Antiulcerogenic activity of the aqueous fraction of *Anacardium humile* St. Hil (Anacardiaceae)

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Accepted 19 June, 2012

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Anacardium humile St. Hil. is being used in traditional medicine to treat ulcer. The present work evaluated the mechanisms of action involved in the anti-ulcer properties of the aqueous fraction from leaves of *A. humile* (AHQ). Gastroprotection of *A. humile* was evaluated in ethanol and piroxicam models. Mechanisms of action such as mucus production, nitric oxide (NO), sulfhydryl compounds (SH) and the anti-secretory action were evaluated. The acetic acid-induced gastric ulcer model was used to evaluate the *A. humile* healing properties. Results obtained in the ethanol model showed that AHQ provided significant gastroprotection at all the tested doses (50, 100, and 200 mg/kg). Thereby, the following protocols were performed using the dose capable of producing the most effective gastroprotection, which was the AHQ 200 mg/kg (P < 0.001). Moreover, AHQ (200 mg/kg) protected the mucosa from piroxicam-induced damage. Mucus, NO, and SH did not participate on this gastroprotection. AHQ interfere in H⁺ secretion in gastric mucosa, thus exerting an anti-secretory activity. Our results suggest that the gastroprotection and cicatrisation process executed by AHQ occurred due to its anti-secretory activity. This study reinforces its traditional medicinal use. Considering that the current therapies are based on the use of anti-secretory agents, the *A. humile* arises as a promising alternative antiulcer therapy.

Key words: Anacardium humile St. Hil., gastric ulcer, gastroprotection.

INTRODUCTION

Medicinal plants are traditionally used to cure various diseases and for the production of various drugs (Mahmood et al., 2011). Natural bioactive compounds present in medicinal plants have been investigated for their characteristics and health effects (Ghasemzadeh and Ghasemzadeh, 2011). The Anacardiaceae family has been exploited for its medicinal value. Among these uses are anticonvulsant (Marchetti et al., 2011), anti-asthmatic (Hemshekhar et al., 2011), and anti-ulcerogenic (Morais et al., 2010).

Anacardium humile St. Hil. has been used in folk medicine as an alternative treatment for ulcers and gastritis (de Almeida et al., 1998). Considering this popular use, our research group demonstrated that this species possesses antiulcerogenic activity (Luiz-Ferreira et al., 2008). However, this study did not evaluate the mechanisms involved in this activity. Therefore, the aim of this study was to evaluate the pharmacological role of this species, describing the mechanism involved in the gastroprotective action of the aqueous extract (AHQ) of leaves of *A. humile* St. Hil. Special attention was paid to its effects on mucus production and gastric acid...
secretion.

MATERIALS AND METHODS

Animals

Male Unib: WH rats (n = 5 to 7, 150 to 250 g) and male Unib: SW mice (n = 6, 30 to 35 g) from Central Animal House of the Universidade Estadual de Campinas (CEMIBUNICAMP; São Paulo, Brazil) were used. The animals were fed a certified Nuvilab® (Nuvital) diet with free access to tap water under standard conditions of 12 h dark-12 h light, humidity (60 ± 1.0%) and temperature (21 ± 1°C). Fasting was used prior to all assays because standard drugs or essential oil treatment were always administered orally (by gavage) or intraduodenally. Moreover, the animals were kept in cages with raised floors of wide mesh to prevent coprophagy. The experimental protocols were approved by the Institutional Animal Care and Use Committee (no. 538-1, CEEA/IB/UNICAMP).

Plant

A. humile St. Hil. was collected along Monte do Carmo road, in Porto Nacional in Tocantins state, Brazil in November, 2002. The plants were identified by Marcos Alves and Eduardo Ribeiro of the University of Tocantins and a voucher specimen (accession number 1922) was deposited in the University of Tocantins Herbarium.

