Antioxidant and α-glucosidase inhibitory properties of soluble melanins from the fruits of *Vitex mollis* Kunth, *Randia echinocarpa* Sessé et Mociño and *Crescentia alata* Kunth

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**ABSTRACT**

Melanins have important pharmacological properties with limited applications due to their poor solubility. Soluble melanins have been reported in different organisms, but not in plants. In this study, aqueous soluble melanins, Impure (IMe) and Partially Purified (PMe), were prepared from the edible pulp of fruits native to Mexico (i.e. *Vitex mollis*, VM; *Randia echinocarpa*, RE; and *Crescentia alata*, CA). IMe and PMe were tested for their phenolics content (TPC), antioxidant activities (AA), α-glucosidase inhibition (αGI) and spectroscopic features (UV-Vis and IR). Melanins of VM showed the highest TPC (222.23 mg GAE/g) and AA (ABTS 2779.30 μmol TE/g; FRAP 1203.70 μmol TE/g). Purified melanins (PMe) showed better αGI than acarbose (IC50 = 8.38 mg/mL). The spectroscopic features of IMe and PMe corresponded to melanins and solubility may be by their complexation with carbohydrates. For the first time, plant-based soluble melanins and their remarkably high antioxidant and α-glucosidase inhibitory characteristics are presented.

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**ABBREVIATIONS:**
AA, antioxidant activity; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid; αGI, α-glucosidase inhibition; CA, *Crescentia alata*; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalents; IMe, impure melanins, extracted at room (-R) or boiling temperature (-B); PMe, purified melanins, obtained by ethanol precipitation (PMeEP) or by dialysis (PMeD) of melanins extracted at room (-R) or boiling temperature (-B); RE, *Randia echinocarpa*; TE, Trolox equivalents; TPC, total phenolics content; VM, *Vitex mollis*

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1. Introduction

Fruit and vegetable consumption has been associated with a lower risk of developing cardiovascular diseases and increased longevity. Phytochemicals (e.g. carotenoids and flavonoids) with diverse activities (e.g. antioxidant and α-glucosidase inhibitory) have been related with those health beneficial properties (Gironés-Vilaplana et al., 2014).

The α-glucosidase inhibitors are important anti-diabetic drugs that prevent postprandial hyperglycaemia by inhibiting the hydrolysis of oligosaccharides in the gut (Kumar, Ghosh, & Pal, 2013). Several efforts are currently underway to identify new natural sources of α-glucosidase inhibitors and α-glucosidase inhibitors that delay the damage caused by oxidative stress in patients with type II diabetes mellitus (Cerillo, 2008; Coman, Rugina, & Socaciu, 2012) or obesity (Gironés-Vilaplana et al., 2014). Additionally, their potential to treat viral ills (e.g. hepatitis and dengue) (Norton et al., 2005; Zhong, Ma, & Shahidi, 2012) or cancer (Atsumi, Nosaka, Ochi, linuma, & Umezawa, 1993; Gamberucci et al., 2006; Pili et al., 1995) has been suggested; these acting by inhibition of glycoprotein maturation.

Melanins are high-molecular-weight pigments that impart light hues to black colours; they are found in animals, plants and microorganisms and are important for human health due to their functional properties (e.g. antioxidant and immunostimulatory) (Huang et al., 2011; Pugh et al., 2005). Studies about plant melanins are scarce compared to others pigments or secondary metabolites. They have been isolated from Osmanthus fragrans seeds (Wang, Pan, Tang, & Huang, 2006), fruits and seeds of Nyctanthes arbor-tristis (Kannan & Ganjewala, 2009), Nigella sativa seeds (El-Obeid, Al-Harbi, Al-Jomah, & Hassib, 2006) and black tea (Sava, Yang, Hong, Yang, & Huang, 2001).

Melanins are commonly represented as covalent bonded indoles (Bilinska, 1996), although their general structure is unknown (Riley, 1997). It has been suggested that identical melanin structures do not exist in nature (Kerestes, Kerestes, & Vegner, 2001) and their chemical characterization is a complicated task. These pigments have been scarcely studied due to their low solubility in most of the common solvents (e.g. water, methanol, ethanol, ethyl acetate, hexane) and harsh pH extraction-conditions (i.e. ammonium hydroxide and hydrochloric acid) that give low yields. On the other hand, soluble melanins have not been reported in plants, but in bacteria, fungi and animals (Aghajanyan et al., 2005; Mazurkiewicz, 2006), whose solubility has been associated with the formation of complexes with carbohydrates or proteins (Aghajanyan et al., 2011; Seniuk et al., 2010). Thus, the presence of soluble melanins in plants is possible.

