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Effects of hecogenin and its possible mechanism of action on experimental models of gastric ulcer in mice

Gilberto Santos Cerqueira [a], Gabriela dos Santos e Silva [a], Emiliano Rios Vasconcelos [a], Ana Paula Fragoso de Freitas [a], Brinell Arcanjo Moura [a], Danielle Silveira Macedo [a], Augusto Lopes Souto [c], José Maria Barbosa Filho [c], Luzia Kalyne de Almeida Leal [b], Gerly Anne de Castro Brito [d], Caden Souccar [e], Glauce Socorro de Barros Viana [a,*]

[a] Department of Physiology and Pharmacology, Federal University of Ceará, Rua Cel. Nunes de Melo, 1127, CEP 60.431-270, Fortaleza, Brazil
[b] Department of Pharmacy, Federal University of Ceará, Rua Cel. Nunes de Melo, 1127, CEP 60.431-270, Fortaleza, Brazil
[c] Department of Pharmaceutical Sciences, Federal University of Paraíba, CEP 58.100-000, João Pessoa, Brazil
[d] Department of Morphology, Federal University of Ceará, Fortaleza, Brazil
[e] Department of Pharmacology, Federal University of São Paulo, Rua 3 de Maio, 100 , CEP 04044-020, São Paulo, Brazil

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ABSTRACT

This study investigates the gastroprotective effects of hecogenin, a steroid saponin isolated from *Agave sisalana*, on experimental models of gastric ulcer. Male Swiss mice were used in the models of ethanol- and indometacin-induced gastric ulcer. To clarify the hecogenin mechanism of action, the roles of nitric oxide (NO), sulfhydryls (GSH), K^+_{ATP} channels and prostaglandins were also investigated, and measurements of lipid peroxidation (TBARS assay) and nitrite levels in the stomach of hecogenin-treated and untreated animals were performed. Furthermore, the effects of hecogenin on myeloperoxidase (MPO) release from human neutrophils were assessed *in vitro*. Our results showed that hecogenin (3.1, 7.5, 15, 30, 60 and 90 mg/kg, p.o.) acutely administered, before ethanol or indomethacin, exhibited a potent gastroprotective effect. Although the pretreatments with L-NAME, an iNOS inhibitor, and capsazepine, a TRPV1 receptor agonist, were not able to reverse the hecogenin effect, this was reversed by glibenclamide, a K^+_{ATP} blocker, and indomethacin in the model of ethanol-induced gastric lesions. The hecogenin pretreatment normalized GSH levels and significantly reduced lipid peroxidation and nitrite levels in the stomach, as evaluated by the ethanol-induced gastric lesion model. The drug alone increased COX-2 expression and this effect was further enhanced in the presence of ethanol. It also decreased MPO release and significantly protected the gastric mucosa. In conclusion, we showed that hecogenin presents a significant gastroprotective effect that seems to be mediated by K^+_{ATP} channels opening and the COX-2/PG pathway. In addition, its antioxidant and anti-inflammatory properties may play a role in the gastroprotective drug effect.

1. Introduction

Peptic ulcer pathophysiology has been centered on an imbalance between aggressive (chloridric acid and pepsin) factors and protective ones represented by mucus and bicarbonate secretions, as well as by prostaglandins, sulphydryl compounds, polyamines, nitric oxide and dopamine. Peptic ulcer disease is a significant cause of morbidity and sometimes mortality among affected individuals, and its most common causes are the use of nonsteroidal anti-inflammatory drugs and the infection by *Helicobacter pylori* (Mynatt et al., 2009).

Although the introduction of H_2-receptor antagonists, proton-pump inhibitors, cyclooxygenase-2-selective (COX-2) anti-inflammatory drugs, and the eradication of *H. pylori* infection have contributed to the decrease of peptic ulcer disease, there is a need for new and safer drugs. The efficacy of different classes of secondary metabolites, including saponins, has been widely demonstrated, and many of them have been the source for the development of new drugs (Harvey, 2008). Saponins are amphipathic glycosides, composed of one or more hydrophilic glycoside moieties, combined with a lipophilic triterpene derivative. According to the structure of aglycone or sapogenin, two types of saponin are recognized: the steroidal and the triterpenoid ones.

The genus *Agave* presents sapogenin steroid-producing species, mainly hecogenin, that are important raw materials for the synthesis of steroidal drugs. Sapogenins are the aglycone nonsugar portions of the saponin molecule used for the semisynthesis of medicinal steroids, as corticosteroids, sexual hormones and steroid diuretics. The most investigated species *Agave americana* L. has shown antibacterial and anti-inflammatory activities (Peana et al., 1997). Anti-inflammatory

* Corresponding author. Tel.: +55 85 3366 8337; fax: +55 85 3366 8333.
E-mail address: gbviana@live.com (G.S. de Barros Viana).

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activities were reported not only for hecogenin but also tigogenin, isolated from the *Agave* genus (Peana et al., 1997). However, the most studied effects of hecogenin, diosgenin and tigogenin are those related to cancer research. Thus, these molecules were found to present an antiproliferative activity in human osteosarcoma cells (Corbiere et al., 2003). Furthermore, over the past decade, a series of studies have been conducted for understanding the role of diosgenin as a chemopreventive/therapeutic agent against several types of cancer (Raju and Mehta, 2009).

Hecogenin (Fig. 1) is the steroidal saponin aglycone (or sapogenin) present in the leaves of species from the *Agave* genus, including *Agave sisalana*, *Agave cantala* and *Agave aurea* (Paik et al., 2005). Extracts from these plants have been available as cardioactive or larvicide, and are popularly used in other ailments (Achenbach et al., 1994). Hecogenin has a wide spectrum of pharmacological activities already studied, including antifungal and hipotensive ones (Gondim, 2006). A recent study (Hashizume et al., 2008) showed that this substance is a selective inhibitor of human UDP-glucuronosyltransferases, enzymes responsible for the detoxification of numerous chemical toxins (Basu et al., 2004). Triterpene saponins showed an inhibitory effect on ethanol-induced gastric mucosal lesions in rats (Morikawa et al., 2006). Some other saponins have shown protective effects on ethanol- and indomethacin-induced gastric damage. Their protective activities are not due to the inhibition of gastric acid secretion, but probably to the activation of mucous membrane protective factors (Ramasubramancaraja and Babu, 2011).

These data lead us to test the possible activity of hecogenin against gastric damage induced by ethanol and indomethacin in mice, and to further assess its antiulcer mechanism of action. The action of hecogenin on lipid peroxidation, oxidative stress and myeloperoxidase, a biomarker of inflammation, was also carried out, as well as its possible protective effect on gastric mucosa, as examined by histological and COX-2 immunohistochemistry studies. This is the first report regarding the antiulcer activity of hecogenin, and may contribute to increase the knowledge on herbal-derived substances, and to offer therapeutic alternatives for the treatment of gastric ulcer.

2. Materials and methods

2.1. Animals

Male Swiss mice, weighing 20–30 g, were used. The animals were maintained in standard cages, at a controlled temperature (23 ± 1 °C), with a 12 h dark/12 h light cycle, and food and water *ad libitum*. Fifteen hours prior to the experiments, the mice were transferred to the laboratory and given only water *ad libitum*. The study was approved by the institutional Committee for Animal Experimentation and the experiments were carried out in accordance with the current law and the NIH Guide for the Care and Use of Laboratory Animals.

