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Chemical composition, antimicrobial and antifungal activities of essential oils of the leaves of *Aegle marmelos* (L.) Correa growing in Egypt

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ABSTRACT

The essential oil obtained by hydro-distillation of *Aegle marmelos* (L.) Correa leaves were analyzed by GC/MS yield (0.9% v/w). Twenty seven components were identified representing 97.76% of the total oil composition. The major components were α-phellenderene (20.97%), α-pinene (17.76%) and δ-carene (16.37%) and other abundant components asγ-cadinene (8.01%), *trans*-2-hydroxycinnmic acid (6.85%) and β-myrcene (4.32%). The essential oil exhibited significant antibacterial activity against Gram-positive bacteria as *Streptococcus faecalis* with inhibition zone (30 mm) and Gram-negative bacteria *as Pseudomonas aeruginosa* (28 mm). Moreover, moderate activity was observed against *Bacillus subtilis* (23mm), *Staphylococcus aureus* (23mm), *Sarcina lutea* (20mm), *Arthrobacter citreus* (20 mm) and *Escherichia coli* (25mm) in comparison with antibiotics. The antifungal activity against *Aspergillus niger* (30 mm) and *Candida albicans* (30 mm) was higher than the antifungal antibiotics. Moreover, the oil inhibited the germination of *Aspergillus niger* and *Fusarium oxysporum* spores at different concentrations.

INTRODUCTION

Aegle marmelos (L.) Correa is a tree belongs to family Rutaceae commonly known as bael; it is native to Northern India but widely found throughout Indian Peninsula and in a Ceylon, Burma, Bangladesh, Thailand and Indo-China(Rahman and Parvin, 2014).It is traditionally used for treatment of various diseases such as dysentery, fever, diabetes, asthma, heart problems, ophthalmia, haemorrhoids and urinary problems (Bansal and Bansal 2011).Moreover,thealcoholic leaves extract used as anti-ulcer (Shenoy *et al*., 2012), anti-diabetic (Bhatti *et al*., 2012),antibacterial and antifungal activities (Venkatesan *et al*., 2009, Kothari *et al*., 2011).

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In addition it has antiproliferative and antioxidant activities (Chockalingam *et al*., 2012).The leaf extracts significantly inhibits the dermatophytic fungilike *Trichophyton mentagrophytes*, *T. rubrum*, *Microsporum canis*, *M.gypseum* and *Epidermophyton floccosum* (Balakumar *et al*., 2011). Various phytochemical constituents have been isolated from the different extracts of the leaves *i.e.*, alkaloids, cardiac glycosides, terpenoids, saponins, tannins, flavonoids and steroids (Sekar *et al*., 2011),in addition to coumarins, polysaccharides, seed oil and carotenoids from different parts of the tree(Dhankhar *et al*., 2011). The antifungal activity of essential oil isolated from leaves was evaluated using spore germination assay. The oil exhibited variable efficacy against different fungi and the inhibition of spore germination was 100% at 500 µg/mL (Rana *et al*., 1997). The insecticidal, larvicidal and nematocidal activities of the oil isolated from *A. marmelos* leaves of Nepal sources due to the presence of high level of limonene(64.1%) (Satyal *et al*., 2012).

Furthermore, the oil isolated from *A. marmelos* leaves of Cuba sources contains δ-cadinene (12.1%) and β-caryophyllene (10%)as major compounds (Pino*et al*., 2005).On the other hand, the oil isolated from *A. marmelos*leaves growing in India composed mainly of α-phellandrene (39.2%) and limonene (26.8%) (Raju *et al*., 1999). The aims of the present study were to isolate the essential oil of the leaves of *Aegle marmelos* (L.)Correa growing in Egypt in order to identify its constituents and to evaluate its antibacterial and antifungal activities.

MATERIALS AND METHODS

Plant materials

Fresh leaves o*f Aegle marmelos* (L.) Correa were collected from El-Zohrya Garden, Giza, Egypt during flowering stage in April 2008.The plant materials were identified by Mrs. Therese Labib, consultant of taxonomy at the ministry of agriculture and the former director of El-Orman Botanical Garden, Giza, Egypt. A voucher specimen (No.00017 1Ac 04-02-05-17) of the plant was kept at the Herbarium of El-Orman Botanical Garden.

