



Original Article

Copaifera langsdorffii: evaluation of potential gastroprotective of extract and isolated compounds obtained from leaves



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ABSTRACT

Gastric ulcer is a prevalent gastrointestinal disease, and the drugs currently used in the treatment produce several adverse effects. In this context, the search for new therapeutic antiulcer agents is essential, and medicinal plants have great potential. Here, we investigated the gastroprotective properties of *Copaifera langsdorffii* Desf., Fabaceae, hydroalcoholic extract obtained from leaves and its isolated compounds. The phytochemistry studies and the compounds isolations were performed using chromatographic and spectroscopic methodologies. The hydroalcoholic extract was evaluated using ethanol/HCl, non-steroidal anti-inflammatory drug, stress-induced-ulcer and chronic ulcer-model. The effects on gastric content volume, pH, total acidity and mucus stomach production were evaluated in the pylorus ligated-model. The *C. langsdorffii* extract obtained from leaves (50, 250 or 500 mg/kg) reduced the injured area compared to control group in all experiments. The extract showed a significant decrease in the total gastric juice acidity and an increase in mucus production (500 mg/kg) when compared to vehicle. Among isolated compounds (30 mg/kg) α -humulene, β -caryophyllene and caryophyllene oxide showed greater gastroprotective activity in the ethanol/HCl induced ulcer model. The data herein obtained shown that *C. langsdorffii* leaves extract and isolated compounds from it, presented gastroprotective properties in different animal models of gastric ulcer. These effects may be associated with the ability of the extract to decrease gastric secretion and increase the mucus production.

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Introduction

Gastric ulcer is a chronic/recurrent disease and currently is the most predominant gastrointestinal disease. Studies have shown that gastric ulcer occurs in at least 10% of the world's population (Freire et al., 2011; Shimoyama et al., 2013). The etiology of disease is not completely understood, however it is known that the extent of injuries to the gastric mucosa depends on the balance between aggressive and protective factors (Banic'et al., 2011). The major protective factors include adequate blood flow, secretion of prostaglandins, mucus, and bicarbonate by resident mucosal cells. Aggressive agents include the increased secretion of hydrochloric acid and pepsin, inadequate dietary habits, the consumption of nonsteroidal anti-inflammatory drugs and alcohol, stressful conditions, and infection by *Helicobacter pylori* (Klein-Júnior et al., 2012).

Due to the high incidence of gastric ulcers, distinct pharmacological therapies have been investigated. The main current therapies are histamine receptor antagonists (H₂-Ras) and/or proton pump inhibitors (PIPs), which acts by inhibiting the gastric secretion (Banic'et al., 2011). However their efficacy is limited by side effects such as increased risk of bone fractures (Vestergaard et al., 2006) and several drug–drug interactions (Ogawa and Echizen, 2010). In this way, there is a clear demand for new therapies for gastric ulcers and plant extracts, or their isolated compounds, provide a potential treatment for the disease (Klein-Júnior et al., 2012).

The *Copaifera* species are popularly known in Brazil as “copaiba.” These species are large trees that grow mainly in the states of Amazonas, Pará and Ceará localized in Northern Brazil (Veiga-Junior and Pinto, 2002). The *Copaifera* oleoresin is a transparent liquid whose color ranges from yellow to light brown. This oil has been used in folk medicine as an anti-inflammatory, antitumor, antituberculosis, urinary antiseptic, and to treat bronchitis and skin diseases (Veiga-Junior and Pinto, 2002; Paiva et al., 2004; Veiga-Junior et al.,

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2007; Gramosa et al., 2010; Leandro et al., 2012). Of the 72 species of trees belonging to the genus *Copaifera* only seventeen species have been chemically studied, and the analyses are limited to the oleoresin (Gramosa et al., 2010; Leandro et al., 2012). Among the volatile compounds of the oleoresin characterized by GC/MS are the sesquiterpenes β -caryophyllene, caryophyllene oxide, α -copaene, α -humulene, γ -muurolene, and β -bisabolol (Veiga-Junior et al., 2007). The main non-volatile components belong to the diterpene class, including kaurenoic acid, kaurenol, copalic acid, agathic acid, and hardwickic acid (Souza et al., 2011). Palmitic and oleic acids from non-polar extract of copaiba seeds have been quantified by GC/MS (Stupp et al., 2008).

Copaifera langsdorffii Desf., Fabaceae, is a medicinal plant found in all regions of Brazil. The extracts obtained from their aerial parts have commercial value and it is an ingredient in beekeeping and in cosmetic products (Veiga-Junior and Pinto, 2002; Gramosa et al., 2010). The hydroalcoholic extract of *C. langsdorffii* leaves have been reported to have leishmanicidal and antimalarial activities (Sousa et al., 2012) and to be active in animal models of urolithiasis (Brancaion et al., 2012) and nephrolithiasis (Oliveira et al., 2013). The antiulcer activity of the oleoresin from *C. langsdorffii* has been previously reported (Paiva et al., 1998, 2004). However this is the first work that reports the therapeutic antiulcer potential of *C. langsdorffii* leaves and its major compounds. This study aim to investigate the gastroprotective and healing properties the *C. langsdorffii* leaves extract and its main isolated compounds using different animal models of gastric ulcer.