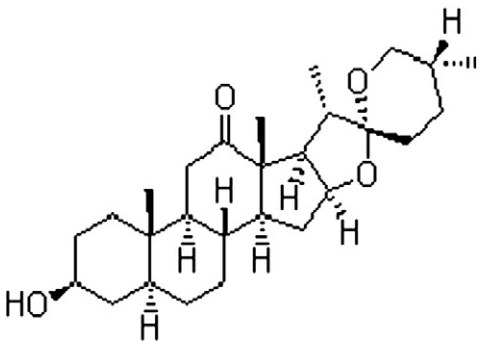

Fig. 1. Chemical structure of hecogenin.

2.2. Plant material and obtention of hecogenin from A. sisalana

The leaves of *A. sisalana* Perr were collected from various sisal cultures, in the municipality of Santa Rita, State of Paraíba, Brazil. A voucher specimen, L.P. Felix 6246 (EAN), is deposited at the Prof. Lauro Pires Xavier Herbarium (JPB), Federal University of Paraíba, Brazil. For the isolation of hecogenin, dry powdered leaves of *A. sisalana* (5 kg) were submitted to extraction (6 l, 95% ethanol) in a Soxhlet apparatus, for 24 h. The solvent was removed under reduced pressure, and the residue hydrolyzed by refluxing, for 4 h, with 1.5 liter 2 N ethanolic hydrochloric acid. The reaction mixture was cooled and filtered. The acid-insoluble material was washed with water, sodium carbonate solution, and water again, until presenting a neutral pH. The dried acid-insoluble residue was extracted with hexane in a Soxhlet apparatus, for 12 h. The hexane extract was left in a freezer for 24 h, the precipitate formed was filtered and its recrystalization in acetone, yielded 1 g (0.02%) hecogenin, m.p. 256–258 °C. Hecogenin was found to be identical to a standard sample (co-TLC and mixed m.p.). ^{1}H and ^{13}C NMR (500 and 125 MHz) spectra of hecogenin were in accordance with the literature (Agrawal et al., 1985). Hecogenin acetate was obtained by the acetylation reaction of hecogenin with an acetic anhydride/pyridine mixture. The supernatant was concentrated, and the residue subjected to column chromatography and PTLC plate over silica gel, yielding more hecogenin (0.35 g, 0.007%) and tigogenin (0,15 g, 0.003%), m.p. 200–202 °C. Tigogenin was found to be identical to an authentic sample (co-TLC and mixed m.p.). The purity of hecogenin as determined by HPLC was 98% and its structure is shown in Fig. 1.

2.3. Drugs and dosage

Hecogenin was emulsified with 0.2% Tween 80 (Sigma-USA) in distilled water, before use. The animals were orally treated with hecogenin, at the doses of 3.1, 7.5, 15, 30, 60 and 90 mg/kg, p.o. (except in the pylorus ligation experiment where it was 100 mg/kg), in the case of ethanol- and indomethacin-induced ulcer models, and at the dose of 90 mg/kg for experiments investigating the hecogenin mechanism of action. In those tests, 0.2 ml of absolute ethanol (CPQ-Brazil) and indomethacin (Sigma-USA), at the dose of 20 mg/kg (suspended in 0.5% carboxymethylcellulose, in distilled water), were orally administered. Controls received vehicle and/or water, and were administered by the same route as the treated groups. L-NAME (N (G)-nitro-L-arginine methyl ester) and glibenclamide (Sigma-USA), both at the dose of 10 mg/kg, were dissolved in distilled water and administered intraperitoneally. Ranitidine (100 mg/kg, p.o.) and N-acetyl cysteine (NAC, 300 or 750 mg/kg, i.p.), both from União Química-Brazil, were dissolved in distilled water.

2.4. Ethanol-induced gastric mucosal lesions

This is a widely used model that seems to cause gastric ulcer, independently from the acid secretion. Acute gastric lesions were induced by the intragastric application of absolute ethanol. For this, male *Swiss* mice were randomly divided into five groups, fasting for 15 h before the experiment, but had free access to water. Absolute ethanol (0.2 ml/animal, p.o.) was administrated orally to mice, 60 min after the vehicle (3% Tween 80 in distilled water, controls) or hecogenin (3.1, 7.5, 15, 30, 60 and 90 mg/kg), while ranitidine (100 mg/kg, p.o.), an antagonist of H_2 receptors, was used as the reference drug. These treatments were performed by gavage, with an orogastric metal tube. Thirty minutes after the administration of ethanol, the mice were killed by cervical dislocation, and the stomach was removed and opened along the greater curvature, for examination. The total and injured stomach areas (glandular portion) were measured by a computer program *Image J*., and expressed in terms of percentages of the ulcerated gastric area.

2.5. Indomethacin-induced gastric mucosal lesions

In this model, the gastric lesions are induced by the inhibition of prostaglandin synthesis. Male *Swiss* mice were randomly divided into four groups, treated orally with vehicle (controls), hecogenin (15, 30, 60 and 90 mg/kg, p.o.) and ranitidine (100 mg/kg, p.o.) as reference. After 60 min, the gastric ulcers were induced in all groups by indomethacin (20 mg/kg, p.o.) suspended in 0.5% carboxymethylcellulose in distilled water. Seven hours later, the animals were sacrificed by cervical dislocation. The stomachs were removed, immersed in 5% formalin for 15 min, and then opened along the great curvature and washed with a saline solution for examination of the lesions. The degree of ulceration was graded, according to an arbitrary scale, as presented in Table 1.

2.6. Pylorus ligature

A pylorus ligature (Shay model) was carefully done in mice under ether anesthesia. Hecogenin (10 to 100 mg/kg, n = 6) or vehicle (water, 0.1 ml/10 g body weight, p.o., n = 6) were injected into the duodenal lumen. After 4 h, the animals were killed, their stomachs opened and the gastric secretion was collected. The final volume and pH were determined after washing the mucosal side of the stomach with 2 ml distilled water. The total acidity of the gastric juice was titrated with NaOH (0.01 N or 0.1 N) using 2% phenolphtalein as an indicator.

2.7. Evaluation of the role of Nitric Oxide (NO), K^+_{ATP} channel, Prostaglandin and Transient Receptor Potential Vanilloid 1 (TRPV1) on the gastroprotective effect of hecogenin, in the ethanol-induced ulcer model

To study the possible mechanism of action of hecogenin, separate experiments were conducted using the following drugs: L-NAME, an inhibitor of the NO synthase activity (10 mg/kg, i.p.), glibenclamide, a blocker of K^+_{ATP} channels (10 mg/kg, i.p.), diazoxide, a K^+_{ATP} channel opener (10 mg/kg, p.o), indomethacin (10 mg/kg, p.o.) and capsazepine, a capsaicin and TRPV1 antagonist (CZP, 5 mg/kg, i.p.). L-NAME and glibenclamide were administrated 15 min before the administration of hecogenin (90 mg/kg, p.o.) with L-arginine (600 mg/kg, p.o.) or with diazoxide (3 mg/kg, p.o.), respectively. Indomethacin was administrated 2 h before the administration of hecogenin (90 mg/kg, p.o.) or misoprostol (70 μg/kg, p.o.). Capsazepine (5 mg/kg, i.p.) was given 30 min before the administration of hecogenin (25 mg/kg, p.o.) or capsaicin, a gastroprotective drug (CPS, 0.3 mg/kg, p.o.), and 1 h after the ethanol-induced ulcer. All animals received absolute ethanol (0.2 ml) for the ulcer induction. Thirty minutes after the administration of ethanol, the mice were killed, and their stomachs removed for examination, as previously described. The dose selections for these drugs were based on pilot experiments, and on literature findings (Peskar et al., 2002).

