BOLETIN LATINOAMERICANO Y DEL CARIBE DE PLANTAS MEDICINALES Y AROMÁTICAS
© / ISSN 0717 7917 / www.blacpma.ms-editions.cl

Artículo Original / Original Article

Hypolipidemic, hypoglycemiant and antioxidant effects of *Myrcia splendens* (Sw.) DC in an animal type 2 diabetes model induced by streptozotocin

[Efectos hipolipemiante, hipoglucemiante y antioxidante de *Myrcia splendens* (Sw.) DC en un modelo animal de diabetes tipo 2 inducido por estreptozotocina]

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Section **Biological activity**

Received: 9 December 2019
Accepted: 11 April 2020
Accepted corrected: 28 April 2020
Published: 30 March 2021

Citation:
Medeiros S, Maia TP, Lima AB, Pscheidt LC, Delmonego L, Vincenzi KL, Custodio BV, Dal Magro DD, Barauna DC, Alberton MD, Pereira EM, Delwing-de Lima D. Hypolipidemic, hypoglycemiant and antioxidant effects of *Myrcia splendens* (Sw.) DC in an animal type 2 diabetes model induced by streptozotocin **Bol Latinoam Caribe Plant Med Aromat** 20 (2): 132 - 146 (2021). https://doi.org/10.37360/blacpma.21.20.2.11

Abstract: We investigated the effects of dichloromethane extract (DME) from *Myrcia splendens* on alterations caused by type 2 diabetes in the blood and kidney of rats, in order to reduce side effects caused by synthetic drugs. Rats received streptozotocin (60 mg/kg), 15 minutes after nicotinamide (120 mg/kg) or water. After 72 hours, the glycemic levels were evaluated to confirm diabetes and the animals received (15 days) DME (25, 50, 100 or 150mg/Kg) or water. DME partially reversed hyperglycemia and (100 and 150 mg/kg) reversed hypertriglyceridemia. Histopathological findings elucidated that DME reduced damage to pancreatic islets. DME 150 mg/kg reversed the increases in TBA-RS, the reduction in the sulfhydryl content, 100 and 150 mg/kg increased CAT, reversed the decrease in GSH-Px and increased it activity in the blood. DME 150 mg/kg reversed CAT and GSH-Px reductions in the kidney. We believe that DME effects might be dependent on the presence of phenolic compounds.

Keywords: Myrtaceae; Diabetes mellitus type II; Oxidative stress; Phenolic compounds; HPLC-ESI-MS/MS.

Resumen: Investigamos los efectos del extracto de diclorometano (DME) de *Myrcia splendens* sobre las alteraciones causadas por la diabetes tipo 2 en la sangre y los riñones de las ratas, para reducir los efectos secundarios causados por las drogas sintéticas. Las ratas recibieron estreptozotocina (60 mg/kg), 15 minutos después de la nicotinamida (120 mg/kg) o agua. Después de 72 horas, se confirmo la diabetes y los animales recibieron (15 días) DME (25, 50, 100 o 150 mg/Kg) o agua. DME revierte parcialmente la hiperglucemia y revierte la hipertrigliceridemia. DME redujo el daño a los islotes pancreáticos. DME revirtió los aumentos en TBA-RS, la reducción en el contenido de sulfhidrilo, aumentó la CAT, revirtió la disminución en GSH-Px y aumentó su actividad en la sangre. Además, DME revirtió las reducciones de CAT y GSH-Px en el riñón. Creemos que los efectos provocados por DME pueden depender de la presencia de compuestos fenólicos.

Palabras clave: Myrtaceae; Diabetes mellitus tipo II; Estrés oxidativo; Compuestos fenólicos; HPLC-ESI-MS/MS

INTRODUCTION

Diabetes mellitus (DM) is a health problem in the current world. In 2013, more than 382 million of people were victims of diabetes, characterized by blood hyperglycemia, resulted from a disorder in secretion or in insulin action or both, that can be associated with obesity, high blood pressure, hypercholesterolemia, hypertriglyceridemia and sedentary lifestyle (Ferreira *et al.*, 2014; Vareda *et al*., 2014).

The prevalence of diabetes shows a world increase and most of the cases are from type 2 diabetes (DMII) (Wallenius & Maleckas, 2015). DMII occurs due resistance to the insulin action, associated with a deficiency in its secretion due to a partial destruction of β-cells (Martin *et al*., 2016). Diabetes treatment requires a change in the daily routine of the individual, since the medical therapy gets in a second moment, when there is the incapacity of controlling the glycemic levels, through balanced diet and physical activities, to minimize the risk of cardiac, neuropathic, renal damage, besides dyslipidemia and oxidative stress (Nathan *et al*., 2009; Ramos *et al.,* 2011; Hou *et al*., 2018).

Among the existent medical options for the diabetes therapy, there are available the insulin and the oral hypoglycemic agents, mainly, biguanides, sulfonylureas, dipeptidyl peptidase 4 inhibitors, thiazolidinediones, GLP-1 receptor agonists and sodium co-transporter glucose-2 inhibitors (SGLT2) (Nathan *et al*., 2009; Ramos *et al*., 2011). The current international guidelines of treatment (2013), recommend the use of metformin as an initial treatment for patients with DMII. If the glycemic control keeps inappropriate, it is recommended a combination of metformin with another class of antidiabetics (Shestakova *et al*., 2018).

The research for new treatments continuous because the existent synthetic drugs show limitations (Arumugam *et al.,* 2013). According to Negri (2005), there are evidences that aqueous and vegetable extracts have hypoglycemic effect. The population has already used the species of the genus *Myrcia* as hypoglycemic, as an empirical form (Batista *et al*., 2011; Cerqueira *et al*., 2013). With the presence of triterpenes, steroids, flavonoids and flavonoid glycosides, these compounds present different activities, being the inhibition of aldose reductase and alpha-glucosidase the most relevant (Matsuda *et al*., 2002; Ferreira *et al*., 2006).

In Brazil, some *Myrcia* species have been used in folk medicine, specially the leaves of these plants are used to treat diabetes (Cascaes *et al.,* 2015). Some studies have shown that *Myrcia* extracts are stronger inhibitors of alpha-glucosidase than acarbose, with 90–500 times the efficacy. Based on these results, extracts from *Myrcia* species were considered promising agents against diabetes (Wubshet *et al.*, 2015; Figueiredo-González *et al.*, 2016). Studies also demonstrate the antioxidant potential of flavonoid extracts, most of which are attributed to myricitrin, and a compound present in some fractions obtained from the *Myrcia* extracts (Moresco *et al*., 2014). Magina *et al.* (2010) and Lima *et al.* (2017) demonstrated the *in vitro* and *in vivo* antioxidant activity of *Eugenia brasiliensis*, possibly due to the total phenolic and flavonoid content of plant extracts and fractions. Another study conducted by Siebert *et al.* (2019), reported the existence of flavonoids quercetin, mycetin, catechin, isoquercetin, rutin and galangin in the crude hydroalcoholic extract and the presence of the compounds catechin, isoquercetin, galangin and apigenin in the ethyl acetate fraction of *Eugenia brasiliensis* (Siebert *et al*., 2019).

