Polyphenolic content and bactericidal effect of Mexican *Citrus limetta* and *Citrus reticulata*

Andrés Alejandro Damián-Reyna¹ · Juan Carlos González-Hernández² ·
Rafael Maya-Yescas¹ · Consuelo de Jesús Cortés-Penagos³ ·
Ma. del Carmen Chávez-Parga¹

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Abstract In this study, total phenolics, total flavonoids, hesperidin and ascorbic acid contents in bagasse, juice and seed of mexican sweet lime (*Citrus limetta*) and mandarine (*Citrus reticulata*) were determined at two commercial maturity stages (maturation index), as well as their bactericidal effect on *Escherichia coli* and *Staphylococcus aureus*. The results showed that bagasses had the highest total phenolics, total flavonoids, and hesperidin content for both, *C. limetta*, and *C. reticulata*; highest ascorbic acid contents were found in *C. limetta* juice ($3.36 \pm 0.25 \text{ mg g}^{-1} \text{ DW}$) and *C. reticulata* bagasse ($3.83 \pm 0.37 \text{ mg g}^{-1} \text{ DW}$). All tested extracts showed bacterial growth inhibition at 50 and $800 \mu\text{g mL}^{-1}$. Bagasse extracts of both fruits showed the highest inhibitions (>90%) on tested bacteria. Total phenolics, total flavonoids, and hesperidin contents, as well as bactericidal effect increased with maturity. Results indicated that both Mexican citric fruits (*C. limetta* and *C. reticulata*) were good sources of antioxidant and bactericidal agents.

Keywords Flavonoids · Phenolics · Hesperidin · Citrus · Maturity · Bactericidal

Introduction

Polyphenolic compounds from fruits, vegetables, cereals, herbs and spices have shown beneficial effects on human health, some extracts of polyphenol-rich plants have been used in functional foods or as supplements. Among polyphenolic compounds, there is an important group, the flavonoids, which consist of flavanones, flavones, flavan-3-ols, flavonols and anthocyanins. It has been found that flavonoid compounds can function as direct antioxidants and free radical scavengers, and are able to modulate enzymatic activities and inhibit cell proliferation (Vikram et al. 2010).

Citrus flavonoids, in particular, are recognized as antioxidants (Procházková et al. 2011; Yu et al. 2014; Asikin et al. 2015), antifungals (Buer et al. 2010), antimicrobials (Vikram et al. 2010; Cushnie and Lamb 2011; Céliz et al. 2011), and even accelerating wound and disease healing (Neves et al. 2010; Arab and Liebeskind 2010; Codoñer-Franch and Valls-Bellés 2010; Wang et al. 2014). In plants, flavonoids appear to play defensive roles against pathogens, including bacteria, fungi and viruses; these are generally found in glycosylated forms in plants, and the sugar moiety is an important factor determining their bioavailability (Agati et al. 2012).

Due to their properties, flavonoids could be used as preservatives for food. Currently, there is a trend that avoids chemical preservatives for foods, which provokes a growing interest in natural compounds exhibiting antimicrobial activity and helping to extend shelf life of food products (Knorr et al. 2011). Citric fruits are also among

✉ Ma. del Carmen Chávez-Parga
cparga@umich.mx

¹ Facultad de Ingeniería Química, Universidad Michoacana de San Nicolás de Hidalgo, Ciudad Universitaria, Francisco J. Mújica S/N, Col. Felicitas del Río, 58030 Morelia, Michoacán, Mexico

² Laboratorio de Bioquímica del Departamento de Ing. Bioquímica del Instituto Tecnológico de Morelia, Av. Tecnológico # 1500, Colonia Lomas de Santiaguito, 58120 Morelia, Michoacán, Mexico

³ Facultad de Químico Farmacobiología, Universidad Michoacana de San Nicolás de Hidalgo, Tzintzuntzan #173, Colonia Matamoros, 58240 Morelia, Michoacán, Mexico

the most important dietary sources of bioactive compounds (Vikram et al. 2010). In particular, the phenolic and flavonoid compounds (Barreca et al. 2013) have attracted the attention of the scientific community due to the well-established connection between flavonoids intake and disease prevention (Abeyasinghe et al. 2007). Therefore, knowledge of content of phenolic compounds in citric fruits and its application as antimicrobial agent is very attractive. One important source of citrus bioactive compounds is the residue from the orange juice industry (Romero-Lopez et al. 2011). Extraction and analysis of phenolic and flavonoid compounds in citrus peels, juice, seeds and bagasse have been reported recently (Damián-Reyna et al. 2016). However up to now, limited studies have analyzed and compared the phenolic composition in bagasse, juice and seeds simultaneously to support their rational utilization.