Aqueous fraction preparation

Air-dried and powdered leaves (650 g) of A. humile St. Hil. were exhaustively extracted by successive maceration at room temperature with dichloromethane (DCM, 5 L) and methanol (MeOH, 5 L) (130:1, w/v, one week for each solvent). Solvents were evaporated at 60°C under reduced pressure to yield the DCM (6 g) and MeOH (193 g) extracts. A portion of the MeOH extract (80 g) was partitioned between a mixture of EtOAc/water (5 L, 1:1, v/v) to and MeOH (193 g) extracts. A portion of the MeOH extract (80 g) was partitioned between a mixture of EtOAc/water (5 L, 1:1, v/v) to yield the DCM (6 g) (MeOH, 5 L) (130:1, w/v, one week for each solvent) . Solvents were exhaustively extracted by successive maceration at room temperature with dichloromethane (DCM, 5 L) and methanol (MeOH, 5 L) (130:1, w/v, one week for each solvent). Solvents were evaporated at 60°C under reduced pressure to yield the DCM (6 g) and MeOH (193 g) extracts. A portion of the MeOH extract (80 g) was partitioned between a mixture of EtOAc/water (5 L, 1:1, v/v) to yield 74 g of aqueous fraction (AHQ) utilized in this study.

Drugs and chemical

Lansoprazole (proton-pump inhibitors; Medley, Campinas, Brazil), piroxicam (non-selective COX inhibitor; Hexal, São Paulo, Brazil), cimetidine (histamine H2-receptor antagonist; Sigma Chemical Co., St. Louis, MO, USA), carbonoxalone (drug that enhance mucus production; Sigma Chemical Co., St. Louis, MO, USA), N-ethylmaleimide (a sulphhydryl blocker; Sigma Chemical Co., St. Louis, MO, USA), and N (G)-nitro-L- arginine methyl ester (nitric oxide synthase blocker; Sigma Chemical Co., St. Louis, MO, USA) were used in this study. The reagents for buffers and other solutions were all of analytical grade. All the other chemicals and reagents used in this study were of analytical grade.

Antiulcer action

Based on their respective specifications, the groups under each experimental model included positive (lansoprazole, carbonoxalone, or cimetidine) and negative (Saline 0.9%) controls. After each experiment, the animals were killed; the stomachs were opened along the greater curvature and pressed onto a glass plate. Ulcerative lesion (U.I) was calculated according to the methodology described by Szelenyi and Thiemer (1978). The antiulcerogenic activity of AHQ was assessed on two experimentally induced gastric ulcer models:

Ethanol-induced gastric lesion in rats.

After a total of 24 h fasting, three groups of rats (n = 5) received an oral administration of AHQ (50, 100, and 200 mg/kg), lansoprazole (30 mg/kg) or vehicle (10 ml/kg). One hour after treatment, all rats received, orally, 1 ml of 99.5% ethanol to induce gastric ulcers (Morimoto et al., 1991). The animals were killed by CO2 gas 1 h after treatment with the ulcerogenic agent and their stomachs were removed to determine the gastric damage as described previously.

Non-steroidal anti-inflammatory drug (NSAID)-induced ulcer

Animals (n = 6) were fasted for 24 h. The gastric injuries were induced by subcutaneous administration of piroxicam 30 mg/kg in male mice. Treatments (p.o.) with vehicle, cimetidine (100 mg/kg), and AHQ (200 mg/kg) were carried out 30 min before administration of the NSAID. Four hours after the NSAID administration, the animals were killed by CO2 gas and their stomachs were removed for lesion quantification (Puscas et al., 1997).

Evaluation of mucosal protective factors

Determination of mucus in gastric content

Rats (n = 6 to 7) had fasted for 24 h, under anesthesia, the abdomen was incised and the pylorus ligated. The vehicle, carbonoxalone (200 mg/kg) or AHQ (200 mg/kg) was administered orally after the pylorus ligation. The animals were killed by CO2 gas 4 h after the drug treatments. The stomach content was immersed in 10 ml 0.02% Alcian blue in 0.16 M sucrose/0.05 M sodium acetate, pH 5.8, and was incubated for 24 h at 20°C. The Alcian blue binding extract was centrifuged at 2000 x g for 10 min. The absorbance of supernatant was measured at 615 nm using a light spectrophotometer U/2000 (Hitachi, Japan). The free mucus in the gastric content was calculated from the amount of Alcian blue binding [mg/wet tissue (g)] (Rafatullah et al., 1990).

Evaluation of the gastric juice parameters

Animals (n = 5) were fasted for 24 h with free access to water. One hour after oral treatment or immediately after intraduodenal administration of AHQ (200 mg/kg), cimetidine (100 mg/kg) or vehicle, pylorus ligation was performed (Shay et al., 1945). Four hours later, the animals were sacrificed by CO2 gas, their stomachs were removed, inspected internally, and their contents were drained into a graduated centrifuge tube and were centrifuged at 2000 x g for 10 min. The supernatant volume and pH were recorded with a digital pH meter (PA 200, Marconi S.A., Piracicaba, Brazil).

