Chemical Composition of the Essential Oils of Variegated Pink-Fleshed Lemon (*Citrus* x *limon* L. Burm. f.) and their Anti-Inflammatory and Antimicrobial Activities

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Z. Naturforsch. **68 c**, 275 – 284 (2013); received December 18, 2012/May 13, 2013

The volatile secondary metabolites of essential oils from fruit peel and leaves of variegated pink-fleshed lemon (*Citrus* x *limon*) were investigated using GLC and GLC-MS (gas-liquid chromatography-mass spectroscopy). Altogether 141 compounds were identified and quantified, accounting for 99.59% and 96.33% of the total hydrodistilled peel and leaf oil, respectively. Limonene occurred in higher amounts in fruit peel (52.73%) than in leaf oil (29.13%). Neral (12.72%), neryl acetate (8.53%), *ρ*-menth-1-en-7-al (4.63%), *β*-pinene (6.35%), and nerol (4.42%) were the most abundant constituents in leaf oil, whereas *γ*-terpinene (9.88%), *β*-pinene (7.67%), geranial (4.44%), and neral (3.64%) dominated in the fruit peel oil. The antioxidant, anti-inflammatory, antitrypanosomal, and antimicrobial activities of the fruit peel essential oil were evaluated. The oil had a low antioxidant activity with an IC_{50} value of (26.66 ± 2.07) mg/ml as compared to the efficient antioxidant ascorbic acid [IC_{50} (16.32 ± 0.16) *μ*g/ml]. The oil moderately inhibited soybean 5-lipoxygenase (5-LOX) with an IC_{50} value of (32.05 ± 3.91) *μ*g/ml and had moderate antitrypanosomal activity [IC_{50} (60.90 ± 0.91) *μ*g/ml]. In addition, moderate antimicrobial activities were detected against Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus capitis*, *Micrococcus luteus*), one Gram-negative bacterium (*Pseudomonas fluorescens*), and yeasts (*Saccharomyces cerevisiae*, *Candida parapsilosis*).

Key words: Pink Lemon, Essential Oil, Bioactivities

Introduction

Products from *Citrus* species (Rutaceae) have been used not only for food and drinks but also in perfumes, soaps, and many other commodities (Crupi *et al.*, 2007; Ladaniya, 2008; Tranchida *et al.*, 2012). In addition, plants from this genus have been extensively studied for their antimicrobial, antioxidant, anti-inflammatory, antiedemic, cardiovascular, antihyperglycaemic, and antitumour properties (Johann *et al.*, 2007; Benavente-Garcia and Castillo, 2008; Fisher and Phillips, 2008; Shen *et al.*, 2012; Mencherini *et al.*, 2013; Wesołowska *et al.*, 2012).

Variegated pink-fleshed Eureka lemon (pink lemon) is a small fragrant tree (4 – 5 m in height). The variegated green and white leaves are of ornamental interest. Fruits are produced throughout the year, but mainly in late winter, spring, and early summer. The fruit peel has a striped green and cream colouration. When fully ripe, the stripes fade and the peel turns yellow with distinct pink oil glands. The flesh is light pink at full maturity; it has few seeds but the taste would be sour (Shamel, 1932).

In the context of our chemical and biological investigation of *Citrus* species cultivated in Egypt (El-Readi *et al.*, 2010; Hamdan *et al.*, 2010, 2011a, b), essential oils from the fruit peel and leaves of variegated pink-fleshed lemon were analysed by high-resolution capillary GLC and GLC-MS (gas-liquid chromatography-mass spectroscopy). In order to understand their biological properties, antioxidant, anti-inflammatory, antitrypanosomal, and antimicrobial activities were assessed.

Material and Methods

Plant material

The fresh ripe fruits and leaves of variegated pink-fleshed lemon (*Citrus* x *limon* L. Burm. f.) (Rutaceae) were collected on the Research Station of the Faculty of Agriculture, Benha University, Benha, Egypt in March 2009 and 2010. The identity of the plant was confirmed by Dr. B. M. Houlyel, Faculty of Agriculture, Benha University, Benha, Egypt. A voucher specimen of the plant material was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt (accession no. P-78, 79).

Sample preparation

The fresh fruit peel and leaves (100 g of each) were separately subjected to hydrodistillation for 6 h using a Clevenger-type apparatus; the yields were 2% and 0.2%, respectively. Both oils were dried over anhydrous sodium sulfate and kept in brown vials at 4 °C until further analyses.

