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Chemical profile, antimicrobial and antioxidant activities of *Citrus reticulata* and *Citrus clementina* (L.) essential oils

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Abstract

The present investigation reports on the chemical composition of three citrus fruit essential oils (mandarin [*Citrus reticulata*], wilking [*Citrus reticulata* cultivar wilking] and clementine [*Citrus clementina*]) from Algeria, and examines their antioxidant and antimicrobial activity against eight spoiling and pathogenic microorganisms. The chemical composition of the essential oils obtained from the peels, by hydrodistillation, was analyzed by Gas chromatography–mass spectrometry (GC-MS). 12 compounds were identified and limonene was the common major component for the three essential oils (77- 97%).The disc agar diffusion technique indicated mandarin essential oil (EO), as evidenced by their zones of inhibition, as the best growth inhibitor followed by clementine and wilking essential oils. Among the tested microorganisms, the oils was very active against *Candida albicans*, *Escherichia coli*, *Lysteria innocua*, Methicillin-Resistant *S. aureus* and *Staphylococcus aureus* with an inhibition zone varied from 9.16 to 27.63 mm. Minimal inhibitory concentration (MIC) of different EOs was for all 5 µL/mL against the sensitive microorganisms studied. All citrus oils studied exhibited antioxidant activity as DPPH free radical scavenger and reducing power in dose dependent manner. Mandarin oil showed the strongest activity compared to clementine and wilking essential oils. The oils may be recommended as safe plant based antimicrobials as well as antioxidants for enhancement of shelf life of food commodities.

Introduction

The growing awareness of consumers concerning the relation between food and health is revolutionising the food industry that look for new ingredients improving health. The reduction of additives used in a wide variety of foods is demanded, while 'natural additives are seen as a benefit for both quality and safety. EOs of some plants have recently been proven to be successful eco-friendly bio-control agent (Chutia *et al*., 2006; Sokovic and Griensven, 2006). Moreover, Citrus EOs are complex mixtures of different compounds that have shown a wide spectrum of biological activities such as antioxidant (Misharina and Samusenko, 2008), anti-inflammatory, anxiolytic (De Moraes Pultrini *et al*., 2006), antimicrobial (Chanthaphon *et al*., 2008; Fisher and Phillips, 2008; Jafari *et al*., 2011), and antifungal (Viuda- Martos *et al*., 2008; Chutia *et al*., 2009).These biological activities may be of great importance in several fields, from food chemistry to pharmacology and pharmaceutics (Cristani *et al*., 2007). The main advantage of essential oils is that they can be used in any foods and are considered generally recognized as safe (GRAS).

Citrus peel accounts for approximately 45% of the total fruit weight, which is available as a byproduct of citrus processing, that create environmental problems (Farhat *et al*., 2011). Although, citrus fruits are mainly used for dessert, it has significant economic value for its essential oil (EO) due to their aromatic compounds (Minh *et al*., 2002). Citrus spp. EO's are present in great quantities and they are used in beverage, confectionary, cookies and desserts (Buchel, 1989; Dharmawan *et al*., 2007). Among citrus fruit, mandarin group (*Rutaceae*

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family, *Citrus* genus) predominate with oranges the fresh fruit Market and the citrus production in Algeria, they represent together more than 93% of the total production in 2012. Species and Cultivars of mandarin group are numerous and present a great diversity of morphological and hortical characters (shape, volume and colour of fruit, adherence of peel, ripeness, distinctive flavor and aroma.) namely mandarin, clementine, wilking, tangerine, etc. (Tanaka, 1961).

Few studies have already reported the chemical composition of *C. reticulata* varieties and wilking peel oil (Shaw, 1979; Lawrence, 1992). Nevertheless, in most cases, the species and/or the varieties remained unspecified. Meanwhile, It is interesting to note that the chemical composition of the oil is significantly affected by the vegetative stage of plant, storage condition and extraction method and citrus peel oil is particularly prone to quantitative and quantitative changes due to genotype, origin, climate, season, ripening stage, etc. (Caccioni *et al*., 1998; Venkateshwarlu and Selvaraj, 2000; Vekiari *et al*., 2002; De Pasquale *et al*., 2006; Njoroge *et al*., 2006; Espina *et al*., 2011; Bourgou *et al*., 2012 ; Cheong *et al*., 2012). Regarding the inhibition and reduction in numbers of foodborne pathogens by mandarin group EOs, scarce reports have been carried out (Viuda-Martos *et al*., 2008; Chutia *et al*., 2009; Espina *et al*., 2011; Bourgou *et al*., 2012).

