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Anti-*Helicobacter pylori* activity and oxidative burst inhibition by the naphthoquinone 5-methoxy-3,4-dehydroxanthomegnin from *Paepalanthus latipes*

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Abstract: *Helicobacter pylori* is a bacterium recognized as the major cause of chronic gastritis and peptic ulcers. Infection by *H. pylori* induces inflammatory responses and pathological changes in the gastric microenvironment. The host immune cells (especially neutrophils) release inflammatory mediators and large amounts of reactive oxygen species (ROS), which are associated with an increased risk of developing gastric cancer. In this study, we evaluated the anti-*H. pylori* and antioxidant activities of a 1,4-naphthoquinone-5-methoxy-3,4-dehydroxanthomegnin. The antimicrobial activity was assessed using a spectrophotometric microdilution technique, and antioxidant activity was assessed by noting the effect of 5-methoxy-3,4-dehydroxanthomegnin on the neutrophil oxidative burst using luminol- and lucigenin-amplified chemiluminescence. The results showed that 5-methoxy-3,4-dehydroxanthomegnin is a potent anti-*H. pylori* compound (MIC 64 µg/mL and MBC 128 µg/mL) and a strong antioxidant. 5-Methoxy-3,4-dehydroxanthomegnin decreased luminol- and lucigenin-amplified chemiluminescence, with ED50 values of 1.58±0.09 µg/mL and 5.4±0.15 µg/mL, respectively, reflecting an inhibitory effect on the oxidative burst. These results indicate that 5-methoxy-3,4-dehydroxanthomegnin is a promising compound for the prevention and treatment of diseases caused by *H. pylori* infection, such as gastritis, peptic ulceration, and gastric cancer, because reactive oxygen intermediates are involved in the pathogenesis of gastric mucosal injury induced by *H. pylori* infections.

Article

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Introduction

Helicobacter pylori is a spiral-shaped bacterium that colonizes the stomach in 50% of the world's population (Stege et al., 2006). *H. pylori* infection is recognized as an important causative agent of gastroduodenal diseases, including chronic gastritis, peptic ulceration, gastric adenocarcinoma, and gastric lymphoma (Park et al., 2006; Ding et al., 2007; Wang et al., 2008; Pastene et al., 2009). Studies have shown an association between long-term infection with *H. pylori* and the development of gastric adenocarcinoma. The World Health Organization has classified *H. pylori* as a group I carcinogen, with an attributable risk for gastric cancer of 50-60% (Smoot et al., 2000). This bacterium induces inflammation, infiltration and activation of immune cells, accumulation of reactive oxygen species, and oxidative DNA damage in the gastric mucosa (Ernst, 1999; Bagchi et al., 2002; Arend et al., 2005). A characteristic feature of this infection is the pronounced and sustained accumulation of neutrophils in the gastric mucosa (Allen & McCaffrey, 2007). The results of several studies indicate that *H. pylori* can activate neutrophils *in vitro*. Moreover, neutrophil density *in vivo* correlates directly with the ability of *H. pylori* to cause severe disease, and the available data suggest that neutrophils and bacteria act in concert to induce gastric ulceration (Allen et al., 2005). Eradication of the organism has been shown to result in ulcer healing and prevention of peptic ulcer recurrence and may also reduce the prevalence of gastric cancer in high-risk populations (Zaidi et al., 2009).

Treatment of *H. pylori* infection consists of 1-2 weeks of 1 or 2 effective antibiotics, including amoxicillin, tetracycline, metronidazole, or clarithromycin, plus ranitidine bismuth citrate, bismuth subsalicylate, or a proton pump inhibitor (Howden & Hunt, 1998; Duynhoven & Jonge, 2001; Park et al., 2006). However, this triple therapy is not always successful in eradicating the infection, and the acquisition of antibiotic resistance by *H. pylori* could reduce treatment efficacy and present a serious problem (Bonacorsi et al., 2009). As eradication therapies can be ineffective and undesirable side effects may occur, the search for new drugs for use in alternative therapies is very important (Stege et al., 2006; Zaidi et al., 2009).

In the course of this search, a number of different products from the plant kingdom have been tested, including antimicrobials belonging to different phytochemical groups (Pastene, 2009).

