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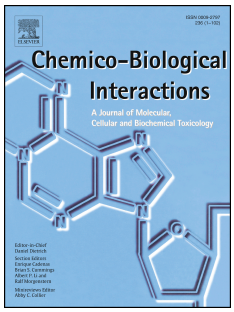

Gastroprotective properties of Lupeol-derived ester: Pre-clinical evidences of Lupeol-stearate as a potent antiulcer agent

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Gastroprotective properties of Lupeol-derived ester: pre-clinical evidences of Lupeol-stearate as a potent antiulcer agent

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Abstract

Lupeol (**1**) was isolated from hexane branch extract of *Maytenus salicifolia* and the Lupeol stearate (***2***), Lupeol palmitate (***3***), Lupeol myristate (***4***), Lupeol laurate (***5***) and Lupeol caprylate (***6***) were obtained reacting ***1*** with an adequate carboxylic acid. *Swiss* mice were treated with vehicle, carbenoxolone or Lupeol esters before administration of ethanol/HCl or indomethacin. Additionally, the involvement of nitric oxide (NO), sulfhydryl compounds (NP-SH), α-2 adrenergic receptors (α2-AR) and prostaglandins (PGE) in antiulcer effects was investigated using appropriate inhibitors or antagonist. Oxidative and inflammatory parameters were measured after euthanasia and anti-secretory effects was evaluated in pylorus-ligated rats. Ethanol/HCl ulcerated the gastric mucosa by 64.45 ± 6.58 mm^2, which the oral treatment with ***1, 4*** and ***6*** (10 mg/kg), and ***3*** and ***5*** (30 mg/kg) reduced the lesion area. Interestingly, ***2*** reduced the gastric ulcer by oral route in a potent and dose-dependent manner (ED_{50}= 0.40 mg/kg), which was accompanied by the increase in reduced glutathione levels and by the reduction of lipids peroxidation and myeloperoxidase and superoxide dismutase activities. Moreover, ***2*** (0.1 mg/kg) also prevented the ulcerogenesis by intraperitoneal route. The participation of NO, NP-SH, α2-AR and PGE in ***2***-mediated gastroprotection was confirmed. In indomethacin-induced ulcer, ***2*** (1 mg/kg, p.o) also reduced the ulcer area and increased the PGE_2 levels. However, ***2*** did not alter the gastric acid secretion. Therefore, these findings indicate that the obtention of ***2*** potentiated the antiulcer activity of ***1*** and that this compound can elicit gastroprotective action due a diversified mode of action.

Keywords: gastric healing; oxidative stress; esterification; semisynthetic compounds.

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1. INTRODUCTION

The gastric ulcer is a global pathology characterized by a decrease in the protective factors of the gastric mucosa, including the mucus, bicarbonate, blood circulation, and antioxidants); and/or an increase in aggressive factors, such as alcohol, nonsteroidal anti-inflammatory drugs (NSAIDs), reactive oxygen species (ROS), pepsinogen activation [1]. The treatment for this disease has been based on anti-secretory drugs such as histamine type 2 receptor antagonists (ranitidine and congeners) or proton pump inhibitors, such as omeprazole. However, the discontinuation of these treatments may lead to ulcer recurrence, whereas a prolonged treatment period has been associated with several adverse effects [2].

Lupeol is a pentacyclic triterpene found in several plants including *Maytenus salicifolia* Reissek (Celastraceae) [3]. The gastroprotective activity of this compound has already been described by Lira et al. [4] at a dose of 3 mg/kg by the oral route. Besides, this triterpene presents many biological activities, including anti-inflammatory, antiarthritic, antimutagenic, antitumor, hepatoprotective and antioxidant properties [5, 6, 7, 8, 9]. In addition to these biological activities, several recent studies show the therapeutic potential of lupeol in different experimental models. Zingue et al. [10] have demonstrated that lupeol has estrogenic properties in ovariectomized rats, an effect that may be attributed to estrogen receptor transcriptional activity. Moreover, Pereira Beserra et al. [11] have described, by using in vitro wound healing assays and human neonatal foreskin keratinocytes and fibroblasts, the therapeutic potential of lupeol for accelerating wound healing and tissue repair. Extending this knowledge about the healing process, this same group of authors also demonstrated the wound healing activity of lupeol in streptozotocin-induced hyperglycemic rats, highlighting the potential of this triterpene in healing processes and tissue repair [12].

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The structural modification of natural compounds has been an interesting tool to obtain analogous more effective and safer, which can lead to the identification of the pharmacophoric group [13]. This practice is already used for pentacyclic triterpenes [14, 15]. In this context, Silva et al. [15] described the synthesis of Lupeol esters; and in continuity, this study evaluated the gastroprotective and anti-secretory effects of Lupeol esters and investigated the mode of action of the most potent derivative.

2. MATERIAL AND METHODS

2.1 Esters obtaining

Lupeol **(*1*)** was isolated from the hexane extract of *Maytenus salicifolia* and the esters: Lupeol stearate **(*2*)**, Lupeol palmitate **(*3*)**, Lupeol myristate **(*4*)**, Lupeol laurate **(*5*)** and Lupeol caprylate **(*6*)** were obtained reacting 1 with an adequate carboxylic acid, as described early by Silva et al. [15].

2.2 Animals

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

2.3 Ethanol-HCl induced-gastric ulcer in mice

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The mice were randomly separated into groups (n = 6) and treated with vehicle (1% DMSO, 10 mL/kg, p.o), carbenoxolone (positive control, 200 mg/kg, p.o) or Lupeol derivatives (0.1-30 mg/kg, p.o) before administration of ethanol-HCl (10 mL/kg, p.o). After 1 hour of the treatments, 60% ethanol/0.3 M HCl (0.1 mL/10g) was given to the induction of gastric ulcer, as described by Hara and Okabe [16]. Further, the animals were euthanized in the CO_2 chamber after 1 hour of ulcerogenic agent intake, the stomachs were removed and opened by the greater curvature and the lesion area was quantified by the EARP® program.

In another set of experiments, mice were divided into groups (n = 6) and treated with vehicle (1% DMSO, 10 ml/Kg, p.o), carbenoxolone (200, mg/kg p.o) and compound ***2*** (0.1 mg/kg, i.p). After 30 min from intraperitoneal or 1 hour from oral administrations, the mice received ethanol-HCl as described above. The animals were euthanized after 1 hour in a CO_2 chamber, the stomachs were removed, and the lesion area was quantified as described above.

