Canthin-6-one ameliorates TNBS-induced colitis in rats by modulating inflammation and oxidative stress. An *in vivo and in silico* approach

Running title: **Canthin-6-one ameliorates ulcerative colitis in rats**

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Bullet Point Summary

What is already known?

- Canthin-6-one has pharmacological properties that could be useful for ameliorating inflammatory disorders, such as IBD.

What this study adds?

- In *in vivo/silico* studies, canthin-6-one reduced pro-inflammatory mediators and oxidative stress, relieving greatly ulcerative colitis.

What is the clinical significance?

- Findings suggest canthin-6-one as novel candidate with better efficacy than current drugs for IBD.

ABSTRACT

Background and Purpose: Canthin-6-one (**Cant**) is an indole alkaloid found in different medicinal plants, reported to be gastroprotective, anti-inflammatory, anti-microbial, anti-diarrheal and anti-proliferative. We aimed to explore **Cant** in the management of ulcerative colitis (UC) using a trinitrobenzenesulfonic acid (TNBS)-induced rat model.

Experimental Approach: Cant (1, 5 and 25 mg/kg) was administered by oral gavage to Wistar rats followed by induction of colitis with TNBS. Macroscopic and histopathological scores, myeloperoxidase (MPO), malondialdehyde (MDA) and reduced glutathione (GSH) were assessed in colon tissues. Pro- (TNF-α, IL-1β and IL-12p70) and anti-inflammatory (IL-10) cytokines, and vascular endothelial growth factor (VEGF) were also quantified. Mitogen-activated protein kinase 14 (MAPK14) and Toll-like receptor-8 (TLR8), as putative targets, were considered through *in silico* analysis.

Key Results: Cant (5 and 25 mg/kg) reduced macroscopic and histological colon damage scores in TNBS-treated rats. MPO and MDA were reduced by up to 61.69% and 92.45%, respectively, compared to TNBS-treated rats alone. Glutathione concentration was reduced in rats administered with TNBS alone (50.00% of sham group), being restored to 72.73% (of sham group) under **Cant** treatment. TNF-α, IL-1β, IL-12p70 and VEGF were reduced, and anti-inflammatory IL-10 was increased following **Cant** administration compared to rats administered TNBS alone. Docking ligation results for MAPK14 (p38α) and TLR8 with **Cant**, confirmed that these proteins are feasible putative targets.

Conclusions and Implications: Cant has an anti-inflammatory effect in the intestine by down-regulating immune molecular mediators and decreasing oxidative stress. Therefore, **Cant** could have therapeutic potential for the treatment of inflammatory bowel disease and related syndromes.

KEYWORDS: Canthin-6-one, inflammatory bowel disease, TNBS, MAPKs, TLRs, cytokines, VEGF, ulcerative colitis

ABBREVIATIONS

IBD - Inflammatory bowel diseases; UC – ulcerative colitis; TNBS – 2,4,6 trinitrobenzenesulfonic acid; MPO – myeloperoxidase; GSH - reduced glutathione; MDA – malondialdehyde; **Cant** - canthin-6-one; PAS - Schiff's periodic acid; H&E, haematoxylin and eosin; MAPK14 - mitogen-activated protein kinase 14 (p38α kinase); TLR8 - Toll-like receptor 8; VEGF - vascular endothelial growth factor.

1. INTRODUCTION

Inflammatory bowel diseases (IBD) are predominantly categorised into Crohn's disease (CD) and ulcerative colitis (UC) that are chronic idiopathic diseases and commonly affect colonic and rectal mucosal layers (Matkowskyj *et al*., 2013). The clinical symptoms of UC are abdominal pain, recurrent bowel inflammation, haematochezia and diarrhoea that often contain blood or mucus, malabsorption, weight loss and fatigue. Usually, the course of the disease comprises periods of worsening, such as chronic and recurrent inflammation, alternating with periods without inflammation (remission), which occur throughout the patient's life (Flynn and Eisenstein, 2019).

IBD has pathology of unknown aetiology. However, it is widely accepted that several factors including genetic susceptibility, alterations in intestinal cell processes, dysregulation of the immune system, intolerance to microbiota, and environmental factors all contribute, in an environment of oxidative stress, to IBD pathogenesis (Tian *et al.*, 2017).

In the last decade, IBD has emerged as a growing worldwide public health challenge and in Westernised countries is associated with morbidity, mortality and substantial costs to the healthcare systems. IBD affects more than 2 million people in Europe and is similarly prevalent in North America (Kaplan, 2015). Recent epidemiological studies suggest that IBD incidence is now fast rising in South America, Eastern Europe, Asia and Africa, but the true prevalence in these regions remains uncertain (Kaplan and Ng, 2017).

As IBD aetiology remains to be elucidated, pharmacological treatments aim to attenuate clinical symptoms in the acute phase and to promote remission of the disease.

The treatment of mild to moderate exacerbations is made with oral or topical administration of 5-aminosalicylate (mesalazine), sulphonamide (sulfasalazine), or corticoids. Severe conditions require the use of intravenous (iv) corticosteroids and maintenance with mesalazine or sulfasalazine. When the response is inadequate, cyclosporine or infliximab iv is used. With the exception of mesalazine and sulfasalazine, the other therapies are intended to induce immunosuppression, by blocking mainly TNF-α or NF-κB-mediated inflammation (Tian *et al*., 2017). In refractory IBD, surgical intervention may become necessary. However, the currently available treatments have several disadvantages, such as low remission rate when using sulfasalazine or mesalazine, harmful side effects (loss of tissue function, increased risk of infections, lymphomas, and skin cancer) when using steroid anti-inflammatory drugs, and the economic cost associated with the biological therapy with antibodies *(Wright et al.*, 2018). Thus, there is a necessity to find new treatments that are either adjunctive or complementary therapies for UC, with better efficacy and reduced potential side-effects. In this way, herbal immunomodulatory agents may represent a promising approach for UC therapy, as demonstrated by the variety of preclinical and clinical studies currently in progress (Ke *et al*., 2012).

