American Journal of Health Research
2017; 5(3): 57-64
http://www.sciencepublishinggroup.com/j/ajhr
doi: 10.11648/j.ajhr.20170503.12
ISSN: 2330-8788 (Print); ISSN: 2330-8796 (Online)

Anti-ulcerogenic Action Mechanism of *Maytenus ilicifolia* Detected by mRNA RT-PCR and Free Radical Scavenging Activity Evaluated by ESR

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To cite this article:

Dalva Pazzini, Elder Bruno Fontes, Ana Paula D Hory, Adevailton B Santos, Oswaldo Baffa, Rosângela Bezerra, Silvana Pasetto, Eduardo José Crevelin, Luiz Alberto Beraldo Moraes, Angela Kinoshita, Ana Cláudia B. Paula-Zurron. Anti-ulcerogenic Action Mechanism of *Maytenus ilicifolia* Detected by mRNA RT-PCR and Free Radical Scavenging Activity Evaluated by ESR. *American Journal of Health Research*. Vol. 5, No. 3, 2017, pp. 57-64. doi: 10.11648/j.ajhr.20170503.12

Received: February 24, 2017; **Accepted**: March 24, 2017; **Published**: March 30, 2017

Abstract: *Maytenus ilicifolia*, known as "espinheira-santa", is used in the popular medicine for peptic ulcer treatment. This work evaluates the preventive and curative actions of *M. ilicifolia* in animals, the healing activity by EGF expression detected by RT-PCR as well as the anti-oxidative activity by Electron Spin Resonance spectroscopy (ESR). Oral administration of *M. ilicifolia* (300 and 500 mg/kg, p.o.) one hour before the ulcerogenic agent application prevented the ulcer formation 42 ± 0.98% and 72 ± 1.2% ($p < 0.05$ and $p < 0.001$, respectively), in comparison with the negative control tween 80, preserving the cytoprotection characteristics of the gastric mucosa and assuring the integrity of gastric glands and gastric fossets. The animals treated with tween, had the epithelium and the mucosa layer damaged and accentuated vascularization. The healing activity of *M. ilicifolia* (500mg/kg, p.o.) was 71 ± 1.4% ($p < 0.001$) in chronic ulcer experiments induced by acetic acid. EGF expression detected by RT-PCR confirmed the healing activity. Histological analysis showed the recovery of the mucosal layer and the epithelium surface harmed by the acetic acid. Studies *in vitro* by Electron Spin Resonance spectroscopy assessed the anti-oxidative action of *M ilicifolia*. Experiments with DPPH (2.2-Diphenyl -1-picrylhydrazyl) demonstrated that the polar fraction of *M. ilicifolia* presents an IC_{50} of 0.68±0.09 mg/ml. The value of 9±2 mg/ml was found for IC_{50} in experiments with the radical OH, produced by the Fenton Reaction and detected through spin adduct DMPO-OH. Mass spectrometry analysis of crude extracts and fractions from *M. ilicifolia* was carried out and compounds of pentacyclic triterpenes class were identified.

Keywords: *Maytenus ilicifolia*, Anti-oxidative Activity, Antiulcerogenic Activity, Epidermal Growth Factor, Gastric Ulcer, Electron Spin Resonance

1. Introduction

Maytenus ilicifolia (Celastraceae) popularly known in Brazil as "espinheira-santa" (holy spines) is a native plant from southern Brazil, Paraguay, Uruguay and northern Argentina [1]. It is used in folk medicine for the treatment of dyspepsia as well as gastric ulcers and potentially on cancer treatment [2]–[4]

Cipriani et al. demonstrated, after several purification steps, that the tea (infusion) of *M. ilicifolia* leaves releases a polysaccharide (arabinogalactan). The pre-treatment with arabinogalactan practically abolished the ethanol-induced gastric damage, suggesting a potential ability to bind to the surface mucosa and function as a protective coating, antisecretory activity, and mucosal protection by increased mucus synthesis, and radical scavenging [5].

Triterpenes, isolated from *Maytenus spp*. and identified in hexane extract, have compounds potentially antiulcerogenic due to their ability of stimulating the synthesis of mucus or the maintenance of the prostaglandin contents of gastric mucosa at high levels. Antiulcerogenic effect was also observed in the ethyl acetate extract and the presence of condensed tannins and flavonoids was observed. Because of anti-inflammatory activity, without gastric irritation and of mucosa protection, hexane and ethyl acetate extracts of *Maytenus ilicifolia* may represent an important clinical alternative both in inflammation and in antiulcerogenic therapeutics [6].

Paula *et al*. described that the mechanism of action of the essential oil obtained from the bark of *Croton cajucara* in chronic gastric ulcer occurred by enhancing EGF expression detected by RT-PCR in rats. Considering that EGF and TGFα are effective at protecting the gastric mucosa from acetic cid-induced gastric lesions, it is reasonable to assume that these factors limit mucosal damage caused by ulcerogenic agents, aiding early mucosal restoration [7].

