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To cite this article: C. Torres-Alvarez, A. Núñez González, J. Rodríguez, S. Castillo, C. Leos-Rivas & J. G. Báez-González (2017) Chemical composition, antimicrobial, and antioxidant activities of orange essential oil and its concentrated oils, *CyTA - Journal of Food*, 15:1, 129-135, DOI: 10.1080/19476337.2016.1220021

To link to this article: <https://doi.org/10.1080/19476337.2016.1220021>



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Published online: 07 Nov 2016.



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Chemical composition, antimicrobial, and antioxidant activities of orange essential oil and its concentrated oils

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ABSTRACT

In this study, the chemical composition, antimicrobial, and antioxidant activities of orange essential oil (AN), its folded orange oils (5×, 10×, 20×) and d-limonene (LN) were investigated. The results observed in the chemical composition showed a decrease in the major component LN, in contrast to other minor components, which increased their concentration, such as decanal, linalool, and α -terpineol. The antimicrobial activity was determined for foodborne pathogens using the disk diffusion method followed by the minimum inhibitory concentration and minimum bactericidal concentration. Results showed that folded orange oils (5×, 10×, 20×) had better antimicrobial activity than AN and LN. The antioxidant activity was carried out by 2,2'-azinobis-3-ethylbenzthiazoline-6-sulphonate and 1,1-diphenyl-2-picrylhydrazyl methods; folded orange oil 20× presented significantly better results ($p \leq 0.05$) than other oils studied. Using folded oils from AN could be a natural alternative in food processing as ingredients with antimicrobial and antioxidant effect.

Perfil químico, actividad antimicrobiana y antioxidante del aceite esencial de naranja y sus aceites concentrados

RESUMEN

En este estudio, se investigó la composición química, actividad antimicrobiana y antioxidante del aceite esencial de naranja (AN), sus concentrados (5×, 10×, 20×) y d-limoneno (LN). Los resultados en la composición química mostraron una disminución del componente mayoritario d-limoneno, contrario a otros componentes minoritarios que aumentaron su concentración como es el decanal, linalol y α -terpinol. La actividad antimicrobiana fue determinada para bacterias de importancia en alimentos por el método de difusión en disco seguido de la concentración mínima inhibitoria (CMI) y concentración mínima bactericida (CMB). Los resultados mostraron que los aceites concentrados (5×, 10×, 20×) presentaron significativamente mayor actividad antimicrobiana que el AN y LN. La actividad antioxidante se realizó por los métodos de ABTS y DPPH obteniendo que la fracción 20×, presentó actividad antioxidante significativamente mayor ($p \leq 0,05$) que los demás aceites estudiados. Los aceites concentrados del aceite esencial de naranja podrían ser una alternativa en la aplicación en alimentos como ingredientes con efecto antimicrobiano y antioxidante

ARTICLE HISTORY

Received 19 April 2016
Accepted 28 July 2016

KEYWORDS

Fold oil; orange essential oil; composition; antimicrobial; antioxidant

PALABRAS CLAVE

Aceite concentrado; aceite esencial de naranja; composición; antimicrobiano; antioxidante

Introduction

Essential oils (EOs) from aromatic and medicinal extracts obtained from a wide variety of plants have recently become popular and raised scientific interest as potential natural agents for food preservation because of their biological activity (Velázquez-Núñez, Avila-Sosa, Palou, & López-Malo, 2013). Citrus EOs have been a part of the human diet for hundreds of years, and they belong to the group of substances generally recognized as safe (GRAS) by U.S. Food and Drug Administration (FDA) (Nannapaneni et al., 2009). It has been suggested that the use of such compounds as additives in foods may act as preservatives, preventing the growth of pathogens and spoiling microorganisms of the product concerned (Fisher & Phillips, 2006; Velázquez-Núñez et al., 2013).

In recent years, consumers have become aware of the health problems (oxidative-stress-related disease) caused by synthetic chemical ingredients used in processed foods.

Therefore, a natural alternative to the chemical preservation of foods is the use of essential oils. Industrially, citrus EOs have been widely applied in a variety of products, including foods, beverages, cosmetics, and medicines because of their flavor, fragrance, and certain properties (Liu, Chen, Liu, Zhou, & Wang, 2012; Settanni et al., 2012). Citrus EOs are complex mixtures containing volatile (85–99%) and nonvolatile (1–15%) compounds mainly from monoterpenes, sesquiterpene hydrocarbons, and their oxygenated derivatives, including aldehydes, ketones, acids, alcohols, and esters. D-Limonene (LN), (1-methyl-4-(1-methylethenyl) cyclohexane) is one of the main component of citrus EOs (25–98%), and its content varies significantly between species and/or varieties of the same (Fisher & Phillips, 2008; Jing et al., 2014). The nonvolatile residue may contain hydrocarbons, sterols, fatty acids, waxes, carotenoids, coumarins, psoralens, and flavonoids; this latter group of compounds is useful for differentiating

between species (Jing et al., 2014; Tranchida, Bonaccorsi, Dugo, Mondello, & Dugo, 2012).

