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Detection of myenteric plexus neurons in dyslipidemic, smoking, and diabetic rats treated with carqueja

Detecção de neurônios do plexo mientérico em ratos dislipidêmicos, tabagistas e diabéticos tratados com carqueja

Detección de neuronas del plexo mientérico en ratas dislipidémicas, tabaquistas y diabéticas tratadas con carqueja

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Abstract

The plant species *Baccharis trimera* presents antioxidants that may have neuroprotective effects on the neurons of the myenteric plexus. Thus, the aim of the present study was to investigate possible quantitative alterations in the myenteric plexus neurons and in the glycemic and lipid profile of 25 rats with 90 days old, exposed to smoking, a hypercholesterolemic diet, and with diabetes mellitus induced by streptozotocin during four weeks, and then treated with different doses of carqueja extract for two weeks. The myenteric plexus neurons were stained with basic Giemsa and using the NADPH-diaphorase histochemistry protocol. In the study conditions, there was a significant reduction in the number of total neurons between the groups treated with carqueja and the positive control, stained with the Giemsa. In contrast, there was no significant difference in the number of neurons of the inhibitory subpopulation between the groups treated with carqueja and the negative control, evidenced by the NADPH-diaphorase histochemistry.

At the 30mg/kg dose there was a reduction in the cholesterol and triglyceride levels. Based on the results, *Baccharis trimera* presented no neuroprotective or hypoglycemic effect, although the nitric subpopulation has proven more resistant to the deleterious effects of diabetes, smoking, and the hypercholesterolemic diet.

Keywords: *Baccharis trimera*; Hypercholesterolemia; Hyperglycemia; Myenteric plexus.

Resumo

A espécie vegetal *Baccharis trimera* apresenta antioxidantes que podem ter efeitos neuroprotetores sobre os neurônios do plexo mioentérico. Assim, o objetivo do presente estudo foi investigar possíveis alterações quantitativas nos neurônios do plexo mioentérico e no perfil glicêmico e lipídico de 25 ratos com 90 dias de idade, expostos ao tabagismo, dieta hipercolesterolêmica e com diabetes mellitus induzido por estreptozotocina durante quatro semanas, e depois tratada com diferentes doses de extrato de carqueja por duas semanas. Os neurônios do plexo mioentérico foram corados com Giemsa básico e usando o protocolo de histoquímica NADPH-diaforase. Nas condições do estudo, houve uma redução significativa no número de neurônios totais entre os grupos tratados com carqueja e o controle positivo, corado com Giemsa. Em contrapartida, não houve diferença significativa no número de neurônios da subpopulação inibitória entre os grupos tratados com carqueja e o controle negativo, evidenciada pela histoquímica da NADPH-diaforase. Na dose de 30mg/kg houve redução dos níveis de colesterol e triglicerídeos. Com base nos resultados, *Baccharis trimera* não apresentou efeito neuroprotetor ou hipoglicemiante, embora a subpopulação nítrica tenha se mostrado mais resistente aos efeitos deletérios do diabetes, tabagismo e dieta hipercolesterolêmica.

Palavras-chave: *Baccharis trimera*; Hipercolesterolemia; Hiperglicemia; Plexo mioentérico.

Resumen

La especie vegetal *Baccharis trimera* presenta antioxidantes que pueden tener efectos neuroprotectores sobre las neuronas del plexo mientérico. El objetivo del presente estudio ha sido investigar posibles alteraciones cuantitativas en las neuronas del plexo mientérico y en el perfil glicémico y lipídico de 25 ratas con 90 días de edad, expuestas al tabaco, dieta de hipercolesterolemia y con diabetes mellitus inducido por estreptozotocina durante cuatro semanas. Las neuronas del plexo mientérico fueron coloreados con Giemsa básico y utilizado el protocolo de histoquímica NADPH-diaforase. En las condiciones del estudio, hubo reducción significativa en el número de neuronas totales entre los grupos tratados con carqueja y el control positivo, colorado con Giemsa. En cambio, no hubo diferencia significativa en el número de

neuronas de la subpoblación inhibitoria entre los grupos tratados con carqueja y el control negativo, evidenciada por la histoquímica de NADPH-diaforase. En la dosis de 30mg/kg hubo reducción de niveles de colesterol y triglicéridos. Con base en los resultados, *Baccharis trimera* no presentó efecto neuroprotector o hipoglucemiante, aunque la subpoblación nítrica se haya mostrado más resistente a los efectos deletéreos del diabetes, tabaquismo y dieta de hipercolesterolemia.