Canthinones are a subclass of β-carboline alkaloids with an additional D ring that are often found in Simaroubaceae and Rutaceae plant extracts (Devkota *et al*., 2014). Canthinones have been reported to have a wide range of potential therapeutic properties, shared with their corresponding source plant extracts, such as anti-pyretic, analgesic, gastric ulcer protection, anti-microbial (for bacteria, viruses and parasites), anti-diarrhoeal and anti-inflammatory. They also display efficacy as anti-proliferative and/or cytotoxic agents against several human cancer cell lines, suggestive that they will have therapeutic properties for cancer chemoprevention (Dejos *et al.*, 2014a). Within this class of alkaloids, canthin-6-one (**Cant**) is in the spotlight by showing efficacy against *Trypanosoma cruzi* (Ferreira *et al.*, 2011) and chloroquine resistant strains of *Plasmodium falciparum* (Cebrián-Torrejón *et al*., 2013), anti-ulcerogenic activity (de Souza Almeida *et al*., 2011), and cytotoxic activity against many cancer cell lines (Dejos *et al*., 2014b; Vieira Torquato *et al*., 2017).

Because of this, there is a growing pharmaceutical interest in the production of canthin-6-one, however, it is difficult to extract from natural sources. Great efforts have been made to increase the production to a commercial level of industrial value. *In vitro*

suspensions of cultured plant cells supplemented with tryptophan, the natural biosynthetic source, have recently been used to efficiently maximise the yield *(Wagih et al*., 2008). A synthetic chemical pathway has now also been described starting from tryptamine (Cebrián-Torrejón *et al*., 2013).

Therefore, the purpose of this study was to evaluate the anti-ulcerative colitis activity of **Cant** in a 2,4,6 trinitrobenzene sulfonic acid (TNBS)-induced experimental rat colitis model. This is a well-established model that mimics human colitis on the basis of its histological and biochemical characteristics, further supported by studies on biochemical and inflammatory markers (Antoniou *et al*., 2016). It is widely used for preclinical testing of chemical or natural compounds to determine their possible anti-colitis potential, focusing primarily on their anti-inflammatory and antioxidant activity.

In this model, we analysed colon damage through macroscopic and histologic scoring, colonic mediators involved in the inflammatory response including interleukins (IL-1β, IL12p70, IL-10), tumour necrosis factor -alpha (TNF-α), vascular endothelial growth factor (VEGF), and oxidative stress parameters, such as glutathione (GSH), malondialdehyde (MDA) and myeloperoxidase (MPO) were also measured. Additionally, an *in silico* analysis, directed towards known molecular targets involved in gut inflammatory signalling such as Toll-like receptor 8 (TLR8) and mitogen-activated protein kinase 14 (MAPK14 or p38α) was conducted to strengthen the findings, concluding all together than **Cant** has potential as an anti-ulcerative colitis agent.

2. METHODS

2.1. Chemicals

Due to the low yield of **Cant** attained when isolated from plant extracts, the alkaloid used was purchased from Alpha Chimica (Chatenay-Malabry, France), CAS no. 479-43-6, purity > 96%. To confirm purity and, in order to ensure that the commercial product is the same previously described in the isolation from plant sources, we compared both compounds by gas chromatography-mass spectrometry (GC-MS), hydrogen-1 nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) (s*ee the supplementary material for additional information*). For

that, we prepared a sufficient quantity of the natural compound, using rhizome methanol extracts of *Simaba ferruginea* A. St.-Hil., followed by dichloromethane fractionation, using methods described by Noldin *et al.* (2005).

Unless otherwise specified, the other chemicals used in this work were purchased from Sigma (St. Louis, MO, USA), as mesalazine, 5% TNBS solution, 5.5-dithiobis-(2-nitrobenzoic) (DTNB), thiobarbituric acid, trichloroacetic acid, sodium bicarbonate, carboxymethylcellulose (CMC), potassium chloride, sodium chloride, dibasic potassium phosphate, monobasic potassium phosphate, reduced glutathione, and Griess reagent.

2.2 Animals

Male Wistar rats (*Rattus norvegicus,* adults), 180 to 200 g, were procured from the "Biotério Central" (animal house), Universidade Federal de Mato Grosso (UFMT), Cuiabá, Brazil. The animals were maintained in cages at 21 ± 2 °C, humidity of 50 ± 1%, in a light/dark cycle of 12 h, receiving standard feed (NUVILAB®, Quimtia, Paraná, Brazil) and water *ad libitum*. The rats were fasted for 12 h prior to the experiment but with free access to water. Experimental procedures were carried out in accordance with the guidelines of the European Commission (2010/63/EU), and licensed by the "Comitê de Ética no Uso de Animais, UFMT" (No. CEUA/UFMT-23108,914446/2018-58).

2.3 Evaluation of Cant effects in TNBS-induced ulcerative colitis

UC was induced by TNBS in animals as previously described (Morampudi et al., 2014). Animals were randomly placed into six groups of 8 rats and orally administered with either vehicle (2% Tween 80, 1 mL/100 g), **Cant** (1, 5 or 25 mg/kg p.o., dissolved in 2% Tween 80) or mesalazine (500 mg/kg, p.o., dissolved in carboxymethyl cellulose at 0.3 mg/mL) for 72, 48, 24 and 2 h before UC-induction by TNBS. Following pre-treatment, rats were anaesthetised by a solution of ketamine/xylazine (60/8 mg/kg, i.p.) and induction of UC was conducted by rectal administration of 30 mg/mL TNBS in 20% EtOH solution (250 µL) into the lumen, through a polyurethane catheter (medical-grade). The sham group received distilled water (10 mL/kg, p.o.) during pre-treatment, and 250 µL of 0.9% saline by rectal instillation instead of TNBS. Subsequently, rats were held in a head down position for 2 min to allow the uniform distribution of TNBS and to avoid leakage back out of the colon (Arunachalam *et al*., 2020).