The chemical composition of the citrus EOs is influenced by several factors, such as the genetic differences between varieties and species, which are the main determinants of the composition and content of essential oils. Environmental factors, such as soil type, cropping practices, stages of maturity, and types of weather can contribute to quantitative variations in the content of essential oils; in addition, they could affect the biological activity of the oils (Jing et al., 2014). Citrus EOs contain a high amount of terpene hydrocarbons; however, these compounds do not contribute much to the flavor and fragrance of the oil. In addition, they are unstable when exposed to heat or light and the solubility of the whole oil in alcohol decreases. Because of this, in order to stabilize the final product, these kinds of compounds should be removed. Furthermore, the oxygenated fraction provides much of the intense characteristic flavor of citrus EOs and consists mainly of alcohols, aldehydes, ketones, and esters (Lopes, Raga, Stuart, & Oliveira, 2003).

Industrially, orange essential oil (AN) is produced from orange peel through the cold-pressed method. Different procedures can be applied for the reduction of undesirable compounds, such as terpene hydrocarbons (including LN), which conventionally may be reduced by vacuum distillation (O'Bryan, Crandall, Chalova, & Ricke, 2008), solvent extraction, and adsorption chromatography. During vacuum distillation, it is difficult to indicate general limits for obtaining defined physicochemical properties of the concentrated fractions because these depend on the degree of concentration, operating conditions, applied technology, relative proportions of the oxygenated components of origin, and economic factors (Lopes et al., 2003). Commercially, the main folding degrees available are 2–5-fold for lime and mandarin oils, 2–10-fold for lemon and grapefruit oils, and 2–20-fold for orange oil. Folded oils are less prone to oxidation, they have a high solubility in water, and they have high organoleptic qualities (López-Muñoz & Balderas-López, 2014).

Moreover, the application of antioxidants plays an important role in inhibiting oxidative reactions in various products; in addition, these could prevent diseases related to the oxidative stress in the human body (Liu et al., 2012). Application of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), has resulted in the appearance of significant side effects, which explains the growing interest in search for natural antioxidants (Olmedo, Nepote, & Grosso, 2014).

Another important issue among the food, cosmetics, and pharmaceutical industries is microbial contamination. The continuous use of synthetic preservatives can cause the development of resistance in some microorganisms, as well as residual toxicity. To solve these problems, natural and safe antimicrobial additives have been suggested. Recently, natural products, such as essential oils or compounds obtained from plants have gained scientific interest and acceptance by these industries, since they have shown little or no harm to human health (Char, Cisternas, Pérez, & Guerrero, 2016; Liu et al., 2012). Biological activities of citrus EOs as antifungal, antioxidant, and antimicrobial among others, have been studied (Espina et al., 2011; Jing et al., 2014; Liu et al., 2012; Singh et al., 2010). However, there are few reports on the antioxidant and antimicrobial activity of the individual components and/or folded oil obtained from the AN.

The aim of this research was to compare and evaluate the chemical composition of AN, its folded oils (5×, 10×, 20×) and LN (as main component) as well as evaluating their antimicrobial and antioxidant activity.

Materials and methods

AN and folded oil

AN was provided by Frutech International (NL, Mexico), industrially obtained through cold-pressed method; subsequently, the folded oils were prepared in a vacuum-distilled column with structured packing equivalent to 20 theoretical plates. The equipment was operated in a batch mode. The conditions of the parameters used during vacuum distillation were adjusted based on the initial composition of AN in order to obtain a final product with a desired composition according to the specification of each folded oil with the following parameters: pressure range 5–20 mbar and the reflux at temperatures between 80°C and 100°C. Table 1 shows some physicochemical characteristics of the AN and folded orange oils (5×, 10×, 20×). LN 97% and Trolox were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA). The reagents used were analytical grade.

Gas chromatography–mass spectrometry (GC–MS) analysis

The analysis for AN and its folded oils (5×, 10×, 20×) was performed according to Liu et al. (2012), with some modifications. These oils were analyzed by GC–MS (7890B, Agilent Technologies, Santa Clara, CA, USA). GC was conducted on HP-5 MS (30 m × 0.25 mm × 0.25 µm) capillary column. The GC conditions were as follows: injection temperature 250°C; the oven temperature was controlled at 70°C for 1 min with the heating rate from 10°C/min to 200°C for 2 min, and finally from 10°C/min to 300°C for 5 min. Helium gas was used as carrier gas at a constant flow rate of 1 mL/min, sample size at 1 µL. Parameters for MS analysis 5977A were with EI ion source, electron energy 70 eV, the temperature of quadrupoles 150°C, temperature of interface 230°C, m/z = 30–400 amu. Identification of compounds was carried out by comparing their mass spectra with those of Wiley 7 n.L library, considering a quality match >85%. In addition, essential oils' analyses were performed in one more capillary column (DB-1 MS, 60 m × 0.25 mm × 0.25 µm) for a more accurate identification. Results were confirmed with the injection of some standards of essential oils components (LN, linalool, α -terpineol, decanal, and valencene) (Sigma-Aldrich) (data not shown).