To our knowledge, there is no report on the antimicrobial and antioxidant activities of wilking EO as well as of Citrus EO from Algeria despite their above mentioned biological properties. Therefore, this study was undertaken in order to investigate the chemical composition and variability of mandarin (*Citrus reticulata* L.), clementine (*Citrus clementina* L.) and wilking (*Citrus reticulata Blanco*, wilking cultivar) EOs and their effectiveness, in vitro, on survival and growth of eight selected spoiling and pathogenic microorganisms commonly associated with food spoilage. On the other hand, to study their antioxidant properties using DPPH and reducing power methods and to determine how the antioxidant activity of essential oils is correlated with changes in their composition.

Materials and Methods

Materials

Clementine, mandarin, and wilking fruits were harvested from Oued-Ghir location (Bejaia region, North East of Algeria) (latitude 36°42’15.76”N; longitude 4°58’55.78”E) on 26 November, 30 September and 20 December 2013, respectively. Only mature fruits were selected, from healthy trees, cultivated under the same climatic and cultural conditions in the field. The fruits were then cleaned with deionized water before use, their thin peels (flavedo parts) were peeled off carefully and cut into small pieces manually then were immediately used for extraction of essential oils.

Essential Oil Isolation.

The fresh peels (150 g) were submitted to hydrodistillation for 150 min using a Clevenger type apparatus. This time was fixed after a kinetic study. The vapor produced by the steam generator crosses citrus peel, charged with essential oil and then passes through the condenser then the essential oil is collected; The isolated fractions exhibited two distinct layers, an upper oily layer and the lower aqueous layer, both layers were separated. The oils obtained were dried over anhydrous sodium sulphate and were stored in glass vials with Teflon sealed caps at -20°C in the absence of light until analyzed.

Gas chromatography/mass spectrometry (GC–/MS) analysis

The essential oils were analyzed by gas chromatography coupled to mass spectrometry (GC–MS) (Shimadzu QP2010) using a ZB5MS column (30 m x 0.25 um x 0.25 um) GC–MS spectra were obtained using the following conditions: carrier gas He; flow rate 1.03 mL/min; split-less mode; injection volume 1 µL; injection temperature 230°C; The oven temperature program was initially 60° C and increased at 3°C/min to 240°C and held at 240°C for 5 min;

The ionization mode used was electronic impact at 70 eV. The ion source temperature is 200°C and the interface temperature is 245°C. The relative percentage of the components was calculated from GC–MS peak areas. Most constituents were tentatively identified by comparison of their GC Kovats retention indices (RI), determined with reference to an homologous series of C5–C28 n-alkanes and with those of authentic standards available in the authors’ laboratory. Identification was confirmed when possible by comparison of their mass spectral fragmentation patterns with those stored in the MS database (National Institute of Standards and Technology and Wiley libraries).

Antioxidant activity tests

Antioxidant activity using DPPH radical scavenging method

The free radical scavenging activity using the

1,1-di-phenyl-2-picrylhydrazil (DPPH) reagent was determined by the reduction of the reaction colour between DPPH solution and sample extracts (Gülçin *et al*., 2010; Chikhoune *et al*., 2013).

Fifty microliter volumes of various concentrations of essential oils in methanol (0.1–1.0 mg/mL) were added to aliquots (2 mL) of a 60-µM methanolic solution of DPPH. Absorbance measurements were read at 517 nm, after 30 minutes of incubation time in the dark at room temperature. Absorption of a blank sample containing the same amount of methanol and DPPH solution acted as the negative control. All determinations were performed in triplicate. The percentage inhibition of the DPPH radical by the samples was calculated according to the formula:

DPPH scavenging activity (%) = [(Abs (t = 0) - Abs (t = 30) / Abs (t = 0)] x100;
Where Abs (t = 0): absorbance of DPPH radical + methanol at t = 0 min;
Abs (t=30): absorbance of DPPH radical + essential oils at t = 30 min.

Reducing power determination

The capacity of essential oils to reduce iron (III) to iron (II) was determined according to the method of Oyaizu (1986). Briefly, the sample (1 mL) was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium hexacyanoferrate III (1%, w/v). After 30 min of incubation at 50◦C in the dark, 2.5 mL trichloroacetic acid (10%) were added and the mixture kept at room temperature for 10 min. Afterwards, 2.5 mL of this mixture was added to 2.5 mL water and 0.5 mL ferric chloride (0.1%), vigorously mixed, and the absorbance was measured at 700 nm in a spectrophotometer. Essential oils were diluted in methanol and all determinations were performed in triplicate.

Antimicrobial activity

Strains and growth conditions

The antibacterial activity tests of different mandarin group EOs included eight food-borne pathogenic bacteria and one species of yeast, supplied by Pasteur institute, identified with the ATCC number (American Type Culture Collection). The Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimirium* ATCC 13311, *Vibrio cholerea* ATCC 14035 and the Gram-positive bacteria: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, Methicillin-Resistant *S. aureus* ATCC 43300 (MRSA), *Listeria innocua* CLIP 74915 and a yeast: *Candida albicans* ATCC 10231 were studied.

