ACUTE AND SUB-CHRONIC EFFECTS OF *COCHLOSPERMUM VITIFOLIUM* IN BLOOD GLUCOSE LEVELS IN NORMOGLYCEMIC AND STZ-NICOTINAMIDE-INDUCED DIABETIC RATS

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This paper is dedicated to Professor Doctor Rachel Mata for her 60th birthday

**ABSTRACT**

The aim of the present study was to determine the acute and sub-chronic hypoglycemic and antidiabetic effects of methanol extract from *Cochlospermum vitifolium* (MECv), a medicinal plant used in Mexican folk medicine for the treatment of hypertension, diabetes and liver damage-related diseases. Intragastric administration of MECv (100 mg/Kg) induced a significant decrease on plasma glucose in normoglycemic and STZ-nicotinamide-induced diabetic rats in acute and sub-chronic models compared to control (p< 0.05). The increase in plasma glucose after sugars (glucose and sucrose) administration was partially suppressed by MECv (p< 0.05). Moreover, extract induced *in vitro* α-glucosidase activity inhibition in a concentration-dependent manner (IC\(_{50}= 1.9\) mg/mL). Furthermore, biochemical profiles as Glucose, total cholesterol, HDL and triglycerides (TG) were modified in serum blood after sub-chronic MECv treatment on normoglycemic and diabetic rats. Finally, it was established the pre-clinical security and tolerability parameters of MECv through toxicological evaluation in mice and rats using OECD protocols (LD\(_{50}> 5000\) mg/Kg). Findings suggest that MECv might exert its hypoglycemic and anti-diabetic effect by extra-pancreatic action through both partial suppression of carbohydrate absorption on intestine lumen and acting as α-glucosidase inhibitor to reduce post-prandial rise of blood glucose.

Keywords: acute toxicity; α-glucosidase inhibitor; antidiabetic; *Cochlospermum vitifolium*; Mexican medicinal plants.

**RESUMEN**

El objetivo del presente proyecto fue determinar el efecto hipoglucemiante y antidiabético *in vivo* del extracto metanólico íntegro de *Cochlospermum vitifolium* (EMCV),

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Acute and sub-chronic effects of Cochlospermum vitifolium

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INTRODUCTION

Diabetes mellitus (DM) is a chronic disease caused by inherited and/or acquired deficiency of insulin production by pancreas as well as modification of insulin sensitivity on tissues such as muscle, liver and adipose tissue. These metabolic alterations trigger toxic glucose accumulation in serum blood causing hypertriglyceridemia, impaired HDL/LDL ratio, blindness, impaired blood coagulation and weight loss. In addition, long-term hyperglycemic process is an important risk factor for development and progression of micro- and macrovascular complications for cardiovascular disease outcome (Altan, 2003; Adisakwattana et al., 2005). The underlying goal of pharmacological therapy against DM is to maintain an adequate plasmatic glucose concentration during extended time periods. In this context, there exist four major classes of oral antidiabetic agents for the treatment of these illnesses: insulin secretagogues, (sulfonylureas, meglitinide class agents, glucagon-like peptide 1 analogs, etc.) antihyperglycemic (biguanides), insulin sensitizers (thiazolidinediones) and α-glucosidase inhibitors (Davis, 2005). These type of drugs work on different target sites including insulin secretion stimulation in beta pancreatic cells, suppression of gluconeogenesis process in liver, increase of insulin sensitivity in peripheral tissues as muscle, liver and adipose tissue and reducing intestinal absorption of starch, dextrin, and disaccharides by inhibiting the α-glucosidase in the intestinal brush border, respectively. Thus, ethnomedical studies in Mexico have reveal that some plant remedies have been used to improve diabetes and its complications (Monroy-Ortiz and Castillo, 2007; Andrade-Cetto and Heinrich, 2005). However, only few medicinal plants have been scientifically evaluated to confirm its therapeutic properties and toxicological security in animal models in order to establish pharmacological/toxicological basis to extrapolate for future human clinical trials (Andrade-Cetto and Heinrich, 2005; Ortiz-Andrade et al., 2005; 2007).

