

Phenolic profile, antimicrobial and anti-inflammatory activity of *Carum copticum* L. essential oil

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Ajowan (*Carum copticum*) is an annual herbaceous plant belonging to the *Umbelliferae* family. The seeds of *Carum copticum* have a therapeutic effect on some cutaneous, neural, and urinary tract disorders. The present study reports the chemical composition, antimicrobial activity, antioxidant and anti-inflammatory properties of *Carum copticum* essential oil and its main compounds. The essential oil was obtained from the seeds of the *Carum copticum* by hydrodistillation and analysed by GC/MS. The major components were carvacrol (1%), *p*-cymene (23%), γ -terpinene (23%), β -pinene (5%), α -thujene (3%), sabinene (0.51%), α -phyllanderene (1.6%), α -pinene (1%), terpinene-4-ol (0.1%), thymol (26.03%), β -phyllanderene (1%), myrcene (1%) and limonene (1.05%). The essential oil was also subjected to antifungal and antibacterial tests, using the minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) method. The essential oil was particularly active against *Candida parapsilosis*, with the lowest MIC and MBC value (1.05 mg/ml). Furthermore, the essential oil and its main compounds showed a potent NO scavenging effect and inhibited inducible NO synthase expression. In conclusion, these results support the use of the essential oil and its main compounds for their anti-inflammatory properties and antimicrobial activity.

Keywords: *Carum copticum*, Essential oil, Antimicrobial activity, Antioxidant activity, Anti-inflammatory activity.

INTRODUCTION

In recent years, essential oils have been traditionally used for their antimicrobial, antioxidant, anti-inflammatory and antimicrobial activity. The antimicrobial and anti-inflammatory properties of essential oils and their constituents from a wide variety of plants have been assessed [1]. Antimicrobial and antioxidant properties of certain plant essential oils towards human and animal pathogens have been well documented and reviewed by several authors [2,3]. Chevallier [4] reported that essential oils are used safely in herbal medicine as antimicrobial, antioxidant and anti-inflammatory compounds. Ajowan (*Carum copticum*) is an annual herbaceous plant belonging to the *Umbelliferae* family which grows in India, Iran, and Egypt with white flowers and small, brownish seeds [5]. During the past centuries, several therapeutic effects including anti-vomiting, antiasthma and anti-spasm, are postulated in the Iranian traditional medicine for *Carum Copticum* fruits [6]. The major component of its fruit is essential oil which is composed of γ -terpinene, *p*-cymene, α -pinene, β -pinene, and other substances such as thymol and carvacol [7]. The seeds of *Carum copticum* have a therapeutic effect on some cutaneous, neural, and urinary tract disorders [5].

Thangam and Dhananjayan [8] reported that *Carum copticum* fruit oil has diuretic, carminative, analgesic, anti-dyspnoea and anti-inflammatory properties. Singh *et al.* [9] found that *Carum copticum* is very effective against some bacteria and its effect on the digestive tract may be due to its antibacterial activity. Due to the application of *C. copticum* as an annual herbaceous plant in folk medicine, the purpose of the present work was to evaluate the anti-inflammatory properties, antioxidant and antimicrobial activities of *Carum copticum* essential oil and relate them with the chemical composition, for further application in pharmaceutical industries as natural valuable products.

MATERIALS AND METHODS

Plant material and oil isolation

The plant materials were collected from the mountains at the city of Ilam-Iran in July 2013. The Ajowan seeds were ground and the resulting powder was subjected to hydrodistillation for 3 h in an all glass Clevenger-type apparatus according to the method recommended by the European Pharmacopoeia [10]. The obtained essential oils were dried over anhydrous sodium sulphate and after filtration, were stored at +4 °C until tested.

