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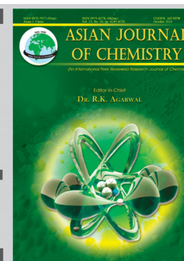

Chemical Composition of the Essential oil, Antibacterial and Antioxidant Activities, Total Phenolic and Flavonoid Evaluation of Various Extracts from Leaves and Fruit Peels of *Citrus limon*

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The essential oils obtained from the dried leaves, flowers, fruit peels of *Citrus limon*. were analyzed using GC and GC/MS. Thirty six compounds were identified in the oil of leaves with β-pinene (20.6 %), limonene (16.8 %), neryl formate (14.8 %) and geraniol (9.3 %) as main components. Twnety six compounds were identified in the oil of fruit peels with limonene (60.2 %), β-pinene (12.1 %) and γ-terpinene (11.8 %) as main components. Also, evaluating of *in vitro* antibacterial and antioxidant properties of the methanol and chloroform extracts from the leave of *Citrus limon*, were performed. The antibacterial test results showed that the chloroformic extracts of the leave medially inhibited the growth of the microorganisms studied especially the Gram-negative strains. The antioxidant potential of the samples was evaluated using DPPH method, inhibition of free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), the sub-fractions of the chloroform extract were able to reduce the stable free radical DPPH with an IC_{50} of 29.197. The highest total phenolic content was observed (0.155 mg gallic acid equivalents (GAE)/g fresh matter (FM) for methanolic extract. The highest total flavonoid content was observed in methanolic extract (2.77 mg quercetin equivalents (QE)/g FM).

Key Words: Citrus limon, Essential oils, Antibacterial, Antioxidant, Flavonoid.

INTRODUCTION

Plants have been used for medicinal purposes across history and cultures and even across species. Over the past several decades, scientific literature, popular media articles on adverse drug effects. A majority of the world still relies heavily on herbal remedies for their primary health care. With the increasing movement of people across countries, there is an accompanying movement of their respective traditional medicines. With the increasing demand for herbal medicinal products, nutraceuticals and natural products for health care all over the world, medicinal plant extract manufacturers and essential oil producers have started using the most appropriate extraction technologies in order to produce extracts and essential oils of defined quality with the least variations from batch to batch[1].

Citrus spp. are an important source of bioactive compounds including antioxidants such as ascorbic acid, flavonoids, phenolic compounds and pectins that are important to human nutrition[2-4]. Epidemiological studies on dietary citrus flavonoids improved a reduction in risk of coronary heart disease[5,6].

Citrus fruits belong to six genera (Fortunella, Eremocitrus, Clymendia, Poncirus, Microcitrus and Citrus), which are native to the tropical and subtropical regions of Asia, but the major commercial fruits belong to genus citrus. Citrus essential oils are a mixture of volatile compounds and mainly consisted of monoterpene hydrocarbons[7]. Citrus oils are mixtures of over a hundred compounds that can be approximated into three fractions: terpene hydrocarbons, oxygenated compounds and non-volatile compounds. Citrus essential oils could represent good candidates to improve the shelf life and the safety of minimally processed fruits[8].

Several studies have demonstrated the antibacterial and/or antioxidant properties of these plants, mainly using *in vitro* assays. Moreover, some researchers reported that there is a relationship between the chemical structures of the most abundant compounds in the plants and their above mentioned functional properties[9,10]. The peel which represents almost one half of the fruit mass, contains the highest concentrations of flavonoids in the Citrus fruit[11-13].

This project was attempted to investigate the anti bacterial and anti oxidant activity and total phenolic and flavonoid contents of *Citrus limon* from Chaloos, Iran. In addition, it was clarified the relation maybe consists between content of constituents and their activation.

EXPERIMENTAL

The samples of the leave and fruit of *Citrus limon* were collected from Chaloos Mazandaran, Iran in Novemberm 2010.

Essential oils isolation procedure: Air-dried leaves of *Citrus limon* (100 g) were hydrodistilled for 4.5 h using a Clevenger-type apparatus according to the method recommended in British Pharmacopoeia (2009). The oil was dried over anhydrous sodium sulfate and kept in a sealed vial at 4 ºC[14].

