Ethanol Extract of *Passiflora cincinnata* Seeds Posses Antidiabetic, Antiglycant, and Antioxidant Activities *in vitro*

Flávia Adaís Rocha dos Santos¹, Elaine Luiza Santos Soares de Mendonça¹, Felipe Cabral da Silva¹, Jadriane de Almeida Xavier⁴*, J. P. Jose Merlin², Marília Oliveira Fonseca Goulart¹, H. P. Vasantha Rupasinghe²*

¹Institute of Chemistry and Biotechnology, Federal University of Alagoas; Alagoas, Maceió, Brazil; ²Department of Plant, Food, and Environmental Sciences, Faculty of Agriculture, Dalhousie University; Truro, New Scotland, Canada

This work aimed to investigate the antidiabetic, antiglycation, and antioxidant potentials of the ethanol extract of seeds of *Passiflora cincinnata* (EPCIN) *in vitro*. The EPCIN was evaluated from the following assays: total phenolic content (TPC – mg of Gallic Acid Equivalents (GAE)/g o f dry extract), Radical Scavenging Assays (DPPH•, HOCl-scavenging assay), and protective effects against glycation of bovine serum albumin (BSA) with methylglyoxal (MGO) or a mixture of reducing sugars, fructose and glucose, as well as the potential for MGO capture by derivatization with ortho-phenylenediamine (OPD). To evaluate the antidiabetic activity of EPCIN in vitro, we used the assays of enzymes α-amylase (4 U/mL), α-glucosidase (0.25 U/mL), and dipeptidyl peptidase-4 (DPP-4 – 3.125 mU). The cell viability of EPCIN-pretreated normal human bronchial epithelial cells (BEAS-2B) alone or in the presence of the carcinogen 4-[(acetoxymethyl)nitrosamine]-1-(3-pyridyl)-1-butane (NNKOAc) was measured using MTS assay. Quercetin (QCT), piceatannol (PIC), acarbose (ACB), and sitagliptin (STG) were used for comparison purposes. EPCIN had an average of TPC 157.0 ± 1.5 mg of GAE/g of dry extract, exhibited IC50 for DPPH• and HOCl of 11.9 ± 1.8 μg/mL and 6.9 ± 0.9 μg/mL, respectively. EPCIN and AMG inhibited the formation of advanced glycation end-products (AGE) with IC50 of 574 ± 8.7 and 31.9 ± 2.7 μg/mL for the initial stage and 542.6 ± 2.7 and 52.8 ± 8.1 μg/mL for the intermediate stage of glycation, respectively. EPCIN showed IC50 for α-amylase and α-glucosidase of 218.2 ± 15.9 μg/mL (p<0,05) and 242.0 ± 25 μg/mL (p<0,05), respectively. EPCIN did not show cytotoxicity for BEAS-2B cells at 10 and 50 μg/mL concentrations. In addition, it was also able to protect cultured human cells from oxidative stress caused by the NNKOAc at 100 μM. The *in vitro* evidence of the potential antioxidant, antiglycant, and antidiabetic effects warrants further investigation of the antidiabetic potential of *Passiflora cincinnata* seeds.

Keywords: Passion Fruit. Phytochemicals. Oxidative Stress. Type 2 Diabetes. Cytotoxicity.

Introduction

Since ancient times, phytochemicals have been used worldwide as alternative/complementary therapies to treat several pathologies due to the presence of biologically active compounds - secondary metabolites, sometimes biosynthesized, in plants, after some chemical, physical or biological injuries [1]. Among them, polyphenols have been highlighted, mainly regarding their antioxidant activity. These compounds, among other mechanisms of molecular action, have decreased NF-kB expression pathways, attenuating the excessive production of reactive oxygen species (ROS) and decreasing lipoperoxidation and damage of biomacromolecules. Additionally, they stimulate the NRF2 signaling pathway, which leads to an increase of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) [2].

Although polyphenols have well-established antioxidant activity, a particular group of non-flavonoid compounds, the stilbenes, has increased interest in the scientific community. Among the stilbenes - resveratrol, piceatannol (PIC), hesperidin, hesperetin, pterostilbene, polidatin, stilbestrol, and pinosylvine have shown multiple spectra of biological activities, with a special emphasis in PIC. PIC’s relevance is probably related to the presence of the catechol moiety.

