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Evaluation of hypoglycemic and antioxidant effects of *Brickellia eupatorioides*, *Citrus limettioides* and *Gochnatia hypoleuca*

Silvia Guadalupe Trevino-Moreno[1#], Diana Patricia Moreno-Peña[2#], Ezequiel Viveros-Valdez[1], María Julia Verde-Star[1], Catalina Rivas-Morales[1] and Paula Cordero-Perez[2*]

[1]Department of Chemistry, College of Biological Sciences, Universidad Autónoma de Nuevo León
[2]Department of Internal Medicine, University Hospital Dr. Jose E. Gonzalez, Universidad Autonoma de Nuevo Leon

Abstract: Diabetes is a chronic metabolic condition with a rapidly increasing prevalence. It comes with a rise in the generation of free radicals, potentially leading to additional health issues. Further studies and creative approaches are required to address this. Natural products are potential new antidiabetic drugs that are worth exploring. The aim of the present study is to assess the antihyperglycemic and antioxidant effects of ethanolic extracts of *Brickellia eupatorioides*, *Citrus limettioides* and *Gochnatia hypoleuca*. The antihyperglycemic activity of the extracts was tested on Wistar rats (diabetes induced by alloxan, 150mg/kg), as well as the inhibitory effect on α-glucosidase and α-amylase (*in vitro* assay). The antioxidant potential was evaluated using DPPH and ABTS assays. The total phenolic and flavonoid contents were also determined. The results indicated that ethanolic extracts of *B. eupatorioides* induced a powerful hypoglycemic *in vivo* effect with a significant decrease at 6 h after administration, similar to that produced by glibenclamide; the decrease could be related to α-glucosidase inhibition. Moreover, the extract exhibited a potent scavenging activity (IC_{50} values 33±6μg/mL and 15±2μg/mL in the DPPH and ABTS methods, respectively). The results demonstrated antihyperglycemic and antioxidant activity of ethanolic extracts of *B. eupatorioides*.

Keywords: Hypoglycemic, antioxidant, enzyme inhibition, medicinal plants.

INTRODUCTION

Diabetes mellitus (DM) is a multi-cause chronic disease that affects 463 million people worldwide and results in a significant number of fatalities each year making DM a serious public health concern (Saeedi *et al.,* 2019). The International Diabetes Federation reports that by 2030 and 2045, an estimated 643 and 783 million people, respectively, will be affected by this aetiology (IDF 2021). Mexico is one of the countries with the highest prevalence: 16.8% in 2018 and 15.7% in 2020 (Basto - Abreu *et al.,* 2021). DM remains a serious and incurable disease. Typical therapies applied to treat DM include a variety of drugs and behavioral modifications to regulate glucose levels in the body. The inhibition of digestive enzymes such as α-amylase and α-glucosidase is a common target for therapies to treat type II diabetes mellitus (DM2) (Kumar *et al.,* 2021). Glucosidase inhibitors have been reported to control the release of insulin, while the inhibition of amylase circumvents or reduces the hydrolysis of starch to maltose, and subsequently glycemia (Srisongkram *et al.,* 2022). Both of these enzymes are therefore excellent therapeutic targets for the development of antidiabetic products. Along with the inhibition of digestive enzymes, it has been proposed that they result in increased consumption of antioxidants in patients with DM2 (Zhang *et al.,* 2020). A close relationship has been reported between the metabolic disorder of glucose absorption with the generation of free radicals and the subsequent development of oxidative stress (Pathikkal *et al.,* 2022).

The systematic search for antidiabetic and antioxidant compounds is a current area in the pharmacopoeia of medicinal plants, as their empirical uses could support their biological activity and perhaps low toxicity (Governa *et al.,* 2018). Extensive exploratory studies have been carried out in Mexico to determine the possible hypoglycemic effect of medicinal plants (Huerta-Reyes *et al.,* 2022). The potential of certain little-studied species of the genera *Brickellia* (Pérez *et al.,* 2022), *Citrus* and *Gochnatia* is highlighted.

Brickellia eupatorioides is a species with potential therapeutic value for the treatment of metabolic diseases, and our research group has previously demonstrated its remarkable hypocholesterolemic effect (Moreno-Peña *et al.,* 2017). *Citrus limettioides* is reported to have antioxidant, antimicrobial, and anticancer activity (Janoti *et al.,* 2014, Vasudeva and Sharma 2012, Jayaprakasha *et al.,* 2013). *Gochnatia hypoleuca* has shown cytotoxic activity in prostate cancer (Shaffer *et al.,* 2016) and is useful for the treatment of respiratory diseases (Piornedo *et al.,* 2011).

