Iranian Journal of Microbiology
Volume 12 Number 6 (December 2020) 577-583

ORIGINAL ARTICLE

Surveying the chemical composition and antibacterial activity of essential oils from selected medicinal plants against human pathogens

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Received: July 2020**, Accepted:** October 2020

ABSTRACT

Background and Objectives: Essential oils (EOs) with different biological activities, such as antibacterial properties, are a valuable resource for developing new drugs.
Materials and Methods: Ingredients of six medicinally important EOs, including *Artemisia dracunculus, Anethum graveolens, Citrus limon, Citrus sinensis, Cinnamomum zeylanicum* and *Zingiber officinale*, were identified using GC-MS analysis. Moreover, their five major compounds were also listed. Furthermore, the half-maximal inhibitory concentration (IC_{50}) against four important human bacteria was also investigated using the 96-well plate microdilution.
Results: *C. sinensis* EO with IC_{50} of 1.0 and 4.7 mg.mL^{-1} have the most effect on the growth of *S. aureus* and *P. aeruginosa.* Moreover, EOs of *Cinnamomum zeylanicum* (IC_{50}: 1.0 mg. Ml^{-1}) and *Artemisia dracunculus* (IC_{50}: 1.3 mg.mL^{-1}) significantly showed better inhibitory effect on *E. coli* and *K. pneumoniae.*
Conclusion: These EOs could be used for developing inexpensive, potent, and green antibacterial agents.

Keywords: Essential oil; Antibacterial activity; Pathogens; Microdilution

INTRODUCTION

Essential oils (EOs) are a concentrated mixture of hydrophobic compounds in the oil phase, characterized by a strong odor (1). They are secreted as secondary metabolites from different parts of aromatic plants, such as flowers, fruits, seeds, stems, and roots (2). Hydrodistillation using the Clevenger type apparatus is the most common approach for the extraction of EOs (3). Recently, a growing number of studies on different medical properties of EOs have been being performed (4). For example, as flavorings in the food (5), larvicidal activity (6), anticancer drug discovery (7), antioxidant properties (8), and antifungal bioassays (9). In addition to such uses, EOs possess antibacterial effects against human pathogens such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (10, 11).

In the past, antibacterial properties were mainly reported by minimum inhibitory concentration (MIC) which is described as the lowest concentration of an agent to prevent bacterial visible growth (12). For instance, the MIC of *Artemisia dracunculus* EO on *S. aureus* was 62.4 mg.mL^{-1} (13). Besides, *Anethum graveolens* EO showed a good antibacterial effect on *E. coli* with MIC of 2.5 mg.mL^{-1} (14). However, by developing optical density (OD) dependent technics, the growth of microorganisms was observed as turbidity, determined by analytical instruments (15).

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By investigating the antibacterial activity of active agents at various concentrations and using software such as CalcuSyn, half-maximal inhibitory concentration (IC_{50}) is measurable. This value is defined as observing a 50% decrease in bacterial growth in the treated sample compared to the control group. It is a reliable and quantitative unit with upper and lower confidence limits (16).

In this study, ingredient and antibacterial activities of six EOs, including *Artemisia dracunculus* (ADEO), *Anethum graveolens* (AGEO), *Citrus limon* (CLEO), *Citrus sinensis* (CSEO), *Cinnamomum zeylanicum* (CZEO), and *Zingiber officinale* (ZOEO) were investigated. Then for the first time, their IC_{50}s were calculated.

MATERIALS AND METHODS

Materials. Standard species of bacteria, including *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), and *K. pneumoniae* (ATCC 13883) were provided by the laboratory of microbiology, Fasa University of Medical Sciences (FUMS). ADEO was bought from Zardband Pharmaceutical co, Iran. Barij Essence Pharmaceutical Co, Iran, provided AGEO and CLEO. Moreover, Green Plants of Life Co. Ltd, Iran, supplied CSEO, CZEO, and ZOEO. Muller Hinton Broth (Bacterial culture media) was purchased from Merck Chemicals, Germany.

