Lupeol Stearate Accelerates Healing and Prevents Recurrence of Gastric Ulcer in Rodents

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Abstract

The gastric healing and gastric ulcer recurrence preventive effect of Lupeol Stearate (**LS**) was measured in this study. To evaluate the gastric healing effect, rats were submitted to the 80% acetic acid-induced ulcer model and treated with vehicle (1 ml/kg, p.o.), **LS** (1 mg/kg, p.o.) or omeprazole (20 mg/kg, p.o.) twice a day for seven days. The gastric injury was evaluated macroscopically, histologically and histochemical; and biochemical parameters were also quantified. To evaluate the effects of **LS** on gastric ulcer recurrence, mice were ulcerated by gastric instillation of 10% acetic acid and treated with vehicle (1 ml/kg, p.o.), **LS** (1 mg/kg, p.o.) or ranitidine (20 mg/kg, p.o.) twice a day for ten days. Then, the ulcer recurrence in these animals was induced by IL- 1β (1 μg/kg i.p) at five day after the end of the treatment period. The area of the lesion recurred were measured, as well as the activity of myeloperoxidase and TNF levels. Oral treatment with **LS** accelerated gastric healing by 63% compared to the group treated with vehicle, which was also evidenced by histological improvement and increased production of mucin in the gastric epithelium. **LS** elevated the activity of the glutathione S-transferase and reduced the activity of myeloperoxidase, but did not change the levels of reduced glutathione or the activity of superoxide dismutase and catalase at the ulcer site in rats. Regarding the recurrence, the **LS** treatment reduced the recurred lesions, reducing MPO activity but not TNF levels at ulcer site. It can be concluded that **LS** promotes the healing of gastric lesions by favoring the mucus production and reducing the migration of neutrophils and that it can reduce the severity of the ulcer recurrence.

1 Introduction

Gastric ulcer is a global problem that affects millions of people all over the world with etiology associated with an imbalance between the protective and aggressive factors of the gastric mucosa (Gurusamy and Pallari, 2016). Some exogenous factors that lead to this imbalance are excessive alcohol consumption, prolonged treatment with non-steroidal anti-inflammatory drugs (NSAIDs), infection with *Helicobacter pylori*, and the social stress associated with modern lifestyle (Cheung et al., 2018). Given the potentiality of gastric acid secretion and pepsin as endogenous aggressors, the treatment of gastric ulcer is often based on gastric acid suppression using histamine 2 receptor antagonists (H_2-RAS) such as ranitidine, or proton pump inhibitors (IBP) such as omeprazole (Albaayit et al., 2016; Yu et al., 2017).

Since the discovery of omeprazole almost 39 years ago, the therapeutic resources aim to reduce gastric acidity, a crucial aggressor factor, but not the strengthening of mucosal protective factors, including the mucus and bicarbonate barrier, cell proliferation, proper blood flow, or antioxidant defenses. Although effective, prolonged anti-secretory therapy is associated with different adverse effects, including a high risk of gastric cancer in patients infected by *H. pylori* (Cheung et al., 2018; Fox and Muniraj, 2016; Mouli and Ahuja, 2011). Another important issue is the quality of gastric healing achieved by IBP therapy, which does not give quality healing and favors the recurrence of the lesion after the therapy interruption, which lead the patient to prolong the treatment time favoring the appearance of adverse effects (Mouli and Ahuja, 2011).

One of the major problems related to the gastric ulcer is the capacity for recurrence and chronification. This process is complex and involves mediators associated with the inflammatory process, such as proinflammatory cytokines including tumor necrosis factor (TNF), interleukin- (IL)-1β, and nuclear factor (NF)-(Nam and Choo, 2021). The increase in these inflammatory cytokines results in gastric oxidative stress, amplifying the lesion in the mucosa (Brito et al., 2018). Therefore, the basis for the treatment of chronic gastric ulcers may be associated with compounds capable of modifying pro-inflammatory factors and reducing gastric oxidative stress.

Lupeol is a very studied pentacyclic triterpene found in several plants with gastroprotective activity already described (Lira et al., 2009). Indeed, this triterpene has several biological activities, including anti-cancer properties (Abu-Lafi et al., 2019; Shen et al., 2019), anti-diabetic effects (Giacoman-Martínez et al., 2019), anti-inflammatory actions (Milani et al., 2019), antimalarial potency (Singh et al., 2019) and is a candidate as a non-hormonal male contraceptive (Arifuzzuman et al., 2018). Recently, through the esterification of Lupeol, it was possible to obtain Lupeol stearate (**LS**), a derivative with potent gastroprotective activity (Somensi et al., 2021).

Indeed, the confirmation of the potent gastroprotection provided by **LS** in acute gastric ulcer models through a preventive and non-therapeutic approach by our research group has shed light on new questions regarding the anti-ulcer potential of this ester. Therefore, this study was designed to evaluate: (1) the therapeutic efficacy of oral treatment with **LS** in chronic gastric ulcer healing and (2) the ability of **LS** treatment to reduce the ulcer recurrence or the severity of this recurrence in rodents.

2 Materials And Methods

2.1 Obtaining Lupeol Stearate (LS) and choice of dose

Lupeol was isolated from *Maytenus salicifolia* Reissek (Celastraceae) hexanic extract as described in details by Magalhães et al. (2011) and esterification with appropriate reagents gave rise to **LS** was performed as described in details by Silva et al. (2017). The oral gastroprotective dose of **LS** of 1 mg/kg was chosen to be employed in this study based in findings from Somensi et al. (2021).

2.2 Animals

Male *Wistar* rats (200-250g) and Male *Swiss* mice (25-30g) were obtained from the central laboratory of the Universidade do Vale do Itajaí and kept in polypropylene boxes at 22 ± 2ºC in 12-hour light-dark cycles with free access to water and feed. The animals were deprived of food eight hours before the experiments. All protocols were approved by the Institutional Committee on Animal Ethics of UNIVALI (CEUA / UNIVALI, approval number 056/2017 and 15/15p), conducted according the ARRIVE guidelines, and were performed in accordance with the International Standards and Ethical Guidelines on Animal Welfare.

