**Name of Journal: *World Journal of Gastroenterology***

**ESPS Manuscript NO: 30382**

**Manuscript Type: ORIGINAL ARTICLE**

***Basic Study***

**Anti-inflammatory intestinal activity of *Combretum duarteanum* Cambess. in trinitrobenzene sulfonic acid colitis model**

de Morais Lima GR *et al*. *Combretum duarteanum*

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**Institutional review board statement:** The protocol was approved by Committee for Ethics in Animal Experimentation, Laboratory of Pharmaceutical Technology, Federal University of Paraíba (CEPA/LTF/UFPB), João Pessoa, Paraíba, Brazil.

**Institutional animal care and use committee statement:** The experimental protocols were approved by the Committee for Ethics in Animal Experimentation (CEPA/LTF/UFPB) under number 1112/10.

**Conflict-of-interest statement:** Authors declare no conflict of interest exists.

**Data sharing statement:** No additional data are available.

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**Manuscript source:** Unsolicited manuscript

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**Received:** September 29, 2016

**Peer-review started:** October 1, 2016

**First decision:** October 20, 2016

**Revised:** November 8, 2016

**Accepted:** December 8, 2016

**Article in press:**

**Published online:**

**Abstract**

***AIM***

To evaluate the anti-inflammatory intestinal effect of the ethanolic extract and hexane phase obtained from the leaves of *Combretum duarteanum*.

***METHODS***

Inflammatory bowel disease was induced using trinitrobenzenesulfonic acid (TNBS) in acute and relapsed ulcerative colitis models in rats. Damage scores, biochemical, histological, and immunohistochemical parameters were evaluated.

***RESULTS***

Both *Cd-*EtOHE and *Cd-*HexP caused significant reductions in macroscopic lesion scores and ulcerative lesion areas. The vegetable samples inhibited myeloperoxidase (MPO) increase, as well as pro-inflammatory cytokines TNF-α and IL-1β. Anti-inflammatory cytokine IL-10 also increased in animals treated with the tested plant samples. The anti-inflammatory intestinal effect is related to decreased expression of cyclooxygenase-2 (COX-2), proliferating cell nuclear antigen (PCNA), and an increase in SOD.

***CONCLUSION***

The data indicate anti-inflammatory intestinal activity. The effects may also involve participation of the antioxidant system and principal cytokines relating to inflammatory bowel disease.

**Key words:** Medicinal plants; Combretaceae; *Combretum duarteanum*; inflammatory bowel disease; anti-inflammatory intestinal activity

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**Core tip:** Inflammatory bowel diseases are chronic inflammatory disorders that include Crohn's disease and ulcerative colitis. The genetic, immunologic and environmental factors are postulated as possible etiologic agents. Their conventional treatment is centered in reducing inflammation and abnormal symptom relief. A variety of herbal medicines have demonstred to produce promising results in the treatment of those diseases. *Combretum duarteanum* is a species popularly used to treat inflammation in folk medicine. Thus, the present study was proposed to evaluate the intestinal anti-inflammatory effect in the ulcerative colitis model, contributing to the safe use and collaborating with the scientific knowledge of natural products.

de Morais Lima GR, Machado FDF, Périco LL, Faria FM, Luiz-Ferreira A, Souza Brito ARM*,* Pellizzon CH,Hiruma-Lima CA, Tavares JF*,* Barbosa Filho JM, Batista LM. Anti-inflammatory intestinal activity of *Combretum duarteanum* Cambess. in trinitrobenzene sulfonic acid colitis model. *World J Gastroenterol* 2016; In press

**INTRODUCTION**

The inflammatory bowel diseases (IBDs) are chronic disorders of the gastrointestinal (GI) tract characterized by alternating periods of remission and relapse[1]. These diseases are a large group of inflammatory disorders, the most common being Crohn's disease (CD) and ulcerative colitis (UC)[2,3].

CD can affect any part of the gastrointestinal tract and has the classic symptoms of fatigue, prolonged diarrhea (with or without bleeding), abdominal pain, weight loss, and fever[4,5]. UC is a type of chronic inflammation restricted to the colon, however the entire large intestine may be affected[6]. Affected patients show symptoms such as rectal bleeding, frequent bowel movements, tenesmus, rectal mucus discharge, and abdominal pain[5].

The etiology of IBDs is still not fully understood, however it is believed that environmental, genetic and immunologic factors have an important role in their occurrence and progression[4,7].

Emerging models in the study of IBD pathogenesis suggest three key disease development factors: (1) breaking the intestinal barrier function; (2) lamina immune cell exposure to luminal contents; and (3) exacerbation of immune response. However, the factors responsible for initiation and perpetuation of the cycle leading to exacerbation of the disease are still unclear[8,9].

A combination of genetic and environmental factors may foment changes in the intestinal mucosal barrier function; this allows translocation of luminal antigens (commensal bacteria or microbial products) into the intestinal wall, and consequent immune cell activation and excessive production of cytokines, causing the acute phase of inflammation. If the acute inflammatory process is not resolved by anti-inflammatory mechanisms and suppression of pro-inflammatory cytokines, chronic intestinal inflammation develops. This can lead to tissue destruction and complications of the disease[10].

Conventional treatments are aimed at reducing inflammation and consequent abnormal symptom relief. Patients with ulcerative colitis are treated with amino-salicylates, corticosteroids, and immunomodulatory drugs[11].

Natural products have become the most attractive source of new drugs for the treatment and prevention of diseases and their use is constantly expanding worldwide. A variety of herbal medicines have been shown to produce promising results in the treatment of peptic ulcer and inflammatory bowel disease [11–14].

*Combretum duarteanum* (*C. duarteanum*)Cambess., the species selected for this study, is popularly known as “mufumbo, cipiúba, cipaúba”. This shrubby species is exclusive to South America with registrations in Bolivia, Paraguay, and Brazil. It occurs in the northern and northeastern regions of Brazil, being associated with the “caatinga” biome[15,16].

In folk medicines, *C. duarteanum* is used to treat pain, inflammation and gastrointestinal tract disorders, which justifies its selection, using ethno-pharmacological indication as the criterion of choice. Phytochemical studies suggest the presence of flavonoids, and triterpenes, whose pharmacological effects have been attributed[16,17].