Essential oil isolation

Fresh leaves of *A. marmelos* (L.)Correa(300 g)were subjected to hydrodistillation method using Clevenger apparatus (Egyptian Pharmacopoeia, 2005), which was repeated three times and then the mean percentage of each yield was calculated. The obtained oil was dried over anhydrous sodium sulphate and stored at -20ºC.The essential oil was diluted (1:10 hexane, v/v), 0.2 μL of the oil was analyzed by GC-MS using the following conditions.

Analysis of the essential oil

The oil was analyzed by trace GC ultra-Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass spectrometer). The capillary column of fused silica (30m x 0.25mm ID x0.25μm (film thickness) was used. Analyses were carried out using helium as carrier gas at a flow rate of 1.0 mL/min at a split ratio of 1:10 and the following temperature program:50ºC for 1 min; rising at 5.0ºC/min to 180ºC and held for 6 min; rising at 5ºC/min to 250ºC and held for 1 min the injector and detector were held at 250ºC and 240ºC, respectively. Mass spectra were obtained by electron ionization (EI) at 70 eV using a spectral range of 40-450*m/z*. The identification of the components was based on comparison of their mass spectral fragmentations pattern with those of the data reported in Wiley and NISTLibraries and those described by (Adams, 2001).

Microorganisms

The bacteria used in this study were Gram-positive bacterial strains *i.e., Bacillus subtilis* NRRL 543, *Staphylococcus aureus* NRRL B-313,*Sarcina lutae* NRRL B-1018,*Streptococcus faecalis* NRRL 537and *Arthrobacter citreus* NRRL B-1258,Gram-negative bacterial strains *i.e., Escherichia coli* NRRL B-210, *Klebsiella pneumonia* NRRL B-117and *Pseudomonas aeruginosa* NRRL B-23. Fungi *i.e., Aspergillus niger* NRRL 599, *Fusarium oxysporum* NRRL 28184 and *Candida albicans* NRRL Y-477. These micro-organisms obtained from Northern Utilization Research and Development Division, United State Department of Agriculture, Peoria, Illinois, USA. The bacterial strains were revived for bioassay by sub-culturing in fresh nutrient broth medium for 24 hours before test. While fungi were cultured on potato dextrose agar (PDA) (2.5% w/v agar) for 7 days at 28°C before the experiment was carried out.

Agar diffusion method

The antibacterial and antifungal activities of the essential oil were tested using agar diffusion method (Linday, 1962).0.1 mL suspension of 24 hours bacterial cultures in sterile distilled water was added to 40 mL sterile nutrient agar media. For fungal strains, 0.1 mL suspension of seven days cultures was added to 40 mL sterile PDA (potato dextrose agar) at 45°C. The mixture was transferred to sterile Petri dishes and allowed to solidify. Holes of 9 mm in diameter were made using a cork borer. Amounts of 0.1mL of the diluted essential oil with paraffin oil were poured inside the holes. A hole filled with paraffin oil only was also used as control.

The plates were left for 1 hour at room temperature as a period of pre-incubation diffusion. The diameters of the inhibition zone were measured and compared with that of the standard and the values were tabulated. Plate cultures were kept in an incubator at 28^{o}C for 48 hours for fungi and at 37^{o}C for 24 hours for bacteria.

Erythromycin, methicillin, oxacillin, bacitracin and nystatin were included during the experiment as references antibiotics. Following incubation, the zone of inhibition for each sample was recorded in mm (including the hole). Microorganisms with zone size $\geq$ 28 mm were classified as strongly sensitive, with a zone diameter of < 28 to 16 mm as moderately sensitive, with a zone diameter of < 16to 12 mm as weakly sensitive and isolates with zone diameter of < 12 mm as resistant (Elgayyar *et al*., 2001). Plates were done in triplicate and an average + SD was recorded (Table 2).

