PharmacologyOnLine April 30, 2014

Archives • 2014 • vol.1 • 36-43

Gastroprotective property of *Plinia edulis* (Vell.) Sobral (Myrtaceae): The role of triterpenoids and flavonoids

Ishikawa, T.[1*]; Navarro, L. B.[2]; Donatini, R. S.[3]; Bacchi, E. M.[3]; Kato, E. T. M.[3]; Vilegas, W.[4]; Yoshida, M.[5]

[1]*Department of Medicine and Food, Faculty of Pharmaceutical Sciences, Federal University of Alfenas*
[2]*Department of Fundamental Chemistry, Institute of Chemistry, University of São Paulo*
[3]*Department of Pharmacy, Faculty of Pharmaceutical Sciences, University of São Paulo*
[4]*Experimental Campus of São Vicente, São Paulo State University*
[5]*Coordination of Analytical Center, Amazon Biotechnology Center*

*tati@unifal-mg.edu.br

Abstract

Based on traditional uses and on previous pharmacological assays, the ethanol leaf extract of *Plinia edulis* and its fractions were evaluated *in vivo* for gastroprotection in HCl/ethanol induced ulcers. In addition, due to our ulcerative model, the antioxidant activities of the polar fractions of the extract were analyzed by the DPPH method. The ethanol extract showed significant antiulcer activity in rats at doses of 100 mg/kg p.o. and was more active than the reference drug lansoprazole at 30 mg/kg. Among the fractions obtained by solvent partition, the hexane fraction was the most effective (100 mg/kg p.o.), but was not as effective as the original extract. The chromatographic procedures for the hexane fraction yielded β-amyrin, lupeol, ursolic acid, maslinic acid, and corosolic acid; the ethyl acetate fraction yielded gallic acid, quercitrin, myricitrin and quercetin. The ursolic acid reduced the injurious area at a 50 mg/kg dose p.o., but the antiulcer activity was not significant in our model. The polar fractions exhibited prominent antioxidant activity and high contents of phenolic compounds. These results suggest that the promising gastroprotective effects of aqueous ethanol extract from *P. edulis* leaves could be due to the mixture of triterpenes and flavonoids.

Key words: *Plinia edulis*, Myrtaceae, gastroprotection, triterpenoids, flavonoids, ursolic acid

Introduction

The ulcer etiopathology affects 5% of the global population and has become a disease of modern times, related to inappropriate diets and stressful lifestyles [1,2] The growing consumption of alcoholic beverages and non-steroidal anti-inflammatory drugs (NSAID) has contributed to increasing numbers of this painful disease [3].

Plants have often been used to treat gastric ulcers, and the phytochemical analyses of some species have afforded several gastroprotective compounds [4]. The preference for use of natural anti-ulcer agents encourages the search for gastroprotectors from vegetal foods such as herbs, fruits or vegetables [5].

Plinia edulis is popularly known as "cambucá" and used by the native people of the Southeastern Brazilian seashore for the treatment of stomach disorders, throat afections and diabetes [6,7]. This species is a small tree with 5-10 m height, and it is endemic of the Atlantic Rain Forest, dispersed from Rio de Janeiro to Rio Grande do Sul States [8].

In our previous studies, the crude extract of *P. edulis* did not show acute toxicity in mice but exhibited significant antiulcer activity in rats, with higher activity than the reference drug lansoprazole [9]. Based on these preliminary results, the extract and its fractions were evaluated for this activity with the aim of correlating the secondary metabolites present in the extract with crude drug efficacy in gastroprotection. The present work also reports the protective effects of ursolic acid on HCl/ethanol-induced gastric mucosa damage, the antioxidant activity evaluated by DPPH (2,2-diphenyl-1-picryl-hydrazylhydrate) and the quantification of total phenolic content in the aqueous ethanol extract.

Methods

Plant Material

The leaves of *P. edulis* were collected at Trindade, Rio de Janeiro State, Brazil. The plant material was identified by Dr. Lúcia Rossi from Instituto de Botânica de São Paulo, São Paulo State, Brazil. The voucher specimen was deposited in the Herbarium of the same Institute under n° SP 356.472.