The crude ethanolic extract of *M. ilicifolia* has a powerful antioxidant potential [3]. Polyphenols and flavonoids are plant constituents with possible action in the free radical scavenging action, and they are present in *M. ilicifolia* [8]. Free radicals and reactive oxygen species are involved in a variety of pathological events like diabetes, cancer and aging. Oxygen-derived free radicals have recently been postulated to play an important role in the pathogenesis of acute gastric mucosal injury induced by ischemia-reperfusion, stress, ethanol and anti-inflammatory drugs in rats. Furthermore, it has been suggested that free radicals generated by neutrophils may be important factors in delaying the healing of acetic acid-induced chronic gastric ulcers in these animals [9]. This work evaluates the preventive and curative actions of *M. ilicifolia* in animals, the healing activity by EGF expression detected by RT-PCR as well as the anti-oxidative activity by Electron Spin Resonance spectroscopy (ESR). In addition, mass spectrometry (MS) analysis of *M. ilicifolia* was carried out to identify its main class of compounds present in the crude ethyl acetate extract.

2. Material and Methods

2.1. Chemicals and Biochemical

Tween, acetic acid, ethanol solution (Sigma Chemical São Paulo, Brazil), lanzoprazole, ranitidine (Glaxo Smith Kline, Rio de Janeiro, Brazil), xylazine hydrochloride, ketamine (Pfizer, São Paulo, Brazil), *Maytenus ilicifolia* (Espinheira Santa Herbarium Laboratories®-Curitiba, Paraná, Brazil). DPPH (2,2-diphenyl-1-picryl-hydrazyl) and DMPO (5,5-dimethyl-1-pyrroline N-oxide) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and have purity of 95% and 97%, respectively. All other reagents used for ESR experiments and Mass spectrometry were of analytical grade. Guanidium iso thiocyanat|phenol chloroform kit, moloney murine leukemia virus reverse transcriptase (MMLV-RT) and oligo-(dt)- primers, RNase Block Ribonuclease Inhibitor were obtained from Stratagene, Heidelberg, Germany). Stand buffer, Taq polymerase (Pharmacia, Germany), MgCl2, KCl, HCl (Takara, Shiga, Japan) also were used in this experiment. EGF antisense primer was made by GIBCO BRL| Life Technologies-Eggenstein, Germany. Rat β actin was obtained by Clon Tech, Palo Alto, CA.

2.2. Plant Material

Maytenus ilicifolia (Espinheira-Santa®) was acquired from Herbarium Laboratories (Curitiba, Paraná, Brasil), and one capsule of *M. ilicifolia* contain 13.3 mg (3.5%) of tannins.

For *in vivo* experiments, *M. ilicifolia* (300 and 500 mg/kg) was diluted in tween (Tween 80®), resulting in solutions with 30 and 50mg/ml, and administered by oral route in rats. Following, *M. ilicifolia* was diluted in water, heated, filtered, lyophilized and used for radical scavenging experiments by Electron Spin Resonance.

2.3. Animals

All experiments were performed on male Wistar rats (200-250g) from the Central Animal House of Universidade do Sagrado Coração (USC). The animals were fed a certified Nuvilab CR-a® (Nuvital) diet and had free access to tap water. Mice were kept in the animal house under a standard 12h light/12h dark cycle, 50% humidity and temperature of 24 ± 1°C. Experimental protocols were approved by the Ethics Committee of USC and were conducted according to recommendations of the Canadian Council on Animal Care All experiments were performed in the morning, according to guidelines for laboratory animal care and ethical guidelines for the investigation of experimental inflammation in conscious animals [10] [11].

2.4. Ethanol Ulceration

Wistar rats (200-250 g) were subjected to an ethanol-induced ulcer assay [12]. These rats were fasted for 24 h (free access to water) before the experiment. The animals then

randomly received oral Tween 80® (10 ml /kg, N=7), lanzoprazole Losec ® (30 mg /kg, N=7) or *Maytenus ilicifolia* (300 and 500 mg/kg, N=7). One hour after treatment with the Tween, lanzoprazole and *M. ilicifolia*, 1ml of absolute ethanol was orally administered to the rats. Thirty minutes later, the animals were euthanized and their stomachs were removed to determine ulcer index [12].

2.5. Chronic Ulcer Induced by Acetic Acid

Wistar rats (200-250 g) were randomly allocated into one of 3 treatment groups, in which ulceration was induced by acetic acid according Takagi *et al*. [13]. Rats were then treated with *M. ilicifolia* (500 mg/kg), ranitidine (100 mg/kg) or Tween 80, as a vehicle (10 ml/kg). Rats were anesthetized with xylazine hydrochloride (50 mg/kg) and ketamine (180 mg/kg) for the application of 50 ml of 30 ml/L acetic acid solution into the subserosal stomach layer of each animal. Two days after surgery, the respective treatment drugs were orally administered once daily for 14 consecutive days. The animals were euthanized on the day after stopping drug administration. The ulcer area (mm^2) and curative ratio (%) were measured [12].

