Study of the gastroprotective action and healing effects of *Kalanchoe pinnata* (Lam.) against acidified ethanol- and acetic acid-induced gastric ulcers in rats

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Estudo da acção gastroprotectora e dos efeitos curativos de *Kalanchoe pinnata* (Lam.) contra úlceras gástricas induzidas por etanol acidificado e ácido acético em ratos

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Flávia Sobreira
Doutorado
Instituição: Centro Universitário São Camilo
Endereço: Avenida Nazaré, 1501 São Paulo
E-mail: flavia.sobreira.smg@gmail.com

Guilherme Sobreira
Mestrado
Instituição: Sanofi
Endereço: Rua Augusto Soares, 130 SBC
E-mail.: guilhermesobrera@yahoo.com.br

Ingrit Elida Collantes Díaz
Doutorado
Instituição: Universidade Nacional de Engenharia, Lima, Perú.
Endereço: Av. Tupac Amaru 210, Rimac Lima, - Peru
E-mail: ingrit_uni@hotmail.com

Fúlvio G. Corazza
Mestrado
Instituição: Unifesp, Campus Diadema
Endereço: Rua São Nicolau, 210 Diadema.
E-mail: ffcorazza@gmail.com

Leandro Hernandes Santoro
Doutorado
Instituição: Universidade de São Paulo
Endereço: Av. Lineu Prestes, 580
E-mail: lesantorofarma@gmail.com

Patricia Gama
Doutorado
Instituição: Universidade de São Paulo
Endereço: Av. Lineu Prestes, 2415
E-mail: patgama@usp.br

Felipe R. Lourenço
Doutorado
Instituição: Universidade de São Paulo
Endereço: Av. Lineu Prestes, 580
E-mail: feliperl@usp.br

Jorge Mancini-Filho
Doutorado
Instituição: Universidade de São Paulo
Endereço: Av. Lineu Prestes, 580
E-mail: jmancini@usp.br

Matheus Aparecido dos Santos Ramos
Doutorado
Instituição: Universidade Estadual de São Paulo
Endereço: Rod. Araraquara-Jaú Km 1 - Machados - Araraquara/SP
E-mail: matheus.s.ramos@unesp.br

Tais Maria Bauab
Doutorado
Instituição: Universidade Estadual de São Paulo
Endereço: Rod. Araraquara-Jaú Km 1 - Machados - Araraquara/SP
E-mail: bauabtm@fcfar.unesp.br

Patrícia S. Lopes
Doutorado
Instituição: Unifesp, campus Diadema
Endereço: Rua São Nicolau, 210 Diadema-SP
E-mail: patslopes@hotmail.com

José Guerra
Doutorado
Instituição: Unisa
Endereço: Rua Isabel Schmidt, 349 São Paulo
E-mail: guerra@usp.br

Elfriede M. Bacchi (in memorian)
Doutorado
Instituição: Universidade de São Paulo
Endereço: Av. Lineu Prestes, 580
E-mail: elfriede @usp.br

ABSTRACT

Kalanchoe pinnata (Lam.) Pers. (Crassulaceae) is a commonly used species in traditional medicine in Brazil for the treatment of various diseases, including gastric ulcers. This research aims to evaluate the antiulcer aspects of *Kalanchoe pinnata* leaves. The LD_{50} value of the hydroethanolic extract (HE) of *K. pinnata* was 1341.46 mg/kg, after the *in vitro* cytotoxicity assay. In the phytochemical analysis, several flavonoids were identified in the HE and ethyl acetate fraction (EAF) of *K. pinnata*. It was verified that the gastroprotective activity of the HE of *K. pinnata* involved prostaglandins and sulfhydryl

compounds. However, the mechanism of gastroprotection of the EAF of *K. pinnata* is dependent on prostaglandins and nitric oxide. The ulcer healing activity of the HE of K. *pinnata* was also evaluated. According to the macroscopic results, doses of 200 mg/kg and 400 mg/kg reduced the injury area, with rates of 33% and 39%, respectively, after 7 days of treatment ($p < 0.05$). Histological analysis showed regeneration of the gastric mucosa and re-establishment of the glandular architecture in groups treated with the HE (200 and 400 mg/kg). Antioxidant enzymes (CAT, SOD and GPx) were evaluated in the mechanism of gastric ulcer healing. The results showed that the antiulcerogenic activity was mediated by SOD. The anti-*Helicobacter pylori* activity was also evaluated; however, the HE did not show anti-*H. pylori* activity. Analysis of the results suggests that *K. pinnata* has therapeutic potential against gastric ulcers and that the flavonoids may be linked to the biological effects.

Keywords: *kalanchoe pinnata*, gastric ulcer, flavonoids.

RESUMO

Kalanchoe pinnata (Lam.) Pers. (Crassulaceae) é uma espécie comummente utilizada na medicina tradicional no Brasil para o tratamento de várias doenças, incluindo úlceras gástricas. Esta investigação visa avaliar os aspectos antiulcerosos das folhas de *Kalanchoe pinnata*. O valor LD50 do extracto hidroetanólico (HE) de K. *pinnata* era de 1341,46 mg/kg, após o ensaio in vitro de citotoxicidade. Na análise fitoquímica, foram identificados vários flavonóides na fração HE e acetato etílico (FEA) de K. *pinnata*. Foi verificado que a actividade gastroprotectora do HE de K. *pinnata* envolvia prostaglandinas e compostos sulfidrílicos. No entanto, o mecanismo de gastroprotecção da FEA de K. *pinnata* depende das prostaglandinas e do óxido nítrico. A actividade de cura de úlceras da FEA de K. *pinnata* também foi avaliada. De acordo com os resultados macroscópicos, doses de 200 mg/kg e 400 mg/kg reduziram a área lesada, com taxas de 33% e 39%, respectivamente, após 7 dias de tratamento ($p < 0,05$). A análise histológica mostrou regeneração da mucosa gástrica e restabelecimento da arquitectura glandular em grupos tratados com o HE (200 e 400 mg/kg). As enzimas antioxidantes (CAT, SOD e GPx) foram avaliadas no mecanismo de cicatrização da úlcera gástrica. Os resultados mostraram que a actividade antiulcerogénica foi mediada pela SOD. A actividade anti-*Helicobacter pylori* também foi avaliada; contudo, a HE não mostrou actividade anti-*H. pylori*. A análise dos resultados sugere que o K. *pinnata* tem potencial terapêutico contra úlceras gástricas e que os flavonóides podem estar ligados aos efeitos biológicos.

Palavras-chave: *kalanchoe pinnata*, úlcera gástrica, flavonóides.

