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Original Article

Chemical Composition and Biological Activities of Lemon (*Citrus limon*) Leaf Essential Oil

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ABSTRACT

Background and Objectives: Essential oils and their compounds are getting increasing interest due to their multipurpose functionals as alternatives to the artificial preservatives. The aim of this study was to extract and identify the volatile constituents of essential oil of *Citrus lemon* leaf and to evaluate its antioxidant and antibacterial abilities.

Materials and Methods: The essential oil was extracted using Clevenger apparatus and characterized by means of gas chromatography mass spectrometry (GC-MS). Folin-Ciocalteu method was used to determine the total phenolic equivalent (TPC). The antioxidant properties of the essential oil were evaluated by 2,2- diphenyl-picrylhydrazyl (DPPH) assay and the detection of inhibitory effect of the essential oil at various concentrations (0.025-10 mg/mL) on *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus faecium*, *Escherichia coli*, *Salmonella typhi* and *Shigella dysentery* was carried out by agar disc-diffusion method and then Minimum Inhibitory Concentration (MIC) assay was evaluated.

Results: GC-MS analysis allowed the identification of twenty-seven compounds and linalool (30.62%), geraniol (15.91%), *α*-terpineol (14.52%), and linalyl acetate (13.76%) were the main constituents. TPC of essential oil was 14.73 mg gallic acid equivalent/g dry plant material and its IC_{50} value was found to be 0.98 mg/mL in DPPH test, so lemon leaf essential oil showed good free radical scavenging capacity at all studied concentrations. However the antioxidant capacity increased with increasing concentration of the essential oil. The results showed that increasing the essential oil concentration, increases the zone of inhibition, so that the highest antibacterial activity was observed at 10 mg/mL of essential oil. The results of antimicrobial activity by disc diffusion method showed that essential oil exhibited maximum zone inhibition against Gram-positive bacteria including *B. cereus*, *S. aureus* and *S. faecium*, whereas the minimum zone inhibition was shown by *S. typhi* and *S. dysentery* as Gram-negative pathogens at the same concentrations. The highest MIC showed by *S. aureus* and *S. dysentery*, whereas the least MIC value was observed with *B. cereus* and *S. typhi*.

Conclusions: The results obtained in this study have shown the moderate biological potential of essential oil of *C. limon* leaf is probably due to its particular chemical composition, mainly the high amounts of linalool. Therefore, this essential oil could be used to raise the shelf life of foods as a natural preservative ingredient.

Keywords: Antibacterial, Antioxidant, Citrus, Essential oil, GC-MS

Introduction

Lemon [*Citrus limon* (L.) Burm. F.] is one of the most important members of the large Rutaceae family, including about 130 genera in seven subfamilies that used are for several purposes throughout the world (1, 2).

Lemon is a small evergreen tree generally produced in temperate climates, with a world production totaling more than 160 million tons in 2014. It is also adapted to drier climates such as Iran. During this year, commercial production of lemons

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(together with limes) was approximately 4.3 million tons that harvested from 25734 hectares in Iran (3).

Nowadays, the interest in essential oils and their application in food preservation have been amplified as the natural additives to improve the shelf-life and the safety of food products, due to the risk in using synthetic preservatives. Also, food-borne diseases are a growing public health problem worldwide, calling for more impressive preservation tactics (4). Lemon has many bioactive components such as citric acid, Ascorbic acid, minerals, flavonoids and essential oils (5).

Citrus essential oils are generally recognized as safe (GRAS) and a complex mixture of about 400 constituents consisting of 85-99% volatile and 1-15% non-volatile components (6). The volatile components contain a mixture of monoterpene, sesquiterpene and their oxygenated derivatives and the non-volatile compounds include hydrocarbons, flavonoids, sterols, fatty acids, coumarins, waxes, carotenoids and psoralens (6, 7).

In addition lemon juice production, essential oils which acquired by cold pressing of the peel or distillation of leaves are broadly applied as an aroma enhancer in beverages, bakery and food products, also serves as a flavoring agent to mask the unpleasant taste of drugs in pharmaceutical, and as fragrance in perfumery and cosmetic industries (1, 8).

Numerous studies have been performed on the chemical composition, antimicrobial, antifungal, antioxidant and radical scavenging abilities of the essential oils of peel and leaf of various species and/or cultivars of lemon around the world, where limonene has been always the prominent component found in all peel essential oils, whereas the leaf essential oil was generally rich in limonene, in some case other compounds were the major constituents (5, 6, 9-22).