Palabras clave: *Baccharis trimera*; Hipercolesterolemia; Hiperglicemia; Plexo mientérico.

1. Introduction

The gastrointestinal tract has an intrinsic innervation that controls the functions of the intestine independent of the central nervous system, named Enteric Nervous System (ENS), divided into plexuses, with the main ones being the myenteric and submucous plexuses (Furness, 2012).

The ENS plexus may be affected by different diseases, including diabetes (American Diabetes Association (ADA), 2019; Silverio, Mari, Clebis & Scoz, 2009) and may promote long-term gastrointestinal disorders (Camilleri, Bharucha & Farrugia, 2011), such as nausea, diarrhea, abdominal pain, constipation, dysphagia, and heartburn (Fonseca & Rached, 2019).

The density of myenteric plexus neurons varies along the length and the intestinal circumference (Miranda-Neto, Molinari, Natali & Sant'Ana, 2001), animals that suffered from diabetes showed changes in the enteric neuronal subpopulations, and both degeneration and reduction in the amount of neurotransmitters (Chandrasekharan & Srinivasan, 2007).

Neurons in the myenteric plexus have neurotransmitters responsible for TGI movement (Furness, 2006). These neurotransmitters can be excitatory, such as acetylcholine (ACh), which acts on the smooth musculature, as well as inhibitory, which promotes relaxation of gastrointestinal muscles such as nitric oxide (NO), the main non-adrenergic and noncholinergic neurotransmitter (NANC).

Diabetes Mellitus (DM) is a chronic disease affecting about 440 million people worldwide (Zhao et al., 2018). DM is characterized by increased blood glucose, because of defects in the action of insulin (ADA, 2019; Silverio et al., 2009) or by peripheral cellular resistance to this hormone. This may lead to acute complications such as ketoacidosis, and systemic chronic diseases such as nephropathies, neuropathies, retinopathies, atherosclerosis, and other diseases (Asmat, Abad & Ismail, 2016).

In addition to hyperglycemia, other changes play an important role in the pathogenesis of diabetes, such as oxidative stress, an imbalance between antioxidants and prooxidants that leads healthy cells to lose their structure and function (Asmat, Abad & Ismail, 2016).

Smoking is an important factor in the emergence of chronic inflammatory diseases such as inflammatory bowel disease, Crohn's disease and ulcerative colitis (Bernstein et al., 2016). Although the pathogenesis of these diseases is not yet fully understood, smoking, in addition to genetic factors, results in a disruption of the intestinal immune balance and may lead to the development of lesions (Bastida & Beltrán, 2011).

The nicotine present in cigarettes is a psychoactive substance and affects several neurotransmitters of the CNS, being also able to stimulate the production of acetylcholine by the myenteric plexus, which can lead to diarrhea, nausea, and vomiting (Fochi, 2003).

Atherosclerosis is one of the etiologies of the chronic mesenteric ischemia (CMI) (Carver, Vora & Taneja, 2016). The most frequent symptoms of CMI are postprandial pain, such as cramps in the epigastric and umbilical region, which might result in sitophobia and irrational fear of feeding, worsening malnutrition and cachexia. Laboratory tests show anemia, leukopenia, and electrolyte disorder (Cronenwett & Johnston, 2014; Kolkman & Greelkerken, 2017; Kanamori et al., 2014; Pecoraro et al., 2013).

It is well known that DM causes several alterations in the digestive system and consequently in the myenteric plexus. However, there are few studies about the association between smoking and hypercholesterolemia with DM. Part of the population is exposed to these factors, which alter the functionality of the digestive tract and reduce the life quality of the individuals.

Alternative therapies involving phytotherapy and the use of medicinal plants have been widespread, mainly because these plants have antioxidant substances in their composition, such as *Baccharis trimera* (Less.) DC, popularly known as carqueja, which contains in its composition flavonoids, such as quinic acid, apigenin-6,8-di-C-glucoside, apigenin C-pentosidehexoside, 3,5-diO-cafeoilquinic acid and 5/7-methyl-apigenin (Souza et al., 2019), which may have an antioxidant action on the oxidative stress (Pádua et al., 2013; Pádua et al., 2010).