2.3 Chronic ulcer induced by acetic acid 80% in rats

The rats were intraperitoneally anesthetized with xylazine and ketamine (10 mg/kg and 50 mg/kg, respectively) and a laparotomy were performed to expose the stomach serosa. Then, 500 µL of acetic acid (80%) were instilled into the gastric serosa using a plastic cylinder (6 mm diameter) to induce the ulcer. After 1 min, the acid was aspirated, the serosa was washed with 0.9% saline solution, the stomach was carefully relocated to the abdominal cavity and the incision was sutured. After recovery, the animals were randomly divided into different groups (n = 8), and on the second day of ulcer induction they were orally treated with vehicle (10% DMSO, 1 mL/kg, p.o.), omeprazole (20 mg/kg, p.o.) or **LS** (1 mg/kg, p.o.), twice a day, for seven days. Furthermore, one group of animals was not submitted to ulcer induction, named non-ulcerated or naïve group, which was used to allow comparison of the treatment groups to a non-ulcerated group. At the end of the treatment period, the animals were euthanized in a CO_2/O_2 chamber, the stomach was removed, and the gastric ulcer area was measured using a ruler (Okabe et al, 1971).

2.4 Histological and histochemical evaluation

Histological and histochemical procedures were performed following the same protocols used by Da Silva et al. (2015) and Somensi et al. (2017). The ulcer site was embedded in a fixative solution (85% alcohol, 10% formalin, 5% acetic acid). Then the samples were soaked in paraffin and cut into sections of 5 µm. After, a part of the histological segments was stained by hematoxylin and eosin, while the other part of the segments was submitted to Schiff Periodic Acid histochemical method to measure the amount of gastric mucin using ImageJ® software.

2.5 Preparation of the subcellular fraction and quantification of proteins

The acetic acid- ulcerated gastric mucosa was homogenized with 200 mM potassium phosphate buffer (pH 6.5). The homogenate was used to measure reduced glutathione levels (GSH). After the homogenate was centrifuged at 9000 × g for 20 minutes and the supernatant was used to evaluate the activity of glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT) and the precipitate was used to measure the activity of myeloperoxidase (MPO). The protein concentrations were determined in all samples using Bradford reagent and bovine albumin as standard following manufacturer's instructions (Biorad®).

2.6 Quantification of GSH levels

50 µL of homogenate and 40 µL of 12.5% trichloroacetic acid were added in a conical plastic tube and centrifuged (4000 × g by 15 min). After centrifugation, 20 µL of supernatant was added to 270 µL of TRIS buffer (pH 8.9) plus 10 µL of 5.5 'ditiobis-2-nitrobenzoic acid. The absorbance was measured after 5 min at 415 nm. After quantification, the values were interpolated in a standard GSH curve (1.25-10.00 µg/mL) and the results were expressed in µg/mg of tissue as described by Sedlak and Lindsay (1968).

2.7 Determination of SOD, CAT and GST activity

To determine SOD activity, the samples of the supernatant (20 µL) were incubated with 200 mM Tris-HCl-EDTA (pH 8.5) and 1 mM pyrogallol for 20 min. After the absorbance was measured at 405 nm and the SOD activity was expressed as U/mg of protein as demonstrated by Marklund and Marklund (1974). Another aliquot of the supernatant (5 µL) was used to verify the CAT activity as described by Aebi (1984); for this it was added to 295 µL of reaction medium [200 mM Tris-HCl-EDTA (pH 8.5) plus 47.35 mL of ultrapure water and 172.5 µL H_2O_2]. The absorbance was measured at 240 nm and the results expressed in mmol/min/mg of protein. Finally, 50 µL of the supernatant was incubated to 250 µL of the reaction medium [(1 mM 1 - chloro – 2.4 dinitrobenzene plus 1 mM GSH in phosphate buffer (pH 6.5)], the absorbance was measured at 340 nm and results were expressed in mmol/min/mg of protein Habig et al. (1974).

2.8 Recurrence of gastric ulcer 10% acetic acid- induced in mice by administration of IL-1β.

The mice were anesthetized with xylazine and ketamine (10 mg/kg and 50 mg kg, i.p., respectively). The abdominal wall was opened, the stomach exposed, and a plastic cylinder (2 mm diameter) was applied to the serosa for instillation of 10% acetic acid. After 1 min, the acetic acid was aspirated and replaced by 0.9% saline solution. The saline solution was aspirated, the cylinder was removed from the serosa, the stomach was replaced, and the abdominal wall was sutured. After recovery from anesthesia, the mice were randomly divided into groups (n = 6) and treated with vehicle (1% DMSO, 10 mL/kg, p.o), ranitidine (20 mg / kg, p.o), or **LS** (1 mg/kg, p.o) as described by Okabe et al. (1971) with modifications to mice. The treatments were started on the second day after the surgery, performed twice a day for ten days, on the 11th to 14th day the animals did not receive treatment. For induction of recurrence of the lesion, on the 15th day after induction of the ulcer, interleukin (IL)-1β was administered at a dose of 1 µg/kg (i.p) as described by Watanabe et al. (1977) with modifications to mice. To control the recurrence, a group of ulcerated animals treated with a vehicle did not received saline instead IL-1β. After 24 hours the animals were euthanized in a CO_2/O_2 chamber.

2.9 Determination of MPO activity

To determine the MPO activity in vivo, the precipitate obtained as described in Sect. 2.5. The samples were resuspended in 80 mM (pH 5.4) potassium phosphate buffer containing 0.5% hexadecil trimethylammonium bromide and re-centrifuged at 11000 × g for 20 min at 4°C. The activity of MPO in the supernatant was determined at 620 nm through the reaction performed between the samples and H_2O_2 plus 3.3',5.5'-Tetramethylbenzidine and expressed in units of milli optical density (mO.D)/mg of protein as proposed by Bradley (1982) and De Young (1989).

The in vitro activity of MPO was verified using a homogenate obtained from stomachs of an ulcerated mice treated with a vehicle, i.e with high MPO activity. The supernatant samples were incubated with **LS** (0.1 to 1000 µg/ mL) at 25ºC for 15 minutes. The MPO activity was measured in a similar manner to in vivo and the results were expressed in the same manner.

