Chemical Composition and Antifungal Activity of *Trachyspermum copticum* Essential Oil Against *Alternaria alternata* (In-Vitro Study)

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Abstract

Background: ***Alternaria alternata*** **(AA) is a fungus that has wide range of plant host and can cause diseases in humans and produces a number of mycotoxins. Exposure to high numbers of AA spores can trigger severe asthma exacerbations. Resistance to antifungal drug has been shown in this species.** ***Trachyspermum copticum*** **(Ajowan) is an annual herbaceous essential oil (EO) bearing plant . The ripening seeds of this plant contain 2- 4% essential oil that is rich in monoterpenes like Thymol and is mainly used as an antiseptic agent as well as a drug component in medicine . Anti** ***Alternaria*** **agents can be used in agriculture products or food preservatives, homes and industrial disinfectants and use in infection treatment.**

Objective: In the present study, chemical composition of ***Trachyspermum copticum*** **essential oil were analyzed and examined for antifungal activity against** ***Alternaria alternata.***

Methods: The EO was extracted by distillation method.GC- MS analysis was performed using gas chromatograph with a HP-5MS column. GC analyses were carried out on a system equipped with a HP-5 column. Six isolated ***Alternaria alternata*** **and standard strain ATCC6663 were cultured on to Sabouraud's Dextrose slant agar and condia harvested. The MIC and MFC values of EO for** ***Alternaria alternata*** **isolates were determined by the broth micro dilution and macro dilution methods according the M38-A2 CLSI method (This process was repeated three times for all isolated).**

Results: Of number 11 compounds representing 94.48 % of EO were identified. The major constituents of Ajowan oil were Thymol (46.19%) and, γ-terpinene (18.26%), and ρ-cymene (25.53%). MIC value in five Isolate was 0.09 mg/ml and in two isloted was 0.19 mg/ml. Range of MFC was 0.19- 0.78 mg/ml.

Conclusion: This study confirms that Ajowan essential oil possess in vitro antifungal activity against ***Alternaria alternata*****.**

Keywords: ***Alternaria alternata, Trachyspermum copticum*****, Antifungal, Essential oil**

Introduction

Alternaria alternata belong to the class *Deuteromycetes*, spread globally and can be isolated from plants, soil, food and indoor air. It grows in humid places with Temperature range 20 to 30° C, *Alternaria alternata (AA)* is a fungus that has wide range of plant host and can cause diseases in humans. AA has emerged as opportunistic pathogens particularly in patients with immunosuppression [1]. *Alternaria* can elicit in school-age children immediate or delayed asthma and bronchial hyperreactivity (BHR) [2]. Exposure to high numbers of AA spores can trigger severe asthma exacerbations and is a risk factor for respiratory arrest in AA-sensitized asthmatic children and young adults [3]. This species of fungus is also considered a cause of phaeohyphomycosis, keratitis, onychomycosis, infectious sinusitis and hypersensitivity pneumonia [1, 4]. *AA* produces a number of mycotoxins, including alternariol, alternariol monomethyl ether, altenuene, altertoxins I, II, III, tenuazonic acid and other less toxic metabolites [5]. Many species of the genus *Alternaria* commonly cause spoilage of various food crops in the field or post-harvest decay. A large number of *Alternaria* metabolites has been reported to occur naturally in food commodities. some methabolic of AA demonstrated have carcinogen activity [5]. Resistance to fungicide has been shown in this species [6, 7]. According to the fungal resistance, adverse effect of current drug and preservative there is an increasing demand for novel antifungal drug. Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads [8]. Herbal plant and traditional medicine are inspired for new medicine. Due to an increasing demand for chemical diversity in screening programs, seeking therapeutic drugs from natural products; interest particularly in edible plants has grown throughout the world [9]. Material derived from plant source later may synthesized by chemical reaction in large amount. *Trachyspermum copticum (*Ajowan) is an annual herbaceous EO bearing plant belonging to the *Apiaceae* family, which grows in India, Iran and Egypt [10]. It has been reported that Ajowan fruit oil has diuretic, carminative, analgesic, anti-dyspnea and, anti-inflammatory compounds [11]. The ripening seeds of this plant contain 2- 4% EO that is rich in monoterpenes like thymol and is mainly used as an antiseptic agent as well as a drug component in medicine [2]. Anti *Alternaria* agent can be used in agriculture product or food preservative, home and industrial disinfectant and use in infection treatment. In the present study, chemical composition and the fungal properties of *Trachyspermum copticum* EO were analyzed and examined for its antifungal activities against *Alternaria alternata.*

Materials and methods

Essential oil

The Ajowan seeds were purchased from the Company of Pakan bazr (Isfahan- Iran) and approved by a botanical specialist. The EO was extracted by distillation method. Three ml of oil was extracted from each 100 grams of seeds. The EO was stored in bottles at 4°c.