Ethanol-induced gastric lesion in N-ethylmaleimide (NEM) and N (G)-nitro-L- arginine methyl ester (L-NAME)-pretreatment rats

Rats were divided into groups of animals that fasted for 24 h. They had previously been treated intraperitoneally with NEM at a dose of 10 mg/kg (n = 5), L-NAME at a dose of 70 mg/kg (n = 6) or vehicle (n = 6). Thirty minutes later, the groups received an oral dose of the vehicle (n = 6), carbonoxalone (n = 6; 100 mg/kg) or AHQ (n = 6; 200 mg/kg). After 60 min, all groups were treated orally with 1 ml of absolute ethanol for gastric-ulcer induction (Arrieta et al., 2003). Animals were killed by CO2 gas 1 h after ethanol administration and the stomachs excised and gastric damage determined as...
Table 1. Effects of AHQ from *A. humile* on two models of acute gastric lesion induced in rats and mice.

<table>
<thead>
<tr>
<th>Gastric lesions models</th>
<th>Treatment (p.o.)</th>
<th>Dose</th>
<th>ULI (mm)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>Saline</td>
<td>10 ml/kg</td>
<td>65.4 ± 17.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Lansoprazole</td>
<td>30 mg/kg</td>
<td>2.2 ± 0.8***</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>AHQ – <em>Anacardium humile</em></td>
<td>50 mg/kg</td>
<td>42.4 ± 17.7*</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mg/kg</td>
<td>39.9 ± 9.9**</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200 mg/kg</td>
<td>5.8 ± 3.6***</td>
<td>91</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>Saline</td>
<td>10 ml/kg</td>
<td>13.7 ± 2.5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cimetidine</td>
<td>100 mg/kg</td>
<td>2.9 ± 1.6***</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>AHQ – <em>Anacardium humile</em></td>
<td>200 mg/kg</td>
<td>8.9 ± 3.1**</td>
<td>35</td>
</tr>
</tbody>
</table>

The results are the mean ± SD ANOVA, followed by Dunnett's test. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the vehicle.

The healing action described earlier.

**Healing action**

**Acetic acid-induced gastric ulcer**

On this gastric ulcer induction, a cicatrisation model, rats were not fasted. Anesthesia (50 mg/kg of ketamine and 10 mg/kg of xylazine) was administered for the application of 100 µl of absolute acetic acid into the subserosal stomach layer of each animal. Two days after surgery, treatments (p.o.) with vehicle (n = 5), cimetidine (n = 5; 100 mg/kg), and AHQ (n = 5; 200 mg/kg) were administered once daily for 14 consecutive days. The animals were sacrificed on the 15th day by CO₂ gas and then their stomachs were removed for lesion quantification (Okabe and Amagase, 2005).

**Statistical analysis**

Parametric data were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test or Tukey's test and were compared to the vehicle group. The results were presented as the mean ± standard deviation (SD). All analyses were performed using GraphPad Prism software. A value of P < 0.05 was considered significant.

**RESULTS**

**Ethanol-induced gastric lesion in rats**

In this model, the results showed that AHQ provided significant gastroprotection in all the tested doses (p < 0.05, p < 0.01, and p < 0.001) (Table 1). Therefore, we focused on the following protocols using AHQ (200 mg/kg) which was the dose capable of producing the most effective gastroprotection.

**NSAID-induced ulcer**

The antiulcerogenic activity of AHQ (200 mg/kg) in piroxicam-induced gastric lesion model is reported in Table 1. When AHQ was administered, it inhibited the lesion formation when compared with vehicle group.

**Evaluation of mucosal protective factors**

**Determination of mucus in gastric content**

We observed the effect of AHQ (200 mg/kg) on adherent mucus production by the gastric mucosa (Figure 1). Pretreatment with carbenoxolone (200 mg/kg) induced significant increase in mucoprotective effect in animals submitted to pylorus ligation. On the other hand, AHQ (200 mg/kg) was not able to increase mucus production.

**Evaluation of the gastric juice parameters**

The gastric juice parameters of the rats that were treated with AHQ (200 mg/kg) administered by intraduodenal route was verified. These results demonstrated that AHQ (200 mg/kg) was able to diminish the H⁺ concentration in the gastric juice without modifying its volume (Table 2). This effect was similar to those of the standard drug cimetidine (100 mg/kg).

