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Evaluation of the safety, gastroprotective activity and mechanism of action of standardised leaves infusion extract of *Copaifera malmei* Harms

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A B S T R A C T

Ethnopharmacological relevance: *Copaifera malmei* Harms (Fabaceae) is a plant that occurs in the central region of Brazil, where the plant's leaves infusion is popularly used to treat gastric ulcer and inflammatory diseases. This study was aimed to investigate the gastroprotective activity and mode of action of the plants' leaves infusion in order to establish the scientific basis for such usage, and to assess its potential as a source of an anti-ulcer agent.
Materials and methods: Leaves infusion extract of the plant (SIECm) was prepared, freeze dried and lyophilised. Its qualitative and quantitative phytochemical constituents were investigated using TLC and HPLC techniques. The safety profile was evaluated on CHO-k1 epithelial cells viability using the Alamar blue assay, and by acute toxicity test in mice. The gastroprotection and anti-ulcer efficacy of the SIECm (25, 100 and 400 mg/kg, p.o.) were tested using acute (acidified ethanol, piroxicam and water restrain stress), and chronic (acetic acid) experimental ulcer models. The plausible mode of action of the SIECm was assessed using gastric secretion, gastric barrier mucus, nitric oxide, and its antioxidant (myeloperoxidase and catalase) effects in mice and rats. The histopathological analyses of the ulcerated tissues as well as the extract's activity on *Helicobacter pylori* were also investigated.
Results: Phytochemical tests indicated the presence of mainly phytosterols, phenolics and flavonoids. The SIECm exhibited no cytotoxic effects on the CHO-k1 cells, and no oral acute toxicity in mice. It prevented against the acute induced ulcerations by enhancing gastroprotection through gastric mucus production, NO modulation, antioxidant, reduced gastric secretion and enhanced chronic ulcers healing process, as shown by reduction/prevention of epithelial and vascular damage, in addition to reduction in leucocyte infiltration. The SIECm however did not exhibit activity against *H. pylori*.
Conclusion: The SIECm is safe, contain useful phytochemicals and exhibited significant gastroprotective/anti-ulcer effects. The results justify its folkloric usage, and provided scientific evidence of its potential as a source of new phytodrug to treat gastric ulcers.

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1. Introduction

Gastric ulcer is a prevalent disorder characterized by mucosal lesions caused by an imbalance between aggressive factors (endogenous or exogenous), and the defensive mechanism factors that work towards the maintenance of mucosal integrity such as mucus, bicarbonate, prostaglandins, blood flow and nitric oxide. The disease often develops into chronic and recurrent lesion with the attendant risks of complications that include bleeding, perforation, gastroduodenal obstruction, and mortality (Lau et al., 2011).

Despite the current progress made in the treatment of the disease, some patients do suffer from recurrence or intractability, indicating that the therapies are not completely effective. There

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has been growing interest in alternative therapies to overcome these problems, especially with medicinal plant products. These products have a long and popular usage over the years as valuable resources in the treatment of gastric ulcer diseases.

The plant genus *Copaifera* occurs in Brazil, along the Amazon and its tributaries, and the Cerrado of Central region as well as in some parts of South America. The genus comprises of 106 species (www.theplantlist.org), about 20 of which are well known. Their woods are durable and produces oily liquid (popularly known as balsam of copaiba) used in folk medicine. Scientific evaluations showed that the oil has anti-bactericidal, anthelmintic, analgesic, anti-inflammatory, gastroprotective, trypanocidal and antitumor activities (Veiga-Júnior et al., 2007).

Copaifera malmei Harms is a plant native to the Central region of Brazil, and is distributed mainly in the states of Mato Grosso and Goiás (Oliveira-Filho et al., 2008; Silva, 2010). The plant is distinguished primarily on the basis of the character of its leaves and leaflets (Dwyer, 1951). It is locally known as 'pau-d'olinho', 'copaibinha' (Oliveira-Filho et al., 2008) 'pau-d' óleo' (Bieski et al., 2012) or 'guaranazinho' (floradobrasil.jbrj.gov.br). Ethnopharmacological studies amongst the inhabitants of some riverine communities in Central Brazil revealed the leaves are used in the form of infusion for the treatment of inflammations and gastric ulcer diseases. In the locals' ethnopharmacy, approximately 40 g of the plant leaves is incubated with hot water, and a cup (150 mL) is taken twice daily to treat gastric ulcer diseases. The plant's leaves infusion is also used for treatment of injury, bronchitis, and kidney disease. The scientific bases for the use of *C. malmei* in the treatment of gastric ulcer disease have not been documented, to the best of our knowledge.

In this report, we investigated the gastroprotective activity and mode of action of the leaves infusion extract of *C. malmei* in order to generate useful data for its safe and efficacious usage, and also assessed its potential as an anti-ulcer prototype. The extract's constituents were standardised using TLC and HPLC technique, and its safety investigated on CHO-k1 epithelial cells using the Alamar blue assay, and acute toxicity test in mice. The gastroprotective efficacy was evaluated in laboratory mice and rats using standard ulcer models. Histopathology of the gastric lesions, and the extract's activity on *H. pylori* were also investigated.

2. Materials and methods

2.1. Plant material

The plant material was collected in April 2014 at the locality of Serra Nova Dourada Municipality (11°58′41″ S, 51°34′57″ W), State of Mato Grosso, Brazil, after obtaining an authorisation (no. 199/2014) from the 'Conselho de Gestão do Patrimônio Genético, Ministério do Meio Ambiente (CGEN/MMA)', Brazil. The plant was identified and authenticated by a taxonomist: Professor Germano Guarim Neto of the 'Departamento de Botânica e Ecologia, Universidade Federal de Mato Grosso' (UFMT), Cuiabá, Brazil. A voucher specimen (no. 40,754) was deposited at 'Herbário UFMT'.

2.2. Animals

Adult Wistar rats (180–220 g) and Swiss albino mice (25–30 g) of both sexes obtained from the 'Biotério Central' of UFMT were used for the studies. The animals were maintained in propylene cages at 25 ± 1 °C in a 12 h dark/12 h light cycle experimental room, with free access to standard laboratory feeds (Purina©,Labina, Goiás, Brasil) and water *ad libitum* for at least three days prior to each experiment. A certificate of approval for the study (no. 23108.036060/14-5) was granted by the Institutional Committee for Ethics in the Use of Animals (CEUA), UFMT after reviewing our experimental designs.

2.3. Drugs and reagents

Methanol, rutin, naringin, gallic acid, catechin, morin, luteolin, stigmasterol, quercetin, kaempferol, benzene, ethyl acetate, formic acid, carbenoxolone, cimetidine, Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), clarithromycin, penicillin, streptomycin, Dulbecco's Modified Eagle's Medium (DMEM), Mueller Hinton (M-H) Broth, Bovine serum albumin (BSA), hexadecyl trimethyl ammonium bromide (HTAB), Foetal Bovine Serum (FBS), and Alcian blue 8GX were obtained from Sigma-Aldrich Co., St. Louis, MO, USA. Other chemicals/reagents used were: Ethanol (Tedia Company, Inc Fairfield, OH, USA), Alamar blue (Invitrogen®, Life Technologies, Grand Island, NY, USA), tetramethylbenzidine (TMB) (eBioscience Inc., CA, USA) sodium acetate, sodium hydroxide (Vetec®, Quimica Fina Ltda, RJ, Brazil); Brain-Heart Infusion (BHI) Broth (Newprov Produtos, Pinhais, PR, Brazil), Foetal Calf Serum (FCS) (Cultilab, Campinas, SP, Brazil), Sucrose (Dinâmica®, Química Contemporânea Ltda, Diadema, SP, Brazil); ketamine, and xylazine (Syntec/R.I. Farmacêutica Ltda, Brasilia, Brazil). All other chemicals, drugs and reagents used were of analytical grade.

2.4. Cell culture

Chinese hamster ovary (CHO-k1) epithelial cells (BCRJ code: 0069) were obtained from 'Banco de Células do Rio de Janeiro', RJ, Brazil. They were cultured in DMEM, supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL), in an oven (Quimis® Aparelhos Científicos, Diadema – SP, Brazil) at 37 °C in a humidified atmosphere with 5% CO_2 and 90% air until confluency.