Extraction and Isolation

Air-dried and powdered leaves (2.9 kg) were percolated with 70% ethanol with an elution rate of 3 mL/min at 25 °C. The eluate was concentrated at 40 °C under low pressure and the aqueous residue was lyophilized to yield the dried crude extract (LE) (1.0 kg). Part of LE (100.0 g) was dissolved in aqueous methanol 50% (1.0 L) and partitioned subsequently with hexane, dichloromethane and ethyl acetate (4 x 330 mL, each solvent). Solvents were evaporated at 25 °C under reduced pressure to yield residues of the hexane fraction (HF) (4.6 g), the dichloromethane fraction (DF) (3.0 g) and the ethyl acetate fraction (EF) (15.9 g). The aqueous methanol solution was lyophilized after methanol evaporation and yielded a residue of methanol fraction (MF) (73.0 g). Three grams of HF was fractionated through a silica gel chromatographic column (CC) (Merck, 60G, 40 cm x 2.5 cm i.d., flow rate 0.5 mL/min), eluted with a gradient of hexane (100 mL) and Hexano/EtOAc (9:1; 8:2; 7:3) (100 mL each), and 198 x 2 mL fractions (HF-1 – HF-198) were collected. These fractions were evaluated by thin layer chromatography (TLC) plates, coated with silica gel SiF254 (Merck), eluted with Hexane/EtOAc (9:1) and revealed with anisaldehyde/sulfuric acid solution [10]. Fractions HF-43 (235 mg), HF-83 (18 mg), HF-101 (13 mg) and HF-104 (16 mg) were submitted for spectrometric analysis. The EF (3.0 g) was submitted to Gel Permeation Chromatography (GPC), Sephadex LH-20 (Pharmacia), in a column (57 cm x 3 cm i.d.), and eluted with MeOH (400 mL, flow rate 0.5 mL/min). A total of 197 fractions (EF-1 – EF-197, 2 mL each) were collected and checked by Thin Layer Chromatography (TLC) on silica gel plates eluted with a mixture of $CHCl_3$/MeOH/H_2O (80:18:2, v/v) and revealed either with NP/PEG reagent or with anisaldehyde/sulfuric acid solution [10]. Fractions EF-66 (157 mg), EF-80 (41 mg), EF-90 (37 mg) and EF-147 (47 mg) were selected to perform spectrometric analysis. . The NMR spectra were recorded in $CDCl_3$, CD_3OD, pyridine-d_5 or DMSO-d_6 solutions (Aldrich), using a Varian INOVA 300 spectrometer, operating at 300 MHz for ^1H and 75.5 MHz for ^{13}C. Chemical shifts are given in δ (ppm) using TMS as an internal standard.

Animals

Female Wistar rats (130–180 g) were obtained from the Animal House of FCF/IQ-USP, São Paulo, Brazil. The animals were housed under standard environmental conditions (22 ± 1 ◦C and 85% relative humidity) on a 12 h light: 12 h dark cycle. They received food and water *ad libitum*. Before the gastroprotection experiments, they were deprived of food during 24 hours but allowed free access to water. The FCF-USP Committee of Ethics in Animal Research, under recommendations of the Canadian Council on Animal Care, approved the employed protocols (proc. n° 128) [11].

Acute gastric ulcer

The previous antiulcerogenic activity of LE was established according to Donatini *et al.* [12]. In this work, the doses of LE and its fractions, as well as ursolic acid, were the same as used by Schmeda-Hirschmann and Yesilada [13] and based on our previous assays. Rats were divided into 5 groups (n = 7) and fasted for 24 h prior to oral administration of aqueous solution of Tween 80 (5%) (10 mL/kg), lansoprazole (30 mg/kg), ursolic acid (50 mg/kg), LE and its fractions (HF, DF, AF and MF) (100 mg/kg). Thirty minutes after the treatments, all animals received a 0.3M HCl/60% EtOH solution (10 mL/kg) orally. After 1 h, the animals were killed in a gas chamber (CO_2). The stomachs were removed and opened along the greater curvature. The Total Lesion Area in mm^2 was calculated through the Image Pro-Plus Program.

