

## Possible Application and Chemical Compositions of *Carum copticum* Essential Oils Against Food borne and Nosocomial Pathogens

<sup>1,2</sup>Kamiar Zomorodian, <sup>3</sup>Mohammad Reza Moein, <sup>1</sup>Mohammad Javad Rahimi,  
<sup>1,2</sup>Keyvan Pakshir, <sup>3</sup>Younes Ghasemi, <sup>2</sup>Sara Abedi and <sup>2</sup>Sanaz Sharbatfar

<sup>1</sup>Center of Basic Researches in Infectious Diseases, School of Medicine,  
Shiraz University of Medical Sciences, Post Code 71348-45794, Shiraz, Iran

<sup>2</sup>Department of Medical Mycology and Parasitology, School of Medicine,  
Shiraz University of Medical Sciences, Post Code 71348-45794, Shiraz, Iran

<sup>3</sup>Department of Pharmacognosy and Pharmaceutical Sciences Research Center,  
Faculty of Pharmacy, Shiraz University of Medical Sciences, P.O. Box: 71345-1583, Shiraz, Iran

**Abstract:** The essential oils (EOs) obtained from *Carum copticum*, harvested in the two different southern parts of Iran, were evaluated for their antimicrobial properties against food borne and nosocomial pathogens. The EOs of air-dried samples were obtained by hydrodistillation and analysed by gas chromatography/mass spectrometry (GC/MS). The antimicrobial activities of the EOs were evaluated by broth microdilution method as recommended by CLSI.  $\alpha$ -Terpinene and *p*-cymene were identified as the main constituents of the both EOs. The both EOs exhibited a broad spectrum antibacterial and antifungal activity against the tested organisms. In addition, the EOs exhibited varying antifungal activities ranging from 0.25 to 16  $\mu$ l/ml. Among the studied EOs, ecotype *A* with high concentration of thymol showed better antimicrobial activities than ecotype *B*. These results suggest that the EOs from *C. copticum* should be investigated further for possible use in antimicrobial products and food preservatives.

**Key words:** *Carum copticum* • Essential Oil • Antimicrobial activity • Foodborne • Broth micro dilution

### INTRODUCTION

There is a growing tendency in producers and consumers to reduce the use of synthetic compounds. Recently, there has been a considerable demand in products from natural resources with a notable antimicrobial activity to be used as a food preservative, antimicrobial agent, detergent or disinfectant [1-3]. These substances such as aromatic plants can be used to inhibit the growth or kill the pathogenic and toxin producing microorganisms in particular the causative agents of food borne and nosocomial infections [1-7]. Unfortunately, many of these pathogenic microorganisms are known to be resistant to the routine antibiotics [8] and therefore, presenting the new antimicrobial agents from natural resources are one of the main priorities of the pharmaceutical and food processing industries.

*Carum copticum* is a grassy, annual plant of Umbelliferae family with a white flower and small, brownish seeds which commonly grows in Iran, India, Egypt and Europe. The seeds have been used for their flavour and spice in food industry [9]. In Persian traditional medicine, the seeds of *C. copticum* (Zenyan in Persian) were used for its therapeutic effects such as diuretic, anti-vomit, carminative, anti-helmentic, expectorant, analgesic, anti-asthma, anti-dyspnea and anti-spasm [10]. *C. copticum* is also useful in treating diarrhea, colic and other bowel problems [10, 11]. The Essential oils (EOs) of *C. copticum* have been reported to have antioxidant [12], anti-cholinergic [13], anti-histaminic activities [14] and analgesic effects [15]. Moreover, the EOs successfully inhibited the growth of bacteria associated with gastrointestinal infections, including *Staphylococcus aureus*, *Enteropathogenic Escherichia*

**Corresponding Author:** Kamiar Zomorodian, Center of Basic Researches in Infectious Diseases, School of Medicine, Shiraz University of Medical Sciences, Post Code 71348-45794, Shiraz, Iran.  
Tel/Fax: +98-711-2305291, Mob: +98-9177144094.

