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Inhibition of gluconeogenesis by *Malmea depressa* root

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ABSTRACT

Ethnopharmacological relevance: *Malmea depressa* is traditionally used in the Mayan communities of southeastern Mexico to treat type 2 diabetes. A root bark infusion is being taken throughout the day, between meals.

Aim of the study: The aim of this study was to determine whether an ethanolic extract of *Malmea depressa* would reduce hepatic glucose production by targeting gluconeogenesis. The effects of the plant extract on gluconeogenesis (*in vivo*) and the activity of GL-6-P (*in vitro*) were examined.

Materials and methods: The plant extract was analyzed by HPLC to confirm its phytochemical composition. The inhibition of gluconeogenesis was tested *in vivo* by performing a pyruvate tolerance test in n5-STZ after an 18-h fasting period. The extracts effect on glucose-6-phosphatase activity were assayed *in vitro* with intact rat liver microsomes.

Results: Using HPLC-DAD we confirmed that the phytochemical compositions of the tested extract were similar to those previously reported. We proved that the ethanolic extract of the root bark of *Malmea depressa* dose-dependently inhibits a glucose peak. Furthermore, the gluconeogenesis inhibition was confirmed *in vitro* using a pyruvate test.

Conclusions: The results suggest that administration of *Malmea depressa* can improve glycemic control by blocking hepatic glucose production, especially in the fasting state. These data support its traditional use as an infusion consumed continually throughout the day.

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1. Introduction

Diabetes mellitus type 2 (DMT2) is defined as an elevated blood glucose level associated with an absent or inadequate pancreatic insulin secretion. This may be expressed with or without concurrent impairment of insulin action. Diabetes mellitus type 2 is characterized by tissue resistance to the action of insulin combined with a relative deficiency in insulin secretion. Although patients with insulin resistance still produce insulin in their beta cells, the secretion is inadequate and the blood glucose level increases (American Diabetes Association, 2011).

In DMT2, insulin resistance in the liver reflects the failure of hyperinsulinemia to suppress gluconeogenesis, resulting in fasting hyperglycemia and decreased liver glycogen storage in the postprandial state. Increased hepatic glucose production occurs early in the course of diabetes, although the increase is more pronounced after the onset of insulin secretory abnormalities and insulin resistance in skeletal muscle (DeFronzo and Mandarino, 2009).

Gluconeogenesis is important in preventing an excessive reduction in the blood glucose concentration during fasting. Glucose is the primary substrate for energy in tissues such as the brain and the red blood cells, and adequate amounts of glucose must be present in the blood for several hours between meals. The liver plays a key role in maintaining blood glucose levels during fasting by converting its stored glycogen into glucose (glycogenolysis) and by synthesizing glucose, mainly from lactate and amino acids (gluconeogenesis) (Guyton and Hall, 2006).

In type 2 diabetic subjects with mild to moderate fasting hyperglycemia (i.e., 140–200 mg/dl), basal hepatic glucose production is increased by approximately 0.5 mg/kg per min. Consequently, during the overnight sleeping hours (i.e., 22:00 h to 08:00 h), the liver of an 80-kg diabetic individual with modest fasting hyperglycemia adds an additional 35 g of glucose to the systemic circulation. The increase in basal (hepatic glucose production) HGP is correlated closely with the severity of fasting hyperglycemia. Thus, in type 2 diabetics with overt fasting hyperglycemia (i.e., >140 mg/dl), an excessive rate of hepatic glucose output is the major abnormality that is causes the elevated fasting plasma glucose levels (DeFronzo and Mandarino, 2009).

DMT2 is one of the most prevalent health problems in Mexico (Secretaría de Salubridad y Asistencia, 2011), and a wide variety of

Abbreviations: MD, *Malmea depressa*; CA, chlorogenic acid; G6P, glucose-6 phosphatase; EE, ethanolic extract.

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medicinal products and health food plants are commonly used to treat diabetes (Andrade-Cetto and Heinrich, 2005). In Mexico, more than 306 plant species from 235 genera and 93 families have been suggested as treatments for diabetes.

Malmea depressa (Baill) R.E. Fries, (Annonaceae), also known as the "Elemuy" tree, is highly valued by Mayan communities for the treatment of type 2 diabetes, especially in the state of Yucatan in southwest Mexico (Andrade-Cetto et al., 2006). The root bark, used to prepare herbal teas, is reported to be prepared in two different ways: boiled or cold (macerated) in water. Subsequently, both preparations are ingested orally throughout the day as the so-called "Agua de Uso" between meals (Andrade-Cetto et al., 2005).