1 INTRODUCTION

Peptic ulcers are a typical disease of the gastrointestinal tract that affects six million Americans every year [1]. The pathophysiology is multifactorial, and ulcers occur from imbalanced protective factors (prostaglandins, mucus, bicarbonate, nitric oxide, and sulfhydryls) and lesive factors (pepsin, gastric acid and free radicals). Many exogenous factors contribute to triggering ulcerative lesions, such as *Helicobacter pylori* infection, long-term usage of nonsteroidal anti-inflammatory drugs (NSAIDs), smoking, alcohol and stress. Several drugs, mainly proton pump inhibitors, H_2 receptor antagonists and

antibiotic drugs for *H. pylori* infection, have been used for the treatment of peptic lesions [2,3]. However, studies have demonstrated the incidence of relapse and adverse effects, such as the risk of dementia in the elderly [4], vitamin B12 deficiency [5]and intestinal metaplasia [6]. A new medicine derived from medicinal plants that is more effective and has fewer side effects is an interesting alternative [7].

Kalanchoe pinnata L. belongs to the Crassulaceae family and is a perennial herb native to Madagascar. This species is very useful in traditional medicine, mainly in tropical countries, for its anti-inflammatory, antimicrobial, antifungal, antihypertensive, antiallergic and antiulcer activities [8,9]. In Europe, *K. pinnata* was introduced to anthroposophic medicine to treat hyperactivity disorders [10]. In Brazil, *K. pinnata* is on the list of Renisus that the Public Health System (SUS) has an interest in to generate herbal medicine. The phytochemical analyses of the leaves from *K. pinnata* revealed the presence of bufadienolides [11,12] terpenoids [13] and flavonoids, that represent the class of secondary metabolites most commonly found [14–17]. Our group and other studies have shown the gastroprotective effects of the leaf juice, aqueous and hydroethanolic extracts, and ethyl acetate- and methanol-soluble fractions of *K. pinnata* [16,18–21].

However, to our knowledge, there is a lack of information in the literature about the mechanism of action involved in the gastroprotective activity of the hydroethanolic extract and ethyl acetate fraction of *K. pinnata* against gastric lesions induced by acidified ethanol in rats, and about the mechanism of action involved in healing chronic gastric ulcers induced by acetic acid, which motivated our group to evaluate these observations. Considering the above, the aim of this study was to investigate the mechanisms involved in the gastroprotection and identify new compounds in the hydroethanolic extract and ethyl acetate fraction from the leaves of *K. pinnata*. Furthermore, an *in vitro* cytotoxicity analysis was carried out to provide information about the safety of *K. pinnata* extract use.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL AND EXTRACT PREPARATION

Kalanchoe pinnata (Lam.) Pers. (Crassulaceae) leaves were collected at the Chemical, Biological and Agricultural Pluridisciplinary Research Center-Campinas State University (CPQBA-UNICAMP), Paulínia, São Paulo, Brazil. A voucher specimen was deposited into the Collection of Medicinal and Aromatic Plants (CQBA-UNICAMP) CPMA under register 337. The species was identified by Katia Calago.

The fresh material was oven heated with forced ventilation at 40 °C and macerated with 70% ethanol for seven days. The extract was filtered, concentrated under vacuum and then lyophilized. The hydroethanolic extract was fractionated by liquid-liquid partition into three fractions: a chloroform fraction, an ethyl acetate fraction and a water fraction. We chose the hydroethanolic extract and ethyl acetate fraction for pharmacological and chemical analyses because these samples showed higher biological activities [20].

2.3 CHEMICAL ANALYSES

For HPLC (UV/DAAD), 10 mg of each of the hydroethanolic extract and ethyl acetate fraction were dissolved in 10 mL of methanol (HPLC grade). The samples were filtered through a 0.45 mm filter, and aliquots of 20 µL were injected. The mobile phase used was water acidified with 0.1% formic acid (A) and 25% acetonitrile (B) for 20 minutes at a flow rate of 1 mL/min, with detection at 254 nm in an isocratic run[20]. The analyses were performed on a Shimadzu® model LC-20 AT instrument with a *Diode Array* detector (DAD) (Kyoto, Japan). The separation was carried out on a 250 mm × 4.6 mm, 5 µm Shim-pack VP-ODS C18 reverse-phase column (Shimadzu®, Kyoto, Japan).

The mass spectrometry assay experiment was performed under the same chromatographic conditions described above. The mass spectrometer used was an Esquire 3000 Plus Bruker Daltonics® (Billerica, MA, USA) equipped with electrospray ionization (ESI). Instrument control and data acquisition were performed using Esquire 5.2 software. The ion source temperature was 325 °C, the capillary voltage was set to -4500 V (positive mode) and the plat offset was -500 V. Nitrogen served as the nebulizer gas regulated at 27 psi and a flow rate of 6 L/min. The mass spectrometer was operated in full scan mode monitoring positive ions. Fragmentation of $[M+H]^+$ molecular ions into specific product ions was performed in enhanced product ion (EPI) mode induced by collision with nitrogen.

2.4 QUERCETIN 3-O-A-L-ARABINOPYRANOSYL (1→2) -A-L-RHAMNOPYRANOSIDE QUANTIFICATION IN THE HYDROETHANOLIC EXTRACT FROM K. PINNATA

The compound quercetin 3-O-α-L-arabinopyranosyl-(1→2)-α-L-rhamnopyranoside was isolated from the ethyl acetate fraction of *K. pinnata* [20]]. This flavonoid was chosen for the calibration curve because it was the major compound found

in this hydroethanolic extract. The calibration curve for this flavonoid ranged from 0.16 mg/mL to 0.06 mg/mL, and it was constructed by plotting the peak area against the corresponding concentration. Linearity was verified (r^2=0.996). The hydroethanolic extract and the quercetin 3-O-α-L-arabinopyranosyl-(1→2)-α-L-rhamnopyranoside standard solution were injected three times each. The results are expressed as the mean ± standard deviation.

2.3 CYTOTOXICITY EVALUATION

The cytotoxicity evaluation was performed according to the test validated by OECD 129 [22] in mouse fibroblast cultured BALB 3T3 clone A31 cells, ATCC CCL-163, exposed to the hydroethanolic extract of *K. pinnata* and neutral red for the evaluation of cell viability at 540 nm. The samples were dissolved in DMSO (0.5%) and diluted with DMEM. The concentrations of the hydroethanolic extract were 0.11 to 1.6 mg/mL, and the concentrations of ethyl acetate fraction were 0.03 to 0.4 mg/mL. The results were evaluated by the Statistical Software Phototox®, version 2.0. The cell viability dose-response curves were constructed, and the IC_{10} and IC_{50} values were calculated. The IC_{50} values were used to estimate the median lethal dose (LD_{50}). The acute oral toxicity of the sample was estimated using the United Nations Globally Harmonized System (GSH) [23].

2.4 ANIMALS

Male adult Wistar rats (150-180 g) were obtained after being approved by the Ethical Committee on Animal Use of the Faculty of Pharmaceutical Sciences, University of São Paulo (protocol number CEUA/FCF/426). The animals were housed under standard conditions: 12 h dark-12 h light, temperature 22 ± 2 °C, 55 ± 10% humidity and food and water *ad libitum*. The animals were deprived of food for 8-12 h before the experiment and were maintained in cages with wired mesh at the bottom to prevent coprophagy.