2.4 Macroscopic assessment of colon damage

After 24 h of TNBS-treatment, rats were sacrificed under anaesthesia and an 8 cm length of distal colon was removed, opened longitudinally, rinsed thoroughly in ice-cold saline (0.9%) and weighed. Colon was subjected to macroscopic analysis to assess oedema, necrosis and ulceration of the mucosa that were scored according to a previously described classification criteria (Morris *et al*., 1989). Briefly, (score 0–5): 0 - no damage; 1 - local hyperaemia, but no ulcers; 2 - ulceration without hyperaemia or thickening of the intestinal wall; 3 - ulceration with inflammation in 1 site; 4 - two or more sites of ulceration and/or inflammation; 5 - two or more sites of inflammation and ulceration or one site of inflammation and ulceration > 1 cm along the length of the colon.

2.5. Colonic tissue histological damage analysis

After macroscopic analysis, colons were prepared for microscopic analysis. Approximately 2 cm from the colon was collected and stored in 4% paraformaldehyde solution in phosphate buffer, pH 7.4. Tissues were processed and embedded in paraffin by standard methods using a histological processor (MTP 100 Slee, Mainz, Germany). Tissues were then cut to 3 μm thick sections and placed onto microscope slides using a microtome (Hyrax M60 Carl Zeiss, Oberkochen, Germany). Sections were subjected to haematoxylin and eosin (H&E) staining, for histopathological evaluation of the epithelial layer, submucosa and muscular tissue damage, and to Periodic acid Schiff's (PAS) staining, to assess goblet cell abundance (mucus secreting cells). After the staining, slides were analysed using optical microscopy. Intestinal inflammation was quantified according to a 0-4 scale using a previously reported method (Arunachalam *et al.*, 2020). For that, mucosal damage, presence of inflammatory cells, cell infiltration in the submucosa, ulceration and presence of goblet cells were assessed (0-4: graded according to the intensity of each one). The scores obtained for each parameter were added up to quantify the total microscopic-assessed damage (Arunachalam *et al*., 2020).

2.6 Determination of *in vivo* antioxidant activity

Colon tissues were weighed, placed in 10 mM Tris buffer, pH 7.4, 150 mM NaCl and 1% Triton X-100 and stored in a biofreezer at -86 ° C. Samples containing colon strips were thawed, disintegrated and homogenized (MA102 Turrax type, MARCONI, São Paulo, Brazil) in potassium phosphate buffer (200 mM, pH 7.5) in a 1:10 ratio (w/v). Aliquots of the resulting homogenate were reserved for quantification

of glutathione content (GSH), and the remaining homogenate was centrifuged at 1538 × g for 20 min. The resulting supernatant was used for determination of the concentration of tissue malondialdehyde (MDA). The pellet was used to determine myeloperoxidase (MPO) activity.

The amount of non-protein sulfhydryl groups in the tissue homogenate was determined using methods adapted from those previously reported (Sedlak and Lindsay, 1968). For that, 50 µL of colon homogenate and 40 µL of 12% trichloroacetic acid (TCA) were mixed, vortexed for 10 min and centrifuged 15 min at 1008 x g. Ten (10) µL aliquots of the supernatant, together with 190 µL of 0.4 M Tris-HCl buffer (pH 8.9) were placed in 96-well microplates. Then, the reaction was initiated by the addition of 5 µL of 0.01M 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (a reagent that, when in contact with sulfhydryl groups, produces a yellow colour) 5 min before spectrophotometric reading at 415 nm. Values were interpolated in a standard GSH curve and expressed in nmol GSH/g tissue.

The amount of MDA (lipohydroperoxide) in colon homogenates was determined as described by Mihara and Uchiyama (1978) through the determination of thiobarbituric acid (TBA) reactive substances. In 500 µL of the homogenate, 3 mL of 1% phosphoric acid (H_3PO_4), 1 mL of 0.6% TBA and 0.15 mL of 0.2% butylhydroxytoluene (BHT) in 95% methanol was added. The samples were heated at 90ºC in a water bath for 45 min, cooled, and vigorously stirred after adding 4 mL of butanol. The butanol phase was separated by centrifugation for 10 min at 1,985 x g and the absorbance read at 535 and 520 nm. The amount of MDA was inferred using a standard curve, obtained from known concentrations of 1,1,3,3-tetramethoxypropane (100 - 6.25 µmol/mL). Values were expressed in µmol MDA/g of tissue.

Finally, to determine extent of MPO activity, pellets resulting from colon tissue homogenates were resuspended in 1 mL of 2% NaCl cold solution, vortexed for 30 s, followed by a new centrifugation at 10,000 *x* g for 10 min at 4 °C, and the supernatant discarded. The pellet was resuspended in $NaPO_4$ (0.05 M, pH 5.4) plus 0.5% hexadecyltrimethylammonium bromide (100 mg tissue/mL of buffer), frozen and thawed in liquid nitrogen (3 times), centrifuged for 15 min at 10,000 *x* g at 4 °C, and the supernatant used for the enzymatic assay. In a 96-well plate, 5 µL of the supernatant plus 45 µL of $NaPO_4$ buffer was placed in triplicate, after adding 25 µL of TMB (“3.3”,

5.5′-tetramethylbenzidine) and 100 µL of H_2O_2 solution (0.017%, in 0.05 M phosphate buffer, pH 5.4). The microplate was kept in an oven at 37 °C for 5 min. Then, the reaction was stopped with 50 µL of 4 M sulfuric acid and read at 450 nm (Matos *et al.*, 1999).

2.7 Determination of IBD-related cytokines and growth factor

Weighed colon strips were thawed, disintegrated and homogenized in 0.2 M phosphate buffer and the concentrations of TNF- α, IL-1β, IL-12p70, IL-10, and VEGF determined following the instructions described in a Milliplex kit (Sigma®, St. Louis, MO, USA), using a magnetic-based dual laser detection instrument (Luminex® XMAP Technology, MAGPIX, Texas, USA).

