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Euphorbia dioeca Kunth as a novel source for α-glucosidase inhibitors

[*Euphorbia dioeca* Kunth como una nueva fuente de inhibidores de α-glucosidasa]

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Abstract: *Euphorbia dioeca* Kunth belongs to the Wanderer's herb complex that is traditionally used for skin diseases and recently as antidiabetic. The methanol and aqueous extracts were evaluated for their *in vitro* α-glucosidase inhibitory activity and an oral starch tolerance test. These extracts showed an IC_{50} of 0.55 and 0.85 mg/mL, respectively. In diabetic Long Evans rats, the methanol and aqueous extracts reduced significantly the postprandial hyperglycemia peak in 15.2% and 12.8%, respectively. The α-glucosidase inhibitory activity is related with the presence of glycosides, phenolic compounds and flavonoids. Additionally, the safety parameters of both extracts were assessed by means of an acute toxicity test, being classified as innocuous. The traditional use of *E. dioeca* to control type 2 diabetes was confirmed, being an important source of α-glucosidase inhibitors.

Keywords: *Euphorbia dioeca*, Mexican Traditional Medicine, Type-2 diabetes, Wanderer's herb complex

Resumen: *Euphorbia dioeca* Kunth, pertenece al complejo de plantas medicinales denominado Hierba de la Golondrina; el cual se utiliza para diversos padecimientos, destacando su uso como antidiabético. En dicho marco, se evaluó tanto la actividad inhibitoria de α-glucosidasa *in vitro*, como su desempeño en una prueba de tolerancia a una carga de almidón postprandial. Los extractos inhibieron la actividad de la α-glucosidasa con una CI_{50} de 0.55 y 0.85 mg/mL, respectivamente. Los extractos metanólico y acuoso disminuyeron significativamente el pico hiperglucémico postprandial en un 15.2% y un 12.8%, respectivamente, cuando se evaluó en ratas diabéticas. La actividad inhibitoria de α-glucosidasa, reflejada en ambas pruebas, está relacionada con la presencia de glicósidos, compuestos fenólicos y flavonoides. De manera adicional, ambos extractos fueron evaluados en una prueba de toxicidad aguda, siendo clasificados como inocuos. Se corroboró el uso tradicional de *E. dioeca* para el control de la diabetes tipo 2, siendo una importante fuente de compuestos inhibidores de α-glucosidasa.

Palabras clave: *Euphorbia dioeca*, Medicina Tradicional Mexicana, Diabetes tipo 2, Hierba de la Golondrina

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INTRODUCTION

Type-2 diabetes mellitus (T2DM) is a polygenic disease characterized by a state of glucose intolerance associated with insulin resistance (multiple deficiencies in insulin action in muscular, adipose or hepatic tissues) and relative insulin deficiency due to pancreatic β-cell failure (El-Kaissi & Sherbeeni, 2011; Israili, 2011). In addition, complications from T2DM, such as cardiovascular disease, peripheral vascular disease, stroke, diabetic neuropathy, amputation, renal failure, and blindness result in increasing disability and reduced life expectancy (Israili, 2011).

There are 346 million people worldwide with diabetes. In high-income countries, diabetes primarily affects people over 50 years of age. But in middle-income countries, as Mexico, the highest prevalence is in the most productive age groups. This trend will put a huge pressure on healthcare systems and governments expenditure (Scully, 2012).

The pharmacological strategy for the control of T2DM has focused on increasing insulin levels by oral secretagogues that promote insulin secretion (*e.g.* sulfonylureas), improving tissue sensitivity with an insulin sensitizer (*e.g.* metformin or thiazolidinediones), enhancing the incretins activity (*e.g.* glucagon-peptide-1 mimetic), or reducing the rate of carbohydrate absorption from the gastrointestinal tract (*e.g.* α-glucosidase inhibitors) (El-Kaissi & Sherbeeni, 2011; Israili, 2011).

The α-glucosidase inhibitors block the enzymes bounded to the brush border of the small intestine, which hydrolyzes complex carbohydrates molecules into glucose and other monosaccharides, leading to delayed glucose absorption and lower post-prandial blood glucose peak (El-Kaissi & Sherbeeni, 2011; Israili, 2011; Kumar *et al.*, 2011). The α-glucosidase inhibitors do not affect the serum lipids, and do not cause hypoglycemia when used in monotheraphy, but may increase the risk of insulin or insulin-secretagogue-associated hypoglycemia in combination therapy. The most common side effects of α-glucosidase inhibitors are gastrointestinal symptoms such as flatulence, diarrhea, and abdominal fullness, because the carbohydrates remain in the intestine and the colon where bacteria digest the carbohydrates releasing gases (El-Kaissi & Sherbeeni, 2011; Israili, 2011).

