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Treatment of anal fistula with *Baccharis dracunculifolia* extract. Experimental study in rats.

Tratamento da fístula anal com extrato de Baccharis dracunculifolia. Estudo experimental em ratos.

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A B S T R A C T

Objective: to evaluate the efficacy of *Baccharis dracunculifolia* extract in the treatment of anal fistulas in rats. **Methods:** twenty male Wistar rats were submitted to anal fistula and, after 30 days, were divided into three groups: Control Group, with five animals; Carbopol Group, with five animals; and *Baccharis dracunculifolia* Group, with ten animals. In the Control Group, no treatment was performed. In the Carbopol Group, a daily infusion of Carbopol was performed for 30 days. In the *Baccharis dracunculifolia* Group, a daily infusion of Carbopol plus *Baccharis dracunculifolia* extract was performed for 30 days. Specimens were taken for histological analysis after euthanasia. **Results:** there was no complete closure of the fistulous tract in any of the animals. The mean area of the remaining tract was of 847.2μm², 565.6μm² and 372.7μm², in the Control Group, Carbopol Group, and *Baccharis dracunculifolia* Group, respectively, (p=0.001). The mean of the inflammatory process score was of 2.4, 2.4, and 2.1, in the Control Group, Carbopol Group, and *Baccharis dracunculifolia* Group, respectively, (p=0.285), while the mean values of vascular congestion were of 1.6, 1.4, and 1.1, in the Control Group, Carbopol Group, and *Baccharis dracunculifolia* Group, respectively, (p=0.031). **Conclusion:** *Baccharis dracunculifolia* extract was able to reduce the lumen of the fistulous tracts and the degree of vascular congestion, without, however, reducing the local inflammatory process or totally closing the fistulous tracts.

Keywords: Baccharis. Inflammation. Plants, Medicinal. Phytotherapeutic Drugs. Rectal Fistula.

INTRODUCTION

Anal fistula is a duct formed between an internal opening in the anus and an external orifice in the perianal skin, forming a epithelial fibrosis tract or filled with granulation tissue, almost always resulting from an abscess of cryptoglandular origin[1]. Depending on the spread of the abscess in relation to the anal and rectal spaces, the resulting fistula may have a tract that crosses greater or lesser thickness of the sphincter muscle. Thus, the treatment with the best cure rate of anal fistulas - fistulotomy, which consists of the opening and curettage of the fistulous tract - cannot be applied in all cases, since the greater the number of severed muscle fibers, the greater the risk of fecal incontinence[2,3]. These less frequent trans-sphincter fistulas are challenging because they require treatment that can cure them without causing fecal incontinence. On the other hand, there is no standardized technique for these situations yet.

The use of extracts and formulations of medicinal plants from different Brazilian biomes in folk medicine, mainly as anti-inflammatory and healing in the treatment of anal fistulas, has been increasing, especially in experimental researches, but with great potential for use in clinical practice[4]. Among these species, there is *Baccharis dracunculifolia* (*Asteraceae*), popularly known as field rosemary. There are still no publications in medical literature using this medicinal plant to treat anal fistulas, but, similarly to what has been observed

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in relation to other herbal medicines, its extract seems to have great therapeutic potential. This is because it is believed that anal fistula represents the chronic phase of cryptoglandular abscess and, thus, local control of inflammation could be useful for its treatment[5].

There are evidences that *B. dracunculifolia* is effective in treating infections caused by gastrointestinal tract bacteria, besides selectively modulating the effector functions of human neutrophils, inhibiting the activity of key enzymes, and eliminating oxidative physiological species, thereby exerting immunomodulatory action against neutrophils, useful in the treatment of anal fistulas[6,7].

The anti-inflammatory effect of *B. dracunculifolia* has also been demonstrated in an experimental study of gastric ulcers in rats. It has been observed that it recovers the ulcerated tissue increasing mucus and antioxidant enzyme levels and reducing H+,K+-ATPase activity[8]. Although the physiopathology of gastric ulcer and anal fistula are different, they have in common the chronic inflammatory process which needs to be controlled. Another effect of the plant has been to prevent colonic damage induced by trinitrobenzenesulfonic acid in rats with acute or chronic colitis. This anti-inflammatory effect may be associated with lower intestinal oxidative stress determined by chemical constituents, such as caffeic acid, p-coumaric acid, aromadendrin-4'-O-methyl-ether, 3-prenyl-p-coumaric acid**,** 3.5-diphenyl-p-coumaric acid, and baccarine detected in *B. dracunculifolia* extract[9].

