academicJournals

Vol. 7(18), pp. 1204-1215, 10 May, 2013
DOI: 10.5897/JMPR13.2589
ISSN 1996-0875 ©2013 Academic Journals
http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Anti-inflammatory, insecticidal and antimicrobial activities and chemical composition of the essential oils of different plant organs from navel orange (*Citrus sinensis* (L.) Osbeck var. Malesy) grown in Egypt

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Accepted 24 April, 2013

Comparative study of the essential oils isolated from fruit peel, leaf and flowers of navel orange (*Citrus sinensis* (L.) Osbeck var. malesy) was carried out using gas liquid chromatography (GLC) and gas liquid chromatography/mass spectrometry (GLC/MS). A total of two hundred components were identified and quantified. The major component in the ripe and unripe fruit peel oils was limonene with 80.14 and 80.93%, respectively. In the leaf oil, terpinen-4-ol was the prominent compound (14.1%). The hydrodistilled oil and hexane-ether extract of the flower afforded 92 and 34 identified components, respectively. Sabinene followed by limonene were represented in higher amount in both flower hydrodistilled oil and its hexane-ether extract. Navel orange var. malesy volatile components exhibited high anti-inflammatory activity represented by its effect on tumor necrosis factor-α and nitric oxide. They also showed significant insecticidal activity against the common home mosquito larvae and substantial antimicrobial effect against most common, Gram positive and Gram negative bacteria and some fungi.

Key words: *Citrus sinensis* var. Malesy, essential oils, gas liquid chromatography (GLC), gas liquid chromatography/mass spectrometry (GLC/MS), anti-inflammatory, antimicrobial, larvicidal activity.

INTRODUCTION

Citrus fruits are among the most important horticultural crops, because of their nutritional value and unique flavour. During the last decade large progress has been made in citrus industry in terms of increase in production and yield. The members of this genus are large shrubs or small tress with evergreen leaves, growing mainly in tropical and subtropical areas of the world. The most medicinally active and important classes of secondary metabolites characterized in this genus are flavonoids, particularly flavanone glycosides and polymethoxy flavones, limonoids, coumarins and furanocoumarins, sterols, volatile oils, carotenoids and alkaloids (Ladaniya, 2008). Many members of the *Citrus* genus are well known for their medicinal, physiological and pharmacological activities including antimicrobial, antioxidant, anticancer, anti-inflammatory and hypoglycemic activities (Ladaniya, 2008).

Citrus sinensis (L.) Osbeck (Navel orange) is a hybrid

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of two *Citrus* species: *Citrus maxima* and *Citrus reticulate* (Saleem et al., 2010). The plant contains many medicinally active components from different classes including coumarins, carotenoid, flavonoids (Ortuño et al., 2006) and essential oil (Singh et al., 2010). The analysis of volatile oil constituents of many varieties of *C. sinensis* revealed that limonene is the major component of the oil. Myrcene, linalyl acetate, linalool, citronellal, citronellol, neral and geranial are found in high percentages. Other oil constituents are minor and vary qualitatively and quantitatively from one variety to another. Navel orange contains high percentage of hesperidin which is used for treatment of capillary fragility, haemorrhages and hypertension. It also contains vitamin C which is used for cold and flu conditions. Furthermore, the presence of polymethoxy flavones, namely, nobiletin, heptamethoxy flavone and tangeretin contributes to the antifungal properties of the plant (Ortuño et al., 2006). The alcoholic extract of fruit peel regulates oxidative stress and glucose, insulin and thyroid hormone levels. The plant juice significantly increase high density lipoprotein (HDL) and lower low density lipoprotein (LDL) type-cholesterol and triglyceride plasma level, therefore, the prolonged use of *C. sinensis* juice exhibits a considerable protection against hypercholesterolemia (Parmar and Kar, 2007). The juice also lowers uric acid levels in the plasma and increases its urinary excretion helping in the treatment of gout. *C. sinensis* essential oils have antimicrobial and antifungal activities against moulds commonly associated with food spoilage such as *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum* and *Penicillium verrucosum* (Viuda-Martos et al., 2008). It also exhibits insecticidal properties against mosquito, cockroach and housefly (Ezeonu et al., 2001). To the best knowledge there is only one report that have illustrated the analysis of secondary metabolites in different variety of the Egyptian *C. sinensis* (Karawya et al., 1971). Compounds of each *Citrus* spp. vary according to variety differences. According to taxonomical classification, *C. sinensis* var. malesy is a navel orange with thin yellowish green coloured skin with shiny appearance. A survey of literatures reveals that *C. sinensis* var. malesy has not been a subject of previous study. Thus, the aim of this study is to explore and analyse the composition of the essential oils of the leaf and peel (ripe and unripe) of the Egyptian species. In addition, investigation of the volatile constituents of the flower was done for the first time in this genus. Furthermore, the biological activities of the essential oils include anti-inflammatory, larvicidal and antimicrobial activities, investigated and described in details.

EXPERIMENTAL

Plant material

The fresh leaf and flowers of *C. sinensis* (L.) Osbeck var. malesy (Rutaceae) were collected from the Research Station of the Faculty of Agriculture, Banha University, Egypt in March, 2011. From the same location, the unripe fresh fruit peel was collected in September, 2011, while the ripe fruit peel was collected in January, 2012. The plant was kindly verified by Dr. B. Holyel, Professor of Pomology, Banha University. Voucher specimens were deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University.

Oil isolation

The fresh ripe and unripe fruit peel (100 g each), fresh leaf (100 g) and fresh flower (50 g) of navel orange were separately subjected to hydrodistillation using Clevenger-type apparatus for 6 h producing oils with 1, 1.5, 0.5, and 0.2% yield, respectively. The fresh flowers of the same species were extracted by cold maceration with a mixture of hexane-ether (1:1 v/v) and the solvents were removed subsequently under a nitrogen stream to yield 0.8% of yellowish-brown fragrant residue. The oils and extract were dried over anhydrous sodium sulphate and kept in brown vials in the refrigerator at 4°C until further analyses.

