Journal of Ethnopharmacology 194 (2016) 137–145

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Beneficial properties of *Passiflora caerulea* on experimental colitis

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ARTICLE INFO

Article history:
Received 11 May 2016
Received in revised form
31 August 2016
Accepted 2 September 2016
Available online 3 September 2016

Keywords:
Passiflora caerulea
Ulcerative colitis model
Anti-inflammatory
Antispasmodic
Antioxidant activity
Histological study

ABSTRACT

Ethnopharmacological relevance: Passiflora caerulea L. (Passifloraceae) is a medicinal plant commonly used in traditional medicine in South America for different pathologies associated with the gastrointestinal tract.
Aim of the study: In the present study, the activity of the ethanolic extract of *P. caerulea* on an experimental colitis model related to inflammatory bowel disease has been investigated.
Materials and methods: Colitis was induced by intracolonic instillation of a 2 mL of 4% (v/v) acetic acid solution. Macroscopic scoring, myeloperoxidase activity and thiobarbituric acid reactive substances levels were evaluated on isolated colon mucosae. The histopathological studies of colon mucosae were performed by hematoxylin and eosin and Alcian blue staining. Diarrhoea was induced by the administration of castor oil (0.3 mL/mouse). The first watery defecation time, the total amount of solid, semi-solid and watery stools and the amount of watery stools were determined. The effect of the extract on a cumulative concentration-response curve of acetylcholine and $CaCl_2$ on isolated rat jejunum was also evaluated. The phytochemical analysis was performed.
Results: The extract (250 mg/kg, p.o.) induced a significant reduction in the weight/length ratio, the macroscopic lesion score, TBARS levels and the microscopic tissue damage when compared with the acetic acid-treated group of animals. *P. caerulea* (125 mg/kg, p.o.) decreased significantly the amount of watery stools in the castor oil-induced-diarrhoea model. Moreover, the *P. caerulea* extract antagonized the jejunum contractions induced by Ach (E_{max} for 0.3 mg/mL: 76.25%; E_{max} for 1 mg/mL: 63.47%; E_{max} for 3 mg/mL: 42.01%) and $CaCl_2$ (E_{max} for 0.3 mg/mL: 75.69%; E_{max} for1 mg/mL: 56.1%; E_{max} for 3 mg/mL: 53.4%). Isoorientin, vitexin, isovitexin, and vicenin-2 were identified in the extract.
Conclusion: P. caerulea showed anti-inflammatory, anti-diarrhoeal and spasmolityc activities on preclinical models.

1. Introduction

Nowadays, diseases that affect the digestive system are among the most prevalent health issues. Chronic inflammation of the gastrointestinal tract is observed in inflammatory bowel disease (IBD). This condition encompasses two major conditions, known as Crohn's disease (CD) and ulcerative colitis (UC) (Garrido-Mesa et al., 2011). Although, the pathogenesis of these clinical entities is still not completely understood, patients having IBD suffer chronic diarrhoea, weight loss, abdominal pain, fever, and fatigue. Besides, the IBD increases the risk of developing inflammation-related colorectal cancer (Reber, 2012).

The treatment for IBD is focused on the induction of clinical remission, to prevent a relapse, to maintain adequate nutrition, and to reduce the duration of relapses. To date, no complete response has been achieved with conventional therapies (aminosalicylates, corticosteroids, and antibiotics), therefore, the development of new therapies that combine efficacy and lower side effects is an important goal in the IBD therapy (Algieri et al., 2013). Since controlled trials using herbal therapy for the treatment of IBD have shown encouraging results (Ng et al., 2013; Langhorst et al., 2015; Triantafyllidi et al., 2015), the evaluation of extracts from plants that have long been consumed and used in traditional medicine, is an important approach for the development of novel treatments

Abbreviations: AA, acetic acid; Ach, acethyl choline; CD, Crohn's disease; CMC, carboxymethylcellulose; DSS, dextran sulfate sodium; GAE, gallic acid equivalent; H&E, hematoxylin and eosin; IBD, inflammatory bowel disease; MDA, malondialdheyde; MPO, myeloperoxidase; NF-κB, nuclear factor-kappaβ; NO, nitric oxide; OD, optic density; PGE_2, prostaglandine E_2; SDS, sodium dodecylsulfate; SEM, standard error of the mean; TBA, thiobarbituric acid; TBARS, thiobarbituic acid reactive substances; UC, ulcerative colitis

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http://dx.doi.org/10.1016/j.jep.2016.09.002
0378-8741/

for gastrointestinal inflammatory processes. In this regard, traditional medicine worldwide is nowadays being re-evaluated by extensive research on different plants and their therapeutic principles.

The genus *Passiflora*, which comprises about 500 species, is the largest in the Passifloraceae family and some of their species have been used extensively in the traditional medicine in many countries. As for gastrointestinal ailments, *Passiflora edulis* has been used as *anti*-diarrhoeal, digestive, and as a remedy for infants colics and gastric carcinoma. Specifically, Cazarin et al. (2014) showed that consumption of passion fruit flour from *P. edulis*' peel provided benefits in experimental colitis induced by TNBS in rats. Moreover, *P. incarnata* is used as antispasmodic. Other species such as *P. foetida* and *P. suberosa* are used for inflammatory disorders of the skin (Dhawan et al., 2004).

In particular, *P. caerulea,* that is native of South America, grows in Argentina, Brazil, Bolivia, Chile, Paraguay and Uruguay. It is known by the common names "pasionaria", "mburucuyá", and "flor de la pasión" (Toursarkissian, 1980). Besides its common uses as sedative, against insomnia, it is used as an antihypertensive and diuretic agent, (Ratera and Ratera, 1980; Soraru and Bandoni, 1978; Dhawan et al., 2004). *P. caerulea* is used in popular medicine for different pathologies associated with the gastrointestinal tract: leaves are used against dysentery, the aerial part is used as an antispasmodic agent (Toursarkissian, 1980), and its fruit is used as a eupeptic agent for having good digestion (Alonso and Desmarchelier, 2005). *P. caerulea* is also used for the treatment of some intestinal infections: the root and leaves are use as antihelmintic and leaves´ decoction as vermifuge (Ratera and Ratera, 1980; Toursarkissian, 1980). In addition, it is used as an inflammatory agent (Soraru and Bandoni, 1978). Furthermore, Melipass®, commercial capsules containing *Melissa officinalis* and *P. caerulea* are recommended for irritable bowel syndrome (Knop, 2016).