Material and methods

Drugs, reagents and solvents

Cimetidine, omeprazole, carbenoxolone, nitro-L-arginine methyl-ester (L-NAME), and N-ethyl-maleimide (NEM) were acquired from Sigma Aldrich (St. Louis, MD, USA). All solvents used for chromatographic analysis were HPLC grade (Tedia, Fairfield, OH, USA). Water was obtained using Milli-Q system (Millipore, MA, USA). All other reagents and solvents were of analytical grade.

Plant material

Copaifera langsdorffii Desf., Fabaceae, leaves were collected in the Ribeirão Preto in the campus of the University of São Paulo, Brazil, at the School of Pharmaceutical Sciences (21° 10' 16" S, 47° 50' 47" W), was identified by Dr. Milton Groppo Junior and a voucher specimen (SPFR 10120) was deposited in the herbarium of the Department of Biology of the University of São Paulo, Ribeirão Preto, SP, Brazil.

Phytochemical methods

Dried and powdered *C. langsdorffii* leaves (3 kg) were macerated for 72 h with 70% aqueous ethanol at room temperature and percolated. The procedure was repeated three times, and the combined hydroalcoholic extract was filtered and concentrated under reduced pressure at 50 °C furnishing the crude extract (520 g; 17.30%). Following, 50 g of this extract were dissolved in 1 l of 90% aqueous methanol and sequentially partitioned with 400 ml of hexanes, chloroform and ethyl acetate (EtOAc). The hexanes fraction (20 g) was fractionated by a sequential series of the three classics chromatography on a silica gel 70–230 mesh (Merck) column and the main samples were purified by preparative thin layer chromatography (TLC) furnishing the compounds 1 and 2. The ethyl acetate crude fraction (6 g) was also submitted to classic chromatography similarly as described for the hexanes fraction, and eluted with increasing amounts of EtOAc in hexanes. The obtained fractions were combined based on similarities in TLC.

Ten different fractions were obtained. The fraction F1 (1 g) was fractionated over silica gel 70–230 mesh (Merck) using classic chromatography and isocratic system, hexanes: EtOAc 9:1 v/v as mobile phase, to furnish compound 3. The fraction F2 (2 g) was purified as described for fraction F1 using isocratic system, hexanes: EtOAc 4:1 v/v, giving compound 4. The fraction F6 (1 g) was purified by semi-preparative reverse-phase HPLC, furnishing compounds 5 and 6. The chemical structures of these compounds and its purity levels were determined by GC, HPLC, MS, ¹H, ¹³C, HMBC and HMQC NMR (Bruker ARX 400 spectrometer) analyses.

Animals

Swiss mice (25–30 g) were provided by the Central Animal House of the University of Vale do Itajaí, SC, Brazil. These animals were housed and cared for in accordance with the Federal Government Legislation on Animal Care. All experiments were authorized by the Ethical Committee for Animal Care (protocol number: 10.1.1800.53.3). The animals were housed in groups of six, in standard cages, at room temperature (25 ± 3 °C), with 12 h dark/light cycles, and food and water *ad libitum*. In all the experiments, the mice were kept in cages with wide-mesh, raised floor, to prevent coprophagy. All procedures as described hereafter were carried out with the mice kept in fasting for 18 h with only water *ad libitum*.

Gastroprotective effect against different ulcer models

Ethanol/HCl-induced ulcer model

The mice were divided into five groups of six animals each accordingly to published by Mizui and Douteuchi (1983). The first group was given 10 ml/kg of saline used as vehicle, and the second group was treated with omeprazole (30 mg/kg). The remaining three groups received 50, 250 or 500 mg/kg of *C. langsdorffii* extract, respectively. In another experiment, isolated compounds (α -humulene, β -caryophyllene, caryophyllene oxide, kaurenoic acid, quercitrin and afzelin) were tested at a dose of 30 mg/kg, this dose was employed based on omeprazole dose. All treatments were administered orally. One hour after treatment, all mice received 0.2 ml of an ethanol/HCl solution (60%/0.3 M) to induce gastric ulcer. One hour later, the animals were euthanized and the stomachs were removed and opened along the greater curvature. The stomachs were gently rinsed with water to remove the gastric contents and blood clots, for subsequent scanning. The images obtained were analyzed using specific "EARP" software to measure each lesion point. The ulcers areas were classified as level I ($\leq 1 \text{ mm}^2$); level II, ($> 1 \leq 3 \text{ mm}^2$); level III, ($> 3 \text{ mm}^2$). The following parameters were determined: (a) total area of lesion; (b) percentage of lesion area in relation to total stomach area; (c) ulcerative lesion index (ULI) as $1 \times (\text{number of ulcers level I}) + 2 \times (\text{number of ulcers level II}) + 3 \times (\text{number of ulcers level III})$ and (d) curative index, which was determined as follows: $\% C = 100 - (\text{ULI}_{\text{treated}} \times 100 / \bar{x} \text{ULI}_{\text{control}})$.