Gas liquid chromatography (GLC) and GLC/mass analysis (MS)

The volatile oil constituents were analyzed 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 (30 m × 0.32 mm i.d and 0.25 m film thickness); Helium was used as the carrier gas (2 ml/min); detector flame ionization detector (FID), 300°C temperature, 250°C injection temperature; oven temperature program: initial temperature 45°C, 2 min isothermal, 300°C, 4°C/1 min, 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 (C10-C28). The identified components were quantified using the GLC "Peak Simple" software. GLC-MS data were recorded on a Clarus 600 gas chromatograph (Connecticut, 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 for the mass spectrometer was 70 eV. Split ratio was 1:30; other conditions were identical to those mentioned for GLC.

Identification of components

Compounds were identified by comparing their spectral data and RI 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; Hamdan et al., 2010). Where possible, retention times and mass spectra were also compared with those of authentic pure samples. Most of the non-identified components were present as traces with relative abundances of less than 0.01%. The identified constituents are listed in the order of their elution in Table 1.

Determination of anti-inflammatory properties

Estimation of tumor necrosis factor-α (TNF-α)

TNF-α was induced in Raw murine macrophage (RAW 264.7) cells using lypo-polysacchride (LPS). The macrophage cells were treated with LPS alone together the oil samples (100 µg/ml) for 48 h. The level of TNF-α was quantified in the serum produced from the

Table 1. Volatile constituents from different plant organs of *Citrus sinensis* (L.) Osbeck var. malesy.

Component*	RI	Quantification (Area %)				
		Hydro-distilled oils				Flower
		Leaf	Ripe peel	Unripe peel	Flower	hexan-ether extract
α-Thujene	943	0.73	-	-	1.02	0.64
α-Pienene	947	1.51	0.02	0.02	2.83	1.55
α-Fenchene	955	0.07	0.97	0.98	0.19	0.23
Benzadehyde	967	-	-	-	0.12	-
Camphene	969	0.04	0.04	0.02	-	0.15
Sabinene	977	tr.	0.61	1.08	20.29	37.65
β -Pienene	982	8.73	-	-	3.55	3.09
Myrcene	993	-	2.73	3.07	-	-
n-Octanal	1003	-	1.08	1.59	-	-
δ-2-Carene	1007	-	0.82	0.89	-	0.30
α- Phellandrene	1006	0.12	-	-	0.43	1.74
iso Sylvestrene	1007	6.16	-	-	2.04	-
α-Terpinene	1013	3.09	-	-	1.84	0.60
ρ-Cymene	1021	1.10	-	-	-	2.00
Limonene	1034	10.18	80.14	80.93	16.90	23.33
β-Phellandrene	1034	0.03	-	-	-	-
(*Z*)-β-Ocimene	1045	0.24	2.90	0.19	0.76	0.76
(*E*)-β-Ocimene	1051	3.36	0.16	0.25	7.63	6.35
γ-Terpinene	1059	5.89	0.42	-	3.86	1.27
ortho-Tolualdehyde	1046	-	-	-	0.38	-
cis-Sabinene hydrate	1064	0.86	-	0.02	0.28	-
ρ-Mentha-3.8-diene	1069	0.01	0.02	-	-	0.61
ρ-Mentha-2.4(8)-diene	1083	-	0.13	0.63	-	-
Terpinolene	1086	3.06	2.26	-	1.91	0.29
μ-Cymenene	1086	-	-	-	-	0.50
Linalool	1101	0.03	1.75	3.30	-	10.01
n-Nonanal	1104	-	0.08	0.20	-	-
trans-Sabinene hydrate	1105	8.21	-	-	13.45	-
1.3.8-ρ-Mentha-triene	1109	-	0.02	0.02	-	-
Benzaldehyde. dimethyl acetal	1113	-	0.01	0.03	-	-
dehydro-Sabina ketone	1115	0.04	-	-	-	-
Myrcenol	1116	0.06	-	-	-	-
exo-Phenchol	1118	-	0.02	-	-	-
cis-ρ-Mentha-2-en-1-ol	1118	-	-	0.04	0.22	-
trans-pinene hydrate	1123	tr.	-	-	-	-
allo-Ocimene	1128	0.25	0.02	0.02	0.38	0.17
cis-Limonene oxide	1131	-	-	0.03	-	-
cis-ρ-Mentha-2.8-dien-1-ol	1133	0.11	-	0.02	-	-
trans-Limonene oxide	1135	-	-	0.05	-	-
trans-ρ-Mentha-2-en-1-ol	1137	0.24	-	-	-	-
cis-β-Terpineol	1142	-	-	0.02	0.45	-
neo-Isopulegol	1143	0.14	-	-	-	-
trans-Verbenol	1144	-	-	0.02	-	-
Z-Tagetone	1147	0.05	-	-	-	-
Citronellal	1153	0.54	0.19	0.16	0.04	-
β-Pinene oxide	1158	-	-	tr.	-	-
iso-Isopulegol	1162	0.10	-	-	0.03	-
cis-Chrysanthenol	1165	-	0.02	0.05	-	-
cis-Pinocacamphone	1166	0.07	-	-	-	-

Table 1. Contd.