2.4 Indomethacin induced-gastric ulcer in mice

Indomethacin induced-gastric ulcer was performed according to Fornai et al. [17]. The animals received vehicle (1% DMSO, 10mL/kg, p.o), carbenoxolone (200 mg/kg, p.o) or ester ***2*** (0.3 - 3 mg/kg, p.o). After 1 hour, indomethacin (100 mg/kg, p.o) was given and after 6 hours the animals were euthanized in a CO_2 chamber, the stomachs removed, opened by the greater curvature and ulcers were analyzed using the EARP® program.

2.5 Ethanol-HCl induced-gastric ulcer in mice pretreated with N-Ethylmaleimide (NEM), N-Ω-Nitro-L-Arginine Methyl Ester (L-NAME), Indomethacin or Yohimbine

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This experiment followed the protocol previously described by Matsuda and Yoshikawa [18], Leite [19], and Arrieta et al. [20]. The mice were pretreated with antagonists or inhibitors: NEM (10 mg/kg, s.c), L-NAME (70 mg/kg, i.p), indomethacin (10 mg/kg, i.p), yohimbine (2 mg/kg, i.p) or saline (1 ml/kg i.p). After 30 min., the animals received vehicle (1% DMSO, 10 mL/kg, p.o) or ***2*** (1 mg/kg p.o). Then, the ethanol-HCl (Ethanol 60% + HCl 0.3 M, 10 mL/kg p.o) was given 1 hour later. After another 1 hour, the mice were euthanized in a CO_2 chamber, the stomachs removed, opened by the greater curvature and analyzed as previously described.

2.6 Pylorus ligature in rats

Rats were randomly distributed into experimental groups (n = 6) and anesthetized with xylazine (10 mg/kg, i.p) and ketamine (50 mg/kg, i.p). A laparotomy was performed subsequently, where the pylorus was sampled and ligated. Further, vehicle (1% DMSO, 10 mL/kg) or ***2*** (1 mg/kg) were administered by intraduodenal route, while the positive control group received omeprazole (20 mg/kg, p.o) 30 minutes before the ligature. Subsequently, the abdominal wall was sutured. After 4 hours, the animals were euthanized in a CO_2 chamber, the stomach was removed and contents were collected. The volume of gastric juice (mL) was measured using a graduated cylinder after centrifugation (1500 × g, 15 min, 4 ° C), the pH was determined with a pH meter and total acidity (mEq/ L/ 4 h) per titration with 10 mM sodium hydroxide following the protocol described by [21].

2.7 Measurement of peptic activity

As described by Anson [22], 100 µL of gastric juice from pylorus ligated rats was incubated with 500 µL of bovine albumin (5 mg/mL prepared in 60 µM HCl) at 37

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°C for 10 min. Then, 1 N Folin reagent was added and incubated at 25 °C for 30 min. The absorbance of each sample was inferred at 660 nm and the results expressed in μM/ mL/ 4 h of tyrosine interpolating individual values on a standard tyrosine curve (30-1000 mmol/mL).

2.8 Preparation of the homogenate and protein analysis

The gastric mucosa was homogenized with 200 mM potassium phosphate buffer (pH 6.5). The homogenate was used to measure the levels of reduced glutathione (GSH) and lipoperoxides (LOOH). Thereafter, the homogenate was centrifuged at 9000 ×*g* by 20 minutes and the supernatant was used to assess the activities of glutathione-*S*-transferase (GST), superoxide dismutase (SOD) and catalase (CAT), while the precipitated was used to measure myeloperoxidase (MPO) activity.

Protein concentrations were determined in all samples using Bradford's reagent and bovine albumin as standard following the manufacturer's instructions (Bio-rad®, Hercules, CA, USA).

2.9 Quantification of the GSH and LOOH levels

As described by Sedlak and Lindsay [23], 50 μl of homogenate was added to 40 μl of 12.5 % trichloroacetic acid, then the material was centrifuged at 1.4 × g/ 15 min. After, 20 μl of the supernatant was added to 270 μl of TRIS buffer (pH 8.9) and 10 μl of 5,5' dithiobis-2-nitrobenzoic acid (DTNB). The absorbance was measured after 5 min at 415 nm and the values were interpolated on a standard curve of GSH (1.25-10.00 μg/mL). Results are expressed in μg /mg of tissue.

To evaluate the LOOH amount, the method described by Jiang et al. [24] was performed. Thus, 100 μl of methanol was added into 100 μl of homogenate and

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centrifuged at 9000 × *g* during 20 min at 4°C. Afterward, 30 µl of the supernatant was added to 270 µl of the reaction medium containing 4 mM butylated hydroxytoluene, 250 mM $FeSO_4$, 25 mM H_2SO_4 and 100 mM xylenol orange and incubated for 30 min at 25°C. Absorbances were recorded at 560 nm and the results expressed in mmol/mg of tissue using the extinction coefficient of 46.6 µM/cm.

2.10 Determination of SOD, CAT and GST activities

The SOD activity was quantified as described by Marklund and Marklund [25]. Briefly, samples were incubated with 200 mM Tris-HCl-EDTA (pH 8.5) and 1 mM pyrogallol for 20 min. Subsequently, absorbance was measured at 405 nm and SOD activity was expressed as U/mg protein.

The CAT activity was measured adding 5 µl of supernatant to 295 µl of reaction medium (200 mM Tris-HCl-EDTA, pH 8.5, 47.35 mL of ultrapure water and 172.5 µl of H_2O_2). The absorbance was measured at 240 nm and results expressed as µmol/min/mg of protein, according to Aebi [26].

The GST activity was measured according to Habig et al. [27], where 50 µl of the sample and 250 µl of the reaction medium (0.1 M buffer phosphate, 1-Chloro-2,4dinitrobenzene (CDMB), and reduced glutathione (GSH)) were added. The absorbance was measured at 340 nm and results expressed as mmol/min/mg of protein.

2.11 Determination of MPO activity

To determine MPO activity, the precipitate obtained as described above was resuspended in 80 mM potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyl trimethyl ammonium bromide and centrifuged at 11,000 × *g* for 20 min at 4 ° C. The MPO activity in the supernatant was determined at 620 nm with H_2O_2 and 3,3',5,5'-

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tetramethylbenzidine and expressed in units of mili optical density (mO.D)/ mg protein as described by Bradley et al. [28] and De Young et al. [29].

