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Antiulcer Effect of Bark Extract of *Tabebuia avellanedae*: Activation of Cell Proliferation in Gastric Mucosa During the Healing Process

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***Tabebuia avellanedae* (syn. *Handroanthus impetiginosus*) is popularly known as 'ipê-roxo' and has been used in folk medicine as anti-inflammatory and in the treatment of ulcers, bacterial and fungal infections. This study evaluated the gastric ulcer healing property of the ethanolic extract (EET) of barks from *Tabebuia avellanedae* and investigated the mechanisms that may underlie this effect. Rats were treated with EET (twice a day for 7 days) after induction of chronic gastric ulcers by 80% acetic acid. Following treatment, histological and immunohistochemical analysis were performed in gastric ulcer tissues. Oral administration of EET (100 and 300 mg/kg) significantly reduced the gastric lesion induced by acetic acid in 44 and 36%, respectively. Histopathological evaluation demonstrated a contraction of gastric ulcer size, increase of mucus layer (periodic acid-Schiff stained mucin-like glycoproteins) and cell proliferation (proliferating cell nuclear antigen immunohistochemistry) in animals treated with EET (100 and 300 mg/kg). The results demonstrate that EET significantly accelerates healing of acetic acid induced gastric ulcer in rats through increase of mucus content and cell proliferation, indicating a potential usefulness for treatment of peptic ulcer diseases. Copyright © 2012 John Wiley & Sons, Ltd.**

Keywords: Tabebuia avellanedae; Handroanthus impetiginosus; chronic gastric ulcer; PCNA; mucin.

Supporting information may be found in the online version of this article (Supplementary Material)

INTRODUCTION

Tabebuia avellanedae Lorentz ex Griseb. (syn. *Handroanthus impetiginosus*) belongs to Bignoniaceae family and is popularly known as 'ipê-roxo', 'lapacho' or 'pau d'arco'. This specie has been traditionally used for various ethnopharmacological purposes, including as anti-inflammatory, antibacterial, antifungal and anti-ulcer (Goel *et al*., 1987; Guiraud *et al*., 1994; de Miranda *et al*., 2001). Indeed, some authors have demonstrated several biological activities for *T. avellanedae*, as antifungal (Portillo *et al*., 2001), antinociceptive and anti-oedematogenic (de Miranda *et al*., 2001), anti-inflammatory (Byeon *et al*., 2008), antioxidant (Park *et al*., 2003) and antidepressive (Freitas *et al*., 2010). Some of these activities promoted by the extract of this plant were attributed to its active compounds like β-lapachone and lapachol (Goel *et al*., 1987; de Almeida *et al*., 1990; Guiraud *et al*., 1994). Moreover, our group recently demonstrated that the gastroprotective effect of the ethanolic extract (EET) of *Tabebuia avellanedae* was involved with increasing in gastric mucus and inhibition of the H^+,K^+-ATPase activity *in vitro* (Twardowschy *et al*., 2008).

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It is known that peptic ulcers affect a large number of people in the world (Grob, 2004). Lifestyle factors (alcohol abuse, stress and smoking), use of NSAIDs and infection by *Helicobacter pylori* are among the most common etiologic factors (Dong and Kaunitz, 2006). Despite the availability of current therapy, based on acid suppression by antacids, antagonists of H_2 receptors and proton pump inhibitors, new therapeutic alternatives that promote a better healing process of gastric ulcers, with increasing of speed and quality of this process healing are interesting.

Considering that, the purpose of this paper is to investigate additional healing effects of the EET of barks from *Tabebuia avellanedae* through experimental model of chronic gastric ulcer induced by acetic acid in rats. We also investigate the involvement of mucin-like glycoproteins (mucus) and cell proliferation in the EET activity.

MATERIALS AND METHODS

Plant material and preparation of EET. Barks of *Tabebuia avellanedae* were provided by Chamel Indústria e Comércio de Produtos Naturais Ltda (Campo Largo, Paraná, Brazil), lot 4753. A voucher specimen has been deposited at the Herbarium of the Department of Botany at the Universidade Federal do Parana, Brazil.

The extraction procedure was described in Twardowsky *et al*. (2008). Briefly, air-dried barks were powdered and extracted three times by maceration with 95% ethanol

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for 7 days at room temperature. The EET was filtered and dried by evaporation under reduced pressure to give a red-brown solid (919.2 g dry) with yield of 18.4%. This ethanolic crude extract retained a water content of 3%.

