An Acad Bras Cienc (2021) 93(Suppl. 4): e20210744 DOI 10.1590/0001-3765202120210744
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

BIOMEDICAL SCIENCES

Antidiabetic properties of oral treatment of hexane and chloroform fractions of *Morus nigra* leaves in streptozotocin-induced rats

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Abstract: *Morus nigra* L. has been widely used in Brazilian folk medicine for the treatment of diabetes. We evaluate the chemical composition and antidiabetic properties of the hexane (Hex-Mn) and chloroform (Chlo-Mn) fractions obtained by partition of the crude ethanolic extract from the leaves in rats. Chemical composition analysis of Hex-Mn and Chlor-Mn was performed by gas chromatography-mass spectrometry (CG-MS). *In vivo* and *in vitro* studies were carried out to compare the antidiabetic activities of the Hex-Mn and Chlor-Mn fractions. Most of the compounds identified in Hex-Mn were α-linolenic acid, stigmast-5-en-3-ol and linolenic acid ethyl ester, while in Chlor-Mn, stigmast-5-en-3-ol, palmitic acid and α-linolenic acid were mainly identified. Only Hex-Mn treatment reduced both fasting and postprandial hyperglycemia. Additionally, Hex-Mn preserved body weight gain, preserved the hepatic glycogen content, and also reduced the thiobarbituric acid reactive substances and nitrite levels, as well as restored the superoxide dismutase. Furthermore, digestion of complex carbohydrates and intestinal glucose absorption was prevented by Hex-Mn treatment. Our results suggest that the antidiabetic activity of Hex-Mn may be explained, at least in part, by the insulin sensitivity increase, antioxidant properties and reduction in carbohydrate absorption in the small intestine.

Key words: Diabetes *mellitus, Morus nigra*, antidiabetic effect, antioxidant effect, nutraceutical.

INTRODUCTION

Diabetes *mellitus* (DM) conditions are recognized for persistent hyperglycemia, which is due to compromised insulin synthesis by beta cells of the pancreatic islet and/or loss of insulin action in the target tissue (Júnior et al. 2017, Barbosa et al. 2018). Nowadays, DM has become a worldwide medical emergency in developed and developing countries. It is reported that about 425 million adults are living with DM, whom 1 of out of 2 is undiagnosed (IDF 2019). Despite the pharmacology therapy efficacy in glycemic control, its use has been limited by side effects of medicines. In light of that, the use of plants as functional food or alternative medicines has been widely accepted to prevent and treat DM (Thaipitakwong et al. 2018).

It is noteworthy that postprandial glucose control deteriorates before fasting blood glucose (FBG) (Rizza 2010). There is also evidence showing that postprandial hyperglycemia (PPH) may be an independent risk factor for the development of diabetes comorbidities, such as micro and macrovascular diseases (Rizza 2010, Gerich 2013). In addition, a growing body of evidence indicates that PPH induces severe oxidative stress, which is caused by the generation of

free radicals such as oxygen and nitric species (ROS/NOS) associated to reduce the antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), and non-enzymatic antioxidant defense (Giri et al. 2018, Konda et al. 2019). Oxidative stress in DM may impair insulin signaling and promote glycation of protein, glucose oxidation and lipid peroxidation, which are involved with the progressive degeneration in the diabetes status (Giri et al. 2018, Ogar et al. 2019).

For this, it is critical to monitor and treat PPH as a tool to prevent or retard the manifestation of diabetes-related complications. Synthetic α-glucosidase inhibitors, such as acarbose, have beneficial effects against PPH in diabetic individuals by inhibiting α-glucosidase, which hydrolyzes carbohydrate and releases monosaccharides for absorption in the small intestine (Yusoff et al. 2015). However, according to individual variation and abdominal discomfort, their clinical use has been limited (Fujisawa et al. 2005).

Medicinal plants are particularly interesting because not only can they be used as alternative medicines to prevent metabolic diseases, but also serve as an interesting source of potential drug candidate molecules, including inhibitors of the carbohydrate digestion, insulin sensitizers and antioxidant potential (Yusoff et al. 2015, Bin-Juman, 2019). Among them, species from the genus *Morus* (Moraceae) are widely distributed in Asia, Africa, Europe and America. They have been established as a functional food because of their phytochemicals and nutrients profile (Rodrigues et al. 2019). *Morus nigra* L. (black mulberry) is a native plant from Asia, but it is widely cultivated in Brazil, particularly in the Caatinga biome (Júnior et al. 2017). It is popularly used to treat several diseases, especially diabetes and its comorbidities in Northeast of Brazil (Araújo et al. 2015, Júnior et al. 2017).

The antidiabetic effect of the crude and aqueous extracts of *M. nigra* leaves has been demonstrated (Volpato et al. 2011, Araújo et al. 2015, Júnior et al. 2017). Therefore, the goal of this work was to evaluate the *in vivo* effect of hexane and chloroform fractions obtained by partition of the crude ethanolic extract of *Morus nigra* leaves in hyperglycemia, lipid profile, carbohydrate digestion and absorptive ratio, and oxidative stress in streptozotocin-induced diabetics in rats.

Abbreviations

ALB, albumin; AI, atherogenic index; ATL, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; BBM, brush border membrane; BUN, blood urea nitrogen; CAT, catalase; Chlro-Mn, chloroform fraction; CRE, plasmatic creatinine; DM, diabetes *mellitus*; EDL – *extensor digitorium longus*; EPI – epididymal adipose tissue; FBG, fasting blood glucose; GC-MS, gas chromatography-mass spectrometry; GLUT, glucose transporter; GPx, glutathione peroxidase; GSH, glutathione, GSSG, oxidized glutathione; HDL-C, high-density lipoprotein cholesterol; HGP, hepatic glucose production; Hex-Mn, hexane fraction; ITT, insulin tolerance test; MDA, malondialdehyde; NO, nitric oxide; OGTT, oral glucose tolerance test; OSTT, oral starch tolerance test; OSuTT oral sucrose tolerance test; PPH, postprandial hyperglycemia; RETRO, retroperitoneal adipose tissue; RNS, reactive nitrogen species; ROS, reactive oxygen species; S.E.M., standard error of the mean; SGLT, sodium glucose cotransporter; SOD, superoxide dismutase; STZ, streptozotocin; TBAR, thiobarbituric acid; Ti, tibia.; TC, total cholesterol; TG, triglycerides; VLDL-C, very low density lipoprotein.