Mexico is a producer of citrus fruits, with up to 7,562,376 Ton per year; additional to the cultivation of common citric fruits, such as sweet orange (*Citrus sinensis*), lemon (*Citrus aurantifolia*), and grapefruit (*Citrus paradisi*), which collectively make up most of the local citrus production, several less advertised *Citrus* are grown in significant amount (Russo et al. 2014). For example, *C. limetta* and *C. reticulata* represents less than 5% of total citrus production; they are used in traditional medicine because of their antihypertensive effect (Arab and Liebskind 2010); however, to the best of our knowledge, there is only a single report on the flavonoid content of *C. limetta* (Nogata et al. 2006), where hesperidin is described as the major flavonoid component of the juice. Narirutin, rutin and diosmin are also mentioned as minor components. Hence, characterization of phenolic, and other compounds present in these fruits is an important research target.

The objective of the present study was to determine the composition of phenolic compounds and bactericidal capacity of two Mexican citric fruits (*C. limetta* and *C. reticulata*). The results obtained were compared with those of the mandarin and sweet lime cultivars reported in literature, to provide useful information for future utilization of Citrus varieties.

Materials and methods

Raw materials

Different samples of *C. limetta* and *C. reticulata* fruits were purchased from local markets of Morelia, Michoacán, México, grown at cultivars located in the regions of Michoacán State and México State, during winter station 2014. Juices were obtained using commercial extractors, bagasse and seeds were separated by hand; a maturation

index (IM), determined as the ratio of total soluble solids (°Brix) to titratable acidity (Bartholomew and Sinclair 1943), was defined for juices.

Solid materials (bagasse and seeds) were dried, ground and washed with acetone (Meyer, México) and hexane (J.T. Baker, México) to remove excess of lignin and oil, then were stirred with methanol (J.T. Baker, México) (4 mL methanol per 1 g of dry material) for 30 min at room temperature, vacuum-filtered and stored at 4 °C until analysis.

Quantitative evaluation of total phenolics and flavonoids content

Total phenolics content (TPC) of methanolic extracts was assayed using Folin–Ciocalteu reagent (Hycel, México) with gallic acid (Golden Bell, México) as the standard and final reaction measurements carried out at 750 nm (Vinson et al. 2001).

A 0.1 ml aliquot of methanolic extract was diluted with 0.4 ml of deionized water, then, the obtained solution was mixed with 2.25 ml of 10% Folin–Ciocalteu reagent aqueous solution and 2.25 ml of sodium carbonate (Golden Bell, México) solution (20 g ml⁻¹ distilled water). After 2 h of incubation, the absorbance was measured at 750 nm against a methanol (J.T. Baker, México) blank, using UV/Vis spectrophotometer (Jenway, mod. 7305). Total phenolic content was expressed as mg of gallic acid equivalents (GAE) per g dry matter (DM) of bagasse and seeds, and mg GAE/mL for juice.

Total flavonoid content (TFC) in juice, bagasse and seeds was analyzed by a modification of the spectrophotometric method described by Abeyasinghe et al. (2007). 1.5 mL of methanol was added to 0.1 ml of diluted methanol extract. After that, 0.1 ml of aluminium chloride (Golden Bell, México) aqueous solution (6.804 g/50 ml of distilled water) was added and the resulting solution was diluted with deionized water to a final volume of 5 ml. The mixture was stirred, left to stand for 30 min and the absorbance was measured at 510 nm. The TFC in the extracts was compared to the standard curve for quercetin (Aldrich, USA) solutions and expressed as mg of quercetin equivalents (QE) per g of dry matter (DM) of bagasse and seeds, and mg QE/mL for juice.

HPLC evaluation of hesperidin

The quantitative analysis of hesperidin and ascorbic acid was carried out by high performance liquid chromatography (HPLC) Varian LC920 equipped with a Varian C18 25 cm × 4.6 mm I.D. column, and a diode array detector. Mobile phases of 20 mM KH₂PO₄, 0.1% aqueous

Table 1 Total phenolics (TPC), total flavonoid (TFC), hesperidin (HD) and ascorbic acid (AA) contents for bagasse, seed, and juice, in two stages of maturity (IM)