Capillary electrophoresis analysis of EET. The analyses were conducted in a CE system ($HP^{3D}CE$, Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector set a 200 nm. The measurements were performed at 25 °C in an uncoated fused-silica capillary (48.5 cm × 50 μm I.D. × 375 μm O.D.) obtained from Polymicro (Phoenix, AZ, USA). Capillary were injected with 1.0 M NaOH followed by deionized water for 30 min and between runs, the capillary was rinsed for 5 min with electrolyte solution (20 mmol/L sodium tetraborate and 10% methanol 10% at pH 9.0). Standard solutions and samples (0.5299 g of EET was solubilized into 10 mL of 50% methanol:water) were introduced from the inlet capillary extremity and injected hydrodynamically at 50 mbar (50 mbar = 4996.2 Pa) for 6 s. The applied separation voltage was 30 kV, positive polarity in the injection side. Caffeic acid (100 mg/L) was utilized as internal standard and detection at 330 nm. Data acquisition and treatment were performed with HP Chemstation software.

Estimation of total phenolic content. Total phenols were determined using the Folin-Ciocalteau reagent, with the microscale protocol developed by Arnous *et al*. (2001). EET (50, 100, 150 and 200 μg in 0.5 ml of distilled water) was mixed with 2.5 ml of Folin-Ciocalteau reagent (1:10 dilution) and 2 ml of sodium carbonate (7.5% w/v), incubated at 45 °C for 15 min and the absorbance read at 750 nm using a spectrophotometer. Tannic acid was employed as a calibration standard (10–100 μg/ml), and the results were expressed as tannic acid equivalents (TAE) in μg.

Animals. All experiments were performed on adult female Wistar rats (180–200 g), housed at 22 ± 2 °C under a 12-h light/12-h dark cycle and with access to food and water *ad libitum*. The animals were deprived of food for 18 h prior to experiments. All experimental protocols were previously approved by the Institutional Ethics Committee of the Universidade Federal do Paraná (approval number: 437) and were carried out in accordance with the international standards and the ethical guidelines on animal welfare.

Chronic gastric ulcer induced by acetic acid. Chronic gastric ulcers were induced with acetic acid as previously described by Okabe *et al*. (1971), with minor modifications. Rats were anaesthetized with xylazine/ketamine (7.5 mg/kg and 60 mg/kg, i.p., respectively), the abdomen was opened and the stomach exposed. The acetic acid (0.5 ml, 80% v/v) was instilled into a cylinder (6 mm in diameter) and applied to the serosal surface of the stomach during 1 min. The acetic acid was removed by aspiration and the area was washed with sterile saline. On the second day after the ulcer induction, animals were orally treated with vehicle (water, 1 ml/kg), omeprazole (40 mg/kg) or EET (30, 100 and 300 mg/kg) twice a day for 7 days. On the day following the last administration, the animals were sacrificed, the stomachs removed and the extent of the gastric ulcer was measured as the total injured area (mm^2) = length (mm) × width (mm).

For histopathological examination, gastric ulcers were fixed in Alfac solution for 16 h. After fixation, the tissue samples were dehydrated with alcohol and xylene. Immediately after the dehydration, each sample was embedded in paraffin wax, sectioned at 5 μm using a microtome and stained with hematoxylin/eosin. The gastric sections were observed and photographed under a stereomicroscope at 16-fold magnification (Potrich *et al*., 2010).

Determination of mucus content. Mucin histochemistry was performed according to Mowry and Winkler (1956) and used to verify the alterations on mucus content of gastric mucosa after acetic acid-induced gastric ulcer. The samples were deparaffinized, rehydrated, oxidized in 0.5% periodic acid for 5 min and washed in distilled water. Then, the sections were stained with Schiff's reagent for 20 min and subsequently washed with sulphurous water (three times for 2 min) and in tap water for 10 min. Finally, the slides containing sections were counterstained with hematoxylin for 20 s and dehydrated. Periodic acid-Schiff (PAS)-stained mucin-like glycoproteins positive pixels were quantified with ImageJ® software.