The aim of the present study is to determine the hypoglycemic and antioxidant activity of the ethanolic extracts of *B. eupatorioides*, *C. limettioides* and *G.*

**Corresponding author:* e-mail: paucordero@yahoo.com.mx

[#]These two authors contributed equally to this work

hypoleuca, as well as the phenolic content and inhibitors of digestive enzymes such as α-amylase and α-glucosidase.

MATERIALS AND METHODS

Vegetal material

B. eupatorioides was collected in the municipality of Galeana, Nuevo Leon (Mexico) (25°39′08.23″N, 100°42′40.14″W, 1169m) in 2014 (record number 26846); *C. limmettioides* from Montemorelos, Nuevo Leon (25°12′28.2″N 99°51′34.9″W) (record number 27789); and *G. hypoleuca* from Montemorelos, Nuevo Leon (25°12′28.2″N 99°51′34.9″W) (record number 27797). The plants were identified at the Department of Botany of the College of Biological Sciences, Universidad Autonoma de Nuevo Leon (UANL), Mexico.

Preparation of ethanolic extracts

One hundred grams of dried aerial of each of the plants was successively subjected to extraction with ethanol (Sigma-Aldrich) and maceration (three sessions of 24h each). The ratio of plant to solvent was 1:10 (weight/volume). The ethanolic extracts were filtered, concentrated under vacuum until dry and stored at 4°C until required for use.

Animals

The study was approved by the ethics committee of the UANL School of Medicine (HI11-002), based on the Official Mexican Standard NOM-062-ZOO-1999 technical specifications for the production, care and use of laboratory animals. The animals were housed in a climate-controlled room at 23ºC with 55% humidity and had unrestricted access to food and water. They were subjected to a 12-hour light/dark cycle.

Diabetic induction

DM was induced in 25 female Wistar rats (average weight 250g) who fasted for 12h, with free access to water and then the animals were injected intraperitoneally (ip) with alloxan (Sigma-Aldrich) (150mg/kg). Glycemia was recorded at 72h after injection by monitoring capillary blood from the tail tip, using a glucometer (Accu-Chek Performa, Roche). Diabetic rats were confirmed by fasting blood glucose concentration >250 mg/dL on the seventh day after alloxan administration (Rodriguez-Magaña *et al.*, 2019).

Acute hypoglycemia assay

The animals were distributed randomly into six groups, with five rats in each, as follows. G-I: healthy negative control group [saline solution with 1% of Tween 20 intragastric (ig)]; G-II: diabetic control group (alloxan 150mg/kg ip and saline solution with 1% of Tween 20 ig); G-III: glibenclamide group (alloxan 150mg/kg ip and glibenclamide (Roche) (5 mg/kg body weight ig); G-IV: *B. eupatorioides* group (alloxan 150mg/kg ip and ethanolic extract *B. eupatorioides* 100mg/kg ig); G-V: *C. limettioides* group (alloxan 150mg/kg ip and extract *C. limettioides* 100mg/kg ig); and G-VI: *G. hypoleuca* group (alloxan 150mg/kg ip and extract *G. hypoleuca* 100 mg/kg ig). After a fasting period of 12h, basal glucose in the animals was recorded. Treatment was then administered and glycemia was recorded: Basal and after 6h (Rodriguez-Magaña *et al.*, 2019).

Phytochemical screening

Preliminary phytochemical analysis included screening for the following: unsaturated hydrocarbons (with potassium permanganate), carbonyl groups (with 2,4-dinitrophenylhydrazine) (Dominguez 1979), tannins (ferric chloride test), sterols (Salkowski's test), triterpenes (Liebermann–Burchard's test) (Raaman 2006), coumarins (NaOH test) (Souza *et al.*, 2020), carbohydrates (Molisch's test), flavonoids (Shinoda's test) and saponins (foam test) (Dominguez 1979).

Phenolic content

The Folin–Ciocalteu assay was used to determine the total phenolic content. Accordingly, 100µL of selected samples was mixed with 0.25mL Folin reagent (1N), 1.25mL Na_2CO_3 (20%) and 0.4mL distilled water in test tubes. After 2h incubation, the optical density was assessed at a wavelength of 760 nanometers, with gallic acid serving as the reference standard. The overall phenolic concentration was stated in milligrams of gallic acid equivalent per gram of plant extract (Monroy-Garcia *et al.*, 2021).

Flavonoid content

Using a colorimetric assay with aluminum chloride and $NaNO_2$ solution, the flavonoid concentration. The assessment was conducted at a wavelength of 415 nanometers. The findings are presented in milligrams of (+) catechin equivalent per gram of plant extract.