2.12 Assessement of PGE2 levels

Prostaglandin E_2 (PGE_2) concentration was determined in indomethacin-ulcerated gastric mucosa and performed using commercial Kit for enzyme immunoassay, following the manufacturer's instructions, Cayman Chemical (Ann Arbor, Michigan, USA). For this determination the indomethacin-ulcerated tissue was homogenized with 200 mM potassium phosphate buffer (pH 6.5) and then centrifuged at 9000 × g for 20 minutes. The supernatant was used to evaluate the levels of this eicosanoid.

2.13 Statistical analysis

The results were expressed as means ± standard error o means (S.E.M). One or two-way analysis of variance (ANOVA) followed by the Bonferroni's test was used to determinate the difference between the means using GraphPadPrism 5® Software (GraphPad Software, La Jolla, CA, USA). A value of $P<0.05$ was considered significant in all experiments.

3. RESULTS

3.1 Compound *2* decreased ethanol/HCl-induced gastric ulcer in mice

As shown in figure 1, the acidified ethanol ulcerated the gastric mucosa by 64.45 ± 6.58 mm^2 in the vehicle-treated only group. As expected, the treatment with

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carbenoxolone (200 mg/kg, p.o), the positive control group, reduced the lesion area in 83.84%. The pretreatment with ***1*** (Figure 1A), ***4*** (Figure 1C) and ***6*** (Figure 1E) diminished the lesion area by 60.4, 67.4% and 52.2%, respectively, both at a dose of 10 mg/kg (p.o). In addition, compounds ***3*** (Figure 1B) and ***5*** (Figure 1D), at the dose of 30 mg/kg p.o, reduced the lesion area by 95% and 70%, respectively, when compared to vehicle-treated group.

Interestingly, the pretreatment with ***2*** (Figure 2A) reduced the gastric ulcer by oral route in a dose-dependent manner (ED_{50}= 0.40 mg/kg, with 95% confidence interval = 0.18 to 0.89 mg/kg). Additionally, representative images from these results are depicted in figure 2B. Moreover, the intraperitoneal administration of ***2,*** at a dose of 0.1 mg/kg, decreased the gastric injury by 55.3% (Figure 3).

3.2 Compound *2* decreased the ulcer area and increased PGE_2 in indomethacin-induced ulcer model

As show in figure 4A, indomethacin-induced gastric lesions in an extension equal to 2.40 ± 0.39 mm^2, while the compound ***2***, at the doses of 0.3, 1 and 3 mg/kg, decreased the lesion area by 67%, 77% and 78%, respectively, when compared to the vehicle group. Additionally, carbenoxolone (200 mg/kg, p.o) decreased the gastric lesions by 92%, in comparison with the vehicle group. In parallel, oral admnistration of ***2*** (1 mg/kg) increased the PGE_2 amount in gastric tissue from mice exposed to indomethacin-induced ulcer model, compared to vehicle-treated group ($p<0.01$, figure 4B). In a similar manner, carbenoxolone (200 mg/kg) also increased the PGE_2 in ulcerated tissues when compared to vehicle-treated group ($p<0.01$, figure 4B).

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3.3 Gastroprotective effects of compound *2* in L-NAME, NEM, indomethacin or yohimbine-pretreated mice in the model of ethanol/HCl-induced ulcer

The pretreatment with L-NAME (Figure 5A) and NEM (Figure 5B) augmented the ulcer area by 116% and 212%, respectively, when compared to the group pretreated with saline only (46.57 ± 7.94 mm^2). However, the pretreatment with Indomethacin (Figure 5C) or Yohimbine (Figure 5D) did not alter the ulcerated area in comparison with the saline group. In addition, the pretreatment with L-NAME (Figure 4A), NEM (Figure 4B), Indomethacin (Figure 4C), and Yohimbine (Figure 4D) abolished the gastroprotective effect of compound ***2***.

3.4 Compound *2* did not change gastric secretion parameters

The volume of gastric juice in the vehicle group was 3.9 ± 0.4 mL; whereas in the same group, the pH was equal to 3.16 ± 0.63, reaching a total acidity of 17.75 ± 2.01 Eq[H^+]/mL/4 hours and a peptic activity of 1.96 ± 0.09 μM of tyrosine/4 hours. The administration of ***2*** (1 mg/kg, i.d) did not change the volume, pH, acidity or peptic activity when compared to the vehicle group. As expected, the administration of omeprazole (20 mg/kg) was able to reduce the acidity and peptic activity in 62.3% and 24.5%, respectively. Moreover, the pH of the gastric medium in the group treated with omeprazole was 6.69, as shown in table 1.

3.5 Compound *2* increases GSH and restores LOOH levels in ethanol/HCl-induced gastric ulcer

As shown in table 2, the ulcerated group treated with vehicle presented GSH levels equal to 124.7 ± 6.46 μg/mg of tissue, whereas the non-ulcerated group, presented the GSH amount equal to 173 ± 8.91 μg/mg of tissue. The treatment with ***2*** (1

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mg/kg) or carbenoxolone (200 mg/kg) increased the GSH values by 63% and 42%, respectively (Table 2).

The LOOH content was increased by 13% in the vehicle group, compared to the naive group (non-ulcerated group: 1.80 ± 0.03 mmol/mg of tissue). In contrast, carbenoxolone (200 mg/kg) and ***2*** (1 mg/kg) reduced the LOOH levels by 12% and 10% respectively, compared to the vehicle group (2.04 ± 0.04 mmol/ mg tissue) (Table 2).

3.6 Compound *2* decreases the activity of SOD but does not change CAT or GST activity

The acidified ethanol increased the SOD activity in the vehicle-treated group by 24%, related to basal levels found in non-ulcerated mice (Naive: 7.02 ± 0.1 U SOD/mg of protein). On the other hand, the administration of ***2*** reduced the SOD activity by 44%, compared to the vehicle group (Table 2). However, the administration of ***2*** did not change the CAT or GST activity, compared to the vehicle group.

3.7 Compound *2* reduced the MPO activity

Expectedly, the MPO activity increased by 953% in ulcerated tissue, when compared to non-ulcerated group (Naïve: 1.7 ± 0.20 mD.O/mg of protein). Oppositely, ***2*** (1 mg/kg) and carbenoxolone (200 mg/kg) were able to reduce this parameter by 78% and 77%, respectively, compared to the vehicle group (Table 2).