2.10 Determination of TNF levels in ulcerated tissue in mice

The ulcerated tissue submitted to ulcer recurrence model was homogenized with potassium phosphate buffer 200 mM (pH 6.5) to measure the levels of TNF by enzyme-linked immunosorbent assay (ELISA), using a kit from the BD Biosciences (Franklin Lakes, NJ, USA), and followed the manufacturer's recommendations. The absorbance was measured at 450 and 550 nm, and the results were expressed in pg/mL.

2.11 Statistical analysis

The data were analyzed by the GraphPadPrism 6.0® program represented as means ± standard error. The differences between the means were determined through the one-way analysis of variance (ANOVA- one way) followed by the Bonferroni test. Significance levels for $p < 0.05$ were adopted in all experiments.

3 Results

3.1 LS accelerates the healing of gastric mucosa of rats

As observed in Fig. 1, treatment with **LS** (1 mg/kg p.o.) or omeprazole (20 mg/ kg p.o.) twice a day for seven days reduced the area of acetic acid-induced ulcer by 63% and 68% respectively, when compared to the vehicle- treated group (73.33 ± 5.50 mm^2, Fig. 1A). The data from the macroscopic evaluation were confirmed in histological analysis of the ulcer site, where the omeprazole (Fig. 1D and 1E) and **LS** (Fig. 1F and 1G) treatment accelerated the healing process, increasing the margin and decreasing the ulcer base, compared to ulcerated vehicle group (Fig. 1B and 1C)

3.2 LS elevated mucin levels in ulcerated gastric mucosa

The staining of mucin-like glycoproteins is shown in Fig. 2A, where the administration of the **LS**, but not omeprazole, raised the mucin levels by 391 %, when compared to the group that received vehicle (4.12 ± 0.6 pixels × 10^3 field, Fig. 2A). Representative images from ulcerated groups treated with vehicle, omeprazole or **LS** can be verified in Fig. 2B, C and D, respectively.

3.3 LS did not change GSH levels, but increased GST activity, at ulcer site

As shown in Table 1, the ulcerated group treated with vehicle showed depleted levels of GSH in the gastric mucosa equal to 169.6 ± 16.5 µg of GSH/ mg, whereas the naive group showed levels equal to 618.5 ± 98.1 µg of GSH/ mg of tissue and the treatments with omeprazole or **LS** not avoided this depletion. Regarding the activity of GST enzyme, the ulcerated group treated with vehicle decreased by 65 % this parameter, when compared to non-ulcerated rats (Naive: 119.4 ± 20.9 mmol/ min/ mg of protein). Both

omeprazole and **LS** administration increased GST activity by 332% and 278%, respectively, when compared to the vehicle treated group (41.7 ± 20.8 mmol/ min/mg of protein).

Table 1
Effects of Lupeol Stearate (LS) on biochemical parameters at ulcer site

	MPO	GSH	SOD	CAT	GST
Naive	1.16 ± 0.13	618.5 ± 98.1	3.77 ± 0.21	223 ± 22.4	119.4 ± 20.9
Vehicle (1 ml/kg, p.o)	4.49 ± 0.68[a]	169.6 ± 16.5[a]	7.72 ± 0.34[a]	132.3 ± 37.4	41.7 ± 20.8[a]
Omeprazole (20 mg/kg, p.o)	2.59 ± 0.31[b]	210.8 ± 66.7[a]	8.28 ± 0.27[a]	302.9 ± 53.7[b]	180.2 ± 27.3[b]
LS (1mg/ kg, p.o)	3.07 ± 0.35[ab]	243.8 ± 51.7[a]	7.16 ± 0.15[a]	70.3 ± 25.5[a]	157.8 ± 21.8[b]

Myeloperoxidase (MPO, mD.O/ mg protein); Reduced glutathione (GSH, µg/ mg tissue); Superoxide dismutase (SOD, U/ mg protein); Catalase (CAT, mmol/ min/ mg protein) and Gutationa S-transferase (GST mmol/ min/ mg protein). Values expressed in mean ± E.P.M (n = 6). One way ANOVA followed by Bonferroni's test (n = 8). [a] $P < 0.05$ when compared to the naïve (non-ulcerated) group. [b] $P < 0.05$ when compared to the ulcerated-vehicle group.

3.4 **LS** did not change the activity of the SOD and CAT enzymes, but reduced the activity of MPO at ulcer site

The SOD activity was increased by 105% in the vehicle-treated group, when compared to the non-ulcerated group (Naive: 3.77 ± 0.21 U SOD/mg of protein), while the groups treated with omeprazole or **LS** not showed changes in these values compared to the vehicle-treated group (Table 1). Furthermore, the CAT activity was reduced by 40% in the ulcerated vehicle group in relation to the non-ulcerated group (Naive: 223 ± 22 µmol/min/mg of protein). The group treated with omeprazole presented increase of 30% in the CAT activity, compared to the vehicle treated group. However, the treatment with **LS** did not changes the CAT activity compared to the vehicle group (Table 1).

Moreover, the MPO activity were elevated by 287 % in the acetic acid-ulcerated tissue compared to the non-ulcerated group (Naive: 1.16 ± 0.13 mD.O/ mg of protein). In contrast, the group treated with omeprazole or **LS** reduced this parameter by 42 % and 32 %, respectively, compared to the vehicle-treated group (Table 1). Taking into account the results found, it was evaluated whether this reduction was caused by blocking the neutrophil flux or by direct inhibition of the MPO enzyme. Thus, a homogenate sample obtained from ulcerated tissue (vehicle-treated animal) was incubated with **LS** (0.1–1000 µL/ mL) and MPO activity was measured again. Interestingly, **LS** 100 µL/ mL inhibited the reduction of MPO activity in vitro by 26%, proving that **LS** can weakly inhibit directly the MPO enzyme and in turn reduces neutrophil-mediated ROS formation (Fig. 4C).

