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Original research

Chemical compounds and antibacterial and antioxidant properties of citron (*Citrus medica* L.) peel essential oil

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A B S T R A C T

In this research, essential oil of citron peel was extracted through water distillation by clevenger apparatus and then its chemical composition and antibacterial and antioxidant properties were evaluated. GC-MS analysis showed the major identified components of the essential oil included limonene (33.60%), myristicin (24.36%), carvacrol (8.1%), apiol (5.34%), β-bisabolene (4.40%) and α-bergamotene (2.67%). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for Staphylococcus aureus and Escherichia coli were respectively determined as 62.5, 250, 125 and 500 µg/ml, respectively. The total phenol content of the essential oil was 6.0 ± 0.03 mg gallic acid/g sample. The scavenging activity of DPPH radicals based on IC50 was 19.587 ± 0.011 µg/ml. This result indicated less performance in comparison with BHT, gallic acid and ascorbic acid. Also, although the total flavonoids content of citron fruit extract was 3.4 mg quercetin/g sample, no flavonoid compound was detected in the essential oil.

Keywords: Citron, Essential oil, Chemical composition, Antibacterial, Antioxidant

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1. Introduction

Negative effects of synthetic antioxidants due to their mutagenicity, toxicity and carcinogenesis have led to use the natural antioxidants as substitute for them (Sakanaka et al., 2005; Golshan Tafti & Panahi, 2019). Based on the findings of researchers, essential oils or volatile oils of herbs and fruits are rich sources of phenolic compounds with strong antimicrobial and antioxidant properties (Guo et al., 2003; Azhdarzadeh et al., 2017), and increasing public awareness about the negative impacts caused by excessive use of synthetic chemicals has led to many researches on them (Hyldgaard et al., 2012; Rahati Noveir, 2018).

The essential oils produced from aromatic plants have been used from ancient times as flavoring and preservative in foods and also as medicine for curing diseases. These materials, which are rich in phenolic compounds, increase the duration of shelf life of foods by delaying lipid oxidation or controlling the growth of microorganisms (Shan et al., 2007; Chun et al., 2005; Fadavi et al., 2018).

Citrus belongs to the Rutaceae family and Aurantiodeae subfamily. This fruit is rich in vitamins A, B, and C which have medicinal and nutritional aspects. Citron with scientific name of *Citrus medica* L. is specie of citrus that its history dates back to 1300 B.C. Although the fresh citron fruit does not have many commercial and edible aspects, jam and marmalade produced from its very thick peel have beneficial effects on the purify the blood, strengthen the heart, stomach and liver, promotion of treatment of diarrhea, diabetes and alzheimer disease (Adedeji et al., 2007; Sood et al., 2009; Pasandide et al., 2017; He et al., 2014).

Citron peel, as a rich resource of phenolic compounds, has the beneficial effects on human health and biological activity which, unfortunately, is usually rejected as wastes. Hence, the use those for production of essential oil can be valuable in terms of both nutraceutical parameters and environmentally friendly.

Therefore, according to the importance of essential oils in food and drug industries, the aim of the work was the extraction, identification and evaluation of chemical compounds and antioxidant and antimicrobial activities of citron peel essential oil (CPEO).

2. Material and Methods

2.1. Materials

Citron fruits were prepared from a citrus orchard in Bam, Kerman, Iran. Chemicals such as sulfuric acid, acetic acid, sodium sulfate, sodium carbonate, aluminum chloride, barium chloride,

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ethanol, dimethyl sulfoxide and Folin-Ciocalteu reagent and also, the culture mediums of Brain Heart Infusion, nutrient agar, nutrient broth and Muller Hinton were purchased from Merck Co. (Darmstadt, Germany). Ascorbic acid, gallic acid, stable radical diphenyl picryl hydrazyl (DPPH) and quercetin were obtained from Sigma Co. (St. Louis, MO, USA). Also, Iranian Scientific and Industrial Research Organization supplied bacterial strains include *Escherichia coli* O157:H7 ATCC43894 and *Staphylococcus aureus* ATCC6538.

