Chapter 7

Antioxidant Capacity and Food Pathogenic Bacteria Inhibition of *Citrus limetta* and *Citrus reticulata*

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Abstract

In this study, phenolic compounds in the juice, seed and bagasse of *C. limetta* and *C. reticulata* cultivated in Mexico at two ripening stages were determined, and their antioxidant capacities were evaluated using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), 2,2′-azin-bis-(3-etilbenzotiazolin-6-sulfonic acid) (ABTS) and oxygen radical absorption capacity test (ORAC) methods, as well as their antibacterial growth inhibition. We found that bagasse had the highest total phenol content and the highest total flavonoid content. The dominant flavonoid, hesperidin, was observed to be the highest in bagasse. Ascorbic acid was analyzed and *C. limetta* juice and *C. reticulata* bagasse had the highest contents. Antioxidant capacity showed variations in both, *C. limetta* and *C. reticulata*, juices which had the highest ABTS value; *C. limetta* juice and *C. reticulata* bagasse had the highest DPPH value; *C. limetta* juice and *C. reticulata* bagasse had the highest ORAC value. *C. limetta* and *C. reticulata* extracts showed the bactericidal effect at the range of 4–40 mg/mL, assayed against *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella enterica* and *Staphylococcus aureus*. Overall, ripeness increased total phenol content (TPC), total flavonoid content (TFC), hesperidin content, antioxidant capacity and bactericidal effect. These results may provide useful information for future utilization of *C. limetta* and *C. reticulata*.

Keywords: citrus, phenolic compounds, antioxidant, bactericidal, ripeness

1. Introduction

At present, there is a growing interest in the effective, economic and innocuous obtaining of extracts with antioxidant and antibiotic properties from natural matrices [1], which opens a field of investigation in the extraction fields, purification and characterization of plant extracts obtained from secondary products [2]. Given a large number of bioactive compounds present in plant extracts, several mechanisms of action may act simultaneously. The two main antibacterial effects that have been identified are (i) the opening of pores in the bacterial membrane at concentrations below the minimum inhibitory concentration (MIC), which leads to the leakage of intracellular components in the period initial contact, without causing a decrease in the viability of the bacteria. This effect can be reversed inappropriate cultivation conditions only when there have been short periods of exposure to the bactericidal agent. When the bacteria are exposed to concentrations above the MIC, the second effect occurs, (ii) causing irreversible damage to the membrane, due to the modification of the carboxyl groups of the fatty acids of the membrane or by reaction with the polysaccharides of the cell wall. This effect can also be seen when bacteria are exposed to MIC for an extended period, affecting cell viability [3]. Citrus species such as *C. aurantifolia*, *C. sinensis*, *C. paradisi*, *C. reticulata* and *C. limetta* are widely cultivated in Mexico and other parts of the world; some secondary metabolites of these fruits have shown bactericidal activity, helping to combat some infections in humans [4–6]; they are also useful as antivirals (herpes, influenza, etc.) [6], antifungal [7] and antibacterial [8, 9]. Similarly, these bioactive molecules can form the structural basis for developing new antibiotics, more effective, less expensive and with fewer collateral effects [6, 10]. A group of polyphenols' with microbicide properties present in citrus fruits are flavonoids [6]. In particular, phenolic and flavonoid compounds [11] have attracted the attention of the scientific community due to a well-established connection between the consumption of flavonoids and the prevention of sufferings [12]. These compounds have shown the antibacterial effect at diluted concentrations, with the rupture of the membrane show the mechanism of action [13]. Altering the macromolecular structure of bacterial membranes affected the carboxylic groups of the fatty acid in the membrane [3]. The almost daily obtaining of new evidence of these compounds interfering with several virulence factors in pathogenic bacteria, including enzymes, toxins and signal receptors opens the possibility of using them in anti-infection therapies or even the development of new drugs [5]. Several recent publications report the regular presence of antibacterial activity of the isolated flavonoids [14]; with MIC for heperidine against *E. coli* CCTCC AB94014 de 800 μg/mL and against *S. aureus* CCTCC AB9105 of 200 μg/mL [15]; for naringine against *E. coli* ATCC 35218 of 4 μg/mL, against *P. aeruginosa* ATCC 10145 de 2 μg/mL and against *S. aureus* ATCC 25923 of 16 μg/mL; for nobiletine against *E. coli* CCTCC AB94014 of 1600 μg/mL and against *S. aureus* CCTCC AB9105 of 1600 μg/mL [15]; for quercitine against *E. coli* ATCC 35218 de 4 μg/mL, against *P. aeruginosa* ATCC 10145 de 4 μg/mL and against *S. aureus* ATCC 25923 de 2 μg/mL; for tangeretine against *E. coli* CCTCC AB94014 of 1600 μg/mL and against *S. aureus* CCTCC AB9105 of 1600 μg/mL [15]; of gallic acid against *E. coli* ATCC 35218 of 4 μg/mL, against *P. aeruginosa* ATCC 10145 of 2 μg/mL, against *S. aureus* ATCC 25923 of 16 μg/mL [6], which are similar to bacterial inhibition of grape seed extracts with MIC against *S. aureus* ATCC 25923 of 1.25 mg/mL, against *L. monocytogenes* ZM58 > 10 mg/mL, against *E. coli* O157: H7 of 2.5 mg/mL [16].