2.8 Biological Activity Spectrum Prediction

The 2D structure of **Cant** was computationally designed using ACD/ChemSketch v.2018.2.5 software and submitted to PASS (Prediction of Activity Spectra for Substances) web tool to predict its biological activity spectrum (Parasuraman, 2011) (http://www.way2drug.com/PASSOnline/). PASS is an online server (hosted by the V. N. Orechovich Institute of Biomedical Chemistry under the aegis of the Russian Foundation of Basic Research) that has the ability to predict 3678 kinds of pharmacological activities based on the structure of the compound, with a mean accuracy of 95%, using the leave-one-out cross-validation method (Lagunin *et al.*, 2000).

2.9 Molecular docking analysis between inhibitors and target proteins

Binding affinities of **Cant** against reported targets for inflammatory bowel disease were evaluated using molecular docking (Bikadi and Hazai, 2009). Based on literature searches, mitogen-activated protein kinase 14 (MAPK14) and Toll-like receptor 8 (TLR8) were selected as targets. For IBD, expanding evidence has shown an immune-system dysfunction, particularly TLRs- and MAPKs-mediated innate immune dysfunctions, as key members in the pathogenesis and in the progression of IBD. The motivation behind this docking study is to understand the molecular mechanisms of MAPK14 and TLR8 signalling pathways in IBD, as well as novel possible therapeutic strategies against this pathology (Kordjazy *et al*., 2018; Coskun *et al*., 2011; Lu *et al*., 2018).

Protein Data Bank (PDB) structures of both targets were retrieved from the Research Collaboratory for Structural Bioinformatics (RCSB) (www.rcsb.org/), upon id 3DS6 and 3W3L, respectively. For comparative purposes, along with **Cant,** previously reported drug molecules for IBD treatment, mesalazine and sulfasalazine were also docked against these targets. These two drugs are commonly used for the treatment of IBD, UC and CD, by reducing inflammatory response through several mechanisms (Masuda *et al*., 2012).

The docking study was carried out using a molecular server as previously described (Solis and Wets, 1981; Ganou *et al*., 2018; Eleftheriou *et al*., 2020). Molecular ligations were minimised using the Merck molecular force field 94 (MMFF94), and Gasteiger partial charges were added to the atomic bonds. Nonpolar hydrogen atoms in PDB target structures were merged and rotatable bonds defined. Kollman united atom type charges, solvation parameters and essential hydrogen atoms were added using AutoDock tools (Morris *et al.*, 1998). Grid points (60×60×60) and spacing was defined using the Autogrid programme. To define electrostatic terms, AutoDock parameters for Van der Waals and distance dependent dielectric functions were used. "Lamarckian genetic algorithm" (LGA) and "Solis & Wets local search method" were implemented in the docking studies. The docking experiment was retrieved from ten different runs, which were set to end after a maximum of 250,000 minimal energy evaluations. Flexible docking was selected rather than a rigid docking model, in order to increase the degree of accuracy of the result (Forli *et al*., 2016).

2.10. Data and statistical analysis

Parametric data were expressed as the mean ± standard error of the means (SEM) or mean ± standard deviation (SD). The differences between means were determined through one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post-test. All non-parametric data were expressed as the median and its quartile range (Q_1:Q_3), and compared by the Kruskal–Wallis followed by the Dunn post-test. *P* values less than 0.05 were considered as significant. Analyses were performed using GraphPad Prism v6.07 (GraphPad Software, San Diego, USA).

3. RESULTS

3.1. Protective effects of canthin-6-one on macroscopic and histological changes in TNBS-induced colitis

Macroscopic and histological signs of colitis were observed in intrarectal TNBS-administered rats. In the vehicle group treated only with TNBS, the macroscopic score increased by 99% (4.75 ± 0.16, $p < 0.001$), compared to the sham group. Treatment with **Cant** at doses of 5 and 25 mg/kg decreased the macroscopic scores of colon tissue by 45% (2.62 ± 0.32) and 58% (2.00 ± 0.32, $p < 0.01$), respectively, compared to the vehicle TNBS-treated group. Comparatively, the standard drug mesalazine (500 mg/kg) significantly reduced the macroscopic scores by 47% (2.50 ± 0.32, $p < 0.01$) when compared to the vehicle TNBS-treated control group (Fig. 2), suggesting that 25 mg/kg **Cant** has a better effect on preventing TNBS-induced tissue damage.

Histopathological examination of colon specimens from the TNBS control group showed severe infiltration of acute and chronic inflammatory cells, deposition of fibrin protein, epithelial necrosis and ulcer. Colon histology in **Cant** (25 mg/kg) or mesalazine (500 mg/kg) treated TNBS-administered groups indicated a reduction of oedema and necrosis, compared to the TNBS-treated vehicle control group (Fig. 3 A-F). Histopathological scoring showed significant inhibition of mucosal damage (62.50%), oedema (62.50%), ulcer (68.75%), and necrosis (62.50%) following 25 mg/kg **Cant**. Mesalazine (500 mg/kg) treatment showed similar reductions in pathological changes ($p < 0.05$) of mucosal damage (62.50%), oedema (62.50%), ulceration (68.75%), and necrosis (56.25%), as shown in Fig. 4 A-D.

Histopathological examination of PAS stained colon tissue specimens from the vehicle treated TNBS control group showed destruction of epithelial architecture. In contrast, histological samples from **Cant** (5 and 25 mg/kg) or mesalazine (500 mg/kg) TNBS-treated colons showed epithelial integrity with intense coloration of globet cells (Fig. 5). PAS staining of the colon from the vehicle-treated colitic group showed goblet cells damage compared to the sham group, decreasing the score by 93.75% ($p < 0.01$). In contrast, when compared to the vehicle-treated group, the **Cant** pre-treated colitic group (1; 5 and 25 mg/kg) showed an increased goblet cell score by 31.25% ($p < 0.05$), 56.25% ($p < 0.05$) and 62.50% ($p < 0.01$), respectively. Mesalazine treated rats similarly displayed an increase of goblet cell score by 56.25% ($p < 0.05$) (Fig. 5 G).

