The Protective Potential of *Phyllanthus niruri* and Corilagin on Gastric Lesions Induced in Rodents by Different Harmful Agents

**ABSTRACT**

The gastroprotective effect of the methanol extract of *Phyllanthus niruri* and its main constituent, corilagin, were studied in vivo. The extract (50, 125, or 250 mg/kg, p. o.) inhibited ethanol-induced lesions in rats by 43% (p < 0.001), 69% (p < 0.001), and 99% (p < 0.001), respectively. It also inhibited the formation of indomethacin-induced gastric ulcers in rats by 80% (p < 0.01), 89% (p < 0.01), and 97% (p < 0.01). A decrease in lipid hydroperoxide levels (p < 0.01) and in myeloperoxidase activity (p < 0.05) evidenced a reduction of oxidative damage and neutrophil infiltration in gastric tissues from ulcerated mice using ethanol/HCl. Potent in vitro free radical scavenger activity (IC₅₀ = 0.07) using the DPPH assay was observed. In contrast, the extract (250 mg/kg, i. d.) did not show antisecretory activity in pylorus-ligated rats, and also failed to inhibit the H⁺, K⁺-ATPase activity in vitro. However, in pylorus-ligated rats, the extract (50, 125, and 250 mg/kg, i. d.) increased adhered mucus content by 22% (p < 0.05), 28% (p < 0.01), and 38% (p < 0.01), respectively. The involvement of prostaglandins, nonprotein endogenous sulfhydryl compounds, α₂-receptors, and endogenous nitric oxide in the gastroprotection elicited by the extract was also evaluated. Finally, corilagin reduced the lesion area of ethanol-induced gastric ulcers in mice by 88% (30 mg/kg, p. o.; p < 0.001). Based on these results, it has been concluded that *P. niruri* methanol extract possesses gastroprotective activity by different and complementary pathways, which together promote an improvement in gastric cytoprotection. The presence of corilagin may partially explain the effectiveness of the extract against gastric damage.

**Introduction**

It is estimated that, each year, about 4 million people are affected by peptic ulcer disease [1]. In fact, Lau and colleagues demonstrated that peptic ulcers continue to be a significant health care problem [2]. It is known that several factors are responsible for peptic ulcer formation, such as *Helicobacter pylori* infection, prolonged use of nonsteroidal anti-inflammatory drugs (NSAIDs), cigarette smoking, and alcohol and caffeine consumption [3].

The classical treatment is based on antisecretory therapy, which includes the use of histamine H(2)-receptor antagonists (H2RA), such as ranitidine, and proton pump inhibitors (PPIs), such as omeprazole. However, several adverse effects are linked to this therapy, including hypersensitivity, arrhythmia, impotence, gynecomastia, hematopoietic disorders [3,4], and hypergastrinemia [5,6]. Moreover, the hypothesis that long-term use of PPIs could promote the development of gastric premalignant lesions has been widely investigated, but the results are still inconsistent [7].

In this context, it is known that the search for new alternatives for ulcer disease treatment, with fewer side effects, is increasing nowadays, and natural products are a promising source for this purpose [8,9].

*Phyllanthus* (Phyllanthaceae) plants are widely distributed across the world, comprising about 600 different species, including shrubs, trees, and herbs [10]. This genus is used in the folk medicine of several countries for the treatment of a wide variety of diseases, some of which are supported by preclinical and clinical studies [11,12]. Regarding the gastroprotective potential of this genus, the aqueous extract of the fruits of *Phyllanthus emblica* L. was able to reduce indomethacin-induced lesions in rats [13], while the gallic acid-enriched fraction of *P. emblica* promoted in-
domethacin-induced gastric ulcer healing via the endothelial nitric oxide synthase (e-NOS)-dependent pathway [14]. In addition, the methanol extract of the leaves and stems of P. amarus also inhibited gastric lesions induced by ethanol in rats [15].

*Phyllanthus niruri* L. is probably the most studied species of the *Phyllanthus* genus. It is a small herb that is widely distributed in tropical areas, including South America, South East Asia, and Southern India and China. The plant is widely used in folk medicine, such as in Indian Ayurveda, traditional Chinese medicine, and Indonesian Jamu. In Brazilian herbal medicine, it is known as "Quebra-pedra" and is used for the treatment of kidney disorders and diabetes [10]. Okoli et al. and Abdulla et al. have already described the gastroprotective activity of polar extracts of the aerial parts of this species [16,17]. However, in both studies, the mechanisms involved in its gastroprotective effects have not been reported, neither have the bioactive compounds present in the studied extracts been elucidated.

Several compounds have already been isolated from *P. niruri*, including tannins. These compounds are well known for their anti-ulcer capacity, mainly because of their antioxidant activity and promotion of tissue repair [18]. Corilagin (1-O-galloyl-3,6-(R)-hexahydroxydiphenoyl-β-D-glucose) is a tannin related to several therapeutic activities reported for the plant [19], such as antioxidant [20], hepatoprotector [21], anti-hyperalgesic [22], and anti-*H. pylori* [23] activities.

