

Brazilian spice has anti-diabetic and cardiovascular risk-reducing effects in rats

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Abstract

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia and whose prevalence has been increasing over the years. Diabetes mellitus and atherosclerosis appear to be connected. Natural herbal remedies have proven to be safe and effective alternatives in the treatment of this disease. In Northern Brazil, the species *Lippia origanoides* Kunth (Verbenaceae), used as a spice, is associated with therapeutic activities, however, its antidiabetic potential lacks studies. This work aimed to evaluate the hypoglycemic and cardiovascular risk-reducing effects of the hydroalcoholic extract of *Lippia origanoides* Kunth (ELO) in rats with alloxan-induced diabetes (120 mg/kg). The animals received ELo at doses of 75, 150, and 250 mg/kg p.o. for 28 days, and positive control was performed in comparison with Glibenclamide (5 mg/kg). Treatment with ELo at all studied doses showed hypoglycemic and cardiovascular risk-reducing effects. Flavonoids that are directly related to these pharmacological activities were identified in ELo by LC-MS, coupled to a liquid chromatography system. The extract obtained from this spice has a promising action on diabetes with cardioprotection establishing a basis for the development of toxicological studies to validate the plant in traditional medicine and further investigations in humans.

1. INTRODUCTION

Diabetes is a group of metabolic disorders characterized by a chronic hyperglycemic condition resulting from defects in secretion and/or insulin action (Poznyak et al. 2020). The prevalence of diabetes is rapidly increasing worldwide. According to the International Diabetes Federation (IDF), the total adult population (20 to 79 years old) living with this disease was, in 2019, 463 million people, and it is expected to increase to 578 million by 2030 (IDF Diabetes Atlas 2019). A significant part of the number of people with diabetes is found in countries like China, the USA, Indonesia, India, Brazil, Mexico, Japan, and Pakistan (Tran and Pham 2020).

Diabetes mellitus and atherosclerosis appear to be connected by several pathological pathways. Dyslipidemia associated with diabetes has received much attention in recent years, and diabetic patients have shown an increased risk of the onset and accelerated development of atherosclerosis (Hirano 2018). The alteration of the lipid profile in diabetes is linked to the elevated hepatic production of triglyceride-rich lipoproteins, leading to the increased formation of atherogenic VLDL (Ivanova et al. 2017).

Herbal medicines and traditional medical practitioners are receiving considerable attention from mainstream health officials, international medical research, and training institutions. Traditional medicine is estimated used by up to 80% of the population of most developing countries (Chelghoum et al. 2021). Herbal medicines are thought to be effective, safe, and affordable.

Lippia origanoides Kunth (Verbenaceae) is a promising oregano-like herb, known in northern Brazil as "*alecrim-pimenta*", "*salva-do-marajó*" or "*alecrim-d'angola*" (Sarrazin et al. 2015; Castilho et al. 2019). The aerial parts of this bushy species are used in regional cuisine as flavoring agents (Sarrazin et al. 2015).

Within the genus *Lippia* (L.), it is described that *L. nadiiflora* (Balamurugan and Ignacimuthu 2011) and *L. javanica* (Arika et al. 2015) reveal promising evidence of antidiabetic action, but this effect in *Lippia origanoides* Kunth has not yet been described experimentally.

The presence of phenolic compounds, such as flavonoids, is responsible for the antidiabetic character of some medicinal plants, through the reduction in blood glucose levels. (Kumar et al. 2021). Many chemical constituents have been identified in different species of *Lippia*, among which flavonoids are one of the predominant groups (Chen et al. 2015). Therefore, this work aimed to evaluate the potential hypoglycemic and cardiovascular risk reduction effects of the hydroalcoholic extract of *Lippia origanoides* Kunth – ELo in diabetic rats.

2. MATERIALS AND METHODS

2.1. Plant material

The aerial parts of the plant (*L. origanoides* Kunth) were collected from José de Freitas District (latitude 04°45'23" south and longitude 42°34'32" west), Piauí, Brazil, 2019. The voucher specimen was deposited under no in the herbarium at "Graziela Barroso" of the Federal University of Piauí Biology Department. TEPB 9.205. Registered in Sisgen - MMA Brazil - Sisgen N° - AB30D19.

2.2. Extract of *Lippia origanoides* Kunth (ELo): preparation and chemical characterization

The standardized extract was obtained according to Coelho et al. (2015). The aerial parts from *Lippia origanoides* Kunth were submitted to maceration in ethanol/water (1:1) for three days. The hydroalcoholic extract was filtered and concentrated in a Fisatom® 802 rotary evaporator (coupled to 826T® vacuum pump - Fisatom®, 50 °C, cooling to 11 °C under a pressure of 600 mmHg) and then dried in a lyophilizer L101 (Liotop). The dry extract was further homogenized with mortar and pestle and kept in a desiccator until constant mass.

The characterization of ELo was performed by LC-MS, Amazon Speed ETD – Bruker® coupled to a Shimadzu® liquid chromatography system with 30AD pumps at a flow of 1 ml/min, SPD-20A detector with selected wavelength at 288 nm, CTO-Over 20A for column (Phenomenex Luna C18 250x4.6mm; 5µm) at room temperature and SIL-30AC Autosampler, with an injection volume of 10µL. Chromatographic analysis was performed using Water (Solvent A) and Methanol (Solvent B) solvents in a gradient method starting at 5% B, maintained for 2 minutes, and reaching 100% in 62 minutes of analysis and 100% B maintained for up to 77 minutes.

ELo was analyzed by mass spectrometer performed analysis by electrospray ionization and the multistage fragmentations were obtained in an ion trap analyzer in negative mode, capillary voltage – 4.5kV, capillary temperature 300°C, N₂ carrier gas (12 L. min⁻¹) and nebulizer gas pressure of 30 psi and acquisition range was *m/z* 100–1000. The identification of the constituents was based on the multistage

fragmentation profile, comparison to a database of MS/MS spectra like the Massbank® (<https://massbank.eu/MassBank/>) and a literature review.

2.3. Experimental animals

The study used male Wistar albino rats (3–4 weeks old) that weighed 200-250g. These were bred in the Bioterium at the Biological Sciences Institute of the Federal University of Pará – UFPA, housed in an air-conditioned room in plastic cages with free access to drinking water and with a standard pellet diet, under controlled conditions of humidity ($50 \pm 10\%$), light (12/12 h light/dark cycle) and temperature ($25 \pm 2^\circ\text{C}$). The experiments were conducted in accordance with Brazilian animal protection and welfare laws and the protocol was approved by the local Committee for the Use and Care of Experimental Animals of the Federal University of Pará, Belém - Brazil (CEUA nº 82113001200).

2.3.1. Induction of Hyperglycemia

After seven days of acclimatization, diabetes was induced in the rats, that were fasted overnight, with a single intraperitoneal (i.p.) injection of freshly prepared alloxan monohydrate (2,4,5,6 tetraoxypyrimidine;5-6-dioxuracil) obtained from Sigma® (Steinheim, Switzerland), dissolved in ice-cold saline at a dose of 120 mg/kg (body weight). The rats which had a fasting blood glucose level above 250 mg/dL post 72 h of diabetes induction were chosen for the study. Blood was collected from the tail end and blood glucose was analyzed (at fasting state) using a glucometer (Accu check®) (Elangovan et al. 2019). After confirmation of hyperglycemia, the animals were assigned to two experimental blocks. The first evaluated oral glucose tolerance, and in the second block, the potential hypoglycemic agents were evaluated with repeated doses of the extract for up to 28 days.

