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Antiulcer activity of ethanolic extract of *Encholirium spectabile* Mart. ex Schult & Schult f. (Bromeliaceae) in rodents

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ABSTRACT

This study evaluated the antiulcer activity of an ethanolic extract of *Encholirium spectabile* (ES-EtOH) by using different standard experimental models of induced acute gastric ulceration. ES-EtOH (100 mg/kg p.o) protected the gastric mucosa against ulceration that was induced by absolute ethanol (53%), ethanol/HCl (75 %), ibuprofen (52 %) and ischemia/reperfusion (43 %). It also restored catalase activity and non-protein sulfhydryl group concentration in the gastric wall of mice that had been treated with ethanol. The pre-treatment of mice with N-nitro-L-arginine (70 mg/kg i.p.) abolished the protective activity of ES-EtOH, which indicates that prostaglandins, antioxidant compounds and nitric oxide synthase activity are involved in the gastroprotective activity of the extract.

Key terms: antioxidant, antiulcer activity, *Encholirium spectabile*, Bromeliaceae.

1. INTRODUCTION

Peptic ulcers are caused by an imbalance between the protective (mucus, bicarbonate, nitric oxide and prostaglandins) and the aggressive mechanisms of the mucosa, and are the result of the association of several endogenous factors (such as acid and pepsin) and aggressive exogenous factors that are related to living conditions (such as stress, continuous use of tobacco, alcohol, non-steroidal anti-inflammatory drugs and infection by *Helicobacter pylori*) [Maity *et al.*, 2003; Wallace and Granger, 1996]. Gastric and duodenal ulcers affect a considerable number of people worldwide and some authors consider these lesions to be a new «plague» of the 21st century [Hiruma-Lima *et al.*, 2006; O'Malley, 2003].

Ananas comosus L., a species of the Bromeliaceae family, is used in folk medicine for the treatment of gastric diseases [Monteles and Pinheiro, 2007]. *Encholirium spectabile* Mart. ex Schult & Schult f. (Bromeliaceae), popularly known as «*macambira de flecha*», is a species endemic to Brazil and it is geographically distributed where there are sandy soils and stony or rocky outcrops. This plant is mainly found in the "*cerrado*" and the "*caatinga*" regions of northeastern Brazil [Smith and Downs, 1999]. It is one of the plants that have been included in a conservation program of the "Reference Center for Recovery of Flora in Priority Areas of the San Francisco River Basin - The *Caatinga* Biome".

Little is known about the ethnopharmacology of *Encholirium* species. A phytochemical screening that was carried out with the ethanolic extract of *Encholirium spectabile* detected the presence of flavonoids, tannins, saponins, steroids and triterpenoids [Ribeiro *et al.*, 2006]. A study has also shown that these chemical constituents have antiulcerogenic activity [Lewis and Hanson, 1991].

Therefore, based on the chemical constituents with possible antiulcerogenic activity that were found in the ethanolic extract of *Encholirium spectabile* (ES-EtOH), the aim of this study was to investigate the existence of antiulcerogenic activity of ES-EtOH in ulcers induced by different agents in mice and rats and to find out which mechanisms are involved in this activity.

2. MATERIALS AND METHODS

2.1. Plant material

The aerial parts of *E. spectabile* were collected in the region of the lower San Francisco River (45 km from the city of Petrolina-PE) and were taxonomically identified by André Paviotti Fontana, a Botany specialist at the Federal University of San Francisco Valley. A voucher specimen (6443) was deposited in this institution's herbarium. The dried and powdered aerial parts (2 kg) were extracted three times over 72 h with 95% EtOH at room temperature. The extractive solution was concentrated under vacuum conditions and yielded 206 g (10.3 % yield) of crude ethanol extract (ES-EtOH).

