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ANTIHYPERGLYCEMIC, ANTIOXIDANT AND ANTI-INFLAMMATORY EFFECTS OF AQUEOUS EXTRACT OF MISTLETOE (*Cladocolea loniceroides*) IN STZ-INDUCED DIABETIC MICE

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Abstract

Background: Inhibition of carbohydrate hydrolyzing enzymes, such as α-amylase and α-glucosidase, is a key element in the regulation of diabetes mellitus (DM). The purpose of this work was to study the inhibition of carbohydrate hydrolyzing enzymes, and the antihyperglycemic activity of aqueous extract of *Cladocolea (C. loniceroides*) in streptozotocin (STZ)-induced diabetic mice.
Materials and Methods: The inhibitory activities of *C. loniceroides* aqueous extract on α-amylase and α-glucosidase were investigated *in vitro*. Glucose tolerance test was performed in normoglycemic (NG) mice which were fed with starch or sucrose. The effect of mistletoe aqueous extract (ME) was measured in (STZ)-induced diabetic mice. On day 35 of the treatment, the effect of decreasing oxidative stress (lipid peroxidation, glutathione redox state, GPx and GR specific activities, cytokines and aminotransferases analysis) was assessed.
Results: ME showed a competitive mode of inhibition for the carbohydrate hydrolyzing enzymes (CHE). The maximum antihyperglycemic activity in mice was observed for the unripe fruit aqueous extract (UFAE) for α-amylase and stem aqueous extract (SAE); for α-glucosidase due to the glycemic response reduction by 23% or 35%, respectively. UFAE decreased malondialdehyde (MDA) 1.76 times; GSH/GSSG ratio was mantained (3.08 ± 0.66); GPx activity was reduced (24%); IL-6 was reduced (18%) and the concentration of TNF-α (37%) was leveled with respect to the (STZ)-induced diabetic mice; ALT and AST (liver transaminases) levels were nearly the same compared with those found in the NG mice.
Conclusion: UFAE of *C. loniceroides* exhibited the highest antidiabetic activity in (STZ)-induced diabetic mice.

Key words: Diabetes mellitus, *Cladocolea loniceroides*, antihyperglycemic, antidiabetic, oxidative stress and α-glucosidase.

Abbreviations: CHE: Carbohydrate hydrolyzing enzymes: *C.*: *Cladocolea :* STZ: Streptozotocin:NG: Normoglycemic: ME: Mistletoe extracts: UFAE: Unripe fruit aqueous extract: RFAE: Aqueous extract of ripe fruit: SAE: Stem aqueous extract: LAE: Aqueous extract of leaves: AE: Aqueous extracts:T2D: Type 2 diabetes: GPx: Glutathione peroxidase GR: Glutathione reductase: GSH: Glutathione reduced: GSSG: Glutathione oxidized. AST =Aspartate transaminase: ALT = Alanine aminotransferase.

Introduction

Type-2 diabetes (T2D) mellitus is a chronic degenerative disease that is characterized by a relative or absolute lack of insulin, resulting in hyperglycemia. Recent statisitics show its worldwide prevalence, with a 90% of occurrence mainly in 40 to 59 year-old adults (Sandoval, 2012). Besides that, the prevalence rate is in constant increase and it is characterized by a fasting glycemia greater than 126 mg/mL (Association, 2014). On the other hand, the deterioration in the antioxidant system also has a role on the decline in the clinical state of the patient. Oxidative stress in patients with T2D causes oxidation of macromolecules and nucleic acids which occur in cell membranes (Matsudda and Shimomura, 2013; Bullon et al., 2014; Rochette et al., 2014). Particularly, some literature point to an alteration of antioxidant enzymes such as glutathione peroxidase (GPx) and glutathione reductase (GR), which affect the concentration of glutathione in its reduced form (GSH) and oxidized form (GSSG) (Díaz-Flores et al., 2012). Immunologic and inflammatory mechanisms have a role

in T2D. The main cytokines involved in the pathogenesis are interleukins (IL-1 and and IL-6) and tumor necrosis factor-alpha (TNF-α). Recent studies have demonstrated that inflammation, specifically inflammatory cytokines, are determinant on the development of microvascular diabetic complications, including neuropathy, retinopathy, and nephropathy (Roman-Ramos et al., 2012). In the treatment of T2D, oral hypoglycemic agents like sulfonylureas, meglitinides, thiazolidines, D-phenylalanine and α-glucosidases inhibitors are used in addition to insulin treatment, along with diet and exercise. However, due to inhibitors of α-glucosidase such as acarbose (a pseudotetrasaccharide and inhibitor of α-glucosidase and pancreatic α-amylase with antihyperglycemic activity), excessive inhibition of α-amylase causes side effects such as abdominal pain, diarrhea, flatulence and an increase in liver enzymes, as a consequence of an abnormal bacterial fermentation of undigested carbohydrates in the colon (Alejandro-Espinosa et al., 2013). Drawbacks associated with existing synthetic oral hypoglycemic agents have prompted continued search for alternative agents from plant sources. Consequently, some plants have been used as sources for new antioxidant and antidiabetic agents because of their traditional uses (Adaramoye et al., 2012).

Several studies have reported that over 400 plants have been used in the treatment of large number of diseases, including diabetes (Lepzem et al. 2007). In particular, polyphenols have the ability to modulate blood glucose levels. Recent research has shown that phenolic compounds have the potential to inhibit CHE such as α-amylase and α-glucosidase in the digestive organs, and thus**,** might play a role in the management of T2D (Striegel et al., 2015). In addition, beneficial effects of antioxidants in diabetes include protection of pancreatic β-cells**,** which are vulnerable to glucose toxicity (Lepzem & Togun, 2017). Some parasitic plants such as the mistletoe *Viscum (V.)* or *album coloratum* which belongs to Santalaceae family, have been shown to possess antidiabetic activity with such study, we are also trying to find out a plausible utility of the mistletoe to prevent environmental deterioration of Xochimilco site with mechanism that are critical in the regulation of insulin secretion (kim et al, 2014).