2.3.1.1. Oral glucose tolerance test (OGTT)

The oral glucose tolerance test (OGTT) was employed to examine the animals' ability to tolerate the glucose load. For this, after an overnight fast, a single dose of glucose solution (2.0 g/kg b.w.) was administered orally in 3 groups that received prior oral treatment ($n = 8$): Diabetic animals, control-vehicle; Diabetic animals - ELo 150 mg/kg and Diabetic animals - 5 mg/kg of Glibenclamide. Blood glucose levels were measured at 0 (just before glucose ingestion), 30, 60, 90, and 120 min after glucose ingestion using a glucometer (Accu check®) (Elangovan et al. 2019).

2.3.1.2. Effects of ELo treatment on Fasting Blood Glucose (FBG) in experimental groups

Initially, the experimental animals were categorized into six groups ($n = 10$):

Group 1: Control group (CN) received normal water and feed.

Group 2: Diabetic control (DC) received alloxan monohydrate (120 mg/kg).

Group 3, 4, and 5: Diabetic rats received daily ELo 75, 150, and 250 mg/kg, respectively.

Group 6: Diabetic rats received a daily oral dose of 5 mg/kg of Glibenclamide.

The treatment was given for 15 to 28 days to the diabetic rats. Body weight and fasting blood glucose were estimated at 0, 7, 15 and 28 days. At 15 days, half of the animals in each group (n = 5/group) were fasted overnight and euthanized with ketamine/xylazine 300/30 mg/kg i.p. and immediately, blood samples were collected for biochemical (lipids, renal, hepatic profiles, and MDA levels), and liver/pancreas for histological analysis. All results were compared to values obtained by normal control animals (NC) and diabetic control (DC). The other part of the group continued to receive treatment for 28 days, and fasting blood glucose was estimated at 21 and 28 days. At 28 days, the remaining animals (n = 5/groups) were fasted overnight and euthanized with ketamine/xylazine 300/30 mg/kg i.p. and immediately blood were collected for Measurement of insulin and HbA1c. All results were compared to values obtained by normal control animals (NC) and diabetic control (DC).

2.3.2. Serum biochemical parameters

2.3.2.1. Lipid profile, kidney function and liver function

Serum was separated by centrifuging blood at 4000 rpm for 10 min and was subjected to the biochemical analysis for estimation of serum lipid profile (Total cholesterol, triacyl glycerides, low-density lipoprotein, high-density lipoprotein - mg/dl). The atherogenic index, atherogenic coefficient, and cardiovascular risk index were calculated by using the formula by Harnafi et al. (2008), Kinosian et al. (1994) and Azizan et al. (2018), respectively.

Atherogenic Index (AI) = (Total Cholesterol – HDL Cholesterol) / HDL Cholesterol

Atherogenic Coefficient (AC) = LDL Cholesterol / HDL Cholesterol

Cardiovascular Risk Index (CRI) = Total Cholesterol / HDL Cholesterol

Renal and hepatic functions were evaluated based on serum parameters: urea and creatinine, alanine transaminase (ALT), and aspartate aminotransferase (AST), respectively. These parameters were estimated (mg/dl) using commercial kits (Wiener lab) with the Automatic Clinical Chemistry Analyzer CM200 Wiener ®.

2.3.2.2. Oxidative damage assessment

The thiobarbituric acid-reactive substances (TBARS) were determined as described by Ohkawa et al. (1979) and Pyles et al. (1993). This test indicates the presence of products derived from lipid peroxidation, mainly malonaldehyde (MDA). Plasm 60µL was recovered with 15% trichloroacetic acid, and the supernatant was incubated with 10 mM thiobarbituric acid and heated at 92°C for 120 min. The resulting lightly pink-colored product was placed in a 96-well microplate for spectrophotometer

quantification at 535 nm (BIO-RAD Model 450 Microplate Reader). The results are expressed in μmol MDA.

2.3.2.3. Measurement of insulin and HbA1c

The serum samples at 28 days of ELo treatment were used to estimate insulin levels by Direct Chemiluminescence method - Competitive Immunoassay. The dosage of HbA1c was obtained by HPLC (High-Performance Liquid Chromatography) carried out in partnership with Clinical Laboratory Paulo Azevedo - Belém PA, Brazil.

2.4. Inhibitory Activity against α -Glycosidase

The methodology used was based on and adapted from the methods described by Schmidt et al. (2012). The enzyme α -glycosidase of *Saccharomyces cerevisiae* was resuspended in phosphate buffer 50mM pH 7.0 at a final concentration of 1 mg/ml, and then stored at -20°C until use. The para-nitrophenyl-alpha-glycoside substrate (pNPG- α) was resuspended in the same buffer at a final concentration of 20 mM and stored under the same conditions. As a stop reaction, the Na_2CO_3 solution at 1M is used. For the assay, the enzyme was first diluted to generate activity of 0.04 U/ml in the reaction solution from the phosphate buffer 10mM pH 7.0. The reactional assay of a final volume of 100 μl was used, consisting of 25 μl of the enzyme, 30 μl of phosphate buffer 100mM pH 7.5, and 20 μl of extract, which will be incubated for 15min at 30°C . Subsequently, 25 μl of pNPG- substrate was added to the reaction and incubated for 30 min at 30°C . The stop agent (100 μl) has been added, and then the solution will be read at 405nm in the spectrophotometer. A solution containing 50 μl of buffer, 25 μl of the enzyme, and 25 μl of the substrate was used for the control. The enzymatic activity of α -glycosidases was calculated from the formula:

$$\text{Enzymatic activity} \left(\frac{U}{ml} \right) = \frac{[(|test|) - (|blank|)] * Vt * fd}{18,1 * t * Vs}$$

Abs: Absorbance measured at 405nm

Vt: Total volume (200 μl)

fd: Dilution factor

18.1: Molar para-nitrophenol extinction coefficient in cm/micromolar

t: Reaction time (30min)

Vs: Reaction volume (100 μl)

2.5. Histopathological studies

The pancreas and liver from experimental animals were fixated with 10% formalin and paraffin for embedding the tissues. Thick tissue sections (5 μm) were processed and subjected to hematoxylin and

eosin (H&E) staining.

2.6. Statistical analysis

The statistical analyses were performed using the statistical package *GraphPad Prism* version 9. Kolmogorov Smirnov test (Normality Test, $\alpha = 0.05$) and Analyses of variance were performed by ANOVA procedures and the significance of each group was verified with Tukey's or Dunnett's post hoc *test* ($p < 0.05$).

3. RESULTS

3.1 Chemical profile of ELo by HPLC-PDA-ESI-IT-MS/MSⁿ

The HPLC-PDA-ESI-IT-MS/MSⁿ analyses of ELo showed a flavonoid profile (Fig. 1), using absorbance at 288 nm and total ion chromatogram, typical of flavonoid (Table 1; Fig. 2), have identified flavones as apigenin, orientin, metoxiflavones. Moreover, ELo showed the presence of flavanones such as Naringenin, Cirsimaritin, and Hidroxytrimetoxiflavanone.