Received on 17 September 2022; revised 24 November 2022. Address for correspondence: Jadriane Almeida Xavier. Institute of Chemistry and Biotechnology, Federal University of Alagoas, Maceio, Zip code: 57072-970, Brazil. E-mail: jadrianexavier@iqh.ufal.br. H. P. Vasantha Rupasinghe. Department of Plant, Food, and Environmental Sciences, Faculty of Agriculture, Dalhousie University, 50 Pictou Road, Truro, NS B2N 5E3, Canada, e-mail: vrupasinghe@dal.ca

J Bioeng. Tech. Health 2022;5(4):224-231 © 2022 by SENAI CIMATEC. All rights reserved.
In addition, researchers have identified the presence of PIC in the genus *Passiflora*, which comprises more than 500 species, found predominantly in tropical and subtropical regions of the world [6-8].

In northeastern Brazil, the species *Passiflora cincinnata*, popularly known as “wild passion fruit” or “passion fruit of the Caatinga”, has been less explored compared to other species because it has a specific sensory analysis, with unusual organoleptic characteristics, mainly regarding the pulp of its fruits, being considered to have a stronger flavor than the other species. However, it is worth mentioning the natural beauty of its flowers and its resistance to pests and hydric deficit [8]. In addition, the literature is scarce regarding their chemical composition and biological profile.

In this context, the objective of the present work was to evaluate the *in vitro* antioxidant, antiglycemic, and antidiabetic activities of the ethanol extract of the seeds of *Passiflora cincinnata* (EPCIN). To the best of our knowledge, no study has been conducted so far reported concerning the seeds of Brazilian *P. cincinnata* as an antidiabetic agent through the association of α-glucosidase and DPP-4 inhibition mechanisms, not even as an antiglycemic in the initial and intermediate stages of glycation. In addition, the cytotoxicity of EPCIN was studied in BEAS-2B cell lines, with and without the oxidative stress-causative carcinogen NNKOAc.

**Materials and Methods**

**Preparation of the Ethanol Extracts of *Passiflora cincinnata* Seeds**

We collected the unripe fruits of *Passiflora cincinnata* in November 2019 in a native forest in Olho d’Água do Casado, Alagoas. The seeds were separated from the fruit pulp, washed with distilled water, and dried in an oven at 50 °C for 48 h. The ethanol extract of *Passiflora cincinnata* seeds (EPCIN) was obtained according to the method described by Xavier and colleagues [6].

**Total Phenolic Content (TPC)**

The TPC was estimated using the Folin-Ciocalteu (FC) method, as described by Cicco [9], with some modifications. Briefly, 180 μL of deionized water, 300 μL of FC reagent, and 2.4 mL of 5% sodium carbonate (w/v) were added to 120 μL of diluted samples. After incubation in a water bath at 40 °C in the dark for 20 min, the absorbance of the resulting mixture was measured at 760 nm using a UV–Vis spectrophotometer (Agilent 8453). The results were expressed as milligrams of gallic acid equivalents (mg GAE) per gram of dry extract.

**Radical Scavenging Assay DPPH•**

The antioxidant activity of EPCIN was determined using the DPPH• method [10]. Briefly, aliquots of 0.30 mL of sample dissolved in ethanol (5–25 μg/mL) were mixed with 2.70 mL of DPPH• solution (40 μg/mL in methanol). After incubation in the dark for 30 min, the absorbance was read at 516 nm, using a UV–Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Results were expressed as the half-maximal inhibitory concentration (IC50) in μg/mL.

**Hypochlorous Acid (HOCl) Elimination Assay**

The HOCl elimination activity of EPCIN was determined using the previously described method [11]. Briefly, a new HOCl solution (30 μM) was prepared in 100 mM phosphate buffer (pH 7.4). Then, in a 96-well plate, the following reagents were added at the indicated final concentrations: buffer solution (100 mM, pH 7.4), EPCIN (1, 5, 10, 25, 50, 100, 200, and 300 μg/mL), dihydrorodamine (DHR) (5 μM) and HOCl (5 μM). QCT and PIC were used for comparison purposes. The fluorescence measurements were performed in a microplate reader (Infinite® 200 PRO, TECAN, Männedorf, Switzerland), at 37 °C, at wavelengths of 505 ± 10 nm and 530 ± 10 nm, for excitation and emission, respectively. The results were expressed as IC50 in μg/mL.
Measurement of Intracellular ROS Level

The generation of intracellular ROS in BEAS-2B after treatments with EPCIN was measured as described previously [12]. The cells, pre-treated with EPCIN for 3 h, were exposed to the carcinogen NNKOAc, for 3 h or alone in different experimental groups. DMSO (0.5%) was utilized as a control. ROS was quantified after treatments using the 2',7'-dichlorofluorescein diacetate dye (DCFH-DA) at a final concentration of 5 μM followed by 40 min incubation at dark. Fluorescence measurements were performed in a microplate reader (Infinite® 200 PRO, TECAN, Männedorf, Switzerland), at 37 °C, at wavelengths of 485 nm and 535 nm, for excitation and emission, respectively.

