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Gastroprotective Effect of *Xylopia langsdorffiana* A. St.-Hil. & Tul. (Annonaceae): Involvement of Endogenous Sulfhydryls Compounds and Nitric Oxide

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Abstract: *Xylopia langsdorffiana* A. St.-Hil. & Tul., belonging to the Annonaceae family, popularly known as "pimenteira-da-terra" was selected for study on the basis of chemotaxonomic criteria because various chemical compounds (among these the diterpenes) with pharmacological activities have been isolated. We investigated the acute toxicity of the ethanolic extract (EtOHE) and hexane phase (HexPh) obtained from the leaves of *X. langsdorffiana* (*XL*) and its ability to prevent gastric mucosa ulceration in animal models. The results suggest that *XL*-EtOHE has low toxicity to mice treated with a single dose of 2000 mg/kg (p.o.) and the inhibition the formation of gastric lesions induced by ethanol, restraint-hypothermic stress and NSAIDs. In the pylorus ligature model, *XL*-EtOHE (500 mg/kg) and *XL*-HexPh (250 mg/kg) showed gastric protection with both oral (p.o.) and intraduodenal (i.d.) administration, yet without altering the gastric juice parameters (pH, $[H^+]$, and volume). *XL*-HexPh (250 mg/kg) did not increase mucus production, and both EtOHE and HexPh induced gastroprotection with a certain dependency on sulfhydryls groups and nitric oxide.

Keywords: *Xylopia langsdorffiana*; acute toxicity; gastric ulcers; gastroprotection; endogenous sulfhydryls compounds; nitric oxide.

1. Introduction

Peptic ulcer is one of the most common gastrointestinal disorders with a growing incidence and prevalence. It is attributed to several factors encountered in daily life, such as stress, excessive alcohol intake, smoking, prolonged therapy with non-steroidal anti-inflammatory drugs (NSAIDs) and infection with *Helicobacter pylori* [1,2,3].

Although there are many medicines on the market for the treatment of gastric ulcers, including antacids, proton pump inhibitors, anticholinergics, histamine H_2-antagonists and cytoprotective agents, most of these drugs produce adverse reactions, such as hypersensitivity, hyper-gastrinemia, arrhythmia, impotence, gynecomastia and hematopoietic alterations [4,5].

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Thus, it is necessary to investigate antiulcer agents which are less toxic and more effective [6]. In this context, plants remain an important source of new therapeutic alternatives.

Annonaceae is one of the largest Magnoliid families with 128 genera and 2300 species, most are pan-tropical [7]. In Brazil, Annonaceae comprise 26 genera (seven endemic) and 260 species [8], which are known primarily for its edible fruits, such as pine or fruit of conde (*Annona squamosa* L.) and soursop (*Annona muricata* L.) [9]. The genus *Xylopia* (one of the largest in family), contains approximately 160 species, represented in Brazil by 25 species distributed throughout the country, mainly in the Amazon region [10].

In the literature there are reports of using *Xylopia* species in traditional medicine. The fruits of *Xylopia aethiopica* (Dunal) A. Rich are used to treat stomach disorders, biliary, dysentery and bronchitis, they also possess carminative and postpartum tonic effects [11]. The seeds of *Xylopia frutescens* Aubl. (Annonaceae) in Brazil are used as an antimicrobial and anti-rheumatic. Kaurenoic acid (a caurane diterpene), abundant in the seeds of *X. frutescens* showed antiprotozoal activity against *Trypanasoma cruzi* and *Plasmodium falciparum*, justifying its use in folk medicine [12]. Osorio *et al.* [13] showed that *X. Aromatic* (Lam) presents leishmanicidal and trypanocidal activities.

Xylopia langsdorffiana A. St-Hil. & Tul. is a tree of 5-7 meters [8] and is known in the Brazil as *"pimenteira da terra"* [9]. It was chosen for study based on chemotaxonomic criteria, since from it, various chemical compounds with pharmacological activities have been recently isolated, among these, diterpenes which are considered important biomarkers for differing species of the genus [14].

Phytochemical study performed with the hexane phase from leaves of *Xylopia langsdorffiana* led to two substances: 8(17),12E,14-labdatrien-18-oic acid (labdane 302) [15,16] and the diterpene atisane *ent*-atisan-7α, 16α diol, which was given the name of Xylodiol [16] (figure 1). From the hexane phase of stems of *X. langsdorffiana* were isolated two new diterpenes: *ent*-7-α-hidroxytrachylobane-18-oic acid (trachylobane-318), and the *ent*-7α-acetoxytrachylobane-18-oic acid (trachylobane-360) [17] (figure 1).

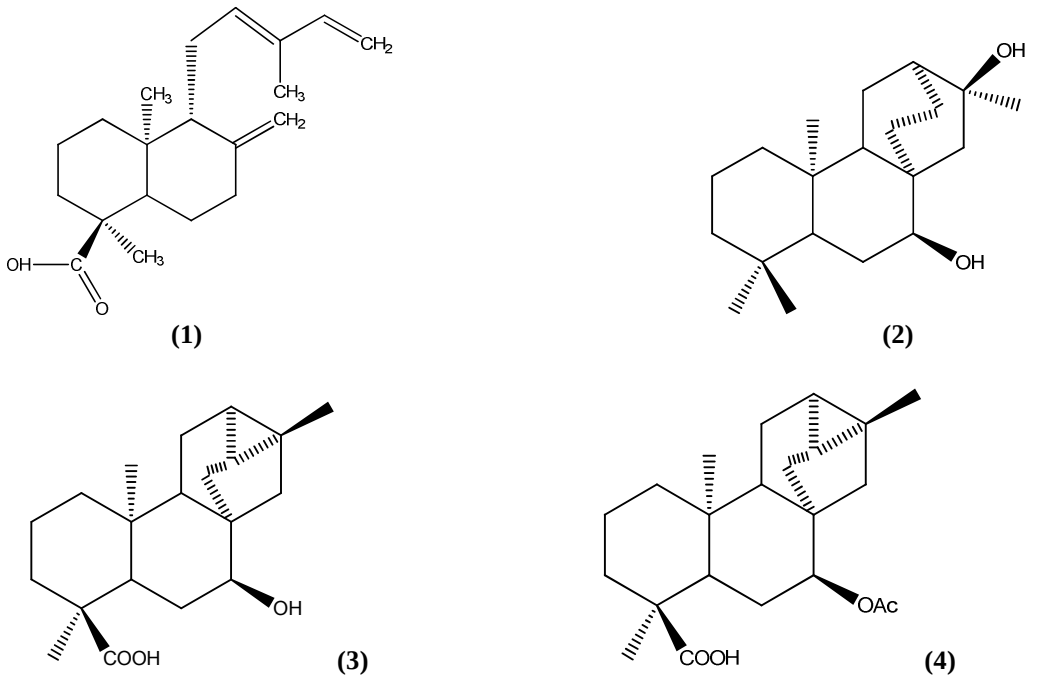


Figure 1. Isolated structures from leaves and stems of *Xylopia langsdorffiana.* **(1)** 8(17),12E,14-labdatrien-18-oic acid (labdane 302); **(2)** ent-atisano-7α,16α-diol (xylodiol); **(3)** *ent*-acid-α-hydroxytrachylobane-18-oic acid (trachylobane-318); **(4)** *ent*-acid 7α-acetoxytrachylobane-18-oic acid (trachylobane-360).

