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Chemical composition of *Citrus aurantium* L. leaves and zest essential oils, their antioxidant, antibacterial single and combined effects

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ABSTRACT

This study aims to investigate the chemical composition, the single and the combined antibacterial effect of zest and leaves Citrus aurantium L essential oils. The identification of chemical compounds was made using chromatography analysis. Furthermore, the antibacterial effect of each essential oil was evaluated using disk-diffusion and the micro-dilution methods against four bacteria strains. While, the antioxidant activity was studied by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. The checkerboard method was used to investigate the antibacterial combined effect of both essential oils. Results showed that the main components are linalyl 2-aminobenzoate (41.87%), β-linalool (32.99%) and α-terpineol (10.53%) for leaves essential oil. However, the major compound was limonene (98.11%) for zest essential oil. Regarding the DPPH radical scavenging activity, the zest essential oil showed higher activity than that of leaves. While, the single antibacterial effect of leaves essential oil was more effective. Moreover, the antibacterial combined effect of both essential oils was demonstrated a remarkable synergistic interaction at the optimal point (1/4 MIC + 1/128 MIC of leaves and zest essential oils respectively). Furthermore, no antagonistic effect was detected between these essential oils.

Keywords: Essential oils, *Citrus aurantium*, Chromatography analysis, DPPH radical scavenging activity, antibacterial effect

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INTRODUCTION

Bacterial resistance to antibiotics has increased recently to a dramatic extend, which protect themselves by developing antibiotic resistance mechanisms [1]. Currently, there are increasing problems worldwide with multidrug-resistant (MDR) bacteria, some of which are capable of resisting all the available antibiotics. These problems are especially evident within hospitals, which have become ‘’hotbeds’’ for highly resistant pathogens and where treatment failures are already taking place in patients with ordinary infections, increasing the risk that hospitalization kills instead of cures[2].

Consequently, this dramatic rise of antibiotic resistance gave good reasons for the scientific community to take many measures, which led to the search for new antimicrobial agents, mainly among plant natural products, in order to find new chemical compounds to overcome the undesirable side effects of some antibiotics [2].

Citrus aurantium L. (*Rutaceae*), commonly known as sour orange, bitter, bigarade, or Seville orange, is generally consumed as marmalade in Mediterranean countries and is used as a flavoring agent [3]. In Haiti, was found to be used medicinally to treat colds, fevers, hepatic disorders, gall bladder problems, rheumatism, epilepsy, emotional shock, bruising internally and externally, skin blemishes and digestive problems[4].

It was reported that volatile compounds from *Citrus* zest exhibit an antifungal activity correlated to monoterpenes and sesquiterpenes content [5]. Anxiolytic and sedative effects have been also reported for both *C. aurantium* extracts and essential oil [6]. Moreover, flavonoids from *Citrus* have been reported as effective cytostatic anticancer agents [7]. The antimicrobial activity of *Citrus* fruit essential oils from three species *C. sinensis*, *C. lemon* and *C. reticulata* were demonstrated [8]. The antioxidant effect was also demonstrated for many *Citrus* essential oils [9].

In recent years, several researchers have focused on drug combinations as a novel approach in controlling resistant pathogens [10]. Therefore, the mixture of essential oils can lead to widen the susceptibility of bacteria. With this in mind, we proposed for this study, firstly the screening of the antibacterial and antioxidant activity of *Citrus aurantium* essential oils, secondly and for the first time, the investigating of the combined antibacterial effect of essential oils obtained from zest and leaves of *Citrus aurantium* L harvested in the National Institute of Medicinal and Aromatic Plants (Morocco).

EXPERIMENTAL SECTION

2.1. Essential Oils Extraction

Zest of fruit and leaves of *Citrus aurantium* were collected from the garden of National Institute of Medicinal and Aromatic Plants (Morocco). The vegetal material were subjected to hydro-distillation for 3 hours using a Clevenger apparatus. The essential oils obtained were stored at + 4°C in dark until use.

2.2. Gas Chromatography / Mass Spectrometry (GC/MS) Analysis Conditions

The essential oils were analyzed by GC/MS. The gas phase chromatographic analyzes were carried out using a Trace GC Ultra instrument equipped with an injector in split mode and a DB-5 column (length 30 m, internal diameter: 0.25 mm, thickness film: 0.25 mm). The flow rate of the carrier gas (helium) was 1.4 mL/min. A sample of 1 µL (solvent: ethyl acetate) was injected, using split mode with an injection temperature of 220 °C. The column temperature was programmed from 40 to 180 °C at a rate of 4 °C/min with a hold of 20 min at 30°C. Coupling with the mass spectrometer Polaris Q MS result with interface temperature 30 °C. The operating conditions are as follows: Type of EI ionization (70 eV), source temperature of ionization 200 °C, ion trap detector. The composition of the essential oils was reported as a relative percentage of the total peak area. The identification of the constituents was performed using NIST MS Search database.