Based on this premise, this study aims to investigate potential influences on the total number of neurons and on the inhibitory subpopulation of the myenteric plexus, present in the jejunum. In addition, it assessed the cholesterol and triglyceride levels, as well as the glycemic profile of rats, exposed to risk factors such as a high cholesterol diet, associated with the

induction of diabetes mellitus, and smoking, that were then treated with the extract of *Baccharis trimera.*

2. Matherial and Methods

Preparation of the *Baccharis trimera* extract

Aerial parts of *B. trimera* were used for the preparation of the extract (registration number 07 - UNIPAR Herbarium), collected in February 2018 at the Medicinal Plant Garden of the Universidade Paranaense (UNIPAR), in Umuarama–PR, Brazil, located 430m above sea level (S23°46'11.3"– W53°16'41.2").

All botanical material was dried in a greenhouse with air circulation at 36°C for three days. For the infusion, 100g of the previously dried and crushed plant were used in one liter of boiling water, remaining at rest for five hours until it reached room temperature (Souza et al., 2019).

The obtained extract was concentrated in a rotary evaporator up to a volume of 200 mL. The residue was separated by filtration and the final product was treated in the proportion of 1:3, using 600 mL of ethanol for the precipitation of proteins and polysaccharides in order to obtain a precipitate (PEI) and an ethanolic supernatant of the infusion (ESI). The obtained ESI was freeze-dried and stored at -20°C until its use, with an extract yield of 16.88%.

Experimental animals

Twenty-five male Wistar rats (n=5 per group) with 90 days were used, the animals were housed at a temperature of 22±2°C, with a relative humidity of 50±10%, with a light/dark cycle of 12 hours.

All procedures were performed in accordance with the Ethics Committee on the Use of Animals (CEUA) of UNIPAR (protocol 1000/2018) in order to respect all the norms and recommendations of CONCEA, in order to provide the welfare of the animals under experimentation.

Experimental design and treatments

Induction of diabetes mellitus

Diabetes was induced after 12 hours of fasting (Souza et al., 2019), after which the rats received 60 mg/kg streptozotocin diluted in a citrate buffer (10mM, pH 4.5, i.p.). Three days after the administration of streptozotocin, the glycemia of the animals was measured by blood collection in the caudal vein on reactive strips using an Accu Check glucometer. Animals with glycemia of ≥250mg/dL were considered diabetic.

Induction of dyslipidaemia

For the induction of dyslipidemia, the animals were given a standard rodent diet (Purina®) plus 0.5% cholesterol ad libitum during the four weeks of the experiment (Jaldin, Falcão Filho, Siqueira & Yoshida, 2006). The diet was prepared weekly, for every 150g of commercial feed, one egg yolk and 13.5ml of corn oil were added. The commercial feed was crushed and mixed with egg yolks, corn oil, and water, and pelleted by a meat mincer. After pelletizing, the feed was kiln-dried at 55°C for 24 hours (Jaldin et al. 2006).

Inducing of smoking

Concurrent to DM induction and dyslipidemia, the animals were exposed to smoke from nine commercial cigarettes (0.8mg nicotine, 10mg tar, and 10mg carbon monoxide) five days a week for one hour a day over four weeks (Cakir et al., 2007).

Experimental groups and treatments

In the last two weeks of the trial, the animals were treated with different doses of *B. trimera* (30, 100, and 300mg/kg) (Souza et al., 2019) via gavage, once a day, resuspended by filtered water, at a volume of 0.28ml. The negative control had normoglycemic, non-dyslipidemic, and not exposed to tobacco rats, treated with vehicle (filtered water). The positive control was made of diabetic, dyslipidemic, and smoking rats, treated with vehicle (filtered water). The BT 30 group had diabetic, dyslipidemic, and smoking rats, treated with 30mg/kg of *B. trimera* whereas the BT 100 group had diabetic, dyslipidemic and tobacco rats treated with 100mg/kg *B. trimera*, and the BT 300 group had diabetic, dyslipidemic, and tobacco rats treated with 30mg/kg *B. trimera.*

Euthanasia and collection of the jejunum

After the four weeks of the experiment, the animals were fasted for 12 hours and then blood samples were collected from the ocular plexus to evaluate the plasma levels of cholesterol and triglycerides. The animals proceeded to euthanasia, performed by deepening anesthesia, using isoflurane in a saturated chamber 1-3%, according to the CONCEA Euthanasia Practice Guidelines and Normative Resolution #37, Law n°. 11,794/2008, Decree n°. 6899/2009.