A previous study demonstrated the acute hypoglycemic effect of water (Aq), butanolic (BE) and ethanolic (EE) extracts on streptozotocin-diabetic rats. EE showed a statistically significant hypoglycemic effect 60 min after oral administration (Andrade-Cetto et al., 2005). Furthermore, we proved that the chronic administration of BE can improve glycated hemoglobin levels in diabetic rats (Andrade-Cetto et al., 2008).

From the pharmacological active fractions (i.e., BE and EE), two phenylbutane derivatives (2-hydroxy-3,4,5-trimethoxy-1-(2′,4′-hydroxy-3′-dihydroxy)butyl-benzene and 2-hydroxy-3,4,5-trimethoxy-1-(2′,3′,4′-hydroxy)butyl-benzene) (Andrade-Cetto et al., 2005) as well as a phenylpropane derivative, 3-(3-hydroxy-2,4,5-trimethoxyphenyl) propane-1,2 diol, were isolated (Andrade-Cetto et al., 2008).

In previous work, we demonstrated the ability of *Cecropia obtusifolia* Bertol and *Cecropia peltata* L. to block hepatic glucose output. Because chlorogenic acid is a specific inhibitor of the glucose-6-phosphate translocase (Gl-6-P translocase) component in rat microsomes, it was used as positive control (Andrade-Cetto and Cárdenas-Vázquez, 2010).

The aim of our study was to test the hypothesis that targeting gluconeogenesis with the ethanolic extract of the root cortex of *Malmea depressa* would result in a reduction of hepatic glucose production; we examined the effects of the plant extract on gluconeogenesis (*in vivo*) and the activity of Gl-6-P (*in vitro*).

2. Materials and methods

2.1. Plant extracts

With the assistance of a traditional healer, samples of *Malmea depressa* were collected in Chikindzonot, Yucatan, Mexico. The identity of the samples was confirmed, and voucher specimens were deposited at the "Instituto Mexicano del Seguro Social" (IMSS) Herbarium in Mexico City (IMSS 14706) as previously reported (Andrade-Cetto et al., 2008).

The Ethanolic extract was prepared as previously reported (Andrade-Cetto et al., 2005) from the root bark (50 g) through fine grinding and extracting with a Soxhlet apparatus for 24 h. The extract was dried with a rotary evaporator resulting in 15 g of EE extract. The ratio of the herbal drug to the native herbal drug preparation (DERnative) was 3.3:1 (Gaedcke and Steinhoff, 2003).

The EE phytochemical composition was confirmed by application on a Nucleosil 60-30 C18 (Macherey & Nagel, Düren, Germany) column and was eluted with H_2O/MeOH/AcCN 70:15:15, 4 mL/min monitored using HPLC-DAD, (Beckman System Gold). The similarities between the compounds were analyzed with Beckman 32 Karat software.

2.2. Diabetic animals and glucose determination

Five-day-old Wistar rats (weighing 10–12 g) received a 90 mg/kg intraperitoneal (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, rats were separated from their mothers and acclimated 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 greater than 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 (Office of Animal Care and Use, of the National Institute of Health, USA, 1996). All methods used in this study were approved by the Internal Council of the "Facultad de Ciencias" of the Universidad Nacional Autónoma de México.

Blood samples were obtained from the tail vein according to Institutional Animal Care and Use Committee, protocol Institutional Animal Care and Use Committee, 99. The glucose concentration was measured in the plasma serum with a Reflotron instrument and was confirmed with Accutrend GC (Roche); 32 μl of blood was used for each assay.

2.3. In vivo pyruvate load test

N5-STZ rats, weighing approximately 250 g, were fasted for 18 hr. Subsequently, the drug extracts were administered orally followed 15 min later by i.p. administration of 2 g/kg of pyruvate (Sigma 2256). 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, metformin (Me), 0.012 mg/kg; group five, EE 80 mg/kg; and group six, EE 60 mg/kg. In all groups, glucose was measured at 0, 30, 60, 90 and 120 min, after the i.p. injections.

2.4. 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 inorganic phosphorus formation from glucose-6-phosphate. We tested the EE at concentrations of 2, 5, 20, 50, 200, 500, 1000 and 2000 μg/ml. Chlorogenic acid was used at 1 mM to assess enzyme inhibition.