Considering that *B. dracunculifolia* is an inexpensive and easily extracted plant, with proven efficacy in reducing the inflammatory process and widely found in Brazilian vegetation, and that there is a need to find an effective treatment for complex anal fistulas without causing fecal incontinence, there has been great interest in the research of this plant.

The aim of this study was to evaluate the efficacy of the ethanolic extract of *B. dracunculifolia* leaves in the closure of anal fistulas and in the reduction of the local inflammatory process.

METHODS

This study was evaluated and approved by the Animal Experimentation Ethics Committee of Anhanguera University - Uniderp (opinion n# 341/2018). The international norms for animal use in experimental surgery, from Brazilian College of Animal Experimentation, were followed.

Collecting and identification of plant compounds

Intact new and ripe leaves of *B. dracunculifolia* were collected at Três Barras Farm School, Uniderp, Campo Grande, MS, Brazil (S20°26'20,64" O54°32'26,78"), from 15 matrices, and a copy was archived in the herbarium of Uniderp (RG: 6766). For collection and research purposes, Genetic Heritage Management Board (GHMB) was authorized to access genetic heritage under registration number 010579/2013-3.

Extraction, phytochemical prospecting, and chromatographic profile (thin-layer chromatography and performance liquid chromatography)

After drying in a circulating oven (40°C), the leaves were crushed, sieved, and the powder (980g) was extracted with ethyl alcohol in an ultrasonic bath (Ultrasonic Cleaner®) for 60 minutes, followed by static maceration for 24 hours at room temperature between 28°C and 30°C ± 2°C. The solution was filtered and the solvent was removed on a rotary evaporator. The process was repeated for seven days and the liquid extracted from each process was pooled and separated on an evaporator; the ethanol extract was dried under reduced pressure. Phytochemical analyses were performed in triplicate and compared with the control sample (ethanolic extracts)[10].

The readings of the results were made by observing the color change and the precipitation of the filtrate, according to Fontoura *et al*.[11].

Confirmatory analyses of chemical constituents' classes of the ethanolic extract of the investigated species were performed using thin-layer chromatography (TLC) with standards, eluents, and developers cited by Wagner and Bladt[12], and the retention factors of the bands formed in the chromatographic areas of the standards and the obtained bands from ethanolic extract were calculated. The scan occurred on a UV-Visible spectrophotometer in the region between 200nm and 700nm and the results were compared with literature[13].

The chemical profile was determined by high-performance liquid chromatography, developed on SCL-10AVP® liquid chromatograph (Shimadzu, Japan), adjusted to 220nm, 254nm, and 340nm, and equipped with LC-10AD pump and DAD SPD-M10A. The chromatographic column used was PR-18 (20x4.6mm ID) and pre-column was RP-18 (250x4.6mm ID, 5µm). The mobile phase was composed of the following solvents: (A) ultrapure water acidified with glacial acetic acid (pH 3.0) / (B) acetonitrile - start (A) / (B) 95/5, 80min (A) (B) 5/95, 80.01min (A) / (B) 95/5, 90min (A) / (B) 95/5. Total analysis time: 90min. Initial pressure: 86psi. Flow: 1ml/min. Oven temperature: 50ºC. Monitoring: 254 and 340 nm. Spectrum analysis was based on literature data[13].

Determination of phenolic and flavonoid total

Dry ethanolic extract (10mg) was diluted in 1ml of spectroscopic grade methanol for solubilization using a 5-minute ultrasonic bath and filtered (0.45µm, Millipore). Ethanolic extract was also used to quantify total phenols by Folin-Ciocalteu method with gallic acid (10 to 350 mg ml -1) as standard (Y= 1.098x-0.011 R2= 0.997), and the flavonoid content by aluminum chloride and quercetin method (Y= 0.0655x-0.0025 R2= 0.985) was used as standard[14].