**Ethanol-induced gastric lesion in NEM and L-NAME pretreatment rats**

The pre-treatment with L-NAME did not affect the gastroprotection exerted by AHQ (200 mg/kg) (Figure 2A). In addition, as presented in Figure 2B, NEM did not affect the gastroprotection exerted by AHQ (200 mg/kg), but increased the ulcer area in animals submitted to ethanol-induced gastric ulcer.

**Healing action**

**Acetic acid-induced gastric ulcer**

Oral administration of AHQ (200 mg/kg) for 14
Figure 1. Effects of AHQ (200 mg/kg) from A. humile on quantification of adherent mucus in gastric mucosa of rats. The results are presented as mean ± SD ANOVA followed by Dunnet’s test, **p < 0.01 compared to the vehicle.

Table 2. Effects of AHQ (200 mg/kg) from A. humile administered by intraduodenal route on biochemical parameters of gastric juice obtained from pylorus ligature mice.

<table>
<thead>
<tr>
<th>Route</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Gastric juice (Content mg)</th>
<th>pH (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraduodenal</td>
<td>Vehicle</td>
<td>-</td>
<td>0.6 ± 0.3</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Cimetidine 100</td>
<td>100</td>
<td>0.5 ± 0.1</td>
<td>3.3 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td>AHQ-A. humile</td>
<td>200</td>
<td>0.5 ± 0.2</td>
<td>3.2 ± 0.3*</td>
</tr>
</tbody>
</table>

The results are the mean ± SD ANOVA followed by Dunnett's test. *p < 0.05 compared to the vehicle.

Figure 2. Effect of oral AHQ (200 mg/kg) from A. humile treatment, under the ethanol-induced gastric lesion model, on rats pretreated with L-NAME (A) or NEM (B). The results are the mean ± SD ANOVA followed by Tukey's test. **p < 0.01, ***p < 0.001 compared to the vehicle.

consecutive days accelerated the healing of gastric mucosa in rats when compared with the control group treated with vehicle (Figure 3).

DISCUSSION

Peptic ulcer (PU) is the consequence of an imbalance between aggressive and defensive factors of gastric mucosa. Tight junctions between epithelial cells, mucus and bicarbonate secretion are protective factors and gastric acid secretion and proteolytic activity of pepsin are, instead, aggressive factors (Tytgat, 2011). The excess of gastric acid secretion and increased production of pepsin have been considered as the main etiological factors in the development of PU (Mózsik, 2010; Tytgat,

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Therefore, the main therapeutic target for PU is the control of this secretion using antacids, H$_2$ receptor blockers or proton pump blockers. Nevertheless, these treatments are often associated with adverse side effects and ulcer recurrence after therapy interruption (Tytgat, 2011). In this study, we used models that resemble the ulcer in humans (ethanol and NSAID) and the antiulcerogenic mechanisms involved. The healing capacity of the aqueous fraction obtained from the leaves of A. humile St. Hil. (AHQ) was also verified with acetic acid-induced ulcers model.

Ethanol is known to be one of many factors that increase the risk of gastric ulcer formation. Hemorrhagic ulceration of the stomach in humans and experimental animals is also caused by ingestion of elevated amounts of ethanol. The main features of ethanol-induced gastric ulcer are epithelial loss, mucosal edema, and subepithelial hemorrhage (de-Faria et al., 2012). Initially, the gastroprotective AHQ was tested in ethanol-induced gastric lesions at these doses (50, 100, and 200 mg/kg). This experimental model provides the screening of doses and efficacy of the treatments against ulcer formation. The ulcerogenic activity of ethanol is driven by its capacity to dissolve the constituent gastric mucus, while concomitantly diminishing the transmucosal action potential, thus, increasing the flow of Na$^+$ and H$^+$ in the lumen and stimulating the secretion of histamine, pepsin and H$^+$ ions (Moraes et al., 2009). Considering that the AHQ (50, 100, and 200 mg/kg) exerted 35, 40, and 88%, respectively protection on the gastric mucosa, it is undeniable that this fraction exert substantial protective action on the gastric mucosa.