2.2. Extraction of CPEO

The fruits were first washed with distilled water and then their peels were separated with a sharp knife and were divided into small pieces. The small pieces were naturally dried in the shade and were ground into powder by electric mill. In the next step, the essential oil of powder was extracted through water distillation for 3 h by Clevenger apparatus. The obtained essential oil was dehydrated by dry sodium sulfate and was passed from a micro-filter (0.45 µm). In final, the extraction yield of CPEO was calculated as follows:

$$\text{Extraction yeild of CPEO} = \frac{\text{Weight of CPEO}}{\text{Weight of initial dried powder}} \times 100 \quad (1)$$

2.3. GC-MS analysis

The analysis of CPEO compounds were carried out on an Agilent GC-MS system (GC 6890; MS 5973, Agilent Technologies, Palo Alto, CA, USA). Separation was performed on an HP-5 capillary column (30 m × 0.25 mm, 0.25 µm thickness). Helium was applied as carrier gas with a flow rate of 0.8 ml/min. The column temperature was programmed as 70° to 220°C with rate of 15°C/min. Also, injector temperature was 290°C. The ionization voltage and temperature of ionization source were 70 eV and 220°C, respectively. Eventually, the CPEO compounds were identified by comparison of the obtained mass spectra with mass spectra libraries.

2.4. Antibacterial activity of the CPEO

In this part, the important gram-positive and gram-negative pathogens in food infection and poisoning including *Staphylococcus aureus* ATCC6538 and *Escherichia coli* O157:H7 ATCC43894 were used in order to evaluate the antibacterial activity of CPEO.

For this purpose, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CPEO on the pathogenic bacteria were evaluated based on the method described by Gulluce et al. (2007), with minor modifications. First, the mentioned bacteria were cultured in brain heart infusion broth for 12 hours, and then the bacterial suspensions were adjusted with standard 0.5 McFarland. The CPEO was dissolved in 10% dimethyl sulfoxide solution at the highest concentrations used in this research (1000 µg/l), and then 7 consecutive dilutions of the essential oil as two-fold were prepared in the range 15.62-1000 µg/l in sterile test tubes containing 10 ml of broth. The value of MIC was determined based on micro-well dilution assay.

The sterile 96-well microplate was used for this purpose. 95 µl nutrient broth and 5 ml culture of each bacterium standardized with 0.5 McFarland were added in each well of the microplate. Then, 100 µl of prepared stock solutions in different concentrations was added to each well. It should also be noted that 1 N acetic acid was used to adjust the pH of the culture mediums.

In the next step, the microplates were shook (20 s, 300 rpm) and incubated at 37°C for 24 h. After exceeding the time of incubation, the turbidity of culture mediums in wells of the microplate was evaluated by naked eye. With the evaluation of turbidity caused by growth bacteria, the MIC was determined as the minimum concentration of CPEO which no turbidity was observed in comparison with the control group. It should be stated that the positive control (well containing sterile nutrient broth medium without the CPEO) and the negative control (well containing sterile nutrient broth medium without the studied bacteria) were considered in each test.

To evaluate MBC, 5 µl of culture mediums without turbidity were cultured in plates containing the Mueller-Hinton agar medium. The plates were placed in incubator (37°C, 24 h) and then the bacterial growth was evaluated. In final, the MBC was determined as the minimum concentration of CPEO which no bacterial growth was observed.

2.5. Total phenolic content

In conducting this experiment, Folin-Ciocalteu solution and gallic acid were used as reagent and standard phenolic compound, respectively. For this purpose, 5 ml Folin-Ciocalteu reagent 10-fold diluted with distilled water was added to 1 ml ethanolic solution of CPEO (5, 10, 20, 40, 80 and 100 µg/ml) and incubated at room temperature for 10 min. Then, 4 ml of sodium carbonate solution (7.5 mg/ml) was added to mixture and again incubated in mentioned temperature. In the final stage, absorbance of sample was measured at 765 nm and also, the phenolic content was determined according to following equation obtained from standard curve of gallic acid solutions (5-100 µg/ml) (Nickavar et al., 2006).

$$\text{Absorbance} = 0.011 \text{ gallic acid } (\mu g) + 0.091;\ (R^2 = 0.998) \quad (2)$$

2.6. Scavenging of DPPH radical

In this experiment, the antioxidant activity of the CPEO was measured based on the ability of the essential oil to give hydrogen to stable radical 2,2-diphenyl-1-picrylhydrazyl. Briefly, 50 µl of various concentrations of CPEO was added to 50 ml of 0.004% methanol solution of DPPH. After 30 min in room temperature, the absorbance of samples was read at 517 nm and compared with the control sample. Inhibition of DPPH was calculated based on the following equation:

$$I\ (\%) = \frac{(A_{blank} - A_{sample})}{A_{blank}} \times 100 \quad (3)$$

where A_{blank} is absorbance of the control solution (containing all compounds except CPEO) and A_{sample} is absorbance of solution containing various concentrations of CPEO.

Eventually, IC50 value, as concentration of the essential oil which inhibits 50% of DPPH radical, was determined. Also, the synthetic antioxidant of butylated hydroxyl toluene (BHT) was applied as the positive control. Gallic acid and ascorbic acid were used as stable antioxidant compounds for comparison (Sahin et al., 2004).