In both assays (the phenolic content and the flavonoid content), the data were reported as mean ± standard deviation (SD) for at least three replicates (Monroy-Garcia *et al.*, 2021).

Antioxidants/free radicals scavenging activity

The antioxidant scavenging capacity of an extract was determined using the ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] (Sigma-Aldrich) and the DPPH (1,1-diphenyl-2-picryl-hydrazyl) (Sigma-Aldrich) assays. Values were reported using the half-maximum effective concentration (EC_{50}). The assays were performed in 96-well microplates; the absorbance of $ABTS^{\bullet+}$ was adjusted to 0.70±0.02. After 6min, the decrease in the absorption was measured at 754nm. In the DPPH assay, 100µl of the radical (2mg/L) was mixed with 100µl of serial dilutions in 96-well microplates. The absorbance at 517 nm was measured in an Agilent BioTek Epoch microplate spectrophotometer. For both tests, mean

Table 1: Phytochemical screening of plant extracts

	B. eupatorioides	*C. limettioides*	*G. hypoleuca*
Yield percentage	6.8%	11.14%	14.6%
Unsaturations	+	+	+
Carbonyl group	+	+	+
Tanins	+	+	+
Sterols	+	+	+
Triterpenes	+	+	+
Cumarins	+	+	+
Carbohydrates	+	+	+
Flavonoids	+	+	+
Saponins	-	-	-

Table 2: Phenolic contents, free radical scavenging, and enzyme inhibition activities of study plants

Sample	Phenols (mg/g)	Flavonoids (mg/g)	DPPH (EC_{50}, µg/ml)	ABTS (EC_{50}, µg/ml)	α-Glucosidase (IC_{50}, mg/ml)	α-Amylase (IC_{50}, mg/ml)
B. eupatorioides	190 ± 31^a	65 ± 7^a	33 ± 6^a	15 ± 2^a	0.48 ± 0.06^a	2.66 ± 0.9^a
C. limettioides	62 ± 13^b	12 ± 0.8^b	>100	>100	>2	>10
G. hypoleuca	114 ± 20^c	35 ± 9^c	68 ± 10^c	53 ± 15^c	1.64 ± 0.3^c	>10
*Control	–	–	18 ± 3^d	7 ± 0.8^d	0.12 ± 0.02^e	0.97 ± 0.08^e

The results are expressed as mean ± SD .*Trolox was used as a positive control in antioxidant and acarbose on enzymatic inhibition assays. n =6. a) $p < 0.05$ *B. eupatorioides* vs other samples; b) $p < 0.05$ *C. limettioides* vs other samples; c) $p < 0.05$ *G. hypoleuca* vs other samples; d) $p < 0.05$ Trolox control vs other samples; e) $p < 0.05$ acarbose control vs other samples according to Tukey's test.

values were obtained from triplicate experiments. Methanol (Sigma-Aldrich) and Trolox (Sigma-Aldrich) were used as negative and positive controls, respectively (Monroy-García *et al.,* 2021).

In vitro enzymatic assay

The inhibition assays were performed using porcine α-amylase (EC 3.2.1.1) (Sigma-Aldrich) and α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20) (Sigma-Aldrich). The absorbance of the inhibition activity of these enzymes was measured using 96-well microplates in an Agilent BioTek Epoch microplate spectrophotometer.

The percentage inhibition was calculated as follows:
Inhibition (%) = [(Aneg control - Ablank) – (Asample – Asample blank)] ÷ (Aneg control - Ablank) × 100%, where A=absorbance. The IC_{50} value (half maximal inhibitory concentration) was thus determined and expressed as means ± SEM of the triplicate measurements.

For the α-glucosidase assay, 50µL of plant extract was mixed with 50µL of enzymatic solution (0.8U/mL of PBS, pH 6.8) and incubation 37ºC/10 min. Thereafter, 50µL þ-nitrophenyl-alpha-D-glucopyranoside (625mM) was added to each well and the solution was further incubated at 37°C for 45 min. The reaction was blocked by the addition of 100µL sodium carbonate solution (0.2M). The absorption was determined at 405 nm.

For the α-amylase inhibition assay, serial dilution of the plant extract or standard was carried out in 96-well microplates with 50µL of α-amylase (1U/mL). After incubation at 37°C for 15min, 50µL of starch solution (0.5%) was added, followed by further incubation at 37 °C for 20 min. Iodine solution (50µL) was then added to the test sample and the absorbance was measured at 750 nm (Caramantin *et al.,* 2021). The reaction was blocked with 20µL hydrochloric acid (1M).