Table 1
Effect of hecogenin (10, 30 and 100 mg/kg, p.o.) on the gastric acid secretion after the pylorus ligation in mice.

Treatment (p.o.)	Volume (ml)	pH	Total acidity (mEq[H+]/L/4 h)
Control	0.92 ± 0.06	3.43 ± 0.36	2.11 ± 0.48
Hecogenin 10 mg/kg	0.80 ± 0.10	4.33 ± 0.61	2.63 ± 0.42
Hecogenin 30 mg/kg	0.66 ± 0.10	2.83 ± 0.27	2.31 ± 0.61
Hecogenin 100 mg/kg	0.70 ± 0.15	3.83 ± 0.54	2.02 ± 0.65
Ranitidine (50 mg/kg)	1.06 ± 0.14	3.83 ± 0.60	2.03 ± 0.61

The results are expressed as means ± S.E.M. of 6–7 animals per group and represent volume, pH and total gastric acid secretion, 4 h after the pylorus ligation.

2.8. Evaluation of the involvement of glutathione (GSH) on gastroprotective effects of hecogenin, in the ethanol-induced ulcer model

The amount of GSH (a non-protein SH) in the gastric mucosa was measured according to the method described by Sedlak and Lindsay (1968), with slight modifications. Briefly, hecogenin (90 mg/kg) or vehicle was administered to mice, 1 h before the administration of water or absolute ethanol (0.2 ml) to each group. Positive controls received N-acetyl cysteine (NAC), an amino acid essential for the formation of GSH, at the dose of 750 mg/kg, i.p., before the ethanol administration. Thirty minutes later, the animals were sacrificed by cervical dislocation, and their stomachs removed. For the assay of GSH, the glandular segment from each stomach was homogenized in an ice-cold 0.02 M EDTA solution (at 10%). Aliquots (400 μl) of tissue homogenates were mixed with 320 μl distilled water and 80 μl 50% (w/v) trichloroacetic acid (50%), in glass tubes, and centrifuged at 3000 rpm, for 15 min. Subsequently, the supernatants (400 μl) were mixed with 800 μl Tris buffer (0.4 M, pH 8.9), and 5,5-dithiobis (2-nitrobenzoic acid) (DTNB; 0.01 M) was added. After shaking the reaction mixture for 3 min, its absorbance was measured at 412 nm, within 5 min of the addition of DTNB, against the blank with no homogenate. The absorbance values were extrapolated from a glutathione standard curve, and expressed in μg GSH/mg of protein.

2.9. Evaluation of lipid peroxidation and nitrite levels on the gastroprotective effect of hecogenin in the ethanol-induced gastric lesions

The antioxidant activity of hecogenin was evaluated by measuring the thiobarbituric acid reactive substances (TBARS) levels, an indicator of lipid peroxidation. Stomach homogenates (10%, 250 μl) were prepared in 10% KCl, incubated at 37 °C for 1 h, and added to 400 μl 35% perchloric acid. The mixture was centrifuged (14,000 rpm, 20 min at 4 °C) and 400 μl of a 0.6% tiobarbituric acid solution were added to the supernatants, followed by the incubation at 95–100 °C for 1 h. After cooling, the supernatant absorbance was determined at 532 nm. For the standard curve, a 1,1,3,3-tetrametoxypropane solution was used. The results are expressed as nmol MDA/mg protein (MDA is a by-product of the lipid peroxidation reaction).

The Griess reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochoride in distilled water and 1% water sulfanilamide in 5% phosphoric acid) is widely used to detect the presence of nitrite in samples of urine, plasma and tissues. NO_2^- is a major unstable product of NO and molecular oxygen reaction, and gives an indication of free radicals generation. The diazotization reaction forms a pink chromophore, presenting a peak of absorption at 560 nm. The 10% stomach homogenate prepared in saline was centrifuged at 3000 for 10 min, and 100 μl of the Griess reagent were added to 100 μl supernatant. The reaction mixture was incubated for 10 min at room temperature. For blanks, 100 μl Griess reagent and 100 μl saline were used. The absorbance was measured at 560 nm in a microplate reader, and the nitrite concentration was determined from a standard $NaNO_2$ curve.

2.10. Histological assessment

For histological assessment, stomachs from all groups (negative control, saline, vehicle + ethanol, hecogenin + ethanol) submitted to the protocol previously described were fixed in 10% neutral-buffered formalin solution, sectioned and embedded in paraffin. Sections (4 μm) were deparaffinized, stained with haematoxylin and eosin, and then examined under a light microscope. The specimens were assessed according to the criteria of Laine and Weinstein (1988). In brief, a 1 cm length of each histological section was assessed by scores for epithelial (0–3), edema in the upper mucosa

(0–4), haemorrhagic damage (0–4) and presence of inflammatory cells (0–3). The sections were analyzed in a blind manner.

2.11. Immunohistochemistry for COX-2

For COX-2 immunohistochemistry assays, the streptavidin-biotin-peroxidase method was used. In brief, paraffin sections were deparaffinized, dehydrated in xylol and ethanol, and immersed in 0.1 M citrate buffer (pH 6.0) under heating for antigen retrieval. After cooling, the sections were washed with PBS, followed by the blockade of endogenous peroxidase with 3% hydrogen peroxide and incubation with primary rabbit anti-COX-2 p65 antibody (1:50). The slides were then incubated with biotinylated goat anti-rabbit secondary antibody (1:400), and again with avidin-biotin-horseradish peroxidase conjugate (ABC Vectastain® complex, Vector Laboratories, USA). COX-2 immunostainings were visualized with the chromogen 3,3 diaminobenzidine (DAB) and counterstained with Mayer hematoxylin, mounted and assessed by light microscopy.

2.12. Myeloperoxidase release from PMA-stimulated human neutrophils in vitro

Following Lucisano and Mantovani (1984), 2.5×10^6 human leukocytes were suspended in buffered Hank's balanced solution salt, containing calcium and magnesium. The preparations contained predominantly neutrophils ($85.0\pm2.8\%$), and the cell viability, as determined by the Trypan blue test, was $97.7\pm0.94\%$. The cells were incubated with hecogenin (1, 10 and 50 μg/ml) for 15 min at 37 °C. Human neutrophils were stimulated by the addition of phorbol myristate acetate (PMA, 0.1 μg/ml) for 15 min at 37 °C. The suspension was centrifuged for 10 min at $2000\times g$ at 4 °C. Aliquots (50 μl) of the supernatants were added to phosphate-buffered saline (100 μl), phosphate buffer (50 μl, pH 7.0) and H_2O_2 (0.012%). After 5 min at 37 °C, TMB (1.5 mM, 20 μl) was added, and the reaction was stopped by 30 μl sodium acetate (1.5 M, pH 3.0). The absorbance was determined using a spectrophotometer (620 nm).

