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Chemical composition, antimicrobial activity and antiviral activity of essential oil of *Carum copticum* from Iran

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Abstract

Objectives: Evaluation of therapeutic effects of *Carum copticum (C. copticum)* has been the subject of several studies in recent years. Thymol the major component of *C. copticum* is a widely known anti-microbial agent. In this study, the antibacterial and anti viral activities of essential oil of *C. copticum* fruit were determined.

Materials and Methods: Essential oil of *C. copticum* was analyzed by means of gas chromatography-mass spectrometry (GC-MS). The antimicrobial activity of the oil was evaluated against six Gram (+/-) bacteria and fungi, using the micro broth dilution technique. Antiviral activity of the essential oil was evaluated using a *Bacillus* phage CP51.

Results: From the ten identified constituents, representing 98.7% of the oil, thymol (72.3%), terpinolene (13.12%) and *o*-cymene (11.97%) were the major components. It was found that the oil exhibited strong antimicrobial activity against *Staphylococcus aureus (S. aureus)* and *Bacillus subtilis (B. subtilis)* (MIC, 0.00025% v/v). Furthermore, the antiviral activity of the oil was evaluated by plaque reduction assay.

Conclusion: The essential oil showed an antiviral activity against phage when phage was pre-incubated with the essential oil prior to its exposure to *B. cereus* and without any pre-incubation with the phage, suggesting that the oil directly inactivated virus particles.

Keywords: *Carum copticum*; Chemical composition; Essential oil; Antimicrobial activity; Antiviral activity.

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Introduction

Carum copticum (C. copticum) is a widely distributed annual herbaceous plant which grows in the east of India, Iran and Egypt. The fruits of *C. copticum*, commonly known in Iran as 'Zenyan', have been used extensively in Iranian folk and traditional medicine to treat several disorders like gastrointestinal, rheumatic and inflammatory disorders (Zargari, 1988). The fruits of *C. copticum* were also used for its therapeutic effects such as diuretic, anti-vomiting, carminative and antihelmetic effects. Evaluation of therapeutic effects of *C. copticum* has been the subject of several studies in recent years. In some studies, muscarinic effect of roasted *C. copticum* (Boskabady, Ramazani, & Tabei, 2003), showed β-adrenergic stimulatory effect and anti-cholinergic properties (Boskabady & Shaikhi, 2000). Bronchodilatory and xanthin-like effects for its essential oil have also been suggested (Boskabady et al., 2003). The essential oil contains thymol, γ-terpinene , ρ-cymene, β-pinene, α-pinene, myrcene, limonene, terpinene-4-ol, α -terpineol, α-thujene, α-terpinene, etc (Boskabady & Shaikhi, 2000; Khajeh et al., 2004; Masoudi et al., 2002). Thymol, the major component of *C. copticum* is a component classified among the group of monoterpenes. The interest in isolated monoterpenes has been growing during recent years due to their eventual pharmaceutical or pharmacological utility. Thymol is a widely known anti-microbial agent.

In this study, the antibacterial and anti viral activities of essential oil of *C. copticum* fruit were determined by micro broth dilution assay and plaque reduction assay, respectively. The chemical constituents of the essential oil were also studied by using gas chromatography-mass spectrometry (GC-MS).

Material and Methods

Plant material

The plant *C. copticum* was collected from Abas abad village area, Torbat e Heydarieh, Khorasan Razavi Province, in North East of Iran in November 1999 and identified by Mr. Joharchi and a voucher specimen deposited in School of Pharmacy herbarium (no 293-0303-19), Mashhad University of Medical Sciences, Iran. The material was air-dried indoors prior to isolation of the essential oil.

Extraction and isolation of essential oils

The air-dried powdered fruits of *C. Copticum* were completely immersed in water and hydrodistilled in a full glass Clevenger-type apparatus. The extraction was carried out for 4 h giving light yellow color oils. When the condensed material was cooled down, the water and essential oils were separated. The oils were decanted to be used as essential oils. To improve the recovery, the essential oils were dried over anhydrous sodium sulphate (Merck) until the last traces of water were removed and then stored in a dark glass bottle at 4 °C prior to GC-MS analyses. The extraction yield for the essential oils was 2% W/V.

Analysis

GC/MS analyses were performed on a Varian Saturn 3 GC-Ms spectrometer equipped with a DB-5 column (30 m, 0.32 mm; J & W Scientific). The oven temperature program was initiated at 60 °C, held for 1 min then raised at 3 °C/min to 240 °C, held for 20 min. Other operating conditions were as follows: carrier gas, He (99.999%), with a flow rate of 2 ml/min; injector mode: split injection. Mass spectra were taken at 70 eV. Mass range was from m/z 40–300 amu. The components of the essential oils were identified by comparing their retention indices and mass spectra fragmentation patterns with those stored on

the MS-data bank (Saturn, version 4) computer library built up using pure substances or with authentic compounds and confirmed by comparison of their retention indices.

