



Gluconeogenesis inhibition and phytochemical composition of two *Cecropia* species

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ABSTRACT

Aim of the study: *Cecropia obtusifolia* and *Cecropia peltata* are plants highly used by the Mexican diabetic population to treat type 2 diabetes. Previous studies have assessed their hypoglycemic effect in animal models and in type 2 diabetic patients. Both plants contain chlorogenic acid, an inhibitor of glucose-6-phosphate translocase. In this work, we found a mechanism by which to understand how these plants could produce the observed hypoglycemic effect according to their traditional use. To test the hypothesis that targeting gluconeogenesis with an inhibitor of Gl-6-P translocase could result in a reduction of hepatic glucose production, we examined the effects of *Cecropia obtusifolia* and *Cecropia peltata* on gluconeogenesis (in vivo) and the activity of the enzyme (in vitro).

Materials and methods: The extracts of the two plants were analyzed by HPLC to confirm their phytochemical composition. To test the inhibition of gluconeogenesis in vivo, a pyruvate tolerance test (2 g/kg) was performed in 18-h fasted n5-STZ rats. The effect of the extracts (*Cecropia obtusifolia* and *Cecropia peltata* 150 mg/kg) on glucose-6-phosphatase activity was assayed in vitro with intact rat liver microsomes.

Results: Using HPLC-DAD, we confirmed that the main components of both species are chlorogenic acid and isoorientin. Diabetic rats treated with the extracts showed a lower glucose curve. The tested extracts were able to reduce the increase in the glucose blood level, and they inhibited the glucose-6-P activity with IC₅₀s of 224 µg/ml for *Cecropia obtusifolia* aqueous, 160 µg/ml for *Cecropia obtusifolia* butanolic, 146 µg/ml for *Cecropia peltata* aqueous and 150 µg/ml for *Cecropia peltata* butanolic.

Conclusions: The results of the experiments presented here suggest that the administration of both plants can improve glycemic control by blocking the hepatic glucose output, especially in the fasting state. These data support the traditional use of the plants as “agua de uso”, a cold infusion of the plant consumed over the course of a day.

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1. Introduction

Type 2 diabetes is one of the most prevalent health problems in Mexico (SSA, 2009). Common treatment options include a wide variety of both medicinal products and health food plants (Andrade-Cetto, 1995; Andrade-Cetto and Heinrich, 2005). In Mexico, at least 306 species from 235 genera and 93 families have been reported for the treatment of diabetes. On a global level, type 2 diabetes is the most common endocrine disorder, and the World Health Organization (WHO, 2009) estimates that by 2030 the number of diabetic people across the world will have increased to 214% of the number of cases in 2000. However, the increase in the prevalence

of this disease is predicted to be even greater in Mexico; the number of diabetic patients is set to increase from 2.2 million in 2000 to more than 6 million in 2030 (an increase of 281%).

The term diabetes mellitus is used to refer to a metabolic disorder of multiple etiologies in which chronic hyperglycemia is caused by defects or alterations in either the secretion or action of insulin. This results in disturbances in carbohydrate, fat and protein metabolism. Type 2 diabetes is caused either predominantly by insulin resistance with a relative deficiency of insulin or by impaired insulin secretion that may or may not be accompanied by insulin resistance (WHO, 1999; Inzucchi, 2003).

2. Background

2.1. Botanical description

Cecropia obtusifolia Bertol (CO), Cecropiaceae, is widespread in México and can be found along both coasts. It is a monopodic Tree

Abbreviations: CO, *Cecropia obtusifolia*; CP, *Cecropia peltata*; CA, chlorogenic acid; IO, Isoorientin; G6P, glucose-6-phosphatase; BuOH, butanolic extract; AE, aqueous extract.

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20–25 m tall, growing as secondary vegetation in the tropical rain-forest. The tree has a tall, straight, hollow trunk. The leaves are in a spiral disposition located at the top of the branches and are simple, peltate or deeply palmate, with a deep green color on the upper surface and grey on the lower surface; the flower is a spike 8–20 cm long.

