Passiflora edulis leaf extract: evidence of antidiabetic and antiplatelet effects in rats

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Abstract

Different *Passiflora* species have been appointed as a promising herbal medicine due to antioxidant properties; however, their effect on oxidative process induced by diabetes is still controversial. We aimed to evaluate effects of hydroethanolic extract 70 % from *P. edulis* leaf on biochemical blood markers, *collagen glycation*, production of oxidant species and platelet aggregation in diabetic rats. The phytochemical analysis of the extract was performed by dereplication using liquid chromatography coupled to the Photodiode Array Detector and Mass Spectrometer detector. Male Wistar rats were assigned to the control group and groups treated with alloxan (150 mg/kg) intraperitoneally, extract (200 mg/Kg/day, for 90 days.) and combination of alloxan and extract. The phytochemical analysis suggested the presence of flavonoids C-glycosides in the extract. The diabetic animals treated with the extract presented improvement in glycaemic control, reduced glycation collagen, levels of non-HDL cholesterol, total cholesterol and creatinine, production of oxidant species and aggregation in platelet in relation to diabetic animals non-treated. Our results showed that *P. edulis* leaf extract presents a health benefit to the diabetic state, preventing the appearance of its complications. Its effect can be associated with flavonoids, among which is the flavonoid C-glycoside isoorientin.

Key words

*Passiflora edulis*, diabetes mellitus, platelet aggregation, biochemical markers, quantification, isoorientin
Introduction

Diabetes mellitus and its complications constitute a serious public health problem due to high incidence, morbidity and mortality rates.\textsuperscript{1, 2} The platelet hyperactivation and hyperaggregation, frequently seen in diabetic patients, play one important role in the development of complications that lead to vascular dysfunction induced by diabetes mellitus. It's known that hyperglycaemia lead to the formation of advanced glycation end products (AGEs) as well as oxidant species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) contribute to the alteration or loss of cellular function.\textsuperscript{3}

Currently, there are several types of glucose-lowering drugs that exert anti-diabetic effects but, due to the high cost of commercial drugs, in addition to the observation of a large number of drug interactions and adverse effect.\textsuperscript{4} Thus much attention has been focused on studies of medicinal plants for treatment of diabetes mellitus and its complications.

In this way, many studies considering \textit{P. edulis} as a promising herbal medicine and experimental studies have suggested that leaf extracts of different \textit{Passiflora} species may have hypoglycaemic or antioxidant properties.\textsuperscript{4, 7} These properties have been attributed mainly to the presence of phenolic compounds, among which are the flavonoid \textit{C}-glycosides.

However, it is important to note that there are divergences in the effects of extracts obtained from the \textit{Passiflora edulis} in diabetic animals and humans. Part of this controversy could be attributed to several factors such as, different experimental model, different analyzed markers, different protocols for the preparation the extracts and consequently different chemical composition of them.\textsuperscript{9-12} Moreover, most studies evaluating the properties of \textit{Passiflora edulis} extract on diabetic complications have been performed with extract obtained from seed or peel of the fruit. There are few studies about the effects of extract
Passiflora edulis leaves in diabetic animals. Therefore, scientific information regarding the use of passion fruit leaves in diabetes treatment demands further investigation.

Thus, the aim of this study was to quantify the major flavonoid of the P. edulis leaf extract and evaluate its effects on glycemic control, formation of advanced glycated end (AGEs) and platelet activation, particularly aggregation and oxidant species production as well as renal function markers and lipid profile, in diabetic rats.