*C. duarteanum* has presented *in vitro* and *in vivo* anti-inflammatory, anti-nociceptive, and antioxidant capacity[15]. Quintans *et al*[18] demonstrated orofacial nociceptive activity as promoted by hexane phase and Fridelin terpenes, isolated from the species studied.

De Morais Lima *et al*[16,19] demonstrated gastro-protective and antiulcer activity in *C. duarteanum* in different models of acute ulcer induction (acidified ethanol, ethanol, NSAIDs, stress, pylorus ligature, acetic acid) in animals. Previous studies demonstrated low toxicity and no change of liver enzymes in animals treated with the tested plant sample for fifteen days in acid acetic induced gastric ulcer model[19].

Given the need for new inflammatory bowel disease therapies, this study aims to evaluate the intestinal anti-inflammatory activity promoted by the species *C. duarteanum*, validating its popular use and contributing to the search for new therapies for diseases that affect the gastrointestinal tract.

**MATERIALS AND METHODS**

***Reagents***

The drugs and reagents were prepared immediately before use. The following drugs were used: Trinitrobenzenesulfonic acid (TNBS) (Sigma-Aldrich), ketamine 5% (Vetanarcol), xilazine 2% (Dorcipec), ethanol (Merck®, Germany), Tween 80 (Merck®, Germany), sodium chloride (Sigma-Aldrich). TNF-a, IL-1β and IL-10 in ELISA kits were provided by R&D systems incorporation, United States.

***Plant material***

Plant samples used in intestinal anti-inflammatory activity research experiments in rats were obtained from the leaves of *C. duarteanum* Cambess, collected at Serra Branca city, Paraíba State, Brazil, in 2010. The species was identified by Dr. Maria Fatima Agra and a voucher specimen (no. 6767) is deposited in the Herbarium Prof. Lauro Pires Xavier (JPB) at the Universidade Federal da Paraíba.

The ethanol extract (*Cd*-EtOHE) and the hexane phase (*Cd*-HexP) obtained from the leaves of *C. duarteanum* Cambess were provided by Dr. Josean Fechine Tavares and his group, all of PgPNSB / UFPB.

The dried leaves (5 kg) were powdered and extracted with ethanol; stirred, and macerated at room temperature for approximately 48 h, with the procedure being repeated three times. The solvent was fully evaporated under reduced pressure, and the extract (yield 200 g) was concentrated. The ethanol extract was subjected to liquid–liquid partition with the following solvents: hexane, chloroform (CHCl3), and ethyl acetate (EtOAc), obtaining their respective phases. This step was repeated to secure the required quantity for the study.

***Pharmacological assays***

**Investigation of Cd-EtOHE and Cd-HexP effects on acute phase intestinal inflammation (TNBS) induced in rats:** The intestinal anti-inflammatory activity of Cd-EtOHE and Cd-HexP was assessed in rats using the Morris *et al*[20] method. Male Wistar rats (*n* = 5-8) fasted for 24 h were divided into four groups: non-colitic, colitic, Cd-EtOHE and Cd-HexP. The animals were anesthetized for rectal administration of TNBS (2,4,6-trinitro-benzene sulfonic acid) - 10 mg solubilized in 0.25 ml of 50% v/v ethanol. The induction of inflammation was performed with the aid of a probe (2-mm diameter) which was inserted about 8 cm into the rectum of the animal. After TNBS administration, animals were maintained upside down for 15 min to enable total absorption of the administered inducing agent. The non-colitic group underwent the same procedures. However, they did not receive TNBS.

Each group of rats was pretreated with vehicle (12% Tween 80), *Cd*-EtOHE (31.25, 62.5, 125, 250 mg/kg) or *Cd*-HexP (31.25, 62.5, 125, 250 mg/kg), at 48, 24 and 1 h prior to administration of TNBS/50% ethanol, and at 24 h after colitis induction. At 48 h after inducing inflammation, the animals were euthanized and colonic segments were removed, opened, washed, and photographed for quantification of ulcerative lesion area (ULA), and macroscopic score evaluation of the intestinal inflammatory process. Analysis of the extent of intestinal injury was performed according to the scale described previously by Bell *et al*[21]. General parameters such as diarrhea and the colon weight/length ratio were also evaluated. The most effective doses obtained in this model were used in the chronic model with relapse of ulcerative colitis in rats.

**Investigation of Cd-EtOHE and Cd-HexP effects in the chronic phase with intestinal inflammation relapse induced by TNBS in rats:** Male Wistar rats (n = 7-9) were divided into non-colitic, colitic, *Cd-*EtOHE effect and *Cd-*HexP groups. After 24 h of fasting, induction of intestinal inflammation was performed with TNBS (10 mg/0.25 ml ethanol 50% v/v, rectally)[20]. At 24 h after initial induction, the animal groups were treated orally with Tween-80 12% (non-colitic and colitic) *Cd*-EtOHE (125 mg/kg) or *Cd*-HexP (62.5 mg/kg). On the day 14after the first induction, the second administration (relapse) was performed with TNBS (10 mg/0.25 ml ethanol 50% v/v, rectally) to mimic recurrent relapses in inflammatory bowel diseases in humans.

General parameters such as diarrhea, water and food intake, and body weight were recorded daily throughout the treatment period. At day 21 all animals were euthanized, the colon removed, opened, and washed for macroscopic lesion analysis and evaluation of the intestinal inflammatory process[21]. Collection of material for biochemical and histological analysis was also performed. The samples were stored at -80 °C for evaluation of myeloperoxidase (MPO), and cytokines involved in intestinal inflammation.

***Histological analysis***

Colonic segments intended for light microscopy were collected. For this, they were fixed in Alfac solution for 24 h at room temperature. Afterwards, the pieces were kept in 80% alcohol until the blocks assembly time. The pieces were dehydrated and embedded in paraplast forming blocks, and then cut to 10 mm thick for mounting on slides. These were stained with hematoxylin and eosin (HE) staining for morphological analysis[22].