2.2. Animals

Swiss albino male mice (20-25 g) and Wistar rats (200-220 g), obtained from the Sectional Vivarium of the Center of Research in Medicinal Plants at the Federal University of Piauí were used. The animals were housed at 24 ± 2 ° C under a 12-h light/12-h dark cycle and had free access to a standard pellet diet and water. After a fasting period of 18 h, they were acclimatized to the test environment for 2 h before the experimentation. All experiments followed the protocols

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that were submitted and approved by the Ethics Committee of the Federal University of Piauí (nº 51/09).

2.3. CHEMICALS AND DRUGS

The following drugs and chemicals were used: absolute ethanol (Quimex, Brazil), carbenoxolone (SIGMA, USA), ibuprofen (MANTECORP, Brazil), cimetidine (GLAXOS MITHKLINE, Brazil), N^G-nitro-L-arginine (SIGMA, USA), L-arginine (SIGMA, USA), EDTA (REAGEN, RJ, Brazil), Tween 80 (RIEDEL,Germany), N-acetylcysteine (ZAMBON, Brazil), DTNB (SIGMA, USA). The ES-EtOH was first dissolved in Tween 80 and subsequently diluted in distilled water and the resulting solutions of ES-EtOH did not have a concentration of Tween 80 higher than 1%. The other drugs were dissolved in distilled water. The ES-EtOH and drug concentrations were adjusted for treatment to result in a volume of 10 mL/kg.

2.4. Absolute ethanol-induced gastric ulcer

Swiss mice (n=8) were orally treated with the vehicle (1% Tween 80 in water, 10 mL/kg), carbenoxolone (100 mg/kg) or ES-EtOH (50, 100 and 200 mg/kg). One hour after this treatment, gastric lesions were induced by using absolute ethanol (0.2 mL/animal p.o.). The animals were killed by cervical dislocation 30 min after ethanol administration, the stomachs were removed and opened along the greater curvature and the area containing the gastric lesions was measured by planimetry, using a transparent grid. The ulcerous area in each animal was measured in mm^2 [Morimoto *et al.*, 1991].

2.5. HCl/ethanol-induced gastric ulcer

Swiss mice (n=7) were orally treated with the vehicle (1% Tween 80 in water, 10 mL/kg), carbenoxolone (100 mg/kg) or ES-EtOH (50, 100 and 200 mg/kg). One hour after this treatment, gastric lesions were induced by using 0.3 M HCl/ 60% ethanol solution (0.2 mL/animal p.o.). The animals were killed by cervical dislocation 1 h after HCl/ethanol administration. The stomachs were then removed, opened along the greater curvature and the area of gastric lesions was measured by planimetry, using a transparent grid. The ulcerous area in each animal was measured in mm^2 [Mizui and Douteuchi, 1983].

2.6. Ibuprofen-induced gastric ulcer

Swiss mice (n=6) were orally treated with the vehicle (1% Tween 80 in water, 10 mL/kg), cimetidine (100 mg/kg) or ES-EtOH (50, 100 and 200 mg/kg). One hour after this treatment, gastric lesions were induced by using ibuprofen (400 mg/kg, 10 ml/kg p.o.). The animals were killed by cervical dislocation 6 h after induction of the ulcers. The stomachs were then removed, opened along the greater curvature and the area of gastric lesions was measured by planimetry, using a transparent grid. The ulcerous area in each animal was measured in mm^2 [Barghava *et al.*, 1973].

2.7. Ischemia and reperfusion-induced gastric ulcer

Wistar rats (n=5) were orally treated with the vehicle (p.o.), N-acetylcysteine (750 mg/kg, i.p.) or ES-EtOH (50, 100 and 200 mg/kg, p.o.). After 30 min, while anesthetized with sodium thiopental (25 mg/ kg, i.p.), the celiac artery blood flow was interrupted using a microvascular clamp. After 30 min, the clamp was removed and reperfusion was established. The animals were killed 1 h after induction of the reperfusion. The stomachs were then removed, opened along the greater curvature and the area of gastric lesions was measured by planimetry, using a transparent grid. The ulcerous area in each animal was measured in mm^2 [Yoshikawa *et al.*, 1989].