Cladocolea (C. loniceroides) is a mistletoe which belongs to the Loranthaceae family. *C. loniceroides* is a killling pest for ahuejote trees**,** and it decreases the natural value of the borough of Xochimilco as a natural habitat within Mexico City. Xochimilco has been declared a world heritage site by the United Nations Educational Scientific and Cultural Organization (UNESCO). Previously, Serrano-Maldonado et al. (2011) have studied the use of *C. loniceroides* as a source of polyphenols with a potential of cytotoxic activity on breast cancer cell-lines. Nevertheless, no reports of antidiabetic properties of *C. loniceroides* are available in the literature. Therefore, the aim of this work centers on the assessment of antidiabetic activity of aqueous extract of *C. loniceroides* in (STZ)-induced diabetic mice, as well as the role and influence of the extract in oxidative stress and inflammation. ALT and AST.

Materials and Methods

Chemical and reagents

The following chemicals were obtained from Sigma-Aldrich (Germany): α-glucosidase type I from Baker Yeast (EC 3.2.1.20), resveratrol, porcine pancreas α-amylase (EC 3.2.1.1, type VI), p-nitrophenyl-α-D-glucopyranose (pNPG; N-1377), 3,5-dinitrosalicylic acid (DNS), streptozotocin, NADPH, Glutathione disulfide, L-glutathione reduced, glutathione reductase and, glutathione peroxidase. Water-solvable starch was purchased from Meyer and acarbose from Glucobay Bayer, Mexico. Serum cytokine levels were quantified using an ELISA kit purchased from Pierce Protein Research Products (Thermo Fisher Scientific, Illinois, USA). Reflotron Test Strips forwith mechanisms that are critical in the regulation of insulin secretion. Such plants may decrease oxidative stress and may also increase insulin secretion and improve glycemic control.

Plant material and aqueous extraction

C. loniceroides (van Tieghemen) Kujit (Loranthaceae) was collected from infested *S. bonplandiana* trees in the area of Xochimilco, Mexico (19 °, 14'N, 99 ° 05 'O, altitude 2273 m) in February, 2013. The identification (URN: catalog: IBUNAM: MEXU: PA1053501) was performed by Dr. David Sebastián Gernandt from the Institute of Biology at the National Autonomous University of Mexico (UNAM). Plant material (stems, leaves, ripe and unripe fruits) was separated and used individually. Samples were dried at room temperature to constant weight; they were milled using an Udy mill (Udy Corporation Fort Collins, Co. USA) until a 420 µm mesh flour was obtained and stored at 5°C for further analysis.

Dried plant material was divided into two batches to process them by two different methods. The first batch was divided into three parts: samples (of 10 g) were macerated with distilled water (solvent:solid ratio of 10:1) with constant stirring at room temperature (20 ± 2 °C) for 12, 24 and 48 h, respectively. The second batch of samples was also split into three parts in exactly the same proportion as aforementioned; then a decoction was prepared at 95 ± 2 °C for 30, 60 and 90 min, respectively. Lastly, all the samples were filtered through Whatman no. 1 filter paper (Whatman International Ltd., Maidstone, U.K.). Filtration was lyophilized for 24 h (Scient-18N Freeze dryer, Shanghai, China).

In vitro assays

Carbohydrate hydrolyzing enzyme inhibitory activity

The α-amylase and α-glucosidase inhibitory activities were measured according to Worthington (1993a and 1993b). Acarbose and resveratrol were used as reference drugs. The percentage of inhibitory activity was calculated for all the samples as:

$$\%\ Inhibition = \frac{Ac - Ae}{Ac}\ X\ 100$$

Where: Ae is the sample absorbance and Ac the absorbance control without sample. Results were expressed by its half maximal inhibitory concentration (IC_{50}) value, which is defined as the sample concentration (mg/L) required to inhibit 50% of the enzyme activity.

Inhibition kinetic of enzymes

The lyophilized aqueous extracts (AE) were tested to determine the kinetic parameters of α-amylase and α-glucosidase enzyme inhibition. The activities were measured by increasing substrate concentrations by the presence/absence of sampling of lyophilized AE of *C. loniceroides*. α–amylase activity was quantified by measuring the maltose equivalents released from corn starch at 540 nm (Rubilar et al. 2011). α–glucosidase activity was quantified by assessing the *p*-nitrophenol equivalents released by pNPG at 400 nm (Jaiswal et al. 2012). The Michaelis–Menten constant (K_m), maximum enzyme reaction rate (V_{max}), and inhibition mode of aqueous extracts of *C. loniceroides*, were obtained by Lineweaver–Burk plots.

Experimental animal, ethics statement and treatment

Male mice strain CD1 of 4-6 weeks old (Charles River) with 35-40 g body weight were supplied by Universidad Autónoma Metropolitana, Campus Iztapalapa (UAM-I). This project was supported by Secretaría de Ciencia, Tecnología e Innovación with the project PINV11-13, contract ICYTDF/295/2014. The handling of laboratory animals was performed in agreement with the statutes of the CICUAL (Institutional Committee for the Care and Use of the Animals) based in the international and national rules established in the "Official Mexican Rule" for the care and use of the laboratory animals" [NOM-062-ZOO-1999]. Mice were individually housed on a 12 h:12 h light-dark cycle (6 AM lights on and 6 PM lights off). The laboratory temperature was 22 ± 1°C and the humidity was 20.5 ± 3.0%. Prior to the experiments, mice were fed with standard food for 1 week in order to adapt them to laboratory conditons. Food and water were available *ad libitum*.

