Anti-Helicobacter pylori and Anti-inflammatory Properties of Eugenia uniflora L.

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HIGHLIGHTS

- E. uniflora MeOH extract has anti-Helicobacter pylori activity;
- E. uniflora showed antioxidant and immunomodulatory effects;
- Some tannins and flavonoids identified are reported to biological activity;
- E. uniflora is source of bioactive compounds for H. pylori infection treatment.

Abstract: Helicobacter pylori is a bacterium that reaches half of the world population and it’s recognized as the main cause of chronic gastritis and peptic ulcer. In this study, we evaluated the anti-H. pylori, antioxidant and immunomodulatory activities of the methanolic (MeOH) extract of Eugenia uniflora leaves and chemical profile. Anti-H. pylori activity was evaluated by spectrophotometric broth microdilution technique by determining the
minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), in addition to the evaluation of the effect on the urease enzyme. The antioxidant activity was evaluated by capturing $O_2^-$, HOCl e NO' radicals. The immunomodulatory effect was evaluated on the cytokines TNF-α, IL-6 and on nitric oxide through inhibition in LPS-stimulated macrophages. The chemical profile was performed by total phenolic, tannin and flavonoid contents and mass spectrometry analysis by ESI-FT-ICR MS. In the anti-\textit{H. pylori} assay the extract showed MIC of 128 μg/mL, however it did not obtain MBC. The extract also showed ability to inhibit the urease enzyme about 20%. The antioxidant activity of the MeOH extract showed EC$_{50}$ values of 29.77 µg/mL, 15.71 µg/mL and 442.10 µg/mL to $O_2^-$, HOCl and NO', respectively. The extract also showed influence on the release of TNF-α, IL-6 and NO in LPS-stimulated macrophages, ranging from 39% to 97% inhibition. Flavonoids, phenylpropanoids, tannins, triterpenoids and carbohydrates were the major classes of compounds present in the MeOH extract as identified by (-)-ESI-FT-ICR MS. The results indicate important anti-\textit{H. pylori}, antioxidant and immunomodulatory activities from \textit{Eugenia uniflora} highlighting its importance in the prevention and treatment of diseases caused by \textit{H. pylori} infection.

**Keywords:** \textit{Eugenia uniflora}; \textit{Helicobacter pylori}; antioxidant; immunomodulation.

**GRAPHICAL ABSTRACT**

**INTRODUCTION**

Gastritis and gastric ulcer are disorders characterized by the disruption of normal gastric mucosal integrity and are associated with complications such as cancer. The incidence of these diseases varies according to the age, gender, geographic location and represents a global health problem due to its high morbidity and mortality. The possible causes are credited to the excessive use of anti-inflammatory drugs, food poisoning, ethanol abuse, tobacco use and infection by the bacteria \textit{Helicobacter pylori}, which is the main etiological factor [1–3].

The infection by \textit{H. pylori}, a gram-negative bacterium, is among the most frequent infections worldwide, affecting about 50% of the global population and about 4.4 billion individuals likely to be infected in 2015 worldwide [4,5]. Known as the most prevalent human pathogen, it reaches 80% of prevalence in developing countries and 25% in developed countries [6].

It is estimated that 10 to 20% of the infected patients develop gastric ulcerative disorders of several degrees and that 1 to 2% of them direct to develop gastric cancer [7–9]. The transmission of the bacteria occurs from person to person orally-oral and / or fecal-oral, and can be transmitted asymptotically [10]. It is well known that there is a higher
prevalence of this infection in childhood due to the constant contact between them. The specific reason for this prevalence is not yet elucidated [11,12].