2.8. Quantification of non-protein sulfhydryl groups (NP-SH)

Stomachs that had been previously treated with the vehicle but not ethanol (SHAM) or vehicle, ES-EtOH (100 mg/kg), carbenoxolone (100 mg/kg) and absolute ethanol were used to analyze the role of non protein sulfhydryl groups (NP-SH) in the protection of the gastric mucosa by ES-EtOH. The amount of NP-SH in the gastric mucosa was measured according to the method described by Sedlak and Lindsay [1968]. The absorbance was measured at 412 nm within 5 min after the addition of 0.05 mL of acid 5.5 dithiobis (2-nitrobenzoic acid) diluted in methanol (DTNB 0.01M) using a white homogenate as a background. Results were calculated from a standard curve of cysteine and expressed as μg NP-SH/g tissue.

2.9. Catalase activity

Stomachs that had been previously treated with vehicle but not ethanol (SHAM) or vehicle, ES-EtOH (100 mg/kg), carbenoxolone (100 mg/kg) and absolute ethanol were used to analyze the role of catalase (CAT) in the protection of the gastric mucosa by ES-EtOH. CAT activity was measured for 6 min according to the method described by Beers and Sizer [1952]. The absorbance was measured at 240 nm and CAT activity was defined as the amount of enzyme required to decompose 1 mmol of H_2O_2 per min. The results were expressed as mmol/min.100 mg tissue.

2.10. Effects of L-arginine (L-ARG) and NG-nitro-L-arginine (L-NOARG) on ES-EtOH gastroprotection

The assessment of the role of nitric oxide in the gastroprotective effect of ES-EtOH (100 mg/kg) was carried out according to the method described by Olinda *et al.* [2008], using an appropriate inhibitor (L-NOARG, 70 mg/kg, i.p.), and substrate (L-ARG, 600 mg/kg, i.p.) of nitric oxide synthase (NOS). In each case, animals were pretreated with the specific substance 30 min before the treatment with ES-EtOH.

2.11. Determination of antioxidant activity by the DPPH free radical scavenging assay

The free radical scavenging activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Mensor et al., 2001; Falcão et al., 2006). A sample stock solution (1.0 mg/ mL) of ES-EtOH was diluted to final concentrations of 250, 125, 50, 25, 10 and 5 μg/mL, in ethanol. One mL of 50 μg/mL DPPH ethanol solution was added to 2.5 mL of sample

solutions with different concentrations, and allowed to react at room temperature. After 30 min the absorbance values were measured at 518 nm and converted into the percentage antioxidant activity (AA) using the following formula: AA% = [(absorbance of the control - absorbance of the sample) / absorbance of the control] x 100.

Ethanol (1.0 mL) plus plant extract solution (2.5 mL) was used as a blank. DPPH solution (1.0 mL) plus ethanol (2.5 mL) was used as a negative control. Ascorbic acid, pyrogallol and quercetin were used as the standard solutions. Assays were carried out in triplicate. The IC_{50} values were calculated by nonlinear regression using the GraphPad Prism 5.0 program (Table II).

2.12. HPLC-DAD analysis of phenolic compounds

The analysis of the profile of phenolic compounds was carried out using a Lachrom Elite liquid chromatography system (Hitachi model) with a LiCospher 100 RP18 (5 mm) column (150 mm x 04 mm) equipped with a Merck Diode Array Detector (DAD). The mobile phase used was a solution of H_2O/H_3PO_4 0.1% (A) and MeOH (B) provided an initial mixture with 75% of A and 25% of B for 25 minutes. The column temperature was kept constant at 30 °C with a flow rate of 1.0 ml/min. An injection volume of 20 µl was used for the extracts. Spectral data were recorded at 320 nm during the whole run.

2.13. Statistical analysis

The data were expressed as mean ± SEM. One-way ANOVA followed by Tukey's test when necessary were used to compare means. The differences between groups were regarded as significant at $p<0.05$.