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Essential oil analysis

The GC/MS analyses were executed on a Hewlett–Packard 5973N gas chromatograph equipped with a column HP-5MS (30 m length × 0.25 mm i.d., film thickness 0.25 μm) coupled to a Hewlett–Packard 5973N mass spectrometer. The column temperature was programmed at 50 °C as an initial temperature, holding for 6 min, with 3 °C increases per min to a temperature of 240 °C, followed by a temperature enhancement of 15 °C per min up to 300 °C and holding at the mentioned temperature for 3 min. Injector port temperature was 290 °C and helium was used as carrier gas at a flow rate of 1.5 ml/min. The ionization voltage of the mass spectrometer in the EI-mode was 70 eV and ionization source temperature was 250 °C. Linear retention indices for all components were determined by coinjection of the samples with a solution containing a homologous series of C₈–C₂₂ n-alkanes and comparing them and their mass spectra with those of authentic samples or with available library data of the GC/MS system (WILEY 2001 data software) and Adams spectral libraries [11].

Antioxidant activity

The efficacy of the essential oils to scavenge 2,20-diphenyl-1-picrylhydrazyl (DPPH) radicals was evaluated using a spectrophotometric method [12,13] based on the bleaching of the bluish-red or purple color of a DPPH solution as a reagent. Briefly, a 50 ml volume of various dilutions of each sample was mixed with 5 ml of 0.004% methanol solutions of DPPH followed by 30 min incubation at ambient temperature. Thereafter, the sample absorbance was recorded against control at 517 nm. The inhibition percentages were measured using Eq. (1). The antioxidant activity of the test samples at a concentration providing 50% inhibition, were considered as IC₅₀ (g/ml).

$$\text{Inhibition percent} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100 \quad (1)$$

Butylhydroxyanisole (BHA) and ascorbic acid were used as positive controls. All experiments were repeated three times and the average results and standard deviations were calculated.

Antibacterial activity

Gram-positive bacteria: *Bacillus cereus* (ATCC 10876), *Enterobacter cloacae* (ATCC 13047), *Enterococcus faecalis* (ATCC 49452), *Listeria monocytogenes* (ATCC15313), *Staphylococcus aureus* (ATCC 25923). Gram-negative bacteria: *Acinetobacter baumannii* (ATCC 19606),

Escherichia coli (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 35659), *Salmonella typhimurium* (ATCC 13311), *Citrobacter freundii* (ATCC 8090). Fungal strains: *Candida albicans* (ATCC 10231), *Candida tropicalis* (ATCC 13803), *Candida parapsilosis* (ATCC 90018), *Aspergillus niger* (ATCC 16404) and *Aspergillus fumigatus* (ATCC 46645).

Screening of antimicrobial activity

The agar disc diffusion assay was employed for determination of the antimicrobial activity of the essential oil [14]. Briefly, a suspension of the test organism (2×10^8 CFU/ml) was spread on the solid media plates. Filter paper discs (6 mm in diameter) were individually impregnated with 15 μl of the diluted oil aliquots (200 mg/ml stock), then placed on the inoculated plates for 2 h at 4 °C. The plates were incubated at 37 °C for 24 h for bacteria, and at 30 °C for 48 h for fungal strains, using a spread restraint method for epiphytes at 30 °C for 48 h [15]. The diameters of the inhibition zones (DD) were measured in millimeters. Each test was carried out in triplicate, repeated three times, and the average was calculated for the inhibition zone diameters.

Determination of minimum inhibitory concentration (MIC)

A broth microdilution method was used to determine the MIC and MBC [16,17]. All tests were performed in Mueller Hinton broth and Sabouraud Dextrose broth, both supplemented with ethanol at a final concentration of 0.5% (v/v) for both bacteria and fungi. Serial doubling dilutions of the oils were prepared in a 96-well plate, ranging from 0.05 to 200.00 mg/ml. The final concentration of each strain was adjusted to 5×10^4 CFU/ml. Plates were incubated at 37 °C for 24 h for bacteria. The MIC was defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth. The microorganism growth was indicated by the turbidity.

Determination of minimum bactericidal concentration (MBC)

To determine MBC, broth was taken from each well and incubated in Mueller Hinton Agar at 37 °C for 24 h for bacteria. The MBC was defined as the lowest concentration of the essential oil at which the incubated microorganism was completely killed [16,17]. Each test was carried out in triplicate and repeated three times.