Nonsteroidal anti-inflammatory drug (NSAID)-induced ulcer in cholinomimetic-treated mice

Five groups of six animals each were randomly separated accordingly to published procedures (Rainsford, 1987). The first group was given 10 ml/kg of saline, and the second group was treated with cimetidine (100 mg/kg). The remaining three groups received 50, 250 or 500 mg/kg of *C. langsdorffii* crude extract, respectively. All the treatments were administered orally. One hour after treatment, all mice received a combination of indomethacin (100 mg/kg, *p.o.*) and bethanechol (5 mg/kg, *i.p.*) to induce gastric ulcer. Five hours after the treatment with NSAID/bethanechol,

animals were euthanized and the stomachs were removed and opened along the greater curvature. The stomachs were gently rinsed with water to remove the gastric contents and blood clots, for subsequent scanning. The images obtained were analyzed using the parameters previously described.

Hypothermic restraint stress-induced lesions

Groups of six animals ($n=6$) were treated, the first group was given 10 ml/kg of saline used as vehicle, and the second group was treated with omeprazole (30 mg/kg). The remaining three groups received 50, 250 or 500 mg/kg of *C. langsdorffii* extract, and 30 min after, each animal was placed in a contensor tube and immersed vertically until the water reached the neck region in a tank with current water at 25 °C for 17 h. After this period, the animals were euthanized and the stomachs were removed, opened along the greater curvature, gently rinsed with water to remove gastric contents and blood clots to be scanned later. The obtained images were analyzed using the parameters described above.

Gastroprotective action mechanism

Determination of gastric secretion

Groups of six animals each were randomly divided and they were anesthetized with ketamine (2 mg/ml; *i.p.*) and xylazine (5 mg/ml; *i.p.*); the abdomen was incised and the pylorus was ligated (Shay et al., 1945). Immediately after pylorus ligation, *C. langsdorffii* crude extract was administered at doses of 50, 250 or 500 mg/kg, respectively. Cimetidine (100 mg/kg) was used as positive control, and 10 ml/kg of saline was administered as negative control. All the samples were administered intraduodenally. This route was used to determine the systemic effect of the extract. Four hours later, the animals were euthanized; the abdomen was opened and another ligation was placed at the esophageal end. The stomachs were removed and the gastric content collected and centrifuged at 3000 rpm (8.000 × g, 25 °C, 10 min). The amount of gastric acid (ml) and the pH values were determined. Total acid secretion in the gastric juice was determined in the supernatant volume by titration to pH 7.0 using a 0.01 mol l⁻¹ NaOH solution, and phenolphthalein as indicator.

Determination of mucus in gastric content

The mice being five groups of six animals each were anesthetized with ketamine (2 mg/ml; *i.p.*) and xylazine (5 mg/ml; *i.p.*); the abdomen was incised and the pylorus was ligated. Immediately after pylorus ligation, *C. langsdorffii* crude extract was administered at doses of 50, 250 or 500 mg/kg, respectively. Carbenoxolone (200 mg/kg) was used as positive control, and 10 ml/kg of saline was administered as negative control. All treatments were administered intraduodenally. The animals were killed four hours after the treatments. The stomach content was immersed in 10 ml of 0.02% Alcian blue 0.16 M sucrose/0.05 M sodium acetate solution, pH 5.8, and incubated for 24 h at 20 °C. The Alcian blue binding extract was centrifuged at 3000 × g for 10 min. The absorbance of supernatant was measured by spectrophotometer at 615 nm. The free mucus in the gastric content was calculated from the amount of Alcian blue binding per gram of stomach tissue (mg/wt stomach tissue g) (Sun et al., 1991).

Role of nitric oxide in gastroprotection

The mice were placed randomly in eight cages, with six animals per cage (Arrieta et al., 2003). Four groups were pre-treated with L-NAME (an inhibitor of nitric oxide synthase), 70 mg/kg (*i.p.*), and the other four groups were pre-treated with saline (1 ml/kg, *i.p.*). Thirty

minutes later, animals received oral doses of the vehicle (saline), carbenoxolone (200 mg/kg) or 500 mg/kg of *C. langsdorffii* crude extract. After 60 min, all groups received 0.5 ml (*p.o.*) of a solution of ethanol/HCl (60%/0.3 M) for gastric-ulcer induction. The animals were euthanized 1 h after the administration of ethanol/HCl, and the stomachs were removed to determine the percentage of gastric lesion area.

Role of sulfhydryl groups in gastroprotection

The mice were placed randomly in eight cages, with six animals per cage. Four groups were pre-treated with NEM (a sulfhydryl depletor) 10 mg/kg (*i.p.*) and the other four groups were pre-treated with saline (1 ml/kg, *i.p.*) (Matsuda et al., 1999). Thirty minutes later, animals received oral doses of saline, carbenoxolone (200 mg/kg) or 500 mg/kg of *C. langsdorffii* crude extract. After 60 min, all the groups received 0.5 ml (*p.o.*) of a solution of ethanol/HCl (60%/0.3 M) for gastric-ulcer induction. The animals were killed one hour after the administration of ethanol/HCl, and the stomachs removed to determine the percentage of gastric lesion area.