Determination of cellular proliferation. Immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) was performed to determine proliferating cells in acetic acid-induced gastric ulcer. Paraffin-embedded sections were deparaffinized in xylene and hydrated through standard graded ethanol solutions. Sections were rinsed two times for 5 min each in PBS (pH 7.4), incubated in H_2O_2 solution (3% in methanol) for 10 min to inactivate endogenous peroxides. Blocking of nonspecific reaction was performed with blocking solution (1% BSA and 0.3% Triton X-100 in PBS) for 30 min. The sections were then incubated for 2 h at 4 °C with goat anti-PCNA (1:100; Santa Cruz Biotechnology Inc., CA, USA). After that, slides were rinsed in PBS (pH 7.4), and the sections were incubated in secondary antibody at room temperature for 1 h. After washing, the immunoreacted cells were then developed utilizing avidin-conjugated horseradish peroxidase with diaminiobenzidine as substrate (BD Biosciences, San Diego, CA, USA). Finally, the specimens were counterstained with hematoxylin. PCNA-containing cells were identified by the presence of a dark reddish-brown chromogen. The nuclear-positive staining cells were observed under microscope (400×) (Potrich *et al*., 2010).

Drugs and reagents. The following substances were used: xylazine (Vet Brands, Miramar, USA), ketamine (Syntec, Cotia, Brazil), omeprazole, hematoxylin, eosin, fuchsin, bovine serum albumin, triton-X (all from Sigma, St. Louis, USA), acetic acid, acetaldehyde, chlorhydric acid, diaminobenzidine, ethanol, hydrogen peroxide, methanol, sodium chloride, sodium metabisulfite, sodium phosphate, periodic acid (all from Vetec, Rio de Janeiro, Brazil).

Statistical analysis. Data were expressed as means ± standard error of mean (S.E.M.). Differences between means were determined by one-way analysis of variance followed by Bonferroni's post hoc test. *P* values less than 0.05 were considered as indicative of significance.

RESULTS

Capillary electrophoresis analysis and estimation of total phenolic conten

The electropherograms for analysis of the EET are shown in Supplementary material 1, which has signals at migration time in the range of 2.1–6.5 min, identified the following compounds: (i) *p*-hydroxybenzoic acid, (ii) anisic acid, (iii) veratric acid and (iv) caffeic acid. However, no signals have been observed at 3–6 min that could be attributed to lapachol.

Table 1 shows the total phenolic content in the EET expressed as TAE (in μg). Total phenolic content varied from 26.89 ± 0.01 to 88.59 ± 0.07 μg TAE.

Effect of EET on chronic gastric ulcer induced by acetic acid

Oral administration of EET (100 and 300 mg/kg) reduced the gastric ulcer induced by acetic acid in 44 and 36%, respectively, when compared to control group (140 ± 13 mm^2). Omeprazole (40 mg/kg, p.o.), positive control of the test, also inhibited the gastric ulcer size in 48% (Fig. 1).

Histological analysis of the gastric ulcers showed that acetic acid promoted an extensive deep tissue injury (Fig. 2A and E). Slices from gastric ulcers treated with omeprazole (40 mg/kg, p.o.) or EET (100 and 300 mg/kg, p.o.) demonstrated an ulcer size regression (Fig. 2B, C and D, respectively), arranged into columnar structures above the granulation tissue (Fig. 2F, G and H, respectively).

Effect of EET on mucus content

The result depicted in Fig. 3A shows that acetic acid application on gastric mucosa decreased the PAS-positive material (mucus). However, oral treatment of animals with EET (100 and 300 mg/kg) or omeprazole (40 mg/kg) increased the staining for glycoproteins corresponding to mucin (Fig. 3C, D and B, respectively). The quantified PAS-staining revealed that the treatment with omeprazole or EET (100 and 300 mg/kg) increased the mucin-like glycoproteins in 120, 173 and 208%, respectively, when compared to control group (C: 31.93 ± 5.41 $\times 10^4$ pixels/field) (Table 2).

