



Antioxidant and Antimicrobial Activities of Essential Oil of Lemon (*Citrus limon*) Peel *in Vitro* and in a Food Model

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HIGHLIGHTS

- The total phenol content was 81.82 ± 8.02 mg gallic acid equivalent/g of lemon peel EO.
- The total amount of flavonoids in the lemon peel EO was 11.72 ± 1.82 mg/g rutin equivalent.
- The MIC and MBC value of lemon peel EO against *Staphylococcus aureus* was 1.25 and 5%.
- Lemon peel EO showed considerable antioxidant and antimicrobial properties both *in vitro* and in food model.

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Acronyms and abbreviations

EO=Essential Oil
MIC=Minimum Inhibitory Concentration
MBC=Minimum Bactericidal Concentration
DPPH=2, 2-diphenyl-1-picrylhydrazyl
CFU=Colony Forming Unit

ABSTRACT

Background: Citrus fruits have some antioxidant and antimicrobial properties. The aim of this study was to determine the chemical compounds, antioxidant, and antimicrobial activities of Essential Oil (EO) of lemon (*Citrus limon*) peel *in vitro* and in a food model.

Methods: The analysis of the lemon peel EO was carried out using gas chromatography-mass spectrometry. Total phenolic and flavonoid content was determined using standard protocols. The antioxidant activity of the EO was also evaluated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Antimicrobial properties of the EO were assessed against *Staphylococcus aureus* using the broth microdilution method. Also, barley soup was chosen as food model. Data were analyzed using SPSS Inc. software version 22.0.

Results: The total phenol content was 81.82 ± 8.02 mg gallic acid equivalent/g of the EO. Also, the total amount of flavonoids in the EO of lemon peel was 11.72 ± 1.82 mg/g rutin equivalent. Lemon peel EO showed 55.09% inhibition of DPPH, showing significant difference with control group ($p < 0.05$). The MIC and MBC value of EO against *S. aureus* was 1.25 and 5%, respectively having significant difference ($p < 0.05$) with control group. A dose-dependent manner was seen in food model revealed significantly lower ($p < 0.05$) bacterial number in EO containing barley soup groups than the control one.

Conclusion: The EO of lemon peel showed considerable antioxidant and antimicrobial properties both *in vitro* and barley soup as food model.

Introduction

In recent years, the consumer desire for natural ingredients and chemical preservative-free foods has increased the popularity of natural antimicrobial agents (Sadaka et al., 2013). For this purpose, aromatic plants and their extracts have been evaluated for their effectiveness for

food safety and preservation applications. Most of their preservative properties are due to their Essential Oils (EOs) and other secondary plant metabolite components (Fisher and Phillips, 2009). EOs have well-recognized properties such as antimicrobial as well as antioxidant properties. Due to their relatively safe status, EOs have some widespread applications in medicine, pharmaceuti-

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cal and cosmetic industries and food industry (Moosavy et al., 2008).

The Rutaceae family consists of about 160 genera that citrus, grown in all continents, is the most important genus of this family (Kamal et al., 2013). About 85 million tons of different types of citrus are produced worldwide annually and the Iran's share of this volume is 650000 million tons which is equivalent to about 9% of global production (Vand and Abdullah, 2012). Citrus fruits and juices are important sources of antioxidants such as ascorbic acid, flavonoids, and also phenolic compounds. Citrus peels, as agro industrial waste, are the potential sources of EOs (Fernandez-Lopez et al., 2005). Since citrus EO is mainly located in the fruit peel; if proper recycling of this peel is performed, a valuable product can be obtained in the form of peel oil.

The aim of this study was to determine chemical compounds, antioxidant, and antimicrobial activities of lemon (*Citrus limon*) peel EO *in vitro* and in a food model.