The Japanese lemon leaf oil consisted of geranial as the main component, followed by limonene and neral (22). It was reported that caryophyllene was the main composition in Egyptian lemon leaf oil, followed by linalool, nerol and limonene (21). While, Italian, Turkish and Chinese lemon leaf oils included limonene, followed by β-pinene and geranial (12, 17, 20). The Benin lemon leaf oils included mainly of limonene, β-pinene and citronellal (11). Furthermore, the antimicrobial and antioxidant potentials of lemon leaf essential oil have been previously reported (18, 19).

Although, it is known that limonene is often the main constituents in lemon leaf oils, but the composition of essential oils are variable by different localities. The quality and quantity of essential oils are influenced by different agents such as genetic, environmental and experimental factors (16, 23).

To the best of our knowledge, research is yet to be done on the composition, antioxidant and antibacterial activities of Iranian origin lemon leaf essential oils. Therefore, the goals of the present study is to identify the volatile constituents of essential oils from the leaf of lemon cultivated in south of Iran using GC-MS and evaluate their antioxidant activities using the DPPH radical scavenging assay. Furthermore, antibacterial properties of essential oils against six Gram-positive and Gram-negative bacteria is to be investigated.

Materials and Methods

Plant material and extraction of essential oil: The fresh leaves of *C. limon* were collected from a garden in the Jahrom region, recognized as one of the most important horticultural locations in South of Iran in November 2016. The harvested leaves were washed with water to obliterate dirt, then were subjected to hydro-distillation extraction using a Clevenger apparatus for 3 h. The extracted oils were dried over anhydrous sodium sulfate to remove any trace of water and stored in sealed glass vials at 4°C until further analysis. The oil yield was calculated according to the volume of obtained essential oil and was expressed on a fresh weight basis (v/w).

Gas chromatography and Mass spectrometry (GC-MS) analysis: The chemical analysis of the essential oils was done by a Shimadzu GC-17A gas chromatograph (Shimadzu Corporation, Kyoto, Japan), coupled with a Shimadzu mass spectro- meter detector GC-MS QP-5050A. The GC-MS system was equipped with a TRACSIL Meta X5 column, 95 % dimethyl-polysiloxane and 5 % diphenyl-polysiloxane (Teknokroma S. Co. Ltd., Barcelona, Spain; 30 m x 0.25 mm i.d., 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 0.9 mL/min in a split ratio of 1:20, and an oven program of (i) 80°C, (ii) an increase at a rate of 3°C/min from 80 to 210°C, holding for 1 min; (iii) an increase at a rate of 25°C/min from 210 to 300°C, and holding for 3 min. Injector and detector were held at 230 and 300°C,

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respectively, and 2 µL of essential oil was always injected. The compounds of essential oils were identified on the basis of GC-MS retention times, Kovats indices in reference to n-alkanes (C_8–C_{24}), and mass spectra (authentic chemicals and NIST 05 spectral library collection). Identification was considered tentative when it was based on mass spectral data only. The quantification of the volatile compounds was performed using percentage peak area calculations by means of a gas chromatograph, Shimadzu 2010, with a flame ionization detector (GC-FID). The column and chromatographic conditions were those previously reported for the GC-MS analysis.

Total phenolic content (TPC): Total phenolic content of the lemon leaf essential oil was measured based on the absorbance values of the oil reacted with Folin-Ciocalteu reagent and compared the results with Gallic acid as standard solution according to the method described previously (24). Briefly, 200 µL of essential oil dissolved in methanol (1 mg/mL) were mixed with 1 mL of 10% Folin-Ciocalteu (Merck, Darmstadt, Germany) reagent in the presence of 2 mL of sodium carbonate (0.75 g/mL) and allowed to stand in the dark at room temperature for 2 h with continuous shaking. The absorbance of the resulting blue color solution was measured at 765 nm using a Spectronic Genesys 5 spectrophotometer (Milton Roy Company, USA) against a blank with distilled water and using Gallic acid as standard. TPC was expressed as milligrams of Gallic acid equivalent per gram of dry weight of plant (mg GAE/g DW).