Also the citrus fruits had been washed, they were cut into some portions and the flesh was removed. The fruit layers were peeled off carefully and discarded. Peel oils were extracted by hand pressing of the flavedo layer with exposed oil sacs and were collected in brine solution kept on ice. The extract was centrifuged (20 min at 6000 rpm) and dried in anhydrous sodium sulphate. The oils were stored at -21 ºC until gas chromatography (GC) and gas chromatograph-mass spectrometer (GC-MS) analyses. Voucher specimen has been deposited in Azad University, Chaloos, Iran.

Extraction procedure: Methanolic extract and then chloroformic fraction of methanolic extracts were obtained by grinding 500 g of leavesto fine powder. The residue was removed by filtration through filter for this purpose the grinded leave samples were extracted at room temperature by percolation with methanol. The extract was concentrated over a rotary vacuum evaporator until a solid extract sample was obtained. Resulting crude extract was freeze-dried. Then chloroformic fraction of methanolic extract was obtained by liquid-liquid extraction from a part of methanolic crude extract. In this manner chloroformic fraction was concentrated over a rotary vacuum evaporator until a solid extract sample was obtained. The extracts were stored at 4 ºC until examination.

GC and GC-MS analysis: GC analysis was performed on a Thermoquest-Finnigan Trace GC instrument equipped with a capillary DB-1 fused silica column (30 m 0.25 mm i. d., film thickness 0.25 µm). The oven temperature was raised from 60 to 250 ºC at a rate of 5 ºC/min, then held at 250 ºC for 10 min.

Nitrogen was used as a carrier gas at a flow rate of 1.1 mL/min. Split ratio was adjusted at 1/50. The injector and detector (FID) temperatures were kept at 250 and 280 ºC, respectively. GC-MS analysis was performed on a Thermoquest-Finnigan Trace GC-MS instrument equipped with a DB-1 fused silica capillary column (60 m 0.25 mm i.d., film thickness 0.25 µm). The oven temperature was raised up from 60 to 250 ºC at a rate 5 ºC/min and then kept at 250 ºC for 10 min. Transfer line temperature was 250 ºC. Helium was used as a carrier gas at a flow rate of 1.1 mL/min with a split ratio of 1/50. A quadrupole mass spectrum was scanned over 45 465 amu with an ionizing voltage of 70 eV and an ionizing current of 150 A. The oil components were identified from their GC retention indices, with either those of the literature[15,16] or with those of authentic compounds available in our laboratories. The identity of the components was assigned by comparing their linear retention indices, relative to C8-C28 *n*-alkanes, under the same operating conditions. Further identification was made by comparison of their MS spectra on both columns, with either stored in NIST 02 and Wiley 275 libraries or with mass spectra from the literature[15,17] and our homemade library.

Antibacterial activity: The antibacterial activity was evaluated by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC), using the broth dilution method[18-20]. Two bacterial species, selected as representative of the Gram (+) and Gram (-) classes, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922,

A series of culture tubes were prepared all containing the same volume of the medium inoculated with test microorganisms. The lowest concentration of sample at which the subculture from test dilution yielded no viable organisms was recorded as minimum bactericidal concentration organisms.

The strains were maintained on Tryptone Soya agar (Oxoid, Milan, Italy); for the antimicrobial tests. Each strain was tested with sample that was serially diluted in broth to obtain concentrations ranging from 100 µg/mL to 0.8 µg/mL. The sample was previously sterilized with a 0.20 µm Millipore filter. The sample was stirred, inoculated with 50 µL of physiological solution containing 5 × 106 microbial cells and incubated for 24 h at 37 ºC.

