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Epiisopiloturine, an imidazole alkaloid, reverses inflammation and lipid peroxidation parameters in the Crohn disease model induced by trinitrobenzenosulfonic acid in Wistar rats

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ABSTRACT

Epiisopiloturine (EPI) is an important imidazole alkaloid because of its pharmacological properties. The aim of this study was to investigate the effects of epiisopiloturine on inflammatory parameters of the colonic mucosa in a rat model of Crohn's disease (CD). For this, we induced colitis using trinitrobenzenosulfonic acid and determined myeloperoxidase (MPO), interleukin 1 β (IL-1β), glutathione (GSH), and malondialdehyde (MDA) levels in the intestinal mucosa. The location and expression of the inflammatory markers in the colon were investigated by immunohistochemistry for NO synthase induced (iNOS), interleukin 1 beta (IL-1β), and cyclooxygenase-2 (COX-2) and western blotting (iNOS and COX-2), respectively. Compared with TNBS alone, epiisopiloturine at 1 mg/kg reduced the macroscopic and microscopic scores, wet weight of the colon, and neutrophilic infiltration and expression of the pro-inflammatory cytokine IL-1β. Epiisopiloturine at 1 mg/kg maintained or restored GSH levels and simultaneously decreased MDA levels. Animals treated with epiisopiloturine exhibited reduced immunostaining for IL-1β, iNOS, and COX-2 and reduced cell count per field. Epiisopiloturine reduced the expression of COX-2 and iNOS in the colon. Based on these findings, we conclude that epiisopiloturine at 1 mg/kg may be an important pharmacological tool against intestinal inflammatory diseases due to its inhibitory action on key enzymes and products involved in inflammation.

1. Introduction

Several species of catalogued plants are reported to have pharmacologic properties, particularly the species *Pilocarpus microphyllus stapf*, popularly known as jaborandi. It is a small, bushy plant belonging to the family Rutaceae, is widely distributed in Northern and Northeastern Brazil, and has great economic importance due to its bioactivity. This species is used as a raw material for the isolation of alkaloids with pharmacological properties, an important class of structurally diverse compounds that possess nitrogen heterocyclic ring atoms and amino acid derivatives [1,2].

One of the main alkaloids extracted from jaborandi is epiisopiloturine (EPI), an imidazole derivative. This alkaloid has several proven pharmacological properties, such as activity against *Schistosoma mansoni* [3], anthelmintic, antibacterial, and cytotoxicity activity [4], protection against naproxen-induced gastrointestinal damage [5], and anti-inflammatory properties [6]. The anti-inflammatory activity of the molecule was evaluated in a model of peritonitis and paw edema

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induced by various agents in Swiss mouse and was shown to significantly reduce the formation of edema, leukocyte migration, myeloperoxidase (MPO) activity, and levels of TNF-α and IL-1β in the peritoneal cavity [6].

Inflammation is part of the etiopathology of Crohn's disease (CD), which affects the entire digestive tract, from the mouth to the anus, and has substantial clinical significance. Its clinical presentation varies according to the extension, intensity, and complications present, starting typically with bouts of diarrhea, fever, abdominal pain, and weight loss, with potential local, systemic, and nutritional complications. The disease worsens and crises become more frequent, generating commitment of general state, and worsening the individual's quality of life [7,8]. In this context, this study aimed to investigate the anti-inflammatory effects of EPI in the colonic mucosa of TNBS-treated rats as a model of experimental Crohn's disease.

2. Material and methods

2.1. Test compound

The organic phase of EPI was submitted to liquid–liquid extraction, alkalinized with ammonium hydroxide solution and precipitated in the neutral form; then, the solution was filtered under reduced pressure (Fig. 1). After further work-up, EPI was confirmed to be pure by high performance liquid chromatography (HPLC) (> 95% w/w), with 1 H nuclear magnetic resonance (NMR) and mass spectroscopy MS/MS. EPI samples was purified from the leaves of *P. microphyllus stapf* in > 98% purity, as described previously by Veras et al [3].

2.2. Animals and ethics

Female Wistar rats (*Rattus norvegicus*) (150–200 g) were kept in a temperature-controlled room at 25 ± 2 °C under a light/dark cycle of 12:00/12:00 h, with free access to food and water, at the Central Federal University Biologist. Animals were fasted for 12 h before all experiments and provided free access to water. Experiments were conducted in accordance with current established principles for the care and useof research animals (National Institutes of Health guidelines) and this study was approved by the Animal Research Ethics Committee (CEUA) of the Federal University of Piaui (approval number, 056/15).