*coli*, *Salmonella thyphimorium* [16], multi-drug resistant *S. typhi* [17] and *Helicobacter pylori* [18]. In addition, it has been shown that *C. copticum* EOs have inhibitory effects against fungal growth [19-21] and aflatoxin production by *Aspergillus parasiticus* [22]. The anthelmintic activity of *C. copticum* seeds against human *Ascaris lumbricoides* and gastrointestinal nematodes of sheep is appreciable [23, 24]. Despite regular (common) usage of *C. copticum*, only a few published reports are available regarding its antimicrobial activities especially against food borne pathogens. In the present study, the chemical constituents of two ecotypes (ECTPs) of *C. copticum* were studied and their components were compared with each other and to previously reported data. In addition, the antimicrobial effects of these EOs were evaluated against standard and clinical strains of the main causative agents of food-borne and nosocomial infections.

## MATERIALS AND METHODS

The plant materials were collected from Kamfirouz (ecotype A) and Eghlid (ecotype B) near Shiraz, Fars Province, Iran in May 2010. Voucher specimens (No: 12) have been deposited in the Herbarium of the Faculty of Pharmacy, the Shiraz University of Medical Sciences, Iran.

GC/MS analysis was carried out using a Hewlett-Packard 6890/5973 operating at 70.1 eV ionization energy, equipped with a HP-5 capillary column (phenyl methyl siloxane, 25 m × 0.25 mm i.d) with He as the carrier gas and split ratio of 1:20. Oven temperature was performed as follows: 60°C (3 min) to 260°C at 3°C/min; detector temperature, 260°C; carrier gas, He (0.9 mL/min). Retention indices were determined by using retention times of *n*-alkanes that were injected after the EO under the same chromatographic conditions. The components of the EOs were identified by comparison of their mass spectra and retention indices (RI) with those given in the literature and by comparison of their mass spectra with the Wiley library or with the published mass spectra [25, 26].

The antibacterial activities of the essential oil against standard species of *S. aureus* (ATCC 25923 and ATCC 700698), *E. coli* (ATCC 25922), enterohemorrhagic *E. coli* (ATCC 43894), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella flexneri* (NCTC 8516), *S. enterica subsp. enterica* (ATCC 14028), *Enterococcus faecalis* (ATCC11700), *Streptococcus pyogenes* (ATCC 8668) and clinical isolates of *S. aureus*, *E. coli*, *En. faecalis*, *En. faecium* and *P. aeruginosa* collected from the Dr. Faghihi Hospital

(Shiraz, Iran) were determined in this study. The antifungal activities of the essential oil against seven American Type Culture Collection (ATCC) strains of fungi including *Candida albicans* (ATCC 10261), *C. tropicalis* (ATCC 750), *C. krusei* (ATCC 6258), *C. glabrata* (ATCC 90030), *C. parapsilosis* (ATCC 4344), *A. flavus* (ATCC 64025) and *A. fumigates* (ATCC 14110) were also determined. In addition, the antimicrobial activities of the EOs against 10 clinical isolates of *C. albicans* identified by PCR-RFLP were also examined [27]. The susceptibility of all the clinical isolates of bacteria and fungi against selected antibiotics were examined by broth microdilution methods [28, 29].

MICs were determined using the broth microdilution method recommended by the CLSI with some modifications [28, 29]. Briefly, for determination of antimicrobial activities against yeast, serial dilutions of the EOs (0.125 to 64.0 µL/mL) were prepared in 96-well microtitre plates using RPMI-1640 media (Sigma, St. Louis, USA) buffered with MOPS (Sigma, St. Louis, USA). To determine the antibacterial activities, serial dilutions of the EOs (0.125 to 64.0 µL/mL) were prepared in Muller-Hinton media (Merck, Darmstadt, Germany). Test yeasts or bacteria strains were suspended in the media and cell densities were adjusted to 0.5 McFarland standards at 530 nm wavelength using a spectrophotometric method (this yields stock suspension of  $1-5 \times 10^6$  cells/mL for yeasts and  $1-1.5 \times 10^8$  cells/mL for bacteria). 0.1 mL of the working inoculums was added to the micotiter plates which were incubated in a humid atmosphere at 30°C for 24 - 48 h (yeast) or at 37°C for 24 h (bacteria). 200 µl of the uninoculated medium was included as a sterility control (blank). In addition, growth controls (medium with inoculums but without essential oil) were also included. The growth in each well was compared with that of the growth control well. MICs were visually determined and defined as the lowest concentration of the essential oil produced  $\geq 95\%$  growth reduction compared with the growth control well. Each experiment was performed in triplicate.

