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Chemical composition of the essential oils of two *Citrus* species and their biological activities

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Essential oils obtained by hydrodistillation of the fruit rinds of *Citrus jambhiri* Lush. (Rough lemon) and *C. pyriformis* Hassk (Ponderosa lemon) were analyzed by capillary gas chromatography (GLC/FID) and gas chromatography–mass spectrometry (GLC/MS). A total of 94 compounds were unambiguously identified from the oils and the (hexane/ether) extracts of the rind and juices representing 98.55% and 97.98% of the total oil composition. The main component of both oils was D-limonene (92.48% and 75.56% respectively). The antioxidant, anti-inflammatory, antitrypanosomal, antimicrobial and cytotoxic activities of the essential oils were evaluated. Whereas *Citrus jambhiri* and *C. pyriformis* have antioxidant activity with $IC_{50} \pm SD$ 37.69 ± 0.21 mg/ml and 28.91 ± 0.09 mg/ml, respectively. Ascorbic acid a known potential inhibitor for DPPH*free radical an commonly used antioxidant showed an antioxidant activity with an IC_{50} value of 16.32 ± 0.16 μg/ml. Both oils inhibited the activity of 5-lipoxygenase (5-LOX) with an IC_{50} of 40 ± 1.63 and 38 ± 0.82 μg/ml, respectively, and could be considered as interesting candidates for antiinflammatory agents. The essential oils of both species showed substantial antimicrobial activity against all tested Gram positive bacteria and yeasts. The essential oil of *C. pyriformis* showed higher cytotoxic activity against tested cell lines than that of *C. jambhiri*. The IC_{50} values were 374.36 ± 43.95 μg/ml and 588.06 ± 27.12 μg/ml in case of HepG2 cells and 213.87 ± 18.50 μg/ml and 512.45 ± 61.46 μg/ml in case of MIA-PaCa-2 cells, respectively.

1. Introduction

Citrus is a large genus of flowering plants in the family Rutaceae. The members of the genus are large shrubs or small tress with evergreen leaves, growing mainly in tropical and subtropical areas of the world. Morphologically, the genus *Citrus* presents considerable taxonomic problems due to the existence of clonally-propagated plants and hybrids. Many species of this genus are cultivated on a commercial scale and in gardens for their edible fruits and characteristic fragrant flowers. The juice contains high levels of citric acid and vitamin C. Biologically important active constituents of different classes of secondary metabolites are reported in this genus such as flavonoids, limonoids, coumarins and furanocoumarins, sterols, volatile oils, carotenoids and alkaloids (Nordby and Nagy 1981; Nornen et al. 1999; Dugo et al. 2005; Njoroge et al. 2005; Gattuso et al. 2007; Pellati and Benvenuti 2007; Manners 2007). Many *Citrus* species are known for their antiseptic, antiviral, antioxidant, antinitrosaminic, anticancer, antiinflammatory, hypocholesterinemic, cardiotonic, sedative, and stomachic activity and effects on capillaries (Benavente-Garcia and Castillo 2008; Ladaniya 2008; Ju-Ichi 2007; El-Shafae and Soliman 1998; Silalahi 2002; Ortuno et al. 1997; Teng et al. 1992; Tanaka et al. 2001; Awad and Fink 2000).
In the context of our chemical and biological investigation of the volatile components of Egyptian plants (El-Shazly et al. 2002a, 2002b, 2004; El-Shazly and Hussein 2004), we studied *Citrus jambhiri* and *C. pyriformis* cultivated in Egypt. So far, only the chemical composition of *C. jambhiri* peel oil has been studied previously and 14 compounds were identified (Shaw and Wilson 1976). The major constituent of the oil from Florida, U.S.A. was limonene; and other components included β-pinene and myrcene (4.9%). In another study with oil from France, 27 constituents were identified, of which limonene (91.4%), sabinene (2%) and myrcene (1.7%) were major constituents (Lota et al. 2002). The oil of *C. pyriformis* has not been thoroughly studied with modern analytical methods. Since 1955, only one report from Taiwan has been dealing with volatile components of peel oil of this plant (Yeh 1955) and also, limonene was the main ingredient (90.6%) of the oil.
The aim of this study was to analyze the composition of the hydrodistilled oil from the fruit rind as well as the solvent extract (hexane/ether) of the rind and juice by capillary GLC and GLC-MS. In addition, the biological properties of the oils (the cytotoxic, antioxidant, antiinflammatory, antitrypanosomal and antimicrobial activities) were investigated in detail.