MATERIALS AND METHODS

Plant material and preparation of the extract

Morus nigra L. leaves were collected in Casa Nova (Coordinates 9°27'74.35" S; 40°85'03.23" W), State of Bahia, Brazil, in October 2016. A voucher specimen (#1764) was deposited at the Vale do São Francisco Herbarium (HVASF) at the Federal University of Vale do São Francisco (UNIVASF). The extract was prepared by maceration of dried and powdered leaves (714 g) with 95% ethanol during three days, at room temperature. The extractive solution was concentrated under vacuum in a rotary evaporator at 50 °C. The crude ethanolic extract was suspended in a mixture of methanol and water (MeOH: H_2O, 3:7, v/v) and partitioned with *n*-hexane and chloroform (250 ml, 3x) to obtain hexane (Hex-Mn) and chloroform (Chlor-Mn) fractions, which were tested for its biological activity. The access to genetic patrimony and associated traditional knowledge was carried out and the project was registered in SisGen (Register #AC34CFC).

Chemicals

Homologous series of *n*-alkanes ($C_{10}H_{22}$ – $C_{40}H_{82}$) were purchased from Merck® (Germany). All solvents (ethanol, hexane and chloroform) were purchased from Synth® (Brazil).

Gas chromatography-mass spectrometry (GC-MS) analysis

The analysis of chemical constituents present in Hex-Mn and Chlor-Mn was performed by GC-MS using a Shimadzu® gas chromatograph (QP-2010 ULTRA) interfaced with a mass spectrometer, employing the following chromatographic conditions: Phenomenex® ZB-5MS Zebron column (30.0 m x 0.25 mm x 0.25 µm); helium (99.999 %) carrier gas at a constant flow of 1.40 ml/min; 1 µl injection volume; injector split ratio of 1:40; injector temperature 260 °C; electron ionization at 70 eV; ion source temperature 250 °C. The oven temperature was programmed from 60 °C to 320 °C in 25 min. A mixture of linear hydrocarbons ($C_{10}H_{22}$ – $C_{40}H_{82}$) was injected under the same experimental conditions.

Identification of compounds

The data were acquired and processed with a PC with Shimadzu GC-MS-Solution software. The identification of the constituents was assigned on basis of comparison of their relative retention indices to a *n*-alkane homologous series obtained by co-injecting the samples, as well as, by comparison of their mass spectra with those of authentic compounds or with reference spectra in the computer library (Wiley7lib and NIST08lib) and other published mass spectra. A similarity index of at least 90 was considered for the identification of compounds.

Animals

Adult male Wistar rats (190-210 g) were housed in individual cages, under controlled conditions, such as 12/12 h light/dark cycle (lights on at 6:00 a.m.) at room temperature (22 ± 2°C). All rats were fed with a standard lab chow (Presence, Purina®) and water *ad libitum*. The protocols were approved by the Animal Ethics Committee of the Federal University of Pernambuco (CEUA-UFPE, process #23076.016693/2014-88) and conducted All experiments were cared for in compliance with the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council for Animal Experimentation.

Experimental diabetes mellitus (DM)

Streptozotocin (STZ, 40 mg/kg, Sigma®, St. Louis, MO, USA) dissolved in citrate buffer (pH 4.5) was injected into the jugular vein of rats that previously fasted for 12 h. Five days after STZ injection, animals with postprandial glycaemia above 250 mg/dL and clear signs of DM (polyuria,

polydipsia and polyphagia) were considered as diabetic, and thus included in the experimental protocol (Barbosa et al. 2018). The glycaemia was measured by One Touch Ultra (Johnson & Johnson®). In order to perform the control group, non-diabetic animals received citrate buffer injection as control. Five days after STZ injection, animals were randomly divided into five different groups (n=5-7 per group): non-diabetic control (C); diabetic (D); diabetic treated with 400 mg/kg of hexane (DHex-Mn) and 400 mg/kg of chloroform (DChlo-Mn) fractions; diabetic treated with insulin (DI). The diabetic and non-diabetic groups (except DI) received the extract samples or water (i.e., control groups) orally by orogastric tube (gavage) once daily for 21 days. DI received 3 U/rat of Insulin NPH – Lilly (s.c.), at 8:00 a.m. and at 6:00 p.m., from day 0 to day 21. Afterwards, the animals were housed in a metabolic cage to measure body weight, urinary volume, as well as food and water intake daily (Barbosa et al. 2018).

Biochemical analysis

On the 21st day of the experiment, the rats were anesthetized using ketamine (150 mg/kg b.w) and xilazin (10mg/kg b.w) and then euthanized for the collection of blood samples and biochemical analysis, such as plasma glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), albumin (ALB), alanine (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP) and total protein (TP), as well as creatinine (CRE), blood urea nitrogen (BUN) and urinary urea. All these biochemical parameters were measured by commercially available kits (Labtest®, Lagoa Santa, MG). The glycerol levels were measured by commercially available kit with minor modifications (Laborclin®, Curitiba, PR).

The globulin levels (total protein – albumin) were calculated. The serum levels of extremely low-density lipoprotein cholesterol (VLDL-C) were calculated using the Friedwald formula (VLDL = TG/5). Atherogenic index (AI) was also calculated using the formula TC/HDL-c (mg/dl) (Zhou et al. 2018).

Urinary glucose was determined by the *orto*-toluidine method (Dubowski, 1962). The glycogen levels in the liver samples were measured as previously described by (Carrol et al. 1956).

Body and organ weight

Selected organs including heart, liver, kidney, skeletal muscle (*soleus* and *extensor digitorium longus* - EDL) and retroperitoneal (RETRO) and epididymal (EPI) adipose tissues were carefully excised and weighed. The right tibia bone was collected and measured for length and used to normalize the organ weight (Barbosa et al. 2018).

Insulin tolerance test (IkTT)

The insulin tolerance test (ITT) was performed on the 21st day of treatment. Insulin (0.75 U/kg body weight) was injected intraperitoneally in rats that previously fasted during 6 hours. Glucose values were measured in venous blood before insulin injection (baseline measurement, 0), as well as at 4, 8, 12 and 16 minutes after insulin administration. In order to measure insulin action, the constant rate of glucose clearance (K_{itt}) was calculated during the 0 – 16 min period by standard preclinical method ($0.693/t_{1/2}$), as previously described (Barbosa et al. 2018).