Materials and Methods

Plant material and extract preparation
The samples, classified as P. edulis Sims species, were collected in the city of Alfenas, Minas Gerais, Brazil (21° 27’ 33” S; 46° 01’ 59” W; altitude 789 m), and identified by Dr. Douglas Carvalho, Federal University of Lavras. Voucher number 22356 was obtained by the Federal University of Lavras, Department of Biology, ESAL herbarium. The extract of P. edulis Sims leaves was prepared according to reported method Rudnick et al.¹³). The P. edulis Sims leaves were dried at 40 °C in an air circulation chamber where their weight was measured in shifts from 5 h until constant weight was obtained. After drying, the plant material passed through a rough division followed by spraying in a Wiley mill. The dry extract was obtained by 70% ethanol maceration in the proportion of one part powder to 10 parts ethanol solution and then it was evaporated under reduced pressure at 46.4 ± 1.9 °C; the organic solvent was removed and the extract dried in a lyophilizer.

Chemical characterization
The LC-ESI-IT-MS analysis was performed in a Thermo Scientific® LCQ Fleet mass spectrometer. UPLC-ESI-MS analysis was used to quantify the isoorientin in the extract and
the compounds present in the extract were identified by HPLC-ESI-IT-MS, on an ACQUITY™ UPLC system (Waters Corp., Milford, MA, USA) with a conditioned auto-
sampler. Validation of the method was performed according to the guidelines for bioanalytical methods.14)

The analysis was performed by online LC-ESI-IT-MS in a Thermo Scientific® LCQ Fleet mass spectrometer. The analytical column used for LC separation was a Kinetex® C18 (2.1 × 100 mm, 100 Å and 5 μm). The analysis was carried out with water containing 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B) added to 0.1% formic acid in an exploratory gradient starting with 10% to 100% B in 32 min at a flow rate of 1.0 mL/min. The sample was infused into the mass spectrometer from the HPLC system, where the sample was analysed online by ESI-MS in negative mode and an associated UV detector. The mass spectra data were obtained in the same Fleet LCQ mass spectrometer from Thermo Scientific®, with direct insertion of the sample device via continuous flow injection analysis (FIA). The sample was ionized with an ESI source and fragmentations were obtained in multiple stages (MSn) in an ion trap (IT)-type interface. The negative mode was chosen for the generation and analysis of all spectra. The experimental conditions were: capillary voltage of −35 V, spray voltage −5000 V, capillary temperature 350 °C, carrier gas N2 and flow 60 (arbitrary units). The track acquisition was m/z 100–2000 with two or more sweep events performed simultaneously in the spectrum.

Standard solution preparation for quantification analysis

Isoorientin (purity > 98%) was purchased from Sigma, dissolved in 1 mL of MeOH/H2O 8:2 (v/v) solution (1 mg/mL) and filtered through a 0.22 μm membrane of a nylon filter. An aliquot (10 μL) was diluted with MeOH/H2O 8:2 (v/v) up to a final volume
of 1 mL (100 ppm, solution A). Solution A was diluted to obtain solution concentrations of 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 ppm.

The analysis was performed on an ACQUITY™ UPLC system (Waters Corp., Milford, MA, USA) with a conditioned auto-sampler. The analytical column used was an ACQUITY™ UPLC XBridge C18 (2.1 × 50 mm, 2.5 µm column; Waters Corp., Milford, MA, USA). The analysis was carried out with water containing 0.1% formic acid (A) and methanol (B) at a flow rate of 0.4 mL/min. For quantification a gradient program was used as follows: 0–4 min, 47–100% B linear; 4–5 min, 100% B. The injection volume was 5 µL in quantification. Mass spectrometric detection was performed using a Waters ACQUITY™ Xevo TQD triple quadrupole tandem mass spectrometer (Waters Corp., Manchester, UK) equipped with an ESI source and ZSpray Probe operated in negative mode. The MS conditions were as follows: capillary temperature 150 °C; capillary voltage 3.5 kV; cone voltage 40 kV; desolvation temperature 300 °C; source gas flow: desolvation 600 L/h. The multiple reaction monitoring (MRM) mode was used for quantification and confirmation. All data were acquired and processed using MassLynx™ v4.1 software.

Method validation

Validation of the method was performed according to the guidelines for bioanalytical methods (US Food and Drug Administration, 2001). Validation parameters such as specificity, sensitivity, accuracy, precision, recovery, matrix effect, and stability were investigated.