Cochlospermum vitifolium (Willd.) Sprenzel is a medicinal tree widely distributed along rain-dry forest in Sierra de Huautla, Morelos. The bark is commonly used to
improve high blood pressure, diabetes and hepatobiliary diseases as jaundice and cirrhosis (Monroy-Ortiz and Castillo-España, 2007; Castillo-España et al., 2009; Sánchez-Salgado et al., 2007). Previous studies performed by our research group showed that methanolic (MECv) and hexanic (HECv) extracts from *C. vitifolium* bark were able to decrease noradrenaline-induced contraction on rat aortic rings about 80 and 90%, respectively. Furthermore, both extracts decrease blood glucose levels in normoglycemic rat model about 15% compared with control group. HECv was more potent than MECv since decreased glucose levels first hour after oral administration while the other showed a hypoglycemic effect until 5 hours after administration (Sánchez-Salgado et al., 2007). These experimental evidences suggest that *C. vitifolium* bark constituted a natural source for bioactive compounds that might improve diabetes and hypertension. Additionally, previous chromatographic analysis of MECv let the isolation of a pure crystalline compound identified as naringenin by X-ray diffraction and NMR techniques (Sánchez-Salgado et al., 2007), a flavonoide with pharmacological effect in diabetes as we described in previous experiments (Ortiz-Andrade et al., 2008). Recent HPLC analysis allowed us to establish that MECv contain 5% of naringenin (Sánchez-Salgado et al., 2009).

In this context, current study aimed to evaluate hypoglycemic and antidiabetic activities of methanolic extract from *C. vitifolium* (MECv) in normoglycemic and streptozotocin-nicotinamide-induced diabetic rats to establish its possible mode of action as well as to determine its toxicological profile.

**MATERIALS AND METHODS**

**Materials**

Glibenclamide, nicotinamide, streptozotocin (STZ), glucose and sucrose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pentobarbital (Anestesal®) was obtained from Glaxo-Smithkline (Mexico City, Mexico). Test kits for enzymatic end-point determinations for glucose, cholesterol, triglycerides (TG) and high density lipoprotein (HDL) were obtained from Wiener Labs (Mexico City, Mexico).

**Plant material and preparation of MECv**

*Cochlospermum vitifolium* (Willd.) Sprengel bark was collected in October 2005. Plant material was collected from its natural habitat in deciduous dry forest in the State of Morelos, Mexico and identified by Dr. P. Castillo-España. A voucher specimen (14628) has been deposited into CEAMISH-Herbarium (HUMO), Universidad Autónoma del Estado de Morelos, in Cuernavaca, Morelos as reference material. The air-dried plant material was ground into powder. Crude extract was prepared by maceration of 2 Kg at room temperature in 4 liters of methanol for 72 h. After filtration, the extract was concentrated *in vacuo* at 40°C and w/w percentage yields was determined.

**Animals**

Male Wistar rats (200-250 g) were obtained from Facultad de Estudios Superiores (FES) Iztacala animal facility, Universidad Nacional Autónoma de México (UNAM). All rats were maintained under standard laboratory conditions (12-h light/dark cycle, 25 ± 2 °C and humidity 45-65 %). Prior to experimentation, rats were fed with standard rodent diet and tap water *ad libitum* during one week in order to adapt them to laboratory conditions. All animal procedures were conducted in accordance with our *Federal Regulations for Animal experimentation and care* (SAGARPA, NOM-062-ZOO-1999, México), and approved by the Institutional Animal Care and Use Committee.

**Induction of Diabetes**

STZ was dissolved in citrate buffer (pH 4.5), and nicotinamide was dissolved in normal
physiological saline solution. Diabetes was induced in overnight fasted rats by a single intraperitoneal (i.p.) injection of 65 mg/kg STZ, 15 min after the i.p. administration of 120 mg/kg of nicotinamide (Masiello et al., 1998). Hyperglycemia was confirmed by the elevated glucose levels in plasma, determined after 10 days. Animals with blood glucose concentration higher than 150 mg/dl were used for the study.