Twelve hours before the experiments, they were fasted overnight, water was always available. Sixty-five mice were used for the study, they were divided into 13 groups, each consisting of 5 animals to assess the inhibition of CHE of the plant aqueous extracts as well as their effect on (STZ)-induced diabetic mice. The fasting blood glucose levels of all the mice were determined before the start of the experiment. Mice were divided into the following groups:

Carbohydrate hydrolyzing enzymes inhibitory activity

Group 1: Normoglycemic (NG) control. Received only vehicle (0.5% carboxymethylcellulose).
Group 2: NG reference. Acarbose was given at a dose of 100 mg/kg.
Group 3. NG reference. Resveratrol was given at a dose of 30 mg/kg
Group 4: NG. Aqueous extract of ripe fruit (RFAE) was given at a dose of 300 mg/kg.
Group 5: NG. Aqueous extract of unripe fruit (UFAE) was given at a dose of 300 mg/kg.
Group 6: NG. Aqueous extract of leaves (LAE) was given at a dose of 300 mg/kg.
Group 7: NG. Aqueous extract of stem (SAE) was given at a dose of 300 mg/kg.

Blood glucose concentration was determined after 30 min when the mice had been intragastrically administered with the vehicle, acarbose or plant aqueous extracts. Afterwards, an oral carbohydrate tolerance test was performed as follows: mice were intragastric administered with soluble corn starch (2 g/kg) or sucrose (4 g/kg). Finally, the blood glucose was assessed at 0.5, 1, 1.5 and 2 h to obtain the glucose curve. Blood samples were collected from the tail tip at the defined times and determined using an Accu-Chek® system (Roche).

Mistletoe aqueous extracts effect on (STZ)-induced diabetic mice

Moderate diabetes was induced by two intraperitoneal injections of STZ (40 mg/kg body weight (b.w.)) freshly dissolved in a citrate buffer (100 mM, pH 4.5), in non-fasted mice on two consecutive days (Soriano-Santos et al. 2015). Blood samples were collected from the tip of the tail at the defined times, the fasting blood glucose levels were determined as previously described. Mice were considered diabetic when the fasting blood glucose level was ≥200 mg/dL.

Five-week subacute study daily, all treatments were administered intragastrically.

Group 1: NG control. Received isotonic saline solution (4 mg/kg), once a day throughout 35 days.
Group 2: STZ-induced diabetes control. Received isotonic saline solution (4 mg/kg), once a day throughout 35 days.
Group 3: STZ-induced diabetic reference: Acarbose was given once a day throughout 35 days at a dose of 100 mg/kg.
Group 4: STZ-induced diabetic reference: Resveratrol was given once a day throughout 35 days at a dose of 30 mg/kg.
Group 5: STZ-induced diabetes. Aqueous extract of SAE was given once a day, by oral gavage procedure, throughout 35 days at a dose of 300 mg/kg.
Group 6: STZ-induced diabetes. UFAE was given once a day, by of oral gavage procedure, throughout 35 days at a dose of 300 mg/kg.

Lipid peroxidation

The 2-thiobarbituric acid reactive substances (TBARS) were measured using the procedure described by Jentzsch et al. (1996). An increase of MDA is linked to a rising of lipid peroxidation. Absorbance was measured at 535 nm in butanolic phase. MDA was used as a standard (0–20 mM).

Glutathione redox state assessment

The GSH redox system is essential to reduce oxidative stress. GSH, a radical scavenger, is converted into oxidized glutathione through glutathione peroxidase, and it is converted back to GSH by glutathione reductase. Measurements of GSH, GSSG and its related enzymatic reactions are important to assess the redox and antioxidant status. The animals were perfused with a phosphate-buffered saline (PBS) solution (0.15 M potassium phosphate, 0.9% NaCl, pH 7.4) through the abdominal aorta to remove residual blood elements. Fragments of liver were removed, washed in cold saline solution and stored at -70 °C for further use. GSH and GSSG measuerments were carried out according the method of Diaz-Flores et al. (2012).

GPx and GR specific activities

Liver fragments were homogenized (10% w/v) in PBS 0.1 M, pH 7.5 using a Polytron PT1200 and were centrifuged at 15 000 x g per 30 min. Supernatants were used for GPx and GR evaluation. GR activity was measured according to the method reported by Beutler (1969) and the protocol published by Lawrence and Burk (1976) was used for GPx. Both assessments were evaluated on NADPH production.

Cytokines analysis

After treatment, the animals were anesthetized using pentobarbital 25 mg/kg, the blood was collected from the orbital plexus in heparinized tubes, plasma was separated wafter 30 min of recollection, using a refrigerated centrifuge for a further estimation of cytokines (IL-6, IL-10 and TNF-α) analysis. Serum cytokine levels were quantified using an ELISA which was purchased from Pierce Protein Research Products (Thermo Fisher Scientific, Illinois, USA) to analyze IL-10, IL-6 and TNF-α.

Aminotransferases analysis

Quantifications of total aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were performed with a Roche Reflotron Plus Chemical System Analyzer (Woodley Equipment Company Ltd, Horwich, UK) and Reflotron Test Strips for ALT and AST using the blood samples that were collected from the tip of tail on the 35th day after the treatment had been completed.

Statistical analysis

The obtained data were analyzed by the Prism program Version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) and expressed as the mean ± standard deviation. To determine statistically significant differences between groups, an ANOVA (one way) was followed by Turkey or Duncan post-hoc test; $p<0.05$ was considered statistically significant.

Results

Carbohydrate hydrolyzing enzymes inhibitory activity

The α-amylase inhibitory activity from different aqueous extracts are displayed in Table 1. The UFAE and RFAE prepared by decoction for 30 min from *C. loniceroides* showed the lowest IC_{50} (µg/mL) values (1.73±0.11 and 5.85±0.05, respectively) of α-amylase inhibitory activity. Acarbose and resveratrol were used as experimental control. Their IC_{50} values = 7.1µg/mL and 111 - 120 µg/mL (Miao et al., 2014), respectively, were of the same order of magnitude as of the samples. On the other hand, the SAE and LAE did not show α-amylase inhibitory activity, regardless of the method. Table 1 also shows the inhibitory activity of α-glucosidase of AE obtained by decoction or maceration of *C. loniceroides.* The extracts with better α-glucosidase inhibitory activity were those obtained after 30 min of decoction from stem (IC_{50} = 14.71 ± 0.43 µg/mL) and leaves (IC_{50} = 37.92± 4.83 µg/mL). α-glucosidase inhibitory activity of acarbose (IC_{50} = 31 µg/mL) and resveratrol (IC_{50} = 1350 µg/mL, (Zhang et al., 2017)) is also of the same order of magnitude, with similar values to those afforded by SAE and LAE.