Estimation of Minimal Inhibitory Concentration

The minimal inhibitory concentration (MIC) of the oil was determined by broth dilution method previously established by (Carson *et al*., 1995) in test tubes containing 5mLnutrient broth. Serial two fold dilutions of stock solution of essential oil were prepared over the range of1.0-200μL/mL. A 50μL spore suspension of each test strain was inoculated in the test tubes and incubated for 24-72 hours at 37°C. The control tube containing the same medium was inoculated only with microorganism's suspension without essential oil. The minimum inhibitory concentrations at which no visible growth observed (100% inhibition) were defined as the MICs which were expressed in μL/mL (Table 3) all dilutions were performed in duplicate.

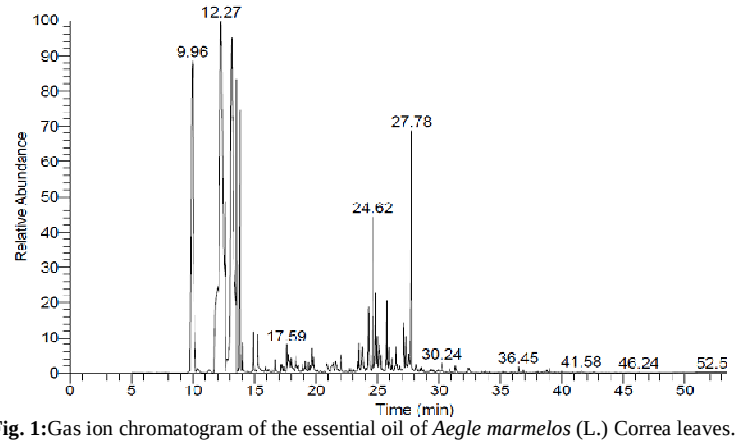

Fig. 1: Gas ion chromatogram of the essential oil of *Aegle marmelos* (L.) Correa leaves.

Table 1: Chemical compositions of the essential oil of *A. marmelos* (L.) Correa leaves.

Peak No.	Compound	MF	Area %	M^+	Main Fragments	R_t	RR_t
1	*α*-Pinene	$C_{10}H_{16}$	17.76	136	93(100),79, 121	9.96	0.8
2	*β*-Myrcene	$C_{10}H_{16}$	4.32	136	41,93,69,81	11.84	0.9
3	*α*-Phellandrene	$C_{10}H_{16}$	20.97	136	93,91,77,105	12.27	1
4	*Iso*sylvestrene	$C_{10}H_{16}$	1.70	136	93,67,121,136	12.37	1.01
5	*δ*-Carene	$C_{10}H_{16}$	16.37	136	93,79,105,121	13.13	1.07
6	*β*-Ocimene	$C_{10}H_{16}$	2.51	136	93,79,105	13.55	1.1
7	*trans*-2-hydroxycinnmic acid	$C_9H_8O_3$	6.85	164	91,77,65,103	13.79	1.12
8	*γ*-Terpinene	$C_{10}H_{16}$	0.66	136	93,91,77,41	14.05	1.14
9	Terpenolene	$C_{10}H_{16}$	1.02	136	93,121,79,41	14.90	1.2
10	Linalool	$C_{10}H_{22}O$	1.08	154	43,71,93,55	15.26	1.24
11	3-*Iso*thujanol	$C_{10}H_{18}O$	0.45	154	43,55,,67,81	16.69	1.36
12	4-Terpineol	$C_{10}H_{18}O$	0.79	154	71,93,55	17.59	1.4
13	Thuj-3-en-10-al	$C_{10}H_{14}O$	0.75	150	79,51,91,107	17.78	1.45
14	*α*-Terpineol	$C_{10}H_{18}O$	0.45	154	59,43,81,93	17.99	1.46
15	Unknown		0.67		126,108,69,55	21.34	1.7
16	Unknown		0.55		111,71,81,126	21.62	1.76
17	*δ*-Elemene	$C_{15}H_{24}$	0.80	204	93,121,41,79	23.52	1.9
18	*α*-Cubebene	$C_{15}H_{24}$	1.70	204	105,119,41,161	24.28	1.98
19	*γ*-Elemene	$C_{15}H_{24}$	3.82	204	121,93,41,67	24.62	2
20	*α*-Humulene	$C_{15}H_{24}$	0.97	204	93,67,79,41	24.96	2.03
21	Unknown		1.02		93,79,67	25.15	2.05
22	*α*-Terpinyl *iso*butyrate	$C_{14}H_{24}O_2$	0.61	224	43,81,9,121	25.30	2.06
23	*γ*-Muurolene	$C_{15}H_{24}$	1.62	204	161,105,41,119	25.72	2.09
24	*γ*-Curcumene	$C_{15}H_{24}$	0.77	204	119,41,93,105	25.82	2.1
25	Valencene	$C_{15}H_{24}$	0.68	204	161,105,91,119	25.97	2.11
26	*β*-Selinene	$C_{15}H_{24}$	0.45	204	93,79,67,41	26.18	2.16
27	*α*-Muurolene	$C_{15}H_{24}$	1.32	204	105,41,161,93	27.31	2.21
28	*β*-Bisabolene	$C_{15}H_{24}$	0.89	204	41,69,93,79	27.53	2.22
29	*γ*-Cadinene	$C_{15}H_{24}$	8.01	204	161,105,91,119	27.78	2.26
30	*β*-Bisabolol	$C_{15}H_{26}O$	0.43	222	41,67,93,82	30.24	2.4

