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Comparison of Chemical and Antimicrobial Studies of Egyptian Mandarin Leaves and Green Branches Volatile Oil

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Author's contribution

The author OAE designed the study, wrote the protocol, wrote the manuscript, managed the literature searches, analyses of the study performed and approved the final manuscript.

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ABSTRACT

Aims: In the present study we compared the chemical composition and the antimicrobial activity of the essential oils isolated from green branches and leaves of mandarin.
Study Design: Extraction of essential oils from mandarin green branches and leaves by hydrodistillation
Place and Duration of Study: leaves and green branches were collected from Benha City, Qualyobia, Egypt in December 2012.
Methodology: The essential oils (EOs) isolated from mandarin (*Citrus reticulata*) were analyzed by GC and GC/MS. The antimicrobial activity was assessed using agar well diffusion method.
Results: Sixteen compounds were identified from the oil of branches which represented about 92% of the total detected constituents. The major components of the oil were dimethyl anthranilate (34.7%), γ-terpinene (33.6%) and limonene (11.2%). Alpha pinene and sabinene were present in considerable amounts (both at 2.8%). Other components were present in amounts less than 2%. From the heavier of layer mandarin leaves oil, thirteen compounds accounting for 95.4% of the oil were identified. Dimethyl anthranilate (65.3%) was the major component, followed by γ-terpinene

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(19.8%) and limonene (4.5%).
On the other hand, fourteen compounds (94.7%) were identified from the lighter layer of mandarin leaves oil including dimethyl anthranilate (60.6%), γ-terpinene (22.8%) and limonene (5.3%).
The antimicrobial activities of the oils were assessed. The antifungal activity was studied against *Trichophyton mentagrophytes*, *Aspergillus fumigatus* and *Candida albicans.* The antibacterial activities were measured against *Staphylococcus aureus, Streptococcus pyogen*es, *Enterococcus faecalis* as gram (+) bacteria*;* and *Klebsiella pneumonia, Escherichia coli, Salmonella typhimurium* as gram (-) bacteria.
Conclusion: The heavier layer of mandarin leaves oil was the most effective layer against all tested microorganisms, followed by the branches oil and finally the lighter layer of leaves oils.

Keywords: Citrus reticulate; hydrodistillation; leaves; green branches; antimicrobial activity.

1. INTRODUCTION

The genus *Citrus* of the family Rutaceae includes about 17 species distributed throughout the tropical and temperate regions [1]. *Citrus* fruits and their by-products are of high economic and medicinal values because of their multiple uses, such as in the food industry, cosmetics and folk medicine [2,3]. EOs of some plants has recently been proven to be successful eco-friendly bio-control agent [4]. Many authors have reported on the antimicrobial, antifungal, antioxidant and radical-scavenging properties of EOs [5,6].

The volatile aroma components of citrus fruits, especially the components of peel, differ depending on the origin of the fruit and the cultivars and hybrids analyzed, have been widely explored [7-10].

Mandarins (Rutaceae family, *Citrus* genus) are cultivated in countries with temperate summers and mild winters, particularly in Mediterranean countries, [11] and predominate, with oranges, the fresh-fruit market.

The peel of mandarin fruits regulates the skin moisture, softens hard and rough skin and has a cleaning effect on oily skin [12]. It also helps skin tone and removes skin blemishes [13]. The effects of the volatile oil of *C. reticulata* have been studied against pathogenic *Schistosoma mansoni* [14] and other microorganisms [15-18]. The volatile oil of *C. reticulata* also shows anti-cancer activity [19]. It was reported that mandarin leaves essential oil produced cytotoxic effect on HL-60 cell line and NB4 cell line (Human promyelocytic leukemia cell lines) [20].

In Egypt, massive amounts of leaves and branches of citrus trees are trimmed and discarded every year. In this study we try to assess the composition and biological activity of oils obtained from mandarin green branches and leaves aiming to find a potential use for this waste product. Gas chromatography- mass spectrometry (GC/MS) was utilized for the chemical analysis of the volatile constituents obtained by hydrodistillation of the green branches and leaves. The antimicrobial activities, against Gram-positive, Gram-negative bacteria and pathogenic fungi have been evaluated also.

2. MATERIALS AND METHODS

2.1 Plant Material

Citrus reticulata, Rutaceae, leaves and green branches were collected from Benha City, Qualyobia, Egypt in December 2012. It was authenticated by Dr. Usama K. Abdel Hameed, Department of Botany, Faculty of Science, Ain Shams University, Cairo, Egypt. A voucher specimen (voucher specimen number; CRR-98) is deposited at the Dept. Pharmacognosy, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt.