Table 1: Inhibitory activity of aqueous extracts from *C. loniceroides* obtained by decoction or maceration on α-amylase and α-glucosidase.

	Decoction			Maceration		
	30 min	60 min	90 min	12 h	24 h	48 h
	IC_{50} α-amylase (µg/mL)					
LAE	*	*	*	*	*	*
SAE	*	*	*	*	*	*
UFAE	1.73 ±0.11^{a}	4.95 ±0.07^{a}	3.25 ±1.17^{a,b}	8.14 ±0.42^{a}	14.78 ±2.56^{b}	40.25 ±0.55^{c}
RFAE	5.85 ±0.05^{a}	6.69 ±0.18^{a}	9.52 ±0.31^{b}	6.63 ±0.50^{a}	23.96 ±0.01^{b}	52.85 ±1.92^{c}
Acarbosa	7.1					
Resveratrol	111-120 (Zhang et al., 2017)					
	IC_{50} α-glucosidase (µg/mL)					
LAE	37.92 ±4.83^{a}	75.25 ±3.67^{b,c}	117.49 ±1.12^{c}	178.92 ±13.02^{b}	95.78 ±2.24^{a,b}	158.95 ±6.25^{b}
SAE	14.71 ±0.43^{a}	82.51 ±1.88^{b,c}	114.50 ±7.21^{c}	56.90 ±0.66^{a}	53.69 ±2.1^{a}	100.02 ±0.56^{a}
UFAE	159.96 ±21.19^{a,b}	116.40 ±5.23^{a}	170.07 ±0.95^{b}	195.78 ±6.93^{a}	469.65 ±4.35^{b}	543.65 ±4.03^{b}
RFAE	168.95 ±11.24^{a}	307.60 ±17.39^{b}	386.15 ±34.86^{c}	1030.75 ±4.35^{a}	680.87 ±55.74^{b}	1030.75 ±30.36^{c}
Acarbosa	31					
Resveratrol	1350 (Miao et al., 2014)					

Values are means ±SD (n=5), means in same row with different superscripts are significantly different ($p<0.05$).
* There is no inhibitory activity.

Inhibition kinetic of enzymes

Inhibition kinetics parameters were assessed. The RFAE and UFAE obtained by decoction at 30 min showed a competitive mode of inhibition for α-amylase (Fig 1a and 1b, respectively), unlike that of the RFAE, a non-competitive mode for α-glucosidase (Fig 1c). On the other hand, UFAE obtained by decoction at 30 min showed a competitive inhibition for the CHE (Fig 1d). Finally, the LAE and SAE obtained by decoction at 30 min only had a competitive inhibition activity against α-glucosidase (Fig. 1e and 1f; respectibly). The acarbose and resveratrol that were used as a control displayed a competitive mode of inhibition for both enzymes.