2.13. Statistical analyses

Values are expressed as means ± S.E.M. For statistical analyses, one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls as the *post hoc* test were used. In the case of ulcer score determinations, the Kruskal–Wallis and the Dunn's tests were used. The results were considered statistically significant at probability (P) values equal or less than 0.05.

3. Results

3.1. Effect of hecogenin on the gastric damage induced by ethanol

The administration of absolute ethanol produced lesions on the gastric mucosa which were significantly and dose-dependently reduced from 46 to 78%, in the animals pretreated with hecogenin at the doses of 15 (10.92 ± 1.17, $P<0.001$), 30 (6.28 ± 1.30, $P<0.001$), 60 (7.90 ± 1.66, $P<0.001$) and 90 mg/kg (4.66 ± 0.40, $P<0.001$). No significant effects were observed with the two smaller doses. Ranitidine (100 mg/kg) and N-acetylcysteine (NAC, 300 mg/kg), used as reference drugs, also significantly reduced the gastric lesions by 83 and 99% (3.58 ± 0.87 and 0.13 ± 0.11, $P<0.001$), respectively, when compared to controls (21.60 ± 1.83) (Fig. 2).

3.2. Indomethacin-induced gastric mucosal lesions

Orally administered indomethacin (20 mg/kg, p.o.) induced severe gastric mucosal damage (score lesion = 9.38 ± 0.324) (Fig. 3). Hecogenin, at the tested doses (15, 30, 60 and 90 mg/kg, p.o.), exhibited a dose-related protective effect against gastric lesions, reducing from 28 to 49% the gastric lesions, as compared to the vehicle group. Ranitidine (100 mg/kg, p.o.), included in the present study as the positive control, also offered significant protection (6.43 ± 0.33), reducing gastric lesions by 31% [hecogenin 90, ranitidine: $F(3, 30)=39.93$, $P<0.05$].

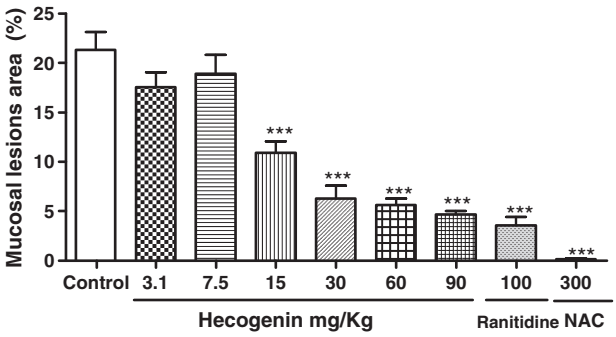

Fig. 2. Effects of the treatment with hecogenin and ranitidine of mice subjected to ethanol-induced gastric ulceration. Absolute ethanol (0.2 ml) was orally administered to 15 h fasting mice. Hecogenin (3.1, 7.5, 15, 30, 60 and 90 mg/kg, p.o.), ranitidine (100 mg/kg, p.o.) or vehicle was administered 60 min before ethanol. The results are presented as means ± S.E.M. *** $P<0.001$ vs. control.

3.3. Pylorus ligature in mice

Acute pyloric ligation (Shay preparation) has been found to increase both gastric histamine formation and total cellular histamine content of the stomachs (Columbus et al., 1974). The peak elevation in gastric cellular histamine content occurs 4 h after the ligation, returning to normal levels 24 h later. This peak elevation period corresponds to observed increases in volume and acidity from gastric secretion. In the present work, control mice were treated with vehicle (distilled water + 0.2% cremophor) or hecogenin (10, 30 and 100 mg/kg) injected into the duodenal lumen at the time of surgery. The gastric secretion collected 4 h after the pylorus ligature presented a volume (ml) of 0.92 ± 0.06, pH 3.43 ± 0.36 and acidity of 2.11 ± 0.48 mEq[H^+]/L/4 h. The treatment with hecogenin did not significantly alter any of these parameters (Table 1).

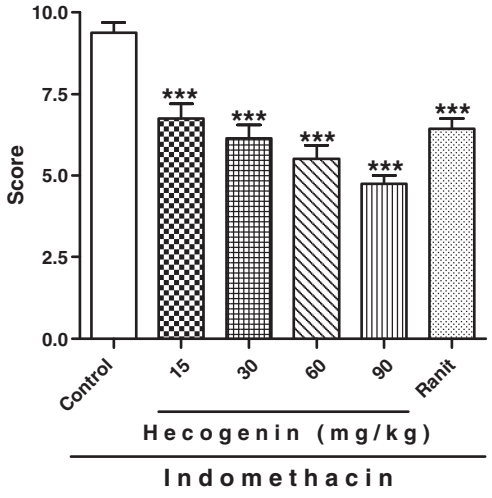

Fig. 3. Effects of hecogenin (15, 30, 60 and 90 mg/kg, p.o.) and ranitidine (100 mg/kg, p.o.) on ulcer scores, in the indomethacin-induced ulcer model (indomethacin 20 mg/kg, p.o.). The results are means ± S.E.M. for 8 mice per group. *** $P<0.001$ vs. control (vehicle + indomethacin).

3.4. Effects of L-NAME on the gastroprotection offered by hecogenin

The results obtained for the gastroprotective effect of hecogenin, after the pretreatment of mice with L-NAME, an inhibitor of the NO synthesis activity, are presented in Fig. 4. In the ethanol group (control), the gastric lesions (20.36 ± 2.06) were significantly reduced (76%) by hecogenin, at the dose of 90 mg/kg, p.o. (5.30 ± 0.96, $P<0.001$). Similarly, L-arginine (L-ARG, 3.87 ± 0.84, $P<0.001$) was able to protect the gastric mucosa (82% inhibition of the lesions). The previous administration of L-NAME (10 mg/kg) did not interfere with the gastroprotection of hecogenin 90 (7.64 ± 0.92; $P<0.001$) as compared to the hecogenin group, indicating that the NO system is probably not involved with the hecogenin effects. On the contrary, the effects of L-ARG were reversed by its combination with L-NAME (13.58 ± 1.77, $P<0.001$), as compared to L-ARG alone, and the values for the gastric lesions score were increased towards those of the ethanol controls.

3.5. Effects of hecogenin on ethanol-induced gastric mucosal lesions in glibenclamide-pretreated mice

The effects of the oral administration of hecogenin on ethanol-induced lesions, in glibenclamide- and diazoxide-pretreated mice, are demonstrated in Fig. 5. Glibenclamide and diazoxide are K^+_{ATP} channel blocker and opener drugs, respectively. The pretreatment with the K^+_{ATP} channel blocker, known to drastically induce gastric lesions, did significantly decrease the gastroprotective effect of hecogenin (11.40 ± 0.69, $P<0.001$), as compared to hecogenin 90 (5.30 ± 0.96) and control groups (18.61 ± 1.97). The co-administration of glibenclamide plus diazoxide (21.43 ± 3.46, $P<0.001$) completely blocked the gastroprotection observed in the diazoxide-treated group (4.52 ± 1.00). These results indicate that the gastroprotection offered by hecogenin is, at least in part, dependent upon the K^+_{ATP} channels activity, and the drug behaves as a K^+_{ATP} channels opener.