The virulence factors of *H. pylori* includes a survival evolutionary mechanism, characterized by the production of urease enzyme which decomposes urea into ammonia and carbon dioxide, creating a less acidic microenvironment around the bacteria. In addition, the presence of polar flagella gives the microorganism the ability of locomotion. Both enhancements help the bacterium survival and colonization in the gastric environment [10,13–17]. The *H. pylori* mechanism activates the innate immune response inducing neutrophils infiltration, macrophages and T and B lymphocytes in the gastric mucosa due to expression of *napA* gene. The *napA* gene encodes the neutrophil activating protein (HP-NAP), which in turn increases the release of proinflammatory cytokines. Activated macrophages produce and release these pro-inflammatory cytokines (IL-1, IL-6, TNF-α), reactive inorganic radicals and reactive oxygen species (ROS) and nitrogen (RNS) such as nitric oxide (NO), a radical that act as a cellular signal and is effective in the microbicidal and cytotoxic response of stimulated macrophages. Neutrophils, in turn, release large amounts of ROS. It is believed that the main cause of tissue damage is the combination of bacterial factors and the host’s inflammatory mediators [18–21].

The treatment against *H. pylori* infection consists in the combination of amoxicillin, clarithromycin, and/or metronidazole, followed by a proton pump inhibitor. According to a study published in 2017 from the World Health Organization (WHO), among several antibiotic-resistant priority pathogens, *H. pylori* is classified as high priority to the development of new drugs [22,23]. The long-term therapy with multiple drugs, its high costs, the high incidence of side effects and the antimicrobial resistance, have led researchers to explore natural products as viable source for treatment of disorders related to *H. pylori* [9,10,24]. In this context, ethnopharmacological studies have contributed for new treatment alternatives, such as the use of the specie *Maytenus ilicifolia*, a high source for tannins and flavonoids, which was evidenced of its antiulcer activity and anti-inflammatory through *in vivo* and *in vitro* tests, being thus available as a herbal medicine [25].

Fruits, bark and mainly the leaves of the species *Eugenia uniflora* L. (Myrtaceae) commonly known as *pitangueira*, are used in traditional medicine for diverse purposes such as treating rheumatism, hypertension, bronchitis, cough, fever, anxiety, worms and dyspepsia [26]. Previous studies have reported antioxidant and antimicrobial potential [27]. The phytochemical analysis of the leaves indicated the presence of polyphenols such as tannins and flavonoids; saponins and sesquiterpenes in the essential oils [13,28–30]. *E. uniflora* was included in the National List of interesting Medicinal Plants of the Brazilian Universal Health System (SUS-RENISUS) which includes vegetal species with high potential to improve the available therapies and to develop herbal medicines to be used on the Unified Health System (SUS) [31]. In addition, this species is also included in the Brazilian Pharmacopoeia [32].

Thus, the aim of this study was to evaluate the potential of *E. uniflora* against *H. pylori* and its influence in the inflammatory overall process associated with the promoted infection, evaluating the antioxidant and immunomodulatory effects of this vegetal specie besides its chemical profile in the search for new drugs against *H. pylori* and its damages in the host.

**MATERIAL AND METHODS**

**Plant Collection**

*Eugenia uniflora* leaves were collected at 20º17'53" S - 40º18'59" O, in Vitoria, Espirito Santo, Brazil. The botanical material was identified by Prof. Dr. Luciana Dias Thomas and deposited in the Herbarium VIES/UFES under number 40586.
Prepare Of The Plant Extract

The collected leaves were submitted to drying in an oven with air circulation at a temperature of 38-40°C. The leaves were then pulverized and extracted by maceration for 10 days with methanol 10% w/v. Then, after filtration, the solvent was evaporated in a rotary evaporator under reduced pressure at 50 °C, the dried extract being then stored at -20 °C. DMSO was used as the solvent in the preparation of the stock solution.

Total Phenolic Content Determination

To determine the total phenol content (TPC) 125 μL of the 10% Folin-Ciocalteau reagent aqueous solution and 25 μL of the stock solution (1 mg/mL diluted 1: 3 in distilled water) were added to a 96-well microplate. After 5 minutes, 100 μL of 4% aqueous sodium carbonate solution were added. The reading was performed at the wavelength of 750 nm in a microplate reader (BioRad, Washington, USA), after approximately 2 hours incubation in the dark. The blank solution was all reagents except methanolic extract. Gallic acid was used as the standard for the calibration curve and for the expression of the result, which was given in milligrams of gallic acid per gram of sample (mg GAE/g) [33,34].