2. Investigations, results and discussion

2.1. Isolation of essential oils and identification of their constituents

The fruit rind of *Citrus jambhiri* and *C. pyriformis* was crushed and steam distilled in a glass Clevenger-type apparatus for 6 h to

Table 1: Chemical composition of the essential oils of *C. jambhiri* and *C. pyriformis* determined by GLC and GLC-MS analysis

Component	RI	*C. jambhiri* area %			*C. pyriformis* area %		
		Distilled oil	Rind extract	Juice extract	Distilled oil	Rind extract	Juice extract
1 α-Thujene	914	-	-	-	Tr	0.48	-
2 α-Pinene*	928	0.01	-	0.15	0.26	0.38	0.01
3 α-Fenchene	943	Tr	Tr	-	0.02	0.01	-
4 Camphene	950	0.42	Tr	-	1.02	2.29	-
5 Sabinene*	963	0.65	Tr	0.15	4.82	0.35	0.05
6 β-Pinene*	966	0.44	Tr	0.16	0.92	0.35	0.04
7 Myrcene*	974	1.50	019	0.34	1.03	1.39	0.40
8 α-Phellandrene*	978	Tr	Tr	-	0.49	0.33	-
9 Δ3-Carene	1010	Tr	Tr	-	0.14	Tr	-
10 Limonene*	1018	92.48	60.81	83.59	75.56	87.69	77.12
11 β- Phellandrene*	1022	0.14	Tr	-	0.16	0.01	-
12 (Z)- β-Ocimene*	1027	0.16	0.09	0.17	8.20	0.02	2.63
13 (E)- β-Ocimene*	1044	0.01	Tr	0.09	0.04	0.18	3.13
14 γ-Terpinene*	1054	0.01	0.03	0.02	0.01	Tr	-
15 Terpinolene*	1078	0.05	Tr	0.04	0.50	0.33	-
16 Linalool*	1083	0.11	0.30	0.73	0.22	0.24	0.87
17 exo-2-Norborneol acetate	1123	0.02	-	0.01	0.03	0.01	-
18 Cis-verbenol	1134	-	-	-	-	0.03	-
19 Citronellal*	1141	Tr	Tr	-	Tr	0.02	-
20 Terpinen-4-ol	1157	0.51	0.93	3.43	0.43	0.02	1.06
21 α-Terpinol*	1168	0.15	0.19	5.59	0.51	0.16	1.07
22 1-Dodecene	1178	0.07	-	-	0.05	-	-
23 γ- Terpinol	1183	0.02	-	-	Tr	-	-
24 trans-Dihydro-carvone	1184	0.02	Tr	-	0.03	Tr	-
25 n-Decanal*	1188	-	Tr	-	-	0.01	-
26 p-Cymen-9-ol	1194	0.01	-	-	0.003	-	-
27 Citronellol*	1211	0.21	0.89	0.08	0.39	0.16	Tr
28 Nerol*	1213	0.01	Tr	0.08	0.11	0.01	-
29 Neral*	1217	0.07	0.64	0.07	0.42	0.01	0.2
30 Geranial*	1232	0.07	Tr	Tr	Tr	0.28	0.2
31 trans-Linalyl oxide acetate	1267	0.01	Tr	-	Tr	Tr	-
32 γ-Terpinen-7-al	1288	0.01	11.94	1.59	-	1.25	5.21
33 Methyl geranate	1327	-	0.02	Tr	-	-	0.05
34 δ-Elemene	1334	0.02	0.35	Tr	-	Tr	0.07
35 neoiso-Carvomenthyl acetate	1336	0.06	0.78	Tr	0.02	0.01	-
36 α-Terpinyl acetate	1339	0.15	Tr	-	0.05	-	-
37 Neryl acetate*	1344	0.05	1.98	0.09	0.14	0.02	-
38 Geranyl acetate*	1361	0.02	0.64	Tr	0.01	0.08	0.07
39 α-Funebrene	1388	0.01	Tr	-	-	-	-
40 β-Longipinene	1404	0.02	0.16	-	0.02	0.04	-
41 α-Cedrene	1407	-	-	Tr	-	-	-
42 α-cis-Bergamotene	1408	0.05	0.53	Tr	0.15	0.01	0.19
43 E-Carophyllene*	1410	Tr	Tr	-	Tr	0.01	-
44 β-Cedrene	1419	-	-	-	-	0.09	-
45 α-trans-Bergamotene*	1428	0.32	2.76	Tr	0.28	0.54	-
46 α-Guaiene	1435	0.01	-	-	0.01	-	-
47 Aromadendrene	1440	0.01	-	-	0.01	-	-
48 (Z)- β-Farnesene	1446	0.02	Tr	-	0.01	0.03	-
49 epi- β-Santalene	1449	0.01	0.16	-	0.21	0.02	-
50 α-Humulene	1451	0.01	0.1	-	Tr	0.01	-
51 (E)- β-Farnesene	1445	0.01	0.27	-	Tr	0.01	-
52 β-Santalene	1455	0.01	0.01	-	Tr	Tr	-
53 Dehydro-aromadendrene	1469	Tr	-	-	Tr	Tr	-
54 9-Epi-(E)-carophyllene	1468	0.02	Tr	Tr	0.01	-	Tr
55 γ-Gurjunene	1470	Tr	-	-	Tr	-	-
56 β-Chamigrene	1473	0.02	Tr	-	0.03	Tr	-
57 Geranyl propanoate	1475	Tr	0.02	-	Tr	0.08	-
58 γ-Muurolene	1482	Tr	Tr	Tr	0.02	0.02	Tr
59 Germacrene D*	1480	Tr	Tr	-	0.01	Tr	-
60 β-Selinene	1482	0.04	Tr	Tr	0.88	0.16	Tr
61 Epi-cubebol	1484	Tr	0.02	-	-	0.04	-
62 Bicyclogermacrene	1490	Tr	Tr	-	0.01	0.07	-
63 (E,E)-α-Farnesene	1493	-	-	-	-	Tr	-

