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In Vitro Antifungal activity of *Raphanus sativus L. var. niger* (Black Radish) and *Trachyspermum ammi* (Ajwain) on resistant and susceptible *Aspergillus fumigatus* isolates

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ABSTRACT

Aspergillus fumigatus is an opportunistic fungal pathogen that causes invasive aspergillosis in immunocompromised patients. *Raphanus sativus L. var. niger* and *Trachyspermum ammi* are two medical herbs which seemed to have an antifungal activity that can be integrated alternative medicine into conventional medicine. The aim of this study was to evaluate the effect of *R. sativus* and *T. ammi* on the resistant and susceptible species of *A. fumigatus*. In the present study, 185 environmental samples from 11 cities of Iran were processed and screened in terms of azole resistance using selective plates. The isolates were confirmed by partial sequencing of the b-tubulin gene. Afterwards, in vitro antifungal susceptibility tests against triazole agents and *R. niger* and *T. ammi* extract were performed based on the CLSI, M38-A2 document. The ingredients in the extract by gas chromatography method were isolated and identified by mass spectrometry. Overall, 51 *A. fumigatus* isolates were detected. According to *in vitro* antifungal susceptibility tests, 45 *A. fumigatus* isolates had high MICs of itraconazole (≥8 mg/L) and voriconazole (>2 mg/L) and 6 *A. fumigatus* isolates were susceptible. The MIC 50 and MIC 90 for *R. sativus* was 1.95 mg/ml and 3.9 mg/ml respectively. Also, The MIC 50 and MIC 90 for *T. ammi* was recorded as 2.30 mg/ml and 4.85 mg/ml respectively. The main identified compounds were Tramadol (58.37%), Butanol (23.42%), Benzofuran (18.21%). Our results indicated that *R. sativus* and *T. ammi* extracts significantly inhibited the growth of *A. fumigatus* isolates and have an appropriate antifungal activity.

1. Introduction

Aspergillus fumigatus is an opportunistic fungal pathogen that causes invasive aspergillosis in immunocompromised patients (Nabili et al., 2012; Nabili et al., 2013). It is ubiquitous worldwide and found in nearly everywhere such as soil, decomposing plant, water and indoor environment especially in hospitals (Balajee et al., 2007; Moazeni et al., 2018). Of course, other species like *A. flavus*, *A. ochraceus*, can also cause infections but due to the small size of *A. fumigatus* asexual conidia, spores are readily transmissible in the air and have a low settling rate compared with other *aspergillus* species (Babamahmoodi et al., 2015; Khodavaisy et al., 2016). Air is considered to be the primary medium for the transport of conidia. These conidia are involved in pulmonary infections in immunocompromised individuals and lead to a spectrum of diseases that range from pulmonary to systemic infection (Latgé

