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Antioxidant and Antimicrobial Activities of Essential Oils and Methanolic Extracts of Tunisian *Citrus aurantium* L.

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Abstract: *This study was designed to examine for the first time the antimicrobial and antioxidant activities of essential oils and organic extracts of Tunisian Citrus aurantium. Essential oils extracted by hydrodistillation from the peels, leaves and flowers were characterized by GC–MS. It results in the identification of three chemotypes: limonene (87,02 %), linalyl acetate (53,76 %) and linalool (39,74 %), respectively, as the major compounds. Phytochemical screening of methanolic extracts (peels, leaves and flowers) lead to identification of tannins, flavonoids, polyphenols, sterols, polyterpenes and alkaloids. Antioxidant activities of both organic extracts and essential oils were examined using DPPH and ABTS methods. Methanol extracts in particular from the peel showed the highest activity compared with essential oils. The antimicrobial activities were tested by the well-diffusion method. Results showed that the tested bacteria were sensitive only to the essential oils.*

Keywords: *Citrus aurantium L., essential oils, methanolic extracts, GC-MS, phytochemical screening, antioxidant and antimicrobial activity.*

I. INTRODUCTION

Sour orange, bitter orange or *Citrus aurantium* L. is a tree that belongs to the *Rutaceae* family. It is grown mainly in regions where the climate and soil are conducive to its development: Italy, Sicily, Spain, North Africa especially Tunisia, Middle East and America [1].

All parts of the plant contain an essential oil with a characteristic smell [2]." Petitgrain oil" is removed from the leaves. This industry was developed in Tunisia, France, Italy, but especially in Paraguay [3].The main compounds in the petitgrain bigarade oil are in descending order linalyl acetate (50%), linalool (17%) and limonene (4%). From the pericarp of the fruit, essences are also extracted and are used, especially in confectionery and perfumery [4]. These essences are composed by two monoterpenoides: limonene (90 to 97%) and myrcene (1 to 2%) [5]. The flowers are used to make an essential oil known as neroli, which is produced in the Grasse region and around the Mediterranean and is used in aromatherapy, as an ingredient of many perfumes, and also as an ingredient of many skin and hair care products. It contains mainly monoterpenes, oxygenated terpenes and nitrogen which together made up 90% of the total volatile composition of flowers of 15 cultivars studied [6].

Essential oils extracted from different parts of sour orange are equipped with various therapeutic effects: they are anti-infectious, energizing and toning the nervous system. In addition, they improve the venous capillary circulation, nourish the skin and regulate the heart [7]. They also have antimicrobial properties. For their part, Caccioni and *al*. [8], have shown the existence of a good antifungal activity of essential oils of *C. aurantium* L. against *Penicillium digitatum* and *Penicillium italicum*. In addition, a study of the antibacterial activity of essential oils of sour orange has found they are very active against bacteria (*Staphylococcus aureus*, *Enterococcus faecalis* and *Escherichia coli*) [9].

Other secondary metabolites extracted from bitter orange such as alkaloids, flavonoids, anthocyanins are also important economically. They have a commercial application in the pharmaceutical and biomedical and part of drugs, dyes and perfumes [10, 11, 12].

There is increasing interest in the radical scavenging activities of some natural antioxidants, especially those found in edible plants, which may play a role in preventing various chronic diseases. Recent works have shown that some *C. aurantium* L. essential oils [13, 14] and extracts [15, 16] have radical scavenging capacity. Yet, although the literature contains extensive reports concerning the antimicrobial activity of citrus species, extracts obtained from *C. aurantium* L. and some essential oils (such as those extracted from the leaves) have so far not been investigated. In fact, this study was the first report regarding antimicrobial activity of extracts (from flowers, leaves and peels) and EO of *C. aurantium* L. leaves from Tunisia. Therefore, the objectives of this study were to (1) analyze the chemical composition of essential oils by a GC-MS system to determine essential oils chemotype of *C. aurantium* L. plants, (2) Identify the main chemical families of methanolic extracts by a phytochemical screening, and (3) Investigate antimicrobial and antioxidant activities of essential oils and various extracts.

