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Antifungal activity of the essential oils of some medicinal plants against human and plant fungal pathogens

Hamzeh Mafakheri[1], Sayid Mahdi Mirghazanfari[2*]

[1] Researcher at AJA University of Medical Sciences, Tehran, Iran
[2] Department of Physiology and Persian Medicine, School of Medicine, AJA University of Medical Sciences, Tehran, Iran

Correspondence to: smmirghazanfari@gmail.com

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Abstract: The present study was conducted to assess the antifungal activity of essential oils of medicinal plants *Mentha piperita* (peppermint), *Foeniculum vulgare*, *Satureja hortensis* (Savory), *Ferula asafoetida* and *Cuminum cyminum* against *Alternaria* sp., *Bipolaris sorokiniana* and *Acremonium sclerotigenum*. The antifungal activity was evaluated by Broth Microdilution Method. Minimum Inhibitory Concentration (MICs) and Minimum Fungicidal Concentration (MFCs) of the essential oils were compared with Amphotricin B and Captan as standard drug. MIC values for all essential oils were between 1 to 8 mg/mL. MIC value of Fennel essential oil was comparable to MFC value obtained from fungicide Captan. Peppermint essential oil exhibited maximum inhibitory and fungicide activity in concentrations of 2 mg/ml and 4 mg/ml against *Alternaria* sp. The essential oil was more effective than Fennel against *Bipolaris,* but MFC values of both essential oils were 4 mg/ml. *C. cyminum* displayed less susceptibility against all of the fungi. Regarding our finding, peppermint and Fennel oil seem to be a promising solution to control plant diseases.

Key words: *Foeniculum vulgare*; *Satureja hortensis*; *Ferula asafoetida*; *Cuminum cyminum*; Captan; Fungicide effect; Traditional iranian medicine.

Introduction

Pathogenic fungi are main infectious agents in plants, causing enormous losses in yield and quality of field crops, fruits, and other edible plant materials. These agents could also lead to serious consequences on human health (1, 2). Mycotoxins, are secreted by phytopathogenic fungi and can directly affect human and animals health, resulting in serious diseases and death (3). On the other hand, several fungal pathogens such as *Aspergillus* spp., *Acremonium* spp., *Alternaria* sp., *Bipolaris* spp., and *Fusarium* spp. can cross kingdoms and infect humans and capable of causing different diseases such as ocular, keratitis, cutaneous, and sinus in human beings (4). To overcome the persistent attack of fungal plant pathogens, various agrochemical products have been developed and used. However, utilization of synthetic chemical fungicides may not always be advantageous (5, 6). The increased knowledge of environmental problems related to fungicides, warrants the search for novel active molecules and antimicrobial substances from various sources including plant bioactive compounds (7). Due to the priority of Iran's health plan that is restoration and development of traditional Iranian medicine and reduce imports of chemical pharmaceutical raw materials (8), the search for novel active molecules from medicinal plants is important. In the last decade, many researches have been done on traditional medicine in the country (9).

Mentha piperita (peppermint) and *Satureja hortensis* (Savory) belong to the *Lamiaceae* family. Peppermint is a species found in Iran, as well as around the world, and is a popular herb that can be used in numerous forms (dried leaves, boiled leaves and essential oil). Essential oil of peppermint has several important properties, such as antibacterial (10, 11, 12), insecticidal (13), and antifungal activities (15, 16), and is nontoxic for humans (14). On the other hand, savoury is an aromatic herb, being used as a spice and natural food preservative (17). The essential oil of savory has antimicrobial, activities due to their high phenolic contents (18).The Apiaceae family is of particular interest in medicinal chemistry and food that include some an aromatic plant such as *Foeniculum vulgare* (fennel), *Cuminum cyminum* (cumin) and *Ferula asafetida* (Asafoetida).The efficiency of fennel seed and bark extracts against fungal pathogen reported in many studies (19, 20, 21).Cumin, commonly used as food additives and pharmaceutical preparations (22). The essential oil of cumin possesses significant biological properties, such as antibacterial and antifungal activity (23). Several biological activities such as antibacterial and antifungal properties have been reported from the essential oil of *Ferula* species (24).