3. RESULTS

3.1. Effects on gastric injury

Table I shows that ES-EtOH had antiulcerogenic activity, which was investigated by using different standard experimental models of induced acute gastric ulceration.

Oral administration of ES-EtOH (100 and 200 mg/kg) and carbenoxolone (100 mg/kg), 1 h before the induction of gastric lesions with absolute ethanol decreased the area of gastric lesions by 53 %, 61 % and 61 % respectively, when compared with the negative control group.

In the HCl/ethanol-induced ulcer model, ES-EtOH (100 and 200 mg/kg, p.o.) and carbenoxolone (100 mg/kg, p.o.) significantly protected the gastric mucosa of mice by 75 %, 66 % and 84 %, respectively, when compared with the negative control group.

In the ibuprofen-induced gastric ulcers, ES-EtOH (100 and 200 mg/kg, p.o.) and cimetidine (100 mg/kg, p.o.) inhibited gastric ulcer formation by 52 %, 60 % and 56 %, respectively.

Table I

Effect of the ethanolic extract of *Encholirium spectabile* (ES-EtOH), carbenoxolone, cimetidine and N-actylcysteine (NAC) in different acute gastric lesion models in rodents.

Gastric lesion model	Treatment	Dose (mg/kg)	Area of gastric lesion (mm^2)	Inhibition (%)
Absolute ethanol (mice)	Control (saline)	-	6.53 ± 0.38	0.0
	ES-EtOH	50	5.50 ± 0.77	16.0
		100	3.10 ± 0.47***	53.0
		200	2.52 ± 0.52***	61.0
	Carbenoxolone	100	2.54 ± 0.19***	61.0
Ethanol/HCl (mice)	Control (saline)	-	2.94 ± 0.42	0.0
	ES-EtOH	50	2.72 ± 0.23	8.0
		100	0.75 ± 0.04***	75.0
		200	0.99 ± 0.07***	66.0
	Carbenoxolone	100	0.47 ± 0.11***	84.0
Ischemia/Reperfusion (rats)	Control (saline)	-	7.48 ± 0.36	0.0
	ES-EtOH	50	6.60 ± 0.81	22.0
		100	4.28 ± 0.35*	43.0
		200	3.11 ± 1.02**	58.0
	NAC	750	3.96 ± 0.58*	47.0
Ibuprofen (mice)	Control (saline)	-	5.68 ± 0.62	0.0
	ES-EtOH	50	4.94 ± 0.49	13.0
		100	2.73 ± 0.69**	52.0
		200	2.27 ± 0.50**	60.0
	Cimetidine	100	2.52 ± 0.29**	56.0

Each group represents the mean ± S.E.M. for 5-8 animals. * $p<0.05$, ** $p<0,01$ and *** $p<0,001$ compared to respective control group (ANOVA one way variance analysis and Tukey's Test).

The administration of ES-EtOH (100 and 200 mg/kg, p.o.) and N-acetylcysteine (750 mg/kg, i.p.), 30 min before the induction of gastric lesions by ischemia and reperfusion decreased the area of lesions by 43 %, 58 % and 47 %, respectively.

3.2. Quantification of non-protein sulfhydryl groups (NP-SH)

When compared to non-treated animals (778.48 ± 38.76 μg NP-SH/g tissue, SHAM), the oral administration of ES-EtOH (100 mg/kg) and carbenoxolone (100 mg/kg) restored the NP-SH levels (808.92 ± 12.78 and 761.80 ± 56.25 μg NP-SH/g tissue, respectively) that had been previously decreased by the oral administration of absolute ethanol (432.05 ± 13.40 μg NP-SH/g tissue) in mice (Fig. 1).