II. MATERIALS AND METHODS

1. Plant Material

Three organs of sour orange were harvested from the region of Nabeul (Northern Tunisia) during the month of February for the leaves and the peel and the month of April for the flowers. Specimens were identified by Dr. Mohamed Larbi Khouja at the National Institute of Research in Rural Engineering, Water and Forest (INRGREF, Tunis, Tunisia) and voucher specimens were deposited at the Herbarium of the Department of Botany in the cited institute.

The samples were then dried in the open air in the shade until constant mass and then stored for the isolation of essential oils (EO) and organic extracts. The dry matter content of plants was determined using an infrared dryer.

2. Extraction

2.1. Isolation of EO

A portion (100 g) of the dry plant material was subjected to hydrodistillation in a Clevenger-type apparatus as described in the European Pharmacopoeia and in the 10^{th} edition of the French Pharmacopoeia [17]. It is an apparatus operating at atmospheric pressure with a cohoba to maintain the ratio plant material/water to its initial level. During each test, the plant material and distilled water were placed in well defined proportions in a flask of one liter capacity. The whole is heated to boiling. The vapors pass through the column and out of the condenser in liquid form. At the end of the distillation two phases are observed, an aqueous phase (aromatic water) and an organic phase (EO) less dense than water. This operation was repeated three times for each sample. The EO was recovered, dried with anhydrous sodium sulphate and stored at 4 °C in the dark until its use.

2.2. Preparation of methanolic *C. aurantium* L. extracts

The plant extracts were obtained by maceration using the protocol developed by Ennajar and *al*. (2009) [18]. The air-dried and finely ground leaves, flowers and peels (5 g) were extracted by stirring with 50 mL of solvent with a low boiling point (methanol) at room temperature for 30 min. Extracts were kept for 24 hours at 4°C then filtered through Wattman Millipore Filter paper (GF/A, 110 mm, 8µm) and evaporated under reduced pressure using a rotary evaporator to obtain a mixture of pasty consistence.

2.3. Determination of chemical composition of EO by GC-MS

The volatile constituent's analyses were achieved on a Hewlett-Packard gas chromatograph GC: 6890 series II and HP 5973 mass selective detector. The fused HP-5MS capillary column (l=60 m, ϕ=0.25 mm ID, 0.25 µm film thickness) was directly coupled to the mass spectrometer. The carrier gas was helium, with a flow rate of 1.2 mL/min. The oven temperature was programmed from 40 °C (1 min) to 280 °C (15 min) at 5 °C/min. The temperature of the injector port was held at 250 °C, the temperature of the detector was set at 280 °C. The mass spectrometer was operating (full scan-mode) in the EI-mode at 70 eV. The injected volume is equal to 1µL in the mode split. Compounds were identified by comparison of their KI (Kovats indices) relative to C5-C24 *n*-alkanes obtained on a non polar DB-5MS column, with those provided in the literature, by comparison of their mass spectra with those recorded in the library database Chem Station HP: HP Wiley 275L and reported in published articles and by co-injection of available reference compounds. The samples were analyzed in duplicate. The percentage composition of EO was computed by the normalization method from the GC peak areas. Results were calculated as mean values of two injections from each EO, without using correction factors. All determinations were performed in duplicate and averaged.

2.4. Phytochemical screening

To get an idea about the main families that can be found in plant material, we have made a phytochemical screening. This one is either based on the formation of insoluble or colored complexes. The observed coloration is usually due to the formation of a conjugation or instauration in a molecule. In such tests of characterization, we cause this instauration using a suitable reagent.

We have characterized the different chemical groups (tannins, cyanogenic hétérozides, flavonoïdes, polyphénols, sterols and polyterpenes, quinones, alkaloids and saponosides) by referring to the techniques described in the work of (Guessan and *al*., 2009) [19] with some modifications.