2.4.1 Ethanol acidified gastric ulcer in indomethacin-pretreated rats

Male rats (n=7) were divided into two major groups. The first group was pretreated with saline, and the second group was pretreated with indomethacin (10 mg/kg) subcutaneously. Thirty minutes after pretreatment, the groups received water (1 mL/100 g), cloprostenol (150 µg/kg), the higher effective dose of the hydroethanolic extract (400

mg/kg) and ethyl acetate fraction (200 mg/kg) orally [20]. After thirty minutes, gastric lesions were induced with 300 mmol/L HCl in 60% ethanol (1 mL/100 g). One hour later, the animals were euthanized, and their stomachs were removed. The incision in each stomach was made along the greater curvature and scanned. Then, the lesion was measured using Image Pro Plus software, and the relative lesion area (RLA, in %) was estimated as RLA= (TLA × 100)/TA, where TLA= total lesion area (mm^2) and TA= total stomach area (mm^2); adapted from Matsuda [24].

2.4.2 Ethanol-acidified gastric ulcers in L-NAME-pretreated rats

Rats were divided into two major groups of seven animals, one group of which was pretreated intraperitoneally with N^G nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich, São Paulo, Brazil), an inhibitor of NO synthesis (70 mg/kg) and the other group was pretreated intraperitoneally with saline (1 mL/100 g). Thirty minutes later, the animals were treated with water (1 mL/100 g), cloprostenol (150 µg/kg), the higher effective dose of the hydroethanolic extract (400 mg/kg) and the ethyl acetate fraction (200 mg/kg) orally. Thirty minutes later, all the animals received an oral dose of 300 mmol/L HCl in 60% ethanol (1 mL/100 g). One hour later, the animals were euthanized, and their stomachs were removed and opened along the greater curvature. The analyses of gastric lesions were measured as described above, adapted from Matsuda [24].

2.4.3 Ethanol-acidified gastric ulcers in NEM-pretreated rats

This experiment was performed according to Matsuda [24] with modification. The animals were pretreated intraperitoneally with saline or NEM (N-ethylmaleimide, Sigma-Aldrich, São Paulo, Brazil), a blocker of sulfhydryl compounds. After thirty minutes, rats received water orally (1 mL/100 g), cloprostenol (150 µg/kg), the higher effective dose of the hydroethanolic extract (400 mg/kg) and ethyl acetate fraction (200 mg/kg). After thirty minutes, gastric lesions were induced with 300 mmol/L HCl in 60% ethanol (1 mL/100 g). One hour later, the animals were euthanized, and the gastric lesions were analyzed as described above.

2.4.4 Healing of the acid acetic-induced gastric ulcer

This method was performed as described by Okabe and Amagase [25]. Eight animals were used in each group. After anesthesia (xylazine 10 mg/kg and ketamine 90 mg/kg i.p.), laparotomy was performed, and the stomachs were exposed. Round forceps

(5 mm in diameter) were used to clamp the stomach, and local application (serosa surface) of 100% acetic acid was performed for 60 seconds. Sterile saline was used to wash the contact area with acetic acid. The abdomen was closed, and the animals were fed normally. Two days after surgery, the animals were orally treated with the hydroethanolic extract (100, 200, or 400 mg/kg), lansoprazole (30 mg/kg) and water (1 mL/100 g) for seven days. After completing the treatment, the animals were euthanized, and the stomachs were removed. The gastric ulcer was analyzed using Image Pro Plus software.

2.4.5 Histological analysis

Tissue samples of the stomach with gastric ulcers induced by acetic acid were preserved in 10% buffered formalin solution, dehydrated in ethanol and clarified in xylene. After this procedure, the samples were embedded in paraffin, sectioned at a thickness of 5 µm and stained with hematoxylin-eosin. Mallory trichrome staining was also performed to observe collagen fibers. The histopathological evaluation was processed under light microscopy.

2.4.6 Measurement of the antioxidant enzymes

The homogenates were prepared using 0.5 g of the gastric tissue sample with 0.1 M phosphate buffer, pH 7.0, in an ice bath with the help of an Ultra Turrax homogenizer. The protein concentration in the supernatant of homogenate was evaluated by the Bradford method [26].

2.4.6.1 Determination of superoxide dismutase activity (SOD)

Superoxide dismutase activity analysis was performed according to the method of McCord and Fridovich [27]. For this purpose, 100 mM cytochrome C, 500 mM xanthine, 1 mM EDTA, and 200 mM KCN in 0.05 M potassium phosphate, pH 7.8 (reaction medium) was used along with xanthine oxidase and the homogenate from each stomach of all the groups. The results were quantified spectrophotometrically at 550 nm and expressed as units per milligram of protein. One unit of SOD activity was the amount of enzyme that induced 50% inhibition of the xanthine reaction at 25 °C, pH 7.8.

2.4.6.2 Determination of catalase activity (CAT)

The measurement of catalase activity was determined according to Beutler [28]. For this experiment, 10 mM H_2O_2, 1 M Tris HCl buffer, 5 mM EDTA pH 8.0 and the

homogenates from each stomach were used. The decomposition of hydrogen peroxide by catalase was measured at 230 nm with a spectrophotometer, and the results are expressed as units per milligram of protein. One unit of CAT activity corresponded to the enzyme activity that hydrolyzed 1 mol of H_2O_2/min at 37 °C, pH 8.0.

2.4.6.3 Determination glutathione peroxidase (GPx)

The evaluation of glutathione peroxidase activity was carried out by the Sies method [29]. The enzymatic activity was measured spectrophotometrically at 340 nm. One unit of enzymatic activity was defined as the amount of enzyme that oxidized 1 µmol of NADPH/min at 30°C at pH 7.0.

2.5 ANTI-*HELICOBACTER PYLORI* ACTIVITY

Helicobacter pylori ATCC 43504 was used. The microdilution technique by CLSI [30], with modifications, was used to determine the minimal inhibitory concentration (MIC) value. *H. pylori* was inoculated on Mueller-Hinton agar plates containing 5% sheep blood and incubated at 36 °C for 72 h in a 10% CO_2 atmosphere. Inoculum were prepared in the same medium supplemented with 10% fetal bovine serum and adjusted to 10^7 cell/mL. Concentration of the hydroethanolic extract was prepared in 2% DMSO, ranging from 0.5 to 1000 µg/mL. Amoxicillin was used as the reference antimicrobial compound, and the MIC was recorded after incubation of the microplates at 36 °C for 72 h in a 10% CO_2 atmosphere. The MIC was registered as the lowest concentration at which no growth was observed. This record was facilitated by the addition of 20 µL of resazurin solution (100 µg/mL) as the colored reactant to each well and incubation for 2 h. A pink color indicated bacterial growth, and a blue color indicated no bacterial growth [31].

2.6 STATISTICAL ANALYSIS

The values were transformed using the square root and Box Cox transformation to normalize the data distribution. The results are reported as the mean ± standard deviation or as the mean ± standard mean error and were analyzed by one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons. All statistical analyses were performed using GraphPad 5.0 software, and statistical significance was set at $p < 0.05$.