In Mexico, like in the rest of the world, T2DM had a widespread incidence. There are more than 6.4 million of diabetics, but the number could be duplicated if we consider the lack of diagnose in many regions (Gutiérrez *et al.*, 2012).

There are several natural products with a α-glucosidase inhibitors activity, such as flavonoids, alkaloids, terpenoids, glycosides and phenolic compounds; being the plants a priceless source for novel compounds aimed to the control of T2DM (Borges de Melo *et al.*, 2006; Wardrop & Waidyarachchi, 2010; Kumar *et al.*, 2011).

There are 383 plant species used in Mexican folk medicine for the treatment of T2DM (Díaz, 1976; Martínez, 1989; Argueta *et al.*, 1994; Aguilar-Contreras & Xolalpa-Molina, 2002; Andrade-Cetto & Heinrich, 2005; Alarcón-Aguilar & Roman-Ramos, 2006; Romero-Cerecero *et al.*, 2009). From these, 11 species belong to the Euphorbiaceae family, being only three from the *Euphorbia* genus. Interestingly, *E. postrata* and *E. maculata* belong to the "hierba de la golondrina" complex (wanderer's herb complex) (Alcocer, 1907); nevertheless, none of the *Euphorbia* species have pharmacological studies aimed to the T2DM control.

The wanderer's herb complex comprises several species of the genus *Euphorbia* in the Euphorbiaceae family: *E. anychioides*, *E. dioeca*, *E. hirta*, *E. maculata* and *E. postrata*. The common features of the species belonging to this plant complex are: their growing as a prostrate herbs; their ancient use for skin diseases as dyes, scabies and dermathosis; and their recent use for the treatment of TII-DM (Alcocer, 1907; Argueta *et al.*, 1994; Aguilar-Contreras & Xolalpa-Molina, 2002; Andrade-Cetto & Heinrich, 2005; Alarcón-Aguilar & Roman-Ramos, 2006).

Euphorbia dioeca Kunth [syn. *Chamaesyce dioeca* (Kunth) Millsp.], a perennial herbaceous plant distributed from northern Mexico to Central America, was chosen to perform, for the first time ever, a pharmacological and a preliminary chemical study aimed to the discovery of new α-glucosidase inhibitors.

In order to establish the safety of the plant material, an acute toxicity test of the crude extracts was performed; the antihyperglycemic activity was quantified by means of an oral starch tolerance test

and an *in vitro* intestinal α-glucosidase inhibition test (Verspohl, 2002; Srinivasan & Ramarao, 2007; Fröde & Medeiros, 2008). Also, a preliminary phytochemical screening was carried out (Domínguez, 1973).

MATERIALS AND METHODS

Plant material

Euphorbia dioeca Kunth (Euphorbiaceae) was collected in La Trocha beach, municipality of Alvarado, Veracruz State, Mexico, in August 2004. Botanical specimens were deposited in the Science Faculty Herbarium (FCME), National Autonomous University of Mexico (voucher specimens 089182 and 089184) and the Instituto Mexicano del Seguro Social Herbarium (IMSSM; voucher specimen 14967).

Preparation of the extracts

The aerial parts of the plant were dried at room temperature in darkness and pulverized. The crude extracts were obtained by means of a Soxhlet extraction with hexane, dichloromethane and methanol (Merck) (200 g × 8h × 3 times), the solvent was eliminated *in vacuo*. The aqueous extracts were prepared by maceration (200 g in 1 L) during 24 hours, filtered, and lyophilized.

Animals

Balb/c mice between 20-25 g were used for the toxicity test. For the oral starch tolerance test Long Evans male rats between 240-250 g were used. The animals were housed under controlled conditions of temperature (25±1° C) and light (14/10h photoperiod) and maintained on water and food *ad libitum* (Rodent Chow 2018S, Harlan Teklad bedding). Procedures involving animals and their care were conducted in conformity with the Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999) and in compliance with international rules on care and use of laboratory animals.

Preliminary phytochemical screening

In order to detect the presence of main groups of secondary metabolites, glycosides (Mölish reagent), alkaloids (Draggendorf and silicotungstic acid), flavonoids (Shinoda), terpenoids (acetic anhydride-chloroform-sulfuric acid), tannins (sodium chloride-gelatin) and phenolic compounds (ferric chloride) were preliminary screened (Domínguez, 1973).

