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The extract of *Schinus terebinthifolius* Raddi intensifies morphological alterations in the colon of diabetic rats

Extrato de Schinus terebinthifolius Raddi intensifica as alterações morfológicas no cólon de ratos diabéticos

Camiliany Pereira da Silva,[1] Marcelo Wecsley Ferreira Araújo,[2] Camila Cavicchioli Sehaber-Sierakowski,[3] Flávia Cristina Vieira Frez,[3] Camila Cristina Iwanaga,[3] Marcelo Biondaro Gois,[4] Jacqueline Nelisis Zanoni,[3] Neide Martins Moreira,[5] Catchia Hermes-Uliana[6]

ABSTRACT

There are several challenges in the treatment of diabetes mellitus. Some of these challenges include the discovery of bioactive compounds with the potential to be adjuncts in the treatment of diabetes mellitus. In this study, the effects of hydroethanolic extract of *Schinus terebinthifolius* Raddi on physiological, morphological, and quantitative parameters of the colon of diabetic rats were investigated. For this purpose, 16 90-day-old rats were randomly divided into four groups: Normoglycemic (N); normoglycemic treated with (50 mg/kg) the hydroethanolic extract of *S. terebinthifolius* Raddi (NA); streptozotocin-induced diabetics (D); and streptozotocin-induced diabetics treated with the hydroethanolic extract of *S. terebinthifolius* Raddi (DA). The diabetic rats had diarrhea, weight loss, increased water and food intake, and high blood glucose levels. In addition, atrophy of the entire intestinal wall and submucosa, hypertrophy of the muscularis mucosae, and changes in the histoarchitecture of the intestinal crypts and enterocytes were observed in groups NA, D, and DA. In addition, changes in collagen remodeling and the ganglia of the enteric nervous system, as well as changes in goblet cells and intraepithelial lymphocytes, were observed only in groups D and DA. Our results show that treatment with *S. terebinthifolius* Raddi extract does not protect the colon from the damage caused by diabetes, nor does it reverse physiological parameters. Moreover, the treatment caused morphological and quantitative changes in the colon of normoglycemic rats and intensified the damage caused by diabetes.

Keywords: Diabetes mellitus; bioactive compounds; antioxidants; goblet cells; intraepithelial lymphocytes.

RESUMO

Existem diversos desafios no tratamento do diabetes *mellitus*. Alguns desses desafios incluem a descoberta de compostos bioativos com potencial para serem coadjuvantes no tratamento do diabetes *mellitus*. Neste estudo foram investigados os efeitos do extrato hidroetanólico de *Schinus terebinthifolius* Raddi sobre parâmetros fisiológicos e morfológicos do cólon de ratos diabéticos. Para esse propósito, 16 ratos com 90 dias de idade foram distribuídos aleatoriamente em quatro grupos: Normoglicêmico (N); normoglicêmico, tratado com (50 mg/kg) o extrato hidroetanólico de *S. terebinthifolius* Raddi (NA); diabéticos induzidos por estreptozotocina (D); e diabéticos induzidos por estreptozotocina, tratados com o extrato hidroetanólico de *S. terebinthifolius* Raddi (DA). Os ratos diabéticos apresentaram diarreia, perda de peso, aumento na ingestão de água e alimentos e níveis elevados de glicose no sangue. Além disso, nos grupos NA, D e DA, observaram-se atrofia de toda a parede intestinal e submucosa, hipertrofia da muscular da mucosa e alterações na histoarquitetura das criptas intestinais e enterócitos. Adicionalmente, foram observadas mudanças na remodelação de colágeno e nos gânglios

[1]Universidade Federal de Mato Grosso do Sul – Campo Grande (MS), Brasil.
[2]Universidade Federal de Mato Grosso do Sul – Coxim (MS), Brasil.
[3]Universidade Estadual de Maringá – Maringá (PR), Brasil.
[4]Universidade Federal de Rondonópolis – Rondonópolis (MT), Brasil.
[5]Universidade Estadual do Oeste do Paraná – Foz do Iguaçu (PR), Brasil.
[6]Universidade Federal de Mato Grosso do Sul - Três Lagoas (MS), Brasil.
Autor correspondente: Marcelo Biondaro Gois
Faculdade de Ciências da Saúde da Universidade Federal de Rondonópolis - Av. dos Estudantes, CEP.: 78736-900, Rondonópolis (MT), Brasil.
E-mail: marcelobiondaro@gmail.com

do sistema nervoso entérico, assim como alterações quantitativas em células caliciformes e linfócitos intraepiteliais somente nos grupos D e DA. Nossos resultados mostram que o tratamento com o extrato de *S. terebinthifolius* Raddi não protegeu o cólon dos danos causados pelo diabetes nem reverteu parâmetros fisiológicos. Além disso, o tratamento causou alterações morfológicas no cólon de ratos normoglicêmicos e intensificou os danos causados pelo diabetes.

Palavras-chave: Diabetes *mellitus*; compostos bioativos; antioxidantes; células caliciformes; linfócitos intraepiteliais.

INTRODUCTION

Diabetes mellitus is a serious public health problem worldwide due to its increasing prevalence and incidence. It is estimated that worldwide the population with diabetes is in the order of 425 million and that it will reach 629 million in 2045. Currently, there are more than 13 million diabetics aged 20 to 79 years and 88,300 children and adolescents in Brazil.[1,2]

In addition the classic symptoms of chronic hyperglycemia, which leads to an imbalance of antioxidants,[3-5] diabetics suffer from complications in the gastrointestinal tract.[6] Dysphagia, abdominal pain, diarrhea, and constipation are symptoms that affect the quality of life of diabetic patients.[7-9] In addition, several studies have reported macrovascular and microvascular complications,[10] ultrastructural,[11] and morphophysiological changes,[12,13] including dysmotility of the colon in diabetic patients.[14]

In experimental models of streptozotocin-induced diabetes, physiologic, morphologic, and quantitative changes, including increased enzyme activity,[4] histomorphometric,[13] and ultrastructural changes,[11] increased proportion of goblet cells and intraepithelial lymphocytes,[15] changes in collagen remodeling,[5,11] and ganglia of the enteric nervous system,[16] have been widely reported. Although the mechanisms are not clear, increased enzyme activity and oxidative stress are responsible for damage to the gastrointestinal tract because endogenous antioxidant activity in diabetic patients is insufficient to maintain intestinal homeostasis.[9] In this context, studies evaluating the efficacy of plant-derived natural products in ameliorating the damage caused by diabetes are of great importance.[17,18]

Recently, Iwanaga *et al.*[4] demonstrated *in vitro* and *in vivo* that the hydroethanolic extract of *Schinus terebinthifolius* Raddi (Anacardiaceae), a plant native to South America and widely distributed in Brazil, popularly known as Aroeira, has the potential to prevent or treat diseases related to oxidative stress, such as diabetes.