	IM	Diameter (cm)	High (cm)	Weight (g)	Bagasse (%)	Juice (%)	Seed (%)
C. limetta	17.7	5.83 ± 0.31[a]	5.73 ± 0.32[a]	92.33 ± 9.06[a]	53.76 ± 3.50[a,b]	39.51 ± 6.15[a,b]	1.93 ± 0.37[a]
	9.9	5.03 ± 0.46[a]	4.97 ± 0.35[a]	64.79 ± 10.81[a]	60.49 ± 6.15[b]	35.02 ± 0.24[a]	1.18 ± 0.64[a]
C. reticulata	9.5	6.23 ± 0.21[a]	5.13 ± 0.21[a]	109.54 ± 17.22[a]	26.76 ± 7.26[a]	52.57 ± 0.26[b]	2.83 ± 0.75[a]
	6.3	6.07 ± 0.40[a]	5.00 ± 0.36[a]	107.32 ± 7.81[a]	31.83 ± 2.53[a,b]	49.46 ± 0.23[a,b]	2.06 ± 0.49[a]

Each value is the average of three replications ± deviation standard. The different superscripts indicate significant differences between samples ($p < 0.05$).

Table 1. Characterization of the used fruits.

This has boosted the development of commercial citrus extracts, with a good bactericidal performance and MIC of 20–80 ppm against *S. enterica* CECT 4300 [3]. Still, some citrus compounds do not inhibit certain bacteria such as *L. monocytogenes* 01/155 and 99/287, *P. aeruginosa* ATCC27853, *S. aureus* ATCC29213, and *E. coli* O157:H7, such as those who showed no inhibition at concentrations up to 0.25 mM of Naringin [17]. Currently, research on citrus-derived antioxidant compounds has neglected the study of low-crop species, such as *C. limetta* and *C. reticulata*, which also have compounds with potential bactericidal effect [11]. The extracts of *C. limetta* and *C. reticulata* have shown good antioxidant capacity in tests with the 2,2-diphenyl-1-picrylhydrazyl-hydrate (DPPH), with values of 261 ± 9 μM Trolox equivalent (TE) in juice of *C. limetta* [11], 210 ± 37.0 mg/mL in seed of *C. reticulata* [18], 48.8 ± 0.91 mg vitamin C equivalent (VCE)/100 ml in *C. reticulata* juice [19], de 9.10 ± 0.68 a 19.75 ± 0.87 μM TE/g, in pulp of *C. reticulata* [20] and 29.04 ± 1.20 to 50.46 ± 3.57 μM TE/g, in shell of *C. reticulata* [21]. By the method with acid 2,2′-azin-bis-(3-etil-benzotiazolin-6-sulfonic acid) (ABTS) have been found 1446 ± 30 μM TE in *C. limetta* juice [11], 59.3 ± 0.23 mg VCE/100 ml in *C. reticulata* juice [19], of 22.92 ± 0.32 to 34.28 ± 1.12 μM TE /g in *C. reticulata* pulp [20]; and of 65.62 ± 1.43 to 108.60 ± 0.24 μM TE/g in *C. reticulata* shell [21]. Currently, research on citrus-derived antioxidant compounds has neglected the study of low-crop species, such as *C. limetta* and *C. reticulata* which also have compounds with potential bactericidal effect [11]. The extracts of *C. limetta* and *C. reticulata* have shown good antioxidant capacity in test with the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), with values of 261 ± 9 μM Trolox equivalent (TE) in juice of *C. limetta* [11], 210 ± 37.0 mg/mL *C. reticulata* seed [18], 48.8 ± 0.91 mg vitamin C equivalent (VCE)/100 ml in juice of *C. reticulata* [19], de 9.10 ± 0.68 to 19.75 ± 0.87 μM TE/g, in pulp of *C. reticulata* [20] and 29.04 ± 1.20 to 50.46 ± 3.57 μM TE/g, in *C. reticulata* shell [21]. With the method of 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), it has been found 1446 ± 30 μM TE in C. limetta juice [11], 59.3 ± 0.23 mg VCE/100 ml in C. reticulata juice [19], de 22.92 ± 0.32 a 34.28 ± 1.12 μM TE/g in C. reticulata pulp [20] and de 65.62 ± 1.43 a 108.60 ± 0.24 μM TE/g in C. reticulata shell [21]. Moreover, these extracts have shown antioxidant capacity of oxygen radicals in the oxygen radical absorption capacity test (ORAC), with values of 126.90 ± 1.96–13.22 ± 231.29 μM TE/g in *C. reticulata* pulp [20] and of 395.66 ± 14.42 to 834.37 ± 16.98 μM TE/g in *C. reticulata* shell [21].

Consequently, citrus fruits are of great importance due to the interest of finding new sources of antibacterial and antioxidant compounds. However, no work has been done in bacterial inhibition and antioxidant capacity of citrus fruits to different stages of maturation [22]. Therefore, in this research, it was determined the bactericidal activity and antioxidant

capacity of bagasse, juice and seeds of two citrus species cultivated in Mexico, *C. limetta* and *C. reticulata*, in two stages of maturity (**Table 1**). The results were compared with those found in the literature, in order to provide innovative information for future applications of these citrus species.