**Experimental protocol**

Thirty rats were divided into six groups, each consisting of five animals. Test samples were suspended in 0.05% of Tween 80 in saline solution (vehicle) and were administered orally by intragastric (i.g.) route using an intragastric tube. Rats were divided into the following groups:

- **Group 1**: Normoglycemic control.
  Received only vehicle (0.5% Tween 80) (5 mL/Kg).
- **Group 2**: Normoglycemic reference.
  Glibenclamide was given at dose of 5 mg/Kg.
- **Group 3**: Normoglycemic treated. MECv was given at dose of 100 mg/Kg.
- **Group 4**: Diabetic control. Received only vehicle (0.5% Tween 80) (5 mL/Kg).
- **Group 5**: Diabetic reference.
  Glibenclamide was given at dose of 5 mg/Kg.
- **Group 6**: Diabetic treated. MECv was given at dose of 100 mg/Kg.

**Acute experimental model**

A protocol previously described was used to evaluate hypoglycemic effect (Ortiz-Andrade et al., 2008). Sixteen hours before the experiments, rats were fasted with free access to water. The body weight and fasting blood glucose levels of all rats were determined before the start of the experiment. Blood samples were collected from the caudal vein at 0, 1, 3, 5, and 7 h after vehicle, MECv and glibenclamide administration. Blood glucose concentration was determined by using a glucose oxidase-based commercial glucometer (Accutrend GCT, Roche Diagnostic®). The percentage variation of glycemia for each group was calculated in relation to initial (0 h) level, according to the equation:

\[
\% \text{Variation of glycemia} = \frac{G_x - G_0}{G_0} \times 100
\]

Where \(G_0\) were initial glycemia values and \(G_x\) were glycemia values at \(+1\), \(+3\), \(+5\) and \(+7\) h, respectively (Verspohl, 2002; Ortiz-Andrade et al., 2007).

**Sub-chronic experimental model**

The samples (vehicle, test samples and glibenclamide) were administered 5 days consecutively. Blood glucose levels were determined 1 h after test samples were administered and body weight was determined before the administration. On the fifth day, all animals were sacrificed and the total blood collected for measurement of biochemical parameters. The percentage variation of glycemia for each group was calculated in relation to initial (first day) day of experiment according to the equation:

\[
\% \text{Variation of glycemia} = \frac{\text{Day}_x - \text{Day}_1}{\text{Day}_1} \times 100
\]

Where \(\text{Day}_1\) was the initial glycaemia value and \(\text{Day}_x\) were the glycaemia values at +second day, +third day, +fourth day and +fifth day respectively (Verspohl, 2002; Ortiz-Andrade et al., 2007).

**Oral Glucose Tolerance Tests**

Thirty minutes after administration of test samples, a dose of 2 g/kg of substrate (glucose and sucrose) solution was administered to each rat. MECv (100 mg/kg), vehicle and acarbosa (3 mg/kg) were administered to rats in the same volume of solution. Blood samples were collected from the tail tip at 0 (before oral administra-
tion), 1, 1.5, 2, 2.5, 3 and 4 h after vehicle, positive control and extract administration. Blood glucose concentration was estimated as described.

**Biochemical Profile**

Blood samples were centrifuged at 3000 g for 10 min at 4 °C. Then, serum was removed for the respective analytical determinations. The serum glucose, total cholesterol, HDL and TG concentrations were determined using commercial kits (Wiener Labs, Mexico) by enzymatic photometric methods.