Considering the promising potential of *P. niruri* as a gastroprotective agent, the present study evaluates the effect of the methanol extract of the whole plant in ethanol- and NSAID-induced gastric lesions, as well as the antisecretory gastric acid activity and the involvement of protective factors of gastric mucosa in the gastroprotection elicited by the extract. Finally, the effect of corilagin, a compound isolated from the methanol extract of *P. niruri*, against ethanol-induced lesions in mice was also evaluated.

### Results and Discussion

*P. niruri* has been the focus of several studies regarding its pharmacological potential as well as its chemical composition. Indeed, some studies had already demonstrated the gastroprotective effect of polar extracts obtained from this species. However, the mechanisms involved in the gastroprotective effects shown by *P. niruri* are still unknown. Taking this into account, the gastroprotective effects of the methanol extract of *P. niruri* are confirmed in the present study. The role of nonprotein endogenous sulfhydryl (NP-SH), nitric oxide (NO), α2-adrenergic receptors, prostaglandins, and antioxidant activity are also demonstrated. However, in contrast to current antiulcer therapy, the extract was not able to inhibit gastric acid secretion. In addition, the antiulcer activity of corilagin was also demonstrated.

The ethanol-induced acute gastric lesion model was chosen to assess the gastroprotective effect of the methanol extract of *P. niruri* in rats. It is well known that ethanol promotes several changes in the gastric mucosa. It stimulates gastric acid secretion at low concentrations, and damages the gastric mucosa at higher concentrations. The pathophysiology is not completely understood, but it is believed that ethanol promotes direct mucosal injury, releasing inflammatory mediators and increasing the levels of granulocytes, reactive oxidizing metabolites, cytokines and vasoactive substances, leading to inflammation, vasoconstriction, and ischemia, which ultimately results in cell death and mucosal damage [24].

In this model, oral treatment with the methanol extract of *P. niruri* (250 mg/kg) and omeprazole (30 mg/kg) significantly reduced the total area of lesion by up to 99% (*p < 0.001*) when compared to the vehicle group (Veh). A at the doses of 50 and 125 mg/kg, the extract was also able to reduce this parameter, though to a lesser extent (43% and 69%, respectively). These results demonstrate that the cytoprotective activity exerted by the extract is dose-dependent. Representative images of this effect are shown in [Fig. 1B](#). Similarly, Abdulla et al. also observed a gastroprotective ratio for a methanol extract from this plant at a dose of 250 mg/kg [17]. However, in contrast to these results, Okoli et al. described no gastroprotective effect of the methanol extract from *P. niruri* in this model, even at the highest dose evaluated (400 mg/kg) [16].

Several models are used experimentally for testing or evaluating antiulcer activity of natural products. In order to evaluate the effects of the methanol extract of *P. niruri* in different harmful agents, the gastric ulcer induced by acidified ethanol was performed in mice in this study. A key factor in ulcer pathogenesis is oxidative stress, characterized by an imbalance between reactive...
oxygen species (ROS) production and antioxidant tissue defenses. ROS production occurs intracellularly by the xanthine oxidase pathway or mitochondrial respiratory chain, or in an extracellular model, which includes the classical production of superoxide by neutrophils [25, 26]. Considering that neutrophils are an important source of ROS in gastric mucosa during ulcerogeneses, and the presence of myeloperoxidase (MPO) in the cytoplasm of these cells, this study assessed neutrophil infiltration indirectly, through the measurement of MPO activity in the gastric tissue. For this purpose, samples from ulcers induced by ethanol/HCl were processed. In this experiment, it is important to note that carbonoxalolone was used as a positive control, similar to Rozza et al. [27] and Schlickmann et al. [28], since it does not have antisecretory activity. Thus, a beneficial effect of the gastric mucosa on oxidative stress is related to the increase in tissue protective factors and not to reduced gastric acidity, as would occur with the use of omeprazole.

As expected, in the ethanol/HCl model, *P. niruri* methanol extract (250 mg/kg) or carbonoxalolone (200 mg/kg) reduced the extent of the gastric lesions by 82% (p < 0.001) and 71% (p < 0.001), respectively, when compared to the vehicle-treated group (24.06 ± 2.68 mm²; ▶ Fig. 2A). At doses of 50 and 125 mg/kg, the extract was also able to reduce the lesion area induced by ethanol/HCl by 46% (p < 0.01) and 53% (p < 0.01), respectively, when compared to the vehicle-treated group (24.06 ± 2.68 mm²; ▶ Fig. 2A). In addition, the MPO activity and lipid hydroperoxide (LOOH) levels were measured in the glandular portion of ulcerated stomach by ethanol/HCl in this study. As shown in ▶ Fig. 2B, gastric MPO activity was significantly increased in the vehicle group, from 0.10 ± 0.01 mD.O/mg of protein (Naive group) to 1.70 ± 0.60 mD.O/mg of protein (p < 0.01). In contrast, the administration of the methanol extract of *P. niruri* (250 mg/kg) or carbonoxalolone (200 mg/kg) reduced the MPO levels by 75% (p < 0.01) and 85% (p < 0.01), respectively (▶ Fig. 2B), demonstrating that the reduction of neutrophil infiltration participates in the gastroprotective effects shown by the methanol extract of *P. niruri*. However, although the extract showed gastroprotective activity at doses of 50 and 125 mg/kg, these doses did not cause any changes in MPO activity.

