculated with fungal spore suspension. The plates were incubated at 30°C for 48 h. At the end of the incubation period, the susceptibility of the test organisms was determined by measuring the diagonal of the zone of inhibition around the disk. The results given are an average of two independent experiments.

B) Broth microdilution method: Minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were performed according to reference documents M27-A3 for yeasts (CLSI, 2008) and M38-A2 for filamentous fungi (CLSI, 2008). Briefly, stock solutions were prepared by dissolving 1 mg dried essential oil in dimethylsulfoxide (DMSO) 5%. The stock solutions were diluted with Roswell park memorial institute (RPMI) 1640 medium containing L-glutamine but no sodium bicarbonate (Sigma Chemical Co., St. Louis, Missouri, USA), buffered to pH 7.0 with 0.165 mol/l MOPS buffer (Sigma Chemical Co., St. Louis, Missouri, USA). Serial two-fold dilutions ranging from 0.1 to 5 mg/ml were tested for EO. One hundred microlitre aliquots of 2-fold diluted EO solution were dispensed into each well of 96-well microtiter plates. Subsequently, 100 µl aliquots of conidial suspensions were added to each well of a microdilution plate, which were incubated at 30°C (for filamentous fungi) and 35°C (for yeasts) for 48 h. The positive control well contained 100 µl of fungal suspension plus 100 µl of RPMI 1640, and the negative one contained 200 µl of RPMI 1640 only. In addition, the reference antifungal compound, fluconazole (Pfizer, New York, USA), was used as the standard antifungal drug. Two-fold serial dilutions ranging from 0.25 to 128 µg/ml for fluconazole were used. MIC90 was defined as the antifungal concentration that inhibited the growth of 90% of the fungal isolates. In addition, MFC was determined by subculturing 100 µl aliquot from all MIC tubes showing no visible growth onto Sabouraud glucose agar plates. All experiments were performed in duplicate.

Statistical analysis: The antifungal activity was analyzed by the two-tailed paired student's t-test. Statistical significance was considered a P value less than 0.05.

Results

Based on disk diffusion method, the diameters of growth inhibition zone of the EO were

Table 1. Antifungal activity of the EO from *Trachyspermum ammi* against different yeasts and filamentous fungi.

Isolates	*Trachyspermum ammi*					Fluconazole	
	Microdilution broth (mg/ml)		Disk diffusion (mm)			Microdilution broth (µg/ml)	
	MIC	MFC	10 µl	20 µl	30 µl	MIC	MFC
Candida glabrata	0.35	0.7	40	52	58	64	128
	0.4	0.8	32	49	55	64	128
	0.45	0.9	39	50	54	64	128
Candida albicans	0.35	0.7	46	50	60	64	128
	0.3	0.6	50	55	60	8	16
	0.4	0.8	40	50	58	32	64
	0.35	0.7	48	49	55	8	16
	0.35	0.7	50	60	60	4	8
Candida tropicalis	0.6	1.2	18	20	22	2	4
	0.5	1	21	22	25	2	4
Candida krusei	0.4	0.8	23	32	41	64	128
Candida parapsilosis	0.5	1	19	22	23	8	16
Aspergillus fumigatus	1	2	10	10.5	12	64	128
Aspergillus niger	1.5	3	9	10	11	64	128
Trichophyton rubrum	2.5	5	13.5	14	15	64	128
Chrysosporium farinicola	2	4	14.5	15	16	32	64

between 11 mm and 60 mm (Table 1, Fig. 1). The EO of *T. ammi* showed a significant degree of antifungal activity against different *Candida* species (mean value: 40.34 mm) in comparison with other fungi (mean value: 13.5 mm) ($p<0.05$). The EO showed little ability to inhibit *Chrysosporium farinicola*, *T. rubrum* and *Aspergillus* strains.

Evaluation of MICs and MFCs showed that *T. ammi* EO was effective against all the tested strains (Table 1). Data from the present study suggested that the conidia of filamentous fungi were comparatively less susceptible to *T. ammi* EO than yeast cells ($p<0.05$). The results showed that MIC values ranged from 0.3 to 0.6 mg/ml and 1 to 2.5 mg/ml against *Candida* isolates and filamentous fungi, respectively. Among different *Candida* isolates, *C. albicans* showed the highest susceptibility (mean value: 0.35 mg/ml) to *T. ammi* EO, followed by *C. glabrata* and *C. krusei* (mean value: 0.4 mg/ml), *C. parapsilosis* (mean value: 0.5 mg/ml) and *C. tropicalis* (mean value: 0.55 mg/ml). *Among filamentous fungi*, *Aspergillus* species were more susceptible than *Chrysosporium farinicola* and *T. rubrum* against the tested EO. For yeasts and filamentous strains, MFC values were higher than MIC values, ranging from 0.6 to 1.2 mg/ml for yeasts and 2 to 5 mg/ml for moulds.