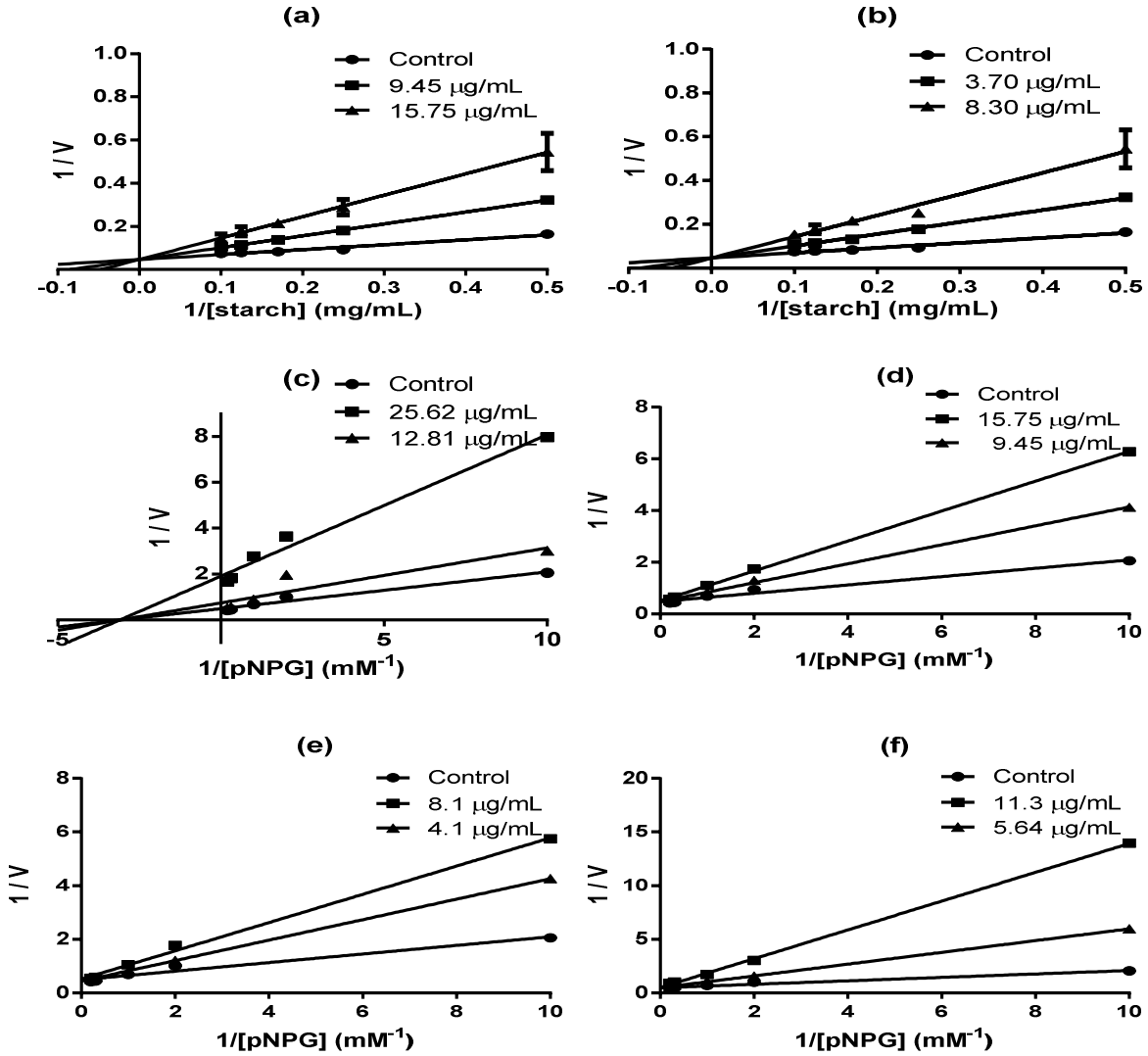

Figure 1: Lineweaver-Burk plot of the effect of aqueous extracts of *C. loniceroides* on the hydrolysis reaction catalyzed by α-amylase for (a) RFAE. (b) UFAE. And by α-glucosidase for (c) RFAE. (d) UFAE. (e) LAE. (f) SAE. All the extracts were obtained by decoction for 30 min. Each plot shows the different concentrations of aqueous extract which were evaluated. Table 2 shows the kinetic parameters from different plant aqueous extracts obtained by decoction at 30 min. Since this is a competitive inhibition mode, except for the inhibition of α-glucosidase by RFAE, Vmax value is roughly the same (21 mg/min and 2 μM/min for α-amylase and α-glucosidase, respectively) when a zero order kinetics is reached. Thus, the comparison is difficult for the inhibition kinetic parameters of α-amilasa and α-glucosidase as obtained of several plant aqueous extracts because there is no standardized way to express these kinetic values. In fact, different inhibitory activity values of CHE have been reported, nevertheless the method has not been accurately described.

Table 2: Kinetics of α-amylase and α-glucosidase inhibition by different aqueous extracts as obtained of *C. loniceroides.*

	α-amylase					α-glucosidase				
	Extract (mg/mL	Km (mg/mL)	Vmax (μg/mL/min)	Ki' (μg/mL)	Inhibition	Extract (mg/mL)	Km (μM)	Vmax (μM/min)	Ki' (μg/mL)	Inhibition
Without extract	0	4.3	21.2	-		0	326.45	2	-	
RFAE 30 min	3.7	12.9	21.2	2.4	Competitive	0.75	323.3	1.3	0.36	Non-competitive
	8.3	20.6	20.8			1.5	343.5	0.5		
UFAE 30 min	1.25	13.9	21.5	0.71	Competitive	0.37	641.9	2.1	0.23	Competitive
	2.5	23.1	21.3			0.63	2429.7	1.9		
LAE 30 min	-	-	-	-	-	0.075	957.2	2.3	0.06	Competitive
						0.15	1199.9	2.1		
SAE 30 min	-	-	-	-	-	0.075	1123.5	2	0.01	Competitive
						0.15	2698.5	2		

- There is no inhibitory activity.

Glucose tolerance test

The starch intake of 2 g/kg bw (Fig. 2a) or the sucrose intake of 4 g/kg bw (Fig. 2b) was administered for the glucose tolerance test. Both assays resulted in a rapid and significant increase in glycemia (88% and 253% for starch or sucrose, respectively) in the NG mice which were used as controls. Both trials showed that the source of carbohydrate does affect the inhibition of CHE due to the AE. On the starch tolerance test, the best antihyperglycemic result was observed on the group treated with UFAE of *C. loniceroides* obtained after 30 min of decoction (Fig. 2a). This extract reduced the glycemic response because of the α-amylase inhibition, by 23% when compared to the group that was only given the starch. This effect was significant ($p<0.05$) and the behavior was similar to that of acarbose and resveratrol. Therefore, the UFAE that inhibited α-amylase was chosen to observe the antidiabetic effect in (STZ)-induced diabetic mice for a period of 35 days. As for the sucrose tolerance test (Fig. 2b), all AEs obtained at 30 min reduced the hyperglycemia of NG mice because of the inhibition of α-glucosidase on the sucrose hydrolysis. The SAE of *C. loniceroides* afforded the largest reduction of hyperglycemia by 35%. This figure was even higher than that of acarbose (13%) or resveratrol (11.4%). After 60 min of treatment, all of the extracts presented the same antihyperglycemic effect without any significant differences ($p > 0.05$). Thus, the SAE was chosen to evaluate the antidiabetic effect in (STZ)-induced diabetic mice for 35 days. Fig. 2c shows the antidiabetic effect of SAE and UFAE on (STZ)-induced diabetic mice. At day 35, UFAE diminished glycemia of (STZ)-induced diabetic mice similarly to that observed for acarbose and resveratrol.