3.2. Oxidative stress parameters are attenuated by Cant during TNBS-induced ulcerative colitis

3.2.1 Myeloperoxidase activity (MPO)

MPO activity in colon isolates was increased by 107.21% ($p < 0.001$) in the TNBS-treated vehicle control group compared to the sham group (Fig. 6A). The pre-treatment of rats with **Cant** administered at 1; 5 and 25 mg/kg, p.o., prior to the induction of colitis by TNBS, reduced MPO activity by 49.37% ($p < 0.05$), 52.85% ($p < 0.001$) and 61.69% ($p < 0.001$), respectively. Pre-treatment with mesalazine (500 mg/kg) showed a comparable reduction in MPO activity, by 59.67% ($p < 0.001$).

3.2.2 Glutathione (GSH) content

Colonic mucosal GSH was partially depleted upon TNBS-induced intestinal colitis in the vehicle group, by 44.56 % ($p < 0.001$) when compared to the sham group (Fig. 6B). Pre-treatment with **Cant** at 1; 5 and 25 mg/kg, reverted this reduction by 29.54%, 26.59% and 26.21% ($p < 0.01$) respectively, when compared to the ulcerated control group (Vehicle). Mesalazine (500 mg/kg) prevented the reduction of GSH levels induced by TNBS to 25.00% ($p < 0.01$).

3.2.3 MDA levels

The vehicle administered TNBS control group demonstrated high lipid peroxidation in colon isolates, as the MDA concentration was increased by 93.60% ($p < 0.001$), compared to the sham group (Fig. 6C). All doses of **Cant** (1; 5 and 25 mg/kg, p.o.) tested reduced lipid peroxidation by 33.75%, 61.63% ($p < 0.01$) and 92.45% ($p < 0.001$), respectively, in a dose-dependent manner, when compared to the colitis-induced vehicle control group. In a similar way to the 25 mg/kg dose of **Cant**, mesalazine (500 mg/kg, p.o.) reduced the MDA concentration by 92.64% ($p < 0.001$) to concentrations near to the observed for the sham group.

3.3. Cant reduces pro-inflammatory cytokines and increases IL-10 during TNBS-induced colitis

As expected, following TNBS-induced colitis, we observed an increase of TNF-α (81.80%, $p < 0.001$), IL-1β (72.77%, $p < 0.001$), IL-12p70 (82.03%, $p < 0.001$) pro-inflammatory cytokines and of VEGF (37.90%, $p < 0.05$) in the colon tissues of rats, compared to the sham group. Colon tissues from the vehicle treated TNBS control

group also demonstrated a reduction in anti-inflammatory IL-10, by 79.28% ($p < 0.001$).

Pre-treatment with **Cant** (5 and 25 mg/kg), significantly reduced the colon tissue concentration of TNF-α (55.25% and 57.26%, $p < 0.01$, respectively), IL-1β (47.98%, $p < 0.05$ and 75.02%, $p < 0.01$), IL-12p70 (58.48%, $p < 0.05$ and 67.06%, $p < 0.01$) and VEGF (45.79%, $p < 0.05$ and 61.39%, $p < 0.01$) when compared to the TNBS-treated vehicle control group. In addition, **Cant** (5 and 25 mg/kg) also increased the concentration of IL-10 by 258.58% and 401% ($p < 0.05$), respectively. **Cant** pre-treatment of 1 mg/kg was capable of ameliorate colon inflammation, however, it did not significantly alter the colon tissue concentrations for IL-12p70 and IL-10 cytokines compared to vehicle treated TNBS control. Mesalazine (500 mg/kg) reduced TNF-α by 56.59% ($p < 0.01$), IL-1β by 52.75% ($p < 0.05$), IL-12p70 by 41% ($p < 0.05$) and VEGF by 27.79% ($p < 0.05$), and increased IL-10 by 66.68% ($p < 0.05$), demonstrating concentrations comparable to those observed following pre-treatment with 5 mg/kg **Cant** (Fig.7 A-E).

3.4 *In silico* experiments

3.4.1 Prediction of Activity Spectrum (PASS analysis) for Cant

The PASS server calculated the molecular scores (the probability to be pharmacologically active, *Pa*, and the probability to be inactive, *Pi*) of **Cant** in its interaction with thousands of enzymes and molecular targets. Of a total of 1494 biological interactions retrieved from the PASS server (with $Pa > Pi$), we selected those with $Pa > 0.7$ and $Pi < 0.05$, as shown in *Supplementary file 2*, with a total of 43 putative targets in several organisms, including humans. From them, 10 have some direct relationship with inflammation inhibition, with "kinase inhibition" ($Pa = 0.719$) as the most relevant pharmacological interaction in this category.

The most frequent interactions are 13 related to the inhibition of various kinds of enzymes implicated in the redox state, including mitochondrial electron-transferring-flavoprotein dehydrogenase ($Pa = 0.767$), two interactions related to P450 reductases, and principally NAD(P)H-dependent oxidoreductases from diverse organisms.

Finally, the other interactions are related to various kinds of biological functions that would explain other recognised properties of **Cant**, such as nociceptive or antitumoral.

3.4.2 Molecular Docking between Cant. and putative candidate target proteins

As an initial screen of the action mechanism of **Cant,** ligand–protein interactions with presumed targets were analysed by molecular docking. The binding sites of each target protein were retrieved from the PDBsum database (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/). From the Hydrogen Bonding plotting, the **Cant** moiety can be placed adjacent to some MAPK14 residues (Fig. 8A), such as TYR 35, LYS 53, GLU 71, LEU 75, ILE 84, LEU 104, THR 106, LEU 167 and ASP 168, and to TLR8 residues (Fig. 8B), such as ASN 262, PRO 264, PHE 265, SER 266, PHE 320, PHE 467 and TYR 468, indicating a strong interaction between drug and target proteins. The crystal structures of the target proteins were obtained from PDB, and the best docked results for each protein, considering the interacting residues with minimal ligation energy, are shown in Fig 8C and D. Among the docked ligands evaluated, for comparative purposes (**Cant**, mesalazine and sulfasalazine), **Cant** has the best docking score, being -7.07 kcal/mol for MAPK14 and -7.41 kcal/mol for TLR8 (Table 1).