2.7. Determination of total flavonoids

Aluminum chloride solution (20 mg/ml) and quercetin were applied as reagent and standard flavonoid compound. For this purpose, 2.5 ml of the CPEO solution was mixed in various dilutions of 2, 5, 10, 20, 40 µg/ml with 2.5 ml of ethanolic solution of the aluminum chloride and incubated at room temperature. After 40 minutes, the absorbance was read at 415 nm. In final, the total flavonoids compounds was calculated based on following equation obtained from quercetin standard curve (2-40 µg/ml) (Nickavar et al., 2006).

$$\text{Absorbance} = 0.041 \text{ quercetin } (\mu g) + 0.078;\ (R^2 = 0.988) \quad (4)$$

2.8. Statistical analysis

The experiments were accomplished in three replicates and reported as mean ± SD. The analysis were conducted in a completely randomized design in various levels of essential oil concentration and data were compared and averaged through Tukey test at level of 5% (SPSS 23.0; Chicago, IL, USA).

3. Results and Discussion

3.1. Extraction yield of CPEO

Performing this test in three replicates showed that the extraction yield of CPEO was 0.75 ± 0.05 %w/w. This observation was higher than the data reported by Wu et al. (2013). These researchers evaluated the extraction yield of *Citrus medica* L. var. *sarcodactylis* essential oil and stated the essential oil of the fruit varied between ~2.5 and ~3.5% in different stages of maturity. This different in observations can be attribute to difference weather conditions of these two countries (Kerman, Iran and Chongqing, China).

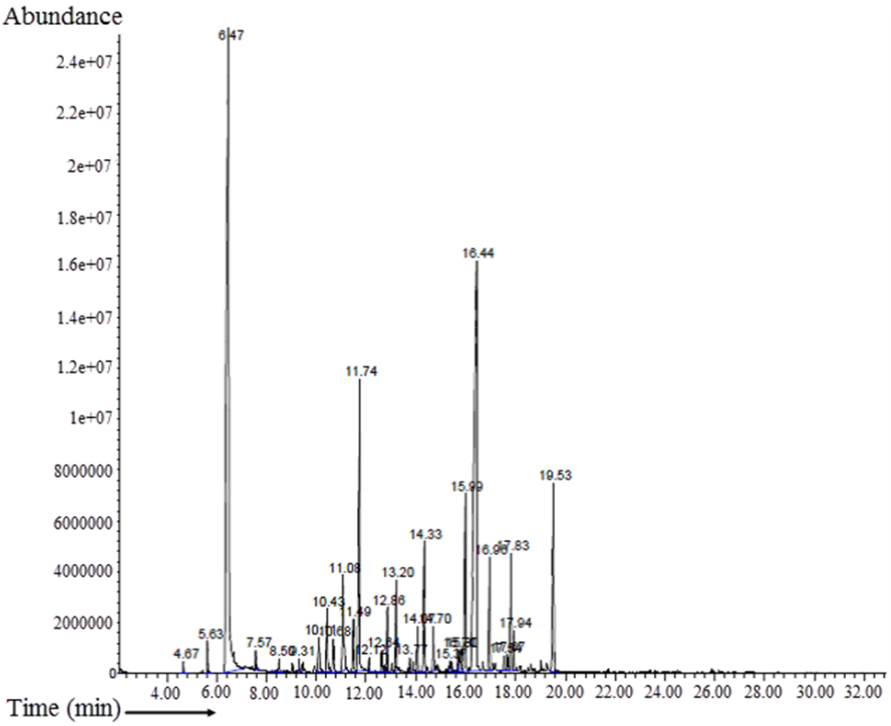

Fig. 1. GC-MS chromatogram of CPEO.

Table 1. Chemical composition of CPEO, obtained by GC-MS.