Total phenolic content

Total phenolic content was determined spectrophotometrically using the Folin-Ciocalteau reagent following a previously reported method with slight modifications [14]. Folin-Ciocalteau reagent was prepared by adding 100 g of sodium tungstate, 25 g of phosphomolybidic acid, 100 mL of phosphoric acid and 50 mL of orthophosphoric acid to 700 mL of distilled water. The solution was refluxed for 10 h, cooled, and then 150 g of lithium sulphate and a few drops of bromine, diluted to 1 L, were added. A standard curve was built with gallic acid (Sigma-Aldrich) reference solutions. Aliquots ranging from 0.25 to 3 mL of standard aqueous gallic acid solution (100 μg/mL) were pipetted into 50 mL volumetric flasks containing 30 mL distilled water. Then, 2.5 mL of Folin-Ciocalteau reagent and 7.5 mL of saturated sodium carbonate solution were added, and the volume was filled with distilled water and mixed. The blank solution was prepared without the gallic acid. After two hours, absorbance was measured at 760 nm. For determination of the total phenolic content of ethyl acetate (EF) and methanol (MF) fractions, 0.1 mL of 70% ethanol solution of each fraction at a concentration of 2 mg/mL was used, proceeding in the same manner as described for the reference solutions. The experiment was performed in triplicate and the results were reported as mean ± SD values.

DPPH antioxidant assay

Antioxidant activity was measured using the DPPH radical photometric assay in a process guided by its discoloration in the presence of antioxidants compounds [15]. The antioxidant activity of the polar fractions (EF and MF) was compared with standard solutions of rutin (Sankyo). The LE was diluted to final concentrations of 1 to 20 μg/mL, in ethanol. One mL of a 0.3 mM DPPH (Sigma-Aldrich) ethanol solution was added to 2.5 mL of sample solutions of different concentrations, and they were 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:

$$AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100]/Abs_{control}\}$$

Ethanol (1.0 mL) and plant extract solution (2.5 mL) were used as blanks. DPPH solution (1.0 mL; 0.3 mM) plus ethanol (2.5 mL) was used as a negative control. The positive controls were those using the standard solutions of rutin (1 to 20 μg/mL, in ethanol). The EC_{50} values were calculated by linear regression from three individual tests.

Statistical analysis

Data are reported as mean ± standard error of the mean (S.E.M.). One-way analysis of variance (ANOVA) was employed for comparison of three or more groups followed by Tukey's contrast. *P*-values less than 0.05 were considered significant.

Results

Identification of isolated compounds

The compounds were confirmed by comparison of observed spectral data with those reported in the literature as being a mixture of β-amyrin (**HF-43a**) and lupeol (**HF-43b**) [16,17], ursolic acid (**HF-83**) [18], maslinic acid (**HF-101**) [19], corosolic acid (**HF-104**) [20], gallic acid (**EF-66**) [21], quercitrin (**EF-80**) [22], myricitrin (**EF-90**) [23] and quercetin (**EF-147**) [24] (Figure 1). The following observed NMR data of the compounds identified:

β-amyrin (**HF-43a**): ^{13}C NMR (75.5 MHz, $CDCl_3$, *δ*): 38.8 (C1), 27.4 (C2), 79.0 (C3), 38.8 (C4), 55.3 (C5), 18.4 (C6), 32.6 (C7), 38.8 (C8), 47.6 (C9), 37.7 (C10), 23.7 (C11), 121.7 (C12), 145.2 (C13), 41.7 (C14), 26.1 (C15), 26.9 (C16), 32.5 (C17), 47.2 (C18), 46.8 (C19), 31.1 (C20), 34.7 (C21), 37.1 (C22), 28.1 (C23), 15.5 (C24), 15.6 (C25), 16.8 (C26), 26.0 (C27), 28.4 (C28), 33.3 (C29), 23.7 (C30).

Lupeol (**HF-43b**): ^{13}C NMR (75.5 MHz, $CDCl_3$, *δ*): 38.7 (C1), 27.4 (C2), 78.9 (C3), 38.8 (C4), 55.3 (C5), 18.3 (C6), 34.2 (C7), 40.8 (C8), 50.4 (C9), 37.3 (C10), 20.9 (C11), 25.1 (C12), 38.0 (C13), 42.8 (C14), 27.3 (C15), 35.5 (C16), 42.9 (C17), 48.2 (C18), 47.9 (C19), 150.8 (C20), 29.8 (C21), 39.9 (C22), 28.0 (C23), 15.3 (C24), 16.1 (C25), 15.9 (C26), 14.5 (C27), 18.0 (C28), 109.3 (C29), 19.3 (C30).

Ursolic acid (**HF-83**): ^{13}C NMR (75.5 MHz, pyridine-d_5, *δ*): 39.2 (C1), 28.2 (C2), 78.2 (C3), 39.6 (C4), 55.9 (C5), 18.9 (C6), 33.6 (C7), 40.0 (C8), 48.1 (C9), 37.4 (C10), 23.7 (C11), 125.7 (C12), 139.3 (C13), 42.6 (C14), 28.9 (C15), 25.0 (C16), 48.1 (C17), 53.6 (C18), 39.6 (C19), 39.4 (C20), 31.1 (C21), 37.3 (C22), 28.9 (C23), 16.6 (C24), 15.7 (C25), 17.6 (C26), 24.0 (C27), 179.7 (C28), 17.5 (C29), 21.5 (C30).