4. DISCUSSION

The gastroprotective effect of Lupeol has been previously described by [4], as well as some gastroprotective mode of action. In continuity to these studies, this research evaluated the gastroprotective activity of the ***1*** and their esters (***2***, ***3***, ***4***, ***5*** and ***6***)

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obtained by Silva et al. [15] through structural modifications in the Lupeol molecule aiming identify if those alterations altered and/or improved the anti-ulcer potential of Lupeol.

The ethanol-induced gastric ulcer is a classical model employed in gastroprotective studies because ethanol enters the gastric mucosa causing an intense vascular injury, decreasing the blood flow causing tissue necrosis and ROS generation [30, 31]. Acidified ethanol undoubtedly caused lesions in the gastric mucosa that were reversed by the action of compound ***2*** by oral and intraperitoneal treatments, suggesting a systemic effect and not just a topical action by due to the oral route. This data corroborates with Navarrete et al. [32] and Liby et al. [33], which described gastroprotective actions to other triterpenes. Similarly, Da Rosa et al. [34] reported the gastroprotective effect of the triterpenes maslinic and ursolic acids against acidified ethanol-induced lesions. The estering is a method used to improve the biological effect, to decrease side effects or to improve the absorption of a molecule. In fact, ***2*** (1 mg/kg) demonstrated a superior anti-ulcer effect than Lupeol (3 mg/kg p.o) already described by Lira et al. [4]. Similarly, Urban et al. [35] demonstrated that esterification in ring A of lupane group may increase or decrease cytotoxic action.

Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) lacking specificity for both cyclooxygenase 1 or 2 (COX-1 or 2), however its use is related to the appearance of gastric lesions due to the inhibition of COX-1 which in turn, decreases the production of endogenous prostanoids, such as PGE_2, a factor that is related to the protection of the gastric mucosa [36]. The pretreatment with indomethacin was able to reduce the gastroprotective effect of ester ***2*** demonstrating that its gastroprotective effect also depends on the effect of prostaglandins. Corroborating with our results, Lira et al. [4] demonstrated that Lupeol also has its effect depleted when

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pretreated with indomethacin. Interestingly, Geetha and Varalakshmi [37] suggested that Lupeol exerts anti-inflammatory actions, but in a different manner compared to NSAIDs, and unlike indomethacin, did not demonstrate the ulcerogenic effect in long term treatment.

Giving a continuity, we evaluated the role of NO and nonprotein sulfhydryl (NP-SH) compounds as contributors to the gastroprotective activity displayed by ***2***. In the ulcer genesis, ROS can cause depletion of NP-SH groups and NO, leading to the damage in the gastric mucosa due to oxidative stress and poor blood circulation [38, 39, 40]. Indeed, it was evidenced that the gastroprotective effect of ***2*** is abolished in mice pretreated with an inhibitor of NO synthase (i.e. L-NAME) or with an NP-SH blocker (i.e. NEM), suggesting that an adequate blood flow and the bioavailability of endogens antioxidants is crucial to antiulcer events elicited by ***2***. As expected, our results corroborate with Lira et al. [4], which demonstrated that the gastroprotective effect of Lupeol was also abolished in mice pretreated with L-NAME and NEM.

According to Gyires et al. [41], the α_2 adrenoceptors are involved in gastric acid secretion and possess crucial roles in other responses in the gastrointestinal tract. Yohimbine is classified as a selective α_2 adrenergic receptor antagonist [42] and in this study was employed to analyze the participation of this receptor in the gastroprotection exerted by ***2.*** In this experiment, it was observed that the antiulcer effect of ***2*** was abolished in mice pretreated with Yohimbine, suggesting that α_2 - adrenergic receptors participate directly in the gastroprotection action exerted by this ester. Confirming our results, Lira et al. [4] showed that the effect of lupeol was also abolished in the presence of Yohimbine.

Besides the mode of actions already discussed herein, it is important to understand the antioxidants mechanisms involved in the gastroprotective action,

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because this mechanism occurs at the cellular level. Ethanol increases oxidative damage in the gastric mucosa by decreasing blood flow, elevating lipid, hydroxyl and superoxide peroxidation [43, 44]. The initial stage of cellular damage caused by ROS is the cell membrane peroxidation [45]. As expected, LOOH levels were elevated in the vehicle-treated group, while these levels were reestablished in the mice treated with ***2*** (1 mg/kg), inferring that oxidative damage was minimized by the action of this ester. Furthermore, ***2*** were also able to raise GSH levels to values greater than those found in the vehicle-treated group. GSH is a tripeptide present within the cell and plays a key role in both non-enzymatic and enzymatic antioxidant pathways [46]. In this way, ester ***2*** is able to restore the oxidative balance.

In parallel, the enzymatic antioxidant defense system includes SOD, an enzyme that promotes the dismutation of superoxide anion; and CAT, which realizes the conversion of hydrogen peroxide to water and oxygen. Moreover, the detoxifying enzyme GST catalyzes the GSH conjugation with various endogenous and exogenous electrophilic compounds [45, 47]. In this way, ester ***2*** reduced the SOD activity, but CAT and GST activities were not altered in groups treated with ***2***, demonstrating that ***2*** does not require these oxidative pathways to exert its gastroprotective effect.

The activity of MPO is classically verified as a marker of neutrophil infiltration in tissues because this enzyme is found in the azurophil granules of these inflammatory cells [48, 49]. As expected, the contribution of the neutrophils to the genesis of the gastric lesion was confirmed by the increase in the levels of MPO activity at the ulcer site in the vehicle-treated group. In contrast, the treatment with ***2*** reduced this parameter in ethanol/HCl-ulcerated tissue. Therefore, we can also infer that ester ***2*** avoided the ulcerogenic process, at least in part, by the reduction of the inflammatory process mediated by neutrophil migration.

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Finally, considering the results obtained, we evaluated the gastric anti-secretory activity of the ***2***. The suppression of gastric acid is the main therapy used for the gastric ulcer treatment. Despite of this, the compound ***2*** was not able to decrease the volume of secretion, total acidity or peptic activity in the gastric juice, suggesting that the mechanism of action of the compound ***2***, as explored in this study, differs from the actions elicited by omeprazole, a classical standard drug used in the clinic due to its inhibitory action of the proton pump.

