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Chemical Characterization and Biological Activities of *Citrus reticulata* and *Citrus aurantium* Essential Oils

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Abstract

This study was conducted to evaluate the chemical composition, antimicrobial and antioxidant activities of *Citrus reticulata* and *Citrus aurantium* essential oils (EOs). The chemical composition was determined by gas chromatography-mass spectrometry (GC/MS). Monoterpene hydrocarbons were found to be the main components. Antibacterial activity was evaluated against *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*. The Gram-positive bacteria *Staphylococcus aureus* was more sensitive than the Gram-negative bacteria. Antifungal properties were determined against *Aspergillus* and *Penicillium* species. The best results were obtained by *Citrus reticulata* and proved to be the most effective inhibitor of all tested fungi. In addition, the screening of the antioxidant activities showed that *Citrus aurantium* was more effective in scavenging DPPH radicals. A reduction of the IC50 values was also noticed when the EOs were used in combination. According to these findings, the tested EOs could be used as a natural source of new compounds for the treatment of fungal and bacterial infections.

Keywords: Antifungal, Antibacterial, Antioxidant, *Citrus*, GC/MS

1 Introduction

Medicinal plants and their extracts are a rich source of bioactive compounds. Among natural plant products, essential oils (EOs) are of particular concern because of their biological activities. EOs are complex mixtures of secondary metabolites, produced by aromatic plants. They are responsible for the odor of plants, although they have other functions such as attracting pollinator insects and protecting plants from microorganisms. They are also known to possess several biological properties including antiseptic, antiviral, antioxidant, anti-parasitic, antifungal, and antibacterial activities [1]. These oily liquids usually obtained by hydrodistillation and steam distillation, are mainly composed of monoterpenes, sesquiterpenes, and their oxygenated derivatives: alcohols, aldehydes, esters, ethers, ketones, phenols, and oxides [2].

Bacterial species have emerged as important opportunistic pathogens responsible for many nosocomial infections. Antibiotic-resistant bacteria are one of the biggest public health challenges of our time [3]. Humans and plants are also susceptible to fungal infections by pathogenic fungi. It has been estimated by the Food and Agricultural Organization (FAO) that, worldwide, about 25% of agricultural crops are affected by molds and their toxins [4]. Synthetic antibiotics and fungicides are known to be effective; however, their continuous use led to increasing resistance to pathogens and limited the efficacy of treatments. Therefore, the use of alternative strategies is needed to maintain public health.

Several compounds of EOs like carvacrol, thymol, menthol, fenchol, borneol, and citronellol demonstrated a noticeable inhibition of fungal growth and mycotoxins production [5]. Furthermore, various studies have shown that EOs are a rich source of antioxidants. The production of reactive oxygen species has been implicated in the development of various chronic and degenerative diseases (cancer, atherosclerosis, malaria, rheumatoid arthritis, neurodegenerative diseases, and aging effects) and the deterioration of food products [6]. The use of EOs as natural antioxidants is regarded as a safe alternative to synthetic ones. Therefore, the present study was carried out to characterize the chemical composition of *Citrus reticulata* and *Citrus aurantium* EOs produced in Algeria and evaluate their efficiency as antimicrobial and antioxidant agents.

2 Materials and methods

2.1 Essential oils

Two commercial EOs isolated from aerial parts of the plants by steam distillation were used in the present work. *Citrus reticulata* and *Citrus aurantium* EOs were purchased from a local producer and stored at +4 °C in the dark until analysis and further use.

