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In vitro investigation of antibacterial properties of *Citrus medica* essential oil against some human pathogenic bacteria

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ABSTRACT

Background & Aim: The medicinal plants are used in treatment of diseases caused by the human pathogenic bacteria due to their antimicrobial compounds. The aim of this study was to investigate antibacterial and antioxidant activity of *Citrus medica* essential oil on some human pathogenic bacteria.

Experimental: The plant samples of *Citrus medica* were collected from North of Iran. Samples were transferred to the biotechnology laboratory, Bu Ali Sina University, Hamadan. The essential oil was extracted by Clevenger device. Antibacterial activity and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by agar well diffusion and by micro dilution broth methods, respectively. Antiradical activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl assay (DPPH).

Results: The highest and lowest inhibitory activity of essential oil was observed on *Bacillus cereus* and *Escherichia coli*, respectively. Leaf essential oil showed the highest IC_{50} value than the skin essential oil. The essential oil of *Citrus medica* skin showed more inhibitory effect than the leaf essential oil. The MIC of leaf essential oil on *B. subtilis*, *B. cereus* and *E. aerogenes* and the MBC on *B. cereus* were found to be 3.12 mg ml^{-1}. The MIC of skin essential oil on *M. luteus* was 1.56 mg ml^{-1} and the MBC on *M. luteus and S. aureus* was 3.12 mg ml^{-1}.

Recommended applications/industries: The results confirmed the efficacy of *C. medica* essential oil as natural antimicrobial and suggested the possibility of employing it in drugs for the treatment of diseases caused by the test organisms.

1. Introduction

Nowadays, the development of drug resistance, high cost of treatment with chemical drugs and observation of the side effects of antibiotics led to extensive research on new antibacterial agents, especially the essential oil of medicinal plants to discover natural compounds with antimicrobial activity (Alamhulu and Nazeri, 2015a).

Essential oils are aromatic oils derived from various parts of the plant such as flower, bud, seed, leaf, branch, skin, wood, fruit, and root (Upadhyay *et al.*, 2010). The essential oils are produced in different species of plants and stored in different organs.

Essential oil has a direct relationship with biosynthesis, metabolism and biological activities of plants.

The essential oils contain volatile aromatic compounds (Ayoola *et al.*, 2008). The results of the researchers' study have shown that the essential oils heve an anti-diabetic (Hamendra and Annand, 2007) antimicrobial (Caccioni *et al.*, 1998), antifungal (Stange *et al.*, 1993), hypotensive agent, antioxidant, urea enhancing agent, insecticide and antiviral activities (Han, 1998). Essential oils of Citrus species are used as antispasmodic, gastric, sedative, diuretic and improved blood circulation (Odugbemi, 2006). The essential oil of Citrus leaves are contain compounds such as DL-limonene, beta-myrsene, alpha-pinene and sabinen (Sharma *et al.*, 2008). Essential oil of orange skin is containing D-limonene with antimicrobial activity and Nobiletin, Narengine, Tangertine and Orantamine with anticancer activity (Ramadan, 1996).

Citrus medica L. is a valuable herbal plant with short thorns, large and rectangular leaves which is used in medicinal field. The aim of this research was to in vitro investigation of antioxidant and antibacterial activity of *Citrus medica* essential oil on some human pathogenic bacteria.

2. Materials and Methods

2.1. Chemicals

Mueller-Hinton Agar (MHA), Nutrient Agar (NA) and Nutrient Broth (NB) culture media, DPPH (2,2-diphenyl-1-picrylhydrazyl) and Ascorbic acid purchased from Merck Co. (Darmstadt, Germany). Ciprofloxacin and Gentamicin antibiotic discs were prepared from Paten Tab Co. (Tehran, Iran).

2.2. Preparation of plant essential oil

The plant of *Citrus medica* was collected from North of Iran. Samples were transferred to biotechnology laboratory and dried at room temperature under shadow. The dried, finely grounded raw material (100g skin and leaf) was submitted to hydrodistillation in a Clevenger-type apparatus for 5h. Obtained essential oils were then dried over anhydrous sodium sulphate, filtered, and stored at 4°C until use (Kamal *et al.*, 2013).