2.5. Free radical scavenging activity: DPPH and ABTS methods

The antioxidant capacity was evaluated by the DPPH and ABTS methods described by (Re and *al*., 1999) [20] and by (Leitao and *al*., 2002) [21], respectively, with some modifications. Different concentrations of organic extracts and EO were prepared in methanol. For the ABTS test, 10 µL of each concentration were added to 990 µL of the solution of $ABTS^{+}$. The measuring of the mixture absorbance was performed at 734 nm after

20 min. For the DPPH test, 1 mL of each concentration was added to 250 µL of DPPH solution. After 30 min, the measuring of the mixture absorbance was performed at 520 nm.

The negative control is consisted of 1 mL of methanol and 250 mL of the ABTS or DPPH solution while the positive control used is the Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid).
The percentage of inhibition is expressed by the following equation given by [22]:

$$\% \text{ inhibition} = \frac{\text{Absorbance of negatif control} - \text{Absorbance of test}}{\text{Absorbance of negatif control}} \times 100. \quad (1)$$

The experiment was repeated for three times. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH or ABTS free radicals.

2.6. Antimicrobial Activity

The *in vitro* antimicrobial activity of different EO and extracts were evaluated against Gram positive bacteria (*Listeria monocytogenes* ATCC 19111, *Staphylococcus aureus* ATCC 25923 (CIP7625), *methicillin-resistant Staphylococcus aureus*) and Gram-negative ones (*Escherichia coli* ATCC 25922 (CIP 7624), *Pseudomonas aeruginosa* ATCC 27853 (CIP 76110), *Salmonella typhimurium*, *Klebsiella pneumoniae* ATCC 1388 and *Klebsiella pneumoniae cefotaxime resistant*) and the yeast culture (*Candida albicans*).

Disk diffusion method: Sterilized antibiotic discs (6 mm) were used following the literature procedure [23, 24]. The discs were impregnated with 10 µL of EO or organic extracts. All the bacteria were incubated and activated at 30 ^{o}C for 24 h inoculation into Nutrient Broth (OXOID) and the yeasts were incubated in Malt Extract Broth (OXOID) for 48 h. Inoculums containing 10^6 bacterial cells or 10^8 yeast cells per cm^3 were spread on Mueller-Hinton Agar (OXOID) plates (1 cm^3 inoculum for each plate). The discs injected with EO or methanolic extracts were placed on the inoculated agar by pressing slightly and incubated at 35^{o}C (24 h) and at 25^{o}C (72 h) for bacteria and yeast, respectively. On each plate an appropriate reference antibiotic disc was applied depending on the test microorganisms. In each case, triplicate tests were performed and the average was taken as the final reading.

Antibiotics activity: On each plate an appropriate reference antibiotic disc was applied depending on the tested microorganisms. Ampicillin and nalidixic acid (10 μg/disk) were used as positive reference standards to respectively determine the sensitivity of Gram-positive and Gram-negative bacteria. Nystatin (10 μg/disk) was used as positive reference standard to determine the sensitivity of yeast and fungi species.

The lowest concentration of extract/EO required to completely inhibit microbial growth after incubation was reported as minimum inhibitory concentration (MIC). The MIC of the active EO and extracts were tested using disk diffusion method at concentrations prepared in methanol: 1000 g / mL, 500 g / mL, 250 g / mL, 125 g / mL and 100 g / mL.

III. RESULTS AND DISCUSSION

1. Chemical Composition of EO

By hydrodistillation of flowers, leaves and peels of *C. aurantium* L., yields (relative to dry weight material) of 0.48% (w/w), 0.08% (w/w) and 1.17% (w/w), respectively, were obtained. These yields are comparable to those demonstrated in other studies [25, 26]. Besides, the high yield of peels EO obtained in our study is in conformity with previous GC-MS studies of *C. aurantium* L. peel oil [27, 28, 29].