Taking into account the folkloric use of *P. caerulea* and of other members of the family, it is expected that this specie possesses beneficial effect on symptoms and/or signs of gastrointestinal inflammatory process. Nevertheless, no scientific evaluation of this plant related to it has been carried out so far. So, on the basis of these considerations, the present study was aimed at examining its effects on the gastrointestinal system in experimental models.

2. Materials and methods

2.1. Plant collection and identification

The leaves of *Passiflora caerulea* L. (Passifloraceae) were collected in Buenos Aires Province, Hurlingham (Jardín Botánico Arturo Ragonese-INTA, Argentina in March 2013, and identified by Engeneer Hernán Bach. A voucher specimen (Herbarium BAB, BAF N° 617) is kept at the Herbarium of the Museo de Farmacobotánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires.

2.2. Plant extraction and purification

The dried aerial parts of *P. caerulea* (139.1 g) were ground into a fine powder and extracted by maceration with 80% ethanol in water (1.4 L) at room temperature for 24 h. This procedure was repeated seven times. Then the extract was concentrated by evaporation and lyophilized (yield: 20.14% w/w).

2.3. Phytochemical study

The total phenol content of the extract was determined by the Folin-Ciocalteu´s colorimetric method described by Singleton et al. (1999). The absorbance was measured at 765 nm and compared with a gallic acid calibration curve. The result was expressed as gallic acid equivalents per gram of extract (GAE/g). The HPLC method was developed according to Filip et al. (2001) and performed with a Varian Pro Star instrument using a diode array detector. Phenomenex Kinetex C18 column (5 μ XB-C18 100 A, 250 × 4.6 mm), solvent A: H_2O/AcOH (98: 2), solvent B: MeOH/AcOH (98: 2). Gradient: 15% B to 40% B, 30 min; 40% B to 75% B, 10 min; 75% B to 85% B, 5 min. Flow rate: 1.2 mL/min. Detection: 365 nm. Rheodyne injector fitted with a 20 μl loop. Identification was achieved by the external standard method by comparison of their retention times and UV spectra with those of authentic standards.

2.4. Drugs

Indomethacine, mesalazine, loperamide, castor oil, Evans blue, acetylcholine, propyleneglycol, hexadecyltrimethylammonium bromide, *o*-dianisidinedihydrochloride, carbachol and $CaCl_2$, isoorientin, vitexin, isovitexin, and vicenin-2 were purchased from Sigma Chemical Co., St. Louis, MO., USA. Acetic acid was purchased from Merck (Darmstadt, Germany). All reagents were of analytical grade.

2.5. Animals

Female Swiss mice weighing 25–30 g and female Sprague Dawley rats (250–270 g) were used. The experiments were carried out following international guiding principles and local regulations concerning the care and use of laboratory animals for biomedical research. The experiments were approved by the local Ethics Committee (Comité Institucional de Cuidados y Usos de Animales de Laboratorio, Exp-FyB: 0738657/11). The animals had free access to a standard commercial diet and water *ad libitum* and were kept in room maintained at 22 ± 2° C with a 12-h light/dark cycle.

2.6. Ulcerative colitis model

2.6.1. Experimental design

Rats were randomly divided into 7 groups of 5 animals each. A group did not receive any treatment (control). The colitis was induced in the other groups. A reference group was treated with mesalazine by oral route (p.o.) (100 mg/kg, dissolved in CMC 1%). Other groups received the *P. caerulea* extract 125 and 250 mg/kg, p.o. (dissolved in CMC 1%) and 0.5 mL of a 4% extract suspension by intracolonic route (i.c.) (vehicle: water: propylenglycol: 2% CMC in water 1:1:2). Animals were treated during 5 consecutive days. On the fourth day, the colitis was induced. All animals were sacrificed on the fifth day (Fatani et al., 2015). The severity of colitis was evaluated by an independent observer blinded to the treatments.

2.6.2. Induction of colonic inflammation in rats

Animals were kept fasting for 36 h, with access to water *ad libitum*. The colitis was induced by administration of 2 mL of a 4% v/v acetic acid in water using a polyethylene tube, inserted into the rectum and along the colon up to a distance of 8 cm (Paiva et al., 2002). Rats were euthanized 24 h later.

2.6.3. Macroscopic scoring

Colons were cut longitudinally and cleansed with saline solution to remove faecal residues. Macroscopic inflammation scores are assigned based on the clinical features of the colon using an arbitrary scale ranging from 0 to 4 as follows: 0 (no macroscopic changes), 1 (mucosal erythema only), 2 (mild mucosal oedema, slight bleeding or small erosions), 3 (moderate oedema, slight

bleeding ulcers or erosions) and 4 (severe ulceration, oedema and tissue necrosis) (Kannan and Guruvayoorappan, 2013).

2.6.4. Histological study

Samples of colon mucosae were fixed in 10% buffered formalin and embedded in paraffin. Sections 5 μm thick were cut in a microtome and stained with hematoxylin and eosin or Alcian blue and the examined under a light microscope. Alcian blue was used for quantifying goblet cell among different experimental groups of colon tissue. Goblet cells were counted using ten crypts per colon tissue section.

2.6.5. Myeloperoxidase activity

The myeloperoxidase (MPO) activity was determined as an indicator of polymorphonuclear leukocyte accumulation according to Paiva et al. (2002). The colon tissue was homogenized in a solution containing 0.5% hexadecyltrimethylammonium bromide dissolved in 50 mM potassium phosphate buffer (pH 6) and sonicated over an ice bath for 10 s. Homogenates were freeze-thawed three times, repeating the sonication step and centrifuged for 20 min at 20,000 rpm at 4 °C. The MPO activity was measured spectrophotometrically. A 0.1 mL volume of the material to be measured was mixed with 2.9 mL of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was then measured for 5 min using a Metrolab spectrophotometer (Metrolab 325 BD). The MPO activity was expressed as the optic density (OD)*100/mg of tissue.

2.6.6. Measurement of TBARS

Thiobarbituric acid reactive substances (TBARS) are low molecular weight compounds formed via decomposition of certain primary and secondary lipid peroxidation products that, at low pH and at high temperature, participate in a nucleophilic addition reaction with thiobarbituric acid, generating a red complex (Fraga et al., 1987).

