SUPPLEMENTARY MATERIAL

Determination of phenolic compounds, in vitro antioxidant activity and characterization of secondary metabolites in different parts of *Passiflora cincinnata* by HPLC-DAD-MS/MS analysis

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Ethanol extracts of different parts of *P. cincinnata* were obtained by maceration. The total phenolic and flavonoid contents were evaluated. The antioxidant activities were determined by β-carotene-linoleic acid bleaching test, 2,2-diphenyl-1-picrylhydrazil (DPPH), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging. The crude ethanol stem extract showed the highest amount of total polyphenols (45.53 mg gallic acid equivalent/g) while the highest total flavonoid contents (1.42 mg of quercetin equivalent/g) were observed in the leaf extract. The lowest IC₅₀ (25.65 μg/ml) by the DPPH method was observed for the stem extract. The ABTS method showed a significant antioxidant activity for all investigated extracts. The secondary metabolite composition of ethanol extracts was assessed by HPLC-DAD-MS/MS analysis, leading to the identification of fourteen secondary metabolites in *P. cincinnata* extracts. These results showed the potentiality of this species as a source of phenolic compounds and antioxidants.

**KEYWORDS:** antioxidant; flavonoid; *Passiflora cincinnata*; phenolic compounds; vitexin.
List of content

Experimental Section

**Table S1.** Total phenolics (TP), total flavonoids (TF), and antioxidant activity of ethanol extracts obtained from different parts of *P. cincinnata.*

**Table S2.** Metabolites detected by HPLC-DAD MS/MS (ESI positive and negative modes) in ethanol extracts of *P. cincinnata* extracts.

**Figure S1.** Antioxidant activity of *Passiflora cincinnata* extracts (fruit peel, flower, leaf, seed and stem) and standards (BHA, BHT and trolox) as a function of the concentration tested.

**Figure S2.** Representative HPLC-DAD-MS chromatogram of ethanol extract from *P. cincinnata* stem (A) at λ340 nm, (B) ESI positive and (C) ESI negative modes.

**Figure S3.** Representative BPC (base peak chromatogram) of *P. cincinnata:* fruit peel extract (A), stems extract (B), leaves extract (C), seed extract (D) and flower extract (E) at ESI positive mode.

**Figure S4.** Representative BPC (base peak chromatogram) of *P. cincinnata:* fruit peel extract (A), stems extract (B), leaves extract (C), seed extract (D) and flower extract (E) at ESI negative.

**Figure S5.** Representative HPLC-UV chromatogram of *P. cincinnata:* fruit peel extract (A), stems extract (B), leaves extract (C), seed extract (D) and flower extract (E) at λ 340 nm.

**Figure S6.** MS/MS spectra of orientin-\(O\)-hexoside (1) in ESI positive and negative modes.

**Figure S7.** MS/MS spectra of apigenin-6,8-di-C-hexoside (2) in ESI positive and negative modes.

**Figure S8.** MS/MS spectra of isoorientin-\(O\)-hexoside (3) in ESI positive and negative modes.
**Figure S9.** MS/MS spectra of orientin-\(O\)-hexoside (4) in ESI positive and negative modes.

**Figure S10.** MS/MS spectra of apigenin-6,8-di-\(C\)-hexoside isomer (5) in ESI positive and negative modes.

**Figure S11.** MS/MS spectra of vitexin-\(O\)-hexoside (6) in ESI positive and negative modes.

**Figure S12.** MS/MS spectra of isoorientin-\(O\)-hexoside isomer (7) in ESI positive and negative modes.

**Figure S13.** MS/MS spectra of apigenin-6-\(C\)-pentoside-8-\(C\)-hexoside (8) in ESI positive and negative modes.

**Figure S14.** MS/MS spectra of orientin-\(O\)-pentoside (9) in ESI positive and negative modes.

**Figure S15.** MS/MS spectra of isovitexin-\(O\)-hexoside (10) in ESI positive and negative modes.

**Figure S16.** MS/MS spectra of vitexin-\(O\)-hexoside isomer (11) in ESI positive and negative modes.

**Figure S17.** MS/MS spectra of vitexin-\(O\)-pentoside (12) in ESI positive and negative modes.

**Figure S18.** MS/MS spectra of quercetin-\(O\)-hexoside-deoxihexoside (13) in ESI positive and negative modes.

**Figure S19.** MS/MS spectra of methoxy-orientin-\(O\)-hexoside (14) in ESI positive and negative modes.
Experimental Section

**Plant material**

Aerial parts of *P. cincinnata* (fruit peel, flower, leaf, seed, and stem) were collected in the city of Uauá (Coordinates: S 09°43′12.4″; W 39°39′33.3″), State of Bahia, Brazil, in may of 2015. The samples were identified by a botanist, and a voucher specimen (#22870) was deposited at the Herbarium Vale do São Francisco (HVASF) of the Federal University of San Francisco Valley (UNIVASF).

**Extraction**

The different parts of the plant were dried in an air circulation oven at a temperature of 45 °C for 72 hours, pulverized in a knife mill and macerated with ethanol 95% at room temperature for 72 h. Three extraction processes were performed. The extractive solution of each part of the plant was concentrated on a rotatory evaporator to get the crude ethanol extract (EEBs-Pc). These extracts were used for further analyses, to perform all tests in the present study.

**Determination of total phenol and flavonoid content**

The content of total phenols was based according to the methodology of Slinkard and Singleton (1977) and Almeida et al. (2011), determined by the colorimetric method using the reagent Folin-Ciocalteu. Gallic acid was used as standard and results were expressed as mg of gallic acid equivalents per gram of sample (mg GAE/ g). The calibration curve was obtained at concentrations with intervals between 50 to 1000 µg/ml. The total flavonoid content present in the extracts was determined according to the methodology proposed by Dewanto et al. (2002), with slight modifications, using quercetin as the standard. The results were expressed as mg of quercetin equivalents per
gram of extract (mg QE/g). The calibration curve was obtained at concentrations with intervals between 2.5 to 20 µg/ml. All tests were carried out in triplicate.