3.3. Catalase activity

Compared to non-treated animals (172.71 ± 13.56 mmol/min.100 mg tissue, SHAM), the reduction of the gastric wall catalase activity (80.51 ± 5.15 mmol/min.100 mg tissue) that was caused by the use of absolute ethanol was prevented by the oral administration of ES-EtOH-100 mg/kg (199.12 ±13.56 mmol/min.100 mg tissue) and carbenoxolone -100 mg/kg (141.55 ± 13.10 mmol/min.100 mg tissue) (Fig. 2).

3.4. Effects L-ARG and L-NOARG on ES-EtOH gastroprotection

The prior administration of the nitric oxide synthase inhibitor (L-NOARG) was able to reverse the gastroprotection provided by ES-EtOH (100 mg/kg) and by the substrate of the enzyme (L-ARG). These data suggest the involvement of NO synthase in the gastroprotection observed (Fig. 3).

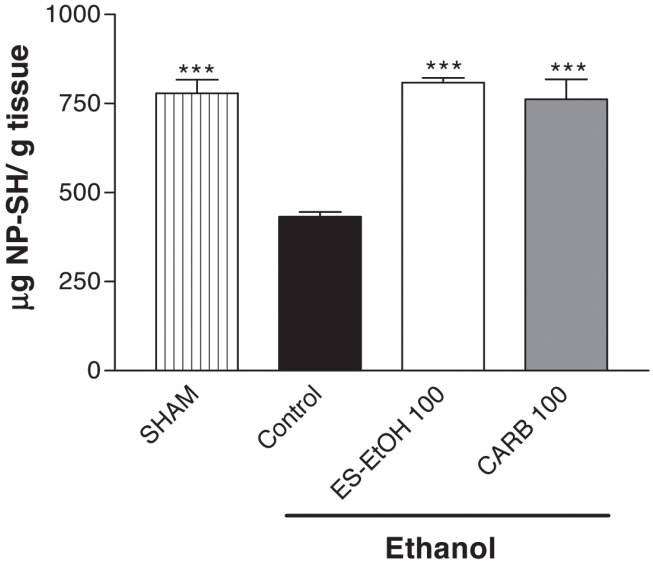

Figure 1: Effect of ES-EtOH (100 mg/kg) and carbenoxolone (100 mg/kg) on the NPSH group concentration of the gastric wall of mice treated with absolute ethanol. The results are shown as means ± S.E.M. of 8 animals/group. ***p< 0.001 (ANOVA and Tukey's test) compared to control.

3.5. Antioxidant activity of ES-EtOH by the DPPH free radical scavenging assay

The effect of ES-EtOH, ascorbic acid, pyrogallol and quercetin on the DPPH free radical scavenging is showed in Table II.

The data show that ES-EtOH had free radical scavenging activity at concentrations of 50, 125 and 250 μg/mL when compared to the positive controls, with an IC_{50} value of 63.47 ± 1.13 μg/mL. It appears that ES-EtOH contains compounds with a strong hydrogen-donating capacity and that it can

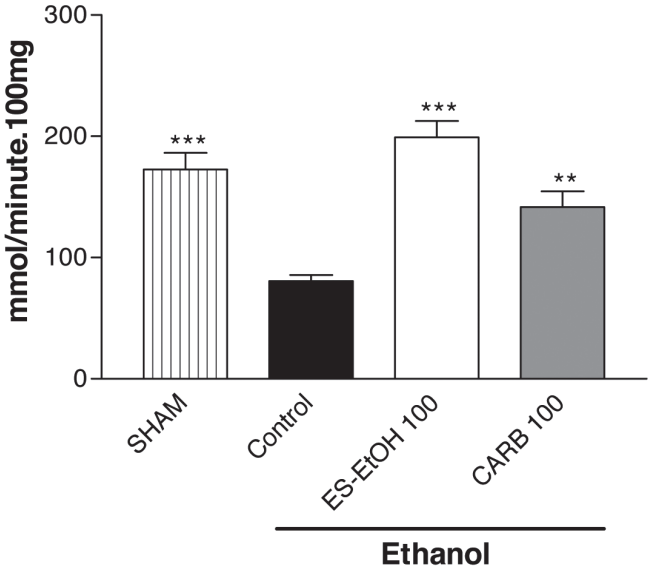

Figure 2: Effect of ES-EtOH (100 mg/kg) and carbenoxolone (100 mg/kg) on the enzymatic activity of catalase of the gastric wall of mice treated with absolute ethanol. The results are shown as means ± S.E.M. of 8 animals/group. ***p< 0.001 and **p<0.01 (ANOVA and Tukey's test) compared to control.