GC/MS analysis

GC-MS analysis was performed in ACECR (Institute of Medicinal Plants) using Agilent 6890 gas chromatograph with a HP-5MS column. The column temperature program was: initial temperature 50 ° C and hold it at

this temperature for 5 min, thermal gradient of 3° C per minute to 240 ° C with a temperature increase rate of 15 degrees per minute, increasing the temperature to 300° C and hold at this temperature for three minutes. Injector and detector temperatures were 290°c. Helium was used as carrier gas with a linear velocity of 0.8 ml/m. Ionization energy was 70 eV, and mass range 50-550 amu. The components of the oil were identified by comparison of their mass spectra with those of a computer library or with authentic compounds or with the data published in the literature .Mass spectra from the literature were also compared.

GC analysis

GC analyses were carried out on a Younglin Acm6000 system equipped with a HP-5 column. Column temperature program was set up this way: At primary oven 50° C and stopped at this temperature for 5 min, thermal gradient of 3° C per minute, increasing the temperature to 240° C with a rate of 15 degrees per minute, increasing the temperature to 300° C for three minutes, stopping at the temperature. Injection chamber temperature was 290° C and helium carrier gas flow rate to be arguably (expense) 0.8 ml/ minute was used.

Fungal isolates

Six isolated *Alternaria alternata* and standard strain ATCC6663 were obtained from the collection of Mycology research center, faculty of veterinary medicine, University of Tehran. Fungal isolated were cultured on to Sabouraud's Dextrose slant agar (Merck Co., Darmstadt, Germany) at 28° C for 7 days.

Preparation of the conidial suspension

Conidia were harvested from seven day old cultures by pouring a sterile 0.1% aqueous solution of Tween 80 onto the culture slants and scraping the medium surface with a bent glass rod to facilitate the release of conidia. The number of conidia in the suspension was adjusted to approximately 1.5×10^8 conidia/ml using a haemocytometer slide.

Antifungal assay

The MIC and MFC values of EO for *Alternaria alternata* isolates were determined by the broth micro dilution and macro dilution methods according the M38-A2 CLSI method [12]. Different EO concentrations (0.006-12.5 mg/ml) diluted with DMSO (2%) was incorporated in RPMI 1640. One ml of fungal conidial suspension (1.5×10^8 conidia) was added to each tube and incubated at 28°c for 48 hours. Positive control tubes containing only broth media and fungal suspension as well as negative control tubes containing broth media accompanied by EO and DMSO were prepared and incubated at the same conditions. The lowest EO concentration that did not permit any visible fungal growth was considered as the MIC. The tubes that did not show visible fungal growth were sub-cultured onto oil-free Sabouraud's Dextrose agar to determine if the inhibition was reversible. The MFC was the lowest concentration that did not permit any growth on the plates. Samples incubated for 48 h at 28° C and then the results were observed. These results did not change in 72 hours. This process was repeated three times for all isolated.

Results

The chemical compositions of Ajowan EO were shown in Table 1. Of number 11 compounds representing 94.48 % of EO were identified. The major constituents of Ajowan oil were Thymol (46.19%) and, γ-terpinene

(18.26%), and ρ-cymene (25.53%). This oil was examined for antifungal activities against six *Alternaria alternata* isolates and standard strain ATCC6663 by the broth micro and macro dilution methods. MIC and MFC value of the EO are shown in Table 2.

Table 1- Chemical compositions (%, w/w) of Ajowan EO

Components	Percent (%)	Retention Index
Thymol	46.19	29.65
ρ- cymene	25.53	16.05
γ-terpinene	18.26	17.82
β- piene	1.34	13.43
β -myrcene	0.49	14.26
α -thujene	0.28	11.01
α -pinene	0.28	11.32
α-terpinene	0.41	15.51
Sabinene	0.69	16.18
Terpinene	0.32	23.63
β-phyllanderene	0.69	16.18β

Table 2- Antifungal activity of different concentrations EO of Ajowan against various *Alternaria alternata* isolates (mg/ml)

MFC	MIC	Isolates
0.19	0.09	A_1
0.19	0.09	A_2
0.78	0.19	A_3
0.39	0.09	A_4
0.78	0.19	A_5
0.19	0.09	A_6
0.19	0.09	A_7 (ATCC6663)
0.09-0.19		MIC Range

Discussion

Antifungal effects of Ajowan EO against *Alternaria alternata* isolates were shown in the present study. The EO showed significant antifungal activities against all examined isolated. Ajowan essential oil led to the collapse of *Alternaria alternata* conidia which shows the essential oil penetrates into the cytoplasm (Figure 1 and 2). Ajowan EO component analysis in this study was consistent with previous studies. Thymol content in Ajowan EO in several studies conducted in Iran was reported between 39.3-45.2%. The main constituents of the EO were thymol (46.19%), ρ-cymene (25.23%) and γ-Terpinene (18.26%) respectively. Similar to previous studies thymol was found to be the major constituent of the Ajowan EO, although the thymol level was higher than previous studies. MIC level was measured in 71.4 percent of the samples 0.09 and in the remaining 28.6 percent 0.19 mg/ml. MFC level was measured in 57.1 percent of the samples 0.19 mg/ml, in 28.6 percent 0.78 mg/ml, and in 14.3 percent 0.39 mg/ml. These data are similar to previous studies. Serivastava et al. were demonstrated that Ajowan EO includes 11 component and thymol and ρ-cymene are main ingredients and also Ajowan has antifungal and antibacterial effects on gram positive and gram negative