***Quantification of MPO activity***

Colon segments, stored at -80°C were used with dosages of MPO and pro-inflammatory and anti-inflammatory cytokines. The samples were homogenized in hexadecyltrimethylammonium bromide buffer (HTAB) (0.5% in 50 mmol/L sodium phosphate buffer, pH 6.0) that acts as a detergent, lysing granules of neutrophils containing MPO which is released. The sample was centrifuged for 10 min at 4 °C. The homogenate was subjected to a threefold freezing and thawing process to facilitate the rupturing of cell structures and the consequent release of the enzyme. On ELISA plates were placed 50 μL of supernatant from each sample and 150 μL of reaction buffer[23].

The results were expressed as MPO units per gram of tissue, where one unit of MPO activity is defined as that degrading 1 µmol of hydrogen peroxide per minute at 25 °C.

***Assessment of the involvement of pro-inflammatory (TNF-α and IL-1β) and anti-inflammatory cytokines (IL-10)***

TNF-α, IL-1β and IL-10 levels were determined from colonic specimens, frozen in -80 °C, and collected in the ulcerative colitis relapse model. For this, we used PBS buffer pH 7.4 (1:5) to homogenize the samples. The homogenate tubes were centrifuged at 12000 rpm for 10 min. The supernatants were frozen at -80 °C until assay. Subsequently, the samples were shaken in water bath at 37 °C for 20 min. and then centrifuged at 10000 rpm for 5 min at 4 °C. The supernatant was collected and the cytokines TNF-α, IL-1β and IL-10 were quantitated using enzyme-linked immunosorbent ELISA assay kits (DuoSet®, R & D Systems). The concentrations of the cytokines in relation to the amount of total protein was quantified by bicinchoninic acid method[24].

***Immunohistochemical analysis (COX-2, PCNA and SOD expression)***

Histological samples were incubated with anti-COX-2 secondary antibody (marker for assessing anti-inflammatory effect), anti-PCNA (cell division marker to assess potential for regeneration), and anti-SOD (marker to evaluate the antioxidant effect). The positively stained cells were counted for the various immunohistochemical reactions in a fixed number of fields by means of an image analyzer Leica Q-Win Standard Version 3.1.0 (United Kingdom) coupled to the Leica DM. They were photographed and analyzed by AVSoft program Bioview Spectra and Seeker 4.0.

***Animal care and use statement***

The experimental protocols were approved by the Committee for Ethics in Animal Experimentation (CEPA/LTF/UFPB) under number 1112/10. Male Wistar albino rats (180-250 g) from the “Prof. Thomas George Vivarium” of LTF/UFPB were fed a certified Presence ® diet, with free access to water under fixed conditions of illumination (12/12 h light/dark cycle), humidity (60% ± 1.0%), and a temperature of (21.5 ± 1.0 °C). Fasting was used prior to all assays since standard drugs were administered orally (by gavage), or by intra-rectal route, using a 12% solution of Tween 80 (10 ml/kg) as the vehicle. The animals were kept in cages with raised, wide-mesh floors to prevent coprophagy.

***Statistical analysis***

Results with parameter values (inflammatory bowel lesion area and weight / length ratio) were subjected to analysis of variance (ANOVA) followed by Dunnett's or Tukey test, and expressed as mean ± SD of the average. In quantitation assays of antioxidant enzymes, cytokines and MPO values obtained were presented as mean ± standard error of mean (SEM).

For nonparametric values (score of intestinal inflammation) the Kruskal-Wallis test (ANOVA, Dunn's post-test) was used. The results were expressed as median (minimum-maximum). Data were analyzed using the software GraphPad Prism 6.0 (San Diego, CA, United States), and the significance level was set at *p* < 0.05.

**RESULTS**

***Investigation of Cd-EtOHE and Cd-HexP effect on induced acute phase intestinal inflammation; TNBS in rats***

A significant reduction in the intestinal ulcerative lesion area for rats treated with *Cd*-EtOHE at doses of 62.5 and 125 mg/kg (46 ± 12, *p* < 0.01; and 19 ± 8, *p* < 0.001 respectively) was observed compared to the colitic group (107 ± 38). In the experimental evaluation of the effect of *Cd*-HexP, a significant reduction was observed at doses of 31.25 and 62.5 mg/kg; 52 ± 18 (*p* < 0.01) and 21 ± 7 (*p* < 0.001), respectively, when compared to the colitic animals (101 ± 45) (Table 1).

For the lesion score, *Cd*-EtOHE at doses of 62.5 and 125 mg/kg significantly reduced the amounts of lesion to 4.0 (1-5) (*p* < 0.05) and 3.0 (2-5) (*p* < 0.01), respectively, compared to the colitic control 6 (5-7). *Cd*-HexP 31.25 and 62.5 mg/kg significantly reduced lesion to 5.0 (1-6) (*p* < 0.05) and 5.0 (4-5) (*p* < 0.01), respectively, compared to colitic group 7 (5-8) (Table 1).

A significant increase in weight/length for the colitic group (148 ± 17, *p* < 0.001, 154 ± 27, *p* < 0.001) was also observed when compared to the non-colitic group (110 ± 8, 102 ± 14, respectively). Treatment with different doses of *Cd*-EtOHE (31.25, 62.5, 125 and 250 mg/kg) did not reduce the weight/length ratio (152 ± 19; 146 ± 19; 134 ± 5 and 152 ± 14, respectively) for the parameter compared to the colitic group (148 ± 17). However, treatment with *Cd*-HexP at a dose of 62.5 mg/kg significantly reduced the ratio to 129 ± 20 (*p* < 0.05) compared to their respective colitic group (154 ± 27) (Table 1).

The administration of TNBS resulted in a diarrhea rate of 100% in the colitic animals. *Cd*-EtOHE at a dose of 125 mg/kg significantly reduced the diarrhea involvement to 14%. For *Cd*-HexP, treatment at dose of 62.5 mg/kg significantly reduced diarrhea to 29% (*p* < 0.05) when compared to their respective colitic control (87%) (Table 1).

Intestines of colitic, non-colitic and treated rats with different tested doses of *Cd*-EtOHE or *Cd*-HexP in the model can be seen in Figures 1 and 2, respectively.

***Investigation of Cd-EtOHE and Cd-HexP effects in chronic phase intestinal inflammation with induced relapse using TNBS in rats***

A significant reduction in macroscopic damage scores was observed for both *Cd*-EtOHE (125 mg/kg) and *Cd*-HexP (62.5 mg/kg), to 1.0 (1-4) (*p* < 0.05) and 1.0 (1-4) (*p* < 0.01), respectively, compared to the colitic control (3-6). Moreover, the tested plant sample reduced the onset of diarrhea by 56% when compared to colitic animals (94%) (see Table 2).