Table 1: (*Continued*)

Component	RI	*C. jambhiri* area %			*C. pyriformis* area %		
		Distilled oil	Rind extract	Juice extract	Distilled oil	Rind extract	Juice extract
64 β-Bisabolene*	1499	0.44	4.52	0.09	0.39	0.41	0.19
65 γ-Cadinene	1506	Tr	-	-	-	Tr	-
66 (E)-α–Bisabolene	1531	0.03	0.18	Tr	0.05	0.01	Tr
67 Germacrene B	1549	Tr	0.29	Tr	Tr	0.01	Tr
68 Longiborneol	1592	-	0.04	-	-	0.04	-
69 (Z)-Bisabol-11-ol	1591	Tr	0.05	-	Tr	0.01	-
70 γ-Eudesmol	1623	-	0.07	-	0.18	0.01	-
71 Daucol	1637	0.02	0.18	Tr	Tr	Tr	Tr
72 Epi-α-Muurolol	1639	0.01	0.32	Tr	Tr	0.03	Tr
73 Himachalol	1642	0.01	Tr	Tr	0.03	Tr	Tr
74 (E)-Bisabol-11-ol	1660	0.01	Tr	-	-	Tr	-
75 Epi-α-Bisabolol	1665	Tr	Tr	-	Tr	Tr	-
76 (Z)-α-trans-Bergamotol	1670	0.02	0.39	-	0.01	0.03	-
77 Caryophyllene acetate	1704	-	-	-	0.09	-	-
78 Cryptomerione	1733	Tr	0.09	-	-	-	-
79 Nootkatone	1763	-	-	-	Tr	0.01	-
80 Methyl hexadecanoate	1909	Tr	0.26	-	-	0.02	-
81 Citropten	1930	-	-	0.03	-	0.38	0.86
82 Osthol	2075	-	-	-	-	0.06	-
83 Methyl octadecanoate	2120	Tr	Tr	-	-	Tr	-
84 Meranzin	2139	-	-	-	-	0.01	-
85 Isomeranzin	2162	-	-	-	-	0.01	-
86 Isopentyloxy-7-methoxy-coumarin	2176	-	-	-	-	0.05	-
87 Bergapten	2367	-	-	Tr	-	Tr	Tr
88 Tangeretin	3052	-	0.19	Tr	-	-	-
89 α-Tocopherol	3062	-	1.29	Tr	-	0.01	Tr
90 Stigmasterol	3169	-	0.05	Tr	-	0.02	0.01
91 Heptamethoxyflavone	3200	-	0.1	Tr	-	-	-
92 Hexamethoxyflavone	3210	-	1.54	Tr	-	-	-
93 Nobiletin	3217	-	1.69	Tr	-	-	-
94 β-Sitosterol	3219	-	0.08	Tr	-	0.02	Tr
Total	——	98.55	95.51	96.5	97.98	98.38	93.36
■Monoterpene hydrocarbons	——	95.87	61.12	84.71	93.17	93.81	83.38
■Oxygen containing monoterpenes	——	1.56	18.31	11.67	2.41	2.31	8.61
■Sesquiterpene hydrocarbons		1.05	9.70	0.09	2.09	1.44	0.45
■Oxygen containing sesquiterpenes		0.07	1.44	-	0.31	0.26	0.05
■Others		-	4.94	0.03	-	0.56	0.87

Compounds are listed in order of OV 1 column elution
RI = retention index were measured relative to *n*-alkanes (C_9–C_{24})
* Previously identified for *C. jambhiri;* Tr = trace (<0.01 %)
- = not detected

give a colourless oil with a strong aromatic sweet fragrance for *C. jambhiri* and a pale yellow aromatic odour for *C. pyriformis* in a yield of 4% and 1.2% (v/w), respectively on the basis of fresh rind.