GLC and GLC-MS

The volatile oil constituents were analysed by high-resolution capillary GLC and GLC-MS. Samples of the oil (1 μl each was dissolved in 1 ml *n*-hexane) were injected (1 μl volume) into a gas chromatograph (TRACE GC ULTRA; Thermo Scientific, Milan, Italy) under the following conditions: column, RTX-5MS® fused silica capillary (Restek, Bellefonte, PA, USA) equivalent to DB-5 (30 m x 0.32 mm i. d., 0.25 μm film thickness); carrier gas, He (2 ml/min); detector (FID) temperature, 300 °C; injection temperature, 250 °C; oven temperature program: initial temperature of 45 °C, 2 min isothermal, at 4 °C/min to 300 °C, then 20 min isothermal; split ratio, 1:15. Kovat's retention indices (RI) were calculated with respect to a set of co-injected standard hydrocarbons (C_{10}–C_{24}). The identified components were quantified using the GLC "Peak Simple" software.

GLC-MS data were recorded on a Clarus 600 gas chromatograph (Shelton, CT, USA) equipped with an identical column used for separation and quantification. The capillary column was directly coupled to a quadrupole mass spectrometer Clarus 600T. The ionization energy of the mass spectrometer was 70 eV and the split ratio 1:30; all other conditions were identical to those mentioned for GLC. Compounds were identified by comparing their spectral data and retention indices with Wiley Registry of Mass Spectral Data 8th edition, NIST Mass Spectral Library (December 2005), and the literature (Adams, 2007; El-Shazly *et al.*, 2004; El-Shazly and Hussein, 2004). The identified constituents are listed in the order of their elution in Table I.

DPPH free radical scavenging activity

Differently graded dilutions of a stock solution (200 μl in 1 ml MeOH) of the peel essential oil were mixed with 500 μl of 0.2 mM diphenylpicrylhydrazine (DPPH) (Sigma-Aldrich, Taufkirchen, Germany), and the final volume was brought to 1 ml. The mixtures were vigorously shaken and allowed to stand in the dark for 30 min at room temperature. The absorbance was measured by spectrophotometry (LKB Pharmacia Biochrom ULTROSPEC PLUS 4054 UV/VIS; Freiburg, Germany) at 517 nm against a blank sample without DPPH (negative control). The antioxidant activity of the essential oil, expressed as IC_{50} (in mg/ml), was compared with that of ascorbic acid as a standard antioxidant (Hamdan *et al.*, 2010).

5-Lipoxygenase inhibition

Inhibition of 5-lipoxygenase (5-LOX) (Fluka, Buchs, Switzerland) by the essential oil was determined as previously reported (Baylac and Racine, 2003) under the following conditions: to 970 μl of phosphate buffer, pH 9.0, 10 μl 5-LOX (1 mg/ml) and 20 μl of twelve different concentrations of the samples (10 – 350 μg/ml) were added and incubated at room temperature for 10 min. The enzymatic reaction was started by adding 25 μl of 62.5 mM sodium linoleate; the absorbance was measured photometrically at 234 nm every 10 s for 3 min. The initial reaction rates were determined from the slope of the linear part of the curve. Nordihydroguaiaretic acid (NDGA) was used as a positive reference compound.

Anti-infective activities

Antitrypanosomal activity

The mature bloodstream forms of *Trypanosoma b. brucei* TC221, the causative agent of Nagana (which can be used as a model for human sleeping sickness), were grown in Baltz medium supplemented with 20% inactivated foetal bovine

serum and 1% penicillin/streptomycin. The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

Trypanocidal activity was determined using resazurin as a cell proliferation indicator dye as previously described (Baltz *et al*., 1985; Nibret *et al*., 2010). Briefly, the samples were serially diluted with the medium in a two-fold fashion to attain final concentrations in the range 250 to 3.9 μg/ml in 96-well plates. *T. b. brucei* was seeded into 96 wells at a density of $1 \cdot 10^4$ cells per 100 μl of medium. The cells were then incubated with samples of various concentrations for 24 h. Ten μl of resazurin were added to each well and the mixture incubated for further 24 h. The absorbance of the plates was recorded using a Tecan® plate reader (Crailsheim, Germany) at dual wavelengths of 492 nm and 595 nm (Huber and Koella, 1993).

Each sample concentration was tested in triplicate, and experiments were independently repeated twice. The maximum content of the solvent dimethyl sulfoxide (DMSO) did not exceed 1.25% in the medium that contained the highest concentration of samples. Diminazeneaceturate was used as a positive control (IC_{50} 0.088 μg/ml).