2. Methodology

2.1. Obtaining the extracts

The fruits of *C. limetta and C. reticulata* were acquired in the city of Morelia, Michoacán, from different regions of the same state. To obtain the extract, the juice, the shells and the seeds were separately dried in the oven at a temperature of 40°C for 48 h and then ground. The materials were washed with acetone (Meyer, México) in proportion 1:1 (g of material: mL of acetone) in a flask. The mixture was stirred for 30 min at 200 rpm, at room temperature and then filtered to vacuum. The operation was repeated with hexane (J.T. Baker, México) in proportion 1:4 (g of material: mL of hexane). For the extraction of phenolic and flavonoid compounds, 4 mL of methanol (J.T. Baker, México) was used for each gram of material, stirring the mixture for 30 min at 200 rpm [23] at room temperature. The mixture is vacuum-filtered and the extracts obtained are stored in refrigeration at 4°C until posters test.

Total phenol content (TPC) was tested using the reagent of Folin-Ciocalteau (Hycel, Mexico) with a standard of Gallic acid (Golden Bell, Mexico), and absorbance was measured at 750 nm [24]. An aliquot of 0.1 mL of the methanol extract is diluted with 0.4 ml of distilled water; then, the solution was mixed with 2.25 mL of Folin-Ciocalteau reagent to 10% and 2.25 mL of solution (Golden Bell, Mexico) (20 g/mL of distilled water) of sodium carbonate.

Absorbance was measured after 2 h of incubation at 750 nm compared to a methanol target (J.T. Baker, Mexico), using a UV/Vis spectrophotometer (Jenway, Model 7305). The TPC was expressed in mg equivalents of Gallic acid (GAE) by g of dry matter (DM) of bagasse and seeds and in mg GAE/mL for the juices. The total flavonoid content (TFC) was analyzed with a modification of the spectrophotometric method described by Abeysinghe et al. [12]. A 1.5 mL of methanol was added to 0.1 ml of the diluted extract, to then add 0.1 mL of solution 6.8 g/50 mL of distilled water of aluminum chloride (Golden Bell, Mexico), and the resulting solution was diluted with distilled water to a final volume of 5 mL. The mixture was stirred, it was left to rest for 30 min and its absorbance was measured at 510 nm. The TFC was calculated using a calibration curve of quercetin (Aldrich, USA) and the results were expressed in mg equivalent of quercetin (QE) per grams of dry matter (DM) of bagasse and seeds and in mg QE/mL for the juices. The quantitative analysis of hesperidin and ascorbic acid was performed in a high-performance liquid chromatography (HPLC) Varian LC920 equipped with a column C18 Varian, 25 cm × 4.6 mm I.D. and a diode array detector. The TFC was calculated using a calibration curve of quercetin (Aldrich, USA) and the results were expressed in mg equivalent of quercetin (QE) per grams of dry matter (DM) of bagasse and seeds and in mg QE/mL for juices. The quantitative analysis of hesperidin and ascorbic acid was performed in a high-performance liquid chromatography (HPLC) Varian LC920 equipped with a column C18 Varian, 25 cm × 4.6 mm I.D. and a diode array detector. Mobile phases of KH_2PO_4 20 mM,

phosphoric acid at 0.1%v, and 1%v methanol for the determination of ascorbic acid were used; 33%v methanol and 67%v water to determine hesperidin. Absorbances were measured at 215 nm and 283 nm, respectively. The samples were analyzed in duplicate, and the calibration curves were constructed from the average areas of the peaks. The contents of hesperidin (HD) and ascorbic acid (AA) expressed in mg/g DM for bagasse and seeds and in mg/mL for juices.

2.2. Determination of minimum inhibitory and bactericidal concentrations

The evaluation of the minimum inhibitory concentration (MIC) and minimum concentration bactericidal (MBC) It was carried out by microdilution of the extract in culture medium [25], in concentrations of 2–40 mg/μL. A 5 mL of bacterial suspension was placed in culture medium (cetrimide broth for *P. aeruginosa*, Muller-Hinton broth for the rest of the bacteria) in a sterile microplate of 96 wells. The volume was completed with the dilutions of the extract in the culture broth to obtain the test concentrations. Control wells were prepared with culture broth, diluted extract and bacterial suspension separately. The microplate was incubated for 24 h at 37°C. The MIC was the lowest concentration where no viability was observed in the well after 24 h [16]. To determine the MBC, 20 μL were taken from wells where no growth was observed and transferred to a plate with solid agar (*Pseudomonas* agar for *P. aeruginosa*, Muller-Hinton agar for the other bacteria) and incubated for 24 h at 37°C. MBC was the lowest concentration of colony-forming units (CFU) growth after the incubation period. Positive controls were wells with bacterial suspension in breeding stock. The negative controls were wells with culture broth and with the dilution of the extract. All the determinations of MIC and MBC were repeated in triplicate.

2.3. Test organisms

Escherichia coli ATCC 15597, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* ATCC 10145, *Salmonella enterica* ATCC 14028 and *Staphylococcus aureus* ATCC 6538 were used as test organisms.

2.4. Antioxidant capacity

The antioxidant capacity was determined by testing the oxidation inhibition of the acid radicals 2,2′-Azino-bis-(3-etilbenzotiazolin-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), as well as the absorption capacity of oxygen radicals (ORAC).

The capacity of the extracts was measured to inhibit the DPPH radical by placing in a microplate 10 μL of the extract solution to the given concentration and added 140 μL of the DPPH radical adjusted to an absorbance of 0.70. The reaction was allowed to elapse for 30 min and the absorbance of the solution was read at 518 nm. The percentage of inhibition was calculated and the results were expressed in μmol equivalents of Trolox per milligram of a sample (μmol TE/mg), using a standard curve of this antioxidant. The tests were carried out in triplicate [26].