3 RESULTS AND DISCUSSION

Medicinal plants are used by humans to treat diseases in a similar proportion in developed and developing countries [32]. In this way, the interest of herbal medicines has increased, and it is estimated that the global market is approximately 20 billion dollars annually [33]. *Kalanchoe pinnata* L. is a very popular medicine in Brazil and other parts of the world that is used to treat gastric ulcers [9,17].

In this study, an *in vitro* cytotoxicity experiment against normal cells was performed to evaluate the safety of the hydroethanolic extract. This assay allowed us to estimate the LD_{50} values, representing the acute oral toxicity. Based on the IC_{50} (0.926 mg/mL), the oral LD_{50} value for the hydroethanolic extract was 1341.46 mg/kg, and it can be placed in category 4 of the United Nations Globally Harmonized System. Further study to assess the safety of the hydroethanolic extract should be performed because the LD_{50} value is approximately three times higher than the therapeutic dose. Fonseca and collaborators [34] performed the *in vivo* toxicity from the extract leaf of *K. brasiliensis.* This extract showed low acute toxicity most evident at the dose of 1000 mg/kg and low relative subchronic toxicity.

The possible mechanism of action involved in the gastroprotection and the healing activity of *K. pinnata* was evaluated. It was evaluated the possible mechanism of action involved in the gastroprotection and the healing activity of *K. pinnata.* In a previous study, Sobreira and collaborators [20] demonstrated that the hydroethanolic extract and ethyl acetate fraction of *K. pinnata* showed gastroprotection in an ethanol/HCl gastric ulcer model, and these results produced motivation to continue the study.

Prostaglandins (PGs), nitric oxide (NO) and sulfhydryl compound (SH) are involved in the mechanism of action of the gastroprotective effect of many medicinal plants [31,35,36]. Therefore, any natural compound that depends on these molecules to promote pharmacological effects will lose its action when a specific blocker is used.

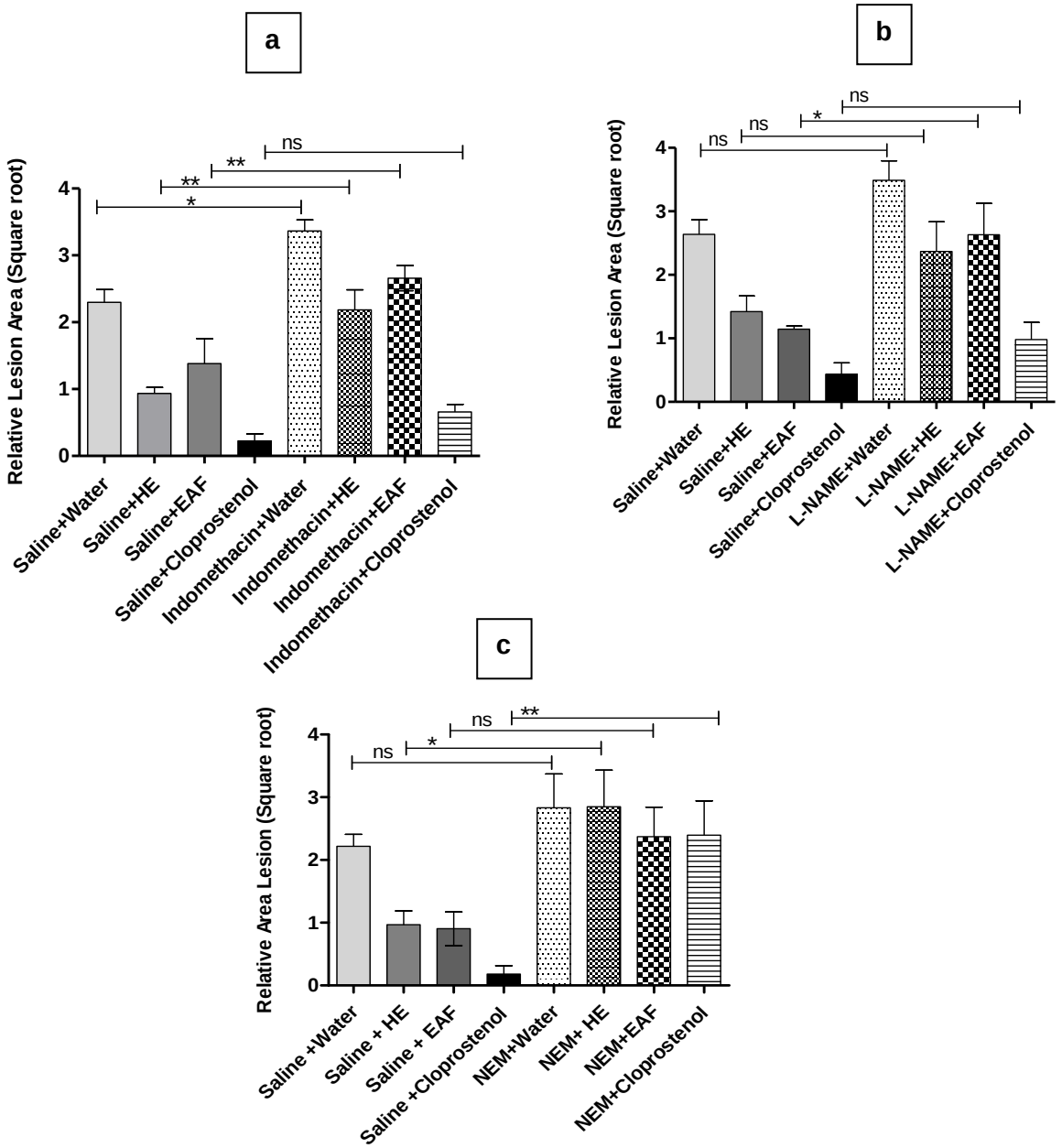
Indomethacin, a nonsteroidal anti-inflammatory drug (NSAID), inhibits the activity of cyclooxygenase (COX), an enzyme involved in prostaglandin synthesis [37]. Prostaglandins make important contributions to maintaining mucosa integrity by inhibiting gastric acid secretion, stimulating the secretion of mucus and bicarbonate, contributing to mucosal blood flow, reducing the permeability of the epithelium and inhibiting neutrophil adherence and activation [38]. Indomethacin pretreatment reversed the gastroprotective effects of the hydroethanolic extract and ethyl acetate fraction from

K. pinnata. These results suggest that the pharmacological effect of both samples is dependent on prostaglandins (Fig. 1).

Nitric oxide (NO) plays an important protective role in the gastric epithelium. NO helps the gastrointestinal tract to maintain homeostasis, including vasodilation and modulation of gastric blood flow, stimulation of mucus and bicarbonate, reduction of superoxide radicals, inhibition of leukocyte adherence and gastric acid secretion [39,40]. The gastroprotection of the hydroethanolic extract was not affected when the animals were pretreated with L-NAME, but the gastroprotective effect of ethyl acetate fraction was blocked when the animals were pretreated with this NO synthase inhibitor, indicating that nitric oxide participates in the gastric defense mechanism (Fig. 1).