Anti-inflammatory activity

To evaluate the anti-inflammatory potential of the oils, NO production in lipopolysaccharide (LPS)-stimulated macrophages was used. Exponentially growing macrophages (RAW 264.7 cells) were placed in 24-well microplates at a density of 2×10^5 cells per well in 400 μ l of culture medium and were allowed to adhere for 24 h at 37 °C under 5% CO₂. Cells were then treated with increasing concentrations of essential oil and pure compounds dissolved in DMSO. The final concentration of solvent in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. Cells were then stimulated with 100 μ g/ml LPS and incubated at 37°C under 5% CO₂. After 24 h, cell-free supernatants were collected and NO was measured by the modified method of Green *et al.* [18]. Griess reagent (50 μ l of 1% sulphanilamide and 50 μ l of 0.1% N-1-naphthylethylenediamine dihydrochloride in 2.5% H₃PO₄) was added in equal volume (100 μ l) to cell supernatant and incubated at room temperature for 30 min. N(G)-nitro-L-arginine methyl ester (L-NAME) was used as a positive control. Absorbance was measured using an ELISA automatic microplate reader at 550 nm and the nitrite concentration was determined by regression analysis performed with serial dilutions of sodium nitrite [19].

Statistical analysis

Main effects indicating significant differences were tested using Duncan's multiple range test. Correlation and regression coefficients were

performed using Statistical Package for the Social Sciences (SPSS).

RESULTS AND DISCUSSION

Chemical composition of the essential oil

The chemical composition of *Carum copticum* essential oil is shown in Table 1.

Thirteen compounds representing 97.29 % of *C. copticum* essential oil were identified. The major organic compounds detected in the seeds oils were: carvacrol (1%), *p*-cymene (23%), γ -terpinene (23%), *b*-pinene (5%), α -thujene (3%), sabinene (0.51%), α -phyllanderene (1.6%), α -pinene (1%), terpinene-4-ol (0.1%), thymol (36.03%), *b*-phyllanderene (1%), myrcene (1%) and limonene (1.05%). Thangam and Dhananjayan [8] reported thymol as the main constituent (36.7%) of the *C. copticum* essential oil. A previous report by Rasooli *et al.*, [20] indicated that the major constituent of the essential oil of *C. copticum* was *p*-cymene and in other studies, γ -terpinene was the second most abundant constituent of the oil [5]. Similar to previous studies [21], thymol was found to be the major constituent of the oil *C. copticum*, while others reported carvacrol as the major constituent of this oil [22]. It has been reported that the chemical composition of the essential oil is highly influenced by climatic conditions and geographical factors [2, 23]. The high level of *p*-cymene and thymol in the essential oil could contribute to the valorization of Iranian *C. copticum* species, since this monoterpene is of great importance in industry as intermediate for the synthesis of fragrances, pharmaceuticals and herbicides.

Table 1. Chemical composition of *Carum copticum* essential oil

	Components	%	Retention Index ^a	Identification Methods
1	Carvacrol	1	1306	MS, RI
2	<i>p</i> -cymene	23	1028	MS, RI, CoI
3	γ -terpinene	23	1060	MS, RI, CoI
4	β -pinene	5	948	MS, RI, CoI
5	α -thujene	3	932	MS, RI
6	Sabinene	0.51	981	MS, RI
7	α -phyllanderene	1.6	1000	MS, RI
8	α -pinene	1	941	MS, RI, CoI
9	Terpinene-4-ol	0.1	1177	MS, RI
10	Thymol	36.03	1294	MS, RI, CoI
11	β -phyllanderene	1	1035	MS, RI
12	Myrcene	1	523	MS, RI
13	Limonene	1.05	610	MS, RI
	Total	97.29		

^aThe retention Kovats indices were determined on a capillary column. MS= Mass Spectroscopy, RI= Retention Index, CoI= Co injection with authentic compounds

Table 2. Antimicrobial activity of the essential oils from *C. copticum* using paper disc-diffusion method and microdilution test