During this investigation, the culture was maintained in cryovials at – 80°C. All strains were subcultured in Brain Heart Infusion (BHI) agar and incubated at 37°C for 18–24 h until the stationary growth phase was reached.

Screening of antimicrobial activity

Antimicrobial activity was analyzed by the disc diffusion method (Rios and Recio, 2005), which is normally used as a preliminary check to select among efficient essential oils, against nine pathogenic microbial strains. All strains were grown on the nutrient agar plate at 37°C and at 30°C for *Pseudomonas aeruginosa* during 18–24 h in order to obtain freshly cultured microbial suspensions for tests. Sterile filter paper disks (6 mm in diameter) impregnated with 20 µL of essential oil were placed on the Mueller Hinton plate (25 mL, pH 7) previously inoculated with a microbial suspension (10^7 CFU mL^{-1}) using a sterile cotton swap. After incubation for 2 h at 4°C, the treated Petri dishes were incubated at 37°C for 18–24 h except for *Pseudomonas aeruginosa* which was incubated at 30°C for the same period. The antimicrobial activity was evaluated by measuring the diameter of the growth inhibition zone around the discs (including the diameter (6 mm) of the paper disk) with an electronic calliper. Each experiment was carried out in triplicate, and the mean diameter of the inhibition zone was recorded.

Minimum inhibitory concentration (MIC)

The MIC was determined by the microdilution plate method as recommended by NCCLS (NCCLS, 1999) with some modifications. The minimal inhibition concentration (MIC) values were determined for the microbial strains which were sensitive to the essential oil in disc diffusion assay.

All tests were performed in Mueller Hinton Broth (MHB) supplemented with Tween 80 detergent (final concentration of 0.5% (v/v), except for yeast (Sabouraud dextrose broth: SDB + Tween 80). Bacterial strains were cultured overnight at 37°C in MHA and at 30°C for 48 h for *Candida albicans* in SDB. Test strains were suspended in MHB to give a final density of $5 \cdot 10^5$ CFU/mL for microbial strains and these were confirmed by viable counts. Different dilutions ranging from 4 µL /mL to 100 µL /mL of the essential oils were prepared in a 96-well plate.

The plate was covered with a sterile plate sealer and was incubated under normal atmospheric conditions at 37°C for 24 h for bacteria and at 30°C for 48 h for Candida albicans isolates. The bacterial growth was indicated by the presence of a white

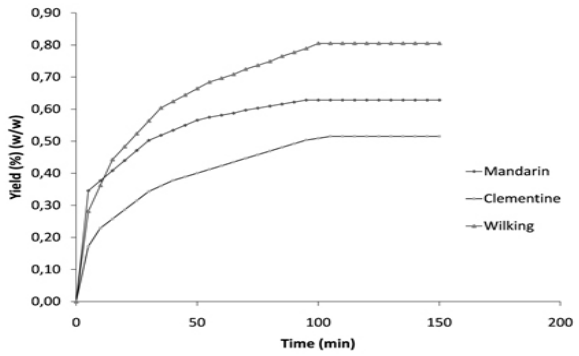

Figure 1. Yield % (w/w) of mandarin, wilking and clementine essential oils as function of the extraction time.

''pellet'' on the well bottom. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms.

Statistical analyses

All data are means ± SD of three measurements. Differences between samples were tested by an analysis of variance (ANOVA) using the STATISTICA software (5.5). The P values of < 0.05 were considered significant.

Results and Discussion

Yields, densities and refraction indices

All investigated citrus peel oils were obtained from trees cultivated under the same climatic and environmental conditions and analyzed using the same methods. So, the influence of the cultural and technical parameters was considered negligible and the main source of variance could be mostly related to genotype. The variations of the extraction yields according to the extraction time are shown in Figure 1. The conventional hydrodistillation method (HD), which is one of the reference methods in essential oil extraction, three phases are recorded. The first stage (Step 1) is represented by an increasing line which characterizes the first quantities of extracted essential oils, accounting for more than 70% of the yield obtained after 35 min. This phase is followed by a second increasing line (Step 2) representing the diffusion of the essential oil from the midst of the particles towards the external medium involved by the intern warming of the water located in the plant cells. The third stage (Step 3) corresponds to a horizontal line which marks the end of the extraction process, which is reached after 120 min, which is in agreement with the results of Sahraoui *et al*. (2011).

Bousbia *et al*. (2009), in their study of different extraction methods of essential oils from lime peel, found that the first drops starts after 20 min of distillation then the yield increase gradually until the yield reached a plateau after 180 min of distillation. The yields of peel essential oils from mandarin, wilking and clementine were found to be 0.63 %, 0.81% and 0.52 % (w/w), respectively.