Table 1. Chemical properties of the oils tested.

Tabla 1. Propiedades químicas de los aceites probados.

Code	Commercial name	Specific gravity (20°C)	Refractive index (20°C)	Solubility in water
AN	Orange essential oil	0.846	1.477	Negligible
LN	D-Limonene	0.840	1.472	Negligible
5×	Fivefold concentrated orange oil	0.884	1.482	Negligible
10×	Tenfold concentrated orange oil	0.920	1.494	Negligible
20×	Twentyfold concentrated orange oil	0.945	1.497	Negligible

Bacterial strains and growth conditions

The following strains used in the study were acquired from American Type Culture Collection (ATCC), *Salmonella typhi* (ATCC 19430), *Bacillus cereus* (ATCC13061), *Staphylococcus aureus* (ATCC 6538), and *Listeria monocytogenes* (ATCC 7644), and kindly provided by the Laboratory of Sanitary Microbiology, FCB, UANL. The strains were stored at -80°C in brain heart infusion with 20% (v/v) glycerol (Difco Laboratories, Sparks, MD, USA). Fresh cultures were obtained in Mueller Hinton broth or agar (MHB and MHA, Difco Laboratories). *L. monocytogenes* was cultivated on trypticase soy broth (TSB). An aliquot (50 μL) was taken from a frozen culture and was added to a tube containing 5 mL of MHB or TSB depending of each strain and were incubated at 37°C for 18 h (30°C for *L. monocytogenes*); thereafter an aliquot (10 μL) of each culture was added to the tubes with MHB or TSB.

Antimicrobial activity assay (disk diffusion assay)

Preliminary antimicrobial activity of AN, LN, and folded orange oils (5 \times , 10 \times , 20 \times) was tested against four food-borne pathogens, using disk diffusion technique (Klančnik, Piskernik, Jeršek, & Možina, 2010). An aliquot (100 μL) of bacterial suspension (adjusted to 0.5 McFarland $\approx 10^8$) was distributed homogeneously on MHA plates. Five disks of 6 mm in diameter were separately impregnated with 10 μL of each oil and placed on the agar surface. The plates were incubated at 37°C for 24 h. Antimicrobial activity was defined as absence of bacterial growth in the area surrounding the disk. The diameter of the inhibition zones was measured with a caliper. A test for *L. monocytogenes* was conducted the same way, but TSA was used instead of MHA, and it was incubated at 30°C . Duplicate analyses were performed at least three times. Disks impregnated with sterile distilled water and/or DMSO served as negative controls and disks with an antibiotic (gentamicin) as positive controls.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

After determining preliminary antimicrobial activity, the MIC and MBC were obtained for the four pathogens. The dilution method according to Castillo, Heredia, Contreras, and García (2011), with minor modifications was carried out. Briefly, an aliquot (50 μL) of fresh cultures adjusted to McFarland standard ($0.5 \approx 1 \times 10^8$) of each strain were added separately in tubes containing 5 mL of MHB with various concentrations of AN, LN, 5 \times , 10 \times , 20 \times (0.25, 0.5, 1.0, 1.5, 2, 2.5 mg/mL final concentration in each tube for every oil). Cultures were incubated at 37°C for 24 h. After that, bacterial survival was determined by plate counting. MIC was defined as the lowest concentration of oil that decreased growth about 90% of the bacterial population after 24 h of incubation, while the MBC was defined as the lowest concentration that completely inhibited growth on the MHA plate after 24 h of incubation (Klančnik et al., 2010). *L. monocytogenes* was tested the same way under the conditions referred above. Triplicate analyses of MIC and MBC were conducted at least three times.

Antioxidant activity assay

The antioxidant activity of the oils was measured using the 2,2'-azinobis-3-ethylbenzthiazoline-6-sulphonate (ABTS) radical and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Trolox (Sigma-Aldrich) a water-soluble analogue of vitamin E, showing a potent antioxidant activity, was used as a standard reference (positive control). ABTS radical scavenging assay was determined following the method of Re et al. (1999), with some modifications, the blue-green ABTS radical cation chromophore ($\text{ABTS}^{\cdot+}$) was prepared by reacting ABTS stock solution (7 mmol) with potassium persulphate (2.45 mmol) and allowing the mixture to stand at room temperature in the dark during 16 h. The solution was diluted with ethanol to obtain an absorbance of 0.700 ± 0.02 at 734 nm in a Genesys 5 (spectronic) spectrophotometer. The DPPH radical scavenging assay was conducted according to Brand-Williams, Cuvelier, and Berset (1995), with some modifications. DPPH solution was prepared with 3.9 mg of radical and 100 mL of ethanol, the solution was measured to 517 nm in a spectrophotometer to obtain an absorbance of 1.000 ± 0.05 . Aliquots of 10–300 μL of solution of each oil with ethanol were mixed with 2.7 mL of $\text{ABTS}^{\cdot+}$ solution. The mixture was allowed to react at room temperature for 7 min in dark conditions. The absorbance was recorded by spectrophotometer at 734 nm. For DPPH assay, different concentrations (200–600 μL) of each oil were added separately to 2.25-mL DPPH solution. The mixture was shaken and left at room temperature for 240 min in the dark. Thereafter, the absorbance was measured at 517 nm in spectrophotometer.