Microorganisms	The essential oil of <i>C. copticum</i>			Antibiotic		
	DD ^a	MIC ^b	MBC ^b	DD ^c	MIC ^b	MBC ^b
<i>Bacillus cereus</i>	24	2	2	18	2 ^f	2 ^f
<i>Enterobacter cloacea</i>	14	2.5	2.5	17	5 ^f	3.5 ^f
<i>Enterococcus faecalis</i>	13	4	3.5	15	3 ^f	3 ^f
<i>Listeria monocytogenes</i>	16	4.5	4.5	16	2 ^f	2.5 ^f
<i>Staphylococcus aureus</i>	NA ^e	NA ^e	NA ^e	14	4.25 ^f	6 ^f
<i>Acinetobacter baumannii</i>	16	5	5	14	4 ^f	4 ^f
<i>Escherichia coli</i>	12	5.5	5.5	16	3.5 ^f	3.5 ^f
<i>Klebsiella pneumoniae</i>	23	4	5	19	2.5 ^f	3 ^f
<i>Pseudomonas aeruginosa</i>	NA ^e	NA ^e	NA ^e	20	4 ^f	3.5 ^f
<i>Proteus mirabilis</i>	22	3.5	3.5	20	3 ^f	3 ^f
<i>Salmonella typhimurium</i>	20	2.5	2.5	17	4 ^f	4 ^f
<i>Citrobacter freundii</i>	21	3	3	18	2.5 ^f	4 ^f
<i>Candida albicans</i>	21	2	3	16	5 ^d	5.5 ^d
<i>Candida tropicalis</i>	22	2.5	3	18	4 ^d	3.5 ^d
<i>Candida parapsilosis</i>	27	1.05	1.05	19	2 ^d	3 ^d
<i>Aspergillus niger</i>	20	2.5	2.5	19	3 ^d	3 ^d
<i>Aspergillus fumigatus</i>	23	3.5	4	18	4.5 ^d	4.5 ^d

DD (mm): disc diffusion; a: tested at a concentration of 1.2 mg/disc; b: values given as mg/ml; c: tested at a concentration of 5.0 µg/disc; d: Streptomycin; e: NA. Not Active. f: Netilmycin.

Antimicrobial activity

The *in vitro* antimicrobial activities of *C. copticum* essential oil against the studied microorganisms were qualitatively and quantitatively assessed by the presence or absence of inhibition zones, disc diameters of zone of inhibition (DDs), minimum bactericidal concentrations (MBCs) and minimum inhibitory concentrations (MICs) values (Table 2).

According to the results given in Table 2, *C. copticum* essential oil exhibited moderate to strong and, in few cases, very weak antimicrobial activity against the tested species. However, the essential oil of *C. copticum* failed to show antibacterial activity toward *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Results obtained by disc-diffusion method and microdilution test, followed by measurements of MIC and MBC values indicated that *Candida parapsilosis* is the most sensitive microorganism tested, with the lowest MIC and MBC value (1.05 mg/ml) in the presence of the oil isolated from *C. copticum*. The *C. copticum* oil showed the strongest activity against both types of microorganisms (MIC 2.5–4.5 mg/ml and MBC 2.5–5 mg/ml for bacteria and MIC 1.05–3.5 mg/ml and MBC 1.05–4 mg/ml for fungi). After this, the goal of the present work was to evaluate how the bacteriostatic and bactericidal effectiveness of the *C. copticum* essential oil was affected by the relative concentration of their major volatile compounds. In our study, most of the

antimicrobial activity of the essential oil from *C. copticum* appears to be associated with phenolic compounds (thymol, γ -terpinene and ρ -cymene); these results agree with those reported by other authors [20, 24]. Trombetta *et al.* [25] reported that thymol might induce antimicrobial action by perturbation of the lipid fraction of the microorganism plasma membrane, resulting in alteration of the membrane permeability and leakage of intracellular materials. ρ -cymene is another major compound identified in *C. copticum* oil that is a hydrophobic molecule and causes swelling of the cytoplasmic membrane [2]. *C. copticum* has shown high antimicrobial activity by incorporating cymene in the lipid bilayer of bacteria, facilitating the transport of phenolic monoterpenes of EOs across the cytoplasmic membrane [26]. The results from the disc diffusion method and microdilution test, followed by measurement of minimum inhibitory concentration (MICs) and minimum bactericidal concentrations (MBCs), indicated that *Bacillus cereus* and *Candida parapsilosis* were the most sensitive microorganisms tested, showing the largest inhibition zones and the lowest MICs and MBCs values. Least activity was exhibited against *Escherichia coli*, with the smallest inhibition zones and the highest MICs and MBCs values. In agreement with our results, Cantore *et al.* [27] reported that the Gram-negative strains of bacteria, especially *Escherichia coli*, have lower sensitivity to essential oils. Food poisoning originating from