The linear calibration curve of isoorientin was equivalent to 0.5, 1, 2, 4, 8, 16 and 32 µg/mL in methanol with three replicates for the intraday and interday assays. Calibration curves for the flavonoid were generated by plotting the ratio of the peak area (y) versus the concentration (x) of the standard by using 1/x-weighted least-square linear regression. The
concentration of the flavonoid was calculated by linear interpolation from the calibration curves. The limit of detection (LDQ) was determined as the signal equivalent to 3.3 times the value of the ratio of the linear coefficient (a) and the slope (b) \( (3.3 \times a/b) \) obtained by the equation of the analytical calibration curve. The limit of quantification (LOQ) was given as 10 times the value of this ratio. Matrix effects were determined by comparing the peak area of analyte (isorientin) in the samples of extract (A) with that of analyte standard solution (B). The matrix effect was considered insignificant when the ratio \( (A/B \times 100)\% \) of the analytes was between 85% and 115%. Recovery of the analytes was determined by comparing the peak area of analyte standard solution (8 ppm) after SPE with that of the analyte standard solution (8 ppm) before SPE which represented 100% recovery. The ratio after SPE/before SPE \( \times 100 \) was determined. Precision and accuracy were assessed on three consecutive days by using mixtures containing low, medium or high concentrations of the analyte standard solution (1, 25 and 50 ppm). Precision was expressed as the percentage relative standard deviation (RSD%), and accuracy was calculated using the following equation: \([\text{(mean measured concentration − spiked concentration)/(spiked concentration)}] \times 100\%\). Intraday and interday precision and accuracy were determined by repeated analysis of the analyte sample.

**Animals**

The study was conducted in accordance with the ethical principles for animal experimentation that have been adopted by the Brazilian College of Animal Experimentation, and the study was approved by the ethics committee on animal research at the Federal University of Alfenas (no. 398/2012). In total, 80 adult male (20 per group) Wistar rats \((\text{Rattus norvegicus})\) weighing 300 ± 25 g obtained from the Unifal-MG vivarium were used.
in this study. The rats were housed in a temperature-controlled room on a 12 h light/dark schedule with food and water available ad libitum.

**Animal experimental protocol**

The animals were allocated into four groups: control (C) – non-diabetic animals that were not treated with dry extract; diabetics (D) – alloxan-treated animals; extract (E) – non-diabetic animals treated with *P. edulis* leaf dry extract; diabetic extract (DE) – alloxan-treated animals also treated with *P. edulis* leaf dry extract. Alloxan (2% solution) was administered intraperitoneally (150 mg/kg) according to Martins et al. In this study, type 1 diabetic rats were defined as those with glycaemia over 250 mg/dL after 7 days of alloxan injection. Blood glucose was monitored regularly using test strips with blood taken from the tail throughout the experiment every 15 days after the diabetic rats were defined.

The dry extract was diluted in water and administered to the animals (200mg/Kg) by gavage for 13 weeks after diabetes induction. The control group received 0.5 mL water. Animal body weight was recorded on a weekly basis. Daily feed and water intake was checked three times a week during the treatment period. The animals were anaesthetized on the 90th day, and blood was collected by puncturing the abdominal aorta.

The blood collected and the determinations of creatinine, urea, glucose, fructosamine, total cholesterol, HDL cholesterol and triglycerides were performed in serum, using automated analyser LabMax Plenno (Labtest).

Renal function was evaluated through the determination of creatinine and urea in serum. The creatinine concentration was determined by modified Jaffe method. Urea levels and blood glucose were evaluated using an enzymatic method, fructosamine levels by a kinetic method and the serum lipid profile was assessed through the determination of total cholesterol, HDL cholesterol, triglycerides and non-HDL cholesterol (LDL and VLDL) using
enzymatic methods. The glycated haemoglobin (HbA1C) levels were determined in blood by HPLC on Bio-Rad equipment.\textsuperscript{17)}

Platelet aggregation was assessed in aliquots of plasma that were rich in platelets, obtained from treated animals according to Baldissera-Jr’s method,\textsuperscript{18)} using 1 μM adenosine diphosphate as an agonist in a dual-channel aggregometer (Qualiterm) standardized at 37 °C.