As shown in Table 1, the MIC values of fluconazole ranged from 2 to 64 µg/ml for *Candida* species and from 32 to 64 µg/ml for filamentous fungi.

Discussion

Fungi are increasingly important causes of acute or chronic deep-seated animal infections, especially recurrent mucosal and cutaneous infections that may be severe in debilitated or immunocompromised animals (Shokri et al., 2010). The small number of drugs available for their treatment (most of them fungistatic) and emerging resistance permanently encourage the search for alternatives and led us to find them among low cost and low toxicity traditional therapies and natural products. Up

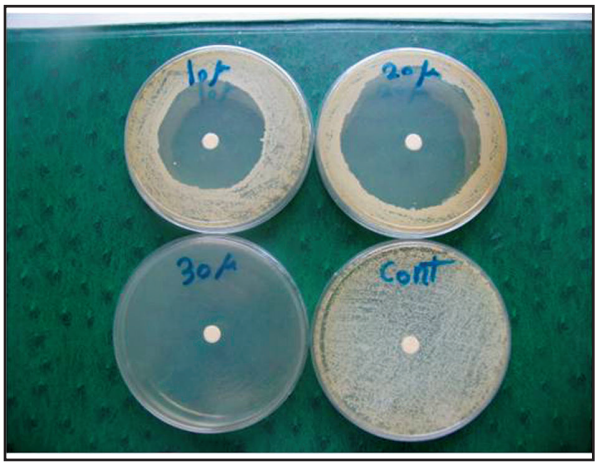

Figure 1. The photo of fungal cultures in disk diffusion method (the diameters of growth inhibition zone of *T. ammi* EO against C. albicans at different concentrations 10, 20 and 30 µl and control [without EO]).

to now, no previous studies have comprehensively investigated the activity of *T. ammi* EO against pathogenic yeasts and filamentous fungi. This study revealed that this EO has both fungistatic and fungicidal activities. *T. ammi* EO disk diffusion method showed the diameters of growth inhibition zone ranging from 11 to 60 mm. There was a statistically significant difference on antifungal activity of *T. ammi* EO against different *Candida* species (mean value: 40.34 mm) in comparison with other fungi (mean value: 13.5 mm) ($p<0.05$). The EO showed little ability to inhibit *Chrysosporium farinicola*, *T. rubrum* and *Aspergillus* strains.

Evaluation of MICs and MFCs showed that *T. ammi* EO was effective against all the tested strains (Table 1). Data from the present study suggested that the conidia of filamentous fungi were comparatively less susceptible to *T. ammi* EO than yeast cells ($p<0.05$). As reported by Pinto et al. (2003), the thickness, composition and density of the conidial wall may be responsible for the reduced susceptibility of conidia to tested EO. Our results showed MIC values ranging from 0.3 to 0.6 mg/ml and 1 to 2.5 mg/ml against *Candida* isolates and filamentous fungi, respectively. The highest susceptibility to *T. ammi* EO was related to *C. albicans* (mean value: 0.35 mg/ml) to *T. ammi* EO, followed by *C. glabrata* and *C. krusei* (mean value: 0.4 mg/ml), *C. parapsilosis* (mean value: 0.5 mg/ml) and *C. tropicalis* (mean value: 0.55 mg/ml). Among filamentous fungi, *Aspergillus* species were more susceptible than *Chrysosporium farinicola* and *T. rubrum* against the tested EO. For yeasts and filamentous strains, MFC values were higher than MIC values, ranging from 0.6 to 1.2 mg/ml for yeasts and 2 to 5 mg/ml for moulds. The studies on the EO of *T. ammi* have been reported to inhibit some of the dermatophytes (Tiwari et al., 2003), *Candida* (Ranjan et al., 2012), *Aspergillus* (Murthy et al., 2009) and *Chrysosporium* (Soni et al., 2014) species. Gandomi et al. (2014) showed that *T. ammi* EO contained a mixture of components, mainly thymol together with a small amount of other volatile compounds. Seven components were identified, which represented thymol (63.4%), p-cymene (19%) and g-terpinene (16.9%) as the major components. Totally, it is difficult to attribute the activity of a complex mixture to particular constituents. Nevertheless, it is reasonable to speculate that the activity of this EO can be related to the presence of thymol. The importance of the phenolic hydroxyl groups for the antifungal activity of the monoterpenoids has previously been reported (Aligiannis et al., 2001; Nostro et al., 2004). In agreement with our results, previous studies have also indicated that thymol as a major component of other herbal plants including *Zataria multiflora* (Ebrahimzadeh et al., 2003) and *Thymus vulgaris* (Soković et al., 2009) was responsible for the strong antifungal activity against a variety of pathogenic yeasts and filamentous fungi, especially fungi with decreased susceptibility to fluconazole (Pinto et al., 2003; Shokri et al., 2012).