The chemical profile of *B. dracunculifolia* a native plant of the Brazilian Cerrado, considered invasive of agricultural areas and pastures[15], has already been determined by several authors[16-18]. It has been evident that this species has strong influence of seasonality, geographical area, and environmental conditions[15-17]. However, regardless of these characteristics, the main constituents are the phenolic and flavonoid compounds[19].

Obtaining the hydrogel formulation

After obtaining the ethanolic extract, 3% were incorporated into the hydrogel, made with 1% Carbopol, 0.1% methylparaben as preservative, and distilled water as vehicle.

Animals and surgical procedure

Twenty adult male and albino Wistar rats, weighing between 250g and 300g each, which received water and ad libitum ration during the research, were studied. Animals were intraperitoneally anesthetized with a solution containing 2ml of 10% ketamine and 1ml of 2% xylazine, applying 0.1ml of the solution for each 100g of body weight.

The fistulous tract was created by introducing a number 1 steel wire into the pectineal line, with exteriorization in the perianal skin, 1cm from the right edge of the anus, crossing the anal sphincter. Then, the steel wire was cut and twisted (Figure 1), remaining for 30 days.

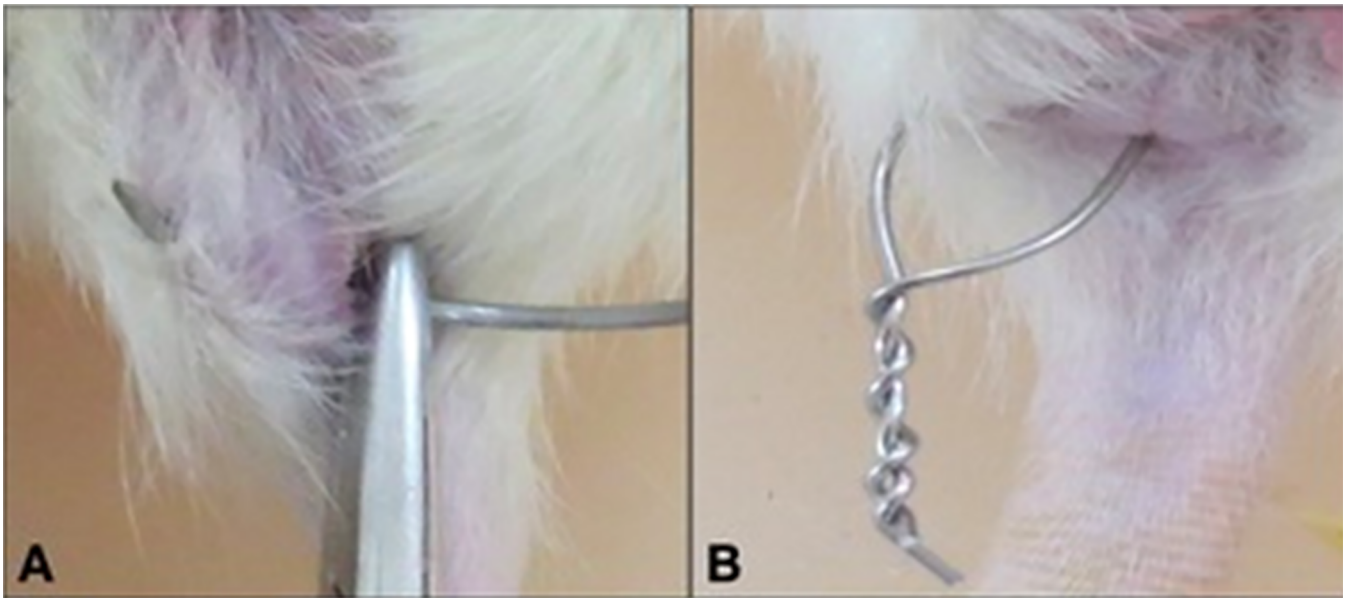

Figure 1. *Fistulous tract confection: (A) transfixation of the anal sphincter; (B) wire twist and maintenance for 30 days.*

After 30 days, the steel wire was removed and the animals were distributed into three groups: Control Group (GCo) with five animals observed for 30 days without treatment; Carbopol Group (GCa) with five animals treated during 30 days with Carbopol (vehicle), and *Baccharis dracunculifolia* Group (GBD) with ten animals treated during 30 days with *Baccharis dracunculifolia* plus Carbopol.