Quinone derivatives are ubiquitous in nature, having been found in plants, fungi, and bacteria, and are associated with antitumor, antibacterial, antimalarial, and antifungal activities (Huang et al., 2002). 5-Methoxy-3,4-dehydroxanthomegnin (**1**), a 1,4-naphthoquinone isolated from the capitula of the *Paepalanthus latipes* Silv. plant belonging to the Eriocaulaceae family, previously, showed a significant cytotoxic index (CI) for McCoy cells compared with cisplatin (Kitagawa et al., 2004).

The purpose of the present study was to investigate both the anti-*H. pylori* activity of this naphthoquinone, and its ability to act as an antioxidant by interfering with the neutrophil respiratory burst.

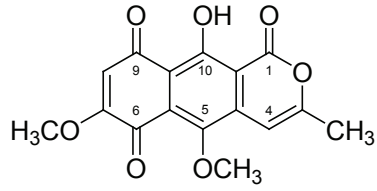
Material and Methods

Plant material

Paepalanthus latipes Silv., Eriocaulaceae, was collected at Serra do Cipó in the Espinhaço Chain, Minas Gerais, Brazil, and authenticated by Prof. Paulo Takeo Sano from Instituto de Biociências, USP, São Paulo. The voucher specimen (CFSC 13846) is on file of the Herbarium in the Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, Brazil.

Chemicals

Dimethyl sulfoxide (DMSO) (D8779), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) sodium salt (A4685), lucigenin (M8010), zymosan A (Z4250), phorbol myristate acetate (PMA) (P8139), glycogen type II from oyster (G8751), RPMI (R7755), and MTT-tetrazolium (M2128) were purchased from Sigma (St. Louis, MO, USA), and Ficoll-Paque Plus d 1077 (17-1440-02) from Pharmacia Biotech (Sweden). 5-Methoxy-3,4-dehydroxanthomegnin (**1**) was isolated from *P. latipes* and characterized as previously described (Kitagawa et al., 2004) and stored as a stock solution of 10.0 mg/mL in DMSO. Eagle medium (Adolf Lutz Institute) with various concentrations of DMSO was used in the experiments. The highest final concentration of DMSO used (2%) does not interfere with cell viability.



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Bacterial strain

H. pylori ATCC 43504, which is resistant to metronidazole (MtzR) and susceptible to amoxicillin (AmxS), was obtained from the American Type Culture Collection (Manassas, VA, USA). The bacteria were cultured in Columbia agar containing 5% sheep blood at 36-37 °C, in a 5% O_2, 10% CO_2, 85% N_2 atmosphere for three days.

Antimicrobial activity

Brain heart infusion (BHI) broth (100 µL) (supplemented with 10% fetal bovine serum) containing various concentrations of 5-methoxy-3,4-dehydroxanthomegnin (4 - 1024 µg/mL) was added to the wells of a 96-well microplate. The same volume of *H. pylori* suspension (about 10^6 cfu/mL in BHI) was added to each well. The absorbance at 620 nm was determined using an automatic ELISA microplate reader (Spectra & Rainbow Readers, Tecan). The microplate was incubated at 36-37 °C under a microaerophilic atmosphere for 72 h and then agitated before the absorbance was read again at the same wavelength. The pre- and post-incubation absorbance values were compared to determine the amount bacterial growth. The lowest concentration of 5-methoxy-3,4-dehydroxanthomegnin (**1**) that inhibited bacterial growth at least 90% was designated as the minimal inhibitory concentration (MIC). All experiments were performed at least three times using three wells for each concentration of the tested chemical. Amoxicillin and metronidazole were used as reference antimicrobials.

The minimal bactericidal concentration (MBC) was determined by culturing one standard loop from each well with no apparent growth in BHI and

incubating at 37 °C for 72 h. The lowest concentration that inhibited colony formation on Columbia agar plates containing 5% sheep blood was designated as the MBC for the tested compound.

Animals

Male rats (*Rattus norvegicus albinus*) weighing 190-200 g were obtained from Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, Araraquara, SP, Brazil, and maintained in a polycarbonate box (at 23±1 ºC, 55±5% humidity, 10-18 air changes/h, and a 12-h light/dark cycle) with free access to water and standard rodent chow. All animals were maintained and handled according to the International Ethical Guidelines for the Care of Laboratory Animals (Faculty Ethics Committee # 06/2005).