DPPH radical scavenging assay: The antioxidant activity of the lemon leaf essential oil was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The ability of the essential oil to scavenge DPPH radical was determined by measuring the bleaching of the methanolic purple-colored solution of DPPH according to the method of Raeisi *et al*. (25). In brief, 50 µL of various concentrations of essential oil (0.025, 0.05, 0.1, 0.5, 1, 2, 5, 10 mg/mL) in methanol was added to 2 mL of a 0.024 % methanolic solution of DPPH. The mixture was incubated for 1 h at room temperature and then the absorbance was read at 517 nm against a blank sample using a spectrophotometer.

The capacity of essential oil to scavenge the DPPH radicals was calculated according to the following equation:

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

Where, A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of sample containing test compound.

The capacity of free radical scavenging was expressed by IC_{50} (mg/mL) value, which represents the concentration required to decrease 50 % of initial DPPH radical.

Butylated Hydroxy Toluene (BHT) and Ascorbic acid as reference antioxidants were used as positive controls in DPPH assay.

Antibacterial screening activity

Microorganisms: Six food borne pathogens including three Gram positive (*Staphylococcus aureus* ATCC 25923, *Bacillus cereus* PTCC 1154, *Streptococcus faecium* ATCC 10541), and three Gram negative (*Escherichia coli* ATTC 25992, *Salmonella typhi* PTCC 1609, *Shigella dysentery* PTTC 188) bacterial species were used for antibacterial activity investigation of lemon leaf essential oil. They were purchased from the Culture Collection Institute of Iranian Research Organization for Science and Technology (Tehran, Iran). All bacterial strains were maintained in a viable state via inoculation on Mueller-Hinton Broth (MHB, Oxoid) and overnight incubation at 37°C.

Disc diffusion method: Antibacterial activity of different concentrations of the essential oils was evaluated with agar disc diffusion assay as described previously with some modification (36). Briefly, Mueller–Hinton Agar (Merck, Darmstadt, Germany) were prepared, autoclaved and transferred aseptically to sterilized petri plates. 100 µl of bacterial suspension (10^8 cfu/mL) was spread on plates and then filter paper disks (6 mm in diameter) (Padtan Teb, Tehran, Iran) were impregnated with 10 µl of various concentrations of the essential oil (0.025, 0.05, 0.1, 0.5, 1, 2, 5, 10 mg/mL) which was prepared by dissolving into 10 µl of dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany) and incubated at 37°C for 24 hours for all bacteria. Chloramphenicol (30 µg/disc) was used as a positive control to determine the sensitivity of one strain in each microbial species tested. After incubation time, the zones of inhibition around each of the discs were calculated by measuring the diameter in mm as a

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measure of the antimicrobial activity. Each test was taken in triplicate.

Minimum Inhibitory Concentration (MIC): The minimum inhibitory concentration (MIC) values of essential oil were obtained using micro-dilution methods. The inocula of the bacterial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The essential oil was dissolved in DMSO to the highest concentration (200 mg/mL), and then serial twofold dilutions were made in a concentration range from 3.55 to 200 mg/mL in 10 mL sterile test tubes including nutrient broth. Ninety five µL of nutrient broth and 5 µl of microbial suspension were added to the each 96-well plates. A 100 µL aliquot from the stock solutions of essential oil initially prepared at the concentration of 200 mg/mL was added into the first wells. Then, 100 µl from the serial dilutions were transferred into six consecutive wells. The last well containing 195 µL of nutrient broth without essential oil and 5 µl of media suspension on each strip was used as the negative control. The final volume in each well was 200 µL. The plates were mixed and then incubated at 37°C for 24 h. Chloramphenicol was used as standard drug for positive control. The lowest concentration of lemon essential oil inhibiting the visible growth of the microorganisms was regarded as the MIC. All experiments were carried out in triplicate.

Statistical Analysis: The experiments were conducted in a completely randomized design with three replications. All data were first subjected to analysis of variance (ANOVA), after checking their normality and later to the Tukey's multiple range test to determine significant differences among the treatments at $p<0.05$. Statistical analyses were carried out using SPSS 20.0 (SPSS Science, Chicago, IL, USA).