Chronic ulcer model

Acetic acid-induced chronic ulcer

The animals were anesthetized with ketamine (2 mg/ml; *i.p.*) and xylazine (5 mg/ml; *i.p.*), and the abdomen was incised. After exposing the stomach, 0.02 ml (v/v) of 20% acetic acid solution was injected into the submucosal layer in the junction between the antrum and the fundus (Takagi et al., 1969). The stomach was bathed with saline to avoid adherence to the external surface of the ulcerated region. Then, the stomach was re-internalized and the cut was sutured. After 24 h, mice were divided into different groups ($n=6$) and treated by gavage with 10 ml/kg of saline, cimetidine (100 mg/kg) or 500 mg/kg of *C. langsdorffii* crude extract once a day during seven days. At the end of the treatments, after 12 h fasting, rats were euthanized. The stomachs were removed and opened along the greater curvature. The stomachs were removed and processed as for ethanol/HCl-induced ulcer used protocol. Also, the curative ratio was calculated by the formula: % C = 100 – (area_{treated} × 100/area_{control}).

Toxicity assay

Acute toxicity study

Acute toxicity study was performed as described in “Guidelines for Testing of Chemicals-Acute Oral Toxicity-Fixed Dose Procedure” (OECD 420, 2001). Five female rats were treated orally with a single dose of 2000 mg/kg of *C. langsdorffii* crude extract. After the administration animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention during the first 4 h) and daily thereafter for a period of 14 days. Once a day the animals were observed, principally in relation to changes in skin and fur, eyes and mucous membrane (nasal) and also autonomic changes (salivation, lacrimation, perspiration, piloerection, urinary volume, and defecation) and alterations to the central nervous system (ptosis, drowsiness, gait, tremors and convulsion). Food and water were provided throughout the experiment.

Statistical analysis

The data are reported as mean ± standard error of the mean (SEM) and were compared using one-way analysis of variance

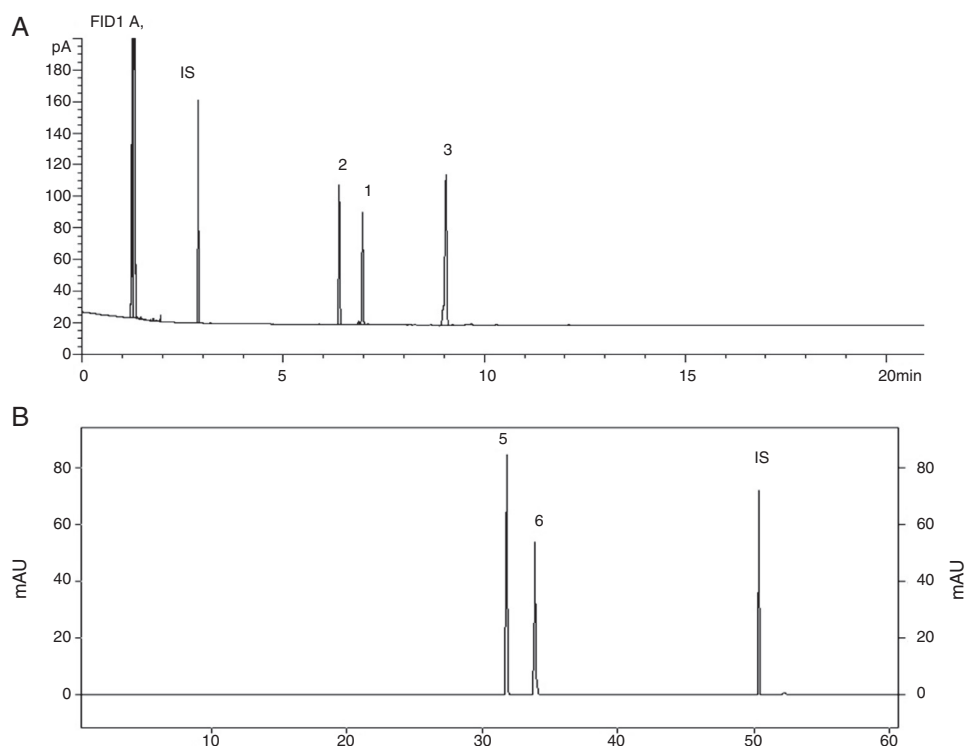


Fig. 1. A. Chromatographic profile by GC-FID of the non-polar purified compounds. 1, 2, 4, 5-tetramethylbenzene is internal standard (IS). B. Chromatographic profile by LC-UV/DAD of the polar purified compounds (5 and 6). Benzophenone is the IS. Kaurenoic acid was evaluated mainly by NMR spectra (see supplementary material) and also HRMS with m/z $[M-1]$ 301.2197 and $C_{20}H_{29}O_2$.

(ANOVA), followed by Dunnett's pairwise test or Tukey test, with p values <0.05 were considered significant.