Antimicrobial activity

Microorganisms and culture media: Antimicrobial activity of the essential oils against bacteria was determined using the microbroth dilution technique. The microorganisms obtained from Persian Type Culture Collection, PTCC, Tehran, Iran were included three Gram-positive microorganisms: *Staphylococcus aureus* (PTCC 1337), *Bacillus subtilis* (PTCC 1023) and *Candida albicans* (PTCC 5027); three Gram-negative bacteria: *Escherichia coli* (PTCC 1330), *Klebsiella pneumoniae* (PTCC 1053) and *Pseudomonas aeruginosa* (PTCC 1047); Standard antibiotic (Gentamycine) was used as controls for the sensitivity of the tested bacteria and ketoconazole were used as control for the tested fungi. Mueller Hinton broth, Sabouraud dextrose broth and Nutrient agar media were used for microdilution assay.

Minimum inhibitory concentrations (MICs): The essential oil was tested against the strains for its inhibitory activity, using a common microbroth dilution method in 24 multiwell plates, in triplicates (Eloff, 1998). For bacterial strains Mueller-Hinton broth (Difco) and for *Candida albicans* Sabouraud dextrose broth were used. The plant essential oil was initially mixed with Tween 80 (4: 6) and then diluted in Mueller-Hinton broth, to reach the proper final concentrations. The assay plates contained 24 wells in a 4 x 6 arrangement. The first column of each plate was considered as growth control, since no essential oil was added. The three remaining wells of each row were used to test each defined dilution of the essential oil. One ml of the broth with the relevant dilution of essential oil, was added to each of the three test wells of each line, and then 1 ml of a growing culture of one of the specified organisms was added to make an inoculum of 10^6 Colony Forming Units per ml ($CFU.mL^{-1}$). Plates were incubated for 24 h at 37 °C. As an indicator of bacterial growth, a 0.5 ml of a solution of three phenyltetrazolium chloride (TTC, 5 $mg.ml^{-1}$) was added to the wells and incubated at 37 °C for 30 min. The lowest concentration of each extract showing no growth was taken as its minimal inhibitory concentration (MIC). The yellow color tetrazolium salt acts as an electron acceptor and is reduced to a red-colored formazan product by biologically active organisms (Eloff, 1998). Where bacterial growth was inhibited, the solution in the well remains yellow after incubation with TTC.

Antiviral activity

Culture media and Bacterial Strain: Phage Assay Broth (PA Broth): nutrient broth 13 gl^{-1} (Merck, Germany), NaCl 5 gl^{-1} (Merck, Germany), at pH 5.6-6.0 was used in all the protocols; Phage Assay Agar, consisted of the above with the addition of 15 gl^{-1} agar (Merck, Germany) was used for *Bacillus cereus (B. cereus)* culture to produce the phage; Phage assay top agar: for plaque assay, the 'soft layer agar' was used where the agar content was reduced to 7 gl^{-1}. All media contained 5 ml of the solution consisting of 40 gl^{-1} $Mg(SO_4)_2.7H_2O$, 10 gl^{-1} $MnSO_4.H_2O$, 30 $gl^{-1}CaCl_2.2H_2O$. Soybean casein digest agar (SCDA): casein enzymatic hydrolysate 15 gl^{-1}, papaic digest of soybean meal 5 gl^{-1}, sodium chloride 5 gl^{-1}, agar 15 gl^{-1}.

B. cereus ATCC 10876 was used throughout the study. Cultures were stored at –20°C in 15% glycerol (Favrin et al., 2003). Prior to investigation a stock culture of the bacteria was maintained on SCDA plate. One loopful of the *B. cereus* was inoculated into a petri dish containing 15 ml of SCDA and incubated for 24 h at 37°C. Then, a few drops of phage suspension were added and the plate

was incubated further for 24 h at 25°C. Bacteriophage: A *Bacillus* phage CP51 was used in this study. The phage stocks were prepared on the host strain, *B. cereus* ATCC 10876, by a plate lysis procedure essentially equivalent to growing bacteriophage Lambda-derived vectors (Atta-ur-Rahman et. al., 2001). Briefly, one loopful of the *B. cereus* was inoculated into a petri dish containing 15 ml of SCDA and incubated for 24 h at 37°C. An aliquot (100 µl) of the phage sample (10-fold serially diluted with PA broth) was mixed with 100 µl of an overnight SCDA culture of *B. cereus* ATCC 10876 in a sterile Eppendorf micro-centrifuge tube (polypropylene; 1.5 ml; Sarstedt) and incubated for 15 min at 37°C to facilitate attachment of the phage to the host cells. The mixture was transferred from the Eppendorf micro-centrifuge tube to a 5 ml Bijou bottle and then 2.3 ml of soft layer agar was added which had been melted and cooled to 40°C in a water bath. The contents of each bottle were then well mixed by swirling, poured over the surface of a plate and allowed to sit for 15 min at room temperature. The plates were incubated for 18 h at 37°C, and a plate showing almost confluent plaques was used to prepare a concentrated phage suspension by overlaying with 5 ml of PA broth. The over layer medium containing the phage CP51 was decanted and filtered through a 0.22 µM filter syringe. The filtrate was used as a phage stock solution. Several dilutions of phage solution were made (Atta-ur-Rahman et. al., 2001).