The determination of the inhibition of the radical ABTS was carried out by adding in a well of microplate 5 μL of extract to the appropriate dilution of test and 245 μL of radical ABTS solution adjusted to an absorbance of 0.70. It was allowed to react for 5 min and the absorbance

was measured at 754 nm. The percentage of inhibition was calculated and the results were expressed in μmol TE/mg using a standard curve of Trolox [1]. Tests were carried out in triplicate.

The reaction mix for the ORAC test was prepared with 150 μL de fluorescein 10 μM, 25 μL of Trolox standard (standard curve of 6.25–200 μM), 25 μL of phosphate buffers (75 μM, pH 7.4) like control and 25 μL of extract; the reaction begin with the addition of AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride, 240 mM). The fluorescein drop was evaluated every 90 s during an hour and a half, at an excitation wavelength of 485 nm and a wavelength of 520 nm with a microplate reader FLUOstar Omega (BMG Labtech Inc., USA). The ORAC values were calculated using a linear regression equation of a standard Trolox curve [27]. The results were expressed as equivalent μmol of Trolox per milligram (μmol TE/mg).

2.5. Analysis of statistics

There were three replicas and at least three independent experiments. The data were presented as mean ± deviation standard (SD). STATGRAPHICS Centurion XVI.I version was used for the statistical analysis. (Statpoint Technologies, Inc., Warrenton, VA, USA). Differences between groups were detected by ANOVA and Tukey multiple comparison tests; *p* values less than 0.05 were considered as statistically significant.

3. Results and discussion

3.1. Effect of maturity on the contents of hesperidin, ascorbic acid, TPC and TFC

The highest values of TPC were found in bagasse, followed by juice and finally in the seed, for both fruits regardless of the level of maturity (**Figure 1**). Changes in the maturation in the TPC showed similar trends in pulp, juice and seed. It was determined an increase of 13.3% 14.4% at the TPC of bagasse for the maturity of 20.38 ± 0.97 to 23.09 2.57 ± and 34.95 ± 0.11 to 39.97 ± 1.25 mg GAE/g DW) for *C. limetta* and *C. reticulata*, respectively. The TPC juice content increased 16.62% and 0.90% during maturation (of 10.98 ± 0.14 to 12.80 ± 1.20 and 17.15 ± 0.42 to 17.30 ± 1.14 mg GAE/g DW). The content TPC in seed increased 2.59 ± 0.95 to 5.63 ± 0.19 and of 6.85 ± 0.95 to 7.46 ± 0.95 mg GAE/g DW, which did not represent a significant difference to $p > 0.05$. It is possible that the variations are due to differences in culture, origin, growth conditions and the same extraction process. Phenolic compounds are secondary metabolites that have mainly been correlated with antioxidant activity in several fruits, vegetables and grains [12]. The presence of a high TPC in pulp and juice *C. limetta* and *C. reticulata* confirms the nutritional value of these fruits and the presence of phenols in the seeds makes them an alternative source for later uses.

The highest values of TFC were found in bagasse, followed by juice and finally in the seed, for both fruits regardless of the level of maturity (**Figure 2**). Changes in the maturation in the TFC showed similar trends in pulp, juice and seed. The higher content of TFC was found in bagasse for both fruits. It measured an increase of 7.11% and 2.23% in the TFC of bagasse during ripening of 1579.03 ± 12.85 to 1691.33 ± 22.83 and of 1892.63 ± 6.89 to 1934.89 ± 7.58 μg QE/g DW) for

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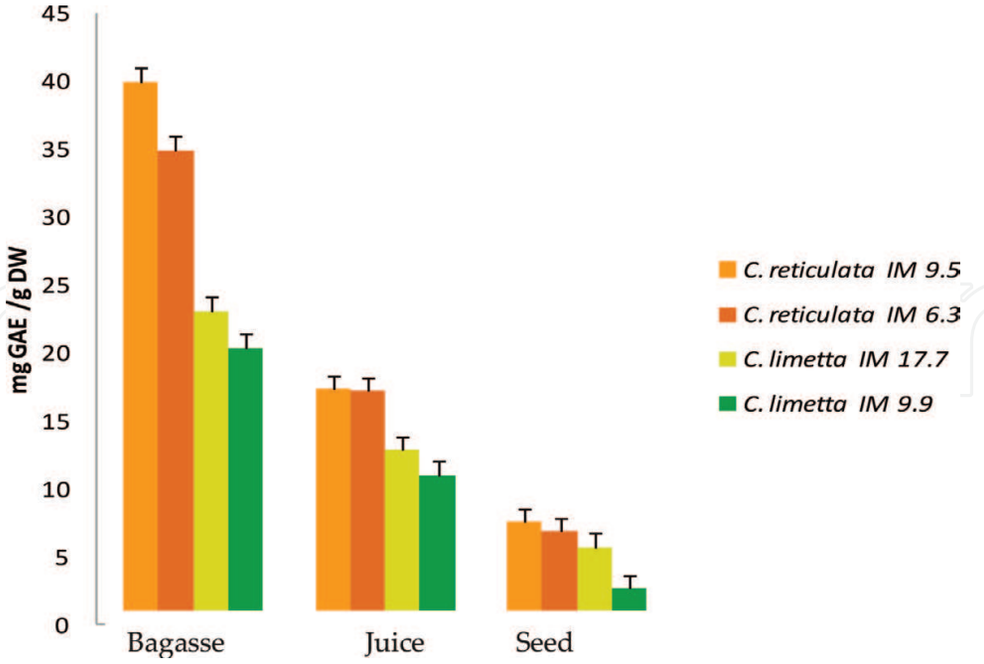