Determination Of Tannin Content

The stock solution of the initial sample was diluted 1:3 and 100 mg casein was added to this solution. Then the solution was stirred for about 1 hour and then filtered. The blank solution was all reagents except methanolic extract. The total non-adsorbed phenols content (NAPC) by the casein is in the filtrate and was determined by the same method used to quantify the TPC. The tannin content was calculated by the difference between TPC and NAPC. Gallic acid was used as the standard for the calibration curve and for expression of the result given in milligrams of gallic acid per gram of sample (mg GAE/g) [35].

Determination Of Flavonoid Content

In a 96-well microplate, were added 99 μL distilled water, 20 μL sample (final concentration 100 μg/mL), 6 μL glacial acetic acid, 100 μL 20% pyridine and 25 μL aluminum chloride 6.5% in methanolic solution. After 30 minutes, spectrophotometric reading was performed on a microplate reader (BioRad, Washington, USA) at 415 nm. Solution with all reagents, except the sample and aluminum chloride, was used as the blank of the reaction. The blank solution consisted of a solution containing all reagents except aluminum chloride. The flavonoid content was expressed as mg quercetin equivalents (QE) per gram of extract (mg QE/g) [36].

Characterization of plant extract by Fourier Transform Ion Cyclotron Resonance Electrospray Ionization Mass Spectrometry (FT-ICR-ESI-MS)

The *E. uniflora* MeOH extract was analyzed in a mass spectrometer (Model 9.4 T Solarix, Bruker Daltonics, Bremen, Germany), which was set to operate in negative ion mode, ESI(-), over a mass range of m/z 200–1300. The parameters of the ESI(-) source were as follows: nebulizer gas pressure of 0.5–1.0 bar, capillary voltage of 3–3.5 kV, and transfer capillary temperature of 250°C. The mass spectrum was processed using the Compass Data Analysis software package (Bruker Daltonics, Bremen, Germany). A resolving power, m/Δm50% ≈ 500,000, in which Δm50% is the full peak width at half-maximum peak height of m/z ≈ 400 and a mass accuracy of <1 ppm, provided the unambiguous molecular formula assignments for singly charged molecular ions. Elemental compositions of the compounds were determined by measuring the m/z values. The unsaturation level of each molecule could be deduced directly from its double bond equivalent (DBE), following...
the equation $DBE = c-h/2 + n/2 + 1$, where $c$, $h$, and $n$ are the numbers of carbon, hydrogen, and nitrogen atoms, respectively. Molecular formula, measured $m/z$ values, DBE, and mass error are shown in Table 1.

**Bacterial Strain**

*H. pylori* INCQS 00390 (ATCC 43629) was obtained from Oswaldo Cruz Foundation (Fiocruz, RJ, Brazil). The bacteria was cultured on Columbia Agar supplemented with 5% sheep blood and subsequently inoculated in BHI (Brain Heart Infusion) (Merck Millipore, Germany) supplemented with 10% (v/v) fetal bovine serum (Sigma, USA), incubated for 72 hours at 37ºC in an atmosphere containing 10% CO$_2$.

**Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Determinations**

MIC and MBC determinations were performed both according to guidelines of the Clinical and Laboratory Standards Institute [37] (CLSI, norm M7-A10, 2015). For MIC determination, each well of the 96 well microplate was added 100 μL of a suspension of *H. pylori* (ATCC 43629) (McFarland 0.5 solution 1:20), about $10^6$ cfu/mL in liquid culture medium BHI supplemented and 100 μL of sample having final concentrations ranging from 32 to 1024 μg/mL in the same culture medium. The microplate was subjected to spectrophotometric reading at 620 nm, and then incubated (37 °C/10% CO$_2$/ 72 hours). After the incubation period, the microplate was homogenized and a new reading was performed to determine MIC.

The MIC was defined as the lowest concentration of the extract that induced an abrupt decline in the absorbance value (90%). Amoxicillin (Sigma, USA) and metronidazole (Sigma, USA) were used as standards antibiotic control.