Materials and methods

Plant material

Fully ripened fresh lemons (*Citrus limon*) were acquired in Tabriz, Iran, in September 2015. The fruit was peeled carefully using a sharp knife. After chopping the peels, they were dried under shade at room temperature. After that, the dried peels were powdered using a mortar as well as electric blender. Then, the powder was stored into closed containers for future use (Tumane et al., 2014).

Extraction of EO

Dried lemon peel powder (100 g) was transferred to a two-L flask; then one L distilled water was added. The EO was extracted using a Clevenger-type apparatus (Germany) by steam distillation over a 3 h period. The obtained oils were filtered through a sterile syringe filter with a 0.45 μm pore size. The EO was dried over anhydrous sodium sulfate (Na_2SO_4) and stored in a sealed dark glass vial at 4 °C (Ghasemi et al., 2014).

Characterization of EO

The analysis of the EO was carried out using gas chromatography-mass spectrometry (Agilent technologies, USA). The chromatograph was equipped with a HP-5MS capillary column (30 m \times 0.25 mm ID \times 0.25 μm film thickness). Column temperature was programmed at 70 °C as an initial temperature and kept at 70 °C for 3 min, then gradually increased to 280 °C at a rate of 5 °C per min, holding at the mentioned temperature for 2-10 min.

The temperature of the injector was 265 °C and helium was used as the carrier gas. The mass spectrometer was operated in electron ionization mode at 70 eV and ionization source temperature was considered at 250 °C. The constituents of the EO were identified and confirmed by comparison the gas chromatography retention indices to n-alkanes (C8–C24) and mass spectra with those of Iran National Institute of Standards and Technology commercial library, as well as with literature data (Adams, 2007).

Total phenolic content

Total phenolic content of the EO was determined using a Folin-Ciocalteu reagent and gallic acid (Sigma–Aldrich Chemie, Steinheim, Germany) as a standard phenolic compound. Briefly, 0.1 ml of the solution containing the EO was mixed with 46 ml distilled water. Then, 1 ml Folin-Ciocalteu reagent was added to the solution and the mixture was shaken vigorously. After 3 min, 3 ml sodium carbonate solution (2%; Na_2CO_3) was added and the mixture was shaken, gently for 2 h. The absorbance of the solution was measured at 760 nm. The same procedures were carried out with gallic acid (as a standard) and a calibration curve was obtained. The total phenolic content was presented as mg of gallic acid equivalent per g of the EO.

Flavonoids content

The flavonoid contents were measured according to the aluminum chloride colorimetric method. Rutin was used to obtain the calibration curve. Various concentrations of the EO were prepared for this test. Each diluted EO of lemon peel (500 μl) was mixed with 500 μl aluminum chloride methanolic solution (2%). Each prepared mixtures was incubated at room temperature for 15 min, then the absorbance of the reaction mixture was measured at 430 nm with a UV–Vis spectrophotometer (Unico Inc, Shanghai, China). Using the same procedure, the calibration curve was obtained for rutin (in the range of 5 to 60 mg/ml). Finally, flavonoids content was expressed in mg of rutin equivalent/g of the EO.

Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The antioxidant activity of the EO was measured using the DPPH radical (Sigma-Aldrich, Germany) scavenging assay. Briefly, 50 μl aliquots of various concentrations of the EO were mixed with 5 μl DPPH solution (0.004% in methanol). The mixture was shaken and incubated at room temperature for 30 min in the dark. The absorbance of the solutions was then measured at 517 nm. DPPH solution was served as negative control while ascorbic acid was used as a positive control. More free radical

scavenging activity was evaluated by lesser absorbance of reaction mixture. The DPPH radical scavenging activity (%) was obtained by the following equation:

$$\frac{A_{\text{control}} - (A_{\text{sample}} - A_{\text{blank}})}{A_{\text{control}}} \times 100$$

Where " A_{control} " is the absorbance of the control reaction (absorbance of DPPH solution) and " A_{blank} " is the absorbance of the blank solution (absorbance of the EO solution) and " A_{sample} " is the absorbance of the test compound (absorbance of the EO with DPPH).