Using GLC and GLC/MS, the compounds were separated and identified and their relative abundance is listed according to their retention indices on an OV-1 fused silica capillary column (Table 1). The constituents were identified by computer library search (data bank NIST), Kovats retention indices and visual interpretation of the mass spectra with those in the literature (Adam 2004; El-Shazly et al. 2002a, 2002b, 2004; El-Shazly and Hussein 2004; Goad and Akihisa 1997; Stremple 1998; Ziegler et al. 1992; Sawamura et al. 2006; Smadja et al. 2005; Alonzo et al. 2000; Dugo et al. 2000; Song et al. 2006). Where possible, retention times and mass spectra were also compared with those of authentic pure samples. Most of non-identified components are present as traces with relative abundances of less than 0.01%. In both species altogether 94 compounds were identified and the major compound was found to be D- limonene. In *C. jambhiri,* a total of 84 components were identified; about 70 in hydrodistilled oil from the fruit rind. On the other hand, we could not find any traces of *trans*-sabinene hydrate, octyl acetate, linalyl acetate, terpineol and 6-methylhept-5-en-2-one which had been described by Lota et al. (2002). 6-Methylhept-5-en-2-one is more likely to be a degradation product of terpenoids, and it appears that the higher steam pressures degraded terpenes, sesquiterpenes and sesquiterpenes alcohols (Doimo et al. 1999). Solvent extracted rind and juice contained 71 and 44 components, respectively. Sixty five compounds were identified in the hydrodistilled oil of *C. pyriformis.* A total of 77 and 31 compounds were identified in the rind and juice extract, respectively. Solvent extraction has the advantage of recovering also the higher molecular weight natural compounds. From these compounds tangeretin, heptamethoxyflavone, hexamethoxyflavone and nobiletin were identified in detectable amounts in the rind extract of *C. jambhiri* while osthol, meranzin, isomeranzin and 5-isopentenyloxy-7-methoxycoumarin were identified (as coumarins) in *C. pyriformis*. Moreover, citropten, bergapten, α-tocopherol, stigmasterol and β-sitosterol were found in both species.

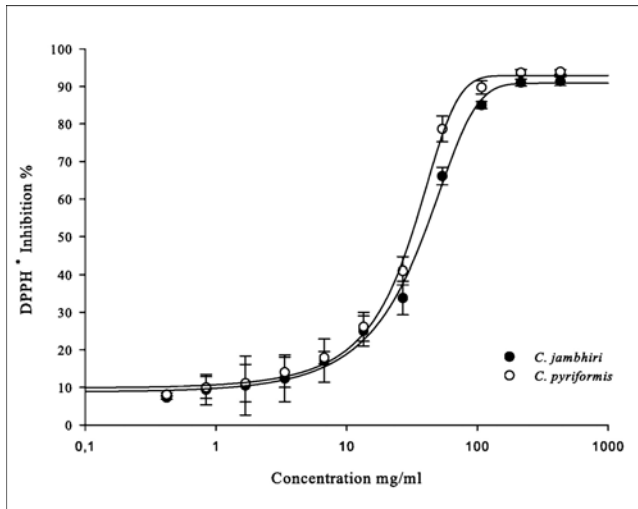

Fig. 1: Antioxidant effects of the *C. jambhiri* and *C. pyriformis* essential oils determined by quantity of DPPH* free radicals $IC_{50} \pm SD$ 37.69 ± 0.21 mg/ml and 28.91 ± 0.09 mg/ml respectively in comparison with an IC_{50} 16.32 ± 0.16 μg/ml for ascorbic acid

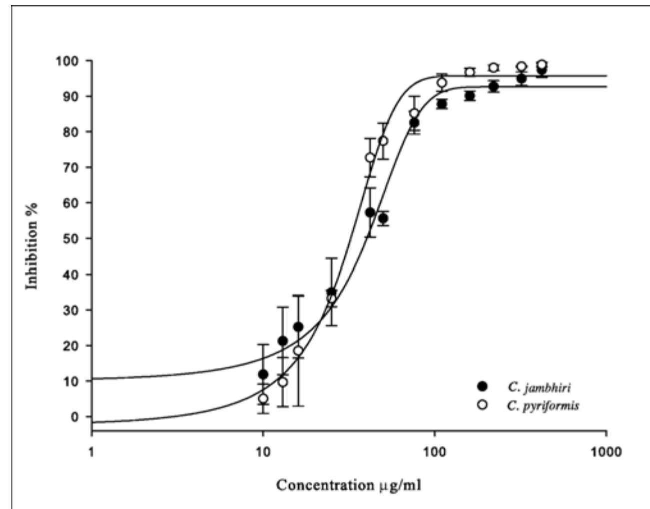

Fig. 2: Inhibition of LOX by *C. jambhiri* and *C. pyriformis* essential oils with an IC_{50} 40 ± 1.63 μg/ml and 38 ± 0.82 μg/ml, respectively; in comparison with nordihydroguaiaretic acid (NDGA) a known inhibitor of soybean 5-lipoxygenase