Peritoneal neutrophils collection

Rat peritoneal neutrophils were obtained by injecting each rat intraperitoneally with 10 mL of oyster glycogen solution (0.5% w/v). Peritoneal exudates were collected 12 h post-injection in Dulbecco's phosphate-buffered saline without calcium (PBS-D) containing 10 IU/mL heparin. The cells were washed twice, carefully layered onto 5 mL of Ficoll-Paque, and centrifuged for 30 min at 700× *g*. The neutrophils were washed twice in Dulbecco's phosphate-buffered saline (PBS) and adjusted to a concentration of 1.0×10^6 cells/mL. This procedure yielded a preparation containing 95% neutrophils, which were 98% viable as measured by the MTT-tetrazolium assay (Freshney, 1994; Mosmann, 1983).

Effect of 5-methoxy-3,4-dehydroxanthomegnin on neutrophil viability

Ninety-six-well tissue culture plates were seeded with 100 µL/well of RPMI containing approximately 6×10^6 cells/mL and incubated at 37 °C. After 1 h, the RPMI was removed and the cells were treated with different concentrations of 5-methoxy-3,4-dehydroxanthomegnin (2-128 µg/mL) in PBS-D. After 1 h, the medium was removed and the plates were prepared for the MTT-tetrazolium assay. The plates were agitated briefly and transferred to a microplate reader (Spectra & Rainbow (Shell) Readers, Tecan, Austria), and the optical density of each well was measured using a 540 nm filter and a 620 nm reference wavelength. All experiments were performed at least four times using three wells for each concentration of the tested chemical. The cytotoxicity data were standardized by determining the absorbance values and calculating the corresponding chemical concentrations. Linear regression analysis with 95% confidence limits was used to define the dose-response curve and compute the cytotoxic midpoint (IC50), the concentration required to obtain a 50% decrease in the spectrophotometric absorbance relative to that of the control (Barile, 1994). This test allowed us to determine the viability of neutrophils after a 1 h incubation with different concentrations of 5-methoxy-3,4-dehydroxanthomegnin.

Chemiluminescence assay

Chemiluminescence was measured on a BioOrbit model 1251 luminometer (Turku, Finland) using polypropylene tubes containing 1.0 mL of the reaction mixture. Briefly, neutrophils at 1.0×10^6 cells/mL were mixed with 2×10^{-5} M luminol or 2×10^{-5} M lucigenin in PBS-D. The mixtures were incubated for 10 min at 37 °C and then mixed thoroughly with 0.7 mg/mL non-opsonized zymosan or 3×10^{-7} M PMA and PBS-D containing 5-methoxy-3,4-dehydroxanthomegnin at non-cytotoxic concentrations (0.6, 1.2, 2.5, 5, 10 and 20 µg/mL). The background signals were measured in the absence of stimulants or 5-methoxy-3,4-dehydroxanthomegnin. The luminol- and lucigenin-enhanced chemiluminescence values were calculated using Multiuse 2.0 software (BioOrbit, Turku, Finland). The chemiluminescence response was quantified as the integrated area (IA) below the resulting chemiluminescence curve from 0 to 40 min for luminol chemiluminescence and from 0 to 15 min for lucigenin chemiluminescence. The values were compared to those of the control curve obtained without 5-methoxy-3,4-dehydroxanthomegnin, *i.e.,* cells/luminol or lucigenin/stimulants.

The ED50, *i.e.*, dose of 5-methoxy-3,4-dehydroxanthomegnin producing 50% inhibition of the control chemiluminescence, was calculated using a log-plot transformation of the data.

Reaction with hypochlorous acid

The reaction with HOCl was studied using the method of Ching et al. (1994) based on the oxidation of 5-thio-2-nitrobenzoic acid (TNB). TNB was obtained by reducing a 1 mM solution of 5,5´-dithiobis(2-nitrobenzoic acid) (DTNB) in a buffer containing 50 mM KH_2PO_4-KOH, pH 6.6, 5 mM EDTA, and 20 mM sodium borohydride. For the assay, 25 µM HOCl was incubated with 50 µM TNB for 5 min in the presence or absence of 5-methoxy-3,4-dehydroxanthomegnin in a final volume of 1 mL. The HOCl-scavenging ability of the 5-methoxy-3,4-dehydroxanthomegnin was determined by measuring the oxidation of TNB to DTNB at 412 nm after pre-incubating the 5-methoxy-

3,4-dehydroxanthomegnin with HOCl and then adding TNB.