Acute toxicity test

The acute toxic category method was carried out following the protocol number 423 of the Organization for Economic Co-operation and Development (OECD, 2001) with the methanol and aqueous extracts. Three Balb/c female mice per extract were used. The extract was dissolved in water and administered orally at a dose of 6 g/kg, using an intragastric cannula. The mice status was evaluated during the each hour until 6 hours, then at 12 and 24 hours and daily until the 14th day.

Alpha-glucosidase inhibition assay

The technique was performed as described by Ortiz-Andrade and collaborators (2007) (Ortiz-Andrade *et al*., 2007). Briefly, rat small intestine was dissected and flushed with 100 mL of ice-cold buffered saline solution. The tissue was externally cleaned, the mucosa exposed and scraped with a glass slide on an ice-cold glass surface. The material from three or more animals was homogenized, adjusted to 1 mg/mL of protein by Lowry assay, aliquoted in 1.8 mL cryotubes and stored at –25º C until used.

The enzymatic assay employed a 0.1 M sodium phosphate buffer (pH 7.0) and 12.5 mg/mL of corn starch (S-4126, Sigma) as substrate. *E. dioeca* methanolic and aqueous extracts solutions (0.08, 0.16, 0.4, 1.6, 4, 16 mg/mL) were added for inhibition measurement; water was used for determine the 100% of activity. Each reaction was initiated adding 10 μL of crude enzyme and incubated at 37º C for 10 minutes, then stopping it with 12.5 μg of acarbose and ice incubation; reactions were performed by quadruplicate.

Released glucose was measured in triplicate, using the glucose oxidase method (GOD-PAP, GL2614, Randox) according with the manufacturer's recommendations.

The absorbance was quantified in a micro-titer plate photometer (StatFax 2100, Awarness Technology, Inc.) at 492 nm, subtracting the absorbance at 630 nm for turbidity compensation.

Experimental model of type 2 diabetes mellitus in male Long Evans rats

Diabetes was induced by the suggested model of the Animal Models of Diabetic Complications Consortium (AMDCC) of the National Institutes of Health of the United States of America (Brosius, 2003).

The animals were fasted for a period of 6 hours before streptozocin (STZ; Axxora; 50 mg/kg) diabetes induction. The STZ was dissolved in a Na-citrate buffer (pH 4.5) and was administered by intraperitoneal injection. Only animals with fasting blood glucose concentration ranging between 250-400 mg/dL were included in the study.

Oral starch tolerance test

Four groups with eight rats each were formed: vehicle, methanol extract, aqueous extract and acarbose (Sincrosa®, 50 mg/kg). The plant extract (500 mg/kg), acarbose, or vehicle was first intragastrically administered, ten minutes later the animals were challenged with a cornstarch suspension (Sigma S-4126; 2 g/kg). Blood samples were taken at 0, 30, 60, 90 and 120 minutes, through a cut at the tip of the tail. Approximately 150 μL of blood were collected using a heparinized capillary pipette. The samples were kept in 0.5 mL refrigerated microtubes until plasma was separated by centrifugation (4500 rpm × 5 minutes) and recovered. The glycemic index was measured with a commercial kit (GOD-PAP, GL2614, Randox) according with the manufacturer's recommendations, following the same procedure described in the α-glucosidase inhibition assay.

Table 1
Main groups of secondary metabolites qualitative tests of *E. dioeca* extracts (- negative result, + lightly positive, ++ positive, +++ strongly positive, secondary metabolite major concentration)

Secondary metabolite	Extract	Result
Glycosides	***Hexane***	–
	Dichloromethane	–
	Methanol	**1×** – | **2×** –
	Aqueous	++
Alkaloids (Draggendorf)	***Hexane***	–
	Dichloromethane	–
	Methanol	–
Alkaloids (Silicotungstic Acid)	***Hexane***	–
	Dichloromethane	+
	Methanol	–
	Aqueous	–
Flavonoids	***Hexane***	++
	Dichloromethane	+
	Methanol	+++
	Aqueous	–
Phenolic compounds	***Hexane***	+
	Dichloromethane	–
	Methanol	++
	Aqueous	++
Tannins	***Hexane***	+
	Dichloromethane	+
	Methanol	+++
	Aqueous	++
Terpenes	***Hexane***	++
	Dichloromethane	+
	Methanol	–
	Aqueous	–

Statistic analysis

The analysis of variance (ANOVA) followed by Dunnett's t-test for comparison with respect to vehicle were performed in order to evaluate the presence of significant differences between the diverse treatments, using the program STATISTICA 6.0.

