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ARTICLE

Effect of Five Different Maturation Stages on the Chemical Composition, Antioxidant and Antimicrobial Activities of Syrian Sour Orange (*Citrus aurantium* L.) Peel Essential Oil

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Abstract: For the first time, the composition of Essential Oils (EOs) from Syrian *Citrus aurantium* L. peel and their antioxidant and antimicrobial activity was studied over five different stages of ripening; (R1: Sep 2021, green; R2: Oct, Yellowish-green; R3: Nov, Greenish-yellow; R4: Dec, Yellow; R5: Jan 2022, orange). EOs were extracted by a hydro-distillation method using the Clevenger apparatus, and their components were identified by Gas Chromatography-Mass Spectroscopy (GC-MS). The antioxidant and antibacterial activities of EOs were determined using 2,2-diphenyl-1-picrylhydrazyl and the dilution method, respectively. The results showed that the highest EO yield (1.68 ± 0.05 mL/100 g peel) was achieved during the fruit ripe stage R5. However, the maturation stage has no significant effect on the limonene proportion (98.5%), which is the most abundant compound in Syrian *C. aurantium* peels EO. Moreover, 2,2-diphenyl-1-picrylhydrazyl inhibition efficacy for EOs was the highest in the R1 stage and had an IC_{50} value of 83.48 ± 0.01 mg/mL. EO from the R1 stage showed the best values of minimum inhibitory concentration MIC (0.0035 ± 0.0011 and 0.0900 ± 0.0023 nL/mL) against *Staphylococcus aureus* and *Escherichia coli*, respectively, suggesting the importance of using these EOs as natural preservatives in food products.

Keywords: *Citrus aurantium* L.; Ripening stages; Hydro-distillation; Essential Oils; Antioxidant activity; Antibacterial activity.

Introduction

The primary industrial purpose of investing in citrus fruits is to produce juice, especially from orange cultivars[1–4] of high content of many bioactive compounds[5]. Indeed, oranges occupy the major portion of world citrus production, where the global production of these fruits, according to the United States Department of Agriculture (USDA), was estimated to be 48.8 million tons per year[6]. This, however, results in large quantities of waste since the peel constitutes about 20% of the orange, and the global release of orange peel was estimated to be 9.8 million tons per year[7]. Recently, researchers became interested in exploiting these wastes for the content of many compounds with high biological activities[1]. These bioactive compounds were found to show antioxidant[3], antiaging, antibacterial, antifungal, anti-aflatoxigenic[4] and anti-inflammatory activities. Some compounds, for example, flavonoids, have also proven to be effective against degenerative diseases[5]. However, the most important of these compounds is hesperidin which was proposed to treat COVID-19 due to its ability to react with the enzyme receptor of the main protein for COVID-19[1,8]. Moreover, many experiments have successfully demonstrated that using the EO extracted from the *C. aurantium* peel, which contains 60% limonene, was effective against

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bacteria that lead to the emergence of cutibacterium acne[6]. Moreover, its interaction with β-cyclodextrin was reported to help reduce the development of Alzheimer's disease[5]. In addition, it has a role in treating cancer cells[9], and protecting against the oxidative action of soybean oil when used as an additive agent[10]. *C. aurantium* peel EO was also reported to show effectiveness against four bacterial strains[2,4,11], as well as insecticidal behaviour against four types of storage-grain insects due to its high content of limonene[12]. Recently, the results indicated that orange peel EO can be used as a natural preservative to improve safety and food quality instead of synthetic preservatives that have shown a detrimental effect on human health[5,11]. Also, EOs are considered among the materials listed in the safety regulations materials[13,14]. This work aimed at obtaining the EOs from the Syrian *C. aurantium* peel during five different maturation stages by hydrodistillation, determining its chemical composition by GC-MS and testing their antioxidant and antibacterial activities against *S. aureus* and *E.coli*, respectively.