The weight/length ratio significantly increased in the colitic, *Cd*-EtOHE, and *Cd*-HexP groups (143 ± 14, *p* < 0.001; 132 ± 11, *p* < 0.001; 122 ± 12, *p* < 0.01, respectively) compared to the non-colitic group (97 ± 9). However, *Cd*-HexP 62.5 mg/kg caused a significant reduction (122 ± 12, *p* < 0.01) compared to the colitic group (143 ± 14). These results are shown in Table 2, and can be best seen in Figure 3.

A significant decrease in water (28 ± 3, *p* < 0.01) and food (19 ± 2, *p* < 0.01) intake was observed in the colitic group compared to the non-colitic animals (31 ± 4 and 22 ± 2, respectively). Only treatment with *Cd*-HexP increased water (31 ± 2, *p* < 0.05) and food (22 ± 3, *p* < 0.01) intake compared to colitic animals (28 ± 3, *p* < 0.01 and 19 ± 2, *p* < 0.01, respectively) (Table 3).

As an additional parameter to the data described above, we evaluated the effect of repeated administrations of *Cd*-EtOHE (125 mg/kg) and *Cd*-HexP (62.5 mg/kg) on the body weights of animals affected with intestinal inflammation. At the end of the experiment, there was a significant reduction in mean body weight for the colitic group (215 ± 21, *p* < 0.001) when compared to the non-colitic group (261 ± 35). However, when the treatments were performed with *Cd*-HexP (62.5 mg/kg) a significant increase in mean body weight (239 ± 17, *p* < 0 05) was observed compared to the colitic animals (215 ± 21) (Table 4).

A significant increase was observed in spleen weight in the colitic group (2.8 ± 0.5, *p* < 0.05) compared to the non-colitic group (2.1 ± 0.2). Analyzing the organs of animals treated with *Cd*-EtOHE or *Cd*-HexP, a significant increase in spleen weight for animals treated with *Cd*-EtOHE as compared to the non-colitic group was demonstrated (Table 5). In other evaluations (heart, liver and kidneys) no significant changes compared to the non-colitic group were observed.

***Histological analysis***

Histological examination of the colon of non-colitic rats showed normal histological structure, highlighting the structure of the mucosal straight intestinal glands with large numbers of goblet cells and lamina propria classical (or normal). The animals belonging to colitic group had transmural inflammation, necrosis of the mucosa with disruption of glands, and loss of goblet and epithelial cells. The presence of granulation, highlighting neutrophilic and lymphocytic infiltration was also observed.

Treatment with *Cd*-EtOHE (125 mg/kg) or *Cd*-HexP (62.5 mg/kg) maintained some areas of the mucosal structure and epithelium intact, reducing the remaining inflammatory lamina propria as compared to the colitic group, suggesting the re-epithelialization of the animals treated with the vegetable samples (Figure 4).

***Quantification of MPO activity***

According to the results obtained can be seen significant increase in MPO to 40270 ± 3077 (*p* < 0.001) in the colitic group when compared to non-colitics (10120 ± 1672). When compared to the colitic controls (40.270 ± 3.077), treatments with *Cd*-EtOHE (15.187 ± 1.158; *p* < 0.001), or *Cd*-HexP (17.620 ± 2.395; *p* < 0.001), significantly reduced MPO (Figure 5).

***Assessment of the involvement of pro-inflammatory (TNF-α and IL-1β) and anti-inflammatory cytokines (IL-10)***

The results showed a significant increase in TNF-α levels in colitic animals (2.0 ± 0.2, *p* < 0.01) compared to non-colitics (1.3 ± 0.1). However, this treatment *Cd*-EtOHE (125 mg/kg) or *Cd*-HexP (62.5 mg/kg) resulted in TNF-α levels reduction (1.2 ± 0.2 and 1.4 ± 0.1) respectively, compared to colitic group (2.0 ± 0.2) (Figure 5).

IL-1β levels increased in colitic animals (63 ± 6, *p* < 0.001) when compared to non-colitic group (32 ± 4.0). Treatment with *Cd*-EtOHE significantly reduced to 41 ± 5 (*p* < 0.01) IL-1-β compared to colitic group (63 ± 6.2). *Cd*-HexP did not cause significant change when compared to the negative control (Figure 5).

The results showed significant reduction of IL-10 in intestinal tissues; colitic group 3.2 ± 0.3 (*p* < 0.05) compared to non-colitic (5.1 ± 0.8). Treatment with *Cd*-EtOHE or *Cd*-HexP caused significant increases (5.0 ± 0.4, p <0.05, 5.8 ± 0.3, *p* < 0.01, respectively) when compared to the colitic group (3 2 ± 0.3) (Figure 5).

***Immunohistochemical analysis (COX-2, PCNA and SOD expression)***

In analyzing the results of COX-2 expression shown in Table 6 and Figure 6, we observed a significant increase in COX-2 expression in colitic group animals to 505 (100-1450) (*p* < 0.05) when compared the non-colitic group 230 (110-700). However, treatment with *Cd*-EtOHE (125 mg/kg) or *Cd*-HexP (62.5 mg/kg) significantly reduced the expression of COX-2 to 90 (10-2590) (*p* < 0.001) and 205 (20-790) (*p* < 0.001) respectively compared to the colitic group 505 (100-1450).

A significant increase in PCNA expression was observed in the animals of colitic group 5380 (850-15960) (*p* < 0.001) when compared to non-colitic 1425 (60-7890). However, treatment with *Cd*-EtOHE (125 mg/kg) or *Cd*-HexP (62.5 mg/kg) significantly reduced PCNA expression to 1,630 (90-14790) (p <0.001) and 1.570 (250-9500) (*p* < 0.001) respectively, compared to 5.380 of the colitic group (850-15960) (*p* < 0.001) (Table 6 and Figure 6).