The TBARS levels were measured in colon samples according Mariotto et al., (2008) with minor changes. Colon tissues were homogenized in phosphate buffer (pH 7.4; 10%). Two hundred μl of the homogenate were mixed with 200 μl of 8.0% w/v SDS and 600 μl of water. Then, 3 mL of 0.4% TBA in 10% acetic acid were incorporated, boiled for 1 h, cooled at room temperature and finally the concentration of malondialdehyde (MDA) formed during lipid peroxidation was measured spectrophotometrically at 635 nm. Results were expressed as nmol MDA/mg protein. The protein content of the extract was determined according to Lowry et al. (1951).

2.6.7. Contractile activity studies

One cm long segments of colonic mucosae were each mounted in 10 mL organ baths containing Tyrode's solution at 36 °C ± 1 °C and oxygenated with 95% O_2, 5% CO_2. The preparations were allowed to equilibrate for at least 30 min at 1 g resting tension. Tissues were connected to a force displacement transducer for the measurement of the isometric force. The cumulative concentration-response curves for carbachol were obtained (Soyer et al., 2009).

2.7. Spasmolytic activity

The experiment was performed according to Gorzalczany et al. (2013). Five independent experiments were performed with jejunum from 5 rats. Briefly, rats were fasted for 24 h with free access to water. Animals were sacrificed without any anesthetic agent in order to avoid any influence on the tissue relaxation response.

One cm long of jejunum were mounted in 10 mL organ baths containing Tyrode´s solution, at 36 ± 1 °C and oxygenated with 95% O_2, 5% CO_2. During 30 min tissues were allowed to equilibrate under 1 g resting tension and were connected to a force displacement transducer for the measurement of isometric force. The cumulative concentration-response curves for acetylcholine (Ach) were obtained in absence and presence of different concentrations of the *P. caerulea* extract (0.1, 0.3, 1 and 3 mg/mL). In other studies, after an initial incubation period, the Tyrode´s solution was replaced by a calcium-free and high potassium concentration medium (K^+ 75 mM). Concentration-response curves were obtained by cumulative addition of $CaCl_2$ ($10^{-4} - 3 \times 10^{-2}$ M) in the absence and presence of different concentrations of the *P. caerulea* extract (0.1, 0.3, 1 and 3 mg/mL).

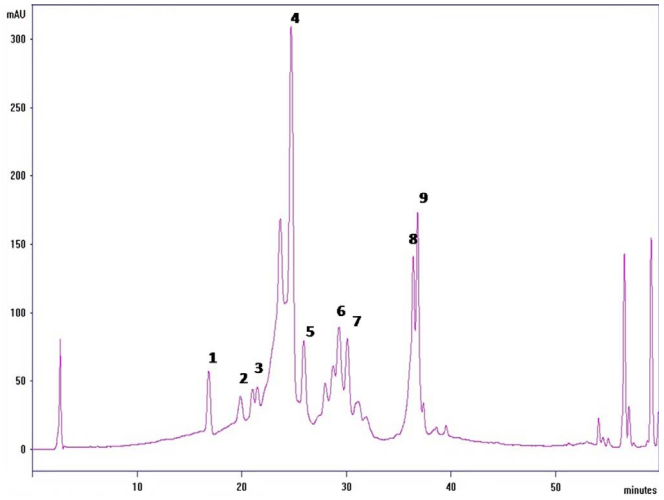

Fig. 1. HPLC chromatogram of ethanol extract of *Passiflora caerulea*. UV = 365 nm. 1: 16.9 min luteolin derivative; 2: 19.9 min vicenin-2; 3: 21.1 min luteolin derivative; 4: 24.7 min isoorientin; 5: 26.2 min vitexin (part of the peak); 6: 29.3 min isovitexin; 7: 30.0 min apigenin derivative; 8: 36.4 min chrysin derivative; 9: 36.8 min chrysin derivative.

Table 1
Amount of constituents identified in the extract.

Constitutent	Amount in the extract (%)
Isoorientin	4.5
Isovitexin	1.8
Vicenin-2	0.4
Vitexin	0.05

2.8. Castor oil-induced diarrhoea

Animals were divided into four groups containing 6 mice in each group. Two groups were treated with the extract (125 and 250 mg/ kg, p.o.), another group with loperamide hydrochloride (3 mg/kg, p.o.), and a fourth group with distilled water (0.1 mL/ 10 g, p.o.) 30 min before the administration of castor oil (0.3 mL/ mouse). Mice were housed individually in cages containing a filter paper and observed for a period of 4 h (Uddin et al., 2005). The following parameters were observed: first watery defecation time, total number of faecal output (solid, semi solid and watery stools), and total number of watery stools.

2.9. Castor oil-induced intestinal motility

The effect of *P. caerulea* on castor oil-induced intestinal motility in mice was tested using the charcoal method described by Mazzolin et al. (2013) and Uddin et al. (2005) with slight modifications. Briefly,