The purpose of this study was to verify the effects of subchronic administration of dichloromethane extract (DME), obtained from the leaves of *Myrcia splendens*, on alterations caused by DMII, inducted by the administration of streptozotocin and nicotinamide, in the hyperglycemia, hypertriglyceridemia and oxidative stress in the blood and kidney of rats, as well as to verify the effect of the DME on the damage caused by DMII in the pancreatic islets.

MATERIALS AND METHODS

Animals and reagents

Male Wistar rats (230-280g), obtained from the Breending and Commercial Laboratory Animals LTDA EPP (ANILAB), Paulínia, São Paulo, Brazil, and were used in the experiments. The animals were weaned at 21 days of age. Before experimentation, the animals were housed and acclimated for 7 days to allow adaptation to the new environment and were maintained on a 12h light/12h dark cycle, at a constant temperature (22±1°C), with free access to water and commercial chow. The animals were housed 4 per cage and the tests were carried out in accordance with law N° 11794, of October 8, 2008, and the other norms applied to teaching and/or research, especially the Normative Resolutions of the National Council of Control of Animal Experimentation – CONCEA.

Environmental conditions, lighting, accommodation and nutrition followed the

recommendations required by the "Guide for the Care and Use of Laboratory Animals", 1996. The experimental protocol was approved by the Ethics Committe for Animal Research of the University of Joinville Region, Joinville, Brazil, under the CEUA protocol number 011/2017.

Plant material

Leaves from *Myrcia splendens* (Sw.) DC, Myrtaceae were collected in Blumenau, Santa Catarina state, Brazil (26°53'55.2"S - 49°04'41.3"W) in October 2017. Botanist Dr. André Luiz de Gasper, from the Natural Sciences Department of Regional University of Blumenau (FURB), identified plant material and Dr. Marcos Sobral, Department of Botany, Federal University of Minas Gerais (UFMG) and a voucher specimen was deposited in the Dr. Roberto Miguel Klein Herbarium of the same institution under number FURB 00607.

Obtaining the crude extract of M. splendens

After collection, the plant material was dried under room temperature, grounded in a knife mill and weight. In order to obtain an extract of medium polarity, this material (1.060 kg) was macerated in dichloromethane for 3 days. The extract was filtered, and the solvent evaporated in a rotary evaporator (below 60°C) coupled with a vacuum condenser and concentrated to a reduced volume. This procedure was repeated one more time and yield crude dichloromethane extract (DME) (Barauna *et al.*, 2018).

Induction of DM II and treatment

For DMII induction, rats in a 12-hour fast received a streptozotocin injection (60 mg/kg; i.p.) solved in 0,1 M citrate buffer (pH 4.5), 15 minutes after the nicotinamide prescription (120 mg/kg; i.p.). Citrate buffer was injected alone in control rats. The nicotinamide preserves the β pancreatic cells (by 40%) from the cytotoxicity of streptozotocin and produces the DMII (Sheela *et al*., 2013). After 72 hours, the glycemic levels were evaluated using a strip-operated blood glucose sensor G-TECH free light. The animals were considered diabetic when the blood glucose in fasting was over 200 mg/dL (Sheela *et al*., 2013).

The rats were divided in groups (n = 10) as follows: **Control group:** received water by intraperitoneal (i.p.) injection (once) and water orally (by gavage) once a day during 15 days; **DMII group:** received an i.p. injection of streptozotocin (60 mg/kg) solved in 0.1 M citrate buffer (pH 4.5), 15 minutes after the nicotinamide prescription (120 mg/kg; i.p.) (once) and water by gavage once a day during 15 days; **Control groups extract:** received an i.p. injection of water (once) and DME (25, 50, 100 or 150 mg/kg) obtained from *M. splendens* species by gavage, once a day during 15 days; **DMII + extract groups:** received an i.p. injection of streptozotocin (60 mg/kg) solved in 0.1 M citrate buffer (pH 4.5), 15 minutes after the nicotinamide prescription (120 mg/kg; i.p.) (once) and DME (25, 50, 100 or 150 mg/kg) by gavage, once a day during 15 days consecutive for subchronic treatment, obeying a 24-hours interval between administrations. After this period, the animals were sacrificed by decapitation in the absence of anesthesia, and blood and kidneys were collected and removed for oxidative stress, triglyceride, glucose and histological analysis. The DME (25, 50, 100 and 150 mg/kg) doses were chosen based on Kar *et al*. (2003), Ravi *et al*. (2004), Ravi *et al*. (2005) studies.

Serum preparation

Serum was prepared from total blood samples obtained from rats. The peripheral blood was rapidly collected and transferred to tubes without anticoagulant, centrifuged at 1,000 rpm for 10 min, after the serum was separated and used to the analyses of biochemical parameters (Lima *et al*., 2017).

Erythrocyte and plasma preparation

Erythrocytes and plasma were prepared from total blood samples obtained from rats. For erythrocyte separation, peripheral blood was collected and transferred to heparinized tubes, which were centrifuged at 1,000 rpm, and the plasma was removed by aspiration and maintained frozen at −80°C until assay. Erythrocytes were washed three times with cold saline solution (0.153 mol/L sodium chloride) and lysates were prepared by the addition of 1 mL of distilled water to 100 μL washed erythrocytes and maintained frozen at −80°C until determination of the antioxidant enzyme activities (Lima *et al*., 2017).

For the determination of antioxidant enzyme activity, erythrocytes were frozen and thawed three times, and centrifuged at 13,500 rpm for 10 min. The supernatant was diluted in order to contain approximately 0.5 mg/mL of protein (Lima *et al*., 2017).

Organ preparation

The kidneys were removed, kept on ice-cold buffered

sodium phosphate (20 mM, pH 7.4, 140 mM KCl) and homogenized in ten volumes (1:10 w/v) of appropriate buffer, according to the technique to be performed. Homogenates were prepared using a Potter-Elvehejem homogenizer (Remi motors, Mumbai, India) by passing 5 pulses and centrifuged at 800 rpm for 10 min at 4°C to discarding nuclei and cell debris. The pellet was discarded and the supernatant was saved in aliquots and stored at −80°C for further determination of parameters of oxidative stress (Lima *et al*., 2017).