The results of analyses of EO by GC-MS are given in Table 1, where the compounds were listed according to their TR.

1.1. EO extracted from flowers

The study of the chemical composition of neroli showed the existence of 21 compounds representing 99.65% of the total oil (Table 1). It is dominated by monoterpenes (98.54%) with a predominance of oxygenated compounds (94.27%) represented mainly by linalool (39.74%) and linalyl acetate (25.44%). This composition does not differ much from that of the oil from Italy [30] and Egypt [31]. The abundance of these two compounds in the oils of citrus flowers demonstrated by (Moraes and *al*., 2009) [5] testifies to their floral odors [32, 33, 34, 35].

Besides these two compounds, other oxygenated terpenes were found such as nerolidol (6.91%), nerol (0.99%) and geranial (0.43%). Acyclic monoterpenes are represented by myrcene (0.36%), while limonene is the major cyclic monoterpene found in this study (2.16%). We have also identified bicyclic monoterpenes including β-pinene (3.01%) is the major component found in this category. These compounds are distributed in all the oils of citrus flowers [5]. Nitrogen compounds present in small amounts (0.66% of methyl anthraniate and 0.45% of

indole) represent the last category. They are reported previously in the oils extracted from flowers of sour orange by (Boelens and *al*., 1994) [36]. They represent potential floral volatile.

1.2. EO extracted from the leaves

12 compounds were identified for leaves oil (Table 1) representing 100% of the total EO composition. It has been shown that the oil of the leaves is rich in oxygenated monoterpenes (93.04%). The most notable are linalool (22.11%) and linalyl acetate (53.76%). The sesquiterpenes are a minority (3.05%).

These two compounds were reported as major constituents of the leaves of Italian *C. aurantium* L. oil [37,38]. However, (Majnooni and *al*., 2012) [13] showed two other major components of *C. aurantium* leaves oil: limonene (57.57%) and linalool (8.01%). The reasons for this variability can be understood if we take into account all the factors influencing the chemical composition of the oils, namely, climatic, seasonal and geographic conditions, harvest period and distillation technique, among others [39].

1.3. EO extracted from the peel

The results of chromatographic analysis of EO of sour orange peels given in Table 1 show that the essence of the peel of *C. aurantium* L. is also completely dominated by monoterpens (100%). The main constituent of this fraction is the limonene (87.02%) which is used as a fragrance material in perfuming household products and as a component of artificial EO [14]. These results are in accordance with those obtained by Moraes and *al.* [5] who found that limonene (97.5 to 98%) is the major constituent in the oils from the peel of Brazlian *C. aurantium* L.

Table 1- Chemical composition (%) of EO from different organs of *Citrus aurantium* L.

			Organs		
N°	RT*	Component	Flowers	leaves	Peels
1	7.83	α-Pinene	0.46	--	--
2	8.62	Sabinene	0.44	--	--
3	8.78	β-Pinene	3.01	0.55	--
4	8.82	Myrcene	0.36	1.01	--
5	9.87	Limonene	2.16	--	87.02
6	9.88	delta-4-carene	--	1.12	--
7	9.98	1,8-cineol	0.54	--	--
8	10.16	Trans-β-ocimene	--	1.23	--
9	10.91	cis-linalool oxide	0.53	--	--
10	11.30	trans-linalool oxide	0.49	--	--
11	11.51	Linalool	39.74	22.11	6.24
12	13.76	Terpinen-4-ol	0.26	--	--
13	13.80	Terpendiol	0.43	--	--
14	14.07	β-frenchyl alcohol	--	8.40	--
15	14.08	α-Terpineol	7.30	--	6.74
16	14.91	Nerol	0.99	1.28	--
17	15.59	linalyl acetate	25.44	53.76	--
18	16.05	Geranial	0.43	--	--
19	16.82	Indol	0.45	--	--
20	18.13	anthraniate méthyl	0.66	--	--
21	18.41	Acetate neryl	1.74	2.61	--
22	18.90	Acétate geranyl	3.03	4.88	--
23	20.36	β-caryophylene	--	2.21	--
24	22.25	Bicyclogermacrene	--	0.84	--
25	23.49	Nerolidol	6.91	--	--
26	27.09	Farnesol	4.28	--	--
Monoterpene hydrocarbons			**4.27**	**3.91**	**87.02**
Oxygenated monoterpenes			**94.27**	**93.04**	**12.98**
Nitrogen components			**1.11**	**3.05**	**--**
Total			**99.65**	**100**	**100**