The MBC was determined by the lowest concentration of extract that inhibited the formation of colonies on Columbia Agar plates containing 5% sheep blood (incubated at 37 °C / 10% CO$_2$/ 72h) corresponding to the microplate well with no apparent growth of BHI.

**Inhibition of Urease Enzyme**

In 96 microplate well were added 25 μL of the protein urease (4UI) (*Jack Bean Urease*, Sigma, USA) and 25 μL of the sample at concentrations ranging from 32 to 1024 μg/mL, and incubated for 2 hours at room temperature. After that, 25 μL 0.02% phenol red and 200 μL 50 mM urea were added in 100 mM PBS buffer solution (pH 6.8). The absorbance of the reaction mixture was measured at 540 nm in a microplate reader (BioRad, Washington, USA) at time 0 and then every 10 minutes to evaluate the reaction kinetics [38]. Boric acid (H$_3$BO$_3$) was used as a standard urease inhibitor.

**Antioxidant Activity Evaluation**

**The Superoxide Anion (O$_2^-$) Scavenging Activity**

In a 96-well microplate, 225 μL of phosphate buffer (PBS) (50 mM, pH 7.4), 30 μL of extract (3.125 to 100 μg/mL), 7.5 μL of phenazine methosulfate (PMS) (0.5 mM) and 30 μL nitroblue tetrazolium (NBT) (0.045 mM) were added. After two minutes, 7.5 μL of NADH (0.125 mM) were added. Finally, the spectrophotometric reading at 540 nm was performed after 10 minutes of incubation at room temperature protected from light [39]. Gallic acid was used as the antioxidant standard of this assay.
Hypochlorous acid (HOCl) scavenging assay

The reaction with HOCl was studied based on the oxidation of 5-thio-2-nitrobenzoic acid (TNB) [40]. TNB was obtained by reducing a 1mM solution of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) in a 50 mM KH<sub>2</sub>PO<sub>4</sub>–KOH buffer (pH 6.6) containing 5 mM EDTA and 20 mM sodium borohydride and then quantified by molar extinction to obtain the final concentration (140 μM). Then, in a 96-well microplate, 20 μL of HOCl (25 μM), 60 μL of 50 mM phosphate buffer (pH 6.6) and 20 μL of sample were added. After 2 minutes, 100 μL of TNB solution (final concentration of 70 μM) were added, and the spectrophotometric reading was performed at 415 nm after 1 minute. Gallic acid was used as the antioxidant standard of this assay.

Nitric Oxide (NO) scavenging assay

The NO production was provided by sodium nitroprusside (SNP) molecules in PBS buffer. The assay was performed according to method provided by Marcocci et al. (1994), with modifications. Thus, SNP (1.25 mM) was prepared in phosphate buffer (0.1 M pH 7.0) in absence of light. In a 96-well plate, 50 μL of SNP and 50 μL of samples at various concentrations (ranging from 25 to 800 µg/mL) were added and incubated for 1 h at room temperature with exposure to light. After incubation, 100 μL of Griess reagent (1% w/v sulfanilamide, 0.1% w/v of naphthylethylenediamine and 2.5% v/v of orthophosphoric acid) were added and the reaction mixture was read at 540 nm. A calibration curve with sodium nitrite (NaNO<sub>2</sub>) was generated to represent the data from NO<sub>2</sub>- formation. Gallic acid was used as the antioxidant standard of this assay.

Immunomodulatory Activity

Cell Culture

Nitric oxide (NO) and cytokine (TNF-α and IL-6) detection assays were performed using the murine macrophages RAW 264.7 (ATCC TIB-71). Cells were maintained in bottles with Dulbecco’s Modified Eagle Medium supplemented with 10% (v/v) fetal bovine serum and incubated for different periods at 37°C in a 5% CO<sub>2</sub> atmosphere until reaching a confluence of approximately 70-90%. After obtaining the desired confluence, the cells were dissociated using a cell scraper and counted in a Neubauer chamber to obtain cell concentration values.