2.5. Microorganism

H. pylori strain ATCC 43504 (vacA and cagA positives) was obtained from Fundação Oswaldo Cruz (FIOCRUZ)', RJ, Brazil. Stock cultures were maintained frozen at −30 °C in Skim Milk broth. For reactivation, the organisms were inoculated on a selective BHI Broth supplemented with 10% FCS and incubated at 37 °C under microaerophilic atmosphere of 5–15% O_2 and 5–10% CO_2, for 3 days.

2.6. Preparation of extracts

Fresh leaves of *C. malmei* were collected, dried at room temperature, and ground with an electric mill (TE-625 Tecnal, SP, Brazil). The plant extract was prepared by infusion: a total of 80 g of the powdered dried material was soaked in 2 L of boiled distilled water for 15 min; in multiples of 40 g/L to mimic the locals' ethnopharmacy. The extract was pooled together, filtered, filtrate kept frozen (Indrel® Ultra freezer IULT 335D, Londrina-Parana, Brazil), lyophilised (Cientifíca-Modelo LJJ02, SP, Brazil) and its constituents standardised as described in Sections 2.7.1–2.7.3), to obtain standardised infusion extract of the leaves of *C. malmei* (denoted SIECm), which was kept in an amber glass container in a refrigerator (Brastemp 350 L, SP, Brazil) until use. The SIECm obtained gave the following physicochemical parameters: yield, 7.68% w/w; colour, brown; pH, 4.93 (1 mg/mL at 25 °C); and solubility, greater than 500 mg/mL in distilled water.

2.7. Phytochemical tests

2.7.1. Qualitative evaluation using thin layer chromatography (TLC)

The qualitative phytochemical constituents of SIECm were evaluated using the TLC method described by Wagner and Bladt

(2001). The test was performed using: as the stationary phase, silica gel plates 0.20 mm Kieselgel 60 Alugram® Xtra Sil G (Macherey-Nagel GmbH & Co., Düren, Germany); mobile phase, benzene: ethyl acetate: formic acid: methanol (60/30/10/3 v/v); and revealing: (1) NP/PEG (1% 2-aminoethyl diphenylborinate diluted in methanol and 5% polyethylene glycol diluted in ethanol), and (2) citroboric (citric acid: boric acid: methanol (12/12/77 w/v)). The preparation was then visualized using a UV camera (Vilber Lourmat CN-15MC, Marne-la-Vallée, France) at 365 nm. The evaluation was carried out along with rutin, naringin, gallic acid, catechin, morin, luteolin, quercetin and kaempferol as reference standards.

2.7.2. Qualitative fingerprint using high performance liquid chromatography (HPLC)

The fingerprints profiles of SIECm was analysed using a HPLC instrument (Shimadzu® chromatograph-LC-10 Avp series, Japan) equipped with a pump (LC-10AD), degasser (DGU-14A), UV–vis detector (SPD-10A), column oven (CTO-10A), manual injector (Rheodyne loop 20 μL) and integrator (CLASS LC-10A). The separation was carried out by a gradient system, using a reverse-phase Phenomenex Luna 5 mm C_{18} column ($250 \times 4.6\ mm^2$) with direct-connect C_{18} Phenomenex Security Guard Cartridges ($4 \times 3.0\ mm^2$) filled with similar material as the main column. The mobile phase consists of: A=2% formic acid in Milli-Q water; and B=2% formic acid in methanol. The system was programmed to give the following gradients: 0.01–0.10 min, 2.5% B; 0.10–5 min, 25% B; 5–10 min, 45% B; 10–16 min, 45% B; 16–20 min, 80% B; 20–25 min, 80% B; 25–30 min, 80% B; 30–35 min, 100% B; and 35–36 min, 100% B. The flow rate was 1 mL/min, UV detection at 280 nm and elution time 37 min. The compounds were identified by comparing the retention times of samples and authentic standards such as gallic acid, rutin, catechin, quercetin and ellagic acid from (Sigma®).

2.7.3. Quantification of total phenolics, total flavonoids and phytosterol contents

These were performed using their specific quantification assays as hereby described. All the quantification assays were performed in triplicate.

Total phenolics content was quantified using the Folin–Ciocalteu method, as described by Amorim et al. (2008). Methanolic solutions (0.2 mL) of SIECm (1 mg/mL, w/v) or the standard used (gallic acid, 0.1–1.0 mg/mL, w/v) were mixed with Folin–Ciocalteu reagent (0.5 mL of 10%, v/v), sodium carbonate (1 mL of 75%, w/v) and Milli-Q water (8.3 mL). The mixture was gently agitated and kept for 30 min in the dark. The absorbance was measured at 760 nm using a UV–visible spectrophotometer (Biochrom® Biowave II^+, Cambridge, UK). Total phenolics were determined by interpolation of the absorbance of the samples against a calibration curve constructed with different concentrations of gallic acid in Milli-Q water ($y=0.0997x+0.004$ adjusted $R^2=0.9956$). The result was expressed as mg gallic acid equivalents (GAE) per gram of SIECm (mg GAE/g).

The total flavonoids content was quantified using the method described by Peixoto-Sobrinho et al. (2008) with slight modifications. Briefly, 0.5 mL of methanolic solutions of the SIECm (1 mg/mL w/v) or standard (rutin, 1–10 mg/mL w/v) was mixed with an aqueous solution of 0.5 mL of 60% acetic acid, 2 mL methanolic solution of 20% pyridine (v/v), 1 mL of 5% aluminium chloride (w/v) and 6 mL of Milli-Q. The mixture was gently stirred and kept for 30 min in the dark and its absorbance was then measured at 420 nm using a spectrophotometer. The total flavonoid contents were determined by extrapolating the absorbance of the samples against a calibration curve constructed with different concentrations of the rutin standard ($y=0.0215x-0.0020$ adjusted $R^2=0.9978$) and expressed as milligrams of rutin equivalents (RE) per gram of SIECm (mg RE/g).

The total content of phytosterol in SIECm was determined using the sulfate phosphate ferric (SPF) method (Lin et al., 2009) with modifications. The SPF reagent was prepared as follows: 2.5 g $FeCl_3 \cdot 6H_2O$ was dissolved in 25 mL phosphoric acid, then 10 mL was taken out and re-dissolved in 100 mL sulfuric acid before the experiment. Ethanolic solutions (1 mL) of the SIECm (0.2 mg/mL, w/v) or the standard (stigmasterol, 0.02–0, 1 mg/mL w/v) were mixed with 1 mL SPF and homogenised. The mixture was stirred and kept in dark place for 1 h. Subsequently, the absorbance at 550 nm was measured using a spectrophotometer. The phytosterol content was determined by extrapolation of the absorbance of the samples against a calibration curve ($y=77.858x+0.0442$ adjusted $R^2=0.9926$) constructed with standard concentrations of stigmasterol. The result was expressed as milligrams of stigmasterol equivalents per gram of SIECm (mg SE/g).

2.8. Safety evaluations

2.8.1. Cytotoxicity assay

This assay was performed by adopting the Alamar blue assay (Nakayama et al., 1997), with slight modification. The CHO-k1 cells (of density 2×10^4 cells/well) were plated on 96-well plates in 200 μL of DMEM and treated with/without SIECm (3.125–200 μg/mL, serial dilution). Doxorubicin (0.0058–58 μg/mL, serial dilution) was used as a positive control, while some wells had the same amount of medium as the negative control. The treatments were removed after 24 or 72 h incubation and 200 μL of 10% Alamar Blue (resazurin) was added to each well and incubated again for 5 h. The conversion of resazurin to resorufin by the cells was measured at 540 nm (oxidised state) and at 620 nm (reduced state) using microplate spectrophotometer (Multiskan EX, Thermo Scientific, Tewksbury, Massachusetts, USA). The cell viability was expressed as inhibitory concentration at 50% inhibition ($IC_{50} \pm$ SEM). IC_{50} values > 30 μg/mL were considered non-toxic (Suffness and Pezzuto, 1990).

2.8.2. Acute toxicity test

The acute toxicity profile of SIECm was evaluated in conscious mice using the method described by Malone (1983). Six mice were weighed, and a single dose of SIECm (5000 mg/kg, p.o.) was administered to each mouse. Another group of mice ($n=3$) was given the vehicle (distilled water, 10 mL/kg) to serve as the control. The mice were individually observed in an open field after the extract or vehicle administration for signs and symptoms of toxicity at 0, 15, 30, 60 min and after 4, 8, 24, and 48 h; and then once daily for 14 days. All observations were systematically recorded with individual records kept for each mouse using the Hippocratic qualitative/semiquantitative screening and toxicity table adopted from Malone.