2.2 GC/MS analysis

The GC-MS analysis was carried out using a Hewlett-Packard Agilent 6890 plus (Agilent Technologies, USA), with an HP-5MS column (30m × 0.25 mm i.d. × 0.25 µm film thickness). The temperature of the injector was held at 280°C. The oven temperature was maintained at 60°C for 8 min and then increased to 250°C at 2°C/min and remained constant at this temperature for 10 min. The carrier gas was helium with a constant flow rate of 0.5 mL/min. An injection volume of 2 µL was diluted in n-hexane (1:10) and split mode 1/80 was

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used. The MS was operated in the electron impact (EI) mode using an ionization voltage of 70 eV and a source temperature of 230°C. The retention indices (RI) of the separated volatile compounds were calculated regarding the retention time of a series of n-alkanes (C8–C28) as external standards, obtained under the same chromatographic conditions. The components were identified based on the comparison of the component mass spectra with those in the library National Institute of Standards and Technology (NIST) and of their retention indices with data from the literature [7]. The relative contents of each compound in the EOs were evaluated from the total chromatogram and were obtained as the percentages of a peak area.

2.3 Antioxidant activity

2.3.1 DPPH free radical scavenging assay

The antioxidant activity was assessed as described by Sahin et al. [8] with some modifications. Briefly, 0.5 mL of different concentrations of EOs in ethanol were mixed with 1.5 mL of DPPH ethanolic solutions (0.004%). A blank was prepared in the same conditions by replacing the EO with methanol. Ascorbic acid was used as a positive control. The mixtures were well shaken and kept at room temperature in the dark for 30 min. The absorbance was measured at 517 nm using a spectrophotometer. The radical scavenging activity was calculated according to the following formula:

$$\% \text{ Free radical scavenging} = (A_{blank} - A_{sample})/A_{blank} \times 100$$

where A_{sample} is the absorbance of DPPH solution after reacting with a given concentration of EOs and A_{blank} is the absorbance of DPPH solution with ethanol. The IC50 value was calculated to determine the concentration of the sample required to inhibit 50% of radical. IC50 values of each extract were calculated from the graph by plotting inhibition percentage against oil concentration.

2.3.2 Determination of antioxidant combination index (CI)

To investigate the possible interaction between the different EOs, the classical isobologram-combination index equation (CI) based on the median effect principle (IC50) was used for analyzing the data [9]. In this study, extracts were paired at 1:1 ratio.

$$CI = (D)1/(Dx)1 + (D)2/(Dx)2$$

where (D)1 and (D)2 are the doses (IC50 values) of two active EOs in combination; (Dx)1 and (Dx)2 are the doses (IC50 values) of two active EOs individually. Based on CI values, the type of antioxidant interactions was interpreted as follows: CI < 1: synergistic; CI = 1: additive; CI > 1: antagonistic.

2.4 Microorganisms

Four reference standard bacterial strains including three species of Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC27853, *Proteus vulgaris* ATCC13315, *Escherichia coli* ATCC 25922), one Gram-positive bacteria (*Staphylococcus aureus* ATCC25923); and six fungal strains (*Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus fumigatus*, *Aspergillus carbonarius*, *Aspergillus tamarii*, *Penicillium* sp.) were used in this study. All strains mentioned above were obtained from the microbial culture collection of the faculty of natural sciences, Algiers (Algeria). The fungal strains were isolated from oil seeds and identified following morphological and biochemical characterization during a mycological survey [10].

2.5 Inoculum preparation

The bacterial strains were cultured on nutrient agar for 48 h at 37°C. Suspensions were prepared in a saline solution (0.85% NaCl) and adjusted to a turbidity of 0.5 McFarland. Fungal pathogens were cultured on Potato Dextrose Agar (PDA) medium. Spores were harvested from 10 days-old cultures in 10 mL sterile distilled water. The spore suspension was adjusted to the desired concentration (10^6 spores per mL) by a spectrophotometer.

2.6 Antibacterial activity

Antibacterial activity was evaluated using the agar well diffusion method on Mueller-Hinton Agar (MHA). Plates were inoculated with bacterial suspensions. Then, two wells were made using a sterilized cork borer into each agar plate. Different concentrations (10 or 30 µL) of EOs were transferred into the wells. Antibacterial activities were determined after 24 hours of incubation at 37°C by measuring the inhibition zone diameters reported in millimeters (mm). Gentamicin (concentration of 10 mg), was included as a positive control. Three replicate trials were conducted for each assay.