5. Conclusion

Together, our results confirmed that the esters ***3, 4, 5*** and ***6*** were able to reduce the area of the ulcer lesion; however, ester ***2*** was able to reduce the ethanol acidified- and indomethacin-induced gastric ulcer in lower doses, evidencing that the stearate group enhanced the gastroprotective potency of Lupeol. Regarding mode of actions, the participation of NP-SH, NO, PGE_2 and α_2-adrenoceptors directly participate in the gastroprotective effect of this compound. Antioxidant properties include the increase in GSH availability and the decrease of LOOH content, as well as a reduction in neutrophil migration. Finally, the chemical modification on the Lupeol structure that provided compound ***2*** increased its pharmacological action.

Conflict of interest

The authors have no conflict of interest.

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Legends for figures

Fig. 1 Effect of Lupeol and esters *3, 4, 5* and *6* on the acute gastric ulcer induced by ethanol/HCl. Panel A - E: The animals received vehicle (Veh: DMSO 1%, 1 ml/kg, p.o), carbenoxolone (Cbx: 200 mg/kg, p.o), Lupeol and its esters *3, 4, 5* and *6* (1 - 30 mg/kg, p.o). Results are expressed as means ± S.E.M. (n=6). One-way ANOVA followed by Bonferronis's test. *P<0.05; ** P<0.01; *** P<0.001 vs. the vehicle-treated group.

Fig. 2 Effect of compound *2* (lupeol stearate) on the acute gastric ulcer induced by ethanol/HCl. Panel A: The animals received vehicle (Veh: DMSO 1%, 1 ml/kg, p.o), carbenoxolone (Cbx: 200 mg/kg, p.o) or ***2*** (0.1 - 3 mg/kg, p.o). Panel B: Representative images of the different experimental groups. Results are expressed as means ± S.E.M. (n=6). One-way ANOVA followed by Bonferronis's test. ** P<0.01 and *** P<0.001 vs. the vehicle-treated group.

Fig. 3 Gastroprotective effect of lupeol stearate (*2*) given by intraperitoneal rout on the ethanol/HCl-induced gastric ulcer in mice. The animals received vehicle (Veh: DMSO 1%, 1 ml/ kg), carbenoxolone (Cbx: 200 mg/kg, p.o) and *2* (0.1 mg/kg, ip). Results are expressed as the means ± S.E.M. (n=6). One-way ANOVA followed by the Bonferroni's test. ** P<0.01 and P<0.001 vs. vehicle-treated group.

Fig. 4 Effect of Lupeol stearate (*2*) on ulcer area (A) and in the PGE_2 levels (B) of ulcerated tissues from indomethacin-induced gastric ulcer in mice. The animals were orally treated with vehicle (Veh: DMSO 1%, 1 ml/kg), carbenoxolone (Cbx: 200

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mg/kg) or *2* (0.3 - 3 mg/kg). Results are expressed as the means ± S.E.M. (n=6). One-way ANOVA followed by Bonferroni's test. ** $P<0.01$ and $P<0.001$ vs. the vehicle-treated group.

Fig. 5 Effects of NEM, L-NAME, Yohimbine, and Indomethacin on the gastroprotective effect of Lupeol stearate (2) against Ethanol/HCl-induced ulcer in mice. The animals were treated with saline (10 ml/kg, i.p), NEM (10 mg/kg, i.p), L-NAME (70 mg/kg, i.p), yohimbine (2 mg/kg, i.p) or indomethacin (10 mg/kg, i.p) 30 min prior to vehicle (Veh: DMSO 1%, 1 ml/kg) or compound ***2*** (C ***2,*** 1 mg/kg, p.o) administration. Results are expressed as the means ± S.E.M. (n=6). Two-way ANOVA followed by Bonferroni's test. * $P<0.05$ vs. vehicle-saline group. # $P<0.05$ vs. C ***2***-saline group.

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Table 1. Effects of compound ***2*** on gastric acid secretion.

	Volume	**Acidity**	**pH**	**Peptic activity**
Vehicle	3.99 ± 0.45	17.75 ± 2.01	3.16 ± 0.63	1.96 ± 0.09
Omeprazole	2.70 ± 0.30	6.69 ± 1.60[a]	5.59 ± 0.65[a]	1.48 ± 0.06[a]
Compound *2*	3.30 ± 0.19	16.63 ± 1.87	3.50 ± 0.40	1.85 ± 0.09

Volume (mL); Acidity (mEq $[H^+]$/mL); Peptic activity (mmol of tyrosine/4 hours/mL). Values are expressed as means ± S.E.M (n=6). One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. [a]$p < 0.05$ when compared with the vehicle-treated group.