2.9. Evaluation of gastroprotective/anti-ulcer effects

2.9.1. Acidified ethanol-induced ulcers

This test was performed using the model described by Mizui and Doteuchi (1983) as modified (Júnior et al., 2014). After 18 h of fasting, mice were grouped into five ($n=6$) and treated p.o. with the vehicle, three dose levels of the extract (25, 100 and 400 mg/kg), and 100 mg/kg of carbenoxolone to serve as the positive control. One hour after the treatment, each mouse received 0.3 mL of 60% ethanol in 0.3 M HCl to induce gastric ulcers. The animals were then sacrificed by cervical dislocation an hour later; and: (1) their stomachs were removed, opened along the greater curvature, distended between two glass plates for better visualization and photographed (Khan, 2004). The ulcerated areas were

evaluated using Image J 1.48v (Java-based image processing program, NIH, USA), and results expressed in terms of percentage inhibition of the induced ulceration; (2) ulcerated portions were carefully cut out for histopathological analysis of ulcerated gastric tissues as described in Section 2.11.

2.9.2. Nonsteroidal anti-inflammatory drugs (NSAIDs)-induced gastric ulcer

In this model, gastric ulcers were induced in rats using piroxicam (Puscas et al., 1997; Balogun et al., 2015). Rats were fasted for 24 h, and then treated p.o. with the vehicle, SIECm (25, 100 and 400 mg/kg) and ranitidine (50 mg/kg). One h later, gastric ulcers were induced with piroxicam (200 mg/kg, p.o.). The rats were sacrificed by cervical dislocation 6 h later and their stomachs were removed, opened along the greater curvature, distended between two glass plates for better visualization, and photographed. The lengths of lesions were measured using Image J 1.48v, and the lesion index expressed as the sum of all lesions in each stomach.

2.9.3. Water restraint-stress (WRS)-induced ulcer

The model described by Takagi et al. (1964) as modified (Yesilada et al., 2014) was adopted for this assay. The rats were fasted for 20 h with water ad libitum, then divided into five groups ($n=6$) and treated p.o. with vehicle, three dose levels of the extract (25, 100 and 400 mg/kg), and 100 mg/kg of Cimetidine. Thirty min after the treatments, each rat was individually immobilised in a restraining cage and immersed vertically up to the xiphoid process level in a water- bath maintained at 19 ± 1 °C; and they remained in that position for 7 h. After this period, the animals were euthanized, their stomachs removed, the length of each lesion was measured using Image J 1.48v, summed up and expressed in terms of ulcerated area (mm^2).

2.9.4. Acetic acid-induced chronic ulcers

The potential effect of SIECm on ulcer healing was tested using the acetic acid-induced method (Takagi et al., 1969; Martins et al., 2014). The mice were first acclimatized in the laboratory by undergoing a period of adaptation against stress involved in the study as follows: the animals received distilled water (10 mL/kg) given orally; followed by food for 2 h per day for three days. They were then fasted for 12 h prior to the commencement of the experiment. On day one (D_1), the mice were anaesthetised with solution of a mixture of ketamine/xylazine (40/50 mg/kg, i.p.). Under the anaesthesia, the abdomen was opened by a midline incision below the xiphoid process, stomach exposed and gastric ulcer induced by carefully injecting 15 μL of 30% acetic acid with a micro syringe/needle in the submucosal layer, glandular part of the anterior portion of the stomach wall, keeping pressed the application site for a moment. The stomach and the abdominal wall were then bathed with sterile 0.9% saline (to avoid adherence), and the incisions were closed by interrupted sutures. A sham group was operated upon; in which 15 μL of sterile saline was injected (in place of acetic acid) and included in the experiment for comparison. All animals were then housed in polypropylene cages to recover from anaesthesia, and received only water on D_1.

Two days after the surgery (D_3), the mice were grouped into five ($n=6$) and treated p.o. twice daily with vehicle, SIECm (12.5, 50 and 200 mg/kg), and Cimetidine (50 mg/kg) at 08:00 and 16:00 hours for 7 days (D_3–D_9). The sham operated group received the vehicle throughout these treatment regimens. On D_{10}, the mice were sacrificed under anaesthesia, their stomachs removed, opened along the greater curvature and the macroscopic ulcer lesion area (width × length) were evaluated (in mm^2) with the aid of a digital calliper (DIGIMESS: 90173020, SP, Brazil).

2.10. Evaluation of gastroprotective mechanism of action

2.10.1. Effect on gastric secretion

This assay was carried out in rats using the Shay et al. (1954) pylorus-ligation gastric ulceration method as modified by Singh et al. (2013). The rats were acclimatised for 3 days, and fasted for 24 h with water ad libitum prior to the experiment. They were then anaesthetised i.p. with a mixture of ketamine (60 mg/kg) and xylazine (7 mg/kg), laparotomy performed, the pylorus ligated and the rats treated intraduodenally with the vehicle (distilled water, 10 mL/kg), SIECm (12.5, 50 and 200 mg/kg) and cimetidine (50 mg/kg). Six hours later, the animals were sacrificed by cervical dislocation; the abdomen opened, another ligature placed around the oesophagus close to the diaphragm, stomach removed, the gastric contents collected and centrifuged at $2500 \times g$ for 10 min (ALC 4239R, ALC International Srl, Cologno Monzese, Italy). After that, the total gastric-juice volume (mL) was measured, 0.5 mL was taken out of it, and made up to 5 mL with distilled water; from which the free acidity was determined using a pH metre (MS Tecnopon Instrumentação, Piracicaba – SP, Brazil) and total acidity (mEq [H^+]/mL) estimated by titration to pH 7.0 with 0.01 N NaOH using 1% phenolphthalein as indicator.

2.10.2. Protective effect on gastric barrier mucus

This assay was carried out in mice by adopting the method described by Corne et al. (1974) with slight modification. The mice were fasted for 18 h, and then grouped into five groups ($n=6$). Group 1 was treated with the vehicle, Groups 2–4 with three dose levels of SIECm (25, 100 and 400 mg/kg, p.o.), while Group 5 received 100 mg/kg, p.o. of carbenoxolone (100 mg/kg). Each mouse was given 0.3 mL of 60% ethanol in 0.3 M HCl, 1 h after the treatments. The animals were sacrificed by cervical dislocation 1 h later and the glandular segments of their stomachs were removed, weighed, and immediately placed in 10 mL solution of 0.02% Alcian blue 8GX in 0.16 M sucrose solution (that was dissolved in 0.05 M sodium acetate buffer, adjusted to pH 5.3 with HCl) and incubated at 20 ± 1 °C for 24 h. After which the mixture was centrifuged at $2500 \times g$ for 10 min (ALC 4239R, ALC International Srl, Cologno Monzese, Italy), and absorbance of the supernatant of each sample measured at 598 nm using a UV–visible Spectrophotometer (Spectronic GENESYS 5, Thermo Electron Coorp., Madison, USA). The concentration of Alcian blue was calculated by linear regression with a calibration curve obtained from standard serial dilutions of different concentrations of the dye ($y=0.0147x+0.1318$ adjusted $R^2=0.9812$), and results were expressed in μg of Alcian blue/g of tissue.

2.10.3. Assessment of the role of nitric oxide (NO) in the gastroprotective action

The role of NO in the gastroprotective action of SIECm was assessed using the method described by Matsuda et al. (1999) with modifications using suboptimal doses of L-NAME (nitric oxidase synthases inhibitor) and carbenoxolone. Mice were fasted for 18 h and grouped into five groups ($n=6$). They were first pre-treated p.o. with 10 mg/kg of L-NAME, and 30 min later, the mice were treated with vehicle (10 mL/kg, p.o.), SIECm (25, and 100 mg/kg) or carbenoxolone (30 mg/kg). One h after the treatments, all the mice were given 0.3 mL of acidified ethanol (60% ethanol in 0.3 M HCl) to induced gastric ulceration; and 1 h after the ulcer induction, the animals were sacrificed, their stomachs removed and the percentage inhibition of gastric ulceration was evaluated as mentioned in Section 2.9.1.

2.10.4. Evaluation of antioxidant activity

Gastric ulcerations were induced in rats using the WRS-induced model described in Section 2.9.2, but in this case, a sham

group was also included. After the ulcer induction, their stomachs were removed, opened along the greater curvature, washed, and the glandular portion of the stomachs was cut into two, weighed and placed into falcon tubes and stored at −80 °C in a biofreezer (Indrel® Ultra freezer IULT 335D) for the CAT (Section 2.10.4.1) and MPO (Section 2.10.4.2) assays.