Materials and Methods

Materials

Samples of Syrian *C. aurantium* were collected from the Damascus University garden during various ripening stages (every month from September 2021 until January 2022), as identified by Dr. G. Babojian (Table 1). Figure 1 shows the *C. aurantium* tree. In addition, the moisture content of the *C. aurantium* peel had been determined to be 72–78%.

Table 1. *C. aurantium* fruit in various ripening stages.

State	Unripe				Ripe
Ripening Stage	R1	R2	R3	R4	R5
Picking month	Sep. **2021**	Oct. **2021**	Nov. **2021**	Dec. **2021**	Jan. **2022**
Colour of Peel	Green	Yellowish-green	Greenish-yellow	Yellow	Orange

Figure 1. Tree of Citrus × aurantium L.

2,2-diphenyl-1-picrylhydrazyl (DPPH) and Ethanol (99.9%) were purchased from Sigma-Aldrich, Darmstadt, Germany, Nutrient Agar from Liofilchem, Roseto degli Abruzzi, Italy and Nutrient Broth from Merk, Darmstadt, Germany. The Gram-positive *Staphylococcus aureus* bacteria and the Gram-negative *Escherichia coli* bacteria were obtained from Al-Mouwasat Hospital, Damascus, Syria.

Extraction of EOs from C. aurantium Peel

After removing most of the white fibres, the peel was cut into 1-cm squares which underwent hydrodistillation for 90 min using a Clevenger apparatus with a distilled water-to-peel ratio of 2:1. The obtained EOs samples were centrifuged at 600 rpm for 10 minutes and then dried over anhydrous sodium sulfate. Separated EOs were kept according to the procedure described in the European Pharmacopoeia, fifth edition, 2004, in the dark at +4 °C until the analysis was conducted on an Agilent 7890A gas chromatograph coupled with a quadruple mass spectrometer (model 5975C). The separation was performed for 1.0 µL of EO diluted in *n*-hexane (1/100; V/V) on an HP-5MS column (5% Phenyl

Methyl Silox), 30 m × 250 µm × 0.25 µm) using helium (Purity 99.999%) as a carrier gas at a flow rate of 1 mL/min with a split ratio of 1:50. The injector and ion source temperature was adjusted at 260 °C and 230 °C, respectively. The oven temperature was constant for 2 min at 40 °C, then ramped at a rate of 2.5 °C/min up to 70 °C, then at 2.0 °C/min to 130 °C, then at 25 °C/min to 220 °C, then run at 240 °C for 2 min (Post Run). The selective mass detector temperature and the interface temperature were 150 °C and 280 °C, respectively. The duration of the analysis was 47.6 min. The chemical constituents of obtained EOs were identified by comparing their mass spectra with standard samples present in the analyzer library (National Institute of Standards and Technology NIST). The retention Index (RI) was determined for all compounds by the Kovats method using the homologous series of *n*-alkanes C_8–C_{22} as standards. The determined values were compared with those of the standards reported in the literature[15,16]. The retention index was calculated by equation 1:

$$RI = \left[n + \frac{RT_{Sample} - RT_n}{RT_N - RT_n}\right] \times 100 \qquad (1)$$

n: the number of carbon atoms in the *n*-alkane preceding the sample.
N: the number of carbon atoms in the *n*-alkane following the sample.
RT: the individual retention time.

EOs Antioxidant Assay

The DPPH assay was used for measuring the total antioxidant capacity of EOs. 1 mL of extracted EO with varying concentrations (0–250 mg/mL), was mixed with 3 mL of freshly prepared ethanolic DPPH solution (0.06 mM) using a vortex mixer (MS1 Mini Shaker by IKA). The mixture was allowed to stay in the dark at room temperature for 30 min. Absorption was recorded at 515 nm (UV-VIS Spectrometer model Humareader HS - Human company), compared to a blank sample of ethanol[13]. The results were compared with a calibration curve of vitamin C solutions in 70%-ethanol (0–10 µg/mL; y = 5.5899 x + 0.1606, Eq. 2, R^2 = 0.9998). The inhibition percentage of free radicals (I_{DPPH}%) was calculated according to the following equation[13]:

$$I_{DPPH}\% = \frac{A_b - A_a}{A_b} \times 100\% \qquad (2)$$

A_a: sample absorption, A_b: blank absorption.