Results

Yield and composition of lemon leaf essential oil: As it can be seen in Table 1, the yield of the hydro-distillation essential oil of lemon leaf was 0.41% (v/w) and it was characterized by a pale yellowish color and pleasant aromatic fragrance. As shown in Table 2, GC-MS analysis of essential oil of lemon leaf identified the twenty-eight volatile components representing 99.5% of the total oil. Lemon leaf oil was found rich in alcohols type compounds (61.55%), followed by esters (24.92%) and monoterpenes (12.10%). Based on our findings, the main constituents of the lemon leaf essential oil were linalool (30.62%), geraniol (15.91%), α-terpineol (14.52%), and linalyl acetate (13.76%). The following major components were geranyl acetate (6.75%), neryl acetate (4.24%), β-pinene (4.51%), and *cis*-ocimene (2.93%).

Table 1. The yield and total phenolic content of *C. limon* leaf essential oil

Essential oil yield (mL/g plant)	Total phenolic content (mg GAE/gDW)
0.41±0.11*	14.73±1.96

*mean values of three replicates ± Standard Error

Total phenolic content (TPC) and antioxidant activity of lemon leaf essential oil: Phenolic compounds are often correlated to the antioxidant activity due to their capability to act as electron donors in free radical reactions. In this study, total phenolic content (TPC) was estimated by the Folin-Ciocalteu colorimetric method using gallic acid as standard. The mean value of TPC of the lemon leaf essential oil as presented in Table 1, was 14.73 (mg GAE/DW). The capability of essential oil to act as a donor for hydrogen atoms or electrons in the transformation of DPPH radical into its reduced form DPPH-H (which is measured spectrophotometrically) gives the antioxidant characteristic. The results of DPPH scavenging activity of lemon leaf oil at different concentrations compared with BHT and ascorbic acid as reference standards are shown in Figure 1.

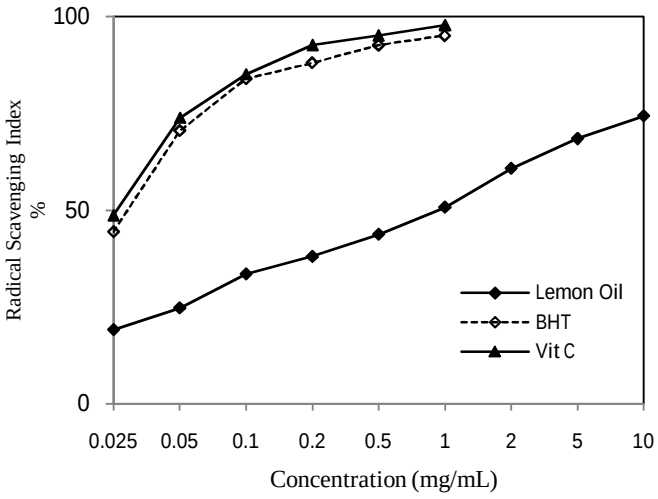


Figure 1. Free Radical scavenging activity of various concentration of *Citrus limon* leaf essential oil

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Table 2. chemical composition of *Citrus limon* leaf essential oil

Volatile compound	Retention Time (min)	Retention Indices[a]		Percent [b]
		Experimental	Literature	
α-Pinene	13.817	939	939	0.2
Sabinene	15.183	978	978	0.41
β-Pinene	15.517	982	980	4.51
Δ-3-carene	16.617	1014	1013	0.11
α-Terpinene	16.892	1016	1016	0.03
p-Cymene	17.325	1026	1026	1.86
Limonene	17.467	1028	1028	1.13
cis-Ocimene	17.867	1036	1034	2.93
γ-terpinene	18.625	1060	1059	0.05
Linalool oxide	19.342	1077	1080	0.07
α-Terpinolene	19.933	1088	1088	0.79
Linalool	20.900	1096	1097	30.62
cis-p-Menth-2-en-1-ol	22.376	1124	1121	0.07
Terpinen-4-ol	24.842	1182	1182	0.43
α-Terpineol	25.983	1193	1195	14.52
Linalyl acetate	27.983	1258	1257	13.76
Geraniol	28.733	1266	1265	15.91
α-Terpinyl acetate	32.592	1350	1350	0.17
Piperitenone oxide	32.950	1359	1363	0.01
Neryl acetate	34.017	1367	1368	4.24
Geranyl acetate	34.883	1374	1372	6.75
β-Elemene	36.092	1393	1393	0.7
trans- Caryophyllene	36.683	1415	1414	0.04
β-Farnesene	37.242	1449	1450	0.01
α-Humulene	38.317	1463	1462	0.1
Germacrene D	39.359	1485	1485	0.06
α-Farnesene	39.608	1503	1504	0.02
Total				*99.50*