2.10.4.1. Determination of Catalase (CAT) activity. The method described by Aebi (1984) was used with modification. The weighed glandular portion of the stomach (Section 2.10.4) was homogenised (MA120, Marconi–Equipamentos, SP, Brazil) with phosphate buffer (pH 7) and centrifuged at 11,180 × *g* for 10 min at 4 °C. A solution of 1 mM H_2O_2 (2 mL) in 50 mM phosphate buffer pH 7.0 was put into a quartz cuvette, 20 μL of sample added; and reading taken immediately at 240 nm (A_{240}=0.380–0.400), and at every minute for 4 min using a UV–visible Spectrophotometer. The absorbance values were extrapolated from a curve obtained from standard concentrations of H_2O_2 in the 50 mM phosphate buffer solution (y=6.7423x – 0.0079 adjusted R^2=0.9959). Protein values were extrapolated from a curve of standard concentration of BSA (y=0.0007x+0.0347 adjusted R^2=0.9751). The results were expressed in μMH_2O_2/min/g protein.

2.10.4.2. Determination of Myeloperoxidase (MPO) action. The MPO activity was determined using the method described by Schierwagen et al. (1990) with modification. The other glandular portion of the stomach (Section 2.10.4) was homogenised on ice in phosphate buffer (0.2 M, pH 6.6). Then, samples were centrifuged at 9000 × *g* for 10 min at 4 °C. The supernatant was discarded and the pellet re-suspended in phosphate buffer (0.08 M, pH 6.6) with HTAB (0.05%) and sonicated for 30 sec. After which the samples were re-centrifuged at 11,000 × *g* for 20 min at 4 °C. In 96-well plates, 30 uL of the supernatant, 220 μL of a solution containing a mixture of: 100 μL of 80 mM phosphate buffer, 85 μL of 22 mM phosphate buffer and 15 μL of 0.017% H_2O_2. The reaction was started by adding 20 μL of TMB, and the sample incubated for 3 min at 37 °C. The reaction was quenched with 30 μL of 1 M H_2SO_4 and the sample absorbance were read using a microplate reader (Biochrom EZ Read 400, Cambridge, UK) at 450 nm. The absorbance values were extrapolated from a curve of standard concentration (y=0.0007x+0.0347 adjusted R^2=0.9751). The enzyme activity was calculated and the results were expressed as U/g tissue.

2.11. Histopathological analysis

The ulcerated gastric tissues obtained (in Section 2.9.1) were subjected to histopathological analysis. The tissues were fixed in 4% buffered paraformaldehyde solution, and dehydrated with mixtures of increasing grades of ethanol, clarified in xylene using a tissue processor (MTP-SLEE, Mainz, Germany) and embedded in paraffin. After processing, two sections of tissues were made from each block using a Hyrax M60 microtome (Carl Zeiss, Oberkochen, Germany) and stained with haematoxylin/eosin (HE). The tissue sections were observed under a Zeiss AxioScope.A1 Microscope (Carl Zeiss, Germany), and images analysed using the AxioVision digital image processing software (www.zeiss.com) for characterisation of histopathological changes (Dixon et al., 1996); using the following criteria: haemorrhagic damage (score: 0–4); oedema in the upper mucosa (score: 0–4); loss of epithelial cells (score: 0–4); haemorrhagic injury (score: 0–4), and the presence of leucocytes (score: 0–4).

2.12. Anti-Helicobacter pylori activity

The activity of SIECm against *H. pylori* was investigated using broth Microdilution assay (McNulty, 2002). BHI broth (100 μL per well) supplemented with 10% foetal bovine serum was inoculated with 6 × 10^8*H. pylori* (McFarland turbidity standard 2). The serial dilutions (Eppendorf Xplorer® Plus, Hamburg, Germany) of the extract dissolved in 0.04% DMSO (100 μL), and BHI was added to each well in the microplate, to reach final concentrations of 6.25–800 μg/mL. Clarithromycin (0.195–50 μg/mL) was used as the standard drug. The microplate was then incubated at 37 °C under microaerophilic conditions in an atmosphere of 5–15% O_2 and 5–10% CO_2, for 4 days. The optical density of the turbidity was measured at 450 nm using a microplate reader. Tests were performed in duplicate and activity assessed based on the criteria for antimicrobial activity of plant extracts. The activity of the extract against the microorganisms was classified as having a good antimicrobial activity MIC ≤ 100 μg/mL; moderate 100 < MIC < 500 μg/mL; weak 500 < MIC < 1000 μg/mL weak; and inactive MIC ≥ 1000 μg/mL (Holetz et al., 2002).

2.13. Data analysis

The results were expressed as mean ± standard error of mean. Parametric one way-analysis of variance (ANOVA) was used to compare the means between groups, followed by the Student–Newman–Keuls test for multiple comparisons using GraphPad Prism Version 5.01 software for Windows (San Diego California, USA). In the case of non-parametric measures, comparisons were made using Kruskal-Wallis test followed by Dunn's test. The inhibition concentrations 50% (IC_{50}) were determined from a linear regression curve relating the percentage of inhibition *vs.* the logarithm of the tested concentrations and assuming a confidence level of 99% ($p < 0.01$) for the curve obtained. For *in vitro* assays that do not involve statistical analysis, we used the mean ± SEM of three independent experiments performed in duplicate.

3. Results

3.1. Qualitative phytochemical constituents

The qualitative phytochemical test of SIECm using the TLC technique detected mainly phenolics, flavonoids and phytosterols as the major constituents. The main components and distinct retention factor (R_f) observed were as shown in Fig. 1.

3.2. HPLC fingerprints

The analysis by HPLC confirmed the qualitative TLC test with regard to the presence of the main phytochemical constituents. In a working time of 37 min, compounds matrixes in SIECm were detected in the intervals between 10 and 31 min. The data show the presence of (peak number,: Retention time): Gallic acid (1,:10.10 min), rutin (16,: 24.88 min), ellagic acid (17,: 25.47 min), catechin (19,: 26.10 min), and quercetin (20,: 27.77 min) as shown in Fig. 2.

3.3. Quantitative phytochemical analysis

The contents of total phenolics, flavonoids and phytosterol in SIECm were: 148.11 ± 1.5 mg GAE/g; 67.07 ± 0.28 mg RE/g and 192.10 ± 2.26 mg SE/g respectively.

3.4. Cytotoxicity assay

The result showed that SIECm did not alter the cellular viability of CHO-k1 cells' line's ability to reduce Alamar blue, with IC_{50} value > 200 μg/mL after both 24 h and 72 h exposure. The standard

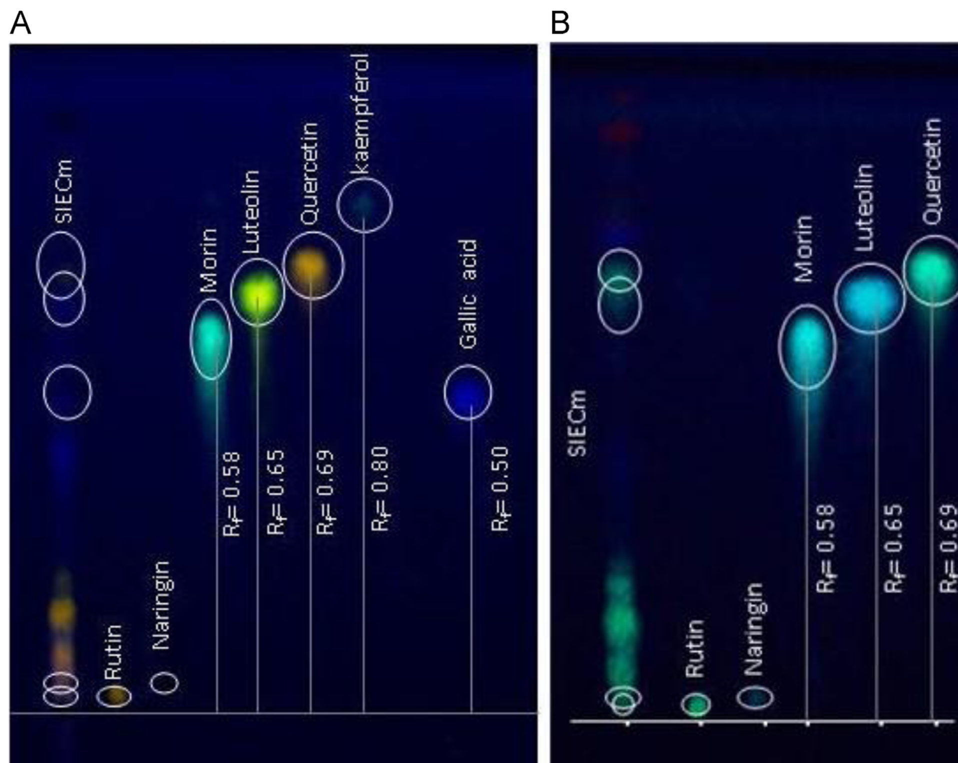

Fig. 1. TLC profiles of infusion extract of the leaves of *Copaifera malmei* (SIECm) under UV camera at 365 nm; revealed using NP/PEG (A), and Citroboric (B).