3.5 LS reduced the severity of gastric ulcer recurrence induced by IL-1β

As shown in Fig. 3A, 10% acetic acid- ulcerated mice treated with vehicle and exposed to IL-1 β showed a unique gastric lesion compatible with ulcer morphology in an extension of 6.8 ± 0.9 mm^2 on the 15th day after induction of the ulcer, indicating recurrence of the lesion because there was no ulcerative lesion in the gastric mucosa in mice ulcerated by 10% acetic acid that did not received IL-1 β on same day after ulcer induction. The group treated with ranitidine and **LS** showed a reduction in the severity of gastric ulcer recurrence, evidenced by a 48% and 78% of reduction in the extent of the lesion, respectively. It is worth mentioning that 25% of the animals in the group treated with **LS** did not present evident lesion. Representative images of 10% acetic acid- ulcerated mice treated with vehicle and not exposed to IL-1 β are shown in Fig. 3B, whereas the macroscopic appearance of ulcer recurrence from mice exposed to IL-1 β and treated with vehicle, ranitidine and **LS** are depicted in Figs. 3C, 3D and 3E, respectively.

3.6 LS increased TNF levels but reduced MPO activity in recurrence of gastric ulcer

As shown in Fig. 4A, TNF levels were 59% higher in of 10% acetic acid- ulcerated mice treated with vehicle and exposed to IL-1 β than in the non-ulcerated group (Naive: 467.8 ± 114.4 pg/ mL). The administration of **LS** ($p < 0.05$) or ranitidine ($p < 0.01$) increased even more the TNF amount compared to the 10% acetic acid- ulcerated mice treated with vehicle and exposed to IL-1 β.

The MPO activity in 10% acetic acid- ulcerated mice treated with vehicle and exposed to IL-1 β was significantly increased compared to the non- ulcerated treated group or the ulcerated group not exposed to IL-1 ($p < 0.001$). In contrast, the groups treated with ranitidine or **LS** showed reduction in this parameter by 61% and 59%, respectively, when compared to the ulcerated vehicle group exposed to IL-1β (Fig. 4B).

4 Discussion

Starting experimental investigations on the antiulcer potential of Lupeol stearate (**LS**), our research group showed that this compound promotes an important gastroprotective effect against gastric lesions induced by ethanol in rodents (Somensi et al., 2021). Given those results, the present study evaluated the capacity of **LS** to accelerates the gastric healing process in chronic and recurrent gastric ulcer model. Here, was possible to show that **LS** promotes the healing of gastric lesions, mainly through the enhancement of the mucus barrier and that it can reduce the severity of the recurrence of lesions especially by preventing inflammation mediated by neutrophils.

The acetic acid instillation in the gastric mucosa produces a lesion that is similar to that found in humans in location, chronicity, and severity, and the healing process presents many similarities too (Takagi et al., 1969). In this lesion, there is a weakening in the protective factors of the gastric mucosa, including the decrease in the microcirculation mediated by nitric oxide, low synthesis of prostaglandins,

and anti-inflammatory cytokines (Kobayashi, et al., 2021). This healing process is extremely complex because it involves migration and cellular proliferation, multiplication of epithelial cells that are located at the margin of the ulcer, which reestablishes the glandular disposition and stimulates angiogenesis at the base of the lesion through the stimulation of granulation tissue (Tarnawski, 2005; Da Silva et al., 2015).

Gastric damage induced by acetic acid results from deep, rounded ulcers that largely resemble human ulcers, and for this reason, is widely used to study the mechanisms involved in the treatment and healing of gastric ulcers. Lesions induced by acetic acid have a multifactorial process that begins with the depletion of the mucous content of the stomach wall, associated with excessive production of free radicals increasing in the pro-inflammatory interleukins TNF-α, IL-1β, and IL − 6, accompanied by an increase in neutrophil infiltration into the gastric mucosa (Orsi et al., 2012; Da Silva et al., 2013).

In our experiments, the efficacy of **LS** was evidenced by the reduction in the lesion area and oxidative and inflammatory parameters, in addition to raising the levels of mucin in the ulcerated gastric mucosa, when administered twice a day for seven days. In previous research Lupeol is effective in the process of skin healing of wounds in diabetic rats (Beserra et al., 2019), in addition to the hydroalcoholic extract of the leaves of *Urtica dioica* L (Urticaceae), which has high concentrations of Lupeol, has shown healing action on the skin (Bouassida et al., 2017). The healing action of the skin performed by Lupeol was similar to that observed in the present study on the gastric mucosa, as there was a significant reduction in the area of 80% acetic acid-induced ulcer (Fig. 1).

Furthermore, studies with oleanolic acid and a pentacyclic triterpene, which are structurally related to **LS**, have shown gastric healing action in both in vitro and in vivo studies, like those found in the present study (Rodríguez, et al., 2003; Sánchez et al., 2006). The in vivo antioxidant value of **LS** was already evidenced using acute ulcer models (Somensi et al., 2021) [17] and research with antioxidants shows that they can interfere with the inflammatory process induced by acetic acid, reducing gastric damage by decreasing the volume and total acidity of gastric secretion and cell proliferation (Silva et al., 2013; Almasaudi et al., 2017; Prazereset al., 2019; Longo et al., 2021).

The increase in production of gastric mucus may be associated with exogenous factors and endogenous factors such as favoring the natural defenses of the gastric mucosa that creates a microenvironment favorable to cell proliferation that may accelerate the healing of the lesion (Laine et al., 2008). Clearly, the levels of mucin at the margins of gastric ulcer were elevated in the group treated with Lupeol stearate (Fig. 2). Previous studies in a model of gastric lesion induced by indomethacin show an increase in PGE_2 levels after treatment of ulcerated animals with **LS** (Somensi et al., 2021) an effect that may be responsible for the increase in mucin levels also observed in the present study. So, the increase in mucin levels induced by **LS** treatment may be related to a key healing factor, since it plays an important role as a barrier against gastric injury caused by luminal acid, bacteria, and physical damage to the epithelium (Chen et al., 2005).