Figure 1. Content of total phenols (TPC) en *C. limetta* y *C. reticulata.*

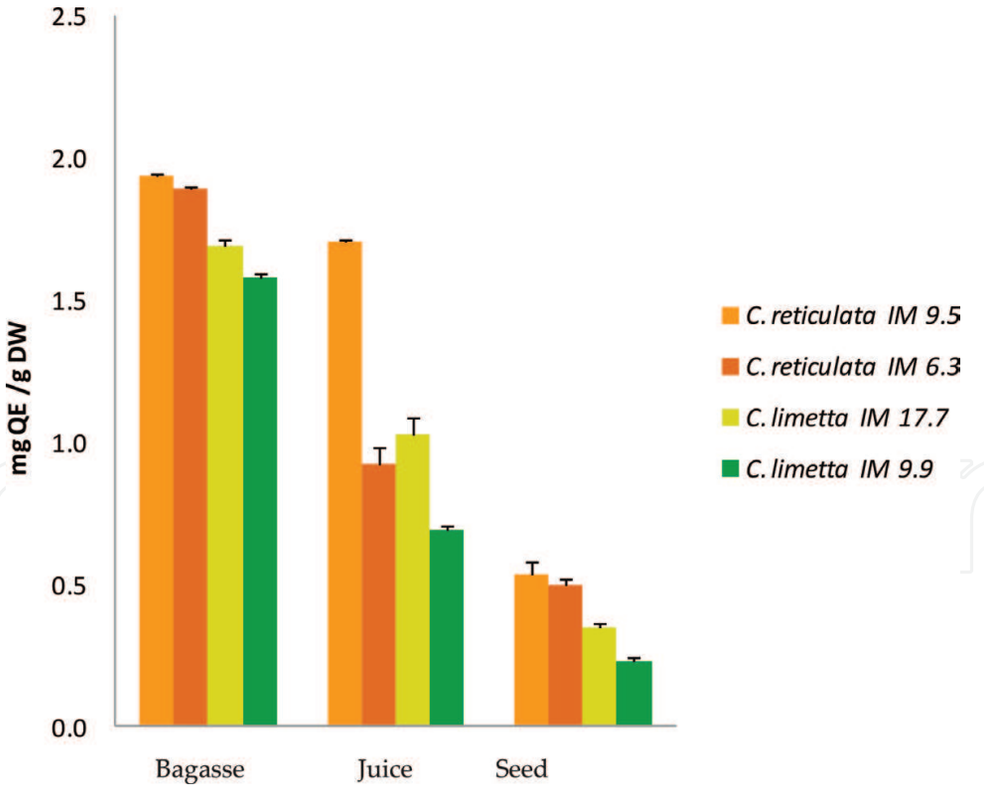

Figure 2. Content of total flavonoids (TFC) in *C. limetta* y *C. reticulata.*

C. limetta and *C. reticulata,* respectively. The content of total flavonoids in juice increased 48.33% and 84.62% during maturation (of 694.70 ± 15.19 to 1030.44 ± 59.58 and from 1709.78 ± 6.23 to 1892.63 ± 6.89 μg QE/ g DW). The TFC in seed increased from 230.74 ± 14.56 to 344.76 ± 15.59

and from 499.65 ± 18.61 to 535.15 ± 40.96 μg QE/g DW, which did not represent a significant difference to $p > 0.05$. It is possible that the variations are due to differences in culture, origin, growth conditions and the same extraction process. Phenolic compounds show flavonoids may inhibit radical free and catch reactive oxygen species (ROS) and therefore provide an effective means to prevent and treat ailments promoted by free radicals [28]. The presence of high TFC in *C. limetta* and *C. reticulata* defines them as a significant source of antioxidants with potential prophylactic applications and in the development of functional foods.

Hesperidin content was determined by an analysis of HPLC-DAD. The presence of hesperidin was obviously different between bagasse, juice and seed and was influenced by maturation (**Figure 3**). In general, the highest value of hesperidin was registered in bagasse, followed by juice and seed. However, the variation was larger in *C. limetta* during maturation. Large amounts of hesperidin were identified not only in bagasse but also in juice and seed, which is consistent with levels reported previously for *C. reticulata* [28–30]. The concentration of hesperidin in bagasse *C. limetta* and *C. reticulata* increasing during ripening of 397.37 ± 20.01 to 617.21 ± 70.73 and 966.49 ± 14.68 to 978.89 ± 43.46 μg/g DW, respectively. Similarly, it increased the amount of hesperidin in the juice of 129.45 ± 24.81 to 444.97 ± 109.57 and 200.69 ± 22.01 to 255.54 ± 37.21 μg/g DW, respectively. Hesperidin is accumulated in seeds of *C. limetta* and *C. reticulata* during maturation, and their quantities increased 90.25 and 14.46%, respectively.