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2001). Three classes of drugs commonly used in the treatment of aspergillosis are azoles, polyenes, and echinocandins. The azoles are the most widely used antifungal drugs (Howard et al., 2010; Snelders et al., 2011). voriconazole is the first choice for the primary treatment of invasive aspergillosis in most patients due to its favorable responses (Walsh et al., 2008). Itraconazole is usually used for the treatment of chronic pulmonary aspergillosis and allergic situation (Denning et al., 2003), and posaconazole is recommended for prophylaxis in immunocompromised patients (Cornely et al., 2007). long-term use of azole drugs in treatment of aspergilloma (Aliyali et al., 2016), chronic pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis (ABPA) (Howard et al., 2009), as well as exposure to *A.fumigatus* strains which become resistant due to use of agricultural fungicides in the environment (Verweij et al., 2009) are the two main causes of treatment failure. (Baddley et al., 2009; Chowdhary et al., 2011). Apart from the emergence of resistant strains, significant toxicity of certain drugs that often prevent their safe use over a prolonged period is another considerable issue because of similar biology of eukaryotic host and fungal cells. Therefore, designing novel medicinal plants with antifungal compounds and fewer side effects to compete with resistance isolates is highly necessary (Webster et al., 2008; de Souza Sales et al., 2017; Kelidari et al., 2018). Plants are known as a safe natural resources for production of antimicrobial agents .There are many medical herbs which seemed to have an antifungal activity that can be integrated alternative medicine into conventional medicine (Moein et al., 2015; Zomorodian et al., 2017). Black Radish (*Raphanus sativus* L. var. *niger*), a subtype of Radish (*Raphanus sativus* L.) is a plant of the cruciferous family that is a root vegetable grown (Gutiérrez and Perez, 2004). This vegetable consumed all over the world and can be used as a salad, even though it is not common in some countries (Jovanović et al., 2016). Biological activities of Radish are diverse and have been determined in many investigations. The Radish extract was shown antibacterial, antifungal and immunological, anticancer activity, due to the presence of the raphanin compound (Singh and Singh, 2013). In addition, Black Rradish contains a high concentration of polar phenolic compounds, including flavonoids, tannins, and quinines (Lourenço et al., 2013). Several in vivo and clinical investigations have reported that the flavonoids show various pharmacological antifungal functions. Flavonoids often inhibit fungal growth with various underlying mechanisms, including plasma membrane disruption, cell wall formation, cell division, RNA and protein synthesis (Aboody and Mickymaray, 2020). Caffeic acid and Ferulic acid in Radish showed antifungal properties against some fungi and bacteria (Gutiérrez and Perez 2004). *Trachyspermum ammi* commonly known as 'Ajwain' is a native of Egypt and is cultivated in Iraq, Iran, Afghanistan, Pakistan, and India. This plant is belonging to apiaceae family that is a high medicinally important seed (Bairwa et al., 2012). In traditional medicine, *T. ammi* is administered as a household remedy for stomach disorders, a paste of crushed fruits is applied externally for relieving colic pains; and a hot and dry fomentation of the fruits applied on chest is used as a common remedy for asthma (Shokri et al., 2016). The seeds of *T. ammi* (L.) are widely used in India and eastern Asia, both in diet and in traditional medicine for instance, applied for the management of ophthalmic and otic infections (Bairwa, Sodha et al., 2012). The seeds contain 2–4.4% brown colored oil known as Ajwain oil. The main components of this oil are phenolic compounds (terpenoids and phenylpropanoids) like thymol, carvacrol or eugenol which are strong germicide and fungicide. So, Ajwain oil, have attributes for antifungal, antibacterial, anti lithiasis, antinociceptive action against wide range of microbes (Cavaleiro et al., 2006; Sharifzadeh et al., 2015). In order to assess the antifungal activity of Ajwain, total essential oil extracted from seeds was showed conventional effect on *Aspergillus niger* and *Curvularia ovoidea* at 5000 ppm as minimum inhibitory concentration (Dwivedi and Singh 1998). According to this distinctive contribution by Ajwain components makes it a source natural antifungal drug with various pharmacological effects (Sharifzadeh, Khosravi et al., 2015; Banihani 2017). The purpose of this study was to determine the chemical components and in vitro antifungal activity of *Raphanus* sativus L. var. *niger* (black Radish) and *Trachyspermum ammi* (Ajwain) on resistant and susceptible *A. fumigatus* isolates.

2. Materials and Methods

2.1. Sample collection and identification of azole resistant A. fumigatus isolates

In the descriptive and cross-sectional present study, 185 environmental specimens were obtained from soil of hospital areas, fields, gardens, composts in 11 cities on north of Iran. To recover *A. fumigatus* strains, 100 gr of specimens was dissolved in 5 mL sterile saline solution containing Tween 40 (0.05%), vortexed, and allowed to settle. According to a previously described protocol, Cultures were prepared on a Sabouraud dextrose agar plate (SDA; Difco), supplemented with 4 and 1mg/L itraconazole and voriconazole, respectively, at 45 °C for 72 h in the dark (Ahangarkani et al., 2020). Identification of *Aspergillus* section Fumigati was performed based on both macroscopic and microscopic characteristics. Moreover, Molecular identification of all *A. fumigatus* isolates that grew on the supplemented plate was performed with sequencing of the partial beta-tubulin gene using TUB2a (5′-TGACCCAGCAGATGTT-3′) and TUB2b (5′- GTTGTTGGGAATCCACTC-3′) as previously described (Nabili et al., 2016).