The first stage of ROS-mediated cellular damage is lipid peroxidation due to peroxidation of the cellular membrane constituents [26,29]. Moreover, in gastric mucosa exposed to oxidative stress, the increase in LOOH levels is accompanied by a decrease in superoxide dismutase (SOD) activity and in a reduced glutathione (GSH) concentration [26, 30]. The LOOH content was determined in gastric mucosa exposed to ethanol/HCl, and the increase in this parameter following ulcer induction was confirmed (p < 0.01), as shown in ▶ Fig. 2C. In contrast, this increase was prevented by the administration of the extract at a dose of 250 mg/kg (p < 0.01), reinforcing the antioxidant hypothesis (▶ Fig. 2C). However, although the extract showed gastroprotective activity at doses of 50 and 125 mg/kg, these doses did not cause any changes in LOOH content. Furthermore, it is noteworthy that the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (▶ Fig. 2D) confirms the antioxidant potential of the methanol extract of *P. niruri*.

Besides alcohol, another well-known factor that is also responsible for the incidence of gastric lesions is the prolonged use of NSAIDs. About 30 to 50% of NSAID users have endoscopic lesions, generally with no clinical significance [31]. Several factors are related to its pathophysiology, including the reduction of PGE2 levels by cyclooxygenase-1 (COX-1) inhibition, the increase in membrane permeability by direct cytotoxicity, and the production of proinflammatory mediators by activation of the lipoygenase pathway and increased synthesis of leukotrienes [32]. Okoli and colleagues have already observed the protective effect of the methanol extract of the aerial parts of *P. niruri* against indomethacin-induced lesions. However, this only occurred at the higher dose of the extract (400 mg/kg) [16]. On the other hand, in the present study, the extract inhibited indomethacin-induced gastric lesions at all of the tested doses (50–250 mg/kg; ▶ Table 1). As expected, omeprazole (30 mg/kg) also inhibited the lesions, reaching a protective effect of 98% (p < 0.01), whereas the methanol extract of *P. niruri* reduced the gastric damage by up to 97% (p < 0.01). These results indicate that the protective effect of *P. niruri* may be related to the prostaglandin pathway and gastric mucus production.
Mucus is a key gastroprotective factor that is deeply mediated by prostaglandin E2 (PGE2) via the prostaglandin E4 receptor. It creates a layer that is responsible for retaining bicarbonate secreted by epithelial cells, also acting as a physical barrier against pepsin and the acid pH of the lumen [33]. According to the results shown in ▶ Table 2, oral treatment with the methanol extract of P. niruri (50, 125, and 250 mg/kg; p < 0.05, p < 0.01, and p < 0.01, respectively) and carbenoxolone (250 mg/kg, p < 0.05) increased the amount of Alcian blue binding to free gastric mucus, indicating that both treatments were able to increase mucus production and confirming the involvement of prostaglandins in its gastroprotective action. Furthermore, as shown in ▶ Fig. 3A, the gastroprotective effect of oral treatment with the methanol extract of P. niruri against ethanol/HCl-induced lesions was impaired after administration of indomethacin, reinforcing the importance of prostaglandins in the mechanism of action of this extract.

The gastroprotective effect of the methanol extract of P. niruri (250 mg/kg) was also obliterated after blockage of NP-SH compounds by N-ethylmaleimide (NEM) administration in ethanol/HCl-induced ulcers in mice (▶ Fig. 3B). Thus, it is possible to infer that NP-SH compounds are required for the gastroprotection shown by this extract. NP-SH compounds, such as GSH, are important for the maintenance of gastric mucosal integrity, due to their ability to bind to the free radicals generated by noxious stimuli [34]. GSH also participates in many antioxidant enzymatic reactions, including the degradation of hydrogen peroxide by glutathione peroxidase [35]. Continuing the elucidation of the underlying mechanisms, as shown in ▶ Fig. 3C, D, respectively, pretreatment with yohimbine and N-ω-nitro-L-arginine methyl ester (L-NAME) effectively blocked the gastroprotective effect of the extract (250 mg/kg) against ethanol/HCl-induced ulcers. These findings suggest that the methanol extract of P. niruri affords gastroprotection by different and complementary mechanisms, which also include the involvement of the α2-receptors and NO. It is well known that presynaptic α2-receptors mediate several responses in