4. DISCUSSION

For this study, doses of 1, 5 and 25 mg/kg of **Cant** were chosen based on previous work carried out by our research group (Noldin et al., 2005; Gazoni et al., 2018). As for safety*, in vitro* tests showed that **Cant** did not cause cytotoxicity using the cell line CHO-k1. Moreover, it is important to note that previous *in vivo* studies in mice of both sexes demonstrated that a single oral administration of **Cant** of 100 mg/kg had no adverse effect (Gazoni et al., 2018), being 100 times higher than the lowest active dose (1 mg/kg) used here in the TNBS-induced ulcerative colitis model, and 4 times higher than the highest dose investigated (25 mg/kg). However, as a cytotoxic and cytostatic agent for tumoral cells, there is likely some cell proliferating genotoxicity and hepatotoxic effects at elevated doses/concentrations (Vieira Torquato et al., 2017; Gazoni et al., 2018).

Although the causal mechanisms of IBD remain to be fully elucidated, accumulating data from both animal experimental models and clinical studies indicate that oxidative stress, along with other inflammatory mediators, is a key factor in the pathogenesis and perpetuation of mucosal damage observed during active IBD (Jaiswal et al., 2018). Oxidative stress and inflammation are closely related pathophysiological processes, each of which can be easily induced by the other, with both processes being simultaneously found in many pathological conditions (Biswas, 2016).

While uncontrolled oxidative stress is destructive for the gastrointestinal mucosa, cellular defences can counteract these effects. Reduced glutathione (GSH) decreases as oxidised glutathione (GSSG) significantly increases in the colon mucosa of UC patients, indicating that tissue is under oxidative stress (Tian et al., 2017). In this sense, in the present work, the administration of **Cant** resulted in increased colonic mucosal non-protein sulfhydryl groups, being represented in the cell mainly by GSH, probably restoring redox balance.

MPO is an inflammatory mediator produced by the degranulation of neutrophils that increased in patients with active UC (Hansberry et al., 2017). Previous studies have shown that colonic MPO is elevated in rats with active colitis induced by TNBS and this is likely to be linked to disease progression (Moura et al., 2015). In humans, activated neutrophils and macrophages are responsible for the generation of both ROS and reactive nitrogen species (RNS), and the levels of reactive radicals may also be correlated with severity of inflammatory injuries in the colon mucosa (Balmus et al., 2016). In our work, **Cant** administration, at all doses tested, displayed an important reduction in colonic MPO activity, plausibly related to a reduced infiltration of neutrophils in this model. Histopathological results support this anti-inflammatory effect of **Cant**, as confirmed by the reduced inflammatory cell recruitment in the injured area (Fig. 6).

MDA is a by-product from the oxidation of polyunsaturated fatty acids and has been established as a biomarker for oxidative stress (Murad *et al*., 2016). Thus, elevated concentrations of MDA have been documented in TNBS-induced colitic tissues from rats (Liu *et al*., 2012), as was also demonstrated in the present study. This finding supports that **Cant** can act mechanistically as an antioxidant, by interfering in the

491 production of free radicals through the interruption of lipid peroxidation, which is one
492 of the main triggers of the inflammatory progression.

493 Hence, the present study indicates that **Cant** can interfere in part with the
494 inflammatory signalling cascade of TNBS-induced colitis, through a direct and/or
495 indirect effect on oxidative stress. In this way, De Souza Almeida *et al*. (2011)
496 demonstrated that **Cant**-gastroprotective activity in rodents depends on MPO and MDA
497 inhibition, corroborating the antioxidant results found here in UC.

498 From PASS analyses, the antioxidant effect of **Cant** appears to be mediated via
499 a direct action through the inhibition of enzymes related to ROS generation, principally
500 NAD(P)H dependent oxidoreductases (NOX-like enzymes) (see *Supplementary Results*
501 *2*). ROS has been reported to activate MAPK in various tissue systems, acting as an
502 intracellular second messenger. In particular, MAPK-activated redox-sensitive
503 transcriptional factors such as nuclear factor-kB (NF-kB) and activator protein-1 (AP-1)
504 play a critical role in the production of cytokines, which mediate several inflammatory
505 responses including IBD (Torres and Forman, 2003; Escoubet-Lozach *et al.*, 2002).
506 Conversely, cytokine activation can prime an oxidative burst initiated by NADPH-
507 oxidases (NOX2) from innate immune cells (Elbim *et al*., 1994).

508 Along with leukocyte migration, the production of cytokines and chemokines by
509 local and migrated cells is one of the main factors that regulate the pathological process
510 of IBD and other inflammatory conditions. Previous studies have reported increased
511 pro-inflammatory cytokine (Il-1β, TNF-α, IL-12) and VEGF expressions in the
512 intestinal mucosa during active ulcerative colitis (Atreya and Neurath, 2005). Therefore,
513 cytokines, by regulating mucosal immune responses, may represent rational targets for
514 therapeutic intervention in IBD (Strober and Fuss, 2011). We therefore evaluated the
515 effect of **Cant** on pro- (TNF-α, IL-1β, IL-12) and anti-inflammatory cytokines (IL-10),
516 as well as on the restoring cytokine VEGF, during TNBS-induced ulcerative colitis in
517 rats. **Cant** (5 and 25 mg/kg) treatment reduced the concentration of pro-inflammatory
518 cytokines and VEGF and increased anti-inflammatory IL-10 in colitic tissues,
519 contributing to dampening the immune response induced by TNBS.