2.4.1. Liver microsomes

A 250-g Wistar rat was anesthetized with pentobarbital (6 mg/100 g, i.p.). The portal vein was perfused with ice-cold saline and the lower vena cava was 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 Dounce homogenizer with a loose pestle moving 8 strokes up and down. 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, and 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. The protein level was determined using the Bradford method with bovine serum albumin as the standard. Notably, we obtained only intact microsomes in these experiments.

2.4.2. Glucose-6-phosphatase assay

Glucose-6-phosphate hydrolysis was evaluated in microsomes using a colorimetric assay at 22 °C 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 of assay buffer (250 mM sucrose, 20 mM Imidazol, pH 7.0) containing 2 mM glucose 6-phosphate in the presence or absence of the test compound (0.2–2000 μg/mL). The reaction was initiated by the addition of

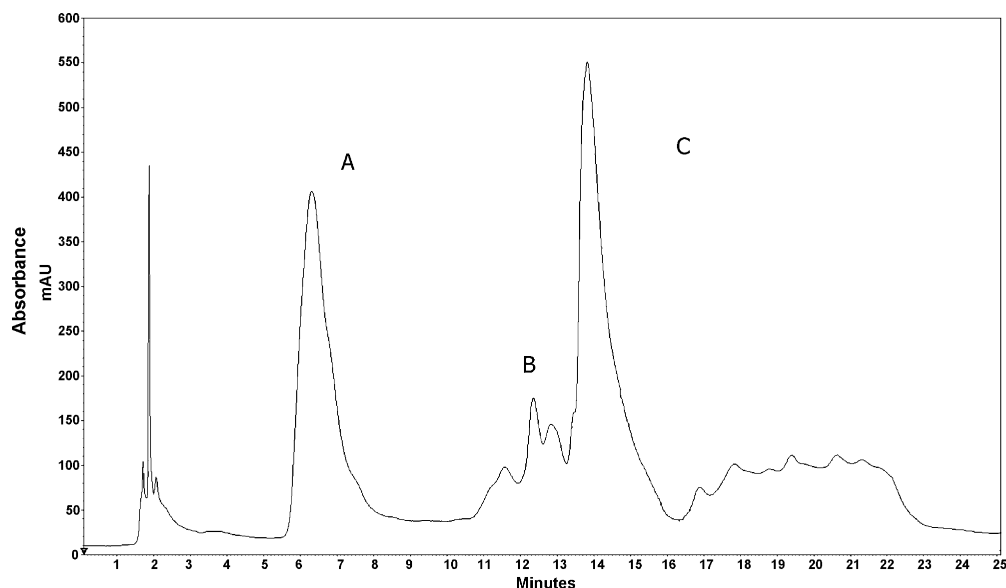

Fig. 1. HPLC-DAAD of the ethanolic extract of *Malmea depressa* root: (A) (2-hydroxy-3,4,5-trimethoxy-1-(2′,4′-hydroxy-3′-dihydroxy)butyl-benzene, (B) 2-hydroxy-3,4,5-trimethoxy-1-(2′,3′,4′-hydroxy)butyl-benzene), (C), 3-(3-hydroxy-2,4,5-trimethoxyphenyl) propane-1,2.

Table 1
The pyruvate tolerance test on n5-STZ diabetic rats. The values represent the mean ± SEM. Superscript letters in the same row indicate statistical differences compared with time 0. Superscript numbers in the same column indicate statistical differences from the control group (diabetic control group compared with non-diabetic control). "a" shows significance at $p < 0.05$, "b" shows significance at $p < 0.01$ and "c" shows significance at least at $p < 0.001$. Gl, glucose; ND, no diabetic; D, diabetic; CA, chlorogenic acid; Me, metformin; Md, *Malmea depressa* ethanolic extract.

Groups	Gl (mg/dl)T0	Gl (mg/dl)T30	Gl (mg/dl)T60	Gl (mg/dl)T90	Gl (mg/dl)T120
1 ND Control	115 ± 1	142 ± 8^{a}	134 ± 6^{a}	125 ± 3^{a}	114 ± 3
2 D Control	144 ± 7^{3}	186 ± 5^{c,3}	221 ± 8^{c,3}	236 ± 13^{c,3}	278 ± 22^{c,3}
3 D + CA	142 ± 6	168 ± 4^{c,1}	154 ± 7^{1}	155 ± 6^{1}	151 ± 7^{1}
4 D + Me	148 ± 3	169 ± 8^{a}	187 ± 14^{c}	172 ± 13^{b,1}	155 ± 8^{3}
5 D + Md80 mg/kg	141 ± 3	149 ± 4^{3}	150 ± 3^{b,3}	146 ± 4^{3}	146 ± 4^{3}
6 D + Md60 mg/kg	142 ± 1	152 ± 4^{b,3}	156 ± 4^{c,3}	147 ± 4^{3}	147 ± 4^{3}