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effects of the methanol extract of P. niruri and omeprazole on NSAID-induced ulcers in rat.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (p. o.)</td>
<td>Dose (mg/kg)</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>30</td>
</tr>
<tr>
<td>Extract</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>250</td>
</tr>
</tbody>
</table>

Results as the mean ± S. E. M. for six rats. Statistical comparison was performed using ANOVA followed by Bonferroni’s test; a p < 0.01 compared with the control group; b p < 0.05 compared with the omeprazole group.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Effect of P. niruri extract and carbenoxolone on Alcian blue binding to free gastric mucus from pylorus ligature in rats.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (p. o.)</td>
<td>Dose (mg/kg)</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
</tr>
<tr>
<td>Carbenoxolone</td>
<td>250</td>
</tr>
<tr>
<td>Extract</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>250</td>
</tr>
</tbody>
</table>

Results as the mean ± S. E. M. for six rats. Statistical comparison was performed using ANOVA followed by Bonferroni’s test; a p < 0.05; b p < 0.01 compared with the control group.

![Fig. 3]( Effect of indomethacin (COX inhibitor; A), NEM (NP-SH blocking; B), yohimbine (α2-receptors antagonist; C) and L-NAME (NOS inhibitor; D) on gastroprotective activity of the methanol extract from P. niruri against HCl/ethanol-induced ulcers. Values are the mean ± S. E.M (n = 6). *P < 0.05, **p < 0.01, and ***p < 0.001 when compared to the ulcerated vehicle group pretreated with saline. Statistical comparison was performed using two-way ANOVA followed by Bonferroni’s test. #P < 0.05, and ##p < 0.01 when compared to the extract group pretreated with vehicle or saline. &P < 0.05 and &&p < 0.01 when compared to the vehicle group pretreated with NEM and L-NAME, respectively. )
The Protective Potential of P. niruri Extract on Gastric Ulceration

Klein-Júnior LC et al.

Table 3 Effects of P. niruri extract and omeprazole on the biochemical parameters of gastric juice obtained from pylorus ligature in rats.

<table>
<thead>
<tr>
<th>Treatment (p.o.)</th>
<th>Dose (mg/kg)</th>
<th>Volume (mL)</th>
<th>pH</th>
<th>[H⁺]/mEq/l/4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>0.91 ± 0.16</td>
<td>2.97 ± 0.15</td>
<td>79.49 ± 3.91</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>30</td>
<td>0.85 ± 0.32</td>
<td>6.90 ± 0.22</td>
<td>22.15 ± 2.68</td>
</tr>
<tr>
<td>Extract</td>
<td>50</td>
<td>0.96 ± 0.16</td>
<td>3.29 ± 0.27</td>
<td>51.63 ± 5.49</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>0.99 ± 0.11</td>
<td>3.27 ± 0.11</td>
<td>54.57 ± 1.87</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.94 ± 0.13</td>
<td>3.70 ± 0.43</td>
<td>63.43 ± 5.46</td>
</tr>
</tbody>
</table>

Results as the mean ± S.E.M. for six rats. Statistical comparison was performed using ANOVA followed by Bonferroni’s test; a p < 0.01; b p < 0.05 compared with the control group; c p < 0.05 compared with the omeprazole group.

Fig. 4 Effect of the methanol extract from P. niruri on in vitro H⁺, K⁺-ATPase activity. The enzyme was incubated with vehicle (Veh: water), omeprazole (Ome: 34.5 μg/mL), ouabain (U: 72.8 μg/mL), or the extract (10–1000 μg/mL). The results are expressed as the mean ± S.E.M. Experiments were performed in triplicate. Statistical comparison was performed using analysis of variance (one-way ANOVA) followed by Bonferroni’s test; **P < 0.01 and ***P < 0.001 compared with the vehicle group; ²P < 0.01 compared with the omeprazole group.

Fig. 5 Chromatographic profile by HPLC/PDA of the methanol extract of P. niruri at 268 nm. The corilagin peak is indicated by the number 1 (A), and the structure of corilagin (B).

The gastrointestinal tract, and are involved in the regulation of gastric acid secretion [36]. NO is involved in the modulation of gastric mucosal integrity and in the regulation of acid and alkaline secretion, mucus secretion, and gastric mucosal blood flow [37].

At this point, it can be inferred that administration of the methanol extract of P. niruri can increase the defensive factor levels in gastric tissue. However, most currently available antiulcer treatments are based on the reduction of acid secretion [38, 39]. Therefore, the pylorus ligature model was performed to assess the reduction of gastric secretion in the mode of action of gastroprotection elicited by the extract. Through this method, it is possible to determine the volume, pH, and H⁺ concentration of gastric acid secretion. Fig. 3 shows that these parameters were not affected by treatment with the extract, in contrast to the results observed for omeprazole. Furthermore, as evidenced in Fig. 4, the methanol extract of P. niruri also failed to inhibit in vitro H⁺, K⁺-ATPase activity, which is also in contrast to the results observed for omeprazole.