Determination of the hepatic antioxidant state

After 21 days of treatment, animals were euthanized and the liver was immediately excised and washed in ice-cold Tris–HCl buffer (0.1 M, pH 7.4). The liver was rinsed again in ice-cold 0.15 M potassium chloride and homogenized (10% w/v) using 0.05% potassium dihydrogen phosphate buffer (pH 7.5 homogenized in 10 volumes of 100 mM KH_2PO_4 buffer containing

1 mM EDTA (pH 7.4), with the addition of 1 mM sodium orthovanadate and 200 µg/ml of phenylmethanesulfonyl fluoride (PMSF), using a digital homogenizer Tissue lyzer (Quiagen®) for no longer than 1 minute. Afterwards, the homogenates were centrifuged at 4,000 rpm for 10 min at 4 °C. The supernatant was collected and used for enzymatic assays. Tissue protein content was estimated by the Bradford method and the absorbance was read at a wavelength of 595 nm, at room temperature (Bradford 1976). A total of 0.3 mg/ml of live homogenate was used to measure malondialdehyde (MDA) levels following reaction with thiobarbituric acid reaction (TBAR, µM/mg protein) at 100 ºC according to the method of Draper et al. (1993). The hepatic nitric oxide levels were estimated by measuring total nitrate/nitrite concentrations (µmol/ml, stable end-products of NO) by the Griess method (Miranda et al. 2001). Reduced glutathione (GSH, µmol/ml) and oxidized glutathione (GSSG, µmol/ml) contents were determined as previously described by Hissin & Hilf (1976). The redox state was estimated by the GSH/GSSG ratio. The activity of superoxide dismutase (SOD) was evaluated according to the method previously described by Misra & Fridovich (1972) following the kinetics of the inhibition of adrenaline auto-oxidation at 480 nm expressed as U/mg protein. The catalase (CAT) activity was assayed by the method of Beers & Sizer (1952). The kinetic analysis of CAT was measured spectrophotometrically at 240 nm after the addition of H_2O_2. The results were expressed as U/mg protein.

Oral carbohydrate tolerance test (OGTT)

On the 21st day of the experiment, the oral glucose tolerance test (OGTT) was performed to assess the glucose tolerance in overnight fasted non-diabetic and diabetic rats divided into 4 groups with 5 rats each: Control group (C) was given distilled water (1 ml/kg); diabetic (D); diabetic treated with a single dose of 400 mg/kg of Hex-Mn (Hex-Mn); and diabetic treated with a single dose of phloridzin (100 mg/kg of b.w., v.o., Sigma®), an inhibitor of SGLT. All animals received 2.5 g of glucose/kg of b.w. (v.o.). Blood glucose was measured in blood samples that were collected from each rat tail. The glycemia was analyzed before (t = 0, baseline) and then after 15, 30, 60, 90 and 120 min (Yusoff et al. 2015).

Oral sucrose tolerance test (OSucTT) and oral starch tolerance test (OSTT) were applied to a similar set of animals. Animals received sucrose or starch respectively, at 4 g/kg of b.w., as well as acarbose (10 mg/kg of b.w., v.o. Sigma®). The glycaemia was analyzed in both tests before (t = 0, baseline) and then 30, 60, 90 and 120 min after the animals received carbohydrate administration (Yusoff et al. 2015).

Intestinal glucose absorption by the everted sac technique

The effect of Hex-Mn on glucose absorption via isolated rat jejunal sacs was studied as previously described by Yusoff et al. (2015). Following an overnight fast, rats were euthanized under anesthesia and the abdominal wall was opened. Small segments (each about 5 cm) close to the duodenum were rinsed with Krebs solution by pushing the solution softly from the syringe. The segments isolated were placed in a well-aerated solution (95% O_2 and 5% CO_2) containing 5.5 mM glucose. The jejunum was everted and cut into 5-cm segments. Each sac was filled with 1 ml of Krebs solution, then formed into a sac by tying both of its ends with cotton threads. Each sac was further incubated for 60 min at 37 °C in a test tube containing a total of 15 ml of Krebs solution. Added to the tubes, the test substances consisted of Hex-Mn (1 mg/ml) in the intestines of control animals and diabetic animals. The tubes containing only Krebs buffer

served as a negative control. The initial and final glucose concentrations following the incubation period were measured and the intestine glucose absorption could be calculated as follows:

Amount of glucose absorbed (mg/g tissue weight) = $(G_{before} - G_{after}) / W_{intestine}$

Where G_{before} and G_{after} represented glucose concentrations (mg/dl) before and after incubation, respectively, and $W_{intestine}$ represented the weight of the intestinal segment in grams.

Statistical analysis

GraphPad Prism 6.01® was used to analyze the data. Results were expressed as mean ± SEM. Kolmogorov-Smirnov was used as a normality test. The one-way analysis of variance, followed by Tukey´s test, was employed to analyze the data between groups. The two-way analysis of variance, followed by Tukey´s test, was employed to analyze intestinal absorption data. When $p < 0.05$, the difference between groups was considered as statistically significant.

RESULTS

Phytochemical profile

The GC-MS chromatogram of Hex-Mn revealed the presence of 71 peaks, of which 15 were identified, corresponding to 77.81% of its total chemical composition. α-linolenic acid (16.04%), stigmast-5-en-3-ol (10.45%) and linolenic acid ethyl ester (9.31%) were the majority of compounds. The GC-MS chromatogram of Chlor-Mn revealed the presence of 77 peaks, of which 13 were identified, corresponding to 53.98% of its total chemical composition. Stigmast-5-en-3-ol (7.28%), palmitic acid (6.18%) and α-linolenic acid (5.33%) were the majority of compounds.

Antihyperglycemic and antidiabetic effect

This study evaluated the antidiabetic properties of both hexane (Hex-Mn) and chloroform (Chlo-Mn) fractions in STZ-induced diabetic rats. STZ induced a sustained high fasting (576.2 ± 59.6 vs 87.6 ± 3.7 mg/dl of control) and postprandial (551.9 ± 43.1 vs 110.0 ± 3.2 mg/dl of control, Table I) glycemia, accompanied with the classical diabetic symptoms such as polydipsia,

Table I. Fasting and postprandial glucose at 21 day of treatment and accumulative effects of Hex-Mn and Chlor-Mn fractions on body weight gain, food and fluid intake, urinary volume, glucose, and urea urinary in control and diabetic treated for 21 days.