The increased use of NSAIDs causes gastrointestinal toxicity, including the PU (Hampton and Hale, 2011). Topical and systemic effects of NSAIDs in the gastrointestinal mucosa are associated with mucosal damage in the upper and lower gastrointestinal tract (Sostres and Lanas, 2011). The systemic effects of NSAIDs involve prostaglandin production inhibition that leads to an impairment of bicarbonate and mucus secretion. In the experimental induction of gastric ulcers by a NSAID, the AHQ (200 mg/kg) presented gastroprotection. Given that the ulcerogenic properties of NSAIDs are due to the fact that they diminish the protective factors of the mucosa such as prostaglandins and mucus (Konturek et al., 2005; Polat et al., 2010), it can be affirmed that the antiulcerogenic activity of AHQ observed in this model must augment these mucosal protective factors. In this sense, aiming this hypothesis, we evaluated the AHQ action on mucus secretion in the gastric mucosa. Figure 1 shows that AHQ (200 mg/kg) was not able to increase mucus production. This result shows that factors cytoprotective do not participate in cytoprotection of AHQ.

Mucosal microcirculation is essential for oxygen and nutrient delivery and toxic substances removal. The epithelial cells lining the microvessels generate potent vasodilators such as nitric oxide (NO) that increases removal and/or dilution of the back-diffusing acid and/or noxious agents when the gastric mucosa is exposed to an irritant agent (Laine, 2008). Thus, NO endogenous and exogenous, plays a major role in mucosal defense by modulating the mucosal circulation. The administration of L-NAME, NO synthesis inhibitor, would certainly be harmful, especially for gastric mucosa. As well as NO, the sulfhydryl compounds (SH) are important for the maintenance of gastric mucosa, because of its protection against pro-oxidant agents. Luiz-Ferreira et al. (2010) reported that the administration of glutathione depletors (NEM) significantly potentiates the effects of ethanol on gastric mucosa injury. AHQ (200 mg/kg) protected the gastric mucosa from the irritant agent when NEM is co...
administered. Thus, our result suggests that the AHQ gastroprotective effect does not depend on the presence of SH compounds in the gastric mucosal barrier. Furthermore, the evaluation of NO participation in the gastroprotection promoted by AHQ demonstrated that despite the inhibition of NO by the action of the L-NMGBlocking NOs, AHQ continued exerting its effect.

After noticing that cytoprotector factors are not involved in AHQ gastroprotection, we verified its ability to inhibit acid secretion. The results suggest that the gastroprotection exerted by AHQ can be due to the inhibition of acid secretion before administration of ethanol, thereby reducing the counter-diffusion and cell disruption in the injured mucosa. Other plants of the Anacardiaceae family showed acid secretion inhibition as gastroprotection mechanisms (Lima et al., 2006; Carvalho et al., 2007; Ajibola et al., 2010; Morais et al., 2010). In addition, Hunt and Yuan (2011) related that acid suppression is an important prevention strategy for NSAID-associated gastric ulcer. Gastric lesions caused by NSAIDs can be treated with anti-secretory agents such as proton pump inhibitors or histamine H₂-receptor antagonists (Satoh and Takeuchi, 2012).

Gastrointestinal ulcers are essentially internal wounds that resist normal healing processes. Superficial mucosal lesions heal by epithelial regeneration, but when ulcers involve the muscularis propria, smooth muscle cells do not divide/regenerate, thus, impaired the process of ulcer healing (Szabo et al., 2011). Application of acetic acid topically to the mucosal or serosal side of the rat stomach healing (Szabo et al., 2011). Application of acetic acid not divide/regenerate, thus, impaired the process of ulcer healing. Furthermore, the evaluation of NO participation in the gastroprotection promoted by AHQ demonstrated that NO participation enhances the healing process of gastric ulcers. This ulcer model highly resembles human ulcers in terms of pathological features induced by acetic acid in rats. This ulcer model highly resembles human ulcers in terms of pathological features and healing processes (Okabe and Amagase, 2005). The results of this study show that treatment with AHQ (200 mg/kg) or with cimetidine for 14 consecutive days in the healing of gastric ulcers induced by acetic acid in rats. This ulcer model highly resembles human ulcers in terms of pathological features and healing processes (Okabe and Amagase, 2005). The acceleration of healing in the current therapies, they are based on the use of anti-secretory drugs; A. humile arises as a promising alternative antilucre therapy.

Conclusions

This study on the pharmacological mechanisms involved in the antilucre activity of A. humile St. Hil. reinforces its traditional medicinal use. The treatment demonstrated to have anti-secretory and healing properties. Considering the current therapies, they are based on the use of anti-secretory drugs; A. humile arises as a promising alternative antilucre therapy.

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