In addition, media from the wells with fungi showing no visible growth were further cultured on sabouraud dextrose agar (Merck, Darmstadt, Germany) and from wells with bacteria showing no visible growth on Muller-Hinton agar (Merck, Darmstadt, Germany) to determine the minimum fungicidal concentration (MFC) and minimum bactericidal concentration (MBC). MBCs and MFCs were determined as the lowest concentration yielding no more than 4 colonies, which corresponds to a mortality of 98% of the microbes in the initial inoculums.

## RESULTS

Components were identified in the essential oil of *C. copticum* presented in Table 1.  $\gamma$ -terpinene (48.07%), *p*-cymene (33.73%) and thymol (17.41%) were the main constituents of the oil from Kamfirouz (ecotype A).  $\gamma$ -terpinene (50.22%), *p*-cymene (31.90%) and nerolidol (4.26%) were the main constituents of the EO from ECTP B and 89.37% of this oil was identified.

The antibacterial activities of *C. copticum* EOs against the tested bacteria are shown in Table 2. The EOs inhibited the growth of all Gram-positive cocci at concentrations of 0.5-16  $\mu$ L/mL. Furthermore, the EOs exhibited the bactericidal activity (MBC) for all of the

above-mentioned Gram-positive cocci at concentrations ranging from 2 to 32  $\mu$ L/mL. Among the studied EOs, ECTP B had better inhibitory activities against the Gram-positive cocci than those of ECTP A.

All of the *E. coli* strains were susceptible to *C. copticum* EOs at concentrations of 2–32  $\mu$ L/mL. Moreover, the EOs inhibited the growth of the isolates of *P. aeruginosa* at concentrations of 8–32  $\mu$ L/mL. The EOs exhibited bacteriostatic activities against the standard strains of *S. flexneri* and *S. enterica* at concentrations of 1  $\mu$ L/mL and 1-8  $\mu$ L/mL, respectively. In addition, the ecotypes had bactericidal activity against all of the strains of Gram-negative bacteria at concentrations of 2-32  $\mu$ L/mL.

Table 1: Composition of the essential oil of *Carum copticum* Benth. And Hook. from Iran

Components	Kamfiruz	Eghlid	KI (HP-5)	Identification
<i>p</i> -Cymene	33.73	31.90	1025	MS, KI
$\gamma$ -Terpinene	48.07	50.22	1064	MS, KI
Thymol	17.41	1.23	1290	MS, KI
Carvarol	-	0.54	1298	MS, KI
Nerolidol	-	4.26	1563	MS, KI
Isophytol	-	0.65	1931	MS, KI
Sclareol	-	0.57	2220	MS, KI
Identification	99.21	89.37		
Monoterpene hydrocarbons	99.21	83.89		
Sesquiterpene hydrocarbons	-	4.26		
Diterpene hydrocarbons	-	1.22		
Phenolic compounds	17.41	1.77		
Alcoholic compounds	-	5.48		

The compounds have been sorted according to retention indices on HP-5 MS capillary column

Table 2: Antibacterial activity (MIC and MBC) of essential oils distilled from *C. copticum*'s ecotypes

Bacteria (Number of Strains)	Ecotype A		Ecotype B		
	MIC ( $\mu$ l/ml) Gm <sup>a</sup> (range)	MBC ( $\mu$ l/ml) GM <sup>a</sup> (range)	MIC ( $\mu$ l/ml) GM <sup>a</sup> (range)	MBC ( $\mu$ l/ml) GM <sup>a</sup> (range)	
Gram positive	MRSA <sup>b</sup> (6)	3.56 (1-8)	14.25 (8-16)	4 (2-8)	11.31 (4-32)
	MSSA <sup>c</sup> (6)	4 (1-8)	8 (2-16)	6.56 (2-16)	13.12 (4-32)
	VR <sup>d</sup> <i>E. faecalis</i>	8	16	16	32
	VR <sup>d</sup> <i>E. faecium</i> (2)	1.41(1-2)	5.65 (4-8)	5.65 (4-8)	22.62 (16-32)
	VS <sup>e</sup> <i>E. faecalis</i> (5)	1.18 (0.5-8)	4.75 (2-16)	4 (2-16)	11.31 (8-32)
<i>Streptococcus pyogenes</i> ATCC 8668	2	4	4	4	
Gram negative	TGC <sup>f</sup> Resistant <i>E. coli</i> (5)	7 (2-16)	7 (2-16)	18.38 (16-32)	21.11 (16-32)
	TGC <sup>f</sup> Sensitive <i>E. coli</i> (7)	3.62 (2-8)	4 (2-8)	10.76 (4-32)	14.49 (4-32)
	Enterohemorrhagic <i>E. coli</i> ATCC 43894	2	2	4	8
	Multidrug resistant <i>P. aeruginosa</i> (5)	21.11 (8-32)	21.11 (8-32)	24.25 (16-32)	27.85 (16-32)
	Sensitive strain <i>P. aeruginosa</i> (6)	20.15 (16-32)	20.15 (16-32)	32	32
	<i>S. flexneri</i> NCTC 8516	0.5	1	1	1
	<i>S. enterica</i> ATCC 14028	1	1	8	64