Test organism and preparation of inocula

Since staphylococcal food poisoning is one of the most important diseases worldwide (Azizkhani et al., 2013), antimicrobial properties of the EO were studied against *Staphylococcus aureus*. For this purpose, *S. aureus* ATCC 6538 was obtained as a lyophilized culture from the Iranian Research Organization for Science and Technology. The lyophilized culture of bacterium was grown twice in tubes containing 10 ml nutrient broth (Merck, Darmstadt, Germany) at 35 °C for 18 h. It was then mixed with sterile glycerol at a ratio of 5:1 and stored at -20 °C (Basti et al., 2007). Staphylococcal inocula were prepared by transferring the cells to the tubes of brain heart infusion broth (Merck, Darmstadt, Germany). After 18 h incubation at 35 °C, the secondary subcultures were prepared and incubated for 18 h at 35 °C. Then, *Staphylococcus* broth cultures were adjusted to absorbance of 0.02 at 600 nm, using a spectrophotometer (Milton Roy, USA). These adjustments gave a cell concentration of 1×10^7 Colony Forming Unit (CFU)/ml for *S. aureus* as determined in preliminary trials. Number of bacterial cells in the suspensions was evaluated using plating twice from 10-fold serial dilutions on the nutrient agar (Merck, Darmstadt, Germany) as well as counting the colonies after incubation of the plates at 35 °C for 24 h (Moosavy et al., 2008).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

A broth microdilution assay was used to determine the MIC in which visible growth of the bacterium was inhibited. Briefly, EO dilutions of 0.75, 1.55, 3.1, 6.2, 12.5, 25, 50, 100, and 200 µl/ml (0.075, 0.155, 0.31, 0.62, 1.25, 2.5, 5, 10, and 20%) (v/v) were prepared using a 96-well microtiter plate (Saxena et al., 2012). Twenty ml EO with specific concentration and 160 µl nutrient broth (Merck, Darmstadt, Germany) were transferred to each well. Then, 20 µl inoculum was added to each well (Hashemi et al., 2017). The final concentration of bacteria in each microwell was 10^5 CFU/ml (estimated using the surface plate counting method). As a positive control, 20 µl inoculum was added to 180 µl nutrient broth

lacking EO (0%). A well containing only 200 µl broth was prepared as a negative control. To evaluate the possible contamination of EO, 20 µl EO and 180 µl broth were also transferred to a well. The microplate was covered with a sterile plate sealer and the contents were mixed for 2 min using a plate shaker (Moosavy et al., 2015). The plates were incubated at 37 °C for 24 h. Bacterial growth was visually determined by turbidity in the wells and the growth in each well was compared with that of the growth control EO free well (Hashemi et al., 2017; Moosavy et al., 2015).

The minimum concentration of EO that reduced 99.9% of the bacterial population after incubation at 35-37 °C for 24 h was considered the MBC (Azizkhani et al., 2013). The wells with no visible growth in the MIC determination assay were used for this test. A sterile swab was applied to the contents of the well and spread on the surface of nutrient agar plates. Then, the plates were incubated at 37 °C for 24-48 h. The concentration of EO in those wells that yielded plates with no visible colonies was determined to be the MBC (Das et al., 2016).

Preparation of barley soup as food model system

A commercial barley soup (Maggi, Iran) was used as a food model in this experiment. According to the manufacturer's instructions, the soup powder was dispensed in one L distilled water and heated for 15 min. The prepared soup was then filtered and distributed into micro-tubes and sterilized at 121 °C for 15 min.