Statistical analysis

The parameters were expressed as the mean (SD). Data were analyzed by analysis of variance (ANOVA), and a $p<0.05$ was considered statistically significant.

Results and Discussion

Helicobacter pylori has been implicated in the pathogenesis of gastritis, peptic ulceration, and possibly neoplasia (Allen et al., 2005). Antibacterial treatment of *H. pylori* is difficult due to the organism's location beneath the layer of mucus adherent to the gastric mucosa. Penetration of antibacterial agents to this site from either the lumen of the stomach or the gastric blood supply is limited. The pH of gastric secretions and sites within the mucosa may also affect drug activity (Park et al., 2006).

However, *H. pylori* antibiotic resistance is thought to be the most important factor in eradication failure. Resistance rates are highest for metronidazole (19.9-39.2%), followed by clarithromycin (1.7-27.7%), and lowest for amoxicillin, tetracycline, and trovafloxacin (0-4.7%) (Wang et al., 2009).

In addition, antibiotic treatments have had undesirable effects on non-target organisms such as intestinal microorganisms and have sometimes caused serious side effects, including diarrhea, nausea, abnormal taste, dyspepsia, abnormal pain/discomfort, headache, and angioedema (Park et al., 2006). Therefore, the increasing difficulties of standard treatment posed by antibiotic resistance, adverse effects, and high cost have led researchers to explore natural products as alternative sources of antimicrobials (Zaidi et al., 2009).

Plants, particularly higher plants, are rich sources of bioactive chemicals and may therefore be alternative sources of materials for *H. pylori* eradication (Park et al., 2006). The antimicrobial compounds from plants may inhibit bacterial growth by different mechanisms than those of presently used antimicrobials and could therefore be of clinical value in the treatment of antibiotic-resistant microbial strains, including *H. pylori* (Bonacorsi et al., 2009). Many anti-*H. pylori* compounds exhibiting significant inhibitory effects including flavonoids, tannins, terpenes, aromatic aldehydes, alcohols, catechins, and quinones, have been identified from plant materials (Funatogawa et al., 2004; Nostro et al., 2005; Shin et al., 2005; Park et al., 2006).

Quinones are ubiquitous in nature and are characteristically highly reactive. In addition to providing a source of stable free radicals, quinones are known to combine irreversibly with nucleophilic amino acids in proteins, often leading to protein inactivation and loss of function. Therefore, the potential range of quinone antimicrobial effects is great. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism (Cowan, 1999).

In the present study, the naphthoquinone 5-methoxy-3,4-dehydroxanthomegnin (**1**) showed selective anti-*H. pylori* activity with an MIC value of 64 µg/mL and an MBC of 128 µg/mL. The antibacterial activity of 5-methoxy-3,4-dehydroxanthomegnin against *H. pylori* measured by spectrophotometer microdilution assay is shown in Figure 1. The anti-*H. pylori* activity of the isolated compound was more potent than that of metronidazole (MIC>254 µg/mL) but less than that of amoxicillin (MIC 2 µg/mL). However, the MBC of amoxicillin for *H. pylori* could not be measured. These data demonstrated that 5-methoxy-3,4-dehydroxanthomegnin exhibited promising activity against *H. pylori.*

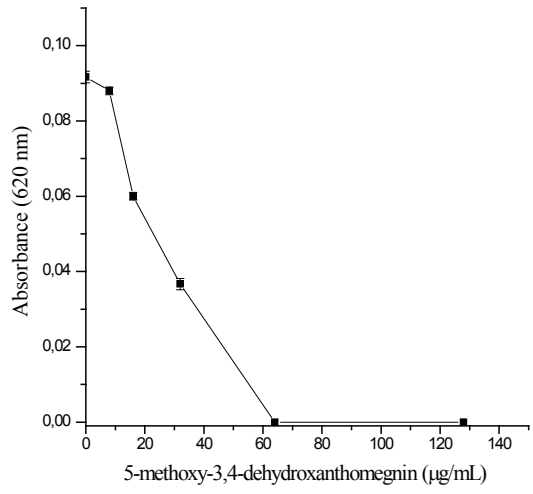

Figure 1. Effect of 5-methoxy-3,4-dehydroxanthomegnin on growth of *Helicobacter pylori* after incubation for 72 h.