Antimicrobial activity

The fruit peel oil (320 mg) was dissolved in 1 ml DMSO from which 10 μl were used for testing. For evaluation of the antimicrobial activity, two methods were used: the agar diffusion method and broth microdilution assay (Mulyaningsih *et al*., 2010). The test microorganisms *Bacillus subtilis* DSM 2109, *Staphylococcus capitis* DSM 20325, *Micrococcus luteus* DSM 1890 (Gram-positive bacteria); *Klebsiella planticola* DSM 4617, *Escherichia coli* ATCC 25922, *Pseudomonas fluorescens* DSM 6147 (Gram-negative bacteria); and *Saccharomyces cerevisiae* DSM 1333 and *Candida parapsilosis* DSM 11224 (yeasts) were obtained from the Institut für Pharmazie und Molekulare Biotechnologie, Universität Heidelberg, Heidelberg, Germany. Kanamycin (1 mg/ml) was used as a standard antibiotic for bacteria while nystatin (100 IU/disc) was used as standard fungicide. The plates were incubated overnight at 37 °C for bacteria and 30 °C for fungi. The diameter (in mm) of inhibition zones was measured as shown in Table II. For minimum inhibitory concentration (MIC) and minimum microbial concentration (MMC) determinations, the broth microdilution assay was employed (Mulyaningsih *et al*., 2010).

Statistical analysis

All experiments were carried out three times unless indicated otherwise in the procedure. Continuous variables were presented as mean ± SD of three individual experiments. The IC_{50} was determined as the drug concentration which resulted in a 50% reduction in cell viability or inhibition of the biological activity. IC_{50} values were calculated using a four-parameter logistic curve (SigmaPlot® 11.0), and all data were statistically evaluated using Student's *t*-test and/or the Kruskal-Wallis test (GraphPad Prism® 5.01; GraphPad Software, Inc., La Jolla, CA, USA) followed by Dunn's *post-hoc* multiple comparison test when the significance value was < 0.05 using the same significance level. The criterion for statistical significance was $P < 0.05$.

Results and Discussion

Analysis of the volatile compounds

A 2% (v/w of the fresh plant material) yield of essential oil was obtained from the peel, while the leaves produced only 0.2% (v/w) essential oil. The essential oils were subjected to GLC and GLC-MS analyses in order to determine the organ-specific variation in their volatile constituents. Altogether 141 secondary metabolites (SM) were identified representing 99.59% and 96.33% of the total compounds in both peel and leaf oils, respectively. The chemical composition of the oils is presented in Table I.

D-Limonene represented the major component in fruit peel oil with 52.73% of the total 1097 identified compounds. In addition, γ-terpinene (9.88%), β-pinene (7.67%), geranial (4.44%), neral (3.64%), α-terpineol (2.19%), nerol (2.14%), neryl acetate (1.79%), geraniol (1.78%), myrcene (1.70%), and α-pinene (1.36%) were the other major SM.

In the leaf oil, 101 compounds were identified. The major constituents were D-limonene (29.13%), neral (12.72%), neryl acetate (8.53%), β-pinene (6.35%), ρ-menth-1-en-7-al (4.63%), nerol (4.42%), *trans*-myrtanol acetate (3.81%), citronellal (2.39%), (*E*)-β-ocimene (2.34%), (*E*)-caryophyllene (2.05%), geranial (1.95%), myrcene (1.80%), linalool (1.36%), and (*Z*)-β-ocimene (1.34%).

The monoterpene hydrocarbons in the peel and leaf oils accounted for 75.39% and 44.22%, respectively; this is attributed to the high amount

Table I. Volatile constituents identified in the essential oils of fruit peel and leaves of variegated pink lemon.