Once dead, the animals were laparatomized and the jejuna were collected. The tissue was processed to stain the total neuron population and the inhibitory subpopulation, using the basic staining technique (Giemsa) and histochemistry (NADPH-diaphorase), respectively.

Basic Giemsa staining - detection of the total population of myenteric neurons

The Giemsa technique, proposed by Barbosa (1978), was used to stain the total population of myenteric neurons (Giemsa+) (Barbosa, 1978). The jejuna were obtained from 25 animals, washed with a 0.9% saline solution, filled and immersed in a fixing solution for 48 hours, and tied at their extremities with suture thread to maintain the filling. For neuronal staining each membrane preparation was immersed in Giemsa staining solution with methylene blue in a Sorensen phosphate buffer (pH 6.9) for up to 12 hours at room temperature.

Histochemistry of NADPH-diaphorase - detection of myenteric NADPH-diaphorase positive neurons (NADPH-d+ neurons)

In order to evidence active NADPH-diaphorase positive neurons (NADPH-d+), the methodology proposed by Scherer-Singler, Vicent, Kimura and McGeer (1983) was used, with the jejunum fragments being washed with phosphate buffer solution (pH 7.4), tied with suture thread at one end, and its interior filled with a phosphate buffer solution (pH 7.4) (Scherer-Singler et al., 1983).

In the next step, the opposite end was also tied and the segment was washed twice (10 minutes each) in sodium phosphate buffer (PBS) and permeated in PBS containing Triton X-100 at 0.3% diluted in sodium phosphate buffer (pH 7.3) for 10 minutes.

After permeation, the jejunum fragments were again washed twice more (10 minutes each) in PBS and incubated for 90 minutes in a reaction medium containing 50 mg of Nitro Blue Tetrazolium (NBT), 100 mg of β-NADPH, and 0.3% of Triton X-100 in a Tris-HCl buffer

(0.1M, pH 7.6). After this period, the end tethers were released and the fragments were immersed in a 4% paraformaldehyde solution to interrupt the reaction, for fixation, and then stored for later use.

Obtaining membrane preparations and photomicroscopy

In order to obtain the membrane preparations, intestinal segments corresponding to the jejunum of 25 rats were used. The jejunum was first sectioned transversally to obtain a fragment of approximately 8 mm long and then sectioned along the longitudinal axis at the level of the mesentery insertion. The fragments were microdissected in a glass plate, with the aid of forceps and stereomicroscope with transillumination to remove the tunica mucosa and submucosa, preserving the muscle tunic where, according to the literature, the myenteric plexus and the serous tunic are located.

Then, they were dehydrated in increasing alcohol series (90%, 95%, and absolute), diaphonized with three consecutive immersions in xylol, and placed between the lamina with synthetic resin and the glass slide.

Quantification of myenteric neurons (neuronal density/mm2 of the jejunum)

Pictures were taken from 120 random microscopic fields per membrane preparation, including the mesenteric, intermediate, and antimesenteric areas of the jejunum using a 200x magnification microscope with an image analysis system (Nikon Eclipse E200) coupled with a high-resolution camera (Moticam 5 of 5.0 megapixels). These images enabled a quantitative analysis of the myenteric neurons stained by the Giemsa technique and by the NADPH-d histochemistry.

The circumference of the intestine at the mesentery insertion (0°) served as a reference to delimit the mesenteric (0° to 60° and 300° to 360°), intermediate (60° to 120°, and 240° to 300°), and antimesenteric (120° to 240°) regions of the intestine guiding the capture of images. This was made taking an equivalent number of images from each of the three areas.

Statistical analysis

The tests were performed using the Bioestat Software. The data analyzed were evaluated for normality using the Liliefors test. After confirmation of data normality and homogeneity of

variance, the results of the morphometric analysis as well as the quantification of myenteric neurons were analyzed by ANOVA, and when significant, the Tukey post-test was used. For all tests the 5% significance level was considered.