The genus *Schinus* comprises approximately 29 species native to South America. Traditionally, this genus has been employed to address a variety of ailments, including rheumatism, hypertension, ulcers, gastric distress, and bronchitis. Numerous phytochemical studies on *Schinus* plants have identified a range of bioactive compounds, including flavonoids, bioflavonoids, phenolic acids, tannins, catechins, terpenoids, and essential oils.[19]

Previous studies have demonstrated its larvicidal,[20] antimicrobial,[21,22] anti-inflammatory,[23,24] antioxidant,[25] antiparkinsonian, anti-allergic, antiviral, chemoprotective, anthelmintic, hepatoprotective,[19] and wound-healing[18] properties.

However, the effects of the hydroethanolic extract of *S. terebinthifolius* Raddi on morphological parameters of the proximal colon of diabetic rats are still unclear.

In view of the search for alternatives that can improve the clinical conditions imposed by diabetes, this study aimed to evaluate the effects of the hydroethanolic extract of *S. terebinthifolius* Raddi on physiological, morphological, histopathological, and quantitative parameters in the proximal colon of diabetic rats.

MATERIALS AND METHODS
Ethical aspects

All procedures described in this study conform to the ethical principles of Sociedade Brasileira de Ciência em Animais de Laboratório – SBCAL. The experimental protocol (n. 158/2014) was approved by the Ethics Committee for the Use of Animals in Experiments at the State University of Maringá.

Plant material

After authorization, leaves of *S. terebinthifolius* were collected in Dourados, Mato Grosso do Sul, Brazil (S22º11'43.7", W54º56'08.5" at 452 meters altitude), and identified by Dr. Maria do Carmo Vieira from the Federal University of Grande Dourados, where the voucher specimen (n. 4602) was deposited. The leaves were collected in April 2013.

Preparation of hydroethanolic extract

The leaves of *S. terebinthifolius* Raddi were dried in a forced-air oven (Quimis®) at a temperature of 35°C for five days. The dried leaves were weighed and crushed in a knife mill (Usiram® model; mesh size of 0.5 mm in diameter). Five hundred grams of the obtained plant powder was subjected to extraction in ethanol and water (9:1 v/v) by maceration at room temperature. The extract was then filtered and concentrated under reduced pressure in a rotary evaporator (IKA®, model RV10) at 40ºC until complete elimination of the organic solvent. The extract was then freeze-dried (Liotop® Lyophilizer), resulting in a hydroethanolic extract with a yield of 29.6% (148.1 g), which was stored in a freezer (-4°C). The phytochemical study of the hydroethanolic extract of *S. terebinthifolius* Raddi was carried out by Iwanaga *et al.*[4]

Experimental design

Sixteen male *Rattus norvegicus* Wistar, 90 days old, weighing 309.08 ± 15.56 g, were randomly divided into four

experimental groups (n = 4): Normoglycemics (N); normoglycemics treated with hydroethanolic extract of *S. terebinthifolius* Raddi (NA); streptozotocin-induced diabetics (D); and streptozotocin-induced diabetics treated with hydroethanolic extract of *S. terebinthifolius* Raddi (DA). Rats were housed in polypropylene cages in a temperature-controlled animal house ($22 \pm 2°C$) with a 12/12-h light-dark cycle and were fed standard rodent chow (Nuvilab, Colombo, Brazil) and water *ad libitum*.

Streptozotocin-induced diabetes

Diabetes was induced at 90 days of age after 14-h fasting in groups D and DA with an intravenous injection of streptozotocin (35 mg/kg body weight; Sigma, St. Louis, MO, USA) dissolved in citrate buffer, pH 4.5 (10 mM). After induction of diabetes, glycemia was measured photometrically, with glucose levels determined by the glucose dye oxidoreductase. For this purpose, an Accu-Chek Active glucometer (Roche Diagnostics GmbH, Germany) was used after two days. The rats were considered diabetic when the glucose level was 250 mg/dL, which confirmed the establishment of the experimental model.[26]

Treatment with the hydroethanolic extract of *S. terebinthifolius* Raddi

Rats in groups NA and DA were treated daily for 60 days (at 90 to 150 days of age) with 50 mg/kg of the hydroethanolic extract of *S. terebinthifolius* Raddi by gavage (5). Rats in groups N and D received water only, by the same route. The dose, safety, and genotoxicity were previously evaluated.[27,28]

Euthanasia and sample collection

Sixty days after the start of the experiment, rats were weighed and anesthetized intraperitoneally with 40 mg/kg thiopental (Abbot, Chicago, USA). Blood was collected by cardiac puncture to determine glucose levels by the glucose oxidase method (Laborclin reagent kit). The rats were killed by deep anesthesia with thiopental. At necropsy, the proximal colon was removed, measured, washed with saline (0.9%), and histologically processed.