The production of oxidizing species in platelets from diabetic and non-diabetic rats in the presence and absence of PMA was evaluated by chemiluminescence, according to Martins\textsuperscript{15)} with modifications. Complete systems (diabetic, diabetic extract, extract control and negative control) contained: platelets (1 × 10\textsuperscript{8} cells/test), luminol (1 mM) and PMA (16 ng/assay). The results were estimated by the integrated area of light emission.

The collagen obtained from tendons of rats was extracted according with Maria and Wada.\textsuperscript{19)} The fibers were dried at room temperature for 24 hours, weighed and rinsed in 70% ethanol. The collagen was solubilized in acetic acid (0.5 M) under stirring for 48 hours at 4 °C. Then the protein was dialyzed in Spectra\textregistered dialysis membrane with a capacity vol / 3.2mL and opening 12-14000 KDa against sterile distilled water at 2 - 8 °C under stirring for 48 hours. A fluorescence assay was used to check the modification of content advanced glycated end (AGEs) in collagen.\textsuperscript{20)}

**Statistical analysis**

The data found for each variable were submitted to analysis of variance. Multiple comparisons between the averages of the different treatments were performed using the Tukey test; P values > 0.05 were significant. Sisvar program version 5.3 was used.
Results and Discussion

Analysis of *P. edulis* extract showed the presence of several flavonoids, of which four were flavonoid C-heterosidglycosides (Table 1). Using an internal standard, we could confirm the presence of 3.9% isoorientin in the *P. edulis* extract.

As expected, fasting glucose was significantly higher in group D animals than in group C and E animals, demonstrating that the diabetes-inducing protocol used in this study was effective in inducing a preclinical type 1 diabetes model in rats. Moreover, there was a reduction in weight gain in diabetic animals compared to non-diabetics animals, but there was no difference in weight gain between D and DE groups.

In diabetic animals, the *P. edulis* extract showed a significant reduction of glucose, creatinine, total cholesterol and non-HDL cholesterol in serum as well as blood HbA1C (Table 2) and AGEs in collagen (Fig. 1). These results showed that *P. edulis* extract was able to reduce glycemic levels, contributing to improvement glycemic control during the 90 days of treatment.

Several studies have shown that different flavonoids such as apigenin and luteolin glycoside derivates, kaempferol, kaempferitrin, rhamnopyranoside and kaempferol-3-neohesperidoside are able to reduce the glucose levels in fasted Wistar rats 2 h after treatment.21,22) It is known that polyphenols can not only reduce the absorption of sugars but also increase glucose uptake by skeletal muscle and adipose tissue, contributing to the reduction of blood glucose levels.23-25)

The inhibition of protein glycation can be facilitated both by improved glycaemic control and by the presence of compounds with antioxidant potential in the extracts evaluated. Martins et al15) demonstrated the antioxidant properties of *Passiflora edulis* leaf ethanolic extract in vitro.
Furthermore, studies have investigated the structure activity of flavonoids such as their antiglycation properties.\textsuperscript{26} This study showed that flavonoids with an OH group at C-3’, 4’, 5 and 7 have increased protein glycation inhibitory ability.\textsuperscript{27} Phytochemical analysis of the \textit{P. edulis} extract using HPLC-ESI-IT-MS confirmed the presence of the flavonoid isoorientin which has an OH group at C-3’, 5 and 7, and suggested the presence of several flavonoids with an OH group at C-3’, 4’, 5 and 7.

One of the major causes of renal disease is the elevation of levels of protein glycation.\textsuperscript{28} Our results demonstrated that it was possible to prevent renal dysfunction in diabetic animals treated with \textit{P. edulis} extract (Table 2). This effect could be attributed to improved glycaemic control and decreased protein glycation in these animals.