On the pacific coast and especially on the Yucatan peninsula, it is possible to find *Cecropia peltata* L. (CP), which can be distinguished from CO only by the size of the flowers that are smaller, only 3–5 cm (Pennington and Sarukhán, 1998).

2.2. Ethnobotany

Cecropia obtusifolia is traditionally called “Guarumbo”, “Chan-carro” and “Hormiguillo”, while *Cecropia peltata* has a traditional Maya name, “X cooch”. Both species are used by Mexican traditional healers for the treatment of type 2 diabetes and are sold at market-places. CO is common in central México while CP is common in the Yucatan area. Traditionally the dry leaves (15 g) are boiled in water (500 ml), and the resulting infusion is cooled in the pot before being filtered and drunk as “agua de uso”. The cold infusion is consumed over the course of a day (Andrade-Cetto, 1999; Andrade-Cetto et al., 2007).

2.3. Phytochemical constituents

From the butanolic extract of CO (Andrade-Cetto and Wiedenfeld, 2001) isolated chlorogenic acid (CA) and isoorientin. These isolated compounds, which are also found in the medicinal tea, were identified as the main constituents of the plant. By an HPLC comparison (Andrade-Cetto et al., 2007) could demonstrate that these compounds are also the main constituents of CP.

2.4. Previous pharmacological data

The hypoglycemic effect of *Cecropia obtusifolia* was demonstrated by our group in streptozotocin diabetic rats (Andrade-Cetto and Wiedenfeld, 2001) and in type 2 diabetic patients (Revilla-Monsalve et al., 2007) and by other researchers in different animal models (Pérez et al., 1984; Román-Ramos et al., 1991) and diabetic patients Herrera-Arellano et al. (2004). These studies were previously reviewed by Andrade-Cetto and Heinrich (2005).

Alonso-Castro et al. (2008) demonstrated that chlorogenic acid and an aqueous extract of the CO plant stimulated glucose uptake in both insulin-sensitive and insulin-resistant 3T3-F442A adipocytes. However, based on the authors data, to obtain the tested in vitro concentration of CA (100 µg/ml) required to increase the uptake of the glucose analog by 35%, approximately 7 g of the plant are needed, when traditionally between 15 and 20 g are used for one 70 kg person.

For *Cecropia peltata*, the hypoglycemic effect was demonstrated by Nicasio et al. (2005) and by our group (Andrade-Cetto et al., 2007).

Andrade-Cetto and Heinrich (2005) stated that the possible mechanism of the activity of the plant is by reducing the hepatic glucose output due to the inhibition of Gl-6-P by chlorogenic acid, which can simultaneously target gluconeogenesis and glycogenolysis.

2.5. Aim of the study

Previously, chlorogenic acid had been identified as a specific inhibitor of the glucose-6-phosphate translocase (Gl-6-P translocase) component in microsomes of rat liver (Hemmerle et al., 1997). Because this acid is one of the constituents of *Cecropia* we test the hypothesis that targeting gluconeogenesis with an inhibitor

of Gl-6-P translocase would result in a reduction of hepatic glucose production, we examined the effects of *Cecropia obtusifolia* and *Cecropia peltata* on gluconeogenesis (in vivo) and the activity of Gl-6-P (in vitro).

3. Materials and methods

3.1. Plant materials and phytochemical composition

With the help of traditional healers, fresh leaves of *Cecropia obtusifolia* and *Cecropia peltata* were collected; CO was collected in the Mexican state of Hidalgo and CP in Yucatan. Their identity was confirmed and voucher specimens were deposited at the IMSS Herbarium in Mexico City (IMSS 14695, IMSS 14696 and IMSS 14697). The butanolic extract (BuOH) and aqueous extract (AE) of both plants were prepared as previously described (Andrade-Cetto and Wiedenfeld, 2001; Andrade-Cetto et al., 2007).

The phytochemical composition of the extracts was confirmed by application on a Nucleosil 60-30 C18 (Macherey & Nagel, Düren, Germany) column and eluted with H₂O/MeOH/AcCN 70:15:15, 4 ml/min monitored by HPLC-DAD, Beckman System Gold. The similarities between the compounds were analyzed with Beckman 32 Karat software.