**α-glucosidase activity assay**

Each animal (n= 4) was sacrificed by cervical dislocation. The small intestine was obtained and flushed several times with ice-cold 0.9% NaCl, sodium phosphate buffer 10 mM (pH 7.0) and 1 mg/mL of ampicillin. The intestine was cleaned of adipose tissue and cut longitudinally. Then, the mucosa was scraped with a glass slide onto an ice-cold glass surface. The material containing α-glucosidase was homogenized with 10 strokes of a Teflon pestle, aliquoted in 1.8 mL cryotubes and stored at -25 °C until required (Ortiz-Andrade et al., 2007). Total protein was determined by the Lowry method (Lowry et al., 1951). The α-glucosidase activity was assayed with cornstarch as substrate (12.5 mg/mL, S-4126; Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M sodium phosphate buffer (pH 7.0) in a volume of 250 µl. Substrates were premixed with MECv at various concentrations (0.01–20 mg/mL), the reaction started by addition of 10 mL of crude enzyme and the mixture incubated at 37 °C for 10 min. The reaction was stopped by addition of acarbose (12.5 mg, enough for 99% inhibition) in an ice-water bath. Released glucose was quantified with a commercial glucose oxidase–based assay (Glucose GOD-PAP, GL 2614; Randox Laboratories, Antrim, UK) following the manufacturer’s instructions and was read at 492 nm and 630 nm for turbidity correction in a microplate reader (StatFax 2100; Awareness Technology, Los Angeles, CA, USA). The reaction was linear until 15 min. Inhibitory activity was reported as percentage of inhibition. Assays were performed in quadruplicate (Ortiz-Andrade et al., 2007).

**LD₅₀ estimation**

The determination of LD₅₀ was carried out using the ‘Acute Toxic Class’ method [Organization for Economic Cooperation and Development (OECD) 423] as described in a previous work (Ortiz-Andrade et al., 2008). Twelve male mice were used (30 - 35 g). The study was conducted with a starting dose of 5 mg/kg. Each dose was tested on three mice (four groups), and the next step was selected depending on the mortality observed during the first 24 h of exposure. In the second step, 12 male Wistar rats (250–300 g) were used. This toxicological study was started at a dose of 50 mg/kg. Three animals were used for tested groups. Finally, the range of LD₅₀ (category) was estimated (OECD, 1996).

**Statistical analysis**

All data are expressed as mean of experiments ± standard error of mean (S.E.M). Concentration–response curves were plotted and experimental data were adjusted by the non-linear, curve fitting program Microcal® Origin 6.0 (Microcal Software Inc., USA). Statistical analysis was performed by one way analysis of variance (ANOVA) and Dunett’s test (sample versus control). p-values less than 0.05 were considered to be statically significant.

**RESULTS AND DISCUSSION**

We have published that hexanic extract from *C. vitifolium* (120 mg/kg) elicits a significantly decrease in blood glucose in normoglycemic rats (about 15%) at first hour after oral administration. Therefore,
MECv also showed an important decrease in plasmatic glucose concentration 3 hours after oral administration (Sánchez-Salgado et al., 2007). Based on last results, we decide to determine the effect of MECv (100 mg/kg) in acute normoglycemic and STZ-nicotinamide induced diabetic rats. Tables 1-2 show that i.g. administration of MECv induced a significant decrease in plasma glucose concentration in normoglycemic rats, during both acute and subchronic time periods compared with control rats (p < 0.05). In acute model, MECv reduced 7% blood glucose (Table 1), which did not return to normal at 7 h after administration (-13%). In addition, sub-chronic treatment with MECv (5 days) also induced a significant hypoglycemic effect at day 2 (approximately -8%) and was sustained during the next three days of treatment (-13%) (Table 2). In DM rats, i.g. administration of MECv (100 mg/kg) also induced a significant reduction in plasma glucose at first hour, and effect was preserved during all time period of the acute experiment compared to control (p<0.05). Moreover, antidiabetic effect was comparable than produced by glibenclamide (positive control). Similarly, sub-chronic treatment with MECv (5 days) produced a significant antidiabetic effect after first day of treatment (-9%). Effect was maintained, and also was significant until the end of the treatment compared with the control group (p< 0.05) (Table 4). As we can see, MECv was more potent in DM than in normoglycemic rats. These findings suggest metabolic regulation in liver processes similar to some approved anti-hyperglycemic drugs as metformin, whose does not has plasmatic glucose reduction in normoglycemic patients but higher glucose decrease in diabetic people. Because of metformin suppress liver glucose production in gluconeogenesis pathway and increase insulin action in muscle and fat, this drug only can act in pathological pro-

### Table 1. Hypoglycemic effect of acute intragastric administration on MECv, glibenclamide and vehicle in normoglycemic rats

