HYPOGLYCEMIC EFFECT OF NEW PECTIN ISOLATED FROM PASSIFLORA GLANDULOSA CAV IN ALLOXAN-INDUCED DIABETIC MICE

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ABSTRACT

Several species from Passiflora has been used to treat Diabetes. This species are source of pectin, a dietary fiber with antidiabetic potential. Studies regarding the chemical characterization and biological effects of Passiflora glandulosa Cav are not reported in the literature. A new polysaccharide was extracted and characterized from the fresh peels of Passiflora glandulosa Cav, isolated pectin is characterized by a low degree of methoxylation and a high molecular weight, in addition to containing galacturonic acid in a carboxilate form in its chemical structure. Therefore, this study aimed to chemically characterize and evaluate in diabetic mice the hypoglycemic effects of pectin isolated from P. glandulosa. The isolation and chemical characterization of the pectin was performed via proton nuclear magnetic resonance (1H NMR) spectroscopy, Fourier transform infrared spectroscopy (FTIR), molecular weight, degree of methoxylation and physicochemical tests. Diabetes was induced in the animals with alloxan and the mice were treated with pectin for a period of 30 days. The results indicate that the studied pectin has a hypoglycemic action at doses of 200, 400 and 600 mg/kg bw in animals with alloxan-induced diabetes. The 200 mg/kg bw dose reduced the concentration of blood glucose and showed no renal toxicity and hepatotoxicity for animals.
This study demonstrates the potential use of plants from the Caatinga biome in the search for novel diabetes treatments.

**KEYWORDS:** Diabetes mellitus; Passiflora; Pectin; Blood glucose.

**INTRODUCTION**
Diabetes mellitus (DM) is a metabolic disorder with multiple etiologies that is characterized by excessive hyperglycemia, which causes alterations in the metabolism of carbohydrates, fats and proteins resulting from a dysfunction in the activity and/or secretion of insulin.\(^1\) When left untreated, DM can have consequences on organs and the basic senses, namely on the heart (heart attack), the kidney (nephropathy), vision (blindness) and peripheral circulation (blood clots), which can culminate in a stroke due to the wear suffered by the arteries and veins.\(^2,3\)

Worldwide, DM affects approximately 371 million people between 20 and 79 years old.\(^4\) The increased incidence of diabetes in the global health scenario has led to an increase in the number and scope of scientific studies focused on the composition and isolation of active substances in foods with therapeutic effects, including the prevention and treatment of chronic diseases.\(^5\) In this context, the appearance of nutraceuticals is particularly relevant; nutraceuticals are foods or parts of foods that can bring health benefits. These products include isolated nutrients, such as pectin (dietary fiber), in the form of capsules or in the concentrated food.\(^6,7,8\)

Passion fruit, which is the common name given to the genus Passiflora, is part of the biodiversity of the Brazilian flora. Studies have shown the hypoglycemic potential of species belonging to this genus. For example, yellow passion fruit peel flour (Passiflora edulis f. flavicarpa Deg) used in the diet of diabetic patients resulted in a decrease in blood glucose levels, an action attributed to the presence of an elevated amount of pectin\(^9,10\), and another study has demonstrated that the flour from yellow passion fruit is not toxic at a dose of 10 g/day.\(^11\) Studies with diabetic rats revealed the hypoglycemic activity of pectin isolated from the Passiflora edulis Sims fruit peel, indicating this treatment for type II DM.\(^12\) Thus, this study aimed to evaluate the hypoglycemic activity of pectin obtained from the peel of Passiflora glandulosa Cav in mice with alloxan-induced diabetes and to relate the chemical characterization of this polysaccharide to its biological effect.
MATERIALS AND METHODS

Plant material
The fruit of P. glandulosa Cav was collected in February 2013 in Nova Olinda, state of Ceará, Brazil (07.1024 S; 39.3537 W). The botanical identification of the species was performed by the Dárdano de Andrade Lima Herbarium, Regional University of Cariri (Universidade Regional do Cariri - URCA) and deposited under number 9983.