Table 1

–Compounds identified in the hydroalcoholic extract of *Lippia origanoides* Kunth – ELo by HPLC-PDA-ESI-IT-MS/MSⁿ

Peak	R _T (minutes)	[M - H] ⁻	Fragment ions [MS ⁿ] (relative abundance, %)	Estimated identification
1	21.9	593	[593, MS ²]: 575, 503, 473 (100), 383, 353; [593→473, MS ³]: 583, 353 (100); [593→473→353, MS ⁴]: 325 (100), 297, 191; [593→473→353→325 MS ⁵]: 297 (100)	Apigenin-6,8-di-C-hexose
2	24.3	447	[447, MS ²]: 429, 357, 327 (100); [447→327, MS ³]: 299 (100), 283, 191; [447→327→299, MS ⁴]: 213, 147	Orientin
3	25	447	[447, MS ²]: 429, 357, 327 (100); [447→327, MS ³]: 299 (100); [447→327→299, MS ⁴]: 271, 225, 188	Orientin (isomer)
4	25,8	623	623, MS ² : 461 (100); [623→461, MS ³]: 391, 315 (100), 135; [623→461→315, MS ⁴]: 179, 135 (100); [623→473→315→135, MS ⁵]:	Isoramnetine-3-rutinoside
5	26.4	879	[915, MS ²]: 879 (100); [915→879, MS ³]: 717 (100), 591, 269; [915→879→717, MS ⁴]: 565, 447, 357, 327, 269 (100)	Morelloflavone-4, 7-di-O-hexose
6	27.5	431	[431, MS ²]: 413, 341, 311 (100); [431→311, MS ³]: 283 (100); [431→311→283, MS ⁴]: 241	Apigen-8-C-hexose
7	31.2	287	[287, MS ²]: 269, 151 (100);	Aromadendrin
8	34.7	271	[271, MS ²]: 176, 151 (100)	Narigenin
9	38.9	269	[269, MS ²]: 267	Apigenin
10	39.2	299	[299, MS ²]: 283 (100); [299→283, MS ³]: 281 (100); [299→283→281, MS ⁴]: 267, 219 (100), 161	Trihidroxi-metoxiflavone
11	41.4	313	[313, MS ²]: 298 (100); [313→298, MS ³]: 283 (100); [313→298→283, MS ⁴]: 255 (100), 239, 226, 183	Cirsimaritin
12	41.9	285	[285, MS ²]: 165 (100)	Sakuranetin
13	45.5	329	[329, MS ²]: 327, 285 (100); [329→285, MS ³]: 271 (100)	Hidroxi-trimetoxiflavanone

3.2. Animal Experiments and biochemical parameters

3.2.1. Effect of ELo on body weight

Table 2 shows the effect of ELo on the body weight of alloxan-induced diabetic animals. The diabetic animals (DC) decrease in body weight (- 23.92%) within 2 weeks of induction compared to control animals (NC). Oral treatment of ELo 75, 150, and 250 mg/kg for 15 days significantly increased body weight by 36.97; 42.70 and 43.94%, respectively compared to diabetic control animals (DC).

Table 2
Effect of ELo on body weight in alloxan-induced diabetic rats

Days	NC	DC	ELo 75 mg/kg	ELo 150 mg/kg	Eo 250 mg/kg	Glib 5 mg/kg
0	226.62 ± 5.6	226.25 ± 3.7	228.63 ± 4.7	218.75 ± 4.5	220.50 ± 4.1	216,50 ± 3.0
7	242.25 ± 6.0 ^a	204.00 ± 4.7	222.75 ± 6.1 ^a	233.12 ± 4.4 ^a	235.25 ± 4.2 ^a	226,00 ± 3.3 ^a
15	253.88 ± 7.3 ^a	172.12 ± 4.5 [*]	235.75 ± 7.5 ^{*a}	245.62 ± 3.6 ^{*a}	247,75 ± 4.8 ^{* a}	234,50 ± 3.7 ^{*a}

All values are mean ± S.E.M (n = 5). * and **a** values deviate significantly from the normal control and diabetic control group ($p \leq 0.05$), respectively.

3.2.2. Effects of ELo in Oral glucose tolerance test (OGTT)

The tolerance test was performed on diabetic rats' control, treatment with ELo (150 mg/kg) and glibenclamide (5 mg/kg). The rats in the diabetic control group had a significant elevation in blood glucose levels throughout the total measurement period (120 min). The treatment with ELo 150 mg/kg reduces by -29.30% glucose level in comparison to the initial value. Moreover, it did not come back to the initial value (0 min) even at the end of the period tested. The glibenclamide (5 mg/kg) reduces by 17%. Both treatments prevented the expressive increase in glucose levels as observed in the diabetic group after significant glucose overload (Table 3).

Table 3

Values of glucose (mean $\pm$ standard error) in tolerance test performed in normoglycemic and diabetic Wistar rats chemically induced by alloxan (120 mg/kg, i.p) pretreated with ELo

Glucose Time	DC	%	ELo 150 mg/kg	%	Glib 5 mg/kg	%
0 (min)	248.60 $\pm$ 8.02		315.63 $\pm$ 12.22		347.13 $\pm$ 5.66	
30 (min)	378.00 $\pm$ 6.03	52.05	433.87 $\pm$ 8.22	37.46	448.22 $\pm$ 11.82	29.00
60 (min)	394.00 $\pm$ 7.22	58.48	344.00 $\pm$ 8.09	8.98	426.00 $\pm$ 8.14	22.72
90 (min)	406.40 $\pm$ 8.26	63.47	300.06 $\pm$ 6.79	-4.93	319.00 $\pm$ 3.14	-8.1
120 (min)	402.80 $\pm$ 10.33	62.03	223.12 $\pm$ 5.22	-29.30	288.00 $\pm$ 3.0	-17.03

3.2.3. Effects of ELo treatment on Fasting Blood Glucose (FBG) in experimental groups

Figure 3 illustrates the effects of ELo on the fasting blood glucose levels of experimental groups. On day-3 post-alloxan induction, there was a significant increase in fasting blood glucose in the entire alloxan-induced diabetic groups (DC) when compared to the Normal control glucose group (NC). Diabetic rats, treated with ELo (75, 150, 250 mg/kg) and glibenclamide showed a decline in fasting blood glucose level from the 15th day of the treatment as compared to the diabetic control group (Fig. 3). Treatment of ELo 250 mg/kg (93.13 ± 2.69 mg/dl) in the diabetic rats significantly brought the blood glucose values to the normal level as compared to the normal control group. (89.17 ± 2.7 mg/dl) on the 15th day of the treatment. Figure 3B shows that the effect is present for other doses (75 mg/kg and 150 mg/kg) and even greater when treatment with 250 mg/kg is maintained for 28 days.

3.2.4. Effect of ELo lipid profile

The level of serum total cholesterol (TC), triacylglycerides (TG), HDL- cholesterol, and LDL- cholesterol was determined significantly higher in diabetic control (DC) than in the normal control rats (NC), while HDL- cholesterol levels were significantly decreased. Continuous treatment with ELo for a 15-day period produced a significant amelioration of deteriorated effects on TC, TG, LDL, and HDL. The ELo 250 mg/kg was the most effective in alleviating TC, TG, HDL, and LDL (mg/dl) levels in diabetic rats. Atherogenic index (AI) and atherogenic coefficient (AC) were significantly elevated in the diabetic control when compared to the normal control and were reduced by all different ELo treatments (Table 4). In comparison with the normal control group (3.57 ± 0.33), the cardiovascular risk (CR) index was higher in the diabetic control – DC (6.93 ± 0.24) ELo reduced these indexes at all doses studied (Table 4). The reduction presented by the ELo treatments was greater than that presented by the glibenclamide group.