Anatomical analysis

To obtain anatomical data, two-centimeter-long rings of the proximal colon from each rat were opened at the mesocolic border fixed in Styrofoam with the aid of tacks, and placed in Bouin's fixative solution for 6 hours. The tissue was then embedded in paraffin, cut into semi-serial 4-μm cross-sections (KEDEE, semi-automatic, microtome KD-3358, China), and subjected to a series of deparaffinization and hydration procedures for staining with hematoxylin and eosin, periodic acid-Schiff, or Picro-Sirius red, which was used to evaluate the histomorphometric and quantitative parameters of the colonic wall.[29,30]

Histomorphometric analysis

Morphometric analysis was performed using images taken with a digital camera connected to a light microscope (Zeiss, Microimaging GmbH, Goettingen, Germany). The thickness (μm) of the entire wall, muscle layer, submucosa, muscularis mucosae, and mucosa was measured under 10× magnification. Thirty measurements were taken for each parameter over the entire circumference of the proximal colon of each rat. In addition, the width and depth of 30 intestinal crypts, the height of 80 enterocytes, and the largest diameter of the nucleus of these cells were measured at 40× magnification in each rat. In addition, images of the ganglia of the myenteric plexus were taken with a 40× objective to measure the area of 10 ganglia in all rats. These results are expressed as the average area of the ganglion profiles of the myenteric plexus in $μm^2$. Morphometric analysis was performed using Image-Pro Plus software (Media Cybernetics, Inc., USA).[29,31,32]

Histopathological score

H&E-stained tissue sections of the proximal colon were scored (blind) by assessing infiltration of the lamina propria with mononuclear cells, distortion and hyperplasia of the crypts, erosions on the epithelial surface, and alteration of the mucosal histoarchitecture, resulting in a score of 0 to 6.[33,34] In addition, the presence of inflammatory infiltrates in the intestinal wall, around and within the myenteric and submucosal plexus ganglia characterizing periganglionitis and ganglionitis, respectively, was assessed. Twenty-five microscopic fields from each rat, from at least 4 rats per group, were examined blindly by a specialist using a light microscope (Olympus® BX43F) and a 20x objective (and 40x or 100x if necessary to confirm structures).

Quantitative analysis

Quantification of intraepithelial lymphocytes (IELs) and goblet cells was performed on tissue samples stained with PAS. All goblet cells and IELs present in 2,500 enterocytes from each rat were counted sequentially. This procedure allowed the calculation of the number of goblet cells or IELs/100 epithelial cells.[35-37] Quantification was performed directly under the light microscope (Zeiss, Microimaging GmbH, Goettingen, Germany) under 40× magnification.

Quantification of type I, type III, and total collagen fibers

Type I and type III collagen fibers stained with Picro Sirius Red were quantified in the submucosa and mucosa. For quantification of collagen types, 32 images per rat were acquired with a 20× objective and a high-resolution camera (Roper Scientific Photometrics®) attached to the microscope and transferred to a microcomputer using image-pro plus software (Media Cybernetics, MD, USA). Polarizing filters (Olympus U-POT Japan) were used for slide analysis. Results were expressed as the percentage of the amount of each collagen per area ($μm^2$) of submucosa and mucosa.[38,39]

Statistical analysis

The distribution of the data was analyzed using the D'Agostino-Pearson normality test. One-way ANOVA analysis of variance followed by Tukey's post hoc test was

used for data with normal distribution, and *p*-values less than 0.05 were considered significant. Results are presented as mean ± standard deviation. Statistical analyzes were performed using GraphPad Prism 5.01 software. Statistical tests are noted in the figure and table legends.

RESULTS
Physiological parameters
At the end of the 60-day experiment, normoglycemic (N) and normoglycemic rats treated with the hydroethanolic extract of *S. terebinthifolius* Raddi (NA) weighed 438.0 ± 29.7 g and 403.5 ± 1.3 g, while diabetic rats induced by streptozotocin (D) and diabetic rats treated with the hydroethanolic extract of *S. terebinthifolius* Raddi (DA) weighed 273.0 ± 29.7 g and 280.0 ± 15.8 g, respectively. In addition to weight loss, the diabetic rats (D and DA) had diarrhea, increased water and food intake, and high blood glucose ($p < 0.05$; Table 1).

Morphological parameters
Histomorphometric analysis showed a decrease in total thickness and submucosa of rats in the groups NA, D, and DA compared to N ($p < 0.05$). There was a decrease in the thickness of the muscle layer and mucosa of the rats in the group NA compared to N ($p < 0.05$). There was an increase in the thickness of muscularis mucosae of rats in the groups NA, D, and DA, compared with N ($p < 0.05$). However, in diabetic rats (DA), treatment with the hydroethanolic extract of *S. terebinthifolius* Raddi did not reverse mucosae hypertrophy ($p > 0.05$; Table 1).

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Table 1. Physiological and morphological parameters assessed in male Wistar rats 150 days of age in the following groups: Normoglycemic (N), normoglycemic treated with hydroethanolic extract of *S. terebinthifolius* Raddi (NA), streptozotocin-induced diabetic (D), and streptozotocin-induced diabetic treated with hydroethanolic extract of *S. terebinthifolius* Raddi (DA).

Physiological parameters	Body mass and blood glucose			
	N	**NA**	**D**	**DA**
Initial body mass (g)	315.33 ± 17.49	321.00 ± 18.77	294.00 ± 16.97	306.00 ± 8.95
Body mass in euthanasia (g)	438.00 ± 29.68	403.50 ± 1.29	273.00 ± 29.70 *	280.00 ± 15.77 *
Final glycemia (mg/dL)	130.00 ± 13.21	114.42 ± 14.67	468.25 ± 66.13 *	422.00 ± 48.04 *
Morphological parameters (μm)	**Proximal colon**			
	N	**NA**	**D**	**DA**
Total wall	328.72 ± 65.35	209.27 ± 39.96 *	303.10 ± 91.67 *	298.26 ± 75.66 *
Muscular	129.41 ± 26.82	93.28 ± 10.94 *	129.59 ± 32.48	124.63 ± 54.07
Submucosa	58.30 ± 15.27	28.29 ± 5.86 *	29.08 ± 8.34 *	30.34 ± 7.84 *
Muscularis mucosae	6.35 ± 1.10	7.59 ± 1.39 *	8.92 ± 1.23 *	8.49 ± 1.66 *
Mucosa	200.24 ± 38.73	125.67 ± 36.28 *	201.57 ± 35.61	188.61 ± 55.55
Crypts depth	184.32 ± 36.57	120.64 ± 19.47 *	190.63 ± 41.65	183.44 ± 36.46
Crypts width	29.12 ± 4.74	29.51 ± 4.15	33.98 ± 6.19 *	34.35 ± 5.63 *
Enterocyte height	47.03 ± 14.34	34.52 ± 12.23 *	47.01 ± 16.27	37.33 ± 13.11 *#
Enterocyte nuclei †	15.70 ± 4.25	12.79 ± 2.87 *	17.02 ± 4.08 *	14.41 ± 3.89 *#