foods contaminated by both bacteria (Gram-positive and Gram-negative) and fungi causes concern to society and to industry. A major problem in antimicrobial chemotherapy is the increasing resistance to antibiotics, which leads to insufficiency of the antimicrobial treatment [28]. Spices and herbs have been safely used since ancient times as food flavoring agents and herbal medicines and are now mainly “generally regarded as safe” (GRAS). Recently, there have been considerable emphasis studies involving essential oils and extracts of spices and herbs on inhibiting the growth of microbes [29].

Antioxidant activity

Antioxidant activity is a complex process usually occurring through several mechanisms. Due to its complexity, the evaluation of the antioxidant activity for pure compounds or extracts should be carried out by more than one test method [30]. The lower IC₅₀ value indicates a stronger ability of the extract to act as a DPPH scavenger while the higher IC₅₀ value indicates a lower scavenging activity, as more scavengers were required to achieve 50% scavenging reaction. The results presented in Table 3 reveal that *C. copticum* essential oil and its main constituents exhibit a remarkable activity. In particular, thymol clearly showed higher activity (IC₅₀ = 19.24± 0.5 µg/ml) followed by *C. copticum* essential oil (20 µg/ml), γ- terpinene (20.11± 0.3 µg/ml) and β- pinene (26.11± 0.8 µg/ml) samples (Table 3). BHT and ascorbic acid as positive controls exhibited IC₅₀ values of 15.33± 0.7 µg/ml and 5.1± 0.4 µg/ml, respectively. It is well-known that oxygenated monoterpenes and monoterpene hydrocarbons are the main antioxidant compounds in plants [31]. The monoterpene hydrocarbons, *p*-cymene and β-pinene were inactive (Table 3), despite previous reports of their *in vitro* antioxidant activities [32].

Table 3. DPPH radical scavenging activity of *C. copticum* seed essential oil and its main constituents. Butylhydroxyanisole (BHA) and ascorbic acid were used as positive controls.

Tested compounds	IC ₅₀ (µg/ml)
<i>C. copticum</i> essential oil	20
<i>p</i> -cymene	25± 1.4 µg/ml
γ- terpinene	20.11± 0.3 µg/ml
β- pinene	26.11± 0.8 µg/ml
Thymol	19.24± 0.5 µg/ml
BHA	15.33± 0.7 µg/ml
AA	5.1± 0.4 µg/ml

Our findings revealed that the presence of oxygenated monoterpenes such as thymol and γ-terpinene could be attributed to strong antioxidant

activity. However, our results indicated that antioxidant activity of the essential oil is mainly due to the action of γ-terpinene and thymol.