2.6. Microscopic Analysis

For morphological ulcer analysis, rat stomachs were fixed in Bouin for 24 h, were dehydrated through ascending concentrations of ethyl alcohol, cleared with xylene, embedded in Histosec (Merck –11609), and prepared for microtomy. The 8 µm-sections were then stained with Hematoxilin and Eosin. Photomicrographs were obtained with microscope Nikon H550L.

2.7. Molecular Assay

Reverse-transcriptase polymerase chain reaction for detection of EGF mRNA

Molecular analyses were carried out only in animals treated with *Maytenus ilicifolia* (treated group) and Tween 80 (negative control group) in the chronic ulcer induced by acetic acid experiment. The total RNA was isolated from mucosal gastric samples and was stored at -80°C [14]. Briefly, total RNA was extracted from mucosal samples using a single-step guanidium isothiocyanate/phenol chloroform extraction kit from Stratagene (Heidelberg, Germany) based on a method described by Chomczynski & Sacchi [15]. After precipitation, RNA was resuspended in RNAse-free TE buffer and its concentration was estimated by absorbance at a wavelength of 260 nm. Furthermore, the quality of each RNA sample was determined by running agarose-formaldehyde electrophoresis. The RNA samples were stored at -80°C until required for analysis.

Single-stranded cDNA was generated from 5 µg of total cellular RNA using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and oligo-(dT)-primers (Stratagene, Heidelberg, Germany). Briefly, 5 µg of total RNA was uncoiled by heating (65°C for 5 min) and was then reversely transcribed (at 37°C for 1 hour) into complementary DNA (cDNA) in a 50 µl reaction mixture containing 50 U MMLV-RT, 0.3 µg oligo-(dT)-primer, 40 U RNase Block Ribonuclease Inhibitor (40U/µl), 2 µl of a 100 mM/l mixture of deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP), 5 µl of 10 X first-strand buffer (all reagents were provided by Stratagene, Heidelberg, Germany). The resultant cDNA (2 µl) was amplified in a 50 µl reaction volume containing 2 U Taq polymerase, dNTP (200 µM each) (Pharmacia, Germany), 1.5 mM MgCl2, 5 µl 10X polymerase chain reaction (PCR) buffer (100 mM KCl, 20 mM Tris-HCl, pH =8.3) and specific primers used at a final concentration of 1 mM (all reagents were from Takara, Shiga, Japan). The mixture was overlaid with 25 µl of mineral oil to prevent evaporation. The PCR mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT). Incubation and thermal cycling conditions were as follows: denaturation at 94°C for 1 min, annealing at 60°C for 45 sec and extension at 72°C for 2 min. PCR amplification proceeded for 33 cycles using AmpliWax® PCR Gem 50 wax beads. The nucleotide sequence of sense primers was 5'-GACAACTCCCCTAAGGCTTA-3' - nucleotides 2804-2823) and the EGF antisense primer (5'-CATGCACAGGCCACCATTGAGGCAGTACCCATCGTACGA-3' - nucleotides 3332-3370) synthesized by GIBCO BRL/Life Technologies (Eggenstein, Germany). Concomitantly, amplification of control rat β-actin (Clon Tech, Palo Alto, CA) was performed on the same samples to assess RNA integrity.

PCR products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. The location of predicted products was confirmed by using a 100-bp ladder (Takara, Shiga, Japan) as a standard size marker. The gel was then photographed under UV transillumination. The intensity of PCR products was measured using a video image analysis system (Kodak Digital Science).

2.8. Free Radical Scavenging Tests

Free radical scavenging activity of the *M. ilicifolia* was determined using stable DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical [16] [17] [18] [19] and hydroxyl radical, produced through Fenton reaction and monitored using DMPO (5,5-dimethyl-1-pyrroline N-oxide) as a spin trap.

A volume of 100µL of the ethanolic solution of DPPH 1mM was mixed to 100µL of aqueous solution of lyophilized extract of *M. ilicifolia* in different concentrations, varying from 0 (reference) to 2.5mg/ml. This mixture was placed in a capillary tube and transferred to an ESR quartz tube. The reactions were carried three times and its spectrum was recorded in a Jeol FA-200 spectrometer, 3 minutes after reaction. The acquisition parameters were: central field 338mT, modulation 0.1mT, scan range 10mT, scan time 1 minute, microwave power 2mW, below to the signal saturation in this system. The signal intensity of the central line was used to monitor the free radical concentration. The average of this intensity related to each extract concentration

was plotted and an exponential function (1) was used to fit them and used to determine the IC_{50}:

$$I = Ae^{-bc} \quad (1)$$

where I is the ESR signal intensity, c the concentration, A and b fitting parameters.