2.2. Antioxidant activity

The antioxidant activity of the essential oil of both species was determined using the scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH). This assay is considered as a valid tool which can easily evaluate antioxidant properties. In addition thin layer chromatography analysis was carried out for the whole samples along with limonene as an authentic reference and sprayed with DPPH solution. In both *C. jambhiri and C. pyriformis*, only one yellow zone, corresponding to limonene showed a strong scavenging of the $DPPH^*$ radical. The essential oil of *C. pyriformis* exhibited significant antioxidant activity with an IC_{50} of 28.91 ± 0.09 mg/ml, while that of *C. jambhiri* was 37.69 ± 0.21 mg/ml in comparison with ascorbic acid IC_{50} 16.32 ± 0.16 μg/ml (Fig. 1).

2.3. 5-Lipoxygenase (5-LOX) inhibition

Inhibition of 5-lipoxygenase (5-LOX) is currently a subject of intense research targeted towards the discovery of novel antiallergic and antiinflammatory agents. Therefore, the prime objective of this study was to evaluate the ability of the isolated essential oils to inhibit, *in vitro*, the 5-LOX enzyme. The initial reaction rate was determined and IC_{50} values were calculated (Fig. 2). Nordihydroguaiaretic acid (NDGA) was used as a positive reference compound because it is widely reported as a strong inhibitor of this enzyme (Safayhi et al. 1992; Abad et al. 1995; Hope et al. 1983). In our study, its IC_{50} was found to be 40 ± 1.63 μg/ml and 38 ± 0.82 μg/ml from *C. jambhiri* and *C. pyriformis*, respectively. It was apparent that *Citrus* essential oils demonstrated a very good inhibitory activity for 5-LOX. It is expected that essential oils rich in limonene would be good inhibitors of 5-LOX and probably good natural antiinflammatory agents as well.

2.4. Antimicrobial activity

The essential oils of *C. jambhiri* and *C. pyriformis* were tested against six bacterial strains and two fungi. Results of antimicrobial activity of essential oils against standard microorganisms

Table 2: Minimum inhibitory concentrations (MIC) and minimum microbicidal concentrations (MMC) of *C. jambhiri and C. pyriformis* essential oils against different pathogens using the broth micro-dilution method

Microorganisms	*C. jambhiri*			*C. pyriformis*			Positive control		
	DD mm	MIC mg/ml	MBC mg/ml	DD mm	MIC mg/ml	MBC mg/ml	DD mm	MIC mg/ml	MBC mg/ml
Gram (+) bacteria									
1. *Bacillus subtilis* DSM 2109	-	4	16	9	4	16	28.3	0.4	0.8
2. *Staphylococcus capitis* DSM 20325	-	8	>16	10	4	>16	30.0	0.2	0.8
3. *Micrococcus luteus* DSM 1890	-	8	8	-	4	8	21.7	0.4	0.8
Gram (-) bacteria									
1. *Klebsiella planticola* DSM 4617	-	-	-	-	-	-	18.3	0.4	3.2
2. *Escherichia coli* ATCC 25922	-	-	-	-	-	-	18.0	0.8	3.2
3. *Pseudomonas fluorescens* DSM 6147	-	16	>16	-	16	>16	18.0	0.8	3.2
Fungi (yeasts)									
1. *Saccharomyces cerevisiae* DSM 1333	12	4	4	12	4	4	26.3	1.6	1.6
2. *Candida parapsilosis* DSM 11224	-	16	>16	-	16	16	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 diffusion method; MIC = Minimum Inhibitory Concentration; MBC = Minimum Bactericidal Concentration
1 mg/ ml of kanamycin was used as standard antibiotic for bacteria and nystatin (100 μg/disc) for fungi
- = no inhibition

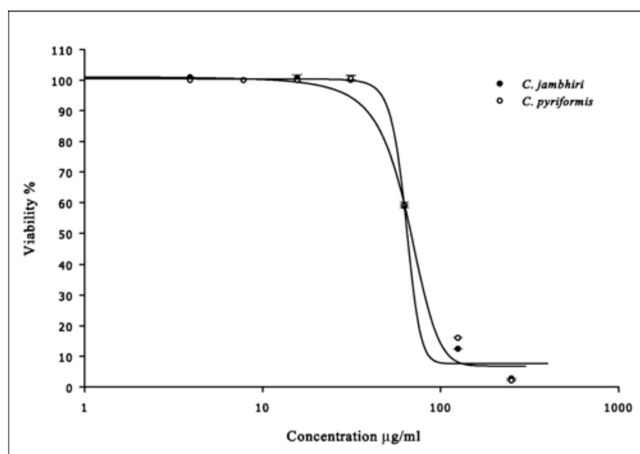

Fig. 3: Antitrypanosomal activity of the *C. jambhiri* and *C. pyriformis* essential oils with an IC_{50} 72.47 ± 0.87 µg/ml and 71.29 ± 0.38 µg/ml respectively in comparison to IC_{50} 0.0832 ± 0.0003 µg/ml for diminazene aceturate (standard antitrypanosomal drug)