Phage inactivation assays

Pre-incubation protocol: The essential oil was mixed with Tween 80 (4: 6) and shaked until homogeneity. Different concentrations of essential oil in PA Broth were prepared and filter sterilized. One loopful of *B. cereus* from overnight culture was taken and inoculated into a PA Broth medium (10 ml). The medium was mixed thoroughly and incubated at 37°C for 5 h. To a 500 µl sterile solution of essential oil, 100 µl of phage in proper dilution was added and the mixture was incubated at 25°C for 2h. Then, 500 µl of bacterial suspension and 3 ml of PA Top Agar medium were added. This mixture was overlaid onto a PA agar plate and incubated at 25°C for 24 h. The negative control contained all above except for the replacement of bacterial suspension with PA Top Agar medium. In the positive control plate the oil was replaced with 500 µl trifluridine.

No pre-incubation protocol: The essential oil was mixed with Tween 80 (4: 6) and shaked until homogeneity. Different concentrations of essential oil in PA Broth were prepared and filter sterilized. One loopful of *B. cereus* from overnight culture was taken and inoculated into a PA Broth medium (10 ml). The medium was mixed thoroughly and incubated at 37°C for 5 h. To a 500 µl sterile solution of essential oil, 100 µl of phage in proper dilution, 500 µl of bacterial suspension and 1.8 ml of PA Top agar were added and the mixture was overlaid onto a PA agar plate and incubated at 25°C for 24 h. The negative control contained all above except for the essential oil. In the positive control plate, the oil was replaced with 500 µl trifluridine.

Statistical analysis

All assays were performed in triplicate. The arithmetic mean ± standard error of the mean (SEM) of control and experimental results were compared using the Student's *t*-test. $p<0.05$ was considered statistically significant.

Results

Essential oil isolation and chemical composition

The essential oil obtained by hydrodistillation of the air-dried powdered fruits of *C. copticum* was light yellow in

color and possessed a distinct sharp odor. The yield was 2 % v/w. The analysis of the volatile constituents was carried out using GC-MS system. The chemical compositions are summarized in Tables 1. The identified components represented 98.7% of all the components found in the oil sample. The major components included: thymol (72.3%), terpinolene (13.12%) and *o*-cymene (11.97%). (Table 1).

Table 1. Chemical composition (%) of the essential oil of *Carum copticum*.

Components	Kovats index[1]	Percentage
Sabinene	974	0.57
Myrcene	993	0.35
α-Terpinene	1019	0.28
o-Cymene	1037	11.97
β-Phellandrene	1044	0.37
Terpinolene	1090	13.12
α-1-Phellandrene	1129	0.16
Terpineol	1138	0.51
Terpinenyl acetate	1180	0.15
Thymol	1294	72.3

[1]Retention indices (KI) on DB-5 MS capillary column.

Antimicrobial activity

The results of the antimicrobial activity (Table 2) showed that the essential oil of *C. copticum* exhibited antimicrobial activities against all the bacteria tested in dilution 0.05% (v/v) except for *P. aeruginosa*. The essential oil showed strong antimicrobial activity against *S. aureus* and *B. subtilis* (MIC, 0.00025% v/v). MIC value for *E. coli* was 0.0005% (v/v) while *K. pneumoniae and C. albicans* appeared to be the microorganisms displaying significant resistance.

Antiviral activity

In order to determine that if essential oil can affect viral particles prior to attachment to host cell or after the virus enters the host cell, we performed our experiment using either protocol where phage was pre-incubated with the essential oil prior to its exposure to *B. cereus* or without any pre-incubation with the phage.

Different dilutions of essential oil were added to the mixture of phage and *B. cerus*, without any pre-incubation of phage CP51 with essential oil and a significant reduction (>%50) in plaque was observed for dilutions of 0.1% and 0.01% (v/v) (Figure 1) ($p<0.001$). After pre-incubation of phage CP51 with different dilutions of essential oil for 2h, a significant reduction (>%50) in plaque number was observed for dilutions of 0.1, 0.01 and 0.001% (v/v) ($p<0.001$) while no significant reduction of plaque was observed at lower dilutions (Figure 1). Comparing the with our without pre-incubation methods, pre-incubation method has shown to be significantly more effective especially in 0.01 and 0.001 dilutions ($p<0.001$).