The procedure of GC-MS analysis. For the identification of ingredients of the EOs, GC-MS analysis was used. Briefly, The GC-MS analyses were performed using a 7890A Network GC system coupled with 5975C VL MSD with Triple-Axis, mass selective detector (Agilent Technologies, Santa Clara, CA, USA). The separation of the components of the EOs was carried out on HP-5MS silica fused columns (30 m length; 0.25 mm i.d.; and 25 µM film thickness). The GC-MS column temp was programmed as follows: the initial temp was set at 40°C and fixed for 1 min, then increased with the rate of 3°C.min^{-1} to the final temperature of 250°C and held for 20 min. Temperature of the injection port and detector fixed at 250 and 230°C, respectively. Other instrument parameters were set as split flow: 100 mL.min^{-1} and column flow rate: 1 mL.min^{-1}. Helium gas with a purity of 99.99% was used as the carrier gas. The EOs components were identified using the method described in our previous report (17).

Evaluation of the antibacterial activity of EOs. 96-well plate microdilution method was used for determining the growth inhibitory effect of EOs against target bacteria with slight modification (15, 18). New cultured bacterial colonies (overnight culture) were suspended in Muller Hinton broth to reach 1.5×10^8 CFU/mL to reach the level of 0.5 McFarland turbidity. Then 20 µL of the bacterial suspension was added to each well using an 8-channel pipette.

A serial dilution of each EO was prepared by dissolving in Muller Hinton Broth (containing 0.5% DMSO) in a concentration range of 10.00-0.39 mg.mL^{-1}. By the addition of 80 µL form serial dilutions to each well, the concentration of EOs eventually fixed at 8.00, 4.00, 2.00, 1.00, 0.50, 0.25, 0.13, 0.06, and 0.03 mg.mL^{-1}. Three control wells were considered in each plate, filled with 20 and 80 µL of the bacteria suspension and the Muller Hinton Broth (containing DMSO 0.5%). Treated plates were then incubated at 37ºC for 24 h. The turbidity of each well was read at 630 nm by a plate reader (Synergy HTX Multi-Mode Reader, USA), and the growth of bacteria was calculated using Equation 1.

$$\text{Growth (\%)} = \frac{\text{Absorption of treated wells} \times 100}{\text{Absorption of control groups}} \quad \text{Equation 1}$$

Statistical methods. Antibacterial tests were performed in triplicates. For calculation of means, standard deviations, and drawing charts, Excel software (Version 2010, Microsoft Corporation, USA) was used. IC_{50} of the EOs was calculated using CalcuSyn software (Free version, BIOSOFT, UK). For comparing determined IC_{50} of the EOs together, independent sample t-test and one-way ANOVA using SPSS software (Version 22, SPSS Inc, USA) were performed. In this study, a confidence interval of 95% (CI 95%) was considered.

RESULTS

GC-MS analysis. The five major constituents of each EO with their retention times and retention indices are listed in Table 1. The most abundant components for EOs were as follow; ADEO: p-allylanisole (67.62%), AGEO: p-cymene (20.81%) and α.phellandrene (20.75%), CLEO: limonene (61.83%),

