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An evaluation on the chemical and biological activities of *Citrus spp*. cultivated in Egypt

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A comparative study of the essential oils isolated from the leaf and fruit peel of Cleopatra mandarin (*Citrus reshni*) was carried out using gas liquid chromatography (GLC) and GLC/mass analysis (MS). Over 140 components were observed in GLC/MS, of which 123 could be identified. 112 compounds were quantitatively analyzed in the leaf oil with linalool as a major component. The total identified components in the fruit peel oil were 69 and limonene was the most prominent. Cleopatra mandarin volatile components showed high anti-inflammatory activity represented by its effect on tumour necrosis factor-α and nitric oxide. They also showed significant anti-microbial activities against most common Gram positive and Gram negative bacteria and some fungi.

Key words: Cleopatra mandarin, essential oils, gas liquid chromatography (GLC), GLC/mass analysis (MS), anti-inflammatory, antimicrobial.

INTRODUCTION

Plants belonging to genus *Citrus* are known for their nutritional value, unique flavour and medicinal properties. The members of this genus are characterized by many biologically active secondary metabolites such as flavonoids (Tripoli et al., 2007), limonoids (Manners, 2007), coumarins and furanocoumarins, sterols (Ladaniya, 2008), volatile oils (Espina et al., 2010; Tranchida et al., 2011), organic acids and alkaloids (He et al., 2010). Many *Citrus* species are recognized for their medicinal, physiological and pharmacological activities including antimicrobial (Espina et al., 2010; Singh et al., 2010), antioxidant (Barros et al., 2012; Goulas and Manganaris, 2011), anticancer (Benavente-Garcia and Castillo, 2008; Manthey and Guthrie, 2002), anti-inflammatory (Menichini et al. 2011) and hypoglycaemic (Aruoma et al., 2012) activities.

The original source of Cleopatra mandarin was in India and introduced to Florida from Jamaica in the 19th Century. The fruit is orange-red in colour with thin and rough peel and acidic flavour. A survey of the literature revealed very few reports on the identification of secondary metabolites in Cleopatra mandarin. Lota et al. (2001) reported the presence of 29 and 44 volatile components in the fruit peel and leaf oil of Cleopatra mandarin growing in France, respectively. The hydrodistilled oil of the fruit peel gave limonene as the major component (93.6%) followed by myrcene (1.7%), sabinene (1.1%) and linalool (1%). Sabinene (49.7%) was the prominent constituent in the leaf oil followed by linalool (13%) and (*E*) β-ocimeme (6.9%) (Lota et al., 2001). Few reports discussed the biological importance of Cleopatra mandarin constituents. The presence of various polymethoxy flavones contributes to the antifungal properties of the plant (Uckoo et al., 2011), in addition, the plant is used for treatment of capillary fragility, haemorrhages and hypertension due to the presence of high percentage

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of hesperidin (Cano and Bermejo, 2011).

There has been no detailed research on the volatile components of Cleopatra mandarin grown in Egypt. Therefore, and in continuing to the investigation of the chemical and biological activities of *Citrus* spp. cultivated in Egypt (El-Readi et al., 2010; Hamdan et al., 2010a, b; Hamdan et al., 2011), the chemical profile of the fruit peel and leaf oils of Cleopatra mandarin were analyzed by GLC and GLC/MS and their anti-inflammatory and antimicrobial activities were also assessed in this study.

MATERIALS AND METHODS

Plant

The fresh ripe fruits and leaves of Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.), family Rutaceae were collected on the Research Station of the Faculty of Agriculture (Benha University, Egypt) in March, 2011. The identity of the plants was confirmed by Dr. B. M. Houlyel, Prof. of Pomology, Faculty of Agriculture, Benha University. Voucher specimens were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt.

Isolation of the oil

The fresh leaves and ripe fruit peel (100 g each) of Cleopatra mandarin were separately subjected to hydro-distillation for 6 h using a Clevenger-type apparatus. Both oils 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 analysed by high-resolution capillary GLC and GLC-MS. Oil samples (1 µl each, 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 equivalent to DB-5 (30 m × 0.32 mm i.d and 0.25 µm film thickness); He as the carrier gas (2 ml/min); flame ionization detector (FID), temperature (300°C), injection temperature (250°C); oven temperature program: initial temperature at 45°C, 2 min isothermal, 300°C, 4°C/1 min, and then 20 min isothermal; split ratio, 1:15. Retention indices (RI) were calculated with respect to a set of co-injected homologous series of saturated hydrocarbon standards (C10 to C28). Components were quantified as area percentage of total volatiles from GC analyses as shown in Table 1. 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 quadruple 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 literature data (Adams, 2007; Hamdan et al., 2010a). Where possible, retention times and mass spectra were also compared with those of authentic pure samples. Most of the non-identified components are 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.