Cell Cultures and Cell Viability Assay

BEAS-2B cells were cultured with LHC-9 media at 37 °C in an incubator with CO₂ (5%). Culture flasks (polystyrene T75) were pre-coated with a mixture of fibronectin (0.01 mg/mL), bovine collagen type I (0.03 mg/mL), and bovine serum albumin (0.01 mg/mL) dissolved in the LHC-9 medium overnight. The MTS assay was used to perform the cell viability assay [13]. For 24 hours, different concentrations of EPCIN were used to examine the viability of the cells. Next, an MTS reagent was applied, and the cells were incubated for 3 hours in the dark. A microplate reader (Infinite® 200 PRO, TECAN, Männedorf, Switzerland) was used, and the absorbance was recorded at 490 nm.

α-Amylase Inhibition Assay

The α-amylase inhibition assay was performed using a previously described method [14] with minor modifications. EPCIN and PIC, in different concentrations, were prepared in 0.01 M potassium phosphate buffer (pH 6.8) containing 2.5% ethanol. All other solutions were prepared in buffer only. The reaction system consisted of adding the samples, α-amylase from porcine pancreas (4 U/mL), and after 10 min of incubation at 37 °C, the substrate 2-chloro-4-nitrophenyl-α-D-maltotrioside (5 mM) was added and then incubated for more 30 min. The reaction was terminated by adding a trisodium phosphate solution of pH 11 (1%, w/v). The amount of 2-chloro-4-nitrophenol released was measured spectrophotometrically at 405 nm in a microplate reader (Infinite® 200 PRO, TECAN, Männedorf, Switzerland).

α-Glucosidase Inhibition Assay

The α-glucosidase inhibition assay was performed using a previously described method [15] with minor modifications. EPCIN and PIC, in different concentrations, were prepared in 0.01 M potassium phosphate buffer (pH 6.8) containing 2.5% ethanol. All other solutions were formulated in buffer only. The reaction system consisted of adding a sample, alpha-glucosidase enzyme (0.25 U/mL), and 4-nitrophenyl-α-D-glucopyranoside (5 mM) substrate. The mixture was then incubated at 37 °C for 15 min, and the reaction was stopped by adding sodium carbonate solution (0.2 M). Acarbose (ACB) was used for comparison purposes. The amount of p-nitrophenol (PNP) released was measured spectrophotometrically at 405 nm in a microplate reader (Infinite® 200 PRO, TECAN, Männedorf, Switzerland).

Dipeptidyl Dipeptidase Enzyme (DPP-4) Inhibition Assay

The DPP-4 inhibition assay was performed according to an established method [14]. STG, a standard DPP-4 inhibitor, was used to compare the effectiveness of PESE and PIC. Briefly, In a 96-well plate, the following reagents were added at the indicated final concentrations: the sample at different concentrations, DPP-4 human recombinant enzyme solution (3.125 μU), and Gly-Pro-7-amido-4-methylcoumarin hydrobromide substrate (2.5 μM). The reaction mixture was incubated for
30 min in the dark at 37 °C. Then, the fluorescent product was recorded using the microplate reader (Infinite® 200 PRO, TECAN, Männedorf, Switzerland) at wavelengths of 350 nm and 450 nm for excitation and emission, respectively.

Inhibition of Advanced Glycation End Products (AGE) Formation

The formation of AGEs was evaluated in the initial (BSA, glucose, and fructose) and final stages of glycation (system containing BSA and MGO). This assay was based on previous methods [6]. Aminoguanidine (AMG) was used as a positive control. We used a mixture of fructose and glucose-reducing sugars to evaluate the initial stage. The reaction system consisted of the addition of adequate volumes of EPCIN or AMG solutions (at different concentrations), D-fructose (200 mM), D-glucose (200 mM), and bovine serum albumin (BSA, 3 mg/mL) in a total volume of 1.5 mL. EPCIN was dissolved in ethanol. All other solutions were dissolved in 0.05 M potassium phosphate buffer (pH 7.4) containing NaCl (100 mM) and NaN3 (0.02% w/v). The reaction system was incubated in the dark at 37 °C for 7 days with constant stirring. For the evaluation of the intermediate stage, the same procedure was followed; however, MGO was used instead of the mixture of reducing sugars. The fluorescent AGEs were measured using a microplate reader (Infinite® 200 PRO, TECAN, Männedorf, Switzerland) at λex= 360 and λem = 440 nm. AMG, a known AGE formation inhibitor, was used as a standard.