3.2. Diabetic animals

Five-day-old Wistar rats (weighing 10–12 g) received a 90 mg/kg i.p. injection of streptozotocin (STZ; Sigma, No. 242-646-8) in acetate buffer 0.1 M, pH 4.5. The non-diabetic control group received only buffer via i.p. injection. At 4 weeks of age, rats were separated from their mothers and acclimatized with free access to food and water in an air conditioned room (23 °C with 55% humidity) under a 12:12 h light/dark cycle. After 12 weeks, animals with fasting glucose values over 150 mg/dl were selected.

The animals were handled according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (OACU, 1996). All methods used in this study were approved by the Internal Council of the “Facultad de Ciencias”, Universidad Nacional Autónoma de México.

3.3. Glucose determination

Blood samples were taken from the tail vein (IACUC, 1999). The glucose concentration was measured in plasma serum with a Reflotron instrument and confirmed by Accutrend GC (Roche). Thirty-two microliters of blood were used for each assay.

3.4. In vivo pyruvate load test

Fifteen minutes after the oral administration of the extracts or the drugs, 2 g/kg pyruvate (Sigma 2256) was administered intraperitoneally to 18-h fasting n5-STZ rats that weighed approximately 250 g. The rats were assigned to one of six groups ($n = 11$ per group): group one – non-diabetic control; group two – diabetic control; group three – CA (Sigma C3878), 5 mg/kg; group four – CO-BuOH, 150 mg/kg; group five – CP-BuOH, 150 mg/kg and group six – Metformin (Me) 0.012 mg/kg. Glucose was measured at times 0, 30, 60, 90 and 120 min.

3.5. Glucose-6-phosphatase activity

The in vivo results were confirmed in vitro using components of the rat hepatic glucose 6-phosphatase system. Intact rat liver microsomes were obtained according to Arion et al. (1997) and the enzymatic activity was calculated by measuring the formation of inorganic phosphorus from glucose-6-phosphate. We tested

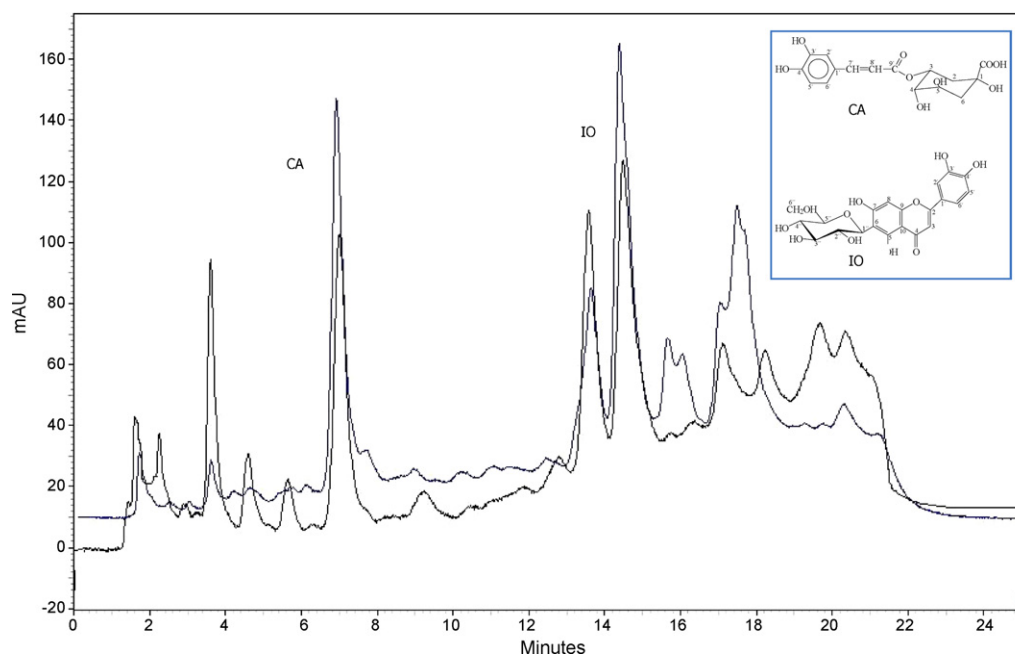


Fig. 1. HPLC comparison of both extracts of *Cecropia*. Upper line – *C. obtusifolia*; lower line – *C. peltata*. In the frame structures of both compounds.

both plants at concentrations of 2, 5, 20, 50, 200, 500, 1000 and 2000 $\mu\text{g/ml}$. Chlorogenic acid was tested at 1 mM (354 $\mu\text{g/ml}$) to assess the enzyme inhibition. To be consistent with our work, we tested the butanolic extracts. We decided to test only the aqueous extract *in vitro* because it is traditionally used and we observed activity.