Histological analysis of the pancreas

Briefly, after sacrificing the animals, the pancreas was removed and fixed in 10% formalin for 24 hours and then transferred to ethanolic solution 70%. After, the organ was passed through solutions with increasing concentrations of alcohol and embedded in paraffin for routine histology procedure. Sections of 7 µm were routinely stained with hematoxylin and eosin (HE). Stained sections of pancreas were analyzed under an Olympus microscope (Olympus CX3).

Histopathological characteristics were described, which may indicate pancreatic islets damage: presence of inflammation, inflammatory infiltrate, asymmetric edges, fibrosis and vacuolar degeneration of nucleus and cytoplasm (Sharma *et al*., 2019).

Biochemical studies

Dosage of glucose and triglycerides

The dosage of glucose and triglycerides were performed using the Glucose and Triglycerides Liquiform kits (Labtest). Absorbance was determined using a UV-vis Shimadzu spectrophotometer, following the manufacturer's package insert.

Catalase Assay (CAT)

CAT activity was determined by the method of Aebi (1984). The method used is based on the disappearance of hydrogen peroxide (H_2O_2) at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0. One CAT unit is defined as 1 µmol of H_2O_2 consumed per minute and the specific activity is calculated as CAT units/mg protein.

Superoxide Dismutase Assay (SOD)

The activity of SOD was assayed by the method described by Marklund (1985), a highly dependent superoxide ($O2^{\bullet-}$) process, which is a substrate for SOD. 15 µL of each sample was added to 215 µL of a mixture containing 50 µM Tris buffer, 1 µM EDTA, pH 8.2, and 30 µM CAT. Subsequently, 20 µL of pyrogallol was added and the absorbance was measured every 30 seconds for 3 minutes at 420 nm using a spectrophotometer. Inhibition of auto-oxidation of pyrogallol occurs in the presence of SOD, the activity of which can be indirectly tested spectrophotometrically. One unit of SOD is defined as the amount of SOD required to inhibit 50% of the auto-oxidation of pyrogallol and the specific activity is reported as SOD units/mg protein.

Glutathione Peroxidase Assay (GSH-Px)

GSH-Px activity was measured by the method of Wendel (1981), using *tert*-butylhydroperoxide as substrate. The decomposition of NADPH was monitored in a spectrophotometer at 340 nm for 4 minutes. The medium contained 2.0 mM GSH, 0.15 U/mL GSH reductase, 0.4 mM azide, 0.5 mM *tert*-butylhydroperoxide and 0.1 mM NADPH. One GSH-Px unit is defined as 1 µmol of NADPH consumed per minute and the specific activity is reported as GSH-Px units/mg of protein.

Total sulfhydryl content

The total sulfhydryl content was determined according to the method of Aksenov & Markersbery (2001), which is based on the reduction of dithionitrobenzoic acid (DTNB) by thiols, generating a yellow derivative (TNB), which is measured spectrophotometrically at 412 nm. For the assay, 50 µL of homogenate was added to 1 mL of phosphate-buffered saline (PBS), pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA). The reaction was started by the addition of 30 µL of 10 mM DTNB and incubated for 30 minutes at room temperature in the dark. Analyses of a blank (DTNB absorbance) was also performed. The results were expressed as nmol TNB/mg protein.

Thiobarbituric acid reactive substances (TBA-RS)

TBA-RS were determined according to the method described by Ohkawa *et al.* (1979). The methodology for the analysis of TBA-RS measures malondialdehyde (MDA), a product of lipoperoxidation, caused mainly by hydroxyl free radicals. Initially, plasma and kidney in 1.15% KCl was mixed with 20% trichloroacetic acid and 0.8% thiobarbituric acid and heated in a boiling water bath for 60 min. TBA-RS were determined by the absorbance at 535 nm. A calibration curve was obtained using 1,1,3,3-tetramethoxypropane as the MDA precursor and each curve point was subjected

to the same treatment as that of the supernatants. TBA-RS content was expressed as nanomoles of MDA formed per milligram of protein.

Protein carbonyl content

Protein carbonyl content was assayed by a method described by Reznick & Packer (1994) [31], based on the reaction of protein carbonyls with dinitrophenylhydrazine to form dinitrophenylhydrazone, a yellow compound that is measured spectrophotometrically at 370 nm. Briefly, 200 µL of homogenate were added to plastic tubes containing 400 µL of 10 mM dinitrophenylhydrazine (prepared in 2M HCl). Samples were kept in the dark for 1h and vortexed every 15 min. Subsequently, 500 µL of 20% trichloroacetic acid were added to each tube. The mixture was vortexed and centrifuged at 14,000 rpm for 3 min and the supernatant obtained was discarded. The pellet was washed with 1 mL ethanol/ethyl acetate (1:1 v/v), vortexed and centrifuged at 14,000 rpm for 3 min. The supernatant was discarded and the pellet re-suspended in 600 µL of 6 M guanidine (prepared in a 20 mM potassium phosphate solution, pH 2.3), before vortexing and incubating at 60ºC for 15 min. Samples were then centrifuged at 14,000 rpm for 3 min and the supernatant was used to measure absorbance at 370 nm (UV) in a quartz cuvette. Results were reported as carbonyl content (nmol/mg protein).

Protein determination

Protein was measured by the Lowry *et al.* (1951) method, using serum bovine albumin as standard.

Analysis of phenolic compounds in the DME by HPLC-ESI-MS/MS

Instrumentation

Analysis of phenolic compounds was performed according Siebert *et al*. (2019). This analysis is conducted using Agilent® 1200 chromatograph, coupled to a mass spectrometry system consisting of a hybrid triple quadrupole/linear ion trap mass spectrometer Qtrap® 3200 (Applied Biosystems/MDS SCIEX, USA) with TurboIonSpray® as the ionization source, in negative ionization mode. The Analyst® software (version 1.5.1) was used for the recording and processing of the data. Pairs of ions were monitored in multiple reaction monitoring (MRM) mode.

Chromatographic conditions

The eluent was formed by mixing solvents A ($MeOH/H_2O$ in ratio of 95:5, v v^{-1}) and B (H2O/formic acid 0.1%) as follows: 1st stage – 10% solvent A and 90% B (isocratic mode) for 5 min; 2nd stage – linear gradient of solvents A and B (from 10 to 90% of A) for 2 min; 3rd stage – 90% solvent A and 10% B (isocratic mode) for 3 min; 4th stage – linear gradient of solvents A and B (from 90 to 10% of A) for 7 min with a flow rate of 250 µL min^{-1} of mobile phase. In all analyses, the injected volume was 5 uL.