Results and discussion

Considering that interaction between phytochemistry and pharmacology is essential for development of studies involving therapeutic potential of vegetable species, the first step of this study was undertake phytochemical analysis of *Copaifera langsdorffii* extract. The GC/MS analyses of the non-polar samples of the leaves of *C. langsdorffii* revealed the presence of sesquiterpenes including α -copaene, α -bergamotene, β -caryophyllene, α -humulene, caryophyllene oxide, bicyclogermacrene, germacrene D, germacrene B, δ -cadinene and α -cadinol. The major compounds in these samples were α -humulene (**1**) and β -caryophyllene (**2**) which are responsible for the anti-inflammatory and antimicrobial activities of Brazilian phytotherapeutic Acheflan® (Fernandes et al., 2007). Caryophyllene oxide (**3**) kaurenoic acid (**4**) and β -caryophyllene (**2**) are reported as the major compounds found in copaiba oil (Brancaion et al., 2012). Kaurenoic acid was the major diterpene isolated from *C. langsdorffii* leaves. Caryophyllene oxide was also identified and isolated during the purification process. The phytochemical investigation of the polar extract using HPLC-UV/DAD allowed the isolation, detection and quantization of the flavononoids quercitrin (**5**) and afzelin (**6**). These two compounds were considered the major polar compounds and were quantified monthly for 14 months in the leaves of *C. langsdorffii*. The structures of the compounds **1–6** were confirmed using HPLC, GC, HRMS and NMR analysis including HSBC, HMBC and comparison with the literature (Leandro et al., 2012; Sousa et al., 2012). The purity of the isolated compounds (Fig. 1) was determined to be more than 97% compared with the peak areas detected by GC-FID or LC-UV/DAD.

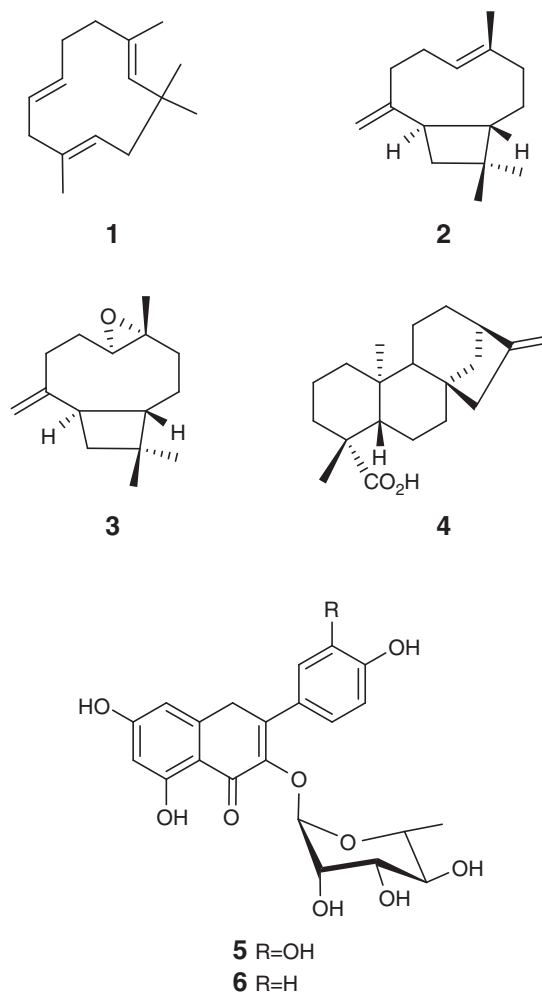


Table 1
Effects of *C. langsdorffii* extract, omeprazole and cimetidine on gastric lesions in mice induced by ethanol, indomethacin and stress.

Assay	Treatment p.o.	Dose (mg/kg)	Total area of lesion (mm ²)	% of lesion area	Ulcerative lesion index (ULI)	Curative index (%)
Ethanol	Vehicle	–	54.18 ± 3.43	13.45 ± 2.43	38.88 ± 3.28	–
	Omeprazole	30	12.06 ± 3.17**	4.49 ± 1.11**	14.57 ± 1.52**	62.52 ± 3.92
	Extract	50	10.11 ± 2.72**	2.80 ± 1.53*	11.12 ± 3.09**	71.38 ± 7.73
		250	9.80 ± 1.71**	2.43 ± 0.39*	9.75 ± 1.54**	74.92 ± 3.97
		500	6.91 ± 2.38**	1.19 ± 1.21*	7.85 ± 1.92**	79.79 ± 4.93
Indomethacin	Vehicle	–	11.14 ± 1.03	3.33 ± 0.36	20.20 ± 2.39	–
	Cimetidine	100	1.25 ± 0.43**	0.33 ± 0.10**	3.60 ± 1.07**	82.17 ± 5.33
	Extract	50	5.38 ± 1.39*	1.03 ± 0.25**	6.83 ± 2.15**	59.40 ± 10.07
		250	5.12 ± 1.00**	0.78 ± 0.24**	6.60 ± 2.35**	67.32 ± 11.67
		500	4.52 ± 1.31**	0.27 ± 0.15**	8.00 ± 1.87**	60.39 ± 9.26
Stress	Vehicle	–	20.29 ± 1.63	5.22 ± 0.45	18.83 ± 1.62	–
	Cimetidine	100	0.10 ± 0.09**	0.02 ± 0.02**	0.40 ± 0.40**	97.87 ± 2.12
	Extract	50	8.52 ± 2.26*	1.87 ± 0.48**	5.80 ± 2.17**	69.19 ± 11.56
		250	5.08 ± 1.43**	1.46 ± 0.48**	4.80 ± 1.68**	74.50 ± 8.95
		500	4.44 ± 1.25**	1.55 ± 0.45**	4.66 ± 1.20**	75.21 ± 6.38

Results are shown as mean ± S.E.M. for six mice. Statistical comparison was performed using ANOVA followed by Dunnett's post test.