In the case of pharmacological activities attributed to diterpenes from *Xylopia langsdorffiana*, labdane 302 presented relaxing effects on both guinea pig trachea and rat aorta [18]. Oliveira [19]

reported hypotensive activity in normotensive rats for labdane 302 mediated by decrease in vascular resistance involving the enzyme NOS and endothelial cyclooxygenase, and also vascular endothelium dependent and independent vasorelaxation.

Xylodiol and trachylobane-360 exhibited potent cytotoxic activity for tumor cells, with lesser cytotoxicity against V79 fibroblasts and hepatocytes [17]. Trachylobane-360 also inhibited growth while inducing differentiation in HL60 and K526 leukemic cells [20] exhibiting antitumor activity *in vivo* without signs of toxicity and *in vitro* for sarcoma 180 cells, this latter result was also observed for trachylobane-318 [21]. Essential oil of the leaves of *X. langsdorffiana* has shown molluscicidal activity [22].

Several terpenoids containing extracts from plants commonly used in the traditional medicine to treat different gastric illnesses have been reported [23]. In the last years terpenes or their derivatives showed to possess gastroprotective activity in different models of induced gastric lesions in animals [24,25] and promoting healing of gastric lesions in rats [26].

Although the mechanism of action of diterpenes has not been well established, they are believed to protect the gastric mucosa mainly through mechanisms that enhance stomach defensive factors such as prostaglandin synthesis and mucus production, while suppressing gastric acid secretion [25].

The present study evaluated the gastroprotective activity of ethanol extract (EtOHE) and hexane phase (HexPh) obtained from leaves of *X. langsdorffiana* (*XL*). Possible mechanisms of action are elucidated using rodents in differing experimental models with the purpose of contributing to a better understanding of the physiopathology of gastric injury.

2. Materials and Methods

2.1. Drugs

Absolute ethanol (MERCK, Germany) , N^G-omega-nitro-L-arginine methyl ester (L-NAME) (SIGMA Chemical Co., St. Louis, MO, U.S.A), carbenoxolone (SIGMA Chemical Co., St. Louis, MO, U.S.A), cimetidine (SIGMA Chemical Co., St. Louis, MO, U.S.A), N-ethylmaleimide (NEM) (Sigma Chemical Co., St. Louis, MO, U.S.A.), piroxicam (Hexal, Brazil), sodium chloride (QUIMEX-MERCK, Brasil), sodium hydroxide (QUIMEX-MERCK, Brasil), phenolphthalein (RIEDEL-DE HAËN, Germany), Tween 80 (MERCK, Germany) alcian blue (SIGMA Chemical Co., St. Louis, MO, U.S.A), ketamine 5% (VETANARCOL), xilazine 2 % (DORCIPEC), magnesium chloride (MERCK), sucrose (SIGMA Chemical Co., St. Louis, MO, U. S. A), ethyl ether, sodium acetate.

XL-EtOHE and XL-HexPh were solubilized in 12% Tween 80 solution. Carbenoxolone and cimetidine (substances used as positive control), NEM and L-NAME as sulfhydryl groups and nitric oxide blockers, respectively, were dissolved in 0,9% NaCl solution. All drugs and reagents were prepared immediately before use.

2.2. Plant Material

The leaves of *Xylopia langsdorffiana* were collected in the city of *Cruz do Espírito Santo*, State of Paraíba, in July 2002. The plant material was identified by Prof. Maria de Fátima Agra, chief of the Botany Section of the "Prof. Delby Fernandes de Medeiros" Laboratory of Pharmaceutical Technology. A voucher specimen (AGRA 5541) has been deposited at the "Lauro Pires Xavier" Herbarium of the Federal University of Paraiba, João Pessoa, Brazil.

2.3. Extraction and Isolation

Dried leaves of *X. langsdorffiana* (4000 g) were powered and exhaustively macerated with 95 % ethanol (EtOH) in three process of extraction, in intervals of 72 hours. Removal of the solvent under

reduced pressure in a rotary evaporator yielded a dark green extract (300 g), from which around 100g of extract was partitioned successively with hexane, chloroform ($CHCl_3$) and ethyl acetate (EtOAc) to yield the hexane (15,79 g), chloroform and ethyl acetate phases, respectively.

2.4. Animals

Male Swiss albino mice (25-35 g) and male Wistar albino rats (180-250 g) from the Thomas George Biotery of the LTF/UFPB were used. The animals were fed a certified Labina (Purina) diet with free access to water and maintained under standard conditions in light/dark cycles of 12 h at 60 ± 1 % of humidity and a temperature of 21 ± 2 ^{o}C. Fasting was imposed prior to all assays because standard drugs and extracts were always administered orally (by gavage) or intraduodenally using a 12% Tween 80 solution (at 10 ml/kg) as the vehicle. All experiments were performed in the morning and followed the recommendations of animal care. The experimental protocols were approved by the Institutional Committee for Ethics in Animal Research of LTF/UFPB and registered under #0106/09.

2.5. Acute Toxicity Study

In the study of acute oral toxicity, a single dose of *XL*-EtOHE (2000 mg/kg, p.o.) was administered in a group of twelve mices (six males and six females) after 12 h fasting. The animals (six males and six females) that received the tween 80 solution 12% served as controls. The parameters of behavior (signs and symptoms) associated with *XL* administration were observed for 30, 60, 90, 120, 180, 240 minutes, 24h, 48h and 72h [27]. Food and water consumption were evaluated in both sexes, within 14 days. At the end of this period the number of survivors was recorded to determine the LD50, it was estimated body weight of mices, then the animals were sacrificed, the macroscopic analyses and weight of vital organs such as liver, heart, kidney and spleen were compared between the animals treated with *XL* and vehicle.

2.6. Absolute Ethanol-induced Gastric Lesions

The experiment was performed according to the method of Morimoto *et al.* [28]. After 24 h fasting, rats (n = 5–7) received an oral administration of *XL*-EtOHE or *XL*-HexPh (at 62,5, 125, 250 and 500 mg/kg), carbenoxolone 100 mg/kg (positive control) or 12% Tween 80 solution at 10 mL/kg (negative control). One hour after treatment, all rats received 1 mL of absolute ethanol to induce gastric ulcer. The animals were euthanized by cervical dislocation 1 hour after treatment with the ulcerogenic agent, the stomachs removed and opened along the greater curvature to determine the ulcerative index (UI), as described by Szelenyi & Thiemer [29].

2.7. Hypothermic Restraint Stress-induced Gastric Lesions

The experiment was performed by the method of Levine [30], with some modifications. After 24 h of fasting, the animals (n = 5–7) received by gavage the *XL*-EtOHE or *XL*-HexPh (at 62,5, 125, 250 and 500 mg/kg), cimetidine 100 mg/kg (positive control) or 12% Tween 80 solution at 10 mL/kg (negative control). Thirty minutes after treatment, mice were immobilized in a restraint cage at 4 $^{\circ}$C for 4 h to induce gastric ulcer. The animals were euthanized by cervical dislocation and the stomachs were removed and opened along the greater curvature to determine the UI in accordance with Szelenyi & Thiemer [29].

2.8. Non-steroidal Anti-inflammatory Drug-induced Gastric Lesions

The experiment was performed according to the method of Puscas *et al.* [31] with modifications. In this model, gastric ulcer was induced using piroxicam (30 mg/kg, s.c.) administered to mice (n = 5–7) after a 24 h fasting. *XL*-EtOHE or *XL*-HexPh (at 62,5, 125, 250 and 500 mg/kg),

cimetidine 100 mg/kg (positive control) or 12% Tween 80 solution at 10 mL/kg (negative control) were administered orally 30 min before the induction of gastric ulcer. The animals were euthanized by cervical dislocation 4 hours after treatment with the ulcerogenic agent. The stomachs were removed and the UI was calculated as described previously by Szelenyi & Thiemer [29].