Table 4

Effect of ELo on lipids parameters, AI, AC and CRI in experimental groups. (Mean $\pm$ standard error)

	NC	DC	ELo 75 mg/kg	ELo 150 mg/kg	ELo 250 mg/kg	Glib 5 mg/kg
TG (mg/dl)	100.4 $\pm$ 3.2	159.2 $\pm$ 2.9 [#]	126.0 $\pm$ 5.8 ^{*#}	120.8 $\pm$ 2.2 [*]	115.2 $\pm$ 6.7 [*]	148.4 $\pm$ 7.9 [#]
TC (mg/dl)	52.8 $\pm$ 3.8 [*]	65.40 $\pm$ 3.3 [#]	59.00 $\pm$ 4.2 [#]	51.80 $\pm$ 2.1 [*]	51.40 $\pm$ 2.6 [*]	59.80 $\pm$ 2.2 [#]
HDL (mg/dl)	14.9 $\pm$ 0.7	9.458 $\pm$ 0.5 [#]	11.25 $\pm$ 0.3 [#]	12.76 $\pm$ 0.3 ^{**}	13.91 $\pm$ 0.5 [*]	12.14 $\pm$ 0.4 ^{**}
LDL (mg/dl)	17,8 $\pm$ 3.4	24.10 $\pm$ 2.7 [#]	22.55 $\pm$ 4.7	14.88 $\pm$ 2.1 [*]	14.45 $\pm$ 2.9 [*]	17.98 $\pm$ 3.2 [*]
AI	2.49 $\pm$ 0.36 [*]	5.92 $\pm$ 0.23 [#]	4.28 $\pm$ 0.48 [#]	3.06 $\pm$ 0.15 [*]	2.92 $\pm$ 0.33 [*]	3.93 $\pm$ 0.18 ^{**}
AC	1.21 $\pm$ 0.26	2.53 $\pm$ 0.24 [#]	2.03 $\pm$ 0.46	1.16 $\pm$ 0.16 [*]	1.04 $\pm$ 0.22 [*]	1.46 $\pm$ 0.25 [*]
CRI	3.57 $\pm$ 0.33 [*]	6.93 $\pm$ 0.24 [#]	5.28 $\pm$ 0.48 ^{**}	4.06 $\pm$ 0.14 [*]	3.71 $\pm$ 0.21 [*]	4.94 $\pm$ 0.19 ^{**}

AI - Atherogenic Index, AC: Atherogenic Coefficient and CRI Cardiovascular Risk Index mean $\pm$ Std. Error.*Concerning DC, # to NC & to glibenclamide (Glib). Values with different superscripts are significantly different from each other at $p < 0.05$ Two-way ANOVA changes according to Tukey's Multiple Range tests.

3.2.5. Effect of ELo liver, renal function parameters and plasm MDA level.

As displayed in Table 5, the serum AST, ALT and plasm MDA levels of diabetic control rats were significantly higher when compared to normal control rats. Of all the doses, ELo at 250 mg/kg revealed the maximum reduction of AST, ALT, and MDA levels in diabetic serum. and glibenclamide groups. As shown in Table 5, the diabetic rats showed a pronounced increase in the urea and creatinine levels compared to the control group. The renal parameters, serum urea, and creatinine have been reduced for ELo treatment.

Table 5

Effect of ELo on liver function test, kidney function tests, and MDA level in experimental groups.

	NC	DC	ELo 75 mg/kg	ELo 150 mg/kg	ELo 250 mg/kg	Glib 5 mg/kg
AST (U/L)	113.7 ± 3.3	218.7 ± 5.2 [#]	127.4 ± 9.6 [*]	109.8 ± 4.9 [*]	107.6 ± 5.9 [*]	116.0 ± 4.8 [*]
ALT (U/L)	27.1 ± 1.6	35.9 ± 1.7 [#]	31.9 ± 0.9	27.5 ± 1.2 [*]	26.34 ± 1.4 [*]	34.83 ± 0.8 [#]
Creatinine (mg/dl)	0.58 ± 0.03	0.78 ± 0.03 [#]	0.70 ± 0.03	0.63 ± 0.03 [*]	0.56 ± 0.03 [*]	0.71 ± 0.03 [#]
Urea (mg/dl)	65.29 ± 1.08	204.4 ± 4.5 [#]	164 ± 8.14 ^{##}	148.6 ± 4.03 ^{##}	153.9 ± 6.72 ^{##}	159.0 ± 5.7 ^{##}
MDA µM	54.5 ± 1.4	444.4 ± 22.3 [#]	224.0 ± 13.8 ^{##}	141.5 ± 7.4 ^{##}	69.0 ± 5.6 [*]	236.7 ± 15.96 ^{##}

*For DC, # for NC & for glibenclamide (Glib). Values with different superscripts are significantly different from each other at $p < 0.05$ ANOVA changes according to Tukey's Multiple Range tests.

3.2.6. Effect of ELo on insulin and glycated hemoglobin (HbA1c) levels

Figure 4 (A/B) illustrates the effects of ELo on insulin levels, glycated hemoglobin, and fasting blood glucose levels. Treatment of diabetic rats with ELo extract and glibenclamide significantly increased the insulin level in a dependent dose way after 28 days when compared to the diabetic control rats – DC 0.347(± 0.021) ELo 75, 150 and 250 mg/kg shows 0.444 (± 0.027), 0.548 (± 0.023)^{*}, 0.738 (± 0.036)^{##} uM/L, respectively. From ELo treatment with 150 mg/kg the insulin values did not differ statistically from normoglycemic rats (NC) – 0.550 (± 0.023) uM/L. ELo 250 mg/kg promotes greater insulin release than the Glibenclamide group (Glib) 5 mg/kg (0.6800 ± 0.0354) (Fig. 4A).

The levels of HbA1c (%) showed higher in diabetic rats -DC (8.30 ± 0.161)^{*} than normoglycemic rats – NC (4.98 ± 0.196) ($p < 0.05$). However, there was a reduction in glycated hemoglobin (HbA1c) levels with treatment with ELo at doses of 150 mg/kg – 6.20 (± 0.295)^{*} and ELo 250 mg/kg -5,2 (± 0.300)^{*} not revealing statistically different from the group Glib 5 mg/kg – 5.3 (± 0.286). * (Fig. 4B).

3.2.7. Relative activity of α-glycosidase in the presence of ELo.

The ELo dilutions (10.0–0.675 mg/ml) show the inhibitory activity of α-glycosidase *in vitro*. (Fig. 5). The anti-α-glucosidase activity was in the range of (98.61% – 64.09%). Hence, it may be suggested that α-glucosidase inhibition is one of the ELo mechanisms of action to reduce serum glucose levels.

3.3. Histopathological findings

Histological observation of the islet cells of the normal control group showed normal acini and islet cells with no structural changes. Histology of the islet cells of the diabetic control showed degeneration and extensive necrotic changes in the islet cells followed by atrophy in comparison with the normal control. Treatment with ELo showed visible positive changes in the histoarchitecture of pancreatic beta cells and the islet tissue section of the glibenclamide-treated group also showed a better appearance of beta cells as evidenced by the histopathological observation (Fig. 6A). The liver section of the alloxan-induced untreated diabetic group showed congestion of central veins and hepatocellular necrosis with vacuolization compared to that of the normal control group. However, diabetic rats treated with ELo and glibenclamide effectively reduced the severity of degenerative changes by attenuating the hepatic necrosis and reversing the hepatic architecture. Histology of the normal (Nave) group showed normal hepatocytes and central veins (Fig. 6A/B)

4. DISCUSSION

The hydroalcoholic extract of *Lippia origanoides* Kunth (ELo) presents hypoglycemic effects. This treatment also promoted an improvement in the clinical conditions of diabetic animals, expressed in laboratory parameters and in lower weight loss, which is so evident in diabetic rats without treatment. This effect observed may be associated with the presence of flavonoids. Other studies also prove the majority presence of these compounds in the chemical composition attributed to this species (Oliveira et al. 2014) and according to the data expressed in Table 1. The flavonoids linked to glucose uptake and metabolism stimulation are widely described in the literature. This regulation of enzymatic activity of carbohydrate metabolism, glucose homeostasis, and protection of pancreatic beta cells is revealed in several plant extracts that have presented antidiabetic potential, acting on glucose homeostasis and protection of pancreatic beta cells (Ullah et al. 2020).