Ethyl benzoate	1169	-	-	-	-	0.98
Terpinen-4-ol	1174	14.01	0.27	0.54	5.31	0.24
iso-Menthol	1183	-	-	0.06	-	-
trans-*ρ*-Mentha-1(7).8-diene-2-ol	1184	0.42	-	-	-	-
μ-Cymen-8-ol	1185	-	-	-	0.03	-
α-Terpineol	1189	1.11	0.46	0.90	1.44	0.62
cis-Dihydro carvone	1193	-	-	0.02	-	-
Dihydro carveol	1194	-	-	-	0.06	-
Myrtenal	1199	-	-	-	0.05	-
cis-Piperitol	1199	0.12	-	0.01	-	-
trans-Dihydro carvone	1203	-	-	0.02	-	-
n-Decanal	1205	-	0.35	0.40	-	-
trans-Piperitol	1206	0.39	-	-	0.07	-
trans-Pulegol	1215	0.04	-	-	-	-
trans-Carveol	1219	0.02	-	0.16	-	-
cis-*ρ*-Menth-(7).8-dien-2-ol	1222	-	-	-	0.01	-
cis-Carveol	1223	0.05	-	-	-	-
Nerol	1229	2.49	0.09	0.40	0.09	-
cis-*ρ*-Mentha-1(7).8-dien-2-ol	1234	0.03	-	-	-	-
Cumin aldehyde	1239	-	-	-	0.02	-
Neral	1242	0.49	0.59	1.03	-	-
Geraniol	1257	0.79	0.08	0.23	0.10	-
trans-Ascaridol glycol	1269	-	-	-	0.09	-
Geranial	1273	0.88	0.77	1.66	0.10	-
α-Terpinen-7-al	1283	0.07	-	-	-	-
cis-Verbenyl acetate	1289	-	-	-	0.02	-
γ-Terpinen-7-al	1291	0.04	-	-	-	-
ρ-Cymen-7-ol	1292	-	-	-	0.01	-
Indole	1295	-	-	-	0.04	-
Carvacrol	1298	0.09	-	-	0.02	-
Perilla alcohol	1299	-	-	0.02	-	-
μ-Acetanisole	1300	-	-	-	0.02	-
Z-Patchenol	1321	0.04	-	-	-	-
Methyl geranate	1326	0.20	-	tr.	-	-
neoiso-Verbanol acetate	1331	0.03	-	-	-	-
cis-Piperitol acetate	1335	0.13	-	-	0.02	-
Citronellyl acetate	1340	0.03	-	-	-	-
Piperitenone	1341	-	-	tr.	-	-
Methyl-anthranilate	1343	-	-	-	0.24	0.57
α-Longipinene	1347	0.02	-	-	-	-
α-Terpinyl acetate	1349	-	-	-	0.03	-
neoiso-Dihydro carveol acetate	1351	0.04	-	-	-	-
4a-*α*.7-*α*.7a-*α*-Nepetalactone	1356	0.26	-	-	-	-
Neryl acetate	1367	0.21	-	-	-	-
α-Ylangene	1373	0.03	-	0.03	-	-
α-Copaene	1377	0.06	-	-	0.02	-
iso-Longifolene	1382	-	-	-	0.12	-
β-Panasinsene	1383	0.22	-	-	-	-
trans-Myrtanol acetate	1386	0.24	-	-	-	-
β-Elemene	1389	5.52	0.12	0.07	2.27	0.57
Z-Jasmone	1398	-	-	-	0.06	-
n-Tetradecane	1399	0.06	-	tr.	-	-

Table 1. Contd.

α-Funebrene	1403	0.54	-	-	-	-
Italicene	1405	-	-	-	0.03	-
Dodecanal	1408	-	0.02	0.02	-	-
(*Z*)-Caryophyllene	1409	tr.	-	-	-	-
β-Funebrene	1416	1.96	-	-	-	-
(*E*)-Caryophyllene	1415	-	0.07	0.03	0.86	0.20
β-Cedrene	1422	0.06	-	-	-	-
β-Copaene	1428	-	0.02	0.02	-	-
γ-Elemene	1431	-	-	-	0.08	-
β-Gurjunene	1434	-	tr.	-	-	-
(*Z*)- β -Farnesene	1443	0.23	-	-	-	-
α-Himachalene	1447	-	0.03	-	-	-
α-Humulene	1450	0.98	-	0.01	0.31	0.21
(*E*)- β -Farnesene	1458	0.69	0.02	0.01	1.52	-
Sesquisabinene	1465	0.08	-	-	0.01	-
cis-Muurola-4(14).5-diene	1467	0.02	-	-	-	-
β-Acoradiene	1471	-	-	-	0.02	-
9-*epi*-(*E*)-Caryophyllene	1472	-	0.03	-	-	-
β-Chamigrene	1474	0.26	-	-	0.04	-
γ-Muurolene	1479	0.02	-	tr.	0.05	-
γ-Curcumene	1482	-	-	-	0.14	-
γ-Himachalene	1484	0.74	-	-	-	-
cis-β-Guaiene	1490	-	1,23	-	-	-
β-Selinene	1492	0.63	-	-	-	-
Viridiflorene	1493	-	0.03	-	0.07	-
α-Alaskene	1494	-	-	tr.	-	-
β-Himachalene	1497	-	-	-	0.14	-
Bicyclogermacrene	1500	0.07	-	-	-	-
α-Muurolene	1500	-	-	tr.	-	-
γ-Patchoulene	1503	0.08	-	-	-	-
(*E.E*)-α-Farnesene	1503	-	-	-	0.08	-
β-Bisabolene	1510	0.25	-	0.02	-	-
Z-γ-Bisabolene	1510	0.02	-	-	0.60	-
β-Curcumene	1515	-	-	-	0.02	-
δ-Cadinene	1522	0.17	0.05	0.05	-	-
β-Sesquiphellandrene	1523	-	-	-	0.15	-
Citronellyl butanoate	1530	0.08	-	-	-	-
1.10-Decanediol	1531	-	0.07	tr.	-	-
(*E*)-γ-Bisabolene	1532	0.03	-	-	0.03	-
Z-Nerolidol	1533	0.02	-	-	-	-
trans-Cadina-1(2).4-diene	1535	0.02	-	-	0.01	-
α-Cadinene	1538	-	-	-	0.02	-
Elemol	1548	0.13	0.01	0.02	0.05	-
Germacrene B	1555	-	-	-	0.10	-
Geranyl butanoate	1564	0.07	-	-	-	-
E-Nerolidol	1565	0.03	-	-	2.09	0.74
Ledol	1574	0.09	-	-	-	-
trans-Sesquisabinene hydrate	1582	0.36	-	-	-	-
Globulol	1583	-	0.14	-	-	-
Viridiflorol	1588	0.15	-	-	0.02	-
Thujopsan-2-β-ol	1589	0.03	-	-	-	-
n-Hexadecane	1598	0.01	-	tr.	0.06	-

Table 1. Contd.

