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CHEMICAL CONSTITUENTS, ANTIMICROBIAL AND ANTIOXIDATIVE EFFECTS OF *TRACHYSPERMUM AMMI* ESSENTIAL OIL

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ABSTRACT

The chemical composition of *Trachyspermum ammi* essential oil (EO) was analyzed by gas chromatography–mass spectrometry, and thymol (63.4%), *p*-cymene (19%) and γ-terpinene (16.9%) were found as the major components. The antimicrobial properties of the EO were investigated against 10 important foodborne pathogenic bacteria and spoilage fungi by disk diffusion and by determining the minimum inhibitory concentration (MIC). The EO exhibited strong activity against both bacteria and fungi, with greater inhibition of bacterial growth compared with fungi; MIC value of EO against all bacteria was evaluated at 500 ppm, whereas the fungal species were inhibited at concentrations of 1000–2000 ppm. The EO also showed antioxidant activity assessed by 2,2-diphenyl-1-picrylhydrazyl, with IC_{50} (concentration providing 50% inhibition) of 34 μg/mL. Similarly, in β-carotene/linoleic acid assay, the EO was effectively able to inhibit the linoleic acid oxidation, exhibiting 82.16% inhibition that was similar to butylated hydroxytoluene. Overall, the EO exhibit potent antimicrobial and antioxidant activity, which supports its potential use for perishable and high fatty foods.

PRACTICAL APPLICATIONS

This study is the first report of the chemical composition of Iranian *Trachyspermum ammi* essential oil and its antimicrobial and antioxidant activities. In conclusion, the obtained data indicate that the essential oil exhibits potent antibacterial and antifungal activity, which supports its use in traditional medicine for its antiseptic properties. The results clearly show that the oil of *T. ammi* presents antioxidant activity and might be useful for high fatty foods.

INTRODUCTION

It has been estimated that as many as 30% of people in industrialized countries suffer from a foodborne disease each year, and in 2000, at least two million people died from diarrheal disease worldwide (WHO 2002). Some bacteria and molds grow and release their own enzymes into the liquid surrounding them, and absorb the products of external digestion. This is the main basis of microbial food spoilage, which lowers its nutritional value and causes financial losses in food industry. Oxidative rancidity is the major cause of food quality deterioration, leading to the formation of undesirable off-flavors as well as unhealthful compounds. Antioxidants are known as molecules capable of inhibiting oxidation process in food and body (Sikorski 2001). Application of some synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), is known to be associated with some malignancies, hepatic damages and toxicities in laboratory animals (Al-Reza *et al.* 2009; Hwang *et al.* 2001).

In recent years, development of antibiotic resistance and negative consumer perception about possible toxicity and other side effects of chemical preservatives shift attentions toward natural alternatives. Many researchers have shown that secondary plant metabolites, such as essential oils (EOs), can exhibit antimicrobial and antioxidant effects

(Carmo and Souza 2010; Gandomi *et al.* 2009; Khosravi *et al.* 2011; Pires *et al.* 2011). These properties are due to the many active phytochemicals including flavonoids, terpenoids, carotenoids, coumarins and curcumines (Koleva and Vanbeek 2001, Singh *et al.* 2004). Moreover, the potential use of EOs as additives to improve food preservation has been researched in several studies (Bei *et al.* 2011; Gandomi *et al.* 2009; Lima 2006; Mahboubi and Kazempour 2011; Saei-Dehkordi *et al.* 2010). *Trachyspermum ammi*, commonly known as *Ajowan*, is one of the aromatic seed spices that originated in the Middle East, India, Iran, Afghanistan and Egypt. In these countries, it is traditionally used as a medicinal plant for its antiseptic, appetizer and carminative properties (Zargari 1989). Thymol, the major phenolic compound present in Ajowan, has been reported to be a germicide, antispasmodic and antifungal agent (Nagalakshmi *et al.* 2000).

The purpose of this study was to assess the chemical composition and antimicrobial effects of Ajowan EO against important foodborne pathogenic bacteria and food spoilage fungi, as well as its antioxidative properties for its potential use as a natural food additive.

MATERIALS AND METHODS

Plant Material

Seeds of *T. ammi* were collected in July 2011 from Isfahan, Iran. Plants were taxonomically identified at the Pharmacognosy Department, Faculty of Pharmacy, University of Tehran, Iran. The seeds were thoroughly washed and dried in the shade at room temperature for 24 h.

Isolation of the Essential Oil

The dried *T. ammi* seeds were submitted to hydrodistillation in a Clevenger-type apparatus at 100C for 5 h. The EO was isolated and dried over anhydrous sodium sulfate and then stored in a dark glass bottle at 4C until required.

