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Der Pharmacia Lettre, 2016, 8 (4):239-245
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

Chemical composition, antioxidant, antibacterial and antifungal activities of peel essential oils of *citrus aurantium* grown in Eastern Morocco

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ABSTRACT

Essential oils (EO) were isolated by steam-distillation from fresh peel of citrus aurantium grown in Eastern Morocco and harvested in February (EO1) or December (EO2). EO were also isolated from peel of citrus aurantium harvested in December and dried at 25°C (EO3) or 50°C (EO4). Soxhlet hexane extract from dried peel of citrus aurantium was also used to isolate EO by steam-distillation (EO5). All these EO were analyzed by gas chromatography (GC) coupled to mass spectrometry (GC–MS). Variation in the yield and chemical composition of these essential oils were determined. Less than nine components were identified and constituting approximately 96 to 100% of the oil. The major component always was limonene for all EO. The antioxidant activity of these EO has been evaluated using in vitro DPPH assay and the results were compared with standard antioxidant(ascorbic acid). The effect of these EO on the growth rate of the yeast Saccharomyces Cerevisiae, gram-negative bacteria (Escherichia coli DH5α and Citrobacter freundii) and gram-positive bacteria (Listeria monocytogenes and Staphylococcus aureus)was studied. Ours findings fromcitrus aurantium peel EO possess very weak antioxidant activity. In contrast, it showed significant antifungal activity and variable antimicrobial activity.

Keyboards: Essential oil, *citrus aurantium* peel, limonene, antioxidant, antibacterial, antifungal

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INTRODUCTION

Natural sourced medicines have become increasingly popular among consumers who search for natural ways to maintain their health[1]. Food processing industries create large quantities ofby-products, which are difficult to dispose of, as they need a high biological oxygen demand. Plant material wastes from these industries sometimes contain high levels compounds that can have an adverse environmental impact[2].

Essential oils are natural products that plants produce for their own needs. In general, they are complex mixtures of organic compounds that give characteristic odor and flavor to the plants. They are mainly made up by monoterpenes and sesquiterpenes whose main metabolic pathway is through mevalonate leading to sesquiterpenes and from methyl-erythritol leading to monoterpenes. They are located in different parts of the plant. They can be found in the root, stems, leaves, flowers, fruit and even seeds[3].These volatile compounds have diverse ecological functions, acting as defensive substances against microorganisms and herbivores, but can also be important to attract insects for the dispersion of pollens and seeds[4].

The essential oil preparations that possess biological activities have been the subject of many investigations resulting in the screening of a wide variety of plant species and have revealed structurally unique biologically active compounds. Again, essential oils of some plants have recently been proven successful eco-friendly bio-control agent. Many authors have reported antimicrobial, antifungal, antioxidant and radical-scavenging properties of essential oils[5].

Citrus aurantium peel essential oils have been used for years in traditional western medicines, Chinese and Japanese herbal medicines, and as flavorings in foods and beverages[6].Historically, the oldest citrus product is the oil. In ancient Sicily, where early Italian citrus industry had just been introduced, lemons were primarily grown for production of lemon oil, and juice was treated as a waste product until its later use for citric acid recovery. Citrus has proven to be a very good option for the oil and essence production[7].

Citrus peel essential oils are reported to be one of the rich sources of bioactive compounds namely monoterpenes and sesquiterpenes. Recently, Citrus peel essential oils have also been searched for their natural antioxidant[8], antifungal[5] and antimicrobial[9] properties. It is widely accepted that biological activities of plant materials are strongly linked with their specific chemical composition[10].

The main objective of the present study was to assess the yields and chemical composition of *citrus aurantium* peel essential oils grown in Eastern Morocco. The effect of the drying pretreatment of peels and the fruit cultivation season on the yields and chemical composition of *citrus aurantium* peel essential oils were also studied. The antioxidant activity of *citrus aurantium* peel essentials oils was investigated. Moreover, the effect of essential oils on the growth rate of yeast *saccharomyces cerevisiae*, gram-negative bacteria (*Escherichia coliDH5α* and *Citrobacter freundii*) and gram-positive bacteria (*Listeria monocytogenes* and *Staphylococcus aureus*)was studied.