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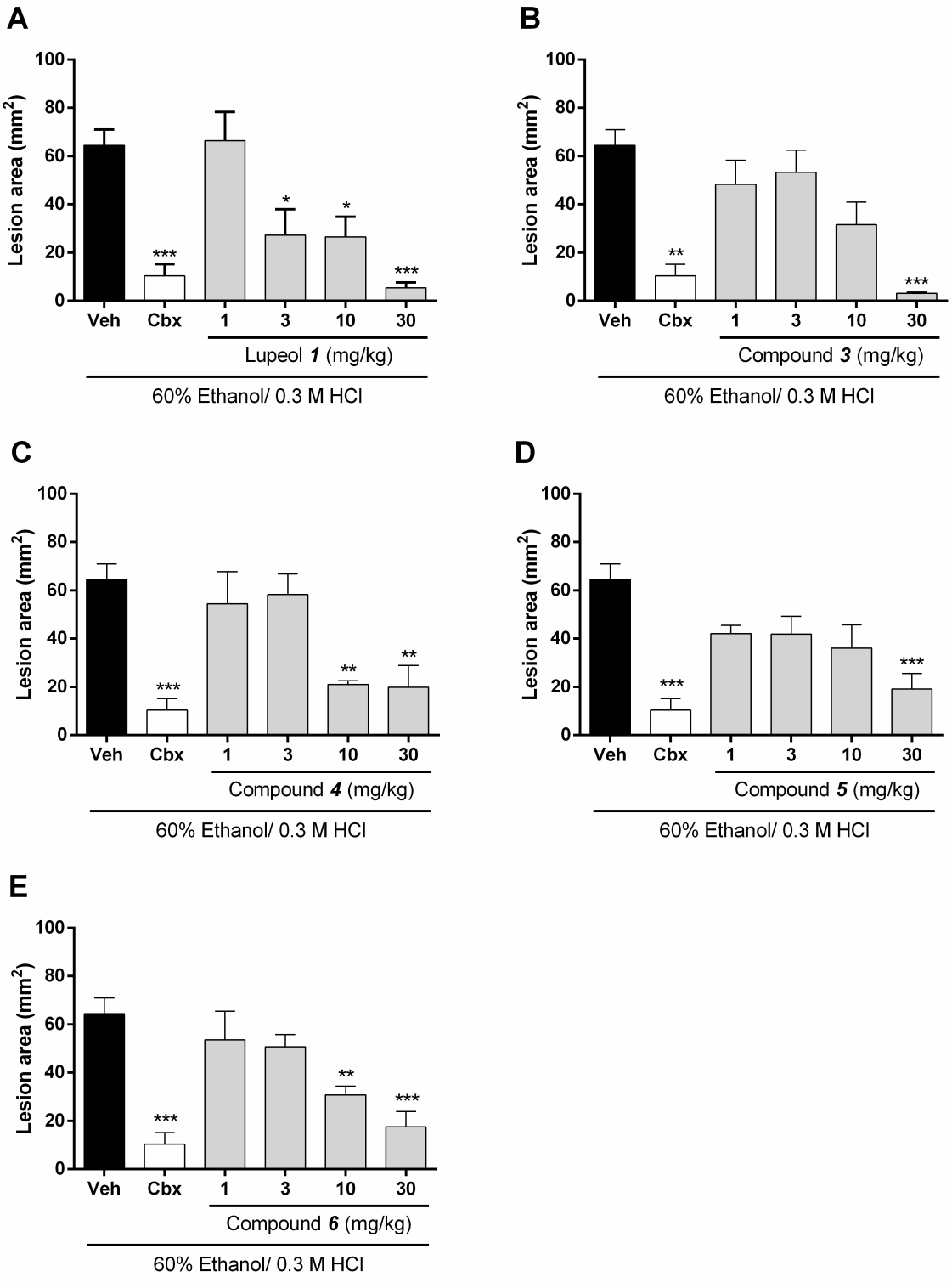
Table 2. Effects of compound ***2*** on oxidative and inflammatory parameters of ulcerated gastric tissue.

	MPO	GSH	LOOH	SOD	CAT	GST
Naive	1.7 ± 0.20	173.9 ± 8.91	1.80 ± 0.03	7.02 ± 0.10	461.4 ± 63.56	979.0 ± 13.03
Vehicle	17.9 ± 5.34[a]	124.7 ± 6.46[a]	2.04 ± 0.04[a]	8.69 ± 0.45	292.2 ± 37.85[a]	736.0 ± 92.70
Carbenoxolone	4.1 ± 0.95[b]	177.2 ± 14.91[b]	1.80 ± 0.04[b]	7.92 ± 0.18	445.9 ± 171.10	1100.0 ± 206.10
Compound *2*	3.9 ± 0.84[b]	203.4 ± 9.49[b]	1.84 ± 0.03[b]	4.85 ± 1.05[b]	468.7 ± 46.12	719.2 ± 177.3

Myeloperoxidase (MPO, mD.O/mg of protein); Reduced glutathione (GSH, µg/mg of tissue); Hydroperoxides lipids (LOOH, mmol/ mg of tissue); Superoxide dismutase (SOD, U/mg of protein); Catalase (CAT, µmol/min/mg of protein) and Glutathione S-transferase (GST, mmol/min/mg of protein). Values are expressed as means ± S.E.M (n=6). One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. [a]$p < 0.05$ when compared with the naive group. [b]$p < 0.05$ when compared to the vehicle-treated group.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Lupeol 1 (mg/kg)
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Lesion area (mm2)
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Compound 3 (mg/kg)
60% Ethanol/ 0.3 M HCl
C
Lesion area (mm2)
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Veh
Cbx
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Compound 4 (mg/kg)
60% Ethanol/ 0.3 M HCl
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Lesion area (mm2)
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Compound 5 (mg/kg)
60% Ethanol/ 0.3 M HCl
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Lesion area (mm2)
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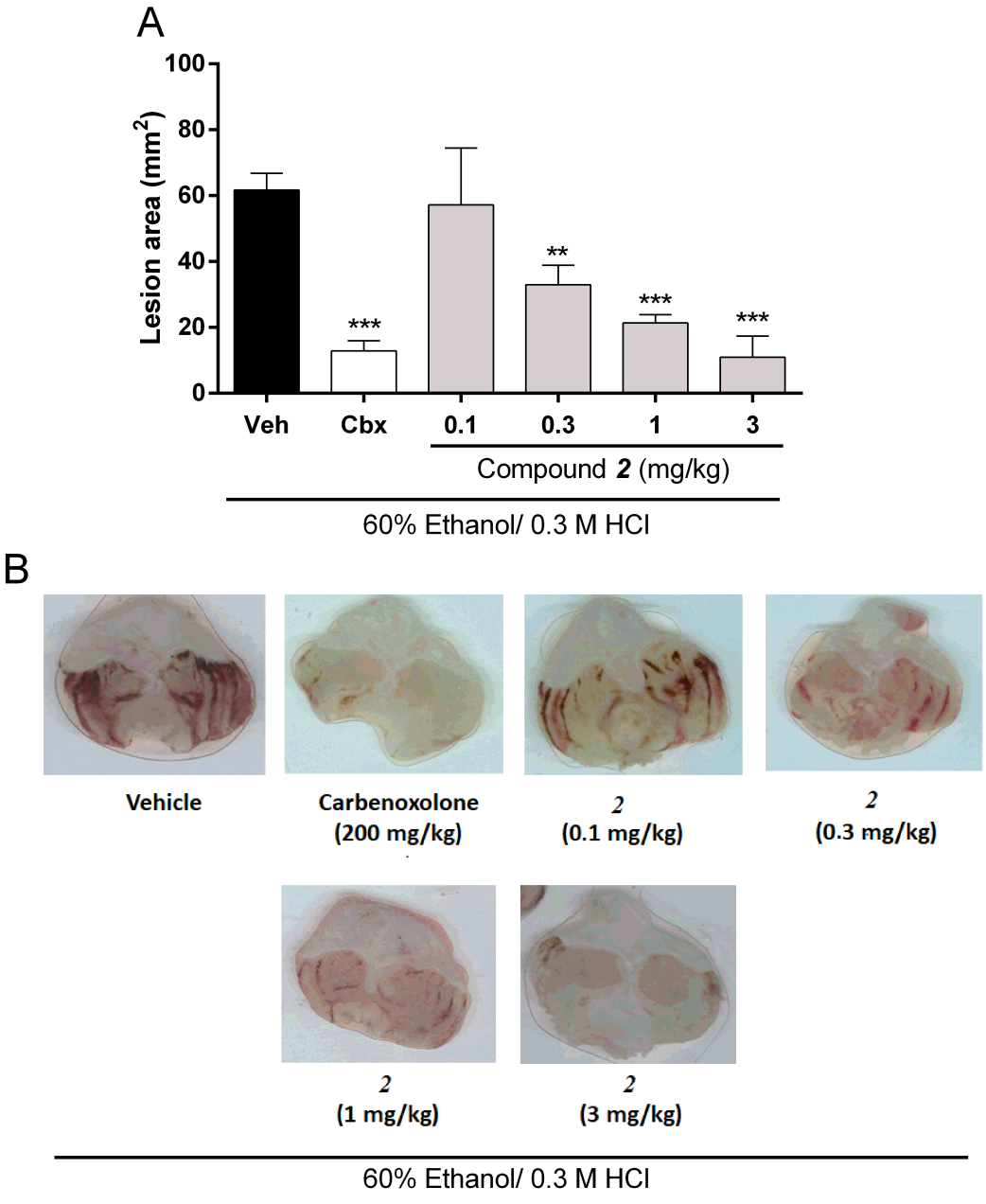
Compound 6 (mg/kg)
60% Ethanol/ 0.3 M HCl