H. pylori infection of the gastric mucosa is associated with an abundant inflammatory response (Smoot et al., 2000). Studies have shown that *H. pylori* stimulates a strong respiratory burst in neutrophils that exceeds that produced by other bacteria (such as *Staphylococcus aureus* and *Salmonella*) on a per organism basis and is comparable in magnitude to that induced by the large yeast cell wall particle zymosan. Neutrophils containing as few as 12 *H. pylori* organisms consume as much oxygen as cells stimulated with the

potent neutrophil agonist PMA. However, *H. pylori* disrupts NADPH oxidase targeting such that ROS are generated in the extracellular space and do not accumulate inside *H. pylori*-containing phagosomes or at other intracellular sites. *H. pylori* thus evades oxidative killing, while neutrophil-derived oxidants induce severe host tissue damage and ulceration (Allen et al., 2005; Allen & McCaffrey, 2007).

Therefore, a new therapeutic approach to treatment of *H. pylori*-associated gastric mucosal inflammation has been proposed, using agents that inhibit ROS production by activated neutrophils or scavenge the ROS produced (Naito & Yoshikawa, 2002).

Pro- and antioxidants can be studied in biological systems using methods such as chemiluminescence assays that measure the production of ROS (including peroxyl radicals, OH., HOCl, H_2O_2, and O_2-, among others) (Yildiz & Demiryürek, 1998; Prior & Cao, 1999).

Chemiluminescence has been widely used as a sensitive assay for monitoring free radicals and reactive metabolites produced by enzymes, cells, or organ systems (Yildiz & Demiryürek, 1998). The chemiluminescence assay allows continual monitoring of ROS throughout different metabolic stages by using different chemiluminescent probes. The use of probes such as luminol and lucigenin-amplifies chemiluminescence by allowing the detection of low levels of light emission, thereby increasing the sensitivity of the reaction (Trush, 1987; Stites, 1994). Several studies have suggested that light emissions from different chemiluminescent probes represent different ROS in different cellular compartments. For example, luminol tracks the production of reactive oxygen species formed in the intra- and extracellular environment, such as HOCl, H_2O_2, O_2^-, and $^1O_2$, while lucigenin, being membrane-impermeable, tracks the production of extracellular O_2^- (Parij et al ., 1998). An antioxidant reduces or prevents ROS oxidation of the substrate (probe), resulting in decreased chemiluminescence (Hirayama et al., 1997).

Significant inhibition of the intensity of luminol- and lucigenin-enhanced chemiluminescence in stimulated neutrophils by 5-methoxy-3,4-dehydroxanthomegnin was observed (Figures 2, 3). Our results showed that 5-methoxy-3,4-dehydroxanthomegnin is able to modulate the phagocyte respiratory burst in a dose-dependent manner, with ED50 values of 1.58±0.09 µg/mL and 5.4±0.15 µg/mL for luminol- and lucigenin-enhanced chemiluminescence, respectively. In the MTT cytotoxicity assay, the neutrophil viability remained above 90% in the presence of all tested concentrations of 5-methoxy-3,4-dehydroxanthomegnin for a period of 1 h (data not shown). Therefore, there is a large difference in the dose-dependency of the protective effects and cytotoxicity of 5-methoxy-3,4-dehydroxanthomegnin for neutrophils. This decrease in the luminol- and lucigenin-amplified chemiluminescence is probably due to an inhibitory effect on one or more reactive oxygen-generating systems, such as the MPO/H_2O_2/Cl_2 and/or NADPH oxidase complexes.