	Groups				
Parameters	**C**	**D**	**Hex-Mn**	**Chlor-Mn**	**DI**
Fasting glycemia (mg/dl)	87.6 ± 3.7 a	576.2 ± 59.6 b	388.4±62.9c	487.1±53.6b	352.8±37.0c
Postprandial glycemia (mg/dl)	110.0 ± 3.2a	551.9 ± 43.1 b	329.8±30.2c	613.9±70.8b	381.9±47.2c
Final body weight (g)	311.7±7.9a	221.2±4.4b	262.9±3.2c	246.0±10.3b	306.3±10.3a
Food intake (g)	524.3±25.9a	859.2±22.6b	744.4±22,8c	799.3±41.2b	672.1±20.3d
Fluid intake (mL)	857.5±52.0a	3671.4±255.4b	2560.9±180.9c	3077.0±254.7d	1365.1±82.2d
Urinary volume (mL)	315.8±19.1a	3060.1±214.2b	2247.7±179.3c	2478.7±282.6d	709.1±50.5d
Urinary urea (mg/dL)	627.1±51.9a	13521.4±1431.2b	9260.5±1194.2c	9802.7±643.9d	1435.0±499.7d
Urinary glucose (mg/mL)	-	12.763±1.4b	9.802±643c	12.821.4±2.42b	1.792±553d

Results are expressed as mean ± SEM of 7 animals per group. Mean values with different superscript letters are statistically different at $p<0.05$ and were analyzed using one-way ANOVA followed by post-hoc Tukey's test.

polyphagia and polyuria, as well as body weight reduction (Figure 1).

Here, only the Hex-Mn treatment reduced both FBG and PPH (~40%) in diabetic rats (Table I) and also the urinary glucose excretion in diabetic treated rats (Table II). Also, Hex-Mn preserved body weight gain and inhibited hyperphagia (Figure 1), which was accompanied by reduced skeletal muscle and adipose tissue loss (Table II). Besides, this fraction also reduced urinary urea (~40%) in STZ-rats. Chlo-Mn preserved only adipose tissue and red skeletal muscle mass and also attenuated urinary urea (~30%) in STZ-rat (Tables I and II). Both fractions studied attenuated the increased fluid intake and urinary volume in diabetic rats (Table I). It is noteworthy that Hex-Mn fraction had a similar effect on hyperglycemia-lowering effects compared to insulin treatment.

Biochemical analysis

As shown in Table III, STZ induced an increase in TC, TG and VLDL-C, without alteration in HDL-C. The damage to the lipid profile contributes to the worst atherogenic index (AI) in diabetic rats. Also, the concentrations of biomarkers of hepatic (ALT, AST and ALP) and kidney (CRE and BUN) dysfunctions were increased in diabetic rats relative to controls. The Hex-Mn treatment exhibited a lipid-lowering action, reducing TC (20%), TG (30%), VLDL-C (30%) and atherogenic index (40%), also increasing the HDL-C levels (~30%). As seen in Table III, this fraction also reduced ALT (50%), AST (35%), ALP (40%) and BUN (60%). Chlo-Mn fraction also ameliorated the TG (60%), VLDL (40%), BUN (35%), ALT (55%), AST (~20%) and ALP (40%). As expected, insulin treatment was efficient to attenuate lipid and hepatic imbalance. Neither DM nor any treatment caused differences in ALB, TP and globulin levels.

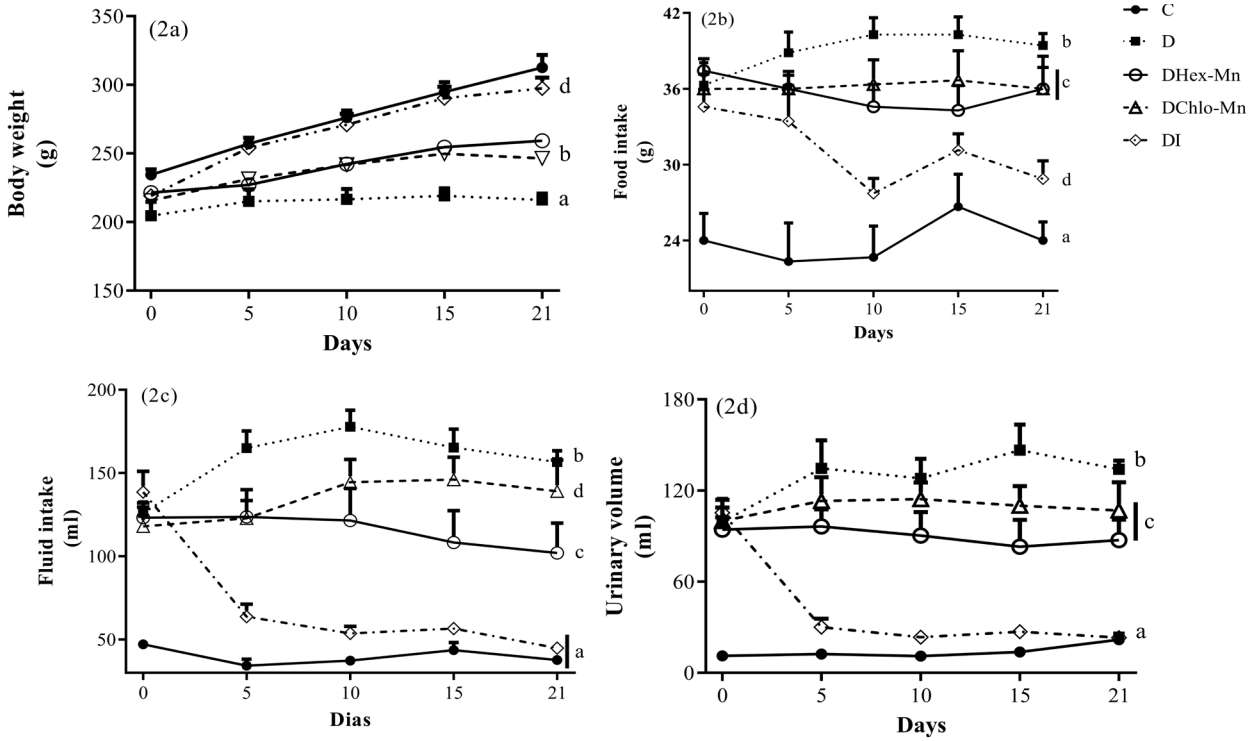

Figure 1. Effect of Hex-Mn and Chlor-Mn fractions on body weight (2a), food (2b) and fluid (2c) intake, urinary volume (2d) in control and STZ diabetic rats for 21-days treatment. Results are expressed as mean ± S.E.M. of 7 animals per group.

Table II. Effects of Hex-Mn and Chlor-Mn fractions on heart, liver, kidney, *soleus*, EDL, EPI and RETRO organ mass in control and diabetic treated for 21 days.