The antioxidant activity was calculated as a percentage of inhibition according to the following equation:

$$\% \text{Inhibition} = \{[\text{Ab} - \text{As}/\text{Ab}] \times 100\},$$

where Ab represents the absorbance of the control (without test oil), and As represents the absorbance of the test oil. A calibration curve was determined for Trolox standard for each radical (ABTS and DPPH) at a concentration range of 10–160 μmol . The curve equations were $y = 0.4447x + 0.3721$, $R^2 = 0.99$ for ABTS and $y = 0.7371x + 0.7321$, $R^2 = 0.99$ for DPPH. Triplicate analyses of each oil were made. The antioxidant activity values were expressed as μmol Trolox equivalent (TE)/mL citrus oil (CO). In addition, the antioxidant activity expressed as half-maximal inhibitory concentration IC_{50} (mg/mL) was defined as the amount of antioxidant necessary to decrease the initial ABTS and DPPH concentration by 50% (Liu et al., 2012).

Statistical analysis

All experiments were performed in duplicate or triplicate at least three times. Statistical analyses were performed using SPSS software (IBM version 22, SPSS Inc, Chicago, IL). Data were analyzed by analyses of variance test (one-way ANOVA) and a post hoc test of Tukey's multiple range. Differences between means were considered significant at p values ≤ 0.05 .

Results and discussion

Chemical composition of the citrus EOs

The analysis of the main components of AN, 5 \times , 10 \times , 20 \times showed differences in the composition and percentage of

Table 2. Chemical composition of AN, 5×, 10×, 20×.**Tabla 2.** Composición química de AN, 5×, 10×, 20×.

No.	Compound	Percentage (%) ^a			
		AN	5×	10×	20×
1	α-Pinene	1.11			
2	Sabinene	0.41			
3	Myrcene	4.11	0.43	1.11	
4	α-Phellandrene	0.42			
5	Octanal	0.15			
6	D-Limonene	91.12	72.08	73.77	23.24
7	β-Ocimene	0.11			
8	Terpinolene	0.10			
9	Octanol	0.10			
10	Linalool	0.65	3.33	3.99	5.53
11	<i>trans</i> -p-Mentha-2,8-dienol		0.80	0.46	0.98
12	<i>cis</i> -p-Mentha-2,8-dienol				1.00
13	<i>cis</i> -Limonene oxide		1.94		
14	<i>trans</i> -Limonene oxide		1.01		
15	Citronellal	0.09	0.52	0.69	1.04
16	α-Terpineol	0.60	1.37	2.16	4.60
17	Decanal	0.44	3.60	4.78	18.46
18	<i>trans</i> -Carveol		1.07		1.59
19	Citronellol				0.61
20	<i>cis</i> -Carveol				0.76
21	Neral		0.67	0.79	1.70
22	Carvone		1.25	0.68	2.01
23	Geranial		1.12	1.33	3.48
24	Perilla aldehyde		0.52	0.57	1.31
25	Geraniol acetate				0.45
26	Dodecanal		0.84	1.09	2.80
27	α-Cubebene		0.71	0.98	2.32
28	β-Cubebene	0.05		0.82	2.22
29	Caryophyllene			0.64	1.35
30	Germacrene D	0.05	0.71	0.94	0.91
31	Valencene		4.89	2.14	6.35
32	γ-Murolene		0.65		1.46
33	δ-Cadinene	0.06	0.98	1.31	3.21
34	Elemol				0.96
35	Caryophyllene oxide				0.77
36	Nootkatone				0.94
Hydrocarbon monoterpenes		97.37	72.51	74.88	23.24
Oxygenated monoterpenes		1.78	17.20	15.45	43.07
Hydrocarbon sesquiterpenes		0.17	7.94	6.83	17.82
Oxygenated sesquiterpenes		ND	ND	ND	2.67
Others		0.25	0.84	1.09	3.25
Total identified components		99.57	98.49	98.25	90.05

^aGiven in the relative peak area percent, mean of triplicate determination. ND: not detected.