Chemical investigation of the P. niruri methanol extract by chromatographic procedures led to the isolation of corilagin. Its structure was confirmed by NMR data comparison [40]. UHPLC/Q-TOF-MS analysis of both the extract and corilagin confirmed its identity by mass spectra, giving a pseudo-molecular ion at m/z 625, calculated for C₂₇H₂₂O₁₈ [M + H]+, which is in accordance with the literature [40]. In addition, corilagin (Fig. 5B) was quantified in the extract by HPLC/PDA (Fig. 5A). An analytical curve was obtained using a series of solutions of the analyte (31.25–500 μg/mL). The curve was linear, as shown by the linear regression coefficient (r²) > 0.999. Taking this into account, the curve was applied and the amount of corilagin determined as 231.5 mg/g in the P. niruri methanol extract.

Finally, the effect of corilagin on gastric lesions induced by ethanol was evaluated. As shown in Fig. 6A, corilagin and omeprazole were able to reduce the total area of lesion and visual lesion area with a protective effect higher than 80% (p < 0.001) in both treatments. Representative images from this experiment are shown in Fig. 6B. In this experiment, the corilagin was only tested in one dose (30 mg/kg), although the extract has been tested in three doses, in order to establish a simple effective comparison between this compound and omeprazole administered in the same dose by oral route, similar to Abreu Miranda et al. [41] and Berté et al. [42]. Furthermore, it is important to emphasize that the tested dose of corilagin is close to its corresponding content in the extract at the dose of 125 mg/kg. Thus, compared to the P. niruri extract, corilagin reduced the ulcerogenic effect of ethanol to a greater extent.

Several tannins have already demonstrated gastroprotective effect. Moreover, purified hydrolysable tannins have also demonstrated antibacterial activity against H. pylori. In fact, corilagin was able to inhibit H. pylori growth with a minimum inhibitory concentration of 6.25 μg/mL [23]. This gram-negative bacillus colonizes the gastric mucosa and is an important causal factor in the pathogenesis of ulcer disease, as it induces gastric inflammation, oxidative stress, and cell cycle deregulation [43]. In view of this, corilagin seems to be a promising molecule for antiulcer drug development.
Plant material
The whole plant was collected at Praia da Esplanada-Jaguaruna, Santa Catarina, Brazil, in January 2011. It was deposited at the Barbosa Rodrigues Herbarium (HBR, Itajaí) under the number VC Filho 075. Professor Oscar Iza authenticated the sample.

Extraction and isolation procedures
The extraction and isolation procedures have already been published by Moreira and colleagues [22]. In brief, the whole dry plant (337 g) was submitted to maceration with methanol for 5 days (yield 30.98 g). Twenty-five g of the crude extract was suspended with methanol/water and extracted three times with dichloromethane and ethyl acetate. After evaporation, 1 g of the ethyl acetate fraction was chromatographed by column chromatography under the silica gel stationary phase and eluted with a dichloromethane/ethyl acetate/methanol gradient, giving six main fractions. The third fraction (272.9 mg) was submitted to isocratic flash column chromatography using dichloromethane/ethyl acetate/methanol (30: 50: 20, v/v/v), giving pure corilagin (88.4 mg; 96% purity). The compound was identified by $^{13}$C-NMR spectral data in comparison with those previously reported (AC-300, Bruker) [40].

HPLC analysis
HPLC experiments were performed with a system consisting of a Model 2695 Waters Alliance analytical module equipped with a 2998 photodiode array detector, and a computerized data station equipped with Waters Empower software. For the development of the method, two different stationary phases were used: a Kromasil 100-5C18 column (5 μm; 150 × 4.6 mm; AkzoNobel) and a XSELECT™ CSH™ Phenyl-Hexyl column (3.5 μm; 100 × 4.6 mm; Waters), conditioned at 30 °C. Different solvent systems were evaluated, with a flow of 0.7 mL/min. The conditions best suited for the corilagin quantification were water (formic acid 0.1%) (A) and methanol (formic acid 0.1%) (B); 0 min, 90: 10 (A: B; v/v); 30 min, 40: 60; 35–40 min 0: 100, using the Phenyl-Hexyl stationary phase. A 10-μl aliquot of the sample was injected, and the absorbance at 268 nm monitored. Both the compound and the extract were diluted in a mixture of water and methanol (1: 1, v/v) by sonication. The solutions were freshly prepared before each experiment and filtered through a 0.45-μm cellulose regenerated membrane filter.

Linearity and quantification in the extract
To obtain the analytical curve, corilagin was injected in five different concentrations, ranging from 31.25 μg/mL to 500 μg/mL. Each elution was prepared and injected in triplicate. The areas of the analyte peak were plotted against the corresponding concentrations of the sample, and linearity was assessed by linear regression analysis using Excel 5.0 software. For the quantification, the extract was injected in triplicate at a concentration of 1 mg/mL.