Isolation and purification of pectin from the ‘in natura’ peel of P. glandulosa Cav
The peel (383 g), seeds (104.47 g) and pulp (200.52 g) were separated from 1 kg of fresh P. glandulosa Cav fruit. The peels were cut and used for the extraction of pectin by applying a 0.25% ammonium oxalate solution (pH 4.6) for 1 hour at 80°C, followed by a solid:liquid extraction at a ratio of 20:1. The extract was filtered after the extraction. The filtered extracts were combined, and the pH was adjusted to 6 with a 0.1 N sodium hydroxide solution; the extracts were then concentrated to a volume of 2000 mL (10:1). Then, 6000 mL of 95% ethanol was added to the concentrate to precipitate the pectin. The pectin was then filtered with a Buchner funnel. The filtration residue was dissolved in 2000 mL of distilled water and filtered through a layer of celite. This operation was repeated until obtaining a transparent liquid that was then lyophilized.

Pectin characterization
The moisture content (%) was determined according to the methodology described in the Manual of Analytic Norms of the Institute Adolf Lutz, and the ash content was determined using the method described by the Association of Official Analytical Chemists. The total soluble protein content was determined according to the method of Bradford, and total carbohydrates were quantified using the method of Dubois et al.

Fourier transform infrared spectroscopy (FTIR) was performed in a Perkin-Elmer 1320 ® device (Waltham, Massachusetts, USA), in KBr pellets. FTIR was used to quantify the degree of methoxylation. Proton nuclear magnetic resonance (1H-NMR) spectroscopy was performed with an Avance DRX-500 Bruker Spectrometers® device (Silberstreifen, Rheinsetetten, Germany), operating at a 1H frequency of 500 MHz and an expansion of 1H-NMR. The thin-layer chromatography (TLC) analyses were performed on silica gel 60 F254 plates (Merck) with a mobile phase of glacial acetic acid:chloroform:distilled water (98:86:16 v/v), and the products were visualized with sulfuric orcinol. The molecular weight of the pectin was measured via gel permeation chromatography (GPC) on a Shimadzu device.
LC-10AD chromatogram with a RID-10A refractive index detector at 40°C with pullulan standards (Shodex Denko®) and a reference interval between $5.9 \times 10^3$ and $7.88 \times 10^5$ g/mol.

**Experimental animals**

Sixty female Swiss mice (Mus musculus), aged between 8 and 12 weeks (25.0-30.0 g), were obtained from the vivarium of the Federal University of Ceará (Universidade Federal do Ceará - UFC). The animals were kept in polypropylene cages at room temperature between 24 and 25°C in light-dark cycles of 12/12 hours. All of the animals received water and food ad libitum. The Ethics Committee on Animal Research (Comitê de Ética em Pesquisa Animal - CEPA) of the UFC approved the experimental protocol (no. 90/10).

**Diabetes induction**

Diabetes was induced with an intraperitoneal injection of 150 mg/kg alloxan monohydrate (Sigma®). After 7 days, diabetes was confirmed by measuring the serum glucose level. Animals with blood glucose levels equal to or greater than 200 mg/dL were considered to be diabetic.[24,25]

**Experimental design**

The animals were divided into the following 6 groups (n=7) and received doses orally for a period of 30 consecutive days, according to the protocol adapted from Barbosa et al. [25]:

- Normal control group (NC): healthy mice that received the vehicle (0.2 mL distilled water/day/animal);
- Diabetic control group (DC): diabetic mice that received the vehicle (0.2 mL distilled water/day/animal);
- PEC 200 Group: diabetic mice treated with pectin [200 mg/kg body weight (bw)] diluted in water;
- PEC 400 Group: diabetic mice treated with pectin (400 mg/kg bw) diluted in water;
- PEC 600 Group: diabetic mice treated with pectin (600 mg/kg bw) diluted in water;
- MET 600 Group: diabetic mice treated with metformin (600 mg/kg bw) diluted in water.

**Measurement of the glucose, urea, creatinine and uric acid levels, bw, food consumption and water intake**

Blood was collected through the retro-orbital plexus using a capillary on days 0, 21 and 30 to determine the glucose concentration; the other biochemical parameters, namely, urea,
creatinine and uric acid, were analyzed on the 30\textsuperscript{th} experimental day. The METROLAB 23300 version 1.7 device was used, which uses the kinetic method for serum samples. The bw of the animals was measured on a scale every week until the end of the experiment. The daily food intake and water intake of the animals are expressed in g of bw and mL, respectively.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of urine**

Urine was collected from the animals at the end of the treatment (30\textsuperscript{th} day). The amount of protein was then measured, and SDS-PAGE was performed in a 4-12\% polyacrylamide gel. \cite{16, 26}

**Histology**

The liver and pancreas were used for the histopathological analysis. The isolated fragments were fixed in 10\% neutral formalin and placed in paraffin blocks for conventional histological processing.\cite{27} Then, 5-\textmu m sections were obtained and stained with hematoxylin-eosin (HE). The slides were examined for the identification of histological alterations with conventional optical microscopy (Nikon YS2), and images representative of each organ were captured with a digital camera (Nikon COOLPIX L14 7.1 megapixels).

