Characterization of Chaya extracts (Cnidoscolus aconitifolius) and evaluation of their hypolipidemic and antiapoptotic effects

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ABSTRACT

Since ancient times, Mexico has been home to a wide variety of plants and herbs, many of them with some medicinal effects used to cure different ailments such as diabetes, cholesterol, and triglyceride diseases, as a weight reduction remedy and as an antioxidant. Tree spinach, also known as Chaya has long been utilized as a traditional medicine to cure multiple ailments. The aim of the study was to characterize the extracts of Chaya (Cnidoscolus aconitifolius (Mill) I. M. Johnst) and to evaluate the hypolipidemic and antiapoptotic effects on blood and cells from Long-Evans rats. Two test groups were made (one to evaluate hypolipidemic and the other to test antiapoptotic effects). The hypolipidemic group received a carbohydrate-rich diet for five months to increase blood glucose and high-density lipoprotein. Once these patterns were altered, the eight-week treatment with the extracts began. The group defined to assess the antiapoptotic effect was injected with 65 mg/Kg of streptozotocin (STZ) to cause the death of pancreatic β-cells. SDS-PAGE and Western blotting evaluated cellular death. C. aconitifolius extracts contain antioxidant compounds such as phenols, hydroxycinnamic acids, and flavonoids. No anti-apoptotic effect on pancreatic cells nor any improvement in lipoprotein values from blood samples were observed. There was an increase in cell death (p=0.014) early apoptosis in the aqueous extracts. After complete treatment with the extract, a tendency to normal behavior is observed in the apoptotic values, such as in the control group.

Keywords: Chaya, anti-apoptotic effect, hypolipidemic effect, antioxidants.

ABBREVIATIONS

HCN: Hydrocyanic acid; HDL: High-density lipoprotein; HPLC: High-performance liquid chromatography; FMVZ: Faculty of Veterinary Medicine and Zootecny; LDL: Low-density lipoprotein; NOM: Official Mexican Standards; MS/MS: Tandem Mass Spectrometry; PI: Propidium iodide; SDS-PAGE: Sodium dodecyl sulphate–polyacrylamide gel electrophoresis; STZ: Streptozotocin; VLDL: Very-low-density lipoprotein.

INTRODUCTION

The Chaya (Cnidoscolus aconitifolius (Mill) I. M. Johnst) belongs to the Euphorbiaceae family and is a shrub of Mesoamerican origin cultivated for its nutritious leaves (Chin-Chan et al., 2021). Evidence suggests that Chaya was an important plant for the ancient Maya culture in Mexico. It has been described that Chaya has therapeutic properties. The boiled leaves have been taken orally to treat eye disorders or discomfort, muscular disorders, fatigue, and even rheumatism or arthritis. However, other parts of the Chaya such as the roots have been taken to treat kidney disorders, back pain, inflammation, or hemorrhoids. On the other hand, sap has been used to treat
Another traditional use is in the treatment of diabetes, high cholesterol and high blood pressure. Chaya is not only used for medicinal purposes, but also for food (Bautista-Cruz et al., 2011). Thus, Chaya seems to be an excellent alternative to control or reverse the effects associated with dyslipidemas (Guevara-Cruz et al., 2021). Another possible benefit is that it may play a crucial role in apoptosis or programmed cell death. It has been proposed that their medicinal properties are due to the photochemical components that the leaves possess (Somade et al., 2021). No studies of this plant related to the antiapoptotic effect have been found, and there are few studies related to the hypolipidemic activity. Therefore, it is intended to carry out a study to evaluate the hypolipidemic and antiapoptotic effects of two types of Chaya extracts cultivated in the locality, as well as to carry out the corresponding characterization that allows us to make the comparison of the components with other varieties of Chaya that are found in the country, which will help us to have the scientific support to be used as a natural and preventive remedy.

MATERIAL AND METHODS

Plant material

Specimens of C. aconitiolius were collected in adjacent region of the State of Durango (25°11'00'' N, 104°34'00'' W) at 1885 m above mean sea level from 2 m tall plants in April 2019. The herbarium voucher was annotated by the herbarium staff of the Interdisciplinary Center of Research for Regional Integral Development of the National Politechnical Institute (CIIHR-IPN Durango) with a specimen number 53591.