	Compound[a]	RI	Composition[b] (%)		Method of identification[c]
			Peel	Leaves	
1.	*α*-Thujene	940	0.33	0.06	MS, RI, AT
2.	*α*-Pinene	944	1.36	0.58	MS, RI, AT
3.	*α*-Fenchene	946	0.12	–	MS, RI
4.	Camphene	949	0.02	0.41	MS, RI, AT
5.	Sabinene	970	0.03	tr.	MS, RI
6.	*β*-Pinene	976	7.67	6.35	MS, RI, AT
7.	6-Methyl-5-hepten-2-one	987	0.06	0.35	MS, RI
8.	Myrcene	989	1.70	1.80	MS, RI, AT
9.	*n*-Octanal	999	0.50	–	MS, RI
10.	*ρ*-Mentha-1(7),8-diene	1004	0.02	tr.	MS, RI
11.	*iso*-Sylvestrene	1010	0.22	–	MS, RI
12.	*ρ*-Cymene	1025	–	tr.	MS, RI, AT
13.	D-Limonene	1031	52.73	29.13	MS, RI, AT
14.	*β*-Phellandrene	1031	tr.	tr.	MS, RI
15.	(*Z*)-*β*-Ocimene	1038	tr.	1.34	MS, RI
16.	(*E*)-*β*-Ocimene	1045	0.26	2.34	MS, RI
17.	*γ*-Terpinene	1056	9.88	0.65	MS, RI
18.	*n*-Octanol	1062	0.05	–	MS, RI
19.	*cis*-Sabinene hydrate	1069	0.19	0.04	MS, RI
20.	*ρ*-Mentha-3,8-diene	1075	tr.	0.07	MS, RI
21.	*ρ*-Mentha-2,4(8)-diene	1082	0.75	0.02	MS, RI
22.	Terpinolene	1092	0.03	0.67	MS, RI
23.	Linalool	1096	0.79	1.36	MS, RI, AT
24.	*n*-Nonanal	1105	0.17	0.23	MS, RI
25.	*trans*-Sabinene hydrate	1107	tr.	–	MS, RI
26.	1,3,8-*ρ*-Mentha-triene	1109	0.02	–	MS, RI
27.	Benzaldehyde dimethyl acetal	1113	–	0.35	MS, RI
28.	*cis*-*ρ*-Mentha-2-en-1-ol	1115	0.07	–	MS, RI
29.	*trans*-*ρ*-Mentha-2,8-dien-1-ol	1127	0.06	–	MS, RI
30.	*trans*-Pinene hydrate	1130	0.02	–	MS, RI
31.	*allo*-Ocimene	1132	0.03	0.45	MS, RI
32.	1-Terpineol	1134	0.04	0.11	MS, RI
33.	*cis*-*ρ*-Mentha-2,8-dien-1-ol	1136	0.06	–	MS, RI
34.	*cis*-Pinene hydrate	1141	0.08	–	MS, RI
35.	Camphor	1144	–	0.57	MS, RI, AT
36.	Citronellal	1149	0.21	2.39	MS, RI, AT
37.	*β*-Pinene oxide	1159	–	0.79	MS, RI
38.	Isoborneol	1160	0.14	–	MS, RI, AT
39.	Terpinen-4-ol	1172	1.19	1.07	MS, RI
40.	*cis*-Pinocarveol	1181	0.08	–	MS, RI
41.	Cryptone	1184	–	0.50	MS, RI
42.	*α*-Terpineol	1188	2.19	0.61	MS, RI, AT
43.	Dihydrocarveol	1191	0.08	–	MS, RI
44.	Myrtenal	1195	0.01	–	MS, RI, AT
45.	Shisofuran	1196	–	0.06	MS, RI
46.	*γ*-Terpineol	1196	0.02	–	MS, RI
47.	*trans*-Dihydrocarvone	1200	0.02	0.22	MS, RI
48.	*n*-Decanal	1203	0.16	–	MS, RI
49.	(2*E*)-Octanol acetate	1211	0.02	–	MS, RI
50.	*trans*-Carveol	1218	0.08	–	MS, RI
51.	4-Methylene-isophorone	1219	–	0.27	MS, RI
52.	Citronellol	1225	–	tr.	MS, RI, AT
53.	*cis*-Carveol	1227	0.06	–	MS, RI
54.	Nerol	1229	2.14	4.42	MS, RI, AT

Table I continued.