Within the genus *Lippia*, it is described that *L. nadiiflora* (Balamurugan and Ignacimuthu, 2011), and *L. javanica* (Arika et al. 2015) also reveal promising evidence of anti-diabetic action. The phytochemical analysis of their respective extracts identified phenolic compounds in common as different flavonoids. Thus, the significant antidiabetic effect of ELo may be due to the presence of more than one antihyperglycemic principle, through the synergy of its properties. The thirteen flavonoids have been identified in the ELo extract (Table 2): According to the literature Naringenin, Apigenin, Vicentine 2, Cirsimaritin, Orientin, Sakuranetin, Vitexin, and Isoramnetin-3-O-rutinosideo reveal hypoglycemic effects.

The Alloxan model of diabetes could result in the destruction of the beta cells of the pancreas with a resultant decline in serum levels of insulin as a result of decreased synthesis. The actions the ELo confirmed in reducing hyperglycemia (Fig. 3) and maintaining insulin levels close to that of normoglycemic animals and glibenclamide group, within 28 days, suggest activation of beta cells in the pancreas of diabetic rats (Fig. 4A). A similar profile is presented by the histological findings (Fig. 6A) where an attenuation in the reduction of the area of the pancreatic islets is observed in the animals submitted to treatment with ELo in relation to the diabetic control group (DC) (Fig. 6B).

Another biochemical marker associated with monitoring diabetes is glycated hemoglobin (HbA1c) and its elevation is related to chronic hyperglycemic states (Stolar, 2010). The values of glycated hemoglobin (HbA1c) were reduced in the groups treated with ELo (150 and 250 mg/kg) and with Glibeclamide (5 mg/kg). This glycation is related to the level of hyperglycemia, erythrocyte lifespan, and patient age. This process occurs continuously throughout the life span of the erythrocyte, which is estimated to be approximately 53–60 days in the rat. The linear and progressive increase of glycated hemoglobin in diabetic animals was observed during the 3 weeks after diabetic induction (De Tata et al. 1996). These factors combined with the rat's high metabolic rate allowed changes in glycated hemoglobin faster than in humans, which allowed observed the reduction of the HbA1c in ELo treatment at 28 days. The ELo treatment should be directly related to the presence of phenolic compounds, such as these identified flavonoids.

Naringenin and its glycosides, found in citrus fruits, for example, present antidiabetic activity (Boudjelal et al. 2015; Nyane et al. 2017). Its ability to reduce hyperglycemia is related to increased messenger RNA expression of the insulin receptor and GLUT4 beta subunit. Thus, insulinotropic effects and the improvement of insulin action can be mediated by increasing the insulin receptor and glut4 transporter (Asmar et al. 2017). Additionally, Apigenin also promotes a positive impact on insulin resistance-related cell signaling in type 2 diabetes. In *in vivo* studies, a significant reduction in the mRNA expression of gluconeogenic enzymes, such as glucose-6-phosphatase, as well as lipogenic enzymes such as acetyl-CoA-carboxylase (Bumke-Vogt et al. 2014) was observed.

Vicentina-2 is also involved in the hypoglycemic activity of ELo via stimulation of insulin secretion from pancreatic islet culture (Boudjelal et al. 2015; Casanova et al. 2016; Antora and Salleh, 2017) and significantly altered the gene expression profile of the main regulatory genes of insulin INS1, INS2, transcriptional factor PDX1, insulin receptor INSR, and IRS1 which is a protein that is part of the insulin signaling pathway acting on the metabolic and mitogenic actions of insulin, as well as glucose transporter 2 (GLUT2), treated islets (Casanova et al. 2016).

Sakuranetin can increase adipogenesis and insulin sensitivity of 3T3-L1 adipocytes by regulating the peroxisome-activated receptor γ 2 (PPAR γ 2) (Saito et al. 2008), stimulating glucose absorption in these cells and thus sensitizing insulin adipocytes. This regulation can also be related to the lower weight loss presented by diabetic animals treated with ELo. Studies with *Lippia graveolens*, that have marked concentrations of Cirsimaritin, as well as this flavonoid alone, were able to inhibit Dipeptidyl-peptidase IV (DPP-IV). This enzyme is a serine protease responsible for the degradation of glucagon-like peptide 1 (GLP-1, Glucagon-like peptide-1). GLP-1 is a potent anti-hyperglycemic that induces stimulation of glucose-dependent insulin secretion while suppressing glucagon secretion. Inhibitors of this enzyme have successfully reduced levels of hyperglycemia and hemoglobin A1C as monotherapy and in combination with other antidiabetic agents (Wu, 2014).

Vitexin and its analogs and through reduction of cellular apoptosis and oxidative stress due to its anti-inflammation and antioxidant properties reveal a multifaceted potential in the diabetes process. This fact

corroborates the ability of ELo to reduce the MDA levels thus revealing its potential under oxidative stress generated in hyperglycemia. Oxidative stress produced by the hyperglycemic state deteriorates the antioxidant defense mechanism of the body and consequently increases MDA levels and reduces the activity of antioxidant enzymes (Wang and Wang, 2017).

The influence on the generation rate of reactive oxygen species (ROS) can act directly on glycemic control since the antioxidant potential of natural products promotes a decrease in ROS generation, and thus protects β cells and improves their function (Mojica et al. 2017). Consequently, the protection of these cells may keep insulin release closer to normoglycemic individuals. For example, Apigenin attenuates β pancreatic cell injury in animals with chemically induced diabetes not only as against free radicals. A free radical hijacker, which also by increasing the cell enzymatic antioxidant system of pancreatic β cells (Wang et al. 2017). And this antioxidant capacity is generally associated with these phenolic compounds. In addition, Naringenin and Apigenin significantly lowering blood glucose, reducing levels of malonaldehyde, ICAM-1 and insulin resistance may also have a direct action on vascular reactivity (Ren et al. 2016). Currently, Apigenin is considered a cardioprotective in heart diseases involving diabetic animals (Liu et al. 2017; Mahajan et al. 2017).

In agreement with the outcomes of the current study, lipidic profiles were found to be diminished in the ELo-treated groups and up-regulated in the diabetic control group. Lipid abnormalities including increased levels of TC, TG, LDL, and decreased HDL levels, are commonly linked with diabetes which contributes the cardiovascular diseases. In the present study, a positive correlation between hyperglycemia and dyslipidemia was observed. Diabetic patients are also more likely to have cardiovascular diseases, because of dyslipidemia. Continuous treatment with three different doses of ELo revealed significant hypoglycemic and hypolipidemic effects. This could be due to the possible stimulatory effect of the extracts on the remnant beta cells by increasing secretion, responsiveness, or insulin-mimicking activity.

The incidence of cardiovascular disease and diabetes mellitus are often correlated (Einarson et al. 2018). The markers of atherogenicity namely atherogenic index and atherogenic coefficient were calculated. Administration of ELo was observed to be prominent in reducing the atherogenic risk predictor indices. The utilization of plant-based antioxidants could lead to protective effects against the formation and progression of atherogenicity (Rafieian-Kopaei et al. 2014). ELo treatment exhibits a reduction in serum in ALT and AST levels compared to the diabetic control group. The significant increase in the serum levels of ALT and AST in the untreated diabetic animals showed the extent of liver injury, which indicates that the impaired liver function might be due to hyperglycemia. Therefore, reduced of these biomarkers closer to normal levels indicates decreased diabetic complications in ELo-treated groups.

These results are in agreement with the histological findings. The histopathological examinations of the pancreas and liver in the diabetic control group showed damaged islets and hepatocytes, respectively. Treatment with ELo revealed a remarkable improvement in the aforesaid histoarchitectural alterations

which indicates the healing potential. Regarding the assessment of renal function, treatment with ELo revealed a reduction in serum creatinine and urea values about those expressed by the diabetes control group and suggests a decrease in the renal impairment associated with diabetic complications due to the inhibition of protein degradation. Studies describe deleterious effects on renal function in the context of diabetes (Alicic et al. 2017). The protective action of ELo on the different metabolic parameters evaluated reveals the multi-active profile of this extract in the context of diabetes.