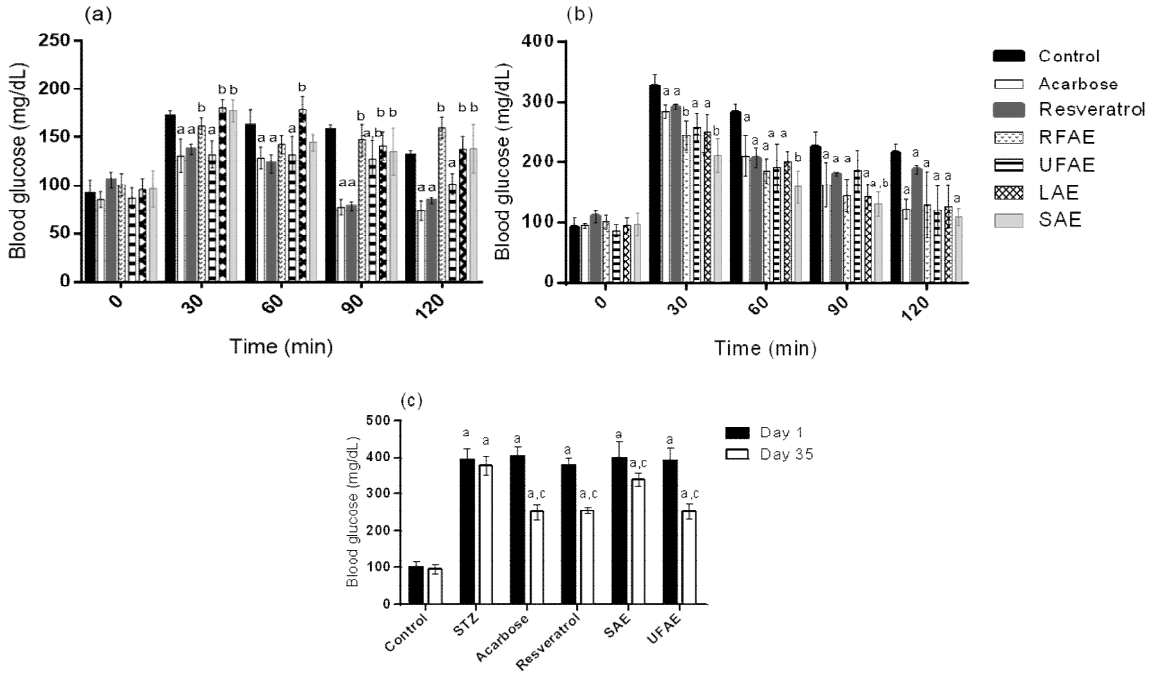

Figure 2: Effect of *C. loniceroides* aqueous extracts on glycemia in normoglycemic mice and STZ-induced diabetic mice. (a) Postprandial blood glucose levels of normoglycemic mice during a starch tolerance test. (b) Postprandial blood glucose levels of normoglycemic mice during a sucrose tolerance test. (c) The blood glucose levels were measured at the beginning and end of the treatment in STZ-diabetic mice (35 d). Values are presented as the mean±S.D. for n=5 mice. Data were analyzed by ANOVA and post-hoc Duncan test.
[a] denote significant difference compared to the control group; [b] statistically significant compared to the acarbose group ($p<0.05$); [c] statistically significant ($p<0.05$) compared to STZ-induced diabetic mice group.

Five-week sub-acute study
Lipid peroxidation

The lipid peroxidation in the different mice groups was evaluated by the TBARS, measured mainly as MDA in samples obtained from liver homogenate (Fig. 3a). The MDA concentration in TBARS increased 1.65 fold in the (STZ)-induced diabetic mice when compared to the NG mice. The MDA concentrations decreased in the mice groups treated with SAE (1.5 times) or UFAE (1.76 times) aqueous extracts obtained by decoction of *C. loniceroides* at 30 min. These MDA levels were similar to those found in the NG mice and the resveratrol group. Finally, the group of mice that was administered with acarbose produced nearly as much TBARS as the (STZ)-induced diabetic mice.

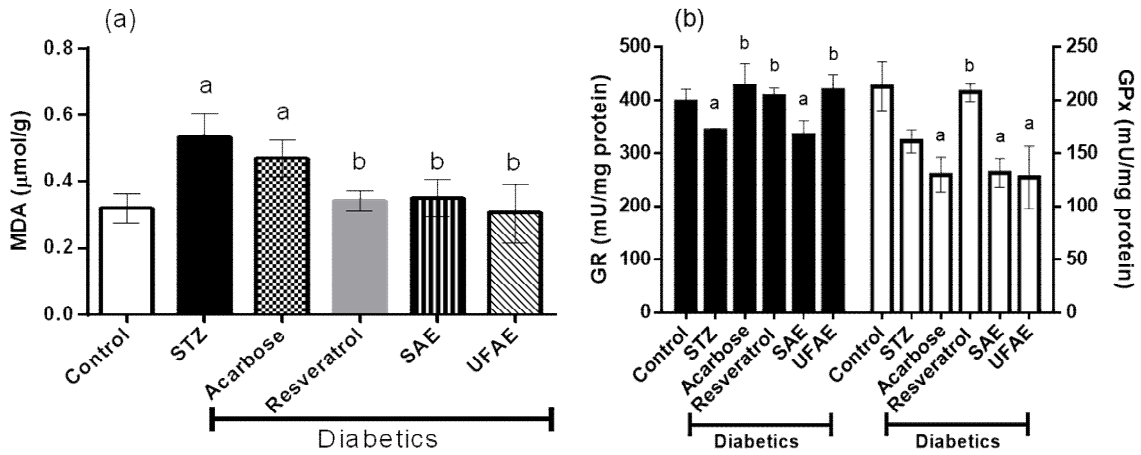

Figure 3: Effect of different *C. loniceroides* aqueous extracts, after a five-week subacute daily dosing, assessed in liver of STZ-induced diabetic mice. (a) MDA concentration and (b) GR and GPx activities. Values are presented as the mean±S.D. for n=5 mice. [a] Statistically significant ($p<0.05$) compared to the normoglycemic mice control group. [b] Statistically significant ($p<0.05$) compared to STZ-induced diabetic mice group.

Effect of *C. loniceroides* on glutathione redox state and GPX and GR

Table 3 shows not only the changes in total pool of glutathione, but also the different forms of glutathione found in the liver homogenate of (STZ)-induced diabetic mice treated with SAE or UFAE. The oxidative stress was observed because the GSH concentration decreased (30%), whereas the GSSG increased (66%) significantly ($p<0.05$) relative to the NG mice. The GSH/GSSG ratio of the (STZ)-induced diabetic mice was the lowest, 2.5 times lower than that of the NG mice. When the UFAE was administered, it was observed that the GSH/GSSG ratio (3.08 ± 0.66) was maintained due to no significant difference found with the control group ($p<0.05$). It even maintained the ratio in a more efficient way than the acarbose or resveratrol group, used as positive controls. Similarly, the total pool of glutathione (GSH+GSSG = 324.47 ± 5.07 µM) of the mice group administered with UFAE increased, possibly due to a rise in GSH production regarding the NG mice. Figure 3b shows the effect of the aqueous extracts of *C. loniceroides* on the antioxidant enzymes. The UFAE also showed a significant effect ($p<0.05$) because it reduced the production of GPx, but increased the production of GR, similarly to that observed for resveratrol, with regard to the (STZ)-induced diabetic mice.