† Largest-diameter. Data were compared by one-way ANOVA followed by Tukey's post-test and presented as mean ± standard deviation. * $p < 0.05$, compared to group N and # $p < 0.05$ compared to D.

There was an increase in the proportion of both goblet cells and intraepithelial lymphocytes (IELs) in the mucosa of the proximal colon of rats in groups D and DA compared to group N ($p < 0.001$; Figure 1A and B).

However, considering diabetic rats, treatment with the hydroethanolic extract of *S. terebinthifolius* Raddi reduced the number of IELs in the DA group compared to D ($p < 0.001$; Figure 1B).
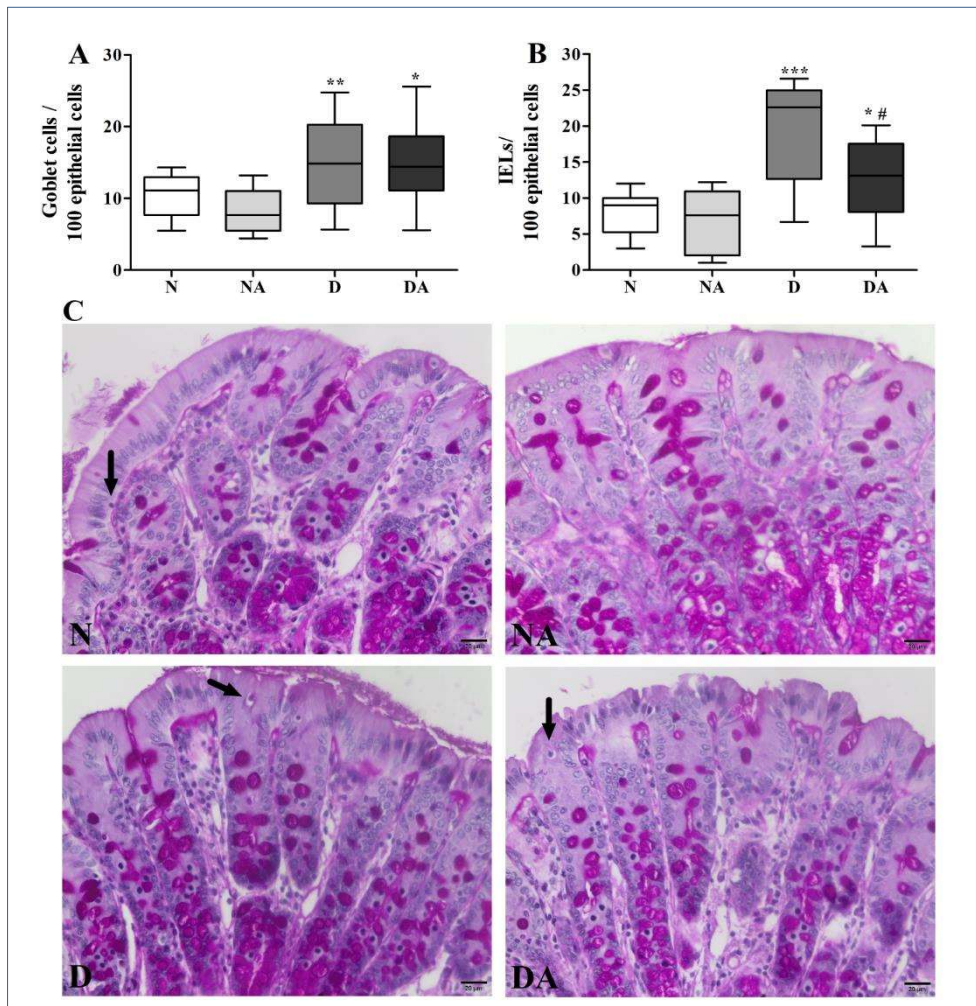

Figure 1. Effects of diabetes mellitus and treatment with a hydroethanolic extract of *S. terebinthifolius* Raddi on the number of goblet cells and intraepithelial lymphocytes (IELs). Diabetes mellitus alters the proportion of goblet cells (A) and IELs (B) in the colon mucosa of streptozotocin-induced diabetic rats (D) and streptozotocin-induced diabetic rats treated with a hydroethanolic extract of *S. terebinthifolius* Raddi (DA). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to N and # $p < 0.05$ compared to D using Kruskal-Wallis test followed by Dunn post-hoc test. Data are presented in box-and-whisker plots with minimum and maximum values, median, and interquartile range. (C) Representative photomicrographs show goblet cells (purple) stained with the Periodic Acid-Schiff histochemical technique (PAS). Note the distribution of IELs (arrows) in the colonic epithelium. N: normoglycemic; NA normoglycemic treated with hydroethanolic extract of *S. terebinthifolius* Raddi. 40x objective; scale bar 20 μm.

Histopathologic analysis revealed that streptozotocin-induced diabetic rats (D group) had erosions on the epithelial surface, distortion and hyperplasia in the intestinal crypts, inflammatory infiltrates in the lamina propria, and loss of histoarchitecture of the colonic mucosa compared with the N group ($p < 0.05$; Figure 2A).