are summarized in Table 2. The essential oils of both species showed substantial antimicrobial activity against all tested Gram positive bacteria and yeasts. However, the oils possess no activity against Gram negative strains except for *Pseudomonas fluorescens*.

2.5. *Antitrypanosomal activity*

Figure 3 shows the effect of essential oils against bloodstream forms of *T. b. brucei*. The essential oils of *C. jambhiri* and *C. pyriformis* were less active than the positive control with an IC_{50} value of 72.47 ± 0.87 µg/ml and 71.29 ± 0.38 µg/ml, respectively, were less active than the positive control in comparison with a diminazene aceturate with an IC_{50} value of 0.0832 ± 0.0003 µg/ml.
To our knowledge, this is the first report of the antitrypanosomal effect of essential oils from *C. jambhiri* and *C. pyriformis* against *T. b. brucei*. Both essential oils showed moderate antitrypanosomal effect and the observed trypanocidal activity of the oils might be due to the principal compound, limonene, which comprises more than 92% or 75% of the two oils, respectively. In other studies, however, when limonene was tested alone in high concentration, it showed weak activities against various types of trypanosomes (Ali et al. 1999; Setzer et al. 2006). The overall trypanocidal activity of the two oils in the presented study is not only due to limonene but also due to the additive or synergistic effects of the minor components of the oils.

2.6. *Cytotoxicity activity*

The essential oils of *C. jambhiri* and *C. pyriformis* were tested against two cell lines, i.e. pancreatic cancer (MIA-PaCa-2) and hepatocellular carcinoma (HepG2) cell lines using MTT assay for cytotoxic activity. The results revealed that the essential oil of *C. pyriformis* exhibited greater cytotoxic activity against the two tested cell lines than that of *C. jambhiri*. The IC_{50} values for the tested oil were 374.36 ± 43.95 µg/ml and 588.06 ± 27.12 µg/ml in case of HepG2 cell line, 213.87 ± 18.50 µg/ml and 512.45 ± 61.46 µg/ml in case of MIA-PaCa-2 cell line, respectively. The cytotoxic effect of *C. pyriformis* essential oil might be due to the presence of *d*-limonene. This compound comprises more than 90% of *C. pyriformis* peel oil. In previous studies, *d*-limonene has been shown to have chemopreventive, chemotherapeutic activities against rodent mammary, liver and pancreatic tumors. In addition, it has been found that; *d*-limonene inhibits the growth of cancer cells (Elegbede et al. 1984: Kaji et al. 2001 and Nakaizumi et al. 1997).

2.7. *Conclusion*

The essential oils of both *Citrus* species exhibited strong antiinflammatory activity. In addition, the essential oil of *C. pyriformis* showed a promising cytotoxicity against two cancer cell lines (HepG2 and MIA-PaCa-2) indicating that the essential oil which inhibit excessive singlet oxygen and exhibit cytotoxicity contained higher amounts of limonene. It is suggested that limonene is involved not only in inhibiting the singlet oxygen production but also expressing the higher cytotoxicity.

3. Experimental

3.1. *Plant material*

The fresh ripe fruits of *C. jambhiri* Lush. (Rough lemon) and *C. pyriformis* Hassk. (syn. *Citrus limon* Burm.f. var. *pyriformis, Citrus limon 'Ponderosa', Citrus limon'* American Wonder' and *C. limon* × *C. medica;* Ponderosa lemon) family *Rutaceae* were collected in March 2004 from the research station of the Faculty of Agriculture, Benha University, Egypt. The identity of the plants was confirmed by Prof. Dr. B. M. Houlyel, Prof. of Pomology, Faculty of Agriculture, Benha University, Egypt. Voucher specimens were deposited in Department of Biology, Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Germany.

3.2. *Sample preparation*

The fresh fruit rind (100 g) of *C. jambhiri* and *C. pyriformis* were separately subjected to hydrodistillation. Six hours produced oils with 4 and 1.2% yield, respectively were obtained. The rind as well as the juice of both species was extracted by centrifugation with a mixture of hexane-ether (1: 1, v/v) and the solvents were removed subsequently under a nitrogen stream. The yield was 1.4% and 1.1% for the rind and juice of *C. jambhiri*, respectively. *C. pyriformis* afforded 0.63% and 0.52% for rind and juice extract, respectively.