Table 1. Chemical composition (% peak area) of Citrus peel oils.

Compounds	RI	Area %		
		Mandarin	Wilking	Clementine
Tricyclene	7 450	0.035	-	-
α-Thujene	7 525	0.4900	-	-
α-Pinene	7 767	1.8052	0.2713	0.1240
Sabinene	9 058	0.1024	0.1946	0.6110
β-Pinene	9 208	1.2467	-	-
β-Myrcene	9 608	1.6673	1.7025	2.0704
α- Terpinene	10 617	0.3094	-	-
ρ- Cymene	10 925	0.6758	-	-
Limonene	11 100	77.8081	97.6991	96.7504
γ-Terpinene	12 275	14.9986	-	-
α-Terpinolene	13 508	0.6623	-	-
Linalool	13 975	0.1993	0.1325	0.3202

Hosni *et al*. (2010) obtained a yield of 4.62 % (w/w on dry weight basis) and 1.13% (w/w fresh weight) found by Bourgou *et al*. (2012) for the Tunisian mandarin, which is higher than our results; while Bousbia *et al*. (2009) and Ferhat *et al*. (2006) found 0.7± 0.1% and 0.39% of essential oils from fresh peel of Algerian clementine and Valencia late species, respectively, moreover a yield of 0.71% and 0.79% has been recorded for Colombian mandarin and orange peel oils (Blanco *et al*., 1995), which is almost in agreement with our results.

In general, the oil content of plant appears somewhat influenced by the genotype, origin, season, environmental factors and extraction method (Asili *et al*., 2007; Blank *et al*., 2007; Bousbia *et al*. 2009; Hosni *et al*., 2010; Demuner *et al*., 2011; Bourgou *et al*., 2012). In fact, Bourgou *et al*. (2012) found that species and harvest time had a significant effect on essential oil yield. Mandarin exhibited the highest yield followed by lemon and orange while bitter orange showed the smallest value. Moreover essential oil yields varied during ripening to reach maximum values during the middle stage of maturity (stage 2) for mandarin.

The refraction index of mandarin peel essential oil showed the highest value of 1.4742, followed by wilking (1.4725) and clementine (1.4713), while their corresponding densities were: 0.968, 0.981 and 0.973, respectively. Viuda-Martos *et al*. (2008) noticed values of 0.85 g/mL and 1.47 for density and refraction index respectively in mandarin oil which is

in accordance with our results

Peel oil constituents

Variation in chemical composition of EOs would definitely alter their biological activity. Hence, determination of chemical profile of essential oil is important before recommending it for antimicrobial or antioxidant activity. Qualitative and quantitative analyses of the EO profiles are listed in Table 1. The table includes the retention indices and the area percentage of 12 identified components, representing more than 99% of all constituents.

As shown in Table 1. 12, 5, and 5 compounds were identified in mandarin, wilking and clementine peel oils, respectively. These results showed that there are many qualitative similarities between the oils although the amounts of some corresponding compounds are different, among them 5 are common to all cultivars. Within the studied cultivars, mandarin oil shows the most complex composition with higher number of volatile components (12), while the cultivar wilking and clementine oils contained fewer constituents (5). In all samples, the monoterpènes hydrocarbons limonene (77.81–97.70%) was the main component; in mandarin case, this was followed by γ-Terpinene (14,99%), α-Pinene (1,80%), β-Myrcene (1,66%), β-Pinene (1,24%) and ρ-Cymene (0,67%); while for wilking oil β-Myrcene (1,70%), α-Pinene (0.27%), Sabinene (0.19%) and Linalool (0.13%) were the most importants, whereas for clementine oil β-Myrcene (2.07%), Sabinene (0.61%), Linalool (0.32%) and α-Pinene (0.12%) were the major constituents.

The composition of essential oils varies with respect to ecological and geographical condition, age of plant and time of harvesting (Bagamboula *et al*., 2004; Huang *et al*., 2009; Msaada *et al*., 2009; Wu *et al*., 2013). When compared with literature data, our results of mandarin peel oil partly agree with those previously reported from the same species. In fact, for *Citrus reticulata Blanco*, the chemical composition of 41 cultivars of *C. Reticulata* peel oils samples consisted almost exclusively of hydrocarbons with limonene as major component (52.2-96.2%) associated with γ-terpinene (tr-36.7%). α-Pinene (0.1-2.1%), linalool (0.1-2.5%), myrcene (1.3-1.8%) and sabinene (0.1-1.3%) were present in almost all samples of French mandarin peel oils (Lota *et al*., 2000). One year later, they found that, a total of the 44 identified components, accounted for 95.8-99.7% of the oil, in other species of mandarin. Even though the composition was dominated by limonene (55.8-96.7%) for 39 samples over 40, the content of the major components varied considerably from sample to sample. Several other monoterpene hydrocarbons were frequently identifed at appreciable contents: γ-terpinene (tr-19.9%), p-cymene (tr-12.0%), myrcène (0.7-24.0%), β-pinene (tr-14.2%), sabinene (0.1-8.7%), α-pinene (0.2-2.2%) and β-phellandrene (0.2-0.8%). Among the oxygenated compounds, linalool was present in all the samples (0.1-10.7%) whereas percentages of octanal, α-terpineol and decanal were not over 0.5% (Lota *et al*., 2001).