Figure 1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Lesion area (mm2)
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3
Compound 2 (mg/kg)
60% Ethanol/ 0.3 M HCl
B
Vehicle
Carbenoxolone (200 mg/kg)
2 (0.1 mg/kg)
2 (0.3 mg/kg)
2 (1 mg/kg)
2 (3 mg/kg)
60% Ethanol/ 0.3 M HCl

Figure 2

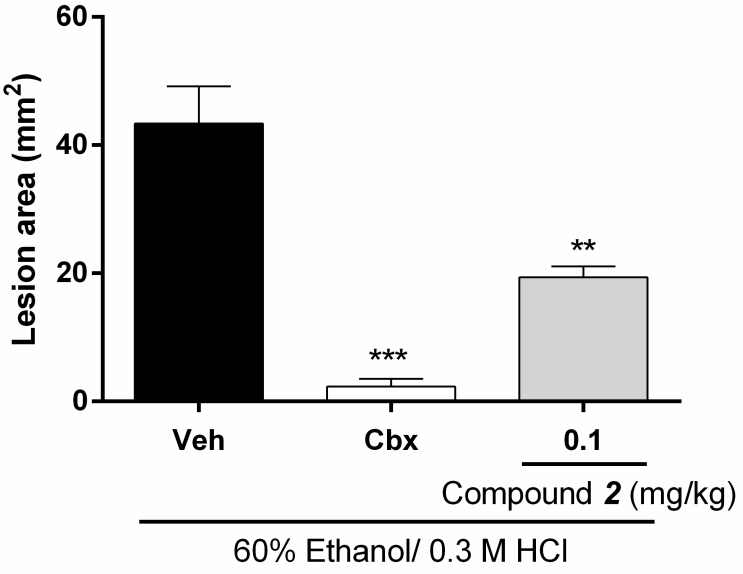

Figure 3

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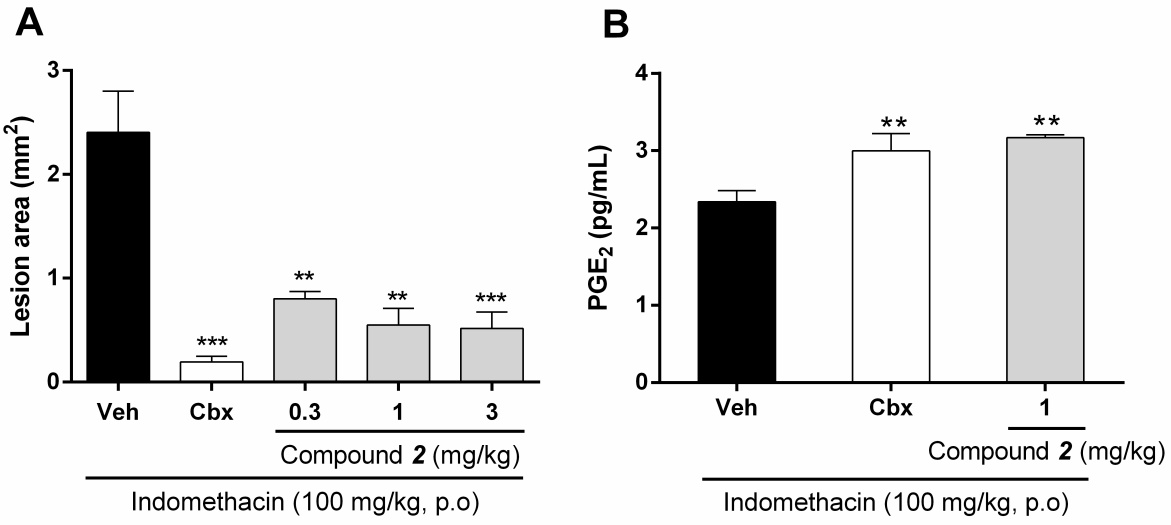