Humulene epoxide II	1605	0.03	-	-	-	-
Davanol D	1612	0.30	-	-	-	-
1-*epi*-Cubenol	1627	0.04	-	-	0.03	-
Eremoligenol	1629	0.06	-	-	-	-
γ-Eudesmol	1630	0.04	-	-	-	-
Caryophylla-4(14).8(15)-diene-5.α-ol	1634	0.02	-	-	-	-
epi-α-Cadinol	1639	-	-	-	0.02	-
epi-α-Muurolol	1640	0.04	-	-	-	-
α-Muurolol	1646	0.03	-	-	-	-
β-Eudesmol	1647	-	0.01	-	0.02	-
α-Eudesmol	1652	-	0.04	tr.	0.08	-
α-Cadinol	1653	0.43	-	-	-	-
14-hydroxy-9-epi-(*E*)-Caryophyllene	1667	0.02	-	-	-	-
β-Bisabolol	1672	0.09	-	-	-	-
n-Tetradecanol	1676	-	-	-	0.58	0.32
α-bisabolol	1687	0.02	-	-	-	-
β-Sinensal	1699	1.35	-	0.04	0.70	-
n-Heptadecane	1700	-	-	-	-	0.16
(*Z*)-*epi*-β-Santalol	1709	0.04	-	-	-	-
(2*Z*.6*Z*)-Farnesol	1714	0.34	-	-	-	-
E-Nerolidol acetate	1716	-	-	-	0.03	-
(2*E*.6*E*)-Farnesal	1725	0.06	0.03	-	1.34	0.25
(2*E*.6*Z*)-Farnesol	1745	0.06	-	-	0.08	-
α-4-oxy-Muurolene	1755	0.02	-	-	0.31	-
α-Sinensal	1757	0.31	-	0.02	-	-
β-bisabolenal	1769	0.06	-	-	-	-
Nootkatone	1812	-	0.04	-	0.02	-
2*E*.6*E*-Farnesyl acetate	1844	0.55	-	-	0.01	-
α-Chenopodiol	1864	0.06	-	-	-	-
n-Hexadecanol	1872	-	-	-	0.02	-
Cubitene	1878	0.04	-	-	-	-
Nonadecane	1899	-	-	-	0.04	-
Cembrene	1935	0.39	-	-	-	-
3*E*-Cembrene A	1943	tr.	-	-	-	-
Palmitic acid	1953	0.29	-	-	-	-
3*Z*-Cembrene A	1983	-	-	-	0.04	-
Ethyl hexadecanoate	1994	-	-	-	-	0.30
n-Octadecanol	2094	0.14	-	-	0.01	-
n-Heneicosanne	2102	0.32	-	-	0.03	-
Methyl octadecanoate	2118	tr.	-	-	0.02	-
1-Docosene	2174	0.03	-	-	-	0.41
n-Docosane	2204	0.01	-	-	0.02	-
n-Tricosane	2303	0.02	-	-	0.39	0.54
n-Tetracosane	2397	tr.	-	-	0.02	-
n-Pentacosane	2502	0.02	-	-	0.16	0.57

Functional group	**Total peak (%, Number of identified components)**				
	Leaf	**Ripe peel**	**Unripe peel**	**Flower**	**Flower hexane-ether extract**
Monoterpene hydrocarbons	44.59 (19)	91.26 (15)	88.08 (12)	63.73 (14)	81.22 (20)
Sesquiterpene hydrocarbons	13.10 (28)	1.59 (11)	0.23 (11)	6.78 (26)	0.98 (3)
Alcohols	32.38 (45)	1.21 (12)	5.73 (20)	24.68 (28)	1 1.60 (4)

Table 1. Contd.

Aldehydes	3.49 (8)	3.05 (8)	4.92 (8)	2.74 (8)	0.25 (1)
Esters	1.82 (10)	-	tr. (1)	0.34 (6)	1.55 (2)
Oxides	0.05 (2)	-	0.09 (3)	0.31 (1)	-
Ketones	0.41 (4)	0.04 (1)	0.04 (3)	0.12 (1)	-
Others	0.82 (10)	-	tr. (2)	0.75 (8)	1.67 (4)
Total identified components	96.66 (126)	97.15 (47)	99.19 (60)	99.45 (92)	97.22 (34)

*In elution order from RTX-5MS® column. RI = identification based on retention index relative to standard *n*-alkanes. tr. = trace (<0.01%). - = not detected.