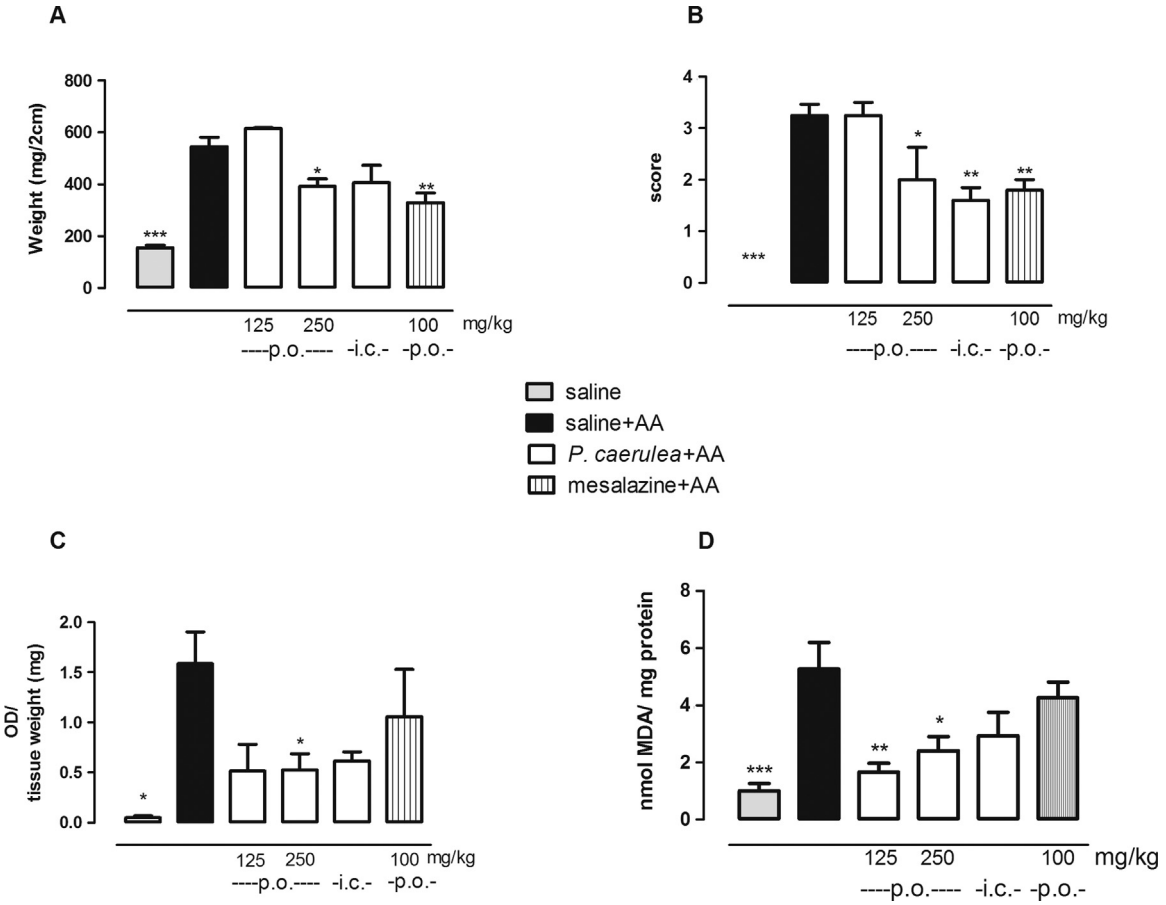

Fig. 2. Acetic acid-induced colitis model. Effect of *P. caerulea* ethanolic extract (125 and 250 mg/kg, p.o., 4% i.c.) and mesalazine (100 mg/kg, p.o.) on (A) colon wet weight/ length ratio (mg tissue/2 cm), (B) score based on macroscopic colon lesions, (C) myeloperoxidase activity expressed as optical density/ tissue weight in mg, and (D) lipid peroxidation levels expressed as nmol of MDA/ mg of protein in colon tissue. Results were expressed as means ± SEM from 5 rats and compared by one way ANOVA, followed by Dunett´s test (comparisons were made with the saline+AA group; $^{*}p < 0.05$, $^{**}p < 0.01$).

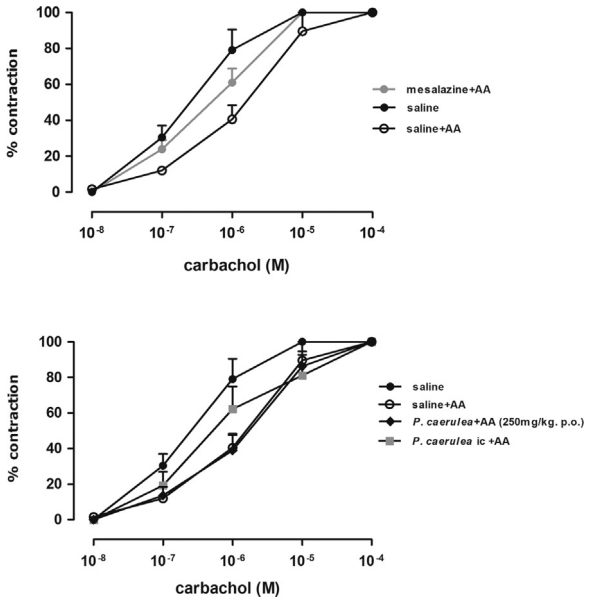

Fig. 3. Cumulative log concentration-response curves of carbachol on mesalazine (100 mg/kg, p.o.)+AA, saline and saline+AA groups (A) and on *P. caerulea* 250 mg/ kg p.o. or ic (4%)+AA (B). Results were expressed as mean ± SEM from 5 rats. (F: 6.62).

mice were fasted for 6 h and randomly assigned to four groups (six animals in each group) that received water 0.1 mL/10 g p.o., *P. caerulea* 125 mg/kg and 250 mg/kg p.o., or atropine 0.1 mg/kg i.p. Every 30 min, castor oil (0.3 mL/animal, p.o.) and then 10% activated charcoal (10 mL/Kg, p.o.) were administered to each mouse. Thirty min later, the distance that charcoal had migrated in the intestine was measured. Intestinal motiliy was quantified by expressing the distance travelled by charcoal in 30 min as a percentage of total small intestine length (from the pylorus to the ileal terminus).

2.10. Vascular permeability

Vascular permeability was assessed according to Yu et al. (2012). Briefly, animals were divided into four groups containing 6 mice each group and pretreated with the *P. caerulea* extract (125 and 250 mg/kg, i.p.), indomethacine (10 mg/kg, i.p.), or saline (0.1 mL/10 g, i.p.), respectively. After 30 min, each mouse received an intravenous injection of 0.5% Evans blue solution (w/v, in saline, 0.1 mL/10 g), and then injected i.p. with 0.8% acetic acid solution (0.2 mL/10 g) to induce inflammation. Twenty minutes after the administration of acetic acid, animals were sacrificed. The peritoneal cavity was washed with 6 mL of cold saline (divided into several washings) with a gentle manual massage. The exudates were collected, taken to 10 mL with saline and centrifuged for 15 min at 3000 rpm. The optical density of the supernatant was measured at 590 nm in a Metrolab 325 BD spectrophotometer. Dyed extravasations were quantified from a standard curve. Indomethacine was used as reference drug.