2.2. hydroalcoholic extract Preparation

Ajowan seeds and black Radish root were collected in the north of Iran (sari, Iran) and collected plants in sari university herbarium received approval and dried at room temperature. The Ajowan seeds and Radish roots were thoroughly washed and black Radish roots were sliced and Ajowan seeds crashed then samples are dried in the room temperature for 24 h. Subsequently, plant samples were ground to a powder and sieved through a sifter (40 mesh). Powdered samples were extracted with 80 (v/v) ethanol on water bath at 70 °C for 6 h. The extracted samples were centrifuged at 1500 rpm for 72 h and the supernatant was transferred into a 50 mL volumetric flask and adjusted the volume to 50 mL with 80% ethanol. The extracts were filtered and dried to remove the solvent prior to the analysis. Dried extracts have been re-dissolved into appropriate sterile solvent before testing. Different concentrations (0.24375- 125 mg/Ml) of each plant extract were prepared for screening anti-fungal activities.

2.3. Isolation and Identification of Extract Ingredients

The ingredients in the extract by gas chromatography method were isolated and identified by mass spectrometry. Identification of the constituents of this extract was obtained by comparing the mass spectrums and the library data of the GC / MSS machine was done.

2.4. In Vitro Antifungal Susceptibility Testing

Minimum inhibitory concentrations (MICs) were determined by broth microdilution susceptibility testing according to the methods in the Clinical and Laboratory Standards Institute (CLSI) reference standard (M38). For the preparation of the microdilution trays, itraconazole (Janssen, Beerse, Belgium) and voriconazole (Pfizer, Sandwich, UK) were obtained from the respective manufacturers as reagent-grade powders (2008). Briefly, the antifungal agents were dispensed into the microdilution trays at final concentrations of 0.016–16 μg/ml for itraconazole, voriconazole. Inoculum suspensions were prepared on potato dextrose agar for 2-3 days by slightly scraping the surface of mature colonies with a sterile cotton swab, soaked in sterile saline including Tween 40 (0.05%). The supernatants were adjusted spectrophotometrically to an optical density range of 0.09-0.13 (0.5×10^4 to 3.1×10^4 CFU/ml) at a wavelength of 530 nm, as determined by quantitative colony count for determining the viable number of colony-forming units (CFUs) per milliliter. Conidial suspensions, which mostly consisted of conidia, were diluted 1:50 in RPMI 1640 medium. Microdilution plates were inoculated with 100 μl of the diluted conidial inoculum suspension, incubated at 35 °C for 48 h and read visually after agitation. Moreover, *Paecilomyces variotii* (ATCC 22319) and *Candida parapsilosis* (ATCC 22019) were used as quality controls. With the aid of a reading mirror, the MIC endpoints were determined as the lowest concentrations of drugs, inhibiting recognizable growth (100% inhibition). Considering the breakpoints for itraconazole and voriconazole (susceptible:<2mg/L; intermediate: 2mg/L; resistant: > 2 mg/L), itraconazole (1 mg/L), voriconazole (1 mg/L) with MICs above the proposed epidemiological cut-off values against

A.fumigatus isolates were selected for further analysis (Rodriguez-Tudela et al., 2008; Verweij et al., 2009; Espinel-Ingroff et al., 2010).