The Fenton reaction was carried using 50µL of $Fe(SO_4)$ (10mM), H_2O_2 (100mM), DMPO (200mM) and phosphate buffer (10mM). To test hydroxyl radical scavenging activity, 50µL of aqueous solution of the lyophilized extract of *M. ilicifolia* in different concentrations (0 to 50mg/ml) was added. The ESR spectra were registered 3 minutes after reaction. The experiment was taken 3 times for each concentration and the acquisition parameters were the same of the DPPH experiment. The signal intensity of the first central line was used as parameter of the free radical concentration. The average of ESR signal intensity was plotted as a function of extract concentration and fitted by equation 1 to IC_{50} determination.

2.9. Mass Spectrometry

Mass spectrometry analysis was carried out with a LC-MS 1200L Triple Quadrupole (Varian) mass spectrometer equipped with a standard ESI (Electrospray Ionization) source operating in positive and negative modes. The ionization of compounds was better in positive mode. The tuning parameters for ESI-MS were: capillary voltage 3.2 kV, cone voltage 40 V, source temperature 100°C, desolvation temperature 200°C. The samples were dissolved in MeOH (Methanol) and injected by direct insertion.

2.10. Extraction of Compounds

The lyophilized extract (0.5 g) of the leaf of *M. ilicifolia* was dissolved in 20 ml of H_2O and partitioned with an equal volume of Hex (Hexane), EtOAc (Ethyl Acetate and BuOH (Buthanol) (3 x) (Figure 1). The organic layer was washed with saturated NaCl solution, dried with anhydrous Na_2SO_4 and concentrated in rotatory evaporator (Büchi B-480) to afford the crude extracts Hex (25 mg), EtOAc (230 mg) and BuOH (30 mg).

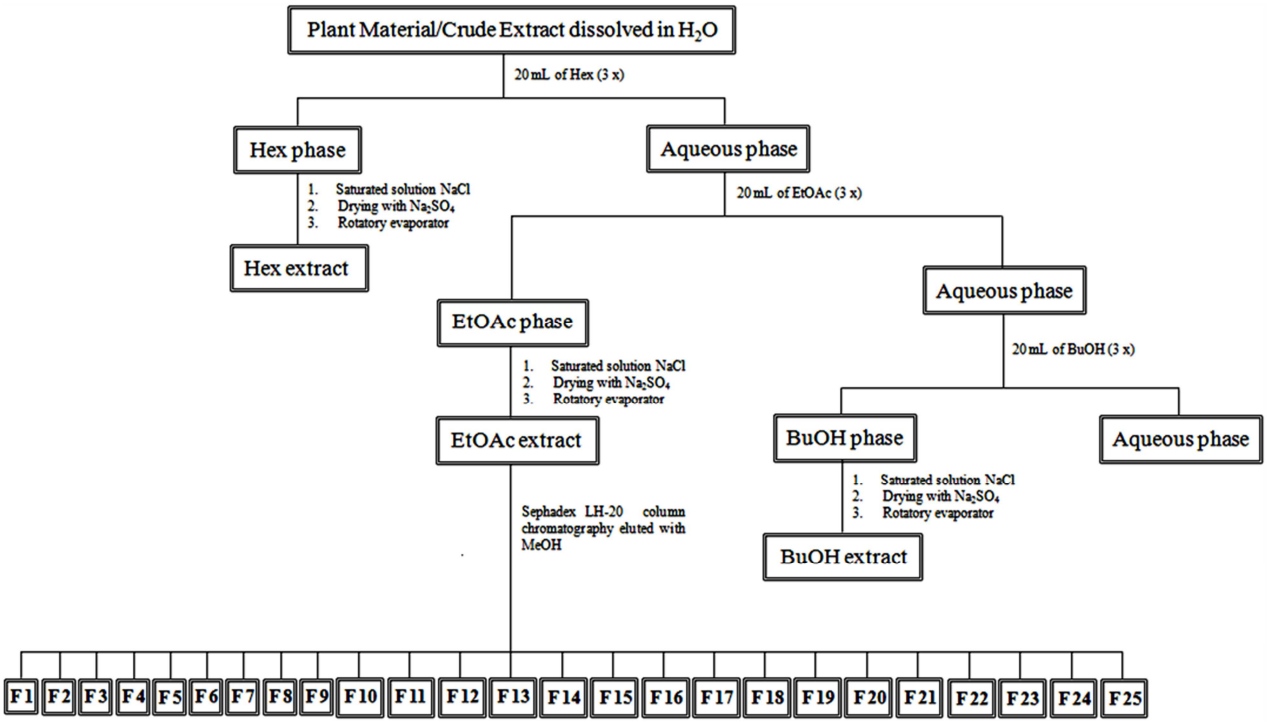

Figure 1. *Methodology used to obtain the fractions of M. ilicifolia. Part of the crude extract EtOAc (215 mg) was fractionated on a Sephadex LH-20 column (1 m x 3 cm i.d., Pharmacia) eluted with MeOH. A total of 25 fractions of 5 ml each were collected.*

2.11. Statistical Analysis

Anti-ulcerogenic results were expressed as mean ± S.E. One-way variance analysis was followed by Dunnett´s, Scheffe's and Tukey's tests. These tests were run in the Statistical 5.1 software (Stat Soft, Inc). The level of significance was set at $p < 0.05$. The experimental data points of ESR signal intensity were fitted using software Microcal Origin 8.5 (Northampton, USA) for IC_{50} calculations and were represented by mean ±S.D.