It was observed that diabetic animals presented lipids concentrations significantly higher than control group. The \textit{P. edulis} dry extract administration prevented the increase of total cholesterol and non-HDL cholesterol levels (Table 2). Considering the effects of \textit{P. edulis} extract glycemic control, as observed in this study, the effects of the extract on the non-HDL cholesterol levels may be associated with improved glycemic control\textsuperscript{29,30} as well as direct effect of phenolic compounds in synthesis or metabolism of LDL cholesterol.\textsuperscript{31,32} These findings are according to other studies that related anti-diabetic activities of others compunds.\textsuperscript{29,33} 

Moreover, it is know that the hyperglycemia prolonged may increase production of reactive species oxygen (ROS) intensifying lipid peroxidation and protein oxidation reactions and changes cellular function, such as in platelets. Thus, the increase of diabetes mellitus-induced platelet aggregation has been demonstrated by different authors.\textsuperscript{25-29} 

Several studies have suggested that increased platelet aggregation might be associated with alterations in ROS production which could directly influence platelet aggregation activation as well as thrombus formation and vascular impairment.\textsuperscript{34-38}
Here we demonstrated, for the first time, that the treatment of diabetic animals with extract of *P. edulis* leaf reduced platelet aggregation and inhibited the production of these oxidizing species in platelets (Figs. 2, 3A and 3B). There was a difference in the kinetics of light emission between the diabetic and non-diabetic animals’ platelets. The reaction system without addition of PMA showed that basal oxidative metabolism is enabled in the platelets of diabetic animals. These effects may be associated with improved glycemic control in diabetic animals treated with the extract.

On the other hand, it should also be considered that most of the beneficial health effects of polyphenolic compounds, in particular flavonoids, are attributed to their ability to scavenge free radicals, chelate metals and activate antioxidant enzyme\(^39\). In this way, considering previous studies that demonstrated the antioxidant properties of *Passiflora edulis* extract,\(^12,13,15\) the effects of the extract on the platelet aggregation may be also associated with a possible antioxidant action of this extract, thus allowing the removal of the ROS from these species in the cellular environment.

It should be noted that some clinical studies using *Passiflora edulis* have been reported negative.\(^40,41\) However, in these studies were evaluated the effects of flour obtained from the peel of passion fruit whose chemical composition may be different from the extract analyzed in the present study.

Thus, this study demonstrates that the extract of passion fruit leaves was able to improve glycaemic control, renal function and lipid profile besides reducing aggregation and oxidizing species production in platelet of diabetic animals. All the results presented in this research show that extract of *Passiflora edulis* leaves could have a beneficial effect on health, especially to prevent chronic diabetes mellitus complications. These effects could be associated to antihyperglycaemic activity as well as antioxidant and antiglycation properties of the compounds present in this extract, particularly, the flavonoid glycosides\(^21-27\). Future
studies are needed to more specifically determine the mechanism of action of this extract. These studies are being developed by our research group.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001. The authors thank FAPEMIG for the Research Scholarships to Salles, BCC and Taniguthi, L.

Conflict of interest

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials.
References


Table 1. Identification of HPLC chromatographic peaks of species from genus *P. edulis* and data taken from HPL-PDA and HPLC-ESI-ITMS/MS analyses