In the next step of this study, we evaluated the involvement of sulfhydryl compounds in gastroprotection. For that, a blocker of sulfhydryl compounds (NEM) was used. The results showed that in the ethyl acetate fraction group, pretreatment with NEM did not increase the gastric lesion compared to the vehicle-pretreated ethyl acetate fraction group. Therefore, these data demonstrated that SH compounds are not associated with the gastroprotective effect of the ethyl acetate fraction. Moreover, gastroprotection promoted by the hydroethanolic extract in the group pretreated with NEM was abolished. This result suggests the importance of sulfhydryl compounds in the gastroprotective activity of the hydroethanolic extract. Additionally, the gastroprotection displayed by cloprostenol, an analog of prostaglandin $F_{2\beta}$, was lost. Szabo and collaborators [41] reported that the SH compounds facilitated the action of prostaglandins. In addition, SH compounds are present in gastric mucus and form disulfide bridges, which can alter the physical state of the SH compounds to scavenge free radicals (Fig. 1).

Fig. 1. Antiulcer activity of the hydroethanolic extract – HE (400 mg/kg) and ethyl acetate fraction - EAF (200 mg/kg) of *Kalanchoe pinnata* in rats pretreated with indomethacin (10 mg/kg) (a), L-NAME (b), and N- ethylmaleimide (c) after induction of gastric ulcers by acidified ethanol. The results are reported as the means ± S.E.M.; n=7. Statistical significance was determined by ANOVA followed by Tukey's test. *p<0.05; ** p<0.01; ns - no significant differences.

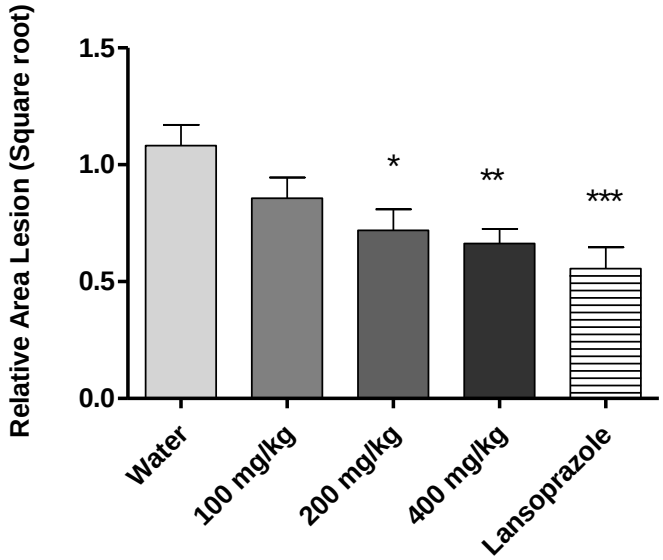


A possible explanation for the different mechanisms of action of the hydroethanolic extract and ethyl acetate fraction is that both samples contain different chemical compounds present in different amounts. For example, quercetin 3-O-α-L-arabinopyranosyl-(1→2)-α-L-rhamnopyranoside is the major component of the hydroethanolic extract, whereas quercitrin is the major component of the ethyl acetate fraction [20]. Another study also found different mechanisms of action between the extract and fraction obtained from the same species. In this paper, the authors demonstrated that the acetonic extract from *Polygala cyparissias* promoted its

gastroprotective effects with participation from NO but without the involvement of SH compounds, while the gastroprotective activity promoted by the methanolic fraction of *P. cyparissias* is dependent on SH compounds. These results must take into account the difference in chemical constituents; for instance, the acetonic extract from *P. cyparissias* contains xanthones as the major compounds; however, these metabolites were not observed in the fraction [42].

Considering the above studies, it is desirable that medicinal plants have both gastroprotective effects and ulcer healing actions [31]. Therefore, it is relevant to evaluate the effects of hydroethanolic extract from *K. pinnata* on the healing of gastric ulcers. A gastric ulcer was therefore induced by acetic acid. This model is well-established and resembles human ulcers in both its pathological and healing features [25]. Acetic acid provokes erosion of the gastric mucosa and changes cellular permeability [43]. Ulcer healing is a process that involves cellular proliferation, re-epithelialization, angiogenesis and matrix deposition, which results in the reconstruction of the mucosal architecture [44,45]. Treatment with the hydroethanolic extract of *K. pinnata* at different doses (100, 200 and 400 mg/kg) was performed for seven days. Treatment with the hydroethanolic extract (200 and 400 mg/kg) promoted a reduction in gastric lesions by 33% and 39%, respectively (Fig. 2).

Fig. 2. Effect of the oral administration of the hydroethanolic extract from *Kalanchoe pinnata* leaves on the healing of ulcers produced by the introduction of acid acetic solution into the stomachs of rats. The results are reported as the means ± S.E.M. ANOVA was carried out followed by Dunnett's test, *p <0.05; ** p<0.01; ***p<0.001, compared to water.



Histopathological analyses of animal slides with gastric ulcers were also performed. The results demonstrated that treatment with vehicle showed extensive lesions, a lack of regeneration in most animal slides and moderate inflammatory infiltrate (mainly polymorphonuclear cells). However, in animals treated with 200 mg/kg and 400 mg/kg hydroethanolic extract from *K. pinnata*, gastric mucosa regeneration (most animal slides), lush of granulation tissue and evidence of fibroblastic proliferation was observed (Fig. 3). Representative slides of each group were also stained with Mallory trichrome. The histopathological analyses of these slides revealed that treatment with 200 mg/kg and 400 mg/kg *K. pinnata* hydroethanolic extract increased the density of collagen fibers compared with the water group. These results demonstrated that treatment with the hydroethanolic extract of *K. pinnata* at 200 mg/kg and 400 mg/kg accelerates the healing process (Fig. 4).

Fig. 3. Effect of treatment with the hydroethanolic extract of *Kalanchoe pinnata* on the histology of acetic acid-induced gastric mucosal damage in rats stained by hematoxylin-eosin. Photomicrographs of the gastric mucosa represent the area with the formation of granulation tissue, fibroblastic proliferation (arrowhead) and neoformed vessels (dashed arrow) and the inflammatory infiltrate (filled arrow). Original magnification: 40×.