*: Retention Time

2. Phytochemical screening of organic extracts

The phytochemical screening performed on flowers, leaves and peels of *C. aurantium* L. has allowed us to highlight the presence of secondary metabolites in the tissues of these organs (Table 2).

Table 2- Main classes of secondary metabolites found in the three organs of *C. aurantium* L.

Chemical Group	Organs		
	Flowers	Leaves	Peels
Catechin Tannins	+++	+++	+++
Gallic Tannins	+++	++	+++
Flavonoïds	+	+	+
Polyphenols	+	+	+
Sterols and Polyterpenes	+++	+++	+++
Quinones	-	-	-
Alkaloïds	+	+	+
Saponozides	-	-	-
Heterozides cyanogenic	-	-	-

- : Absence-: Absence ; +: Low presence ; ++ : Présence moyen+ +: Middle presence ; +++ : Forte présence+ + +: Strong presence

Table 2 shows that all studied organs contain tannins, flavonoïds, polyphenols, sterols, alkaloïds and polyterpenes but we note the absence of quinones, saponozides. Moreover, our results did not reveal the existence of cyanogenic hétérozides in the three organs tested confirming the absence of toxic effects of bitter orange.

3. Antioxidant activity

Antioxidant activity of EO and various extracts of *C. aurantium* L. has been determined by two different systems, namely, DPPH and ABTS assays. All of the data are presented in Table 3.

Table 3- Free radical scavenging capacities of *C. aurantium* L. EO and extracts

Samples	Organs	IC_{50} (µg/mL)	
		DPPH	ABTS
EO	Flowers	> 8000	>15000
	Leaves	2400 ± 0.34	>15000
	Peels	800 ± 0.11	>15000
Methanolic extract	Flowers	57.5 ± 0.05	10000 ± 0.09
	Leaves	68 ± 0.03	4000 ± 0.07
	Peels	25 ± 0.01	3400 ± 0.14
Reference standard	Trolox	2.08 ± 0.08	51.3 ± 0.25

3.1. DPPH assay

The reduction of DPPH radical was followed by monitoring the decrease of absorption of sample extracts and EO at 520 nm. The results obtained from DPPH radical-scavenging activity of *C. aurantium* L. EO (Table 3) showed that all these exhibited moderate to good DPPH radical inhibition activity compared with Trolox. It was observed that peel's EO show higher DPPH free radical scavenging activity than leaves and flower's EO with IC_{50} values of 800 ± 0.11, 2400 ± 0.34 and > 8000 µg/mL, respectively. The data of Siddique et *al.*[14], however, show discordance, it reveals a strong antioxidant potential of peel's oil.

Although the DPPH test is widely applied in the literature, it is based on the use of a very crowded radical. However, the use of a less crowded radical such as ABTS can be more suitable. In addition, the DPPH test is performed in methanol, while the ABTS test is carried out in aqueous ethanol, which provides an environment closer to physiological conditions.

The scavenging ability of leaves EO on DPPH radical was lower than that obtained in Majnooni and *al.* [13] study (IC_{50}=1040 µg/mL). Moreover, radical scavenging activity of *C. aurantium* L. petit-grain is lower than mandarin petit-grain antioxidant capacity with an IC_{50} of 80 µg/mL [40]. The low activity can be attributed to their predominant compound (linalool). Our results contradict with those obtained by Yoko and *al.* [41] who have studied the scavenging activity of neroli and petit-grain oil against singlet oxygen. They found that EO which enhanced the singlet oxygen production has high levels of limonene and low levels of linalool which is a monoterpene alcohol. So, they suggest that limonene and linalool are the causative substances for enhancement and scavenging of singlet oxygen, respectively.