macrophage cells by enzyme-linked immunosorbent assay (ELISA). The assay uses the quantitative sandwich immunoassay technique that uses immobilized monoclonal antibody and biotin-linked polyclonal antibody, both of which are specific against mice TNF-α. Commercially available matched paired antibodies were used (R&D Systems Inc. Minneapolis, MN). The first anti-TNF-α monoclonal antibody (4 µg/ml) and secondary biotin-labeled anti-TNF-α polyclonal antibody (200 ng/ml) were used. The first (capture) antibody was coated onto 96-well flat bottom microtiter plate (Griener Labortechnik, Kremsmunster, Austria) in phosphate-buffered saline (PBS; Sigma Chemical Company, St. Louis, MO, USA), 50 µl/well and incubated 1 h at 37°C then overnight at 4°C in humidified chamber. Plates were washed three times with washing buffer and blocked with 200 µl/well blocking buffer and were incubated at 37°C for 1.5 h. Triplicate assays on 50 µl aliquots of serum samples were quantified by reference to recombinant human standards (R&D Systems, Inc. USA) added to each plate and incubated for 1 h at 37°C. At the end of the incubation period, the plates were washed three times with washing buffer and diluted second biotin labeled antibody was added for 1 h incubation at 37°C. After washing away any unbound substances, the peroxidase-conjugated streptavidin (Jackson Immunsearch Lab, USA) diluted 1:1000 was added to as 50 µl/well, then the plates were incubated for 1 h at 37°C. After an intensive washing, the enzyme reaction was carried out by adding a 50 µl/well of substrate solution. Color development was stopped by addition of 50 µl/well of stopping buffer (1 M HCl) (Surechern Products, Needham Marker, Suffolk, England). The intensity of the developed color was measured by reading optical absorbance at 450 nm using a microplate reader (FLUOstar OPTIMA, BMG LABTECH GmbH, Offenburg, Germany). The ELISA reader-controlling software (Softmax) readily processes the digital data of raw absorbance value into a standard curve from which TNF-α concentration of unknown samples can be derived directly.

Estimation of nitric oxide (NO)

Assay of nitrite accumulation, as an indicator of NO production, was done estimated in macrophage (RAW 264.7) cell culture supernatant based on the Griess reaction according to Green et al., (1982). The macrophage cells were treated with the samples (100 µg/ml) for 48 h and compared with control cells. The NO level of each of the tested cells was expressed using the following equation:

NO level of the tested tissue homogenate ×100/NO level of the control

Larvicidal activity

Culex mosquito was selected for the present study. Experiments were made on the fourth instar larvae of *Culex pipiens*. These larvae were originally collected from Mashtool Alsouk, Sharkia Governorate, Egypt and then kept at 27°C, 70% relative humidity and a 12 h light:2 h dark photo-periods (Kumar et al., 2010). Fourth instar larvae were exposed to 10, 20, 40, 80 and 160 ppm concentrations of oils obtained from fresh fruit peel, leaves and flower in dimethyl sulfoxide (DMSO) for 24 h according to standard WHO procedure (Dharmagadda et al., 2005). The dead larvae were counted and the larval mortality was calculated after 24 h of the exposure period. The lethal concentrations LC_{50} and LC_{90} were calculated using Probit analysis. The percentage mortality was calculated and corrected using Abbott's formula.

Antimicrobial activity

Cup-plate method (Wood and Washington, 1995) was used to detect the preliminary antimicrobial activity. Gram positive bacteria (*Staphylococcus aureus* ATCC 6538), Gram negative bacteria (*Pseudomonas aeruginosa* ATCC 9027*, Klebsiella pneumoniae* ATCC 27736 and *Escherichia coli* ATCC 10536) and fungi (*A. niger* ATCC 16404 and *Candida albicans* ATCC 10231) are the used standard strains obtained from the Department of Microbiology, Faculty of Pharmacy, Zagazig University, Egypt. The nutrient agar or Sabouraud's agar were seeded by about 10^6 microbial cells. 100 µl of peel, leaf and flower oils were dissolved separately in 500 µl dimethyformamide (DMF) and each cup was filled with 100 µl from each extract. Amoxicillin (500 µg/ml) and amphotrricin B (500 µg/ml each) were used as standard antibacterial and antifungal, respectively. The plates were incubated overnight at 37°C for bacteria and 30°C for fungi. Zones of inhibition were measured (mm).

Statistical analysis

The student's unpaired *t-test* was used to detect the statistical significance, where a *p* value less than 0.05 were considered significant.

RESULTS AND DISCUSSION

GLC analysis

Essential oils obtained from fruit peel (ripe and unripe), leaf, and flowers of *C. sinensis* (L.) Osbeck var. malesy were subjected to detailed GLC/FID and GLC/MS analysis. The yield of oil ranged from 0.2 to 1.5% v/w. Altogether, 200 components were identified, quantified in the oils and flower hexane-ether (1:1 v/v) extract from four different plant parts; the leaves, flowers and ripe and

unripe fruit peel. The chemical composition of these oils is as shown in Table 1 according to their elution sequences and RI. The monoterpene hydrocarbons, *α*-pienene, *α*-fenchene, sabinene, limonene, *(Z)*-*β*-ocimene, *(E)*-*β*-ocimene and *allo*-ocimene are present in all studied oils, where *α*-pienene is the major in the leaf, flower oils and flower extract. Moreover, sabinene and *(E)*-*β*-ocimene constitute the most abundant components in both flower oil and extract.

One hundred thirty five peaks were detected in the leaf oil of which 126 were identified and quantified representing 96.66% of the total oil. The most abundant components were monoterpene (44.59%) and sesquiterpene (13.10%) hydrocarbons with limonene (10.18%) and β-pienene (8.73%) as major compounds. Forty five alcohols accounting for 32.38% were also identified, where the level of terpinen-4-ol (14.01%), *trans*-sabinene hydrate (8.21%) and nerol (2.49%) was also considerably high in the leaf oil. In addition, 8eight aldehydes (3.49%), 10 esters (1.82%), 2 oxides (0.05%) and 4 ketones (0.41%) were also identified.

The total identified components in the ripe and unripe fruit peel oils were 47 and 60 compound, accounting for 97.15 and 99.15% of the total oil components, respectively. Ripe and unripe fruit peels were characterized by the presence of high amount of limonene compared with other oils. The major monoterpene was found to be *d*-limonene in fruit peel oil (ripe and unripe) by 80.14 and 80.93%, respectively. It is therefore not surprising that the overall amount of monoterpenes in ripe and unripe peel oil is relatively high, 91.26 and 88.08%, respectively. *γ*-Terpinene (0.42%) is only present in the ripe peel oil. The sesquiterpene fraction is scarcely represented in the peel oil, accounting for 1.59% in ripe peel compared with unripe peel (0.23%). cis-*β*-Guaiene (1.23%) is only present in ripe peel. Oxygenated components show higher percentages in unripe peel oil in comparison with ripe one with 10.12 and 7.48%, respectively. Linalool represents the most abundant alcohol in both unripe and ripe peel oils (3.33 and 1.75%), respectively. Neral (1.03%) and geranial (1.66%) were detected in unripe peel oil only.