In animals belonging to the GCo, no treatment was performed and the animals were only observed for a period of 30 days. Animals from the GCa and GBD groups were treated by daily injection of 0.3ml of solution through the external fistula orifice with a 21G plastic catheter for 30 days. In the GCa Group, the solution contained Carbopol and, in the GBD Group, the solution contained *B. dracunculifolia* extract and Carbopol.

Euthanasia and specimen collecting

After the end of treatment, under anesthesia, all animals were euthanized by deepening the anesthetic plan. Subsequently, the specimens were removed for histological slides. Perianal trichotomy was performed, incision with cold scalpel, removing a cube-shaped fragment that included the anal canal and the entire fistulous tract. The specimens were stored in labeled vials filled with 10% formaldehyde until the preparation of histological slides.

Histological analysis

The slides were stained with hematoxylin-eosin (HE) for histological analysis. The analysed variables were: fistula lumen closure, remnant fistulous tract area, inflammatory process, and vascular congestion. The area of the fistula was measured in pixels and converted to micrometers. The inflammatory process was evaluated according to the following score: (0) absence of inflammatory infiltrate; (1) mild inflammatory infiltrate; (2) moderate inflammatory infiltrate; and (3) intense inflammatory infiltrate. Vascular congestion was classified as: (0) absent; (1) mild; (2) moderate; and (3) intense.

The results were submitted to statistical analysis by the nonparametric Kruskal-Wallis test, considering significant $p<0.05$.

RESULTS

There was no complete closure of the fistulous tract in any of the animals. The mean area of the fistulous tract after treatment was of 847.2μm² in the GCo, 565.6μm² in the GCa, and 372.7μm² in the GBD (p<0.05 between the GCo and the GBD) (Table 1).

Figure 2 shows the measurement of the remnant fistulous tracts in the animals after treatment.

Regarding inflammation, the mean GCo score was 2.4, the same observed in the GCa, while, in the GBD, there was a mean score of 2.1 without statistical difference among all groups (Table 2).

The means of the vascular congestion scores were 1.6 in the GCo, 1.4 in the GCa, and 1.1 in the GBD (p<0.05 between GCo and GBD), as observed in table 3.

Table 1. *Evaluation of the remnant fistulous tract area in the studied groups, according to histological findings (values in square micrometers).*

	GROUPS		
RATS	GCo*	GCa**	GBD***
1	946	422	754
2	501	573	514
3	894	545	259
4	782	677	230
5	1113	611	244
6	-	-	321
7	-	-	409
8	-	-	251
9	-	-	260
10	-	-	485
Mean	847.2	565.6	372.7#

** GCo: control group; ** GCa: carbopol group; *** GBD: Baccharis dracunculifolia group; # p<0.05 between the GCo and the GBD; there was no statistically significant difference between GCoXGCa or GCaXGBD.*