For extracts, the results show that the scavenging effect of extracts on the DPPH radical increased in order with IC_{50}: peel *C. aurantium* L. > flower *C. aurantium* L. > leaf *C. aurantium* L. All the investigated samples have a strong scavenging activity and peel extracts have revealed the strongest one with an IC_{50} value of 25 µg/L. This is in accordance with Bocco and *al.* [42] who showed good antioxidant activity of methanol extracts obtained from the peel of bitter orange. Extracts from the flower showed an antioxidant activity with an IC_{50} value of 57.5 µg/L. This result is better than that obtained by Karimi and *al.,[*15] who found that

methanolic extract from blooms show a DPPH free radical scavenging activity with values of 55.32% at 300 µg/mL. The DPPH scavenging activity of leaf *C. aurantium* L. extracts was lower than that of other extracts (IC_{50} was 68 µg/L) but higher than results obtained by Muthiah et *al.*, [16] with IC_{50} value of 142.25 µg/mL.

These activities are probably related to polyphenols, in particular flavonoïds, which react with the free radical ($DPPH^{\cdot}$) via the phenolic hydroxyl groups, giving hydrogen forming thus stable complexes (DPPH-H) that are not able of initiating oxidation reactions [43]. These observations reinforce those of Jayaprakasha and *al.*,[44] working on the antioxidant capacity of citrus and showed a good antioxidant activity of extracts related to the content of polyphenols. Another study showed a positive correlation between phenolic compounds in citrus, especially flavanones glycosides, and the antioxidant activity [42].

Indeed, phenolic compounds are known as substances with potential antioxidant capacity to trap free radicals and reactive forms of oxygen [45, 46, 47].

3.2. ABTS method assay

Free radical-scavenging capacity was also evaluated by measuring the scavenging activity of EO and methanolic extracts on ABTS radicals. This method was chosen for the excellent stability of the chromogen compound (ABTS+), for its ease, speed and sensitivity and for its ability to measure the hydrophilic and lipophilic antioxidant activity since ABTS•+ of fowers and leaves can be solubilized in aqueous and organic media. The results measured on the EO showed low antioxidant activities and it appeared that the EO of peels had the most important activity (IC_{50} = 5000µg/L) compared to that of flowers and leaves (IC50 values > 10000 µg/L which confirms the results obtained by the DPPH method.

For extracts, the results obtained showed a good antioxidant activity of all tested samples. From Table 3, the extract of the peel is the most active (IC_{50} =3400 µg/) followed by extracts from leaves (IC_{50} =4000 µg/L) and the extracts from flowers (IC_{50} =10000 µg/L). All activities are lower than below the Trolox used as positive control (IC_{50} = 51.3 µg/L).

On comparing the results for antioxidant activity with those of EO, there emerged little correlation between EO content and antioxidant activity of the extracts. This could be due to the occurrence in the methanolic extract of other phytochemical compounds (that constitute the non-volatile fraction of EO such as limonoïds and flavonoïds) that have antioxidant activity and that are not detected by GC analysis. The chemical composition of the non-volatile fraction of peel methanolic extracts requires further study to ascertain the possible correlation between the amounts of bioactive compounds and antioxidant activity of the extracts.

Finally, the differences in the antioxidant activity between the two used methods, DPPH and ABTS, are also considered in the literature [48, 18, 49, 50] without any satisfactory explanation. According Bendaoud et *al*. [48], the differences between the two results can be explained by the reaction mechanisms involved. ABTS radical reactions involve electron transfer and take place at a much faster rate than DPPH radicals whose degree of discoloration is attributed to the hydrogen donation ability of the test compounds.