Table 1. Total phenolic contents of EET[a]

EET (μg)	Tannic acid equivalents (TAE)
50	26.89 ± 0.01
100	47.70 ± 0.05
150	69.50 ± 0.01
200	88.59 ± 0.07

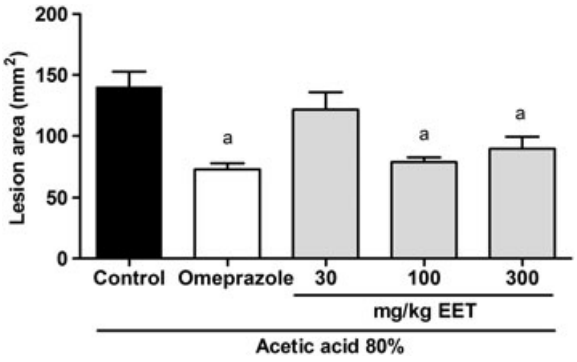

Figure 1. Effect of oral administration of EET on chronic gastric ulcers induced by 80% acetic acid in rats. The animals were orally treated with vehicle (Control: water, 1 ml/kg), omeprazole (40 mg/kg) or EET (30, 100 and 300 mg/kg) twice a day for 7 days after induction of gastric ulcer. Results were expressed as mean ± S.E.M. (n = 8) and statistical comparison was performed using one-way analysis of variance (ANOVA) followed by post hoc Bonferroni's test. [a] p <0.05 when compared to the control group.

Effect of EET on cell proliferation

The animals that received omeprazole or EET (100 and 300 mg/kg) showed a significant increase of PCNA immunoreactivity in ulcerated gastric mucosa (Fig. 4B, C and D, respectively), which is characterized by brown color and indicates the proliferating cells when compared to control group (Fig. 4A). There was an increase in the quantity of PCNA immunoreactivity between omeprazole and EET (100 and 300 mg/kg) treatments by 219, 124 and 337%, respectively, when compared to control group (C: 27.0 ± 3.7) (Table 2).

DISCUSSION

The present study confirms and extends previous reports that EET of bark from *Tabebuia avellanedae* Lorentz ex Griseb. possess antiulcerogenic activity against gastric ulcer induced by acetic acid. It was demonstrated by Twardowsky *et al.* (2008) that this extract accelerated the healing of gastric mucosa in rats, but no mechanisms of action was added. For this reason, in this study, we investigated the possible mechanisms that underlie the gastroprotective action of EET on acetic acid-induced gastric ulcer model.

Since it was introduced in 1969 by Takagi *et al.*, the model of gastric ulcer induced by acetic acid has been proved useful to investigate the pathophysiology of gastric ulcer disease and the efficacy of antiulcer drugs (Tsukimi and Okabe, 1994). In addition, this model has been developed to examine the healing process of peptic ulcers (Okabe and Amagase, 2003). This kind of ulcer induction procedure is simple and resembles human peptic ulcers macroscopically, histologically and in terms of pathology and healing processes (Okabe and Amagase, 2003; Kang *et al.*, 2010). Our results demonstrated that EET promoted the acceleration of