2.9. Shay Ulcer and Evaluation of Biochemical Parameters of Gastric Juice

After fasting for 36 hours, the rats (n = 6-7) were anesthetized with ketamine hydrochloride 5% (muscle relaxant) and xylazine 2% (anesthetic) and subjected to a longitudinal incision below the xiphoid apophysis for pylorus ligature. Afterwards the animals received intraduodenally *XL*-EtOHE (500 mg/kg) or *XL*-HexPh (250 mg/kg) (doses that showed the best results in the ethanol-induced ulcer model), cimetidine 100 mg/kg (positive control) or 12% Tween 80 solution 10 mL/kg (negative control). When the route of administration was oral, thirty minutes after the treatment, pylorus ligature was performed as described by Shay *et al.* [32]. Soon afterwards, the incisions were sutured and four hours later the animals were euthanized by cervical dislocation, the abdomen was opened and another ligature was placed around the esophagus close to the diaphragm. The stomachs were removed and the gastric content was collected to determine the total amount of gastric juice (mL). The gastric content was transferred to a graduated centrifuge tube, distilled water was added and the resultant solution centrifuged at 3000 rpm for 10 min. The pH of the supernatant was recorded with a digital pHmeter. Total acid of the gastric secretion was determined in the supernatant volume by titration to pH 7.0 with 0.01 N NaOH.

2.10. Determination of mucus adhering to gastric mucus

The experiment was performed according to the Raffatullah *et al.* [33] method with modifications. After 36 hours of fasting the rats received *XL*-HexPh (250 mg/kg) orally (dose that presents significant results in all acute experiments), carbenoxolone 200 mg/kg (positive control) or 12% Tween 80 solution 10 mL/kg (negative control). After 1 hour, under anesthesia with ketamine 5 % and xilazine 2% the animals were submitted to longitudinal incision slightly below the xiphoid apophysis for the pylorus ligature. After 4 hours, the animals were euthanized by cervical dislocation, the glandular portion of the stomach was separated, weighed and immersed in 10 mL of the 0.1% alcian blue solution (0.16 M sucrose/0.05 M sodium acetate, pH 5.8). After a 2 hour immersion, excess dye was removed in two successive rinses with 7 mL of 0.25 M sucrose, 15 min and 45 min. Each stomach was sequentially transferred to 10 mL of 0.5 M $MgCl_2$ solution for 2 hours. Four milliliters of dye solution was then shaken vigorously with an equal volume of ether. The resulting emulsion was centrifuged at 3600 rpm for 10 min and the absorbance of the aqueous layer was measured at 598 nm. The amount of blue dye extracted per gram of wet glandular tissue was then calculated. The results expressed as mg of Alcian Blue/g of tissue.

2.11. Involvement of Nitric Oxide (NO) and Sulfhydryl Compounds (SH) in Gastric Protection Against Ethanol-induced Gastric Lesions in NEM or L-NAME Pretreated Rats

The experimental protocols were performed according to the Matsuda; Li; Yoshikawa [34] method for determination of the role sulfhydryl compounds (SH) and the Sikiric; Seiwerth; Grabarevic [35] method for nitric oxide (NO). Rats were divided into groups of 5-7 animals that were fasted for 24 hours. They were pretreated intraperitoneally with 12% Tween 80 solution, NEM (N-ethylmaleimide, 10 mg/kg) a blocker of SH compounds or L-NAME (N-nitro-L-arginine methyl ester, 70mg/kg) an inhibitor of the NO synthesis. Thirty minutes after the pretreatment, the animals received *XL*-EtOHE (500 mg/kg) or *XL*-HexPh (250 mg/kg) by gavage (doses that showed the best results for ethanol-induced ulcer), carbenoxolone 100 mg/kg (as positive control) or 12% Tween 80 solution 10 mL/kg (as negative control). After 60 minutes, all the groups were treated with 1 mL absolute ethanol to induce gastric ulcers. At 1 hour after the administration of ethanol, the animals were euthanized by

cervical dislocation, the stomachs excised and the UI was determined as described previously by Szelenyi & Thiemer [29].

2.12. Statistical analysis

The results are expressed as mean ± S.D. Differences between the means were statistically compared using Student´s t-test for acute toxicity and one-way analysis of variance (ANOVA) followed by Dunnett´s and Tukey's tests for the gastroprotective activity. The values were considered significantly different when the levels of $p < 0.05$. The software used was the GraphPad Prism© 5.0 (GraphPad Software Inc., San Diego CA).

3. Results and Discussion

3.1. Acute Toxicity Study

The use of medicinal plants showed that certain species have potentially dangerous substances and, therefore, should be used carefully, respecting the toxicological risks [36], which can be assessed by preclinical toxicology studies (*in vitro* or *in vivo*) and clinical studies (phases 1-4).

The acute toxicity model was performed using the standardized methodology by Almeida et al. (1999) [27] in order to identify changes in the Central Nervous System (CNS) and Autonomic Nervous System (ANS), recording some signs or behavior changes shown by animals after treatment with the ethanolic extract of *X. langsdorffiana* [37]. The *XL*-EtOHE administered in a single dose of 2000 mg/kg, p.o. induced irritability and hyperactivity only in female mice when compared to respective control group (12% Tween 80 solution - 10 mL/kg) and these signs were reversed in the third hour after treatment, suggesting an action in the CNS, but this data are insufficient to infer toxicity to the *XL*-EtOHE.

When investigated the consumption of food and water and observed an increase in food intake by females and water consumption by males treated with *XL*-EtOHE (Table 1). Although they are important parameters in study of product safety with therapeutic purposes, cannot infer toxicity to the extract.

In addition to these parameters, changes in body weight and organ weights can be an indicator of adverse effects promoted by the drug. It is considered a sign of toxicity when the animal loses more than 10% of initial body weight [38,39]. In evaluating weight was observed an increase in weight of females at the end of the acute test, which confirms the previous result, where was found an increase in food intake by females treated with *XL*-EtOHE (Table 1). During the 14 days of observation, there were no death, making impossible the determination of LD50 and at the end of the experiment no macroscopic change was observed in organs animals (Table 1).

Table 1. Effect of oral administration of ethanolic extract obtained from *Xylopia langsdorffiana* (2000 mg/kg) on water and food consumption, on body and organ weights in mice over 14 days.

Parameters	**Sex**	**Treatments**	
Food consumption (g)		**Control 12% Tween 80 solution**	***XL*-EtOHE (2000 mg/Kg)**
	Female	8.15 ± 1.49	10.78 ± 2.13***
	Male	9.2 ± 2.7	11.21 ± 3.49
Water consumption (mL)			
	Female	7.84 ± 1.19	8.34 ± 1.41
	Male	11.99 ± 2.95	16.10 ± 3.85**
Body weight (g)			
	Female		
Initial		29.63 ± 2.01	32.52 ± 1.37
Final		32.92 ± 2.00	37.62 ± 1.80**
	Male		
Initial		28.38 ± 1.73	31.62 ± 2.61
Final		30.07 ± 2.64	34.32 ± 5.23
Organ weight (g)			
	Female		
Liver		13.6 ± 0.5	13.6 ± 0.7
Heart		4.2 ± 0.8	3.8 ± 0,42
Kidney		6.0 ± 0.5	6.0 ± 0.4
Spleen		4.2 ± 0.75	3.9 ± 0.3
	Male		
Liver		14.24 ± 1.2	14.6 ± 1.28
Heart		3.90 ± 0.34	3.8 ± 0.27
Kidney		6.4 ± 0.27	6.7 ± 0.28
Spleen		3.7 ± 0.6	3.9 ± 0.5

Data are expressed as mean ± S.D. for six mice. Statistical comparison between 12% tween 80 solution and *XL*-EtOHE 2000 mg/kg was performed using Student t-test. **$p < 0.05$ and ***$p < 0.01$. For the evaluation of organ weights, the values are expressed as mean ± S.D. of arcsine of division of weight of the organs by the weight of animals; Student *t*-test, $p > 0.05$.