Vitex mollis Kunth, Randia echinocarpa Sessé et Mocíño and Crescentia alata Kunth, are endemic to Mexico and commonly known as “uvalama”, “papache” and “ayale”, respectively. Their edible pulps are dark, have therapeutic properties (Delgado-Vargas et al., 2010; Santos-Cervantes, Ibarra-Zazueta, Loarca-Piña, Paredes-López, & Delgado-Vargas, 2007; Vargas-Solís & Perez-Gutierrez, 2002), and the spectroscopic characteristics of their un-characterized pigments do not correspond to those commonly found in other fruits (e.g. anthocyanins, carotenoids, chlorophylls, betalains), representing potential sources of melanins. In fact, our research group has compared the aqueous soluble (purified by dialysis) and insoluble (obtained by alkali and acid extraction) melanins of V. mollis fruit; both melanins show similar spectroscopical and biological properties, but the yield of the soluble is higher (Pío-León et al., 2014, submitted for publication). Therefore, we hypothesized that pigments of the black edible pulps of these fruits are soluble melanins extractable by friendly environmental methods.

In this research, we partially purified aqueous soluble melanins from the pulp of the fruits of V. mollis, R. echinocarpa and C. alata, with high extraction yields, using two easy and environmentally friendly methods. Pigments were characterized by spectroscopic techniques and the effect of heating was studied; their total phenolics content and in vitro antioxidant and α-glucosidase inhibitory activities were also evaluated.

2. Material and methods

2.1. Plant material

Vitex mollis (VM), Randia echinocarpa (RE) and Crescentia alata (CA) fruits were collected from the municipalities of Culiacan and Elota (VM), and Badiraguato and Salvador Alvarado (RE and CA), Sinaloa, Mexico. Taxonomic identification was corroborated by Dr. Rito Vega Aviña of the Faculty of Agriculture of the Autonomous University of Sinaloa (UAS) and the assigned numbers for the materials deposited in the herbarium of the Faculty of Agriculture were VM (8258), RE (9035) and CA (9055). The fruits were harvested at maturity as recommended by inhabitants of the rural communities. Once collected, peel (except for VM) and seeds were removed; recovered pulps were frozen (−40 °C), freeze-dried (Model 5L Virtis Co., Gardiner, NY, USA) and milled to obtain flours that passed through the sieve number 30. Vitex mollis, R. echinocarpa and C. alata flours were stored for no more than three months at −20 °C until use.

2.2. Preparation of impure melanin (IMe)

Impure melanins (IME) were obtained by aqueous maceration at room (IME-R, 27 °C) and boiling (IME-B) temperatures. In an amber flask, 5 g of fruit flour and 100 mL of deionized water were mixed and stirred for 30 min at 27 °C. The suspension was centrifuged (20000 g/ 15 min/ 20–25 °C), the supernatant was recovered and then freeze-dried to obtain IMe-R. IMe-B was obtained in the same way as IMe-R, but extraction time (30 min) started after reaching the boiling temperature. Both melanins were stored for no more than 3 months at −20 °C until used.

2.3. Preparation of partially purified melanins (PMe)

2.3.1. Ethanol precipitation

The freeze-dried impure melanins (IME-R and IMe-B) were re-suspended in deionized water (50 mg/mL) and mixed with four volumes of distilled anhydrous ethanol, stirred for 30 min and centrifuged (20000 g/ 5 min/ 20–25 °C); the pellets were recovered and freeze-dried to obtain partially purified melanins (PMe) by ethanol precipitation (PMeEP) at room (PMeEP-R) and boiling (PMeEP-B) temperature; and the yield was calculated. The PMe
were stored for no more than 3 months at −20 °C until use. The supernatants of the centrifugation processes were evaluated for carbohydrates by using the Tollens’ reagent (Clugston & Fleming, 2000).

2.3.2. Dialysis
The freeze-dried impure melanins (IMe-R or IMe-B) were re-suspended in 10 mL of deionized water (50 mg/mL) and dialyzed against 300 mL of deionized water using a 12 kDa pre-hydrated cellulose membrane (Sigma-Aldrich, Seelze, Germany); dialysis was carried out for four days under stirring and the water was changed two times/day. Dialyzed retentates were recovered, freeze-dried and stored at −20 °C until use; yields were calculated. The fractions so obtained were partially purified melanins by dialysis of the aqueous extract at room temperature (PMeD-R) and of the aqueous extract at boiling temperature (PMeD-B).

2.4. Total phenolics content (TPC)
Total phenolics of the melanin samples (IMe and PMe) were determined by the Folin-Ciocalteau reaction (Waterhouse, 2002). Melanin dissolved in deionized water (20 μL) was mixed with 1.58 mL of deionized water and 100 μL of the Folin-Ciocalteau reagent, gently stirred for 5 min and added with 300 μL of a saturated solution of sodium carbonate. The mixture was allowed to stand in darkness for 30 min at 40 °C and the absorbance was measured at 765 nm (Spectronic Genesis 20, Spectronic Instruments, Rochester, NY, USA). A calibration curve of gallic acid in water-methanol (1:1, v/v) (0, 50, 100, 200, 400 and 500 μg/mL) was prepared and TPC was calculated as milligrams of gallic acid equivalents (GAE)/gram of melanin (mg GAE/g).

2.5. Antioxidant activity
Two methods were used: ABTS [2,2’-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid] and FRAP (ferric reducing antioxidant power).