Maslinic acid (**HF-101**): ^{13}C NMR (75.5 MHz, $CD_3OD/CDCl_3$, *δ*): 48.1 (C1), 69.1 (C2), 84.1 (C3), 40.1 (C4), 56.1 (C5), 19.1 (C6), 33.5 (C7), 38.8 (C8), 38.8 (C10), 24.0 (C11), 122.8 (C12), 144.8 (C13), 42.1 (C14), 28.4 (C15), 24.0 (C16), 47.3 (C17), 42.5 (C18), 47.2 (C19), 31.3 (C20), 34.6 (C21), 33.4 (C22), 29.1 (C23), 17.0 (C24), 17.2 (C25), 17.4 (C26), 23.9 (C27), 181.7 (C28), 33.3 (C29), 23.9 (C30).

Corosolic acid (**HF-104**): ^{13}C NMR (75.5 MHz, pyridine-d_5, *δ*): 48.0 (C1), 68.6 (C2), 83.8 (C3), 39.8 (C4), 55.9 (C5), 18.8 (C6), 33.2 (C7), 40.0 (C8), 47.7 (C9), 38.4 (C10), 23.9 (C11), 125.5 (C12), 139.3 (C13), 42.5 (C14), 28.8 (C15), 24.9 (C16), 48.0 (C17), 53.5 (C18), 39.4 (C19), 39.5 (C20), 30.9 (C21), 37.4 (C22), 29.3 (C23), 17.6 (C24), 16.9 (C25), 17.4 (C26), 23.9 (C27), 179.9 (C28), 21.4 (C29), 17.4 (C30).

Gallic acid (**EF-66**): ^{1}H NMR [300 MHz, DMSO-d_6, *δ* (*mult*, *J* in Hz, H)]: 6.78 (*s*, H2/H6) ^{13}C NMR (75.5 MHz, DMSO-d_6, *δ*) 120.9 (C1), 109.2 (C2), 145.9 (C3), 138.5 (C4), 145.9 (C5), 109.2 (C6), 168.0 (C7).

Quercitrin (**EF-80**): ^{1}H NMR [300 MHz, DMSO-d_6, *δ* (*mult*, *J* in Hz, H)]: 6.19 (*d*, 2.0, H6), 6.38 (*d*, 2.0, H8), 7.29 (*d*, 2.0, H2'), 6.86 (*d*, 8.5, H5'), 7.24 (*dd*, 2.0, 8.5, H6'), 5.25 (*d*, 1.5, H1''), 3.15 - 3.97 (*m*, H2'' - H5''), 0.82 (*d*, 6.0, H6'') ^{13}C NMR (75.5 MHz, DMSO-d_6, *δ*) 156.5 (C2), 134.3 (C3), 177.8 (C4), 157.3 (C5), 98.7 (C6), 164.2 (C7), 93.6 (C8), 161.3 (C9), 104.1 (C10), 120.8 (C1'), 115.5 (C2'), 145.2 (C3'), 148.5 (C4'), 115.7 (C5'), 121.1 (C6'), 101.9 (C1''), 70.4 (C2''), 70.6 (C3''), 71.2 (C4''), 70.1 (C5''), 17.5 (C6'').

Myricitrin (**EF-90**): ^{1}H NMR [300 MHz, DMSO-d_6 *δ* (*mult*, *J* in Hz, H)]: 6.18 (*d*, 2.0, H6), 6.35 (*d*, 2.0, H8), 6.86 (*s*, H2'/H6'), 5.17 (*d*, 1.5, H1''), 3.13 - 3.96 (*m*, H2'' - H5''), 0.82 (*d*, 6.0, H6'') ^{13}C NMR (75.5 MHz, DMSO-d_6, *δ*) 156.5 (C2), 134.3 (C3), 177.8 (C4), 161.3 (C5), 98.8 (C6), 164.4 (C7), 93.6 (C8), 157.5 (C9), 104.0 (C10), 119.6 (C1'), 107.9 (C2'), 145.8 (C3'), 136.5 (C4'), 145.8 (C5'), 107.9 (C6'), 102.0 (C1''), 70.4 (C2''), 70.6 (C3''), 71.3 (C4''), 70.1 (C5''), 17.6 (C6'').