2.5. Antifungal susceptibility testing with R. niger and *T. ammi extracts*

Similar to the procedure we used to prepare a 96-well plate, serial dilution test with itraconazole and voriconazole, we repeated this process for the extracts of *R. niger* and *T. ammi*. The only difference was that in the 96-well plate in the first column, instead of the drug, the plant extract was poured and the same microdilution was performed with positive and negative control. Compare the results with each other to see what concentrations of drugs and extracts can influence the growth inhibition of *aspergillus fumigatus*. Because of the opacity of the extract, we had to apply invert microscope to check the growth of fungi in each well. The concentrations of the extracts were 0.24375- 125 mg/mL.

3. Results

Based on the present study, 185 specimens were obtained from 11 cities of Iran. The isolates obtained, which were initially identified as *Aspergillus* species, were confirmed via molecular assessments. The results showed that 95% of the isolates were 99–100% identical to b-tubulin genes of *A. fumigatus*. In total, 51 (27.5%) *A. fumigatus* environmental isolates from 11 cities of Iran including Sari (n=16; 33.3%), Tehran (n= 8; 15.6%), Ghaemshahr (n= 5; 9.8%), Mahmud Abad (n= 5; 9.8%), Amol (n= 5; 9.8%), Sorkhroud (n= 4; 7.8%), Babolsar (n= 2; 3.9%), Fereidunkenar (n= 2; 3.9%), Farah Abad (n= 2; 3.9%), Damavand (n= 1; 1.9%), Amir kola (n= 1; 1.9%), were confirmed (Table 1). After extraction with ethanol under optimum conditions, the extract of the plant was injected to GC / MSS using thermal programming. The main identified compounds in the extract consist of 3 compounds and its major components are Tramadol (58.37%), Butanol (23.42%), Benzofuran (18.21%). (Table 2. Figure 1). According to *in vitro* antifungal susceptibility tests, 45 *A. fumigatus* isolates had high MICs of itraconazole (≥8 mg/L) and voriconazole (>2 mg/L) and 6 *A. fumigatus* isolates were susceptible. (Table 3). In Table 3 summarizes the susceptibility patterns of itraconazole, voriconazole, *R. niger* and *T. ammi* has been shown. It also showed minimum inhibitory concentrations (MICs) including Geometric mean, MIC Range, MIC50, MIC90 against 51 *A. fumigatus* isolates. Both drugs and extracts showed good antifungal activity at different concentrations. The antifungal activity of an extract of *R. niger* and *T. ammi* were assessed against 51 clinical isolates of *A. fumigatus* using a broth microdilution technique. The MIC 50 and MIC 90 for *R. niger* was 1.95 mg/ml and 3.9 mg/ml respectively. Also, The MIC 50 and MIC 90 for *T. ammi* was recorded as 2.30 mg/ml and 4.85 mg/ml respectively. In this setting *R. niger* and *T. ammi* were interpreted as being a potential fungistatic agent, however the antifungal effects these plant extracts are less than those of chemical fungicides. Interestingly, 42 (82.3%) isolates of *A. fumigatus* showed high MIC value for itraconazole >16µg/ml) and 4 (7.8%) isolates of *A. fumigatus* showed high MIC value for voriconazole (>16µg/ml). The geometric mean MIC of Black Radish for strains was 1.639748 µg/ml; a similar finding was reported for the geometric mean MIC of *Trachyspermum* 2.457574 µg/ml. MIC50, MIC90 and G mean of itraconazole, showed the same concentration of 16 µg/ml in contrast MIC50, MIC90 and G mean of Voriconazole were 2, 6.2, 3.5213 µg/ml respectively. The high MIC distributions of itraconazole and voriconazole were shifted approximately more than two log2 dilution steps apart.