Fruit	Part	IM	TPC, mg GAE/g DM	TFC, mg QE/g DM	HD, mg/g DM	AA, mg/g DM
<i>C. limetta</i>	Bagasse	17.7	23.09 ± 2.57 ^e	1.69 ± 0.02 ^{e,f}	0.62 ± 0.07 ^c	3.22 ± 0.28 ^c
		9.9	20.38 ± 0.97 ^{d,e}	1.58 ± 0.01 ^e	0.40 ± 0.02 ^d	3.13 ± 0.41 ^c
	Juice	17.7	786 ± 73.58 mg/L ^c	63.24 ± 3.66 mg/L ^d	27.3 ± 6.73 mg/L ^{b,c}	112 ± 12 mg/L ^{a,b}
		9.9	725 ± 9.14 mg/L ^c	45.91 ± 1.00 mg/L ^c	8.6 ± 1.64 mg/L ^{a,b}	222 ± 16 mg/L ^b
	Seed	17.7	5.63 ± 0.19 ^{a,b}	0.34 ± 0.02 ^{a,b}	0.04 ± 0.01 ^a	1.01 ± 0.01 ^a
		9.9	2.59 ± 0.95 ^a	0.23 ± 0.01 ^a	0.02 ± 0.00 ^a	0.96 ± 0.07 ^a
<i>C. reticulata</i>	Bagasse	9.5	39.97 ± 1.25 ^g	1.93 ± 0.01 ^g	0.98 ± 0.04 ^f	3.46 ± 0.20 ^c
		6.3	34.95 ± 0.11 ^f	1.89 ± 0.01 ^{f,g}	0.97 ± 0.01 ^f	3.83 ± 0.37 ^c
	Juice	9.5	1470 ± 96.50 mg/L ^d	145.24 ± 0.53 mg/L ^{e,f}	21.7 ± 3.16 mg/L ^d	153 ± 14 mg/L ^b
		6.3	1513 ± 37.06 mg/L ^d	81.72 ± 5.18 mg/L ^d	17.7 ± 1.94 mg/L ^c	168 ± 17 mg/L ^c
	Seed	9.5	7.46 ± 0.95 ^b	0.54 ± 0.04 ^{b,c}	0.09 ± 0.03 ^a	1.06 ± 0.08 ^a
		6.3	6.85 ± 0.95 ^b	0.50 ± 0.02 ^{b,c}	0.08 ± 0.01 ^a	1.04 ± 0.06 ^a

Each value is the average of three replications with a standard deviation <10%. Different superscripts between rows represent significant differences between samples ($p < 0.05$)

Table 2 Regression equation and coefficients for % inhibition of growth as a function to maturity and concentration of extracts, Concentration expressed in $\mu\text{g mL}^{-1}$

	<i>C. limetta</i>			<i>C. reticulata</i>		
	Bagasse	Juice	Seed	Bagasse	Juice	Seed
<i>E. coli</i>						
a_0	2.854	4.191	0.376	13.514	−0.770	4.092
a_1	0.155	−0.199	0.008	−0.277	0.087	0.203
a_2	0.095	0.010	0.009	0.139	0.104	0.020
$a_3 \times 10^2$	0.048	0.404	0.194	−0.397	0.227	0.262
R^2	0.9339	0.9692	0.9154	0.9577	0.9846	0.7594
<i>S. aureus</i>						
a_0	1.256	−1.543	−3.146	37.966	16.039	−3.798
a_1	0.799	0.139	0.130	−1.826	−0.827	0.058
a_2	0.118	0.031	0.002	0.088	0.094	0.017
$a_3 \times 10^2$	−0.096	0.145	0.132	0.148	−0.208	0.278
R^2	0.9662	0.9208	0.8654	0.8870	0.8783	0.9228
%Inhibition = $a_0 + a_1IM + a_2\text{Concentration} + a_3IM*\text{Concentration}$						
<i>IM</i> maturity index						

phosphoric acid, and 1% methanol for ascorbic acid content analysis (Nováková et al. 2008), and 33% methanol and 67% water for hesperidin content analysis (El-Shafae and El-Domiaty 2001), and measured at wavelengths of 215 and 283 nm, respectively. In order to confirm the linearity and reproducibility for the quantification of the compounds, the samples were analyzed twice, and linear calibration curves were constructed from the averaged peak areas. Hesperidin (HD) and ascorbic acid (AA) contents were expressed as mg g^{-1} DM for bagasse and seed, and mg mL^{-1} for juice.

Microbial strains and culture conditions

Two common microbial strains, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213, were used as indicators for antibacterial testing. Both microorganisms were obtained from the in-house collection of the Biochemistry Laboratory at Instituto Tecnológico de Morelia. Bactericidal capacity was evaluated through a colony-forming units (CFU's) macrodilution method assay (Andrews 2001), with a initial 250 ± 50 UFC mL^{-1} counting. The colonies developed after incubation were counted and

Fig. 1 Bactericidal effect of *C. limetta* on *E. coli*

Fig. 2 Bactericidal effect of *C. limetta* on *S. aureus*

the inhibitory effect was calculated using the following formula (Rico-Muñoz and Davidson 1983).