The results of this analysis demonstrated a significant decrease in the expression of SOD in colitic controls to 165 (10-1000) (*p* < 0.001) when compared to the non-colitic group 400 (80-1115). The treatment of the animals with *Cd*-EtOHE (125 mg/kg) or *Cd*-HexP (62.5 mg/kg) increased the expression of SOD 400 (50-1480) (*p* < 0.05) and 435 (20-1.650) (*p* < 0.001) respectively compared to the colitic control (Table 6 and Figure 6).

**DISCUSSION**

A promising area for research, many plant species and their chemical constituents exert therapeutic actions. This has led to the development of new, effective and safe drugs for the treatment of various pathological processes. There is an interest in targeted therapy for diseases derived from oxidative stress, such as inflammatory bowel disease[11,25].

IBDs are progressive and destructive chronic disorders of the gastrointestinal tract, the most common being CD, or UC. There is evidence that the pathogenesis of IBD is related to a dysfunctional interaction between the bacteria of the intestinal microflora and mucosal immune system [26,27].

CD and UC are immunologically different diseases. CD is characterized by an exaggerated cellular Th1 response (CD4+) and Th17, characterized by high levels of INF-γ / IL17 and IL-12/IL-23. Ulcerative colitis is characterized by a heightened Th2 response, and excessive IL-5 and IL-13[28–30].

TNBS is a hapten, administered by enema in rats in combination with 50% ethanol to break the mucus barrier and facilitate penetration of the hapten into the intestinal epithelium. TNBS reacts with autologous proteins and stimulates the development of hypersensitivity, leading to the activation of antigen specific T cells. The immune response induced by the hapten causes severe ulceration of the mucosal and epithelial barrier, characterized by trans-mural infiltration of mononuclear cells[20].

Preventive treatment with *Cd*-EtOHE (62.5 to 125 mg/kg) or *Cd*-HexP (31.25 and 62.5 mg/kg) caused a significant reduction in the severity and extent of injury as reflected in the macroscopic lesion score. The macroscopic/microscopic damage scores, and colon weight/length ratio can be considered as sensitive and reliable markers to estimate the severity of the disease and thus the anti-inflammatory effect promoted by the test drug [31].

A low incidence of diarrhea in animals treated with *Cd*-EtOHE and *Cd*-HexP was also observed. Diarrhea is a major symptom of disease in both animals and humans and indicates loss of the absorptive capacity of the colon, which is impaired in an intestinal inflammation[32]. The results suggest that treatment with the plant samples restored the intestinal absorptive capacity.

*Cd*-HexP 62.5 mg/kg significantly reduced the weight/length ratio. This effect is possibly related to the scavenging capacity of free radicals caused by treatment at this dose[33]. The results for the first time demonstrate anti-inflammatory intestinal activity for a species belonging to the genus *Combretum*.

The more effective doses *Cd*-EtOHE 125 mg/kg or *Cd*-HexP 62.5 mg/kg were selected to investigate their effects in the chronic phase with relapse in TNBS-induced ulcerative colitis in rats. This model mimics the disease in humans and can be used to evaluate new treatments potentially applicable in IBD[31].

*Cd*-EtOHE 125 mg/kg or *Cd*-HexP 62.5 mg/kg significantly decreased signs of disease such as macroscopic lesion (lesion area and score), weight/length ratio, and diarrhea; showing the anti-inflammatory effect at 21 d of treatment.

Intestinal inflammation in the TNBS-induced model promoted the loss of 8% to 10% of body weight notably at one week after induction, this is related to reduction in food intake due to abdominal pain and diarrhea during the active phase of the disease[34]. Treatment with *Cd*-HexP 62.5 mg/kg reversed low water and food intake (and body weight loss) caused by disease

The spleen recycles and acts as a reserve red of blood cells. The organ is the center of reticuloendothelial system activity, and is an essential part of the immune system[35]. A significant increase in spleen weight of colitic animals when compared to non-colitic animals was demonstrated. UC and CD are mentioned in lists of factors that can cause increased spleen size, which can occur through lymphoid cell accumulation in immune functions[35,36].

MPO is an enzyme found in neutrophils and has been used as a quantitative index of neutrophil influx into inflamed intestines. The recruitment and activation of neutrophils results in a significant increase in free radical production, capable of overcoming antioxidant protections, and resulting in oxidative stress and inflammation[37,38]. *Cd*-EtOHE 125 mg/kg and *Cd*-HexP 62.5 mg/kg significantly decreased MPO activity when compared to colitic animals. This may be interpreted as a manifestation of anti-inflammatory effect for *C. duarteanum* species.

The inflammation in the TNBS induced colitis model is characterized by a Th1 pathway immune response, in which there is an increase in TNF, IL-1β, IL-12, IL-17, IL-18 and IL-6[39–41].

Up-regulation of the inflammatory state with increased TNF-α and IL-1β levels in colitic rats was observed, which corroborates the literature findings[42]. The treatment with *Cd*-EtOHE 125 mg/kg or *Cd*-HexP 62.5 mg/kg was able to significantly reduce TNF-α levels when compared to the colitic controls, suggesting that TNF-α suppression is related to anti-inflammatory effect promoted by the vegetable samples studied. *Cd*-EtOHE 125 mg/kg was able to decrease IL-1β levels suggesting that compounds of this plant sample may interfere with the synthesis machinery of IL-1β activation by inhibiting production[10,43,44].

IL-10 suppresses production of pro-inflammatory cytokines such as IL-12, IL-6, IL-1 and TNF-α in activated macrophages *in vitro,* and blocks the ability of macrophages stimulating the production of interferon (IFN) by Th1 cells. IL-10 is produced in large amounts by TCD4+ regulatory cells subtype (Tregs). Tregs maintain homeostasis by suppressing the adaptive response of T cells and preventing autoimmunity[45].

A significant elevation in IL-10 levels in animals treated with *Cd*-EtOHE 125 mg/kg or *Cd*-HexP 62.5 mg/kg compared to the colitic group was demonstrated. This increase is attributable to compensatory mechanisms against colonic injury, possibly playing a role in reducing mucosal inflammation and preventing it from becoming uncontrolled. IL-10 down-regulates antigen presentation and thereafter the release of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6[46,47].

Studies have shown that COX-2 is expressed predominantly in experimental colitis. Human and colitic animals show considerable improvement in the inflammatory process when COX-2 inhibitors are used[48]. The vegetable samples tested prevented the increase in expression of this enzyme, and suggest that the intestinal anti-inflammatory effect promoted by *Cd*-EtOHE 125 mg/kg or *Cd*-HexP 62.5 mg/kg is mediated by a reduction in COX-2 expression.