Pick no.	Components	Percentage (%)	RT* (min)
1	α-pinene	0.16	4.67
2	β-myrcene	0.53	5.64
3	limonene	33.60	6.47
4	linalool	0.30	7.58
5	citronellal	0.28	8.50
6	α-terpineol	0.25	9.32
7	nerol	1.30	10.12
8	neral	1.50	10.43
9	geraniol	0.85	10.68
10	geranial	2.53	11.08
11	thymol	1.32	11.49
12	carvacrol	8.10	11.74
13	geranyl acetate	0.19	12.12
14	cis-2,6-dimethyl-2,6-octadiene	0.38	12.64
15	neryl acetate	1.06	12.86
16	geranyl acetate	1.49	13.20
17	methyl-N-methyl anthranilate	0.26	13.77
18	trans-caryophyllene	0.82	14.07
19	α-bergamotene	2.67	14.34
20	trans-β-farnesene	0.77	14.70
21	α-curcumen	0.22	15.37
22	valencene	0.55	15.70
23	cis-α- bisabolene	0.70	15.80
24	β- bisabolene	4.40	15.99
25	myristicin	24.36	16.43
26	elemicin	2.46	16.97
27	espatulenol	0.28	17.54
28	caryophyllene oxide	0.30	17.66
29	1,2,3,4-tetramethyl-5-(2-propenyl) benzene	2.32	17.83
30	carotol	0.71	17.93
31	apiol	5.34	19.53

*RT=Retention time

3.2. *Chemical composition of CPEO*

GC-MS is an analytical method which combines the output of gas-chromatography (GC) and mass spectrometry (MS) to identify different components within various samples. In this work, this method was used to identify chemical compositions of CPEO which its graph was showed in Fig. 1. The results obtained from Table 1 indicated there were 31 compounds in CPEO that the most important of them were limonene (33.60%), myristicin (24.36%), carvacrol (8.1%), apiol (5.34%), β-bisabolene (4.40%) and α-bergamotene (2.67%).

In another study the presence of 36 compounds in citron peel essential oil such as limonene, geraniol, neral and γ-terpinene was proved (Lota et al., 1999). The difference in number and percentage of chemical components in citron peel essential oil is probably due to being different extraction method, citron variety and weather conditions. However, it is certain that limonene is the most important compound in CPEO. This compound, as a monoterpene abundant in citrus fruit peel, has anti-cancer properties and commonly applied as a flavor and fragrance additive in food products (Chaturvedi et al., 2015; Igimi et al., 1976).

3.3. *Antibacterial activity of CPEO*

In this part, the antibacterial effects of CPEO were evaluated by determining minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Table 2 showed MIC value of CPEO for *S. aureus* and *E. coli* was 62.5 and 250 µg/ml. Also, MBC value of CPEO was 125 and 500 µg/ml for these two bacteria, respectively. Therefore, it can be stated that *E. coli* is more resistance against the antibacterial effect of CPEO. The antibacterial properties of CPEO can be due to the presence and synergistic effects of compounds such as limonene, thymol and elemicin in the essential oil (Peterson et al., 2006). The effect on structure of the cell membrane, increase in its permeability, exit of critical compounds and eventually cell death is the action mechanism of these compounds.

So far, many studies have focused on the antimicrobial effects of essential oils, for instance, Fisher and Phillips (2008) indicated that steam of citrus essential oils can greatly reduce bacteria in air from 2500 to 1250 cfu/m3 during 30 min. These researchers believed that the presence of compounds such as linalool (987-496 m3/mg) and D-limonene (69-13 m3/mg) is cause of air bacteria reduction. In another study, Upadhyay et al. (2010) evaluated the antimicrobial activity of the *Citrus lemon* L. essential oil on gram-positive bacteria such as *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus cereus* and gram-negative bacteria such as *E. coli*, and reported that the essential oil of this plant has inhibitory effect on the growth of all these bacteria.

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CPEO on the pathogen bacteria tested.

Species	MIC (µg/ml)	MBC (µg/ml)
Staphylococcus aureus	62.5	125
Escherichia coli	250	500

3.4. Total phenolic content of CPEO

Phenolic compounds, as natural antioxidant, are a kind of plant metabolites with a various numbers of phenol rings. Flavonoids, anthocyanins and tannins are the most important of these compounds (Ainsworth & Gillespie, 2007). Therefore, analysis of these compounds can be indicating the antioxidant activity of the essential oil. For this purpose, total phenolic content of CPEO was calculated based on the comparison with standard curve of gallic acid as phenolic standard compound. The results showed that the total phenolic content of CPEO was 6.0 ± 0.3 µg gallic acid/mg sample.

3.5. DPPH radical scavenging activity by CPEO

As it was mentioned, the ability to inhibit free radicals by CPEO was evaluated through DPPH assay. For this purpose, the natural and synthetic antioxidants were compared with the antioxidant activity of CPEO (Fig. 2). The compounds with high antioxidant activity have a low IC50. As can be seen, IC50 of the essential oil was 19.587 ± 0.011 µg/ml which was lower than the gallic acid, butylated hydroxyl toluene (BHT) and ascorbic acid as stable antioxidant compounds ($p < 0.05$). Unfortunately, no report is available on the scavenging activity of DPPH radicals by CPEO, but by comparing the antioxidant properties of CPEO with other plant resource, it can be said that the DPPH radical scavenging activity of CPEO was higher that essential oil obtained from fennel seeds (IC50=32.32 µg/ml) and was lower than *Hyssopus officinalis* L. (IC50=16.37 µg/ml), *Mentha spicata* L. (IC50=13.3 µg/ml) and *Melissa officinalis* L. (IC50=7.58 µg/ml) essential oils (Anwar et al., 2009; Mimica-Dukic et al., 2004; Hussain et al., 2010; Kizil et al., 2010).