Cell viability by MTT assay

Cytotoxicity assay was performed to determine the non-cytotoxic concentrations of extracts for macrophages. Aliquots (0.2 mL) of medium containing macrophages were seeded into 96-well tissue-culture plates at 2x10<sup>5</sup> cells/mL. After 2h, the medium was removed and the adherent cells were incubated with the extracts for 24 h. After incubation, the medium was removed, and the macrophages were incubated with MTT solution (1 mg/mL) for 3 h [42]. The formazan formed was dissolved in DMSO, and the optical density was measured using a microplate reader with a 540 nm filter and 620 nm reference filter. The optical density of the dissolved formazan in the control (untreated cells) was taken to be 100% viable.

Measurement of cytokine production (TNF-α and IL-6) and NO

The cells were distributed into 24 well plates at a concentration of 5x10<sup>6</sup> of viable cells per mL to the IL-6 and NO assays and 2x10<sup>6</sup> viable cells/mL for the TNF-α assay DMEM solution. After two hours of incubation (37 °C/5% of CO<sub>2</sub>) for cell adhesion, the supernatant was discarded and the cells were stimulated with Escherichia coli lipopolysaccharides (LPS)
Anti-Helicobacter pylori and anti-inflammatory properties of Eugenia uniflora L. (Sigma, USA) at a concentration of 1 µg/mL, receiving at the same time, different concentrations of the extract (previously determined by cytotoxicity assays). The plates were incubated overnight (37 °C/5% of CO₂). After that, the supernatants were collected in order to detect and quantify the present cytokines (TNF-α and IL-6) by enzyme immunoassay (eBioscience, USA) and NO by the Griess method [43]. Additionally, cytotoxicity was evaluated after all assays by MTT method.

Statistical Analysis

Statistical differences were determined by analysis of variance (ANOVA) of two-way with Tukey post-test and p ≤ 0.05 as statistically significant. Linear regression tests were also conducted for the EC₅₀ values.

RESULTS

Phytochemical Analysis

Total Phenols, Tannins and Flavonoids Contents

The values obtained in the total phenol and tannins quantification are 193.06 (19.31%) and 161.29 (16.13%), respectively, presented in milligram equivalent of gallic acid (mgEAG/g) and the values obtained in the flavonoids quantification are 28.64 (2.86%) presented in milligram equivalent of quercetin (mgEQ/g) per gram of extract.

Characterization of plant extract by Fourier Transform Ion Cyclotron Resonance Electrospray Ionization Mass Spectrometry (FT-ICR-ESI-MS)

The (-) - ESI FT-ICR MS technique was applied to analysis of E. uniflora MeOH extract. The exact molecular mass of the compounds present in the extract makes it possible to obtain a reliable chemical profile of the analyzed solutions (Table 1). Because of the ionization method used was electrospray in negative mode, acidic molecules like phenolic acids, flavonoids, acid triterpenes, tannins and alcohols such as carbohydrates were properly ionized to be analyzed. Important Myrtaceae chemical classes present in the extract, were: gallotannins [44], ellagitannins [44], flavonoids [45], phenylpropanoids like caffeoylquinic acid, triterpenoids [46] and sesquiterpenoids [47]. Quinic acid was ionized as its chloride cluster at [M-H]- 227. Myricitrin and myricetin, important flavonoids of E. uniflora were identified at 463, 499 (chloride cluster) and 317 respectively [48]. For further information, access the supplementary material, Figure S1.
Table 1. Molecular formula, DBE, and mass error of the *E. uniflora* MeOH extract by electrospray ionization mass spectrometry

<table>
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<th>[M-H]</th>
<th>Molecular Formula (m/z)</th>
<th>Error (ppm)</th>
<th>DBE</th>
<th>Signal Intensity (%)</th>
<th>Proposed Substance or Class of Substance</th>
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<tr>
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<td>1.44</td>
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<td>2.86</td>
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<td>300.99949</td>
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<td>4.81</td>
<td>Ellagic acid</td>
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<tr>
<td>317.0307</td>
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</table>

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC value of the MeOH extract fraction of *E. uniflora* against *H. pylori* was 128 μg/mL. Despite all concentrations tested, no MBC values were detected since colony formation were identified in the Columbia Agar lamb blood solution (5%). The reference compounds, metronidazole and amoxicillin, presented MIC>512 μg/mL and MIC 0.125 μg/mL, respectively.