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 and with the oil samples (100 µg/ml) for 48 h. The level of TNF-α was quantified in the serum produced from the macrophage cells by 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). 4 µg/ml of anti-TNF-α monoclonal antibody and biotin-labelled 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 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 labelled 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). Enzyme-linked immune sorbent assay (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) in the supernatants of cultured RAW 264.7 based on the Griess reaction was done according to Green et al. (1982). The cells were treated with the samples (100 µg/ml) for 48 h and compared with control. The concentration of nitrate was measured by reading the absorbance at 540 nm. The NO level of each of the tested cells was expressed using the following equation: NO level of the tested cells ×100/NO level of the control.

Antimicrobial activity

Cup-plate method (Wood and Washington, 1995) was used to detect the preliminary antimicrobial activity. The test microorganisms: *Staphylococcus aureus* ATCC 6538 (Gram positive bacteria), *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 27736 and *Escherichia coli* ATCC

Table 1. Volatile oil components identified in the fruit peel and leaf oils of Cleopatra mandarin.

Component	RI	Composition (%)	
		Fruit peel	Leaf
α-Thujene*	943	0.09	0.87
α-Pinene●*	947	1.11	2.03
α-Fenchene	957	0.03	0.06
Camphene●*	962	tr.	tr.
Sabinene●*	977	0.98	22.27
β-Pienene●*	999	-	0.03
Myrcene●*	994	3.45	2.91
α-Phellandrene●*	1002	-	0.27
δ-2-Carene	1005	0.74	0.05
α-Terpinene*	1013	0.01	1.41
ρ-Cymene	1022	-	0.52
Limonene●*	1026	79.46	3.78
(Z)-*β*-Ocimene●*	1037	-	0.37
(E)-*β*-Ocimene●*	1049	0.45	5.39
γ-Terpinene●*	1057	-	3.43
cis-Sabinene hydrate	1064	-	0.70
n-Octanol●*	1076	3.33	tr.
ρ-Mentha-2,4(8)-diene	1086	0.37	1.17
Linalool●*	1092	3.26	23.91
trans-Sabinene hydrate●*	1095	-	0.13
trans-ρ-Mentha-2,8-dien-1-ol	1116	0.06	0.08
cis-*ρ*-Menth-2-en-1-ol*	1121	-	0.27
allo-Ocimene	1129	0.01	0.24
cis-*ρ* -Menth-2,8-dien-1-ol	1132	0.02	0.07
trans-*ρ*-Menth-2-en-1-ol	1138	-	0.19
neo-*allo*-Ocimene	1140	-	0.05
cis-*β*-Terpineol	1145	0.02	tr.
trans-Verbenol	1148	-	0.02
Citronellal	1154	0.02	tr.
Karahanaenone	1156	-	0.01
iso-Isopulegol	1160	-	0.01
neoiso-Isopulegol	1165	-	0.01
Terpinen-4-ol●*	1178	0.52	6.32
trans-*ρ*-Mentha-1(7),8-diene-2-ol	1184	-	0.04
α-Terpineol●*	1190	0.84	1.34
Dihydro carveol	1194	0.05	0.09
n-Dodecane	1199	-	0.02
n-Decanal●*	1206	1.01	-
trans-Piperitol	1207	-	0.21
iso-Dihydro carveol	1210	-	0.01
Octanol acetate	1214	0.05	tr.
trans-Carveol	1219	-	0.01
cis-Carveol	1223	-	0.05
Nerol*	1229	0.3	0.09
Thymol, methyl ether	1235	-	0.04
Cumin aldehyde	1238	-	0.05
Geraniol*	1242	0.07	tr.
Piperitone	1253	-	0.01
Methyl citronellate	1258	-	0.06

Table 1. Contd.