The highest content of Ascorbic acid was found in bagasse, followed by juice and finally the seeds regardless of maturity for both fruits (**Figure 4**). The content of ascorbic acid in the pulp of *C. limetta* and *C. reticulata* decreased slightly during the maturation of 3.13 ± 0.41 to

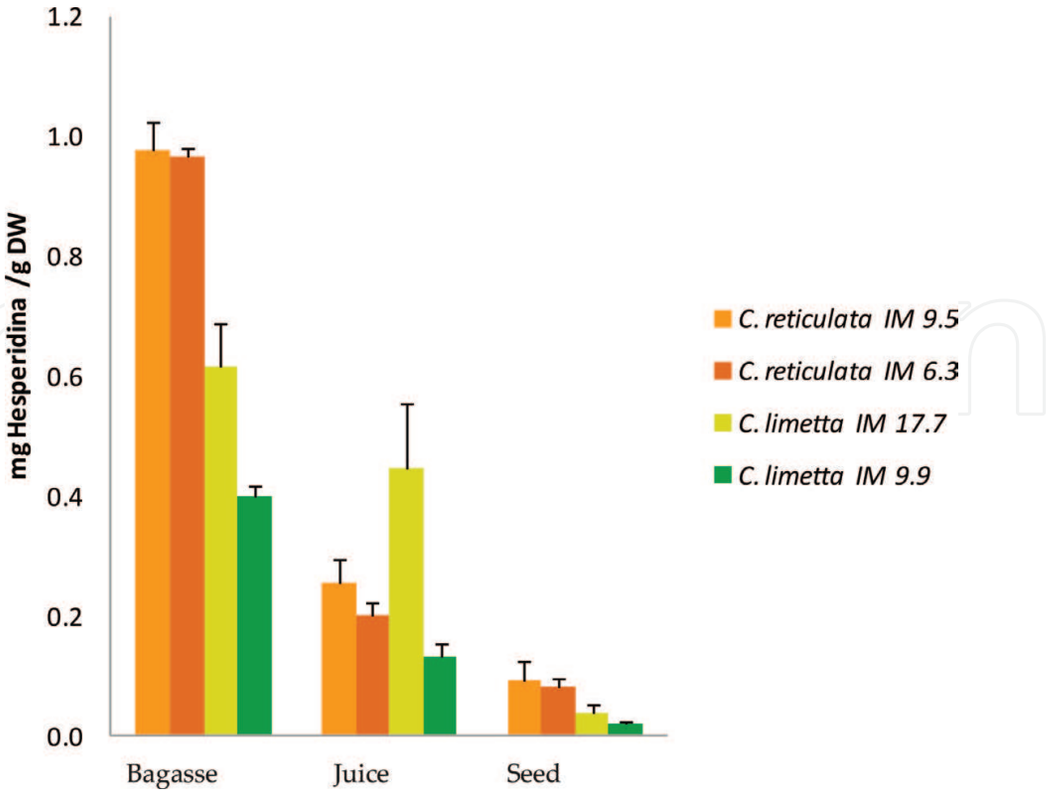

Figure 3. Contents of hesperidin in *C. limetta* y *C. reticulata*.

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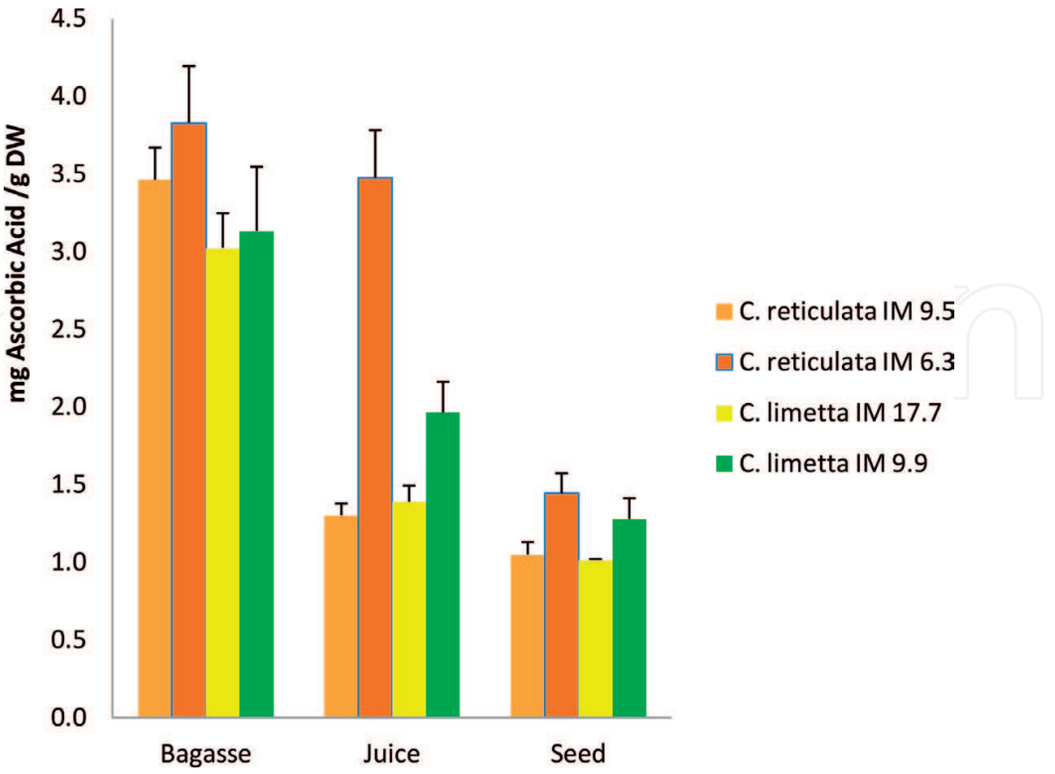