Figure 4

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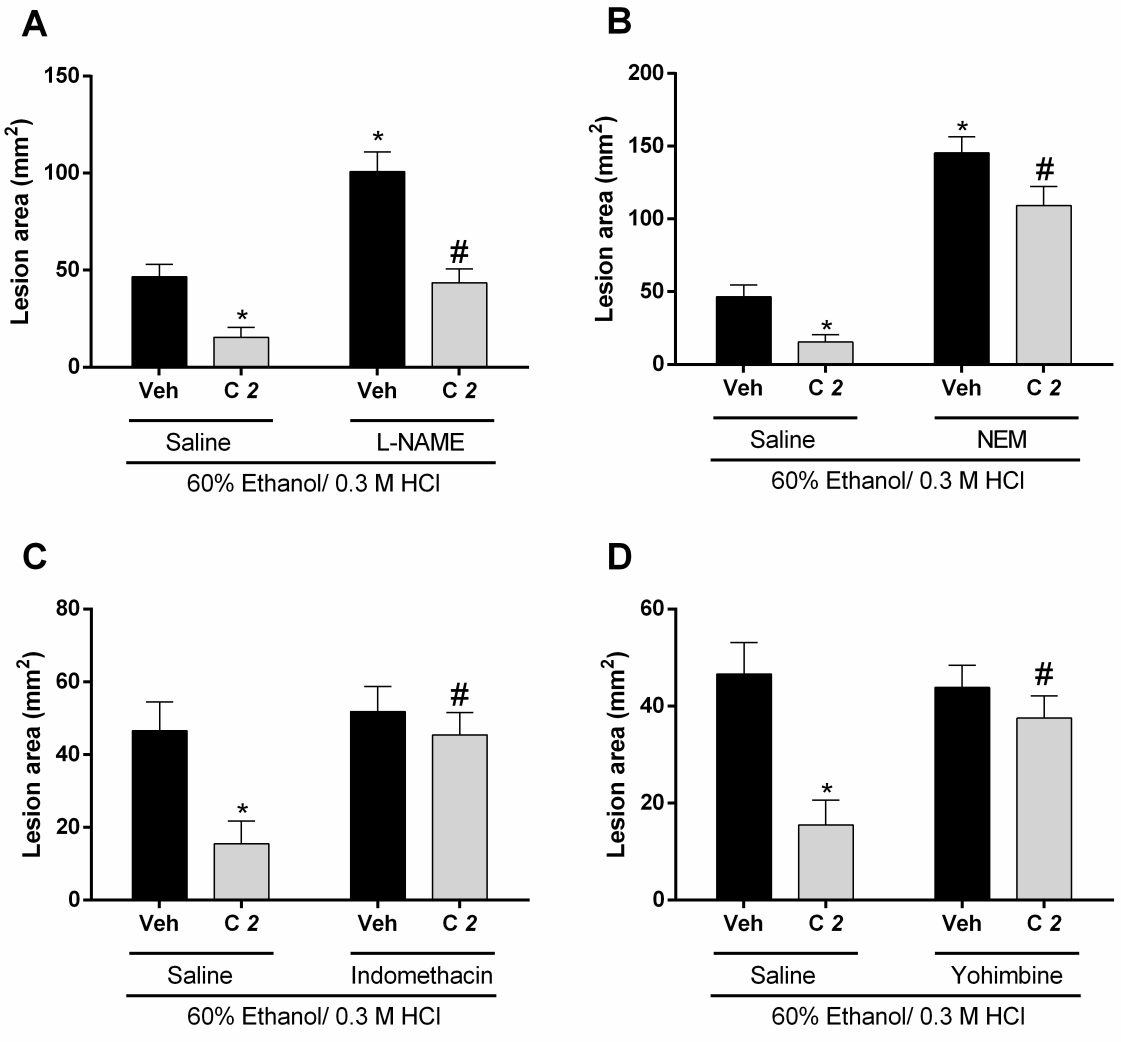
A
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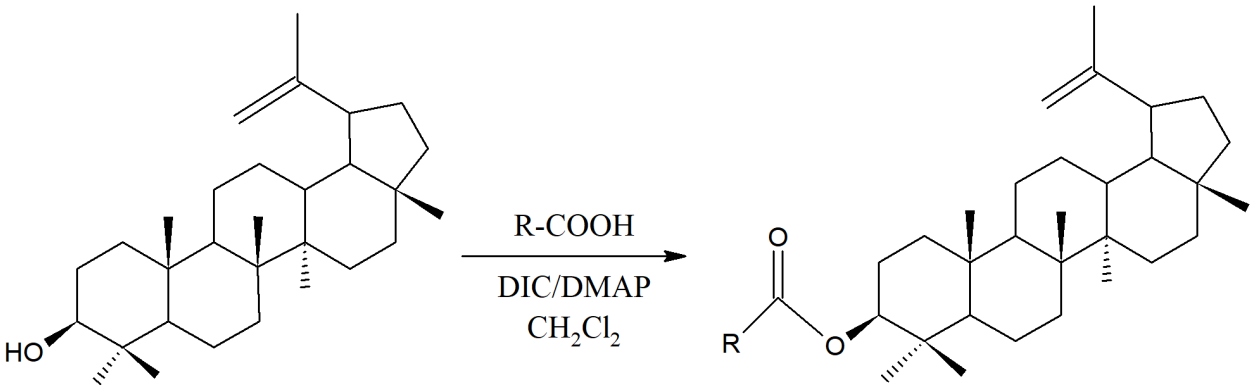
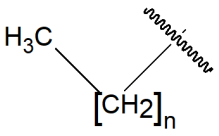
60% Ethanol/ 0.3 M HCl

Figure 5



R	Lupeol esters	
	n = 16	Lupeol stearate (*2*)
	n = 14	Lupeol palmitate (*3*)
	n = 12	Lupeol myristate (*4*)
	n = 10	Lupeol laurate (*5*)
	n = 8	Lupeol caprylate (*6*)

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Highlights

Lupeol-stearate decreased ethanol/HCl-induced ulcer in mice (ED_{50}= 0.40 mg/kg).

Lupeol-stearate increased PGE_2 and decreased the indomethacin-induced ulcer in mice.

Lupeol-stearate did not change gastric secretion parameters

Lupeol-stearate increases GSH and restores LOOH levels in ethanol/HCl-induced ulcer.

Lupeol-stearate decreases the activities of SOD and MPO enzymes.

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AUTHORSHIP STATEMENT

All persons who meet authorship criteria are listed as authors of the manuscript "**Gastroprotective properties of Lupeol-derived ester: pre-clinical evidences of Lupeol-stearate as a potent antiulcer agent"**, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Chemical Biological Interaction.

Lincon Bordignon Somensi was responsible for the acquisition of all pharmacological data; Philipe Costa, Thaise Boeing and Luísa Nathália Bolda Mariano contributed largely to the pharmacological data acquisition; Bruna Longo performed the statistical analyzes; Cássia Gonçalves Magalhães, Lucienir Pains Duarte and Aline Teixeira Maciel e Silva were responsible for all chemical obtaining of the compounds used in this manuscript; Sérgio Faloni de Andrade contributed with the design of study; Priscila de Souza contributed revising the manuscript critically for important intellectual content and Luisa Mota da Silva supervised all pharmacological steps and contributed with Conception and design of study, Writing- Original draft preparation and Writing- Reviewing and Editing. All authors approved the version of the manuscript to be published.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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