The proliferating cell nuclear antigen (PCNA) is an intra-nuclear protein whose expression is related to cell proliferation and DNA repair. It is highly expressed during the S phase of the cell cycle[49]. Studies show that PCNA expression is up-regulated during chronic inflammation inducing the proliferation of epithelial cells to repair the mucous[50,51].

The positive expression of PCNA increased in colitic animals; while *Cd*-EtOHE 125 mg/kg or *Cd*-HexP 62.5 mg/kg treatment significantly decreased expression of this protein. Treatment with the plant samples studied protected against intestinal epithelial cell damage induced by TNBS and the effect was mediated via regulation of PCNA.

Superoxide dismutase is a key enzyme which converts superoxide to H2O2 a more stable metabolite. During oxidative stress and inflammation SOD activity is decreased in inflamed as compared to non-inflamed tissues. The decreased SOD activity allows superoxide accumulation and subsequent oxidative effects in the intestinal tissue, as well as increased expression of adhesion molecules[52].

Treatment with *Cd*-EtOHE 125 mg/kg or *Cd*-HexP 62.5 mg/kg significantly increased the expression of this enzyme when compared to colitic animals, suggesting involvement of an antioxidant effect in the intestinal anti-inflammatory activity promoted by the vegetable samples.

In conclusion, *C. duarteanum* presents promising anti-inflammatory intestinal effects that are related to reduced levels of the pro-inflammatory cytokines (TNF-α and IL-1β), and increased anti-inflammatory cytokine (IL-10), which feature regulatory effects on the immune response, with reduction in the expression of cyclooxygenase-2 (COX-2), proliferating cell nuclear antigen (PCNA), and an increase in the antioxidant enzyme superoxide dismutase (SOD).

**ACKNOWLEDGMENTS**

We are grateful to José Crispim Duarte, Rodrigo de Oliveira Formiga and the members of the Laboratório de Farmacologia do Trato Gastrintestinal of the Programa de Pós-Graduação em Produtos Naturais e Sintéticos Bioativos for technical support. The authors also thank David Peter Harding for the careful language assistance.

**COMMENTS**

***Background***

A variety of herbal medicines have been shown to produce promising results in the treatment of peptic ulcer and inflammatory bowel disease. *Combretum duarteanum* (*C. duarteanum*), the species selected for this study, is popularly known as “mufumbo, cipiúba, cipaúba”. In folk medicine, it is used to treat pain, inflammation and gastrointestinal tract disorders. Given the need for new inflammatory bowel disease therapies, this study aims to evaluate for the first time the intestinal anti-inflammatory activity promoted by the species *C. duarteanum*, validating its popular use and contributing to the search for new therapies for diseases that affect the gastrointestinal tract.

***Research frontiers***

*C. duarteanum* has presented *in vitro* and *in vivo* anti-inflammatory, antinociceptive, and antioxidant capacity. Furthermore, it was demonstrated low toxicity, gastroprotective and antiulcer activity in different models of acute ulcer induction (acidified ethanol, ethanol, NSAIDs, stress, pylorus ligature and acetic acid) in animals.

***Innovations and breakthroughs***

This study evaluated for the first time the intestinal anti-inflammatory activity promoted by the species *C. duarteanum* Cambess.

***Applications***

This study validated the popular use of *C. duarteanum* and contributes to the search for new therapies for diseases that affect the gastrointestinal tract.

***Peer-review***

Authors demonstrated that *Cd-*EtOHE and *Cd*-HexP obtained from leaves of *C. duarteanum* displays anti-inflammatory effect in TNBS colitis model in rats. These results are promising. The vegetal samples may have a role in antioxidant activity and mucosal healing in ulcerative colitis.

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**P-Reviewer:** Capasso R, Gassler N,Lakatos PL **S-Editor:** Gong ZM

**L-Editor:** **E-Editor:**

**Specialty type:** Gastroenterology and hepatology

**Country of origin:** Brazil

**Peer-review report classification**

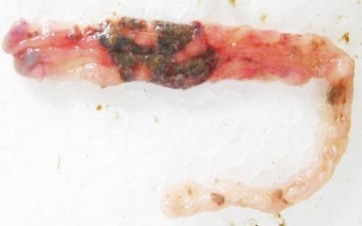
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**A**

**B**

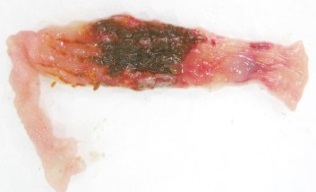
**C**

**D**

**E**

**F**

**Figure 1 Representative macroscopic of rat colonic mucosa in non-colitics (A), colitics (B), *Cd*-EtOHE 31.25 mg/kg (C), *Cd*-EtOHE 62.50 mg/kg (D), *Cd*-EtOHE 125 mg/kg (E), and *Cd*-EtOHE 250 mg/kg (F).**



**A**

**B**

**C**

**D**

**E**

**F**

**Figure 2 Representative macroscopic of rat colonic mucosa in non-colitics (A), colitics (B), *Cd*-HexP 31.25 mg/kg (C), *Cd*-HexP 62.50 mg/kg (D), *Cd*-HexP 125 mg/kg (E), and *Cd*-HexP 250 mg/kg (F).**