Concerning the volatile components isolated from the flower by hydrodistillation and hexane-ether extraction, 92 and 34 identified components were quantified, respectively. The major monoterpene components in hexane-ether extract were sabinene and limonene (37.65 and 23.33%) which were present in higher amount when compared with hydrodistilled oil (20.29 and 16.89%) respectively. Furthermore, the most abundant constituents present in hydrodistilled oil when compared with hexane-ether extract were (*Z*)-*β*-ocimene (7.63 and 6.35%), *β*-pienene (3.55 and 3.09%), *γ*-terpinene (3.86 and 1.27%) and *α*-pienene (2.82 and 1.55%), respectively. *trans*-Sabinene hydrate (13.45%), terpinen-4-ol (5.31%) and *α* -terpineol (1.44%) were considered the three major alcohols from 28 alcohols accounting for 24.68% in flower hydrodistilled oil. *β*-Elemene (2.27%) represents the most abundant sesquiterpene hydrocarbons which accounted for 6.78% in hydrodistilled oil. In addition, 1 oxide (0.31%) and 1 ketone (0.12%) were also detected only in hydrodistilled oil. Alcohols (24.68 and 11.62%), aldehydes (2.74 and 0.25%) and esters (0.34 and 1.55%), were also detected in hydrodistilled oil and hexane-ether extract, respectively. Twenty three compounds were present in both flower oil and extract. Some oxygenated components such as *cis*-*β*-terpineol, ethyl benzoate, myrtenal, cumin aldehyde, *cis*-verbenyl acetate, indole, methyl-anthranilate, etc., were only identified in flower oil and they were responsible for the flower aroma and fragrance. Noteworthy, this is the first report for the volatile constituent of the flower in *Citrus* spp.

Anti-inflammatory activity

Anti-inflammatory activity of *C. sinensis* (L.) Osbeck var. malesy oils was determined relative to its ability of inhibit both TNF-α and NO. TNF-α is a pro-inflammatory cytokine which regulates inflammation and related disorders. Enhanced-production of TNF-α is associated with the development of rheumatoid, psoriasis, arthritis and other inflammatory diseases. Anti-TNF-α are new lines of therapies used in the treatment of all the former inflamematory diseases (Palladino et al., 2003). LPS induced TNF-α production reached up to 50-folds in the control. Both the peel and flower oils at concentration of 100 µg/ml possessed a very high significant inhibitory activity for LPS-stimulated TNF-α level nearly to the extent of the control level ($P<0.001$), as shown in Figure 1. The leaf oil showed high significant inhibitory activity for LPS-stimulated TNF-α level ($P<0.05$) (Figure 1).

NO is produced from the amino acid L-arginine through the effect of the NO synthase. NO is generated through stimulation of many types of cells, especially macrophages, by stimulants such as LPS. NO enhances defence mechanisms due to its antibacterial and anti-viral properties. However, if NO is produced in large quantities out of control, damage of cells occurs due to cell injury. NO is an important inflammatory regulator especially in hepatic inflammatory conditions and measuring NO production is a method for assessing the anti-inflammatory effects of essential oils (Kiemer et al., 2002). LPS induced NO produced up to 2.1 fold of the control as shown in Figure 1. 100 µg/ml leaf or peel and flower oils possessed high significant inhibitory activity against LPS- induced NO ($P<0.001$) nearly to the extent of the control level (Figure 1). The leaf oil possessed a high significant inhibitory activity against LPS-induced NO ($P<0.01$) (Figure 1).

Many natural compounds belonging to various classes have been found to act as Anti-TNF-α and NO inhibitor agents. These naturally occurring drugs has the advantage of being safer and sometimes more-cost-effective

Table 2. The antimicrobial activities of different hydro-distilled oils of *Citrus sinensis* (L.) Osbeck var. malesy.

Tested material	Activity (%)					
	Staphylococcus aureus ATCC6538	***Escherichia coli ATCC10536***	***Klebsiella pneumoniae ATCC27736***	***Pseudomonas aeruginosa ATCC9027***	***Candida albicans ATCC10231***	***Aspergillus niger ATCC16404***
Amoxicillin	100.00	100.00	100.00	100.00	0.00	0.00
Amphotericin B	0.00	0.00	0.00	0.00	100.00	100.00
Leaf oil	64.71	62.96	68.00	83.33	71.43	108.70
Peel oil	50.00	62.96	68.00	70.83	64.29	82.61
Flower oil	58.82	59.26	64.00	66.67	53.57	78.26

Oil samples (100 µl) dissolved in DMF (500 µl) and each cup was filled with 100 µl from each extract. The data is represented as an activity percentage of the standard antibacterial amoxicillin (500 µg/ml) or antifungal amphotericin B (500 µg/ml). The data is the mean of three replicates (n=3).

effective than the chemically-synthesized inhibitors. Examples of naturally occurring TNF-α inhibitors are flavonoids such as naringenin, anthocyanidin and hesperetin. Some terpenes are reported to be anti-TNF-α agents such as abietic acid, acanthoic acid and tanshinone (Paul et al., 2006). Other naturally-occurring products especially *Citrus* essential oils are considered to have an anti-inflammatory activity through inhibition of NO production in target area (Yang et al., 2009). Navel orange oils reduced the levels of TNF-α and NO in Raw murine macrophage cell culture (RAW 264.7) induced by LPS (Figure 1A, B, C and D). The peel oil showed the higher inhibition activity followed by the flower and the leaf oils, respectively. This inhibition activity can be attributed to the presence and the percentage of limonene in the oil. Limonene is reported to suppress the production of TNF-α and NO, thus becoming a potent anti-inflammatory agent especially in skin inflammatory condition (Yoon et al., 2010). TNF-α and NO inhibition percentages of the oils, when compared, are directly proportional to the amount of limonene in the oil and as the amount of limonene increases, its ability to suppress the production of TNF-α increases (Figure 1E) and this can explain the higher TNF-α and NO inhibition activity of the peel oil. Both TNF-α and NO results indicate the ability of using the fruit peel oil in skin preparation as an anti-inflammatory potent drug putting in mind that it is produced in a good yield by hydrodistillation.