2.2 Isolation of Volatile Aroma Compounds

The fresh leaves and branches *C. reticulata* were hydrodistilled in all glass Clevenger apparatus for 5 h as described by Sharma and Tripathi [21]. The hydrodistillation of leaves resulted in two layers, one was heavier than water and the other was lighter. Branches yielded only a single lighter layer of oil. The volatile oils were dried over anhydrous sodium sulphate and stored at 5℃ in the dark.

2.3 Analysis of Volatile Aroma Components by GC and GC-MS

GC analysis was carried out using a Perkin-Elmer Autosystem apparatus equipped with FID

and fused silica capillary columns (30 m X 0.25 mm i.d., film thickness 0.25 µm), HP-5 (diphenyl dimethyl polysiloxane). Oven temperature was programmed from 45°C to 240°C at 3°C/min; injector temperature, 280°C; detector temperature, 250°C; carrier gas, helium (1 ml/min); automatic sample injection, 0.02 µl of the oil; split: 1/60. The relative proportions of the essential oil constituents were expressed as percentages obtained by peak area normalization without using correcting factors.

GC–MS analysis was performed on a Perkin-Elmer quadrupole MS system (Model 910) coupled with the above gas chromatograph, equipped with a HP-5 capillary column and operating under the same conditions described above, except for the carrier gas flow rate (1 ml/min.). The MS operating parameters were: ionization 70 eV; ion source temperature, 230°C; scan mass range, 35–450 Da.

2.4 Compounds Identification

Compounds were identified using their MS data compared to those from the mass spectral library and published mass spectra.

2.5 Biology Experiments

This work has been done in Regional Center for Mycology and Biotechnology, Al-Azhar University, Nasr City, Cairo, Egypt.

2.5.1 Antibacterial activity

Antibacterial activity was investigated using agar well diffusion method. The activity of the tested oils was studied against the *Staphylococcus aureus* (RCMB 010027), *Streptococcus pyogene*s (RCMB 010015), *Enterococcus faecalis* (RCMB 010068) [as Gram positive bacteria] and *Klebsiella pneumonia* (RCMB 0010093), *Escherichia coli* (RCMB 010055), *Salmonella typhimurium* (RCMB 010072) [as Gram negative bacteria]. A solution of 250 µl/mL of each oil and the standard drug in DMSO were prepared to be used against the tested bacteria. Centrifuged pellets of bacteria from a 24 h old culture containing approximately 10^4–10^6 CFU (colony forming unit) per mL were spread on the surface of nutrient agar (tryptone 1%, yeast extract 0.5%, NaCl 0.5%, agar 1%, 1000 mL of distilled water, pH 7.0) which was autoclaved under 121 °C for at least 20 min. Wells were created in medium with the help of a sterile metallic bores and then cooled down to 45°C. The activity was determined by measuring the diameter of the inhibition zone (in mm). Fifty microliters of the oil (concentration: 250 µl/mL) were loaded into the wells of the plates. All the samples were prepared in dimethyl sulfoxide (DMSO), DMSO was loaded as the control. The plates were incubated at 37°C for 24 h and then the plates were examined for the formation of inhibition zone. Each inhibition zone was measured three times by caliper to get an average value. The test was performed three times for each bacterium culture. Ampicillin was used as a positive control for Gram positive bacteria and gentamicin for Gram negative bacteria.

2.5.2 Antifungal activity

Tested samples were screened separately *in vitro* for their antifungal activity against *Aspergillus fumigatus*, *Candida albicans*, and *Trichophyton mentagrophytes*. These species were isolated from the infected organs of some patients on Sabouraud dextrose agar plates. The fungi culture was purified by single spore isolation technique. The antifungal activity was done by agar well diffusion method according to the following procedure: Sabouraud dextrose agar plates: A homogeneous mixture of glucose-peptone-agar (40:10:15) was sterilized by autoclaving at 121°C and 15 lb/cm^2 for 20 min. The sterilized solution (25 mL) was poured in each sterilized Petri dish in laminar flow and left for 20 min to form the solidified sabouraud dextrose agar plate. These plates were inverted and kept at 30°C in the incubator to remove moisture and to check for any contamination. Antifungal assay: Fungal strain was grown in 5 mL Sabouraud dextrose broth (glucose:peptone, 40:10) for 3–4 days to achieve 105 CFU/mL cells. The fungal culture (0.1 mL) was spread out uniformly on the sabouraud dextrose agar plates by sterilized triangular folded glass rod. Plates were left for 5–10 min so that culture is properly adsorbed on the surface of Sabouraud dextrose agar plates. Small wells of size (4-2 mm) were cut into the plates with the help of well cutter and bottom of the wells were sealed with 0.8% soft agar to prevent the flow of test sample at the bottom of the well. 50 µL of the tested samples (250 µL/mL) was loaded into the wells of the plates. All compounds were prepared in dimethyl sulfoxide (DMSO), DMSO was loaded as control. The plates were kept for incubation at 30°C for 3–4 days and then the plates were examined for the formation of inhibition zone. Each inhibition zone was measured three times by caliper to get