In addition to the effects described in glucose metabolism and antioxidant activity of the compounds present in ELo, its hypoglycemic action should be related to its inhibiting capacity of α -glycosidase (Fig. 5). Vitexin and other derivatives such as isovitexin-4'-methyl ether, as isovitexin and 2"-o-xylopyranosil vitexin, may act as natural hypoglycaemic agents due to its inhibiting activity of α -glycosidase. (Shibano et al. 2008; Li et al. 2009; Yao et al. 2011; Choo et al. 2012; Chen 2013; Yang et al. 2016). The results indicate that ELo extract exhibits antidiabetic potential and cardioprotector effects in lipidic profile and attenuating effects on hepatic and renal parameters. These factors directly contribute to an overall improvement in diabetes.

5. CONCLUSION

In conclusion, *Lippia origanoides* Kunth has hypoglycemic activities and protective effects in serum lipidic hepatic and renal profiles. The possible mechanism of action may be through stimulation of insulin release from the remnant pancreatic beta cells and inhibitory Activity against α -glycosidase. These effects may be partly due to its antioxidant activity in particular because of the presence of flavonoids. However, the particular actions of these agents cannot be ruled out. The findings show the promising action of ELo on diabetes and, establish a foundation for the application of toxicological tests to investigate possible toxic effects, to open a perspective for the development of a phytotherapeutic presentation of *L. origanoides* Kunth, as hypoglycemic and cardioprotective in the treatment of diabetes.

Declarations

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DECLARATION OF COMPETING INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTION

Vinicius Carvalho Miranda, Yago Luis Gonçalves Pereira, Allane Patricia Santos da Paz, Keyla Rodrigues de Souza, Nilton Akio Muto contributed to the research design, conduction of

experiments (animal experiments) data collection, interpretation, and manuscript preparation; Márcia Cristina Freitas da Silva contributed to the research design, conduction of experiments (histological analyses); Patrick Romano Monteiro, Agenor Valadares Santos contributed to the research design, conduction of experiments (Inhibitory Activity against α -Glycosidase); Moises Hamoy, Maria das Graças Freire de Medeiros contributed to data interpretation and manuscript revision; Iolanda Souza do Carmo, Maria Eduarda Moraes Silva, José de Sousa Lima Neto contributed to the research design, conduction of experiments (Extract Preparation and characterization); Vanessa Jóia de Mello contributed to the research design, study supervision, funding acquisition, data interpretation, manuscript revision and funding support. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Figures

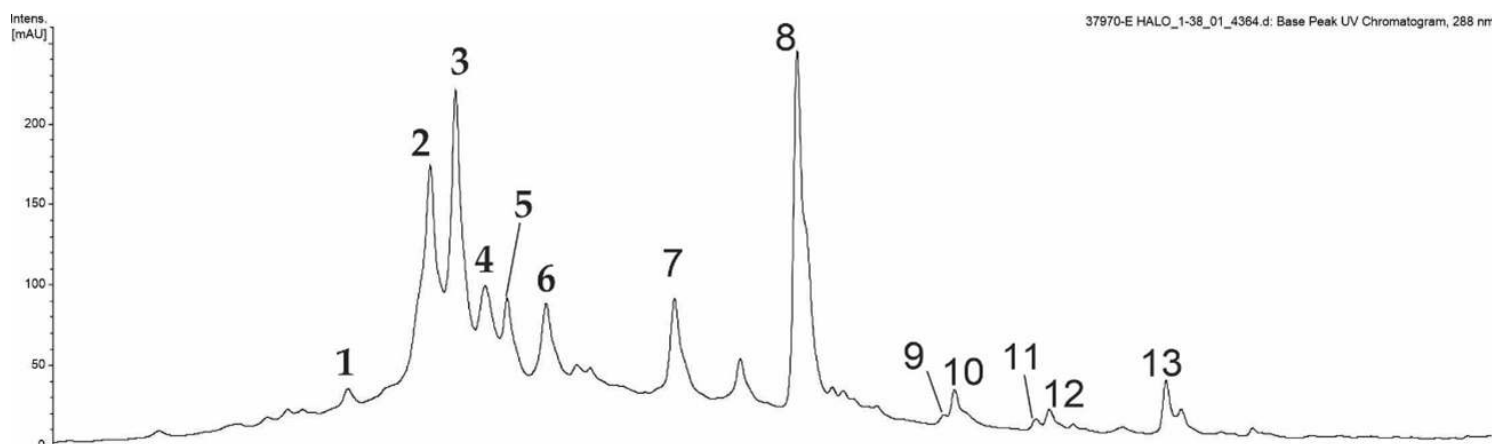


Figure 1

Chromatogram of ELo obtained by HPLC-PDA-ESI-IT-MS/MSⁿ with UV detector at 288 nm. Peak numbers are identified in Table 1 and Figure 2. Experimental Conditions are described in the Materials and Methods