Table 3: Effect of different aqueous extracts of *C. loniceroides* on the glutathione pool in liver of STZ-induced diabetic mice.

	Groups	Liver
GSH (µM)	Control	213.93 ±13.81
	STZ	148.93 ±9.98[c]
	Acarbose	148.21 ±3.51[c]
	Resveratrol	186.12 ±5.81[b,c]
	SAE	152.2 ±5.23[c]
	UFAE	244.89 ±14.94[b,c]
GSSG (µM)	Control	74.05 ±10.18
	STZ	123.59 ±5.39[c]
	Acarbose	53.43 ±3.27[a]
	Resveratrol	83.97 ±5.83[b]
	SAE	78.63 ±18.07[b]
	UFAE	79.58 ±9.97[b]
GSH/GSSG	Control	2.94 ±0.57
	STZ	1.17 ±0.10[c]
	Acarbose	2.48 ±0.10[a]
	Resveratrol	2.22 ±0.14[a]
	SAE	2.25 ±0.53[a]
	UFAE	3.08 ±0.66[a]
GSH+GSSG (µM)	Control	287.97 ±14.14
	STZ	272.52 ±10.38
	Acarbose	201.64 ±6.76[a,c]
	Resveratrol	270.09 ±10.07[d]
	SAE	230.83 ±19.01[b,c]
	UFAE	324.47 ±5.07[b,c]

[a] $p<0.05$ compared to STZ-induced diabetic mice group; [b] $p<0.05$ compared to the STZ-induced diabetic mice group and acarbose; [c] $p<0.05$ compared to the normoglycemic mice control group; [d] $p<0.05$ compared to the acarbose group.

Effect of *C. loniceroides* extracts on cytokines

The serum pro-inflamatory markers, IL-6 and TNF-α, in (STZ)-induced diabetic mice increased when compared to those of the NG mice ($p<0.05$). On the other hand, the level of anti-inflammatory marker IL-10 decreased in (STZ)-induced diabetic mice (Figs. 4a, 4b and 4c). The SAE and UFAE of *C. loniceroides* as well as acarbose and resveratrol were administered orally to mice and all of them decreased IL-6 levels, thus maintaining near-normal IL-6 levels as in the NG mice (Fig. 4a). Similarly, these extracts decreased the concentration of TNF-α with respect to the (STZ)-induced diabetic mice used as control (Fig. 4c), although a reduction of this concentration did not reach the level exhibited by NG mice. No extract of *C. loniceroides* had an influence on IL-10 cytokine levels, whose level remained close to that of (STZ)-induced diabetic mice ($p<0.05$; Fig. 4b). Just resveratrol group could elevate IL-10 regarding (STZ)-induced diabetic mice.

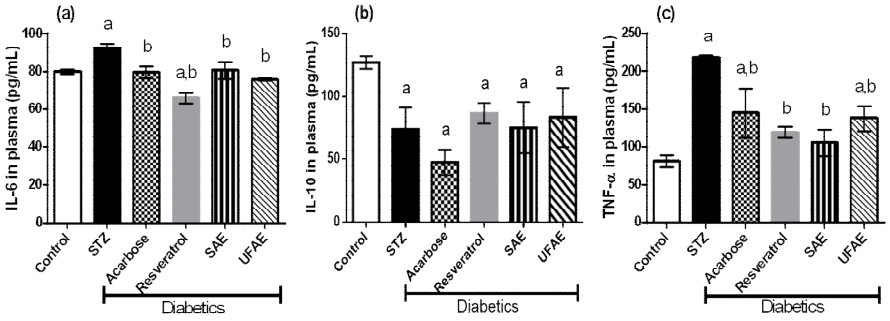

Figure 4: Effects of different *C. loniceroides* aqueous extracts on serum pro-inflamatory (IL-6 and TNF-α) and anti-inflamatory (IL-10) cytokines, after a five-week subacute daily dosing in STZ-induced diabetic mice. (a) IL-6, (b) IL-10 and (c) TNF-α. Mean±S.D. (n=5). [a] Statistically significant ($p<0.05$) compared to the normoglycemic mice control group. [b] Statistically significant ($p<0.05$) compared to STZ-induced diabetic mice group.

Liver transaminases

Figures 5a and 5b show the effect of *C. loiceroides* extracts on hepatic markers (ALT and AST) in (STZ)-induced diabetic mice. They were administered with SAE and UFAE and they maintained nearly the same ALT level as the NG mice. This level also remained the same as in (STZ)-induced diabetic mice. However, administration of acarbose did induce an increase in the serum ALT while resveratrol reduced it ($p<0.05$). On the other hand, AST levels in (STZ)-induced diabetic mice also increased as a consequence of diabetes. Administration of acarbose increased the serum AST level, which was similar to that of diabetic mice, but resveratrol maintained it nearly to NG mice. In contrast, the administration of SAE and UFAE for 35 days restored the level of AST to a level similar to that of the NG mice ($p <0.05$.).

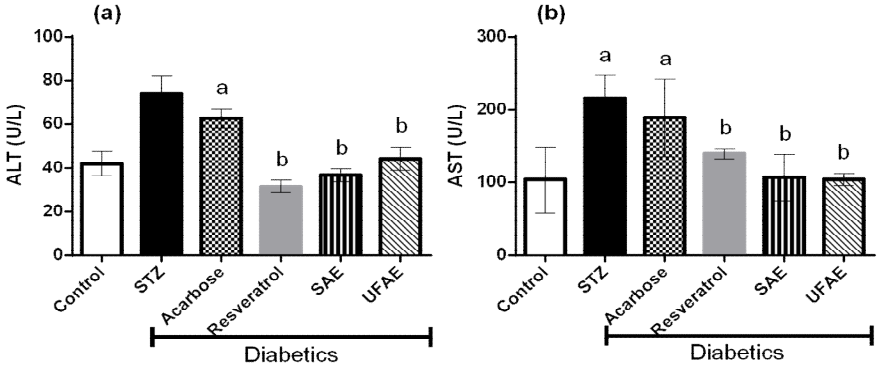

Figure 5: Effects of different *C. loniceroides* aqueous extracts on transaminases, after a five-week subacute daily dosing, assessed as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations in the liver of STZ-induced diabetic mice. (a) ALT and (b) AST. [a] Statistically significant ($p<0.05$) compared to the normoglycemic mice control group. [b] Statistically significant ($p<0.05$) compared to STZ-induced diabetic mice group.