3.6. Evaluation of the involvement of prostaglandins on gastroprotective effects of hecogenin, in the ethanol-induced ulcers model

The results of the pretreatment with indomethacin on the gastroprotective effect of hecogenin are shown in Fig. 6. As described before, the ethanol-induced ulcers were significantly reduced by the pretreatment with hecogenin (5.30 ± 0.96, $P<0.001$) as well as by misoprostol, a PGE_1 analog (9.16 ± 1.13, $P<0.01$), as compared to controls (22.34 ± 1.51). This effect was significantly altered and the ethanol-induced ulcers increased by the previous indomethacin administration (13.47 ± 1.59, $P<0.05$), as compared to hecogenin 90 alone. A similar result was also shown by the combination of indomethacin plus misoprostol (18.53 ± 2.04, $P<0.05$), as compared to the misoprostol group alone. These results indicate that the effect of hecogenin, similarly to that of misoprostol, is at least in part dependent upon the prostaglandin synthesis.

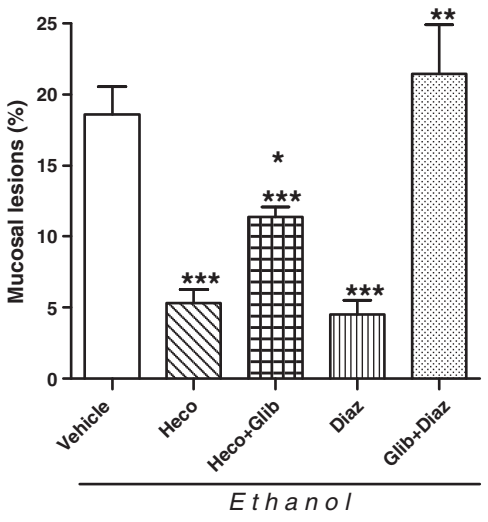

Fig. 5. Effects of the pre-treatment with glibenclamide on the gastroprotection of hecogenin in mice subjected to ethanol-induced gastric ulceration. Glibenclamide (GLIB, 10 mg/kg, i.p.) or vehicle were injected 15 min before the oral administration of hecogenin (90 mg/kg, p.o.) or water to each group. Sixty minutes after, gastric mucosal lesions were induced with absolute ethanol (0.2 ml, p.o.). The results are presented as means ± S.E.M. *** $P<0.001$ vs. Vehicle (control); ** $P<0.001$ vs. Diaz; * $P<0.05$ vs. Heco.

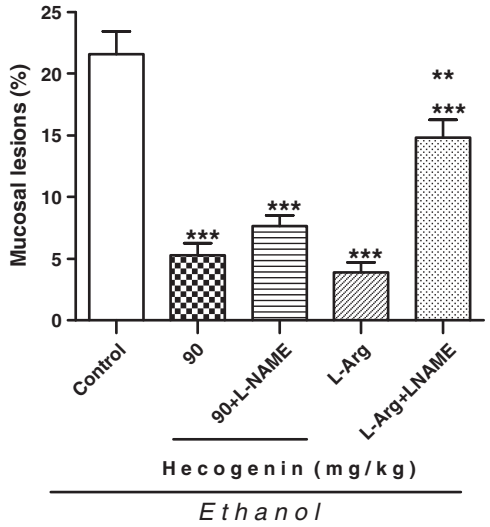

Fig. 4. Effects of nitric oxide synthase inhibition by L-NAME + L-Arg on the gastroprotection of hecogenin in mice subjected to ethanol-induced gastric ulceration. L-NAME (10 mg/kg, i.p.) or vehicle were injected 15 min before the administration of hecogenin (90 mg/kg, p.o.) or water to each group. Sixty minutes after, gastric mucosal lesions were induced with ethanol (0.2 ml, p.o.) in all groups. The results are presented as means ± S.E.M. *** $P<0.001$ vs. control; ** $P<0.01$ vs. L-Arg.

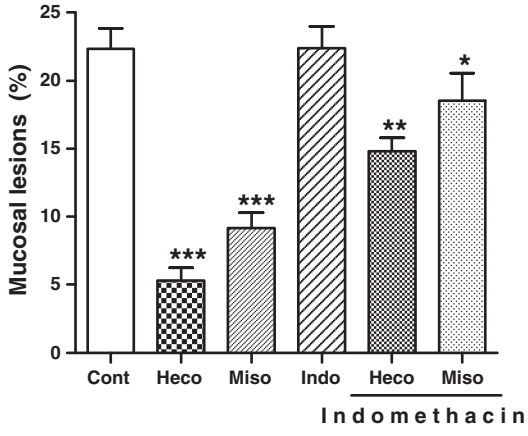

Fig. 6. Effects of the pre-treatment with indomethacin on the gastroprotection of hecogenin in mice subjected to ethanol-induced gastric ulceration. Mice were pre-treated with indomethacin (20 mg/kg, p.o.) or vehicle, 2 h before the oral administration of hecogenin (90 mg/kg, p.o.) or water to each group. Sixty minutes after, gastric lesions were induced with absolute ethanol (0.2 ml, p.o.). The values are expressed as means ± S.E.M. (n = 8 per group). *** $P<0.001$ vs. control and vs. indo; ** $P<0.01$ vs. Heco; * $P<0.05$ vs. Miso.

3.7. Evaluation of the involvement of TRPV1 (Transient Receptor Potential Vanilloid 1) on gastroprotective effects of hecogenin, in the ethanol-induced ulcer model

Capsaicin, a TRPV1 agonist, is known to protect the animal against gastric mucosal injury by stimulating afferent neurons, and these effects are blocked by the previous administration of capsazepine, a TRPV1 antagonist. Our results showed that capsazepine did not alter the gastroprotection promoted by hecogenin (90 mg/kg, p.o.). Thus, the ethanol-induced ulcers were reduced by the pretreatments with hecogenin (8.29 ± 1.34, $P<0.001$) or capsaicin (13.19 ± 1.22, $P<0.01$), as compared to controls (19.93 ± 1.50). While capsazepine did not alter the hecogenin effect (8.89 ± 0.93), it was able to revert the gastroprotection promoted by capsaicin (19.72 ± 2.49, $P<0.05$), as compared to capsaicin alone. These data suggest that TRPV1 receptors are not involved with the hecogenin effects (Fig. 7).

3.8. Effect of hecogenin on the non-protein sulfhydryl (GSH) content in stomach tissues

Table 2 shows the influence of the pretreatment with hecogenin and NAC, a mucolytic and antioxidant agent, on the gastric content of GSH (μg/g protein) in ethanol-induced ulcers in healthy animals. The animals treated with NAC 750 mg/kg, i.p., before the ethanol administration, showed significant increases in the levels of GSH (649.40 ± 16.17, $P<0.001$), when compared to the saline plus ethanol group (285.40 ± 22.13). However, in the group treated with hecogenin (263.60 ± 14.51), the GSH content was not altered. Animals without ulcer showed GSH contents higher than those of controls (399.50 ± 55.29, $P<0.01$). Our results indicate that, in the presence of ethanol-induced gastric lesions, the animals pretreated with hecogenin presented increased GSH contents, as compared to the ethanol group, reducing the ethanol effects and bringing values close to those of controls (without ethanol).