$\% \text{Inhibition} = (1 - T/C) \times 100$, where T is CFU mL⁻¹ of test sample and C is CFU/ml of control. Extracts were tested in concentrations from 50 to 800 µg mL⁻¹, and at two levels of IM for each fruit.

Statistical analysis

Three replicates and at least three independent experiments were performed. Data were presented as mean ± standard deviation (SD) and statistical analyses were performed using STATGRAPHICS Centurion version XVI.I (Statpoint Technologies, Inc., Warrenton, VA).

Comparison of means was performed by one-way analysis of variance (ANOVA) followed by Tukey's test.

P value less than 0.05 were considered to be statistically significant. Correlation coefficient between bactericidal effect and IM and Concentration were done by Pearson's correlation coefficient option.

Results and discussion

The highest phenolic and flavonoids content was observed in bagasse, followed by juice and finally seeds regardless of different ripening stages (Table 1). The changes of total phenolics and flavonoids content in bagasse, juice and seed showed similar trends during ripening. *C. limetta* juice exhibited TPC between 20.38 ± 0.97 and 23.09 ± 2.57 mg GAE/g DW. Amounts obtained for bagasse and seed in *C. reticulata*, were similar to reported data from

Fig. 3 Bactericidal effect of *C. reticulata* on *E. coli* **Fig. 4** Bactericidal effect of *C. reticulata* on *S. aureus*

0.68 to 2.11 mg EGA/g (Londoño-Londoño et al. 2010; Moulehi et al. 2012; Zhang et al. 2014). TFC was the highest for in *C. reticulata* bagasse (1.89 ± 0.01 – 1.93 ± 0.01 mg QE/g DW). In general, TPC and TFC increased proportionally to ripeness for both fruits.

The hesperidin and ascorbic acid composition was evidently different among bagasse, juice and seed and was also influenced by ripening (Table 1). In general, the highest amount of hesperidin and ascorbic acid was measured for bagasse, followed by juice and seed. The presence of hesperidin and ascorbic acid was the highest in *C. reticulata* bagasse (0.98 ± 0.04 mg/g DM and 3.46 ± 0.20 mg/g DM, each). As expected, hesperidin contents increased proportionally with ripeness for both fruits, while ascorbic acid contents decreased. Concentrations in *C. reticulata* juice were in accordance with Abad-García, et al. (2014) and Kelebek and Selli (2014);

and amounts obtained in *C. reticulata* seed with reported hesperidin presence in range from 4.31 to 47.08 mg/100 g FW (Moulehi et al. 2012) while Abeyasinghe et al. (2007) reported high level of hesperidin in Citrus unshiu and Citrus reticulata, 84.5 and 120 mg/100 g FW, respectively.

The index of maturity and concentration of extract, and their interaction showed a significant effect on the inhibition of growth for both bacteria. The linear regression equations are presented in Table 2, along with their correlation coefficient. The regression models explained 75.94–98.46% of variability in % Inhibition of growth on both tested bacteria.

The bactericidal effect of extract from *C. limetta* bagasse, juice and seed during ripening were tested against *E. coli* (Fig. 1) and *S. aureus* (Fig. 2). They effectively inhibited bacterial growth dose-dependently, and variations

were observed among different tissues and different maturity. *C. reticulata* bagasse presented the strongest effect with an inhibition up to 99.7% in *E. coli* (Fig. 3) and 98.6% in *S. aureus* (Fig. 4), at 800 $\mu\text{g mL}^{-1}$. Results of the present study were consistent with the minimum inhibitory concentration at 300 $\mu\text{g mL}^{-1}$ in *S. aureus* and 1200 $\mu\text{g mL}^{-1}$ in *E. coli* reported earlier (Jayaprakasha et al. 2000). It was interesting to notice that this extract showed the highest levels of total phenolics and total flavonoids contents; however it indicated that interaction of several compounds could inhibited microorganism growth. Bactericidal effect of alcohol soluble fractions may be higher than other fractions probably due to higher polymethoxylated flavone content, which affected bacteria growth (Jayaprakasha et al. 2000).

As expected, increase of the bactericidal effect on both bacterias was proportional to fruits ripeness, probably resulted from increase in TPC and TFC. *S. aureus* was more resistant to the tested extracts, showing inhibitions up to 20% less than *E. coli* for all extracts.

Conclusions

Citrus reticulata and *Citrus limetta* showed an important content of total phenolics and flavonoids, which makes them a potential source of polyphenolic compounds. The extracts of bagasses, seed and juice of *C. reticulata* and *C. limetta* were found to show antibacterial activity. Antibacterial effect arise within total flavonoids and total phenolics contents. Bagasse extracts were found to exhibit a high degree of antibacterial activity in both fruits and have the feasibility to be used as a biopreservative.

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