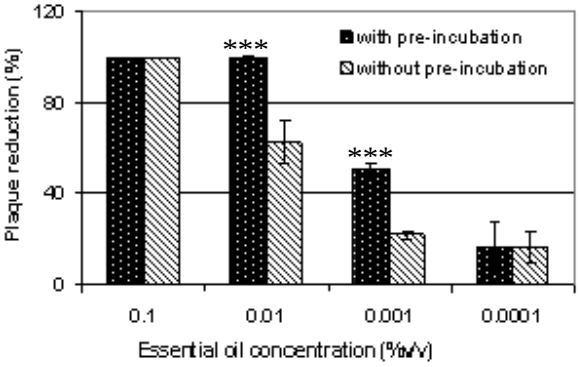

Figure 1. Effect of different concentration of *C. copticum* essential oil on reduction of phage CP51 using pre-incubation and no pre-incubation protocols, ***$p<0.001$.

Table 2. Minimum inhibitory concentrations of the essential oil of *Carum copticum* against test organisms.

	Minimum inhibitory concentrations					
Dilutions (v/v%)	***Staphylococcus aureus***	***Bacillus subtilis***	***Candida albicans***	***Escherichia coli***	***Klebsiella pneumoniae***	***Pseudomonas aeruginosa***
0.05	Y	Y	Y	Y	Y	R
0.005	Y	Y	R	Y	R	R
0.001	Y	Y	R	Y	R	R
0.0005	Y	Y	R	Y*	R	R
0.00025	Y*	Y*	R	R	R	R
0.0002	R	R	R	R	R	R
0.000125	R	R	R	R	R	R

Y No growth (No colour change)
R Growth (Color changed to red formazan)
* Minimum inhibitory concentrations

Discussion

GC study of the essential oil from Iranian *Carum copticum* led to the identification of 10 constituents (corresponding to 98.7% of the total weight) among which thymol, terpinolene and *o*-cymene were the main ones. Other reports indicated that thymol is the major constituent of *C. copticum* essential oil but mostly less than 50% of all the components (Balbaa et al., 1973; Khajeh et al., 2004; Lucchesi et al., 2004; Mohagheghzadeh et al., 2007). Terpinolene was found to be the second main component in our study which is reported by Sahaf et al., 2007 as a constituent of *C. copticum* essential oil. Cymene was the other main component of the oil (11.97%). This value is in accordance with literatures data on *C. copticum* fruit essential oil (Balbaa et al., 1973; Masada, 1976; Lucchesi et al., 2004; Mohagheghzadeh et al., 2007) which *para*-cymene is one of the three major components, however, in our analysis *ortho* isomer of the cymene was present

The oil exhibited strong antimicrobial activities against gram positive bacteria and possessed much better antiviral activity. Considering the fact that thymol possesses antimicrobial activity and has been the subject of several investigations in vitro (Dorman & Deans, 2000; Lambert et al., 2001) and in vivo (Adam et al., 1998; Manohar et al., 2001), we can conclude that the antimicrobial activity of the essential oil from *C. copticum* can be attributed, to a considerable degree, to the presence of thymol. Its mechanism seems to be mainly related to the harmful effects on both the cellular cytoplasmic membrane (perforation) and the generation of ATP (Sanchez et al., 2004). It has been reported that essential oils with high concentrations of thymol and carvacrol e.g. oregano, savory and thyme, usually inhibit Gram-positive more than Gram-negative pathogenic bacteria (Nevas et al., 2004). Our data fit well with the above mentioned study.

It has been reported that essential oils not only show antibacterial and antifungal activities but also have antiviral activity. Essential oils have demonstrated virucidal properties, with the advantage of low toxicity compared with the synthetic antiviral drugs (Baqui et al., 2001; Primo et al., 2001; Schnitzer et al., 2001).

Several phytochemicals act as antiviral agents and have complementary and overlapping mechanisms of action, including antiviral effects by either inhibiting the formation of viral DNA or RNA or inhibiting the activity of viral reproduction. (Jassim & Mazen, 2007). The essential oil of *Santolina*

insularis showed an antiviral activity *in toto* against HSV-1 and HSV-2 *in vitro* and was capable of preventing cell-to-cell virus spread in infected cells (De Logu *et al.*, 2000). From our study it could be concluded that the oil directly inactivated virus particles, thus preventing adsorption of virion to host cells.

The results showed that this plant oil could act as a potential antiseptic agent; however, further investigation should be carried out against new series of pathogenic microorganisms.

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