Compound[a]		RI	Composition[b] (%)		Method of identification[c]
			Peel	Leaves	
55.	Neral	1242	3.64	12.72	MS, RI, AT
56.	Geraniol	1252	1.78	0.63	MS, RI, AT
57.	Piperitone	1254	0.02	–	MS, RI
58.	Geranial	1274	4.44	1.95	MS, RI, AT
59.	*ρ*-Menth-1-en-7-al	1282	0.04	4.63	MS, RI
60.	Limonen-10-ol	1291	0.10	tr.	MS, RI
61.	Carvacrol	1299	0.08	0.05	MS, RI, AT
62.	Terpinen-4-ol acetate	1304	0.01	–	MS, RI
63.	(8*Z*)-Undecenal	1307	0.04	–	MS, RI
64.	Undecanal	1310	–	0.12	MS, RI
65.	*n*-Nonanyl acetate	1313	tr.	–	MS, RI
66.	*Z*-Patchenol	1315	tr.	0.02	MS, RI
67.	*neo*-Verbanol acetate	1322	–	0.03	MS, RI
68.	Methyl geranate	1324	0.01	0.05	MS, RI
69.	*neo-iso*-Verbanol acetate	1330	tr.	tr.	MS, RI
70.	*cis*-Piperitol acetate	1333	tr.	–	MS, RI
71.	(*E*)-Dimethoxy-citral	1336	–	0.04	MS, RI
72.	Piperitenone	1342	–	0.07	MS, RI
73.	Citronellyl acetate	1350	0.09	0.53	MS, RI
74.	Neryl acetate	1366	1.79	8.53	MS, RI
75.	*cis*-Carvyl acetate	1374	0.02	0.04	MS, RI
76.	Geranyl acetate	1384	0.66	0.03	MS, RI
77.	*trans*-Myrtanol acetate	1388	–	3.81	MS, RI
78.	*β*-Elemene	1390	0.02	0.09	MS, RI
79.	*n*-Tetradecane	1397	0.02	0.01	MS, RI, AT
80.	(*Z*)-Caryophyllene	1408	tr.	0.02	MS, RI, AT
81.	*β*-Funebrene	1413	0.48	–	MS, RI
82.	(*E*)-Caryophyllene	1420	0.02	2.05	MS, RI
83.	*α-trans*-Bergamotene	1433	0.68	0.25	MS, RI
84.	(*Z*)-*β*-Farnesene	1444	tr.	0.01	MS, RI
85.	*α*-Humulene	1449	0.03	0.23	MS, RI, AT
86.	(*E*)-*β*-Farnesene	1457	0.10	0.15	MS, RI, AT
87.	9-*epi*-(*E*)-Caryophyllene	1471	tr.	–	MS, RI
88.	Isobornyl *n*-butanoate	1471	–	0.04	MS, RI
89.	Geranyl propanoate	1477	–	0.08	MS, RI
90.	*γ*-Muurolene	1477	0.03	tr.	MS, RI
91.	Germacrene D	1483	0.05	tr.	MS, RI, AT
92.	*β*-Selinene	1491	0.12	–	MS, RI
93.	Neryl isobutanoate	1491	–	0.03	MS, RI
94.	Viridiflorene	1494	0.05	–	MS, RI, AT
95.	*α*-Alaskene	1497	tr.	0.09	MS, RI
96.	*α*-Muurolene	1500	tr.	0.02	MS, RI
97.	(*E,E*)-*α*-Farnesene	1503	0.08	–	MS, RI
98.	*β*-Bisabolene	1509	0.98	0.39	MS, RI
99.	(*Z*)-*γ*-Bisabolene	1515	0.03	0.03	MS, RI
100.	*β*-Curcumene	1519	tr.	–	MS, RI
101.	*δ*-Cadinene	1523	tr.	0.06	MS, RI
102.	(*E*)-*iso*-*γ*-Bisabolene	1529	tr.	tr.	MS, RI
103.	(*E*)-*γ*-Bisabolene	1531	tr.	0.01	MS, RI
104.	*cis*-Sesquisabinene hydrate	1543	–	0.03	MS, RI
105.	Elemol	1550	0.03	–	MS, RI
106.	(*E*)-Nerolidol	1560	–	0.02	MS, RI
107.	Geranyl butanoate	1564	–	0.11	MS, RI
108.	Spathulenol	1575	–	0.18	MS, RI

Table I continued.