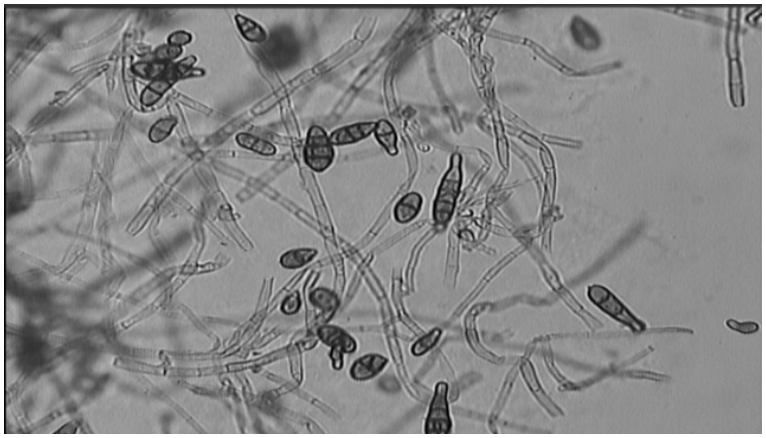

Figure 1- *Alternaria alternata* (ATCC6663) before exposure to Ajowan EO (40X)

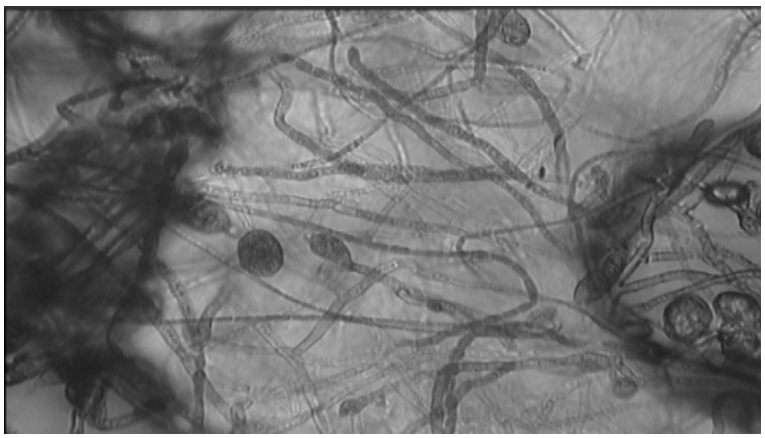

Figure 2- *Alternaria alternata* (ATCC6663) after exposure to Ajowan EO (40X). Ajowan essential oil led to the collapse of *Alternaria alternata* conidia which shows the essential oil penetrates into the cytoplasm

bacteria [13]. Goodarzi et al. investigated the antibacterial activity and combinations of theAjowan EO. The compositions of the EO: thymol 36.%, γ- Terpinene 36.5% and ρ-cymene 21.1 % [10]. Akbari Nia et al. investigated Chemical composition Ajowan EO of produced in Qazvin. The main constituents of the EO were thymol, ρ-cymene and γ- Terpinene [14]. Sirirat Siripornvisal investigated antifungal effects of Ajowan EO against Fusarium oxysporium. MIC 240 µg/ml-1 and MFC 480 µg/ml-1 was reported [15]. Pushpa et al. studied the inhibitory effect of Ajowan EO against Aspergillus ochraceus. At concentrations of 250 ppm, Ajowan EO indicated inhibitory effects on fungal growth and toxin production [16]. Natanzian et al. investigated the antifungal properties of Ajowan EO against *Candida albicans* , the lowest MIC 0.87 and lowest MFC 3.51 µgr/liter was reported [17]. Antifungal of other medicinal plants against *Alternaria alternata* has approved in previous studies. Dalger et al. was reported Sage extract MIC 5.12 mg / liter against Alternaria alternate [18]. Wufeng et al. evaluated antifungal effect of Thyme, Nutmeg, Eucalyptus and Cassia against *Alternaria alternata* by disk diffusion method. Cassia in 300ppm and 500ppm concentrations inhibited of fungal growth. Thyme in 300 ppm concentration inhibiting 62 percent [19]. Zaker and Mossalanejad investigated effect of Mint extract, Eucalyptus, Rubber marigold and Datura against *Alternaria alternata*. Peppermint extract 15%, Marigold 15%, Mint 10%, and Eucalyptus 15% ability to inhibit the myselial growth of *Alternaria alternate* [20] .Comparison of results obtained in this study with previous research some noted indicated that Ajowan EO component analysis used in this study was consistent with previous studies.

In summary, this study confirms that Ajowan EO possess in vitro antifungal activity. However, further studies are still required to investigate its application in medicine and food industries.

Conclusion

In summary, this study confirms that Ajowan EO possess in vitro antifungal activity. However, further studies are still required to investigate its application in medicine and food industries.

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