[a]Retention indices were calculated for a DB-5 column and literature values were obtained from NIST (2016)
[b]Relative percentage obtained from peak area

As can be seen in Figure 1, when DPPH scavenging activity of lemon leaf essential oil was compared with synthetic antioxidant ascorbic acid and BHT, all the oil concentrations showed significantly lower antioxidant activity, and the oil reached its maximum antioxidant activity (77.4%) at a concentration of 10 mg/mL. In the present study, the lemon leaf essential oil showed lower DPPH radical scavenging ability (IC_{50}=0.98 mg/mL) than the ascorbic acid (IC_{50} = 0.265 mg/mL) and BHT (IC_{50} = 0.0312 mg/mL) as standards. It's proven that the essential oil antioxidant capacity relates to their major compounds and the greater the consumption of DPPH in a sample, the lower its IC_{50} and the greater its antioxidant activity. Therefore, in this study, ascorbic acid with lower IC_{50}, had greater antioxidant activity. The correlation between antioxidant activity (%) and the concentration of the lemon leaf oil showed a low IC_{50} value of 0.98 mg/mL, when compared with the standards, BHT and ascorbic acid.

Antibacterial Activity: The potential antibacterial activity of the lemon leaf essential oil at different concentration with the inhibition zone diameters compared with antibiotic and MIC values are presented in Table 3 and Figure 2. The results indicate that the oils have no antibacterial activity against all six tested bacteria at lower concentrations (0.025 - 0.1 mg/mL). Table 3 also revealed that *S. typhi* and *S. dysentery* did not give any activity at the concentration 0.2 mg/mL of the essential oil. Whereas, the essential oil at this concentration had significant activity against *S. faecium*, *B. cereus*, *S. aureus*, and *E. coli* ($p<0.05$).

The highest antibacterial activity of essential oil was observed against *B. cereus* with a zone of inhibition of 33.46 mm followed by *S. aureus*, *S. faecium*, *E. coli*, and *S. dysentery* with inhibition zones of 31.52 mm, 30.21 mm, 29.33 mm, and 18.19 mm, respectively at 10 mg/mL concentration, while, it was less effective against *S. typhi* with zone inhibition

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of 16.11 mm at the same concentration. Essential oil gave the inhibition against Gram-positive bacteria such as *S. faecium*, *B. cereus* and *S. aureus* at the concentration 0.2 -10 mg/mL with the range of 2.93 - 33.46 mm. The smallest and largest inhibition zones were observed at the concentration 0.2 and 10 mg/mL on *S. Areas* and *B. cereus* cultures, respectively.

Table 3. Zone of inhibition for the essential oil of *Citrus limon* leaf and Chloramphenicol against various bacterial strains

	Gram positive			Gram negative		
Concentrations	*S. faecium*	*B. cereus*	*S. aureus*	*S. typhi*	*E. coli*	*S. dysentery*
0.025	nd*	nd	nd	nd	nd	nd
0.05	nd	nd	nd	nd	nd	nd
0.1	nd	nd	nd	nd	nd	nd
0.2	3.19 ± 0.52^{Fb}	3.97 ± 1.07^{Fa}	2.13 ± 0.83^{Fc}	nd	1.02 ± 0.31^{Fd}	nd
0.5	7.01 ± 1.20^{Eb}	8.60 ± 1.32^{Ea}	7.53 ± 0.75^{Eab}	2.06 ± 0.50^{Fe}	5.06 ± 0.94^{Ec}	3.83 ± 1.34^{Fd}
1	11.91 ± 2.43^{Dab}	12.17 ± 0.47^{Da}	11.16 ± 1.47^{Dab}	3.25 ± 0.85^{Ed}	10.22 ± 1.14^{Db}	8.25 ± 1.19^{Ec}
2	17.72 ± 1.62^{Cb}	19.67 ± 0.12^{Ca}	16.92 ± 0.82^{Cb}	7.78 ± 1.34^{Dd}	16.11 ± 2.15^{Cb}	11.72 ± 1.92^{Dc}
5	25.05 ± 1.18^{Bb}	28.12 ± 2.12^{Ba}	26.23 ± 2.04^{Bb}	13.88 ± 1.42^{Cd}	26.12 ± 2.38^{Bb}	16.55 ± 2.44^{Cc}
10	30.21 ± 1.23^{Ab}	33.46 ± 3.33^{Aa}	31.52 ± 1.62^{Ab}	16.11 ± 2.52^{Be}	29.33 ± 2.12^{ABc}	18.19 ± 2.31^{Bd}
Chloramphenicol	28.44 ± 0.77^{Aab}	30.01 ± 1.03^{Aa}	28.51 ± 1.14^{Aab}	20.87 ± 1.31^{Ab}	30.97 ± 0.88^{Aa}	20.01 ± 1.02^{Ab}