Figure 4. Ascorbic acid content in *C. limetta* y *C. reticulata.*

3.02 ± 0.22 and 3.83 ± 0.37 to 3.46 ± 0.20 mg/g DW for *C. limetta* and *C. reticulata*, respectively. During maturation, the content of ascorbic acid in juice decreased 1.96 ± 0.20 to 1.39 ± 0.10 and 3.47 ± 0.31 to 1.30 ± 0.08 mg/g DW. Ascorbic acid decreased from the seeds of *C. limetta* and *C. reticulata* with maturation, and their number was reduced by a 20.80 and 27.31%, respectively. How the ascorbic acid is an essential nutrient to prevent scurvy, cancer, cardiovascular and nervous system diseases [31], they seem to be a beneficial and important source of this compound. The concentrations of metabolites found in the juices of *C. reticulata* are consistent with those reported previously [19, 32, 33]; also the amounts found in seed [18].

3.2. Antioxidant capacity

The results of this study showed, in general, similar trends in the inhibition of radical capacity for pulp, juice and seed and increased with maturation, as shown in **Table 2**. In the ABTS test, the highest values were found in juice, of 72.71 ± 1.72 to 82.19 ± 6.39 μmol TE/g DW from *C. limetta* and of 61.48 ± 1.40 to 62.00 ± 5.47 μmol TE/g DW of *C. reticulata*, being higher than the data reported in the literature [11, 19]; the inhibitory activity of bagasse with ABTS for *C. limetta* increased during maturation, while it came down to the bagasse of *C. reticulata*, and their values agreed with previously reported data [20, 21]. ABTS of seed value did not change significantly with maturation and ranged from 6.06 ± 0.21 to 13.30 ± 1.07 μmol TE/g DW for *C. limetta* and 16.73 ± 1.39 to 42.82 ± 1.67 μmol TE/g DW for *C. reticulata*. The DPPH of juice *C. limetta* is consistent with that reported by Barreca et al. [11]. The seeds of *C. reticulata* showed good DPPH [18], but below that obtained for juice, as expected [19]; the highest DPPH antioxidant capability found in this study were for bagasse of *C. reticulata* (7.56 ± 0.88 μmol

	IM	ABTS	DPPH	ORAC
C. limetta				
Bagasse	17.7	$47.34 \pm 2.84^{c,d,e}$	$1.64 \pm 0.27^{a,b}$	14.82 ± 0.33^{d}
	9.9	$25.26 \pm 2.21^{a,b,c}$	$3.31 \pm 0.11^{a,b}$	12.51 ± 0.17^{c}
Juice	17.7	82.19 ± 6.39^{f}	$5.61 \pm 0.62^{a,b}$	11.97 ± 0.33^{c}
	9.9	$72.71 \pm 1.72^{e,f}$	$3.78 \pm 1.06^{a,b}$	11.33 ± 0.04^{c}
Seed	17.7	$13.30 \pm 1.07^{a,b}$	0.61 ± 0.24^{a}	1.69 ± 0.01^{a}
	9.9	6.06 ± 0.21^{a}	0.13 ± 0.02^{a}	0.79 ± 0.04^{a}
C. reticulata				
Bagasse	9.5	$36.04 \pm 2.27^{b,c,d}$	7.56 ± 0.88^{b}	22.56 ± 0.28^{f}
	6.3	$59.67 \pm 5.31^{d,e,f}$	$3.65 \pm 0.37^{a,b}$	16.97 ± 0.13^{e}
Juice	9.5	$62.00 \pm 5.47^{d,e,f}$	$2.72 \pm 0.86^{a,b}$	22.56 ± 0.36^{f}
	6.3	$61.48 \pm 1.40^{d,e,f}$	$3.50 \pm 1.27^{a,b}$	22.07 ± 0.29^{f}
Seed	9.5	$42.82 \pm 1.67^{b,c,d,e}$	$2.08 \pm 0.13^{a,b}$	3.73 ± 0.12^{b}
	6.3	$16.73 \pm 1.39^{a,b}$	$1.60 \pm 0.60^{a,b}$	$2.26 \pm 0.05^{a,b}$

Each value is the average of three replications ± standard deviation. The different superscripts indicate significant differences between samples ($p < 0.05$).

Table 2. Antioxidant capacity of *C. limetta* y *C. reticulata*, μmol TE/g DW ± SD.

TE/g DW), which is higher than the values reported in previous studies [20, 21]. Although the DPPH values of seeds were lower than those of juice and pulp, this inhibiting activity DPPH implies a potential application as a valuable source of antioxidants. In general, values of DPPH showed similar trends for pulp, juice and seeds during maturation, with the exception of *C. limetta* and of *C. reticulata* juice.