Chemical Composition Analysis of the Essential Oil

Gas chromatography–mass spectrometry (GC-MS) method was used for chemical composition analysis of the EO, using an AGILENT 6890 (Santa Clara, CA) gas chromatography equipped with an AGILENT 5973 series mass selective detector (length, 30 m; inner diameter, 250 μm; film thickness, 25 μm). The injector and selective mass detector temperatures were maintained at 250 and 230C, respectively. The oven temperature program was initiated at 40C, held for 1 min and then raised up to 250C at a rate of 3C/min and held for 20 min. Helium was used as the carrier gas at a flow rate 1.0 mL/min. The compounds of the oil were identified by comparing their retention indices (RIs) and mass spectra fragmentation with those on the stored Wiley 7 n.1 Mass Computer Library.

Antimicrobial Activity

Microbial Strains. The activity of the EO was tested toward 10 different foodborne pathogens: bacterial species including *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 6538), *Listeria monocytogenes* (ATCC 19118), *Salmonella typhimurium phagetype II* and *Escherichia coli O157H7*; and fungal species including *Penicillium citrinum* (PTCC 5304), *Penicillium chrysogenum* (PTCC 5033), *Aspergillus flavus* (ATCC 15546), *Aspergillus niger* (ATCC 16404) and *Aspergillus parasiticus* (PTCC 5286). Bacterial and fungal strains were maintained on brain–heart infusion (BHI) agar and potato dextrose agar (PDA) slants, respectively, and stored at 4C.

Preparation of Inocula. Bacterial inocula were prepared by inoculating a loopful from slant culture in BHI broth which was incubated for 18 h at 35C. Second subcultures were prepared in the same condition. Bacterial suspension was adjusted to an optical density of 0.1 at 600 nm, using a Spectronic 20 spectrophotometer (Milton Roy Company, Warminster, PA) and enumerated by duplicate plating from tenfold serial dilutions on BHI agar and counting the colonies after 24 h of incubation at 35C. Fungal strains were grown on PDA slants at 30C for 7–10 days until sporulate. Spores were harvested by adding 10 mL of sterile distilled water containing 0.05% Tween 20 and scraping the surface of the culture to free the spores. The spore suspension was counted with a hemocytometer and further diluted to give a concentration of $(1–5) \times 10^6$ spores/mL.

Determination of Microbial Susceptibility with Disk Diffusion Method. The antimicrobial activity was carried out using the disk diffusion method. *T. ammi* EO (10 μL) was inoculated to 6-mm-diameter disks and placed on BHI agar or PDA plates inoculated with bacterial suspension or fungal spore and incubated at 37C for 24 h and 30C for 48 h, respectively. 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 as an average of duplicated experiments.

Determination of Minimum Inhibitory Concentration (MIC). MICs were determined using the broth microdilution method recommended by the CLSI, with

some modifications (Schwalbe *et al*. 2007). Stock solution of the tested EO in sterile broth medium (RPMI 1640 for fungi and BHI broth for bacterial pathogens) with 10% dimethyl sulfoxide (DMSO) was prepared. Serial dilutions of the stock solutions in broth medium were prepared (200 μL/well) in a 96-well microtiter plate. Then, all wells were inoculated by 20 μL of fungal spore suspension or bacterial culture (final fungal spore and bacterial concentration was 10^4 spores/mL and 5×10^5 cfu/mL, respectively), and incubated at 37C for 24 h and 30C for 48 h, respectively. The MIC was defined as the lowest concentration of the EO at which the microorganism does not demonstrate visible growth. A positive control containing the bacterial culture and DMSO without the EO and a negative control containing only the medium were performed as well.

Antioxidant Activity

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay. The hydrogen atom or electron donation abilities of the corresponding EO were measured through the bleaching of the purple-colored methanol solution of DPPH. This spectrophotometric assay was carried out using the stable radical DPPH as a reagent according to the method of Burits (2000). Briefly, 50 μL of various concentrations of the EO was added to 5 mL of the DPPH solution (0.004% methanol solution). After 30 min of incubation at room temperature, the absorbance was read against pure methanol at 517 nm. The radical-scavenging activities of the samples were calculated as percentage of inhibition according to the following equation:

$$I(\%) = \left(A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}\right) \times 100$$

where A_{blank} is the absorbance of the control (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. EO concentration providing 50% inhibition (IC_{50}) was calculated from the linear regression algorithm of the graph plotted inhibition percentage against the EO concentration using PHARM/PCS Version 4 (Springer-Verlag New York Inc., New York). Values (mean ± SD) of the EO were compared with those of BHT using Student's *t*-test.