Limonene (93.1–94.2), myrcene (1.93–1.98) and linalool (0.67–0.88%) were reported as the major constituents of the Uruguayan mandarin peel oil (Verzera *et al*., 2000). While *Citrus reticulata* peel oil from Burundi consists mainly for limonene (84.8%), γ–terpinene (5.4%), myrcene (2.2%) and α–pinene (1.1%) as reported by Njoroge *et al*. (2006). Whereas, Chutia *et al*. (2009) reported that a total of 37 components were identified in Indian mandarin peel oil and the major components were limonene (46.7%), geranial (19.0%), neral (14.5%), geranyl acetate (3.9%), geraniol (3.5%), β–caryophyllene (2.6%), nerol (2.3%), citronellal (1.3%), neryl acetate (1.1%) etc.

In 2010, Hosni *et al*. found that at least 34 compounds were determined in Tunisian mandarin peel oils, which are rich in monoterpene hydrocarbons (98.9%). The major components were limonene (92.6%), γ–terpinene (3.39%), β–pinene (1.55%) and α–pinene (0.61%). Oxygenated monoterpenes were found as the second main group components (0.65%). Approximately, the half of this fraction was represented by linalool (0.31%). Six sesquiterpene hydrocarbons were identified in the mandarin peel oils with α–humulene (0.08%) being the most abundant compound. Cubebol and α–sinensal were found to be the major oxygenated sesquiterpenes in mandarin peel oil. Moreover, peel oil composition of Tunisian mandarin during ripening indicated that mature stage was characterized by the predominance of monoterpenes hydrocarbons, where limonene represents (69.00%) followed by γ–terpinene (14.06%), spathulenol (2.5%) , α-pinene (1.25%), α–terpineol (1.25%), E–β–ocimene (1.05%) and myrcène (0.98%) (Bourgou et al., 2012).

On the other hand, for *Citrus clementina Hort*. ex Tan. peel oils, the chemical composition of 16 samples of clementines, 30 identified components accounted for 97.3-99.5% of the total amount of oil. Peel oils consisted almost exclusively of hydrocarbons with limonene as the major component (89.1-95.5%) with sabinene (0.3-4.0%) and myrcene (1.4-2.0%). α–Pinene, β–phellandrene, β–pinene, (E)–β–ocimene, 3–carene and γ–terpinene were identified in almost all samples at low amounts (tr-0.6%). The oxygenated

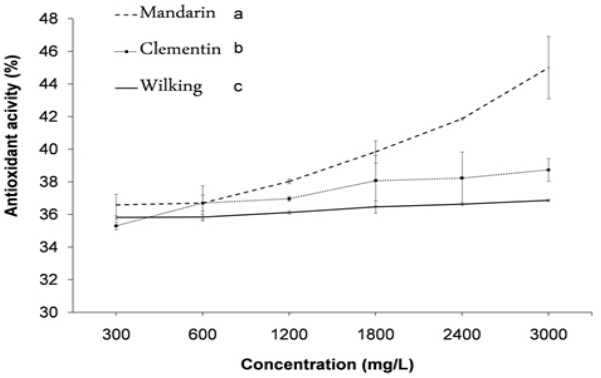

Figure 2. Effect of citrus peel EO concentrations on DPPH scavenging percentage

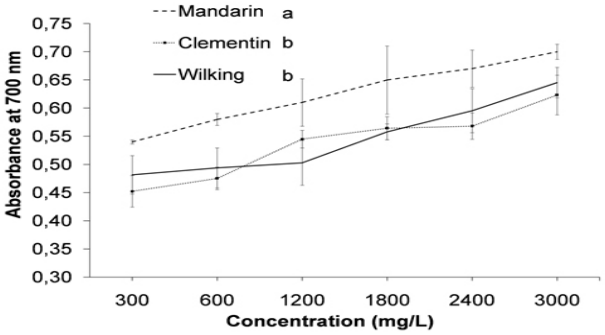

Figure 3. Effect of citrus peel EO concentrations on reducing power

fraction was made up of linalool (0.6-2.3%), octanal, decanal, citronellal, α–terpineol, α–sinensal and β–sinensal (≤ 0.7% for each one) (Lota *et al*., 2001).