Compound[a]		RI	Composition[b] (%)		Method of identification[c]
			Peel	Leaves	
109.	Caryophyllene oxide	1580	tr.	0.42	MS, RI, AT
110.	*n*-Hexadecane	1580	tr.	0.42	MS, RI, AT
111.	Humulene epoxide II	1605	–	0.07	MS, RI
112.	*epi*-Cedrol	1612	0.03	0.06	MS, RI
113.	γ-Eudesmol	1629	0.01	0.03	MS, RI
114.	Caryophylla-4(12),8(13)-dien-5α-ol	1642	–	0.03	MS, RI
115.	*epi*-α-Muurolol	1642	–	0.04	MS, RI
116.	β-Eudesmol	1649	tr.	–	MS, RI
117.	Isoamyl geranate	1652	–	0.02	MS, RI
118.	α-Cadinol	1657	0.11	0.18	MS, RI
119.	(*E*)-Citronellyl tiglate	1665	–	0.01	MS, RI
120.	*epi*-β-Bisabolol	1671	0.08	–	MS, RI
121.	(*Z*)-Nerolidol acetate	1675	–	0.16	MS, RI
122.	*epi*-α-Bisabolol	1685	0.09	–	MS, RI
123.	α-Bisabolol	1688	–	0.12	MS, RI, AT
124.	(*Z*)-α-*trans*-Bergamotol	1694	tr.	–	MS, RI
125.	(2*Z*,6*Z*)-Farnesol	1698	–	0.07	MS, RI
126.	(2*E*,6*Z*)-Farnesol	1717	–	0.04	MS, RI
127.	(2*Z*,6*E*)-Farnesol	1724	–	0.10	MS, RI
128.	(2*E*,6*E*)-Farnesol	1743	–	0.05	MS, RI
129.	β-Bisabolenal	1765	tr.	–	MS, RI
130.	*n*-Octadecane	1797	–	tr.	MS, RI
131.	Nootkatone	1804	0.02	0.02	MS, RI
132.	Hexadecanoic acid	1953	tr.	tr.	MS, RI
133.	*n*-Octadecanol	2071	0.01	–	MS, RI
134.	*n*-Heneicosane	2102	tr.	0.01	MS, RI
135.	Methyl octadecanoate	2120	0.02	–	MS, RI
136.	1-Docosene	2184	tr.	tr.	MS, RI
137.	*n*-Docosane	2204	tr.	tr.	MS, RI
138.	*n*-Tricosane	2303	tr.	tr.	MS, RI
139.	*n*-Tetracosane	2397	–	tr.	MS, RI
140.	Pentacosane	2502	–	tr.	MS, RI
141.	Hexacosane	2605	tr.	–	MS, RI
	Monoterpene hydrocarbons		75.39	44.22	
	Oxygen-containing monoterpenes		20.18	45.96	
	Sesquiterpene hydrocarbons		2.66	3.96	
	Oxygen-containing sesquiterpenes		0.37	1.79	
	Others		0.99	0.40	
	Total identified components		99.59	96.33	

[a] In order of elution from an RTX-5MS® column.
[b] The composition (in %) is calculated as average of three analyses; tr., trace (<0.01%); – not detected.
[c] MS, identification based on mass spectral data; RI, identification based on retention index relative to standard *n*-alkanes; AT, identification based on co-chromatography with authentic samples.

of D-limonene (52.73% and 29.13%). In addition, thujene, α-pinene, camphene, sabinene, β-pinene, myrcene, (*Z*)-β-ocimene, (*E*)-β-ocimene, and γ-terpinene are the major SM in both oils. The overall amount of oxygenated monoterpenes in leaf oil was very high (45.96%) as compared with fruit peel oil (20.18%); this is related to the high content of neral (12.72%), neryl acetate (8.53%), ρ-menth-1-en-7-al (4.63%), and nerol (4.42%). Furthermore, (*E*)-caryophyllene (2.05%) constituted the most abundant sesquiterpene hydrocarbons. In fruit peel oil, β-bisabolene represented the major sesquiterpene constituent (0.98%). These results closely agree with the chemical com-

position of a Portuguese variety of *Citrus limon*, which contains a high content of both monoterpenes, especially limonene. However, American oils contain less D-limonene indicating that varieties, geographical location, and the climate play an important role in the composition of *Citrus* oil (Choi *et al*., 2000; Lota *et al*., 2002).

Scavenging of DPPH radicals

The antioxidant activity of the peel oil, determined by the DPPH assay, was moderate with an IC_{50} value of (26.66 ± 2.07) mg/ml, as compared with ascorbic acid [IC_{50} (16.32 ± 0.16) μg/ml] (Fig. 1). Similar low values were obtained for oils of two other *Citrus* species, *C. jambhiri* and *C. pyriformis*, with IC_{50} values of 37.61 and 28.91 mg/ml, respectively (Hamdan *et al*., 2010). In general, the complete absence of phenolic compounds, which are the main species responsible for the inhibition of the DPPH radicals, is apparently related to the low antioxidant activities of these essential oils (Mulyaningsih *et al*., 2010).

5-Lipoxygenase inhibition

Inhibition of 5-lipoxygenase (5-LOX) by the peel oil is illustrated in Fig. 2. The inhibition was concentration-dependent with an IC_{50} value of (32.05 ± 3.91) μg/ml (the positive control NDGA exhibited an IC_{50} value of 0.24 μg/ml). This 5-LOX inhibition activity may be attributed to the high content of D-limonene, which exhibits an IC_{50} value of 13 μg/ml (Jin *et al*., 2011).