3. Results and Discussion

After four weeks of trial, significant weight gain was found in the C-group animals. During the experimental period a weight reduction was observed in groups C+ and in those treated with carqueja (Table 1), mainly in the BT100 group, in which there was a significant difference. In other studies with rats, as in the work of Silvério et al. (2009) and Furlan, Molinari and Miranda-Neto (2002) weight loss was also observed in diabetic animals (Silvério et al., 2009; Furlan, Molinari & Miranda-Neto, 2002).

The oxidative stress in DM has been reported by several authors (Ksiazek & Wisniewska, 2001; Davison et al., 2002), in association with superoxide accumulation, increased polyol pathway activity, accumulation of advanced glycation end products (AGEs), and changes in the kinase protein activity leading to a progressive cell dysfunction in diabetes that may result in this weight loss (Feldman, Nave, Jensen & Bennett, 2003).

The administration of carqueja has not affected the maintenance of body weight in animals exposed to diabetes (Table 1). The use of natural antioxidants and aldose-reductase inhibitors has been investigated with the purpose of reducing effects such as weight loss (Feldman et al., 2003) and the glycaemia reduction that may restore the metabolic imbalance (Silverio et al., 2009). However, in the period of two weeks of treatment in this trial, these effects were not observed in this study.

Table 1. Averages of initial weight in the first week, final weight of the fourth and last week and weight gain of the negative control, positive control and groups treated with different doses of gorse extract.

Group	Initial weight (g)	Final weight (g)	Weight gain (%)
C-	329.6[a]	379.0[b]	+ 13.03
C+	248.8[a]	244.2[a]	- 1.88
BT30	284.8[a]	257.2[a]	- 10.73
BT100	268.0[a]	233.4[b]	- 14.82
BT300	256.6[a]	250.0[a]	- 2.64

Means followed by different letters in the line direction differ by the T-test. (C-) normoglycemic, non-dyslipidemic, and non-smoking animals; (C+) diabetic, dyslipidemic, and smoking animals; BT30 (diabetic, dyslipidemic, and smoking animals treated with 30mg/kg *Baccharis trimera*); BT100 (diabetic, dyslipidemic, and smoking animals treated with 100mg/kg of *Baccharis trimera*); and BT300 (diabetic, dyslipidemic, and smoking animals treated with 300mg/kg of *Baccharis trimera*). N=5. Source: Authors.

No statistical difference was observed between groups (Table 2) regarding the length of the small bowel. Silverio et al. (2009) mentioned that no differences were found in the bowel area between diabetic and normoglycemic rats, in accordance with our results (Silverio et al., 2009).

Table 2. Averages and standard errors of the means of small bowel length of the negative control, positive control, and groups treated with *Baccharis trimera*.

			Baccharis trimera		
	C-	C+	BT30	BT100	BT300
SB length	1.28±0.01	1.26±0.03	1.32±0.01	1.25±0.02	1.33±0.02

(C-) normoglycemic, non-dyslipidemic, and non-smoking animals; (C+) diabetic, dyslipidemic, and smoking animals; BT30 (diabetic, dyslipidemic, and smoking animals treated with 30mg/kg of Baccharis trimera); BT100 (diabetic, dyslipidemic, and smoking animals treated with 100mg/kg of *Baccharis trimera*); and BT300 (diabetic, dyslipidemic, and smoking animals treated with 300mg/kg of *Baccharis trimera*) without statistical difference by the ANOVA test. Source: Authors.

All animals in the diabetic groups presented a glycaemia higher than in the C- group after induction to DM by streptozotocin (Table 3). Streptozotocin destroys the beta cells of pancreatic islets and consequently makes insulin production impossible (Furman, 2015). In this study the carqueja extract did not affect significantly the blood glucose concentration of the diabetized animals.

Table 3. Averages and standard errors of the glycemia, triglycerides, and cholesterol means of the negative control, positive control, and treated groups.