<table>
<thead>
<tr>
<th>Test samples</th>
<th>Dose (mg/Kg)</th>
<th>Zero hour</th>
<th>First hour</th>
<th>Third hour</th>
<th>Fifth hour</th>
<th>Seventh hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>0±0.0</td>
<td>10.9±3.3</td>
<td>1.4±5.6</td>
<td>1.2±3.6</td>
<td>-3.6±2.7</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>5</td>
<td>0±0.0</td>
<td>-8.3±1.9*</td>
<td>-21.7±3.9*</td>
<td>-24.8±1.0*</td>
<td>-23.8±0.9*</td>
</tr>
<tr>
<td>MECv</td>
<td>100</td>
<td>0±0.0</td>
<td>1.25±5.15*</td>
<td>1.67±3.55</td>
<td>-7.01±3.42*</td>
<td>-13.43±2.5*</td>
</tr>
</tbody>
</table>

### Table 2. Hypoglycemic effect of sub-chronic intragastric administration of MECv, glibenclamide and vehicle in normoglycemic rats

<table>
<thead>
<tr>
<th>Test samples</th>
<th>Dose (mg/Kg)</th>
<th>Day zero</th>
<th>First day</th>
<th>Second day</th>
<th>Third day</th>
<th>Fourth day</th>
<th>Fifth day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>0±0.0</td>
<td>27.2±5.3</td>
<td>21.9±2.2</td>
<td>20.0±0.9</td>
<td>23.0±1.7</td>
<td>28.9±1.4</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>5</td>
<td>0±0.0</td>
<td>-23.5±2.1*</td>
<td>-28.7±2.1*</td>
<td>-33.5±3.5*</td>
<td>-29.8±2.9*</td>
<td>-30.1±2.4*</td>
</tr>
<tr>
<td>MECv</td>
<td>100</td>
<td>0±0.0</td>
<td>5.36±3.08*</td>
<td>8.22±2.2*</td>
<td>8.32±2.4*</td>
<td>11.86±2.93*</td>
<td>12.66±2.52*</td>
</tr>
</tbody>
</table>
In this context, MECv antidiabetic mode of action could be related with an antihyperglycemic action through an enhancing insulin action (ligands for peroxisome-proliferator activated receptors, retinoid X receptor modulators and protein tyrosine phosphatase 1B inhibitors) and/or inhibitor of hepatic glucose production (inhibitors of pyruvate dehydrogenase kinase, liver-selective glucocorticoid receptor antagonists, 11β-HSD1 inhibitors, adenosine A₂B receptor antagonists, glucagon receptor antagonists, glycogen phosphorylase inhibitors, glucose-6-phosphatase inhibitors, fructose-1,6-bisphosphatase inhibitors and glycogen synthase kinase-3 inhibitors) (Sarabu and Tilley, 2005). Another possible related mechanism could be the antihyperglycemic action by regulation of glucose uptake from intestinal lumen, because of inhibition of carbohydrate digestion and absorption, or retardation of postprandial glucose increase by inhibition of α-glucosidases (Ortiz-Andrade et al., 2008). Thus, in order to establish if MECv possessed an in vivo antihyperglycemic effect, we evaluated the effect of the extract on oral glucose tolerance test (OGTT) in normoglycemic rats using glucose and sucrose as substrates. In both experiments, MECv induced a significant suppression in the increase of plasma glucose after substrate administration (Figures 1 and 2) compared to control (p<0.05), indicating that the mechanism of action of MECv is through an anti-hyperglycemic effect, mediated by the regulation of glucose uptake from the intestinal lumen, through the inhibition of carbohydrate digestion or absorption (Ortiz-Andrade et al., 2007). This could be possible by retarding the postprandial glucose by inhibition of glucose co-transporters (SGLT) of by the inhibition of intestinal α-glucosidase complex (Ortiz-Andrade et al., 2008). In order to test the last hypothesis, we determined MECv’s ability to inhibit the α-glucosidases. The extract inhibited α-glucosidase activity in vitro in a concentration-dependent manner (IC₅₀ of 1.9 mg/mL) (Figure 3). Consequently, with in vivo and in vitro experiments, we established that the inhibition of α-glucosidases is one of the mode of action of MECv as antidiabetic agent. Finally, the antihyperglycemic effect induced by MECv seems to be much more effective in vivo than in vitro. This observa-
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Figure 1. Effect of MECv on glycemia after a single oral administration of 2 g/kg of glucose in male Wistar rats. Each plot represents the means ± S.E.M. (n = 5); *p < 0.05; SS vs. control.