The *Acremonium* genus is most saprophytic and pathogenic in some plants. Some *Acremonium* species are recognized as opportunistic pathogens of human and animals, causing eumycetoma, onychomycosis, and hyalohyphomycosis (25). *A. sclerotigenum* is a widely distributed saprophyte (26), reported as epiphytic fungus on barley and causal agent of bagged apple brown spot (27, 28). *Bipolaris* spp. is known as plant and humane pathogens with worldwide distribution. It is the causal

agent of common root rot, leaf spot disease, seedling blight, and head blight in plants. A numerous clinical spectrum of *Bipolaris* spp. including allergic and chronic invasive sinusitis, keratitis, endocarditis, endarteritis, osteomyelitis, peritonitis and otitis media have been reported (29). *Alternaria* sp. is a large genus composed mostly of saprobic or plant pathogenic species, which is responsible for at least 20% of agricultural spoilages. They are also common allergens in humans. They willingly cause opportunistic infections in immunocompromised people such as AIDS patients (30).

The objective of the present study was to investigate the possibility of *in vitro* antifungal activity of essential oil of peppermint, fennel, savory, cumin and asafoetida against *Alternaria* sp., *Bipolaris sorokiniana* and *Acremonium sclerotigenum* which known as human and plant fungal pathogens.

Materials and Methods

Plant materials, preparation and essential oil extraction

The aerial parts of cultivated peppermint, fennel, savory, cumin and asafoetida, were collected at the beginning of the flowering stage from Medicinal Plant Garden of Sadra University, situated in Fars province, Iran. The plant materials were dried at room temperature for 10 days. Air-dried aerial parts (100 g) of the plants were subjected to hydrodistillation for 4 h by using a conventional glass Clevenger-type apparatus. The resulting essential oils were dried with anhydrous sodium sulfate and stored at 4℃ for further use.

Chemical analysis and identification of essential oil components

The essential oils were analyzed by Gas Chromatography-Mass Spectrometry. The analysis was carried out on a Thermoquest-Finnigan Trace GC-MS instrument equipped with a DB-5 fused silica column (60m×0.25mm i.d., film thickness 0.25 mm). The oven temperature was programmed to increase from 60◦C to 250◦C at a rate of 4◦C/min and finally held for 10 min; transfer line temperature was 250◦C. Helium was used as the carrier gas at a flow rate of 1.1 mL/min with a split ratio equal to 1/50. The quadrupole mass spectrometer was scanned over the 35–465 amu with an ionizing voltage of 70 eV and an ionization current of 150 mA (31). Retention indexes (RIs) were calculated by using retention times of n-alkanes (C6–C24) that were injected after the oil at the same temperature and conditions. The compounds were identified by comparing their RI with those reported in the literature, and their mass spectrum was compared with those reported In Wiley Library (31).

Fungal strains and inoculum preparation

Strains of *Alternaria* sp., *B.sorokiniana* and *A.sclerotigenum* were isolated from plants in military garrison, situated in Fars province, Iran. The isolates of organisms were subculture once onto potato dextrose agar (PDA) and incubated for 48 to 72 h at 25 C. the identity of all the fungal strains were confirmed by the Mycology lab in the Department of Plant Protection, at shiraz University, Iran. Pure cultures of the fungal strains were deposited at the fungal culture collection the Mycology lab at the same institute. For the broth microdilution method, the inoculum was prepared by growing the fungi on PDA for 7 days at 25 ℃. fungal spores were washed from the surface of PDA plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was transferred to a sterile tube and diluted to 1:100, and then 1:20 with RPMI1640 medium. The suspensions were mixed for 15 second to ensure homogeneity and subsequently were adjusted to a concentration of approximately 1×10^6 spores/ mL (32).

Antifungal activity assays

To investigate the antifungal activity of essential oil, first, a preliminary test was designed using a disc diffusion method. The essential oils were diluted 1.25 µl/ml to10 µl/ml. A plant essential oil that cannot demonstrate inhibitory effect against fungi in Broth Micro dilution method was eliminated (32). To evaluate the antifungal activity of essential oil, a modified version of the microdilution technique was performed (32)**.** A broth micro dilution method was applied to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). The RPMI 1640 medium (Roswell Park Memorial Institute – 1640) with glutamine and morpholinepropanesulfonic acid (MOPS) buffered to pH 7.0, 0.165 mol/l was used. The essential oil was twofold serially diluted with 2% DMSO that contains 0.0125 to 64 µl/ml of essential oil. In each well, 100 µl of each extract dilution was mixed with 100 µl of the fungal spore suspension (2×10^6 spores/ml for *A. sclerotigenum* and 1×10^5 spores/ml for *B. sorokiniana* and *Alternaria* sp.).