2.5.1. ABTS assay
The radical 2,2’-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS+) was produced by mixing 7 mM ABTS with 2.45 mM potassium persulphate (5 mL of ABTS + 5 mL of potassium persulphate 4.9 mM). The mixture was incubated in darkness at room temperature for 16 h, diluted with 7 mM phosphate buffer pH 7.4 to reach an absorbance between 1.0 and 1.2 at 734 nm. For the ABTS assay, 50 μL of melanin (IMe or PMe), or dissolved as control, were mixed with 1.95 mL of ABTS+ solution, incubated in darkness for 10 min at 37 °C and then the absorbance was measured at 734 nm (Liu et al., 2009). A calibration curve of Trolox in water-methanol (1:1, v/v) (400, 300, 200, 100 and 50 μg/mL) was prepared and the antioxidant activity (AA) calculated as % AA = [(Ac – Am) / Ac] × 100; where, Ac and Am are the absorbances for the control and melanin, respectively. Results were reported as micromoles of Trolox equivalents (TE)/gram of melanin (μmol TE/g).

2.5.2. FRAP assay
The FRAP reagent was prepared by mixing 2.5 mL of 10 mM TPTZ (2,4,6-tris(2-pyridyl)-S-triazine) in 40 mM HCl, 2.5 mL of 20 mM FeCl3•6H2O and 25 mL of 0.3 mM acetate buffer (pH 3.6), followed by incubation for 30 min at 37 °C. For the evaluation, 900 μL of freshly prepared FRAP reagent, 90 μL of deionized water and 10 μL of melanin sample (IMe or PMe, at known concentrations) or appropriate reagent blank were mixed. The mixture was incubated (30 min/37 °C) and the absorbance at 595 nm was read. Results were obtained by linear regression, using a Trolox calibration curve (500, 400, 300, 200, 100 and 50 μg/mL), and expressed as micromoles of TE/gram of melanin (μmol TE/g) (Benzie & Strain, 1996).

2.6. α-Glucosidase inhibition (αGI)
In 96 microwell plates, 50 μL of melanin sample (IMe or PMe) or acarbose (positive control) at different concentrations were incubated for 10 min at 37 °C with 100 μL of α-glucosidase from Saccharomyces cerevisiae (Sigma-Aldrich, Toluca, Estado de Mexico, Mexico) (1 U/mL) in phosphate buffer (0.1 M, pH 6.9). Then, 50 μL of 4-nitrophenyl β-D-glucopyranoside (5 mM in phosphate buffer pH 6.9) were added and the mixture was incubated again for 10 min at 37 °C (Stat Fax-2200, Awareness Technology, Palm City, FL, USA). For correction of sample interferences, a blank was prepared by mixing 100 μL of α-glucosidase, 50 μL of melanin sample and 50 μL of phosphate buffer. As control, 50 μL of solvent was used instead of the melanin or acarbose. Absorbance at 405 nm was measured with a Multiskan Biochromatic (SmartSpect™ 3000, Bio Rad, Philadelphia, PA, USA). The inhibition was calculated as αGI (% = [(Aα – Aαm) / Aα] × 100; where, Aα and Aαm are the absorbance for the control and melanin, respectively (Pinto et al., 2008). For each sample, the half maximal inhibitory concentration (IC50) of the α-glucosidase activity was determined with the Software GraphPadPrism Inc. version 5.03 (La Jolla, CA, USA).

2.7. Ultraviolet-visible spectroscopy (UV-Vis)
Melanin solutions were prepared at 0.5 mg/mL and the UV-Vis spectra were obtained (200–800 nm) (SmartSpect™ 3000, Bio Rad, Philadelphia, PA, USA).

2.8. Absorbance ratios (Aα500/Aα600)
The Aα500/Aα600 values of melanins were determined by measuring the UV-Vis absorption spectra of different dilutions (at least in triplicate). All dilutions followed the criteria 2.5>Aα500>0.25 (SmartSpect™ 3000, Bio Rad, Philadelphia, PA, USA); these limits were established to ensure all spectra gave measurable Aα500 and Aα600 values, and to reduce errors due to instrument saturation at high absorbance measurements (Haywood, Lee, & Linge, 2006). Thus, the melanin concentrations (mg/mL) were as follows: IMe-R-RE and IMe-B-RE (5.5); IMe-R-CA and IMe-B-CA (6); IMe-R-VM (3); IMe-B-VM (1.75); all PMeEP (0.5), except PMeE-B-CA (2); all PMeD (0.4), except PMeD-R-RE (0.5) and PMeD-B-CA (1.0). Absorbances of the dilutions at 300 nm were around 2.0 (1.95≤Aα500 ≤2.38).

2.9. Infrared spectroscopy (IR)
The IR spectra of melanin solid samples were obtained at room temperature by attenuated total reflection with a Fourier
transform infrared spectrometer (FTIR-ATR Cary 660; Agilent Technologies, Santa Clara, CA, USA) in the range 4000–400 cm⁻¹ (10 scannings, 1 cm⁻¹ resolution).