3.5.1. Liver microsomes

A 250 g Wistar rat was anesthetized with pentobarbital (6 mg/100 g b.w., i.p.). The portal vein was perfused with ice-cold saline and the lower vena cava excised. The liver was dissected, weighed and minced. A 20% homogenate in 0.25 M sucrose, 1 mM EDTA and 5 mM HEPES pH 7.4, was prepared in a Duncce homogenizer with 8 strokes up and down of a loose pestle. The homogenate was filtered through a nylon mesh and centrifuged at 1000 g for 10 min. The supernatant was spun at 12,000 g for 10 min. The post-mitochondrial supernatant was centrifuged at 100,000 g for 1 h. The microsomes were suspended in homogenizing media. All procedures were performed at 4 °C. Protein was determined by the method of Bradford using bovine serum albumin as standard. It is important to remark that in these experiments we obtained intact microsomes.

3.5.2. Glucose-6-phosphatase assay

Glucose-6-phosphate hydrolysis was determined in microsomes at 22 °C using a colorimetric assay described by Arion (1989) with some modifications. Briefly, 100 μg microsomal protein was incubated for 20 min at 22 °C in a total of 100 μl assay buffer (250 mM sucrose, 20 mM Imidazol, pH 7.0) containing 2 mM glucose 6-phosphate in the presence or absence of test compound (0.2–2000 $\mu\text{g/ml}$). The reaction was started by the addition of 900 μl phosphate color reagent (Arion, 1989) and the formation of inorganic phosphate was quantified colorimetrically after incubation at 45 °C for 20 min by reading the extinction at 830 nm. For each concentration of test compound, background extinction was determined in parallel incubations by adding phosphate color reagent before addition of the microsomes. Stock solutions of test compounds were prepared in water for aqueous extracts and in ethanol

for butanolic extracts and diluted with assay buffer. The resulting maximal ethanol concentration of 5% (v/v) did not affect the phosphatase activity (data not shown). IC_{50} values were determined by the graphic method.

4. Results

4.1. Phytochemical composition

Using HPLC-DAD we confirmed that the main components of both species are chlorogenic acid and isoorientin (Fig. 1), the other main peaks are precursors of this molecules as previously reported (Andrade-Cetto and Wiedenfeld, 2001; Andrade-Cetto et al., 2007).

The similarity of the UV spectra between the original isolated chlorogenic acid (Andrade-Cetto and Wiedenfeld, 2001) and the CA present in the extracts was 1 for CO and .9929 for CP. The similarity between the original isolated isoorientin and the extracts was .9915 for CO and .983 for CP (data not shown).

4.2. *In vivo* pyruvate load test

Between 30 and 90 min after pyruvate administration, glucose levels in the non-diabetic control group were significantly higher than at time 0 of the same group and then returned back to the initial values by 120 min. In the diabetic group, glucose values rose beginning at 30 min and they did not return to the initial value. Both plant extracts and CA were able to block this increase in the blood glucose level concentrations starting at 60 min, while the effect of metformin was observed until 120 min (Table 1).

4.3. Glucose-6-phosphatase activity

To assess the degree of inhibition of the hydrolysis of glucose-6-phosphate, we plotted a dose-response curve and reported the results as the IC_{50} . The measured IC_{50} s are CO; Aqueous 224, CO; Butanolic 160, CP; Aqueous 146 and CP; Butanolic 152. All of the extracts tested of both plants showed an IC_{50} within the same order of magnitude. The aqueous extract of CO showed the highest value.

Table 1
Pyruvate tolerance test on n5-STZ diabetic rats.