2.7 Antifungal activity

2.7.1 Poisoned food technique

The antifungal activity of the EOs was undertaken by the poisoned food (PF) method using Czapek Yeast Agar (CYA) medium. The requisite amount of EO was dissolved in 0.5 mL acetone and added to 9.5 mL molten CYA to achieve final concentrations of 2 µL/mL. Plates containing CYA and acetone (0.5 mL) only served as negative controls [11]. After solidification, plates were inoculated with mycelial discs (6 mm diameter) from 7-days-old cultures and incubated for 7 days at 25°C. Fungal inhibition was recorded as mycelial diameter reduction over time. The plates were used in triplicate for each treatment and the percentage of inhibition (%) was calculated as follows [12].

$$\text{Inhibition (\%)} = (1 - Da/Db) \times 100$$

where: Da = Average diameter of the fungal colony in treatment and Db = Average diameter of the fungal colony in control.

2.7.2 Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined by the broth dilution method [13]. Different concentrations of EOs were prepared by two-fold serial dilutions in acetone. EOs dilutions were mixed with sterile Potato Dextrose Broth (PDB) to obtain final concentrations ranging from 4 to 0.03 µL/mL. A 100 µL spore suspension (10^6 conidia/mL) of each test strain was inoculated in all tubes containing PDB medium and incubated for 7 days at 25°C. The control tubes containing PDB medium were inoculated only with the fungal suspensions. The MIC was defined as the lowest concentration at which no visible growth was observed.

2.8 Statistical analysis

All experiments were performed in triplicate and the results reported are represented as the mean ± standard deviation.

3 Results and discussion

3.1 Chemical composition of essential oils

The chemical composition of the EOs was investigated by GC/MS. The main components and their percentage values are presented in Table1. The analysis of *C. aurantium* EO revealed the presence of monoterpene hydrocarbons; limonene (65.3%), β-pinene (10.45%), and β-caryophyllene (8.45%) representing together 84.2% of the total EO composition. The EO of *C. reticulata* was constituted mainly of monoterpene hydrocarbons and sesquiterpene hydrocarbons β-pinene (22.53%), α-humulene (20.92%), linalool (10.96%), limonene (8%) and α-terpinene (5.39%).

The composition of *C. aurantium* and *C. reticulata* EOs has been previously reported in the literature. Our findings are similar to those of Majnooni et al. [14], who reported limonene, linalool, and *trans*-beta-ocimene as major components of *C. aurantium* L. collected in Iran. Oppositely to our results, the analysis of *C. reticulata* EO by Kasali et al. [15], showed some variations, with sabinene, γ-terpinene, p-cymene, δ-3-carene, and (E)-β-ocimene being the major compounds. These variations in essential oils composition are associated with many factors including radiation, humidity, soil condition, temperature, and the extraction method [16]. All these factors are not controlled or known when dealing with commercial samples.

3.2 Antioxidant activity

Free radical scavenging activities of EOs were measured by DPPH assay. The obtained results are summarized in Table 2. The IC50 values of the EOs were compared to a positive control (Ascorbic acid). Ascorbic acid exhibited a potent antioxidant activity with an IC50 value of 15 µg/mL.

The two tested essential oils had antioxidant activity. Based on the IC50 values, *C. aurantium* EO was more effective in scavenging DPPH radicals with an IC50 of 77.6 µg/mL. However, the potential of our samples to inhibit lipid peroxidation remains lower compared to ascorbic acid. The antioxidant activity of plant extracts is majorly due to their chemical composition. The major compounds obtained from our samples were monoterpenoids and several sesquiterpenes. Monoterpenes such as α-pinene and sesquiterpenes are known to have the capacity to neutralize free radicals [17, 18]. In our study, synergistic interactions were observed between *C. aurantium* and *C. reticulata* EOs. Indeed, the EOs were more effective when used in combination. (Table 2).

Table 1: Chemical constituents (%) of *C. aurantium* and *C. reticulata* essential oils.