The antioxidant activity for tested compounds was determined by estimating the IC_{50} value from the inhibition curves versus antioxidant concentration.

Antibacterial Activity

The nutrient broth solution was prepared by dissolving 1.3 g of the nutrient broth in 100 mL of distilled water in a glass beaker with stirring, followed by autoclaving at 121 ºC and 15 bar for 15 min. The final solution was stored below 25 ºC. The culture media were prepared by dissolving 2.8 g of nutrient agar in 100 mL of distilled water in a glass beaker with heating, followed by autoclaving at 121 ºC and 15 bar for 15 min before pouring in Petri dishes.

The bacterial suspension was prepared by culturing the bacteria directly in nutrient broth NB to get a concentration of 0.5 McFarland (1–2×10^8 CFU/mL). Concentrations down to 10^6 CFU/mL were then prepared by serial dilution with sterile saline (physiological water)[17].

Evaluation of the *S. aureus* and *E. coli* antibacterial activity of extracted EOs was investigated by the agar dilution method[18,19]. Different concentrations were prepared in 20%-ethanol to form 0.01 nL/mL to 10 µL/mL of these extracts, so sterile water and 2% ethanol were used as a blank sample. Autoclaved nutrient agar (18 mL) was prepared, poured at 60 ºC into sterile petri plates and mixed with 2 mL of *C. aurantium* EOs to obtain final concentrations from 0.001 nL/mL to 1 µL/mL of the extracts. Moreover, all plates were incubated (Memert incubator (JSGI-250DT –JSR)) on a solidified surface of an agar plate with 100 µL of 10^6–10^7 CFU/mL bacterial suspension and spread using a sterile glass swap at 37 ºC for 24 h. Six replicates were run for each concentration. Briefly, the number of bacterial colonies was used to calculate the percentage bacterial inhibition according to equation 3:

$$\text{growth inhibition}\% = \frac{CFU_b - CFU_t}{CFU_b} \times 100\% \quad (3)$$

CFU_b: CFU of a blank sample plate
CFU_t: CFU of a plate containing EO.

The antibacterial activity of the EOs was determined by estimating the IC_{50} value from the percentage of bacterial inhibition curves versus EO concentration.

Statistical Analysis

Statistical analysis was performed by an IBM® SPSS® statistics program (Version 22.0.0.0) and expressed as a mean ± standard deviation. All experiments were conducted in six replicates. Differences were considered statistically significant if $p < 0.05$.

Results and Discussion

EOs Chemical composition

Tables 2 and 3 represent, respectively, the yield and chemical composition of extracted EOs for the five ripening stages. It can be seen from Table 2 that the yield of EO increases with increased maturation. Table 3 shows the main constituents of EOs during the five maturation stages (R1, R2, R3, R4, R5). Although there is a significant change in composition depending on the maturation stage, the predominant constituent in all maturation stages was limonene (98.6%). The analysis also showed the presence of 7 compounds (limonene, linalool, delta 3-caren, β-ocimene (E), β-myrcene, sabinene and α-pinene). The EOs are 99.8% monoterpenes (which includes 0.2% oxygenated monoterpenes). These compounds are important to protect against mutations. Also, previous studies have approved that these compounds have the ability to inhibit free radicals as antioxidants [14].

Table 2. Effect of *C. aurantium* maturation stage on the yield of peel EOs.