β-Carotene–Linoleic Acid Assay

In this assay, the antioxidant capacity was determined by measuring the inhibition of volatile organic compounds and the conjugated diene hydroperoxides arose from linoleic acid oxidation according to the method of Dapkevicius *et al.* (1998). In this regard, stock solution of β-carotene–linoleic acid mixture was prepared as follows: 0.5 mg β-carotene (K15555836, Merck, Whitehouse Station, NJ) was dissolved in 1 mL of chloroform (HPLC grade)and then 25 μL of linoleic acid (L1376-500MG, Sigma, St. Louis, MO) and 200 mg Tween 40 (822185, Merck) were added. After the evaporation of chloroform, 100 mL of oxygen saturated distilled water was added with vigorous shaking. Then, 2500 μL of aliquots was dispensed into the test tubes, 350 μL of the EO (2 g/L) was added and the emulsion system was incubated for 48 h at room temperature. The same procedure was performed for both BHT (as positive control) and blank. After this incubation period, absorbance of the mixtures was obtained at 490 nm. Afterward, the antioxidative capacity of the EO was compared with those of BHT and blank. Further, all inhibition percentages were compared using one-way analysis of variance.

TABLE 1. CHEMICAL COMPONENT (%) OF THE *TRACHYSPERMUM AMMI* ESSENTIAL OIL ANALYZED BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

Components	Rt	RI[a]	%
Thymol	27.64	1235	63.42
α-Thujene	9.41	852	0.07
α-Pinene	9.64	857	0.06
Sabinene	11.34	899	0.44
p-Cymene	14.06	956	19.01
β-Ocimene X	14.12	958	0.10
γ-Terpinene	15.85	993	16.89
Total			99.99

[a] The retention indices (RIs) were calculated for all volatile constituents using a homologous series of *n*-alkanes C8–C16.
Rt, retention time.

RESULTS AND DISCUSSION

The results of the GC-MS analysis of *T. ammi* volatile oil are given in Table 1. The oil was shown to contain a mixture of components, mainly thymol together with a small amount of other volatile compounds. Seven components were identified, which represented 99.99% of the total oils. As shown in Table 1, thymol (63.4%), *p*-cymene (19%) and γ-terpinene (16.9%) were found as the major components. Some previous studies have already reported that thymol is the major component of this oil. Although in our study (Iranian species) thymol represents 63.4% of the total oil, in Indian species (Singh *et al.* 2004), it appears to represent 39%, which may reflect variations due to time of plant growing, preparation process, cultivar differences and geographical location from which the plants were collected (Saei-Dehkordi *et al.* 2010).

The oil was tested against 10 important food spoilage microorganisms, including five pathogenic bacteria and five foodborne fungi, by a qualitative and quantitative evaluation expressed in growth inhibition zone diameters and MIC values, respectively. The results of the inhibitory activities of EOs on the growth of bacterial species are presented in Table 2. As shown in the disk diffusion results, the oil was

TABLE 2. AVERAGE INHIBITION ZONE (MM) AND MINIMUM INHIBITORY CONCENTRATIONS (MIC) (PPM) OF *TRACHYSPERMUM AMMI* ESSENTIAL OIL AGAINST FIVE IMPORTANT FOODBORNE BACTERIA

	Diameter of inhibition zone (mm)	MIC (ppm)
Bacillus cereus	35	500
Staphylococcus aureus	33.5	500
Listeria monocytogenes	36	500
Salmonella typhimurium	34	500
Escherichia coli	39	500

TABLE 3. AVERAGE INHIBITION ZONE (MM) AND MINIMUM INHIBITORY CONCENTRATION (MIC) (PPM) OF *TRACHYSPERMUM AMMI* ESSENTIAL OIL AGAINST FIVE IMPORTANT FOOD SPOILAGE FUNGI

	Diameter of inhibition zone (mm)	MIC (ppm)
Penicillium citrinum	>80	2000
Penicillium chrysogenum	>80	2000
Aspergillus flavus	>80	2000
Aspergillus niger	>80	1000
Aspergillus parasiticus	>80	3000

found to be effective at a 10-μL dose and based on broth microdilution method, the MIC of the volatile oil was 500 ppm against all the tested bacteria (Table 2).

Table 3 shows the antifungal activity of *T. ammi* EO on food spoilage fungi. All assayed fungi were sensitive to the EO presenting large growth inhibition zones with a diameter of more than 80 mm using direct contact disk diffusion method at 10-μL dose (Table 3), which could be due to fungi static activity of its vapor phase. The researchers believe that the antifungal activity of the vapor phase of EOs is the result of their indirect effects on mycelium, and lipophilic properties of the EOs provide the opportunity to be absorbed by mycelium (Vesaltalab and Zafari 2012). MIC values were between 1000 and 3000 ppm for fungal strains. Fungal MIC levels for *T. ammi* were more than that of bacterial strains, which may be related to different outer membrane structures.