RESULTS

Preliminary phytochemical screening

The hexane, methanol and aqueous extracts displayed a positive reaction to flavonoids, phenolic and tannins in secondary metabolites qualitative tests; nevertheless, the methanol and aqueous extracts of E. dioeca showed more intense reaction. In addition only the hexane extract exhibited a positive reaction to terpenes. It should be noted the high content of flavonoids in methanol extract and the presence of glycosides in the aqueous extract (Table 1).

Acute toxicity test

In agreement with the phytochemical results, the acute toxicity test was performed in order to establish the safety parameters of the aqueous and methanol extracts. The mice administered with methanol and aqueous extracts, even at the dose of 6 g/kg, did not present any physiological or behavioral disturbances. Accordingly to the protocol 423 of OECD, the extracts belong to the innocuous class.

Alpha-glucosidase inhibition assay

Tests were run with the methanol and aqueous crude extracts at a concentration of 2 mg/mL, for comparison with other plant species; these extracts inhibited the α-glucosidase activity in 79% and 57% respectively; further pharmacological characterization was scheduled for the most active preparations. The dose-response curves were obtained in a wide range of concentrations (16, 4, 1.6, 0.4, 0.16, and 0.08 mg/mL). The IC_{50} values were calculated as 0.55 and 0.85 mg/mL for methanol and aqueous extracts (Table 2, Figure 1).

Table 2
Alpha-glucosidase inhibitory effect of *E. dioeca* methanolic and aqueous extracts. Each measure was performed by quadruplicate.

	α-glucosidase inhibitory effect (%)						
Extract *(mg/mL)*	**0.08**	**0.16**	**0.4**	**1.6**	**4**	**16**	***IC_{50} (mg/mL)***
Methanolic	**14**	**27.82**	**42.79**	**77.54**	**93.96**	**96.64**	**0.55**
Aqueous	**25.66**	**32.51**	**38.65**	**59.88**	**76.20**	**88.82**	**0.85**

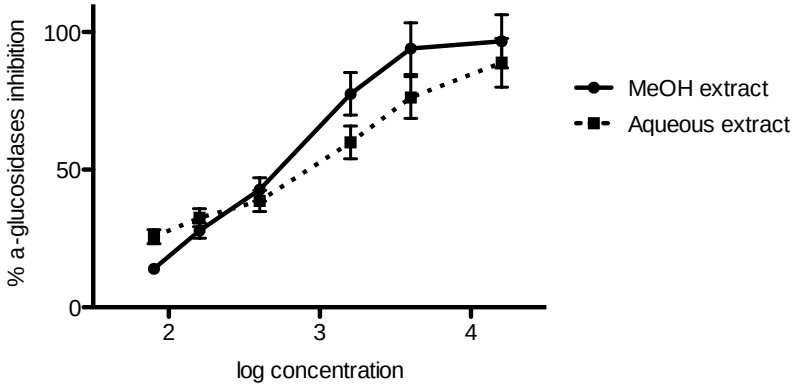

Figure 1
Concentration-response curve of α-glucosidase inhibitory effect of *E. dioeca* methanolic and aqueous extracts. Each value is the mean ± SEM for four independent repetitions of respectively concentration.

Oral starch tolerance test

Once the high inhibition of intestinal glucosidases was shown in rat intestinal preparations, the *in vivo* activity of the extracts was tested in the model of oral starch tolerance curve in STZ-diabetic rats. The methanol and aqueous extracts reduced the postprandial hyperglycemia peaks in 15.2% and 12.8% respectively (Figure 2).

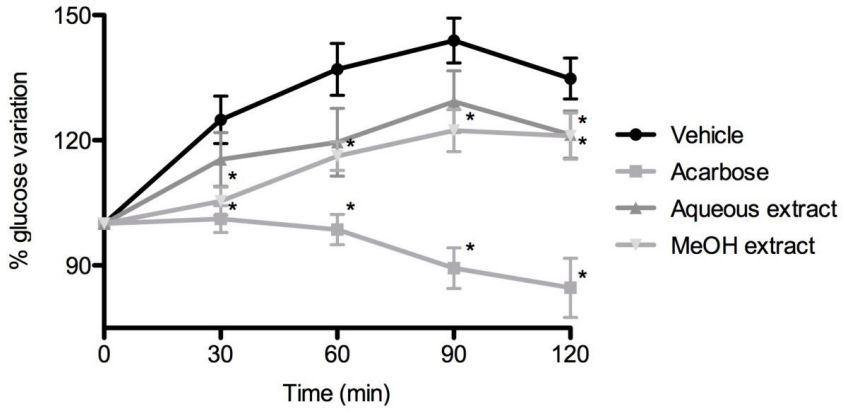

Figure 2
Oral starch tolerance curve for *E. dioeca* methanolic and aqueous extracts (500 mg/kg) in STZ-diabetic Long Evans rats (2 g/kg; each value is the mean ± SEM for 8 rats in each group *$p < 0.05$ significantly different ANOVA followed by Dunnett's *t*-test for comparison with respect to vehicle at the same time).