^aDada en el porcentaje de área relativa del pico, con una media de determinación por triplicado. ND = No detectado.

the compounds observed (Table 2). These results agree with results of previous studies (Espina et al., 2011; Ruiz & Flotats, 2014; Singh et al., 2010) in which differences in the chromatographic profile of AN were found depending on the type of extraction, retaining its major components, such as the LN. This finding is consistent with our results, which showed LN as majority compound. Lopes et al. (2003) performed a study with different parameters during vacuum distillation and they found quantitative variations in the chromatographic profiles. Our results showed the same tendency indicating that these variations might be influenced by the type of extraction used. It is well known that vacuum distillation process produces folded oils from orange EO, through a selective decrease of LN (main constituent) and other terpene hydrocarbons; therefore, the concentration of oxygenated compounds, primarily aldehydes and alcohols are increased (Lopes et al., 2003; O'Bryan et al., 2008). Terpene hydrocarbons (including LN) must be removed in order to obtain a stable final product because they do not contribute much to the flavor and fragrance of the oil; also, they are unstable when exposed to heat or light and their oil

solubility is decreased as well (Beneti et al., 2011; Liu et al., 2012; Lopes et al., 2003). This study analyzed the chemical composition of AN and its folded oils 5×, 10×, and 20× to estimate the differences between the constituents, since the folded orange oils, provide greater flavor intensity by increasing the concentration of oxygenated compounds. In addition, they are less susceptible to oxidation and off-flavor development, showing better stability with greater industrial interest (Beneti et al., 2011; Lopes et al., 2003). Considering the information mentioned above, it is preponderant to evaluate the antimicrobial and antioxidant activity of the folded orange oil. Analysis by GC-MS gave the chemical composition and relative abundance of the major components that comprise AN, 5×, 10×, 20×. The components are grouped into several classes: hydrocarbon monoterpenes, oxygenated monoterpenes, hydrocarbon sesquiterpenes, oxygenated sesquiterpenes, and others (Table 2). These results showed that there are qualitative similarities between components present in oils although some of them showed quantitative differences when tested. For AN, 5×, 10×, and 20×, were identified 16, 20, 18, and 26 compounds, respectively, representing the 99.57%, 98.49%, 98.25%, and 90.05% of the total detected constituents, respectively. There was a decrease in the mixture of hydrocarbon monoterpenes (including LN) in all fractions compared with AN (97.37%), keeping the 20× fraction the minor amount (23.24%) followed by 5× and 10× with 72.51% and 74.88%, respectively. For oxygenated monoterpenes, an increase in the concentration of folded orange oils with respect to AN was observed, being these 1.78% (AN), 17.20% (5×), 15.45% (10×), and 43.07% (20×), coinciding with Lopes et al. (2003) who reported an inversely proportional diminution on hydrocarbon compounds, such as LN, myrcene, among others, respect to the folded oils, while for oxygenated compounds as decanal and linalool, an inversely proportional increase with respect to the folded oils was reported. It is important to note the variation about the quantity of oxygenated monoterpenes between the oils because some compounds from this group are recognized with biological and antimicrobial activity (Espina et al., 2011). According to some reports (Espina et al., 2011; Njoroge, Koaze, Karanja, & Sawamura, 2005; Singh et al., 2010), LN is found at around 90%, recognized as the major component of AN, coinciding with our results, in which AN presented 91.12% of LN; however in the folded oils, a decrease of this compound was observed, resulting in 72.08% and 73.77% of LN in 5× and 10×, respectively, while in the 20×, a lower amount was observed (23.24%). Nevertheless, LN was still the leading component in all oils analyzed. Other compounds detected over 1%, as decanal, an aldehyde that provides fragrance (Liu et al., 2012) increased significantly in 5×, 10× and 20× with 3.60%, 4.78%, and 18.46%, respectively, while in AN it was detected only 0.44%. In addition, compounds such as linalool, recognized for its importance in fragrance and biological activity (Fisher & Phillips, 2006) presented an increment in 5× (3.33%), 10× (3.99%), and 20× (5.53%), with respect to AN (0.65%). The same tendency was observed for hydrocarbon sesquiterpenes, in which an increase was detected for 5× (7.94%), 10× (6.83%), and 20× (17.82%) compared to AN (0.17%), being the 20× the major concentration of these compounds. Finally, oxygenated sesquiterpenes were detected only in 20× (2.67%), while in AN, 5× and 10× were not.

Antimicrobial activity by the disk diffusion assay and determination of MIC and MBC

Antimicrobial activity was performed for AN, LN, 5×, 10×, and 20× by disk diffusion method against four foodborne pathogens. The antimicrobial activity of the oils studied is summarized in Table 3. The oils tested showed inhibition zone in ranges of 12–28 mm. *B. cereus* and *S. aureus* showed greater inhibition at 10× and 20×, *L. monocytogenes* showed inhibition with 5× and 20×, while for *S. typhi*, the greatest inhibition was in 5× and 10×, and it was comparable with positive control (gentamicin). When comparing the antimicrobial activity of AN and LN, with 5×, 10×, and 20× there were significant differences ($p \leq 0.05$) in inhibition; the major inhibition zones were for the folded orange oils in most cases. These results are similar to those previously reported by Espina et al. (2011), who found that lemon and orange EOs showed lower inhibition against foodborne pathogen than other essential oils, which could be attributed to the portion of oxygenated monoterpenes.