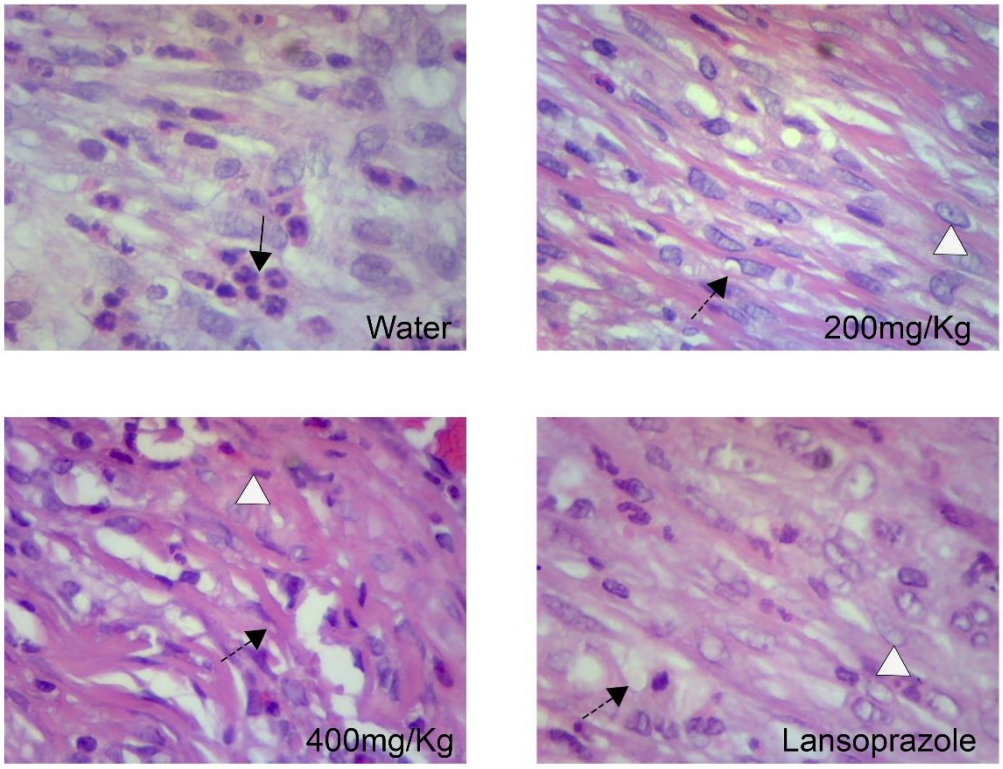
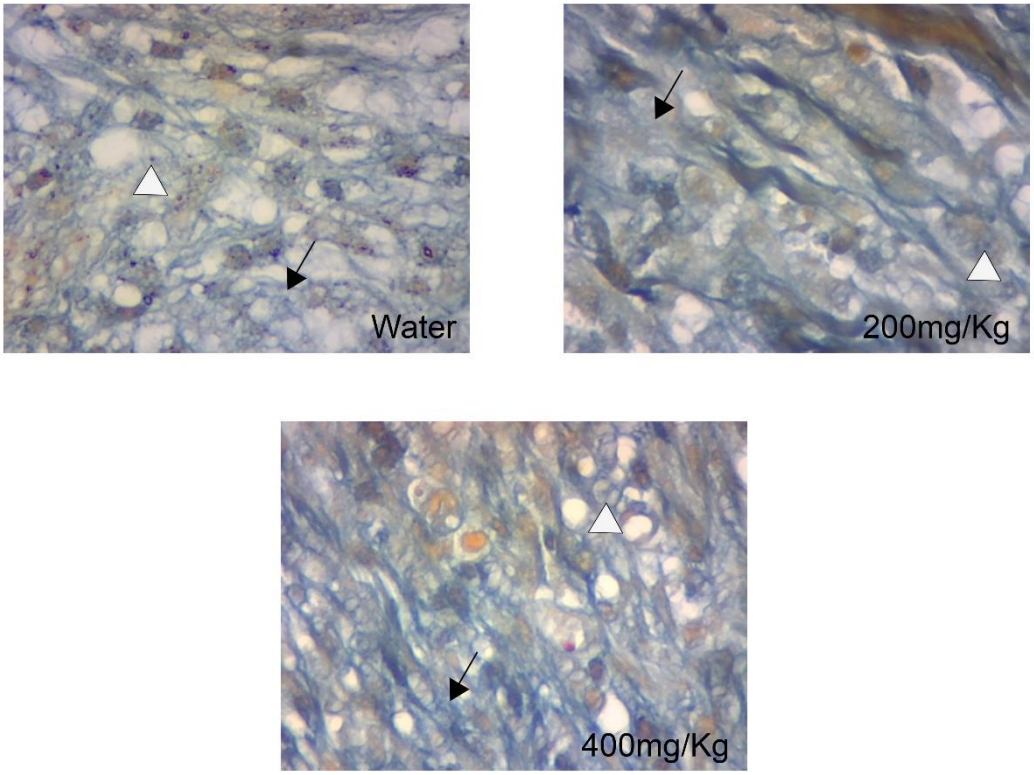


Fig. 4. Effect of treatment with the hydroethanolic extract of *Kalanchoe pinnata* on the gastric mucosa after damage induced by acetic acid. Representative images of the histological sections after the Mallory trichrome reaction. Representative images of different conditions: the water group shows discreet fibroblast proliferation (triangle arrow) and collagen fiber deposition (filled arrow). The histological analysis of gastric mucosa of rats treated with 200 mg/kg and 400 mg/kg hydroethanolic extract showed an apparent increase in fibroblast proliferation (triangle arrow) and collagen fiber deposition (filled arrow). Original magnification: 40×.



Vascular endothelial growth factor (VEGF) is a growth factor involved in gastric ulcer healing. VEGF stimulates angiogenesis, epithelialization and collagen deposition [46]. Therefore, its expression in gastric tissue was evaluated. The immunohistochemical analysis showed VEGF expression at the ulcer margins in all the treatment groups. At *K. pinnata* hydroethanolic extract doses of 200 mg/kg and 400 mg/kg, the expression of VEGF increased, but there was no significant difference ($p>0.05$) compared to the vehicle group. The methodology for this assay and a graphic representation of the results are provided in Supplementary Material (Fig. S1). Therefore, administration of hydroethanolic extract of *K. pinnata* that promotes gastric ulcer healing did not induce the expression of VEGF. Niu and collaborators [43] reported that healing activity of gastric ulcers induced by acetic acid in cod (*Gadus macrocephalus*) skin collagen

peptides increased the expression level of VEGF, but a significant difference was not reached.

Oxidative stress plays an important role in mucosal damage. Gastric cells have antioxidant defense systems, and enzymatic and nonenzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT) and the glutathione redox cycle [47,48]. Many studies have reported the involvement of antioxidant enzymes in both gastroprotection and gastric ulcer healing promoted by medicinal plants [49,50]. The administration of hydroethanolic extract at a dose of 400 mg/kg increased the SOD activity ($p<0,05$) compared to the negative control, indicating the involvement of this system in the mechanism of gastric ulcer healing presented by hydroethanolic extract at the 400 mg/kg dose evaluated. However, the CAT and GPx activities were not altered by the treatment with the hydroethanolic extract of *K. pinnata* (see Table 1). *Helicobacter pylori* is also associated with the development of gastric ulcers [51], but assays with the hydroethanolic extract showed no promising results (MIC > 1000 µg/mL).

Table 1. Antioxidant enzymes in rat stomach tissue treated with hydroethanolic extracts (100, 200 and 400 mg/kg) for seven days after the induction of gastric lesions with acetic acid. The results are expressed as the mean ± S.D. ANOVA followed by Dunnett's test: *p<0.05 represents a difference in relation to the control group.