Although there have been some changes in the CNS and food intake by females, these data are insufficient to infer toxicity to extract and thus suggest that *XL*-EtOHE has low toxicity to mice treated orally in a single dose of 2000 mg/kg (p.o.), under the conditions evaluated, ensuring safety in the continuity of the study, starting to investigate the pharmacological properties of the species in question, especially the gastroprotective activity.

3.2. Absolute Ethanol-induced Gastric Lesions

Ethanol is used experimentally for causing intense gastric mucosa damage by promoting solubilization of gastric mucus, microcirculation disturbances and after absorption is oxidized and produce toxic metabolites that promotes mitochondrial damage and leads to oxidative stress [40]. In this model ulcer induction in rats, *XL*-EtOHE and *XL*-HexPh, in defined doses, significantly inhibited ulcers when compared to the respective negative controls, suggesting gastroprotective activity (Table 2, figures 2 and 3). Doses of 500 mg/kg for *XL*-EtOHE, and 250 mg/kg for *XL*-HexPh were found to be the most effective.

Table 2. Effect of oral administration of ethanolic extract (EtOHE), hexane phase (HexPh) obtained from *Xylopia langsdorffiana* (*XL*) and carbenoxolone under models of gastric ulcer induced by absolute ethanol in rats.
Data are expressed as mean ± S.D. ANOVA: $F_{(5,29)} = 11.68$ (n=5-6) for *XL*-EtOHE and $F_{(5,30)} = 46.63$ (n=5-7) for *XL*-HexPh in the ethanol model. Dunnett's test: *p<0.05, **p < 0.01, ***p<0.001 for significant differences in relation to control group.

Experimental model	Treatments	Dose (mg/Kg)	UI (mm)	Inhibition (%)
Absolute etanol	**12% Tween 80 solution**		250.8 ± 51	-
	Carbenoxolone	100	151 ± 55.4**	40
	***XL*-EtOHE**	62.5	158.8 ± 56.6*	37
		125	149 ± 60.7**	41
		250	89.8 ± 26.7***	64
		500	46 ± 10.15***	83
	12% Tween 80 solution		251 ± 20.63	-
	Carbenoxolone	100	157.3 ± 28.46***	37
	***XL*-HexPh**	62.5	194 ± 60.96*	23
		125	122.2 ± 18.06***	51
		250	47 ±11.8***	81
		500	41.33 ±14.6***	84

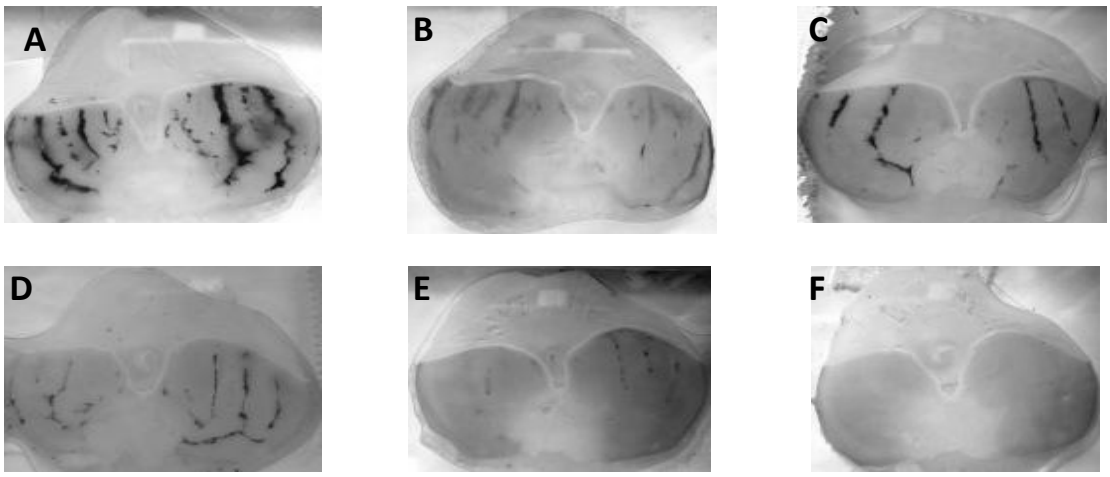


Figure 2. Absolute ethanol-induced gastric lesions model. Stomachs of rats pretreated (p.o.) with 12% Tween 80 solution **(A)**, carbenoxolone 100 mg/kg **(B)**, *XL*-EtOHE (62,5 mg/kg) **(C)**, *XL*-EtOHE (125 mg/kg) **(D)**, *XL*-EtOHE (250 mg/kg) **(E)**, *XL*-EtOHE (500 mg/kg) **(F)**.

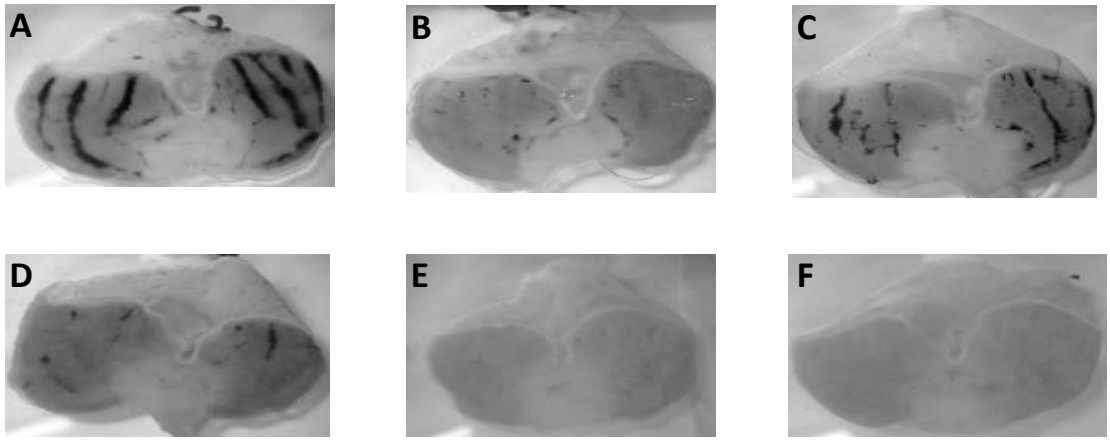


Figure 3. Absolute ethanol-induced gastric lesions model. Stomachs of rats pretreated (p.o.) with 12% Tween 80 solution **(A)**, carbenoxolone 100 mg/kg **(B)**, *XL*-HexPh (62,5 mg/kg) **(C)**, *XL*-HexPh (125 mg/kg) **(D)**, *XL*-HexPh(250 mg/kg) **(E)**, *XL*-HexPh(500 mg/kg) **(F)**.