In addition to the significant increase in mucin levels associated with the gastric healing effect observed after treatment with **LS**, other mechanisms could be present to ensure the healing of ulcerated tissue. The involvement of free radicals in gastric injury induced by acetic acid is well described, as well as for the maintenance of ulcerative injury (Amagase et al., 2003; Potrich et al., 2010), in this context compounds that improve antioxidant defenses can reduce gastric injury caused by acetic acid.

Although it was expected that **LS** would reverse the depletion of GSH levels in the gastric mucosa when compared to the vehicle group, this was not observed. In an ethanol-induced acute ulcer model, **LS** avoided a reduction in GSH levels (Somensi et al., 2021), however, the difference in the nature of the lesions between studies may be directly related to these differences. On the other hand, oral treatment with **LS**, twice a day by seven days, increased the activity of the GST enzyme suggesting that the compound helps in the reduction of free radicals. Interestingly, this increase in GST activity may be related to the reduction of GSH levels since the detoxifying effects of this enzyme occur to the detriment of GSH as an important enzymatic cofactor.

In addition to GST, SOD and CAT are part of the enzymatic antioxidant defenses of the gastric mucosa. SOD causes dismutation of the superoxide anion, while CAT converts hydrogen peroxide into water and oxygen (Kwiecien et al., 2014). However, the levels of the SOD and CAT enzymes were not altered in the groups that received the **LS**, and it can be assumed that stearate does not need these routes to guarantee its gastric healing effect against acetic acid-induced ulcers in rats.

In gastric lesions induced by acetic acid, there is a strong inflammation in the gastric mucosa and activated neutrophils associated with increases in MPO activity, since they are found in the azurophil granules present in the neutrophils (Amagase et al., 2003; Lu et al., 2018). Therefore, MPO activity is considered an index of neutrophil infiltration, which produces large amounts of free radicals that damage the gastric mucosa, contributing to the development of the ulcer (Potrich et al. 2010). Indeed, neutrophil infiltration contributed to the origin of the injury, being confirmed by the increased levels of MPO activity at the site of gastric injury in the ulcerated group treated with a vehicle. The contrary, the treatment with **LS** reduced this parameter in the ulcerated tissue, being possible to infer that the inflammatory process mediated by neutrophil migration is minimized in the presence of the **LS**. These results corroborate those found in the ethanol-induced ulcer model, where pretreatment with **LS** also reduced the activity of the MPO (Somensi et al., 2021).

Considering the results found so far, follow-up was given through the evaluation of the effects of **LS** administration on the recurrence of gastric ulcers in rodents through the administration of IL-1β. The IL-1β is an important pro-inflammatory interleukin in the gastric mucosa, which comes from activated macrophages and has a function to mediate the cellular migration, as an example, the migration of neutrophils (Machado et al., 2003). Allied to this, our results showed that after 24 hours of administration of IL-1β, animals previously treated with **LS** presented a lower degree of reappearance of a gastric lesion, and in 25% of this group there was no evident lesion (result found in triplicate of experiments).

Regardless of the effects observed, TNF levels were increased in the group treated with ranitidine or **LS**. It is known that TNF is directly related to the recruitment of pro-inflammatory cells in ulcerated tissue, which therefore supports the inflammatory process and favors gastric injury (Brzozowska et al., 2004). However, even in the presence of increased TNF, the levels of MPO activity in ulcerated tissue of animals treated with **LS** or ranitidine remained reduced, indicating that although there is chemotactic stimulus mediated by TNF, it is possible to infer that there is no efficient leukocyte recruitment. The hypothesis of reduced neutrophil migration is strengthened by the findings of in vitro inhibition of MPO, which demonstrated weak direct inhibition performed by the **LS** on the enzymatic action and would not justify a direct inhibition of the enzyme in vivo, but that the in vivo findings reflect the reduction in neutrophil migration.

The inflammatory process occurs due to the recruitment and infiltration of inflammatory cells in the injured tissue for protection and tissue repair against an aggressive stimulus. Tissue restructuring is considered successful when the inflammatory process is completed, as low recruitment of inflammatory cells may increase the healing time of the injured tissue. In contrast, swollen infiltration of these cells can result in chronic wounds, making tissue repair and scar formation difficult. The nuclear factor (NF)-κB plays a crucial role in controlling the expression of some inflammatory genes, such as TNF, cell adhesion molecules such as E-selectin, and vascular adhesion molecules (Beserra et al., 2018). These adhesion molecules are present in the cytoplasm disposed of as a pair of dimers (p50 and p65), that when activated by some inflammatory stimulus these dimers of NF-κB, are reallocated in the nucleus of the cell connecting to the DNA, to which it induces the transcription of several genes responsible for the proliferation, migration, cellular cycle, inhibition of apoptosis, besides the inflammation (Monkkonen and Debnath, 2018).

A study with triterpenes reveals that these compounds modulate the production of ROS in the microenvironment of the wound, accelerating the process of tissue repair, since inducing cell proliferation, cell migration, and collagen deposition (Agra et al., 2015). Based on this point, can assume in the present study that there is a relationship between the inflammatory process and cell migration since TNF levels are high in the group treated with LS, but there is no stimulus for inflammatory cells to migrate to the tissue.

5 Conclusion

In conclusion, these results amplify the knowledge about the antiulcer effect of Lupeol stearate, confirming that this ester has gastric healing activity in the mucosa already ulcerated and its effect is mediated by the reduction of myeloperoxidase activity which reflects the reduction in neutrophil migration, and by its cytoprotective action mediated by the favoring of GST activity, the increase in mucus production. Moreover, the Lupeol stearate can reduce the severity of the ulcer recurrence, proving a good quality of gastric healing mediated by this compound evidencing its potential in the search for therapeutic alternatives in the treatment of gastric ulcer and its recurrence.

Declarations

Conflict of interest

The authors have no conflict of interest.