The antifungal medication and commercial fungicide respectively, Amphotericin B and Captan (N-(trichloromethylthio) cyclohex-4-ene-1, 2- dicarboximide) were used as a standard fungicide, while the final solvent concentration (1%) DMSO, without oil or the standard drug, was used in the test as a negative control. The assays for all essential oils were repeated at least twice. The microplates were incubated at 25 ◦C and read visually after 72 h. The MIC was defined as the lowest essential oil concentration that caused complete inhibition of visible microorganism's growth in the microdilution wells, as can be detected by the unaided eye. The minimum inhibitory concentration (MIC) readings were performed spectrophotometrically with a microplate reader at 595 nm using ELISA plate reader after 48 h. Also, 10 µl of each well was poured onto the slide and examined by microscope to the determination of germination or non-germination of spores. MICs values were calculated by comparing growth in control wells and the extract blank, which consisted of un-inoculated plates.

The minimum fungicidal concentration (MFC) was determined by sub-culturing the negative wells on potato dextrose agar (PDA). For determined of MFC, after 72 h of incubation 20 µl of contents of each well that showed complete inhibition (100% or an optically clear well), and from the growth control (essential oil -free medium) was sub-cultured onto PDA plates. The plates were incubated at 25 °C until growth was seen in the growth control subculture. The lowest concentration of the essential oil with no visible growth after 48 h was

defined as the MFC, indicating 99.9% killing of the original inoculum compared to Amphotericin B and Captan, used as a positive control (33, 34).

Results

Chemical analysis of the essential oils

The major identified components of the essential oils of peppermint, fennel, savory and cumin are enlisted in Table 1. GC/MS analyses showed that the main constituent of the essential oils was menthol (53.10%) followed by menthyl acetate (15.1%) and menthofuran(11.18%). The major compounds in the essential oils of fennel were (E)-anethole (75.8%), fenchone (7.2%), α-phellandrene (4.6%) and α-Thujone (4.3%). Thymol (28.5%), p-cymene (18.9%), γ-terpinene (16.2%) and carvacrol (11%) were predominant component of savory essential oil. Major component of cumin belonged to cuminaldehyde (48.9%), p-cymene (16.9%), β-pinene (6.1%) and γ-terpinene (6.5%).

Antifungal activity assay

Our finding of in the primary test (disc diffusion method) showed that because of the inadequacy of the inhibition of asafoetida essential oil against the fungi, it was excluded in the next steps. MIC was determined by microscope for each essential oil based on observation of germination or non-germination of fungal spores in all essential oil concentrations (Figures 1-3; essential oils that have had MIC fewer than 2mg/ml are shown). The MFC was determined by sub-culturing the negative wells on PDA. The MICs and MFCs of the selected essential oils on the fungi are shown in Table 2. The results indicated that essential oils of the plants were effective on pathogenic fungal species with a different degree in the following range of concentrations. MIC

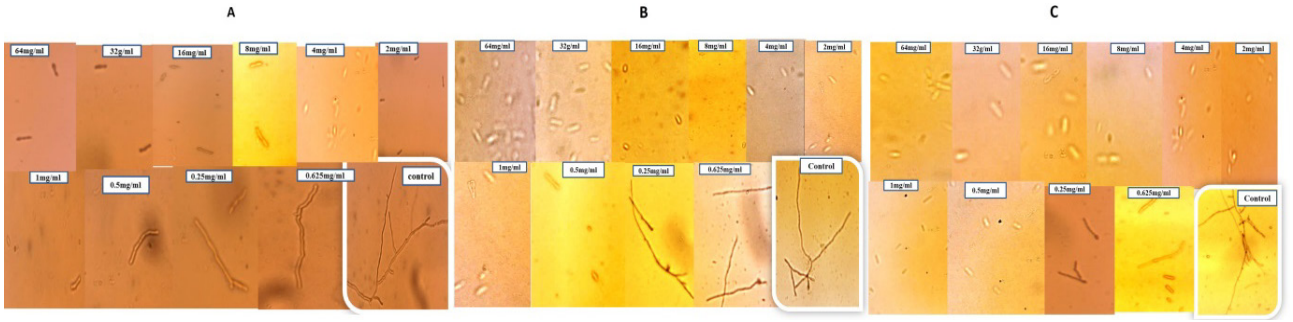

Figure 1. Inhibition and fungicide effect of essential oil of *Foeniculum vulgare* (A), captan (B), and amphotricin B (C) against *Acremonium sclerotigenum*.