Discussion

In diabetes disease, oxidative stress plays an important role in the development of insulin resistance and its effects (Evans et al. 2005; Verdile et al. 2015). Therefore, antioxidants can be used to manage diabetes due to their biological properties. Treatment with polyphenols could enhance the effectiveness of diabetes management. *C. loniceroides* is a

source of polyphenols, especially when the fruit is unriped. Several biological and beneficial health effects have been demonstrated by phenolic compounds in plants. There is evidence that these compounds modulate carbohydrates hydrolysis by inhibition of the enzymes α-amylase and α-glucosidase (McDougall et al., 2005). Within the Loranthaceae family, mistletoes containing large numbers of polyphenols, have already been reported, and also have antidiabetic activity *in vivo* and *in vitro* (Osadebe et al 2004, Osadebe et al., 2010). The UFAE of *C. loniceroides* showed remarkable competitive inhibition of α-glucosidase and α-amylase. Consequently, in an acute study, antidiabetic effect *in vivo* is observed by inhibiting the hydrolysis of starch, possibly by the polyphenols action that has been reported (Serrano-Maldonado et al. 2011). Also, in the subacute study, a decrease in hyperglycemia was observed in mice treated with *C. loniceroides* compared with the (STZ)-induced diabetic mice group. Then, carbohydrate hydrolyzing enzymes inhibitors may be an attractive therapeutic modality in diabetic patients (Jaiswal et al. 2012).

The group of (STZ)-induced diabetic mice showed an increase in the levels of MDA and AST, but GSH/GSSG ratio, GR and GPx activity decreases. The GSH/GSSG ratio is inversely related to oxidative stress and it is often used as a sensitive index of oxidative stress *in vivo* (Díaz-Flores et al. 2012). The alterations in GR and GPx activity produce changes in the redox state (Al-Dallen et al. 2004). Then, there is an increase in oxidative stress in the (STZ)-induced diabetic mice group, which could cause chronic hyperglycemia, that is responsible for oxidative stress because of an excessive ROS production from auto-oxidation of glucose, glycated proteins, and glycation of antoxidative enzymes which impair their capacity to detoxify the free radicals (Martín-Gallán et al. 2003).

The inhibition of intracellular ROS formation would serve as a therapeutic strategy to prevent oxidative stress in diabetes. Several studies have demonstrated that antioxidants like vitamin C, vitamin E, and polyphenols can reduce oxidative stress and lipid peroxidation in T2D patients and animals (Jaiswal et al. 2012). Therefore, in the present study, in addition to evaluating the antihyperglycemic activity of *C. loniceroides*, the mistletoe extracts effect on the oxidative stress was determined. In the five-week subacute daily dosing, the state redox was improved because the GSH/GSSG ratio and GR activity significantly increased with respect to the group of (STZ)-induced diabetic mice, the values were similar to the NG mice. It could be an indirect indicator of ROS reduction. As a result, lipid peroxidation decreased, this was determined by the levels of MDA. On the other hand, when the polyphenols exert their antioxidant action they reduce ROS (Rice-Evans et al. 1997; Martinez and Moreno 2000). Accordingly, the mistletoe study should be proposed in the characterization of phenolic compounds that could have the antioxidant effect. Thus, SAE and UFAE of *C. loniceroides* treatment could be an alternative to decrease or prevent oxidative stress in diabetes mellitus.

In previous studies, it was observed in the (STZ)-induced diabetic mice an inflamatory state due to a decrease in the concentration of IL-10, which triggered the production of IL-6 and TNF-α. The TNF-α stimulates hyperlipidemia and hepatic lipogenesis simultaneously reducing the sensitivity to insulin in muscle tissues and finally the necrosis of target organs (Khanra et al. 2015). In the present study, this inflammatory state is observed in STZ-diabetic mice. As mentioned before, hyperglycemia induces oxidative stress, and also causes an inflammatory state (Rosado Pérez and Mendoza Núñez 2007). The ROS generated by hyperglycemia induce the activation of NF-κB, which is an activating factor that regulates the expression of different inflammatory cytokines. At the same time, inflammation and oxidative stress can cause liver damage (Mittal et al. 2014) as observed in diabetic mice, through the determination of transaminases (AST y ALT).

After the administration of SAE and UFAE of *C. loniceroides* in (STZ)-diabetic mice, the concentration of IL-6 and TNF-α was reduced with respect to the group of diabetic mice. However, the concentration of IL- 10 was not improved in the diabetic group. The decrease in inflammatory cytokines could be explained by the antioxidant effect of the extracts. The polyphenols contained in the extracts can also inhibit NF-κB activation by decreasing ROS (Bhattacharya and Sil 2018). Therefore, they inhibit the expression of cytokines like TNFα and IL-6, as well as the decrease of transaminases such an AST and ALT.

In order to develop and construct knowledge about the antidiabetic activity of *C. loniceroides*, further studies should be performed to confirm whether this mistletoe may display a similar antidiabetic mechanism as the one found in other medicinal plants.

Conclusion

The UFAE of *C. loniceroides* showed an antihyperglycemic effect through the inhibition of carbohydrate hydrolyzing enzymes. Besides having this activity, further study can be interesting for the treatment of diabetes due to its effects on oxidative stress and its anti-inflammatory activity. These effects *C. loniceroides* could be due to its high polyphenol composition. However, more research is needed to confirm and evaluate these effects at the *in vivo* and clinical levels.

Competing interests: The authors declare that they have no competing interests.

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