4. Antimicrobial activity

The *in vitro* antimicrobial potential of *C. aurantium* L. EO and extracts against microorganisms (Gram-positive and Gram-negative bacteria and *Candida albicans*) along with control antibiotic was quantitatively assessed by the presence or absence of inhibition zones (Table 4).

4.1. Antimicrobial activity of EO

In the present study, the biological activity of *C. aurantium* EO was evaluated against five Gram-negative bacteria including *Echerichia coli* ATTC 25922, *Salmonella typhimurium*, *Pseudomonas aeruginosa* ATTC 27853, *Klebsiella pneumonia* ATTC 1388, *Klebsiella pneumonia* cefotaxime resistant and three Gram-positive bacteria including: *Listeria monocytogenes* ATCC, *Staphylococcus aureus* ATCC, *Staphylococcus aureus MR*.

All the oils tested in the disk diffusion study showed a moderate activity. The EO of the peel exhibited the lowest antibacterial activity.

The results indicated that most tested Gram-negative bacteria were resistant to *C. aurantium* peel EO as shown in Table 4 except *Klebsiella pneumonia* ATTC 1388, which inhibition zone was 8mm. While considering the efficacy of the *C. aurantium* peel's EO against Gram-negative bacteria, present results are not on one hand in conformity with Siddique et *al.*, [14] who don't report an antimicrobial activity of *Citrus* peel oil against *Klebsiella pneumonia* and on another hand correlated with Quintero et *al.*, [51] who showed that *Pseudomonas aeruginosa* and *E. coli* were resistant to *C. aurantium* peel oil.

Present study indicated that peel oil was more effective against Gram-positive bacteria which are in accordance with Kirbaslar et *al*., [27] who reported strong antimicrobial activity of Citrus peel oil against tested Gram-positive. Inhibition zones were also obtained for *Candida albicans* 8 mm.This result joins those of

Caccioni and *al.*, [8] showing antifungal activity of oils from the peel of *C. aurantium* L. and a positive correlation between the content other than hydrocarbon monoterpenes limonene and antifungal activity of these oils.

Leaf's oil did not show inhibition against *Pseudomonas aeruginosa* ATTC 27853 and *Klebsiella pneumonia* cefotaxime resistant.

The EO which showed the best antibacterial activity in disc-diffusion method where oils isolated from flowers (9 - 23 mm). *Pseudomonas aeruginosa*, a highly pathogenic and resistant bacteria, showed a zone of inhibition of 9 mm. However, it was highly sensitive to Nalidix acid (19 mm). It can be seen that neroli possess a higher antibacterial effect than Ampicillin against *Staphylococcus aureus* ATCC (22 mm vs. 20 mm). Neroli and Nystatin possessed similar inhibition zones against *Candida albicans* (25 mm). This is consistent with the observations of Rao and Nigam [52] showing an antibacterial effect of neroli. It would be advisable for a large-scale exploitation for therapeutic purposes.

It is obvious that the antimicrobial potential of *C. aurantium* EO is proportional to their doses. Therefore, we have determined the minimum inhibitory concentrations (MIC) of each of EO showing activity against the indicator strains (Table 5). The data pertaining to the MIC of *C. aurantium* EO showed that neroli has the lowest MIC (75 µg /mL for most strains considered). Thus, it was the most effective among all the oils tested. There was an inhibitory action against *Pseudomonas aeruginosa* even it has an outer membrane giving it an intrinsic resistance to a wide range of biocides [53].

It is clear that neroli containing the highest quantity of oxygenated compounds, possess a higher antimicrobial potential, while peels essential oils containing the highest quantity of hydrocarbon monoterpenes show the lowest antibacterial activity. This is in accordance with Soković and *al.* [54] who showed that oxygenated monoterpenes, exhibit strong antimicrobial activity, especially pronounced on whole cells, while hydrocarbon derivatives possess lower antimicrobial properties, as their low water solubility limits their diffusion through the medium. Griffin and *al.* [55] have shown that hydrocarbons tend to be relatively inactive regardless of their structural type, and this inactivity is closely related to their limited hydrogen bound capacity and water solubility.