<table>
<thead>
<tr>
<th>Nº</th>
<th>Molecule</th>
<th>[M–H]−</th>
<th>Fragment ions (m/z)</th>
<th>UV max (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Quercetin-3-O-β -glucopyranosyl-7-O-α-rhamnopyranoside and Quercetin-8-O-β-glucopyranosyl-6-O-α-rhamnopyranoside</td>
<td>609</td>
<td>519; 489; 462 447; 301</td>
<td>268, 330 sh, 335</td>
</tr>
<tr>
<td>2</td>
<td>6,8-di-C-β-glucupyranosylapigenin (vicenin-2)</td>
<td>593</td>
<td>575; 503; 473; 353</td>
<td>269, 330 sh, 335</td>
</tr>
<tr>
<td>3</td>
<td>Apigenin-6-C-arabinoside-8-C-glicoside</td>
<td>563</td>
<td>504; 473; 443</td>
<td>267, 290 sh, 335</td>
</tr>
<tr>
<td>4</td>
<td>Isorientin</td>
<td>447</td>
<td>327; 357</td>
<td>269, 330</td>
</tr>
<tr>
<td>5</td>
<td>Isovitexin</td>
<td>431</td>
<td>341; 311</td>
<td>268, 290sh, 328</td>
</tr>
<tr>
<td>6</td>
<td>4-O-beta-glucopyranosyl caffeic acid</td>
<td>325</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Fenolic Acid</td>
<td>195</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Fenolic acid derivated</td>
<td>133</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Effect of *P. edulis* leaf dry extract on biochemical markers in non-diabetic and diabetic animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Extract</th>
<th>Diabetic</th>
<th>Diabetic Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>171.9 ± 14.4 a</td>
<td>177.7 ± 39.1 a</td>
<td>486.8 ± 18.9 b</td>
<td>353.7 ± 99.6 c</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>11.5 ± 0.51 a</td>
<td>10.6 ± 0.99 a</td>
<td>21.2 ± 2.43 b</td>
<td>16.9 ± 2.60 c</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>92.7 ± 14.5 a</td>
<td>65.8 ± 15.5 a</td>
<td>114.1 ± 29.1 b</td>
<td>116.7 ± 47.8 b</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>114.1 ± 9.30 a</td>
<td>55.99 ± 10.0 c</td>
<td>265.5 ± 29.7 b</td>
<td>84.10 ± 8.67 c</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>47.39 ± 7.84 a</td>
<td>47.01 ± 7.27 a</td>
<td>68.98 ± 6.88 b</td>
<td>67.44 ± 9.83 b</td>
</tr>
<tr>
<td>N-HDL-C (mg/dl)</td>
<td>66.7 ± 8.21 a</td>
<td>8.98 ± 4.43 c</td>
<td>174.8 ± 50.6 b</td>
<td>16.65 ± 6.75 c</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.57 ± 0.05 a</td>
<td>0.51 ± 0.07 a</td>
<td>0.82 ± 0.09 b</td>
<td>0.50 ± 0.15 a</td>
</tr>
</tbody>
</table>

Data are expressed by mean ± S.E. statistics for 16-18 determinations per treatment. Different letters in same line indicate statistical significance, P<0.05. TG=Triglycerides, TC= Total Cholesterol, HDL-C= Cholesterol of high density lipoprotein, N-HDL-C= non-HDL Cholesterol
Fig. 1. Effect of *P. edulis* leaf dry extract on AGEs levels in type I collagen from tendons of non-diabetic and diabetic animals. The results represent the average ± standard deviation of 16-18 determinations per treatment. Different letters indicate statistical significance, p<0.05. C=control group; D= diabetic animals; E= *P. edulis* dry extract-treated non-diabetic animals; DE= *P. edulis* dry extract-treated diabetic animals.
Fig. 2. Effect of *P. edulis* leaf dry extract on platelet aggregation in non-diabetic and diabetic animals.

The results represent the average ± standard deviation of 6-8 determinations per treatment. Different letters indicate statistical significance, p<0.05. C=control group; D= diabetic animals; E= *P. edulis* dry extract-treated non-diabetic animals; DE= *P. edulis* dry extract-treated diabetic animals.
Fig. 3. Effect of *P. edulis* leaf dry extract on the kinetics of light emission in systems containing platelets from diabetic and non-diabetic rats. Data are expressed by mean ± S.E. statistics for 6-8 determinations per treatment. #P<0.05 vs. Control, *P<0.05 vs. Diabetic. C=control group; D=diabetic animals; E= *P. edulis* dry extract-treated non-diabetic animals; DE= *P. edulis* dry extract-treated diabetic animals. A) PMA stimulation; B) Without PMA stimulation.