Figure 2. Effect of MECv on glycemia after a single oral administration of 2 g/kg of sucrose in male Wistar rats. Each plot represents the means ± S.E.M. (n = 5); *p < 0.05; MECv vs. control.

tion could be related with a possible blockade of glucose co-transporters as Glut-2 and Glut-4, main carriers of glucose from intestine to circulation (Li et al., 2006). This last asseveration is based on the presence of naringenin (NG) on the methanolic extract of C. vitifolium (Sánchez-Salgado et al., 2007). NG exhibited a strong inhibitory action on glucose uptake in normoglycemic rats and in diabetic rats reverted intestinal sleeves through the inhibition of intestinal and renal Na+-GLU co-transporters (SGLT) (Li et al., 2006). Therefore, we suggest that the main antidiabetic principle from MECv is naringenin, since its pharmacological properties have been related with antidiabetic activity. So, it was reported that NG induced hypoglycemic and antidiabetic activities in STZ-nicotinamide-induced diabetic rat models, and also, inhibited
Figure 3. Concentration-response curve of the inhibitory activity of MECv on in vitro activity of intestinal α-glucosidases. Each point represents the means ± S.E.M. (n = 4.)

Figure 4. Blood biochemical profiles in normoglycaemic and streptozotocin (STZ)–nicotinamide (Nc) diabetic rats treated with MECv, glyben clamide and vehicle during 5 days. *p < 0.05; MECv vs. control.
11β-hidroxysteroid dehydrogenase in vitro activity type 1 (Ortiz-Andrade et al., 2008). Moreover, Harmon and Patel (2003) described that NG inhibited insulin-stimulated glucose uptake in 3T3-L1 adipocytes, in a dose-dependent manner by inhibiting the phosphoinositide 3-kinase, a key regulator of insulin-induced glucose transporter (GLUT-4) translocation. Taking these data, we propose that MECv induced its antidiabetic effect by extrapancreatic events via inhibition of α-glucosidase Activity, and also by a possible blockade of glucose co-transporters.

On the other hand, STZ-nicotinamide-induced diabetic rats treated sub-chronically with MECv were anesthetized, and blood was withdrawn from the heart. Then, the following biochemical metabolites were determined: glucose, total cholesterol, HDL cholesterol and triglycerides (TG) (Figure 4). In STZ-nicotinamide-induced diabetic rats glucose blood concentration was significantly reduced to control values (p< 0.05); while TG were unexpectedly increased (p< 0.05). On the other hand, in normoglycemic rats HDL values were augmented than normoglycemic control group (p< 0.05).

Finally, a preliminary oral toxicological study of MECv was conducted. The LD₅₀ estimation using the ‘Acute Toxic Class’ method required to determine the effect of MECv at doses of 2000, 300, 50 and 5 mg/kg. Following 24 h of observation at each of these doses, all animals survived. LD₅₀ was determined and was assigned to category 5, that is >5000 mg/kg. MECv possesses a high LD₅₀ at doses given and can be considered as a non-toxic candidate for future drug detection and isolation.

In conclusion, findings allowed us to establish C. vitifolium as an important anti-diabetic agent. Furthermore, inhibition of intestinal α-glucosidase enzymes by MECv, suggest that this plant species is an alternative for the control of DM, and also it seems to be non toxic for its uses in traditional medicine. Finally, based on its capability to augment HDL-cholesterol in normal rats, and by the presence of NG that is able to diminish TG levels and to inhibit activity of 11βHSD type 1, MECv could be used for prevention and control of the metabolic syndrome.

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