3.3. *Capillary GLC/ and GLC/MS Analysis*

The constituents of the volatile oils were analyzed by high-resolution capillary GLC and GLC-MS. Samples of the oil and hexane extracts (1 mg each was dissolved in 1 ml *n*-hexane) were injected (1 µl volume) into a gas chromatograph (Varian 3400, HP 5890 Series II) under the following conditions: column, OV1 fused silica capillary (30 m × 0.25 mm i.d and 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 45 °C, 2 min isothermal, 300 °C, 4 °C/1 min, then 20 min isothermal; split, 1: 15. Kovats retention indices (RI) were calculated with respect to a set of co-injected standard hydrocarbons (C_9–C_{24}). Percentage of the identified components was computed from GLC "Peak Simple" software.
GLC-MS data were recorded on a Finnigan MAT equipped with a fused silica column (OV1; 30 m, 0.25 mm i.d. and film thickness 0.25 µm). The capillary column was directly coupled to a quadrupole mass spectrometer Finnigan MAT SSQ 7000. The ionization energy for the mass spectrometer was 70 eV. Split is 1: 30; other conditions were identical to those mentioned above. The identified constituents are listed in their order of elution from the OV1 column (Table 1).

3.4. *Antioxidant activity*

Different aliquots from stock solution (200 µl in 1 ml MeOH) of essential oils were mixed with 500 µl of 0.2 mM diphenylpicrylhydrazine (DPPH) and the final volume 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 BIOCHROM® ULTROSPEC PUS 4054 UV/VIS) at 517 nm against a blank sample without DPPH (negative control). The antioxidant activity of the essential oil of both species, expressed as IC_{50} (mg/ml), was compared with standard antioxidants such as ascorbic acid (Ricci et al. 2005).
For TLC screening of antioxidants in essential oil, dilutions of the volatile oils (5 µl, 1:10 in hexane) were spotted onto silica gel sheets and developed in toluene – ethyl acetate (97 – 3 v/v). The plates were sprayed with 0.2 mM of the stable DPPH solution (Dordevic et al. 2007). Active compounds were detected as yellow spots on a purple background. Zones where the colour changed within 30 min (after spraying) were taken as positive results.

3.5. *5-Lipoxygenase inhibition*

The essential oils of *C. jambhiri* and *C. pyriformis* were tested for 5-lipoxygenase (5-LOX) inhibition as reported (Racine 2003). Inhibition of

soybean lipoxygenase by the essential oils of *C. jambhiri* and *C. pyriformis* was determined spectrophotometrically under the following conditions: to 970 of phosphate buffer pH 9.0, 10 µl 5-LOX (1 mg/ml) and 20 µl of 12 different concentrations of the tested samples (10–420 µg/ml oils were added and incubated at room temperature for 10 min. The enzymatic reaction was started by adding 25 ml of 62.5 mM sodium linoleate and absorbance measured spectrophotometry at 234 nm every 10s for 3 min using a LKB BIOCHROM® spectrophotometer. The initial reaction rates were determined from the slope of the straight-line portion of the curve. Nordihydroguaiaretic acid (NDGA) was used as a positive reference compound for studies of 5-LOX inhibition.

3.6. Antimicrobial activity

The hydrodistilled oils of *C. jambhiri* and *C. pyriformis* (320 mg essential oil) were dissolved in 1 ml dimethyl sulfoxide (DMSO) from which 10 µl was used for testing. For the evaluation of antimicrobial activity, two methods were used: the agar diffusion method (Acar and Goldstein 1996) and broth microdilution assay (Candan et al. 2003). The test microorganisms were *Bacillus subtilis, Staphylococcus capitis, Micrococcus luteus* (Gram positive bacteria); *Klebsiella planticola, Escherichia coli, Pseudomonas fluorescens* (Gram negative bacteria) and *Saccharomyces cerevisiae* and *Candida parapsilosis* (yeasts) were obtained from the Institut für Pharmazie und Molekular Biotechnologie, Universität Heidelberg, Germany. Kanamycin 1 mg/ml was used as a standard antibiotic for bacteria while nystatin 100 units/disc was used as standard antifungal. The plates were incubated overnight at 37 °C for bacteria and 30 °C for fungi. The diameter (mm) of inhibition zones were measured as shown in Table 2. Broth microdilution assay was performed for MIC determination (Candan et al. 2003). Stock solution was 10 µl subjected to two fold serial dilution and 96-well microtiter plates were prepared in different dilutions range 4–8 µg/ml.