Methylglyoxal Capture Potential

MGO capture was evaluated by derivatization with o-phenylenediamine (OPD) using a previously described procedure [6]. The reaction system was composed of MGO, phosphate buffer (negative control), or EPCIN (2 mg/mL) or AMG (2 mM, positive control) incubated at 37 °C for 1 h. After incubation, the OPD solution (4 mM) was added, and the tubes were kept for 30 min for the derivatization reaction between MGO and OPD to complete. Then, the formation of the derived product 2-methylquinoxyline (2-MQ) was monitored by high-performance liquid chromatography (HPLC) and detected at 315 nm. Formic acid (0.1% v/v, solvent A) and methanol (solvent B) were used as a mobile phase, with a flow rate of 1.0 mL/min. The linear gradient for elution was starting at 5% of solvent B, 0–3 min, 5 to 50% B; 3–16 min, isocratic in 50% B; 16–17 min, 50–90% B; 17–19 min, isocratic in 90% B and 19–19.5 min, 90–5% B. The percentage of MGO capture was calculated concerning the peak area corresponding to the 2-MQ product in the systems with and without treatment.

Statistical Analysis

All analyses were performed in triplicate (n = 3) using Graph-Pad Prism software (GraphPad Software Inc., San Diego, CA, USA). Data were presented as the mean ± standard deviation (SD), and analyses of variance, one-way ANOVA, followed by Tukey test, and p ≤ 0.05 were considered significant between experimental groups.

Results and Discussion

The EPCIN yield was 1.9% from 12 g of dried and ground seeds. Extraction of extract yields is crucial in assessing the cost and benefits associated with potential nutraceutical development. Xavier and colleagues [7], when studying the ethanol extraction of the seeds of Passiflora edulis, obtained 4 times higher yields (7.9%). The degree of maturation of the fruit seems to be a factor that could influence the composition of the seeds.

Table 1 shows the levels of total phenols and antioxidant activity of EPCIN compared to QCT. Particularly concerning the DPPH• and HOCl elimination assays, we observed that although the antioxidant capacity of EPCIN was lower than that presented by the QCT (control), the IC50 values are close to those proposed by the standard.
Table 1. Total phenol content (TPC) and antioxidant potential of the ethanolic extract of seeds of Passiflora cincinnata in comparison to quercetin (QCT).

<table>
<thead>
<tr>
<th>Sample</th>
<th>TPC dry extract of EAG (mg/g)</th>
<th>DPPH• IC₅₀ (μg/mL)</th>
<th>HOCl IC₅₀ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPC</td>
<td>157.0 ± 1.5</td>
<td>11.9 ± 1.8</td>
<td>6.9 ± 0.9</td>
</tr>
<tr>
<td>QCT</td>
<td>-</td>
<td>5.5 ± 0.6 (18.1)</td>
<td>2.2 ± 0.3 (7.2)</td>
</tr>
</tbody>
</table>

EPC: ethanolic extract of seeds of Passiflora cincinnata; QCT: Quercetin; DPPH•: 1,1-Diphenyl-2-Picrylhydrazyl; GAE: Gallic Acid Equivalents. Results in Parentheses (μM).

Table 2. In vitro antidiabetic activity of ethanol extracts from seeds of Passiflora cincinnata and Piceatannol (PIC), in comparison with acarbose (ACB) and sitagliptin.

<table>
<thead>
<tr>
<th>Sample</th>
<th>α- Amylase IC₅₀ (μg/mL)</th>
<th>α- Glucosidase IC₅₀ (μg/mL)</th>
<th>DPP-4 IC₅₀ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPCIN</td>
<td>218.2 ± 15.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>242.0 ± 25.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>PIC</td>
<td>85.9 ± 1.8 (339.4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.5 ± 8.3 (88.0)</td>
<td>1,300.3 ± 20.0 (5,323.9)</td>
</tr>
<tr>
<td>ACB</td>
<td>0.6 ± 0.1 (0.9)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>251.6 ± 4.5 (389.7)</td>
<td>-</td>
</tr>
<tr>
<td>STG</td>
<td>-</td>
<td>-</td>
<td>0.005 ± 0.0005 (0.01)</td>
</tr>
</tbody>
</table>

EPC: ethanol extract of seeds of Passiflora cincinnata; PIC: Piceatannol; ACB: acarbose; STG: sitagliptin; NA, it was not possible to calculate the IC₅₀. Data in parentheses (μM).