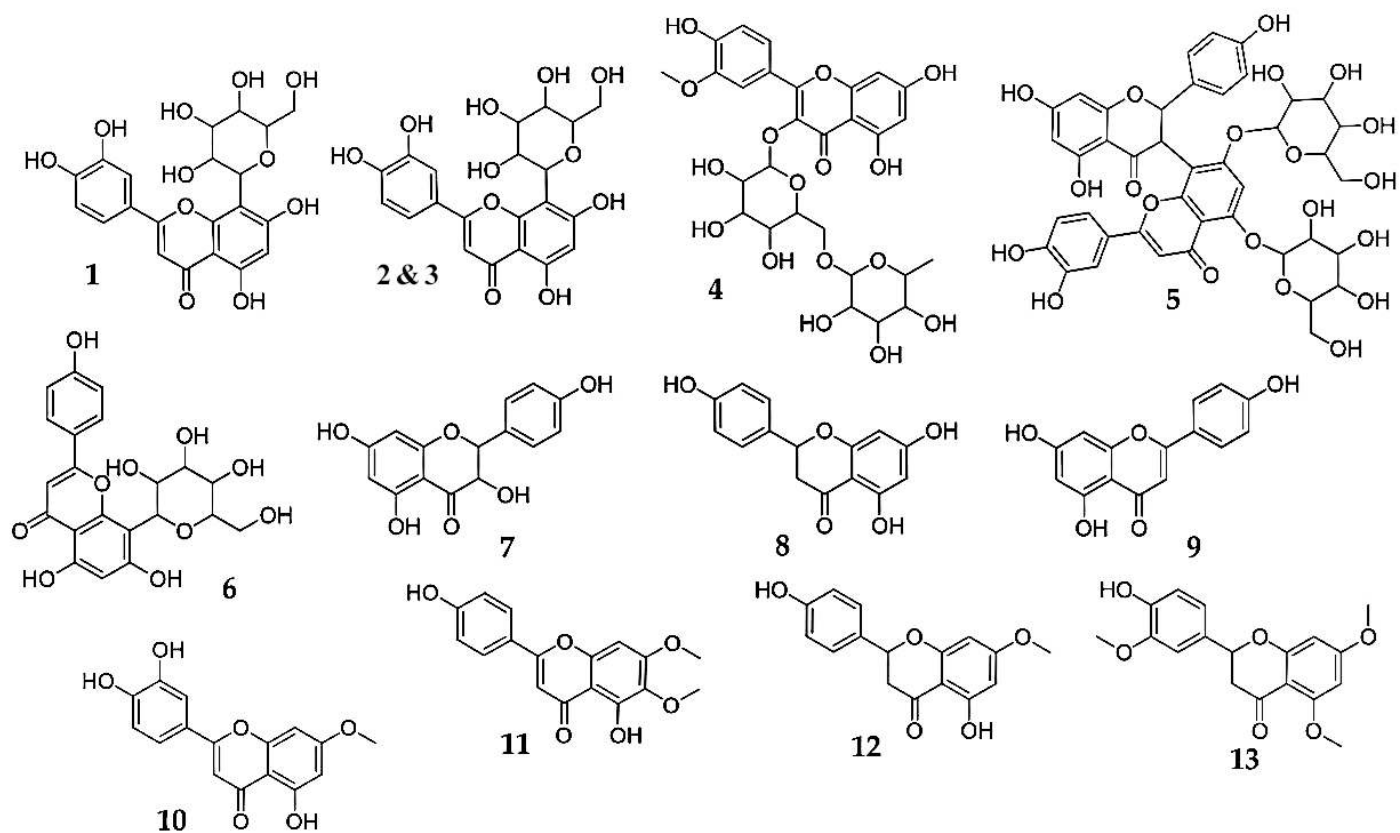


Figure 2

Chemical structures identified in the ELo chromatogram

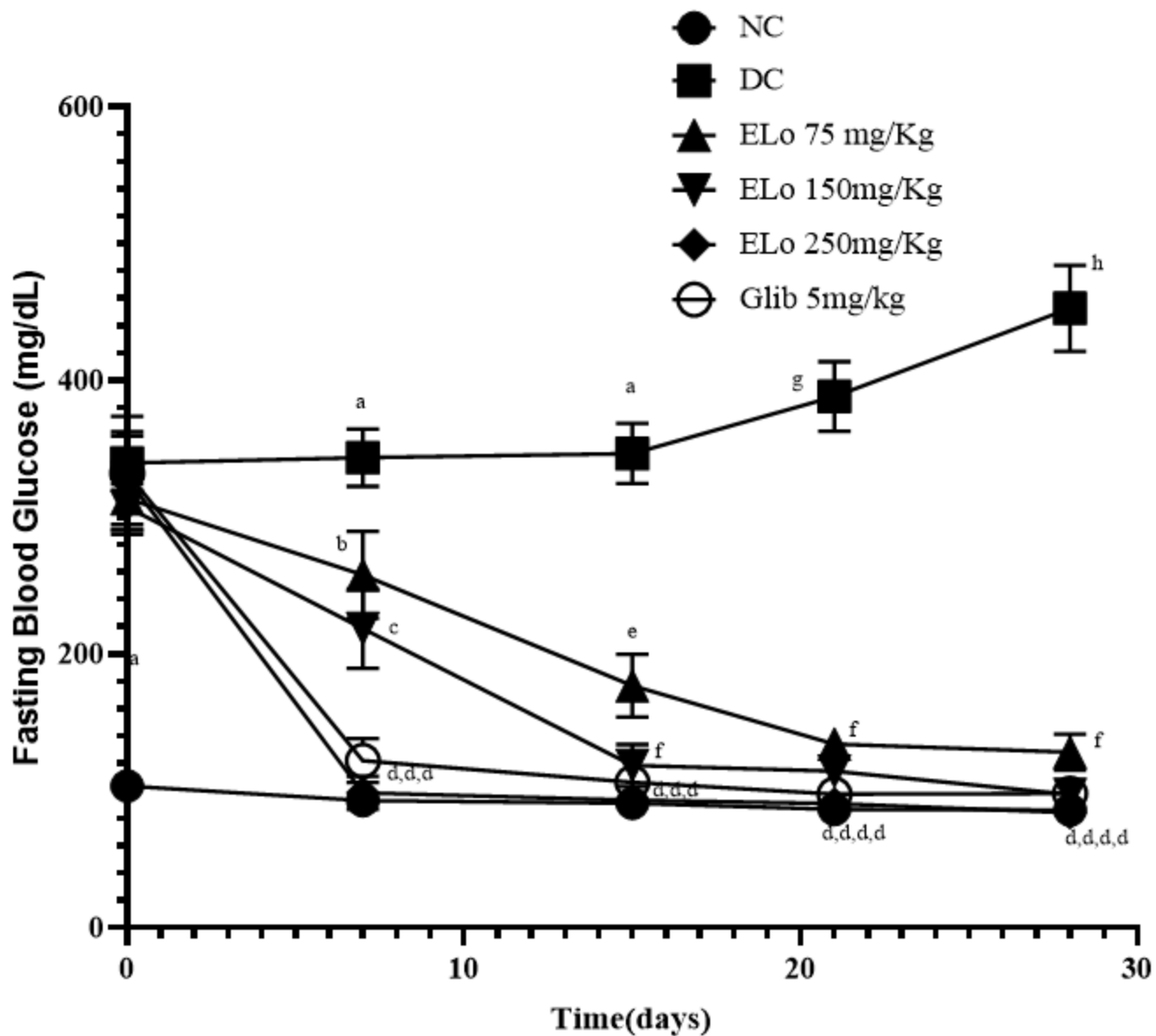


Figure 3

Effects of ELo treatment on Fasting Blood Glucose (FBG) in experimental groups. ELo treatment 15 days (n=10); 21 and 28 days (n=5). Data are expressed as mean $\pm$ SEM. Values with different superscripts (a–h) are significantly different from each other at $p < 0.001$ and with the same alphabets have insignificant changes according to Two-way ANOVA and Tukey's Multiple Range tests

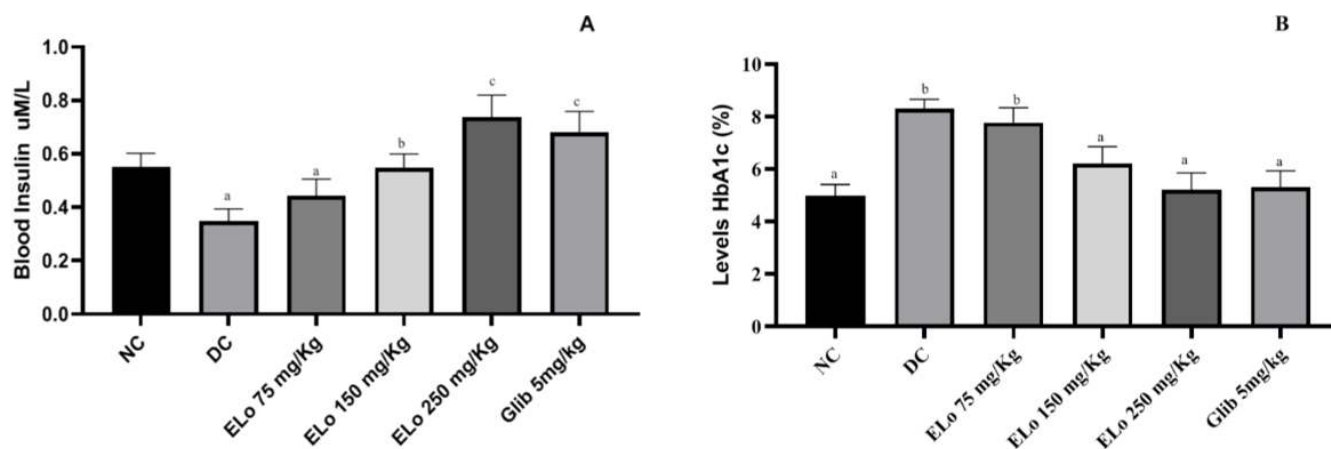


Figure 4

(A-B) Effects of ELo treatment on insulin and glycated hemoglobin (HbA1c) levels. ELo Treatment 28 days (n=5). Data are expressed as mean $\pm$ SEM. Values with different superscripts (a-c) are significantly different from each other at $p < 0.05$ and with the same alphabets indications have insignificant changes according to Two-way ANOVA and Tukey's Multiple Range tests