For the identification and quantification, 47 standard phenolic compounds were analyzed under the same conditions described above, being them: 4-aminobenzoic acid, 4-hydroxymethylbenzoic acid, apigenin, aromadendrin, caffeic acid, carnosol, catechin, chlorogenic acid, chrysin, cinnamic acid, coniferaldehyde, ellagic acid, epicatechin, epigallocatechin, epigallocatechin gallate, eriodictyol, ferulic acid, fustin, galangin, gallic acid, hispudulin, isoquercetin, kaempferol, mandelic acid, methoxyphenylacetic acid, myricetrin, naringerin, naringin, p-anisic acid, p-coumaric acid, pinocembrin, protocatechuic acid, quercetin, resveratrol, rosmarinic acid, rutin, salicylic acid, scopoletin, sinapaldehyde, sinapic acid, syringaldehyde, syringic acid, taxifolin, umbelliferone, vanillic acid and vanillin dissolved in methanol (0.02 to 6 mg L^{-1}).

Sample of extract was prepared by dissolving 50 mg of the freeze-dried material in a 5 mL solution of hydrochloric acid at pH 2. These solutions were extracted three times with 2 mL of ethyl ether each time and the three extracts were combined. After drying the combined extract, it was stored in a sealed container at -20°C. Prior to analysis, the dried material was dissolved in 1 mL of MeOH and centrifuged at 12,000 rpm for 120 s.

Statistical analysis

The Kolmogorov-Smirnov normality test was performed to confirm a parametric distribution; data were then analyzed by ANOVA, followed by Duncan's *post-hoc* test, when the F-test was significant. All analyses were performed using the IBM Statistical Package for the Social Sciences (SPSS) for Windows version 20.0 using a PC compatible computer (IBM Corp. Armonk, NY, USA). Values of $p<0.05$ were considered significant. Results are expressed as means ± SD for 7-8 independent experiments (animals) performed in duplicate. ***$p<0.001$, **$p<0.01$ and *$p<0.05$, compared to the Duncan's multiple range test.

RESULTS

Effects of subchronic administration of DME from M. splendens on glucose and triglyceride levels on streptozotocin-nicotinamide-induced type-2 diabetic in rats

We investigated the effects of subchronic administration of DME (25, 50, 100 and 150 mg/kg) obtained from the leaves of *M. splendens* on glucose and triglyceride levels in the serum of type-2 diabetics rats. The Figure No. 1 shows that streptozotocin-nicotinamide administration signify-cantly enhanced glucose (A) [F (9,52) = 37.850; $p<0.001$] and triglyceride (B) levels [F(9,52) = 8.881; $p<0.001$]in the serum of rats, when compared to the control groups. Post hoc analyzes showed that chronic administration of DME at doses of 25, 50, 100 and 150 mg/kg per se did not alter glucose and triglyceride levels. DME (25, 50, 100 and 150 mg/kg) administration partially reversed, at dose dependent, the hyperglycemia induced by streptozotocin-nicotinamide administration. In addition, DME at dose of 50 mg/kg partially reversed and at doses of 100 and 150 mg/kg totally reversed hypertriglyceridemia induced by streptozotocin-nicotinamide administration.

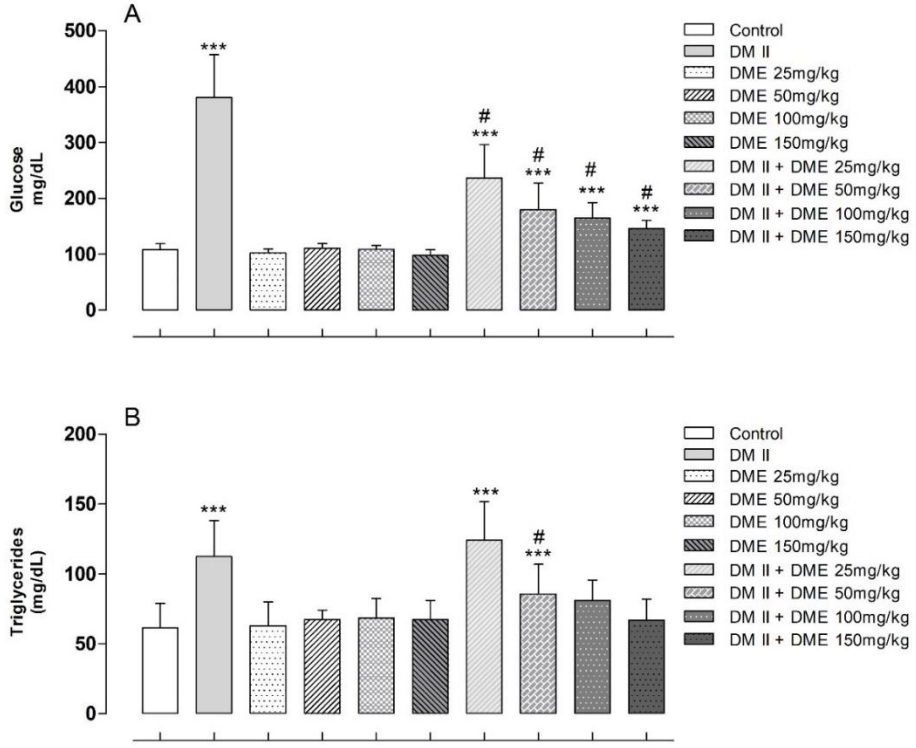

Figure No. 1
Effect of different DME doses (25, 50, 100 or 150mg/kg) on the effects elicited by DMII on glucose levels (A) and triglycerides levels (B) in the blood of 60-day-old Wistar rats. Results are expressed as mean ± standard deviation for 7-8 independent (animal) experiments performed in duplicate. ***$p<0.001$, compared to the control. #: Partial reversal

Effects of subchronic administration of DME from M. splendens on histological analysis of the pancreas on streptozotocin-nicotinamide-induced type-2 diabetic in rats

A qualitative analysis of the pancreatic islets showed differences in the morphological aspects analyzed. As shown in Figure No. 2, the streptozotocin administration induced degeneration of the pancreatic islets, since the group that received streptozotocin and was treated with vehicle show pancreatic islets with asymmetrical edges, a decreased in the islets size and the presence ofcytoplasmic vacuolization. The diabetic groups treated with DME of the *M. splendens* at all doses

(25 mg/kg, 50 mg/kg, 100 mg/kg and 150 mg/kg) showed the same characteristics of the diabetic group treated with vehicle, however, a decreased in cytoplasmic vacuolization can be observed, indicating less cell damage. The diabetic groups that received DME of the *M. splendens*, at doses of 25 mg/kg and 150 mg/kg, presented fibrosis areas, indicating tissue regeneration.