Preparation of extracts from Chaya for treatment

The aqueous extract was prepared using 4.8 g of dry leaves in 300 mL of water, boiling for 5 min. The volume was filled with de-ionized water until 1 L. The aqueous extract was stored at -20°C in amber containers until used. To prepare the methanolic extract, 5.7 g of dry leaves were mixed in 1 L of pure methanol (99.8%) and stored in amber containers for eight days. After, 100 mL of the extract was separated in a flask connected to a vacuum pump (30–40 cm Hg pressure) at 30–40°C until evaporation. The aliquot was gauged at 1 L of de-ionized water and stored at -20°C in amber containers.

Preparation of aqueous and methanolic extracts for chromatography

The aqueous extract was prepared with 200.6 mg of dry leaves in 200 mL of de-ionized water, boiling for 5 min. 100 μL was diluted in 50 mL of HPLC grade water. For the methanolic extract 200.6 mg of Chaya dry leaves were mixed in pure methanol, and stored in amber container for 8 days with occasional stirring. After 100 μL of the extract were diluted in 50 mL of HPLC grade water.

HPLC-MS/MS

Both the aqueous and methanolic extracts were analyzed on an Agilent 1200 chromatograph equipments (Agilent Technologies, Waldbronn, Germany) coupled to Agilent 6410 triple quadruple mass-mass spectrometry detector with ESI ionization chamber (Agilent Technologies, Waldbronn, Germany).

Experimental animals

Long-Evans rats, male and female, weighing 160 g were used. The animals were subjected to an adaptation period of one week, under 12 h light-dark cycle at 25 ± 3°C. They were fed with Purina® Roden Chow pellets with free access to water. The ethical guidelines established by the experiments reported in this study were performed according to established by the Official Mexican Norm(NOM-062-ZOO-1999 (2001)).

Induction of diabetes of Long-Evans rats

Diabetes was induced by a single intra-peritoneal administration of STZ [65 mg/kg] (Sigma-Aldrich, Missouri, United States) freshly dissolved in 0.1 M citrate buffer (pH 4.5). At 72 h after the administration of STZ blood glucose level of fasting rats was verified. A blood glucose level of 140 mg/dL or more was considered diabetes.

Determination of glucose levels

The glycemic measurement was carried out through glucose test strips on the Roche Accu-Chek Performa (Roche Diagnostics, Basel, Switzerland). The range of glucose detection was 60–600 mg/dL. For practical purposes, the times were determined as follows: (t0) sample before subjecting animals to experimentation, (t1) 6 months of exposure to a diet high in fat and calories without Chaya treatment, (t2) 5 months of exposure to diet and treatment with Chaya extracts.

Model of diet-induced obesity and dyslipidemia in Long Evans rats

For six months, rats were fed with a high-triglyceride, high-
sugar, high-cholesterol diet. They had two lipid and sugar profiles: before and after diet to corroborate glucose, lipoproteins, and triglycerides values. Animals were divided into two groups according to Chaya treatment; (g1) aqueous extract and (g2) methanolic extract. The treatment lasted five months. During this period, the diet was not modified.

### Blood and pancreatic β-cells samples

Venous blood samples were drawn from each rat after 8 h fasting, under ether inhalation anesthesia, and collected in BD Vacutainer Serum Separation Transport Tube. Serum was separated by centrifugation for 5 min at 1500 rpm. Surgical removal of the pancreas was macerated with 5 mL of Hanks’ solution. Pancreas cells were obtained by centrifugation for 5 min at 1500 rpm.

### Flow cytometry-based apoptosis measurement

The effect of each treatment on cell apoptosis was determined using flow cytometric analysis following fluorescent detection of annexin V according to Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, Missouri, United States) following the manufacturer's instructions. The cells were co-staining with propidium iodide (PI) distinguished from necrotic cells.