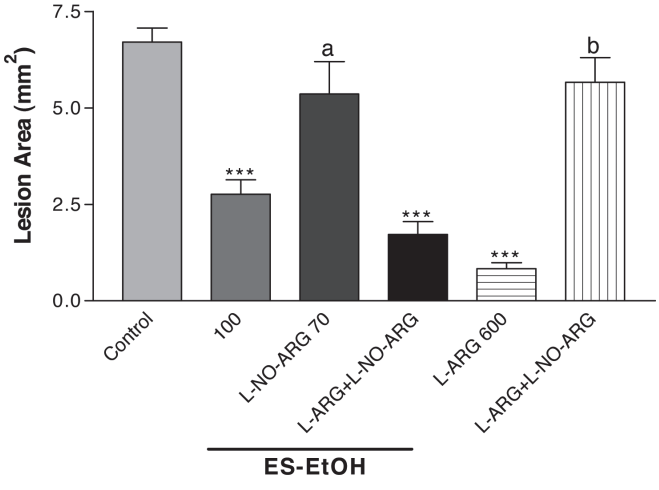

Figure 3: Effect of the pre-treatment of mice with L-NO-ARG (70 mg/kg) in the gastroprotective activity of ES-EtOH (100 mg/kg) and L-ARG (600 mg/kg). The results are shown as means ± S.E.M. of 7 animals/group. ***p< 0.001 compared to control, [a]p<0.01 compared to ES-EtOH and [b]p<0.001 compared to L-ARG (ANOVA and Tukey's test).

efficiently scavenge DPPH radicals. The presence of phenolic compounds in the extract was found to be essential for free radical scavenger properties.

3.6. HPLC-DAD analysis of phenolic compounds

The phenolic profile of ES-EtOH that was evaluated by HPLC-DAD analysis at 320 nm is presented in Fig. 4, which confirms the results found in the preliminary phytochemical screening.

4. DISCUSSION

The results of this study show that the crude ethanolic extract of *Encholirium spectabile* (ES-EtOH) is an effective antiulcerogenic agent. This protection seems to occur due to the activation of antioxidant systems and the involvement of prostaglandins and the NO synthase pathway.

Ethanol causes necrotic lesions of the gastric mucosa in a multifactorial way. It can reach the mucosa by disruption of the mucus-bicarbonate barrier and cause cell rupture in the

Table II

DPPH free radical scavenging of ES-EtOH, ascorbic acid, pyrogallol and quercetin.

Concentration (μg/mL)	Activity % Antioxidant			
	ES-EtOH	Ascorbic acid	Pyrogallol	Quercetin
250	87.03	96.22	76.77	82.58
125	75.61	95.64	76.48	79.48
50	14.52	92.74	72.80	76.09
25	0	95.06	70.67	75.17
10	0	86.64	72.12	76.28
5	0	76.48	72.27	75.02
[a]IC_{50} ± SD	63.47 ± 1.13	< 5	< 5	< 5

[a] Value obtained from nonlinear regression with 95% of confidence level. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%, SD standard deviation.