2.3. DPPH Radical Scavenging Assay

The ability of *C. aurantium* essential oils to scavenge free radicals was assayed with the use of a synthetic free radical compound 1,1-diphenyl-2-picrylhydrazyl (DPPH), according to the method employed by [11]. Briefly, essentials oils were serially diluted (0.5, 1, 2.5, 10 and 20 mg/mL (w/v)) in methanol. A solution of DPPH (0.004% (w/v)) was prepared in the same solvent. Then 3 mL of each dilution were mixed with 3 mL of DPPH solution. The mixtures were kept in the dark for 30 minutes and the optical density was measured at 517 nm. Butylhydroxytoluene (BHT) was used as standard. Each test was performed in triplicate.

The antioxidant activity was calculated as follows:

$$AA\% = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} * 100$$

AA: antioxidant activity
Abs: absorbance

2.4. Antibacterial Activity

2.4.1. Bacterial strains and Inoculum Preparation

The *in vitro* antibacterial effect of essential oils was tested against the following bacterial strains: *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 3366, *Staphylococcus* aureus ATCC 29213, *Escherichia coli* ATCC 25922. All of these strains were maintained in 20 % glycerol at - 20 °C and sub-cultured before use. The bacteria were inoculated in the nutrient agar (NA) at 37 °C for 24 h. 2-3 colonies were peaked up by wire loop aseptically into physiologic saline solution. The turbidity was adjusted to 0.5 McFarland scale prepared according to the protocol previously described [12].

2.4.2. Disk-Diffusion Method

A primary antibacterial screening was performed using the disk-diffusion method according to [12]. Briefly, Petri dishes containing Mueller Hinton Agar (MHA) culture medium were inoculated with the bacterial inoculum previously prepared. The disks (filter paper, 6 mm of diameter) placed in the center of each plate were impregnated with 10 µL of each essential oil. Petri dishes were placed at 4 °C for 2 h to allow a better diffusion of molecules. After were incubated at 37 °C for 18-20 h. The diameters of the inhibition zones were measured in (mm). Each assay was carried out in triplicate.

2.4.3. Determination of Minimal Inhibitory Concentration (MIC)

The determination of MIC was performed in 96 well-microplate using the micro-dilution assay according to the protocol previously described [13], with slight modifications. Firstly, each essential oil was serially diluted in Mueller Hinton broth (MHB) supplemented with agar 0.15% (w/v), used as an emulsifier. The 12^{th} well was considered as growth control. Then, 50 µL of bacterial inoculum were added to each well at a final concentration of 10^6 CFU/mL. After incubation at 37° C for 18-20 hours, 10 µL of rezasurin were added to each well as bacterial growth indicator. After further incubation at 37 °C for 2 h, the bacterial growth was revealed by the change of coloration from purple to pink. The MIC value was determined as the lowest concentration that prevented a change in resazurin color. Experiments were carried out in triplicate.

2.4.4. Determination of Minimal Bactericidal Concentration (MBC*)*

The MBC value corresponded to the lowest concentration of essential oil yielding negative subcultures after incubation at 37 °C for 24 h. It was determined by spreading 5 µL from negative wells on Luria Bertani (LB) plates. Experiments were carried out in triplicate.

2.4.5. Determination of Fractional Inhibitory Concentration (FIC)

The antibacterial combined effect of *C. aurantium* leaves and zest essential oils was evaluated using the checkerboard method [14]. The concentrations of both essential oils were prepared in MHB supplemented with bacteriological agar (0.15% w/v). Along the x-axis across the checkerboard plate, 50 µL of each essential oil concentration were added into each well from the first to the 11^{th} well. As for the y-axis, 50 µL of each *Citrus* essential oil concentration were added into each well. The 12^{th} –A well was considered as growth control.