3.2. Hypothermic Restraint Stress-induced Gastric Lesions

Stomach damage of animals caused by stress involves the neuroimmunendocrine system, which stimulates the autonomic nervous system, activates the hypothalamic-pituitary-adrenal axis (HPA) and stimulates the thermogenic system in the brain, which results in immune system modulation and an inflammatory response [41].

In this experimental gastric ulcer induction model, *XL*-EtOHE and *XL*-HexPh (at all doses) prevented the development of gastric lesions (Table 3). This suggests that the gastroprotective activity of *X. langsdorffiana* might be involved in the regulation of acid secretion, production of both mucus and bicarbonate, restoration of blood flow or the maintenance of endogenous antioxidants to decrease the formation of free radicals.

3.3. Non-steroidal Anti-inflammatory Drug-induced Gastric Lesions

Piroxicam, a non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic activity causes hemorrhagic gastric mucosa lesions in about 20% of patients with osteoarthritis [42]. NSAIDs induce injury/bleeding by two key pathways: direct cytotoxic effects (local action) on the epithelium and (most importantly) systemic inhibition of cyclooxygenases (COX-1 and COX-2) which induces gastric ulceration by suppressing prostaglandin synthesis [43].

In the model of ulcer induction by NSAIDs it was found that *XL*-EtOHE (at 125, 250 and 500 mg/kg) and *XL*-HexPh (at 62.5, 125, 250 and 500 mg/kg) significantly inhibited gastric lesions induced by Piroxicam (Table 3), suggesting that *X. langsdorffiana* gastroprotective activity works via cytoprotective mechanisms, such as prostaglandin mediators.

Table 3. Effect of oral administration of ethanol extract (EtOHE), hexane phase (HexPh) obtained from *Xylopia langsdorffiana* (*XL*) and cimetidine under models of gastric ulcer induced by hypothermic restraint stress and non-steroidal anti-inflammatory (NSAIDs) (Piroxicam).

Experimental models	Treatments	Dose (mg/Kg)	UI (mm)	Inhibition (%)
Stress	**12% Tween 80 solution**		178.3 ± 12.4	-
	Cimetidine	100	89.8 ± 16.5***	50
	***XL*-EtOHE**	62.5	93.8 ± 11.7***	47
		125	88.7 ± 17.12***	50
		250	86 ± 2.8***	52
		500	93.2 ± 9.7***	48
	12% Tween 80 solution		167.6 ± 33.2	-
	Cimetidine	100	117 ± 27.15**	30
	***XL*-HexPh**	62.5	113.7 ± 23.1**	32
		125	95.3 ± 21.4***	43
		250	73.9 ± 15.3***	56
		500	41.6 ±11.2***	75
NSAIDs	**12% Tween 80 solution**		97 ± 11.8	-
	Cimetidine	100	48.5 ± 6.3***	50
	***XL*-EtOHE**	62.5	67.1 ± 12.2***	31
		125	29.5 ± 10.3***	70
		250	28.3 ± 2.9***	71
		500	27.2 ± 1.6***	72
	12% Tween 80 solution		120.7 ± 8.9	-
	Cimetidine	100	67.83 ± 20.56***	44
	***XL*-HexPh**	62.5	110 ± 20.49	9
		125	69.33 ± 20.98***	42
		250	50.3 ± 24.5***	58
		500	47.4 ± 13***	61

Data are expressed as mean ± S.D. ANOVA: $F_{(5,29)}$ = 47.88 (n=5-6) to *XL*-EEtOH and $F_{(5,31)}$ = 22.37 (n=5-7) to *XL*-FaHex in the hypothermic restraint stress model and $F_{(5,29)}$ = 54.43 (n=5-7) to *XL*-EEtOH $F_{(5,29)}$ = 15.14 (n=5-6) to *XL*-FaHex in the NSAIDs (Piroxicam) model. Dunnett's test: **p< 0.01, ***p<0.001 for significant differences in relation to control group.

3.4. Shay Ulcer and Evaluation of Biochemical Parameters of Gastric Juice

In view of the gastroprotective activity of *X. langsdorffiana*, the next step was to investigate the action of plant samples on gastric secretion through biochemical parameters (pH, concentration of H^+ and volume of gastric juice) and to assess the UI in the pylorus ligature-induced gastric lesions model. In this protocol, secretion and accumulation of gastric acid are the most important factors for causing auto-digestion of the gastric mucosa and break down of the gastric mucosal barrier and it also causes an increase in calcium levels, which in turn is known to stimulate free radical generation [32,44].

Administrations (oral and intraduodenal) of both *XL*-EtOHE (500 mg/kg) and *XL*-HexPh (250 mg/kg) significantly reduced the rate of ulcerative lesion (Table 4). This occurred without altering any of the biochemical parameters of gastric juice assessed (pH, $[H^+]$, and gastric juice volume), as compared to the negative control group in both treatments (p.o. or i.d.). The data suggests that the gastroprotection promoted by *XL*-EtOHE and *XL*-HexPh is not linked to anti-secretory mechanisms.

To better understand the mechanisms involved in the gastroprotective activity of EtOHE and HexPh from *Xylopia langsdorffiana*, experimental models were performed in order to investigate the involvement of mucus, sulfhydryl groups and nitric oxide.

Table 4. Effects of administration of ethanol extract (EtOHE), hexane phase (HexPh) obtained from *Xylopia langsdorffiana* (*XL*) and cimetidine administered orally (p.o.) and intraduodenally (i.d.) on the ulcerative index and biochemical parameters of gastric juice obtained from pylorus-ligature rats.

Model	Treatments	Dose (mg/Kg)	pH	$[H^+]$ (mEq/mL/4h)	Gastric volume (g)	UI (mm)	Inibition (%)
[a] Pylorus ligature (p.o.)	**12% Tween 80 solution**		3.1 ± 0.2	11.8 ± 1.9	1.6 ± 0.57	156.2 ± 22.77	---
	Cimetidine	100	6.5 ± 0.9***	4.7 ± 1.5***	0.62 ± 0.25***	117.3 ± 14.98**	25
	***XL*-EtOHE**	500	3.09 ± 0.2	13.9 ± 1.3	1.84 ± 0.3	72.57 ±18.19***	54
	12% Tween 80 solution		3.3 ± 0.3	14.3 ± 2.8	1.2 ± 0.4	134.4 ± 19.01	---
	Cimetidine	100	6.7±0.42***	6.6 ± 2.3***	0.69 ± 0.3*	104.2 ± 11.09*	22
	***XL*-HexPh**	250	3.1 ± 0.35	14.4 ± 4.2	1.6 ± 0.3	88.6 ± 15.32***	34
[b] Pylorus ligature (i.d.)	**12% Tween 80 solution**		3.7 ± 0.50	12.5 ± 2.5	0.6 ± 0.2	213.7 ± 18.52	---
	Cimetidine	100	4.5 ± 0.70*	7.7 ± 2.9**	0.57 ± 0.2	140.3 ±20.12***	34
	***XL*-EtOHE**	500	3.4 ± 0.08	13.2 ± 1.3	0.60 ± 0.2	137.5 ±23.14***	36
	12% Tween 80 solution		2.8 ± 0.3	13.8 ± 1.7	0.9 ± 0.3	185.2 ± 9.9	---
	Cimetidine	100	3.3 ± 0.1***	9.3 ± 1.3***	0.63 ± 0.18*	129 ± 12.5***	30
	***XL*-HexPh**	250	2.7 ± 0.2	14.7 ± 0.6	0.7 ± 0.2	101.2 ±18.19***	45

[a]Data are expressed as mean ± S.D. ANOVA: $F_{(2, 17)} = 86.15$ (EtOHE) / $F_{(2, 17)} = 224.2$ (HexPh) to pH; $F_{(2, 15)} = 53.82$ (EtOHE) / $F_{(2, 16)} = 13{,}47$ (HexPh) to $[H^+]$; $F_{(2,18)} = 17.88$ (EtOHE) / $F_{(2, 14)} = 12.83$ (HexPh) to volume; $F_{(2,15)} = 30.09$ (EtOHE) / $F_{(2,13)} = 11.8$ (HexPh) to UI (n=5-7 / p.o.).