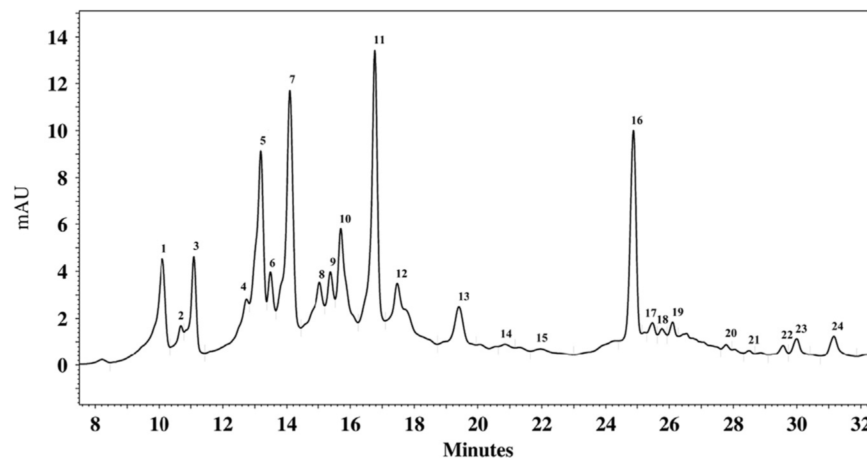

Fig. 2. HPLC fingerprints of infusion extract of the leaves of *Copaifera malmei* (SIECm) showing a total of 24 peaks due to metabolic classes in the extract or their synergistic interactions.

agent used (doxorubicin) gave an IC_{50} value > 58 μg/mL at 24 h, which was highly cytotoxic at 72 h with IC_{50}=0.3 ± 0.04 μg/mL.

3.5. Acute toxicity test

SIECm at 5000 mg/kg, p.o. showed no significant alteration in behaviour. There was no significant difference in the weight gain, and none of the mice died throughout the 14 day duration test/ observation period.

3.6. Acidified ethanol-induced gastric ulcers

The acidified ethanol caused severe gastric ulceration as expected in the vehicle control group (6.14%). Treatment with SIECm inhibited the gastric lesions induced by the ulcerogenic agent dose dependently with maximal inhibition of 71.28% ($p < 0.01$) at 400 mg/kg. The standard drug used (carbenoxolone, 100 mg/kg) in this model inhibited the induced ulceration by 74.92% ($p < 0.01$) as shown in Fig. 3.

3.7. Piroxicam-induced gastric ulcer

Piroxicam induced gastric ulceration was characterized by mucosal haemorrhage in the gastric body and antrum, and was significantly attenuated by treatment with SIECm in all the doses tested, with 400 mg/kg of the extract producing a comparable effect (80%; $p < 0.001$) with the standard drug used (ranitidine 50 mg/kg, p.o.) which caused 90% ($p < 0.001$) inhibition (Table 1).

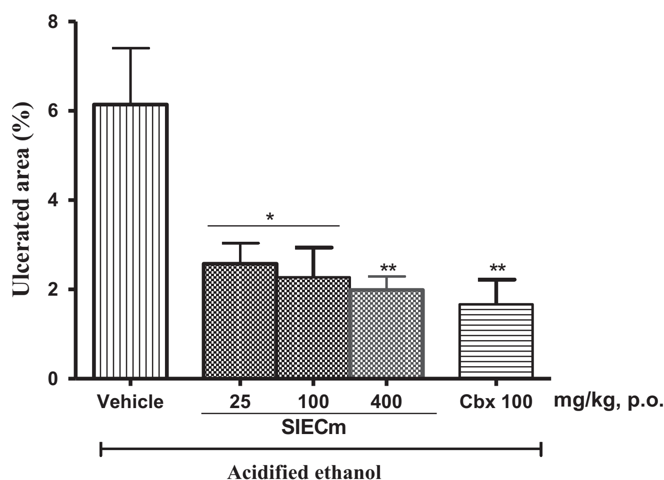

Fig. 3. Effect of oral administration of vehicle (10 mL/kg), infusion extract of the leaves of *Copaifera malmei* (SIECm; 25, 100 and 400 mg/kg) and carbenoxolone (Cbx, 100 mg/kg) against acidified ethanol-induced gastric lesion in mice. Each bar represent mean ± SEM, $n=6$; one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls test for multiple comparison. $^{*}p<0.05$; and $^{**}p<0.01$ vs. vehicle.

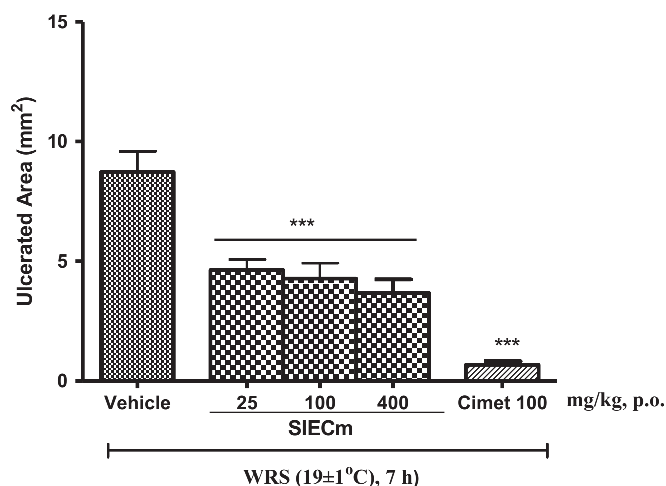

Fig. 4. Effect of oral administration of vehicle (10 mL/kg, p.o.), infusion extract of the leaves of *Copaifera malmei* (SIECm; 25, 100 and 400 mg/kg, p.o.) and cimetidine (Cimet, 100 mg/kg, p.o.) on water restrained stress-induced gastric lesion in rats (WRS). Each column represent mean ± SEM, $n=6$; one-way ANOVA, followed by Student-Newman–Keuls test for multiple comparison. $^{***}p<0.001$ vs. vehicle.

3.8. Water restraint stress-induced ulcer

Subjecting the rats toWRS caused gastric bleeding characterised by the mucosal haemorrhage and lesions; with an ulcerated area of 8.73 mm^2 in the vehicle control group. Oral administration of SIECm (25, 100 and 400 mg/kg) significantly inhibited the ulcerative lesions dose dependently, with maximal inhibition of 57.91% ($p<0.001$) at 400 mg/kg. The standard drug (cimetidine, 100 mg/kg) gave a superior activity with 92.28% ($p<0.001$) inhibition, compared to the vehicle group, as seen in Fig. 4.

3.9. Acetic acid induced-chronic ulcer

The surgically induced acetic acid chronic ulcer caused ulceration in the mice, with the vehicle group presenting an ulceration of 8.79 ± 1.14 mm^2. The post-operative treatment with SIECm for 7 days, accelerated healing of the induced chronic ulceration with a peak effect at 200 mg/kg (62.00%; $p<0.001$), similar to the standard drug cimetidine (50 mg/kg) (69.74%; $p<0.001$) when compared to the vehicle control group (Table 2).

3.10. Effect on gastric secretion

Table 3 shows the results of the effect of vehicle, SIECm (12.5, 50 and 200 mg/kg) and cimetidine (50 mg/kg) on the secretory parameters in rats with pylorus ligation. SIECm (12.5, 50 and 200 mg/kg) significantly reduced the volume of gastric secretion in a dose-dependent manner, with maximal inhibition of 75.10% ($p<0.001$) for 200 mg/kg; while cimetidine (50 mg/kg) inhibited the secretion of gastric juice by 79.82% ($p<0.001$) when compared with the vehicle group. A significant increase (67.93%, $p<0.05$) in the free acidity (pH) was observed only at the dose of 200 mg/kg of SIECm when compared with the vehicle group (pH=2.37 ± 0.26), while the reference drug (cimetidine) inhibited the pH by 82.92%. In the case of total acidity, reduction were observed as a result of treatment with SIECm (50 and 200 mg/kg) and cimetidine (50 mg/kg) by 26.15% ($p<0.05$), 43.13 and 54.50% ($p<0.01$), respectively.