UHPLC/Q-TOF-MS analysis
UHPLC/Q-TOF-MS analysis was performed on a Q-TOF Micro Micromass mass spectrometer from Waters with an ESI interface coupled with an Acquity UPLC system from Waters. Detection was performed in the positive ion mode in the range of m/z 100–10000.

Material and Methods

Chemicals and reagents
Ascorbic acid, carbenoxolone, cimetidine, indomethacin, omeprazole, and ouabain were purchased from Sigma-Aldrich. All of the reagents and solvents used were of analytical grade.

Taken together, the findings of this study show that the methanol extract of P. niruri exerts its gastroprotective effect via several pathways. The ability of the extract to reduce oxidative damage and neutrophil infiltration, while increasing mucus production, indicates the protective effect of the methanol extract of P. niruri against aggressive factors. Furthermore, the participation of SH compounds, NO, α2-receptors, and prostaglandins, as well as a high radical scavenging activity, were verified in the underlying mechanisms of the gastroprotective effect of the studied extract. In contrast to the antisecretory therapy commonly used today, the extract is not able to alter the gastric juice biochemical parameters or the H+ ·K+-ATPase activity. The presence of corilagin also partially explains the effectiveness of the extract against gastric damage.
in the centered mode with a scan time of 0.2 s and an interscan delay of 0.3 s for polarity switching. ESI conditions were capillary voltage 3000 V, cone voltage 30 V, source temperature 100 °C, desolvation temperature 250 °C, cone gas flow 70 L/h, and desolvation gas flow 350 L/h. The separation was performed on a 150 mm × 2.1 mm i.d., 1.7 μm, Acquity BEH C18 UPLC column (Waters) in gradient mode at a flow rate of 0.3 mL/min with the following solvent system: water (formic acid 0.1%) (A) and methanol (formic acid 0.1%) (B): 0 min, 90:10 (A:B; v/v); 6 min, 40:60; 7–8 min 0:100. The data were analyzed using Masslynx V 4.1 software. MS data for corilagin was compared to that reported in the literature [40].

Animals

Wistar male rats (250–350 g) and Swiss male mice (20–30 g) were provided by the Central Animal House of the Universidade do Vale do Itajaí (UNIVALI), Itajaí – SC. One adult albino rabbit (Oryctolagus cuniculus) weighing ~ 2 kg was used for the gastric H⁺K⁺-ATPase assay. The rats were housed in groups of six, in standard cages, at room temperature (25 ± 3 °C), with 12-h dark/12-h light cycles. All of the cages were supplemented with food and water ad libitum. Twelve hours prior to the experiments, the animals were transferred to the laboratory and given only water ad libitum. To prevent coprophagy, the animals were kept in cages with raised floors and wide mesh. The animals used in the present study were housed and cared for in accordance with the international standards and the ethical guidelines on animal welfare. The experiments were authorized by the Ethical Committee for Animal Care of the University of Vale do Itajaí, Itajaí, Santa Catarina, Brazil, under approval certificate number 022/11 (approval date:07/10/2011).

Ethanol-induced ulcers

The experiment was carried out according to the method described in Morimoto et al. [44]. After 12 h of fasting, rats were randomly divided into six groups of six animals each. The first group was given the vehicle (1 mL/kg, 1% Tween-80 aqueous solution; p.o.) and the second group was treated with omeprazole, the positive control of the test (30 mg/kg; p.o.). The remaining groups received 50, 125, and 250 mg/kg (p.o.) of P. niruri extract. One hour later, all of the animals received 99.5% ethanol (5 mL/kg) to induce gastric lesions. After one hour, the animals were euthanized and the stomachs were removed and opened along the greater curvature, and then the gastric contents and blood clots were removed for subsequent scanning of the gastric mucosa. The images obtained were analyzed using specific “EARP” software to measure each lesion point, as described by Klein-Júnior et al. [45].

In another experiment, mice fasted for 12 h were randomly divided into three groups of six animals each and treated with vehicle (10 mL/kg, 1% Tween-80 aqueous solution; p.o.), omeprazole (30 mg/kg; p.o.), or 30 mg/kg of compound 1 (p.o.). After 1 h, all of the groups received 99.5% ethanol (10 mL/kg) to induce gastric lesions. After 1 h, the animals were euthanized and the stomachs were removed to measure the lesion area, as described above.

HCl/ethanol-induced ulcers

Acute gastric ulcers induced by HCl-ethanol were performed as described earlier [46]. Mice were randomly divided into five groups of six animals each and pretreated with vehicle (1 mL/kg, 1% Tween-80 aqueous solution, p.o.), carbenoxolone (CBX: 200 mg/kg, p.o.) or P. niruri methanol extract (250 mg/kg, p.o.) 1 h before oral administration of a 0.3 M HCl/60% ethanol solution (HCl/ethanol, 10 mL/kg) to induce gastric ulcers. After 1 h of HCl/ethanol administration, the mice were euthanized; the stomachs were removed and processed to measure the ulcer area, as described previously.