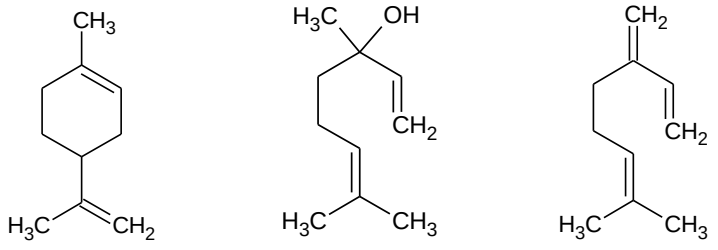
EOs	Yield (%)
R1	0.65 ± 0.09^{a}
R2	0.83 ± 0.12^{b}
R3	1.05 ± 0.18^{c}
R4	1.44 ± 0.03^{d}
R5	1.68 ± 0.05^{e}

a,b,c,d,e values differ significantly from each other according to the Bonferroni test ($p < 0.05$).

Table 3. Peel EOs composition during *C.aurantium* maturation stages

No.	Time (min)	Compounds	RI_{cal}*	RI_{ref}**	Area %				
					R1	R2	R3	R4	R5
1	10.0814	**α-pinene**	0928	0932	0.1975	0.1591	0.1461	1020.1	0.1054
2	12.1041	**sabinene**	0969	0969	0.0168	0.0070	0620.0	0.0055	0.0026
3	13.1428	**β-myrcene**	0990	0988	0.7511	1.1636	1.2694	1.3140	1.1909
4	13.8693	**delta 3-caren**	1005	1008	0.0334	0.0304	0.0485	0.0176	0.0098
5	15.1997	**limonene**	1027	1024	98.8634	98.5902	98.2824	98.3727	98.6338
6	16.2995	**β-ocimene (E)**	1046	1044	0.0282	0.0167	0.0428	0.0294	0.0172
7	17.7794	**γ-terpinene**	1073	1054	0.0026	---	---	---	---
8	17.9152	**m-cymenene**	1076	1082	0.0026	---	---	---	---
9	19.4766	**linalool**	1094	1095	0.0778	0.0330	0.1908	0.0861	0.0432
10	25.0095	**dihydrocarvone**	1191	1191	0.0089	---	0.0044	0.0034	---
11	26.048	**β-cyclocitral**	1208	1217	0.0032	---	---	---	---
		total identified			≈99.97	≈100.00	≈100.00	≈100.00	≈100.00

RI_{cal}*: calculated retention indices, determined on HP- 5MS column, using the homologous series of *n*-alkanes (C_8–C_{22}) standard; RI_{ref}**: Reference retention indices on a polar DBS capillary column (literature Adamsc DB5)[20].



Limonene **linalool** ***β*-myrcene**

Figure 3. Components of monoterpenes in EOs.

The components of extracted EOs differed with the ripening stages, so there were also differences in their biological effectiveness. An important result of this research work is the presence of limonene in abundance (about 98.6%), which is much higher compared with a previous work in which the proportion of limonene does not exceed 91.1%[21]. These differences in the proportion of limonene can be attributed to the nature of the habitat and the extraction method applied.

Antioxidant Activity

Table 4 shows the IC_{50} values for the free radicals inhibitory capacity of *C. aurantium* peel EOs. The results showed that different maturation stages have statistically significant different HPPD IC_{50} values, with the EO of maturation stage R1 exhibiting the best inhibition effectiveness with a minimum IC_{50} value of 83.48 ± 0.01 mg/mL. The observed difference in antioxidant effectiveness may be attributed to the difference in EO chemical composition. On the other hand, this difference in activity can be explained by the presence of synergistic actions between the main constituent, limonene, and the other components. With this respect, we note that sabinene, α-pinene and dihydrocarvone have their highest concentrations in R1 and lowest in R5, and that γ-terpinene, *m*-cymenene and β-cyclocitral are present in R1 while they are absent in the EOs of other maturation stages. Obviously, the differences in these concentrations do affect the antioxidant effectiveness.

Our results indicate that the extracted EOs studied in this work have better antioxidant effectiveness than those studied previously, where the DPPH inhibition effectiveness was almost constant at 37% at EO concentrations in the range of 300–3000 mg with limonene percentage of 98%[22].

Table 3. DPPH IC_{50} values of *C. aurantium* peel EOs.