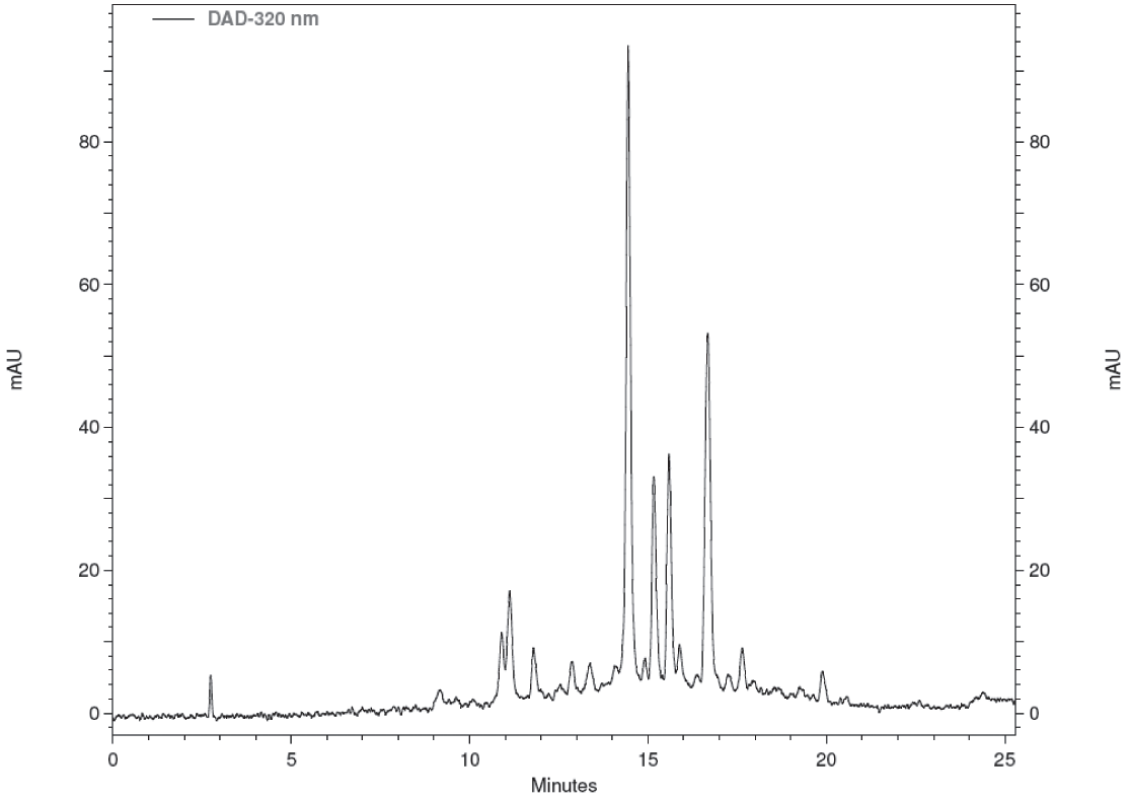

Figure 4: HPLC-DAD phenolic profile for ES-EtOH recorded at 320 nm.

wall of blood vessels [Mincis *et al.*, 1995]. These effects are probably due to biological actions, such as of lipid peroxidation, formation of free radicals, intracellular oxidative stress, changes in permeability and depolarization of the mitochondrial membrane prior to cell death [Repetto and Llesuy, 2002; Bagchi *et al.*, 1998; Hirokawa *et al.*, 1998].

According to Mizui and Douteuchi [1983], oral administration of HCl/ethanol produces necrotizing lesions in the gastric mucosa, and the lesions are more severe than those caused by absolute ethanol, mainly due to a reduction in the protective layer of mucus and an increase in peptic acid secretion. Like ethanol, ethanol/HCl causes oxidative stress, increases the release of histamine and pepsin, and reduces the levels of DNA, RNA and proteins in the tissue, which results in tissue injury [Batista *et al.*, 2004]. The results obtained with ES-EtOH, in these two models of acute ulcer presented cytoprotectant activity, with a possible increase in the release of endogenous protection factors, such as secretion of mucus, bicarbonate and antioxidants agents.