Assessment of lipid hydroperoxide levels and myeloperoxidase activity in HCl/ethanol-induced ulcers

Tissue samples from the stomachs ulcerated by HCl/ethanol solution were homogenized with 200 mM potassium phosphate buffer (pH 6.5), and the homogenate was used to measure the LOOH levels. After this procedure, the remaining homogenate was then centrifuged at 4000 rpm for 20 min at 4 °C, and the resulting precipitate was used to determine MPO activity. All procedures were performed at 4 °C.

LOOH content was determined by the method of Ferrous Oxidation-Xylenol Orange (FOX2) as previously described [47]. Briefly, 100 μL of the sample were mixed with 100 μL of methanol and centrifuged at 13 000 rpm for 5 min. The supernatant plus FOX2 reagent [4 mM butylatedhydroxytoluene (BHT), 250 mM FeSO₄, 25 mM H₂SO₄ and xylenol orange at 100 mM] was incubated in the dark for 30 min at room temperature. The absorbance was determined at 560 nm, and the concentration of LOOH was calculated by the molar extinction coefficient (ε = 43 mM/cm) and expressed as mmol hydroperoxide/mg of tissue.

MPO activity was measured as described previously [48,49]. Briefly, samples were resuspended in 80 mM potassium phosphate buffer (pH 5.4) plus 0.5% hexadecyltrimethylammonium bromide (HTAB), and again centrifuged at 12 000 rpm for 20 min. MPO activity in the supernatant was determined at 620 nm in the presence of H₂O₂ and 3,3′,5,5′-tetramethylbenzidine (TMB), and expressed as units of milli-optic density (m.O.D.)/mg of protein.

Protein concentrations were determined by the Bradford method (Bio-Rad) and interpolated in a bovine serum albumin (0.1–0.0125 μg/mL) standard curve.

Nonsteroidal anti-inflammatory drug-induced ulcers

The experiment was carried out according to the method described by Nwafor et al. with a few modifications [50]. After 12 h of fasting, rats were randomly divided into five groups of six animals each. The first group received vehicle (1 mL/kg, 1% Tween-80 aqueous solution), and the second group was orally treated with omeprazole (30 mg/kg). The other groups received 50, 125, or 250 mg/kg of the P. niruri methanol extract. After 1 h, all of the rats received indomethacin (100 mg/kg) to induce gastric lesions. All of the treatments were administered orally. Four hours after administration of the ulcerogenic agent, the animals were euthanized; the stomachs were removed and processed to measure the ulcer area, as described previously.
Effects of indomethacin, N-Ethylmaleimide, yohimbine, and N-ω-nitro-L-arginine methyl ester pretreatments

In mechanistic studies, separate experiments were performed to examine the role of prostaglandins, nonprotein endogenous sulf-hydryl (NP-SH) compounds, α2-receptors, and NO on the gastroprotective effect of the *P. niruri* methanol extract (250 mg/kg) by pretreatment with appropriate antagonists or inhibitors: indomethacin (10 mg/kg, p.o.), NEM (70 mg/kg, i.p.), yohimbine (2 mg/kg, i.p.), and L-NAME [10 mg/kg, intraperitoneal (i.p.)]. The dose selections were based on our pilot experiments and on the literature. In each case, animals were pretreated with the specific antagonist or inhibitor 30 min (i.p administration) or 1 h (p.o. administration) before vehicle (1 mL/kg, 1% Tween-80 aqueous solution) or *P. niruri* methanol extract (250 mg/kg) treatment. A gastric ulcer was then induced by oral administration of HCl/ethanol solution (10 mL/kg) 1 h after administration of the vehicle or extract. One hour after HCl/ethanol administration, the mice were euthanized; the stomachs were removed and processed to measure the ulcer area, as described previously.

**Evaluation of gastric acid secretion parameters**

The assay was performed using the method described by Shay et al., with a few modifications [51]. The animals were divided into groups (n = 6) according to the treatment used, as described previously. After 24 h of fasting, the animals were anesthetized with typanal sodium (10 mg/kg, i.p.), the abdomen was incised, and the pylorus carefully ligated. Immediately after this procedure, the methanol extract of *P. niruri* was administered at doses of 50, 125, and 250 mg/kg, respectively. Omeprazole (30 mg/kg) was used as a positive control, and the vehicle (1 mL/kg, 1% Tween-80 aqueous solution) as a negative control. Omeprazole was administered orally 30 min prior to the ligature procedure and all of the remaining treatments were performed intraduodenally immediately after pylorus ligation. Four hours later, the animals were euthanized; the abdomen was opened, and the esophageal end was clamped. The stomachs were removed and the gastric contents collected and centrifuged at 2000 rpm, at 25 °C, for 10 min. The amount of gastric juice acid (mL) and the pH values were determined. Total acid secretion was determined in the supernatant volume by titration at pH 7.0, using a 0.01 M NaOH solution, and phenolphthalein as an indicator.