2.10. Statistical analysis

Data from every measured property were analyzed by one way analysis of variance and the means were compared by the Fisher test ($\alpha = 0.05$). Relationships between the measured properties were analyzed by simple linear regression and the Pearson correlation coefficient. All the analyses were carried out with Statgraphics 5.1 (StatPoint Inc., Warrenton, VA, USA). Property evaluations were done at least in triplicate.

3. Results

3.1. Impure melanins (IMe)

3.1.1. Extraction yields

The extraction yields of IMe ranged from 69% dry weight (d.w.) (IMe-B-VM) to 84% d.w. (IMe-R-RE) and, comparing for every fruit, were not affected by the thermal treatment (Table 1).

3.1.2. Total phenolics content (TPC) and antioxidant activity (AA)

The IMe of VM showed the highest TPC and AA; their TPC values were at least 4.7 higher than those of RE and CA; whereas their AA values were approximately 7 and 4 times higher than those of RE and CA, respectively.

The AA values by ABTS were higher than those obtained by FRAP (Table 1). Moreover, boiling extraction increased both TPC and AA for VM and RE (IMe-B-VM) and, comparing for every fruit, were not affected by the thermal treatment (Table 1).

3.1.3. α-Glucosidase inhibition (αGI)

The order of activities for αGI of impure melanins (IMe) were VM > RE > CA. The IC₅₀ values for VM (3.11 mg/mL and 3.79 mg/mL) were better than that of acarbose (8.38 mg/mL). By contrast with TPC and AA, heating during extraction did not affect the αGI of IMe from the same fruit (Table 1).

3.2. Purified melanins (PMe)

3.2.1. Extraction yield

The extraction yields (% of IMe) of PMe obtained by ethanol precipitation (PMeEP) varied from 6.05 to 19.3%; whereas lower values were obtained for melanins purified by dialysis (PMeD) (3.63–13.55%). The decreasing order of extraction was VM > CA > RE for both PMeEP and PMeD. Contrasting the effect of heating, the extraction yields of PMeEP were not affected (PMeEP-R = PMeEP-B), but increased for the PMeD of V. mollis and C. alata (Table 2).

The supernatants obtained in the preparation of the PMeEP gave a positive result for carbohydrates by the Tollens’ test (Clugston & Fleming, 2000). In addition, the solubilities of the PMe were lower than those of their corresponding IMe, considering the absorption at the $\lambda_{\text{max}}$ and the ease of dissolution.

3.2.2. Total phenolics content (TPC) and antioxidant activity (AA)

The TPC values for melanins purified by ethanol precipitation (PMeEP) were from 22.31 (PMeD-R-RE) to 217.85 (PMeD-B-VM) mg GAE/g with a decreasing order of VM > RE > CA. The IC₅₀ values for VM (3.11 mg/mL and 3.79 mg/mL) were better than that of acarbose (8.38 mg/mL). By contrast with TPC and AA, heating during extraction did not affect the αGI of IMe from the same fruit (Table 1).

<table>
<thead>
<tr>
<th>Impure melanin (IMe)</th>
<th>Extraction yield (% d.w.)</th>
<th>Total phenolics content (mg GAE/g)</th>
<th>Antioxidant activity (µmol TE/g)</th>
<th>αGI (IC₅₀) (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMe-B-VM</td>
<td>73.24 ± 1.31</td>
<td>29.48 ± 0.46</td>
<td>429.21 ± 28.53</td>
<td>146.98 ± 18.81</td>
</tr>
<tr>
<td>IMe-B-VM</td>
<td>69.45 ± 0.25</td>
<td>40.15 ± 0.46</td>
<td>531.14 ± 5.17</td>
<td>217.17 ± 7.15</td>
</tr>
<tr>
<td>IMe-B-VM</td>
<td>84.07 ± 1.98</td>
<td>4.84 ± 0.05</td>
<td>53.59 ± 1.12</td>
<td>23.65 ± 0.13</td>
</tr>
<tr>
<td>IMe-B-VM</td>
<td>80.52 ± 1.78</td>
<td>6.28 ± 0.19</td>
<td>76.06 ± 0.69</td>
<td>37.90 ± 0.13</td>
</tr>
<tr>
<td>IMe-B-VM</td>
<td>70.49 ± 6.26</td>
<td>4.51 ± 0.37</td>
<td>120.69 ± 0.45</td>
<td>67.42 ± 9.71</td>
</tr>
<tr>
<td>IMe-B-VM</td>
<td>77.25 ± 1.95</td>
<td>4.71 ± 0.03</td>
<td>124.86 ± 1.05</td>
<td>64.97 ± 2.85</td>
</tr>
</tbody>
</table>

Table 1 - Extraction yields, total phenolics content, antioxidant activity and α-glucosidase inhibition (αGI) of the impure melanins (IMe), obtained by aqueous extraction at room and boiling temperature, from the Vitex mollis, Randia echinocarpa and Crescentia alata fruits.

a Impure melanins obtained at room (IMe-R) and boiling temperature (IMe-B) from Vitex mol (VM), Randia echinocarpa (RE) and Crescentia alata (CA) fruits.