MATERIALS AND METHODS

1. Sample preparation and essential oil extraction:

The fruits were harvested from *Citrus aurantium* trees grown in Eastern Morocco in February and December. The fruits were washed and peeled. Firstly, 100g of fresh peel, or peel dried at 25°C and 50°C were submitted for 4 hours to steam-distillation using the Clevenger type-apparatus. The EO were dried over anhydrous sodium sulphate and then stored in sealed glass vials at 4 to 5°C prior to analysis. Secondly, 2.5 g of the hexane extract obtained from the Soxhlet extraction of dried peel were submitted for 2 hours to the steam-distillation by Clevenger type-apparatus. The hydrosol containing EO extracted is decanted three times with diethyl ether. The organic portion is dried with anhydrous sodium sulphate then filtered and evaporated in a rotary evaporator at 30°C[11]. The residue obtained is an essential oil that will be stored away from light at 4°C.

2. GC and GC-SM analysis:

The EO were analyzed using gas chromatography couples to mass spectrometry (Shimadzu QP 2010).

3. DPPH essay:

Radical scavenging activity of the essential oilwas measured using the stable radical DPPH(2,2-diphenyl-picryl-hydrazil). Theprocedure followed was according to Sanchez-Moreno et al (1998)[12].50µl of essential oil at different concentrations (from 0.3125 to 5 mg/ml) are added to 1.95 ml of DPPH solution (DPPH in methanol 0.025g/l). In parallel, a negative control was prepared by mixing 50µl of methanol in 1.95 ml DPPH solution. After 30 min of incubation in the dark at room temperature, the absorbance measured at 517nm. The results were expressed as percent inhibition (I%) and IC_{50} values are graphically determined by linear regression. The absorbance is measured by the UV-Vis spectrophotometer Shanghai mapada instrumants Co-Ldt model V-1200).The ability to scavenge the DPPH• radical was calculated using the equation below, where A_0 is the absorbance of the control at30min and A_1 is the absorbance of the sample at 30min. All samples were analyzed in triplicate, and a solution of ascorbic acid was used as a positive control.

$$I(\%) = 100 \times \frac{A0 - A1}{A0} \qquad (1)$$

4. Bacterial and fungal strains used:

The yeast *Saccharomyces cerevisiae* strain (BY4741)[13], was used to evaluate the antifungal activity of *Citrus aurantium* essential oils. Gram-negative bacterial stain (*E-coliDH5α and Citrobacter freundii*) and gram-positive bacteria strain (*Listeria monocytogenes* and *Staphylococcus aureus*)were used to evaluate the antibacterial activity of *Citrus aurantium* essential oils. All bacteria stains were kindly provided from Institute Pastor Morocco.

5. Antibacterial essay:

Essential oils of *citrus aurantium* peels were tested for their antibacterial activity against the various bacterial stains. The antibacterial activity was determined by the disc diffusion method[14].Briefly, a fresh colony was cultivated overnight in liquid Luria-Bertani medium (LB) at (37°C) under aeration. After that, a suspension containing 10^8(CFU / ml) of bacteria cells was prepared (0.5 McFarland) [15], and used to inoculate Petri plates containing solid (LB) medium[16]. The plates were then allowed to dry for 15minutes. Then paper discs (6mm in diameter) were placed on the inoculated agar plates. Then, 10µl of tested essential oils were deposited on the paper discs and then allowed to diffuse into medium by incubating the plates for one hour at room temperature. These were then incubated at 37°C. Twenty-four hours later. The antibacterial activity was evaluated by measuring the inhibition zone diameters in millimeter. The measurements of inhibition zones were performed three times for each essential oil including streptomycin as positive control (PC).

6. Antifungal essay:

Growth rate of yeast cells was measured as the optical density of cells at 600 nmas a function of time (hours) in rich medium. Yeast cells were diluted from an overnight culture to an O.D (600nm)of ~0.08 and allowed to grow until the O.D (600nm)reached ~0.14,ensuring that the cells were in logarithmic phase. EO was then added and growth rate was measured. All EO were diluted in 100% DMSO, and all assays, including the "no drug" control, contained 1% DMSO.