3. Results and Discussion

3.1. Ethanol Ulceration

Oral treatment with ethanol causes focal hyperemia, edema, necrosis and submucosal hemorrhage, as well as circulatory disturbances [20]. The extent of ethanol-induced gastric mucosal damage in rats correlates with the number of degranulating mast cells since these cells are a source of several neuropeptides and inflammatory mediators, including histamine and leukotrienes [21].

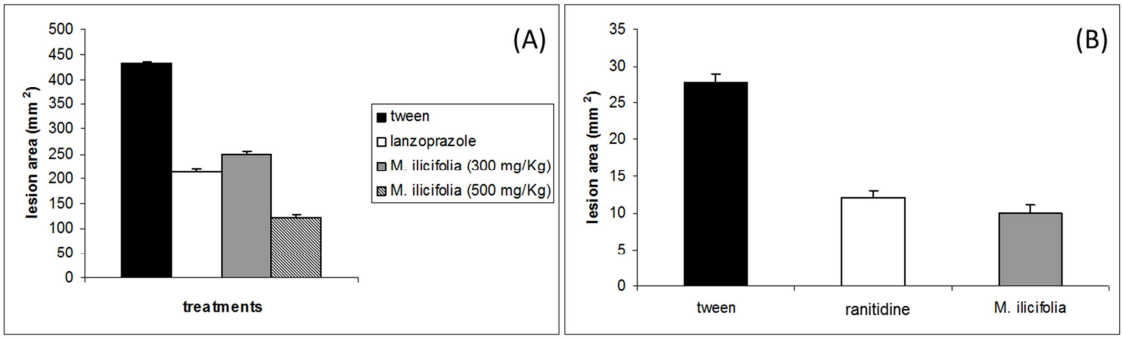

Figure 2. *(A) Preventive effect of Maytenus ilicifolia (300 and 500mg/kg) and the positive control lanzoprazole in ethanol acute gastric ulcer model. Each value is the mean ± S.E.M for 7 animals. ANOVA $F_{(3, 24)}$= 3,20 para ILU (mm^2). Teste de Tukey: * p<0.05 e ** p<0.001. (B) Lesion area due to acetic acid administration in gastric mucosa of rats. The animals were treated by 14 days with tween (negative control), ranitidine (positive control) and Maytenus ilicifolia. Each value is the mean ± S.E.M for 7 animals. ANOVA $F_{(2, 18)}$= 2,84 para ILU (mm^2). Teste de Dunnett's: ** p<0.001.*

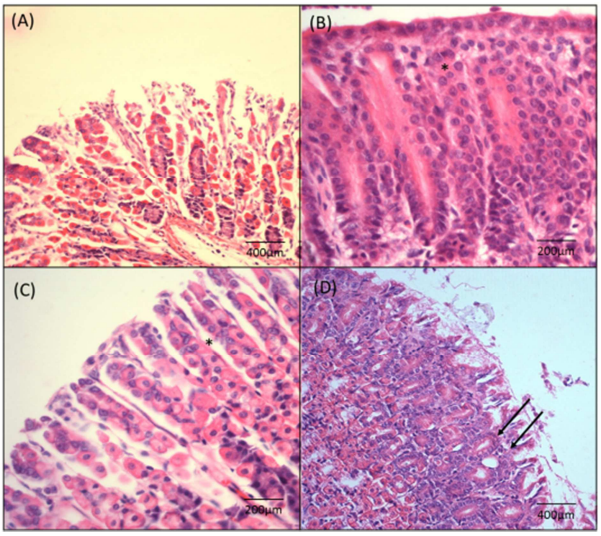

Figure 3. *Photomicrographs of ulcer treatments induced by ethanol solution (A) negative control group (tween), glandular epithelium partially destroyed (open arrows). Pre-treatments with extract of Maytenus ilicifolia; 300 mg/kg (B) and 500 mg/kg (C): to observe intact gastric mucosa, with glands and gastric pits (asterisks) very visible. (D) pre-treatment with anti-ulcerogenic drug lanzoprazole 30 mg/kg, intact and continuous glandular epithelium (black arrows). Haematoxiline & Eosine.*

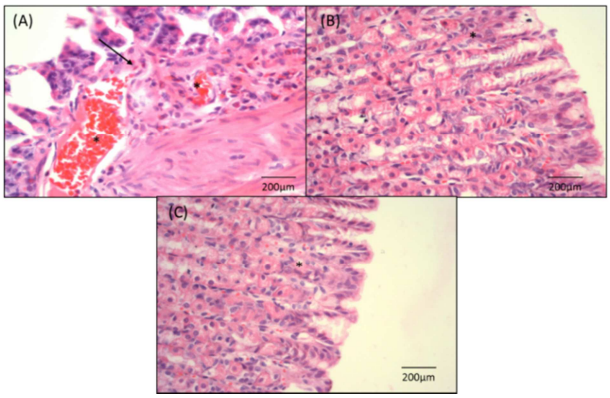

Figure 4. *Photomicrographs of the treatments of chronic ulcer induced by acetic acid solution. Negative control group (tween) (A): atrophic epithelium glandular with intense hyperemia (asterisks). Treatment with Maytenus ilicifolia at the dose of 500 mg/kg (B); and treatment with ranitidine 100 mg/kg (C): to observe the glands and gastric pits (asterisks) continuous, without sign of atrophy of the tissue. Haematoxiline & Eosine.*