2.3. Bacterial strains

All bacteria were obtained from Clinical microbiology, Bu Ali Sina University, Hamadan, Iran. Antibacterial activity of essential oils were tested against gram positive bacteria such as *Streptococcus pyogenes* (PTCC-1447), *Bacillus subtilis* (PTCC-1156), *Bacillus cereus* (PTCC-1247), *Micrococcus luteus* (ATCC 10987) and *Staphyllococcus aureus* (PTCC-1189), and gram negative bacteria such as *Escherichia coli* (ATCC-25922), *Shigella boydii* (PTCC1744), *Salmonella typhi* (PTCC-1609), *Pseudomonas aeruginosa* (PTCC-1181), *Klebsiella pneumoniae* (ATCC700603) and *Enterobacter aerogenes* (PTCC-1221). The bacterial suspension concentration was determined equivalent of 0.5 McFarland standard (1.5×10^8 CFU/ml) (Shojaemehr and Alamholo, 2019).

2.4. Agar well diffusion assay for antibacterial activity

The concentrations of 100, 200 and 400 µg ml^{-1} of leaf and skin essential oil were prepared in dimethyl sulfoxide (DMSO). The wells with 5 mm diameter were created in Petri plates on MHA and NA media, and then 50 µl of essential oil was poured into the wells (Okunowo *et al.*, 2013). Petri plates were incubated at 37˚C for 24h (Alamhulu and nazeri, 2015b). Gentamicin (10 µg) and Ciprofloxacin (0.005 µg) antibiotics were used as positive controls (Ayoola *et al.*, 2008). The inhibitory zone (mm) formed around each well was measured.

2.5. Determination of MIC and MBC

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of leaf and skin essential oil with 96- well plate was determined by Microdilution broth method (Sokovic *et al.*, 2007).

Essential oils dilutions were prepared to achieve in the well each of the following concentrations: 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 and 0.195%. A volume of 95µl of NB medium was poured into each 96- well plate. Then 100µl of essential oil dilution was added. Finally, 5µl of bacterial suspension (0.5 Mcfarland) was added to each test tube. The tubes were incubated at 37 °C for 24 h. The lowest dilution of essential oil with no growth of bacteria was considered as MIC. To measurement of MBC, 5 µl of each well, in which no human bacterial growth was seen, was spread

into MHA culture and incubated at 37 °C for 24 h. The minimum concentration with no bacterial growth on the plates was considered as MBC.

2.6. Investigation of free radical scavenging activity by DPPH

The free radical scavenging activity was measured according by Stojicevic *et al.* (2008). Different concentrations (0.2, 0.4, 0.6, 0.8 and 1 mg ml^{-1}) of leaf and skin essential oil were prepared (Sahin *et al.*, 2004). The ascorbic acid and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were used as standard and reagent, respectively. The absorption of samples was recorded with spectrophotometer at 517 nm after 30 min. The IC_{50} of leaf and skin essential oil and also ascorbic acid were measured. The experiments were performed by three replications.

2.7. Statistical analysis

The experiments were performed in completely randomized design with using SPSS 16 software. The results were expressed in means ± standard. The average comparisons were performed using Duncan method at 5% level ($P<0.05$).

3. Results and discussion

3.1. Antibacterial activity

After incubation, the diameter of the holes of bacterial growth inhibition around the wells was measured and recorded. DMSO was used as negative control and Gentamicin and Ciprofloxacin antibiotics were used as positive control. The diameter of inhibitory zone of the leaf and skin essential oils in different concentrations against human pathogenic bacteria has been presented in Table 1. The inhibition zone diameter increased by increasing of essential oil concentration. The essential oil of the leaf and skin showed inhibitory effect on all tested bacteria. The highest and lowest inhibitory effect of essential oil was observed on *B. cereus* and *E. coli*, respectively. The essential oil of skin showed more inhibitory effect than the leaf essential oil. The bacteria such as *B. cereus*, *S. aureus* and *E. aerogenes* showed more sensitivity to the leaf essential oil than gentamicin.