520 TNF-α contributes to the pathogenesis of IBD and mediates multiple biologic
521 effects, including recruitment of neutrophils to local sites of inflammation (Baumgart
522 and Sandborn, 2007). IL-1β is also implicated in the development of colitis and Th17-

associated responses in the gut (Coccia *et al*., 2012). A recent work has demonstrated that IL-12 orchestrates the initial innate immune-cell-driven inflammatory reaction in response to intestinal barrier disruption (Eftychi *et al*., 2019). VEGF is a growth factor that is involved in IBD pathogenesis by regulating inflammatory-driven angiogenesis and mucosal inflammation (Scaldaferri *et al*., 2009). Finally, IL-10 is a key anti-inflammatory cytokine that limits and terminates immune responses (Iyer and Cheng, 2012).

Previously, Cho *et al.* (2017) demonstrated that, in RAW 264.7 macrophagic cells, treatment with **Cant** suppressed the LPS-stimulated transcriptional activation by NF-κB, suggesting that **Cant** may inhibit the expression of inflammatory mediators such as iNOS, COX-2, and pro-inflammatory cytokines, by preventing the phosphorylation of IκB. Also, using a NF-κB-driven luciferase reporter gene assay, it was demonstrated that **Cant** is a potent direct NF-κB inhibitor (Tran *et al*., 2014). The production of inflammatory mediators and cytokines, via the NF-κB transcription factor in activated macrophages, is regulated by upstream kinases such as Akt and p38α MAPK. **Cant** markedly inhibited the LPS-induced phosphorylation of Akt (Cho *et al*., 2017), suggesting that the inhibition of this pathway may be associated with its anti-inflammatory effect in UC. Together with direct oxidative stress modulation, the PASS biological activity spectrum points to a possible anti-inflammatory effect of **Cant**, by inhibiting cellular kinases.

Receptors of the innate immune system, such as TLRs, impact many aspects of IBD aetiology. TLR8 is upregulated in colon epithelial cells from patients with active UC (Steenholdt *et al.*, 2009; Cohen, 2014) and can mediate TNF-α and IL-1β release from immune cells. TLR8 can also regulate the IL-8 chemokine secretion from colon epithelial cells (Steenholdt *et al*., 2009). Along with TLR8, MAPK14 (p38α) is important in regulating intestinal inflammation (Broom *et al*., 2009). MAPK upregulates the expression of some genes participating in intestinal inflammation, such as those coding for TNF-α, IL-1, IL-6, IL-8 and COX-2 (Bermudez-Brito *et al*., 2013). In this context, TLR8 and MAPK14 were hypothesised as putative targets for **Cant** as previous preclinical studies with specific inhibitors of both TLR8 and MAPKs have provided strong treatment efficacy in colitis experimental models (Broom *et al*., 2009).

Therefore, in this study the impact of **Cant** on intestinal inflammation was further strengthened by a molecular docking approach. Mesalazine and sulfasalazine were used as standard therapeutic drugs to treat IBD in humans, for comparative purposes. Molecular interaction showed high binding affinity of **Cant** for MAPK14 (-7,07 kcal/moL) and TLR8 (-7.41 kcal/moL) residues, with low docking energies, which were more favourable than for mesalazine or sulfasalazine. Many studies confirmed that molecules having high binding affinity with low docking energy for its target proteins exhibit potential therapeutic efficacy (Yadav *et al*., 2013). So, our findings suggest that **Cant** may also plausibly exert its therapeutic effect in IBD by modulating TLR8 and/or MAPK14 or structurally and functionally similar target proteins.

In conclusion, **Cant** may exert potent activity against UC by modulating inflammatory mediators, by decreasing oxidative stress and by promoting the balance between pro- and anti-inflammatory cytokines. Molecular docking results showed a good interaction of **Cant** with residual domains of protein targets upstream of the signalling pathway for these inflammatory mediators, such as the MAPK14 (p38α) enzyme and the TLR8 receptor, suggesting a putative molecular mechanism for **Cant** by inhibiting these targets. Therefore, **Cant** may be a template or a potential drug candidate for the treatment of IBD. Further clinical studies are necessary to validate the use of **Cant** for IBD treatment and to establish further detail about its mechanisms of action.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interests.

AUTHOR CONTRIBUTIONS

KA - participated in the design of the experiments, conducted the experimental work and the data analysis, and contributed to manuscript writing. **ASD** - contributed to the histopathological analysis and experimental work. **AM** - contributed to the cytokine analysis and writing and revision of the manuscript. **MSM** - contributed in the molecular docking studies and data analysis. **EP** – participated in the *in vivo* and manuscript writing. **FFF** – participated in the *in vivo* experimental work. **DMO** - participated in the *in vivo* experimental work. **CAD** - critically evaluated and revised the manuscript. **PT**- evaluated the data and revised the manuscript. **ML** - evaluated the data and revised the manuscript. **DTOM** - designed the study, supervised the work, discussed the results, data analysis and contributed to the manuscript writing. All authors read and approved the final version of manuscript.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration recognises that this paper complies to the standards for straightforward announcing and logical thoroughness of pre-clinical exploration as expressed in the BJP rules for Experimental Design and Data Analysis, Animal Experimentation and Molecular Docking as suggested by financing source, publishers and different organisations.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends:

Figure. 1. Structure of canthin-6-one (6H-Indolo[3,2,1-de][1,5]naphthyridin-6-one), molecular formula: $C_{14}H_8N_2O$, exact mass: 220.06.

Figure. 2. Effect of Cant pre-treatment on TNBS-induced colonic gross pathology: vehicle (Veh, 2% Tween-80 aqueous solution 10 mL/kg p.o.), canthin-6-one (**Cant**, 1; 5 or 25 mg/kg p.o.), mesalazine (Mesa 500 mg/kg p.o.) was administered to Wistar rats prior to colitis induction by TNBS. The results are expressed as medians and quartiles (Q_1;Q_3) for 8 animals/group. Statistical comparisons were performed using analysis of Kruskal-Wallis followed by Dunn's test. ** $p < 0.01$ vs vehicle control. $^{###}p < 0.001$ vs sham.