Geranial	1273	0.29	0.04
n-Decanol	1275	0.5	-
α-Terpinen-7-al	1283	-	0.03
Limonen-10-ol	1289	0.06	0.04
ρ-Cymen-7-ol	1293	-	0.01
ρ -Mentha-1-en-9-ol	1297	0.03	-
Terpinen-4-ol acetate	1300	0.11	-
n-Tridecane *cis*-	1301	-	0.01
Piperitol acetate *δ*-	1326	-	0.02
Elemene	1336	0.05	1.67
α-Cubebene *α*-	1347	-	0.04
Terpinyl acetate●	1351	-	tr.
Eugenol	1359	-	tr.
Longicyclene *α*-	1368	-	0.03
Ylangene *β*-	1372	0.03	0.05
Panasinsene	1382	tr.	0.12
(E)-*β*-Damascenone	1385	0.02	-
β-Elemene* *n*-	1390	-	1.73
Tetradecane *β*-	1398	0.01	0.02
Longipinene	1402	-	0.16
Dodecanal	1408	0.14	-
β-Funebrene	1414	0.03	-
(E)-Caryophyllene●*	1416	-	3.65
β-Cedrene *γ*-	1424	0.02	0.16
Elemene *β*-Copaene	1431	0.29	-
α-Guaiene	1433	-	3.67
	1437	0.01	0.10
Aromadendrene	1441	tr.	0.07
α-Humulene●*	1451	0.05	0.78
cis-Muurola-4(14),5-diene	1460	0.02	0.05
γ-Gurjunene	1475	-	0.23
γ-Muurolene	1479	0.24	1.01
γ-Himachalene	1483	tr.	-
β-Selinene	1484	-	0.07
trans-Muurola-4(14),5-diene	1489	tr.	-
cis-*β*-Guaiene	1490	-	0.06
γ-Amorphene	1494	0.01	-
α-Alaskene	1495	-	0.57
α-Muurolene	1500	tr.	0.05
γ-Patchoulene	1503	-	0.08
(E,E)-*α*-Farnesene*	1504	0.04	-
δ-Amorphene	1507	-	0.17
γ-Cadinene	1513	tr.	0.06
δ-Cadinene*	1523	0.06	0.31
Z-Nerolidol	1527	0.13	0.03
α-Cadinene	1533	-	0.04
trans-Cadina-1(2),4-diene	1537	0.01	-
cis-Sesquisabinene hydrate	1538	-	0.35
α-Calacorene	1542	-	0.03
Elemol*	1549	0.04	0.11
Germacrene B	1555	0.14	1.77
E-Nerolidol*	1564	-	0.12

Table 1. Contd.

Spathulenol*	1575	-	0.07
Caryophellene oxide*	1579	-	0.09
Carotol	1590	tr.	0.06
n-Hexadecane	1597	tr.	0.04
β-Atlantol	1604	-	0.04
Z-Bisabolol-11-ol	1615	0.01	0.01
10-*epi*-*γ*-Eudesmol	1623	-	0.03
γ-Eudesmol	1628	0.02	0.37
epi-*α*-Muurolol	1642	tr.	0.17
β-Eudesmol	1648	0.02	0.19
α-Eudesmol	1651	0.03	0.11
α-Cadinol*	1653	tr.	0.12
Khusinol	1679	-	0.02
n-Heptadecane	1700	tr.	0.01
Cedroxyde	1713	-	0.02
Mint sulfide	1733	-	0.01
Z-Lanceol	1758	-	tr.
14-hydroxy-*α*-Muurolene	1766	-	0.02
Guaiazulene	1772	-	0.02
α-Eudesmol acetate	1793	tr.	tr.
n-Octadecane	1797	tr.	0.02
Nootkatone	1806	tr.	tr.
Monoterpene hydrocarbons	-	87.04	54.31
Oxygen containing monoterpenes	-	5.97	13.48
Sesquiterpene hydrocarbons	-	1.22	24.15
Oxygen containing Sesquiterpenes	-	0.41	4.27
Others	-	5.04	0.08
Total	-	99.68	96.29

In elution order from RTX-5MS® column. RI = identification based on retention index relative to standard *n*-alkanes. tr. = trace (<0.01 %). - = not detected. *Previously reported in the leaf oil of Cleopatra mandarin (Lota et al., 2001). •Previously reported in the peel oil of Cleopatra mandarin (Lota et al., 2001).

10536 (Gram negative bacteria), *Aspergillus niger* ATCC 16404 and *Candida albicans* ATCC 10231(fungi) are 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 fruit peel and leaf oils were separately dissolved in 500 µl dimethyl formamide (DMF). Each cup was filled by about 100 µl from each solution. Amoxicillin (500 µg/ml) and amphotericin B (500 µg/ml) 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 was considered significant.