Table1. Antibacterial activity (mm) of leaf and skin essential oil of *C. medica* on human pathogenic bacteria

Bacteria	Leaf (mg mL^{-1})			Skin (mg mL^{-1})			Gentamicin	Ciprofloxacin
	100	200	400	100	200	400		
B subtilis	23±1	25.5±0.66	24.5±0.5	26.5±1.5	28.5±0.5	32.5±0.5	29±0.57	29.5±0.33
B. cereus	27.5±0.5	25.05±0.33	28.5±0.5	28.5±0.5	35.5±0.33	35.5±0.66	19.66±0.33	28.5±0.66
S.aureus	18.5±0.5	18.5±0.33	21.5±0.66	26±1	24.5±0.66	31.5±0.33	20±1	28.5±0.66
M.luteus	15±1	11±1	14.5±0.33	11.5±0.5	18.5±0.33	13.5±0.5	22±0.33	30±1
E.aerogenes	17.5±0.5	18.5±0.88	18.5±88	24.5±0.5	25.5±0.5	32±1	11±0.33	28±0.33
S. typhi	23.5±0.5	19.5±1.5	23.5±1.5	24±1	27.5±0.33	26.5±0.88	29.5±1	33±0.57
P.aeruginosa	11.5±0.5	9.5±0.5	13.5±1.5	13.5±.033	17±1	25.5±0.5	20±0.33	24.5±0.66
E. coli	10.5±0.5	9.5±0.5	12.5±0.5	10±1	16±1	15±0.5	19.5±1	24.5±0.57
S. pyogenes	16.5±0.5	16±1	18.5±0.66	22.5±1.5	22.5±0.5	28.5±0.5	20±0.57	31.5±0.33
S. boydii	15.5±0.33	14.5±0.5	16.5±1.5	19.5±0.5	23.5±0.33	24.5±0.66	19±0.57	37.5±0.66
K. pneumoniae	14±0.57	15.5±0.33	18±1.5	15±0.88	16.5±0.33	18±0.57	27±0.33	32.5±0.66

3.2. MIC and MBC

The results of minimum inhibitory concentration and minimum bactericidal concentration of essential oil were shown in Table 2. The MIC of leaf essential oil on *B. subtilis*, *B. cereus* and *E. aerogenes* and MBC on *B. cereus* were observed at 3.12%. MIC of skin essential oil on *M.luteus* was 1.56% and MBC on *M. luteus and S. aureus* was 3.12%. The essential oil showed greater inhibitory activity on Gram-positive bacteria than Gram-negative bacteria. Gram-negative bacteria due to the presence of lipopolysaccharide layer are more resistant than Gram-positive bacteria (Delaquis *et al.*, 2002). Essential oil causes bacterial cell death by inhibiting microorganisms respiration (Walsh *et al.*, 2003). In this context, factors such as

bacterial strain, plant genotype, experimental conditions and chemical properties of essential oil can be effective (Badar *et al*., 2008; Alamhulu and Nazeri, 2016).

Table 2. MIC and MBC of leaf and skin essential oil of *C. medica* against human pathogenic bacteria

Organ		Bacteria										
		B. subtillis	*B. cereus*	*S. aureus*	*M. luteus*	*E. aerogenes*	*S. typhi*	*P. aeruginosa*	*E. coli*	*S. pyogenes*	*Sh. boydii*	*K. pneumoniae*
Leaf	MIC	3.12	3.12	6.25	6.25	3.12	6.25	12.5	6.25	12.5	6.25	6.25
	MBC	6.25	3.12	6.25	12.5	6.25	6.25	25	12.5	12.5	12.5	12.5
Skin	MIC	6.25	6.25	3.12	1.56	6.25	12.5	6.25	12.5	6.25	6.25	6.25
	MBC	6.25	12.5	3.12	3.12	12.5	12.5	6.25	12.5	12.5	6.25	6.25

3.3. Assessment of anti-radical activity by DPPH

Table 3 indicates the free radical scavenging activities of the examined essential oils relative to ascorbic acid, at the same concentration. The radical scavenging activities increased by increasing of essential oil concentration. Leaf essential oil showed the highest IC_{50}. Significantly difference was observed between IC_{50} of *C. medica* essential oil and ascorbic acid.