	Groups				
Parameters	**C**	**D**	**Hex-Mn**	**Chlor-Mn**	**DI**
Heart (g/mm Ti)	25.41±1.27a	20.53±0.79b	22.30±0.91a	20.76±0.66a	25.67±1.47a
Liver (g/mm Ti)	276.9±16.9a	252.6±6.7a	284.5±12.5a	266.2±7.6a	294.7±9.3a
Kidney (g/mm Ti)	65.08±2.88a	65.28±2.79a	66.67±2.97a	62.10±1.55a	60.48±2.19a
Soleus (g/mm Ti)	0.758±0.059a	0.536±0.019b	0.656±0.039c	0.690±0.023c	0.685±0.016 c
EDL (g/mm Ti)	0.980±0.038a	0.630±0.040 b	0.761±0.030c	0.634±0.035b	0.923±0.019 a
EPI (g/mm Ti)	62.8±2.9a	20.6±4.1b	47.7±4.6c	42.9±5.5c	66.4±5.6a
RETRO (g/mm Ti)	66.24±8.48a	4.31±1.81b	32.44±2,24c	24.34±4.52c	77.08±6.74a

Results are expressed as mean ± SEM of 7 animals per group. Mean values with different superscript letters are statistically different at $p<0.05$ and were analyzed using one-way ANOVA followed by post-hoc Tukey's test.

Table III. Effect of Hex-Mn and Chlor-Mn fractions on biochemical and lipid profile in control and diabetic treated for 21 days.

	Groups				
Parameters	**C**	**D**	**Hex-Mn**	**Chlor-Mn**	**DI**
TG (mg/dL)	35.3±3.2a	168.2±10.1b	116.1±5.2c	71.7±15.9d	56.6±4.9e
TC (mg/dL)	44.87±3.13a	64.03±4.12b	52.88±4.08c	59.62±3.42	61.31±2.68
HDL-C (mg/dL)	34.7±2.4a	31.9±2.2a	40.9±3.1b	31.4±3.0a	36.9±2.9d
VLDL-C (mg/dL)	6.45±0.15a	33.6±2.0b	23.7±1.1c	19.9±3.6c	11.3±0.9c
Atherogenic Index (AU)	1.2±0.1a	1.9±0.2b	1.2±0.06c	1.9±0.2b	1.5±0.1c
ALT (U/mL)	50.6±3.2a	255.6±31.3b	124.0±6.7c	115.4±8.9c	102.0±6.3c
AST (U/mL)	139.3±25.1a	254.9±22.5b	219.8±17.1c	198.9±15.6c	205.8±8.3c
ALP (mg/dl)	32.9±4.2a	138.5±11.8b	86.7±5.0c	86.3±12.1c	66.3±6.6c
BUN (mg/dL)	7.33±1.3a	104.6±17.2b	41.8±3.7c	68.9±10.6d	10.0±0.43c
CRE (mg/dL)	0.46±0.03a	0.60±0.03b	0.63±0.02b	0.64±0.05b	0.59±0.07b
Albumin (mg/dL)	2.8±0.2a	2.43±0.2a	2.49±0.01a	2.4±0.1a	2.6±0.1a
Total protein (mg/dL)	5.8±0.3a	5.9±0.2a	6.8±0.2a	6.2±0.3a	6.1±0.4a
Globulin (mg/dL)	3.5±0.3a	3.4±0.1a	4.4±0.2a	4.3±0.4a	4.5±0.3a

Results are expressed as mean ± SEM of 7 animals per group. Mean values with different superscript letters are statistically different at $p<0.05$ and were analyzed using one-way ANOVA followed by post-hoc Tukey's test.

Glycerol, glycogen and IkTT levels

As shown in Table IV, STZ induced both a rise in glycerol levels (3.4-fold) and a reduction in hepatic glycogen (~85%). The Hex-Mn reduced glycerol levels (~60%) and preserved the hepatic glycogen content as much as insulin. Chlor-Mn fraction did not cause any improvement in these parameters. The insulin treatment elevated the hepatic glycogen content (3.3-fold). In addition, the reduction in insulin tolerance (IkTT) induced by STZ was ameliorated by Hex-Mn treatment.

Table IV. Effect of Hex-Mn and Chlor-Mn fractions on IkTT, serum glycerol and hepatic glycogen in control and diabetic treated for 21 days.

	Groups				
Parameters	**C**	**D**	**Hex-Mn**	**Chlor-Mn**	**DI**
IkTT	-	2.7±0.5b	4.4±0.4c	-	-
Glycerol (mg/dL)	23.1±3.3a	79.4±7.4b	32.4±6.3c	56.8±1.3d	
Hepatic glycogen (%/g of tissue)	5.10±0.23a	0.72±0.11b	2.6±0.17c	1.21±0.27d	2.48±0.21c

Results are expressed as mean ± SEM of the mean of 5 - 7 animals per group. Mean values with different superscript letters are statistically different at $p<0.05$ and were analyzed using one-way ANOVA followed by post-hoc Tukey's test.

Antioxidant activities

Considering that the antioxidant properties are one of the most important mechanisms involved in the anti-hyperglycemic effects of plant extracts, we decided to analyze the antioxidant effects only in Hex-Mn fraction, mainly because the Chlo-Mn did not improve hyperglycemia. The hepatic lipid peroxidation was determined by evaluating the concentration of thiobarbituric acid reactive substances (TBARS), which were expressed regarding malondialdehyde (MDA) content. STZ induced the pro-oxidant status in the liver, which was accompanied by both increasing in TBARS and nitrite content (60%), and GSH:GSSG ratio (20%) and reduction in SOD (40%) antioxidant activity (Table V). Hex-Mn treatment reduced the TBARS and nitrite levels, as well as restored the SOD activity back to normal in diabetic rats (Table V), but it did not improve the GSH:GSSG ratio. Catalase activity was not modified by any conditions evaluated.

Oral carbohydrates tolerance tests

Since only Hex-Mn had an antihyperglycemic effect in STZ diabetic rats, we estimate the *in vivo* effect of Hex-Mn fraction on postprandial hyperglycemia reduction. As seen in Figure 2, this fraction inhibited the rise in the blood glucose levels after carbohydrate overload. The baseline of fasting glucose level was higher in diabetic non-treated animals as expected. After the overload of glucose, the peak blood glucose level was 231.75 mg/dl in the control rats. Diabetic non-treated rats (2a) reached the peak blood glucose level at 579.8 mg/dl and its glycemia remained higher throughout the experimental period. The control animals returned glycemia to basal levels at 120 minutes of the experiment. Hex-Mn treatment attenuated the peak of blood glucose levels (25%). The increase in the glucose absorption induced by diabetes (3.3-fold) was reduced by Hex-Mn diabetic rats (30% lower in the area under the curve, Figure 2b). Phloridzin, a competitive inhibitor of SGLT1, reduced the glucose absorption (50%) in diabetic rats.