3.11. Protective effect on gastric barrier mucus

The administration of acidified ethanol caused a 56.26% ($p<0.001$) reduction in the gastric mucus as shown by the dye recovery compared to the sham group. Treatment with SIECm prevented the gastric mucus depletion at doses of 100 ($p<0.05$) and 400 mg/kg ($p<0.001$) compared to the vehicle control group. For 100 mg/kg of carbenoxolone, reduction in the depletion was slightly higher (69.04%, $p<0.01$) than observed in the highest dose of the SIECm (Fig. 5).

Table 1
Effect of orally administered vehicle (10 mL/kg), infusion extract of the leaves of *Copaifera malmei* (SIECm; 25, 100 and 400 mg/kg), ranitidine (50 mg/kg) on piroxicam induced gastric ulcer in rats.

Treatment	Dose (mg/kg)	Ulcerated area (mm^2)[a]	% Inhibition
Vehicle	–	15.25 ± 0.14	–
SIECm	25	10.02 ± 0.04	34.30*
	100	6.94 ± 0.05	54.49**
	400	2.91 ± 0.02	80.91***
Ranitidine	50	1.42 ± 0.01	90.69***

[a] Each value represents mean ± SEM, $n=6$ animals; one-way ANOVA, followed by Student–Newman–Keuls test for multiple comparison.
* $p<0.05$.
** $p<0.01$.
*** $p<0.001$ vs. vehicle.

Table 2
Effect of oral administration of the vehicle (10 mL/kg), infusion extract of the leaves of *Copaifera malmei* (SIECm; 12.5, 50 and 200 mg/kg) and cimetidine (50 mg/kg) on the gastric ulcers induced by acetic acid in mice.

Treatment	Dose (mg/kg)[a]	Ulcerated area (mm^2)[b]	% Cure rate
Vehicle	–	8.79 ± 1.14	–
SIECm	12.5	4.09 ± 0.35	53.47**
	50	3.52 ± 0.50	59.95***
	200	3.34 ± 0.23	62.00***
Cimetidine	50	2.66 ± 0.37	69.74***

[a] Twice daily × 7 days.
[b] Each value represents the mean ± S.E.M. 6 animals per group; One-way ANOVA, followed by the Student–Newman–Keuls test.
** $p<0.01$.
*** $p<0.001$ vs. vehicle.

Table 3
Effect of intraduodenal (id.) administration of vehicle 10 mL/kg, infusion extract of the leaves of *Copaifera malmei* (SIECm; 12.5, 50 and 200 mg/kg) and cimetidine (50 mg/kg) on gastric secretion on the pylorus-ligation-induced ulcers in rats.

Treatment	Dose (mg/kg, id.)	Gastric volume (mL)	Free acidity (pH)	Total acidity (mEq [H^+]/mL)
Vehicle	–	4.66 ± 0.85	2.37 ± 0.26	12.31 ± 0.80
SIECm	12.5	3.07 ± 0.48*	2.89 ± 0.37	10.24 ± 0.78
	50	2.19 ± 0.67***	3.14 ± 0.27	9.09 ± 0.37*
	200	1.16 ± 0.18***	3.98 ± 0.59*	7.00 ± 0.68***
Cimetidine	50	0.94 ± 0.18***	6.44 ± 0.32**	5.60 ± 0.43***

Each value represents mean ± SEM, $n=6$; one-way ANOVA, followed by Student-Newman–Keuls test for multiple comparison.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$ vs. vehicle.

3.12. Assessment of the role of NO in the gastroprotective effect of SIECm

Pre-treatment with L-NAME to evaluate the role of NO production in SIECm gastroprotection, showed that it inhibited ($p < 0.001$) the induced increments in the ethanol-induced gastric mucosal lesions by L-NAME. As expected, the gastroprotection of carbenoxolone was also partially attenuated ($p < 0.05$) when compared to its respective control group (Fig. 6).

3.13. Catalase activity

Analysis revealed an increased in catalase enzymatic activity in the vehicle by 83.34% ($p < 0.001$) compared to the sham group. Treatment with SIECm prevented the deleterious increase in the catalase activity dose-dependently, with maximum effect being 66.14% ($p < 0.01$). Cimetidine also confers similar protection, inhibiting by 72.62% ($p < 0.01$) as shown in Fig. 7.

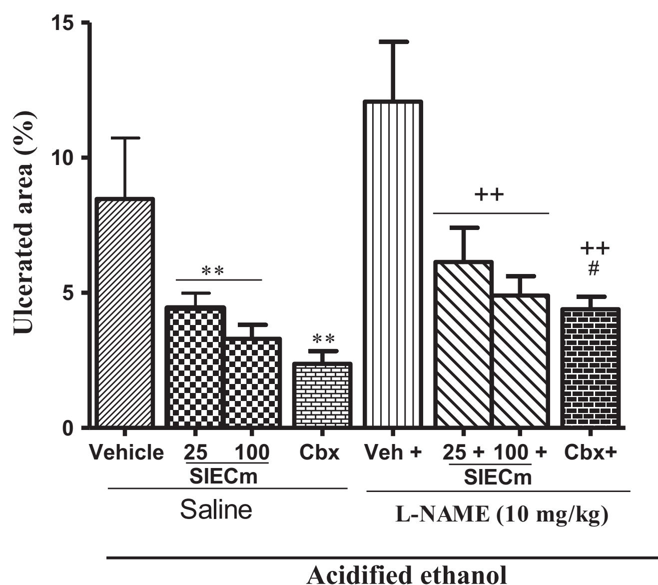

Fig. 6. Effect of pre-treatment with L-NAME (10 mg/kg) on gastric damage, and on gastroprotective effect of infusion extract of the leaves of *Copaifera malmei* (SIECm; 25, and 100 mg/kg) and carbenoxolone (Cbx, 30 mg/kg). Each column represents the mean ± S.E.M. of 5–6 animals. One-way ANOVA followed by Student-Newman-Keuls test. ** $p < 0.01$ vs. vehicle; ++ $p < 0.01$ vs. control values; #$p < 0.05$ vs. Cbx.

3.14. Myeloperoxidase (MPO) action

Subjecting the rats to WRS showed that the MPO action increased in the vehicle group by 63.08% ($p < 0.001$) compared to the sham. Treatment with SIECm attenuated this increase at the doses of 100 and 400 mg/kg, with maximum effect being 38.99% inhibition ($p < 0.05$). Cimetidine also confers similar protection, inhibiting the increase MPO activity by 47.19% ($p < 0.01$) as shown in Fig. 8.

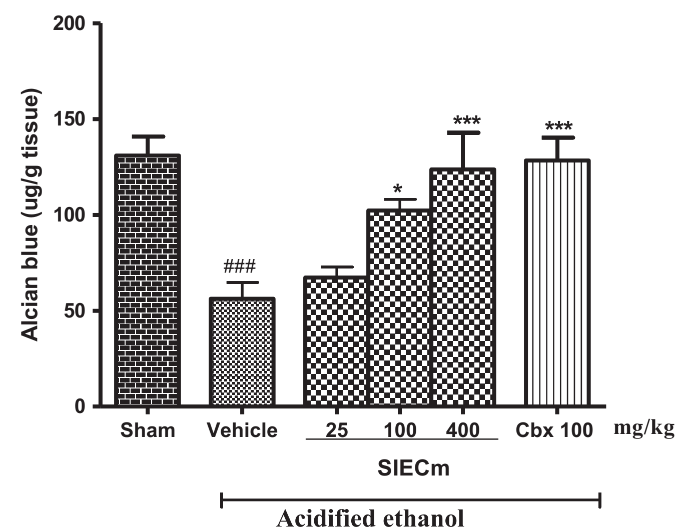

Fig. 5. Effect of orally administered vehicle (10 mL/kg), infusion extract of the leaves of *Copaifera malmei* (SIECm; 25, 100 and 400 mg/kg) and carbenoxolone (Cbx, 100 mg/kg) on gastric mucus in mice with gastric ulcer induced by acidified ethanol. Each bar is mean ± SEM, $n=6$ mice; one-way ANOVA, followed by Student-Newman–Keuls test for multiple comparison. *$p < 0.05$; and ***$p < 0.001$ vs. vehicle; ###$p < 0.001$ vs. sham.