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism software v. 9.0 (GraphPad, San Diego, CA, USA). Data were analyzed using a one-way ANOVA test with a Tukey post hoc. The results are expressed as mean ± SD. Differences between means were considered to be significant at $p<0.05$.

RESULTS

Results of phytochemical screening and the percentage yield of plant extracts are presented in table 1.

Hypoglycemic activity

Figure 1 shows the hypoglycemic effect induced by the ethanolic extract of *B. eupatorioides.* Alloxan ip (150mg/kg weight) induced a significant increase in blood glucose, from 118.6±6.28 to 478.8±59.17mg/dL

(compared with G-I). This effect was maintained throughout the test. In G-III (glibenclamide), there was a significant reduction in the blood glucose levels (388.8±43.21mg/dL); in G-IV, treatment with *B. eupatorioides* (100mg/kg) at 6 h showed a significant reduction in glucose levels (385.3±38.52); G-V G-VI showed no hypoglycemic activity.

Phenolic and flavonoid contents, free radical scavenging, and enzyme inhibition activities of the plants studied

Table 2 shows the antioxidant effect of *B. eupatorioides*. A powerful effect against the free radicals DPPH and ABTS was observed: EC_{50} values were 33±6 and 15±2 µg/mL, respectively. This could be related to its high content of phenols [190±31gallic acid equivalent/g (GAE/g)] and flavonoids [65±7 catechin equivalent/g (CE/g)]. The table also shows a marked effect on the α-glucosidase, IC_{50} value 0.48±0.06mg/mL and a poor effect on the α-amylase, IC_{50} value 2.66±0.9mg/mL.

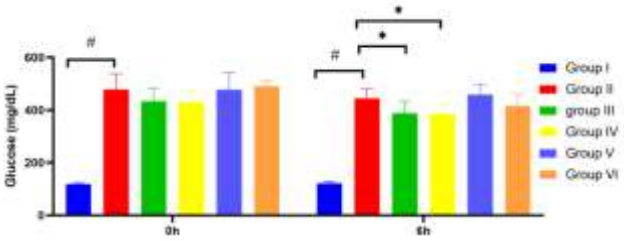

Fig. 1: Glucose levels, at 0h and 6h after the administration of glibenclamide or the extracts under study. # p<0.0001 vs. G-II; * p<0.05 vs. G-II. Values are expressed as mean ± SD.

DISCUSSION

DM2 is a global public health problem which has acquired considerable dimensions in developing countries such as Mexico (IDF 2021, Tinajero and Malik 2021). In this country, medicinal plants play an important role in the treatment thereof and exploratory studies have been carried out to validate their hypoglycemic effect (Torres-Vanda *et al.,* 2023). Based on this, the present study addresses the hypoglycemic and antioxidant activity of the selected plant species.

In the search for natural hypoglycemic compounds, the potential of the *Brickellia* genus has already been reported. A hexanic extract of *Brickellia veronicaefolia* (concentration 300mg/kg) decreased blood glucose levels by 72.13%, 4.5h after its oral administration (Pérez-Gutiérrez *et al.,* 1998). Furthermore, a compound isolated from this plant showed hypoglycemic activity in mice treated with alloxane (Perez *et al.,* 2000). Lyophilized *Brickellia cavanillesii* tea extract exhibited antidiabetic activity *in vitro* on HepG2 cells exposed to 0.2 mg/mL of tea extract for 2, 4, 6 and 24h, suggesting that facilitated glucose transporter protein 2 (GLUT-2) expression was increased (Eshiet *et al.,* 2014).

We note that the relevant effect of polar extracts of medicinal plants in models of DM induced by alloxan has already been demonstrated. For example, a methanolic extract of *Morus mesozygia* leaves at a concentration of 200mg/kg decreased blood glucose levels to 273±16.23 mg/dL at 4h after administration (Tirwomwe *et al.,* 2019); an aqueous extract of *Tectaria heracleifolia* at a dose of 100 and 300mg/kg showed a decrease to levels similar to those obtained with glibenclamide at 3h and 5h after ingestion (Luna-Rodríguez *et al.,* 2019); aqueous and ethanolic extracts of *Caralluma attenuata* at 100mg/kg decreased blood glucose levels to 162±2.76 and 150±3.94 mg/dL, respectively, at 3h after oral administration (Venkatesh *et al.,* 2003). In the present study, the ethanolic extract of *B. eupatorioides* (concentration 100mg/kg) also induced a potent hypoglycemic effect in diabetic rats-there was a significant decrease in glucose levels at 6h after administration, similar to that produced by the positive control glibenclamide.