[b]$F_{(2, 18)} = 9.3$ (EtOHE) / $F_{(2, 17)} = 15.11$ (HexPh) to pH; $F_{(2, 17)} = 10.59$ (EtOHE) / $F_{(2, 15)} = 31.44$ (HexPh) to $[H^+]$; $F_{(2,15)} = 0.04203$ (EtOHE) / $F_{(2, 17)} = 3.051$ (HexPh) to volume; $F_{(2,15)} = 26.15$ (EtOHE) / $F_{(2,15)} = 56.40$ (HexPh) to UI (n = 6 / i.d.). Dunnett's test: *p<0.1, **p < 0.01, ***p<0.001 for significant differences in relation to control group.

3.5. Determination of mucus adhering to gastric mucus

Gastric mucus consists of a viscous, elastic, adherent and transparent gel formed from water and glycoproteins that covers the entire gastrointestinal mucosa. The protective properties of the mucus barrier against proteolytic digestion and acidity depend not only on the gel structure, but also on the layer thickness or quantify which covers the mucosal surface [45,1,46].

In assessing the involvement of gastric mucus, it was observed that *XL*-HexPh did not increase its levels (Figure 4), suggesting that the gastroprotective activity of *XL*-HexPh has no connection with the increase in the production this protective agent.

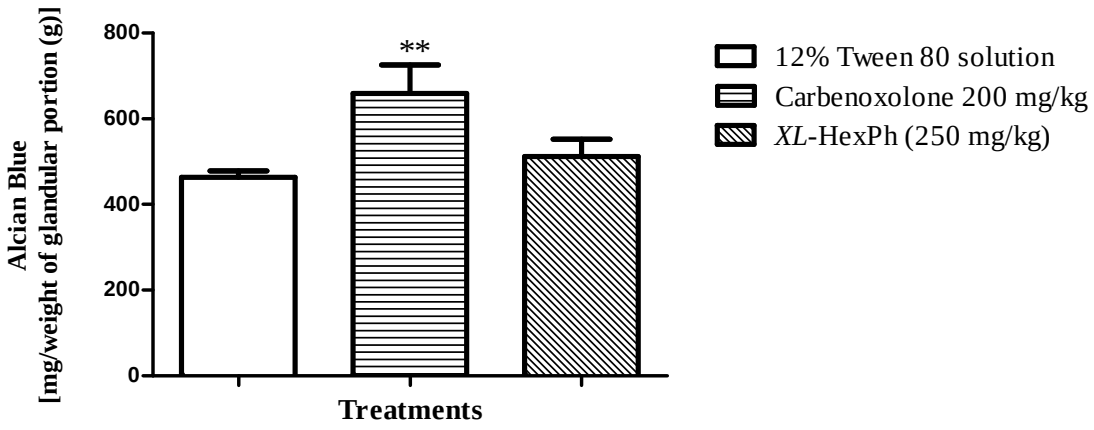


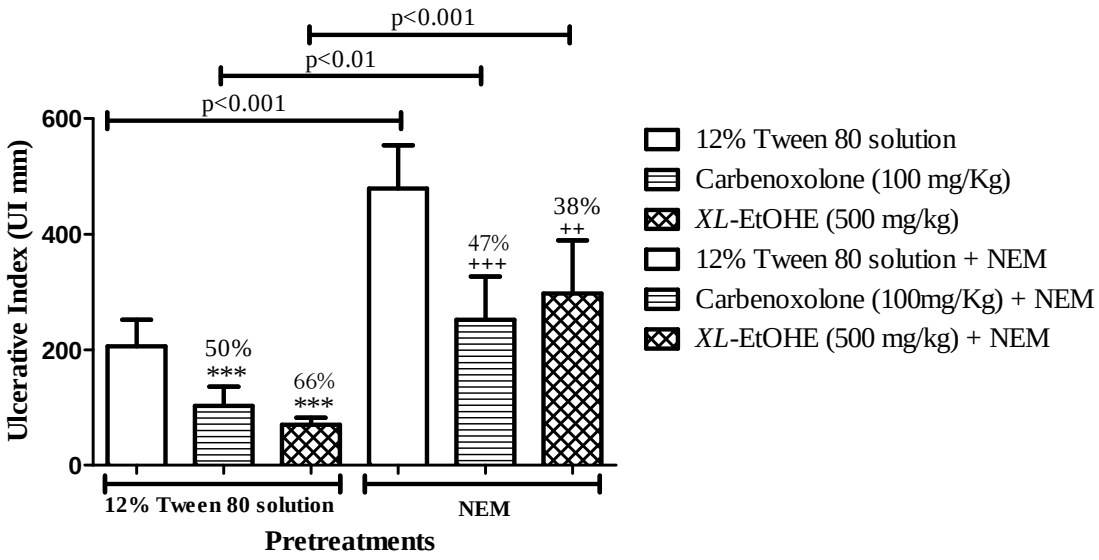
Figure 4. Effect of oral administration of hexane phase (HexPh) obtained from *Xylopia langsdorffiana* (*XL*) and carbenoxolone under mucus content in pylorus-ligature rats. Data are expressed as mean ± S.D. ANOVA: $F_{(2, 19)} = 5.47$. Dunnett's test: **p < 0.01 (n = 7-8) for significant differences in relation to control group.

3.6. Involvement of Nitric Oxide (NO) and Sulfhydryl Compounds (SH) in Gastric Protection Against Ethanol-induced Gastric Lesions in NEM or L-NAME Pretreated Rats

The roles of endogenous sulfhydryl compounds (SH) and nitric oxide (NO) were also investigated because these substances are involved in gastric mucosa protection. These factors maintain the gastric mucosa as well as the condition and integrity of local microcirculation. Among other factors, locally released SH group mediators are involved in the regulation of gastric function, while NO is a vasoactive agent that may play a central role in mucosal defense and also ulcer healing thru stimulation of growth factors [47].

Glutathione is the major non-protein sulfhydryl (NP-SH) of the gastric mucosa and is one of the most important cytoprotective mechanisms [48]. It prevents tissue damage by keeping ROS at low levels and at certain cellular concentrations [47]. GSH and GSH-related enzymes are accepted as antioxidant protective factors in tissues, preventing injuries from luminal acid, bacteria and noxious agents [50].

One way to investigate the participation of SH groups in protecting the gastric mucosa is through administration of N-ethylmaleimide (NEM), an SH blocking agent which results in a higher incidence of ulcerative lesions induced by absolute ethanol [51,34]. In this experiment the *XL*-EtOHE (500 mg/kg, p.o.) and *XL*-HexPh (250 mg/kg, p.o.) significantly inhibited the ulcerative lesions induced by absolute ethanol, both in the absence or presence of NEM. There was a significant difference between groups pretreated with 12% Tween 80 solution and NEM (Figure 5 and 6; Figure 7), suggesting that the gastroprotective effect of XL-EtOHE and XL-HexPh gastroprotective effect depend on the production and/or presence of SH compounds and revealing that antioxidant drugs play an important role in the treatment of peptic ulcers.