The effect of *M. ilicifolia* on gastric ulcers induced by ethanol is shown in Figure 2A. In this acute experiment, the lesion areas of rats undergoing Tween 80 treatment were 7.42±0.40 mm^2 and animals undergoing *Maytenus ilicifolia* (300 and 500 mg/kg) treatment showed smaller lesion areas: 3.12 ±0.80 mm^2 and 5.34 mm^2 ($p<0.05$ and $p<0.001$, respectively), when compared with the negative control tween 80. The oral administration of ethanol solution on the negative control group (pre-treated with tween) caused necrotizing gastric mucosa lesions, observed by the tissue disorganization, lack of continuous glandular epithelium and own lamina formed by connective tissue (Figure 3 A and B).

Maytenus ilicifolia (300 and 500 mg/kg, p.o.) and the positive control lanzoprazole (30 mg/kg, p.o.) significantly inhibited the lesions formation in the gastric mucosa. The treated animals with *M. ilicifolia* showed intact gastric glands and mucosa, with the continuous coating epithelium (Figure 3 C, D and F). Intact glandular epithelium was also observed in the lanzoprazole group (Figure 3 G and H). This protection could reflect the inhibition of gastric secretion or an increase in the release of protective substances by the mucosa as reported by Jorge et al. [6] that related an anti-inflammatory and antinociceptive activities and protection against gastric lesions, including cytoprotection and healing.

3.2. Chronic Ulcer Induced by Acetic Acid

The healing effect of *M. ilicifolia* was demonstrated because the healing of chronic gastric ulcer induced by acetic acid in rats was accelerated. Postoperative treatment with ranitidine and *M ilicifolia* (500 mg/kg) for 14 consecutive days accelerated the ulcer healing. On day 14 after surgery, the percentage of rats with cicatrized ulcers in both experimental groups was significantly higher than in the negative control group (Figure 2B). In addition, *M. ilicifolia* and ranitidine significantly healed the chronic gastric ulcer reducing its area in 71.3 ± 1.4% and 54.5 ± 1.6% (respectively) when compared with the negative control tween 80 ($p<0.001$).

The ulcer induction by acetic acid solution was verified in the negative control group (treated with tween) for 14 consecutive days. The ulcerogenic agent caused

disorganization on part of the mucosa, with atrophy of the glandular epithelium and intense hyperemia (Figure 4 A and B).

Maytenus ilicifolia (500 mg/kg) and the anti-ulcerogenic drug ranitidine (100 mg/kg) were efficient on the treatment of gastric mucosa lesions. The treatment with 500mg/kg of *Maytenus ilicifolia* for 14 consecutive days caused gastric mucosa regeneration, intact and continuous superficial epithelium, with well defined glands and gastric pits. The tissue also presented hyperemia (figure 4 C and D). The same was observed in the animals treated with ranitidine (100mg/kg) (figure 4 E and F): continuous superficial epithelium, own lamina and complete gastric gland. But the hyperemia was more intense than in the treated group.

3.3. Molecular Assay

Several authors associate the antiulcerogenic process with healing of chronic ulcer and participation of protein of epidermal growth factor (EGF). EGF is a 53-amino acid peptide originating mainly from the salivary glands. EGF mRNA has not been detected in the intact gastric mucosa of rodents and humans [22].

Growth factors and their receptors play important roles in cell proliferation and migration, repair of tissue injury and ulcer healing [23]. Paula *et al*. [7] related that the essential oil obtained of *Croton cajucara* healed the chronic gastric ulcer increasing EGF mRNA expression detected by RT-PCR and also increased somatostatin plasma levels and consequent gastrin plasma levels.