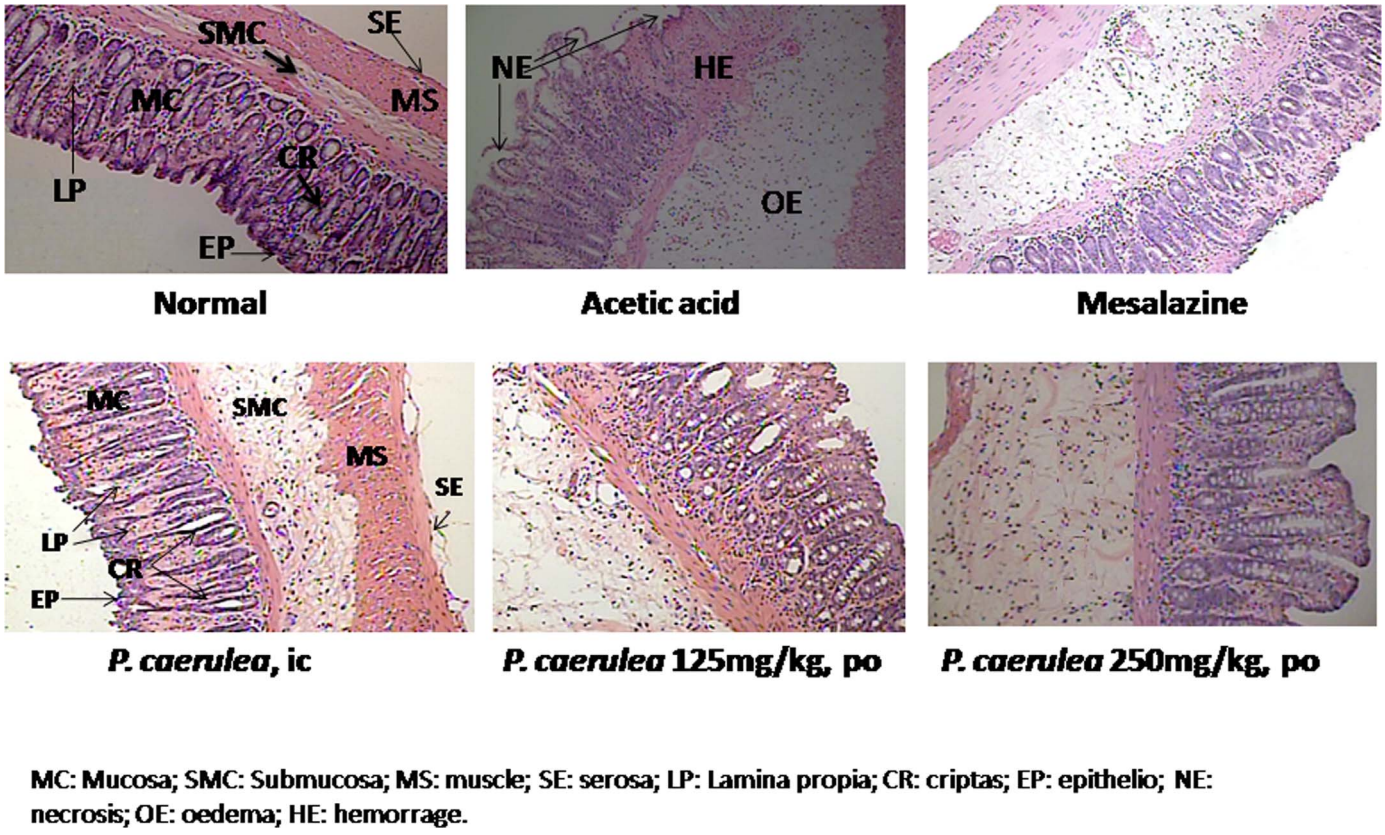

Fig. 4. Effect of *P. caerulea* extract (125 and 250 mg/kg, p.o.; 4%, i.c.), saline and mesalazine (100 mg/kg, p.o.) on colon morphology 24 h after acetic acid intra-colonic administration and normal group. Representative hematoxylin and eosin-stained sections of the rat colons for at least 4 independent analysis (H&E 10 ×).

2.11. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). The statistical significance of differences between groups was assessed by means of analysis of variance (one way ANOVA, two way ANOVA) followed by Dunnett's test or Bonferroni´s test. *P* values of 0.05 were considered significant. The statistical analysis was carried out using the Instant statistical package (Graph Pad software, Inc., version 5 USA).

3. Results

3.1. Phytochemical analysis

The phytochemical analysis showed that the total phenol content expressed as GAE/g of the *P. caerulea* extract was 61.5 ± 0.17 GAE/g. Isoorientin, vitexin, isovitexin, and vicenin-2 were identified in the extract (Fig. 1). Their amount in the extract was quantified (Table 1).

3.2. Ulcerative colitis model

No mortality was observed in any rat. Unlike the treatment with mesalazine, acetic acid caused an increase in the wet weight/length ratio. Besides, *P. caerulea* (250 mg/kg), administered by the oral and the intracolonic routes, decreased the wet weight/length ratio significantly (Fig. 2A). The macroscopic inflammation score showed a similar pattern between acetic acid-treated animals treated with either the extract or mesalazine (Fig. 2B). The effect of *P. caerulea* on MPO levels is shown in Fig. 2C. MPO levels were found to be significantly increased in the ulcerative colitis group compared to the control group. The treatment with *P. caerulea* induced a decrease in the colon MPO levels, without affecting the mesalazine group. Lipid peroxidation was significantly reduced after the treatment with *P. caerulea* extract and mesalazine (Fig. 1D). Groups that received 1% CMC by oral route and water: propylenglycol: 2% CMC in water by intracolonic route, displayed no evidence of pharmacological activity.

Carbachol produced concentration-dependent contractions of colon segments. The concentration – response curves to carbachol in colon segments from experimental groups are shown in Fig. 3. The colon contraction induced by carbachol was reduced in the colitis group. Mesalazine (Fig. 3A) and *P. caerulea* i.c. groups reversed the decrease in carbachol-induced contraction associated with acetic acid i.c. group (Fig. 3B).

3.2.1. Histology

The effect of *P. caerulea* on the colon morphology is shown in Fig. 4. Control animals showed a normal colon structure without neither inflammation nor necrosis foci (Fig. 4A). Acetic acid caused severe injury to the colon mucosae, degeneration and necrosis of epithelial cells was observed, crypt distortion, diffuse polymorphonuclear leukocytes infiltration in the submucosa and lamina propia and evidence of massive hemorrhage with a significant accumulation of erythrocytes in the lamina propia. The treatment with *P. caerulea* extract (i.c. and 250 and 125 mg/kg p.o.) and mesalazine proved to reduce the severity of tissue damage.