After describing the cytoprotectant effect of ES-EtOH, the role of prostaglandins (PGs) in this effect was investigated using the model of ulcer induced by non-steroidal anti-inflammatory drugs (ibuprofen). Ibuprofen damages the gastric mucosa by inhibiting cyclooxygenase I and II (COX-1 and COX-2) in order to promote reduction of the production of prostaglandins [Wallace, 2001]. The prostaglandins E2 and I2 are involved in the synthesis of mucus and bicarbonate and in the regulation of acid secretion and gastric mucosal blood flow [Halter *et al.*, 2001; Curtis *et al.*, 1995]. Other factors, such as activation and infiltration of neutrophils, appear to be involved in the initial processes of formation of the lesion. The reduction in the levels of PGs compromises the barrier of mucus and facilitates the formation of lesions caused by gastric secretions (acid and enzyme) [Suzuki *et al.*, 2000]. In the experiment where ulcers were induced by ibuprofen, the data showed that ES-EtOH (100 mg/kg) had gastroprotective activity, which suggests a prostaglandin-dependent effect.

In the experiment of ischemia and reperfusion in the stomach of rats, ischemia alone induced lesions and cell death. However, the reperfusion caused a number of tissue changes, which resulted in the appearance of lesions [Ribeiro and Yoshidaw, 2005]. Among all these changes, the most prominent are the increased capillary permeability and the production of free radicals [Ueda *et al.*, 1989], which attack and damage the cell membranes, attract neutrophils and initiate the inflammatory response [Ribeiro and Yoshidaw, 2005]. The administration of ES-EtOH (100 mg/kg, p.o.) reduced the lesions induced by ischemia and reperfusion, which demonstrates the extract's antioxidant effect. The chemical constituents of ES-EtOH may be involved in the antioxidant paths, modulating and maintaining the antioxidant enzymes or by directly scavenging the free radicals produced [Ribeiro and Yoshidaw, 2005].

There are many enzyme systems that capture reactive oxygen species and prevent their destructive action [Kwiecien *et al.*, 2002]. Usually, NP-SH serves as a scavenger of free radicals and toxic substances ingested with food or produced directly in the gut [Shirin *et al.*, 2001]. Non-protein sulfhydryl groups protect the mucosa by binding to free radicals and forming bridges of disulfide between the subunits of mucus in order to prevent its cleavage [Avila *et al.*, 1996]. CAT is an enzyme that accelerates the degradation of H_2O_2 into water and oxygen [Halliwell, 1990].

The quantification of free radical scavengers showed that the gastroprotective effect of the extract seems to be related to the increased amount of NP-SH and the increased activity of catalase in the gastric mucosa, and this fact prevents cellular damage caused by free radicals.

Studies indicate that nitric oxide (NO) has recently been recognized as a key mediator in gastric defense mechanisms, due to its ability to increase blood flow of the gastric mucosa and the production of mucus, and inhibit the adhesion of neutrophils to endothelial cells [Coruzzi *et al.*, 2000]. The endogenous NO seems to be involved in the regulation of postprandial gastric acid secretion. This effect may be mediated by changes in the release of gastrin and somatostatin. The endogenous NO also delays gastric emptying and antral motor activity without affecting gastric myoelectrical activity [Konturek *et al.*, 1999].

The production of NO depends on the activity of the enzyme NO synthase, from the oxygen (O_2) and L-arginine. The results of the experiment with the NOS inhibitor (L-NOARG) demonstrated that the gastroprotective effect of ES-EtOH was reversed by the prior administration of the inhibitor, suggesting that the extract depends upon the NOS pathway.

In conclusion, the results of this study indicate that ES-EtOH has gastroprotective activity against gastric mucosal damage induced by ethanol, HCl/ethanol, ibuprofen and ischemia and reperfusion, which suggests that the extract may activate cytoprotective mechanisms that increase the release of prostaglandins, increase the concentration of NP-SH groups, increase catalase activity and activate the NOS pathway. The confirmation of the presence of phenolic compounds that was shown by the chromatographic profile and the presence of free radical scavenging activity contribute to the gastroprotective activity of ES-EtOH.

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