Quercetin (**EF-147**): ^{1}H NMR [300 MHz, DMSO-d_6, *δ* (*mult*, *J* in Hz, H)]: 6.11 (*d*, 1.4, H6), 6.33 (*d*, 1.4, H8), 7.60 (*d*, 1.4, H2'), 6.81 (*d*, 8.5, H5'), 7.46 (*dd*, 1.4, 8.5, H6') ^{13}C NMR (75.5 MHz, DMSO-d_6, *δ*) 146.8 (C2), 135.7 (C3), 175.8 (C4), 160.7 (C5), 98.2 (C6), 163.9 (C7), 93.3 (C8), 156.1 (C9), 103.0 (C10), 122.0 (C1'), 115.1 (C2'), 145.1 (C3'), 147.7 (C4'), 115.6 (C5'), 120.0 (C6').

Acute gastric ulcer induced by HCl/ethanol

LE showed significant antiulcer activity in comparison to the control. Among fractions, HF was the most effective and showed significant activity. LE provided higher gastroprotection than lansoprazole. LE reduced the area of injury to 73.8% compared to control, while lansoprazole reduced the damaged area to 70.3%.

Both fractions, AF and MF, showed considerable activity compared to the control. Although animals treated with ursolic acid and DF showed a reduction in total lesion area (26.1% and 43.3% respectively), the results were not statistically significant in comparison to the control group. The results are shown in Figure 2.

Total Phenolic Content and Antioxidant Activity

EF and MF showed high content of total phenolics: 52.2 ± 0.4% and 45.3 ± 0.9% respectively. The antioxidant activity shown by both fractions was equally elevated with EC_{50} of 2.5 ± 0.1 μg/mL, superior than that showed by rutin, which was used as a positive control (EC_{50} 7.9 ± 0.5 μg/mL).

Discussion

Our previous phytochemical study reported triterpenes from *P. edulis* [9]. In the current study, a bioassay-guided fractionation of LE of this species led to isolation of other triterpenes (corosolic acid, lupeol), flavonoids (quercetin, quercetrin, myricitrin) and gallic acid. Although the isolated flavonoids as well as triterpenes have been found in other Myrtaceae [25-29], this is the first report of the isolation of these compounds from *P. edulis*. They are probably related to gastroprotective activity of its extract, thus confirming the traditional use.

These isolates were not obtained in sufficient quantity for *in vivo* tests, therefore ursolic acid was purchased for this assay. Lupeol and β-amyrin have already been assayed for this activity [30-33].

Peptic ulcer is related to various factors. In ulceration induced by ethanol and acidified ethanol, strong vasoconstriction followed by vigorous arteriolar dilation induced the production of oxygen free radicals [34].

Compounds with antioxidant or scavenging abilities could be potential antiulcerogenic agents. Since the Folin-Ciocalteau assay used to measure the total phenolic content in the polar fractions of the extract is based on an oxidation-reduction reaction, it can be considered another antioxidant method in addition to DPPH [35]. In both methods, the polar fractions showed extremely high antioxidant activity that probably had contributed to the extract gastroprotection observed.

The antiulcerogenic effects of triterpenes have been known for a long time [36] and seem to be related mainly to the reinforcement of defensive factors of the gastric mucosa, as in the increase in prostaglandin synthesis [33]. Different gastroprotective mechanisms have been attributed to flavonoids, especially those related to the increase in defense and inhibition of aggressive factors, such as histamine secretion [37]. In fact, many authors have reported *in vivo* gastroprotective effect of lupeol [30,31] and quercetin [38,39], both substances identified in *P. edulis* extract.

Therefore, the triterpenoids and flavonoids characterized in HF and EF are probably involved in the gastroprotection of this model of induction. Although they must play an important role, the gastroprotection by *P. edulis* probably occurs due to synergic action of different classes of substances, because better results were observed in the original extract when compared with its fractions.

Despite the structural similarity of ursolic acid with β-amyrin, α-amyrin and oleanolic acid, which have been reported as cytoprotectors in different models of evaluation in gastric ulcers [32,40], including the model of induction by acidified ethanol [33,41], ursolic acid protected the mucosa but did not shown significant activity when compared with the control group.