**In vitro antioxidant activity**

**β-Carotene bleaching test**

In order to evaluate the antioxidant activity, it was used the methodology proposed by Oliveira-Junior et al. (2013), using as standards, butyl-hydroxy-anisole (BHA) and butyl-hydroxy-toluene (BHT). To determine the % AA the following equation was used:

\[
\% \text{ AA} = \left[1 - \frac{(A_0 - A_i)}{A_0^0 - A_i^0}\right] \times 100
\]

where \(A_0\) is the initial absorbance and \(A_i\) is the final absorbance measured for the test sample, \(A_0^0\) is the initial absorbance and \(A_i^0\) is the final absorbance measured for the negative control (blank). All tests were carried out in triplicate and the results are expressed as % AA.

**DPPH free radical scavenging assay**

The elimination test by free radical 2,2-diphenyl-1-picrylhydrazil (DPPH) was carried out using as standard BHA and BHT (Oliveira-Junior et al. 2013). The measurement was determined by absorbance values, in triplicate, measured at 518 nm and converted into IC\(_{50}\) and % AA, using the following formula:

\[
\% \text{ AA} = \left[\frac{(\text{absorbance control} - \text{absorbance sample})}{\text{absorbance control}}\right] \times 100
\]

**Free radical scavenging assay ABTS**

\(ABTS^{++}\)
In this analysis, it was used the methodology of free radical ABTS$^{•+}$ (2,2’-azinobis (3-sulfonic acid ethylbenzthiazoline-6) (Camargo et al. 2016), with slight modifications, observing the gradual change of dark green to light green of the ABTS solution. For this, Trolox® was used as standard, and the measurement was determined by absorbance values, measured at 734 nm and converted into IC$_{50}$ and % AA, through the same calculation formula of DPPH. All tests were carried out in triplicate.

**Identification of chemical compounds by HPLC-DAD-MS/MS**

For this purpose, 2.0 mg of each sample were dissolved in 1.0 ml of ACN/H$_2$O (1:1, v/v, 0.1% of acetic acid). For the removal of non-polar molecules, each solution was purified by solid phase extraction (SPE), using C18 cartridges, previously activated with 5.0 ml of ACN and equilibrated with 5.0 ml of ACN/H$_2$O (1:1, v/v). The resulted samples were dried in N$_2$ atmosphere and dissolved at final concentration 2 mg/ml, then filtered through a 0.45 μm GHP filter and injected into the HPLC.

HPLC-MS/MS analyses were performed using an UFLC (Shimadzu) containing two LC20AD solvent pumps, a SIL20A$_{HT}$ auto sampler, a CTO20A column oven and a CBM20A system controller, coupled with an Ion Trap Mass Spectrometer (AmaZon SL). LC experiments were performed using a C$_{18}$ column (Phenomenex® – 250 mm x 4.6 mm x 5 μm) and the following gradient elution: solvent A = H$_2$O and acetic acid (0.1% v/v); solvent B = ACN and acetic acid (0.1% v/v); elution profile = 0.0 - 30.0 min (10 - 100% B); 30.0 - 40.0 min (100% B); 40.0 - 45.0 min - (100 - 10% B); 45.0 - 60.0 min (10% B), column oven at 35 °C, volume injection 20 μl and flow rate of 1.0 ml/min. Ion Trap acquisition parameters were set as follows: capillary 3.5 kV, positive and negative ESI modes, end plate offset 500 V, nebulizer 40 psi, dry gas (N$_2$) with flow of 8 L/min and temperature of 300 °C. CID fragmentation was achieved in autoMS/MS mode using Enhanced resolution mode for MS and UltraScan mode for
MS/MS acquisition. Possible putative structures were proposed based on MS/MS fragmentation pattern. The spectra (m/z 50-1000) were recorded every 2 s.

**Statistical analysis**

The data obtained were analyzed using the softwares GraphPad Prism version 6.01® and Class-VP control version 6.14. The IC$_{50}$ and % AA values were calculated by linear regression and expressed as mean values ± standard deviation. Values of p < 0.05 were considered to be significantly different.
Table S1. Total phenolics (TP), total flavonoids (TF), and antioxidant activity of ethanol extracts obtained from different parts of *P. cincinnata*.

The IC<sub>50</sub> values were obtained by interpolation from linear regression analysis with 95% of confidence level. IC<sub>50</sub> is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%. Values are given as mean ± SD (n = 3). *n.d = not determined. All data were analyzed using the unpaired student t test, where a (vs. fruit peel), b (vs. flower), c (vs. seed), d (vs. BHA), e (vs. BHT) and f (leaf) indicate p<0.05.

<table>
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<tr>
<th>Sample</th>
<th>TP (mg GAE/g)</th>
<th>TF (mg QE/g)</th>
<th>β-Carotene (%AA)</th>
<th>DPPH (IC&lt;sub&gt;50&lt;/sub&gt;, µg/ml)</th>
<th>ABTS (IC&lt;sub&gt;50&lt;/sub&gt;, µg/ml)</th>
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<td>Fruit peel</td>
<td>19.53 ± 1.41</td>
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<td>Leaf</td>
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<td>1.42 ± 0.01&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>63.77 ± 0.03&lt;sup&gt;c,d,e&lt;/sup&gt;</td>
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Figure S19. MS/MS spectra of methoxy-orientin-O-hexoside (14) in ESI positive and negative modes.
References