Treatments	CAT[a] (U/mg protein)	SOD[b] (U/mg protein)	GPx[c] (U/mg protein)
Water	5.66±1.74	6.56±0.85	0.34±0.05
100 mg/kg	5.57±2.01	7.90±2.54	0.37±0.11
200 mg/kg	6.54±1.50	7.38±0.49	0.39±0.08
400 mg/kg	6.50±2.65	9.87±3.80*	0.36±0.15
Lansoprazole	7.27±1.46	8.19±2.40	0.49±0.15

[a]CAT catalase, [b]SOD superoxide dismutase, [c]GPx peroxidase glutathione.

Quantification analyses of the flavonoid quercetin 3-O-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside from *K. pinnata* by HPLC were performed to contribute to the production of a standardized extract. This flavonoid corresponds to 9.649 ± 0.169 g/100 g of hydroethanolic extract, which corresponds to 0,114 ± 0.002 % (w/w) in the fresh leaves. This flavonoid is the major compound found in the hydroethanolic extract, which is in agreement with other studies, and it can be used as a chemical marker for quality control of this species [14,52,53]. Quercetin 3-O-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside was described for few other species from different botanical families, however not as their major compound [17]. Muzitano and collaborator [54] carried out an HPLC analysis of the total flavonoids in the aqueous extracts of *K. pinnata*

leaves under different cultivation conditions. In this study, rutin was used as a standard because it contains quercetin aglycone as the major flavonoid from *K. pinnata*. The yield of the flavonoid quercetin 3-O-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside corresponded to 2.09 ± 0.03% / 100 g of aqueous lyophilized extract, calculated as rutin. In our study, the standard used was quercetin 3-O-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside. Because of the different standard, extractive processes and solvents used, the value found by our group was higher than that in the other study.

Chemical characterization was performed to identify compounds by liquid chromatography mass spectrometry (LC/ESI-MS^n) analysis in positive mode (see Table 2) in the hydroethanolic extract and ethyl acetate fraction of *K. pinnata* because they showed gastroprotective and healing effects. Eight flavonoids were identified by analysis of fragment ions: myricetin-3-*O*-α-L-arabinopyranosyl-(1→2)-α-L-rhamnopyranoside (1), myricetin-3-*O*-rhamnopyranoside (1.1), mearnsetin pentosyl rhamnopyranoside (2), quercetin 3-O-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (3), quercetin 3-*O*-β-D-xylopyranosyl-(1→2)-α-L-rhamnopyranoside (4), quercetrin (5), 4',5,7-trihydroxy-3',8-dimethoxyflavone 7-O-β-D-glucopyranoside (5.1), and kaempferol-3-*O*-α-L-arabinopyranosyl-(1→2)-α-L-rhamnopyranoside (6). Chromatograms are available in the Supplementary Material (Fig. S2-S3). Previous studies have already identified quercetin 3-O-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (3) and quercetrin (5) in *K. pinnata* leaves [14,20,55]. The compounds identified in the hydroethanolic extract were 1, 2, 3, 4, 5 and 6, and the compounds identified in the ethyl acetate fraction were 1, 1.1, 2, 3, 4, 5, 5.1 and 6.

Myricetin-3-*O*-rhamnopyranoside (1.1) and 4',5,7-trihydroxy-3',8-dimethoxyflavone 7-O-β-D-glucopyranoside (5.1) were only identified in the ethyl acetate fraction. Mearnsetin pentosyl rhamnopyranoside (2) and quercetin 3-*O*-β-D-xylopyranosyl-(1→2)-α-L-rhamnopyranoside (4) were identified for the first time in *K. pinnata*. Compound 4 is an isomer of quercetin 3-O-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside. The presence of compounds 1.1 and 5.1 in the ethyl acetate fraction is because the fraction was preconcentrated with respect to the hydroethanolic extract.

Table 2. Identification of the chemical constituents of *K. pinnata* from hydroethanolic extract and ethyl acetate fraction by LC-ESI-MS^2 in positive mode.

Compound	MW (Da)	Hydroethanolic extract					Identification	Ethyl acetate fraction					References
		RT (min)	UV λ_{max}(nm)	$[M+H]^+$ *m/z*	MS^2	Fragment ions		RT (min)	UV λ_{max}(nm)	$[M+H]^+$ *m/z*	MS^2	Fragment ions	
1	596	5.4	209 263 349	596.9	542.8 464.7 428.9 318.6	$[M+H-3H_2O]^+$ $[M+H-Ara]^+$ $[M+H-Ara-2H_2O]^+$ $[M+H-Ara-Rham]^+$	Myricetin 3-*O*-α-L-arabinopyranosyl-(1→2)-α-L-rhamnopyranoside	5.9	208 264 341	597.3	465 447 319	$[M+H-Ara]^+$ $[M+H-Ara-H_2O]^+$ $[M+H-Ara-Rham]^+$	[52]
1.1	464	*	*	*	*	*	Myricetin 3-*O*-rhamnopyranoside	6.5	208 260 349	465.2	318.9	$[M+H-Rham]^+$	[52]
2	610	7.1	206 266 348	611	583.9 478.8 442.7 332.6	$[M+H-H_2O]^+$ $[M+H-pentose]^+$ $[M+H-pentose-2H_2O]^+$ $[M+H-pentose-Rham]^+$	Mearnsetin pentosyl rhamnopyranoside	7.7	202 269 347	611.2	479 333	$[M+H-pentose]^+$ $[M+H-pentose-Rham]^+$	[56,57]
3	580	7.8	205 255 348	581	448.8 302.6	$[M+H-Ara]^+$ $[M+H-Ara-Rham]^+$	Quercetin 3-*O*-α-L-arabinopyranosyl-(1→2)-α-L-rhamnopyranoside	8.7	205 255 348	581.2	449 302.9	$[M+H-Ara]^+$ $[M+H-Ara-Rham]^+$	[14,]
4	580	8.5	206 255 348	581.2	476.7 448.7 412.6 302.6	 $[M+H-Xyl]^+$ $[M+H-Xyl-2H_2O]^+$ $[M+H-Xyl-Rham]^+$	Quercetin 3-*O*-β-D-xylopyranosyl-(1→2)-α-L-rhamnopyranoside	9.5	205 255 347	581.3	449 431 413 303	$[M+H-Xyl]^+$ $[M+H-Xyl-H_2O]^+$ $[M+H-Xyl-2H_2O]^+$ $[M+H-Xyl-Rham]^+$	[58]
5	448	9.1	205 255 349	448.7	430.7 302.5	$[M+H-H_2O]^+$ $[M+H-Rham]^+$	quercitrin	10.2	204 255 348	449.1	302.9	$[M+H-Rham]^+$	[55]
5.1	492	*	*	*	*	*	4',5,7-trihydroxy-3',8-dimethoxyflavone 7-O-β-D-glucopyranoside	13.2	254 349	493.2	331	$[M+H-Glc]^+$	[10,14]
6	564	11.8	206 264 339	565	432.8 396.6 286.6	$[M+H-Ara]^+$ $[M+H-Ara-2H_2O]^+$ $[M+H-Ara-Rham]^+$	Kaempferol-3-*O*-α-L-arabinopyranosyl-(1→2)-α-L-rhamnopyranoside	13.6	201 263 341	565.3	433 415.1 397 286.9	$[M+H-Ara]^+$ $[M+H-Ara-H_2O]^+$ $[M+H-Ara-2H_2O]^+$ $[M+H-Ara-Rham]^+$	[52]

RT: retention time; MW: molecular weight; $[M+H]^+$: molecular ion.
*: Not identified in the hydroethanolic extract.