Table 3. Antioxidant activity and IC_{50} of leaf and skin essential oil of *C. medica* relative to ascorbic acid

Organ	DPPH Inhibition at different concentrations (mg ml^{-1})					IC_{50}
	0.2	0.4	0.6	0.8	1	
Skin	18.53	21.11	30.3	35.74	43.72	0.54^{b}
Leaf	13.85	16.31	19.57	25.64	43.88	0.72^{a}
Ascorbic acid	25.58	49.02	56.51	79.85	92.04	0.39^{c}

Same letters are not significantly different at $P<0.05$.

The essential oils contain phenolic compounds with broad antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria. Gonzalez *et al*. (2002) reported the antimicrobial activity of *C. milon*, *C. paradisi*, *C. restaurantium* and *C. grandis* essential oil against *E. coli*, *S. aureus* and *P. aeruginosa* bacteria. They showed that *S. aureus* was more sensitive than other bacteria. The antimicrobial activity of *C. grandis* essential oil on *E. coli* was investigated by disk diffusion and dilution method. Accordingly, the inhibition rate on *E. coli* was 13.5 mm and minimum bactericidal concentration was 4.10 mg mL^{-1} (Oh *et al.*, 2007). Antimicrobial activity of essential oil of *C. grandis* skin was reported on *B. subtilis*, *S. aureus* and *E. coli* (Tao and Liu, 2012). The essential oil of *C. garndis* skin has strong antioxidant properties due to having compounds such as caffeicacid, coumaric acid and nomilin (Mokbel *et al.*, 2006). Theanphong *et al*. (2008) reported the antimicrobial activity of *C. medica* leaf essential oil against *S. aureus*, *B. subtilis*, *M. luteus* and *E. coli*, which was similar to our results. Upadhyay *et al*. (2010) investigated the antimicrobial activity of *C. lemon* essential oil on *S. aureus*, *M. luteus*, *B. cereus* and *E. coli*. Accordingly, the essential oil showed inhibitory activity on the growth of all bacteria and *B.cereus* showed the most sensitivity to essence. Srisukh *et al*. (2012) reported the inhibitory effect of leaf and skin essential oil of *C. hystrix* on growth of *S.aureus.* Jafari *et al.* (2011) reported the antimicrobial activity of *C. aurantifolia* essential oil on *B. subtilis*. Okunow *et al.* (2013) investigated the antimicrobial activity of *C. paradisi* essential oil by agar well diffusion method against *B. cereus*, *P. aeruginosa*, *S. aureus* and *E. coli.* Their results showed the highest inhibition effect of essence on *B. cereus*, which was similar to the present study.

According to Menichini *et al.* (2011), the IC_{50} of *C. medica* essential oil was reported as 0.156 to 0.176 mg ml^{-1}, which was lower than the IC_{50} value of present study. The factors such as region conditions, genotype and plant species, and also tissue extraction methods can affect the antioxidant activity (Geyas *et al.*, 1996). According to Sarrou *et al.* (2013) study, the essential oil of *C. aurantium* showed the highest free radical inhibition percentage (53.98%) due to the presence of

alpha-terpinene, alpha-terpinolene and geraniol. The difference between their results and the present results can be due to the differences in species and climatic conditions. Choi *et al.* (2000) investigated the antioxidant activity of essential oil of 34 Citrus fruits and reported the DPPH inhibition from 17.7 to 64%. The free radical scavenging activity of *C. reticula*, *C. paradise* and *C. sinensis* essential oils has been reported to be as 24.08, 18.47, and 14.05%, respectively (Kamal *et al.*, 2013).

4. Conclusion

According to obtained results, *C. medica* essential oil has potential to be used for developing antibacterial drugs due to the presence of compounds with antimicrobial properties. This study suggests that Citrus essential oil can be a suitable alternative in medicine for prevention and treatment of many bacterial diseases.

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