4. Discussion

Aspergillus is found in the environment and in the soil, vegetables, decaying organic matter and food debris, as well as, they are part of saprophytic fungi. Aspergillosis is a type of opportunistic human and animal fungal infection caused by different *Aspergillus* species. the important species is *A.fumigatus*, extremely heat resistant and grows at 45°C. (Denning et al., 2003). In the present study, 185 isolates of which 51 were identified as *A. fumigatus*, and according to in vitro antifungal susceptibility 45 samples were resistant and 6 susceptible to itraconazole, voriconazole. Also, we determine the antifungal effects of two extracts of *T. ammi* and *R. niger* on resistant and susceptible *A. fumigatus* isolates and found that a good response to the antifungal effects of these extracts.

Table 1. Frequency distribution of sources of screened samples

sources	Number of samples	Number of resistant isolate	Species type
commercial and home-made compost	16	15	*A.fumigatus*
flower shops soil	9	8	*A.fumigatus*
Hospital Garden Soil	10	9	*A.fumigatus*
Garden soil and agricultural land	16	13	*A.fumigatus*
Total	51	45	

Table 2. Results of GC / MSS of R. niger Extract

PK	Material name	Area %	KI
1	1-Butanol (CAS) $$ n-Butanol $$ n-Butyl alcohol $$ Hemostyp $$ n-But an-1-oldl-Glyceraldehyde dimer 1-PROPANOL-O-D	23.42	**1136**
2	Tramadol	58.37	**1984**
3	Benzofuran-2-one, 3-methyl-3-aza-2 ,3-dihydro- Benzene-1,2- dicarboxylic acid, mon oamide, N-(1-cyano-1-methylethyl)-(E)-3,13-Tetradecadien-2-one	18.21	**2564**