3.9. Effect of hecogenin on lipid peroxidation (nmol/g protein) and nitrite (ng/g protein) concentrations in homogenates of mice stomachs, as evaluated in the ethanol-induced gastric lesions model

The lipid peroxidation results (Table 2) showed that ethanol (control) significantly increased by 70% the lipid peroxidation in mice stomachs exposed to that ulcerating drug. Hecogenin brought values to normality (saline), and the data were similar to those observed in the presence of N-acetylcysteine (NAC) used as the reference drug. These results suggest the participation of oxidative stress and its blockade, in the anti-ulcer effect of hecogenin. On the contrary, while in ethanol exposed stomachs there was a significant increase in nitrite concentrations (63%), the results observed in the presence of hecogenin were not different from those of saline, indicating that the blockade of free radicals production may be also involved with the anti-ulcer action of hecogenin.

Table 2
Effects of hecogenin on the levels of non-protein sulfhydryls (NP-SHs), lipid peroxidation (MDA) and nitrite concentrations, in stomachs of ethanol-treated mice.

Treatment	Dose (mg/kg, p.o.)	NP-SH (μg/g protein)	MDA (nmol/mg protein)	Nitrate/nitrite (μg/g protein)
Saline	–	197.4 ± 15.43	$0.13 \pm .22$[a]	8.61 ± 0.86
Control	–	122.7 ± 13.17	0.18 ± 0.01	9.85 ± 1.18
Hecogenin	90	178.4 ± 15.44[a]	0.15 ± 0.01	6.90 ± 0.43[a]
NAC	750	184.1 ± 19.29[a]	0.13 ± 0.00[a]	–

The results represent means ± S.E.M. of 8 animals per group.
[a] vs. control, $P<0.05$.

3.10. Effect of hecogenin on human neutrophil-released mieloperoxidase (MPO) in vitro

The addition of Tween 80 (2%) to PMA-stimulated human neutrophils drastically increased in 8.5 times the MPO release, as compared to cells exposed to the negative controls represented by the Hanks solution (0.084 ± 0.0224), as observed by the significant increase in absorbance values (0.713 ± 0.068). The addition of hecogenin (1, 10 and 50 μg/ml) reduced dose-dependently the myeloperoxidase release from 19 to 65%, in the presence of its highest concentration (0.249 ± 0.027, $P<0.001$), as compared to neutrophils exposed to 2% Tween 80 (Fig. 8).

3.11. Histological evaluation of the hecogenin effect on the gastric mucosa in the model of ethanol-induced gastric ulcer in mice

While the results (Fig. 9 and Table 3) showed normal gastric epithelia in saline-treated mice (saline with 3% Tween 80, used as vehicle), the ethanol treated stomachs presented inflammation, edema, moderate hemorrhage and a great loss of epithelium cells. On the

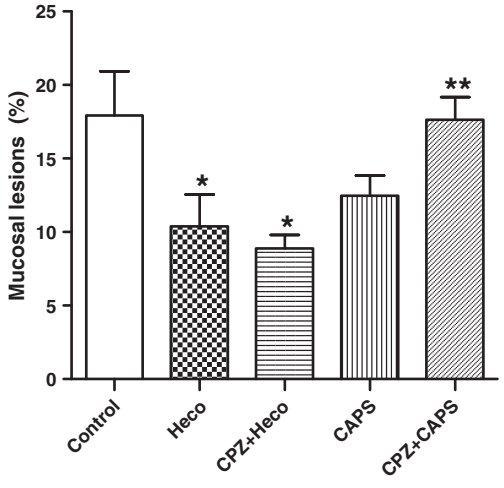

Fig. 7. Role of vaniloid receptors (TRPV1) on the gastroprotective effect of hecogenin in the model of ethanol-induced gastric lesion in mice. The values are means ± S.E.M. of the percentage of ulcerated gastric area (vehicle 2% Tween 80 in distilled water, 10 ml/kg). Eight animals per group were used. * $P<0.05$ vs. control; ** $P<0.05$ vs. Heco and vs. CPZ + Heco.

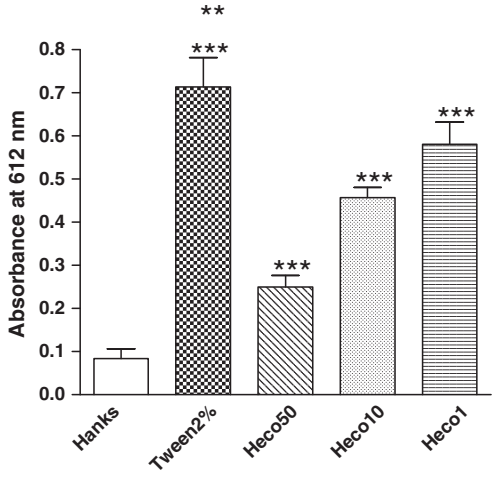

Fig. 8. Effects of hecogenin on the release and activity of human neutrophil myeloperoxidase (MPO) stimulated by PMA. Freshly isolated cells were pre-incubated with indicated concentrations of hecogenin, prior to the addition of PMA (100 nM). The data are expressed as percentages of inhibition by hecogenin on the release of MPO. The values represent means ± S.E.M. *** $P<0.05$ to 0.001 vs. Hanks (control); ** $P<0.05$ to 0.001 vs. Heco (1, 10, 50).