The MIC and MBC were determined for the four foodborne pathogens for the oils tested, the values were compared finding differences between AN and LN with 5×, 10×, and 20× (Table 4). The MIC varied among folded orange oils ranged from <0.25 to 1 mg/mL while the MBC from 0.25 to 2.5 mg/mL. Major values of MIC and MBC for AN and LN were evidenced. The MIC for these two oils ranged from 1.5 to 2.5 mg/mL and MBC from 2 to >2.5 mg/mL, depending on the strain tested. MIC and MBC values were lowest in the entire folded orange oils fractions being the 20× with the higher antimicrobial potential. However, the values varied very little between the folded orange oils for the same

microorganism, but on comparing the MIC and MBC values with AN and LN, significant differences were observed. LN showed the lowest antimicrobial activity with MIC values of 0.5–2 mg/mL and MBC values >2.5 mg/mL. Even AN presented lower values of MIC and MBC than LN, no significant differences were evidenced in most cases. These results agree with Frassinetti, Caltavuturo, Cini, DellaCroce, and Maserti (2011) and Nannapaneni et al. (2009), who reported a low or null antimicrobial activity of LN compared with citrus essential oils against foodborne pathogens.

When MIC/MBC were determined, slight differences in susceptibility with respect to the results obtained in disk diffusion assay were noted; the most sensitive bacteria was *L. monocytogenes* with MIC/MBC (<0.25/0.5 mg/mL), followed by *B. cereus* (0.5/1.5 mg/mL), *S. aureus* (0.5/2 mg/mL) while the more resistant one was *S. typhi* (1.0/2.5 mg/mL). This could be attributed to the disk diffusion method, which is limited due to changes in the diffusivity of the active compounds in agar; for this reason, the size of the inhibition zone did not necessarily predict the MIC/MBC determined in liquid medium (Nannapaneni et al., 2009; O'Bryan et al., 2008). On the other hand, MIC/MBC values varied between bacteria, confirming the observations of Kim, Marshall, and Wei (1995); Rivera, Bocanegra-García, and Monge (2010) who reported that essential oils could vary in their antimicrobial potential among different microorganisms. This will depend on several factors including the structure of bacterial cell wall and variability of compounds present in the oil, which sometimes could exert a synergism between them (O'Bryan et al., 2008). In previous reports, it has been demonstrated that compounds present in low concentrations have a crucial role in antimicrobial activity due to a synergic effect that potentiates biological activity. In this study, the folded orange oil 20× presented the best antimicrobial activity followed by 10× and 5×; all of them had major activity than AN and LN. This could be attributed to the increment of oxygenated monoterpenes considering that this portion was lower for AN (1.78%) than the fractions 5× (17.21%), 10× (15.45%), and 20× (43.07%). In addition, other minority compounds, which have been reported to possess antimicrobial activity (linalool, decanal, geranial, among others), could be implicated in synergism or biological activity (Jing et al., 2014; Nannapaneni et al., 2009; Singh et al., 2010). These findings were consistent with those found in this study, in which these components are present, and are actually increased in the concentrated oils.

There are some studies about fractions from cold-pressed orange oil, terpeneless orange oil, terpenes from orange juice essence, or fivefold orange oil (Nannapaneni et al., 2009; O'Bryan et al., 2008), and also of isolated compounds

Table 3. Zones of inhibition of AN, LN, 5×, 10×, 20× against foodborne pathogens by a disk diffusion assay.

Tabla 3. Zonas de inhibición del AN, LN, 5×, 10×, 20× contra patógenos transmitidos por alimentos mediante ensayo de difusión en disco.

Oil	Zone of inhibition (mm)			
	<i>B. cereus</i>	<i>S. aureus</i>	<i>L. monocytogenes</i>	<i>S. typhi</i>
AN	13 ± 1 ^d	13 ± 1 ^d	12 ± 0.7 ^d	16 ± 0.7 ^c
LN	15.4 ± 1.1 ^d	17 ± 1 ^c	14 ± 0.7 ^d	17 ± 0.7 ^c
5×	23.4 ± 2.0 ^c	17.4 ± 0.5 ^c	24.4 ± 1.3 ^{b,c}	28 ± 1.4 ^a
10×	28 ± 1.2 ^b	20.4 ± 2.4 ^b	22.6 ± 1.8 ^c	27.2 ± 1.6 ^a
20×	27.8 ± 1.3 ^b	18.6 ± 0.8 ^{b,c}	26.2 ± 1.1 ^b	24.8 ± 1.1 ^b
Gentamicin	45.2 ± 0.8 ^a	37 ± 1.4 ^a	35.2 ± 0.8 ^a	28 ± 0.7 ^a

Zone of inhibition are average values of three replicates ± the standard deviation of the mean. ^{a–d}Mean values with different letter in the same column are significantly different ($p \leq 0.05$).