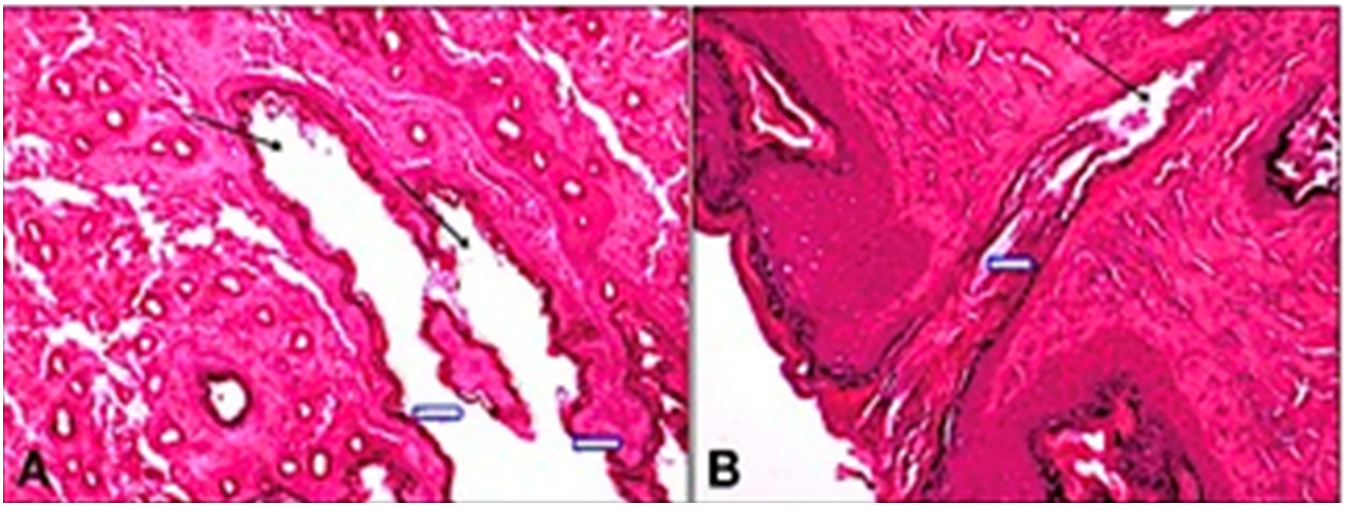

Figure 2. *Photomicrography demonstrating the measurement of the fistulous tract area after treatment. (A) GCo animal; (B) GBD animal; arrows indicate the pervious tract (HE 400X).*

Table 2. *Inflammatory process scores around the fistulous tracts in the studied groups.*

	GROUPS		
RATS	GCo*	GCa**	GBD***
1	2	2	3
2	2	3	2
3	3	2	3
4	2	2	2
5	3	3	3
6	-	-	2
7	-	-	1
8	-	-	1
9	-	-	1
10	-	-	3
Mean	2.4	2.4	2.1

** GCo: control group; ** GCa: carbopol group; *** GBD: Baccharis dracunculifolia group; there was no statistically significant difference between GCoXGCa, GCoXGBD, or GCaXGBD.*

Table 3. *Vascular congestion scores around the fistulous tracts in the studied animals.*

	GROUPS		
RATS	GCo*	GCa**	GBD***
1	0	1	2
2	1	2	1
3	3	2	2
4	1	0	1
5	3	2	2
6	-	-	2
7	-	-	1
8	-	-	0
9	-	-	0
10	-	-	0
Mean	1.6	1.4	1.1

** GCo: control group; ** GCa: carbopol group; *** GBD: Baccharis dracunculifolia group; p<0.05 between the GCo and the GBD; there was no statistically significant difference between GCoXGCa or GCaXGBD.*

DISCUSSION

The goal of any treatment for anal fistulas is to lead to complete healing of the fistulous tract without causing fecal incontinence. In the model used here, there was no complete closure of the fistulous tract in any of the animals. However, it was observed that, in the GBD, there was a significant reduction of the lumen of the tracts, which allows us to infer that perhaps in a longer treatment could occur total occlusion. This is because, since there is no similar research on *B. dracunculifolia* in the treatment of anal fistulas, we used a 30-day treatment period based on researches with other drugs[4]. However, the treatment period with *B. dracunculifolia* could have been longer and the evaluation of its efficacy in a prolonged treatment should be subject of future researches.

Considering that anal fistula is the chronic phase of the abscess[2], that is, there is persistence of local inflammation, the reduction of the inflammatory process could be beneficial for the complete closure of the fistulous tract, but in this respect there was no difference among the groups. However, as it is known that the reduction of vascular congestion is one of the early stages of the reduction of inflammation[20], and since there was a significant difference in favor of the GBD in this regard, it can be assumed that a longer treatment could lead to a reduction in inflammation and, consequently, to the closure of the fistula.

Baccharis dracunculifolia has a proven anti-inflammatory action. In a study on tissue inflammation with and without *B. dracunculifolia* in the rat diet, the authors have confirmed that those treated with the plant have had a significant reduction in the inflammatory process, characterized by a reduction in neutrophil concentration and a decrease in vascular congestion[21,22]. In the present research, a reduction in vascular congestion was also observed, demonstrating the effectiveness of the treatment at least in the initial phase of inflammation reduction.