			Baccharis trimera		
	C-	C+	30mg/kg	100mg/kg	300mg/kg
Glycemia	87.1±4.1^{a}	515.7±28.1^{b}	478.6±15.1^{b}	556.1±11.4^{b}	430.4±28.6^{b}
Triglycerides	55.77±14.1^{a}	1476.0±215.7^{b}	204.1±34.1^{c}	276.5±66.2^{c}	432.7±154.3^{c}
Cholesterol	79.73±6.4^{a}	1776.0±178.9^{b}	180.5±26.3^{c}	411.3±69.4^{bc}	750.4±45.1^{bc}

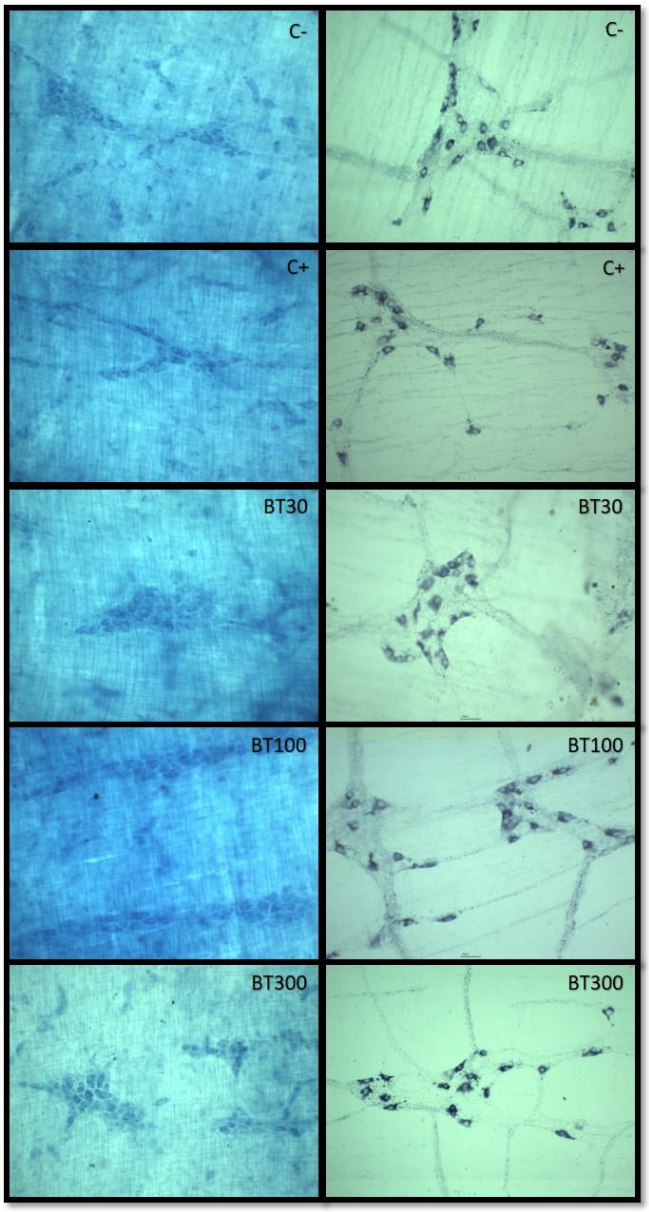
Means followed by different letters in the line direction differ by the T-test. Glycemia, cholesterol, and triglycerides are expressed in mg/dL. (C-) normoglycemic, non-dyslipidemic, and non-smoking animals; (C+) diabetic, dyslipidemic, and smoking animals; BT30 (diabetic, dyslipidemic, and smoking animals treated with 30mg/kg of Baccharis trimera); BT100 (diabetic, dyslipidemic, and smoking animals treated with 100mg/kg of *Baccharis trimera*); and BT300 (diabetic, dyslipidemic, and smoking animals treated with 300mg/kg of *Baccharis trimera*). ANOVA statistical analysis. Source: Authors.

However, *B. trimera* reduced the values of the lipid profile at 30 and 100 mg/kg when compared to group C+, both for triglycerides and cholesterol (Table 3). The study of Lívero et al. (2016) in mice with hepatic steatosis treated with 30mg/kg of *B. trimera* showed reduced plasma levels of triglycerides and total cholesterol, since the carqueja extract protects hepatocytes against lipids and changes caused by ethanol (Lívero et al., 2016).

In this same experimental model, Souza et al. (2019) have also observed a reduction in the lipid profile of cholesterol and triglycerides at a dose of 30mg/kg of carqueja extract during four weeks (Souza et al., 2019). Therefore, *B. trimera* was able to reverse these changes, which might be attributed to its hypolipemiant and inhibitory action on the generation of free radicals.