Bacterial inoculum was then added into all of the wells at a final concentration of 10^6 CFU/mL. The 96-well plate was then sealed and incubated at 37 °C for 18-20 h. After incubation, 10 µL of resazurin were added to each well to assess bacterial growth. And after further incubation at 37°C for 2 h, the FIC index values were calculated using the following formula:

$$\sum \text{FICI} = \text{FIC(A)} + \text{FIC(B)}$$

Where

$$\text{FIC (A)} = \frac{\text{MIC (A) in combination}}{\text{MIC (A) alone}}$$

And

$$\text{FIC (B)} = \frac{\text{MIC (B) in combination}}{\text{MIC (B) alone}}$$

The ∑ FICI values are interpreted as follows: ≤ 0.5= synergistic; 0.5-0.75 = partial synergy; 0.76-1.0 = additive; >1.0-4.0 = indifferent (non-interactive); > 4.0 = antagonistic.

RESULTS AND DISCUSSION

3.1. Chemical Composition of the Citrus Essentials Oils

Extraction yields of 0.8 and 1.85% (v/w) were obtained from *C.aurantium* leaves (CaL) and *C.aurantium* zest (CaZ) respectively. As shown in table 1, sixteen volatile compounds accounting for 99% of CaL essential oil were detected. The main components are linalyl 2-aminobenzoate (41.87%), β-linalool (32.99%) and α-terpineol (10.53%) which presents 85.39% of total volatile constituents detected. Similar chemical composition has been previously found for this essential oil, especially for the content of β-linalool and α-terpineol [15].

However, as shown in figure 1, the composition of CaZ essential oil presents only three compounds corresponding to 98.11% of the volatile constituents were detected. Limonene was the major compounds with a percentage of 96%.

Sabinene and delta-3-carene were found with a percentage of 0.45 % and 1.43 % respectively. These results are in agreement with those obtained in other studies [16], [17].

Table1. Chemical composition of *C. aurantium* leaves essential oil obtained by GC/MS analysis

Retention time	Compounds	Area %
6.5251	Sabinene	0.1226
6.9596	β-pinene	1.9247
8.2872	Limonene	0.3614
8.4723	(E)- β-Ocimene	0.339
8.8477	(Z)-β-Ocimene	0.7267
10.2933	α-Carene	0.096
10.8726	β-Linalool	32.9918
14.1258	Terpinen-4-ol	0.1166
14.7534	α-Terpineol	10.5356
15.9442	Nerol (cis-geraniol)	1.5577
17.0224	Linalyl 2-aminobenzoate	41.8797
17.7814	Geranial (α-Citral)	0.1489
19.2189	O-Cymen-5-ol	0.4648
21.6246	Geraniol butyrate	2.8847
22.4560	Geranylisobutyrate	5.6753
24.0518	Trans-γ-Caryophyllene	0.1744
Total		99.99