2.3. Induction of colitis

Animals were divided into six groups (n = 6/group): the negative control group received sterile 0.9% saline solution rectally; the control group received TNBS solution rectally; the treated groups received EPI solution at doses of 0.1, 0.3, or 1 mg/kg, (intraperitoneally; *i.p.*) or dexamethasone 2 mg/kg subcutaneously (*s.c.*), 1 h prior to induction of colitis by TNBS and once daily for 3 days after the induction of colitis. To induce colitis, animals were anesthetized with previously used ketamine (250 mg/kg *s.c.*) and positioned in the left lateral decubitus. Colitis was induced by administering (800 μL) 20 mg of TNBS dissolved in 50% ethanol solution to the rat colon, rectally, by means of a polyethylene catheter n° 6 that was introduced 8 cm per pathway anal. Immediately after, the animals were placed in a vertical position, upside down, for 30 s and then placed back in the boxes with free access to water and food. After the third day, the animals were euthanized 1 h after treatment and samples were obtained for subsequent analyses. A portion of the colon was excised, rinsed with sterile 0.9% saline and the wet weight was evaluated using an analytical scale. Samples were then embedded in wax blocks and fixed for macroscopic score assessment according to the criteria described by Morris [9] and adopted by Brito et al [10].

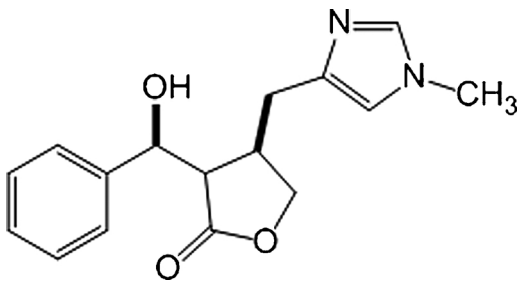

Fig. 1. Chemical structure of Epiisopiloturine.

2.4. Histopathological analysis

Histopathological scores were used to evaluate colitis according to the criteria presented by Appleyard and Wallace [11]. Intestinal samples were fixed in 10% formalin solution for 24 h. Then, samples were transferred to a solution of 70% alcohol. The material was then embedded in paraffin and sectioned; 4 μm thick sections were stained with hematoxylin/eosin and evaluated by an experienced pathologist (P.M.G.S). Histological criteria evaluated included: mucosal architecture loss (0–3), cellular infiltrate (0–3), muscle thickening (0–3), crypt abscess (0, absent; 1, present), and goblet cell depletion (0, absent; 1, present).

2.5. Myeloperoxidase (MPO) activity assay

The tissue was homogenized in 1 mL of potassium buffer with 0.5% of hexadecitrimetilamônio (HTAB). Then, homogenatewas centrifuged at 40.000 *g* for 10 min at 4 °C. MPO activity inthe resuspended pellet was assayed by measuring the changein absorbance at 450 nm using o-dianisidine dihydrochloride and 1% hydrogen peroxide. mlThe results are expressed as MPO units per milligram of colon tissue [12].

2.6. Malondialdehyde acid (MDA) assay

To quantify the levels of MDA, colon samples were homogenized in cold 1.15% KCl to prepare 10% homogenates. Immediately after, 250 μL of each homogenate was added to 1.5 mL of 1% H_3PO_4 and 0.5 mL of 0.6% tert-butyl alcohol (aqueous solution). Then, this mixture was stirred and heated in a boiling water bath for 45 min. The preparation was then cooled immediately in an ice water bath, followed by the addition of 2 mL of n-butanol. This mixture was shaken, and the butanol layer was separated by centrifugation at 1200 *g* for 10 min. Absorbance was determined at 520 and 535 nm, and the difference between the two values was calculated [13]. MDA concentrations are expressed as millimoles per gram of tissue.

2.7. Glutathione assay

Samples were homogenized in cold 0.02 M EDTA solution (1 mL/100 mg of tissue). Aliquots (400 μL) of tissue homogenate were mixed with 320 μL of distilled water and 80 μL of 50% (w/v) trichloro acetic acid in glass tubes and centrifuged at 3000 rpm for15 min. Next, 400 μL of each supernatant was mixed with 800 μL of Tris buffer (0.4 M, pH 8.9), and 20 μL of 0.01 M 5,5-dithio-bis(2-nitrobenzoic acid). After shaking the preparation, absorbance was measured at 412 nm on a spectrophotometer [14]. The results are expressed as micrograms of GSH per gram of tissue.

2.8. Cytokine determination

Samples of intestinal tissue were collected and homogenized in sterile saline. After that, the interleukin (IL)-1β levels were measured using (Enzyme-Linked Immunosorbent Assay) ELISA kits according to the manufacturer's recommendations [8]. The homogenates were centrifuged at 0.8 *g* at 4 °C for 10 min, and supernatants were stored at