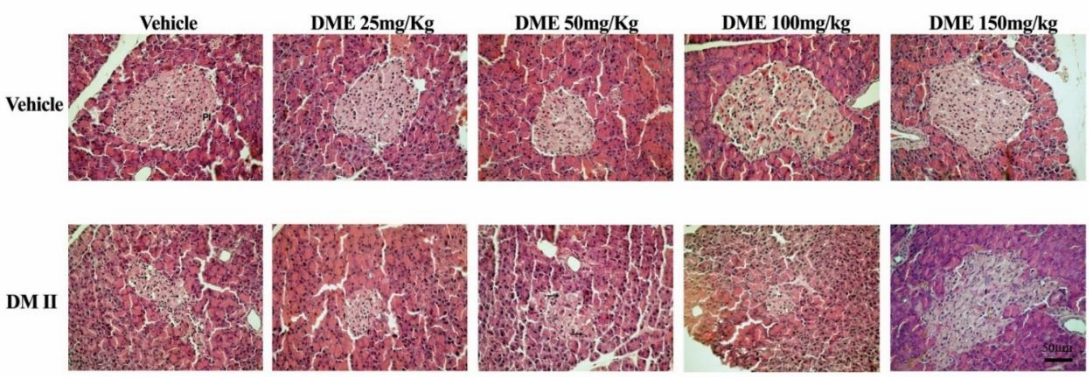

Figure No. 2
Pancreatic islets of control group streated with vehicle or DME at doses of 25 mg/kg, 50 mg/kg, 100 mg/kg or 150 mg/kg presenting normal appearance and symmetrical borders. Pancreatic islets of diabetic groups treated with vehicle or DME at doses of 25 mg/kg, 50 mg/kg, 100 mg/kg or 150 mg/kg showing asymmetrical borders. PI: pancreatic islet, v: blood vessels, arrow: cytoplasmic vacuolization, f: fibrosis. Scale bar = 50 µm. Olympus optical microscope

Effects of the subchronic administration of DME from M. splendens on the alterations in the blood oxidative stress parameters caused by streptozotocin-nicotinamideinduced type-2 diabetic in rats

We next investigated the effects of the subchronic administration of DME (25, 50, 100 and 150 mg/kg) on the alterations in TBA-RS, total sulfhydryl content, protein carbonyl content and antioxidant enzymes activities caused by type-2 diabetes in the blood of rats. As shown in Figure No. 3, streptozotocin-nicotinamide DMII model increased TBA-RS (A) [$F(9,52) = 30.966$; $p<0.001$], reduced total sulfhydryl content (B) [$F(9,52) = 22.302$; $p<0.001$], did not alter protein carbonyl content (C) [$F(5,32) = 1.601$; $p>0.05$] and SOD (D) activity [$F(5,32) = 0.821$; $p>0.05$], increased CAT (E) [$F(9,52) = 12.683$; $p<0.001$] and reduced GSH-Px (F) [$F(9,52) = 13.190$; $p<0.001$] activities.

Post hoc analysis showed that DME per se did not alter TBA-RS levels, total sulfhydryl content, protein carbonyl content and SOD activity, when compared to the control groups. Moreover, chronic administration of DME at doses of 25 and 50 mg/kg didn't reverse, 100 mg/kg partially reversed and 150 mg/kg totally reversed the increases in TBA-RS levels (Figure No. 3A) and the reduction in the sulfhydryl content (Figure No. 3B) in the blood of rats. With regard to the antioxidant enzymes CAT (Figure No. 3E) and GSH-Px (Figure No. 3F), subchronic administration of DME at doses of 100 and 150 mg/kg led to a significant increase in the CAT and GSH-Px activities, when compared to the control group. DME treatment at doses of 25 and 50 mg/kg didn't revert CAT increases and partially reversed GSH-Px reduction, at doses of 100 and 150 mg/kg, when administrated in animals that received streptozotocin-nicotinamide, led to a significant increase in the CAT activity, when compared to diabetics group. Furthermore, reversed the decrease in GSH-Px activity induced by diabetes and increased this enzyme activity.