The location and organization of the myenteric plexus observed in all the studied groups (Figure 1) was that also described by different authors, being organized in ganglia interconnected by nerve fibers, with a mesh or ganglion net appearance (Irwin, 1931; Silverio et al., 2009; Serenini et al., 2020). This has not been altered by the diabetic condition, dyslipidemic status, smoking, or in the groups that were treated with carqueja.

Figure 1. Photomicroscopy showing ganglia of the myenteric plexus of the jejunum of rats of different groups, stained by the Giemsa method and by the histochemistry of the NADPH-diaphorase. (Bar 100 µm. Objective de 20x).



Groups: (C-) normoglycemic, non-dyslipidemic, and non-smoking animals; (C+) diabetic, dyslipidemic, and smoking animals; BT30 (diabetic, dyslipidemic, and smoking animals treated with 30mg/kg of *Baccharis trimera*); BT100 (diabetic, dyslipidemic, and smoking animals treated with 100mg/kg of *Baccharis trimera*); and BT300 (diabetic, dyslipidemic, and smoking animals treated with 300mg/kg of *Baccharis trimera*). Source: Authors.

In the total population of neurons stained by the Giemsa technique, significant reduction was observed in groups C+ and in the group treated with the carqueja extract, when compared to group C- (Table 4). In a study performed by Hernandes, Bazotte, Gama and Miranda-Neto

(2000), after 19 weeks with diabetes, the animals presented neuronal reduction (Hernandes et al., 2000).

However, in six weeks it was already possible to observe a reduction in the intensity of basophilia of these neurons, which reveals a reduced neuronal activity. In the present study, the reduction of the neurons stained by the Giemsa technique was already significant after four weeks of exposure to diabetes. This may be due to the association of the diabetic state with two other risk factors: hypercholesterolemia and smoking, accelerating the process of neuronal reduction.

Table 4. Averages and standard errors of the means of the number of positive NADPH-diaforase and Giemsa neurons found in mm² of jejunum membrane preparations of rats of the negative control, positive control, and treated groups.

Groups	NADPH-d^+/mm²	Giemsa/mm²
C-	47.58±0.60	163.82±7.94[a]
C+	32.27±2.97	109.21±10.17[b]
BT30	42.73±4.23	91.46±9.64[b]
BT100	47.35±2.54	81.29±1.72[b]
BT300	42.34±2.71	94.03±5.34[b]

Means followed by different letters in the line direction differ by the T-test. (C-) normoglycemic, non-dyslipidemic, and non-smoking animals; (C+) diabetic, dyslipidemic, and smoking animals; BT30 (diabetic, dyslipidemic, and smoking animals treated with 30mg/kg of Baccharis trimera); BT100 (diabetic, dyslipidemic, and smoking animals treated with 100mg/kg of *Baccharis trimera*); and BT300 (diabetic, dyslipidemic, and smoking animals treated with 300mg/kg of *Baccharis trimera*). N=5. Statistical analysis using ANOVA and the Tukey post-test. Source: Authors.

The Giemsa method was used in this research to show the total population of myenteric neurons, which according to Barbosa (1978) is a technique that uses methylene blue to stain all the myenteric neurons (Barbosa, 1978; Sant'ana, Araújo, Ramos, Hermes-Uliana & Natali, 2012). This staining is the result of the affinity of the dye for the acid polyrribosomes of the nerve cells, allowing their identification (Góis et al., 2016; Sant'ana et al., 2012).

Further, in this study the Giemsa technique was used to evaluate the nitrergic subpopulation of neurons compared to the total number of myenteric neurons in the jejunum of rats, in order to estimate the number of active neurons in this subpopulation.

In the histochemistry of NADPH-diaphorase, the density of myenteric neurons did not show any significant differences between the groups (Table 3), which might be attributed to the

resistance of the nitrergic neurons to oxidative stress (Belai, Cooper & Burnstock, 1995; Furlan, Molinari & Neto, 2004). NO has a protective role on myenteric plexus neurons. Thus, neurons that use NO constantly improve their defense mechanism against free radicals (Cowen, Johnson, Soubeyre & Santer, 2000; Belai, Cooper & Burnstock, 1995; Phillipes, Kiefer & Powley, 2003), which are yielded in diabetes and smoking.