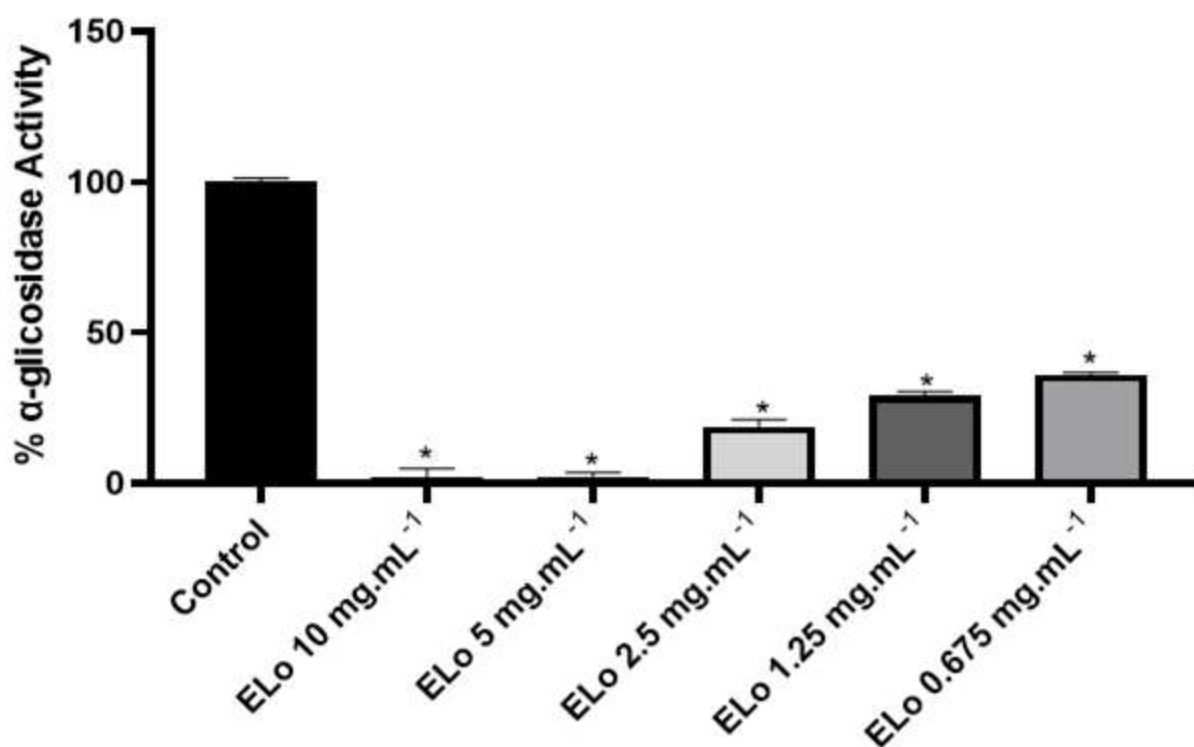


Figure 5

Relative activity of α -glycosidase in presence of ELo. The ELo dilutions (10.0 - 0.675 mg/ml) show the inhibitory activity of α -glycosidase *in vitro*. Two-way ANOVA Dunnett's * $p < 0,05$

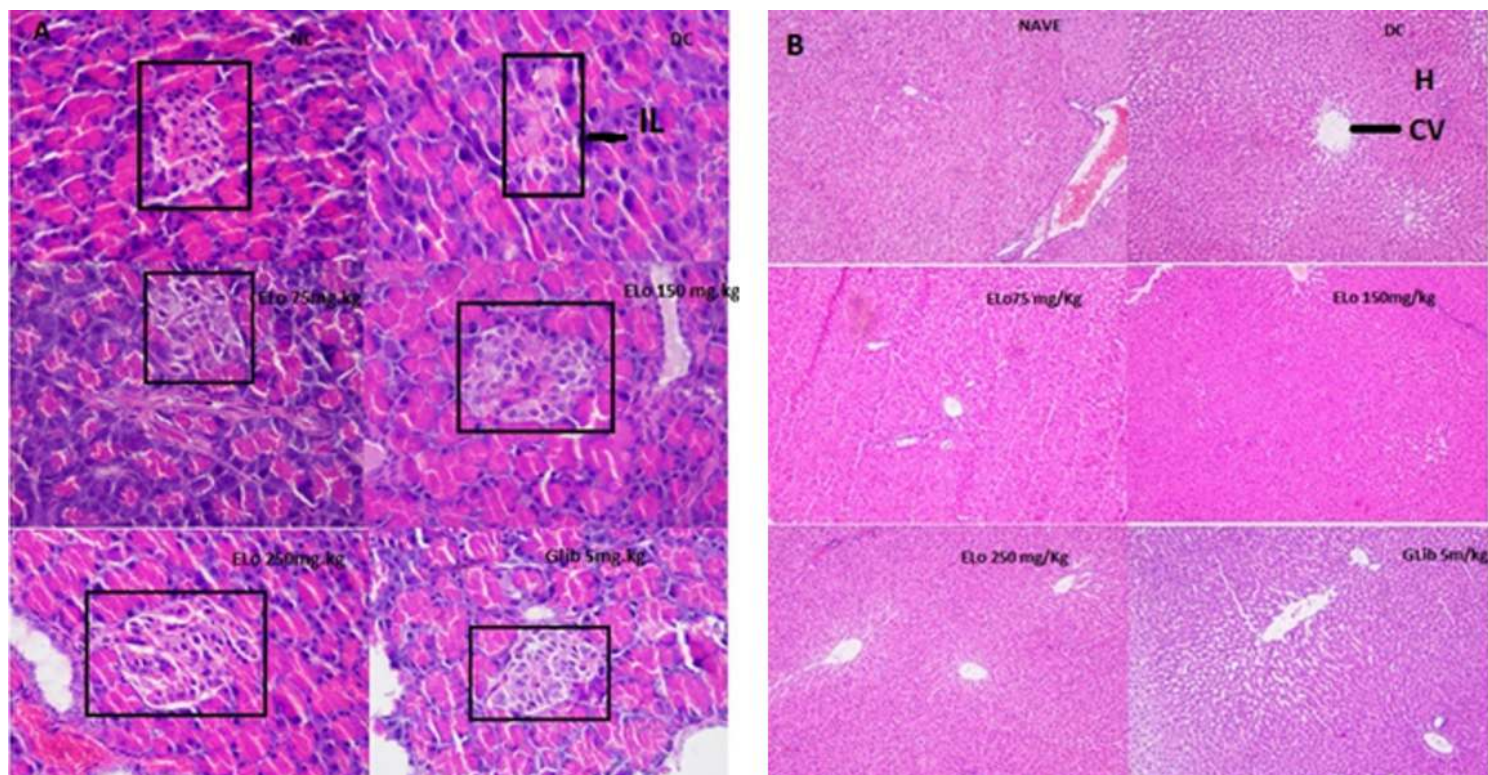


Figure 6

(A/B) Histopathological observations of the pancreas (**6A**) and liver tissues (**6B**) from experimental groups after treatment with ELo. Photomicrographs (40X). **6A** presents pancreatic beta cells and the islet tissue section (IL). **6B** shows the liver tissue sections (H: hepatocytes and CV: Central vein). For both images: NC - Normal Control, DC – Diabetic Control; ELo 75, 150 at 250 mg/kg and Glib-Glibenclamide 5 mg/kg

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [renamed234f4.tif](#)