b Dry weight basis (d.w.).

c Expressed as milligrams of gallic acid equivalents per gram of melanin (mg GAE/g).

d Expressed as micromoles of Trolox equivalents per gram of melanin (µmol TE/g).

e α-Glucosidase inhibition (αGI), expressed as inhibitory concentration of 50% of the enzyme activity (IC₅₀), in milligrams of melanin per milliliter (mg/mL) (IC₅₀ for acarbose = 8.38 mg/mL).

f Data shown are the mean of at least three independent experiments and different superscript letters (h–k) in the same column indicate significant differences ($p < 0.05$).
higher than those of any impure melanin (IMe) (Table 1). The PMe of V. mollis showed the highest AA; PMeD-B-VM was the most active, being at least 3.9 (ABTS) and 4.1 (FRAP) times higher than any of the impure melanins (IMe) and acarbose, with that PMeD-B-CA being the most active, followed by those of RE and CA. Moreover, the AA ABTS-values for PMe were only different with strong absorances in the range from 289 to 304 nm. All PMe showed higher absorption units (hyperchromic effect), at the same concentration, than their corresponding IMe; also, the A\textsubscript{max} were higher for the PMe (bathochromic effect), except for PMeEP-B-CA, which also showed the lowest increment in the absorption.

### 3.3. Spectroscopical analyses of impure (IMe) and partially purified (PMe) melanins

#### 3.3.1. UV-Vis spectroscopy

Figure 1 shows the UV-Vis absorption spectra of the melamins obtained at boiling temperature and partially purified by dialysis (PMeD-B) from VM, RE and CA. They exhibit absorption peaks in the region from 353 to 296 nm and low or null absorption in the visible region. The spectral behavior of IMe and PMeEP (R and -B) was very similar to that shown in Fig. 1, with strong absorances in the range from 289 to 304 nm. All PMe showed higher absorption units (hyperchromic effect), at the same concentration, than their corresponding IMe; also, the A\textsubscript{max} were higher for the PMe (bathochromic effect), except for PMeEP-B-CA, which also showed the lowest increment in the absorption.

#### 3.3.2. Absorbance ratios (A\textsubscript{300}/A\textsubscript{600})

Partially purified melanins (PMe) showed lower A\textsubscript{300}/A\textsubscript{600} values than their corresponding impure melanins (IMe), except for the PMeD-R-RE sample, which was slightly higher. Moreover, contrasting the same fruit and thermal treatment of extraction, ethanol precipitated melanins (PMeEP) showed higher A\textsubscript{300}/A\textsubscript{600} ratios than those obtained by dialysis (PMeD) (Table 3). For VM and RE, boiling during extraction decreased the A\textsubscript{300}/A\textsubscript{600} ratios, but an opposite effect was registered for the CA mela-
ins (Table 2).

#### 3.3.3. IR spectroscopy

The infrared (IR) spectra of the impure melanins (IMe-R and IMe-B) from the three fruits were characterized by bands within the following wave numbers: 3400–3200, 2940–2920, 1630–1610, 1590, 1440–1340 and 1250–990 cm\textsuperscript{-1} (Fig. 2), and heating during extraction did not change the melanin spectra. The other evaluated melanins showed similar IR spectra to those of Fig. 2; nevertheless the IR spectra of partially purified melanins (PMe)
(mostly PMeD) showed spectral lines more defined than those of IMe (Fig. 3). The PMeD IR spectra of VM and CA showed a higher number of spectral lines in the range of wavelengths 1500–1000 cm\(^{-1}\). The IR spectra of CA melanins (IMe and PMe) showed a more intense spectral line around 1700 cm\(^{-1}\) (Figs. 2 and 3), and the spectrum of PMeD-B-CA, compared with those of PMeD-B of VM and RE, showed a less extended peak in 3347 cm\(^{-1}\).

### 4. Discussion

#### 4.1. Extraction yields of the impure (IMe) and purified (PMe) soluble melanins

The extraction yields obtained for the IMe (69.45–84.07% dw) (Table 1) were higher than that reported for impure soluble melanins.
melanins from the fungus *Inonotus obliquus* (20%) (Mazurkiewicz, 2006). In the case of PMe, the extraction yields in this work (3.63–19.03% dw) were higher than those reported for pure melanins of black tea (1.36%) (Sava et al., 2001) and fruits and seeds of *Nyctanthes arbor-tristis* (0.05%) (Kannan & Ganjewala, 2009), obtained by alkaline extraction. Because we used water for extraction, our methodology is easier, cheaper and more environmentally friendly.

The improved water solubility of melanins from different sources may be caused by the formation of complexes with carbohydrates or proteins (Aghajanyan et al., 2011; Seniuk et al., 2010). Results of this work suggested that plant soluble melanins IMe and PMe showed high water solubility by the formation of complexes with carbohydrates; i.e., fruits are rich in carbohydrates; high extraction yields for the obtained soluble melanins (IMe and PMe) (Tables 1 and 2); positive Tollens’ test for the supernatants obtained during the preparation of the PMeEP; purified melanins (PMe) showed lower water solubilities than impure melanins (IMe); and spectroscopic evidence (see Sections 3.3. and 4.4.).