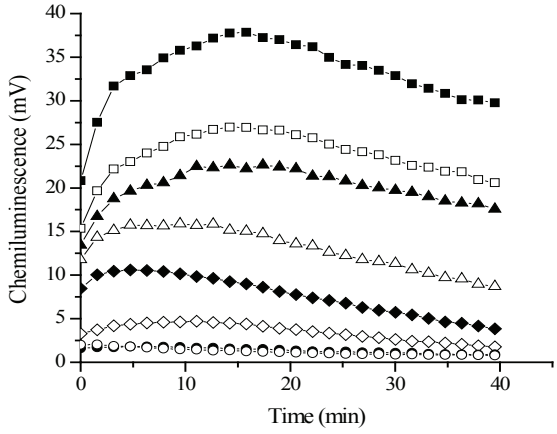

Figure 2. Influence of different doses of 5-methoxy-3,4-dehydroxanthomegnin on non-opsonized zymosan-induced chemiluminescence of polymorphonuclear neutrophils. Reactive oxygen species (ROS) production in neutrophils (1.0 × 10^6 cells/mL) is represented kinetically as millivolt (mV) values detected by luminol-enhanced chemiluminescence assay. ROS production in the cells treated with non-opsonized zymosan only as a control (solid rectangles) or phosphate-buffered saline without calcium (PBS-D) only to measure the background (open circles) was measured from 0 to 40 min post-treatment. The cells were treated with 5-methoxy-3,4-dehydroxanthomegnin at 0.6 µg/mL (open rectangles), 1.2 µg/mL (solid triangles),* 2.5 µg/mL (open triangles),* 5 µg/mL (solid diamonds),* 10 µg/mL (open diamonds)* and 20 µg/mL (solid circles).*p<0.05.

Myeloperoxidase catalytic activity is complex and involves two distinct pathways: myeloperoxidase has HOCl generation activity in addition to its regular peroxidase activity (Kitagawa et al., 2003). We investigated the scavenger effect of 5-methoxy-3,4-dehydroxanthomegnin on HOCl using the TNB test, where oxidation of TNB by HOCl results in a loss of absorbance at 412 nm. Any HOCl scavenger present in the system will compete with TNB for HOCl and limit the change in absorbance at 412 nm (Marshall et al., 1996). Incubation of HOCl with 5-methoxy-3,4-dehydroxanthomegnin before the addition of TNB did not diminish the amount of TNB oxidized, indicating that 5-methoxy-3,4-dehydroxanthomegnin does not react with HOCl (Table 1). Therefore, it seems that the inhibitory activity of 5-methoxy-

3,4-dehydroxanthomegnin on luminol-enhanced chemiluminescence is a consequence of interference with the enzymatic system rather than scavenging activity.

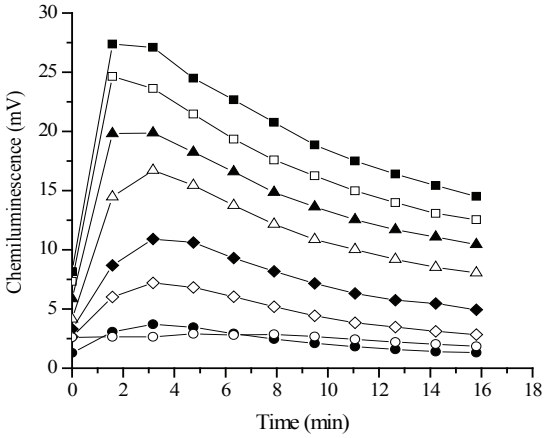

Figure 3. Effect of 5-methoxy-3,4-dehydroxanthomegnin on phorbol myristate acetate (PMA)-stimulated lucigenin-enhanced chemiluminescence of polymorphonuclear neutrophils. Superoxide production in cells treated with PMA only as a control (solid rectangles) or with PBS-D only to measure the background (open circles) was measured from 0 to 15 min post-treatment. The cells were treated with 5-methoxy-3,4-dehydroxanthomegnin at 0.6 µg/mL (open rectangles), 1.2 µg/mL (solid triangles), 2.5 µg/mL (open triangles),* 5 µg/mL (solid diamonds),* 10 µg/mL (open diamonds)* and 20 µg/mL (solid circles).*$p<0.05$.

Table 1. Effect of 5-methoxy-3,4-dehydroxanthomegnin on HOCl-dependent TNB oxidation.

Additions	A412[a)]
None	0.473
HOCl	0.097
HOCl and 5-methoxy-3,4-dehydroxanthomegnin	
5 µg/mL	0.108
10 µg/mL	0.113
20 µg/mL	0.119

[a)]Values are the means of two independent measurements that varied by <5%.

In conclusion, we have described an anti-*H. pylori* effect exhibited by 5-methoxy-3,4-dehydroxanthomegnin associated with antioxidant activity due to interference with the neutrophil respiratory burst. These results suggest that 5-methoxy-3,4-dehydroxanthomegnin (**1**) exerts a protective effect, inhibiting the synergistic mechanism by which *H. pylori* and neutrophils cause gastric mucosal damage.

Acknowledgments

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