**Statistical analysis**

The data are expressed as the means ± standard deviation (SD). The significance of differences between animals from the groups was assessed using an analysis of variance (ANOVA), followed by the Newman-Keuls test. A value of p<0.05 was considered significant.

**RESULTS AND DISCUSSION**

A new polysaccharide was extracted and characterized from the fresh peels of *Passiflora glandulosa* Cav, isolated pectin is characterized by a low degree of methoxylation and a high molecular weight, in addition to containing galacturonic acid in a carboxylate form in its chemical structure.

The yield of pectin extracted from the fresh peel of *P. glandulosa* Cav was 5.73\%, and the moisture, ash, total protein and total carbohydrate contents on a dry basis are listed in Table 1.
Table 1 Moisture, Ash, Total Protein and Total Carbohydrate Contents of Pectin From P. glandulosa Cav.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>19.6</td>
</tr>
<tr>
<td>Ash</td>
<td>7.77</td>
</tr>
<tr>
<td>Total Protein</td>
<td>0.023</td>
</tr>
<tr>
<td>Total Carbohydrate*</td>
<td>59.91</td>
</tr>
</tbody>
</table>

*Galacturonic acid used as reference

FTIR is an effective tool for the structural analysis of polysaccharides and was therefore used to analyze pectin. Based on the FTIR analysis (Figure 1) of the pectin from P. glandulosa, it is possible to confirm the presence of absorption bands that are characteristic of the polysaccharide: 3425 cm\(^{-1}\) (OH); 1751 cm\(^{-1}\) (C=O) of methyl ester COO\(\text{CH}_3\); 1603 cm\(^{-1}\) (C=O) of carboxylate COO\(^-\); 1427 cm\(^{-1}\) and \(\delta\) (C-OH) of carboxylic acid COOH; 1104 cm\(^{-1}\) (CO)(CC); 630 cm\(^{-1}\); 673 cm\(^{-1}\) low frequency vibration of pyranoid ring. Moreover, the expansion of the FTIR scan of pectin enabled the degree of methoxylation to be calculated as 35%. \(^{19}\)

![FTIR absorption spectrum of the pectin obtained from P. glandulosa Cav. (KBr).](image)

Figure 2 and Table 2 show the analysis and expansion of the \(^1\)H-NMR spectrum of the pectin from P. glandulosa. Distinguishable line patterns and the intensity of signal arise in the NMR spectra and result from different degree of methoxylation values and sequential arrangements.
of carboxylate and methyl esterified carboxylic groups along the polymer chains. The results confirmed that pectin is a polymer of poly-\(\alpha-(1\rightarrow4)\)-D-galacturonic acid. The analyzed TLC chromatogram confirmed the presence of galacturonic acid, galactose and arabinose in the extracted pectin. The molecular mass obtained via GPC was \(1.6 \times 10^5\) g/mol.

![Figure 2](image)

**Figure 2** (A) \(^1\)H NMR absorption spectrum of pectin obtained from *P. glandulosa* Cav (\(D_2O, 500.13\) MHz) and (B) expansion of 4.55-5.35 (\(D_2O, 500.13\) MHz); (C) representation of the chemical structure of the pectin.

**Table 2 Assignment of \(^1\)H NMR Chemical Shift ppm for the Isolated Pectin**

<table>
<thead>
<tr>
<th>(ppm)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.9-5.2</td>
<td>H-1</td>
</tr>
<tr>
<td>3.74</td>
<td>H-4</td>
</tr>
<tr>
<td>3.97</td>
<td>H-3</td>
</tr>
<tr>
<td>4.90</td>
<td>H-4</td>
</tr>
<tr>
<td>4.9-5.4</td>
<td>H-5 (COOMe)</td>
</tr>
<tr>
<td>4.6</td>
<td>H-5 (COO)</td>
</tr>
<tr>
<td>3.75</td>
<td>OCH(_3)</td>
</tr>
</tbody>
</table>