Antioxidant activity

The antioxidant activities of the essential oil of citrus peels and its main components studied here were determined by two complementary test systems, namely DPPH and reducing power test. The methods chosen are the most commonly used for the determination of antioxidant activities of plant essential oils, because it is generally accepted that a mix of methods should be used for assessing antioxidant activities *in vitro* so that all aspects of antioxidant efficacy are covered (Viuda- Martos *et al*., 2010).

DPPH scavenging activity

The DPPH• scavenging activity of EOs was reported as the percent inhibition of DPPH•, with a higher value of percent of inhibition associated with a stronger antioxidant activity. The DPPH• scavenging ability of all EOs is shown in Figure 2. It can be seen that EOs presents different scavenging capacities. A concentration-dependent scavenging activity was found for all the EOs studied. The mandarin EO had the strongest ($p<0.05$) radical-scavenging effect at all concentration assayed. This activity was followed in decreasing order by clementine and wilking EOs.

Reducing power

Figure 3 shows the reducing power of the EOs as a function of their concentrations. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of the EOs. The presence of reducers causes the conversion of the Fe^{3+}/ferricyanide complex used in this method to the ferrous form. This reducing capacity occurs in a concentration-dependent manner. mandarin EO showed the greatest reducing power while those of wilking and clementine showed the lowest reducing capacity with no statistically differences ($p>0.05$).

The antioxidant nature of the citrus EOs in terms of free radical scavenger may be due to antioxidant activity of limonene, the major constituent of the oils (Junior *et al*., 2009). Nevertheless, antioxidant activities of essential oils may vary based on the variations in chemical composition. Antioxidant activity of an essential oil is attributed mainly to its major components, although the synergistic or antagonistic effect of one compound in minor percentage of mixture has to be considered. In fact Misharina and Samusenko (2008) found that essential oils exhibit antioxidant activity that largely depends on their composition in their study of antioxidant properties of essential oils from lemon, grapefruit, coriander, clove, and their mixtures. Furthermore, Misharina *et al*. (2011) found that the antioxidant activity of lemon peel essential oils depended on the system composition and concentrations of essential oils. Individual citral and limonene displayed the lowest antioxidant activity, whereas the activity of their mixture was higher, which explained the synergetic effects in the antioxidant activity of the components.

The relatively weak activity of citrus essential oils compared to other plant oils, such as the values obtained by Chikhoune *et al*. (2013) in their study of *Tetraclinis articulata* (Vahl) Masters essential oils antioxidant activity was confirmed by Teixeira *et al*. (2013), that found that the antioxidant activity of 17 different essential oil including grapefruit and lemon EOs, measured with the ferric reducing power assay revealed similar results to those obtained with the DPPH technique. The highest antioxidant activities were obtained for clove and origanum essential oils, followed by thyme and citronella, whereas the remaining essential oils including grapefruit and lemon oils had relatively the lowest activities. According to Liyana-Pathirana and Shahidi (2006),

Table 2. Zones of growth inhibition (mm) showing antimicrobial activity for mandarin, wilking and clementine EOs; disk diameter 6.0 mm

	Strains tested								
	L.i.	S.a.	M.R.S.a.	B.s	E.c	S.t.	V.c.	P.a.	C.a.
EOs	Inhibition zone (mm)								
Mandarin	+++ 17.13	+++ 15.06	+++ 16.11	-	+++ 21.23	-	-	± 8.76	+++ 21.81
Clementine	++ 14.01	++ 12.62	++ 13.24	-	++ 13.09	-	-	-	+++ 27.63
Wilking	+ 9.87	+ 9.16	+ 10.35	-	+ 10.52	-	-	-	+++ 17.63

E.c.: *Escherichia coli* O157:H7; L.i.: *Lysteria innocua*; S.a.: *Staphylococcus aureus*; M.R.S.a.: Methicillin-Resistant *S. aureus*; B.s.: *Bacillus subtilis*; S.t.: *Salmonella typhi*; V.c.: *Vibrio cholerea*; P.a.: *Pseudomonas aeruginosa*; C.a.: *Candida albicans*.
The scale of measurement was the following: -, no inhibition; ±, inhibition (<9 mm diameter); +, clear inhibition (9-12 mm diameter); ++, strong inhibition (13-15 mm diameter); +++, huge inhibition (>15 mm diameter).

phenolic and terpenoid compounds present in the chemical composition of EOs are closely associated with their antioxidant function, mainly due to their redox properties exerted by various possible mechanisms: free radical-scavenging activity and hydrogen Donors, etc.