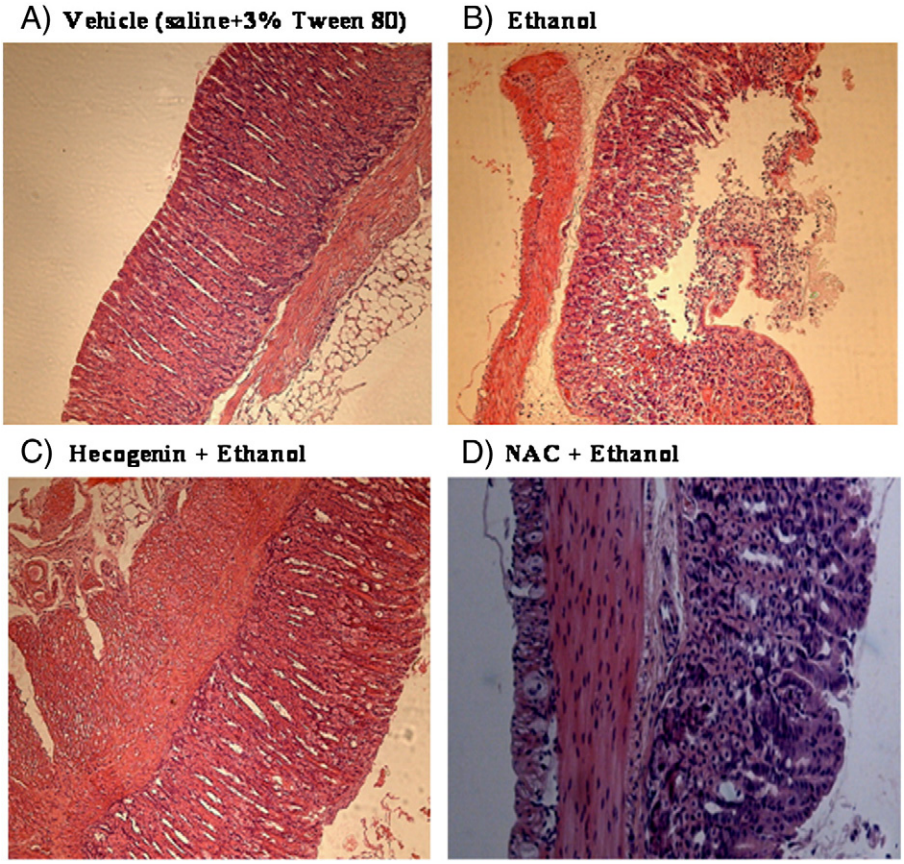

Fig. 9. Photomicrographs of HE staining of gastric mucosa (magnification × 100). A. Vehicle (normal mice); B. Animals treated with absolute ethanol, showing disruption of the superficial region of the gastric gland with epithelial cell loss, edema and intense hemorrhage; C. Animals treated with absolute ethanol-hecogenin (90 mg/kg, p.o.), showing preservation of gastric mucosa; and D. Animals treated with absolute ethanol-NAC (300 mg/kg), showing a profile similar to normality. Quantitative results are shown in Table 2.

other hand, the pre-treatment with hecogenin of the ethanol-treated animals (90 mg/kg, p.o.) reversed these alterations, and the histological aspect was similar to those observed in the ethanol group pre-treated with N-acetylcysteine (NAC, 300 mg/kg, p.o.), used as the reference drug.

3.12. Immunohistochemistry for COX-2

Fig. 10 shows the effects of hecogenin on COX-2 immunoreactivity, in the model of ethanol-induced gastric damage in mice. The results show a normal gastric epithelium, not only in the negative control (A) but also in the saline-treated (B) groups. No immunostainings for COX-2 were observed in the gastric gland base. On the contrary, the vehicle + ethanol group (C) presented an intense gastric damage of epithelium cells, associated with immunostaining for COX-2. A similar effect was observed in the group treated with hecogenin only (D). Furthermore, the hecogenin + ethanol group (E) also presented an enhanced immunostaining for COX-2.

4. Discussion

The therapeutic management of peptic ulcer includes several classes of drugs, such as proton pump inhibitors, histamine receptor blockers, drugs affecting the mucosal barrier, and prostaglandin analogs. However, the development of tolerance and side effects, as well, make the efficacy of these treatments questionable, pointing to the need for the development of new anti-ulcer drugs (Mard et al., 2008). On the other hand, there are very few studies showing the anti-inflammatory activity of hecogenin (Peana et al., 1997) and none on its gastroprotection. Most of the literature data focus on diosgenin, also a steroidal sapogenin as hecogenin (Liagre et al., 2005, 2007a, 2007b; Moalic et al., 2001; Shishodia and Aggarwal, 2006). Thus, the present study demonstrates, for the first time, the anti-ulcer property of hecogenin from *A. sisalana*, on experimental models of gastric ulceration in mice.

We showed that hecogenin was effective in both ethanol- and indomethacin-induced gastric ulceration. In the model of ethanol-

Table 3
Effects of hecogenin (90 mg/kg) and NAC (300 mg/kg) on ethanol-induced microscopic damage in gastric mucosa.

Experimental group (n = 6)	Hemorrhagic damage (score 0–4)	Edema (score 0–4)	Epithelial cell loss (score 0–3)	Inflammatory cells (score 0–3)	Total score (0–14)
Saline	0[b]	0[b]	0[b]	0	0
Ethanol	3.5 (3–4)[a]	3 (3–3)[a]	3 (3–3)[a]	0.5 (0–1)	
Ethanol + Hecogenin	0 (0–2)[b]	0 (0–3)[b]	0 (0–2)[b]	0 (0–1)	
NAC (300 mg/kg) + Ethanol	0[b]	0[b]	0[b]	0[b]	0

The data are medians with minimal and maximal scores shown in parentheses. For histological analyses, the Kruskal–Wallis nonparametric test followed by the Dunn's test were used.
[a] $P<0.05$ vs. saline.
[b] $P<0.01$ vs. ethanol.

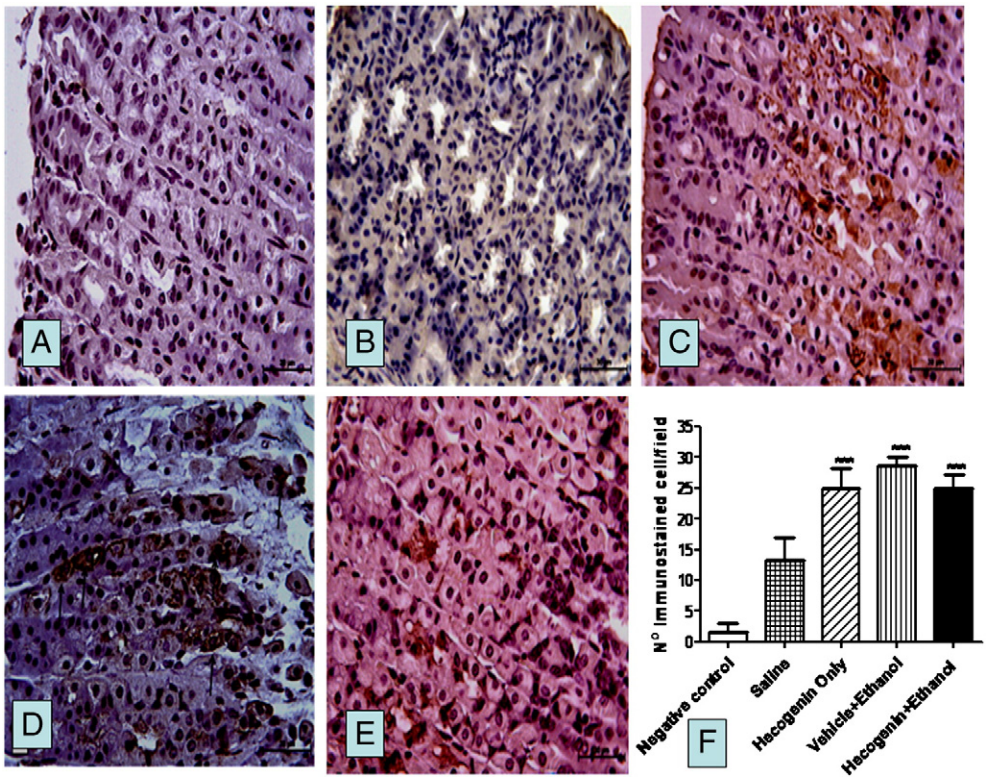

Fig. 10. Representative micrographies (40×) showing the effect of hecogenin on COX-2 immunoreactivity in the ethanol-induced gastric damage model in mice. Hecogenin (90 mg/kg) increased COX-2 expression, and this effect was further enhanced in the presence of ethanol. A: negative control; B: saline group; C: hecogenin (90 mg/kg) only; D: vehicle + ethanol showing ethanol-induced gastric damage associated with immunostaining for COX 2 (arrows), also observed in E: hecogenin + ethanol group; F: counting of COX-2 positive immunostained cells (3 animals from each group). The results are presented as means ± S.E.M. *** $p<0.001$ vs. negative control and saline.

induced gastric damage, hecogenin at the higher dose showed a gastroprotective effect similar to that observed with ranitidine and NAC, used as reference drugs. This model was shown to be associated with a significant reduction in NP-SH contents in the gastric mucosa, as well as with other mechanisms, such as the mucosal leukotriene release (Peskar et al., 1986) and submucosa venular constriction (Oates and Hakkinen, 1988). The NP-SH compounds exert their functions in gastroprotection by maintaining the integrity of the mucosal barrier and binding free radicals formed (Szabo and Vattay, 1990).