* nd: non detected; [a] Values represent mean ± Standard Deviation of three replicates; [b] Within columns and rows, Means followed by the same capital and small letter are not significantly different according to Tukey's test (0.05), respectively.

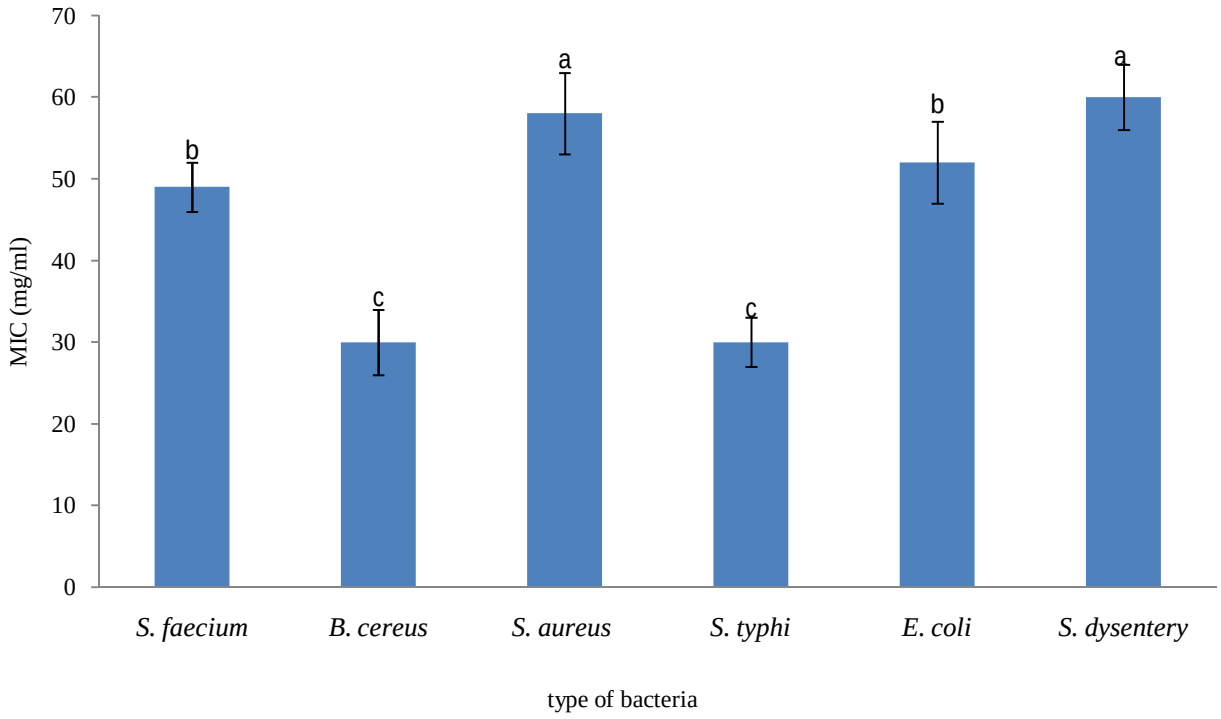


Figure 2. Minimum Inhibitory Concentration (MIC) values of *Citrus limon* leaf essential oil on various bacteria