Antimicrobial assay

Preliminary screening of the in vitro antimicrobial activity of mandarin, wilking and clementine EOs was carried out against 9 spoiling and pathogenic microorganisms using the filter paper disc agar diffusion technique. Table 2 summarizes the antimicrobial activity of the three citrus fruit EOs studied. Citrus EOs are complex mixtures of different compounds and their antimicrobial activity depends on their chemical composition. Preliminary results obtained using the filter paper disc agar diffusion technique showed great differences among the antimicrobial activity of the three EOs. Overall, the three essential oils displayed a broad antimicrobial spectrum against only gram-positive bacteria with no effect against gram-negative bacteria, an exception was about mandarin EO which showed slight effect against *Pseudomonas aeruginosa* and *Escherichia coli* which was sensitive to different oils while *Bacillus subtilis* proved to be resistant to the different essential oils.

Mandarin EO exerted the strongest antimicrobial effect against gram-positive bacteria and Escherichia coli, followed by clementine and wilking EOs. In general, our results indicate, in the decreasing order that *Candida albicans*, *Escherichia coli*, *Lysteria innocua*, Methicillin-Resistant *S. aureus* and *Staphylococcus aureus* were found to be the most sensitive to the three EOs because their growths were affected, while *Bacillus subtilis*, *Salmonella typhi*, *Vibrio cholerea* and *Pseudomonas aeruginosa* were the most resistant bacteria.

It is well known the antifungal and antibacterial properties of citrus oils, though the degree of inhibition of microorganisms varies considerably with the bacteria tested. In fact, Martinez *et al*. (2003) found that mandarin oil (*Citrus reticulata Blanco*) variety Dancy showed an antibacterial activity against *B. subtilis*, *S. aureus* and *L. monocytogenes*. Furthermore, Teixeira *et al*. (2013) found that Mean radius of inhibition zones (mm) of 20 µL undiluted essential oils of grapefruit and lemon tested against *E. coli* and *L. innocua* were not detected for the first strain and 7 and 5 (mm), respectively for the second strain. Whereas Espina *et al*. (2011) found that the inhibition zones of mandarin EO against *S. aureus*, *E. coli* O157:H7 and *P. aeruginosa* were 18.8, 20.0 (mm) and no inhibition, respectively, which is almost in accordance with our results.

Viuda-martos *et al*. (2008) study showed an antifungal activity of lemon, mandarin, grapefruit and orange essential oils on the growth of moulds commonly associated with food spoilage namely: *Aspergillus niger*, *Aspergillus flavus*, *Penicillium chrysogenum* and *Penicillium verrucosum*, using the agar dilution method. In addition, Hammer *et al*. (1999) reported that sporulation of *Candida albicans* was completely inhibited by *C. reticulata* oil at 2 µL/mL.

Minimal inhibitory concentration (MIC) of mandarin, wilking and clementine EOs was for all 5 µL/mL against the three Gram-positive microorganisms studied (namely *Lysteria innocua*; *Staphylococcus aureus*; Methicillin-Resistant *Staphylococcus aureus*), *Escherichia coli* O157:H7 and *Candida albicans*. Espina *et al*. (2011) found that the MIC of mandarin EO were 1, 5 and 5 (µL/mL) for *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *P. aeruginosa*, respectively, which is almost in agreement with our results. On the other hand, Sharma and Tripathi (2008) suggested that citrus EO exhibits among other properties, antifungal activity by reducing or totally inhibiting fungal growth in a dose response manner, this was confirmed by Velázquez-Nuñez *et al*. (2013) that found the growth of *Aspergillus flavus* decreased when increasing orange peel EO concentration.

Chemical composition of individual EO constituents affects the mode of action and antibacterial activity of the plant extracts (Dorman

and Deans, 2000) thus, this antimicrobial activity could be provoked by the major compounds of the EOs or due to a synergistic effect or antagonistic effect among the major compounds and the minor ones (Viuda-Martos *et al*., 2008; Carovic-Stanko *et al*., 2010). A comparison of the constituents among the EOs, and their amount in each case, may help to understand the key points in the antimicrobial activity of EOs, this suggested that the compounds present in the greatest proportions (limonene) are not necessarily responsible for the greatest share of the total activity. Thus, the involvement of the less abundant constituents should be considered; so, the activity could be attributed to the presence of compounds like linalool, α-pinene that have antifungal and antibacterial activity (Matasyoh *et al*., 2007) which are found in appreciable amounts in Citrus oils.

The chemical characterization of the three EOs demonstrated the presence of a significantly higher proportion of γ-terpinene, β-pinene, ρ-cymene, α-terpinolene, α-thujene in mandarin EO, in contrast to wilking and clementine Therefore, these monoterpènes might be involved in the higher antimicrobial activity of mandarin EO. In fact, a positive correlation between monoterpenes other than limonene and sesquiterpene content of the citrus fruit essential oils and the pathogen fungi inhibition was found by Caccioni *et al*. (1998). Whereas Settanni *et al*. (2012) found that the efficacy of some citrus EOs may be imputable to the oxygenated monoterpenes, in particular to α-terpineol, cis-geraniol, β-citral, nerol and α-citral, exhibits strong antimicrobial activity than hydrocarbon monoterpènes, the latter are characterized by a low water solubility which limits their diffusion through the medium and their inactivity is closely related to their limited hydrogen bound capacity (Griffin *et al*., 2000).