No.	Compound	RI	*C. aurantium*	*C. reticulata*
1	α-Thujene	925	-	0.34
2	α-Pinene	931	1.2	1.3
3	β-Pinene	973	10.45	22.53
4	β-Myrcene	991	1.23	1.66
5	δ-3-Carene	1009	1.82	0.49
6	α-Terpinene	1023	-	5.39
7	p-Cymene	1025	-	3.79
8	Limonene	1028	65.3	8.01
9	(E)-β-Ocimene	1050	0.97	3.58
10	γ-Terpinene	1059	1.2	3.26
11	*Trans*-Sabinene hydrate	1066	0.12	0.39
12	*α-Terpinolene*	1087	0.31	1.87
13	Linalool	1103	3.23	10.96
14	*cis*-p-Menth-2-en-1-ol	1120	0.25	0.1
15	Terpineol	1134	0.56	0.09
16	*cis*-β-Terpineol	1142	0.16	0.25
17	Citronellal	1153	0.11	1.92
18	Terpinene-4-ol	1176	0.41	1.26
19	α-Terpineol	1189	0.15	0.39
20	*cis*-Piperitol	1194	0.13	0.07
21	Methyl chavicol	1199	0.13	-
22	Citronellol	1218	0.26	0.08
23	Nerol	1230	0.32	0.91
24	Neral	1243	0.22	0.09
25	Linalyl acetate	1257	0.51	0.11
26	Geranial	1273	0.12	0.07
27	Myrthenyl acetate	1327	0.15	0.09
28	δ-Elemene	1340	0.23	-
29	Eugnol	1362	-	0.11
30	Geranyl acetate	1382	0.21	-
31	β-Caryophyllene	1418	8.45	2.44
32	α-Humulene	1445	-	20.92
33	δ-Cadinene	1523	-	0.08
34	Caryophyllene oxide	1580	0.22	0.28
35	α-Bisabolol	1689	-	1.65
36	α-Bisabolol oxide A	1752	-	1.36
	Total identified (%)		98.42	95.84

RI: values of calculated retention indices

Table 2: Antioxidant effects of *C. aurantium* and *C. reticulata* essential oils and their combination.

	IC50 (µg/mL)	CI1=D1/DX1	CI2= D2/DX2	CI=CI1+CI2	Effect
C. aurantium	77.6 ± 0.94	-	-	-	-
C. reticulata	87.6 ± 0.53	-	-	-	-
C. reticulata+ C. aurantium	36.7 ± 0.78	0.41	0.47	0.88	synergistic

Table 3: Diameters of inhibition zones (mm) of the EOs and gentamicin against the selected bacteria strains

EO	*C. aurantium*		*C. reticulata*		Gentamicin
Concentration	10 µL	30 µL	10 µL	30 µL	10 mg
Escherichia coli	-	-	9± 0	25.2±1.2	22±0
Staphylococcus aureus	8 ± 0.5	12.2±0.7	12.4±0.5	21.5±1.4	28±0
Proteus vulgaris	-	-	-	8 ± 0	26±0
Pseudomonas aeruginosa	-	-	8 ± 0	10 ± 0.6	23±0

(-): Resistant strains

In general, when extracts share similar compounds their combination will have an additive rather than a synergistic effect [19]. In our case, *C. aurantium* and *C. reticulata* EOs were characterized by the presence of similar compounds at different concentrations; which may explain their synergistic effect.

3.3 Antibacterial activity

C. reticulata and *C. aurantium* EOs were tested against four bacterial strains. The inhibition zones obtained are shown in Table 3. *C. reticulata* EO presented an interesting antibacterial activity against all strains at a concentration of 30 µL/mL, with inhibition zones varying from 8 mm to 25.2 mm. Gram-negative bacteria showed good resistance to *C. aurantium*. *S. aureus* was sensitive to all EOs even at a concentration of 10 µL/mL, while *Proteus vulgaris* and *Pseudomonas aeruginosa* appeared to be the most resistant strains. In general, the Gram-positive bacteria are more sensitive than the Gram-negative bacteria due to differences in their cell wall composition. The presence of a lipopolysaccharide layer in Gram-negative bacteria limits the permeability of EOs through the outer membrane. The absence of this barrier in Gram-positive bacteria allows direct contact with the plasma membrane and the entry of EOs compounds [20].