Table 1. Identified components in the EOs using GC-MS analysis

EOs	Major components	[a]RT	[b]RI	%
ADEO	Limonene	10.73	673.23	4.34
	cis-Ocimene	11.32	696.48	8.69
	β-Ocimene Y	11.90	712.26	7.58
	p-Allylanisole	19.18	876.22	67.62
	3-Methoxycinnamaldehyde	34.25	1166.13	1.49
AGEO	α-Phellandrene	9.73	634.08	20.75
	p-Cymene	10.80	675.94	20.81
	Dill ether	17.38	839.99	9.88
	cis-Sabinol	18.21	856.67	3.61
	Carvone	20.25	897.85	10.97
CLEO	α-Pinene	9.45	643.87	3.46
	Sabinene	11.35	800.60	16.99
	Limonene	13.98	764.62	61.83
	Limonene oxide, cis-	18.57	864.00	2.27
	Limonene oxide, trans-	18.80	868.71	3.08
CSEO	Limonene	13.97	764.32	71.26
	trans-p-2,8-Menthadien-1-ol	18.60	864.66	4.96
	Limonene oxide, cis-	18.77	868.04	2.59
	Limonene oxide, trans-	18.82	869.09	2.29
	trans-Carveol	22.69	943.77	2.91
CZEO	Linalool	17.23	837.05	6.96
	Cinnamaldehyde	25.76	1001.60	62.04
	trans-Caryophyllene	31.36	1108.55	6.60
	transS-Cinnamyl acetate	32.57	1132.76	4.30
	Benzyl Benzoate	44.52	1383.62	3.33
ZOEO	Camphene	10.11	1625.67	6.73
	α-Curcumene	34.00	1161.19	11.61
	Zingiberene	34.70	1175.25	30.28
	β-Bisabolene	35.07	1182.57	10.69
	β-Sesquiphellandrene	35.73	1195.68	12.37

[a]Retention Time, [b]Retention index

CSEO: limonene (71.26%), CZEO: cinnamaldehyde (62.04%), and ZOEO: zingiberene (30.28%).

Effect of the EOs on the growth of bacteria. The effect of ADEO at different concentrations (0.03-8.00 mg.mL^{-1}) on the targeted bacterial growth is depicted in Fig. 1. The best result was observed at a concentration of 8.00 mg.mL^{-1} against *S. aureus*; the growth was reduced to ~ 17%, while *K. pneumoniae*, *P. aeruginosa* and *E. coli* were decreased to 36, 47 and 69%, respectively. From the literature, MIC of ADEO on *S. aureus* and *E. coli* were reported as 1.25 and 2.50 mg.mL^{-1} (19). Moreover, its zone of inhabitation in the disk diffusion approach was reported as 8 mm for *E. coli* and 10 mm for *S. aureus* (8).

Fig. 2 shows the antibacterial activity of AGEO at various concentrations. The highest antibacterial activity was achieved at 8.00 against *S. aureus*, with inhibition in 34% growth. However, other bacterial growth was 54, 61, 73% for *P. aeruginosa*, *K. pneumoniae* and *E. coli*, respectively. Some reports on the MIC of ADEO against many bacteria have been found; For example, *E. coli* 1.25 mg.mL^{-1}, *P. aeruginosa* 1.5 mg.mL^{-1}, and *S. aureus* 0.62 mg.mL^{-1} (20). In another study, the MIC of AGEO on *K. pneumoniae* was reported as >10 mg.mL^{-1} (21).

Results of the growth inhibitory effect of CLEO on some bacteria are demonstrated in Fig. 3. With the maximum growth of 14%, *S. aureus* was more affected after 24 h exposure with CLEO at a concentration of 8.00 mg. mL^{-1}; observed growth for three other bacteria was ~ 60%. Antibacterial effect (MIC) of CLEO on *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus* was reported previously. These values were 6.4, 12.8, 12.8 and 12.8 mg.mL^{-1}, respectively (22).

The antibacterial effect of CSEO is shown in Fig. 4. Totally, by increasing the concentration of EO, the growth of bacteria was reduced. At the highest level (8.00 mg.mL^{-1}), the growth of *S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumoniae* decreased to 13%, 57%, 43% and 35%, respectively. Like the previously mentioned EOs, *S. aureus* was more susceptible than other examined bacteria. Reviewing the literature, MIC of CSEO against *S. aureus*, *K. pneumoniae* and *E. coli* was reported as 0.062, 0.25, and 0.12 mg.mL^{-1} (23). The related value for *P. aeruginosa* was 0.75 mg.mL^{-1} (24).