4.2. Total phenolics contents (TPC) and antioxidant activities (AA) of the impure (IMe) and purified (PMe) soluble melanins

The TPC of the *V. mollis* impure melanin IMe-B-VM (40.15 mg GAE/g) (Table 1) was higher than the TPC reported for water-ethanol extracts from skin of grapes (*Vitis vinifera* cv. “Tempranillo”) (<1.6 mg catechin/ g) (López de Lerma, Peinado, & Peinado, 2013). The IMe-B-VM also showed the highest antioxidant activities (Table 1); their values were similar to those reported for aqueous extracts of roasted and non-roasted coffee beans (ABTS, 450–630 μmol TE/g) (Liu & Kitts, 2011) and higher than those reported for the methanol-water extracts of several red cabbage (*Brassica oleracea* L. var. capitata L. f. rubra) varieties (ABTS, 87–169 μmol TE/g) (Wiczkowski, Topolska, & Honke, 2014). The high antioxidant activity of melanins was expected because the protection against UV-radiation and free radicals generation are within their main functions (Huang et al., 2011). The edible portion of *V. mollis* fruits is more exposed to solar radiation and weather changes than those of *R. echinocarpa* and *C. alata*, which are protected by a hard-opaque pericarp. The highest TPC and AA of *V. mollis* could be associated with this characteristic.

In vegetables, the total phenolics content (TPC) and the antioxidant activity (AA) tend to decrease after cooking (Kaur & Kapoor, 2001), contrasting with the increased values for the IMe of VM and RE (Table 1). Melanins are very stable, probably not so much affected by heating and considering that temperature of extraction did not affect the extraction yield (Table 1), thus, the improved TPC of the mentioned fruits could be associated with differences in the profiles of the impure melanins obtained at room (IMe-R) and boiling (IMe-B) temperatures. *Vitex mollis* is traditionally consumed after cooking (Flores-Islas, 1999; Montiel-Herrera, Camacho-Hernández, Rios-Morgan, & Delgado-Vargas, 2004), and by contrast with raw fruit, boiled

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**Fig. 2 – Infrared spectra of impure melanins (IMe) obtained at room (IMe-R) (a, b, c) and boiling temperature (IMe-B) (d, e, f) of Vitex mollis (a, d), Randia echinocharpa (b, e) and Crescentia alata (c, f). Wave number values (cm⁻¹) of the most representative peaks are shown.**
preparation may be characterized by high TPC and AA improving its beneficial properties.

Melanins are high molecular weight polymers and less contaminated samples must have higher TPC and AA values, as it was registered, the PMe showed higher values than IMe (Tables 1 and 2). Moreover, the highest AA of the dialysis purified melanins (PMeD) suggested their higher purities in contrast with the ethanol precipitated (PMeEP). Probably, ethanol precipitation induced the co-precipitation of other components non-bound to melanins (e.g. proteins and oligosaccharides) and if they were characterized by low or null AA, the observed high extraction yields and the low AA values for the PMeEP may be explained.

In the ABTS assay, AA values of purified melanins from RE and CA were similar to those reported for melanoidins (another aqueous-soluble pigment) of roasted coffee beans (480-730 μmol TE/g) (Liu & Kitts, 2011), but the AA for the V. mollis PMe was superior (Table 2). Contrasting with the FRAP method, the highest ABTS values (Tables 1 and 2) could be associated with differences in sample composition and in the mechanisms on which the assays are based on. The FRAP method only detects ferric reducing compounds with potentials lower than 0.7 V and is not sensitive to antioxidants acting by proton-transfer reaction. Whereas, the ABTS method measures both electron- and proton-transfer reactions, sensing lipophilic and hydrophilic compounds (Re, Pellegrini, Proteggente, & Pannala, 1999). Consequently, synergic interactions among antioxidants impact our results.

The positive correlation of the AA and TPC for the impure (IMe) and the purified (PMe) soluble melanins could be due to their structures; melanin (IMe and PMe) molecules have phenolic groups (Harborne, 1984; Marcano & Hasegawa, 2002; Riley, 1997) and their quantification could estimate the pigment concentration in the sample.

Natural antioxidants (e.g. green tea extracts) are important in the food industry to avoid oxidative reactions in diverse products (e.g. meat, poultry, emulsions, beverages, snacks) and to increase their shelf-life (Senanayake, 2013). Some desirable characteristics of water-soluble antioxidants are an easy incorporation to diverse food matrices, heat stability, low toxicity and cost; in particular, heat stability is important to use such antioxidants in cooked foods. Soluble melanins reported in this work have the aforementioned characteristics but their lack of toxicity must be corroborated in order to recommend them as a food ingredient.