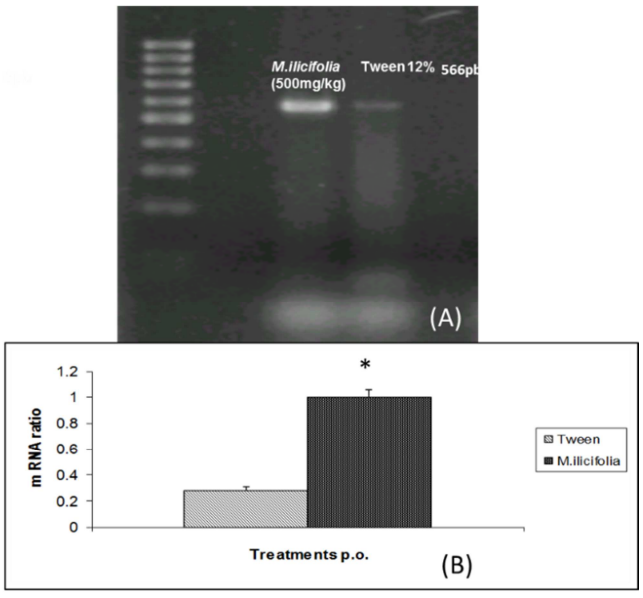

Figure 5. *(A) Expression of mRNA of EGF after the treatment of Maytenus ilicifolia (500mg/kg) by 14 days on acetic-acid chronic ulcer and analyzed by RT-PCR in rats. (B) Densiometric data Expression of mRNA of EGF. Results are presented as mean ± SD of 9 rats in each group. ANOVA, Dunnett's test: *p<0.01.*

In experiments with chronic acetic acid-induced ulceration, mRNA was identified in the mucosa of rats treated with *Maytenus ilicifolia*. The EGF expression in areas surrounding the ulcers in rats treated with *M. ilicifolia* was more pronounced than in the Tween 80 ($p<0.05$) treatment group (Figure 5A and B). This healing activity evidenced by epidermal growth factor expression is associated with the antiulcerogenic process of chronic ulcer. So, the healing promoted by the *Maytenus ilicifolia* through increased EGF mRNA expression detected by RT-PCR, determined the action mechanism of the anti-ulcer activity.

3.4. DPPH (2,2-Diphenyl -1 –picrylhydrazyl) and Hydroxyl Radical Scavenging Activity

Oxygen-derived free radicals have recently been postulated to play an important role in the pathogenesis of acute gastric mucosal injury induced by ischemia-reperfusion, stress, ethanol and anti-inflammatory drugs in rats. Furthermore, it has been suggested that free radicals generated by neutrophils may be important factors in delaying the healing of acetic acid –induced chronic gastric ulcers in these animals [9].

It is well known that medicinal plants act as free radical scavengers, and as antilipoperoxidants and are helpful in protecting collagen from degradation caused by superoxide anion radicals [24]. Scavenging test have been extensively used to monitor biological activities of medicinal plants used in traditional medicines [25]. Traditionally, these studies are carried through spectrophotometry, with reactions involving free radicals and the most used substance for this purpose is the DPPH (1,1-diphenyl-2-picrylhydrazyl). Its "natural" color in ethanolic solution is dark violet, with a band of optic absorption centered at 520nm. This color changes to dark yellow, when it reacts with a substance that can donate a hydrogen atom [26]. However this transition of colors is usually difficult to detect because the optical bands of absorption are wide. So, in this work, Electron Spin Resonance Spectroscopy (ESR) was used, allowing the direct measurement of free radical concentration, with high sensitivity, to test the scavenging activity of *Maytenus ilicifolia*. Ito *et al*. [27] related that, among the free radicals involved the gastric ulcer pathogenesis, the oxygen composites with 4 electrons, the superoxide anion (O_2), the hydrogen peroxide (H_2O_2) and the hydroxyl radical are the most important.

Figure 6A shows the ESR spectrum of DPPH after reaction with water (0mg/ml) and different concentrations of *M ilicifolia*. The intensity of the central line was used as the indicator of the free radical concentration. The fitting parameters from equation (1) of signal intensity (average of 3 spectrum) as function of extract concentrarion are: $A=(4.6 \pm 0.3).10^{-5}$ and $b= (1.0 \pm 0.1)$ mg/ml. Using this function, a value of 0.68±0.09 mg/ml was found to IC_{50}.

Figure 6B shows ESR spectra of spin-trapp DMPO-OH after reaction with *Maytenus ilicifolia* in different concentrations. The first central line was used as the indicator of free radical concentration. The fitting parameters from equation (1) of signal intensity (average of 3 spectrum) as function of extract concentrarion are: $A=(2.2 \pm 0.2).10^{-4}$ and $b= (7 \pm 1)$ mg/ml. Using this function, a value of 9 ± 2 mg/ml was found to IC_{50}.

The anti-oxidative activity of the initial three crude extracts Hex (25 mg), EtOAc (230 mg) and BuOH 30 mg (Figure 1) were tested by ESR using the DPPH radical (500µM) and EtOAc was the most antioxidant. Part of the crude extract EtOAc (215 mg) was fractionated on a Sephadex LH-20 column (1 m x 3 cm i.d., Pharmacia) eluted with MeOH. A total of 25 fractions of 5 ml each were collected (figure 1). These fractions were also submitted to ESR experiments with DPPH to determine the most antioxidant fraction. (After TLC (Thin Layer Chromatography) analysis (Si gel 60 plates - Merck; mobile phase Hex/EtOAc 4:6; reagents vanillin and phosphomolybdic acid) fractions with similar Rfs (Retardation Factor) were combined.