After 24 h exposure with CZEO (8.00 mg.mL^{-1}), the growth of bacteria had a substantial difference from each other (see Fig. 5). For instance, the observed growth for *S. aureus* was around 15%, while this amount for *K. pneumoniae* was 71%. This value for the other bacteria falls between those values (*P. aeruginosa*: 53% and *E. coli*: 40%). Antibacterial effect (MIC mg.mL^{-1}) of CZEO on such bacteria, i.e., *E. coli* (1.6), *K. pneumoniae* (3.2), *P. aeruginosa* (0.8), and *S. aureus* (3.2) was reported previously (22).

As shown in Fig. 6, only the growth of *K. pneumoniae* decreased to <50% after treatment with ZOEO. *E. coli*, with a growth of 74%, was more resistant than others. In previously published papers, MIC of ZOEO on targeted bacteria, including *P. aeruginosa* 31.25, *S. aureus* 7.81, *E. coli* 62.5 (25), and *K. pneu-*

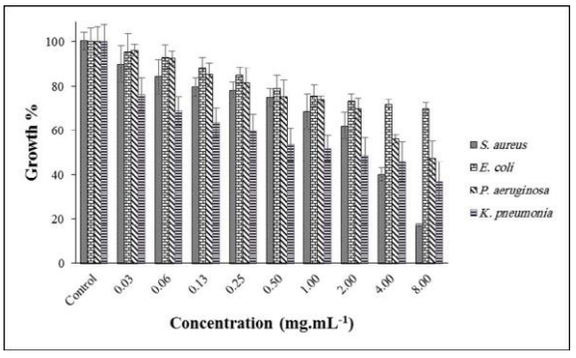

Fig. 1. Effect of ADEO on the growth of targeted bacteria

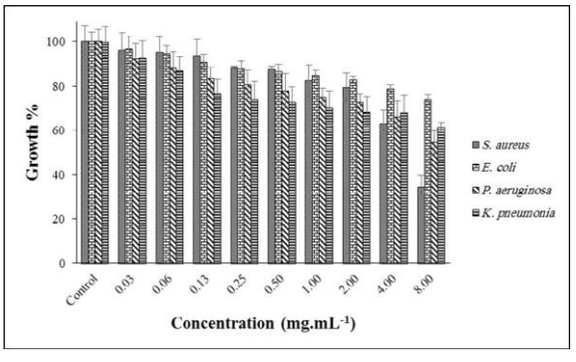

Fig. 2. Effect of AGEO on the growth of targeted bacteria

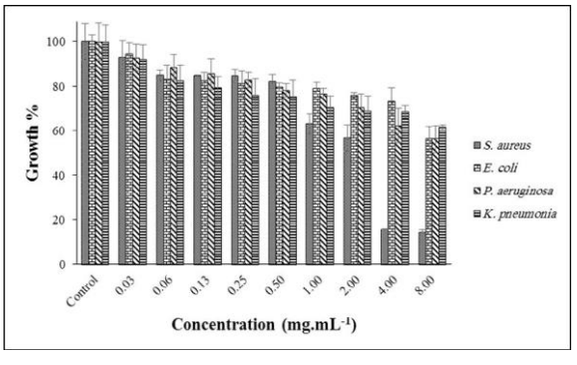

Fig. 3. Effect of CLEO on the growth of targeted bacteria

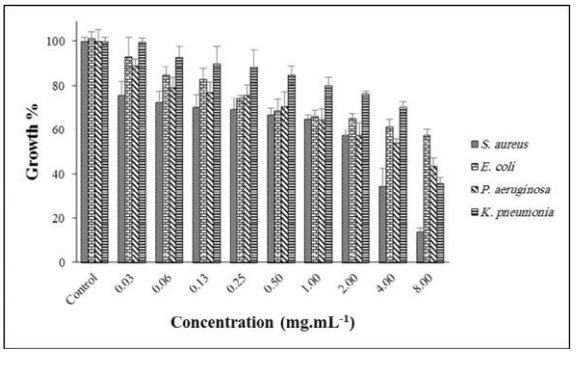

Fig. 4. Effect of CSEO on the growth of targeted bacteria

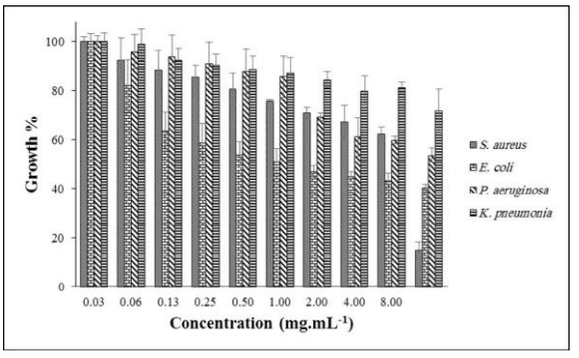

Fig. 5. Effect of CZEO on the growth of targeted bacteria

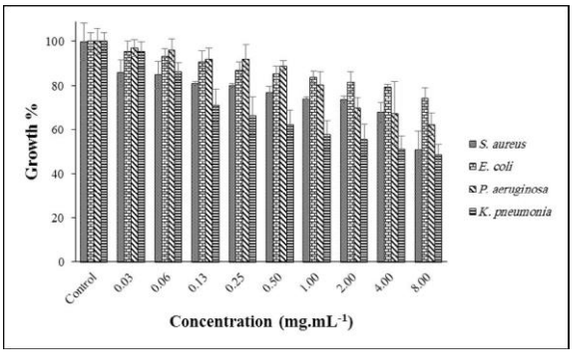

Fig. 6. Effect of ZOEO on the growth of targeted bacteria

moniae 20 (26) were reported.

In Table 2, IC_{50}s (with lower and upper confidence limits: LCL and UCL) of the EOs against four human pathogens are summarized.