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Figures

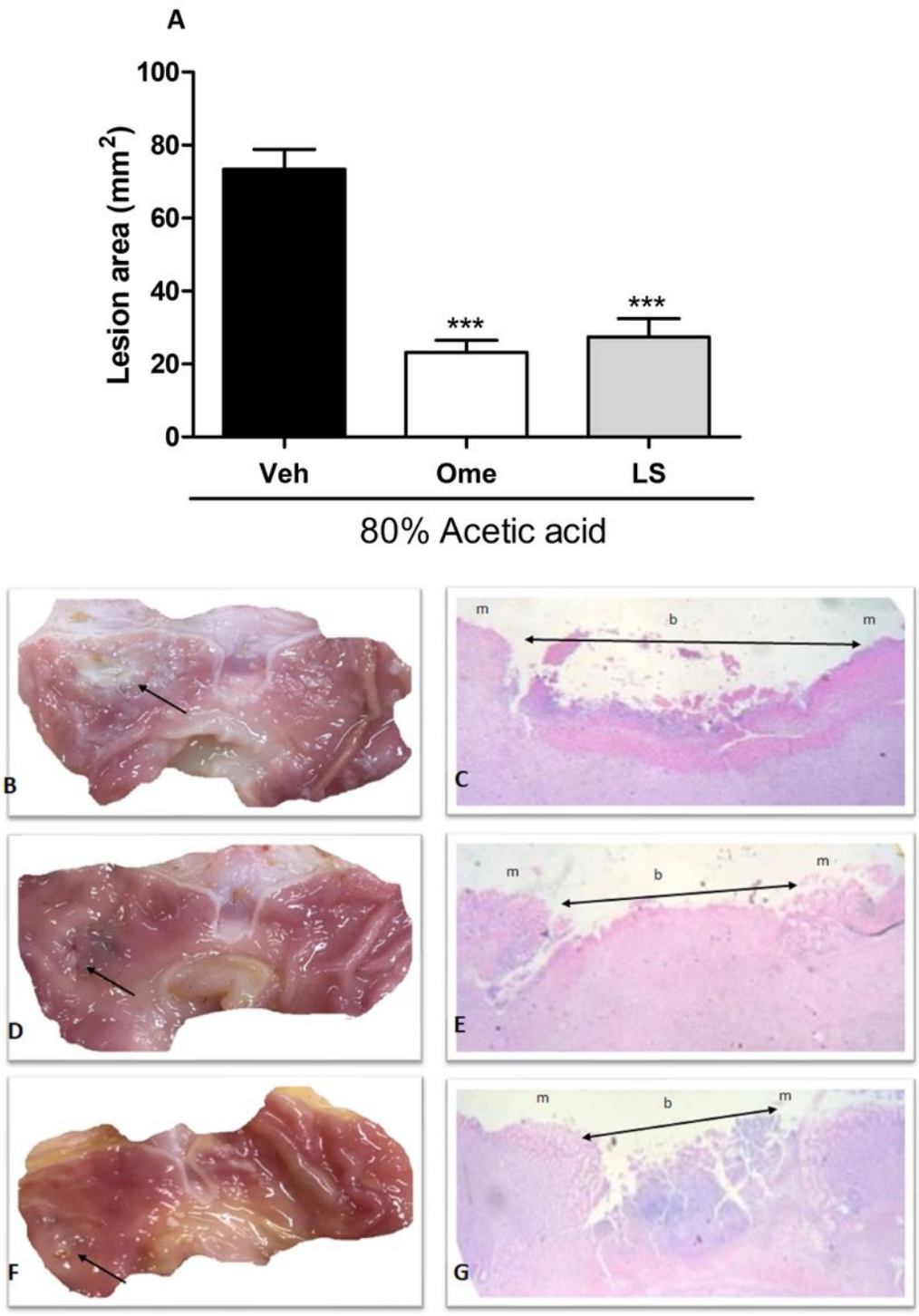

Figure 1

Oral administration of Lupeol Stearate (LS) accelerates the healing of gastric mucosa in rats. The rats were orally treated with vehicle (Veh: 10% DMSO,1 ml/kg), omeprazole (Ome: 20 mg/kg) or LS (1 mg/kg) twice a day by 7 days after the gastric ulcer induction. Panel A shows the gastric ulcers area (mm2) and results are expressed as mean ± S.E.M. (n=8) analyzed using One-way ANOVA followed by Bonferroni's test and ***P< 0.001 compared to Veh group. Representative macroscopic images of gastric mucosa

from ulcerated rats treated with vehicle, omeprazole or LS are shows in panels B, D and F, respectively, with black arrows indicating the ulcer site. Histological hematoxylin/eosin appearence of ulcer site from ulcerated rats treated with vehicle, omeprazole or LS are shows in panels C, E and G, respectively, where m indicate margin of ulcer and b indicate base of ulcer.