Sample	IC_{50} (mg/ml)
R1	83.48 ± 0.01^{a}
R2	95.51 ± 0.01^{b}
R3	102.59 ± 0.02^{c}
R4	125.87 ± 0.05^{d}
R5	147.16 ± 0.05^{e}
Vit. C	8.92 ± 0.05 µg/mL

Different letters a, b, c, d, and e indicate significant differences in IC_{50} values for R1, R2, R3, R4, and R5 extracts of EO.

Antibacterial Activity

The antibacterial activity of obtained EOs is summarized in Tables 5 and 6, where gentamicin is used as a reference. The bacteria inhibition efficacy depends obviously on the concentration of EO as well as on the bacterial strain type. However, the EO of unripe *C. aurantium* peel (R1) had higher efficacy than that of ripe *C. aurantium* peel (R5), apparently because of the higher concentrations of sabinene, α-pinene, dihydrocarvone and delta 3-caren in R1, compared with R5. Moreover, synergistic action is believed to be present between limonene and the other compounds. Table 5 shows that the EO of R1 effectively inhibited bacterial growth of *S. aureus* and *E. coli* at a minimum inhibitory concentration (MIC) of 0.0035 nL/mL and 0.0900 nL/mL, respectively.

Table 5. MIC of EOs against *S. aureus and E. coli*.

Sample	MIC	Bacteria	
		S. aureus	***E.coli***
Gentamicin[23]	(µg/mL)	0.125	0.25
R1		0.0035 ± 0.0011^{a}	0.0900 ± 0.0023^{e}
R2		$0.0075 \pm 0.0020^{a,b}$	0.1775 ± 0.0035^{f}
R3	(nL/mL)	0.0100 ± 0.0055^{b}	0.2525 ± 0.0188^{g}
R4		0.0250 ± 0.0112^{c}	0.5550 ± 0.0218^{h}
R5		0.0370 ± 0.0223^{d}	0.7100 ± 0.0355^{i}

Different letters (a, b, c, d, e, f, g, h, i) indicate significant differences, while (ab) indicates no significant difference than a or b in the MIC values for R1, R2, R3, R4 and R5 extracts of EOs.

Table 6. IC_{50} of EOs against *S. aureus and E. coli*.

	Sample	Bacteria	
		S. aureus	***E.coli***
IC_{50} (nL/mL)	R1	00.750 ± 0.050^{a}	012.525 ± 0.060^{f}
	R2	07.250 ± 0.080^{b}	020.500 ± 0.100^{f}
	R3	12.125 ± 0.110^{c}	050.600 ± 0.125^{g}
	R4	30.200 ± 0.250^{d}	150.250 ± 0.350^{h}
	R5	50.400 ± 0.500^{e}	250.500 ± 0.750^{i}

Similar letters indicate no significant differences, while different letters indicate significant differences in IC_{50} values for R1, R2, R3, R4 and R5 extracts of EOs.

Conclusion

In this work, the chemical composition of Syrian *C. aurantium* peel EOs obtained over five ripening stages was studied for the first time. The study showed that the greatest yield of EO (1.68 ± 0.05 ml / 100 g) was obtained for the peel of the ripe *C. aurantium* L. fruit (R5). Moreover, the antibacterial efficiency of obtained EOs was determined by the agar dilution method against the Gram-positive *Staphylococcus aureus* bacteria and the Gram-negative *Escherichia coli* bacteria. The EO of unripe fruit peel in the R1 stage was thereby the most active EO with MIC values of 0.0035 ± 0.0011 and 0.0900 ± 0.0023 nL/mL for *S. aureus* and *E. coli*, respectively. These values were much lower than those of the EO of ripe fruit peel (R5) which were equal to 0.0370 ± 0.0223 and 0.7100 ± 0.0355 nL/mL against *S. aureus and E. coli*, respectively. Based on previous experiments, it is believed that monoterpenes are the reason of biological efficiency.

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Conflicts of Interest

The authors declare no conflict of interest.

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