Burt (2004) and Al-Reza *et al*. (2010) studies showed that Gram-positive bacteria were more susceptible to EOs of different origin, including citrus fruits, than Gram-negative bacteria. The presence of the outer membrane provides a strong impermeable barrier for the Gram-negative bacteria (Nikaido, 1994). In fact, Settanni *et al*. (2012) found that activity of the essential oils (EOs) extracted from 23 fruit peel of several citrus genotypes (pummelo, grapefruit, orange, kumquat, mandarin and lemon) was evaluated against 92 foodborne pathogen bacteria (43 strains of *Listeria monocytogenes*, 35 strains of *Staphylococcus aureus* and 14 strains of *Salmonella enteric*), EOs were more effective against the Gram-positive bacteria rather than *Salmonella*. Whereas previous other studies showed that the inhibition of some Gram-negative (*Escherichia coli* and *Campylobacter jejuni*) strains was comparable with that of Gram-positive bacteria (Fisher and Phillips, 2006); They suggested that the antibacterial effects of citrus EOs are not uniform among bacteria and could depend on the compounds and the species/ isolate under study.

Although the EOs showed antimicrobial activity, the reason behind this capacity are not well documented. According to (Viuda-Martos *et al*., 2008; Tyagi and Malik, 2011; Xing *et al*., 2012),The antimicrobial mechanisms of essential oils may be an attack on the cell membrane's phospholipid bilayer, the disruption of the enzymatic systems, the compromising of the genetic material of the bacteria/ yeast, the formation of fatty acid hydroperoxidase by the oxygenation of unsaturated fatty acids, the coagulation of the cytoplasm, the damage of lipids and proteins, the distortion of the proton motive force (PMF), the electron flow and/or the active transport; they can also inhibit the activity of protective enzymes and sequentially inhibit one or more biochemical pathways. EOs are hydrophobic, thus they can enter in the phospholipid bilayer of the cell membrane and of the mitochondria distorting the structure and making them more susceptible to the cell leakage of vital intracellular constituents.

Cristani *et al*. (2007) reported that the antimicrobial activity is related to ability of terpenes to affect not only permeability but also other functions of cell membranes, these compounds might cross the cell membranes, thus penetrating into the interior of the cell and interacting with critical intracellular sites. In fact, the antimicrobial effects of citrus essential oils have been mainly explained through the presence of C10 and C15 terpenes with aromatic rings and a phenolic hydroxylic group able to form hydrogen bonds with active sites of target enzymes (Belletti *et al*., 2004). Furthermore, the antimicrobial activity of EOs on yeasts may be due to the disturbance in several enzymatic complexes involved in the energy production and in the synthesis of structural components (Xing *et al*., 2012). Additionally, the EOs can inhibit completely the germ tube formation of yeast (Zuzarte *et al*., 2012).

On the other hand, target cell damage caused by acid lime essential oil was investigated under transmission electron microscopy. Destructive alterations of plasma and nucleus membrane, loss of cytoplasm, vacuole fusion, and detachment of fibrillar layer were clearly exhibited in essential-oil-treated cells of *Aspergillus flavus* and *Aspergillus parasiticus* (Rammanee and Hongpattarakere, 2011).

Conclusion

In conclusion, differences in yield and chemical composition of the studied Citrus peel essential oils reflect genetical differences among studied species and cultivars. Based on the present study, citrus essential oil showed interesting results; the different samples namely mandarin, wilking and clementine showed remarkable antioxidant activities and may be rich sources of antioxidants. In DPPH and in reducing power assay, mandarin essential oil showed a higher antioxidant activity than wilking and clementine essential oils. On the other hand, it could be concluded that essential oil from *Citrus reticulata* and *clementina* possess antimicrobial activities inhibiting the growth of food borne pathogens specially *Candida albicans*, *E. coli*, *lysteria innocua* and *staphylococcus aureus*, where mandarin essential oil being the best performing one.

In view of their potential as inhibitory of pathogenic microbial growth as well as their antioxidant activity, the citrus peel essential oils may be recommended for formulation of plant based preservatives for enhancement of shelf life of food items by controlling their losses from bacterial contamination and lipid peroxidation during storage, Additionally, the abundance of citrus peel byproducts as raw materials from food industry and their renewable nature makes the use of citrus peel essential oils economical for practical application, thus they can be considered as potential possible alternatives to synthetic preservatives.

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