4.3. α-Glucosidase inhibition (αGI) of the impure (IMe) and purified (PMe) soluble melanins

Soluble melanins (IMe and PMe) of the studied fruits were better inhibitors than acarbose (Tables 1 and 2), a drug used to treat diabetes mellitus type II (Clissold & Edwards, 1988). The αGI

Fig. 3 – Infrared spectra of partially purified melanins (PMe) by ethanol precipitation (PMeEP) (a, b, c) and dialysis (PMeD) (d, e, f) of Vitex mollis (VM) (a, d), Randia echinocharpa (RE) (b, e) and Crescentia alata (CA) (c, f), obtained from impure melanins extracted at boiling temperature (IMe-B). Wave number values (cm⁻¹) of the most representative peaks are shown.
of extracts from different strawberry cultivars (*Fragaria × ananassa Duch.*) were evaluated by the same methodology used in this work (IC₅₀ = 25 mg/mL) (Pinto et al., 2008); our values for the IMe of VM were more than six times lower and those for RE and CA were comparable. All the PMe were better inhibitors than IMe suggesting an association of melanin components with the αGI. However, αGI values were neither correlated with TPC nor with AA, although both of these parameters increased with purification (Tables 1 and 2); consequently, sample composition was affecting differentially the mentioned parameters.

The *V. mollis* melanin obtained at room temperature and by dialysis (PMeD-R-VM) was one of the most potent α-glucosidase inhibitors reported in the literature; it was 43 times better than acarbose. Li et al. (2009) isolated different flavonoids (e.g. apigenin, luteolin, vitexin, isovitexin) with such activity from *Crateagus* *soxyacantha* leaves, which were 9 to 17 times superior to acarbose. In another study, the α-GI by (+)-α-viniferin isolated from *Carex bacans*, a plant used to treat diabetes, was 1.5 times higher than acarbose (Kumar, Gupta, Ghosh, Gaonkar, & Pal, 2013).

Inhibitors of α-glucosidase, like acarbose, decrease the post-prandial absorption of glucose after eating complex carbohydrates, helping with the prevention/ control of the diabetes illness. In patients with diabetes, glucose oxidation increases the oxidative stress and the risk of cardiovascular diseases (Ceriello, 2008). The melanins evaluated here could have a therapeutic advantage over acarbose since they combine high αGI and AA activities (Tables 1 and 2).

On the other hand, natural α-glucosidase inhibitors have been suggested as antivirus and antitumor agents. Glycoproteins are involved in crucial steps of tumor progression and viral reproductive cycle; thus, blocking protein glycosylation is a strategy against these illnesses. In particular, the plant alkaloid castanospermine is one of the best studied -glucosidase inhibitors (Atsumi et al., 1993; Pili et al., 1995; Whitby et al., 2005); moreover, dietary green tea phenolics inhibit the α-glucosidase II of rat liver microsomes (Senanayake, 2013). However unlike castanospermine and green tea phenolics, soluble melanins reported in this work are characterized by high molecular weights, so their bioavailability must be previously demonstrated.

Plants, fungi and microorganisms have been sources of several compounds with α-glucosidase inhibitory activity (e.g. flavonoids, alkaloids, organic acids, and peptides) (Coman et al., 2012). However, we are reporting for the first time the existence of soluble melanins with α-glucosidase inhibitory activity in different plant sources.

### 4.4. Spectroscopical analyses of impure (IMe) and partially purified melanins (PMe)

The UV-Vis absorption spectra of the impure (IMe) and purified (PMe) soluble melanins (Fig. 1) were similar to those reported in the literature. Melanins obtained from black tea, *Nyctanthes arbor-tristis*, *Cinnamomum burmannii*, *Osmanthus fragrans* and marine *Pseudomonas* sp. showed λₘₐₓ values in the region from 200 to 300 nm and lower absorption at higher wavelengths (Huang et al., 2011; Kannan & Ganjewala, 2009; Sava et al., 2001; Tarangini & Mishra, 2013).

This phenomenon is a characteristic of melanins and has been associated with their complex structure, the presence of phenolics groups as the main chromophores and the formation of complexes with substances of low or no absorption in the visible region (Aghajanyan et al., 2011; Seniuk et al., 2010).

As suggested in Section 4.1., water solubility of melanins (IMe and PMe) may be caused by the formation of complexes of melanin–carbohydrates. Supporting this proposal, the UV-Vis absorption spectra of the evaluated melanins suggested that proteins are not part of the soluble melanin complexes; they lacked a well defined absorption-peak for aromatic amino acids (320–380 nm), which should be evident when comparing the spectra of impure (IMe) and partially purified (PMe) melanins.

In relation with IMe, the UV-Vis absorption spectra of the PMe showed hyperchromic and bathochromic effects (Fig. 1) that could be explained by the elimination of components during the purification process; purer melanins must induce an additive effect of chromophores characterized by a higher molar absorption (hyperchromic effect), as well as higher interactions among these chromophores (bathochromic effect).