DISCUSSION

IC_{50} of four EOs on *S. aureus* was around 2 $mg.mL^{-1}$, CSEO (1.0), CLEO (1.3), ADEO (1.9), and CZEO (2.9). Their IC_{50} is not significantly different from each other (one-way ANOVA, sig > 0.05), but substantially better than AGEO and ZOEO (one-way ANOVA, sig < 0.05). *S. aureus* is Gram-positive cocci, which is usually found in the nasal cavity and on the skin. Although most *S. aureus* strains often act as normal flora of the human microbiota, it can become an opportunistic pathogen, a common cause of various infections, such as skin infections and food poisoning. *S. aureus* is one of the most common reasons for hospital-acquired infections and is usually the cause of wound infections following surgery (27, 28).

Effect of CZEO on *E. coli* was significantly better than the other examined EO (one-way ANOVA, sig <

Table 2. Antibacterial effect (IC_{50}[a] (LCL[b] and UCL[c])) of each essential oil against bacteria

Bacteria	ADEO	AGEO	CLEO	CSEO	CZEO	ZOEO
S. aureus	1.9 (1.1-3.6)	8.0 (4.1-15.6)	1.3 (0.7-2.3)	1.0 (0.4-2.5)	2.9 (1.2-7.1)	37.3 (11.2-124.2)
E. coli	29.8 (10.5-85.1)	101.9 (33.3-311.4)	41.7 (5.7-303.9)	10.0 (3.8-26.1)	1.0 (0.5-2.0)	189.8 (75.0-480.8)
P. aeruginosa	6.1 (3.6-10.3)	19.1 (10.5-35.0)	16.2 (11.1-23.7)	4.7 (3.1-7.3)	7.2 (4.7-10.9)	14.0 (8.9-22.0)
K. pneumoniae	1.3 (1.0-1.8)	22.2 (4.5-108.9)	33.1 (7.3-150.0)	5.8 (2.0-16.9)	42.9 (5.4-343.2)	3.0 (1.1-8.2)

[a] The half-maximal inhibitory concentration, [b] Lower Confidence Limit, [C] Upper Confidence Limit