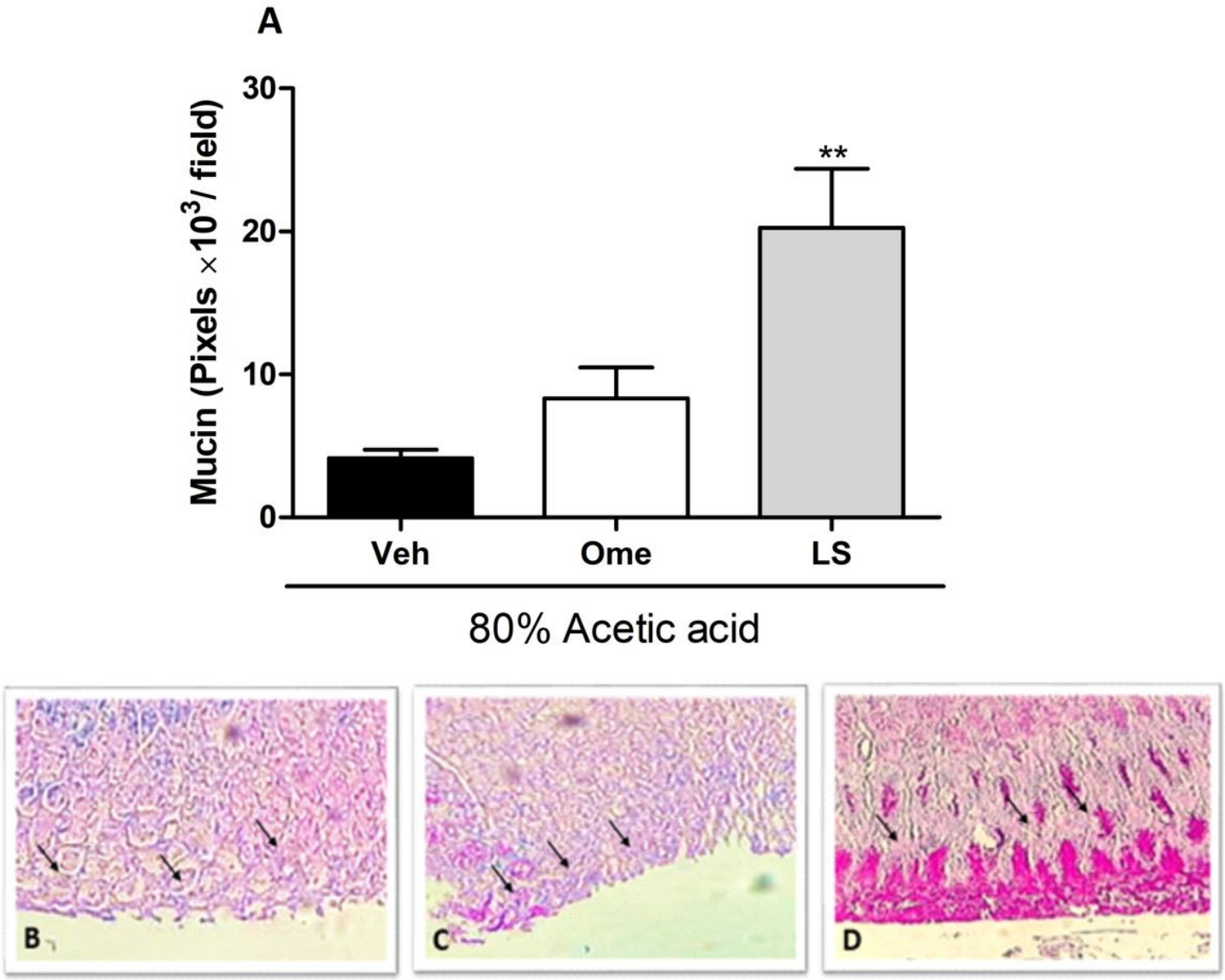

Figure 2

Lupeol Stearate (LS) elevated staining for mucin-like glycoproteins in ulcerated gastric mucosa of rats. The rats were orally treated with vehicle (Veh: 10% DMSO,1 ml/kg), omeprazole (Ome: 20 mg/kg) or LS (1 mg/kg) twice a day by 7 days after the gastric ulcer induction. Panel A shows the quantification of PAS-staining and results are expressed as mean ± S.E.M. (n=8) analyzed using One-way ANOVA followed by Bonferroni's test and **P< 0.01 compared to Veh group. Representative images of groups orally treated with vehicle (Panel B), omeprazole (Panels C) or LS (Panels D). Panels B–D: magnification=400×.