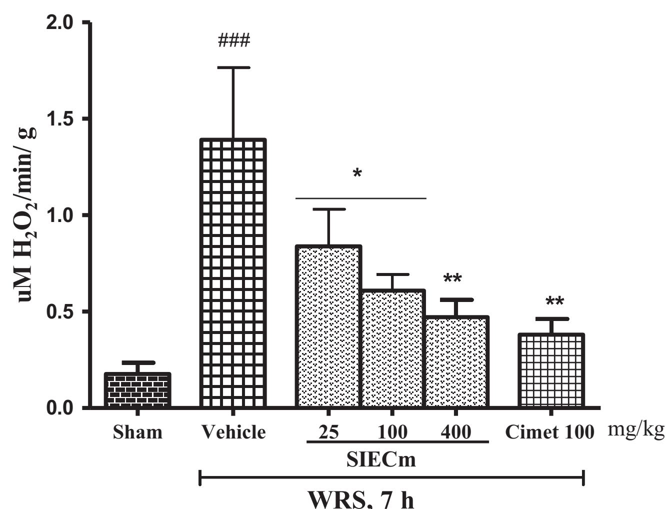

Fig. 7. Effect of oral administration of vehicle (10 mL/kg), infusion extract of the leaves of *Copaifera malmei* (SIECm; 25, 100 and 400 mg/kg), and cimetidine (Cimet, 100 mg/kg) on the activity of catalase in rats subjected to water restrained stress (WRS). Each column represents the mean ± S.E.M. of 6 animals. One-way ANOVA followed by Student–Newman–Keuls test. *$p < 0.05$; and ** $p < 0.01$ vs. vehicle; ### $p < 0.001$ vs. sham.

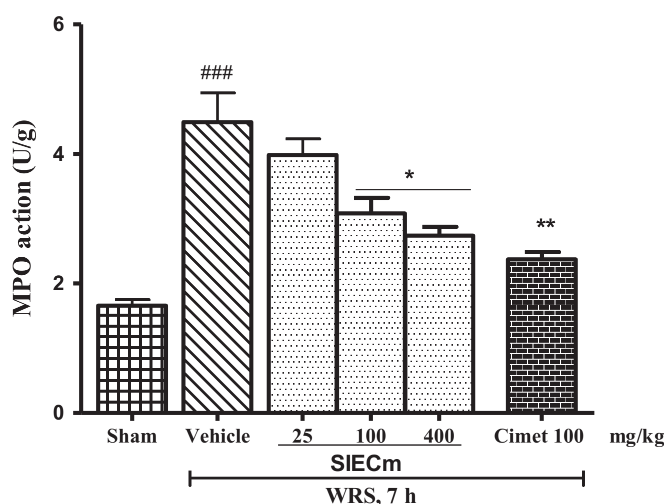

Fig. 8. Effect of oral administration of vehicle (10 mL/kg), infusion extract of the leaves of *Copaifera malmei* (SIECm; 25, 100 and 400 mg/kg), and cimetidine (Cimet, 100 mg/kg) on the MPO action in rats subjected to water restrained stress (WRS). Each column represents the mean ± S.E.M. of 6 animals. One-way ANOVA followed by Student–Newman–Keuls test. *$p<0.05$; and ** $p<0.01$ vs. vehicle; ###$p<0.001$ vs. sham.

3.15. Histopathological analysis

As shown in Table 4, no damage to the gastric tissue was observed in the Sham group. However, the oral administration of acidified ethanol caused a marked haemorrhagic injury, oedema, epithelial cells loss and leucocytes infiltration. Treatment with SIECm (100 and 400 mg/kg) reduced the severity of damage to the gastric tissues by 44.82 ($p<0.05$) and 56.90% ($p<0.01$), respectively, while the reference drug (carbenoxolone, 100 mg/kg) caused a reduction of 67.24% ($p<0.01$). The qualitative representative of the gastric tissue of each group is presented in Fig. 9.

3.16. Anti-Helicobacter pylori activity

In concentrations range of 6.25–800 μg/mL, infusion extract of the leaves of *C. malmei* (SIECm) showed no anti *H. pylori* activity. Clarithromycin, the standard for this test showed an intense activity with MIC of 0.78 μg/mL.

4. Discussion

The phytochemical constituents' profile of SIECm was investigated using TLC and HPLC techniques to identify relevant secondary metabolites groups and useful chemical markers. Such multiconstituent profile markers are used for quality control of herbal preparations and subsequent validity checks. An important finding in the phytochemical analysis of SIECm is the presence of phenolics, flavonoids and phytosterols; which were subsequently quantified separately using their specific assays.

Literature reports revealed that the tannins (polyphenolics) act as haemostatic in the wound healing process, by forming a protective layer (tannin-protein/tannin-polysaccharide complex) over the injured epithelial gastric mucosa; leading to the formation of a coated-barrier against gastric juice and permitting the ulcer healing process (Vasconcelos et al., 2010). Flavonoids exhibit anti-inflammatory action and induce angiogenesis and cell proliferation in addition to their gastroprotective effects (Vasconcelos et al., 2010). Sterols have been reported to have a cytoprotective effect on the gastroduodenal mucosa (Tovey et al., 2013).

One of the toxicity tests more frequently used for the evaluation of drugs and herbal extracts is tests on cell viability and cytotoxicity, for the fact that many drugs can cause irreversible damage at the cellular level. The CHO-k1 cell lines are widely used to screen for cytotoxic substances, and to study mechanism (s) leading to cytotoxicity (Węsierska-Gądek et al., 2005). Our result showed that SIECm did not alter the cellular viability of CHO-k1 cell lines ability to reduce resazurin after both 24 and 72 h exposures. Plant extract is considered to have an active cytotoxicity effect if the IC_{50} value following incubation between 48 and 72 h is <30 μg/mL and <4 μg/mL for pure substances (Suffness and Pezzuto, 1990). This indicates that SIECm has no cytotoxic effect. Although SIECm is devoid of cytotoxic effect, such in vitro assay is not enough to predict its in vivo effect (Houghton et al., 2007). Therefore, acute toxic effect of SIECm was also evaluated using in vivo model to affirm its safety. Result showed that SIECm is safe, with LD_{50} greater than 5000 mg/kg, p.o. in mice.

The SIECm gastroprotective activity was first assessed on the acidified ethanol-induced gastric ulcer. The ulcerogenic agent exerts direct toxic effect on epithelium and solubilises the gastric mucus (Martins et al., 2014) and is characterized by multiple haemorrhagic streaks along the glandular part of the stomach. This process of gastric ulceration can be blocked or successfully treated with potent gastroprotective agent, making the model a useful experimental tool to evaluate the capacity of a test agent in protecting the gastric mucosa (Júnior et al., 2014). SIECm effectively protects against gastric ulceration in this model and thus demonstrate its potential as a gastroprotective agent.

Induction of gastric ulcer and potentiation or impairment of the healing of pre-existing ulcers is one of the drawbacks in the use of NSAIDs. Our evaluation of the effect of SIECm on ulceration induced by piroxicam shows that SIECm attenuated the induced ulcerations, indicating that the extract may be acting by increasing

Table 4
Effect of orally administered vehicle (10 mL/kg), infusion extract of the leaves of *Copaifera malmei* (SIECm; 25, 100 and 400 mg/kg) and carbenoxolone (Cbx, 100 mg/kg) on gastric mucus in mice with gastric ulcer induced by acidified ethanol.

Treatment (mg/kg, p.o.)	Dose	Haemorrhagic damage (max. 4)	Oedema (max. 4)	Epithelial damage (max 4)	Leucocytes (max. 4)	Total (max. 16)
Sham	–	0	0	0	1.00	1.00
Vehicle	–	4.00	4.00	4.00	2.50	14.50
SIECm	25	4.00	4.00	4.00	2.25	14.25
	100	2.25	2.25	2.25	1.25	8.00*
	400	1.75	1.75	1.50	1.25	6.25**
Cbx	100	1.25	1.25	1.25	1.00	4.75**

Data shown are the mean scores. ANOVA of Kruskal–Wallis non-parametric test followed by Dunn's test.
* $p<0.05$.
** $p<0.01$ vs. vehicle.