4.2. Antimicrobial activity of methanolic extracts

The methanol extracts of the leaves and the peels were inactive against all tested microorganisms except from flowers extract that was active only against *Staphylococcus aureus* ATCC (Table 4). A study by Athamena et *al.*, [43] showed a specific sensitivity of this bacterium to the tested extracts.

The hypersensitivity of *Staphylococcus aureus* ATCC may due to the absence of the outer membrane, external environmental changes, such as temperature, pH, and natural extracts [56].

To the best of our Knowledge, the evaluation of the antimicrobial activity of methanol extracts of *C. aurantium* L. has never been published. Only one published work, was developed by Del Río et *al.*, [57] on flavonoïd extracts of *C. aurantium* L. and showed a good antifungal activity of these extracts against several fungal species *(Phytophthora citrophthora, Penicillium digitatum, and Geotrichum).* However, these results are hardly comparable with our ones because the difference of the used methods may be the process of drying. Previous study of Chanthaphon et *al.*,[58] comparing antimicrobial activities of dried lime peels and fresh lime peels extracts showed that the extracts from dried peels had lost some antimicrobial activity.

Table 4. Antimicrobial activity (diameter of the inhibition zones in mm) of EO and extracts of *C. aurantium* L.

Samples		Gram-positive bacteria			Gram-negative bacteria					yeast
		Listeria monocytogenes ATCC	*Staphylococcus aureus ATCC*	*Staphylococcus aureus MR*	*Escherichia coli ATCC*	*Pseudomonas aeruginosa ATCC*	*Salmonella typhimurium*	*Klebsiella pneumoniae ATCC*	*Klebsiella Pneumonia CR*	*Candida albicans*
EO	Flowers	9	22	9	9	9	8	23	12.5	25
	Leaves	8	8	8	9	--	8	23	--	23
	Peels	7.5	7.5	7.5	--	--	--	8	--	8
Methanolic extracts	Flowers	--	1.2	--	--	--	--	--	--	--
	Leaves	--	--	--	--	--	--	--	--	--
	Peels	--	--	--	--	--	--	--	--	--
Reference standard	Ampicillin	31	20	15						
	Nalidix acid				30	19	17	28	18	
	Nystatin									25

--No inhibition zone

Table 5- CMI of different *C. aurantium* L. EO (µg / mL)

Samples	EO		
	Flowers	leaves	peels
Staphyloccocus aureus MR	75	500	-
Staphylococcus aureus ATTC	<75	500	-
Salmonella	75	250	-
Listeria monocytogenes ATTC	500	75	-
E. coli ATTC	75	250	-
Pseudomonas aeruginosa ATTC	75	-	-
Klepsiella pneumoniae CR	75	-	-
Klepsiella pneumoniae ATTC	75	75	500
Candida albicans	75	250	250

IV. CONCLUSION

The results of this study show that methanol extracts of *C. aurantium* L. were a strong radical scavengers in both DPPH and ABTS assays especially those obtained from the peels, that suggests the possibility of using them as a potential source of antioxidant ingredients for the food, cosmetic and pharmaceutical industries. While EO of all tested organs possessed high antimicrobial activities against the most investigated bacteria, therefore which represent a natural alternative and a safe method for controlling bacterial and fungal infections in food.

Following these results, it would be interesting to conduct a detailed investigation of the fractions of organic extracts and EO demonstrating antioxidant and antimicrobial activity *in vitro*, to identify the chemical species or molecule responsible for this activity. In fact, this study is only a first step in the search for biologically active natural substances. Follow-up *in vivo* are needed to confirm the activities identified by *in vitro* tests.

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ABREVIATIONS

EO: Essential Oils.
C. aurantium: *Citrus aurantium* L.

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