Conclusion

The gastroprotective effects obtained of *P. edulis* extract and fractions, allied with the secondary metabolites of pharmacological interest, indicate it is a promising antiulcerogenic herbal drug. However, further studies are required to establish the mechanisms involved in gastroprotection, because the observed effect could be related to triterpenes and phenolics isolated from this species.

Acknowledgements

The authors thank CAPES, CNPq, FAPESP, FAPEMIG and FAPEAM for fellowships and financial support.

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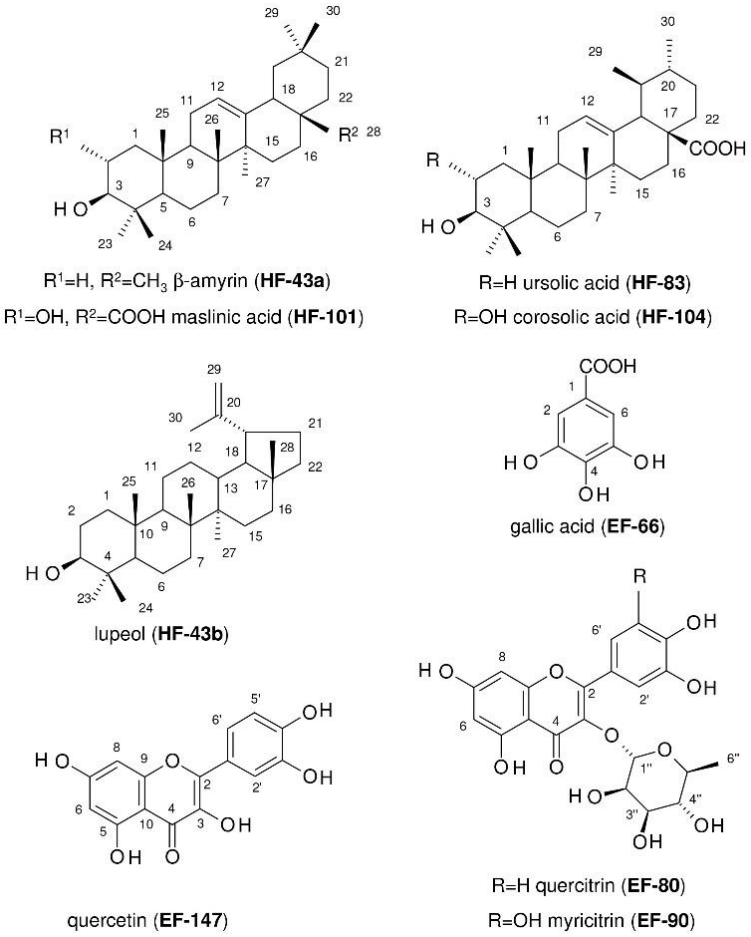

Figure 1: Structures of secondary metabolites identified in aqueous ethanol leaf extract of *Plinia edulis* after chromatographic fractionation of the hexane and ethyl acetate fractions obtained by solvent partitions.

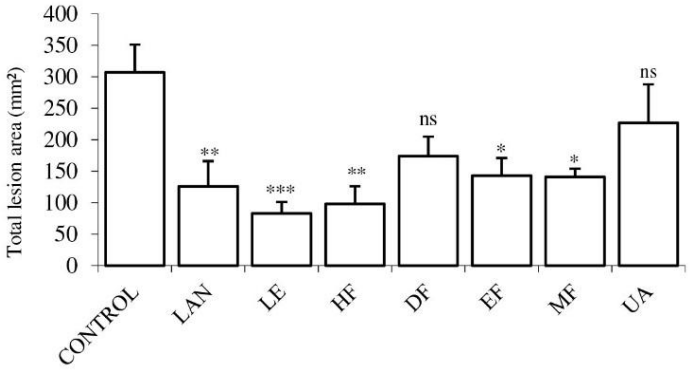

Figure 2: Effects of lansoprazole (LAN) (30 mg/kg), ursolic acid (UA) (50 mg/kg), aqueous ethanol extract of *Plinia edulis* (LE) (100 mg/kg) and the fractions obtained from the partitioned LE (100 mg/kg): hexane fraction (HF), dichloromethane fraction (DF), ethyl acetate fraction (EF) and methanol fraction (MF), on HCl/ethanol induced gastric lesions in rats. Results are expressed as mean ± S.E.M. (*n*=7). Asterisks indicate a significant difference from control (ANOVA followed by Tukey test). *ns* P>0.05; *** P<0.001; ** P<0.01; * P<0.05.