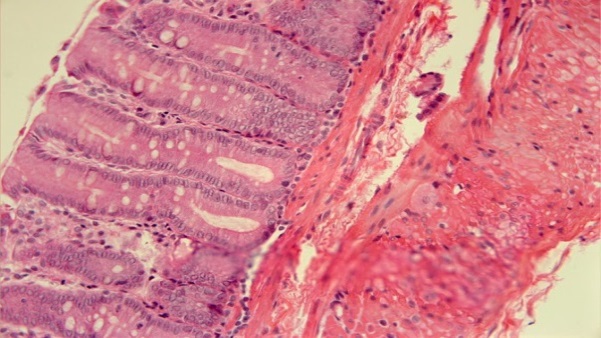
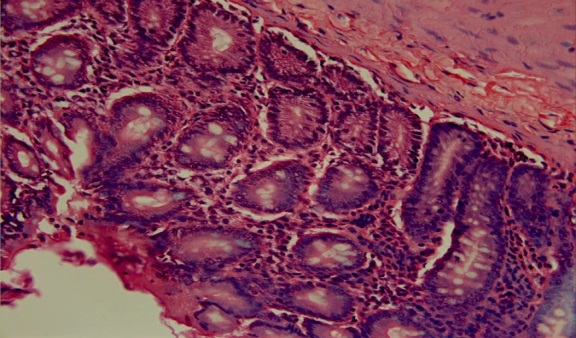
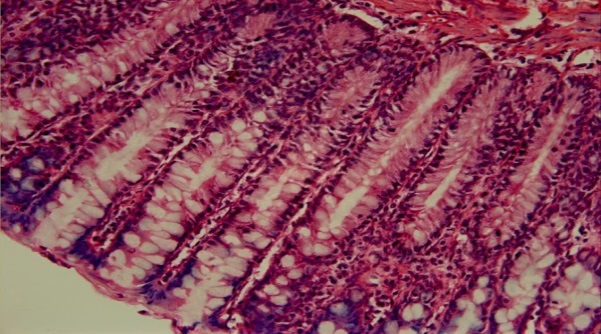
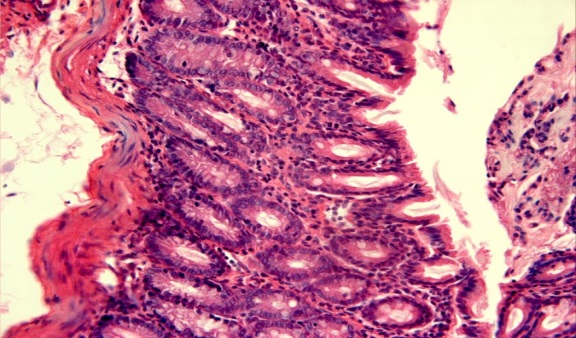
**A**

**B**

**C**

**D**

**Figure 3 Representative macroscopic of rat colonic mucosa in non-colitics (A), colitics (B), *Cd*-EtOHE 125 mg/kg (C), *Cd*-HexP 62.5 mg/kg (D).**



\*

**\***

**A**

**B**

**C**

**D**

**Figure 4 Representative histological appearance of rat colonic mucosa in non-colitic group (A), colitic group (B), *Cd*-EtOHE (C), and *Cd*-HexP (D).** aumento 40 × (\*goblet cells; → ulceration region; 50 μm). Colonic tissue sections were stained with hematoxylin and eosin (H&E), and observed under light microscope (40 ×).

A



B



**C**



D



Figure 5 Effect of acute administration of *Cd-*EtOHE 125 mg/kg and *Cd-*HexP 62,5 mg/kg on myeloperoxidase activity (MPO, U/mg protein) (A), tumor necrosis factor-alpha (TNF-α, pg/mg protein) (B), interleukin-1β (IL-1β, pg/mg protein) (C), interleukin-10 (IL-10, pg/mg protein) (D) in trinitrobenzene sulfonic acid-induced colitis model with relapse in rats (TNBS,10 mg/animal). Data are expressed as the means±S.E.M. a*P* < 0.05; b*P* < 0.01 and c*P* < 0.001 *vs* colitic group.d*P* < 0.05; e*P* < 0.01 and f*P* < 0.001 *vs* non-colitcs.

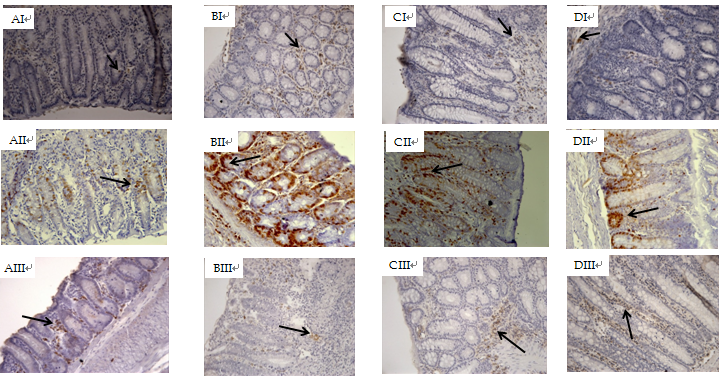


Figure 6 Photomicrograph of colonic samples from rats submitted to itrinitrobenzene sulfonic acid-induced colitis model with relapse in rats after 21 d of treatment. A: Non-colitic group, B: colitic group, C: *Cd*-EtOHE 125 mg/kg, D: *Cd*-HexP 62.5 mg/kg. Immunohistochemical localization of Line I- COX-2; Line II- PCNA; Line III SOD (→ COX-2, PCNA and SOD, respectively). COX-2: Cyclooxygenase-2; PCNA: Proliferating cell nuclear antigen; SOD: Superoxide dismutase.

**Table 1 Effects of oral administration of *Cd*-EtOHE or *Cd*-HexP in acute phase of intestinal inflammation in TNBS-induced ulcerative colitis in rats**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Groups** | **Dose (mg/kg)** | **ULA (mm2)** | **Inhibition (%)** | **Lesion score** | **Weight/length (mg/cm)** | **Diarrhea (%)** |
| Non-colitics | - | - | 100 |  | 110 ± 7.7 | 0 |
| Colitics | - | 107 ± 38 | - | 6.0 (5-7) | 148 ± 17f | 100 |
| *Cd*-EtOHE | 31.25 | 79 ± 31 | 26 | 5.0 (3-7) | 152 ± 19f | 100 |
|  | 62.5 | 46 ± 12b | 57 | 4.0 (1-5)a | 146 ± 19f | 57 |
|  | 125 | 19 ± 8e | 82 | 3.0 (2-5)b | 134 ± 5c | 14b |
|  | 250 | 76 ± 23 | 29 | 6.0 (4-7) | 152 ± 14f | 86 |
| Non-colitics | - | - | - | - | 102 ± 14 | 0 |
| Colitics | - | 101 ± 45 | - | 7.0 (5-8) | 154 ± 27f | 87 |
| *Cd*-HexP | 31.25 | 52 ± 18b | 49 | 5.0 (1-6)a | 142 ± 20c | 87 |
|  | 62.5 | 21 ± 7e | 79 | 5.0 (4-5)b | 129 ± 20a | 29a |
|  | 125 | 95 ± 33 | 7 | 6.0 (5-6) | 154 ± 10f | 71 |
|  | 250 | 80 ± 12 | 22 | 5.0 (4-7) | 155 ± 16f | 86 |

Results expressed as mean ± S.D or median (minimum-maximum) of the parameters analyzed (*n* = 5-8). For the parametric data was used mean ± S.D. ANOVA and a posteriori Dunnet test. Nonparametric data (median, minimum-maximum) Kruskal Wallis test and a posteriori Dunn. a*P* < 0.05, b*P* < 0.01 and e*P* < 0.001 *vs* colitic group; c*P* < 0.05, d*P* < 0.01 and f*P* < 0.001 *vs* non-colitic group.