3.4 Antifungal activity

The effects of the EOs on mycelial growth after an incubation period of 7 days at 28°C are summarized in Table 4.

Table 4: Mycelial growth inhibition (%) of the essential oils at a concentration of 2 µL/mL after 7 days of incubation.

	C. aurantium	*C. reticulata*
A. flavus	0 ± 0	24 ± 1.1
A.parasiticus	3.9 ± 0.7	7.8 ± 0.9
A.fumigatus	4.4 ± 0	13.3 ± 1.1
A.carbonarius	0 ± 0	9.4 ± 0.9
A. tamarii	10.4 ± 1.3	22.9 ± 1.1
Penicillium sp.	54.1 ± 1.2	73.1 ± 1.9

The obtained results revealed interesting activities of the tested EOs at a concentration of 2 µL/mL. The EOs of *C. reticulata* and *C. aurantium* exhibited a strong antifungal effect against *Penicillium* sp. with mycelial growth inhibition percentages of 73.1% and 54.1%, respectively. The tested EOs were less effective in inhibiting the growth of *Aspergillus* species. The fungal pathogens *A. flavus* and *A. tamarii* displayed a moderate susceptibility to the EO of *C. reticulata* with inhibition rates reaching 24% and 22.9%, respectively. A loss of pigmentation in fungal conidia was also noticed after treatments with EOs. It has been previously reported that pigments are important virulence factors for many pathogenic fungi. Thus, the virulence of the pathogens could be affected by the loss of mycelial pigmentation [21].

In order to determine the MIC of the EOs against fungal isolates, the broth macro-dilution method was used. The results obtained for each EO are shown in Table 5. Acetone as a control did not inhibit the spore germination of the pathogens tested. *Penicillium* sp. showed more sensibility to all EOs in comparison with *Aspergillus* strains. The growth of *A. flavus* and *A. parasiticus* was uniformly inhibited by the tested oils. Besides, *A. fumigatus* was the most resistant strain to the studied EOs.

Table 5: Minimum inhibitory concentration (µL/mL) of essential oils against the fungal strains.

	C. aurantium	*C. reticulata*
A. flavus	0.125	0.03
A.parasiticus	0.125	0.06
A.fumigatus	0.5	0.25
A.carbonarius	0.25	0.06
A. tamarii	0.5	<0.03
Penicillium sp.	<0.03	<0.03

The lowest MIC values were recorded with *C. reticulata*. The chemical analysis of this EO revealed the presence of linalool and limonene. Limonene and linalool are components with low antifungal activity. The strong antifungal activity of *C. reticulata* was probably a result of a synergistic effect between major and minor constituents of the EO [22].

4 Conclusions

Based on our results, the main compounds in the investigated EOs were represented by monoterpene hydrocarbons. The EOs of *C. aurantium* and *C. reticulata* exhibited different degrees of effectiveness when used as antimicrobial and antioxidant agents. *C. reticulata* EO was found to be more effective against all the tested bacterial strains and had an interesting antifungal activity against *Aspergillus* and *Penicillium* isolates. The strongest antioxidant activity was exhibited by *C. aurantium* EO. Besides, a reduction of the IC50 values was observed when *C. aurantium* and *C. reticulata* were used in combination. These findings suggest that these commercial essential oils can be used for their antioxidant and antibacterial properties to control pathogens and as preservatives for stored food products.

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Ethical issue

Authors are aware of and comply with, best practices in publication ethics specifically about authorship (avoidance of guest authorship), dual submission, manipulation of figures, competing interests, and compliance with policies on research ethics. Authors adhere to publication requirements that the submitted work is original and has not been published elsewhere in any language.

Competing interests

None declared.

Authors' contribution

All authors have made substantial contributions to the conception, acquisition of data, or analysis and interpretation of data.

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