* $p < 0.05$ and

** $p < 0.01$ compared to control group

Table 2
Effects of *C. langsdorffii* extract and cimetidine, administered intraduodenally, on the biochemical parameters of gastric juice in the pylorus-ligature assay.

Treatment (p.o)	Dose (mg/kg)	Volume (mL)	pH	[H ⁺] mEq/l/4h
Vehicle	–	0.57 ± 0.09	3.56 ± 0.30	69.59 ± 4.34
Cimetidine	100	0.32 ± 0.03 [†]	4.86 ± 0.39*	25.63 ± 4.67 [†]
Extract	50	0.26 ± 0.04 [†]	6.38 ± 0.36*	25.62 ± 6.01 [†]
	250	0.23 ± 0.03 [†]	6.25 ± 0.27*	23.29 ± 7.05 [†]
	500	0.21 ± 0.03 [†]	5.56 ± 0.33*	32.97 ± 6.41 [†]

Results are shown as mean ± S.E.M. for six mice. Statistical comparison was performed using ANOVA followed by the Dunnett's test.

[†] $p < 0.05$ compared to control group.

The experimental models of gastric ulcer have been employed to investigate the pathogenesis of the disease and the efficacy of new drugs. The administration of ethanol/HCl solution to mice causes severe damage and disturbances in the gastric mucosa that can lead to blood vessels rupture, bleeding, sub-epithelial cell exfoliation, generation of reactive oxygen species and a severe inflammatory process (Shimoyama et al., 2013). In this context, the oral treatment with *C. langsdorffii* extract significantly reduced the percentage of gastric injury caused by administration of ethanol/HCl solution when compared to control group (vehicle) ($p < 0.05$) (Table 1). In

relation to curative ratio, the lowest dose (50 mg/kg) showed a curative ratio of 71.4%, which was higher than omeprazole (62.5%) at 30 mg/kg. The results described in Table 1 showed that *C. langsdorffii* leaves extract has gastroprotective effect. This effect may be related to the cytoprotective activity of the extract, since it reduced the severity of ethanol-induced lesions.

It is known that indomethacin and other NSAID display ulcerogenic effects associated with the blockade of Cyclooxygenase-1 (COX-1) in gastric epithelial cells, leading to inhibition of prostaglandins synthesis (Deoda et al., 2011). Prostaglandins such

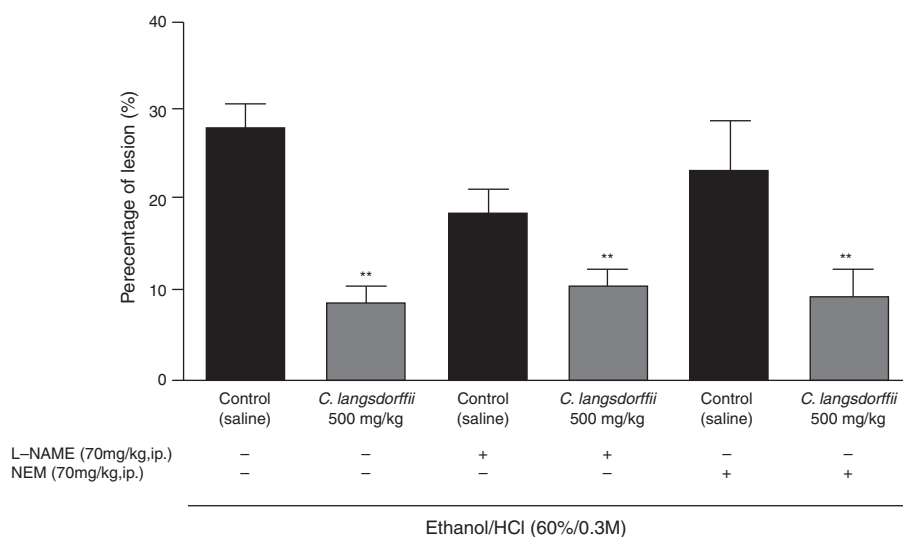


Fig. 2. Effects of *C. langsdorffii* extract, administered orally, on gastric lesions in mice pre-treated with the inhibitor of nitric oxide synthase (L-NAME) and sulfhydryl depletter (NEM) on the ulcer model induced by ethanol/HCl. Results are shown as mean ± S.E.M. for six mice. Statistical comparison was performed using ANOVA followed by the Dunnett's test. ** $p < 0.01$ compared to respective control group.

Table 3

Effects of *C. langsdorffii* extract and carbenoxolone, administered intraduodenally, on the binding to free gastric mucus in the pylorus-ligature assay.

Treatment (<i>p.o</i>)	Dose (mg/kg)	Alcian blue bound (mg/wt tissue stomach (g))
Vehicle	–	3.19 ± 0.62
Carbenoxolone	200	4.30 ± 0.32*
Extract	50	3.93 ± 0.12
	250	4.19 ± 0.31*
	500	4.57 ± 0.25*

Results are shown as mean ± S.E.M. for six mice. Statistical comparison was performed using ANOVA followed by the Dunnett's test.