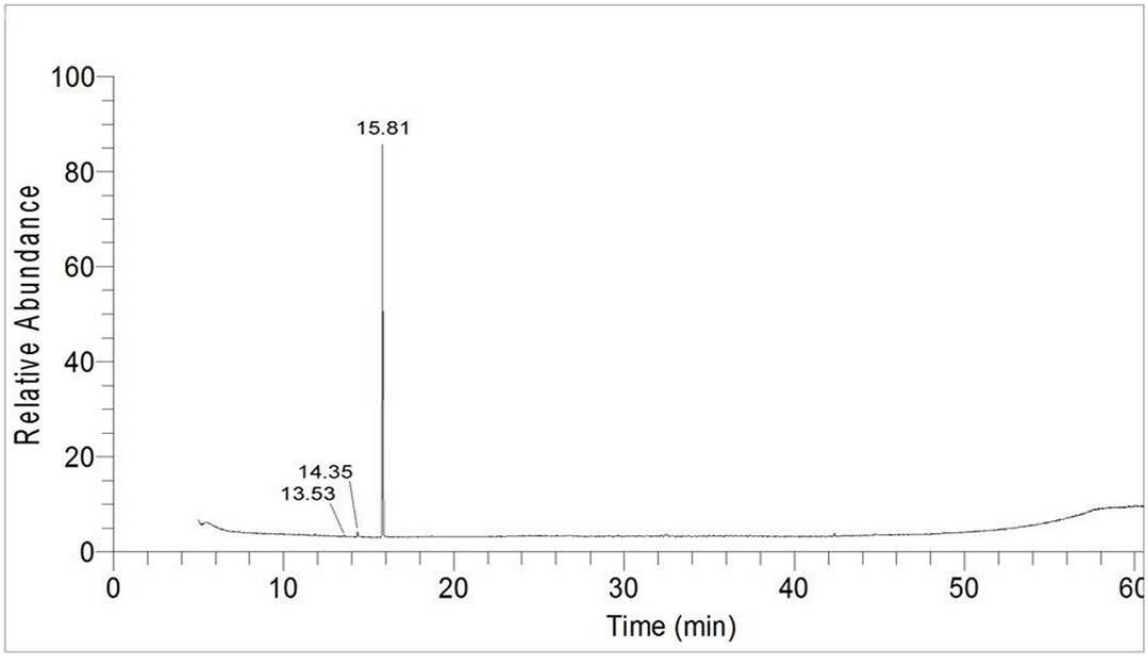

Fig.1. Chromatogram profile of *C. aurantium* zest essential oil obtained by GC/MS analysis

3.2. Antioxidant activity of Citrus essential oils

The assessment of the antioxidant activity of natural products *in vitro* can be performed with different methods. Therefore, DPPH radical scavenging assay was used in this study. The results were compared with the synthetic antioxidant BHT.

As shown in figure 2, CaZ essential oil exhibited a high antioxidant activity compared to that of leaves, with an IC50 respectively of 26.99 mg/mL and 496.2 mg/mL. Furthermore, the BHT was exhibit the greater radical scavenging activity compared to the both essential oils studied. However, the synthetic antioxidants have fallen out of favor because of their carcinogenicity [18]. Therefore, these essential oils particularly that of zest, are a promising alternative which makes them important in health maintenance and disease protection.

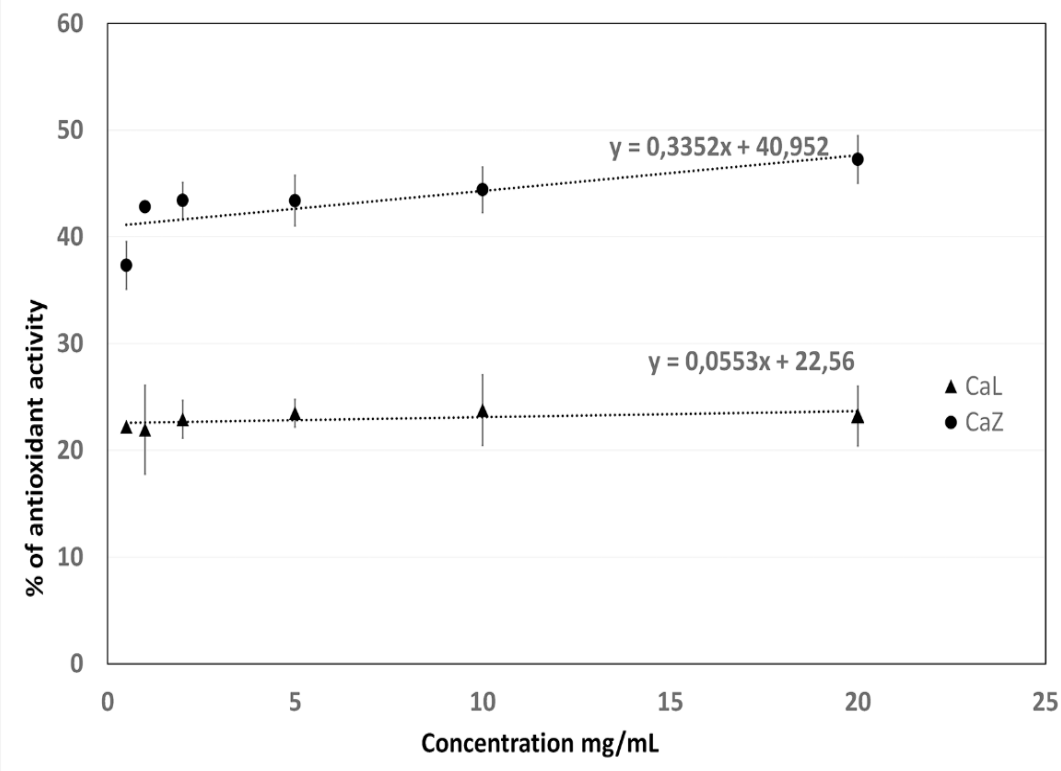

Fig. 2. DPPH radical scavenging activity of *C. aurantium* zest and leaves essential oils

3.3. Antibacterial activity of essential oils

The antibacterial activity of the *Citrus* essential oils was evaluated by disk-diffusion and microdilution methods. The CaL essential oil showed strong antibacterial effect against *B. subtilis*, *S. aureus* and *E. coli* as shown in Table 2. It was highly effective against Gram positive bacteria. However, CaZ essential oil was exhibit an inhibitory effect only against *B. subtilis*, theother strains were found resistant to this essential oil (Table 2). Moreover, *P. aeruginosa* was found as the more resistant strain against both essential oils. Regarding the findings of CaL essential oil, a quite similar results were also reported by other authors[15]. This activity could be due to their major compounds, linalyl 2-aminobenzoate with its ester function, β-linalool and α-terpineol with their alcohol function, known for their antimicrobial activity [19] [20].