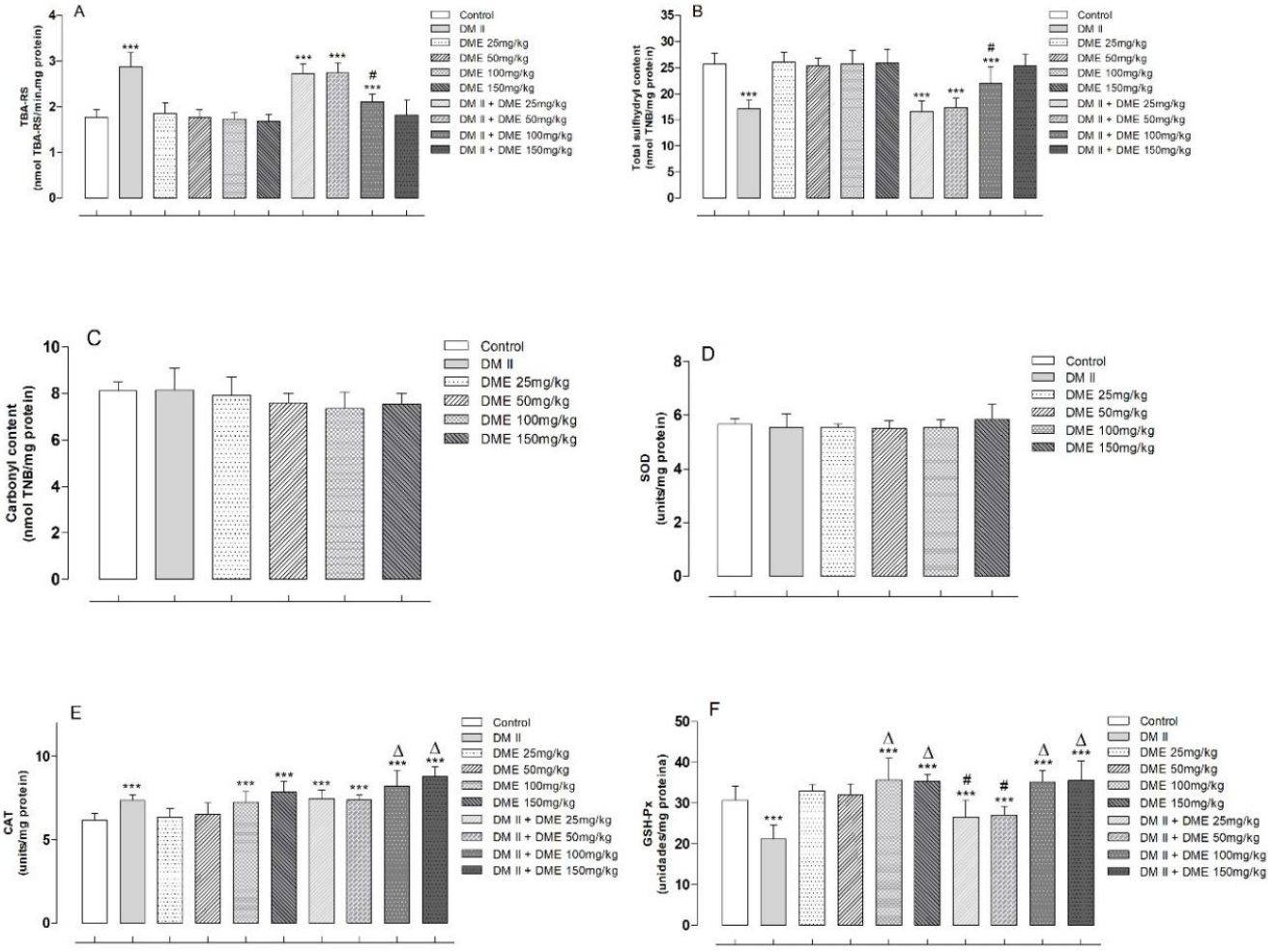

Figure No. 3
Effect of different DME doses (25, 50, 100 or 150mg/kg) on the effects elicited by DMII on TBA-RS (A) levels, total sulfhydryl content (B), carbonylated proteins (C), SOD (D), CAT (E) and GSH-Px (F) in the blood of 60-day-old Wistar rats. Results are expressed as mean ± standard deviation for 7 independent (animal) experiments performed in duplicate. ***$p<0.001$, compared to controls. #: Partial reversed. Δ: Potentiation of the effect

Effects of the subchronic administration of DME from M. splendens on the alterations in the kidney oxidative stress parameters caused by streptozotocin-nicotinamide induced type-2 diabetic in rats

We also investigated the effects of the subchronic administration of DME (25, 50, 100 and 150 mg/kg) on the alterations in oxidative stress parameters induced by type-2 diabetes in the kidney of rats. Figure No. 4 shows that streptozotocin-nicotinamide DMII model did not alter TBA-RS (A) [$F(5,32) = 0.525$; $p>0.05$], total sulfhydryl content (B) [$F(5,32) = 0.724$; $p>0.05$], protein carbonyl content (C) [$F(5,32) = 25.351$; $p<0.001$] and SOD (D) activity [$F(5,32) = 0.120$; $p>0.05$] in the kidney of rats. In contrast, decreased CAT (E) [$F(9,52) = 34.774$; $p<0.001$] and GSH-Px (F) activities [$F(9,52) = 25.410$; $p<0.001$] in this organ. *Post hoc* analysis

showed that DME (25, 50, 100 and 150 mg/kg) *per se* did not alter TBA-RS levels, total sulfhydryl content, CAT and SOD activities, when compared to the control groups, while DME at doses of 100 and 150 mg/kg reduced protein carbonyl content and DME at dose of 150 mg/kg increased GSH-Px activity, when compared to the control groups.

Additionally, DME treatment at doses of 25 and 50 mg/kg did not reversed CAT (Figure No. 4E) and GSH-Px (Figure No. 4F) reductions caused by DMII, at doses of 100 mg/kg partially reversed the reduction in CAT activity and at dose of 150 mg/kg totally reversed CAT and GSH-Px reductions.