Considering the mucosa protective role of goblet cells and taking into account that the number of such cells is reduced in ulcerative colitis (Kim and Ho, 2010), the Alcian blue staining was performed in colon samples. A significant decrease in the number of goblet cells and mucin production were observed in colitis group. These effects were reduced by *P. caerulea* and mesalazine treatments, showing a higher number of goblet cells in those experimental groups (Fig. 5).

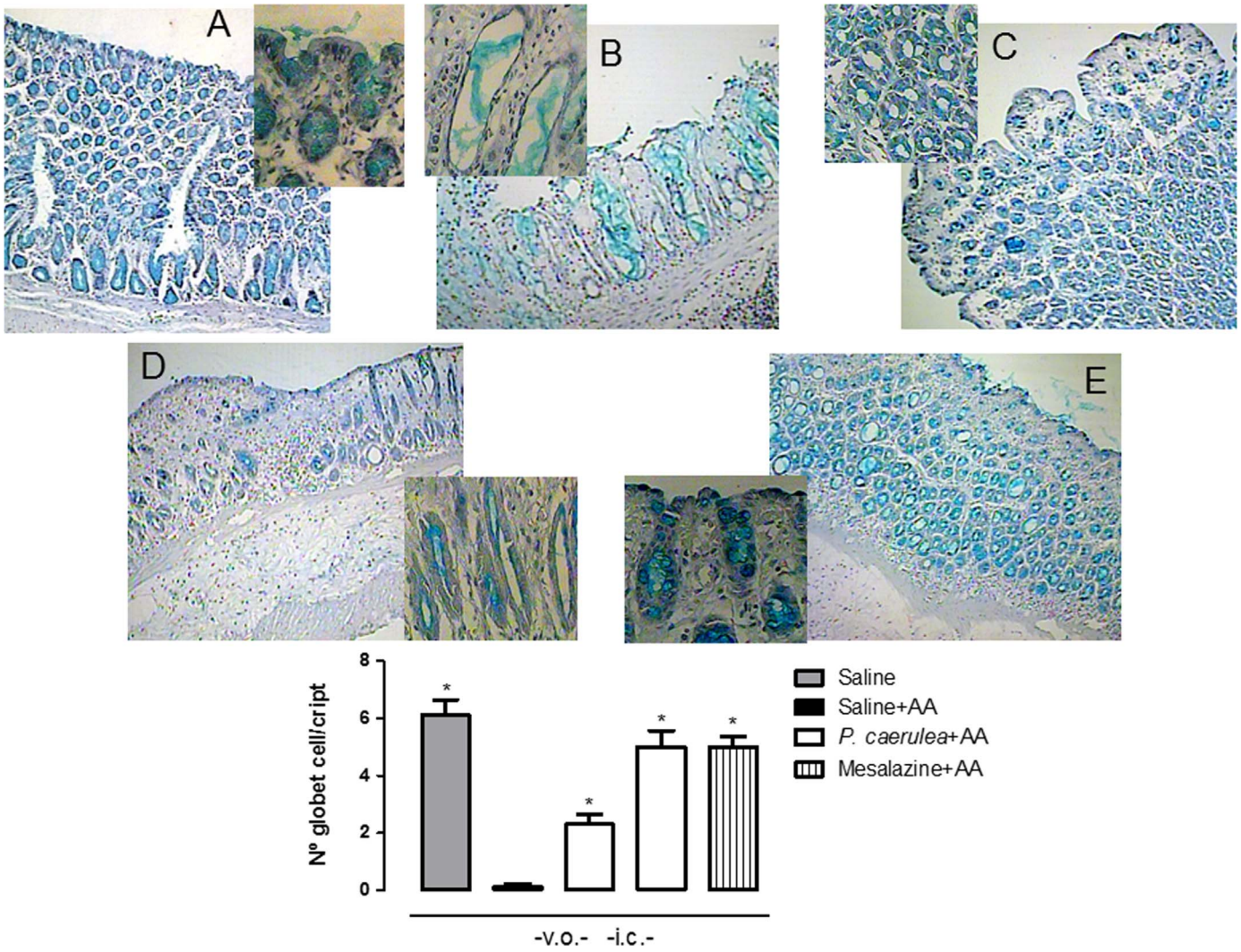

Fig. 5. Effect on goblet cells and qualitative alteration in mucus layer and storage of mucin on the colonic mucosa in: (A) Saline group, (B) Saline+AA group, (C) AA+*P. caerulea* i.c. (4%), (D) AA+*P. caerulea* 250 mg/kg p.o. and (E) Mesalazine group (100 mg/kg, p.o.). (Alcian blue, 10 × and 40 ×). Representative hematoxylin and eosin-stained sections of the rat colons for at least 4 independent analysis.

3.3. Spasmolytic activity

The *P. caerulea* extract antagonized the jejunum contractions induced by Ach ($1 \times 10^{-9} - 1 \times 10^{-5}$ M). Higher concentration of the extract significantly reduced the maximal response of the agonist in a concentration-dependent manner (E_{max} for 0.3 mg/mL, 76.25%; E_{max} for 1 mg/mL, 63.47%; E_{max} for 3 mg/mL, 42.01%), without inducing any change in potency (pD_2 for 3 mg/mL, 6.28; pD_2 for 1 mg/mL, 6.45; pD_2 for 0.3 mg/mL, 6.12), suggesting a spasmolytic effect of the extract (Fig. 6A).

In order to investigate if the observed effect was due to the blockade of calcium channels, the effect of *P. caerulea* extract on the muscle contractions induced by $CaCl_2$ ($1 \times 10^{-4} - 3 \times 10^{-1}$ M) was analyzed. The extract non-competitively inhibited the response-concentration induced by $CaCl_2$ and higher concentrations reduced the maximal response in a 50% (Fig. 6B).

3.4. Effect of P. caerulea on castor oil-treated mice

In the castor oil-induced diarrhoea test in mice, no differences were found on the total number of stools between control and test groups (Fig. 7A). Unlike the dose of 250 mg/kg; the dose of 125 mg/kg of the extract reduced significantly the number of watery stools (Fig. 7B). Both doses caused a slight increase in the time elapsed between the administration of the castor oil and the excretion of the first diarrhoea faeces as compared to the diarrhoea control group (Fig. 7C). In the loperamide-treated animals, a significant decrease in the number of total and watery stools and a significant increase in the latency time, until the development of diarrhoea were observed.