Larvicidal activity

Citrus plants have been known for their use as mosquitocidal agents. The ethanolic extracts of *C. sinensis* peel demonstrated high insecticidal activity against the larvae of the yellow fever mosquito *Aedes aegypti* (Amusan et al., 2005). Peel oils of lemon, grapefruit and navel orange showed high insecticidal activities against larvae and adults of *C. pipiens* (Shalaby et al., 1998). Here, in this study, *C. sinensis* oils from different parts showed good activity against 4th larval instars of *C. pipiens*. The values indicated that the peel oil was the most effective one compared with leaves and flower oils as shown in Figure 2. The high larvicidal activity of peel oil is probably attributed to the presence of monoterpenes which are known for their larvicidal activities towards *C. pipiens*, for example, limonene, γ-terpinene, carvone, geraniol and cuminaldehyde (Zahran and Abdelgaleil, 2010). These results suggest that the essential oils isolated from navel orange have the potential to be used as an ideal eco-friendly approach for the control *C. pipiens*.

Antimicrobial activity

Essential oils, particularly those of *Citrus* spp. are known for their bactericidal and fungicidal properties. Many studies confirmed the antimicrobial activity of fruit peel oil of many *Citrus* spp. including *C. sinensis* (L.) Osbeck (Espina et al., 2010), however, these studies were neither done on the Egyptian variety of the orange nor on the oils of parts other than the fruit peel. This study evaluated the antimicrobial activity of the essential oil produced by hydrodistillation of the fruit peel, the leaf and the flower of the Egyptian variety of navel orange. The results indicated moderate antibacterial activity ranging from 50 to 83% activity of amoxicillin as a standard broad spectrum antibiotic (Table 2). The antimicrobial activity was nearly the same on Gram positive and Gram negative bacteria; however, the oils showed very good activity against *Pseudomonas aeruginosa* which is known for causing infection in lungs and urinary tract. The examined oils showed relatively strong antifungal activities especially on *A. niger* which exceeded the activity of amphotericin B as an antifungal standard (Table 2). Surprisingly, leaf oil has the best antimicrobial activities on both the bacteria and the fungi followed by the peel then the flower oil. Terpinen-4-ol and α-terpineol are considered the major alcohols in leaf and flower oils in comparison with other type of oils and terpinen-4-ol is known for its broad anti-microbial activities (Carson and Riley, 1995) which can explain the better antimicrobial