MF: Molecular formula
R_t: Retention time
RR_t: Relative Retention Time to *α*-phellandrene (12.26).

Table 2: Antimicrobial activities of the essential oil from *A. marmelos*(L.)Correa.

Test organism	Inhibition zone diameter(mm)					
	Essential oil (1:50v/v)	Erythromycin 150*μ*g/mL	Methicillin 50*μ*g/mL	Oxacilin 10*μ*g/mL	Bacitracin 100*μ*g/mL	Nystatin 100*μ*g/mL
Gram positive						
B. subtilis	23	26	14	-ve	18	11
S. aureus	23	16	-ve	-ve	12	12
S.lutea	20	25	24	24	26	-ve
S. faecalis	30	20	-ve	-ve	19	-ve
A. citreus	20	28	19	24	12	12
Gram negative						
E. coli	25	16	11	12	12	-ve
K. pneumoniae	-ve	-ve	12	14	-ve	-ve
P. aeruginosa	28	35	17	15	12	-ve
Fungi						
A. niger	30	-ve	-ve	-ve	-ve	15
C. albicans	30	-ve	-ve	-ve	-ve	15

Spore germination assay

Spore germination assay was carried out according to (Rana *et al*., 1997). Three concentrations of the essential oil (4, 2, 1 % v/v) and controls (one sterile distilled water and other 0.1 % (v/v) methanol in sterile distilled water) were tested for spore germination of *Aspergillus niger* and *Fusarium oxysporum*. Aliquots of 0.1 mL from each sample were mixed with fungal spores obtained from 10 days cultures of the fungi and placed on separate glass slides in triplicate. Slides containing the spores were incubated in a moist chamber at 28 °C for 24 hours. Each slide was then fixed in lactophenol-cotton blue and observed under the microscope for spore germination (Table 4)a spore was considered germinated when the germ tube length was 1.5 times the spore diameter (Plascencia-Jatomea *et al.*, 2003).

RESULTS AND DISCUSSION

The percentage of the volatile oil from fresh leaves of *A. marmelos* (L.) Correa was 0.9% (v/w). GC/MS analysis of oil revealed the presence of 30 components as shown (Table 1&Figure 1), from which 27 compounds were identified represent (97.76%) of the total oil composition, classified into; monoterpene hydrocarbon represented the most abundant constituents of the oil (65.31%) *i.e.*, α-phellandrene (20.97%), α-pinene (17.76%) and δ-carene (16.37%) were major components identified, and sesquiterpenes hydrocarbons represented (21.03%) *i.e.*, γ-cadinene (8.01%) was the major component, oxygenated compounds representing (11.41%) *i.e.*, monoterpens alcohols (2.77%), sesquiterpene alcohols (0.43%), aldehyde (0.75%), ester (0.61%) and *trans*-2-hydroxycinnmic acid (6.85%) reported here for the first time from *A. marmelos* (L.) Correa. Previous report by (Raju *et al*., 1999) confirmed the high content of monoterpenes in the leaves oil which agreed with our results. However, the concentration of α-phellandrene (39.2%), α-pinene (6.6%) were quite different that our study, in which the percentages of α-phellandrene and α-pinene were (20.97%) and(17.76%) respectively.