### Statistical analysis of data

In order to compare the effects, ANOVA tests were applied using the IBM-SPSS statistics v.22 software. All values are reported as mean ± SD, *p* < 0.05 was considered statistically significant.

### RESULTS

#### Characterization of Chaya extracts by HPLC MS/MS

Different phenolic compounds and vitamins were identified during the analysis, which was identified by comparing the ion products and their relative abundances identified in the aqueous and methanolic extracts of Chaya (Table 1). Around 60 different masses were detected, of which those with abundances equal to or greater than 1x106 (mAU). Table 2 shows the five most important phenolic compounds.

#### Hypolipidemic effect of the aqueous and methanolic extracts

The weight gain of the rats throughout the experimental

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**Table 1:** Compounds found in the aqueous and methanolic extract of Chaya.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular weight (g/mol)</th>
<th>Extract type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocatechicacid</td>
<td>154.1</td>
<td>Methanolic and aqueous</td>
</tr>
<tr>
<td>Caffeicacid</td>
<td>180.1</td>
<td>Methanolic</td>
</tr>
<tr>
<td>Ferulicacid</td>
<td>194.1</td>
<td>Aqueous</td>
</tr>
<tr>
<td>Myristicacid</td>
<td>228.3</td>
<td>Aqueous</td>
</tr>
<tr>
<td>Palmiticacid</td>
<td>256.4</td>
<td>Aqueous</td>
</tr>
<tr>
<td>Naringenin</td>
<td>272.2</td>
<td>Methanolic and aqueous</td>
</tr>
<tr>
<td>Chlorogenicacid</td>
<td>354.3</td>
<td>Methanolic</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>356.8</td>
<td>Aqueous</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>376.3</td>
<td>Aqueous</td>
</tr>
<tr>
<td>Astragalin</td>
<td>448.3</td>
<td>Aqueous</td>
</tr>
<tr>
<td>Rutin</td>
<td>610.5</td>
<td>Methanolic and aqueous</td>
</tr>
<tr>
<td>Chlorogenicacid</td>
<td>354.3</td>
<td>Methanolic</td>
</tr>
</tbody>
</table>

**Table 2:** Phenolic compounds and vitamins found in Chaya extracts.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular weight (g/mol)</th>
<th>Extract type</th>
</tr>
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<tr>
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<td>Aqueous</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>356.8</td>
<td>Aqueous</td>
</tr>
</tbody>
</table>
Figure 1: Monthly weight gain in rats subjected to a hypercaloric diet.

Table 3: Initial and final average of blood chemistry in aqueous Chaya extract.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Sample in t0 (mg/dL)</th>
<th>Sample in t1 (mg/dL)</th>
<th>Sample in t2 (mg/dL)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>134.33 ± 40.31</td>
<td>204.10 ± 76.59</td>
<td>159.51 ± 15.56</td>
<td>0.199</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>76.83 ± 8.08</td>
<td>109.67 ± 7.64</td>
<td>124.2 ± 28.57</td>
<td>0.325</td>
</tr>
<tr>
<td>HDL</td>
<td>67.58 ± 11.24</td>
<td>38.03 ± 1.58</td>
<td>37.86 ± 1.44</td>
<td>0.724</td>
</tr>
<tr>
<td>LDL</td>
<td>10.35 ± 8.31</td>
<td>57.38 ± 6.24</td>
<td>51.73 ± 32.05</td>
<td>0.712</td>
</tr>
<tr>
<td>VLDL</td>
<td>18.48 ± 3.03</td>
<td>14.25 ± 4.25</td>
<td>34.63 ± 15.84</td>
<td>*0.013</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>92.33 ± 15.18</td>
<td>71.21 ± 21.44</td>
<td>172.67 ± 79.27</td>
<td>*0.013</td>
</tr>
</tbody>
</table>