A few studies have reported that *T. ammi* is among the most potent medicinal plant because of its antimicrobial properties (Hajare and Sharma 2005; Murthy *et al.* 2009; Singh *et al.* 2004), which have been confirmed and extended in this study. Murthy *et al.* (2009) reported the inhibitory effects of *T. ammi* ethanolic extract on the mycelial growth and spore germination of toxigenic fungi *Aspergillus ochraceus*, showing the immense antitoxigenic potential of this oil (Murthy *et al.* 2009). Previous studies showed that thymol has a high microbicidal and anti-aflatoxigenic effects due to the presence of a phenolic –OH group (Farag *et al.* 1989).

In addition, the antioxidant activity of the EO and the amount of EO which induced 50% inhibition (IC_{50}) was estimated and shown in Table 4. The results indicated that an increase in *T. ammi* EO concentration resulted in an increase of the free radical-scavenging activity. In this regard, the antiradical activity of the EO was less than that of BHT ($P < 0.05$); however, *T. ammi* EO showed good antiradical activity in DPPH assay. In the present study, the decrease in DPPH due to the EO (IC_{50}: 34 μg/mL) of *T. ammi* was higher than that reported by Khanum *et al.* (2011) for the alcohol extract (around 37 μg/mL) of *T. ammi* and also lower than its water extract (around 29 μg/mL) in the work mentioned.

It seems that the high percentage of oxygenated monoterpenes, particularly thymol, and the high level of γ-terpinene and *p*-cymene of *T. ammi* EO might be related to its high antiradical activity (Cao *et al.* 2009).

The antioxidant activity of *T. ammi* EO determined in terms of percent inhibition in β-carotene–linoleic acid system is presented in Fig. 1. As can be seen, the EO effectively inhibited the linoleic acid oxidation as much as 82.16%. In this regard, with the same concentration, the EO showed higher inhibition compared with the control (5.83%) and similar to BHT by as much as 86.53%.

The results of this test were in good agreement with Khanum *et al.* (2011) on *T. ammi* extracts. These characteristics of the *T. ammi* EO can be attributed to its main components. In this regard, Cao *et al.* (2009) presented the oxygenated monoterpenes and monoterpene hydrocarbons

TABLE 4. *IN VITRO* ANTIOXIDANT ACTIVITIES OF *TRACHYSPERMUM AMMI* ESSENTIAL OIL AND BUTYLATED HYDROXYTOLUENE (BHT) IN DPPH (2,2-DIPHENYL-1-PICRYLHYDRAZYL) ASSAY

Sample	IC_{50} (μg/mL)
Essential oil	34 ± 0.45
BHT	11 ± 0.2

Values (mean ± SD) were expressed as IC_{50}.

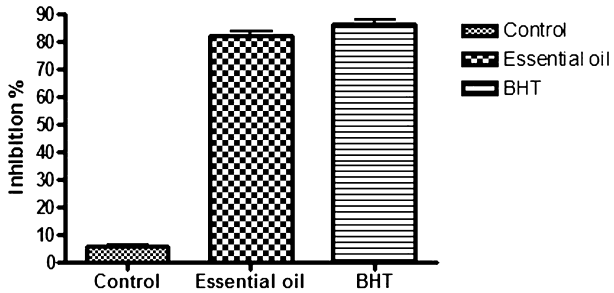

FIG. 1. ANTIOXIDANT ACTIVITY OF *TRACHYSPERMUM AMMI* ESSENTIAL OIL IN TERMS OF INHIBITION OF PEROXIDATION IN LINOLEIC ACID SYSTEM
BHT, butylated hydroxytoluene.

as the principal antioxidant compounds in the plants, and thymol, carvacrol, γ-terpinene and *p*-cymene as the main antioxidant compounds have been reported (Matkowski 2008; Cao *et al*. 2009; Conforti *et al*. 2009; Loizzo *et al*. 2009). The antioxidant activity of these phenolic compounds could be due to their proton-donating effects. In this regard, they can retard or stop the oxidation of other molecules by inhibiting the chain reaction of oxidation (Sepici-Dincel *et al*. 2007).

This study is the first report of the chemical composition of Iranian *T. ammi* EO and its antimicrobial and antioxidant activities. In conclusion, the obtained data indicate that the EO exhibits potent antibacterial and antifungal activity, which supports its use in traditional medicine for its antiseptic properties. The results clearly show that the oil of *T. ammi* presents antioxidant activity and might be useful for high fatty foods.

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