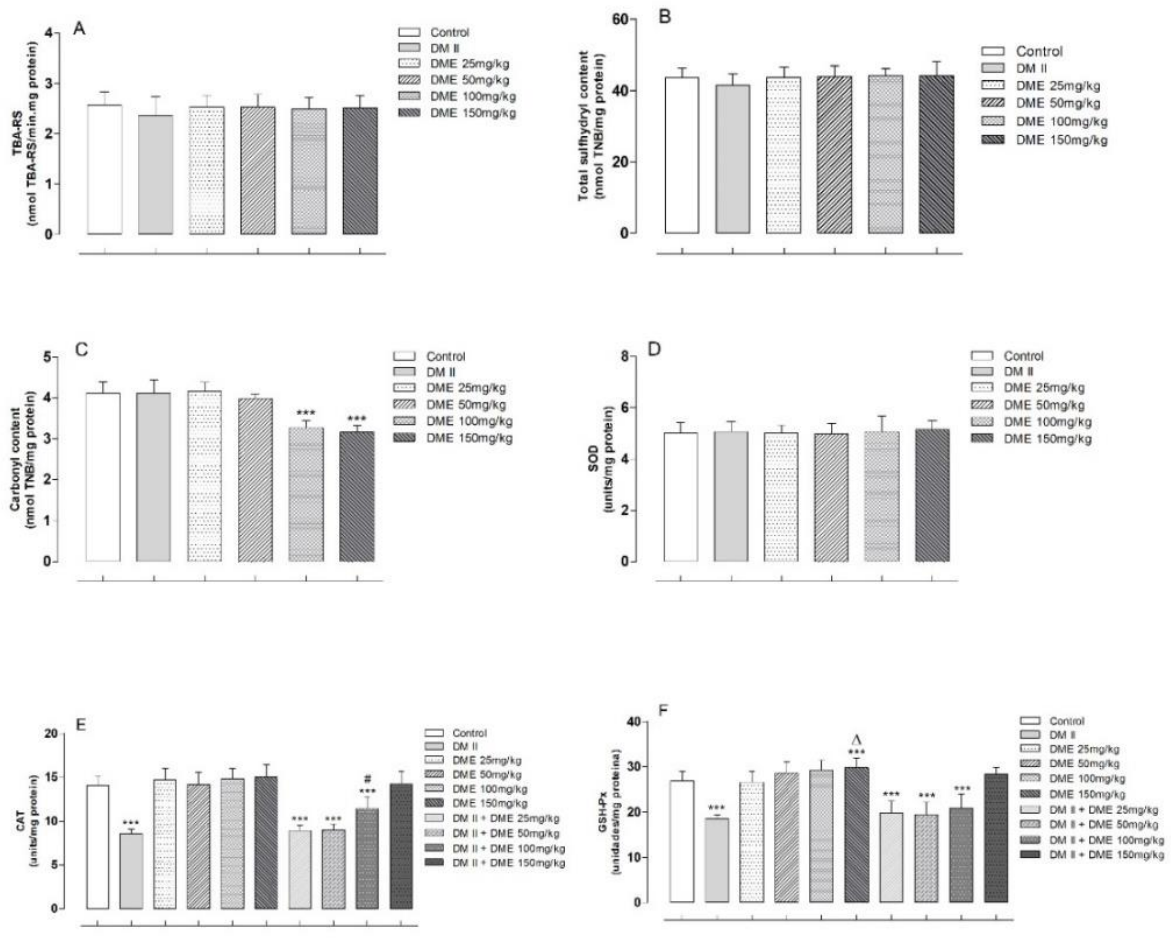

Figure No. 4

Effect of different DME doses (25, 50, 100 or 150 mg/kg) on the effects elicited by DMII on TBA-RS (A) levels, total sulfhydryl content (B), carbonylated proteins (C), SOD (D), CAT (E) and GSH-Px (F) in the kidney of 60-day-old Wistar rats. Results are expressed as mean ± standard deviation for 7 independent (animal) experiments performed in duplicate. *** $p<0.001$, compared to the control. #: Partial reversed. Δ: Potentiation of the effect

Identification of the compounds present in the DME by qualitative analysis using HPLC-ESI-MS/MS

After complete drying of samples, the extract yields were calculated. Leaf EBDM yielded 0.7%. Table No. 1 shows the results obtained by HPLC-ESI-MS/MS analysis. This technique provides superior specificity and sensitivity when compared to direct injection methods, since modern mass spectrometers are highly sensitive than LC-MS assays. Another advantage is its ability to multiplex multiple analytes in a single analytical run (Pitt, 2009). From forty-seven investigated standards, eleven phenolic compounds were identified in the extract, although

the low yield of the EBDM. The compound with the highest amount found was ellagic acid. Further, protocatecuic acid, syringic acid, *p*-coumaric acid, salicylic acid, isoquercetin ferulic acid, umbelliferone, coniferaldehyde, sinapaldehyde and carnosol were also identified.

Table No. 1
Idenfied phenolic (μg g^{-1}) in *M. splendens* leaves extracts

Phenolic compound	Rt (min)	TM (Da)	EM (M-H, *m/z*)	MS/MS (*m/z*)	EBDM
Protocatecuic acid	6.27	154.12	152.92	109.00	0.03 ± 0.005
Syringic acid	9.91	198.17	196.93	121.10	1.18 ± 0.030
***p*-Coumaric acid**	10.32	164.05	162.92	119.10	0.28 ± 0.008
Ferulic acid	10.65	194.18	192.95	134.00	0.03 ± 0.001
Salicylic acid	10.75	138.12	136.94	93.00	1.09 ± 0.008
Umbelliferone	10.78	162.14	160.94	133.10	0.05 ± 0.001
Isoquercetin	10.79	464.38	463.15	300.00	< LOQ
Coniferaldehyde	11.24	178.18	177.01	162.00	0.24 ± 0.002
Synapaldehyde	11.33	208.21	207.04	177.00	0.77 ± 0.010
Ellagic acid	11.78	302.19	300.95	145.00	3.84 ± 0.080
Carnosol	13.87	330.42	329.16	285.20	0.24 ± 0.014

Rt = Retention time (min); TM = Theoretical mass (Da); EM = Experimental mass (*m/z*); MS/MS = MS/MS Fragments (*m/z*); <LOQ = Less than limit of quantification

DISCUSSION

The main characteristic of DM is hyperglycemia, causing disturbances in metabolism (Subramanian & Chait, 2012). One of the causes of diabetes complications is oxidative stress, a condition in which antioxidant defenses are not enough to inactivate the reactive species generated (Rains & Jains, 2011). Diabetes is characterized by a series of complications that affect different organs and oxygen free radicals are involved in the pathogenesis of this disease (Maritim *et al*., 2003). During persistent hyperglycemia of diabetes, there is an increase in oxygen free radical production through glucose self-oxidation, and these radicals exert their cytotoxic effects on membrane phospholipids, resulting in the formation of malondialdehyde (MDA), an end product of lipid peroxidation, which reacts with thiobarbituric acid (Ayoub *et al*., 2000).

Streptozotocin causes irreversible damage to pancreatic β-cells, generating inability to secrete insulin, justifying its use in research (Zafar *et al*., 2009). Increased hypertriglyceridemia is associated with diabetes due to metabolic syndrome (Subramanian & Chait, 2012).

Our results showed that administration of streptozotocin/nicotinamide increased serum glucose and triglyceride levels in treated rats, generating diabetes and HTG. Subchronic administration of DME for 15 days at doses of 25, 50, 100 and 150 mg/kg did not alter glucose and triglyceride levels and partially reversed, depending on the dose, leading us to believe that a higher dose would be able to reverse hyperglycemia induced by streptozotocin-

nicotinamide administration. In addition, DME at 50 mg/kg partially reversed and at 100 and 150 mg/kg completely reversed HTG. Corroborating our data, the study of Bongiolo (2008) showed that treatment with hydroalcoholic extract of *Eugenia uniflora* (500 and 1000 mg/kg), a Myrtaceae species, caused reduction and/or reversed of HTG, suggesting that this hypolipidemic effect was mediated by the flavonoids quercetin and myricetin present in the extract. Furthermore, Santana (2016) and Kala *et al.* (2012) demonstrated that other plants of the same genus, including *Eugenia dysenterica* and *Eugenia floccosa*, also demonstrated a significant reduction in HTG. Also, Prince *et al.* (2003) highlights that the aqueous extract of *Syzygium cumini* (*Eugenia jambolana*) (Myrtaceae) also caused a reduction in HTG and emphasized that aqueous extracts and ethanolic agents exerted a more significant hypoglycemic effect than glyburide. Yet, the studies by Grover *et al.* (2001), elicit that *Momordica charantia* and *Eugenia jambolana* (Myrtaceae) extracts reduce hyperglycemia, protect a basal glomerular membrane from the deleterious effects of diabetes and inhibit increased urinary volume, albuminuria and renal hypertrophy. Additionally, *Mormodica charantia* extract has been shown to exert a hypoglycemic effect even in the presence of almost complete destruction of pancreatic β cells, signaling a direct insulinomimetic effect (Grover *et al*., 2001). Our data corroborate with the studies of Rabbani *et al*. (2010), which showed that glibenclamide reduces hyperglycemia and according to the studies of Ashraf *et al.* (2014), which showed that glibenclamide reduces hyperglycemia and HTG caused by streptozotocin-nicotinamide induced type-2 diabetic in rats. According to Lima *et al.* (2017), the chronic administration of the hydroalcoholic and acetonic extracts of *Eugenia brasiliensis* reversed HTG, decreased lipid accumulation in the hepatocytes, decreased lipid peroxidation and protein damage in the blood and inhibited alterations in the activities of antioxidant enzymes in the blood and liver of coconut oil-induced HTG rats.