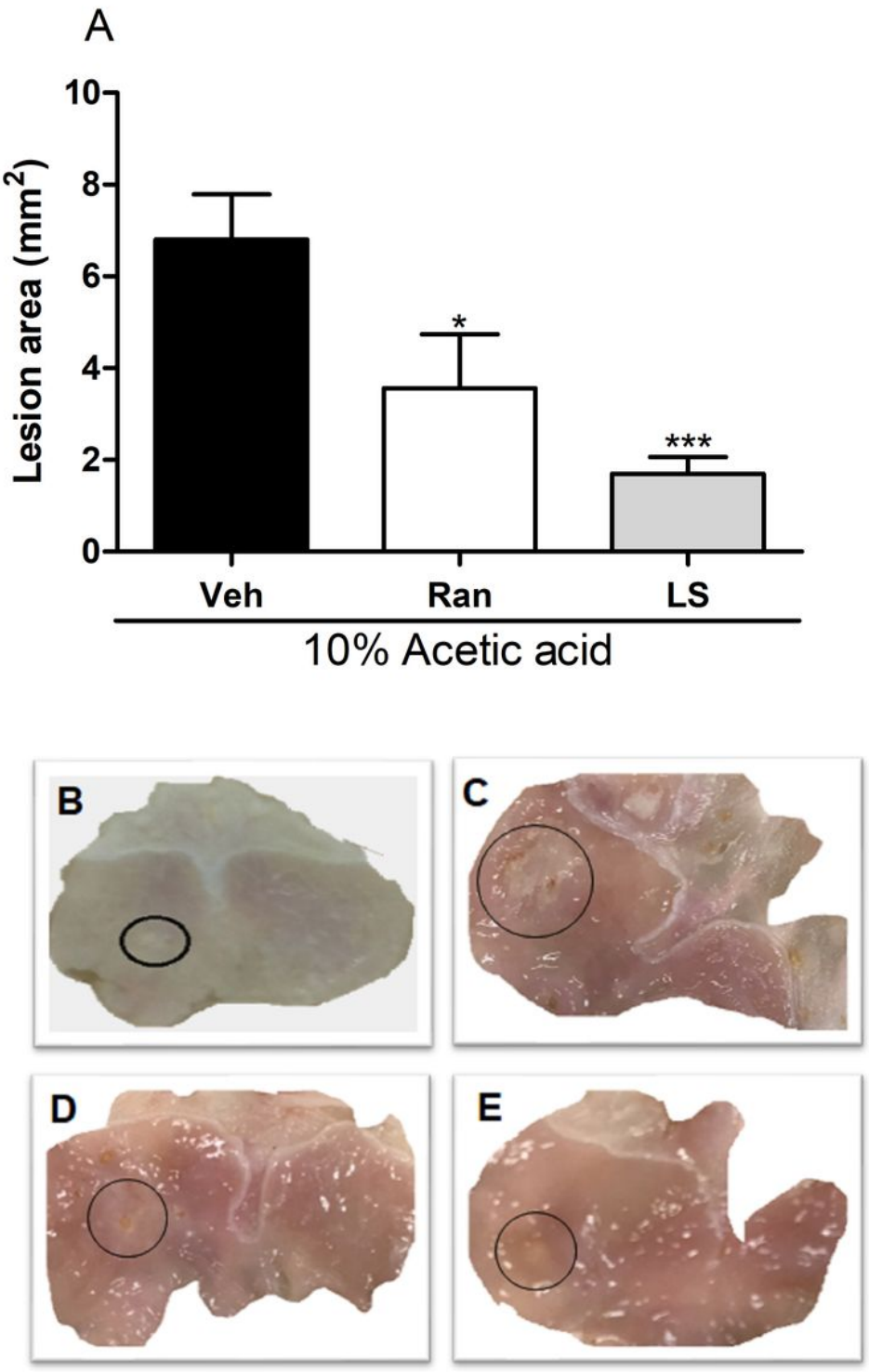

Figure 3

Lupeol Stearate (LS) reduced the severity of gastric ulcer recurrence induced by IL-1β in mice. The animals were orally treated with vehicle (Veh: 10% DMSO,1 ml/kg), ranitidine (Ran: 200 mg/kg) or LS (1 mg/kg) twice a day by 10 days after the gastric ulcer induction and IL-1β (1 µg/kg, i.p) was given at 15th day after ulcer induction to ulcer recurrence. Panel A shows the gastric ulcers area (mm2) and results are expressed as mean ± S.E.M. (n=8) analyzed by One-way ANOVA followed by Bonferroni's test and *P<

0.05; ***P< 0.001, compared to Veh group. Representative macroscopic images of gastric mucosa from ulcerated rats treated with vehicle without IL-1β exposure, vehicle with IL-1β exposure, ranitine with IL-1β exposure, or LS with IL-1β exposure are shows in panels B, D, F and E, respectively, with circles indicating the ulcer site.

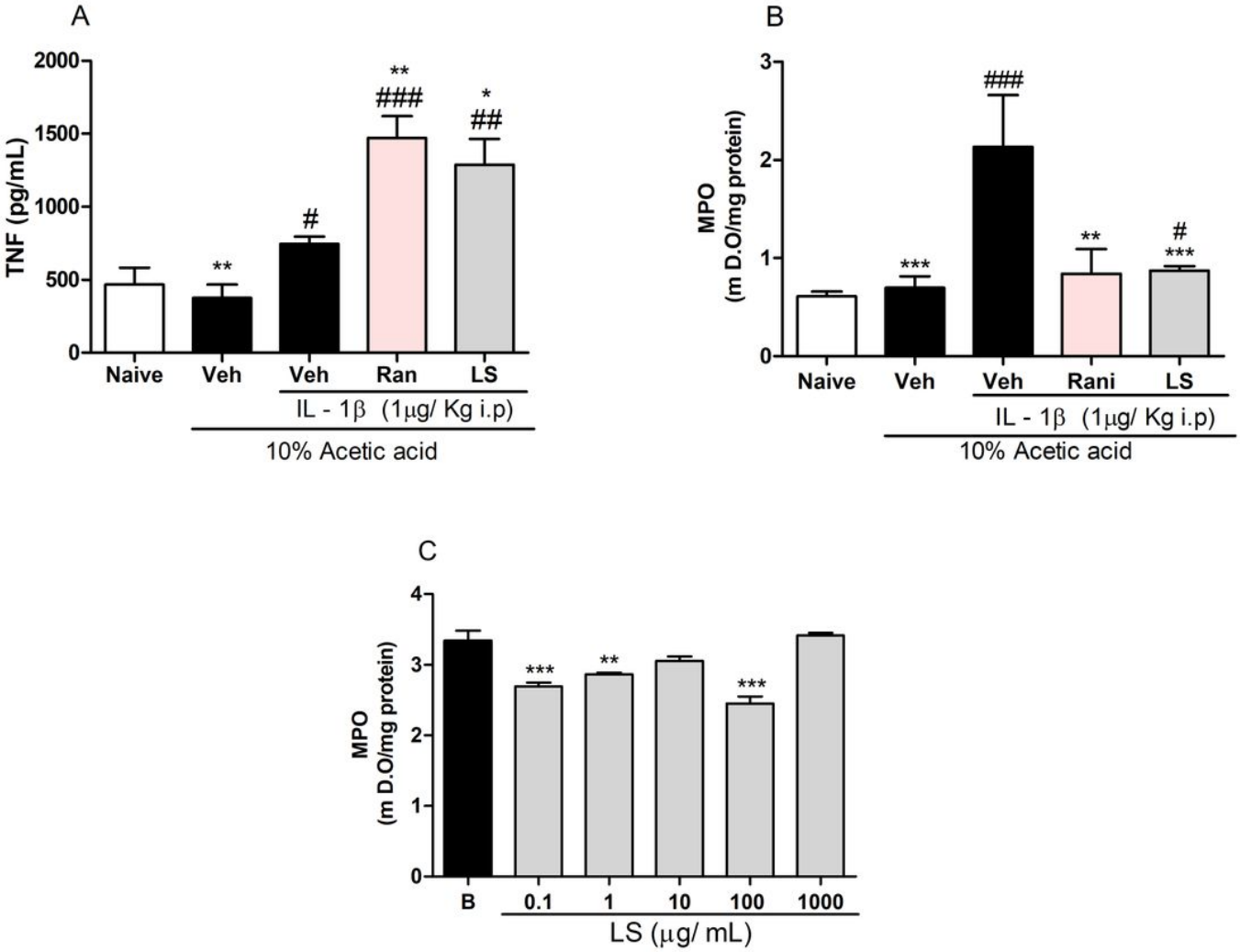

Figure 4

Lupeol Stearate (LS) increased TNF levels (A) but reduced MPO activity (B) in recurrence of gastric ulcer and partially inhibits the MPO activity in vitro (C). results are expressed as mean ± S.E.M. (n=8) analyzed by One-way ANOVA followed by Bonferroni's test. # P< 0.05, ## P< 0.01 and ## P< 0.01 compared to the naive (non-ulcerated group). *P< 0.05; **P< 0.01, ***P< 0.001, compared to Veh ulcerated group exposed to IL-1β.