Zona de inhibición son valores promedio de tres repeticiones ± desviación estándar de la media. ^{a–d}Los promedios en una columna con diferente letra difieren significativamente ($p \leq 0.05$).

Table 4. Minimal inhibitory concentration (MIC) and minimal bactericide concentration (MBC) (mg/mL) of AN, LN, 5×, 10×, 20×.

Tabla 4. Concentración mínima inhibitoria (CMI) y concentración mínima bactericida (CMB) (mg/mL) de AN, LN, 5×, 10×, 20×.

Oil	Microorganisms							
	<i>B. cereus</i>		<i>S. aureus</i>		<i>L. monocytogenes</i>		<i>S. typhi</i>	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
AN	1.5 ± 0.3 ^{a,b}	>2 ^a	1 ± 0.3 ^b	>2 ^a	0.5 ± 0.2 ^a	2 ± 0.3 ^b	2 ± 0.5 ^a	>2.5 ^a
LN	>2 ^a	>2 ^a	2 ± 0.2 ^a	>2 ^a	0.5 ± 0.2 ^a	>2 ^a	2.5 ± 0.3 ^a	>2.5 ^a
5×	1 ± 0.3 ^{b,c}	1.5 ± 0.3 ^b	0.5 ± 0.2 ^b	1.5 ± 0.3 ^b	0.25 ± 0.1 ^a	0.5 ± 0.2 ^c	1 ± 0.3 ^b	2.5 ± 0.3 ^{a,b}
10×	1 ± 0.3 ^{b,c}	1.5 ± 0.3 ^b	0.5 ± 0.3 ^b	2 ± 0.3 ^{a,b}	0.25 ± 0.1 ^a	0.5 ± 0.2 ^c	1 ± 0.3 ^b	2.5 ± 0.3 ^{a,b}
20×	0.5 ± 0.3 ^c	1 ± 0.3 ^b	0.5 ± 0.2 ^b	1.5 ± 0.3 ^b	< 0.25 ^b	0.25 ± 0.1 ^c	1 ± 0.6 ^b	2 ± 0.3 ^b

MIC and MBC are average values of three replicates ± the standard deviation of the mean. ^{a–c}Mean values with different letter in the same column are significantly different ($p \leq 0.05$).

CMI y CMB son valores promedio de tres repeticiones ± desviación estándar de la media. ^{a–c}Los promedios en una columna con diferente letra difieren significativamente ($p \leq 0.05$).

Table 5. Antioxidant activity of AN, LN, 5×, 10×, 20×.**Tabla 5.** Actividad antioxidante de AN, LN, 5×, 10×, 20×.

Method	μmol TE/mL CO				
	AN	LN	5×	10×	20×
ABTS	23.25 ± 0.84 ^d	ND	86.02 ± 1.12 ^c	141.53 ± 0.16 ^b	156.25 ± 0.62 ^a
DPPH	3.01 ± 0.20 ^d	ND	7.69 ± 0.35 ^c	16.96 ± 0.81 ^b	21.24 ± 0.32 ^a

Mean values of three replicates ± the standard deviation of the mean.

^{a-d}Mean values with different letter in the same row are significantly different ($p \leq 0.05$). ND = Not detected.

Valores promedio de tres repeticiones ± desviación estándar de la media.

^{a-d}Los promedios en la misma fila con diferente letra difieren significativamente ($p \leq 0,05$). ND: No detectado.

from orange EO (Liu et al., 2012), a different variety of orange (Settanni et al., 2012; Singh et al., 2010) and a different variety of citrus EOs (Espina et al., 2011; Frassinetti et al., 2011; Settanni et al., 2012). However, there are no studies about folded oils (5×, 10× and 20×) from AN obtained by vacuum distillation, as studied in this work.