Figure

Figure 5. Effect of oral administration ethanol extract (EtOHE) obtained from *Xylopia langsdorffiana* (*XL*) and carbenoxolone on gastric lesions induced by ethanol in rats pre-treated with NEM (a blocker of SHs). Results expressed as mean± S.D. ANOVA followed by Dunnett's test. Pre-treatment: 12% Tween 80 solution ***p < 0.001, $F_{(2,17)} = 28,61$; NEM $^{++}p < 0.01$, $^{+++}p < 0.001$, $F_{(2,14)} = 13,82$ and Tukey's test ($F_{(5,31)} = 41,19$).

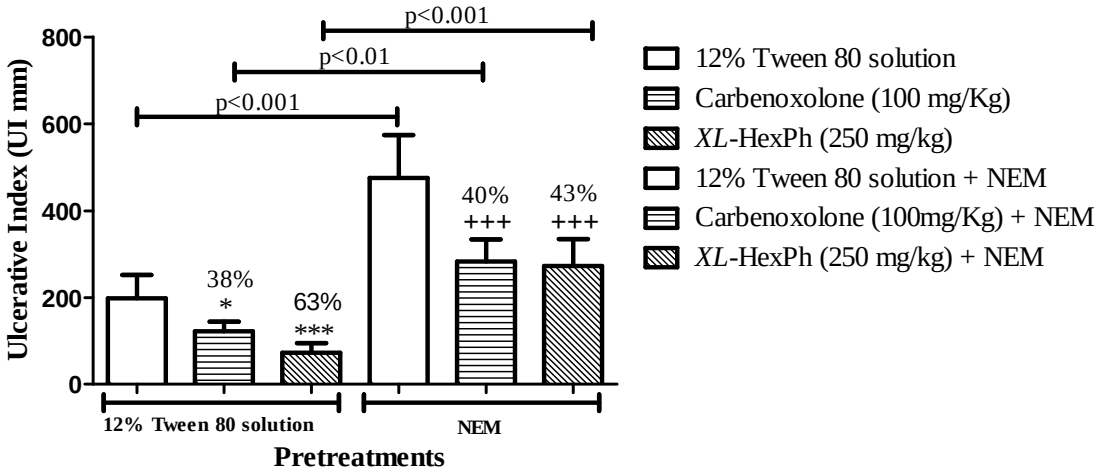


Figure 6. Effect of oral administration hexane phase (HexPh) obtained from *Xylopia langsdorffiana* (*XL*) and carbenoxolone on gastric lesions induced by ethanol in rats pre-treated with NEM (a blocker of SHs). Results expressed as mean± S.D. ANOVA followed by Dunnett's test. Pre-treatment: 12% Tween 80 solution *p<0,05, ***p < 0.001, $F_{(2,12)} = 15,47$; NEM, $^{+++}p < 0.001$, $F_{(2,15)} = 14,48$ and Tukey's test ($F_{(5,27)} = 31,73$).

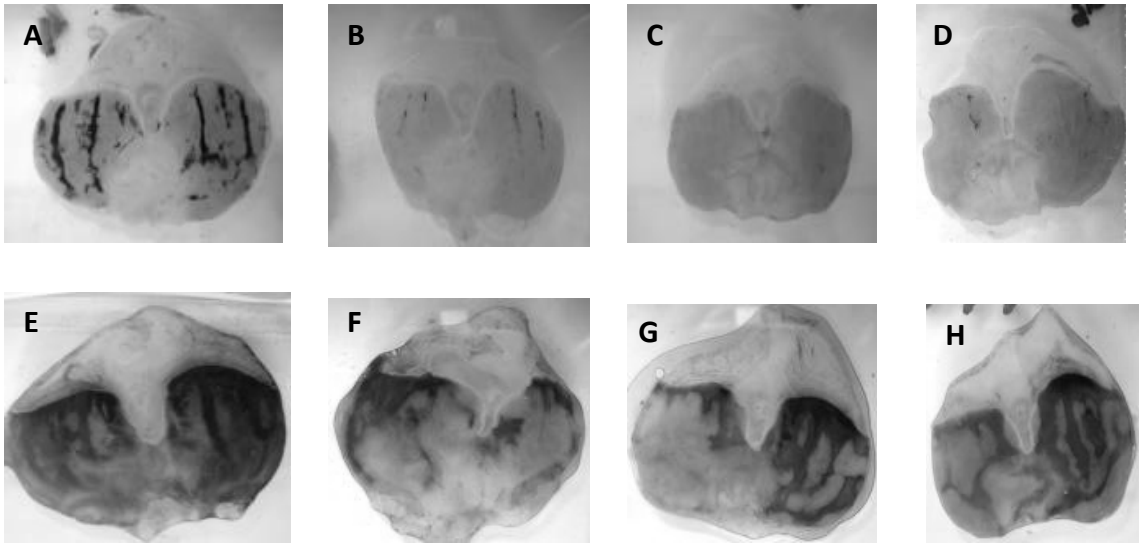


Figure 7. Involvement of sulfhydryl compounds (SH) in gastric protection in Ethanol-induced Gastric Lesions NEM pretreated rats. Stomachs of rats treated (i.p./p.o.) with tween/tween **(A)**, tween/carbenoxolone **(B)**, tween/EtOHE **(C)**, tween/HexPh **(D)**, NEM/tween **(E)**, NEM/carbenoxolone **(F)**, NEM/EtOHE **(G)**, NEM/HexPh **(H)**.

It's well known that NO, presents a short half-life, is produced by nitric oxide synthase, and is implicated in mechanisms that regulate the gastric blood flow, it also reduces lipid peroxidation [52] and stimulates protective gastric mucus secretion due to the activation of the guanylate cyclase enzyme [53]. NO protects the gastric mucosa against ethanol-induced lesions and endothelin. L-NAME inhibits NOS, resulting in endogenous mediator decreases and consequent lesion of the stomach lining [34].

Thus, it was observed that *XL*-EtOHE (500 mg/kg, po) and *XL*-HexPh (250 mg/kg, p.o.) significantly inhibited the ulcerative lesions induced by absolute ethanol, both in the absence or presence of L-NAME, which blocks NO synthesis. There was a significant difference between groups pretreated with 12% Tween 80 solution and L-NAME (Figure 8, 9 and 10), there being an UI exacerbation in the latter group, which suggests that NO is involved in the gastroprotetion promoted by *X. langsdorffiana*.