Anti-infective activities

Antitrypanosomal activity

Fruit peel essential oil moderately inhibited the growth of bloodstream forms of *Trypanosoma b. brucei* [IC_{50} (60.90 ± 0.91) μg/ml] (Fig. 3) as compared to diminazeneaceturate as a positive control. Based on our previous work, this activity is quite promising; however, the selectivity of this oil towards the causative protozoa remains the main obstacle for the utilization of these drugs against trypanosomiasis (Hamdan *et al*., 2010; Mulyaningsih *et al*., 2010).

Antimicrobial activity

The peel oil was tested against six standard bacterial strains and two fungi (Table II). The essential oil showed a moderate antimicrobial activity against all tested Gram-positive bacteria and yeasts with MIC values ranging between 2 and 16 mg/ml. However, the oil possessed no activity against Gram-negative strains, except for *Pseudomonas fluorescens*.

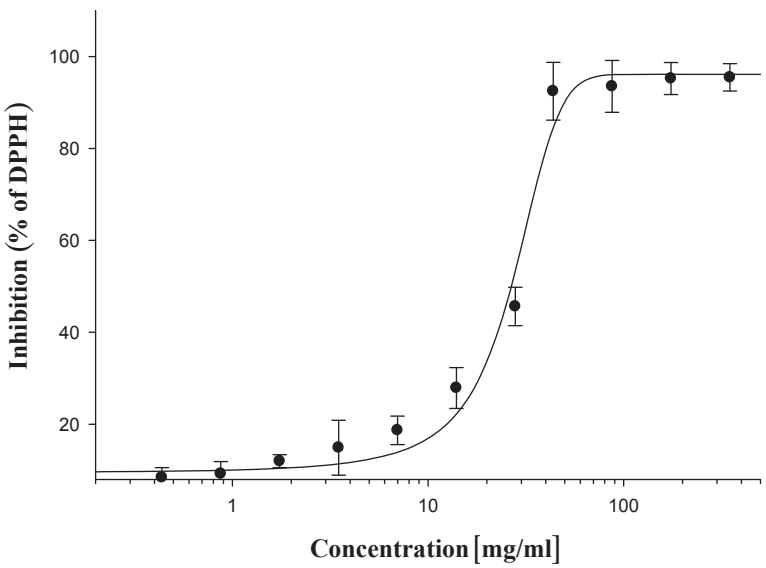

Fig. 1. Scavenging of DPPH radicals by the essential oil of pink lemon (*Citrus* x *limon*) fruit peel [IC_{50} (26.66 ± 2.07) mg/ml]. Data are expressed as the mean ± SD of three individual experiments.

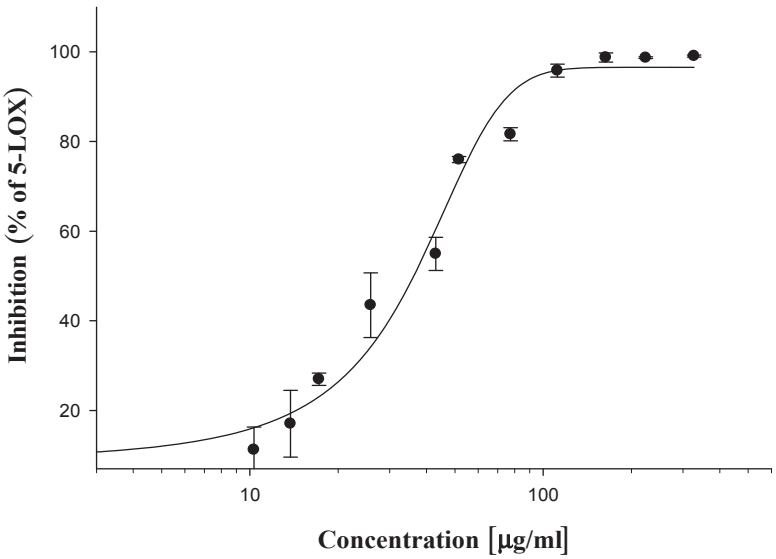

Fig. 2. Dose-dependent inhibition of soybean 5-LOX activity by the essential oil of pink lemon (*Citrus* x *limon*) fruit peel [IC_{50} (32.05 ± 3.91) μg/ml]. Data are expressed as the mean ± SD of three individual experiments.