There was an increase in glucose levels after sucrose overload (Figure 2c), reaching its peak within 30 min (510 mg/dl) in the diabetic group. In Hex-Mn-treated animals, the peak was delayed to 60 minutes of experiment with a lower value (421 mg/dl). After the starch overload (Figure 2e), the increase in glycemia was much lower than the increase observed in glucose and sucrose overload. Diabetic rats increased glycemia after the starch overload, which was not observed in the Hex-Mn-treated group (Figure 2). The reduction in the area under the curve of the OSuTT (Figure 2d, 25%) and starch (Figure 2f, 50%) in Hex-Mn-treated rats may represent an inhibition of digestion of the complex carbohydrates by disaccharidases.

Table V. Effects of Hex-Mn and Chlor-Mn on SOD, catalase, nitrite, TBARS, GSH and GSSH in the liver in control and diabetic treated for 21 days.

	Groups		
Parameters	**C**	**D**	**Hex-Mn**
SOD (U/mg protein)	29.9±3.8a	18.5±0.8b	26.5±3.2a
Catalase (U/mg protein)	0.026±0.004 a	0.027±0.003b	0.025±0.005b
Nitrite (µM)	2.3±0.2a	3.7±0.2b	3.3±0.1c
TBARS (µM/mg protein)	2.7±0.18a	4.3±0.5b	2.3±0.04c
GSH (µmol/ml)	8.6±0.2a	9.4±0.6a	7.8±0.2a
GSSG (µmol/ml)	2.8±0.3a	3.8±0.2b	4.0±0.5b
Ratio GSH:GSSG	3.1±0.4a	2.5±0.3b	2.0±0.3b

Results are expressed as mean ± SEM of 5 animals per group. Mean values with different superscript letters are statistically different at $p<0.05$ and were analyzed using one-way ANOVA followed by post-hoc Tukey's test.

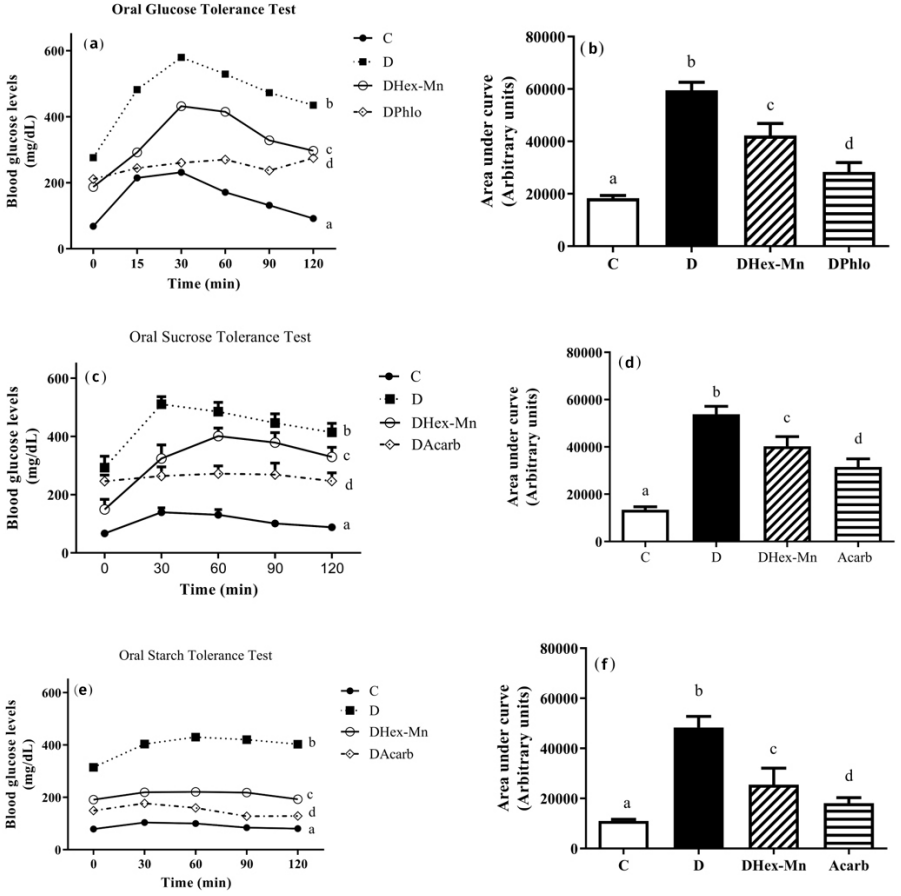

Figure 2. Effect of Hex-Mn fraction on carbohydrate oral tolerance test in control and STZ diabetic rats treated for 21 days. (a, c, e) Glycemia levels before (t = 0) and after glucose load in rats treated for 21 days; (b; d, f) area under the curve (AUC) of the oral carbohydrate tolerance test. Results are expressed as mean ± S.E.M. of 7 animals per group. Mean values with different superscript letters are statistically different at $p<0.05$ and were analyzed using one-way.

Intestinal glucose absorption

Next, we attempted to study the role of Hex-Mn in modulating jejunal glucose absorption. As shown in Figure 3, diabetes caused the elevation in intestinal glucose absorption (~80%), which was attenuated by Hex-Mn treatment (60%).

DISCUSSION

Diabetes is an important public health emergency for the 21st century, with a frightening increase in morbidity and mortality for the coming decades (IDF 2019). The role of medicinal plants in the health care system worldwide has been recognized, mainly in low- and middle-income countries (Srujana et al. 2018). The glycemic control is therefore a major challenging task because of side effects associated with synthetic drugs used in the clinic, such as biguanide (metformin), sulfonylurea, rosiglitazone and α-glucosidase inhibitors (Jiao et al. 2017). Plants have been used to treat several diseases, especially DM. The antidiabetic effect of *Morus nigra* species or blackberry has been demonstrated. The ethanolic extract of *M. nigra* has antidiabetic, anti-hyperglycemic, antioxidant and lipid-lowering effects, with a few adverse effects (Volpato et al. 2011, Araújo et al. 2015, Júnior et al. 2017). Here, we show, for the first time, that the hexane and chloroform fractions from the ethanolic extract of *M. nigra* leaves may be a source of antidiabetic and antihyperglycemic molecules that may be used in the treatment of DM.