3.7. Antitrypanosomal activity

Trypanosoma brucei brucei TC221 cells (causing Nagana in animals) were grown in Baltz medium (Baltz et al. 1985) supplemented with 20% inactivated fetal bovine serum and 1% penicillin-streptomycin. The cells were incubated in a humified atmosphere containing 5% CO_2 at 37 °C.
The essential oils were dissolved in dimethyl sulfoxide (DMSO). The oils were further serially diluted with the medium in a two-fold fashion into seven different concentrations so as to attain final concentrations ranging from 250 to 3.91 µg/ml in 96-well plates. Diminazene aceturate, the standard trypanocidal drug, was also included as positive control. *T. b. brucei* cells were seeded into 96 wells at a density of 1×10^4 cells. The cells were incubated together with the test drugs for a total of 48 h and the antitrypanosomal activity of the essential oils was determined from the degree of bioreduction of resazurin with some modifications from the method that was used by Rolón et al. (2006). Briefly, 10 µl of resazurin was added to trypanosome culture and the culture was incubated with the resazurin for 24 h before measuring the 96-well plates after 48 h of incubation. The absorbance of the plates was read using Tecan® plate reader at dual wavelengths of 570 nm and 600 nm.

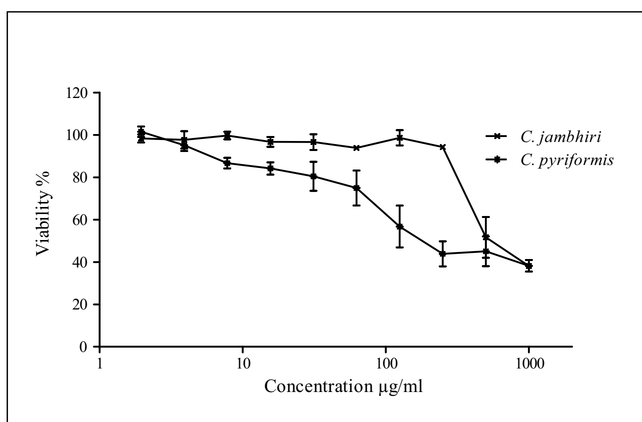

Fig. 4: Cytotoxicity of the essential oil of *C. pyriformis* and *C. jambhiri* in MIA-PaCa-2 cells. Viability of MIA-PaCa-2 cells after 24 h was assessed with the MTT assay. Data are means of three independent experiments; bars represent standard deviation and IC_{50} values for tested oil were µg/ml 213.87 ± 18.50 and 512.45 ± 61.46 µg/ml, respectively

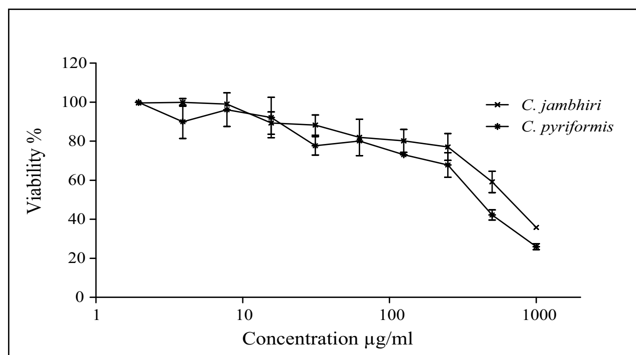

Fig. 5: Cytotoxicity of the essential oil of *C. pyriformis* and *C. jambhiri* in HepG2 cells. Viability of HepG2 cells after 24 h was assessed with the MTT assay. Data are means of three independent experiments; bars represent standard deviation and IC_{50} values for tested oil were 374.36 ± 43.95 µg/ml and 588.06 ± 27.12 µg/ml, respectively

3.8. Cytotoxicity and cell proliferation assay

3.8.1. Cell culture

MiaPaCa-2 and HepG2 cells were maintained in DMEM (glutamate I) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 atmosphere at 37 °C.

3.8.2. MTT assay

Sensitivity of the cells to drugs was determined in triplicate using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) cell viability assay where the MTT is reduced by mitochondrial dehydrogenases of viable cells to a purple formazan product (Marks et al. 1992). The subconfluent MiaPaCa-2 and HepG2 cells (2×10^4 cell/well) were seeded in a 96-well plate Greiner Labortechnik® (Frickenhausen, Germany) after trypsinisation. After incubation for 24 h the cells were incubated with various concentrations of *C. jambhiri* and *C. pyriformis* at 37 °C for 24 h. The cells were incubated with 0.5 mg/ml MTT for further 4 h. The formazan crystals formed were dissolved in 200 µL DMSO. The plates were shaken 15 min at room temperature, and the absorbance was detected at 570 nm with a spectrophotometric plate reader (Bio-Rad®).

3.9. Statistical analysis

All experiments were repeated at least three times. Results are reported as means ± SD. 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) .

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