Histopathological findings are according to Konda *et al.* (2019), which observed asymmetrical edges and a decreased in the islets size in diabetic animals due to decreased in β-cells number. Pancreatic β-cells express GLUT-2, which is identified by streptozotocin, so these cells are affected by the toxic effects of streptozotocin (Eleazu *et al.*, 2013). Our results showed that administration of DME from *M. splendens*, at all doses tested showed less cell damage when compared with the diabetic control group. It can be due to antioxidant effect of this extract. A study by Krishnasamy *et al.* (2016) demonstrated that *Syzygium densiflorum*, a Myrtaceae specie, promotes decreased damage caused by streptozotocin in pancreatic tissue.

The extract of *M. splendens* studied here demonstrated similarity on the chemical composition with others of the genus *Myrcia*. Despite this, since phenolic compounds are ubiquitous in most medicinal plants and constitute an essential part of the human diet due to their antioxidant and many other beneficial health properties, the quantification of already known compounds, opens the possibility for further studies of biological potential and it contributes to a better understanding of the secondary metabolism in the *Myrcia* genus (Balasundram *et al*., 2006).

Although some of the compounds identified in this work have already been cited in the *Myrcia* genus, syringic acid, *p*-coumaric acid, ferulic acid, umbelliferone, isoquercetin, coniferaldehyde, synapaldehyde and carnosol are reported for the first time in the genus. In addition to the previously mentioned compounds, this was also the first report of the presence of protocatecuic acid and ellagic acid in extracts in leaves of *M. splendens*.

The solvent used during extraction process are reported to have an influence on the nature and the number of secondary metabolites extracted from medicinal plants. Commonly, polar solvents are used to extract phenolic compounds and their glycosides (Dirar *et al*., 2019). In this work, we used a solvent of medium polarity, and apolar compounds between standards searched, for example ferulic acid and carnosol, were found only in DME. This relation can be attributed to its more apolar structure, to have a greater carbonic chain or smaller number of hydroxyls.

Our results also show that streptozotocin-nicotinamide induced DMII increased TBA-RS and reduced total sulfhydryl content but did not alter the protein carbonyl content in rat plasma. With regard to the kidney, DMII model did not alter TBA-RS, total sulfhydryl and protein carbonyl contents. In addition, DME extract "per se", at doses of 100 and 150 mg/kg, reduced protein carbonyl content and, at dose of 150 mg/kg, increased GSH-Px activity. Moreover, subchronic administration of DME at dose of 100 mg/Kg partially reversed and 150 mg/kg totally reversed the increases in TBA-RS levels and the reduction in the sulfhydryl content in the blood of rats. TBA-RS is a lipid oxidation parameter and reflects the amount of malondialdehyde formation, a

product of membrane fatty acid peroxidation. Also, is an important parameter of lipoperoxidation and to measure damage to membrane lipids. Since lipoperoxidation is responsible for change the membrane lipids structure, reducing its selectivity in ionic transport and signaling, imparing its permeability and cellular transport, these alterations in excess, may result in cell death by the release of hydrolytic enzymes and formation of cytotoxic products, such as malondialdehyde (Aksenov & Marksberry, 2001). In addition, total sulfhydryl content is a parameter, which is very important to measure damage to protein. Alterations in protein structures lead to the increase of its fragmentation, aggregation, and susceptibility to degradation by proteasomes (Wendel, 1981; Aebi, 1984).

With regard to the antioxidant enzymes CAT and GSH-Px, subchronic administration of DME, at doses of 100 and 150 mg/kg, increased CAT and GSH-Px activities in the blood of rats. Furthermore, DME treatment (25 and 50 mg/kg) did not reversed CAT increases and partially reversed GSH-Px reduction and at doses of 100 and 150 mg/kg, when administrated in animals that received streptozotocin-nicotinamide, increased CAT activity. Also, reversed the decrease in GSH-Px activity induced by diabetes and increased this enzyme activity. Results also showed that DMII model decreased CAT and GSH-Px activities in the kidney of rats. The antioxidant enzymes protects organ from highly reactive hydroxyl radicals. Thus, a reduction in kidney SOD and CAT activities, observed in our study, may lead to an increase in the availability of superoxide ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) in biological systems, enabling the generation of hydroxyl radicals ($OH^{\bullet}$), resulting in the onset and spread of lipid peroxidation (Prince *et al.*, 2003). The DME *per se*, at dose of 150 mg/kg, increased GSH-Px activity. Additionally, DME treatment at doses of 25 and 50 mg/kg did not reversed CAT and GSH-Px reductions caused by DMII, at doses of 100 mg/kg partially reversed the reduction in CAT activity and at dose of 150 mg/kg totally reversed CAT and GSH-Px reductions. Corroborating to our data, Pontes *et al.* (2019), also showed antioxidant activity in different solvents of the leaves of *M. splendens*.

In summary, this study shows that streptozotocin-nicotinamide administration in rats causes DMII and HTG, in association with oxidative stress in the blood and kidneys. Subchronic administration of DME of *M. splendens* was able to partially reversed hyperglycemia, reversed HTG totally, and to reduce lipid peroxidation and protein damage in diabetics rats. In addition, this extract also reversed alterations in the activity of antioxidant enzymes in the blood and kidneys of rats. The data indicate that DME obtained from *M. splendens* leaves, may have hypoglycemic and hypolipidemic effects and high antioxidant capacity, probably mediated by the action of phenolics present in this extract. However, further studies are needed to assess whether *M. splendens* could be useful as a potential adjuvant for the treatment of hyperglycemia, HTG and oxidative stress in DMII patients.

ACKNOWLEDGEMENTS

The authors would like to thank Fundação Universidade da Região de Joinville (UNIVILLE), Fundação Universidade Regional de Blumenau (FURB) and Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC) – 88887.178154/2018-00 for the financial support and Universidade Federal de Santa Catarina (UFSC) for the partnership.

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