The alloxan-induced diabetes increased the blood glucose levels in the mice to above 200 mg/dL. The hypoglycemic action of the pectin isolated from *P. glandulosa* significantly reduced blood glucose levels in the diabetic animals treated with doses of 200 mg/kg bw, 400 mg/kg bw and 600 mg/kg bw by 45.7%, 42.2% and 26.1%, respectively (p<0.05). Pectin had a hypoglycemic effect beginning at day 21 of treatment, and this effect increased at the end of the treatment (Table 3). Doses of 200 and 400 mg / kg bw reduced blood glucose levels similar to standard drug (metformin).
Table 3 Effect of Pectin from P. glandulosa Cav on the Glucose Concentration in Animals with Alloxan-Induced Diabetes

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glucose level (mg/dl)</th>
<th>% Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 21</td>
</tr>
<tr>
<td>Normal control</td>
<td>70.0±5.1</td>
<td>92.6±19.3</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>464.6±97.9*</td>
<td>364.6±46.4*</td>
</tr>
<tr>
<td>PEC (200 mg/Kg)</td>
<td>449.0±15.9</td>
<td>329.9±25.1</td>
</tr>
<tr>
<td>PEC (400 mg/Kg)</td>
<td>446.6±29.2</td>
<td>317.6±66.3</td>
</tr>
<tr>
<td>PEC (600 mg/Kg)</td>
<td>544.4±39.2</td>
<td>396.6±3.3</td>
</tr>
<tr>
<td>Metformin (600 mg/Kg)</td>
<td>611.8±26.5a</td>
<td>337.5±54.5</td>
</tr>
</tbody>
</table>

Values expressed as means ± standard deviation (n=7), *diabetic control group vs. normal control group; a treated groups vs. diabetic control group, significance of p<0.05, analysis by One-Way ANOVA followed by the Newman-Keuls test.

These results are in agreement with those found by Silva et al. (2011) in diabetic Wistar rats using pectin from the peel of P. edulis Sims, obtained a 65% reduction in blood glucose levels. Lima et al. [28] showed that the fasting serum blood glucose levels of alloxan-induced diabetic rats treated for 21 days with feed enriched with 20% and 40% flour from the mesocarp of P. nitida Kunth were reduced by 38.7% and 40%, respectively. A phase II clinical trial with 43 diabetic patients used supplementation with 30 g/day of P. edulis f. flavicarpa Deg peel flour for 60 days, and the results revealed a reduction in the fasting blood glucose levels, which was explained by the presence of pectin. [10] The results of these studies clearly show that pectin reduces blood glucose levels. Studies for the isolation of new sources of pectin provide different chemical structure and may increase the hypoglycemic action by producing an effective and safe product, and further research is needed in addition to the aggregate value to the native plants. The feed consumption and water intake of the animals in the study were significantly reduced, while the bw remained stable, except in the diabetic group treated with a pectin dose of 600 mg/kg bw, according to the data in Table 4.

Diabetic animals treated with pectin at doses of 600 mg/kg bw, 400 mg/kg bw and 200 mg/kg bw did not have a significant difference (p<0.05) in the concentration of uric acid and urea compared to healthy animals. Similar results were observed with the standard drug (metformin). The creatinine levels were different at the tested pectin doses (Figure 3).
Table 4 Change in Body Weight and Food Consumption and Water of Diabetic Mice Treated with Pectin Extracted from P. glandulosa Cav

<table>
<thead>
<tr>
<th>Groups</th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
<th>Feed (g/mouse/day)</th>
<th>Water (mL/mouse/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>28.5±2.1</td>
<td>31.1±2.5</td>
<td>18.0±1.6</td>
<td>30.1±7.2</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>29.4±2.0</td>
<td>32.6±3.7</td>
<td>44.3±2.4</td>
<td>137.8±8.1</td>
</tr>
<tr>
<td>PEC (200 mg/Kg)</td>
<td>29.6±0.9</td>
<td>31.0±3.0</td>
<td>31.0±1.7*</td>
<td>42.5±4.9*</td>
</tr>
<tr>
<td>PEC (400 mg/Kg)</td>
<td>29.5±1.8</td>
<td>30.2±2.8</td>
<td>36.7±1.3*</td>
<td>105.0±18.0*</td>
</tr>
<tr>
<td>PEC (600 mg/Kg)</td>
<td>27.1±1.3</td>
<td>22.5±0.9*</td>
<td>37.8±2.8*</td>
<td>133.5±8.0</td>
</tr>
<tr>
<td>Metformin (600 mg/Kg)</td>
<td>28.1±2.3</td>
<td>28.2±2.0</td>
<td>27.0±1.9*</td>
<td>86.5±17.2*</td>
</tr>
</tbody>
</table>