Antioxidant activity determination: The method consisted of spectrophotometric measurement of the intensity of the change in solution depending on the amount of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The reaction was initiated by mixing 1 mL of the extracts with 3 mL methanol and then by adding 1 mL of DPPH$^{\hat{}}$ (0.012 g/100 mL). The absorbance at λ_{max} 517 nm (UV-VIS spectrophotometer SP 8001, Metertech Inc.) was checked at 0, 0.5 and every 0.5 min until the reaction reached a steady state. This plateau was reached within 15 min. The activity of the extract in scavenging DPPH$^{\hat{}}$ was calculated as follows:

$$\% \text{ DPPH scavenging} = \left[\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right] \times 100$$

The amount of sample needed to decrease the initial DPPH concentration by 50 %, IC_{50}, was calculated graphically. The antiradical power (ARP) of extracts calculated as[21-23].

$$\text{APR} = \frac{1}{(\text{IC}_{50})}$$

Determination of total phenolic contents: Total phenolic contents of extracts were determined by the Folin-Ciocalteu method[24,25] Briefly, aliquots of 0.1 g lyophilized powder of fruit and leaf were dissolved in 1 mL of deionized water. This solution (0.1 mL) was mixed with 2.8 mL of deionized water, 2 mL of 2 % sodium carbonate (Na_2CO_3) and 0.1 mL of 50 % Folin-Ciocalteau reagent after incubation at room temperature for 0.5 h, the absorbance of the reaction mixture absorbance was measured at 750 nm against a deionized water blank on a spectrophotometer (Thermo, Model Nicolet 100 UV-VIS) gallic acid was chosen as a standard. Using a seven point standard curve (0-200 mg/L), the total phenolic contents in extracts were determined and results expressed as mg gallic acid equivalents (GAE) g1 dry weight (DW).

Determination of total flavonoid content: Colorimetric aluminum chloride method was used for flavonoid determination[22,26-28]. Briefly, 0.5 mL solution of each plant extracts were separately mixed with 1.5 mL of methanol, 0.1 mL of

10 % aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a double beam Perkin Elmer UV/Visible spectrophotometer (USA). Total flavonoid contents were calculated as quercetin from a calibration curve. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg mL^{-1}.

RESULTS AND DISCUSSION

GC-MS analysis: Chemical compositions of the peel and leaf oils were determined with GC-MS instrument and identification of component was based on retention times, computer matching with Wiley 275.L data library, comparison of the fragmentation pattern with those reported in the literature and conjunction with authentic sample in case of major components. The results are presented in Table-1.

TABLE-1
PERSENTAGE COMPOSITION OF THE LEAF OIL AND FRUIT PEEL OIL OF *Citrus limon*

Component	RI	Leaf oil	Fruit peel
Nonane	900	0.1	0.1
α-Thujene	930	0.2	0.6
α-Pinene	939	1.6	1.9
Camphene	954	0.1	-
Sabinene	975	0.1	3.8
β-Pinene	979	20.6	12.1
Myrsene	991	1.5	1.6
α-Phellandrene	1003	0.2	-
Iso-sylvestrene	1009	0.5	-
α-Terpinene	1017	0.2	0.3
Limonene	1029	18.8	60.2
Z-β-ocimene	1037	0.4	0.3
E-β-ocimene	1050	0.8	0.2
γ-Terpinene	1060	0.5	11.8
Terpinolene	1089	0.2	0.6
Linalool	1099	7.5	0.2
citrinellal	1153	1.5	-
Z- chrysanthenol	1164	0.5	0.1
Terpinene-4-ol	1177	0.2	-
Z-dihydro carvone	1193	0.8	-
α-terpineol	1188	0.5	0.4
n-Decanal	1202	0.1	-
Neral	1238	8.1	1.1
Geraniol	1253	9.3	-
Geranial	1267	4.4	1.5
Neryl formate	1282	14.8	0.1
Undecanal	1307	0.1	-
Citronellyl acetate	1353	0.1	0.1
Neryl acetate	1362	2.3	0.8
Geranil acetate	1381	1.9	0.3
β-Caryophyllene	1419	1.4	0.4
E-α-bergamotene	1435	0.2	0.5
α-Humulene	1455	0.1	0.2
Bicyclogermacrene	1500	0.5	0.2
β-Bisabolene	1506	0.4	0.6
δ-Cadinene	1523	0.1	-
Total		98.6 %	100 %

Retention index (RI) values are calculated from retention times relative to that of n-alkanes on the non polar DB-5 column

The identity of the spectra above 95 % was needed for the identification of compounds. In this investigation about 100 % constituents of peel oils were determined and on the whole 36 compounds were identified. Four compounds constituting about 87.7 % of the essential oil and the major constituents of *C. limon* peel oils were limonene (60.2 %), β-pinene (12.1 %) and β-terpinene (11.8 %) sabinene (3.6 %).