an average value. The test was performed three times for each fungus. Amphotericin was used as a standard for the antifungal activity.

2.5.3 determination of the minimum inhibitory concentrations (MIC)

MIC was determined by using the microdilution broth method. This was done following the procedures recommended by the National Committee for Clinical Laboratory Standard [23, 24]. Ampicillin, gentamycin and amphotericin B were used as the reference compounds for bacteria and fungi. The reference compounds were dissolved in sterile distilled water. The microtiter plates were incubated at 37℃ for tested bacteria and 28 for tested fungi and were read using microplate reader after 24 h for bacteria and 48 h for fungi.

3. RESULTS AND DISCUSSION

Qualitative and quantitative analysis of the essential oils volatile profile are listed in Table 1. These results showed that there are many qualitative similarities between the three oils although the amounts of some corresponding compounds are different.

The green branches produced dark yellow oil in a high yield 0.4% (v/w). Sixteen compounds were identified, which represented about 92% of the total detected constituents. The major constituents of the oil were dimethyl anthranilate (34.7%), γ-terpinene (33.6%) and limonene (11.2%). Alpha pinene and sabinene were present in a considerable amount (both at 2.8%). Other components were present in amounts less than 2%.

From the heavier layer of leaves oil (pale yellow oil, 0.2% v/w) thirteen compounds, accountings for 95.4% of the oil were identified. Dimethyl anthranilate (65.3%) was the major component, followed by γ-terpinene (19.8%) and limonene (4.5%).

The lighter layer (pale yellow oil, 0.3% v/w) of mandarin leaves oil; fourteen compounds were identified (94.7%). Dimethyl anthranilate (60.6%) was also the major component, followed by γ-terpinene (22.8%) and limonene (5.3%).

Table 1. Volatile components isolated by hydrodistillation of mandarin green branches, leave heavier and lighter layers

No.	Compounds	Area [%][a]		
		Leaves layers		
		Heavier	Lighter	Branches
1	α-Thujene	0.3	0.8	1.2
2	α-Pinene	1.7	1.9	2.8
3	Sabinene	1.7	0.2	2.8
4	β-Pinene	0.5	0.6	0.9
5	*p*-Cymene	0.2	0.1	0.2
6	**Limonene**	**4.5**	**5.3**	**11.2**
7	*cis*-Ocimene	0.2	0.2	0.3
8	Trans-Ocimene	0.4	0.5	0.8
9	**γ-Terpinene**	**19.8**	**22.8**	**33.6**
10	α-Terpinolene	0.6	0.6	1.4
11	4-Terpineol	0.1	0.2	0.1
12	Isoterpinolene	n.d.	0.1	0.1
13	Thymol methyl ether	n.d.	n.d.	0.4
14	Thymol	0.1	n.d.	n.d.
15	**Dimethyl anthranilate**	**65.3**	**60.6**	**34.7**
16	*trans*-Caryophyllene	n.d.	0.8	1.3
17	(-)-Caryophyllene oxide	n.d.	n.d.	0.2
Total		**95.4**	**94.7**	**92**

*[a]: Values are expressed as relative area percentage; n.d: Not detected; The major components are highlighted in bold. (*values expressed as relative area percentages to the total identified components)*

It is obvious from the table that the oil of mandarin branches contains higher amounts of α-thujene, α-pinene, β-Pinene and sabinene than that of the leaves; however the situation is reversed in case of dimethyl anthranilate. Also limonene, γ-terpinene and α-terpinolene constituted a higher percent in branches than in leaves. Thymol was not detected in branches and in the lighter layer while thymol methyl ether was detected only in the oil obtained from branches. *Trans*-Caryophyllene was only detected in the oil from branches and the lighter layer. Moreover, caryophyllene oxide was only detected in the oil from branches.