Hecogenin presented a significant gastroprotection, similar to that observed in the N-acetyl cysteine (NAC) group and controls, indicating the involvement of hecogenin protective mechanisms on the oxidative stress induced by ethanol in the gastric mucosa. The role of reduced glutathione (GSH) as an endogenous antioxidant is to protect the gastric mucosa (Tanaka and Yuda, 1996), and it functions as a scavenger of $O^{2\bullet}$, protecting thiol groups from oxidation. Evidences showed that the GSH redox cycle is catalyzed by endogenous antioxidative enzymes (Fesharaki et al., 2006).

It is well known that NO is involved in the modulation of the gastric mucosal integrity, and is important to the regulation of acid and alkaline secretions, mucus secretion, and gastric mucosal blood flow (Andreo et al., 2006). In order to investigate the role of endogenous NO in the cytoprotection, the NO synthase inhibitor (L-NAME) was used to access the protective effect of hecogenin on ethanol-induced gastric hemorrhagic lesions. Our data showed that the pretreatment with L-NAME did not change the cytoprotection induced by hecogenin. Nitric oxide (NO) appears to be a key mediator of gastrointestinal mucosal defense. NO, produced via activity of NO-synthase (NOS), is one of the major factors involved in the regulation of the gastric blood flow and gastric microcirculation (Olinda et al., 2008; Rocha et al., 2009).

In addition, it has been shown that prostaglandin mediated gastroprotection involves the opening of K_{ATP} channels, which are a class of ligand-gated proteins that seems to be involved with a variety of physiologic functions of the stomach, such as gastric blood flow regulation, acid secretion, and contractility (Garcia et al., 1997). In fact, Peskar et al. (2002) demonstrated that endogenous prostaglandins act as activators of K_{ATP} channels, and this mechanism, at least in part, mediates gastroprotection. Recent studies have also suggested the participation of K_{ATP} channels in the indomethacin- (Silva et al., 2009) and ethanol-induced (Medeiros et al. 2008) ulcer models, in which prostaglandins were shown as probable activators of these channels.

Our results showed that the gastroprotection mechanism of hecogenin was K_{ATP} channel-dependent, since its gastroprotective effects were reverted by the pretreatment with glibenclamide, a potent antagonist of these channels. We can then suggest the participation of K_{ATP} channels in the gastroprotective effects of hecogenin, and prostaglandins could be involved in the activation of these channels.

Previous studies demonstrated that prostaglandins and NO cooperate with sensory nerves to the mechanism of gastric mucosal integrity and cytoprotection (Brzozowski et al., 1999). Furthermore, NO generated from iNOS was shown not only to participate in ulcer formation, but also to play a beneficial role in ulcer healing (Akiba et al., 1998). We postulate that hecogenin, as prostaglandins, participates in the mechanism of gastric mucosal integrity through the activation of K_{ATP}-channels. Prostaglandins play an important role in modulating the integrity of the gastric mucosa in the presence of gastric acid secretion (Curtis et al., 1995). Our results showed that the pretreatment with indomethacin prevents the protective effect of hecogenin in the model of ethanol-induced gastric ulcer, suggesting that an increase in prostaglandin synthesis is probably involved with the drug gastroprotective action.

Furthermore, we also investigated the possible involvement of TRPV1 receptors on the gastroprotective effects of hecogenin. It has been reported that capsaicin, a TRPV1 agonist, prevents the

indomethacin- and ethanol-induced gastric mucosal injuries (Mózsik et al., 2007). In the present work, we showed that while capsazepine, a capsaicin antagonist, completely blocked the gastroprotective effect of capsaicin, it did not alter the hecogenin gastroprotection, suggesting that TRPV1 receptors are not involved with the drug action.

In the indomethacin-induced ulcer model, we showed that the pretreatment with hecogenin also promoted protection against gastric damage, similarly to ranitidine, an antisecretory drug, suggesting that the hecogenin antiulcer activity might be also related to its antisecretory effect. Considering that indomethacin-induced ulcerations are mainly attributed to the inhibition of prostaglandin synthesis, these results corroborate the findings mentioned above, and strongly suggest the involvement of prostaglandins in the gastroprotection offered by hecogenin.

Furthermore, an earlier work (Yamada et al., 1997) showed that diosgenin attenuated indomethacin-induced intestinal inflammation. The cytoprotection observed in the stomach is highly dependent upon the products of the arachidonic acid pathway and the peroxidative–antioxidative balance. It has been firmly established that the oxidative stress and impaired prostaglandin synthesis contribute to gastric mucosal damage, in experimental models of gastric lesions induced by both ethanol and indomethacin (Chattopadhyay et al., 2004; Olinda et al., 2008).

Hecogenin alone increased COX-2 expression and this effect was further enhanced in the presence of ethanol. Ethanol induces gastric lesions by increasing gastric MDA levels, MPO activity, and COX-2 expression, as well as by decreasing PGE2 synthesis (Zhao et al., 2009). Our results suggest that the hecogenin beneficial effect in this model of gastric injury occurs through mechanisms that may involve inhibition of inflammatory cell infiltration and lipid peroxidation, and upregulation of the COX-2/PG pathway. The expression of COX-2 was shown to be markedly upregulated in gastic ulcers, and its inhibition leads to a delay of the ulcer healing process (Perini et al., 2003). Thus, COX-2 may promote ulcer healing (Guo et al., 2003).

Furthermore, our results also show that the MPO (a biomarker for inflammation) activity was significantly decreased in the presence of hecogenin, and a similar effect was demonstrated by others (Yamada et al., 1997) with diosgenin. Reactive oxygen metabolites may play an important role in the pathophysiology of acute ulceration induced by ethanol. It is also reasonable to assume that MPO may be an important source of reactive oxygen species. Indomethacin which induces gastric lesions was reported (Akar et al., 1999) to increase MPO activity in the gastric mucosa, an effect reversed by cromakalin and diazoxide, known K^+_{ATP} channel openers. On the contrary, glibenclamide, a K^+_{ATP} channel blocker, abolished their effects, and increased MPO activity.

In summary, the results of the present study demonstrated that hecogenin shows significant gastroprotective effects, in both ethanol- and indomethacin-induced ulcer models, that may be mediated by K^+_{ATP} channels. These gastroprotective effects were confirmed by histological and COX-2 immunohistochemistry data. Furthermore, the drug presents antioxidant properties, as observed by the increase in GSH contents and the blockade of lipoperoxidation and of the production of free radicals. Hecogenin also showed an anti-inflammatory action, as demonstrated by the inhibition of MPO release from human neutrophils. Although further studies are still needed in order to better elucidate the exact mechanism of the hecogenin action, the participation of the COX-2/PG pathway is also probably involved.

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