<sup>a</sup> Geometric mean, <sup>b</sup>Methicillin Resistant *S. aureus*, <sup>c</sup>Methicillin Sensitive *S. aureus*, <sup>d</sup>Vancomycin resistant, <sup>e</sup>Vancomycin Sensitive, <sup>f</sup>Third Generation Cephalosporin

Table 3: Antifungal activity (MIC and MFC) of the essential oils of *Carum copticum*'s ecotypes

	Ecotype A (µl/ml)	Ecotype B (µl/ml)
Isolates (number)	MIC90 GM <sup>a</sup> (Range) MFC GM <sup>a</sup> (Range)	MIC90 GM <sup>a</sup> (Range) MFC GM <sup>a</sup> (Range)
<i>C. albicans</i> (n=11)	1.63 (0.25-4) 7.05 (1-16)	1.94 (0.25-4) 7.51 (1-16)
<i>C. tropicalis</i>	0.5 1	1 8
<i>C. parapsolosis</i>	8 16	16 32
<i>C. glabrata</i>	0.5 2	1 8
<i>C. krusei</i>	4 16	4 16
<i>A. flavus</i>	2 16	4 16
<i>A. fumigatus</i>	1 16	2 16

<sup>a</sup> Geometric mean

The antifungal activities of *C. copticum* EOs against yeasts are shown in Table 3. For the clinical and standard yeasts tested, the MICs for the EOs were 0.25–16 µL/mL. Among the examined EOs, ECTP B had better fungicidal activities with minimum fungicidal concentration (MFC) values ranging from 1 to 32 µL/mL.

## DISCUSSION

Frequent variations between geographical regions in the chemical composition of EOs have been reported [30, 31]. In contrast to the studies that reported carvacrole [32] or thymol [19, 22, 33-37] as the major constituent of the oil of *C. copticum*, in the both examined ecotypes (A and B),  $\gamma$ -terpinene was identified as the main constituent. Although the percentage of monoterpene hydrocarbons was high in both ecotypes, sesquiterpene and diterpene hydrocarbons were found in the EO from the Eghlid region (ecotype B). Similar to previous reports [19, 22, 36, 37], a high amount of phenolic compounds was identified in the EO from Kamfiruz region (ecotype A), whereas the alcoholic compounds were only found in the EO from Eghlid (ecotype B).

*Staphylococcus aureus* is one of the etiological agents of food-borne and nosocomial infections [38]. In addition, there is major concern about this species due to the rapid development of methicillin resistance which poses a greater treatment challenge. Similar to previous reports [32], the growth of the standard and clinical isolates of MRSAs and MSSAs was inhibited by both ECTPs at concentrations of 1-8 µL/mL. In another study, Goudarzi

*et al.* found a chemotype of the *C. copticum* oil with a better antibacterial activity (MIC = 0.3 µL/mL) against *S. aureus* which may be due to a higher concentration of thymol in the EO, about twice as much [16].

Enterococci are opportunistic pathogens that are normal inhabitants of human gastrointestinal and genitourinary tracts and have been identified as a common cause of nosocomial infections. Vancomycin-resistant *Enterococci* is a problematic pathogen with few treatment options [39, 40]. The tested ECTPs exhibited strong antimicrobial activities against vancomycin-resistant *E. faecium* as well as both vancomycin-resistant *E. faecalis* and vancomycin-sensitive *E. faecalis*. The MICs of ECTP A of *C. copticum* EO against VREfs and VSEfs in this study were lower than those of ECTP B. Interestingly the tested EOs had bactericidal activities and killed all of the Gram-positive bacteria at concentrations less than 16 µL/mL of the ECTP A and 32 µL/mL of the ECTP B.