4 CONCLUSION

The results of this study suggest that the pharmacological mechanism involved in the gastroprotection of the hydroethanolic extract from *Kalanchoe pinnata* is due to stimulation of the endogenous prostaglandin and sulfhydryl compounds. However, this effect promoted by the ethyl acetate fraction from *K.* pinnata seems to be mediated by endogenous prostaglandins and NO. Treatment for seven days with the hydroethanolic extract showed healing properties with an increase in the antioxidant defense system represented by the enzyme superoxide dismutase (SOD). This effect could be attributed to the different flavonoids identified in the samples.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no conflicts of interest.

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APENDICE

1. Supplementary Method

Immunohistochemical Staining of Vascular Endothelial Growth Factor (VEGF)

For this analysis, sections of gastric tissue were deparaffinized and rehydrated. Citrate buffer (10 mM, pH 6) was used for antigen retrieval in a pressure cooker (Pascal, DakoCytomation, USA). Blocking of the endogenous peroxidase reaction was performed with 6% hydrogen peroxidase in methanol. Then, slides were incubated with a background suppression reagent (CAS-Block^{tm}, Invitrogen), which was followed by the primary antibody, a mouse monoclonal anti-VEGF (VG-1) antibody (1,25 µg/mL; Abcam ab 1316) for one hour. For the detection of the complex antigen/antibody, the sections were incubated with Picture Max (Invitrogen) followed by staining using diaminobenzidine chromogen (DAB, DakoCytomation) and counterstaining with Harris-hematoxylin. The expression of VEGF was analyzed using an Olympus BX51 associated with Image Pro Plus® *version 5.1.2.* Measurements were performed in 3-5 fields per animal.

2. Supplementary Figures

Figure S1. Immunohistochemical analysis of VEGF expression in the gastric mucosa in acetic acid-induced gastric ulcers in rats. The results are reported as the mean ± S.E.M. ANOVA was carried out followed by Dunnett's test.

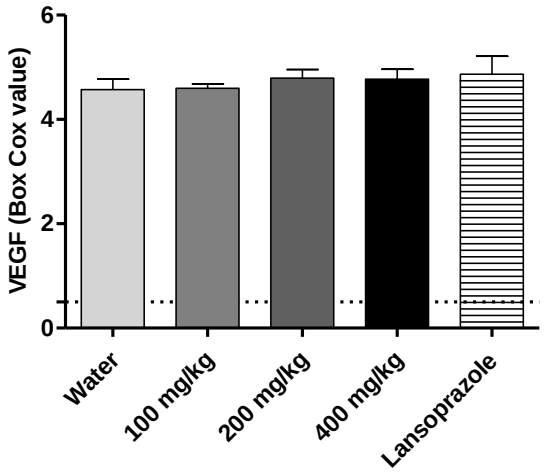
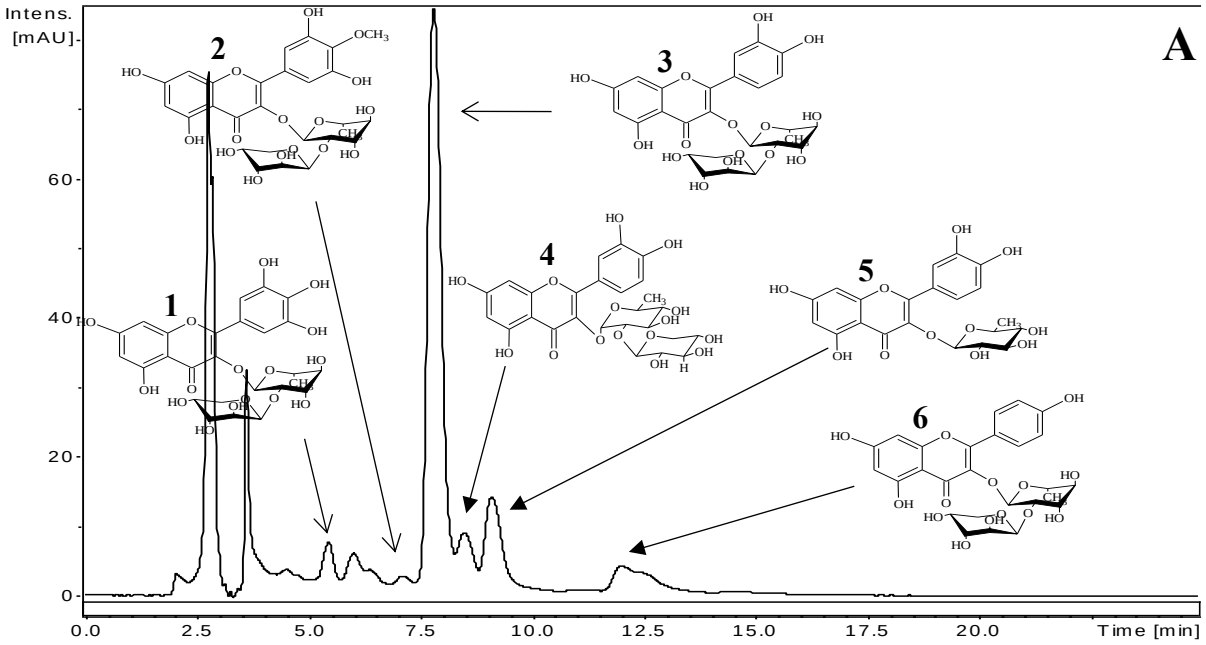


Figure S2. (A) HPLC-MS chromatogram of the hydroethanolic extract of *Kalanchoe pinnata*. (1) myricetin-3-*O*-α-L-arabinopyranosyl-(1→2)-α-L-rhamnopyranoside; (2) mearnsetin pentosyl rhamnopyranoside; (3) quercetin 3-O-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside; (4) quercetin 3-*O*-β-D-xylopyranosyl-(1→2)-α-L-rhamnopyranoside; (5) quercetin; and (6) kaempferol-3-*O*-α-L-arabinopyranosyl-(1→2)-α-L-



rhamnopyranoside

Figure S3. (B) HPLC-MS chromatogram of the ethyl acetate fraction of *Kalanchoe pinnata.* (1) myricetin-3-*O*-α-L-arabinopyranosyl-(1→2)-α-L-rhamnopyranoside; (1.1) myricetin-3-*O*-rhamnopyranoside; (2) mearnsetin pentosyl rhamnopyranoside; (3) quercetin 3-O-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside; (4) quercetin 3-*O*-β-D-xylopyranosyl-(1→2)-α-L-rhamnopyranoside; (5) quercetin; (5.1) 4',5,7-trihydroxy-3',8-dimethoxyflavone 7-O-β-D-glucopyranoside; and (6) kaempferol-3-*O*-α-L-arabinopyranosyl-(1→2)-α-L-rhamnopyranoside.