Values expressed as means ± standard deviation (n=7), *diabetic control group vs. normal control group; a treated groups vs. diabetic control group, significance of p<0.05, analysis by One-Way ANOVA followed by the Newman-Keuls test.
Figure 3 Biochemical markers (A) uric acid, (B) creatinine and (C) urea from diabetic animals treated with pectin isolated from *P. glandulosa* Cav after the 30th day of treatment

Grover et al. [29] found that diabetic animals that received PolyGlycopleX (a soluble fiber) exhibited metabolic homeostasis. In this study, the renal biomarkers, urea, creatinine and uric acid, displayed values in the normal range, according to international parameters for mice. [30]

The SDS-PAGE profile of the urine samples from the diabetic control group, the 400 and 600 mg/kg pectin groups and the 600 mg/kg metformin group had a band corresponding to the molecular weight of albumin (~67 kDa), indicating the loss of this protein in the urine. Conversely, the presence of this protein was not observed in samples from the group treated with pectin at 200 mg/kg bw or the normal group (Figure 4).

Figure 4 Electrophoretic profile of urine from healthy and diabetic mice treated with pectin extracted from *P. glandulosa* Cav on the 30th day of treatment
Legend: NC (normal control), DC (diabetic control), P. (Pectin) and M. (Metformin)

Figure 5 Photomicrographs of the livers of diabetic animals treated with pectin isolated from P glandulosa Cav – (A) Normal control (B) Diabetic control (C) PEC 600 (D) PEC 400 (E) PEC 200 (F) MET 600 – Vacuolated areas in hepatocytes representative of hydropic degeneration and steatosis (white arrows) – HE staining, 400X magnification

This result is relevant because albuminuria is a powerful predictor of morbidity and mortality in patients with diabetes.\cite{31} In particular, the degree of albuminuria is strongly associated with both the progression of diabetic renal disease and the risk of strokes.\cite{32} Furthermore, this finding suggests that the electrophoresis technique is effective for the analysis of animals with experimental diabetes.
The pancreatic and hepatic histopathological analyses of the diabetic animals treated with pectin at doses of 200, 400 and 600 mg/kg bw indicate that pectin at these doses is suitable for consumption, because no architectural alteration was observed in the livers of the animals treated with pectin or the standard drug (metformin) (Figure 5 c, d, e, f). However, hydropic degeneration and steatosis were observed in all of the groups, including the healthy group, suggesting that this finding was not caused by treatment with pectin (Figure 5a).

No alterations were observed in the pancreases of the animals, except in the group that received metformin, which exhibited a focus of cellular degeneration in the pancreatic islets (Figure 6f).

Figure 6 Photomicrograph of the pancreas of diabetic animals treated with pectin isolated from P glandulosa Cav – (A) Normal control (B) Diabetic control (C) PEC 600 (D) PEC 400 (E) PEC 200 (F) MET 600 – Vacuolated areas in the pancreatic islets represent cellular degeneration (white arrow) – HE staining, 400X magnification.
A study performed by Gupta et al. [33] is in agreement with the present study because the pancreatic tissue of diabetic animals treated for 21 days with a methanol extract from Moringa oleifera (Moringa) exhibited regeneration of the islets of Langerhans. Therefore, the plant extract is non-toxic. The treatment of diabetic animals with the ethanol extract of Citrullus Colocynthis also did not show an alteration in the cellular architecture of the pancreatic and hepatic tissues.[34]

CONCLUSIONS
In conclusion, pectin isolated from P. glandulosa has hypoglycemic action at doses of 200, 400 and 600 mg/kg bw in animals with alloxan-induced diabetes. The 200 mg/kg bw dose reduced the concentration of blood glucose and showed no renal toxicity and hepatotoxicity for animals. The results showed that the isolated pectin has effect on glycemic control by their chemical structure (polysaccharides) with low degree of methoxylation and gelation capability by reducing the absorption of carbohydrate.

Future studies are required to clarify the mechanism of action of pectin in the diabetic organism, as well as its metabolism and excretion. This study demonstrates the potential use of plants from the Caatinga biome in the search for novel treatments for diabetes.

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