RESULTS AND DISCUSSION

1. Essential oil yields and compositions:

EO were isolated by steam-distillation from fresh peel of *citrus aurantium* grown in Eastern Morocco and harvested in February (EO1) or December (EO2). EO were also isolated from peel of *citrus aurantium* harvested in December and dried at 25°C (EO3) or 50°C (EO4). Soxhlet hexane extract from dried peel of *citrus aurantium* was also used to isolate EO by steam-distillation (EO5). The EO1, EO2, EO3, EO4 and EO5 were fragrant, colorless, with a density of 0.85, 0.85, 0,83, 0.85 and 0,71 respectively. These density values are similar with those reported in literature[7]. Yield and chemical composition of these EO were also determined. Yields of EO1, EO2, EO3, EO4 and EO5 were respectively 1.01%, 1.02%, 1.04%, 2.13% and 4.5% (Figure 1).

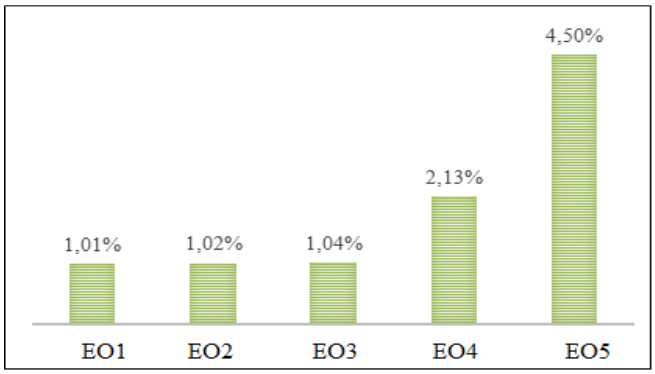

Figure 1: *Citrus Aurantium* essential oil yields

Our results are similar to those finding by Hosni et al (2010) in Tunisia, where the yield of the EO from dried peel of *Citrus aurantium* was around 1.24%[17], but contrary with results of Hamadani et al (2015) in Algeria and Essadik (2015) in Morocco, where the yield of fresh *citrus aurantium* peel EO was 0.73% [18]and 0.3%[19] respectively. Therefore, the highest yield of EO was obtained from dried hexane extract followed by peel dried at 50°C. The yield of EO obtained from fresh peel or dried at 25°C were two times lower than the one from peel dried at 50°C, and four times lower than the one from dried peel hexane extract. The results of our present study regarding effect of drying conditions on peel essential oil yields are in agreement with finding of Kamal et al (2011)[10], where higher yield of EO was obtained from oven-drying peel of citrus species. The chemical composition of EO is shown in table1.

Table 1 : Compounds of fresh and dried peel *Citrus aurantium* EO

Chemical compounds	(%)EO1 February	(%)EO2 December	(%)EO3 (25°C)	(%)EO4 (50°C)	(%)EO5 (Hex)
β-Myrcene	4.02	1.34	2.97	3.01	2.9
D-Limonene	**88.97**	**92.62**	**82.58**	**64.06**	**94.12**
Linalool Oxide	----	----	2.32	3.91	---
Linalyl Acetate	0.54	----	----	----	---
p-Linalool	0.36	----	5.19	12.55	---
α-Terpineol	----	0.40	----	2.69	---
Perillaldehyde	----	----	----	5.90	---
Carvacrol	---	----	----	4.54	---
Geranyl acetate	0.25	----	----	1.88	---
6-Methyl-2-(2-oxiranyl)-5-hepten-2-ol	0.42	0.38	1.56	----	---
β-Linalool	----	1.98	5.38	----	---
β-Pinene	0.69	0.50	----	-----	---
α-Pinène	0.84	0.49	----	----	---
(+)-Sabinene	----	0.24	----	----	---
cis-Ocimene	----	0.21	----	----	---
Total	96.09%	98,16%	100%	98.54%	97,02%

The total number of compounds identified in EO1, EO2, EO3, EO4 and EO5 were respectively, 8, 9, 6, 9 and 2, and which represented respectively 96.09%, 98.16%, 100%, 98.54% and 97.02% of the total oil. These results are different from those reported previously. For example, 13-37 compound were found in dried and fresh peel essential oil in Tunisia[8, 17]. In Turkish fresh *Citrus aurantium* peel essential oil, 29 compounds were identified [20]. 32-33 compounds were found in peel essential oil in Algeria [18, 21].