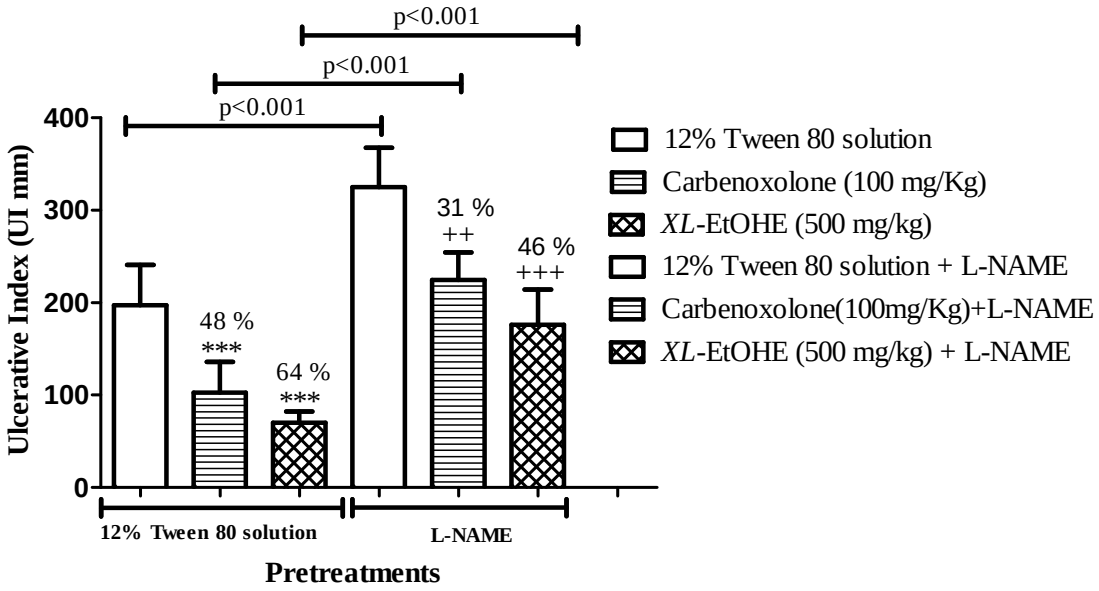


Figure 8. Effect of oral administration ethanol extract (EtOHE) obtained from *Xylopia langsdorffiana* (*XL*) and carbenoxolone on gastric lesions induced by ethanol in rats pre-treated with with L-NAME (an inhibitor of NO synthase). Results expressed as mean± S.D. ANOVA followed by Dunnett's test (Pre-treatment: 12% Tween 80 solution ***$p < 0.001$, $F_{(2,16)} = 25,07$; NEM, $^{++}p< 0.01$, $^{+++}p < 0.001$, $F_{(2,13)} = 22,47$) and Tukey's test ($F_{(5,29)} = 41,29$).

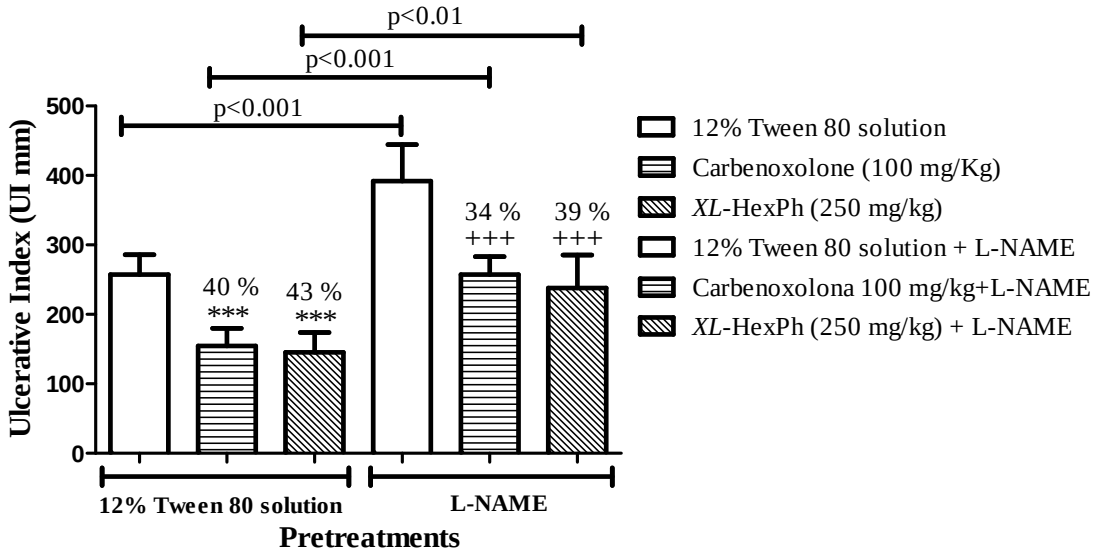


Figure 9. Effect of oral administration hexane phase (HexPh) obtained from *Xylopia langsdorffiana* (*XL*) and carbenoxolone on gastric lesions induced by ethanol in rats pre-treated with with L-NAME (an inhibitor of NO synthase). Results expressed as mean±S.D. ANOVA followed by Dunnett's test (Pre-treatment: 12% Tween 80 solution ***$p < 0.001$, $F_{(2,16)} = 34,13$; NEM, $^{++}p< 0,01$, $^{+++}p < 0.001$, $F_{(2,15)} = 22,19$) and Tukey's test ($F_{(5,31)} = 36,75$).

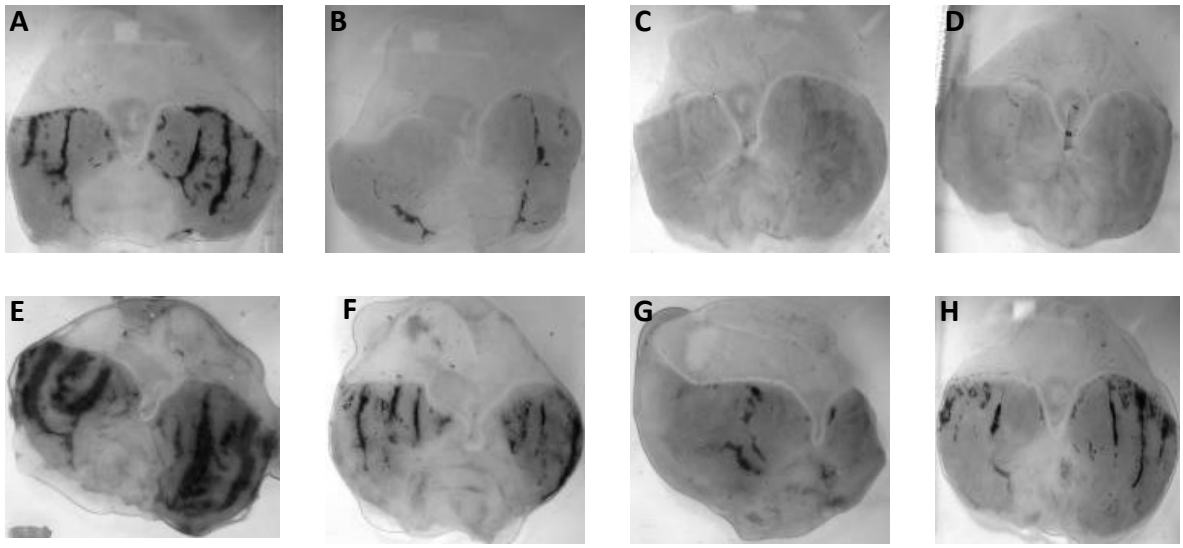


Figure 10. Involvement of nitric oxide (NO) in gastric protection in Ethanol-induced Gastric Lesions in L-NAME-pretreated rats. Stomachs of rats treated (i.p./p.o.) with tween/tween **(A)**, tween/carbenoxolone **(B)**, tween/EtOHE **(C)**, tween/FaHex **(D)**, L-NAME/tween **(E)**, L-NAME/carbenoxolone **(F)**, L-NAME/EtOHE **(G)**, L-NAME/HexPh **(H)**.

Ethanol extract and hexane phase obtained from the leaves of *Xylopia langsdorffiana* showed gastroprotective activity in varied models of ulcer induction with the participation of sulfhydryl groups (as antioxidants) and the nitric oxide pathway (in gastric microcirculation). Anti-secretory mechanisms and increased mucus production were most likely not involved. It is probable that the observed actions occur at least in part, because of the presence of diterpenes, substances that phytochemically characterize the species studied.

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