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As observed in Table 3, the essential oil had a significant inhibitory effect on all tested bacteria at the concentration 0.5, 1, 2, and 5 mg/mL and in all concentrations, the greatest and smallest inhibition zones were founded in *B. cereus* and *S. typhi*, respectively. Table 3 showed with increasing the essential oil concentration from 0.2 to 10 mg/mL, the zone of inhibition increased, so that higher antibacterial activity was observed at 10 mg/mL of essential oil on the all examined bacteria. Also, as observed in Table 3, the essential oil was less effective against Gram negative bacteria compared to Chloramphenicol as a synthetic antibiotic at all concentrations. However, the activity of essential oil at higher concentration (5-10 mg/mL) was found to be quite comparable with Chloramphenicol against Gram positive bacteria. Figure 2 showed MIC values of essential oil on the tested bacteria. In this study, *S. aureus* (59 mg/mL) and *S. dysentery* (57 mg/mL) had the greatest MIC, , than other tested pathogens. As shown in Figure 2, *S. aureus* and *S. dysentery* (58 and 60 mg/mL) had the greatest MIC of essential oil, followed by *E. coli* and *S. faecium* with MIC values of 52 and 49 mg/mL respectively. Also, *S. typhi* and *B. cereus* had minimum MIC values with 30 mg/mL of essential oil. Based on the present results, the essential oil exhibited maximum zone of inhibition against *B. cereus*, whereas the minimum MIC was observed in this bacteria.

Discussion

The hydro distillation method and GC/MS apparatus were used to isolate and identify the essential oils from leaves of lemon collected from south of Iran. The yield of lemon leaf essential oil in present study was in close agreement with authors (12), who reported that the yield of the essential oil from Turkish lemon leaves was 0.49%. Also, Vekiari *et al*. (2002) observed that Greek lemon leaf oil yield varied from 0.35-0.53% depending on the time of harvest. In agreement with our GC-MS analysis, Lota *et al*. (2002) noted that linalool (30.1-47.3%) was the main compound in the leaf oil of some lemon species which cultivated in the island of Corsica in South-East of France. Furthermore, Hamdan and El-Shazly (2014) reported that caryophyllene, linalool and nerol were the main compounds in rough lemon leaf oil. The chemical profile of the oil of lemon leaf in the present study is poor in limonene (1.13%) and is similar to that reported in the literature by Waikedre *et al*. (2010) who analyzed leaf oil of *C. macroptera* and *C. hystrix*. Also, it's similar to results of Huang *et al*. (2000) who reported that the leaf oils from seventeen cultivars of *C. aurantium* L. which cultivated in China were rich in linalool (31.93-39.75%) and linalyl acetate (24.18-34.74%), while these results are disagreeing with those of Vekiari *et al*. (2002), Ayedoun *et al*. (1996), and Kirbaslar and Kirbaslar (2004), who showed that lemon leaf oil of various origin is characterized by high concentrations of limonene. The chemical composition and yield of essential oils vary significantly depending on different factors. Qualitative and quantitative differences in the components of essential oils between this study and others may be due to genetic, geographic, seasonal variation, organs of plants, cultivar, species, ripening stage, cultural practices, extraction methods, environmental and climate conditions (9, 16, 23).

The TPC value of the present work is in close agreement with the findings of Khue *et al*. (2013) who worked on capacity of phenolic compounds of some plants including lemon leaf (15.49 mg GAE/DW) which grown in Vietnam. However, the result of this study farther exceeded the TPC of lemon peel (9.7 mg GAE/DW) and pomace (12.97 mg GAE/DW) reported by Agarwal *et al*. (2012) and Papoutsis *et al*. (2016), respectively.

It is difficult to compare our results with others data. Indeed, the extraction of phenolic compounds from their natural matrix is complex by their diversity and their susceptibility to oxidation and hydrolysis. Similarly, several factors influence amount of phenolic components such as variety, environmental conditions, the mode of keep substrates extraction, the degree of ripening and genetic agents as well as many parameters related to the extraction methods such as temperature, time, solvent to solid ratio and solvent type (31).

Although the antioxidant activity of lemon leaf oil is less than ascorbic acid and BHT, it's higher than the bitter orange leaf and peel lemon oils were harvested from the same geographical area. Majnooni *et al*. (2012) and Golmakani *et al*. (2015) observed the IC_{50} values of bitter orange leaf and peel lemon oils which were collected from south of Iran, were 1.04 mg/mL and 44.06 mg/mL, respectively. Furthermore, radical scavenging ability of peel oil from pink-fleshed lemon (IC_{50} = 26.66 mg/mL), *C. jambhiri* (IC_{50} = 37.69 mg/mL) and *C. pyriformis*

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(IC_{50} = 28.91 mg/mL) were significantly lower than lemon leaf (33, 34). In general, the citrus peel oil exhibited moderate antioxidant activity and citrus leaf oil had higher antioxidant activity than peel (35), and our results also confirmed this. It was indicated that the essential oil of citrus leaves may contain components with strong proton-donating potentials (5).