The moderate antibacterial activity of CaZ essential oil could be explained by the weak antibacterial effect of its major compound (limonene), which is previously reported by [21]. While, it was reported by the same authors that linalool possess a higher antibacterial effect compared to limonene, which explain the effectiveness of CaL essential oil demonstrated by our study.

Table 2 Antibacterial activity of *C. aurantium* leaves and zest essential oils

	Antibacterial screening Inhibition zone (mm)		Minimal inhibitory concentration (% v/v)		Minimal bactericidal concentration (% v/v)	
Essential oil	Leaves	Zest	Leaves	zest	Leaves	zest
S. aureus	12.33±1.52		1		>2	
B. subtilis	14.33±1.52	11.33±2.3	1	2	1	2
E. coli	8±1		2		>2	
P. aeruginosa	-	-	-	-	-	-

3.4. Antibacterial combined effect of *Citrus* essential oils

Bacillus subtilis was identified as the more sensitive strain to both *Citrus* essential oils. In order to amplify this sensitivity, the evaluation of antibacterial combined effect of these essential oils was carried out following the checkerboard method.

As shown in Table 4, FIC index values of the combined application of both *Citrus* essential oils ranged from 0.25 to 1.12. Also as can be noted from this Table, the combination (1/4 MIC CaL + 1/128 MIC CaZ) was displayed a remarkable synergistic effect against *B. subtilis* with a FIC index of 0.25. Furthermore, the combination (1/2 MIC

CaL + 1/256 MIC CaZ) was exhibited an inhibitory activity toward the strain tested with a FIC index of 0.5039, indicating a partial synergistic interaction. The other combinations stayed the inhibitory effect with a FIC index of 1.125 and 1.06 indicating an indifferent outcome, which is defined as absence of interaction [22]. In other word, all of the combined applications tested between both essential oils did not display any antagonism interaction. It is imperative to emphasize that the optimal combination reduces the MIC value of CaL essential oil to one quart, which is an important aspect of valorization of this essential oil obtained by low yield compared to that of zest.

Table 3 Determination of FIC, FIC index and outcome of interactions of *C. aurantium* leaves and zest essential oils combination against *B. subtilis* ATCC 3366

Essential oil	MIC % (v/v)		FIC % (v/v)	FICI	Outcome
	Alone	In combination			
Leaves	1	0.5	0.5	0.5039	Partial synergy
Zest	2	0.007813	0.0039		
Leaves	1	0.25	0.25	0.257	Synergy
Zest	2	0.015625	0.007813		

The antibacterial combined effect of *C. aurantium* zest and leaves essential oils has not been reported previously. While, Sonboli et al. [21] reported that linalool exhibit high antibacterial effect compared to limonene and that these two compounds have no antagonistic interaction, which are in agreement with our findings. The synergistic effect between both essential oils studied could be explained by the capacity of hydrocarbons monoterpenes, to facilitate the penetration of the oxygenated monoterpenes (that of CaL essential oil) into the cell by interacting with the cell membrane [23]. Furthermore, other reports showed that combinations including monoterpene hydrocarbon (α-pinene) with limonene or linalool showed additive and synergistic effects [24], [25].

CONCLUSION

This study revealed that essential oil of *C. aurantium* leaves exhibited high single antibacterial effect against bacterial strains tested, except *P. aeruginosa* which resist to both essential oils studied. This remarkable activity is due to several majors components particularly linalool and α-terpineol. Moreover, the combined effect of the essential oil obtained from the zest and that obtained from leaves of *C. aurantium* against *B. subtilis* has demonstrated a remarkable synergistic effect by the combination (1/4 MIC CaL + 1/128 MIC CaZ). This optimal combination offer huge potential as alternative phytotherapy against bacteria at suitably low concentrations and lead to new research on the morphological and ultrastructural analysis of strains treated.

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