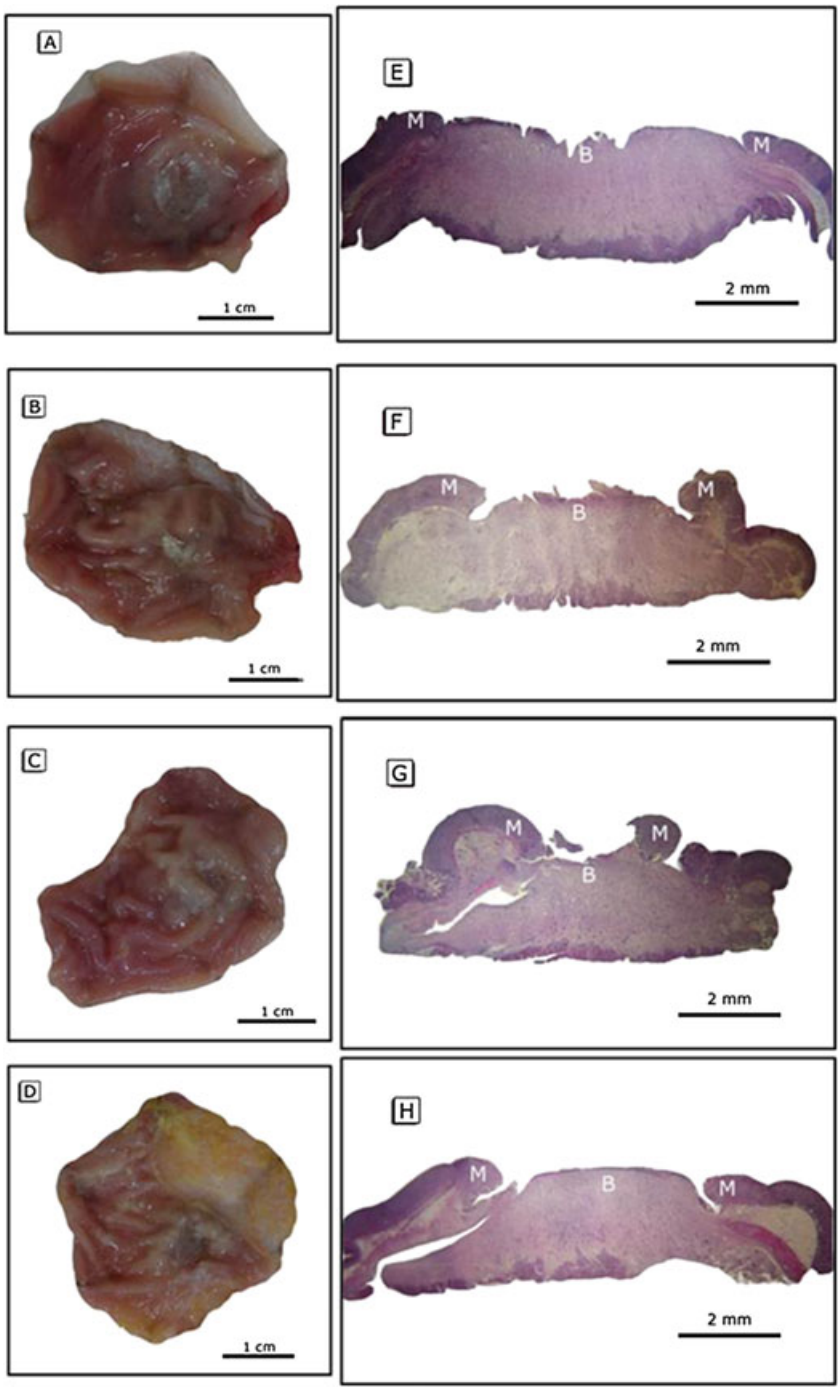

Figure 2. Effect of oral administration of EET on the regeneration of gastric mucosa 10 days after injury induced by 80% acetic acid in rats. The images representing macroscopic photograph of the control group water 1 ml/kg (A), omeprazole 40 mg/kg (B), EET 100 mg/kg (C) or EET 300 mg/kg (D); and the histological sections (16X) of the control group water 1 ml/kg (E), omeprazole 40 mg/kg (F), EET 100 mg/kg (G) or EET 300 mg/kg (H). Bars = 1 cm (A–D) and 2 mm (E–H), where M indicates margin of ulcer and B indicates base of ulcer. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

healing process, similar to data obtained by Twardowsky *et al*. (2008).

Ulcer healing, a genetically programmed complex repair process, includes inflammation, cell proliferation, re-epithelization, formation of granulation tissue, angiogenesis, interactions between various cells and the matrix and tissue remodeling, all resulting in reconstruction of gastric mucosal architecture (Tarnawski, 2005). This process consists in two phases: the quick healing that depends on contraction of the ulcer basis and the slow healing that needs mucosa regeneration (Vasconcelos *et al*., 2010). The healing action presented by EET was confirmed by histological analysis where it was observed an extensive repair of gastric ulcer caused by the administration of acetic acid, with contraction of the ulcer base. Similarly, Coelho *et al*. (2010) observed that an ointment containing the aqueous extract of *T. avellanedae* presented skin wound healing activity, accelerating the epithelization in rats.