In this manner, 35 compounds in totally 98.6 % of essential oil from the leaf of *C. limon* were identified. The most abundant components found in the leaf oil were β-pinene (20.6 %), limonene (16.8 %), neryl formate (14.8 %), geraniol (9.3 %), neral (8.1 %) and linalool (7.5 %). (Table-1).

Antibacterial activity: The minimum inhibitory concentration (MIC) and the minimum bacterial concentration (MBC) values of the various extracts against two selected microorganisms are reported in Table-2. The chloroformic sub-fraction showed action mainly (> 256 mg/L) against the Gram-negative pathogens.

According to the results given in Table-2, the extracts of *C. limon* had great antibacterial activity against two type of bacteria and most activity against Gram-negative ones. The proportion of non-polar sub fraction of the methanol extract (chloroformic) was also found to be effective against both Gram-negative and positive strains probably due to the presence of similar compounds in these two extracts.

TABLE-2
MIC AND MBC VALUES (mg/L) OF EXTRACTS FROM *Citrus limon*

Microorganisms	Antibacterial activity as:	Methanolic extract	Chloroformic extract
		(mg/L)	
S. aurous	MIC	32	128
	MBC	128	> 256
E. Coil	MIC	128	> 256
	MBC	256	> 256

Total phenol compounds: Total phenol compounds, as determined by Folin-Ciocalteu method, are reported as gallic acid equivalents by reference to standard curve.

The total phenolic contents were higher in methanolic extract (0.155) respect to chloroformic subfraction (0.16) as mg Gallic acid equivalent/g of extract powder (Table-3). These results showed that phenolic components were not only reason for antibacterial activity and may be presence of other compounds ware more effectiveness.

TABLE-3
TOTAL PHENOLIC COMPOUND OF *C. limon*

Sample	Chloroformic extract (mg/g)	Methanolic extract (mg/g)
Total phenolic compounds	0.155	0.16

Total flavonoid contents: The total flavonoid contents were higher in methanolic extract (2.7) respect to chloroformic subfraction (trace) as mg quercetin equivalent/g of extract powder by reference to standard curve (Table-4).

Antioxidant activity: Free radical scavenging properties and the inhibition effects on the lipid peroxidation were determined in this stage. The inhibition rate of the plant extracts is

comparatively closed to the synthetic antioxidant BHT. Free radical scavenging activity of the extracts is concentration dependent and lower IC_{50} value reflects better protective action. The chloroformic extract was able to reduce the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) to the yellow-coloured diphenylpicrylhydrazine with an IC_{50} of 29.197 µg/mL exhibiting a little decrease from activity to the synthetic antioxidant agent BHT (8 µ/mL). It seems that this activity is mostly related to the presence of flavonoids and phenolic acids in the this fraction. The key role of flavonoids compounds as scavengers of free radicals is emphasized in several reports[29-31]. Moreover, radical-scavenging activity is one of various mechanisms to contribute overall activity, thereby creating a synergistic effect. This activity was less for methanolic extract (Table-5).

TABLE-4
TOTAL FLAVONOID COMPOUND FROM *C. limon*

Sample	Chloroformic extract	Methanolic extract
Total flavonoides as quercetin	Trace	2.77 mg/g

TABLE-5
ANTIOXIDANT ACTIVITY (DPPH test) as IC_{50} VALUE

Sample	Chloroformic extract	Methanolic extract	BHT
IC_{50} (µg/mL)	29.19759	48.17238	8

Conclusion

In conclusion, present study can be considered as the confirmation in antibacterial and spatially antioxidant effectiveness of *C. lemon* and necessity to place limen in our daily nutrition programs.

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