In the castor oil-induced intestinal motility test in mice (Fig. 7D), *P. caerulea* (250 mg/kg, p.o.) significantly reduced the intestinal motility, decreasing the peristalsis by a 35.1% compared to the control group (72.6%). Similar results were obtained with the reference drug, atropine (31.7%).

3.5. Vascular permeability

The reduction induced by *P. caerulea* extract on the acetic acid-induced increase of abdominal capillary permeability was evaluated. The dye leakage into the abdominal cavity was suppressed significantly in the *P. caerulea* 125 mg/kg i.p. group and indomethacine groups. The values of extravasation of Evans blue obtained were: saline group: 8.92 ± 0.93 μg/mL,*P. caerulea* 125 mg/kg i.p.:4.90 ± 0.98 μg/mL, *P. caerulea* 250 mg/kg i.p.: 6.61 ± 0.88 μg/mL, indomethacine: 5.20 ± 0.75 μg/mL).

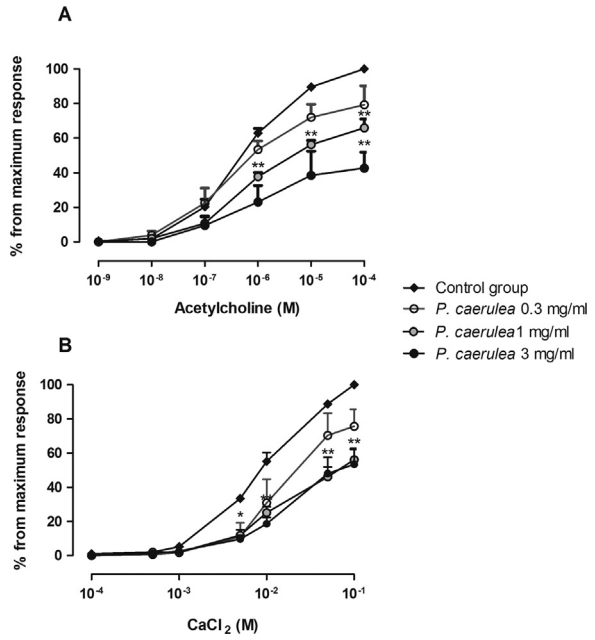

Fig. 6. Effect of *P. caerulea* ethanolic extract (0.3, 1 and 3 mg/mL) on (a) cumulative log concentration-response curves for Ach and (b) cumulative log concentration-response curves for $CaCl_2$. Results were expressed as mean ± SEM, from 5 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ (*P.caerulea vs* control group) (one way ANOVA, followed by Bonferroni *a posteriori* test).

4. Discussion

In this work we have studied the effect of the ethanolic extract of *P. caerulea* on intestinal disorders. Firstly, the acetic acid-induced acute colitis test was used since it is considered an experimental model with some pathological and clinical features that are similar to the human ulcerative colitis (Minaiyan et al., 2014) which includes neutrophil infiltration into the intestinal tissue, necrosis of mucosal or submucosal layers, vascular dilation, oedema and submucosal ulceration (Randhawa et al., 2014). The protective effect of *P. caerulea* extract on experimentally induced colitis was observed by its ability to decrease the mucosal MPO activity, the TBARS levels, and the colon weight/length ratio, to reduce the colonic damage..

The IBD is a gastrointestinal inflammatory process that has been associated with an intense local immune response followed by the release of cytokines and inflammatory mediators. The wet weight of the inflamed colonic tissue is considered an indicator of the severity and extent of the inflammatory response. Although neutrophils are critical in the mechanisms of host's defence, they can also produce tissue damage in inflammatory processes. Neutrophils secrete enzymes and release reactive oxygen species that are harmful for the colonic tissue. In this sense, the MPO activity from the inflamed tissues is a marker of neutrophil recruitment and an indicator of acute intestinal inflammation (Vinod Prabhu et al., 2014). In the present study, the *P. caerulea* extract significantly reduced the colon wet weight/length radio and the MPO activity, suggesting that the extract could contribute to the attenuation of the inflammation induced by acetic acid and to the regulation of neutrophil trafficking. This anti-inflammatory activity was also reinforced by the low macroscopic score and the histological findings obtained in the animals treated with the extract. Microscopically, the acetic acid group showed loss of epithelial cells, tissue necrosis with crypt atrophy. Moreover, the number of goblet cells, the storage of mucin and the mucus layer