Our peel essential oils were dominated by monoterpenes hydrocarbons with 94.52%, 95.40%, 85.55%, 67.07% and 97,02%for EO1, EO2, EO3, EO4 and EO5, respectively. Limonene was always the major component with variable percentage. This result is similar to those reported for EO from Tunisia and Turkey, where Limonene was also the major component with 90.25-96.9% and 94.1% respectively [8, 17, 20]. However, in Algeria limonene was not the dominant constituent (2.5-7.18%), but linalool was the major constituent (12-63.03).

In this paper, we found that the chemical composition of our EO showed noticeable difference. EO1, which was obtained from peel of *citrus aurantium* harvested in February, showed higher content of β-Myrcene, but lower content of D-Limonene than EO2, which was obtained peel of *citrus aurantium* harvested in December. Thereby, the oil contents are influenced by season of harvest or ripening stage. Similarly, drying treatments influenced the chemical composition of EO. EO3, which was obtained from peel of *Citrus aurantium* dried at 25°C, showed higher content of D-Limonene than EO4, which was obtained peel of *Citrus aurantium* dried at 50°C (82.58% versus 64.06%). In addition, a number of minor components are present in EO obtained from peel dried at 50°C, but absent in EO obtained from peel dried at 25°C. Therefore, these results could help establish the optimum harvest date and the best methods for preparing EO from peel of *citrus aurantium*.

2. Antioxidant activity:

DPPH can be used to determine the free radical scavenging activity as it forms a stable molecule on accepting an electron or hydrogen atom[22].There was a reduction in the concentration of DPPH due to the scavenging effect of extracts. The extracts and standard antioxidants reduced DPPH to yellow colored product in a concentration dependent manner [23].The free radical scavenging activity of essential oils has been studied. *Citrus aurantium* EO displayed weak DPPH• radicals scavenging capability. The DPPH scavenging capacity of all EO was ranging from 7-15%. The IC_{50} values were much higher than the control ascorbic acid (Table 2).

Table 2: DPPH scavenging capacity (I%) and effective concentration (IC50) of EO

EOs	I%	IC_{50}(mg/ml)
EO1	15.33±0.433	1.49±0,156
EO2	7.47±0.115	4.20±0,476
EO3	7.00±0,529	2.57±0,840
EO4	14.60±0,115	1.81±0,208
EO5	12.38±0,595	0.68±0.070
Ascorbic Acid	95,52±0,570	0.018±0.09

Our results are consistent with work of Choi et al (2000),where 34 kinds of *citrus* oils obtained from Japan, Korea and Italy exhibited weak DPPH radical scavenging effect ranging from 12% to 17.7%. In their work, they showed that *citrus aurantium* EO have low DPPH radical-scavenging activity. As this EO was mainly composed of limonene in proportion of >90%, it is considered that limonene would not play the principal role in determining the scavenging activity for radical and also the same for Myrcene in which there was non-direct correlation between

Myrcene content and the radical scavenging activity[24].Ming-ChiuOu et al (2015) also reported that *citrus grand is* EO and cold-pressed *citrus paradisii* EO displayed weak DPPH radical-scavenging capability. However, DPPH scavenging capacity of cold-pressed *citrus paradisii* EO was less than 20% and distilled *citrus paradisii* EO exhibited the potent DPPH scavenging capacity among 4 *citrus* EO. And where the IC50 value was more than 40mg/ml[25].

3. Antibacterial activity:

The effect of our EO on the growth rate of gram-negative bacteria (*Escherichia coli DH5α*, and *Citrobacter freundii*) and gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) is represented in Table 3.

Table 3: *Citrus aurantium* peel EO effect on gram-positive and negative *bacterial stain*

	Inhibition Zone (IZ) (mm)			
	Gram positive		Gram negative	
Samples	*Listeria monocytogenes*	*Staphylococcus aureus*	*E-coli DH5α*	*Citrobacter freundii*
EO1	10	11	15	11
EO2	-	10	-	-
EO3	08	10	07	11
EO4	07	13	-	10
EO5	-	10	-	-
Streptomycin	08	11	20	22

(-) non-activity; 7< IZ <9.9mm: Lower activity; 10 < IZ< 11.9 mm: modest activity; 12 < IZ< 15mm: Higher activity.