The results revealed that various concentrations of essential oil from *C. limon* leaf showed significant degrees of antibacterial activity toward all tested microorganisms ($p<0.05$). The data proved that essential oil displayed a variable level of antibacterial activity against both Gram-positive and Gram-negative investigated bacteria. Although the growth of all examined microorganisms was inhibited by different concentrations of lemon leaf essential oil, the result of disc diffusion showed *B. cereus* and *S. typhi* were the most sensitive and resistant bacteria against the essential oil, respectively. Similar results were also reported by others indicating that the essential oil of citrus leaf was active against both Gram positive and Gram negative bacteria, thought it was more effective on Gram-positive strains (33, 36-38). Indeed, the lemon leaf essential oil was less effective against Gram-negative compared to Gram positive bacteria. The result obtained in this work is consistent with the result observed by Saeb *et al*. (2016) in which *S. typhi* was the resistant bacteria against the essential oil of *C. limon* leaf. A similar study was conducted by Hamdan *et al*. (2013) on the antimicrobial activity of the essential oil of the peel and leaf of the Egyptian Cleopatra mandarin. They reported that essential oil had moderate antibacterial activity against bacteria including *S. aureus, E. coli, K. pneumonia, and P. aeruginosa* (33). The biological and chemical activities of the essential oils are completely dependent on their chemical ingredients and it was demonstrated that alcohols, aldehydes, terpenes, phenols, ketones, ethers and hydrocarbons in natural essential oil were largely responsible for inhibition of the bacteria. In the present study, the predominant constituent of the leaf essential oil composition was linalool. Linalool is an alcoholic monoterpene and have shown strong bacteriostatic properties with low MIC values for pathogenic bacteria (39). The antimicrobial action of monoterpenes suggests that they can easily diffuse into or penetrate through the damage cell membrane structures of microorganisms. Therefore, essential oils rich in terpenes have been shown to possess good antibacterial activity. However, the weak activity against Gram-negative microbes could be due to the presence of their double membrane that protects the Gram-negative bacteria from the effect of the oil components.

As can see in Table 3 and Figure 2, the antibacterial activity results obtained by disc diffusion (inhibition zone) were not correlated with serial dilution method (MIC). Indeed, essential oil that produced high inhibition zone showed less MIC. On the other hand, some bacteria showed small inhibition zone, but possessed high MIC value, and vice versa. Our findings are in agreement with Kivanç, and Akgül (1986) who mentioned that the antibacterial data obtained by agar diffusion and serial dilution methods were not always comparable, and therefore in testing the bacteriostatic effects of essential oils it is advisable to carry out both techniques. In addition, disc diffusion is a screening method usually used as a preliminary check for antibacterial activity prior to the more detailed study in liquid medium to determine MIC and MBC (37). There are many factors that may affect the results of disc-diffusion test such as volume of essential oil placed on paper discs, thickness of agar, diffusion ability of oil in agar and variation of essential oil concentration. Therefore, disc-diffusion method is not suitable for comparing the efficacy of essential oil. The broth microdilution method measures the strength of antibacterial activity and it is better suited for comparison (37).

Conclusion

The chemical analysis of hydro-distilled essential oil from *C. limon* leaves showed that linalool is major component followed by geraniol, α-terpineol and linalyl acetate. According to the findings, the inhibition of DPPH free radicals was obtained less for lemon leaf essential oil than ascorbic acid and BHT and therefore the IC_{50} of this oil was higher than ascorbic acid and BHT. Also, TPC of this oil was 14.73 (mg GAE/DW), so the essential oil of lemon leaf is good as a natural antioxidant. In the light of these findings, the oil of lemon leaf inhibits the growth of pathogenic bacteria to a variable extent and the moderate antibacterial activity of the oil was confirmed by the results of agar disc diffusion method and micro-dilution assay. The antibacterial capacity of the oil could be attributed to the presence of

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adequate amounts of monoterpenes. Finally, the essential oil of lemon leaf could be considered as an interesting natural alternative for finding new substances with antioxidant and antimicrobial properties in combating oxidative spoilage and pathogenic microorganisms.

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