In addition, during the healing process the newly formed cells must be protected against damage caused by acidic pH and the proteolytic potential of gastric

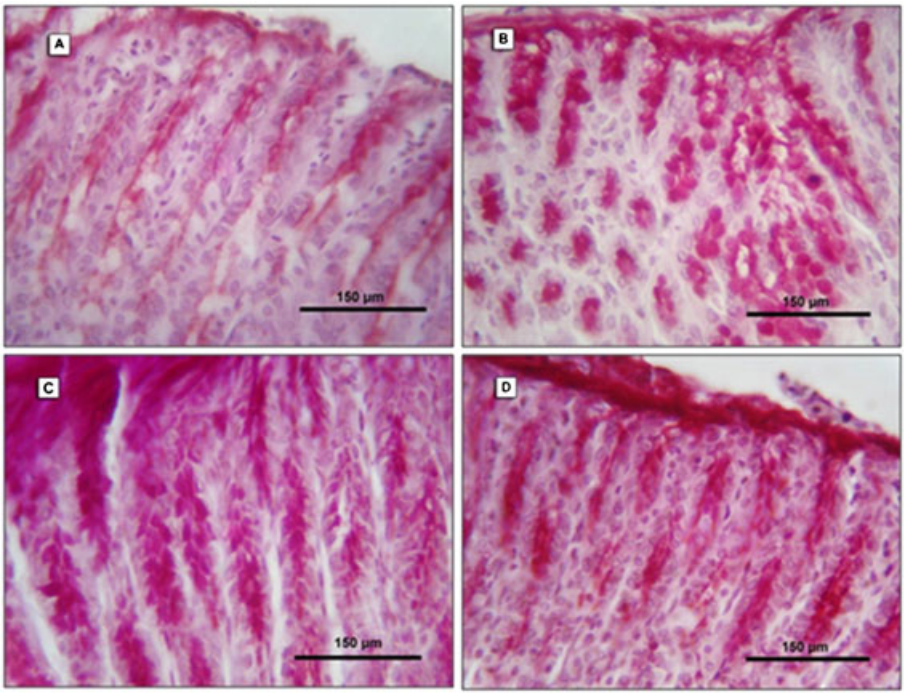

Figure 3. Effect of oral administration of EET on histochemical staining for mucin-like glycoproteins (PAS) in gastric mucosa after injury induced by 80% acetic acid in rats. Representative images of groups orally treated with water 1 ml/kg (A), omeprazole 40 mg/kg (B), EET 100 mg/kg (C) or EET 300 mg/kg (D). Magnification = 400X, bars = 150 µm. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

Table 2. Effect of oral administration of EET on the histochemical staining for mucin (PAS) and on the immunohistochemical staining for PCNA in gastric mucosa after injury induced by 80% acetic acid in rats

Treatment	PAS stained mucin (pixels/field $\times 10^4$)	PCNA (number of proliferating cells)
Control (water, 1 ml/kg)	31.93 ± 5.41	27.41 ± 3.75
Omeprazole (40 mg/kg)	70.46 ± 11.32^a	87.55 ± 7.27^a
EET (100 mg/kg)	87.18 ± 7.68^a	61.60 ± 4.88^a
EET (300 mg/kg)	98.55 ± 9.61^a	120.00 ± 9.87^a

The results were expressed as mean ± S.E.M. ($n = 12$), and statistical comparison was performed using one-way analysis of variance (ANOVA). [a] $p < 0.05$ when compared with the control group (vehicle).

[a]Values represent means of triplicate determinations ± S.E.M. at 95% confidence interval.

secretions (Tarnawski *et al.*, 2001; Vasconcelos *et al.*, 2008). This protection was performed by the mucus layer, which constitutes the first line of mucosal defense and consists of a viscous, elastic, adherent and transparent gel formed by 95% water and 5% glycoproteins (mucins) (Repetto and Llesuy, 2002). Staining with PAS is used to detect glycoproteins in tissues where the reaction of periodic acid selectively oxidizes glucose residues, creates aldehydes that react with Schiff's reagent and creates a purple-magenta color, a pattern for detection of neutral mucins. As expected and reported in literature, the gastric mucosa exposure to acetic acid reduced the mucus secretion (Vasconcelos *et al.*, 2008). Moreover, the treatment of animals with EET was able to prevent the reduction of mucins that are critical cytoprotective glycoproteins, and consequently the gastric mucus layer also responsible for the healing activity of the extract.