The genus *Morus* has a long history in Traditional Chinese Medicine and also became valued by the ethnopharmacology of several other cultures and countries. The GC-MS analysis of the hexane fraction of *M. nigra* extract led to the identification of fatty acids and their esters, such as elaidolinoleinic acid, 24-ethylcholestol and ethyl linolenate. The major molecule class present in the hexane fraction is polyunsaturated fatty acids (PUFA), which prevents diabetes and their comorbidities, such as cardiovascular disease and dyslipidemia (Derosa et a. 2012). In addition, the linoleic acid (LA) and ethyl linolenate (EL) described here and also described in *M. alba* fruits extract, which exhibits a potent anti-inflammatory effect through the inhibition of nitric oxide production in murine macrophage stimulated by lipopolysaccharide (Qin et al. 2015). Likewise, stigmast-5-en-3-ol has shown an *in vitro* insulin-like effect, which stimulates glucose transport in L6 myotubes and inhibits the protein tyrosine

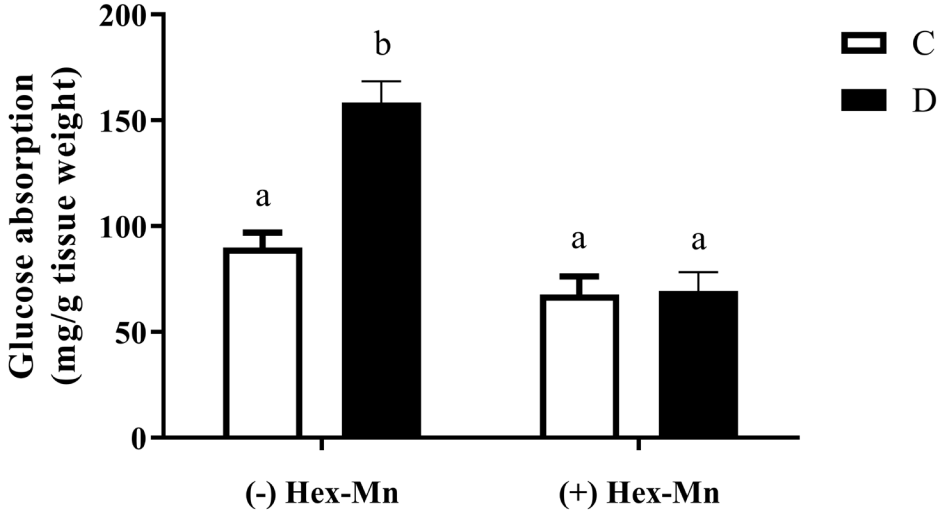

Figure 3. Effect of Hex-Mn fraction on intestinal glucose absorption by everted sac technique in control and STZ diabetic rats treated for 21 days. Results are expressed as mean ± S.E.M. of 7 animals per group. Mean values with different superscript letters are statistically different at $p<0.05$ and were analyzed using two-way.

phosphatase 1B (PTP1B), the major negative regulator of insulin signaling (Sujatha et al. 2010, SarathKumar & Lakshmi 2019). Besides, it exhibits antiadipogenic activity by maintaining the glucose and lipid homeostasis (SarathKumar & Lakshmi 2019).

The GC-MS analysis of the chloroform fraction led to the identification of loliolide acid, a member of benzofurans; stigmast-5-en-3-ol and ascorbic acid, a natural antioxidant. It is well known that heterocycle molecules, such as benzofuran, have attracted attention because of the presence in natural products, biologically active compounds and potential applications as anti-inflammatory, antidepressant, antihyperglycemic and lipid-lowering agent (Nguyen et al. 2010, Shamsuzzaman 2015). The presence of ascorbic acid in the extract of *Morus nigra* leaves was previously demonstrated by Zeni et al. (2017), while the loliolide presence was identified in the dichloromethane-soluble fraction from *M. alba* (Hunyadi et al. 2013). Taken together, this is the first description of hexane and the chloroform fraction of the *Morus nigra* leaves, which clearly shows that this species may be used as a source of molecules for adjuvant therapy targeting the treatment of diabetes. Besides that, the chemical characterization of the species belonging to the *Morus* genus has shown not only medicinal, but also dietary purposes with promising nutritional support.

Previous reports from our group, as well as others, have shown clearly that the ethanolic extract of *M. nigra* leaves has glucose-lowering effect (Volpato et al. 2011, Júnior et al. 2017, Araújo et al. 2015). For that, we fractionated the ethanolic extract of *M. nigra* leaves in solvent polarity gradient to analyze the antidiabetic effects of the hexane and chloroform fractions. Also, for the first time, we report that Hex-Mn fraction reduced both FBG and PPH in STZ-induced diabetic rats. Also, Hex-Mn ameliorated all canonical signs of DM such as polyuria, polydipsia, food intake and weight gain. Antihyperglycemic and antidiabetic effects of certain plants have been explained by several mechanisms such as the increase in insulin secretion in-pancreatic cells, improvement in insulin sensibility by peripheral tissues and inhibition of intestinal glucose absorption (Srujana et al. 2018, Silva et al. 2020).

It is noteworthy that the long-term treatment with Hex-Mn exhibited the anticatabolic action in the skeletal muscle and adipose tissue, indicated by urinary urea reduction, plasma glycerol and organ weight. It is well known that insulin has a classical anabolic and anticatabolic effect. So, Hex-Mn treatment had an anticatabolic effect in diabetic rats, which may be explained, at least in part, by stimulation of the anabolic pathways in similar manner to insulin (Barbosa et al. 2018, Tokarz et al. 2018).

The major endocrine regulator of hepatic glucose production (HGP) is insulin, which controls gluconeogenesis (new synthesis of glucose), glycogenesis (glucose storage as glycogen) and glycogenolysis (glucose release via the breakdown of glycogen) (Rizza 2010, Hatting et al. 2019). It is well established that insulin inhibits HPG. Insulin enhances hepatic glycogen content by stimulating glycogen synthase and suppressing glycogen phosphorylase (Srujana et al. 2018). Excessive HGP is the most important contributor to both fasting and postprandial hyperglycemia in DM (Rizza 2010). Lactate, glycerol and amino acids account for 90% of gluconeogenic substrates. Here, Hex-Mn improved glycogen content in the liver from diabetic rats, as well as reduced glycerol levels, what suggests that the reduction in hyperglycemia is due to the inhibition of HGP by stimulating glycogen synthesis and storage and/or decreased gluconeogenesis. This effect of Hex-Mn fraction was confirmed by the augment

of the insulin sensitivity index. In addition, Chlo-Mn reduced moderately the serum levels of gluconeogenesis substrates but, in turn, did not improve hyperglycemia in diabetic rats. It is therefore suggested that this fraction may possess molecules which, at higher levels, may not contribute to the glycemic control.