Anti-inflammatory activity

The anti-inflammatory activities of *C. copticum* essential oil and its major constituents were evaluated by measuring their capacity to inhibit cellular NO generation. Nitric oxide is an endogenous free radical species that is synthesized from L-arginine by nitric oxide synthase (NOS) in various tissues. The anti-inflammatory activity of *C. copticum* essential oil was evaluated on RAW 264.7 macrophages which were stimulated to induce overproduction of NO. As presented in Table 4, the essential oil showed a strong inhibitory effect on LPS-induced NO secretion with 84.0±0.3% inhibition observed at 45.0 µg/ml. Comparatively, the L-NAME, used as positive control inhibited NO release by 40.31± 0.3%. Thymol was found to be the most active compound inhibiting NO production by 88.0±0.7 % at 45.0 µM. The effect of thymol alone was similar to that of the essential oil. Therefore, this compound may be responsible for the anti-inflammatory activity of the oil. Nitric oxide inhibition was also demonstrated at 45.0 µM by *b*- pinene (43.1± 0.1%), γ - terpinene (61.11± 0.2 %), γ- and *p*-cymene (39.45± 0.5%). The anti-inflammatory potential of the *C. copticum* essential oil or its main compounds may be directly related to its scavenging ability and/or capacity to inhibit iNOS expression, the enzyme responsible for the release of high amounts of NO, during inflammatory conditions. However, our results (Table 4) suggest that the anti-inflammatory capacity of *C. copticum* essential oil could be mediated, at least in part, by its strong direct antioxidant activity as an effective ROS scavenger. Our results are concomitant with literature data indicating the potent anti-inflammatory activity of oxygenated terpenes and thymol [33-35]. It has been shown that the activity of the enzyme NOS is consistent in human cancer and its selective modulation has been suggested as a potential strategy for chemoprevention and reduction of cancer cell proliferation [36-37]. Indeed, inflammatory mediators, such as NO have been reported to contribute to mutagenesis [38]. This radical is an important regulator of physical homeostasis, whereas large amounts have been closely correlated with the pathophysiology of a variety of diseases and inflammations [38]. Therefore, the inhibition of NO production may be a useful strategy for the treatment of various inflammatory disorders [39]. Inflammation is

involved in many chronic diseases and several types of cancers [40]. Essential oils seem to be a good source of antioxidant and anti-inflammatory natural products. In conclusion, the essential oil of *C. copticum* revealed antimicrobial, antioxidant and anti-inflammatory effects and these results support the traditional use of this plant in antimicrobial activity, relieving pain and inflammation.

Table 4. Effects of *C. copticum* seed essential oil (45.0 µg/ml) and its main constituents (45.0 µM) on NO production in LPS-stimulated RAW-264.7 macrophages. Values are mean±S.D. of three replicates.

Tested compounds	NO inhibition (%)
<i>C. copticum</i> seed essential oil	84± 0.3
<i>p</i> - cymene	39.45± 0.5
γ- terpinene	61.11± 0.2
β-pinene	43.1± 0.0
Thymol	88± 0.7
L-NAME	40.31± 0.3

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ХИМИЧЕН СЪСТАВ, АНТИМИКРОБНО И ПРОТИВО-ВЪЗПАЛИТЕЛНО ДЕЙСТВИЕ НА ЕТЕРИЧНО МАСЛО ОТ *CARUM COPTICUM* L.

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(Резюме)

Аджоуан (*Carum copticum*) е едногодишно тревисто растение, принадлежащо към семейство Umbelliferae. Семената на *Carum copticum* имат терапевтично действие при някои кожни, нервни и урологични смущения. Настоящата работа съобщава за химичния състав, антимикробното, антиоксидантното и противовъзпалителното действие на маслото от *Carum copticum* и неговите по-важни съставки. Етеричното масло се добива от семената на *Carum copticum* чрез хидродестилация и е анализирано чрез GC/MS - техника. Основните компоненти са карвакрол (1%), ρ - кимен (23%), γ - терпинен (23%), β - пинен (5%), α -тужен (3%), сабинен (0.51%), α -филандерен (1.6%), α -пинен (1%), терпинен-4-ol (0.1%), тимол (26.03%), β -филандрен (1%), мирцен (1%) и лимонен (1.05%). Етеричното масло е изпитано с тестове за фунгицидно и бактерицидно действие, използвайки метода на минималните концентрации за инхибиране (MIC) и за бактерицидно действие (MBC). Етеричното масло е особено активно спрямо *Candida parapsilosis*, с най-ниски стойности за MIC и MBC (1.05 mg/ml). Освен това, етеричното масло и неговите основни компоненти показват потенциален NO-отстраняващ ефект и инхибират експресията на индуцирана NO-синтаза. В заключение, тези резултати подкрепят употребата на етеричното масло и неговите основни компоненти поради техните противовъзпалителни свойства и антимикробна активност.