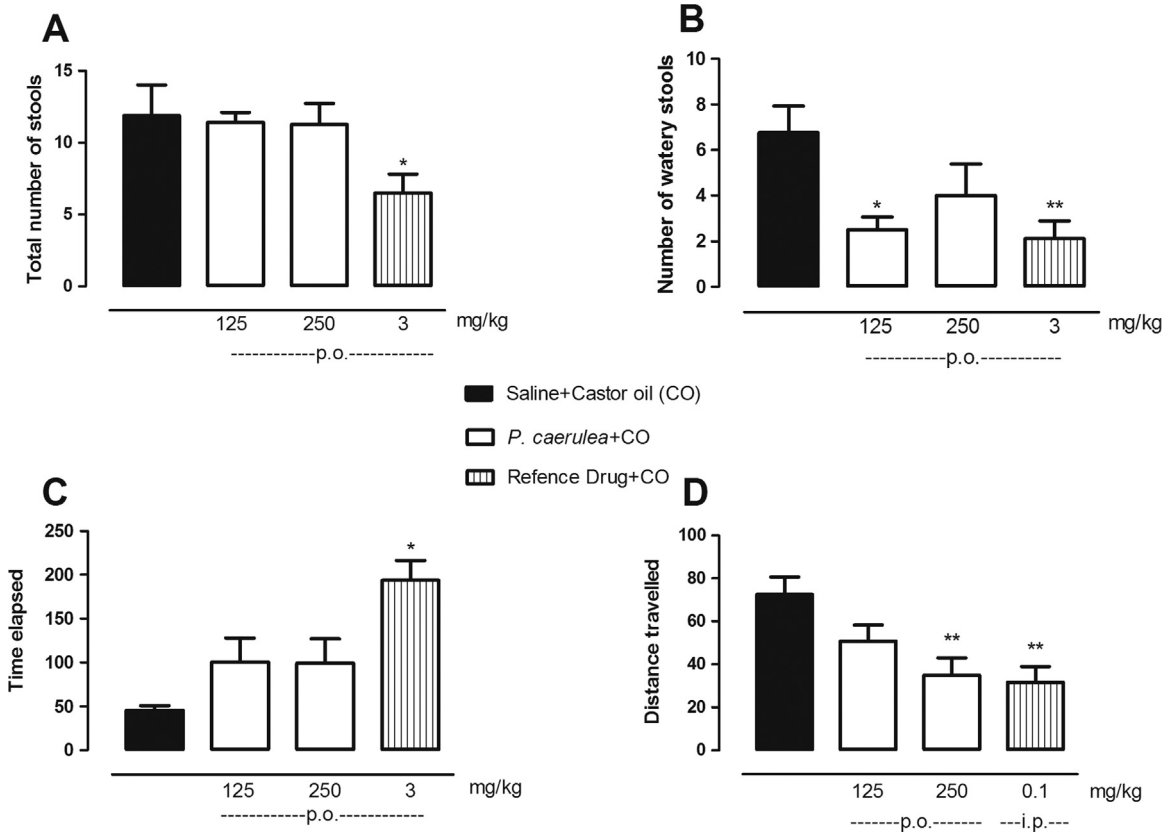

Fig. 7. Castor oil-induced diarrhoea and intestinal motility model in mice. Effect of *P. caerulea* ethanolic extract (125 and 250 mg/kg, p.o.) or reference drug (3 mg/kg, p.o.) on. (A) Total number of stools (solid semi-solid and watery stools), (B) number of watery stools,(C) time elapsed between the administration of the castor oil and the excretion of the first diarrhoea faeces, and (D) distance travelled by the charcoal marker expressed as % of total length of the small intestine. Results were expressed as mean ± SEM, from 6 mice. $^{*}p < 0.05$, $^{**}p < 0.01$, one way ANOVA, followed by Dunett's test.

overlying the epithelium were diminished by the administration of acetic acid. Since mucus is the first line of defence against physical and chemical injuries, the beneficial effects of the extract, mainly when administrated by the intracolonic route, could represent an useful strategy to protect the integrity of the intestinal mucosa.

On the other hand, although the pathophysiology of IBD has not been fully studied, the increase of reactive oxidative species in colonic tissues plays a pivotal role in the pathogenesis of IBD and could contribute to the initiation and/or propagation of the disease (Rezaie et al., 2007). Since lipid peroxidation is a biomarker of oxidative stress, the reduction of TBARS levels in colon induced by *P. caerulea* extract would indicate that the extract could possess an antioxidant effect in this experimental model.

The endothelial barrier dysfunction, that is known to induce an increased vascular permeability, is critical for the initiation and perpetuation of an inflammatory response. It is known that the development of an oxidative state contributes to the inflammatory response, featuring leukocyte recruitment and vascular protein leakage. There are evidences indicating that polymorphonuclear neutrophil-derived proteases can disrupt inter endothelial junctions, inducing endothelial cell retraction and paracellular permeability. The widening of the inter endothelial junctions results in microvascular protein and fluid leakage into the interstitium, inducing oedema (Kvietys et al., 2012). The effect of the extract on vascular permeability could contribute to the reduction of oedema in the experimental model, adding a beneficial effect to the pharmacological profile of *P. caerulea.*

Clinical and *in vitro* studies suggest that colitis can induce alterations in the motility of the smooth muscle from the inflamed area (Myers et al., 1997). In this study, acute colonic mucosal inflammation produced a reduction of the contractile response induced by carbachol. Although intracolonic administration of *P. caerulea* could not induce a significant recovery of the motility in colonic tissue, taking into account the *in vitro* activity of the extract, further studies will be necessary to clarify the pharmacological role of the extract in other parts of intestine on chemical induced colitis.

Changes in the colonic motility and the epithelial barrier dysfunction produce an increase in the intestinal permeability which could contribute to induce diarrhoea in IBD. The castor oil is a suitable substance to induce diarrhoea, since its active principle, recinoleic acid, has proved to be able to alter the permeability to electrolytes in the intestine (Uddin et al., 2005). The effect induced by *P. caerulea* in this model, would indicate that the extract exerts a beneficial antisecretory effect in intestinal diseases related to inflammation.

The intestinal motility induced by castor oil was also evaluated upon administration of the *P. caerulea* extract. The reduction of the distance travelled by charcoal in the *P. caerulea* treated animals suggested that the extract may exert its antidiarrhoeal action by reducing the intestinal motility. At the same time, this reduction of peristalsis could be related to a decrease of *in vitro* contractions induced by Ach and calcium in the isolated jejunum. These results would indicate that the extract possesses antidiarrhoeal and antispasmodic actions, and these activities could lead to a relief from symptoms such as gastrointestinal discomfort.

As similar to other species of *Passiflora*, flavonoids isoorientin, vitexin, isovitexin, and vicenin-2 were found in aerial parts of *P. caerulea* (Müller et al., 2005; Pereira et al., 2004). Evidences indicate that phenolic compounds have an antioxidant activity and that the decrease in the level of reactive oxygen species might lead to an anti-inflammatory status (Chouhan and Singh, 2011), since the extract total phenol content is considerable, these compounds could be implicated in the extract´s activity. Isoorientin, vitexin and vicenin-2 have shown antinociceptive and anti-inflammatory properties (Küpeli et al., 2004; Gorzalczany et al., 2011; Marrassini et al., 2011; Prabhakar et al., 1981). Moreover, vicenin-2 have shown antispasmodic activity trough inhibition of neurotropic and musculotropic activity (Verspohl et al., 2013). Although none of the compounds have been tested in experimental models of colitis, those activities could be related to beneficial effects on intestinal inflammatory processes and could explain, at least in part, the observed effect of the whole extract.

Safety and efficacy of herbal medicines in clinical trials, in many cases, remains problematic because many of them do not contain all the information recommended by CONSORT (Consolidated Standards of Reporting Trials) guidelines (Izzo et al., 2016). It is important to note that preclinical assays using animal models, like this work, can be useful to support clinical decisions.

In conclusion, this study demonstrated, for the first time, that *P. caerulea* possesses antiinflammatory, antidiarrhoeal, spasmolityc, and antioxidant effects in preclinical models, suggesting that the extract and/or its active principles might represent a potential therapeutic alternative for IBD, thus contributing to the relief of symptoms. Further studies are required to elucidate the precise mechanism involved in the effects induced by *P. caerulea* extract.

Acknowledgment

This work was supported by the grant 20020130200265BA of the Universidad de Buenos Aires, Argentina.

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