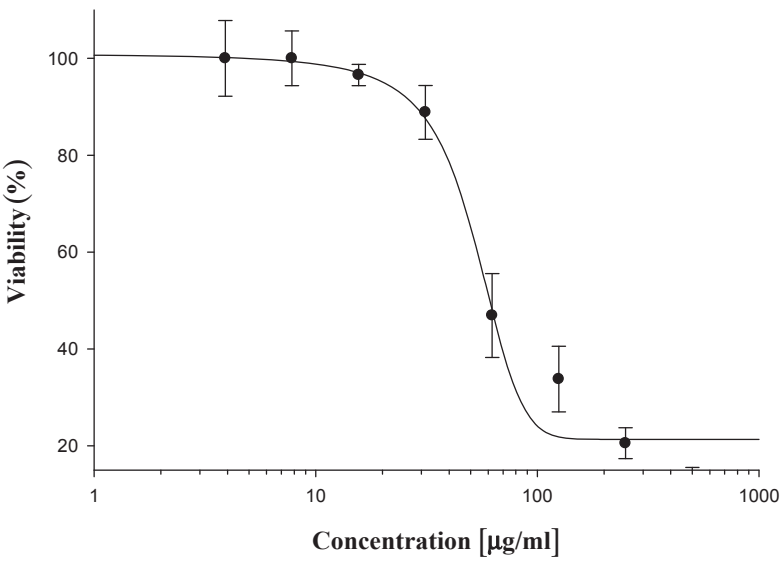

Fig. 3. Dose-dependent growth inhibition of *Trypanosoma b. brucei* (resazurin assay) by the essential oil of pink lemon (*Citrus* x *limon*) fruit peel [IC_{50} (60.90 ± 0.91) μg/ml]. Data are expressed as the mean ± SD of three individual experiments.

The antimicrobial and trypanocidal activities of the oil could be attributed to the monoterpenes D-limonene and γ-terpinene. Both the oil and the two isolated compounds are lipophilic substances that can dissolve in the biomembrane of the causative microbes and interact with membrane lipids and proteins, resulting in disruption of cellular integrity, leakage of cell content, and finally cell death (Wink, 2008; Wink *et al.*, 2012). However, the weak activity against Gram-negative microbes could be due to the presence of their double membrane that protects the Gram-negative

Table II. Minimum inhibitory concentrations (MIC) and minimum microbicidal concentrations (MMC) of essential oils of pink lemon fruit against different pathogens using agar diffusion and broth microdilution methods.

Microorganism	Pink lemon fruit oil			Positive control		
	DD [mm]	MIC [mg/ml]	MMC [mg/ml]	DD [mm]	MIC [mg/ml]	MMC [mg/ml]
Gram-positive bacteria						
Bacillus subtilis DSM 2109	11	2	4	28.3	0.4	0.8
Staphylococcus capitis DSM 20325	14	4	16	30.0	0.2	0.8
Micrococcus luteus DSM 1890	9	4	8	21.7	0.4	0.8
Gram-negative bacteria						
Klebsiella planticola DSM 4617	–	–	–	18.3	0.4	3.2
Escherichia coli ATCC 25922	–	–	–	18.0	0.8	3.2
Pseudomonas fluorescens DSM 6147	6	4	16	18.0	0.8	3.2
Fungi						
Saccharomyces cerevisiae DSM 1333	18	4	4	26.3	1.6	1.6
Candida parapsilosis DSM 11224	14	8	8	22.3	3.1	12.5

All assays consisted of 320 mg essential oil in 1 ml DMSO, and 10 μl were applied. DD, diameter of inhibition zone as measured by the agar diffusion method; – no inhibition.
Kanamycin (1 mg/ml) was used as standard antibiotic for bacteria and nystatin (100 IU/disc) for fungi.

bacteria from the effect of the oil components (van Wyk and Wink, 2004).

Conclusions

Citrus fruits and their products are major sources of vitamins and minerals; however, their essential oils have hardly been investigated. Based on the fact that a high yield of essential oil can be obtained from *Citrus* fruit peel, we have studied the possible antioxidant, anti-inflammatory, and anti-infective effects of variegated pink-fleshed lemon oil. The peel oil showed moderate inhibitory activity against 5-lipoxygenase (as a measure for anti-inflammatory activity) and moderate antimicrobial activity. These properties can improve the conditions associated with common cold, cough, and upper respiratory tract infections. However, future studies are required to investigate the oil and the activity of some of its major components in animal experiments in order to determine the most active compound(s) and potential synergistic effects.

Acknowledgement

The authors would like to thank Dr. E. Nibret for valuable help in carrying out the antitrypanosomal tests.

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