± = standard deviation, * = p < 0.05

Table 4: Initial and final average of blood chemistry in methanolic Chaya extract.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Sample in t0 (mg/dL)</th>
<th>Sample in t1 (mg/dL)</th>
<th>Sample in t3 (mg/dL)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>145.16 ± 13.46</td>
<td>173.86 ± 19.27</td>
<td>163.41 ± 28.15</td>
<td>0.286</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>76.66 ± 9.54</td>
<td>110.33 ± 14.67</td>
<td>110.01 ± 14.67</td>
<td>0.889</td>
</tr>
<tr>
<td>HDL</td>
<td>62.5 ± 17.48</td>
<td>38.75 ± 3.25</td>
<td>42.98 ± 17.01</td>
<td>0.726</td>
</tr>
<tr>
<td>LDL</td>
<td>9.53 ± 8.76</td>
<td>57.71 ± 12.02</td>
<td>42.98 ± 17.2</td>
<td>0.188</td>
</tr>
<tr>
<td>VLDL</td>
<td>16.9 ± 4.03</td>
<td>13.86 ± 4.075</td>
<td>30.01 ± 6.83</td>
<td>0.503</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>94.5 ± 20.15</td>
<td>69.36 ± 20.36</td>
<td>150.91 ± 34.19</td>
<td>*0.007</td>
</tr>
</tbody>
</table>

± = standard deviation, * = p < 0.05

The control group did not have a significant variation in weight compared with the hypercaloric diet group. Lipid profiles of both groups treated with aqueous and methanolic extracts at the beginning of the treatment and the end of the treatment are shown in Table 3 and Table 4 respectively. The results of biochemical variables before the administration of carbohydrate-rich diet (t0), 6 months of exposure to a diet high in fat and calories without treatment (t1), and 5 months of exposure to diet and treatment with aqueous extract (t2) are shown in Table 3 and treatment with methanolic Chaya extract in Table 4. The comparison of biochemical variables between rat model in obesity/dyslipidemia condition (t1) and exposure to treatment with Chaya extracts (t2) showed no differences in glucose, cholesterol, HDL, LDL values. However, the very-low-density lipoprotein (VLDL) showed a significant increase in the aqueous extract treatment group (p = 0.013). Besides, triglyceride values were significantly different in both group (p = 0.013 in aqueous group; p = 0.007 in methanolic group).

Antiapoptotic effects of the aqueous and methanolic extracts

The flow cytometry data, showed no significant protective
Figure 2: Flow cytometry analysis of apoptosis. Dot Blots of treatment with C. aconitiiolius at A) Week two B) Week six C) Week eight

**DISCUSSION**

The phytochemical analysis helps to evaluate the organic compounds of plants such as *C. aconitiiolius* and to identify the components that could respond to therapeutic use. The present study showed that Chaya presents many antioxidant compounds, such as phenolic acids, flavonoids,
and tannins. It has been reported that some antioxidants may be beneficial in the treatment of chronic degenerative diseases such as diabetes mellitus (Bajaj and Khan, 2012), obesity (Abdali et al., 2015), and cancer (Menon et al., 2016). In this study, we observed that the aqueous extract showed a more significant number of compounds, possibly because many antioxidants are soluble in water. The highest phenolic content was recorded in the aqueous extract; this result is comparable to *C. aconitifolius* aqueous extract collected in the region of southeastern Mexico (Medina et al., 2020). We observed the presence of ferulic acid and palmitic acid in the aqueous extract. Ferulic acid is a hydroxycinnamic acid that has been shown anti-inflammatory properties (Yin et al., 2019). However, the presence of palmitic acid has been reported that palmitic acid increased metastasis (Sarwat et al., 2019) and apoptosis (Yuan et al., 2013). Caffeic and chlorogenic acid were found in the methanolic extract of *Chaya*; this compound has been implicated in antiproliferative activity (Nagaoka et al., 2002) and improving glucose tolerance and insulin resistance (Meng et al., 2013) in obese rats. The presence of caffeic acid, chlorogenic acid, and ferulic acid is in concordance with the observations by Godínez-Santillán et al. (2019) *C. aconitifolius* leaves. Naringenin flavonoid was found in both extracts, and it has been shown to improve insulin sensitivity and glucose tolerance (Li et al., 2019) and reduce oxidative stress (Wang et al., 2017). Rutin detected in the aqueous and methanolic extract has been associated with a cholesterol decrease (Liang et al., 2021).