* $p < 0.05$ compared to control group

as E_2 and I_2 enhance the synthesis of mucus and bicarbonate, regulate the acid secretion and maintain the integrity of the gastric blood flow in the stomach (Gargallo and Lanas, 2013). The oral treatment with *C. langsdorffii* extract significantly reduced the gastric injury caused by NSAID/bethanechol administration in comparison with the control group (vehicle). The curative ratio of the extract at 50, 250 and 500 mg/kg were 59.4, 67.3, and 60.4% respectively (Table 1).

Stressful condition is another important factor in the pathology of gastric ulcer. Stress promotes an increase in the gastric secretion, which causes severe damage to the mucus layer that covers the gastric epithelium (Gupta et al., 2013). Gastric secretion is stimulated by the activation of the vagus nerve and by the interaction of acetylcholine with muscarinic receptors in parietal cells and histamine-secreting cells (Schubert and Peura, 2008). Herein we simulated this situation using the stress-induced ulcer model. The results showed that oral treatment of mice with *C. langsdorffii* extract reduced the gastric injury when compared to control group (Table 1). This result indicates that the extract may act by increasing the gastric mucosa defense factors (e.g.: mucus) or by antagonizing the histamine H_2 receptor.

To investigate the first hypothesis the pylorus ligature was performed in mice. This procedure allows measuring the volume, pH and H^+ ions concentration present in the gastric juice, as well as the production of mucus. The results showed that the extract decreased

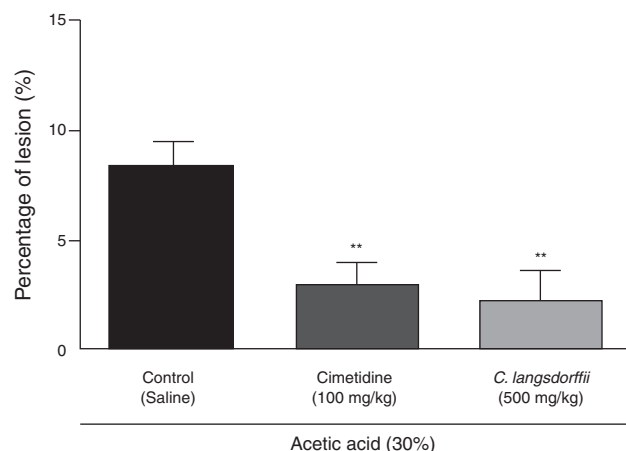


Fig. 3. Effects of *C. langsdorffii* extract and cimetidine, administered orally, on gastric lesions induced by acetic acid (30%). The results are shown as mean ± S.E.M. for six mice. Statistical comparison was performed using ANOVA followed by the Dunnett's test. ** $p < 0.01$ compared to control group.

the volume of gastric juice in the stomach (0.26, 0.23 and 0.21 ml at 50, 100 and 500 mg/kg respectively). The extract also increased the pH of the gastric juice and decreased the concentration of H^+ ions compared to control (Table 2). Regarding mucus production, the extract increased mucus production in comparison to the vehicle-treated group (Table 3). At 500 mg/kg of extract, mucus production was similar to the carbenoxolone (positive control).

These results are important because the success of the gastric ulcer treatment relies not only on the blockade of acid secretion, but also on the augmentation of the protective factors of the gastric mucosa. The mucus also plays a role in keeping the mucosa pH near neutral and protecting it from the action of the gastric acid (Santin et al., 2010).

Other important factors to consider in gastroprotection are nitric oxide (NO) and sulfhydryl groups. Nitric oxide regulates the inflammatory and vascular process in the stomach, controls the integrity of the gastric mucosa and it inhibits gastric acid secre-