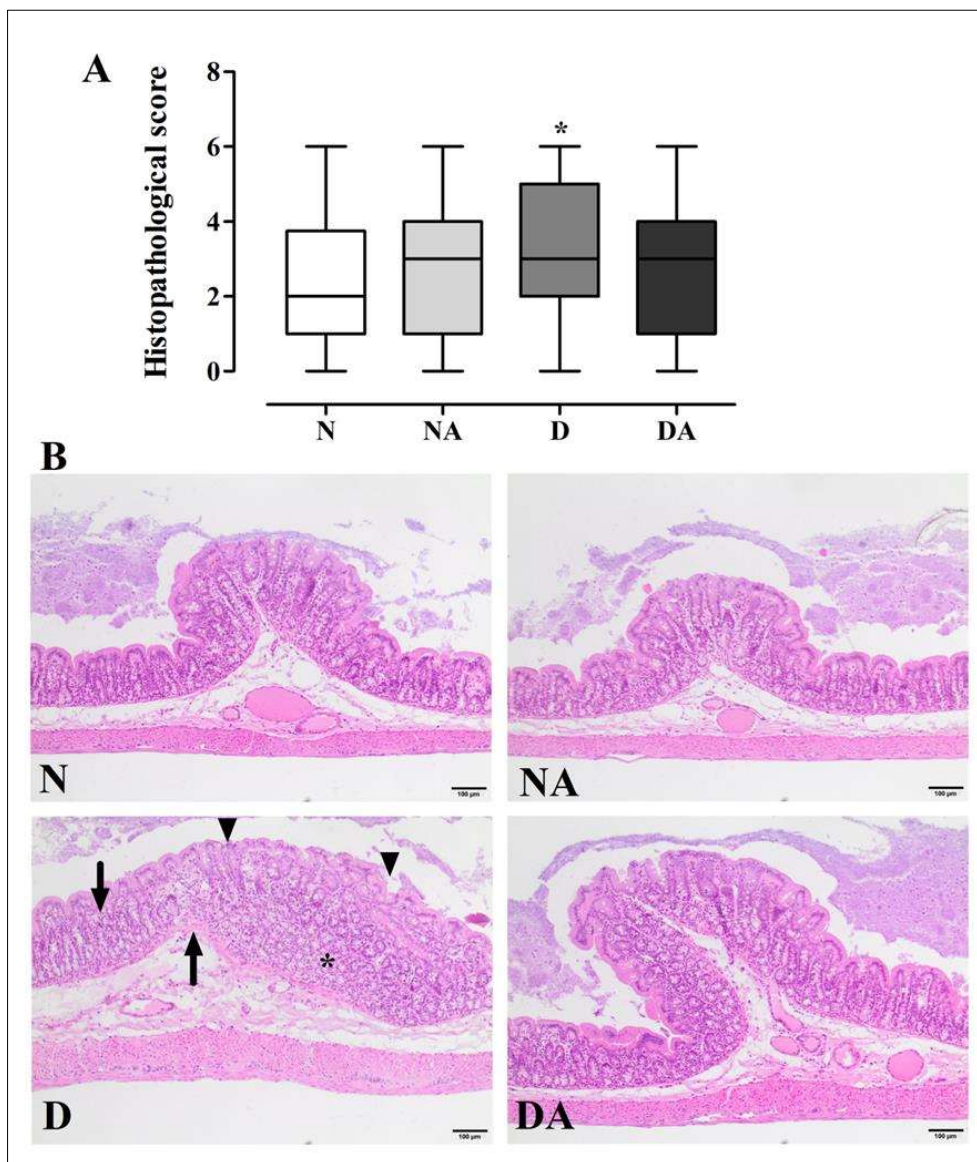Periganglionitis or ganglionitis were not observed (Figure 2B).

Figure 2. Effects of diabetes mellitus and treatment with a hydroethanolic extract of *S. terebinthifolius* Raddi on the histoarchitecture of the mucosa of the proximal colon of rats. (A) Diabetes mellitus causes histopathological changes in the mucosa of the proximal colon of streptozotocin-induced diabetic rats (D). * $p < 0.05$ compared with N by Kruskal-Wallis test followed by Dunn post-hoc test. Data are presented in box-and-whisker plots with minimum and maximum values, median, and interquartile range. (B) Representative photomicrographs show erosions on the epithelial surface with focal ulcerations (arrowhead) and the strong presence of diffuse inflammatory infiltrates in the lamina propria (arrows), leading to loss of mucosal histoarchitecture (group D). Note the distortion and hyperplasia in the intestinal crypts (asterisks). N: normoglycemic; NA normoglycemic treated with hydroethanolic extract of *S. terebinthifolius* Raddi; DA: streptozotocin-induced diabetic rats treated with hydroethanolic extract of *S. terebinthifolius* Raddi. HE Staining; scale bar, 100 μm.

We studied the remodeling of type I and III collagen fibers in the proximal colon wall of normoglycemic and streptozotocin-induced diabetic rats and the effects of treatment with a hydroethanolic extract of *S. terebinthifolius* Raddi.