Regarding the *Citrus* genus, various species have shown a hypoglycemic effect. *Citrus pseudolimon (*Naim *et al.,* 2012), *Citrus paradisi* (Adeneye *et al.,* 2008), *Citrus aurantifolia* (Ramya *et al.,* 2020), and *Citrus sinensis* (Parmar and Kar 2007) are reported to reduce elevated fasting blood glucose and lipid levels in alloxan-induced diabetic rats (in some cases similar to as produced by glibenclamide). In the present study, *Citrus limettiodes* slightly reduced glucose levels in diabetic rats induced with alloxan at 6h post-treatment, but this was not significant.

The genus *Gochnatia* has mainly been associated with anti-inflammatory and antimicrobial activity. *Gochnatia pulchra* is reported to have antileishmanial activity (Lucarini *et al.,* 2012), whereas *Gochnatia polymorpha* has antispasmodic (Piornedo *et al.,* 2011) and anti-inflammatory activity (Moreira *et al.,* 2000). In addition, *G. polymorpha* is reported to have the ability to inhibit the activity of aldose reductase, an enzyme that catalyzes the conversion of glucose into sorbitol, whose accumulation in nervous tissue has been suggested as a contributing factor in the development of diabetic neuropathy (Ferro and Degen 2011). However, in the present study, the *G. hypoleuca* species did not present any hypoglycemic effect in rats treated with alloxan.

For the complementary treatment of DM, the use of inhibitors of postprandial carbohydrate absorption is common. These drugs inhibit some enzymes, such as α-amylase and α-glucosidase (Ríos *et al.,* 2015) however, they bring unwanted effects. Therefore, the search for new and/or better inhibitors of digestive enzymes is included

in research into natural products (Governa *et al.,* 2018). In this regard, *B. eupatorioides* has a poor effect on the inhibition of α-amylase (IC_{50}: 2.66±0.9mg/mL), but an important effect on the inhibition of α-glucosidase (IC_{50}: 0.48±0.06mg/mL). This may be related to the hypoglycemic effect shown in the murine model. Moreover, a wide variety of secondary metabolites with an inhibitory effect on α-glucosidase have been reported, including phenols and flavonoids (Ćorković 2022). The ethanolic extract of *B. eupatorioides* had a total phenol content of 190±31 mg/g, a value similar to that reported by Aryal *et al.,* 2019 in different species (72.66–292.65 mg/g) and a high content of flavonoids (65±7 mg/g), higher than that found by Corkovick, which reported content in the range 6.61–39.38mg/g. The importance and relevance of flavonoids in the *Brickellia* genus have already been reported by Goodwing *et al.* in 1984. The flavone 5,7,3′-trihydroxy-3,6,4′-trimethoxyflavone (centaureidin) with antioxidant and hypoglycemic effects was isolated from *B. veronicaefolia* (Perez *et al.,* 2020). As is evident from the above discussion, it is relevant and important to continue with the study of species of the *Brickellia* genus, such as *B. eupatorioides*, for the complementary treatment of DM.

The alloxan used to induce hyperglycemia in mice is a structural analogue of glucose that accumulates in the beta cells of the pancreas through the GLUT-2 glucose transporter, generating reactive oxygen species, then triggering oxidative stress that causes the death of beta cells (Lenzen 2008). This clarifies the relevance of and the fundamental role that antioxidants play in the development and progression of DM and its related complications (Yaribeygi *et al.,* 2020).

In the present study, the antioxidant capacity of the ethanolic extracts was also determined by the DPPH and ABTS radical-scavenging assays. For *B. eupatorioides*, the results showed a mean effective concentration (EC_{50}) of 33±6μg/mL for the DPPH assay similar to that reported for medicinal plants (35.8-47.7μg/mL) (de la Cruz-Jimenez *et al.,* 2022) and edible plants (9.89-45.68μg/mL) (Monroy-Garcia *et al.,* 2021). For the ABTS assay, *B. eupatorioides* and *G. hypoleuca* gave results comparable to values reported for other medicinal plants (15.7-75.6μg/mL) (de la Cruz-Jimenez *et al.,* 2022).

CONCLUSION

Medicinal plants show important biological activities that can be used in the search for therapies aimed at treating metabolic problems such as DM. The antihyperglycemic and antioxidant effects achieved with *B. eupatorioides* were demonstrated. We also recorded promising results pertaining to the importance of the content of phenolic compounds and the enzymatic inhibition of α-glucosidase, which are involved in glycemic control (shown in an *in vivo* assay). This is the first report describing the potential of *B. eupatorioides* in inhibiting hyperglycemia. Our results suggest that the administration of *B. eupatorioides* ethanolic extract may be helpful in the prevention of diabetic complications, associated with oxidative stress-thus offering a promising source of new agents to treat DM.

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