*Values are presented in $mg.mL^{-1}$

0.05); IC_{50} (LCL-UCL): 1.0 (0.5-2.0) $mg.mL^{-}1$. However, the calculated IC_{50} for ZOEO (189.8) and AGEO (101.9) differ substantially against this bacterium, but they were also larger than the total IC_{50}s calculated in this study. *E. coli* is a Gram-negative, facultative anaerobe rod and a genus of Enterobacteriaceae. Most strains of *E. coli* are harmless and are part of the normal microbiota of the gut. Still, some strains (pathotypes) can cause severe infections in humans, usually through food contamination. *E. coli* is one of the most important bacteria in a hospital and community-acquired infections in humans. Fecal–oral transmission is the usual route through which pathotypes of the *E. coli* cause disease (29, 30).

CSEO has the lowest IC50 (4.7 $mg.mL^{-1}$) against *P. aeruginosa*, this amount significantly better than AGEO (19.1), CLEO (16.2), and ZOEO (14.0) (one-way ANOVA, sig < 0.05). Furthermore, ADEO and CZEO with IC_{50} of 6.1 and 7.2 mg. mL^{-1}, respectively, showed good antibacterial activity, and their IC_{50} were not significantly different from CSEO (one-way ANOVA, sig > 0.05). *P. aeruginosa* is a Gram-negative rod found in soil, water, and skin flora. An opportunistic microorganism in which severe infection often occurs during existing diseases or conditions, such as damaged tissues, cystic fibrosis, and wound burns, is common in acute illness, especially hospital-acquired infections. Treatment of *P. aeruginosa* infections can be difficult due to its natural resistance to antibiotics (multidrug-resistant pathogen) (31, 32).

The lowest observed IC_{50} (LCL-UCL) against *K. pneumoniae* was related to ADEO: 1.3 (1.0-1.8) $mg.mL^{-1}$. ZOEO, CSEO, AGEO, CLEO and CZEO with IC_{50} of 3.0, 5.8, 22.2, 33.1 and 42.9 $mg.mL^{-1}$ were situated in other ranks. *K. pneumoniae* is a Gram-negative rod, facultatively anaerobic, found in the intestine normal flora. *K. pneumoniae* can cause destructive changes to the human lungs if aspirated, resulting in bloody sputum. In recent years, *Klebsiella* species have become important pathogens in hospital-acquired infections (33, 34).

In other researches, MIC of ADEO on *S. aureus* and *E. coli* were reported as 1.25 and 2.50 $mg.mL^{-1}$ (19). MIC of CLEO on *E. coli, K. pneumoniae, P. aeruginosa* and *S. aureus* was 6.4, 12.8, 12.8 and 12.8 $mg.mL^{-1}$, respectively (22). MIC of CSEO against *S. aureus, K. pneumoniae* and *E. coli* was reported as 0.062, 0.25 and 0.12 $mg.mL^{-1}$ (23). The related value for *P. aeruginosa* was 0.75 $mg.mL^{-1}$ (24). MIC of ZOEO on targeted bacteria, including *P. aeruginosa* 31.25, *S. aureus* 7.81 *E. coli* 62.5 (25), and *K. pneumoniae* 20 (26) were reported.

CONCLUSION

Antibacterial activity of six EOs was investigated in a quantitative approach on four important human pathogens. CSEO (IC_{50} : 1.0 $mg.mL^{-1}$), CZEO (IC_{50} : 1.0 $mg.mL^{-1}$), CSEO (4.7 $mg.mL^{-1}$), and ADEO (IC_{50}: 1.3 $mg.mL^{-1}$) were the most effective against *S. aureus, E. coli, P. aeruginosa* and *K. pneumoniae*, respectively. These EOs could be used for developing inexpensive, potent, and green antibacterial agents.

ACKNOWLEDGEMENTS

The authors appreciate Fasa University of Medical Sciences for financial support of this work (Grant No. 97090). Also, this research has been ethically approved (IR.FUMS.REC.1397.152).

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