Futhermore, as previously cited, cell proliferation plays an important role in gastric wound healing (Tarnawski, 2005). However, acetic acid is known to reduce DNA synthesis in the gastric mucosal epithelium, which served as the principle cause of the decreased proliferation (Jainu *et al.*, 2010). In this context, PCNA is a nuclear polypeptide and plays a fundamental role in early cell cycle, so their quantity correlates with cell proliferation (Czyzewska *et al.*, 2009). In this study, we observed that treatment with EET increases the number of PCNA-positive nuclei, suggesting an acceleration of ulcer healing by stimulation of cell proliferation in gastric epithelial cells. It is important to know that β-lapachone, a natural compound extracted from *T. avellanedae*, also accelerated the skin wound healing in rats and increased the proliferation of several cell lines *in vitro* (Kung *et al.*, 2008). However, capillary electrophoresis analysis of EET presented no traces of β-lapachone or lapachol. In this regard, it is important to point out that our results resembled previously described data that were unable to detected lapachol in *T. avellanedae* bark extracts (Steinert *et al.*, 1995; Queiroz *et al.*, 2008).

We also investigated the total phenolic contents of EET because it has been long recognized that phenols are very important constituents of plants with scavenging ability, contributing directly to antioxidant action (Kahkonen *et al.*, 1999). It is known that reactive oxygen species are involved in the development of gastric ulcers induced by acetic acid (Ishihara *et al.*, 2008) and, therefore, antioxidants seem to be involved in the healing of this type of gastric ulcer (Potrich *et al.*, 2010). Hence, the phenolic compounds in the EET could provide a favorable environment for tissue healing, contributing to cicatrization property of the extract.

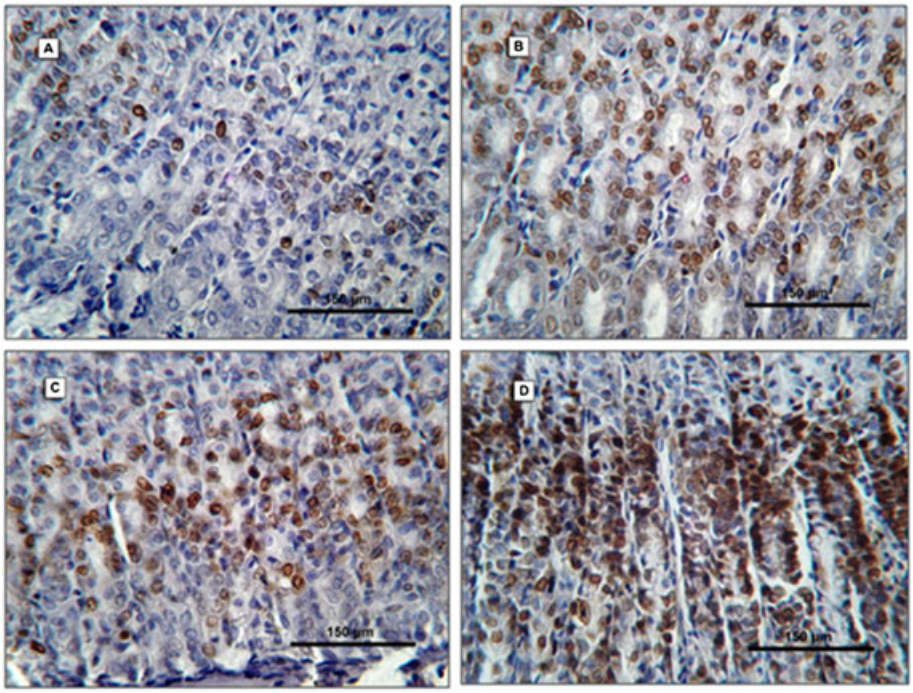

Figure 4. Effect of oral administration of EET on the immunohistochemical staining for PCNA in gastric mucosa after injury induced by 80% acetic acid in rats. Photomicrographs represents PCNA immunoreactivity in the control group water 1 ml/kg (A), omeprazole 40 mg/kg (B), EET 100 mg/kg (C) or EET 300 mg/kg (D). Magnification = 400X, bars = 150 μm. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

Collectively, the present study confirms the healing property of the EET of barks from *Tabebuia avellanedae* and adds the possible mechanisms of action of this extract. The healing process promoted by EET could be attributed to maintenance of gastric mucus layer and stimulation of cell proliferation. Moreover, we demonstrated that, besides the gastroprotective effect of EET, the extract also possesses gastric ulcer healing activity. However, further studies are required to investigate the active compound(s) and complementary mechanisms involved in the effects produced by *T. avellanedae*.

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Conflict of Interest

The authors disclose that there are no conflicts of interest that could innapropriated influence that outcome of the present study.

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