Figure. 3. Representative images of the histological analysis of the colon and histopathological changes of the mucosa (arrow), submucosa (arrowhead), muscle layer (curve arrow) in ulcerative colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS): (A) Colon cross section of sham animals demonstrating normal tissue architecture. (B) TNBS-treated colon sections showing intense inflammatory cell infiltration into the submucosa and disrupted epithelial and muscular layers. (C) Colon cross sections from rats pre-treated with 1 mg/kg canthin-6-one (**Cant**) followed by TNBS showing intense inflammatory cell infiltration. (D and E) Rats pre-treated with **Cant** at 5 and 25 mg/kg p.o., respectively followed by TNBS showing reduction of the inflammation in the lamina propria. (F) Animals pre-treated with mesalazine (500 mg/kg p.o.) followed by TNBS showing a prominent reduction of immune cell infiltration into the submucosal layer, and also having intact epithelium and musculature. The sham group received distilled water (10 mL/kg, p.o.) and 250 µL of 0.9% saline rectal instillation instead TNBS. Haematoxylin and eosin (H&E) staining. Bar = 50 µm.

Figure. 4. **Histopathological changes in the colon mucosa following Cant pre-treatment and TNBS-induced colitis**. Effects of vehicle (Veh, 2% Tween-80 aqueous solution, 10 mL/kg, p.o.), canthin-6-one (**Cant,** 1; 5 and 25 mg/kg, p.o.) or mesalazine (Mesa, 500 mg/kg, p.o.) on histological changes in colon tissue from TNBS-induced colitis in rats. Histopathological changes were determined by damage scores from H&E stains: (A) mucosal damage score; (B) oedema score; (C) ulceration score; (D) necrosis score. The results are expressed as medians and quartiles (Q_1;Q_3) for 8 animals/group. The sham group received distilled water (10 mL/kg, p.o) and 250 µL of 0.9% saline rectal instillation instead TNBS. Statistical comparisons were performed using Kruskal-Wallis analysis followed by Dunn test. *$p < 0.05$ vs vehicle group, $^{##}p < 0.01$, $^{###}p < 0.001$ vs sham. Bar = 50 µm.

Figure. 5. Representative PAS stained images of the colon following Cant. pre-treatment and TNBS colitis induction: (A) Colon of sham rats exhibiting normal epithelial architecture and presence of goblet cells. (B) Colon sections from rats administered vehicle followed by TNBS alone, showing disruption of epithelial architecture and reduction of the presence of goblet cells. (C) Colon sections from rats pre-treated with 1 mg/kg canthin-6-one (**Cant**) followed by TNBS, showing epithelial

destruction and reduction of the presence of goblet cells. (D and E) Colon sections from rats pre-treated with **Cant** at 5 and 25 mg/kg respectively, followed by TNBS showing intact epithelium and intense presence of goblet cells. (F) Animals pre-treated with mesalazine (Mesa, 1 mg/kg p.o.), showing intact epithelium with intense presence of goblet cells. (G) Effects of vehicle (Veh, 2% Tween-80 aqueous solution, 10 mL/kg, p.o.), canthin-6-one (**Cant** 1; 5 and 25 mg/kg, p.o.) or mesalazine (Mesa, 500 mg/kg, p.o) pre-treatment on goblet cell abundance in colon tissue from TNBS treated rats. The sham group received distilled water (10 mL/kg, p.o.) and 250 µL of 0.9% saline rectal instillation instead TNBS. The results were expressed as medians and quartiles (Q_1;Q_3) for 8 animals/group. Statistical comparisons were performed using Kruskal-Wallis analysis followed by the Dunn test. *$p < 0.05$ and **$p < 0.01$ vs vehicle group, $^{\#\#}p <$ 0.01 vs sham. Bar = 100 µm.

Figure. 6. Evaluation of antioxidant capacity: effects on stress oxidative parameters, (A) myeloperoxidase activity (MPO), (B) reduced glutathione levels (GSH) and (C) malonaldehyde accumulation (MDA), after vehicle (Veh, 2% Tween-80 aqueous solution, 10 mL/kg, p.o.), canthin-6-one (**Cant** 1; 5 and 25 mg/kg, p.o.) or mesalazine (Mesa, 500 mg/kg, p.o.) pre-treatments, followed by TNBS-induced colitis, in colon tissues from rats. The sham normal control group received only distilled water (10 mL/kg, p.o). Values represent the mean ± standard error (S.E.M.) from 8 animals/group. Statistical comparisons were performed using a one-way ANOVA followed by Student-Newman-Keuls test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle control. $^{\#\#\#}$ $p < 0.001$ vs sham.

Figure. 7. **Quantification of cytokine production:** effects of vehicle (Veh, 2% Tween-80 aqueous solution, 10 mL/kg, p.o.), canthin-6-one (**Cant** 1; 5 and 25 mg/kg, p.o.) or mesalazine (Mesa, 500 mg/kg, p.o.) on cytokines produced by rat colon tissues in response to TNBS-induced ulcerative colitis. (A) TNF-α, (B) IL-1β, (**C**) IL-12p70, (D) VEGF, and (E) IL-10**.** The sham group received distilled water (10 mL/kg, p.o) and 250 µL of 0.9% saline rectal instillation instead TNBS. Statistical comparisons were performed using a one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, vs vehicle control. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs sham.

Figure. 8. Molecular docking analysis of the hydrogen bonding (HB) plots between Cant and (A) **MAPK14 and** (B) **TLR8 residues**. Best docking positions between canthin-6-one (**Cant**) (C) MAPK14 and (D) TLR8. The interacting residues of MAPK14 and TLR8 (grey, blue, red and yellow) and the structure of **Cant** (green) are represented using a "Cylinder" model. The residues of target proteins are represented using black dots whereas the red dots represent HB putative interactions with **Cant**. Colours' in the Figure (C) and (D) represents the atoms: Carbon (grey), Oxygen (red), Nitrogen (blue), Sulphur (yellow).