We found that type I collagen fibers increased in the D group compared with the N group. In contrast, type I collagen fibers decreased in the DA group compared with the D group (Figure 3 A). There was no change in type III collagen fibers (Figure 3 B).
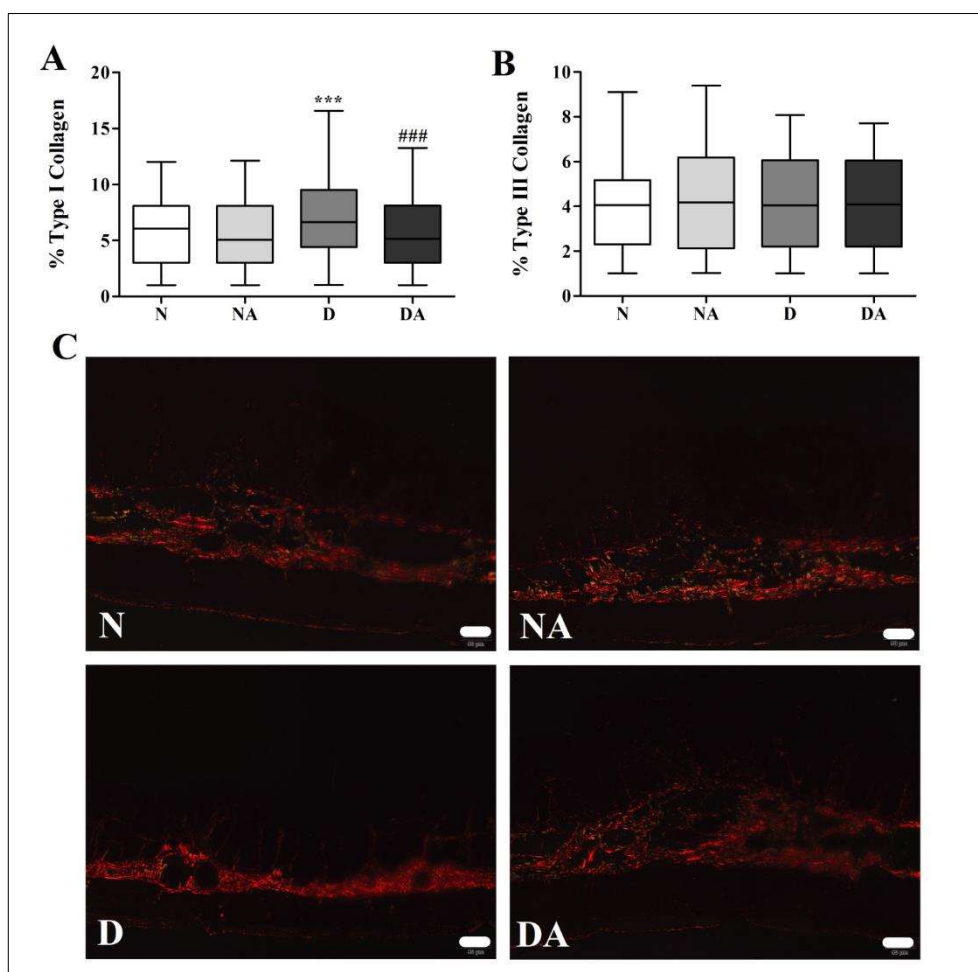

Figure 3. Effects of diabetes mellitus and treatment with a hydroethanolic extract of *S. terebinthifolius* Raddi on collagen remodeling. Distribution of type I (A) and type III (B) collagen fibers in the proximal colon of normoglycemic rats (N) and normoglycemic rats treated with a hydroethanolic extract of *S. terebinthifolius* Raddi (NA), streptozotocin-induced diabetic rats (D), and streptozotocin-induced diabetic rats treated with a hydroethanolic extract of *S. terebinthifolius* Raddi (DA). ***, ### $p < 0.001$ compared with N and D, respectively, by Kruskal-Wallis test followed by Dunn's post hoc test. Data are presented in box-and-whisker plots with minimum and maximum values, median, and interquartile range. (C) Representative photomicrographs of rat colon stained with Picro Sirius Red showing collagen type I (red color) and collagen type III (green color) in the submucosal layer. Scale bar, 50 µm.

Regarding the morphology of the ganglia of the myenteric plexus, we observed a significant reduction in the ganglion areas in the proximal colon of streptozotocin-induced diabetic rats (D) and of streptozotocin-induced diabetic rats treated with a hydroethanolic extract of *S. terebinthifolius* Raddi (DA) compared with group N (Fig. 4A).

There were no significant changes in the ganglia of rats in group DA compared with group D, indicating that the treatment did not improve ganglion atrophy. In addition, no periganglionitis or ganglionitis was observed (Figs. 4B and 2B).
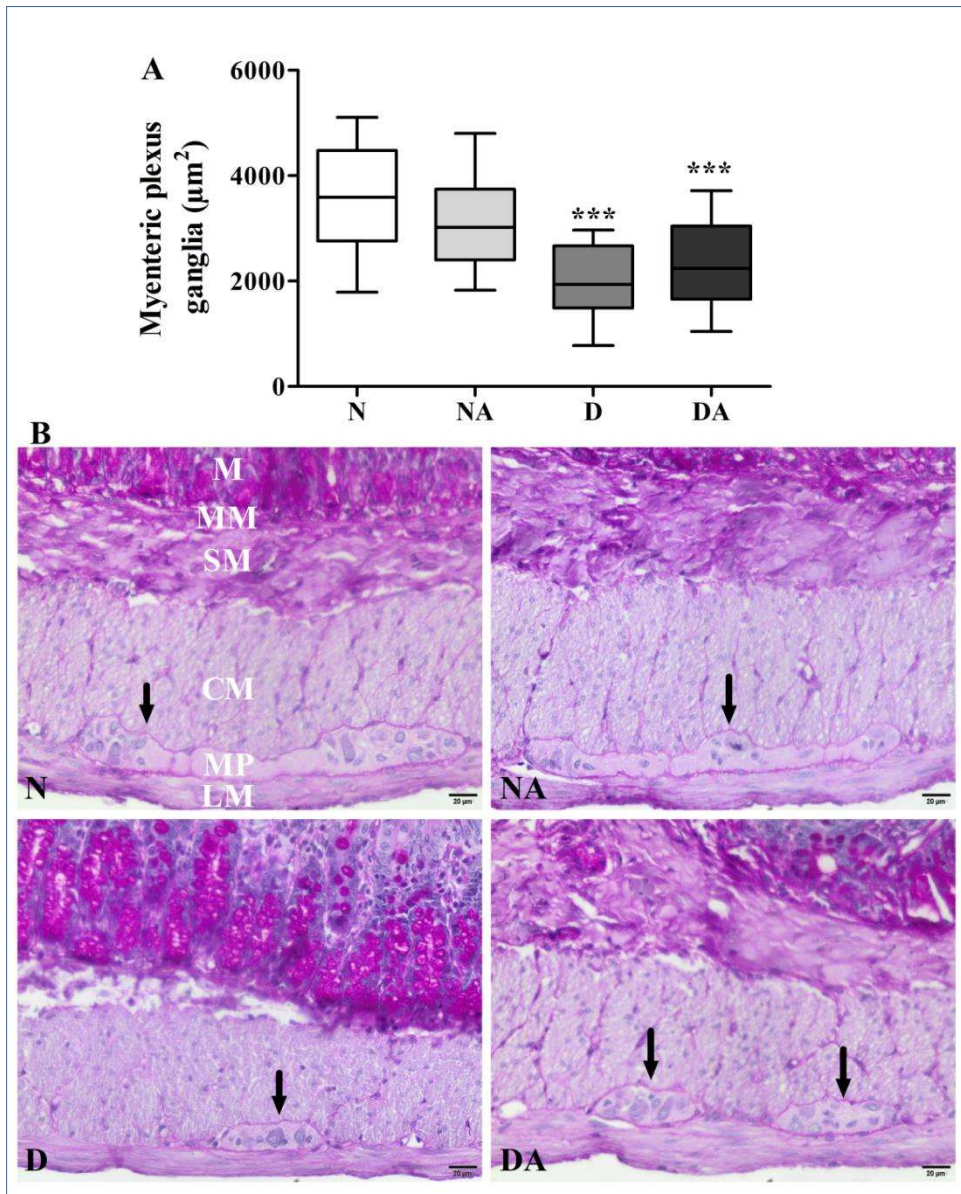
Figure 4. Effects of diabetes mellitus and treatment with a hydroethanolic extract of *S. terebinthifolius* Raddi on ganglion morphology of the myenteric plexus. (A) Ganglion profiles (μm^2) of the myenteric plexus in the proximal colon of normoglycemic rats (N) and normoglycemic rats treated with a hydroethanolic extract of *S. terebinthifolius* Raddi (NA), streptozotocin-induced diabetic rats (D), and streptozotocin-induced diabetic rats treated with a hydroethanolic extract of *S. terebinthifolius* Raddi (DA). *** $p < 0.001$ compared with N by Kruskal-Wallis test followed by Dunn post-hoc test. Data are presented in box-and-whisker plots with minimum and maximum values, median, and interquartile range. (B) Representative photomicrographs of the myenteric plexus (MP; arrows) between the longitudinal (LM) and circular (CM) layers of the muscular tunic. M: mucosa; MM: muscularis mucosa; SM: submucosa. Objective: 40x. PAS Staining; scale bar, 20 μm.