Table 1. Quantitative composition of four most abundant compounds identified in essential oils of *Mentha piperita*, *Foeniculum vulgare* and *Satureja hortensis* by GC-MS.

Essential oil	Chemical compound	RIa	Abundance (%)
Mentha piperita	Menthol	1171	53.28
	Menthyl acetate	1295	15.10
	Menthofuran	1164	11.18
	1,8 Cineole	1031	6.69
Foeniculum vulgare	Anethole tranc	1279	75.8
	fenchone	1071	7.2
	α-phellandrene	1000	4.6
	α-Thujone	935	4.3
Satureja hortensis	thymol	1119	28.5
	p-cymene	944	18.9
	γ-terpinene	986	16.2
	Carvacrol	1180	11.0
Cuminum cyminum	Cuminaldehyde	1241	48.9
	p-cymene	1024	16.9
	β-pinene	979	6.1
	γ-terpinene	1059	6.5

Table 2. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) (mg/mL) values for essential oil of plants.

Fungi	MP		FV		SH		CC		Amphotericin B		Captan	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Alternaria sp.	2	2	2	4	4	4	-	-	0.5	1	1	2
Bipolaris sorokiniana	2	4	4	4	8	8	-	-	1	2	0.5	1
Acremonium sclerotigenum	4	8	1	2	4	8	8	8	0.5	1	0.5	1

MIC: minimum inhibitory concentrations; MFC: minimum fungicidal concentrations. MP: *Mentha piperita*, FV: *Foeniculum vulgare,* SH: *Satureja hortensis,* **CC***: Cuminum cyminum.*

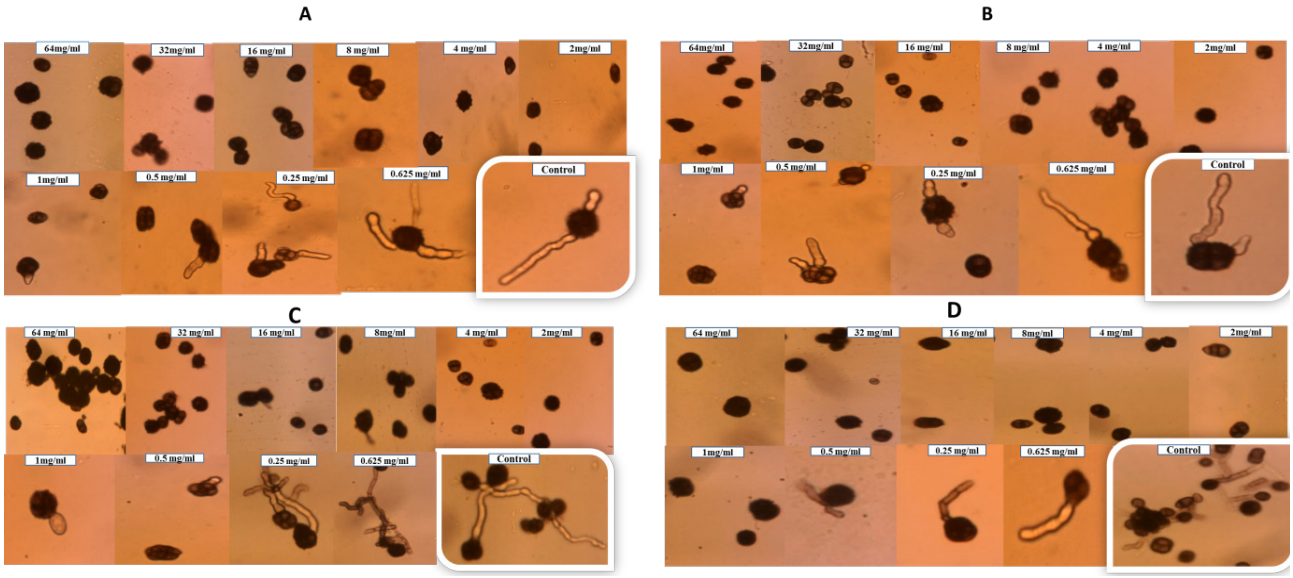

Figure 2. Inhibition and fungicidal effect of essential oil of *Mentha piperita* (A), *Foeniculum vulgare* (B), captan (C) and amphotricin B (D) against *Alternaria* sp.