Groups	Gl (mg/dl), T0	Gl (mg/dl), T30	Gl (mg/dl), T60	Gl (mg/dl), T90	Gl (mg/dl), T120
1-Control ND	118 ± 2	145 ± 8	137 ± 6	128 ± 3	118 ± 3
2-Control D	154 ± 7	171 ± 6a	207 ± 12b	212 ± 19b	212 ± 20b
3-D + CA	139 ± 8	164 ± 4	150 ± 6c	151 ± 5c	148 ± 5c
4-D + CO	148 ± 4	156 ± 5	161 ± 9c	147 ± 11c	141 ± 6c
5-D + CP	145 ± 4	157 ± 12	148 ± 14c	129 ± 6c	137 ± 7c
6-D + Me	146 ± 3	172 ± 8	190 ± 14	173 ± 13	154 ± 8c

The values represent the mean ± SEM as compared with control time intervals. Letter “a” shows significance versus Control (ND) group at $p < 0.05$, letter “b” shows significance versus control (ND) group at $p < 0.001$, letter “c” shows significance versus control (D) group at each sample time at least at $p < 0.01$. CA, chlorogenic acid; CO, *Cecropia obtusifolia*; CP, *Cecropia Peltata*; Me, *Metformin*.

5. Discussion

On the basis of the phytochemical results, we can conclude that the compositions of the butanolic extracts of both plants are similar, with chlorogenic acid and isoorientin as the main components. We have previously demonstrated that the aqueous extract has a similar composition as the butanolic extract (Andrade-Cetto and Wiedenfeld, 2001).

Insulin resistance involving both muscle and liver are characteristic features of the glucose intolerance in type 2 diabetic individuals. In the basal state, the liver represents a major site of insulin resistance, and this is reflected by overproduction of glucose despite the presence of both fasting hyperinsulinemia and hyperglycemia. This accelerated rate of hepatic glucose output is the primary determinant of the elevated fasting plasma glucose concentration in type 2 diabetic individuals (DeFronzo and Mandarino, 2009).

In the postabsorptive state, approximately 85% of endogenous glucose production is derived from the liver, and the remaining amount is produced by the kidney (Ekberg et al., 1999). Approximately half of basal hepatic glucose production is derived from glycogenolysis and half from gluconeogenesis. Glucose-6-phosphatase is an enzyme that hydrolyzes glucose-6-phosphate, which results in the creation of a phosphate group and free glucose. Glucose is then exported from the cell via membrane glucose transporter proteins. This catalysis completes the final steps in gluconeogenesis and glycogenolysis and therefore plays a key role in the homeostatic regulation of blood glucose levels; this is particularly so in the fasting state.

Because pyruvate (together with citric acid) is the main source of hepatic glucose production after a long fasting period, we can conclude that CA, CO and CP are able to block this pathway. Metformin did not prevent the glucose elevation at the beginning of the experiment, the hypoglycemic effect was observed until 120 min. We suggest that CA is a good control drug for this kind of experiments.

The IC₅₀s of aqueous and butanolic extracts of both plants are similar and within the same order of magnitude. This suggests that the same compound(s) is/are responsible for glucose-6-phosphatase inhibition and that both plants have similar concentrations.

In the clinical trials of CO (Revilla-Monsalve et al., 2007), we observed a hypoglycemic effect that began after 4 weeks of treatment and became stable after 18 weeks. As this effect was paralleled by changes in the Hb1Ac levels, we assumed that the regular consumption of this plant can control high plasma glucose levels. The results of the experiments presented here suggest that the administration of CO or CP can improve the glycemic control by blocking the hepatic glucose output, especially in the fasting state. This can explain previous observations and supports the traditional use as “agua de uso,” a cold infusion of the plant consumed over the course of a day.

From an ethnopharmacological perspective we have to understand that the administration of a plant extract at traditional doses

normally involves low concentrations of the active principles. For this reason, to explain a hypoglycemic effect for these extracts, more than one mechanism of action must be involved. In this case, in addition to other possibilities, the ability of *Cecropia obtusifolia* and *Cecropia peltata* to block hepatic glucose output can explain their hypoglycemic effect.

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