Diabetes is often associated with lipid abnormalities consisting of elevated plasma concentration of triglyceride (TG), small dense low-density lipoprotein (LDL) and reduced low high-density lipoprotein (HDL) cholesterol (Chait & Goldberg 2017). Diabetic dyslipidemia is directly associated with the development of cardiovascular disease in diabetic patients (Vergès 2015, Zhou et al. 2018). STZ-induced diabetic rats show the classical diabetic dyslipidemia, including the worsening of the atherogenic index, usually calculated by the TG/HDL ratio (Zhou et al. 2018). Curiously, Hex-Mn fraction not only attenuated diabetic dyslipidemia but also the atherogenic index, while Chlo-Mn weakly ameliorated the TG and VLDL content. As known, insulin is a key hormone in the regulation of lipid metabolism. In adipose tissue, insulin stimulates lipid synthesis and storage while inhibits the VLDL-TG production by the reduction in circulating levels of free fatty acids (FFA), which are substrates for VLDL synthesis in hepatocytes (Vergès 2015). This effect was due to the improvement of insulin sensitivity induced by Hex-Mn fraction. In addition, the presence of stigmast-5-en-3-ol, an antilipidemic agent, could explain, at least in part, the lipid-lowering effect of both fractions studied here.

In addition to the well-known metabolic function of the liver, this organ is compromised by DM on a long-term basis, which can be observed from the increase in alanine (ALT) and aspartate (AST) aminotransferase and alkaline phosphatase levels (ALP), classical biomarkers of liver functioning (Júnior et al. 2017, Barbosa et al. 2018). ALT is a cytosolic enzyme of the hepatocyte and its increased plasma content reflects damage in cell permeability commonly associated to cell death. Extracts of species from the *Morus* genus have demonstrated hepatoprotective effects, such as *M. alba* (Jiao et al. 2017), and *M. nigra* (Diab et al. 2020). However, it is the first scientific report which demonstrated the hepatoprotective action of hexane and chloroform fraction of *M. nigra* leaves in STZ-rats. Our data show data both Hex-Mn and Chlo-Mn fractions are able to protect hepatocytes probably because of the stability of cell membrane and/or parenchymal cell regeneration.

The underlying mechanism of these anti-diabetes effects required further exploration. We decided to analyze the possible anti-diabetic mechanism of the hexane fraction, the most effective fraction evaluated here. Hyperglycemia and hyperlipidemia lead to the excess production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which, in turn, cause lipid peroxidation and cell damage (Konda et al. 2019, Ogar et al. 2019). In addition, DM suppresses the enzymatic and non-enzymatic antioxidant defense, which together contributes to intense oxidative status seen in diabetes (Araújo et al. 2015, Júnior et al. 2017). Consistent with previous studies, the diabetic liver exhibited an increase in MDA and nitrite, coupled with reduced GSH and activity of antioxidant enzymes (Althunibat et al. 2019, Sheweita et al. 2020). The antioxidant activity of *M. nigra* leaves has been demonstrated (Araújo et al. 2015, Turgut et al. 2016, Júnior et al. 2017); however, this is the first report of the antioxidant activity effect of Hex-Mn. The antioxidative effect may explain, at least in part, the most important antidiabetic action of the hexane fraction when compared with the chloroform one.

Clinical studies have indicated that postprandial hyperglycemia is an important risk for micro and macrovascular diseases in several organs such as the brain, retina, heart, liver and kidney (Gerich 2013, Silva et al. 2020). Furthermore, good management of postprandial glycemic control is crucial in the treatment of DM to prevent diabetes comorbidities. The reduction of postprandial hyperglycemia induced by Hex-Mn in STZ rats suggested a blockage of the enzymatic digestion and absorption of the carbohydrate in the gut. The intestinal mucosa hyperplasia induced by diabetes was described in STZ-rats, which was associated with the increase in total activity of disaccharidases in the entire small intestine which, in turn, results in the enhancement of the glucose absorption (Adachi et al. 2003). Postprandial hyperglycemia controls may be achieved by modulating two physiological pathways: (1) the digestion of complex carbohydrates into absorbable monosaccharides by carbohydrate-digestive enzymes (pancreatic α-amylase, and intestinal α-glucosidase); and (2) intestinal absorption of those monosaccharides via intestinal glucose transporters (SGLT1 and GLUT2). The first one, Silva et al. (2020) showed that Hex-Mn acutely delayed the carbohydrate digestion, but not the glucose transport through the brush border membrane of the small intestine. Here, *in vitro* glucose absorption, using isolated and rat jejunum everted technique, was reduced by Hex-Mn treatment during 21 consecutive days. It is reasonable to suggest that Hex-Mn was able to modulate the activity of the glucose transports in the enterocyte membrane. Similar inhibitory effects were seen for Hex-Mn and acarbose *in vivo,* during the carbohydrate oral tolerance test, which may be hypothesized that Hex-Mn, at least in part, delays the intestinal carbohydrate digestion and consequently reduces glucose absorption. These results show that Hex-Mn has also an antihyperglycemic effect regardless of the insulinotropic properties.

CONCLUSIONS

Our results suggest that fractions of the ethanolic extract of *M. nigra* leaves bear positive metabolic effects in STZ-induced diabetic rats. Hex-Mn showed potent antihyperglycemic and antidiabetic effects through different mechanisms, such as more insulin-sensitive action, antioxidant property and delay of the digestion and absorption of complex carbohydrates by the small intestine. This study reaffirms the ethnomedicinal use of the Brazilian *M. nigra* for the treatment of DM and its most common comorbidity. In summary, the hexane fraction of the *M. nigra* leaves may represent an important source of new molecules for the treatment of DM in the future.

Acknowledgments

The authors are grateful for the financial support of Coordenação de Aperfeiçoamento de Pessoa de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Instituto Nacional de Ciência e Tecnologia em Etnobiologia, Bioprospecção e Conservação da Natureza (FACEPE APQ 0562-2.01/17).

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How to cite
DA SILVA DHA, BARBOSA HM, DA SILVA JF, MOURA CA, GOMES DA, ALMEIDA JRGS & LIRA EC. 2021. Antidiabetic properties of oral treatment of hexane and chloroform fractions of *Morus nigra* leaves in streptozotocin-induced rats. An Acad Bras Cienc 93: e20210744. DOI 10.1590/0001-3765202120210744.

Manuscript received on January 13, 2021;
accepted for publication on July 28, 2021

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Author contributions

D.A.G., J.R.G.S.A and E.C.L. designed the study, analyzed the data and drafted wrote the manuscript. D.H.A.S, H.M.B. and J.F.S. performed the antidiabetic and biochemical assays. C.A.M and J.R.G.S.A made the ethanolic extract of M. nigra leaves and their fraction as well as the phytochemical analysis. Each author should have collaborated sufficiently in the work to take public responsibility for appropriate portions of the content.