On the other hand, our obtained results were different from the leaves oil isolated from Cuba sources, which constituted mainly of sesquiterpenes *i.e.*, δ-cadinene (12.1%) and β-caryophyllene (10%)as reported by (Pino*et al.*, 2005). Satyal *et al*.,(2012) reported that, the essential oil isolated from Nepalsources mainly contain slimonene (64.1%).The qualitative and quantitative variations between our results and the previous reports for the constituents of the leaves oil may be attributed to the difference in geographical location, climate conditions and time of harvest. Which comes in accordance with Verma *et al*., (2014) who reported that the growing location and time of harvest (season) had a close relation to the yield and quality of *A. marmelos* essential oil.

The essential oil isolated from *A. marmelos* (L.)Correa at dilution 1:50(v/v)showed antibacterial activity against all tested microorganisms; except for *K. pneumoniae* no activity was detected. A significant activity of the essential oil was observed against *S. faecalis* with inhibition zone(30mm) and *P. aeruginosa* (28mm). However, the oil revealed a moderate activity against *B. subtilis* (23mm), *S. aureus* (23mm), *S. lutea* (20mm), *A. citreus* (20 mm) and *E. coli* (25mm).A strong inhibitory effect against *A. niger* and *C. albicans* was recorded (30mm) as compared with nystatin (100 μg/mL) (Table 2).

The lowest minimal inhibitory concentration (MIC) value (Table 3) was against the most susceptible species of *A. niger* and *C. albicans* marked 50μL/mL, this was followed by *E. coli* and *B. subtilis* with 100 μL/mL and 150 μL/mL respectively. These values suggested that the essential oil possessed bacteriostatic and bactericidal activities and demonstrated great potential as an antifungal compound with potent *in-vitro* fungicidal activity against *C. albicans*, an opportunistic pathogen responsible for both superficial and systemic mycoses. Furthermore, the essentiall oil of *A. marmelos* (L.)Correa leaves (Table 4) demonstrated a significant inhibition of *A. niger* at concentration (4, 2, 1 % v/v) and *F. oxysporum* spores at concentrations (4, 2 % v/v) with no response at concentration 1% (v/v).Previous studies reported the antifungal activity of essential oil isolated from the leaves of *Aegle marmelos* (L.) Correa, with inhibition of the growth of dermatophytes and *Fusarium* species at concentration of 500 μg/mL (Rana *et al*., 1997).

Table 3: MICs of the essential oil of *A. marmelos* (L.) Correa leaves.

Test organism	MIC (μL/mL)
B. subtilis	150
E. coli	100
A. niger	50
C. albicans	50

Table.4: Effect of essential oil from *A. marmelos* (L.)Correa leaves on spore germination of *A. niger* and *F. oxysporum*.

Conc. of oil mL %	*Aspergillus niger*	*Fusarium oxysporum*
4%	-ve	-ve
2%	-ve	-ve
1%	-ve	+ve

Our obtained results for the antifungal activity of the oil come in accordance with Balakumar *et al*., (2011) who reported that the essential oil of *A. marmelos* (L.) Correa leaves have potent antifungal activity against the clinical isolates of dermatophytes. The mode of action of the essential oil as antimicrobial agents may be due to inhibition of respiration and disrupting the permeability barriers of the cell membrane structures (Cox *etal*.,2000).Furthermore, İşcan *et al*., (2012) reported that α-phellandrene is the responsible for the antimicrobial activity, while the antibacterial and fungicidal activities *Wedelia prostrata* was mainly attributed to α-pinene.

CONCLUSION

The biological activity of the essential oil isolated from the leaves of *Aegle marmelos* (L.)Correa was due to the synergism between the total components of the oil and mainly attributed to the presence of α-phellandrene and α-pinene as major constituents. The broad spectrum antimicrobial and antifungal activities of the

oils against variety of pathogenic fungi and bacteria, can recommend its incorporation in different pharmaceutical preparations.

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