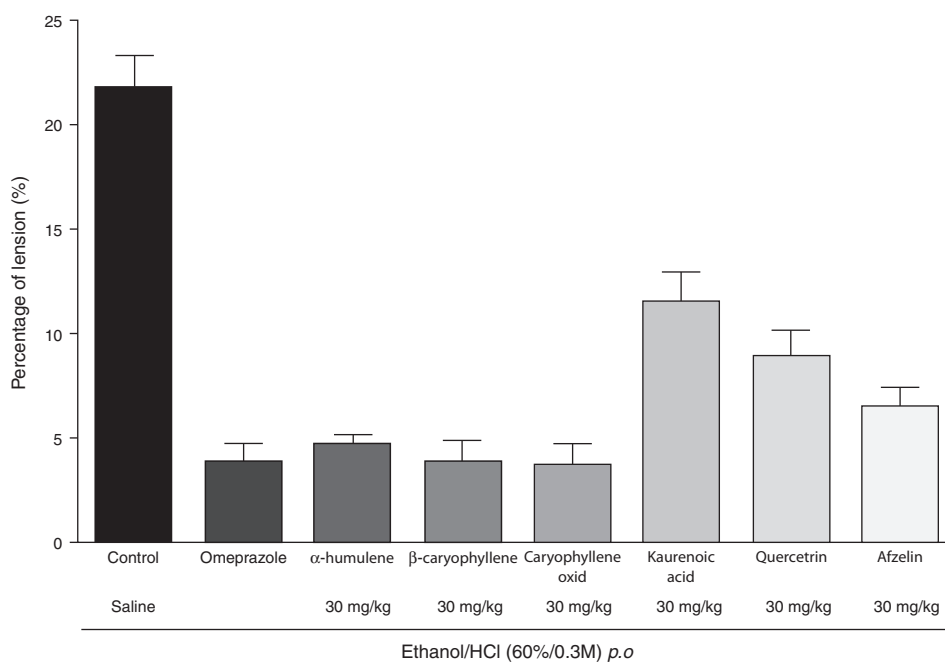


Fig. 4. Effects of compounds of the *C. langsdorffii* extract and omeprazole, administered orally, on gastric lesions induced by ethanol/HCl. Results are shown as mean ± S.E.M. for six mice. Statistical comparison was performed using ANOVA followed by the Dunnett's test. ** $p < 0.01$ compared to control group.

tion (Berg et al., 2004). The sulfhydryl groups are responsible for protecting the gastric mucosa against oxidative damage (Konturek and Konturek, 1995). To investigate the participation of NO and sulfhydryl groups in the gastroprotective activity of *C. langsdorffii* animals were pre-treated with L-NAME and NEM, a NO inhibitor and a thiol blocker, respectively. The data showed that treatment with L-NAME and NEM did not affect the gastroprotective effects of the extract (Fig. 2), suggesting that the gastroprotection presented by *C. langsdorffii* extract is not mediated by nitric oxide and sulfhydryl groups in this case.

Recurrent gastric ulcer can evolve into a chronic inflammatory process. Autoimmune disorders and infection by *Helicobacter pylori* are among the main factors associated with the recurrence of this disease, leading to a gradual destruction of the stomach homeostasis (Feldman, 2013). To evaluate the effects of *C. langsdorffii* extract on chronic ulcer we used the acetic acid chronic ulcer model. This model mimics the gastric lesions found in human chronic ulcers caused by the release of histamine, which increases the capillary permeability and back diffusion of HCl. Treatment with *C. langsdorffii* extract at the dose of 500 mg/kg once a day for seven days significantly reduced the size of the injury produced by acetic acid when compared to control group (Fig. 3).

In the final series of experiments the gastroprotective activities of the main compounds isolated from *C. langsdorffii* extract were evaluated using the ethanol/HCl-induced ulcer model. The data obtained showed that kaurenoic acid (4), quercitrin (5) and afzelin (6) exhibited gastroprotective activity. The three compounds reduced gastric lesions by 51.45, 60.48 and 60.83%, respectively (Fig. 4). The compounds α -humulene (1), β -caryophyllene (2) and caryophyllene oxide (3) showed a greater gastroprotective activity, reducing the gastric lesions by 76.20, 76.57 and 70.17% respectively (Fig. 4). The gastroprotective activity of β -caryophyllene (2) has been previously reported (Tambe et al., 1996). Literature data show that the compounds quercetin, caryophyllene oxide and α -humulene are present in the chemical composition of extracts and essential oils with gastroprotective activity (Esteves et al., 2005; Lima et al., 2012; Zakaria et al., 2014). It is important to emphasize that is the first time that the gastroprotective activity of (1), caryophyllene oxide (3), kaurenoic acid (4), quercitrin (5) and afzelin (6) as pure compounds is showed. These results corroborate with the literature, which shows that terpenes and flavonoids exhibit protective effects on gastric ulcer and its activities are associated with cytoprotective mechanisms (Hiruma-Lima et al., 2006; Siqueira et al., 2012; Klein-Júnior et al., 2012; Santin et al., 2014). In addition, in the acute toxicity study, no signs of toxicity were observed after *C. langsdorffii* extract administration at the dose of 2000 mg/kg, at any time during the observation. The rational of OECD 420 (2001) permits an acute toxicological classification, using the globally harmonized system (GSH), without pre-determination of LD50. The GSH classification system suggests that if any mortality was observed when the substance or mixture of substances were tested up to category 4 (2000 mg/kg), it is classified as category 5 or low acute toxicity with a LD₅₀ superior to 2000 mg/kg.

Taken together, the data herein obtained shown that *C. langsdorffii* leaves extract displayed gastroprotective and healing properties in different animal models of gastric ulcer. The gastroprotective effects may be associated with the ability of the extract to decrease gastric secretion and increase the production of mucus. The extract was also effective in a chronic ulcer model and it significantly reduced the size of the gastric lesion. The gastroprotective effects of the *C. langsdorffii* extract may be associated with the gastroprotective activity shown by its major compounds α -humulene, β -caryophyllene, caryophyllene oxide, kaurenoic acid, quercitrin and afzelin. Therefore *C. langsdorffii* extract and its isolated compounds have potential as antiulcer therapy.

Conflict of interest

The authors declare no conflicts of interest.

Authors' contributions

Conceived and designed the experiments: JRS SFA JKB ML. Performed the experiments: JRS ML TB JPBS DN CSM. Analyzed the data: JRS ML CM JPBS DN. Contributed reagents/materials/analysis tools: SFA JKB CSM. Wrote the manuscript: JRS SFA CSM ML.

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