**Table 2 Effects of oral administration of *Cd*-EtOHE or *Cd*-HexP in chronic phase with relapse of intestinal inflammation in TNBS-induced ulcerative colitis in rats**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Groups** | **Dose (mg/kg)** | **Lesion score** | **Weight/length (mg/cm)** | **Diarrhea (%)** |
| Non-colitics | - |  | 97 ± 9 | 0 |
| Colitics | - | 4.0 (3-6) | 143 ± 14f | 94 |
| *Cd*-EtOHE | 125 | 1.0 (1-4)a | 132 ± 11f | 56a |
| *Cd*-HexP | 62.5 | 1.0 (1-4)b | 122 ± 12b,d | 56a |

Results expressed as mean ± S.D or median (minimum-maximum) of the parameters analyzed (*n* = 7-9). For the parametric data was used mean ± S.D. ANOVA and a posteriori Dunnet test. Nonparametric data (median, minimum-maximum) Kruskal Wallis test and a posteriori Dunn. a*P* < 0.05, b*P* < 0.01 and e*P* < 0.001 *vs* colitic group; c*P* < 0.05, d*P* < 0.01 and f*P* < 0.001 *vs* non-colitic group.

**Table 3 Effect of oral administration of *Cd*-EtOHE or *Cd*-HexP for 21 d on water and food consumption in TNBS-induced ulcerative colitis in rats**

|  |  |  |  |
| --- | --- | --- | --- |
| **Groups** | **Dose** | **Water intake (mL)** | **Food intake (g)** |
| Non-colitics | - | 31 ± 4 | 22 ± 2 |
| Colitics | - | 28 ± 3d | 19 ± 2d |
| *Cd*-EtOHE | 125 mg/kg | 30 ± 4 | 20 ± 3c |
| *Cd*-HexP | 62.5 mg/kg | 31 ± 2a | 22 ± 3b |

Values are expressed as mean ± SD (*n* = 7-9). One-way ANOVA, followed by Dunnett's test, a*P* < 0.05 and b*P* < 0.01 *vs* colitic group; c*P* < 0.05 and d*P* < 0.01 *vs* non-colitic group.

**Table 4** **Effect of oral administration of *Cd*-EtOHE or *Cd*-HexP for 21 d on body weight on TNBS-induced ulcerative colitis in rats**

|  |  |  |  |
| --- | --- | --- | --- |
| **Groups** | **Initial weight (g)** | **Final weight (g)** | Average increase (g) |
| Non-colitics | 202 ± 32 | 261 ± 35 | 51 ± 10 |
| Colitics | 189 ± 19 | 215 ± 21f | 35 ± 12c |
| *Cd*-EtOHE | 178 ± 13 | 230 ± 7 | 43 ± 6 |
| *Cd*-HexP | 184 ± 14 | 239 ± 17a | 48 ± 10a |

Values are expressed as mean ± SD (N = 7-9). One-way ANOVA, followed by Dunnett's test, a*P* < 0.05 *vs* colitic group; c*P* < 0.05, f*P* < 0.001, *vs* non-colitic group.

**Table 5 Effect of oral administration of *Cd*-EtOHE or *Cd*-HexP for 21 d on weight organs in TNBS-induced ulcerative colitis in rats**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Groups** | **Heart** | **Liver** | **Kidneys** | Spleen |
| Non-colitics | 4.0 ± 0.2 | 41 ± 1.5 | 8.6 ± 0.6 | 2.1 ± 0.2 |
| Colitics | 4.0 ± 0.5 | 43 ± 5.3 | 8.6 ± 1.0 | 2.8 ± 0.5c |
| *Cd*-EtOHE | 4.0 ± 0.3 | 43 ± 3.1 | 8.6 ± 0.2 | 2.8 ± 0.4d |
| *Cd*-HexP | 4.0 ± 0.3 | 44 ± 1.2 | 8.4 ± 0.5 | 2.4 ± 0.2 |

Values are expressed as mean ± SD (*n* = 7-9). One-way ANOVA, followed by Dunnett's test, c*P* < 0.05 and d*P* < 0.01 *vs* non-colitic group.

**Table 6 Effect of oral administration of *Cd*-EtOHE or *Cd*-HexP for 21 days on the expression of cyclooxygenase-2, proliferating cell nuclear antigen and superoxide dismutase in TNBS-induced ulcerative colitis in rats**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Groups** | **Dose (mg/kg)** | **COX-2 (µm2)** | **PCNA (µm2)** | **SOD (µm2)** |
| Non-colitics | - | 230 (110-700) | 1425 (60-7890) | 400 (80-1.115) |
| Colitics | - | 505 (100-1.450)c | 5380 (850-15.960)c | 165 (10-1.000)f |
| *Cd*-EtOHE | 125 | 90 (10-2.590)e | 1630 (90-14.790)e | 400 (50-1.480)e |
| *Cd*-HexP | 62.5 | 205 (20-790)e | 1570 (250-9.500)e | 435 (20-1.650)e |

Results are expressed as median (minimum-maximum) of the analyzed parameters (*n* = 7-9). For nonparametric data (median, minimum-maximum) Kruskal Wallis and Dunn’s test *a posteriori.* e*P* < 0.001 *vs* colitic group; c*P* < 0.05 and f*P* < 0.001 *vs* non-colitic group. COX-2: cyclooxygenase-2; PCNA: Proliferating cell nuclear antigen; SOD: superoxide dismutase.