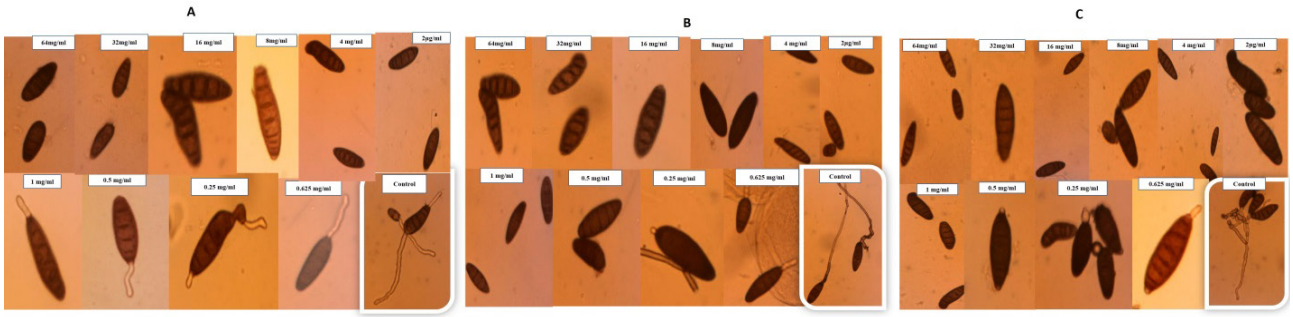

Figure 3. Inhibition and fungicidal activity of essential oil of *Mentha piperita* (A) captan (B), and amphotricin B (C) against *Bipolaris sorokiniana.*

values for all essential oils of peppermint, fennel, savory and cumin were between one to eight mg/ml. The lowest MIC and MFC of fennel essential oil against *A. sclerotigenum* was in concentrations of one and two mg/ml (Figure 1). *B. sorokiniana* with the highest MIC and MFC was a persistent strain but *A. sclerotigenum* was sensitive to the lowest MIC and MFC against the fennel essential oil. The highest inhibitory and fungicidal effect of peppermint essential oil was obtained in concentrations of two mg /ml against *Alternaria* sp.(Figure 2). The MFC values of peppermint essential oil and captan was equal against this fungus. The MIC values of fennel essential oil were two mg/ml and in four mg/ml had fungicide activity against *Alternaria* sp. The lowest activity of essential oils again *Alternaria* sp. was determinate in the essential oil of savory. Cumin essential oil did not show inhibitory and fungicide potential again this fungus.

Peppermint essential oil was more effective against *B. sorokiniana* than fennel essential oil (Figure 3). Fennel essential oil at a concentration of four mg/ml showed a fungicidal and inhibitory effect against *B. sorokiniana* and its fungicidal effect was equal to MFC of peppermint essential oil. The inhibitory effect of peppermint essential oil was equal to the MIC of amphotericin B (2mg/ml) and among the essential oils in this study; it has the same effect with standard fungicide. The highest inhibitory and fungicidal activity of savory essential oil were obtained in concentrations of four mg/mL against *Alternaria* sp. Essential oil of cumin displayed less susceptibility against all of the fungi.

Regarding obtained results, peppermint and fennel essential oils proved to have great fungicidal properties, which their compounds as an efficient bio-preservative system can be used to control plant diseases. Amphotericin B and captan used as positives control, their MIC range obtained from 0.5 to 1 mg/ml. These results demonstrated that a MIC value of fennel essential oil was quite comparable to MFC values obtained from fungicide captan and amphotericin B. The results confirmed the antimicrobial potency of these plants essential oil, especially in case of the peppermint and fennel.