In a trial with diabetic rats induced by streptozotocin and supplemented with oral ascorbic acid, an important antioxidant, for 120 days, Zanoni, Buttow, Bazotte e Neto (2003) have not found any significant differences between the treated groups and the control (Zanoni et al., 2003).

In another study, Silverio et al. (2009) have not observed differences between the groups in the jejunum of diabetic rats supplemented orally with ascorbic acid for 90 days, corroborating the results of this study with the supplementation of the carqueja extract, which contains in its composition different antioxidants (Silverio et al., 2009).

Preliminary studies that evaluated the phytochemical composition of the ethanol soluble fraction of the *B. trimera* extract used in the present study identified the presence of phenolic compounds, mainly the flavonoids quinic acid, apigenin-6.8-di-C-glucoside, apigenin C-pentoside-hexoside, 3.5-diO-cafeoilquinic acid, and 5/7-methyl-apigenin (Souza et al., 2019), which have an antioxidant action (Souza et al., 2019).

According to studies that evaluate the expression of nitrergic neurons the amount of NADPH-d+ neurons might vary from 23.0% to 34.0% among the total population of myenteric neurons in humans, rats, mice, and also guinea pigs (Ferezin et al., 2017; Furness, 2006; Qu et al., 2008; Serenini et al., 2020; Gagliardo et al., 2008).

In the present study, 29.03% of nitrergic neurons were found in the C- group, which is within the estimated population. In contrast, the diabetic groups C+ (36.07%), BT30 (46.71%), BT100 (58.24%), and BT300 (45.02%) presented the highest percentage of neurons of this myenteric subpopulation, which may bring symptoms such as intestinal stasis.

The changes caused by certain diseases do not affect the neuronal population simultaneously since some types of neurons are more resistant to the imposed experimental conditions than other nerve cells. This is the case of the cholinergic neurons, which are more susceptible to the deleterious effects of aging (Gagliardo et al., 2008).

Nitrergic neurons are more resistant to the effects of aging and diabetes (Phillips, Kieffer & Powley, 2003; Belai, Cooper & Burnstock, 1995; Cowen et al., 2000; Gagliardo et al., 2008; Silva Porto et al., 2012; Zanoni et al., 2003). The complications of diabetes occur mainly in tissues that are independent of insulin for glucose uptake. This condition generates

hyperglycemia and consequently the excess formation of ROS. Since in diabetes the antioxidant system acts inappropriately (Feldman et al., 2003; Giri et al., 2018; Shakeel, 2015; Oyenihi, Ayeleso, Mukweyho & Masola, 2015; Rocha, Teixeira, Pereira & Kaplan, 2006).

In DM there is an increase in the formation of glycosylated compounds and the stimulation of glucose flow in the aldose-reductase pathway, which are reduced to sorbitol suffering from oxidation and generating fructose. It should be noted that the polyol pathway is a normal process of the cell. However, when there is hyperglycemia, the amount of fructose in neurons significantly increases, leading to their destruction (Feldman et al., 2003; Giri et al., 2018; Shakeel, 2015; Oyenihi et al., 2015; Rocha et al., 2006).

The inhibitory (nitrergic) and excitatory (cholinergic) neuronal populations of the myenteric plexus work together to mediate relaxation and contraction of the digestive tract through peristalsis. When there is a reduction or increase in one of these populations, where nitrergic neurons survive more than cholinergic, the gastrointestinal motility may be impaired, presenting an interrupted or reduced response (Phillips, Kieffer & Powley, 2003).

4. Conclusion

In this experimental model that investigated the association of DM, dyslipidemia, and smoking, the rats with induced diabetes showed no reduction in blood glucose when treated with the carqueja extract. With a 30mg/kg dose of carqueja there was a significant reduction in serum cholesterol and triglyceride levels. The different doses of carqueja did not present protective effects on the neurons of the myenteric plexus, presenting a significant reduction in the total population of neurons (Giemsa+) from the positive and treated control groups compared to the negative control group. No significant quantitative changes were found in the nitrergic subpopulation (NADPH-d+) of neurons between the groups, which proved to be more resistant to the deleterious effects of diabetes, smoking, and a hypercholesterolemic diet.

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