Santos and colleagues [6], when evaluating the antioxidant activity of the ethanolic extract of Passiflora edulis seeds, observed a TPC of 227 ± 3.9 mg of GAE/g, approximately 2x that observed in the present study, which was reflected in the antioxidant activity in scavenging DPPH• (20.4 ± 2.1 μg/mL), with lower activity than EPCIN; however, the antioxidant activity, obtained through the ability to eliminate HOCl, of Passiflora edulis (1.7 ± 0.3 μg/mL) was approximately 4 times superior to that of EPCIN. The results suggest that EPCIN may be an essential source of phenolic compounds and that EPCIN has antioxidant activity, decreasing the formation of ROS and other metabolites.

The antidiabetic potential of EPCIN can be analyzed through the ability to inhibit α-amylase and α-glucosidase, in addition to the DPP-4 enzyme. This study used acarbose (ACB) and sitagliptin (STG) for comparison purposes. EPCIN showed a concentration-dependent activity against α-amylase and α-glucosidase inhibition, and the IC₅₀ values are shown in Table 2. EPCIN and PIC, one of the compounds present in the seeds of Passiflora cincinnata, showed higher potential towards α-glucosidase than α-amylase. We noted that EPCIN showed toward α-glucosidase an IC₅₀ value equivalent to that presented by ACB. However, it caused a low α-amylase inhibition. On the other hand, PIC showed excellent α-glucosidase inhibition potential, with IC₅₀ about 4.5 times lower than ACB, suggesting that PIC plays an essential role in the antidiabetic potential of EPCIN.

BSA glycation was performed in the presence and absence of EPCIN. In the initial stage, IC₅₀ values presented by EPCIN and AMG were 574.4 ± 8.7 and 31.9 ± 2.7 μg/mL, respectively. For the intermediate stage, where BSA was treated with MGO, the IC₅₀ values were 542.6 ± 2.7 and 52.8 ± 8.1 μg/mL, respectively. EPCIN inhibited the formation of fluorescent AGEs; however, AMG showed higher activity when compared to EPCIN. Gomes and colleagues [16] evaluated...
the hydroethanolic extract of *P. cincinnata* aerial parts. They observed a 68.17% inhibition of AGEs formation for a concentration of 10 mg/mL, while Santos and colleagues [6] found an IC₅₀ of 360 μg/mL for the ethanol extract of *P. edulis* seeds.

When investigating the MGO capture potential in the reaction system, after 1 h incubation, EPCIN could capture 47.9% of the MGO present in the solution, while AMG presented 89.2% of MGO capture. The results show that EPCIN may act in the intermediate step of the glycation reaction, stabilizing the dicarbonyl species and thus inhibiting the response with amino groups of biomolecules through the formation of AGEs [17].

It has been recognized that when the cell viability is greater than 90%, the sample is considered non-toxic at a given dose. Figure 1A shows a dose-responsive decline in cultured BEAS-2B cells with increasing concentrations of EPCIN (p ≤ 0.05). EPCIN did not alter cell viability up to a concentration of 50 μg/mL. Xavier and colleagues [7], when testing the cell viability (human placental HTR-8/SVneo) in the presence of the ethanol extract of *Passiflora edulis* seed, observed that there was no reduction in cell viability, even at the concentration used of 100 μg/mL.

Additionally, Figure 1B shows the effects of EPCIN on the BEAS-2B cells exposed to NNKOAc (100 μM), evidencing that the carcinogen was able to stimulate the production of ROS and that the pre-treatment with EPCIN was able to protect cultured BEAS-2B cells against oxidative stress. EPCIN was also added to cells in the absence of NNKOAc, and again no increase in ROS production was observed when compared to the control.

**Acknowledgments**

The authors are grateful for the financial support provided by Global Affairs Canada Student...
Exchange Program and the International Office of Dalhousie University and by the Brazilian research funding agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support (435704/2018-4) and fellowships (MOFG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brazil (CAPES)—Finance Code 001, CAPES/RENORBIO/PROAP (23038.011373/2017-31), INCT-Bioanalítica (process 465389/2014-7), and Fundação de Apoio à Pesquisa de Alagoas (FAPEAL).

Conclusion

EPCIN showed potential antioxidant, antiglycanc, and antidiabetic effects. Furthermore, EPCIN was shown to be non-toxic to BEAS-2B cells at the concentration effective to exhibit antidiabetic and antioxidant activities, in addition to protecting the BEAS-2B cells from oxidative stress caused by NNKOAc. Based on this study, further studies need to be conducted using experimental animal models to assess the extract’s safety and confirm its antioxidant, antiglycanc, and antidiabetic activities. In conclusion, EPCIN has the potential to be used as an alternative therapy for managing type 2 diabetes mellitus.

References