RESULTS AND DISCUSSION

Essential oils obtained from plants have many potential applications, including their use as food additives, preservatives, in perfumes and in pharmaceuticals. These applications are usually due to the medicinal and pharmacological activities of these oils such as antimicrobial, antioxidant and anti-inflammatory activities. Volatile components of the Egyptian variety of Cleopatra mandarin were separated and identified using GLC and GLC/MS and their relative abundance is listed according to their retention indices in Table 1. The oil yields from the fruit peel and leaf were 2 and 0.8% (v/w), respectively. A total of 123 compounds were identified in the fruit peel and leaf oils. The composition of the fruit peel and leaf oils is very different. From the compounds

detected, 67 and 110 components have been identified in the respective oils, representing 99.86 and 96.29% of the oils. In the peel, limonene being the most abundant component (79.46%), this is typical for the orange oils. Only 19 of the identified components were previously reported in the literature and 48 compounds are now reported for the first time as volatile constituents of Cleopatra mandarin. On the other hand, we could not find any traces of *β*-phellandrene, terpinolene, octanal, nonanal, *cis*-limonene-1,2-oxide, octyl acetate, α-copaene, trans-α-bergamotene, neral, germacrene-D and bicyclogermacrene as described by Lota et al. (2001). The main constituents of the leaf oil were linalool (23.91%), sabinene (22.27%), terpinen-4-ol (6.23%) and E-(*β*)-ocimene (5.39%). It is worth noting that 81 compounds are now reported for the first time as volatile constituents of Cleopatra mandarin leaf oil. The compounds 3-carene, *β*-phellandrene, terpinolene, ρ-cymene, bicyclogermecrene, 6-methylhept-5-ene-2-one, nonanal, cis- and *trans*-limonene-1,2-oxide, (E)-*β*-farnesene and manoyl oxide identified previously in the leaf oil of France species (Lota et al., 2001), were not detected in the oil of leaf specimen analyzed in the work. It is evident from the oil yield that seasonal variation, nutrition and temperature have an influence of oil production and its composition.

Anti-inflammatory activity

The anti-inflammatory activity of Cleopatra mandarin oils was determined relative to its ability to inhibit both TNF-α and NO production. TNF-α is an inflammatory mediator which is associated with the development many inflammatory diseases such as rheumatoid, psoriasis and arthritis. Anti-TNF-α is a new approach which is now used in the treatment of many of the former inflammatory diseases (Palladino et al., 2003). NO is another inflammatory regulator which is produced from the amino acid L-arginine through the effect of the nitric oxide synthase. NO is generated through stimulation of many types of cells, especially macrophages, by stimulants such as LPS. If NO is produced in high concentrations out of control, damage of cells occurs due to cell injury. Measuring NO production is a method for assessing the anti-inflammatory effects of essential oils (Kiemer et al., 2002). Many classes of natural compounds 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 than the chemically-synthesized inhibitors. *Citrus* essential oils are considered to have an anti-inflammatory activity through inhibition of NO production in target area (Yang et al., 2009).

Oils isolated from Cleopatra mandarin reduced the levels of TNF-α and NO in Raw murine macrophage cell culture (RAW 264.7) induced by LPS. The results indicated that the fruit peel oil at concentration of 100 µg/ml, possessed a very high significant inhibitory activity for LPS-stimulated TNF-α and NO levels to the extent nearly of the control level ($P<0.001$). The leaf oil also showed high significant inhibitory activity for LPS-stimulated TNF-α and NO levels ($P<0.01$) as shown in Figure 1. The peel oil showed the higher inhibition activity than leaf oil, respectively as an inhibitor for TNF-α; however, both oils showed nearly the same activity as NO inhibitors. 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). Both TNF-α and NO, results and the good yield of oil by hydro-distillation suggests the ability of using Cleopatra mandarin fruit peel oil in skin preparation as an anti-inflammatory potent drug.

Antimicrobial activity

Bactericidal and fungicidal activities are known properties for volatile oils, particularly those of *Citrus* spp. Many studies confirmed the antimicrobial activity of fruit peel oil of many *Citrus* spp. including Cleopatra mandarin (Caccioni et al., 1998; Espina et al., 2010; Fisher and Phillips, 2008). This study investigates the antimicrobial activity of the hydro-distilled essential oil of the fruit peel and the leaf of the Egyptian Cleopatra mandarin. The results revealed that all the tested essential oils had moderate antibacterial effect against all tested Gram positive and Gram negative bacteria as shown in Table 2. The activity ranged from 50 to 66% activity of amoxicillin as a standard broad spectrum antibiotic. The antimicrobial activity was nearly the same on Gram positive and Gram negative bacteria; however, the oils showed good activity against *P. aeruginosa* ATCC 9027 which is known for causing infection in lungs and urinary tract. The examined oils showed relatively strong antifungal activities especially on *A. niger* which reached to 82.6% activity of amphotericin B as an antifungal standard (Table 2). The difference between leaf and fruit peel oils in antimicrobial activities on both the bacteria and the fungi cannot be distinguished. These findings encourage the use of these oils as antimicrobial agents topically or internally which can be considered as a reuse and recycling of a waste product from *Citrus* spp. industries.