The immunomodulatory/anti-inflammatory effect of *B. dracunculifolia* and its main compound, caffeic acid, on the production of cytokines (IL-1ß, IL-6, and IL-10) by murine macrophages was investigated. Increased serum concentration of IL-1ß and inhibited production of IL-6 and IL-1022 were observed. As these interleukins are implicated in the inflammatory process of intestinal diseases, such as anal fistula, it is possible that this fact explains the beneficial action of the plant in the initial phase of inflammation reduction in the present research.

There is a series of evidences that anal fistulas can be completely cured with the use of stem cells, which have been tested mainly on complex fistulas in Crohn's disease[23]. A very interesting research has shown that *B. dracunculifolia* can promote stem cell proliferation, differentiation, and in vitro migration to the applied site[24], which could theoretically contribute to the closure of the fistulous tracts. Although this did not occur in the present research, there was a significant reduction in lumens, which may have occurred due to the influence of this factor. There was also a reduction in the pulmonary inflammatory process of rats treated with *B. dracunculifolia*, confirmed by the inhibition of proinflammatory cytokines and increase of anti-inflammatory cytokines, suggesting an immunomodulatory activity[25].

Considering these evidences in favor of *B. dracunculifolia* as an anti-inflammatory and healing agent, the fact that there was no reduction in the GBD inflammation may be explained by the method used to apply the extract. The experimental model of anal fistula in rats is relatively new, therefore, researches on herbal medicines with this model are very limited. Thus, the best method of applying the treatment is not well-defined yet. We opted to daily inject the extract through the external orifice of the fistula, without the use of a seton stitch, according to other similar investigations. The trauma of daily application may also have contributed to the persistence of the inflammatory process, despite the reduction of the lumen of the tracts. Perhaps an interval of seven to ten days between the last application and euthanasia could eliminate treatment trauma inflammation, persisting only anal fistula inflammation.

The reduction of the anal fistula lumen alone is already a good result, because it leads to less purulent drainage, since this one is proportional to the lumen of the tract. But this result certainly serves more as a stimulus for future researches using *B. dracunculifolia* with longer treatment periods, since the result found here is very promising. The reduction of vascular congestion also observed here, the initial stage in inflammation control, is another stimulating result for the use of this drug in the treatment of anal fistulas, and future researches should demonstrate the best method of its use.

We conclude that *B. dracunculifolia* extract was able to reduce the lumen of the fistulous tracts and the degree of vascular congestion, without, however, reducing the local inflammatory process or totally closing the fistulas.

R E S U M O

Objetivo: *avaliar a eficácia do extrato de Baccharis dracunculifolia no tratamento de fístulas anais em ratos.* **Métodos:** *vinte ratos Wistar machos foram submetidos à confecção de fístula anal e, após 30 dias, foram distribuídos em três grupos: Grupo Controle, com cinco animais; Grupo Carbopol, com cinco animais; e Grupo Baccharis dracunculifolia, com dez animais. No Grupo Controle, não se realizou nenhum tratamento. No Grupo Carbopol, realizou-se infusão diária de carbopol, e no Grupo Baccharis dracunculifolia, infusão de extrato de Baccharis dracunculifolia com carbopol, ambos por 30 dias. Foram retirados espécimes para análise histológica após a eutanásia.* **Resultados:** *não houve fechamento completo do trajeto fistuloso em nenhum dos animais. A média da área do trajeto resultante foi de 847,2μm², 565,6μm² e 372,7μm², respectivamente, nos Grupos Controle, Carbopol e Baccharis dracunculifolia (p=0,001). A média do escore de processo inflamatório foi de 2,4, 2,4 e 2,1, respectivamente, nos Grupos Controle, Carbopol e Baccharis dracunculifolia (p=0,285), enquanto a média de congestão vascular foi de 1,6, 1,4 e 1,1, respectivamente, nos Grupos Controle, Carbopol e Baccharis dracunculifolia (p=0,031).* **Conclusão:** *o extrato de Baccharis dracunculifolia foi capaz de reduzir o lúmen dos trajetos fistulosos e a congestão vascular, sem reduzir, no entanto, o processo inflamatório local ou fechar totalmente os trajetos fistulosos.*

Descritores: *Baccharis. Inflamação. Plantas Medicinais. Medicamentos Fitoterápicos. Fístula Retal.*

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