In general, the antioxidant properties of essential oils are based on their compounds which the most important of them are phenolic compounds (Kizil et al., 2010). Choi et al. (2000) evaluated radical scavenging activity of 34 species of citrus essential oils and observed between 17.7 and 64% of the essential oils have DPPH radical scavenging power. These researchers reported that the maximum antioxidant properties were due to essential oils containing geraniol, terpinolene and γ-terpinene.

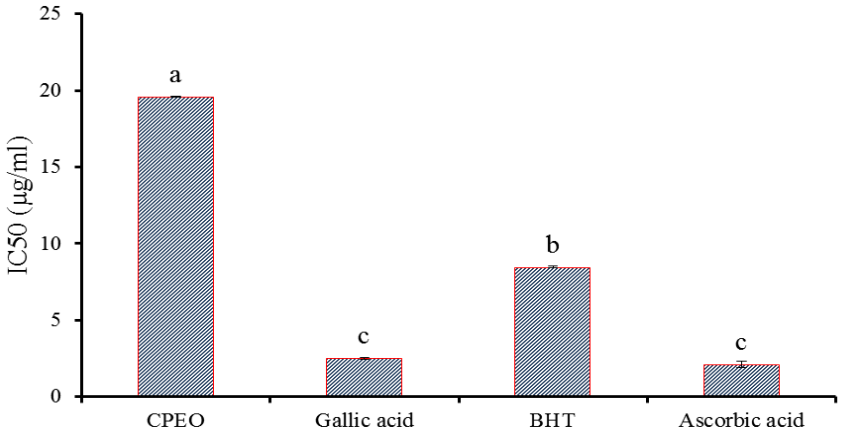

Fig. 2. Scavenging activity of DPPH radicals of CPEO, gallic acid, BHT and ascorbic acid based on IC_{50} (the columns with different letters are significantly different at $p < 0.05$).

3.6. Determination of total flavonoids content of CPEO

Flavonoids are a class of plant and fungus secondary metabolites containing two phenyl rings and a heterocyclic ring. So, these compounds can be effective on antioxidant properties of essential oils. In this stage, total flavonoids content of CPEO was measured through aluminum chloride colorimetry and comparing with the standard curve of quercetin as standard flavonoid compound. The observations showed that CPEO did not respond even in the highest possible concentration (3000 µg/ml) in terms of total flavonoid content. Considering the absence of flavonoid compounds in the results of analyzing the essential oil by GC-MS, it can be expressed that flavonoid compounds was not separated by essential oil extraction process which can be attribute to the heavy of the components. However, the results of our study on the hydroalcoholic extract of the citron peel were showed that the flavonoid content of this extract was 3.4 mg quercetin per gram of the sample. In a similar study, Yu et al. (2007) investigated the presence of routine (a flavonoid compound) in peel essential oil and extract of grapefruit. These researchers also reported that routine are not found in the peel essential oil but are found in extract of the fruit (0.32 mg/ml).

4. Conclusion

Citron peel, as a rich resource of antioxidants and antibacterial compounds is usually discarded as waste. Therefore, the aim of this study was extraction and also, the evaluation of chemical compositions and antibacterial and antioxidant properties of CPEO. A total of 31 compounds were identified in CPEO by GC-MS analysis, which the most important of them were limonene, myristicin, carvacrol, apiol, β-bisabolene and α-bergamotene. The results of antibacterial test indicated that MIC was 62.5 and 125 µg/ml for *S. aureus* and *E. coli* and MBC for these bacteria were 250 and 500 µg/ml, respectively. Measuring antioxidant activity of the CPEO showed that the scavenging of DPPH radicals based on IC50 and total phenol content of this essential oil were 19.587 ± 0.011 µg/ml and 6.0 ± 0.03 mg gallic acid/g sample, respectively.

Also, the observations indicated that the total flavonoid content of the mentioned essential oil did not respond even in the highest concentration of the essential oil (3000 µg/ml). In final, considering the suitable antibacterial and antioxidant properties of CPEO, it seems that it can be used as a functional additive in food products.

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