Inoculation and storage of barley soup

The effect of lemon peel EO with designated concentrations of EO ($1/2\times$, $1\times$, $2\times$ and $4\times$ MBC; v/v) was evaluated on the growth of *S. aureus* at 4 ± 1 °C for 15 days in commercial barely soup. After sterilization and cooling of the soup, the EO was added at varying concentrations into tubes containing 900 µl soup. They were inoculated with the test organisms at a final level of 10^5 CFU/ml by adding 100 µl *S. aureus* suspension. Tubes containing soup and initial inoculum served as the positive control. Tubes containing only soup sample were prepared as a negative control. The samples were studied by counting on brain heart infusion agar at different intervals, including 0, 1, 2, 3, 6, 9, 12, and 15 days (Moosavy et al., 2008).

Statistical analysis

The experimental results were expressed as mean \pm standard error of three replicates. Statistical analysis was performed using Graph Pad Prism software, version 3.0 for windows (Graph Pad Software Inc., CA, USA). The effect of the EO concentrations on *S. aureus*

in soup was evaluated by repeated measure analysis using SPSS Inc. (Chicago, IL, USA) software version 22.0. *P* value less than 0.05 was considered statistically significant.

Results

Distillation of the dried lemon peel gave a yellow-colored and transparent EO with the yield of 1.33%. The chemical analysis of the EO showed that the EO contained a complex mixture of several components (Table 1). The main constituents were DL-limonene (46.93%), γ -terpinene (16.89%), tri-cyclen (6.67%), 1-beta-pinene (4.69%), and 2-beta-pinene (3.86%), respectively.

The total phenol content was 81.82 ± 8.02 mg gallic acid equivalent/g of EO. Also, the total amount of flavonoids in the EO of lemon peel was 11.72 ± 1.82 mg/g rutin equivalent. The lemon peel EO showed 55.09% inhibition of DPPH while ascorbic acid (as positive control) exhibited 5.18% activity, showing their significant difference ($p < 0.05$).

The MIC and MBC value of EO against *S. aureus* was 1.25 and 5%, respectively having significant difference ($p < 0.05$) with control group. The results in Figure 1 showed that different concentrations of lemon peel EO could affect the growth of *S. aureus* in a dose-dependent manner, revealing significantly lower ($p < 0.05$) bacterial number in EO containing barley soup groups than the control one.

Table 1: Chemical analysis of the major components* of lemon peel essential oil

No.	Compound	Retention time (min)	%
1	DL-limonene	6.59	46.93
2	γ -terpinene	6.89	16.89
3	tri-cyclen	7.27	6.67
4	1-beta-pinene	5.46	4.69
5	2-beta-pinene	5.29	3.86
6	Beta-bisabolene	15.55	3.15
7	3-cyclohexenone	9.19	2.19
8	bicycloheptene	13.96	2.19
9	alpha pinene	5.54	1.81
10	neryl acetate	12.6	1.63
11	6, 2-Octadien	12.92	1.29