It is clearly that all EO exhibited antibacterial activities, but with different specificity. EO1 and EO3 showed antibacterial activity against all bacterial strains tested (Table3). However, EO2 and EO5 showed antibacterial activity only against the gram-positive bacteria *Staphylococcus aureus* (Table 3). On the other hand, EO4 was not active against the gram-negative bacteria *Escherichia coli*, but showed antibacterial activity against the other bacterial stains. These finding are different from that reported by Ullah et al (2012)and Teixeria et al (2013), where EO from peel *citrus sinensis* grown in Pakistan and EO from peel of grapefruit and lemon had no antimicrobial activity against the *E. coli* gram-negative bacteria[4, 26]. Similarly, Chanthaphon et al (2008) showed that all gram-negative tested including *Salmonella sp* and *E. coliO157:H7* were resistant to EO from peel of *citrus* grown Thailand at the concentration tested (200µg/ml)[27],

4. Antifungal activity:

Our EO were tested for antifungal activity, against budding yeast (*Saccharomyces cerevisiae*) cells. Cells were cultured in the presence of 100µg/ml each EO and assayed for growth inhibition in liquid culture as described in Materials and methods. The results are show in figure1.

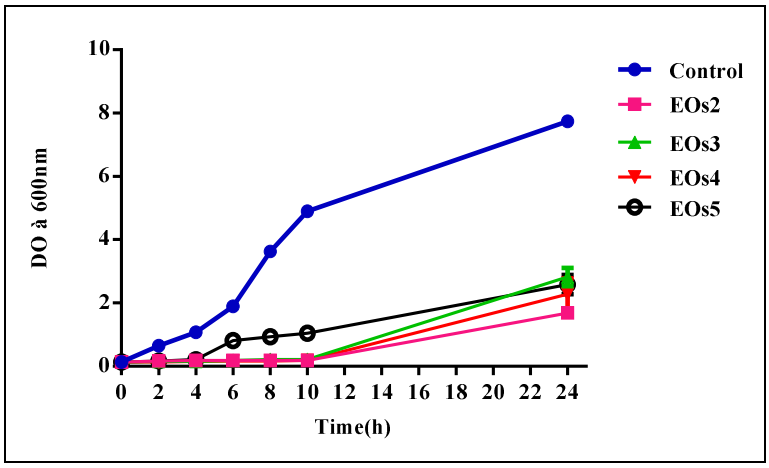

Figure 2:Citrus Aurantium peel EO effect against cell growth rate of the yeast of *Saccharomyces cerevisiae*, 100µg/ml in DMSO

All the EO showed a significant inhibition of cell growth rate of yeast (Figure 2). This result is similar to other studies, where strong antifungal activity of citrus EO and essences against *Saccharomyces cerevisiae* was observed [27-28]. EO from citrus grown in Algeria showed also antifungal activity against stains such as Fusarium, Aspergillus, Penicillium, Alternia[18, 29]. Considerable studies on antifungal activities of EO components were previously reported. Kurita et al (1981) tested antifungal activity of different EO components, such as aliphatic aldehydes in which there is one or more double bonds conjugated to their carbonyl group, as Perillaldehyde. Those components showed higher antifungal activity, unlike tertiary alcohol such as linalool which does not inhibit the

growth of any fungi. In addition, seven hydrocarbons among them D-limonene, α-pinene and β-myrcene were examined and were almost ineffective in inhibiting the growth of any of the fungi employed at a concentration of as high as 2mM[30]. In contrary, Rančić et al (2003) showed that limonene possessed antibacterial and antifungal activities[31].

CONCLUSION

Generally, citrus EO are complex mixtures, constituted by monoterpenes, oxygenated monoterpenes and sesquiterpenes. They originate from the plant secondary metabolism and are responsible for their characteristic aroma. Many factors influenced yields and chemical compositions of essential oils such as season, drying temperature pretreatment, extraction methods. Those factors have direct effect on antifungal, antibacterial and antioxidant activities as a result of chemical composition variation. Biological activities of essential oils complex mixtures cannot easily be explained through the action of a single or few molecules.

Eastern Moroccan peel *Citrus aurantium* EO is rich in monoterpenes in which limonene is often the predominant compound. Drying pretreatment on peel influenced yield of the essential oils on the one hand and its chemical compositions and percentage in the other hand. The essential oils showed antibacterial activity against Gram (+) and Gram (-) bacteria, and also effect of essential oils on *Saccharomyces cerevisiae* yeast in which present higher antifungal activity. Essential oils don't show antioxidant activity.

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