*E. coli* are inhabitants of the intestinal tract. However, some toxicogenic serotypes of *E. coli* such as enterotoxigenic and Enterohemorrhagic (O157:H7) *E. coli* can cause food poisoning to life-threatening complications like gastroenteritis, haemorrhagic colitis and hemolytic uremic syndrome [41]. In this study, the EOs exhibited both bacteriostatic and bactericidal activities against enterohemorrhagic, TGCsR and TGCsS *E. coli* at concentrations of 2-16 µL/mL and 4-32 µL/mL for ECTPs A and B, respectively, which can be best compared to the study reported by Goudarzi *et al.*, on the same enterohemorrhagic strain [16].

*Salmonella* and *Shigella* are important causes of acute gastrointestinal illness [42]. Recently, high rates of antimicrobial resistance have been described for these two pathogens [42, 43]. The EOs exhibited inhibitory and bactericidal activities against standard strains of *S. enterica* and *S. flexneri* at concentrations ranging from 0.5-8 to 1-64  $\mu\text{L}/\text{mL}$ . The antibacterial activity of ECTP B in this study is most comparable to that of Goudarzi *et al.*, who reported significant antibacterial effects against *S. typhimurium* from the EO of *C. copticum* [16].

One of the Gram-negative bacteria examined, *P. aeruginosa*, is an opportunistic bacterium with low susceptibility to many antibiotics [44]. In the present study, the essential oil exhibited bactericidal activity against standard and clinical isolates of *P. aeruginosa* at concentrations ranging from 8-32  $\mu\text{L}/\text{mL}$ . This result is comparable with the recent study by Goudarzi *et al.*, who reported an MIC of 10  $\mu\text{L}/\text{mL}$  against a standard strain of *P. aeruginosa* [16]. Of the tested Gram-negative bacteria, *P. aeruginosa* revealed the least sensitivity to the EOs and this may be caused by the complex structure of its membrane [30, 31].

*Candida* species are endogenous flora of human and under certain circumstances they may cause a wide range of diseases from simple dermatosis to life threatening fungemia [45, 46]. In the past two decades, the emergence of azole resistance species in particular among non-albicans species increased dramatically. Among the yeasts tested here, 5 isolates were resistant to fluconazole and itraconazole. The essential oil inhibited the growth of all the tested yeasts at concentrations ranging from 0.25-16  $\mu\text{L}/\text{mL}$ . These results are consistent with those of Natanzian *et al.* who reported the same MICs for susceptible and resistant isolates of *C. albicans* [19]. The EOs inhibited the growth of *A. fumigatus* and *A. flavus* at concentrations ranging from 1 to 4  $\mu\text{L}/\text{mL}$  which is comparable to the previous studies [21, 22].

We found no obvious differences in the MICs between sensitive and resistant species, suggesting that modes of action of the EOs are distinct from the antibiotics tested here and cross-resistance with these antibiotics may be minimal. Among the tested ECTPs, ECTP A had the lower MICs against both susceptible and resistant strains than those of ECTP B which may be caused by (due to) the higher concentration of thymol in the this ECTP. It has been shown that the phenolic monoterpenes have hydroxyl groups at different positions around the phenolic ring and exhibit their antimicrobial activities through perturbation of the cytoplasmic membrane, resulting in alteration of membrane

permeability and leakage of ions and intracellular materials [30, 47, 48]. It has also been reported that thymol and carvacrol have fungicidal activity through inhibition of ergosterol biosynthesis and the disruption of membrane integrity [48].

In addition to considerable antimicrobial effects of the tested EOs, it had been show that the EO of *C. copticum* has a high antioxidant activity [12]. The combination of these two properties may act in a synergistic manner by reducing lipid oxidation and extending the shelf life of perishable foods. On the other hand, concerning significant antimicrobial activities of the EOs against nosocomial pathogens, in particular resistant strains, they might be the candidate for further investigation to be used in the antimicrobial products.

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