Antioxidant activity

The antioxidant activity values obtained for AN, LN, and folded orange oils were evaluated by the methods of ABTS and DPPH (Table 5). These were expressed as [μmol Trolox equivalent (TE)/mL citrus oil (CO)]. The values obtained by ABTS method were 23.25–156.25 μmol TE/mL CO and DPPH method showed values between 3.01 and 21.24 μmol TE/mL CO. The folded orange oil 20× showed the highest concentration equivalent Trolox with 156.25 μmol TE/mL CO in ABTS and 21.24 μmol TE/mL CO with DPPH, followed by 10× and 5× with values 141.53 and 86.02 μmol TE/mL CO, respectively, for ABTS, and 16.96 and 7.69 μmol TE/mL CO, respectively, for DPPH. In the case of AN, it showed values of 23.25 and 3.01 μmol TE/mL CO in ABTS and DPPH, respectively. Finally, in LN, activity for any of the two methods was not detected. The IC₅₀ values are shown in Table 6, where the folded orange oil 20× showed higher antioxidant activity with values of 3.80 and 10.25 mg/mL to inhibit 50% of ABTS and DPPH radicals, respectively, followed by 10× and 5× with values 6.49 and 16.27 mg/mL, respectively, for ABTS while 15.50 and 37.25 mg/mL, respectively, for DPPH. The AN showed values of 68.40 and 70.17 mg/mL for ABTS and DPPH, respectively. The LN did not present antioxidant activity for inhibiting the radicals studied. The results showed significant differences in antioxidant activity between 20×, 10×, and 5× with respect to AN and LN, highlighting the best antioxidant activity for 20× compared to the others oils studied. The folded orange oils (5×, 10× and 20×) are mainly used in the food and cosmetics pharmaceutical industries for their flavor and fragrance; however, few studies have determined their antimicrobial and antioxidant activities. According to the results obtained in this work, the folded orange

oils showed better antioxidant activity than AN. This could be attributed to the potentiation of minority compounds and reduction of LN, which in normal conditions remain at 90%.

In some studies, it has been proposed that the antioxidant activity of citrus essential oils could be related to the presence of LN and other monoterpenes as γ-terpinene and terpinolene (Choi, Song, Ukeda, & Sawamura, 2000; Frassinetti et al., 2011). However, in this study the best results were obtained by the folded orange oils (5×, 10× and 20×), which according to the chromatographic profile, the amount of LN decreased while other compounds such as decanal and linalool increased. These results agree with Choi et al. (2000), who found that although LN was the majority compound in ANs, it would not play the main role in determining antioxidant activity.

Some investigations have studied the antioxidant activity of individual compounds. According to Liu et al. (2012), the antioxidant activity of AN was major compared to individual compounds, such as linalool, decanal, octanal, and valencene; this evidence could confirm, based on the results obtained in this study, that antioxidant activity is not due to a single compound as LN, but a mixture of compounds, that alter their amount when the orange EO is processed by vacuum distillation. Some of them were found in lesser proportion while others increased their concentration; this effect could potentiate biological or synergistic action among compounds that derive in higher antimicrobial and antioxidant activities. In that case, the folded orange oil could provide different biological characteristics of those found in the AN.

A major concern in this context is the toxicity of the compounds. A variety of EO components have been registered by the European Commission for being used as flavorings in food-stuffs. Therefore, presently no risk to human health, including the AN and LN (LN), which are GRAS. Meticulous studies of toxicology support the safety of the substance for its intended use (Gavarić et al., 2015). Studies in rats revealed the oral LD₅₀ for AN and LN and is established at >5 g/kg, also for the concentrated oils used in this study. According to the health hazard definition, the AN and its folded oils are classified as non-toxic (Tisserand & Young, 2014).

In addition, further research could address these results' focus on strategic applications of these compounds, considering their efficiency in antimicrobial and antioxidant activities (Choi et al., 2000).

Conclusions

The folded orange oils showed the best potential as antimicrobials, as well as in their antioxidant activity. The food-borne pathogens were affected at significant low concentrations than the AN and LN when treated with the concentrated oils, attributing these differences to their chemical composition. The results of this research may provide the knowledge of the antimicrobial and antioxidant potential of folded orange oils obtained from AN, and these could be used for control of foodborne pathogens as an alternative to replace the additives used today. Finally, the study of the enormous range of biological activities of essential oils and their prospective industrial applications in order to increase the food safety is suitable; at the same time, it may provide alternatives for the development of new, safer products that are accepted by consumers who prefer natural ingredients rather than synthetic ingredients.

Table 6. Inhibition concentration (IC₅₀) of AN, LN, 5×, 10×, 20×.**Tabla 6.** Concentración Inhibitoria (CI₅₀) de AN, LN, 5×, 10×, 20×.

Method	IC ₅₀ (mg/mL)				
	AN	LN	5×	10×	20×
ABTS	68.40 ± 0.39 ^a	ND	16.27 ± 0.32 ^b	6.49 ± 0.10 ^c	3.80 ± 0.06 ^d
DPPH	70.17 ± 5.15 ^a	ND	37.25 ± 2.85 ^b	15.50 ± 1.20 ^c	10.25 ± 0.50 ^c

Mean values of three replicates ± the standard deviation of the mean.

^{a-d}Mean values with different letter in the same row are significantly different ($p \leq 0.05$). ND = Not detected.

Valores promedio de tres repeticiones ± desviación estándar de la media.

^{a-d}Los promedios en la misma fila con diferente letra difieren significativamente ($p \leq 0,05$). ND: No detectado.

Acknowledgements

We would like to thank Frutech International (N.L., Mexico) for providing the commercial EO samples and Juan José Ledezma for his support and the technical information provided.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported by Consejo Nacional de Ciencia y Tecnología de México (CONACyT) [No. 269983/275113].

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