The A₃₀₀/A₆₀₀ ratios offer us information about the oxidation state and the range size of the melanin molecules. Melanin oxidation induces lower absorbance values at 600 nm (A₆₀₀), and the A₃₀₀/A₆₀₀ absorbance ratio was proposed as a measure of the oxidation extent, high values corresponded to more oxidized melanin molecules. Also, it was argued that during the oxidation of melanins, the phenolics are converted to semiquinones or quinones, which produces more oxidized (higher A₃₀₀/A₆₀₀ absorbance ratios) and smaller melanins (molecular weight < 1000 Da) (Haywood et al., 2006). Thus, impure soluble melanins (IMe) showed higher A₃₀₀/A₆₀₀ ratios than purified ones (PMe); and as expected for PMe the ratios for PMeD were higher than PMeD (Table 3). These data support that IMe are a more complex mixture of melanin molecules than that of PMe, with variability in the size and degree of oxidation; as it happened with PMeEP compared with PMeD. Thus, the differences in their A₃₀₀/A₆₀₀ values were associated with the purification process (IMe > PMe) and the range in the size of melanins (PMeEP > PMeD), with PMeEP containing more oxidized and smaller melanins; this last point may be partially associated with the negative correlation of the A₃₀₀/A₆₀₀ ratio with the antioxidant activity (AA) for VM and CA. Moreover, soluble melanins of VM and RE obtained by boiling may be better reduced and characterized by lower A₃₀₀/A₆₀₀ ratios (Table 3). In melanins synthesized by oxidation of (D)-3-(3,4-dihydroxyphenyl)-alanine, free protons found in their intermolecular spaces mediate the equilibrium between semiquinones, indolquinones, and hydroquinones forms (Goncalves, Filho, & Graeff, 2006). The thermal treatment induces the capture of H⁺ by the COO⁻ groups present in the molecule (according the IR spectra), which produces more reduced melamins. This phenomenon could be associated with higher antioxidant activities (AA) of VM and RE for boiled than room temperature extracted soluble melanins (Tables 1–3). The pattern found for purified melanins (PMe) of C. alata (CA) was opposite. Data suggested that PMe of CA were more heat sensitive; thus, boiling during extraction produced lower values for
TPC and AA (Table 2), associated with higher \( \frac{A_{300}}{A_{600}} \) ratios (negative correlation) and αGI (Table 3).

The IR bands of the soluble melanins obtained in this work (Figs. 2 and 3) were similar to melanins of black tea (Sava et al., 2001), fungi and humic substances (Bilinska, 1996) and Nyctanthes arbor-tristis fruits (Kannan & Ganjewala, 2009). The assigned groups for the infrared (IR) spectra of impure melanins (Ime-R and Ime-B) from the three fruits (Fig. 2) were as follows: 3400–3200 cm\(^{-1}\), associated with –OH and –NH\(_2\) groups; 2940–2920 cm\(^{-1}\) corresponding to stretching vibrations of C–H (from aromatic rings) and C–H (from aliphatic compounds); 1630–1590 cm\(^{-1}\), caused by oscillation of coupled double bonds of C=C and C=O types; 1440–1340 cm\(^{-1}\), attributed to methylene vibration; 1250–990 cm\(^{-1}\), caused by oscillation of C–O groups of carbohydrates, alcohols and phenols. Moreover, the additional and more defined bands for the purified melanins (PMe), and in particular of the PMeD of VM and CA, at 1500–1000 cm\(^{-1}\) were possibly associated with alcohol groups (C–OH from aromatic rings, CH\(_2–\)OH, CH–OH) and with CH\(_2–\), CH\(_3–\), or C–N peaks. Peaks around 1100 cm\(^{-1}\) were associated with a carbohydrate moiety in the water soluble chitin-melanin-glycan complex (Veleshko et al., 2012). The IR band at 1700 cm\(^{-1}\) for CA melanins (Ime and PMe) (Figs. 2 and 3) was characteristic of C=O group; whereas differences in the peak in 3347 cm\(^{-1}\) for the PMeD-B-CA corresponded to the presence of O–H groups (Pretsch, Bèuhlmann, & Badertscher, 2009). This information could suggest that the CA melanins were polymers of lower-molecular-mass and less moisture; only during the partial purification by dialysis of CA melanins, colored pigments of lower sizes (molecular mass ≤12 kDa) were eliminated, the molecular interactions were reduced and best defined spectral lines were registered. However, \( \frac{A_{300}}{A_{600}} \) ratios of the CA melanins (Table 3) did not correspond with the suggested explanation. Thus, more information about the structure of these melanins must be generated.

5. Conclusions

The soluble melanins from the fruits of V. mollis, R. echinocarpa and C. alata were extracted and partially purified by facile and environmentally friendly methods. The analyses suggested that the aqueous solubility of the obtained melanins could be due to their presence as melanin-carbohydrate complexes. Based on the high antioxidant and \( \alpha \)-glucosidase inhibitory activities of these soluble melanins, they could be used as nutraceutical ingredients for the food and the pharmaceutical industries; e.g. their use could prevent the oxidative stress and avoid the increase of postprandial glucose, helping with the prevention/treatment of diabetes mellitus type II. This is the first report contrasting the \( \alpha \)-glucosidase inhibitory and antioxidant activities attributed to soluble melanins obtained from fruits.

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