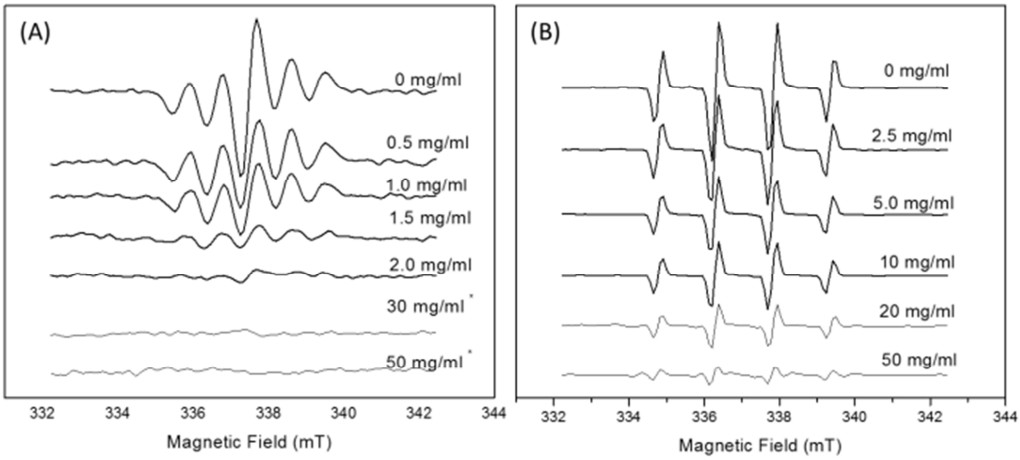

Figure 6. *(A) ESR spectra of DPPH (1mM) after reaction with aqueous solutions of Maytenus Iliciofolia using lyophilized plant extract and dried leaves content of capsules diluted at same concentration used for in vivo experiments (30 and 50mg/ml). The DPPH signal intensity diminishes after reaction, demonstrating the antioxidative activity of plant. The fitting of experimental data of ESR signal intensity of DPPH radical as a function of extract concentration with equation (1) give a value of 0.68±0.09 mg/ml for* IC_{50}. *(B): ESR spectra of DMPO-OH after reaction with aqueous solutions of Maytenus ilicifolia lyophilized extract in several concentrations demonstrating the free radical scavenging activity of plant. The fitting of experimental data of ESR signal intensity of DPPH radical as a function of extract concentration with equation (1) give a value of 9 ± 2 mg mg/ml for* IC_{50}.

The most active fraction F 14-15 was analyzed by ESI-MS via direct insertion. Figure 7 shows the mass spectrum of the most active fraction F 14-15. This spectrum shows adduct ions of sodium $[M + Na]^+$ and potassium $[M + K]^+$ as well as ammonium adduct ions $[M + NH_4]^+$ giving the same molecular mass at *m/z* 459, *m/z* 475, *m/z* 489, *m/z* 505 and *m/z* 534. Thus, with the molecular mass of the compounds obtained by ESI-MS, the search across natural product databases, particularly the Dictionary of Natural Products (Chapman & Hall, version online) [28] was carried out. This information was cross with the genus *Maytenus* and was possible identify the compounds which belong to the pentacyclic triterpenes class. The presence of compounds of the triterpenoids class in the most active fraction of *Maytenus ilicifolia* reinforce the relationship between the anti-oxidative and anti-ulcerogenic properties of this class of compounds. [29] evaluated antiulcerogenic activity of friedelan-3β-ol extracted from *Maytenus ilicifolia* (Celastraceae) and reported that these compounds did not decrease gastric ulcer. However, in our study, these triterpenes were not observed by mass spectrometry in the most active fraction.

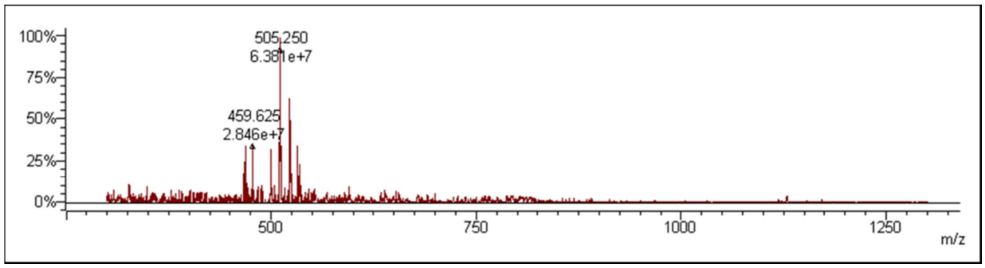

Figure 7. *Mass spectrum of the active fraction F 14-15 from M. ilicifolia.*

4. Conclusion

In conclusion, the results of this work reinforce the antiulcer and gastroprotective effect of *Maytenus ilicifolia* and this pharmacological feature can be associated to its antioxidant properties and presence of triterpenes. The anti-ulcer action mechanism of *M. ilicifolia* involve epidermal growth factor expression detected by RT-PCR.

Acknowledgements

Thank to Wilson Orcini and Maira Couto for technical assistance, CNPq, CAPES and FAPESP for partial financial support.

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