Conclusion

Mandarins are one of the fruits which are highly consumed by human due to its nutritional and medicinal values. However, the cultivation and consumption of these fruits generate wastes by-products such as leaves and peel which could bring environmental problems if not properly dealt with. This study was carried out as a step towards reusing and recycling these by-products and

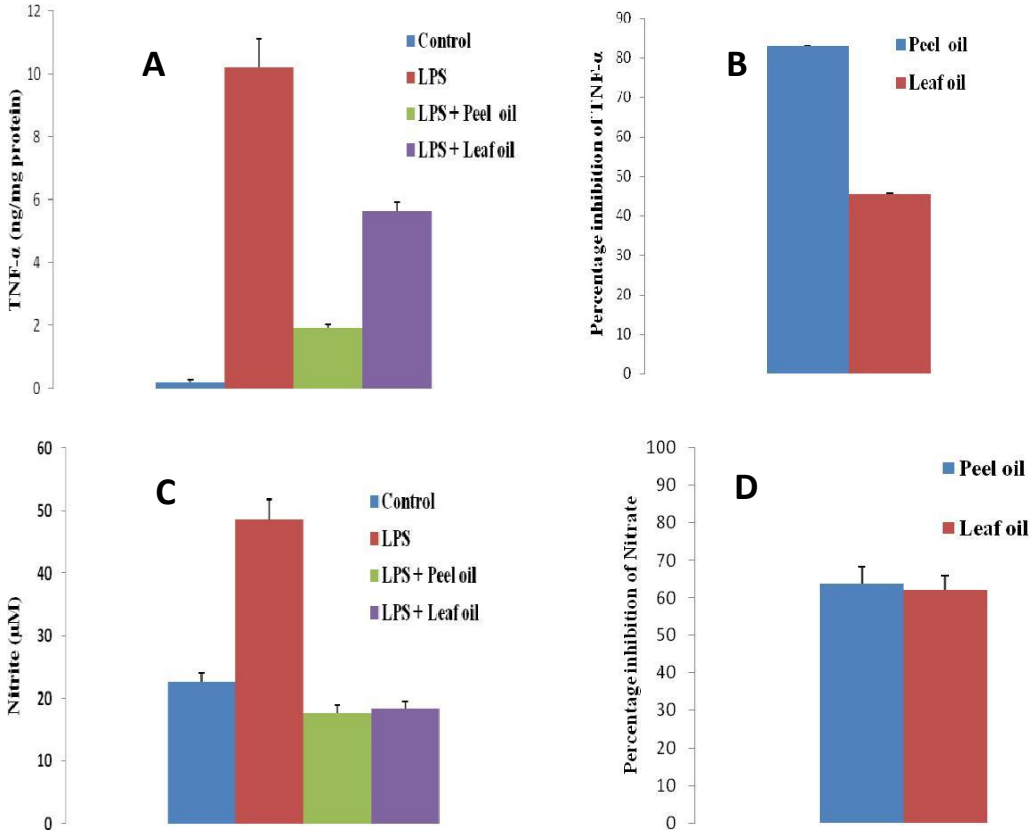

Figure 1. The anti-inflammatory activity of hydro-distilled essential oils of the fruit peel and leaf of Cleopatra mandarin. A: The level of tumour necrosis factor-α (TNF-α) protein in RAW 264.7 cells after treatment with the samples (100 µg/ml) for 48 h compared with lypo-polysacchride (LPS) treated cells as measured by ELISA assay. B: Percentage inhibition of TNF-α by leaf and peel oils. C: The level of nitric oxide (NO) in RAW 264.7 cells supernatant after the treatment with the samples (100 µg/ml) for 48 h compared with LPS treated cells as measured by Griess reaction according to Green et al. (1982). D: Percentage inhibition of NO by the leaf and peel oils. The data are presented as absorbance (mean ±SE) of three replicates (*n*=3).

Table 2. The antimicrobial activity of the fruit peel and leaf oils of Cleopatra mandarin.

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	58.82	52.86	60.20	62.50	60.71	82.61
Fruit peel oil	44.12	55.56	66.00	64.56	53.57	65.22

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).

focuses on the identification of new flavors that could have been used in perfumery, food industries and medicinal application.

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