The second objective of the present work was to evaluate the effect of mandarin essential oils composition on their antimicrobial.

Tables 2 and 3 showed a comparative study on the qualitative and quantitative antimicrobial activities of the three essential oils. *Aspergillus fumigatus, Streptococcus pyogene*s, *Salmonella typhimurium* were the most affected fungi, G (+ve) and G (-ve) bacteria by the three oils, respectively.

The heavier layer oil of the leaves was the most effective one against the tested bacteria and fungi, followed by the oil from branches and the lighter layer oil of the leaves.

The antimicrobial effect increased proportionally with the amount of oxygenated constituents in the oil [25]. It was reported that the inhibitory effect of dimethyl anthranilate and limonene was in proportional with increment of their concentrations [26-27]. As the heavier layer contains the highest concentration of dimethyl anthranilate, so the highest antimicrobial activity was attributed to it. The lighter layer oil contains higher percent of dimethyl anthranilate compared to the oil from branches but the later showed higher antimicrobial activity than the former. This observation was attributed to the presence of higher concentration of limonene in oil from branches compared to the lighter layer oil suggesting a synergistic effect with dimethyl anthranilate. However, further investigation is required to evaluate the synergistic effect of dimethyl anthranilate and limonene.

Table 2. Antimicrobial activity of mandarin volatile oils expressed as inhibition zone diameter (mm)[a,b,c]

Tested microorganism	Branches	Leaves layers		St.
		Heavier	Lighter	
Fungi				**Amphotericin B**
Trichophyton mentagrophytes	21.4±0.5*	23.4±05	**18.6±03**	25.4±0.2
Aspergillus fumigatus	24.6±02	25.8±0.4	**20.3±0.4**	22.8±0.2
Candida albicans	19.6±0.3	20.1±0.4	**18.7±0.2**	20.7±0.3
Gram (+) Bacteria				**Ampicillin**
Staphylococcus aureus	25.4±0.5	26.8±0.2	**22.2±0.3**	28.9±0.1
*Streptococcus pyogene*s	27.3±05	28.9±0.2	**25.3±0.5**	29.8±0.1
Enterococcus faecalis	19.6±0.1	20.8±0.1	**16.2±0.2**	20.4±0.1
Gram (-) Bacteria				**Gentamicin**
Klebsiella pneumonia	23.1±0.6	25.4±0.5	**20.1±0.4**	26.3±0.15
Escherichia coli	20.6±0.3	22.3± 0.2	**18.6±0.2**	22.8±0.3
Salmonella typhimurium	24.3±0.2	26.4±03	**22.6±0.3**	28.8±0.2

[a]Mean zone of inhibition in mm ± standard deviation beyond well diameter (6 mm) produced on a range of environmental and clinically pathogenic microorganisms
The concentration used for the standard antibiotic was (30 µg/mL).
[b]The test was done using the diffusion agar technique. Well diameter: 6.0 mm
[c]Data are expressed in the form of mean ± SD

Table 3. Antimicrobial activity as MIC (µg/ml) of mandarin volatile oils

Tested microorganism	Branches	Leaves layers		St.
		Heavier	Lighter	
Fungi				**Amphotericin B**
Trichophyton mentagrophytes	**0.98**	0.24	7.8	0.06
Aspergillus fumigatus	**0.12**	**0.12**	3.9	0.24
Candida albicans	7.8	**3.9**	15.63	0.98
Gram (+) Bacteria				**Ampicillin**
Staphylococcus aureus	0.12	**0.06**	0.98	0.015
*Streptococcus pyogen*es	0.03	**0.015**	0.12	0.007
Enterococcus faecalis	7.8	**1.95**	31.25	1.95
Gram (-) Bacteria				**Gentamicin**
Klebsiella pneumonia	0.49	**0.12**	3.9	0.06
Escherichia coli	1.95	**0.98**	15.36	0.24
Salmonella typhimurium	0.24	**0.06**	0.98	0.007

4. CONCLUSION

In this work we reported for the first time: (1) The chemical composition of mandarin branches essential oil; (2) A comparative study on the chemical composition of mandarin oil green branches and leaves; (3) The antibacterial and antifungal activities of the essential oils from leaves and green branches of Egyptian mandarin. Our findings demonstrated the significant antimicrobial effect of the heavier oil layer from mandarin leaves. Moreover, the potent antimicrobial agent, dimethyl anthranilate, which comprises up to 65% of the oils from leaves, suggests the potential application of the essential oils from Egyptian mandarin waste products as a good natural source for this valuable compound.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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