* The minor components have not been shown

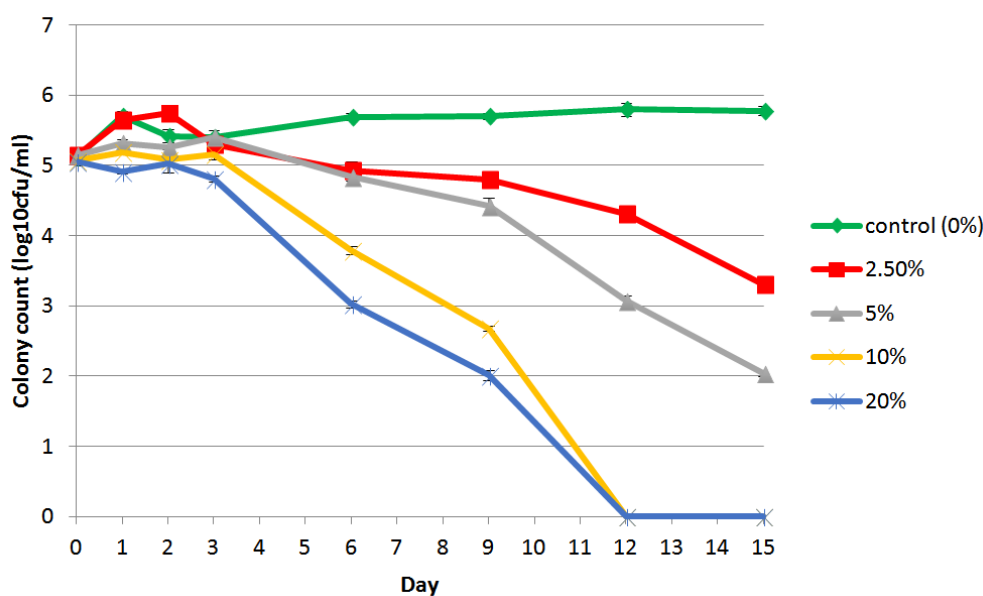


Figure 1: Colony counting of *Staphylococcus aureus* cultured at different concentrations of lemon peel essential oil in barley soup during 15 days of storage at 4 ± 1 °C

Discussion

In the present study, the yield of the EO obtained by hydro distillation of dried lemon peel was 1.33% which is in accordance with the study carried out by Bourgo et al. (2012) who reported a yield of 1.30%. Chemical analysis of lemon peel EO identified limonene and γ -terpinene as the major compounds which are also in close agreement with the findings of previous studies. For instance, Kamal et al. (2013) found that β -myrcene and limonene were the most abundant components in peels of three *Citrus* species, including *C. reticulata*, *C. sinensis*, and *C. paradisi*. Lota et al. (2001) and Djenane (2015) reported limonene as well as γ -terpinene as the major monoterpenes in peel EO of *C. reticulata* (mandarin) and lemon EO, respectively. However, compositions of the EOs can vary depending on various factors, such as plant variety, geographical region, age of the plant, the EO drying and extraction method, etc. (Bagamboula et al., 2004).

A high level of phenolic and flavonoid components was found in lemon peel EO in the present study which is similar with some previous reports (Ghasemi et al., 2009; Guimarães et al., 2010). The phenolic and flavonoid compounds of *C. lemon* peel oil in this study may correlate with the antioxidant activity of the EO. The relation between total phenol content and antioxidant activity has been widely studied in different foodstuffs, such as fruit and vegetables (Kiselova et al., 2006; Klimczak et al., 2007) indicating that the free radical scavenging activity of fruits and vegetables significantly increases with a high concentration of total polyphenol content (Ghasemi et al., 2009). Meanwhile, the phenolic compounds have the ability to scavenge free radicals by donating a hydrogen atom from their phenolic hydrogen groups (Thitilertdech et al., 2008). As found in this work, lemon peel EO was able to reduce the stable, purple-colored radical DPPH into yellow-colored DPPH-H by 54.67% and it showed a good antioxidant capacity comparable to that of ascorbic acid. This result is in agreement with those obtained by Frassinetti et al. (2011), demonstrating the scavenging abilities ranging from 20 to 70% of *Citrus* spp. EOs. The significant antioxidant activity of the tested EO might be related to the presence of monoterpenes, particularly limonene and γ -terpinene, which are the main compounds of EO and have been reported to have a good antioxidant activity (Conforti et al., 2007).

In this study, the EO of the lemon peel was found to have a significant inhibitory effect against *S. aureus* and the MIC and MBC values of 1.25 and 5% were obtained, respectively. This antimicrobial effect might be related to limonene and γ -terpinene in EO, which exert their toxic effects through the disruption of the bacterial membrane and the inhibition of respiration and ion

transport processes (Martins et al., 2000). Recently, the *in vitro* antimicrobial activity of lemon peel EO was evaluated using the disk diffusion method against *S. aureus* and the EO was found to have an inhibitory effect against this microorganism (Roy et al., 2012). In this study, the effect of lemon peel EO was evaluated on the growth of *S. aureus* in a food model. Furthermore, the antimicrobial activity of the EO was investigated using broth microdilution method which is generally recognized as the more reliable method for antimicrobial susceptibility testing. Another advantage of this test is that quantitative results are obtained, which are of importance in epidemiological studies, as changes in susceptibility of bacteria can be detected (Meyer et al., 2011).