Discussion

The MIC of peppermint essential oil in proposed bio-preservatives system proved to be equal with a MIC of captan fungicide (two mg/ml) against *Alternaria* sp. Dellavalle (2011) has reported the another essential oil , savory seed, is equal with captan Fungicide (35). Based on our results, peppermint essential oil showed the best fungicide efficiency to control *Alternaria* sp., following by fennel and savory.

Peppermint essential oil with concentrations of two mg/ml exhibited the best inhibitory effect on *B. sorokiniana*. The essential oil showed the best fungicidal properties against *Alternaria* sp. and *B. sorokiniana* compared to other essential oils. Our results were in

good agreement with other researches (36, 37, 38, and 39). Furthermore, several studies investigated the inhibitory and fungicidal effect of peppermint essential oil on plant pathogens (40, 41). Recent structural studies revealed that menthol has a hydroxyl group around the phenolic ring, which acts as an effective antimicrobial agent by disruption the cytoplasmic membrane of the microorganisms (42). The antimicrobial property of this essential oil related to the amount of menthol content (43).Possibly the strong fungicidal potential of peppermint essential oil in the present study related to the high content of bioactive compound such as Mentol. Fennel essential oil after Peppermint essential oil showed the best inhibitory and fungicidal effect on *Alternaria* sp. and *B.sorokiniana* which were in agreement with(44, 45,46, 47), studied the effect of this essential oil on plant pathogens such as *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *A.alternata* , *Botrytis cinerea and Colletotrichum acutatum*.

The inhibitory effect of fennel essential oil on 12 human pathogenic and food-degrading fungi has been investigated (48).In addition, the essential oil showed better antifungal properties against *A. sclerotigenum* compare to other essential oils, and its inhibitory concentration was similar to that of captan fungicide. Since the *Acromonium* sp. has been identified as a common human and plant pathogen and no studies have been conducted on the effect of plant essential oils on this fungus, proving the inhibitory and fungicidal effect of fennel essential oil against this fungus in our research was very promising. The antifungal property of fennel essential oil is related to anethole, pinene and fenchone(48). Anethole has been reported as the most efficient bio-preservative compound in the essential oil (49). Verity in the antifungal value of different fennel essential oil samples related to differences in anethole content (48).

In the present study, the best inhibitory and fungicidal effect of the savory essential oil was found on *Alternaria* sp., which was in agreement with other's results (50) who investigated the inhibitory effect of savory essential oil on this fungus. Although in this study, it was found that the inhibitory and fungicidal property of savory essential oil is not as strong as fennel essential oil and peppermint essential oil, but in other studies, it has been reported that this essential oil has a strong inhibitory effect on fungus such as Aspergillus *flavus* and *B. cinerea* (51).

Thymol and carvacrol are recognized as the most important antimicrobial compounds of savory essential oil. The inhibitory effect of thymol against pathogenic fungi has been proved previously (52). Carvacrol is an isotyomyol which inhibits the activity of ATPase and enhance the non-specific permeability of the cell membrane of microorganisms .The difference in the content of bioactive compounds can reduce or increase the inhibitory and fungicidal properties of the essential oil. (53).Cumin essential oil exhibited the lower antifungal potential, which only affects the *A. sclerotigenum* at a concentration of eight µg/ml. Previous studies investigated the fungicidal activity of cumin essential oil on *Candida albican* is lower than other essential oils (54), which are in good agreement with our study. Although, another study proved the stronger fungicidal potential of cumin essential oil against Aspergillus spp.(55). Differences observed in various studies may relate to different constituents of plant essential oils in terms of geographical area, plant variety and age, environmental and seasonal conditions, type of culture, harvesting time, drying and extraction methods, genetic difference, and finally the different of studied microbial strains. The antimicrobial effect of cumin essential oil attributed to the presence of terpinene and cuminaldehyde compounds (56).

Finally, results of our study showed the peppermint and fennel essential oil as a promissory natural fungicidal which could be used to control plant diseases. The essential oil could safely be used as an organic fungicide to replace synthetic fungicides in the prevention and cure of some human disease. These data, together with high yield and nontoxicity, justify their use for those purposes. However, the mechanism of inhibitory effects of these plant's oils against infectious fungi is still unclear. Further investigations regarding the in vitro and in vivo should be conducted in order to decipher the mechanistic pathways and develop such products.

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