ORAC testing revealed that the pulp and juice of *C. reticulata* had ORAC values significantly higher than pulp and juice of *C. limetta*, probably due to the higher levels of phenolic compounds and flavonoids in *C. reticulata* [11, 20, 21] which can lead to further investigation of this fruit. The ORAC values of seeds did not change significantly during maturation and were 0.79 ± 0.04 to 1.69 ± 0.01 μmol TE/g DW for *C. limetta* and 2.26 ± 0.05 to 3.73 ± 0.12 μmol TE/g DW for *C. reticulata*. A comparative evaluation indicates that the seeds of *C. limetta* and *C. reticulata* may be an attractive source of antioxidants for future applications. The results suggest that both *C. limetta* as *C. reticulata* possess a remarkable inhibitory activity of radical and therefore a significant antioxidant capacity.

3.3. Bacterial inhibition

Table 3 shows testing of antibacterial susceptibility of extracts against some Gram-positive and Gram-negative bacterial strains. Observed growth inhibition varied from one organism

		Gram positive				**Gram negative**					
		Listeria monocytogenes		***Staphylococcus aureus***		***Escherichia coli***		***Pseudomonas aeruginosa***		***Salmonella enterica***	
	IM	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
C. limetta											
Bagasse	17.7	18	22	31	40	13	22	3	4	13	22
	9.9	9	13	31	40	13	22	3	4	9	13
Juice	17.7	40	>40	>40	>40	36	40	31	40	31	40
	9.9	40	>40	>40	>40	>40	>40	36	40	36	40
Seed	17.7	>40	>40	>40	>40	>40	>40	36	40	36	40
	9.9	>40	>40	>40	>40	>40	>40	36	40	40	>40
C. reticulata											
Bagasse	9.5	22	31	40	>40	31	40	3	4	18	22
	6.3	22	31	40	>40	31	40	9	13	18	22
Juice	9.5	27	31	40	>40	31	40	13	22	27	31
	6.3	31	40	40	>40	36	40	31	40	31	40
Seed	9.5	>40	>40	>40	>40	>40	>40	36	40	40	>40
		>40	>40	>40	>40	>40	>40	>40	>40	>40	>40

MIC, Minimum inhibitory concentration; MBC, minimum bactericidal concentration. Each value is the mean of three replicates with standard deviation <10%.

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts, mg/mL.

to another and a summary to another. Gram-positive strains showed values higher than the Gram-negative strains MIC and MBC. Extracts that showed greater bactericide effect were those obtained from bagasse for both fruits, with up to 3 mg/mL and MIC MBC 4 mg/mL (**Table 3**), which is consistent with the reported values of for essential oil of *C. sinensis* [34], this effect may be due to the increase in phenolic compounds and flavonoids during the ripening of fruit. *S. aureus* was the microorganism tested that showed greater resistance to the extracts, with MIC above 31 mg/mL and MBC greater than 40 mg/mL for the majority of tests. *L. monocytogenes* had MIC from 18 mg/mL and MBC from 13 mg/mL. *E. coli* showed MIC from 13 mg/mL and MBC from 22 mg/mL. *P. aeruginosa* recorded MIC from 3 mg/mL and MBC from 4 mg/mL. *S. enterica* recorded MIC from 9 mg/mL and MBC from 13 mg/mL. Extracts of *C. reticulata* showed one inhibitory effect greater than the *C. limetta*, but still below that reported for extracts of green tea [16]. Lower bacterial inhibition was in seed for both fruits extracts, with MIC values higher to 36 mg/mL, but still below those obtained with extracts from grape seed [16]. Even so, extracts showed better antibacterial compounds isolated citruses such as quercetin, gallic acid [6] and naringin [17]; still better than the performance shown by some commercial fruit extracts [3].

4. Conclusions

We investigated the content and antioxidant activity of the phenolic compounds and flavonoids from two Mexican varieties of *C. reticulata* and *C. limetta*. Bagasse, juice and seeds of *C. reticulata* and *C. limetta* contain phenolic compounds and flavonoids. Its content increases as the maturity increases. The extracts show microbicide effect on microorganisms of study under in vitro conditions. The effect increases when rising the ripening of the fruit. Extracts of *C. reticulata* have a higher bactericide effect than those obtained *C. limetta*, test microorganisms. As a result, the inhibition is directly related to the content of phenolic compounds and flavonoids. The extracts show antioxidative effect in vitro tests. The effect increases when rising the ripening of the fruit *C. reticulata* has antioxidant capacity and content of secondary metabolites greater than *C. limetta*. Inhibition of oxidation is directly related to the content of phenolic compounds and flavonoids. Our findings suggest that *C. reticulata* and *C. limetta*, especially its bagasse, are good sources of antioxidant and antibacterial compounds. The results of citrus species analyzed in this study can motivate more widely used them in the pharmaceutical and food products.

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Conflict of interest

I confirm there are no conflicts of interest.

Nomenclature

AAPH	2,2′-azo-bis-(2-aminopropane)-dihydrochloride)
ABTS	2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	analysis of variance
ATCC	American Type Culture Collection
CCTCC	China Center for Type Culture Collection

DPPH	2,2-diphenyl-1-picrylhydrazyl-hydrate
DW	dry weight
GAE	gallic acid equivalents
HPLC	high performance liquid chromatography
MBC	minimum bactericidal concentration, mg mL^{-1}
MIC	minimum inhibitory concentration, mg mL^{-1}
ORAC	oxygen radical absorption capacity, μmol TE/g DW
QE	quercetin equivalents
SD	standard deviation
TE	Trolox equivalents
TFC	total flavonoid content, mg GAE g DW^{-1}
TPC	total phenol content, mg QE g DW^{-1}
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UFC	colony forming units

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