glucose-6-phosphate. The reaction was halted by the addition of 900 μl of phosphate color reagent (Arion, 1989), and the formation of inorganic phosphate was colorimetrically quantified by reading the result at 830 nm after incubation at 45 °C for 20 min. Background extinction was determined in parallel incubations by adding phosphate color reagent before the addition of the microsomes at each concentration of the test compound. Stock solutions of the test compounds were prepared in water for aqueous extracts and in ethanol for butanolic extracts and were then diluted with the 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 using the graphic method.

2.5. Statistical analysis

The data were statistically analyzed with one-way ANOVA followed by a Tukey's test. The plasma glucose levels were expressed as the mean ± S.E.M.

3. Results

3.1. Phytochemical composition

Using HPLC-DAD, we confirmed that the phytochemical compositions of the tested extract was similar to those previously reported (Andrade-Cetto et al., 2005, 2008; Fig. 1).

3.2. 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 and returned to baseline by 120 min after pyruvate administration. In the diabetic control group, glucose increased beginning at 30 min and did not return to baseline. After 60 min, the chlorogenic acid inhibited the glucose peak more than in the diabetic control group and relative to its own baseline. The metformin control group also showed more pronounce inhibition of the peak at 90 min compared to the diabetic control. Both *Malmea depressa* BE concentrations highly inhibited the glucose peak at 30 min. The higher extract concentration also produced a statistically significant inhibition at 30 min compared to baseline (Table 1).

3.3. Glucose-6-phosphatase activity

To assess the degree of inhibition of glucose-6-phosphate hydrolysis, we plotted a dose–response curve and reported the results as the IC_{50}, as shown in Table 2.

Table 2
IC_{50} values tested at 1 mM.

Extract	IC_{50} (μg/ml)
Ethanolic extract	267.62
Chlorogenic acid	354

4. Discussion

The phytochemical results confirmed that the (2-hydroxy-3,4,5-trimethoxy-1-(2′,4′-hydroxy-3′-dihydroxy) butyl-benzene, the 2-hydroxy-3,4,5-trimethoxy-1-(2′,3′,4′-hydroxy)butyl-benzene) and the 3-(3-hydroxy-2,4,5-trimethoxyphenyl) propane-1,2 are present in the tested EE.

We confirmed with the *in vitro* studies that chlorogenic acid inhibits the hydrolysis of glucose-6-phosphate, preventing glucose-6-phosphatase activity; thus, chlorogenic acid is a valid control drug for this type of study and can be used as a reference inhibitor of G-6-Pase.

In the postabsorptive state, approximately 85% of endogenous glucose production occurs in the liver, and the remaining amount occurs in the kidney (Ekberg et al., 1999).

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 in the overproduction of glucose despite the presence of both fasting hyperinsulinemia and hyperglycemia. This accelerated rate of hepatic glucose output is the primary cause of the elevated fasting plasma glucose concentration in type 2 diabetic individuals (DeFronzo and Mandarino, 2009).

Approximately half of the basal hepatic glucose production occurs via glycogenolysis and the other half via 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 particularly 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 CO and MD are able to block this pathway.

The hypoglycemic effect of metformin was not observed until 120 min. The *in vivo* studies showed that the EE of *Malmea depressa* root bark dose-dependently inhibits the glucose peak under a pyruvate test, the probable effect of the extracts as an inhibitor of gluconeogenesis was confirmed *in vitro*.

As mentioned previously, insulin resistance in the liver during type 2 DM reflects the failure of hyperinsulinemia to suppress gluconeogenesis, resulting in fasting hyperglycemia. This effect correlates with the liver of an 80-kg diabetic individual with modest fasting hyperglycemia and adds an additional 35 g of glucose to the systemic circulation.

In addition to other possible mechanisms (e.g., stimulation in the insulin secretion), *Malmea depressa* acts by blocking gluconeogenesis in the fasting state. Furthermore, this effect is correlated with the plant's traditional use, where an infusion is prepared and consumed throughout the day.

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