DISCUSSION

The antidiabetic activity of a medicinal plant can be explained from various pharmacological approaches, in the case of *E. dioeca*, the inhibitory activity of α-glucosidase is, at least, one of the mechanisms that allow us to confirm its traditional use.

α-Glucosidase is membrane-bound enzyme of the GH31 family that hydrolyzes larger carbohydrate molecules to glucose and related monosacharides. Most of these enzymes are located in the brush border of the small intestine where they catalyze the final step in the digestive process of carbohydrates. Hence, α-glucosidases inhibitors can delay the liberation of glucose from dietary complex carbohydrates, retarding glucose absorption and lowering the postprandial blood glucose peak, being a useful pharmacological tool aimed to prevent the progression of the disease and for treating pre-diabetic conditions (El-Kaissi & Sherbeeni, 2011).

The best-known α-glucosidase inhibitors are acarbose and miglitol; the former is a natural product isolated initially from an *Actinoplanes* strain, and the second is the N-hydroxyethyl analogue of 1-deoxynojirimycin, isolated from *Morus* spp. (Borges de Melo *et al.*, 2006; El-Kaissi & Sherbeeni, 2011; Israili, 2011).

The highest *α*-glucosidase inhibitory activity of the methanol extract showed in the *in vitro* inhibition assay and the oral starch tolerance curve (Table 2, Figures 1 and 2), is in agreement with the chemical composition. A report of *Euphorbia drummondii*, demonstrates that the highest amount of phenolic compounds is related with their α-glucosidase inhibitory activity (Gulati *et al.*, 2012). This biological activity could be explained with the role of phenolic compounds as antioxidants, and its relation in diabetes prevention and control (Borges de Melo *et al.*, 2006; Wardrop & Waidyarachchi, 2010; Kumar *et al*., 2011; Aboul-Enein *et al.*, 2013; Gulcin & Beydemir, 2013; van Dam *et al.*, 2013).

In addition, the high content of flavonoids in the methanol extract could be associated with the lowest α-glucosidase inhibitory EC_{50} and the highest reduction of the postprandial hyperglycemia peak. The reports of α-glucosidase inhibitory activity exerted by the isolated flavonoids from *Euphorbia humifusa* extracts (Kang *et al*., 2012), as well as several flavones reported in *Origanum majorana* (Borges de Melo *et al.*, 2006), support the importance

of the chemical composition of *E. dioeca* methanol extract, showing their value as an aid for decrease the postprandial hyperglycemia, involved in the mechanism of type 2 diabetes mellitus and its complications (Wardrop & Waidyarachchi, 2010; El-Kaissi & Sherbeeni, 2011; Israili, 2011; Kumar *et al*., 2011).

Nevertheless, the aqueous extract had a complex phytochemical composition, the presence of glycosides in this extract indicate that different compounds mediate the α-glucosidase inhibitory activity; being acarbose and miglitol the most outstanding examples of this group of inhibitors (Borges de Melo *et al.*, 2006).

Additionally, the active extracts of *E. dioeca* fulfill the safety parameters, since the acute toxicity test reveals that the methanol and aqueous extracts of *E. dioeca*, were innocuous (OECD, 2001) this result may be related to the absence of alkaloids and terpenes, secondary metabolites frequently associated to the toxic properties (Bruneton, 2001; Gershenzon & Dudareva, 2007).

CONCLUSION

To conclude, *E. dioeca* methanol and aqueous extracts showed strong capabilities as an aid to control type 2 diabetes mellitus, by the reduction of the postprandial hyperglycemia peak; their action is mediated, at least, through the α-glucosidase inhibition. The biological activity is related with the presence of glycosides, phenolic compounds and flavonoids. This study provides pharmacological and chemical evidence for the popular use of the wanderer's herb complex for the treatment of diabetes; however, future phytochemical studies focused in the isolation and identification of the pharmacologically active compounds are required for the standardization and description of the antidiabetic mode of action.

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