In agreement with our findings, Mahmud et al. (2009) showed the antimicrobial activity of the EO of *C. acida* var. Sour lime peel. Smith-Palmer et al. (1998) studied the antimicrobial activity of the EO of lime against *Salmonella enteritidis* and *S. aureus*, using the agar diffusion method. Their results showed that the lime EO has an antibacterial effect against *S. enteritidis* and *S. aureus* with MIC of 6.40 mg/ml and 12.80 mg/ml, respectively. It should be noted that the comparison of the antimicrobial effect of the EO through different studies is difficult. Some factors that may explain conflicting results from different studies include the chemical composition and the relative proportions of the various constituents in EO (influenced by genotypes, chemotypes, etc.), the technique used to evaluate the antimicrobial activity, the choice of organism, microorganism growth, the period of exposure of the microorganism to the EO, and the choice of the emulsifier to solubilize the EO (Djenane, 2015). It has been reported that the inhibitory effect of volatile components such as linalool, citral, and limonene occurs in the first three h, and then rapidly decreases over time (Inouye et al., 2006). The antimicrobial effect of EO in food depends on several factors such as the availability of nutrients that can quickly repair damaged cells; the presence of fat, protein, antibacterial substances, salt, and the other substances in food; pH; temperature; type of packaging; and the characteristics of the microorganism (Djenane, 2015). Low pH and salt increase the hydrophobicity of EO, which improve its dissolution in the phospholipid of the membranes. While proteins and fats in foods protect the bacteria against the activity of EO, carbohydrates appear to have little or no effect on this activity (Mejlholm and Dalgaard, 2002).

In recent years, the potential applications of EOs have been studied to reduce or control some food-borne pathogens in food models. Similar to the present research, Moosavy et al. (2008) evaluated the effect of different concentrations of *Zataria multiflora* Boiss. EO on the growth of *S. aureus* in commercial barley soup during the 21 days storage at 8 °C and showed that the viable count

of *S. aureus* was significantly decreased by treated groups. Also, Mahmoud et al. (2004) studied the antibacterial activity of citral and linalool (the two abundant components in lemon and orange) at a concentration of 2% at 2 °C for 48 h on the surface of fish microflora. These researchers indicated that linalool was more effective than citral against *Acinetobacter*, *Morexella*, Enterobacteriaceae, as well as Vibrionaceae. The results obtained in the present study showed that the different EO concentrations inhibited growth of *S. aureus* during 15 days of storage at 4±1 °C and the antimicrobial effect of EO was improved with increasing its concentrations in barely soup. So, the EO concentration of 10 and 20% had more pronounced effect on the growth of *S. aureus* and these concentrations could completely inhibit the growth of organism in food model after 12 days of storage showing bactericidal effect. Our results are in agreement with findings reported by Djenane (2015), who found that the application of lemon peel EO at 4×MIC values resulted in a considerable reduction in the growth of *S. aureus* in *Sardina pilchardus* during storage at 8 °C for 9 days. Ibrahim and Abu Salem (2013) also reported that lime peel oil reduced significantly the aerobic plate count in chicken patties during a storage period of 9 days at 4 °C as compared to control group.

Conclusion

This study indicated that the EO of *C. lemon* peel can be used as a potential natural antimicrobial as well as antioxidant agent in the food industry. It would also be of interest to evaluate the antimicrobial activity of lemon peel EO against other food-borne pathogens. Further studies using different food models and storage conditions are suggested to improved utilization of this EO as a natural alternative instead of synthetic preservatives.

Conflicts of interest

The authors declare no conflict of interest.

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