In comparison with us, Valenzuela-Soto et al. (2015) detected Rutin in the aqueous extract of Chaya. The antioxidants found in both extracts (caffeic acid, chlorogenic acid, and ferulic acid) coincide with those found in other studies such as Chaya from Querétaro, Mexico, except for naringenin (Godínez-Santillán et al., 2019). The variability in compounds may be due to the origin region. In this sense, we suggested evaluating the results using the standards of the corresponding compounds to confirm the data. Our biochemicals test showed that aqueous and methanolic extracts no affect cholesterol values in Long Evan rats, these results contrast with the findings by Miranda-Velasquez et al. (2010). In BalB/C rats where a decrease in cholesterol was found from 27.9 to 31.1% with the aqueous extract, but strict control of the daily dose of 50-100 mg/kg respectively was managed. However, the type of diet was different from that of our study. In addition, the fact that the mice fasted for five days, it is unclear whether the decrease in cholesterol was caused by the Chaya extract or by diet (Miranda-Velasquez et al., 2010). The extracts used were not effective in reducing the concentration of low-density lipoproteins or triglycerides. The LDL concentration increased with the high-calorie and high-fat diet, but the treatment began to decrease. However, it was not reached to normalize. HDL presented a decrease in blood, which is expected by the type of food supplied; at the end of the treatment, there was a tendency to stabilize the HDL levels. We suggest prolonging the study for at least six months of treatment to increase LDL and HDL concentrations and thus obtain conclusive results. Chaya consumption did not reduce the concentration of triglycerides. Even our result is opposite to that shown by Guevara-Cruz et al., 2021, where a reduction of the lipid profile was observed in patients with dyslipidemia submitted to the consumption of 500 ml Chaya for six weeks. The no-effect of extracts may be due to the difference in the concentrations supplied between the two studies (Guevara-Cruz et al., 2021).

Our results are controversial because naringenin and rutin antioxidants found in both extracts have been associated with the decrease in the concentration of VLDL and LDL and the concentration of serum triglycerides...
(Mulvihill et al., 2009) and increased anti-hypercholesterolaemic effects in an animal model (Ziaee et al., 2009), respectively. The antiapoptotic effect of C. aconitifolius extracts was evaluated by flow cytometry. At the moment, there are no reports of the antiapoptotic capacity of Chaya. However, being a plant rich in antioxidants, it would be expected a similar result to the treatment of extracts of A. koraensis in STZ-induced diabetic rats, where the antiapoptotic effect restored the expression of the Bax and Bcl-2 proteins (Sohn et al., 2010). However, our results are contradictory, suggesting that the treatment of methanolic and aqueous extract increases the percentage of early apoptosis and a considerable decrease in viable cells. Although Chaya extract contains some antioxidants that may contribute to the antiapoptotic effect, the observed apoptotic effect may be due to the palmitic acid apoptosis, which is activated via activating c-Jun N-terminal kinase (Yuan et al., 2013). However, it is notable that the values at the end of the treatment with the aqueous extract (the eighth month), the early and late apoptosis values have a tendency to normalize as in the control group.

CONCLUSIONS

The characterization of the extracts evaluated by liquid chromatography HPLC-MS/MS showed the existence of antioxidant compounds corresponding to hydroxycinnamic acids and flavonoids in the extracts. The evaluation of the hypolipidemic capacity of Chaya extracts, both aqueous and methanolic, showed not to reduce the concentration in blood of total cholesterol, triglycerides, and lipoproteins. On the contrary, they continued to show an increase throughout the experiment. Regarding the evaluation of antiapoptotic effects determined by flow cytometry, there was no significant protective effect against apoptotic damage because the groups in the other weeks did not present statistically significant differences. p > 0.5 in all cases. In the aqueous extracts, there is a difference. However, it is not an antiapoptotic effect but an increase in cell death (p = 0.014 early apoptosis). After complete treatment with the extract, a tendency to normal behavior is observed in the apoptotic values, such as in the control group.

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