DISCUSSION

The experimental model of streptozotocin-induced diabetes was effective. The rats in groups D and DA showed diabetic syndrome after 60 experimental days, including classic signs such as polydipsia, polyuria, polyphagia, decreased body mass, diarrhea, and increased blood glucose. However, the physiological parameters altered by diabetes

were not reversed by treatment with the hydroethanolic extract of *S. terebinthifolius* Raddi, as no blood glucose-lowering effect was observed in the rats of the DA group compared with healthy rats. Supplementation with L-glutamine, glutamine dipeptide,[13,40] or quercetin[16] also failed to reverse the effects of diabetes mellitus. It is noteworthy that quercetin is one of the bioactive components of the hydroethanolic extract of *S. terebinthifolius* Raddi.[4]

Regarding the histomorphometric parameters, we have shown that diabetes causes atrophy of the total wall and reduces the thickness of the proximal colon of rats in groups D and DA by 7.8% and 9.3%, respectively. Considering the results of histomorphometric analysis, we suggest that the atrophy of the total wall mainly resulted from the reduction of the thickness of the submucosa by 50.1% and 47.9% in the rats in groups D and DA, respectively. Moreover, in evaluating the effects of the hydroethanolic extract of *S. terebinthifolius* Raddi, we showed that the treatment reduced the thickness of the muscular, mucosal, and submucosal layers of the proximal colon of the rats in the NA group by 27.9%, 37.3% and 51.5%, respectively. Taken together, the atrophy of these layers caused a 36.4% reduction in the total thickness of the proximal colon wall of the rats in the NA group. Based on our results, we suggest that the treatment damaged the colon wall four times more than diabetes, although we found no similar reports in the literature.

In addition to atrophy in the mucosa of rats in the NA group, we show histomorphometric changes in cells and tissues that form this layer. Atrophy of the mucosa, followed by a decrease in the depth of the crypts, was observed only in the NA group. Considering that these were normoglycemic rats (NA), treatment with the hydroethanolic extract of *S. terebinthifolius* Raddi possibly acted as a prooxidant and increased lipoperoxidation and oxidative stress. Under certain conditions, antioxidant compounds can act as pro-oxidants and cause oxidative damage to DNA, proteins, carbohydrates, and lipids.[4] Yet, there is no evidence that the hydroethanolic extract of *S. terebinthifolius* Raddi has an inhibitory effect on epithelial cell proliferation. The crypts provide a suitable site for the proliferation of stem cells that promote cell renewal in response to injury, as induced by diabetes. Regardless of the reduction in-depth, we showed an increase in the width of the intestinal crypts (in groups D and DA), similar to that reported by da Rosa *et al.*,[13] who also demonstrated an increase in cell proliferation in the intestinal mucosa of diabetic rats. Alterations in the epithelium of the intestinal crypts have been associated with increased proliferation of epithelial cells in diabetic rats[41] or predisposition to diabetes.[15] The increase in proliferative activity of epithelial cells is directly related to the rate of epithelial renewal (turnover).

Although we did not evaluate the expression of cell proliferation markers, we demonstrated by morphometric and quantitative analyzes that both *S. terebinthifolius* Raddi and diabetes treatment-induced epithelial changes. While the height of enterocytes was not altered in diabetic rats, their nuclei were. However, when we examined non-diabetic and diabetic rats treated with *S. terebinthifolius* Raddi, we found

that there was atrophy of the same parameters. Therefore, we can suggest that the treatment caused atrophy of both the height and the largest diameter of the enterocyte nucleus. Sukhotnik *et al.*[41] showed that streptozotocin-induced diabetes increased enterocyte turnover (which can alter cell morphology) in the jejunal mucosa and that oral insulin administration reversed the effect. However, the effect of hydroethanolic extract of *S. terebinthifolius* Raddi on enterocyte morphology remains to be investigated.