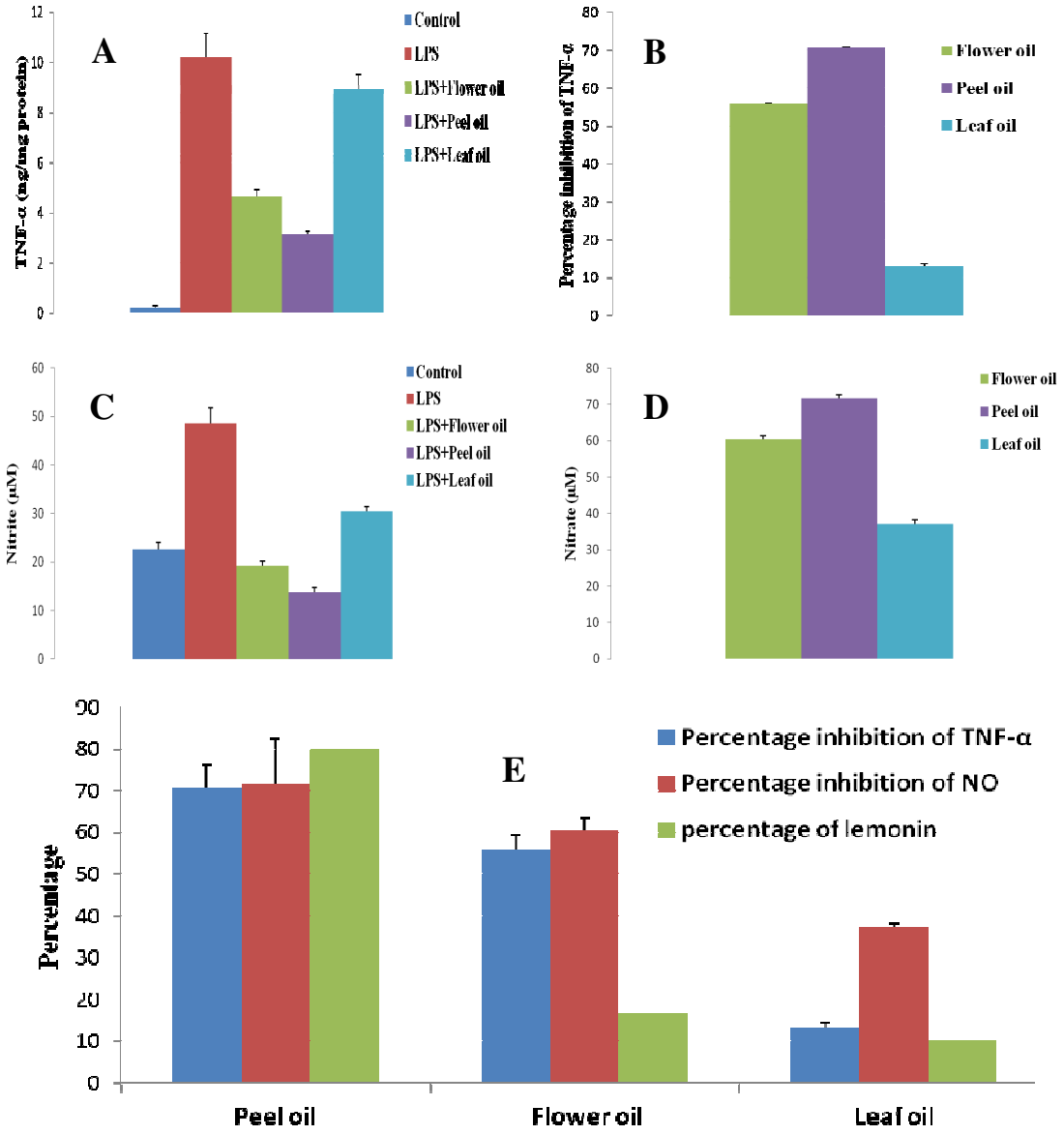

Figure 1. The anti-inflammatory activity of *Citrus sinensis* (L.) Osbeck var. malesy oils. **(A)** Level of TNF-α protein in RAW 264.7 cells lysate after the treatment with the samples (100 µg/ml) for 48 h compared LPS treated cells, as measured by ELISA assay. **(B)** Percentage inhibition of TNF-α by different oil samples. **(C)** The level of NO in RAW 264.7 cells lysate after the treatment with the samples (100 µg/ml) for 48 h compared LPS treated cells, as measured by Griess assay. **(D)** Percentage inhibition of NO by different oil samples. **(E)** Comparison between the percentage constituent of limonene in the oil and the percentage inhibition of TNF-α an NO by the same oil. The data are presented as absorbance (mean±standard error (SE)) of three replicates (*n*=3).

activities attributed to the leaf oil. These findings encourage the use of leaf oil as an antimicrobial agent topically or internally which can be considered as a reuse and recycling of a waste product from *Citrus* spp. industries.

Conclusion

Orange fruits have been used as a human diet for centuries due to its nutritional and medicinal values. However, the cultivation and consumption of orange generates wastes by-products such as leaves and peel which could bring about environmental pollution if not properly managed. A step towards reusing and recycling of these wastes and by-products should be proceeding. Hydro-distilled oils from different parts of *C. sinensis* (L.) Osbeck variety malesy was analysed (GLC and GLC-MS) and many components of the oils were identified on the basis of their mass analysis and RI. Monoterpene

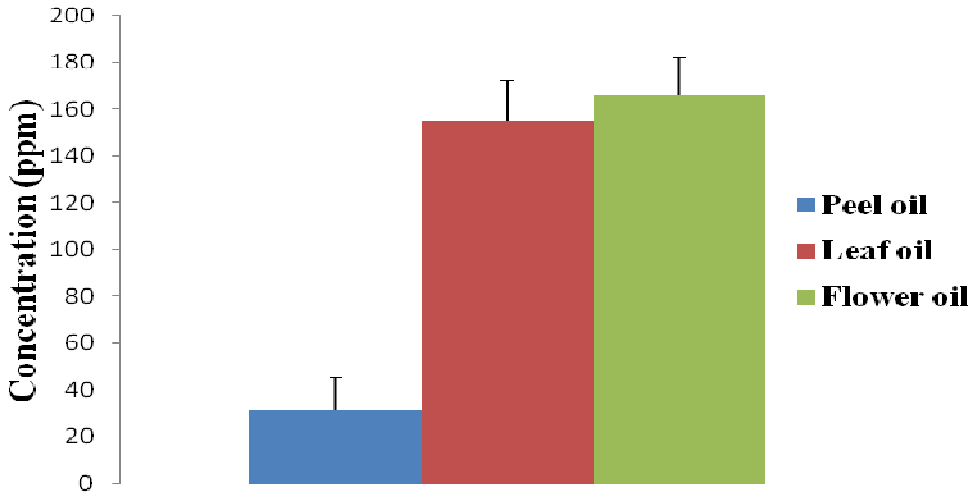

Figure 2. Larvicidal activity against fourth instar larvae of *Culex pipiens*. LC_{50} were calculated using Probit analysis. The data are presented as LC_{50} (ppm), mean±standard error (SE) of three replicates (*n*=3).

hydrocarbons accounted for 44.59% of the leaf oil, having limonene and *β*-pienene as major components (10.18 and 8.73%, respectively). In hexane extract of the flower, the major monoterpene components are sabinene and limonene (37.65 and 23.33%) which are present in higher amount compared with hydrodistilled oil (20.29 and 16.89. respectively). The fruit peel oil shows d-limonene as a major component in ripe and unripe peels (80.14 and 80.93%, respectively). The oils of different parts showed different biological activities such as anti-inflammatory, larvicidal, bactericidal and fungicidal activities. Beside using navel orange by-product for medicinal uses, it can be used as a source of limonene; the very well-known monocyclic monoterpene hydrocarbon. Limonene is known for its medicinal and pharmacological actions such as antitumor, anti-inflammatory and larvicidal activities and it has many industrial uses in cosmetic products, in food manufacturing as flavoring agent to mask the bitter taste of many drugs and as a fragrant in perfume industry. It is added to cleaning products to give orange- or lemon-like fragrance. Limonene is found in fruit peel oil up to 80% of total oil components and the oil yield is 1.5% (v/w) making the Egyptian variety of *C. sinensis* (L.) Osbeck var. Malesy a good natural source for this valuable compound.

ACKNOWLEDGEMENTS

The authors thank Mr. Nader Shawky, Department of Immunology and Microbiology, Faculty of Pharmacy, University of Zagazig for performing the anti-microbial analyses of the oils. The authors thank the University of Zagazig for providing the fund for this project (Grant No. 5K2/M4 to AE).

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