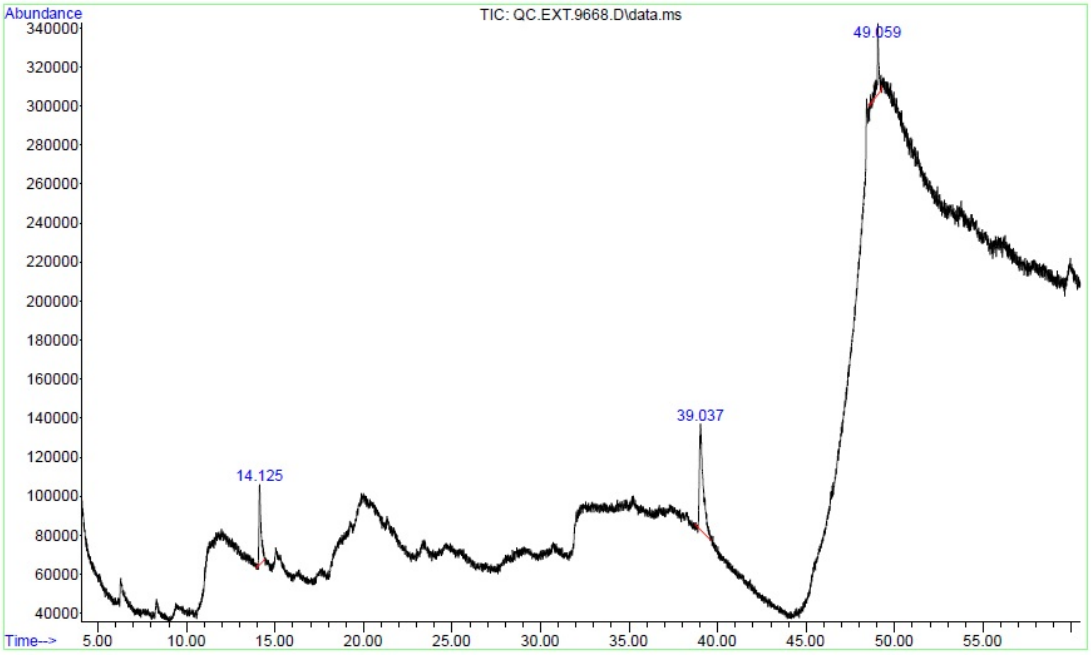

Figure 1. Results of GC / MSS of Raphanus niger Extract

Table 3. In vitro antifungal susceptibility of 51 *A. fumigatus* strains

Source of origin	Number	Anti fungal agent/ plant extract	MIC									MIC rang	MIC 50	MIC 90	Gmean
			16	8	4	2	1	0.5	0.25	0.125	0.0625				
A. fumigatus	n=51	Voriconazole	4	0	0	40	2	4	1	0	0	0.25 – 16	2	6.2	3.5213
Environmental	n=51	Itraconazole	42	0	0	1	2	3	0	3	0	0.125 – 16	16	16	16
			MIC												
			125	62.5	31.25	15.6	7.8	3.9	1.95	0.975	0.487	0.24375			
	n=51	Black radish	0	0	0	0	0	20	25	3	3	0	0.24375-7.8	1.95	3.9	1.639748
	n=51	Trachyspermum	0	0	0	0	0	25	20	3	3	0	0.24375-7.8	2.30	4.85	2.457574

According to Yahyaabadi et al that investigated the effect of a number of herbal extracts on the growth of *A.fumigatus and A. flavus,* the most effective antifungal compounds studied include aquatic extract Anethum graveolens, Thyme, Coriander, Rosa damascena, respectively (Yahyaabadi et al., 2011). Janjua et al, analyzed root peel extract of *Raphanus sativus* L. var niger for its phytochemicals and *in vitro* antimicrobial activity. They showed *the peel of R. sativus* L. var niger had most of the important phytochemicals like tannins, saponins, flavonoids terpenoids, glycosides that each of them had strong potential for medicinal use (Janjua and Shahid, 2013). Some studies reported that *R. sativus niger* roots, leaves and seeds have antimicrobial agents indicating its pharmaceutical potential for development of new alternative medicine (Hanlon and Barnes, 2011; Kim et al., 2011; da Silva et al., 2020). Terras et al, reported that the cysteine-rich peptides (Rs-AFP1 and Rs-AFP2) isolated from *R. sativus* showed substantial antifungal activity against several fungal species with minimal inhibitory concentration (MIC) of 30–60 µg/ml which was consistent with our study (Terras et al., 1992). Similar to Shin et al study that reported, Radish has many useful biological properties such as: alkaloids, nitrogen compounds, coumarines, enzymes, gibberellins, organic acids, phenolic compounds, polysaccharides and sulfur compounds , we found that some antifungal activities of *R. sativus* niger extract are due to these phytochemicals (Shin et al., 2015). Shokri et al. assessed the antifungal activity of Ajwain essential oil against the most frequent pathogenic fungi including *Candida*, *Aspergillus*, *Chrysosporium* and *Trichophyton* species. They indicated that *T. ammi* essential oil has considerable antifungal activity (Shokri, Sharifzadeh et al., 2016). In the antifungal prospecting, our results are in agreement with the findings of other authors that showed *R. niger* and *T. ammi* extracts, Voriconazole and Itraconazole have good effect on *A. fumigatus* species. Drugs at lower concentrations (due to the active ingredient of the drug being pure) and extracts at higher concentrations (due to their other compounds) showed good response. *R. niger* and *T. ammi* extracts with identical MIC had similar effect on *A. fumigatus*. Voriconazole had more sensitive to *A. fumigatus* and more effective and applicable in the treatment of aspergillosis. Due to the high resistance of compost and farmers' use of azole drugs, bio-organic drugs and safer composts to be replaced by farmers. Accurate identification of the causative agent and evaluation of drug susceptibility profiles on strains isolated from samples can be very helpful in treating these types of infections quickly and avoiding the extra costs and unsuccessful treatments.

Conclusion

According to our assessment, *R. niger* and *T. ammi* extracts can be very effective antifungal herbal remedies, so further studies should be conducted to understand the mechanisms of action involved in the antifungal activity of this extract in order to produce a novel plant-based antifungal drug.

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Refereces

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