Furthermore, histopathological analysis revealed that streptozotocin-induced diabetic rats exhibited erosions on the epithelial surface, distortion and hyperplasia in the intestinal crypts, inflammatory infiltrates in the lamina propria, and loss of histoarchitecture of the colonic mucosa compared with healthy rats. Furthermore, the epithelial changes observed suggest that the integrity of the epithelial barrier was compromised by the hydroethanolic extract of *S. terebinthifolius* Raddi, as enterocytes were altered along with goblet cells and IELs, which form a line of defense against luminal antigens. Here we show that the number of goblet cells was increased in the colon mucosa of diabetic rats. Goblet cells are responsible for the production and release of mucins that form mucus upon contact with water in the lumen, which lubricates and protects the intestinal epithelium.[36,37,42] Remedio *et al.*[11] showed that the proliferation of goblet cells impairs water transport into the cells and alters the secretion of electrolytes and the consistency of mucus in the colon, contributing to the worsening of symptoms in diabetic rats. The increase in goblet cells may lead to increased mucin secretion, which in turn leads to more mucus. Considering the significant increase in water intake, it is possible that the greater amount of mucus in diabetic rats led to the diarrhea observed in the present study. However, treatment with the hydroethanolic extract of *S. terebinthifolius* Raddi did not reverse the goblet cell hyperplasia.

Another interesting finding observed in the present study was the increase in the proportion of IELs in the proximal colon of diabetic rats. These data suggest that the colonic mucosa was inflamed at the time of euthanasia. Like our findings, an increase in the proportion of lymphocytes has also been reported in the jejunum of diabetes-prone rats[15] and the small intestinal mucosa of diabetic patients.[35] IELs help regulate the immune response through the secretion and release of various chemical mediators and are in close contact with epithelial cells.[37,43] When we investigated the effects of the hydroethanolic extract of *S. terebinthifolius* Raddi, we observed a decrease in the number of IELs in the colonic mucosa of treated compared to untreated rats. The hydroethanolic extract of *S. terebinthifolius* Raddi is rich in bioactive compounds,[4,27,28] among which the anti-inflammatory activity stands out.[23,24] Duek *et al.*[18] demonstrated significant skin regeneration in rats. This study corroborates the efficacy of the pharmacological properties of *S. terebinthifolius* Raddi. The reduction of IELs in the mucosa of the rats in the DA group therefore indicates an anti-inflammatory effect achieved by the extract of *S. terebinthifolius* Raddi.

We studied the remodeling of type I and III collagen

fibers in the proximal colon wall. We showed that type I collagen fibers increased in group D compared with healthy rats (N); however, treatment with *S. terebinthifolius* Raddi (group DA) decreased the deposition of type I collagen fibers to a level similar to that of the control group (N). A similar result was obtained by Yassa and Tohamy[5] who reported a reduction in total collagen in the pancreas of rats with streptozotocin-induced diabetes treated with *Moringa oleifera*. Type I is the most abundant form, and its main function is to resist tensile stresses by allowing elongation in both longitudinal and transverse directions, limiting excessive expansion in both cases.[44] Furthermore, collagen fibers are involved in regulating the transport of water and electrolytes from the intercellular compartment of the epithelium to the interior of the intestinal cells and support the local structures, in addition to their involvement in healing.[45] Thus, the observed changes could damage the colon in some way, as the amount of collagen type I fibers is increased in diabetic patients. In contrast, Remedio *et al*.[11] showed a decrease in total collagen fibers in the colonic submucosa in diabetic rats. The authors suggested that this change may be due to dysfunction of intestinal distensibility, which correlates with diarrhea.

Concerning the myenteric plexus, the present study was limited to the evaluation of the areas of the ganglionic profiles. We observed a significant reduction of ganglion areas in the proximal colon of streptozotocin-induced diabetic rats (D) and streptozotocin-induced diabetic rats treated with a hydroethanolic extract of *S. terebinthifolius* Raddi (DA) compared with healthy rats. The neurons and glial cells that form the ganglia of the myenteric plexus are responsible for intestinal motility and, together with the submucosal plexus, modulate the integrity of the intestinal barrier. Considering that changes in the ganglionic areas are directly correlated with the neural structures there,[46] we hypothesized that the release of chemical mediators was impaired in the diabetic groups (D and DA). The oxidative stress and inflammation in the intestinal wall may cause inflammatory neuropathy and lead to neuronal degeneration, which in turn decreases the release of neurotransmitters required for cell and tissue repair in the intestinal wall.[47,50] Therefore, we can suggest that alterations in ganglion profiles impair intestinal wall repair in diabetic rats and that treatment with *S. terebinthifolius* Raddi (DA) does not ameliorate damage to the myenteric plexus. Previously, the reduction of cell body profiles of total population neurons (HuC/D) and nitrergic neurons (nNOS) in the jejunum myenteric plexus of quercetin-supplemented diabetic rats[16] and the loss of HuC/D and nNOS neurons per ganglion in the ileum myenteric plexus of L-glutamine-supplemented diabetic rats[50] were reported.

CONCLUSION

Taken together, our results suggest that treatment with the hydroethanolic extract of *S. terebinthifolius* Raddi did not improve the physiological parameters altered by diabetes. Moreover, the treatment caused histomorphometric and quantitative changes in the colonic wall of normoglycemic rats and intensified the damage caused by streptozotocin-induced diabetes. We conclude that the use of the hydroethanolic extract of *S. terebinthifolius* Raddi should be carefully evaluated under healthy conditions, as its use may lead to gastrointestinal complications of varying severity, which need further investigation.

CONFLICT OF INTEREST
The authors do not have any conflicts of interest.

Author contributions
CH-U and JNZ: designed the research study; CPS, MWFA, CCSS, FCVF, and CCI: collected the data; CPS, MBG, NMM, and CH-U: analyzed the data and wrote the manuscript; CH-U and MBG Review & Editing.

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