As shown in Table 1, the MIC values of fluconazole ranged from 2 to 64 µg/ml for *Candida* species and from 32 to 64 µg/ml for filamentous fungi. It should be noted that the EO tested by the disk diffusion method appeared

less active than in the test carried out in a liquid medium and these facts might be explained by the more limited diffusion of the EO in a solid medium.

Ergosterol is the major sterol component of the fungal cell membrane, and is responsible for maintaining cell function and integrity. The primary mechanism of action of azole antifungal drugs, such as fluconazole, is to inhibit the fungal cell growth and disruption of normal sterol biosynthetic pathways, leading to a reduction in ergosterol biosynthesis (Alcazar-Fuoli et al., 2013). Pinto et al. (2003) demonstrated that the large spectrum of activity of this EO acting on *Candida*, *Aspergillus* and dermatophyte agrees with the mechanism of fluconazole action, representing a considerable impairment of the biosynthesis of ergosterol and a marked reduction of the ergosterol content. This effect is also superior to the effect of most azole antifungals, as most of them are fungistatic.

In conclusion, the findings of the present study indicated that *T. ammi* EO had considerable antifungal activity. The oil inhibited the filamentous fungi including dermatophyte, *Chrysosporium* and *Aspergillus* species and different *Candida* species. It is necessary to mention that *T. ammi* exhibited remarkable inhibitory effect against fluconazole-resistant *Candida* isolates such as C. krusei and C. glabrata, which were intrinsically resistant to fluconazole or whose resistance was easily inducible. The results presented should stimulate studies on toxicity, improved formulations and the determination of optimal concentrations for clinical applications, as well as comparative studies alongside currently used drugs of the therapeutic efficacy of EO to control fungal infections.

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چکیده

زمینه مطالعه: مقاومت رو به رشـد نسـبت به داروهای ضدقارچی و کاهش تعداد داروهای موجود سبب شد تا بررسی در زمینه داروهای جایگزین درمانی میان گیاهان آروماتیک و اسانس‌های روغنی‌شان به‌ویژه با اثرات ضدقارچی انجام شود. **هدف:** هدف مطالعه حاضر، ارزیابی فعالیت ضدقارچی اسانس روغنی زنیان (*Trachyspermum ammi*) در برابر شایع‌ترین قارچ‌های بیماریزا نظیر گونه‌های کاندید\ آسپرژیلوس، کریپتوکوکوس نئوفورمانس و تریکوفایتون بود. **روش کار:** اسانس روغنی از دانه‌های گیاه با روش تقطیر آبی به دست آمد. آزمایش‌های حساسیت به صورت هالهٔ مهار رشد (قطر) با روش دیسک دیفیوژن و حداقل غلظت مهاری (MIC) و حداقل غلظت قارچ‌کشی (MFC) با روش میکرودایلوشن براث بیان شدند. **نتایج:** نتایج آزمایش‌های حساسیت نشان دادند که روغن زنیان در مقابل تمام سویه‌های آزمایش شده فعال بود. قطرهای هالهٔ مهار رشد روغن بین ۱۱mm و ۶۰ بودند. همچنین روغن به ترتیب با مقادیر MIC و MFC بین ۰/۳ mg/ml تا ۲/۵ و ۰/۶ mg/ml تا ۵ بسـیار مؤثر بود. اسـانس روغنی زنیان فعالیت معنی‌داری را در مقابل گونه‌های مختلف کاندید\در مقایسه با سایر قارچ‌ها نشان داد ($p<0/05$). **نتیجه‌گیری نهایی:** مطالعه حاضر نشان داد که اسانس روغنی زنیان فعالیت قابل ملاحظه ضدقارچی دارد و شایسته است تحقیقات گسترده‌تری برای کاربرد بالینی آن جهت درمان عفونت‌های قارچی انجام پذیرد.

واژه‌های کلیدی: فعالیت ضدقارچی، آسپرژیلوس، کاندید\ زنیان، تریکوفایتون

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