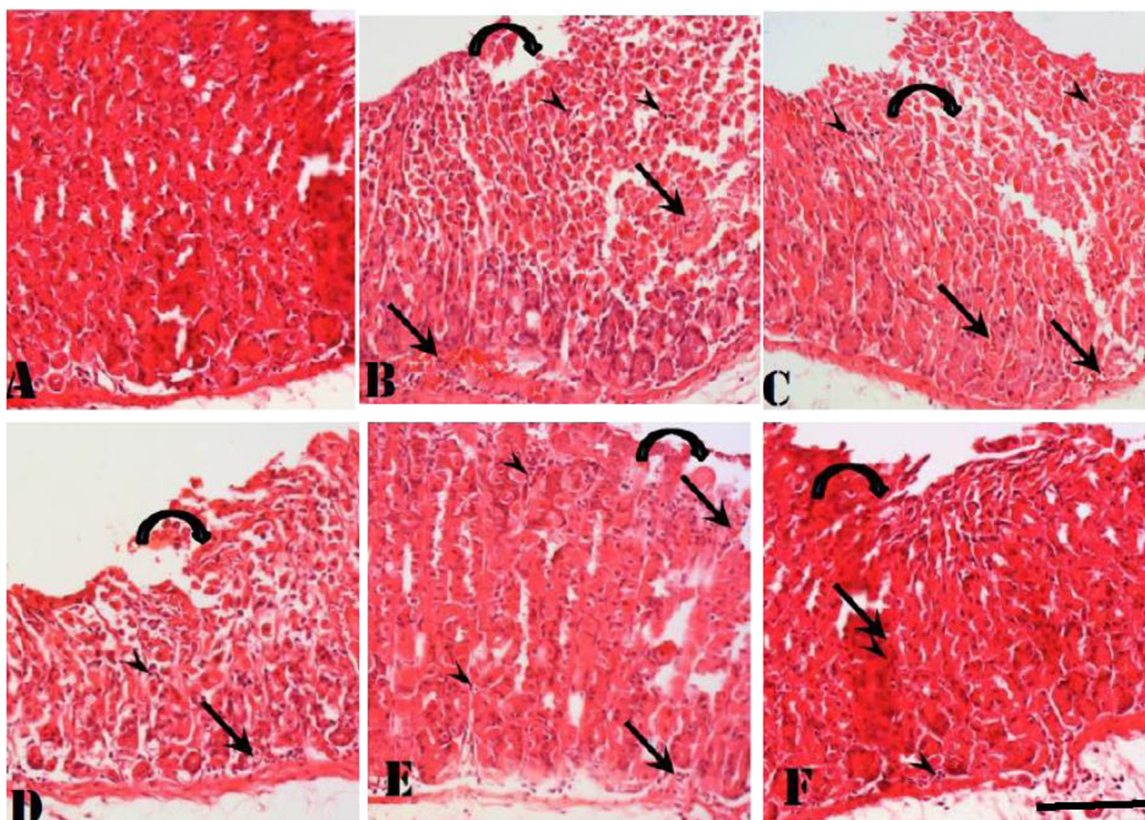

Fig. 9. Histopathological analysis of the Sham (A); vehicle (B); infusion extract of the leaves of *Copaifera malmei* [SIECm, 25 (C), 100 (D), and 400 (E) mg/kg], and carbenoxolone (F) on gastric mucosa lesions induced by acidified ethanol in mice. The arrows indicate epithelial damage (curve arrow), vascular damage (straight arrows), and leucocyte infiltration (arrowheads). Analysis of the tissue revealed normal gastric mucosa (A), extensive damages to the gastric mucosa (B), mild damage areas (C), and significant reduction in the epithelial damage (D, E and F). Stain: Haematoxylin and eosin. Bar = 50 μm.

the production of mucosal prostaglandins (Musumba et al., 2009) and thereby exhibiting gastroprotection through mucus production, and/or increased blood flow.

The ulcerogenic processes of the WRS is attributed to activation of peripheral autonomic pathways, characterised by neurotransmitters release leading to alteration of gastric blood, stimulation of gastric motility, enhanced acid secretion and reduced mucus production (Murakami et al., 1985). The SIECm exhibited gastroprotective activity in this model. The activity of the extract on the model is a very important criterion to assess the extract's potential efficacy as an anti-ulcer prototype due to its pathophysiological similarity to human gastric ulcer (Paré, 1989).

The acetic acid ulcer model has been used to investigate gastric ulceration and the ulcer healing efficacy of drugs against the disease. The model mimics the pathophysiology of human gastric ulcer disease in both necrosis of the gastric mucosa and its healing process (curative mechanisms) whereby the two distinct pathological characteristics: ulcer border and granulation tissue are expressed. The SIECm caused reduction of the ulcerated area by acetic acid at all doses tested, in a manner comparable to cimetidine. This result indicates that the extract contain potent constituent with gastric ulcer curative potentials (Martins et al., 2014). The plausible mechanisms of the gastroprotective activity of SIECm were also assessed. Currently, gastric ulcer treatment is in two main approaches: reduction of gastric acid secretion and re-enforcement of gastric mucosal protection, along with the eradication of *H. pylori* in infected patients. We tested the efficacy of SIECm in achieving these objectives.

Gastric ulceration in the pylorus-ligation model results from auto-digestion of mucosa by secretion of gastric acids that eventually leads to the activation of the gastric proton pump (H^+, K^+-ATPase), an important factor in the genesis of ulceration. The activity of SIECm in this assay indicates its anti-ulceration potency, plausibly via an antisecretory mechanism through the inhibition of H^+, K^+- ATPase activity. The extract exhibited significant activity on the model suppressing the accumulation of: gastric juice, free acidity (pH), and total acidity (mEq [H^+]/mL). This effect is important since suppression of gastric acid remains the main therapeutic approach to promote ulcer healing.

The gastric barrier mucus plays an important role as a first line defence against gastrointestinal damage. Ulcerogenic substances cause dissipation of the mucus gel and phospholipid layer, leading to mucosal injury. Histological dye has been used to explore the effects of treatments on the gastric barrier mucus based on the principle that a reduction in dye recovery indicates depletion of mucus barrier (Corne et al., 1974). As seen from the result, the gastroprotective effect of SIECm involves stimulation of gastric mucus production/protection.

The gastric lesions induced by the acidified ethanol model are also known to be associated with the reduction of endogenous derived NO level in the gastric mucosa. This subsequently deprived the NO role as one of the endothelial mediators essential for mucosal defence against acute damage by decreasing gastric mucosal blood flow and gastric mucus secretion (Tarnawski et al., 2012; Júnior et al., 2014). The partial attenuation of SIECm gastroprotective effect by L-NAME suggests that replenishment of NO levels in the gastric mucosa by the SIECm is one of the possible gastroprotective mechanisms of action.

The generation of free radicals are actively involved in the pathogenesis of gastric ulcers, particularly when its production

overwhelms the endogenous anti-oxidant system. We thus evaluated the antioxidant effect of SIECm. Our finding shows that SIECm curtailed the excessively elevated catalase activity, and decreased the MPO action in the WRS. This indicates that the gastroprotection effect of the SIECm is partly due to its antioxidant action.

Histopathological evaluation showed that SIECm caused reduction or prevention of epithelial and vascular damage, and leucocyte infiltration. Contraction of fibrous tissue formed at ulcer sites by fibroblast (cicatrisation) that leads to the ulcer healing process is performed by undifferentiated epithelial precursors that migrates from the ulcer border to the granulation tissue and eventually covering the base of the ulcer (Vasconcelos et al., 2010). The reduction/prevention of epithelial cell loss as confirmed by histological analysis is an indication of its potent anti-ulcer activity.

In conclusion, the study demonstrated that the SIECm has low oral acute toxicity, and exhibited gastroprotective and ulcer healing effect. This justifies the folkloric use of *C. malmei* leaves infusion in the treatment of gastric ulcers and also provided scientific evidence for the plant's potential as an anti-ulcer drug prototype. The mechanism in which SIECm exerts these effects is multi-targeted: plausibly mediated by complementary effects of protection/production of the gastric mucus, inhibition of gastric juice secretion, positive modulation of NO production and antioxidant effect. These gastroprotective activities might be attributed to the phytochemicals detected in the plant extracts, most likely acting as moiety (synergistically). The extract, however might have a limitation in cases in which *H. pylori* is the main aetiological factor since our results showed that SIECm was inactive against the organism.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2015.09.027.

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