**Determination of mucus in the gastric content**

This assay was performed according to the methodology previously described by Sun et al., with a few modifications [52]. Rats were divided into groups (n = 6). After 12 h of fasting, the abdomen was incised under anesthesia and the pylorus ligated. Immediately after pylorus ligature, the methanol extract of *P. niruri* was administered intraduodenally at doses of 50, 125, and 250 mg/kg, respectively. Carbexonolone (250 mg/kg) was used as a positive control due to its well-established effect on mucus production, and 1 mL vehicle (1% Tween-80 aqueous solution) was used as a negative control. The animals were euthanized 4 h after the drug treatments. The stomach content was immersed in 10 mL of 0.02% Alcian blue 0.16 M sucrose/0.05 M sodium acetate solution at pH 5.8 and incubated for 24 h at 20 °C. The Alcian blue binding extract was centrifuged at 13 000 rpm for 10 min. The absorbency of the supernatant was measured by spectrophotometry at 615 nm. The free mucus in the gastric content was calculated based on the amount of Alcian blue binding (mg/g of tissue).

**In vitro H⁺,K⁺-ATPase activity**

To prepare the H⁺,K⁺-ATPase enzyme sample, microsomes from rabbit gastric mucosal homogenates were obtained by a gradient of separation, as described by Kubo et al. [53]. Reaction mixtures containing 50 mM Tris-HCl buffer (pH 7.4), 20 mM KCl, 2.5 mM MgCl₂ and 100 μL of H⁺,K⁺-ATPase enzyme sample with or without *P. niruri* methanol extract (1–100 μg/mL), ouabain (72.8 μg/mL), or omeprazole (34.5 μg/mL) were incubated at 37 °C for 10 min. Omeprazole was incubated in 50 mM Tris-HCl buffer (pH 5.5) for its protonation. The reaction was initiated by adding substrate 1 mM ATP, and after 30 min of incubation at 37 °C, the reaction was stopped by adding ice-cold 50% trichloroacetic acid followed by centrifugation at 2000 g for 10 min. Inorganic phosphate released from ATP was measured spectrophotometrically at 620 nm by following the method of Fiske and Subbarow [54]. The experiments were performed in triplicate and the enzymatic activity was calculated using the molar extinction coefficient of inorganic phosphate (P) (ε = 11 mM/cm) and expressed in μM Pi/mg of protein/min.

**DPPH free radical scavenging**

The DPPH assay is widely used in plant biochemistry to evaluate the properties of plant constituents for scavenging free radicals. The method is based on the spectrophotometric measurement of the DPPH concentration change resulting from the reaction with an antioxidant [54]. Briefly, aliquots of the extracts at different concentrations (0.3–300 μg/mL), ascorbic acid (50 μg/mL, positive control, purity ≥ 99.0%), or vehicle (distilled water, negative control) were mixed with DPPH (40 μg/mL) methanol solution. The decrease in absorbance at 517 nm was measured after 5 min and interpolated in a DPPH standard curve (10–60 μM) [55, 56].

**Statistical analysis**

The data are reported as the mean ± standard error of the mean (SEM) and were compared using one- or two-way of analysis of variance (ANOVA), followed by Bonferroni’s post hoc, where applicable, using the software GraphPad Prism 5®. In all experiments, p < 0.05 was considered to be significant.

**Acknowledgments**

L.C. K., A. T. H., S. F. A., and V. C. F. acknowledge the fellowships from CNPq/Brazil, and APB and JARR from UNIVALI/Brazil. L. M. S. is grateful for the postdoctoral scholarship and financial support from PNPD/CAPES. This work was supported by CNPq, FAPESC, and CAPES.
Conflict of Interest

The authors report no conflicts of interest.

References


Berté PE, da Silva Lopes J, Comandulli NG, Rangel DW, Monache FD, Filho VC, Niero R, de Andrade SF. Evaluation of the gastroprotective activity of the extracts, fractions, and pure compounds obtained from aerial parts of Rubus imperialis in different experimental models. Naunyn Schmiedebergs Arch Pharmacol 2014; 387: 313–319


Morimoto Y, Shimohara K, Oshima S, Sukamoro T. Effects of the new anti-ulcer agent KB-5492 on experimental gastric mucosal lesions and gastric mucosal defensive factors, as compared to those of terpenone and cimetidine. Jpn J Pharmacol 1991; 57: 495–505


Hara N, Okabe S. Effects of gefarnate on acute gastric lesions in rats. Nihon Yakurigaku Zasshi 1985; 85: 443–446


De Young LM, Khelfets JB, Ballaron SJ, Young JM. Edema and cell infiltration in the porcine eschar-treated ear are temporarily separate and can be differentially modulated by pharmacologic agents. Agents Actions 1989; 26: 335–341


Pyrzynska K, Pekal A. Application of free radical diphenylpicrylhydrazyl (DPPH) to estimate antioxidant capacity of food samples. Anal Methods 2013; 5: 4288–4295