Inhibition Of Urease Enzyme

Figure 1 shows the results for urease inhibition profile, evidencing an inhibition constant value of *E. uniflora* MeOH extract at all tested concentrations (32 to 1024 μg/mL) ranging from 20.501 to 24.363%, which is (32 - 512 μg/mL) showed no statistical difference in relation to boric acid (inhibition control).
Anti-Helicobacter pylori and anti-inflammatory properties of Eugenia uniflora L.

Figure 1. Urease inhibition assay. Results of the urease enzyme inhibition assay (% inhibition vs. sample concentration). Results presented as mean ± standard deviation of at least three independent experiments. *p<0.05 compared to boric acid.

Antioxidant Activity Evaluation

The EC_{50} of O_2•−, HOCl and NO• for the E. uniflora MeOH extract were 29.77, 15.71 and 442.10 μg/mL, respectively, while the gallic acid, showed values of 30.84, <3.125 and 228.40 μg/mL, respectively, evidencing the ability of the extract to block the action of reactive oxygen species as O_2•− and HOCl.

Immunomodulatory Activity Evaluation

The extract concentrations used for the macrophage assays were defined according to the previously cytotoxicity assay performed, selecting those with no influence on cell viability. The effect of E. uniflora MeOH extract of LPS-stimulated macrophages are shown in Figure 2 which presents the quantification of TNF-α, IL-6 and NO•. The inhibition of IL-6 and NO (80 and 100%, respectively) at concentration of 100 μg/mL, was observed in a dose-dependent manner. Inhibition for TNF-α at the same concentration was 40%.

Figure 2. Effects of E. uniflora MeOH extract (μg/mL) on LPS-stimulated macrophages on: TNF-α release (A); IL-6 release (B); NO release (C). Cells incubated just with LPS were used as a positive control and cells in culture medium (DMEM) as a negative control (C-). *p<0.05 vs. LPS control. Results presented as mean ± SD of at least three independent experiments.

DISCUSSION

H. pylori have been associated with the pathogenesis of gastritis, peptic ulcer and gastric cancer. The microorganism has been classified as a Group I carcinogen by the International Agency for Research on Cancer (IARC) since 1994, and in 2014 it accounts for 78% of the world's cases of gastric cancer. The infection of the gastric mucosa by H. pylori is associated with excessive inflammatory response and tissue damage, since the
microorganism stimulates the respiratory burst in neutrophils increasing ROS production, furthermore modulates NADPH oxidase so that the radicals are released in the extracellular environment and not within the phagosomes where the bacterium is found [18,21].

The high prevalence of *H. pylori* strains resistant to clarithromycin and metronidazole and especially the difficulty of antimicrobial action in the stomach environment due to the acid pH associated with the difficulties to access to the microorganism region have led to a need for alternative solutions to diminish or control of the infection [49].

Previous reports with plants of the same family (Myrtaceae), indicates that the extract evaluated in this study presented better anti-*H. pylori* activity than species such *Myrciaria cauliflora* (MIC 256 μg/mL), *Myrtus communis* (MIC 500 μg/mL) and *Syzygium aromaticum* (MIC 500 μg/mL) [50]. Additionally, *E. uniflora* did not show MBC at 1024 μg/mL. The absence of MBC suggests bacteriostatic activity. The bacteria proliferation inhibition is of great importance since the *H. pylori* has several defense mechanisms against the host cells and thus an attenuated bacteria or a metabolically weakened one, it is more susceptible to the immune system [51].

The *E. uniflora* MeOH extract demonstrated ability to inhibit the enzyme urease, as in previous studies with other species of the same family as *Myrciaria cauliflora, Eucalyptus grandis* and *Myrtus communis* [52]. These results were in accordance with the (-) - ESI FT-ICR MS technique applied to analysis of the methanolic extract of *E. uniflora* leaves indicating the major presence of tannins and flavonoids, which have the capacity of produce complexes and consequent inhibition of bacterial enzymes [53]. In this study, inhibition of urease may be related mainly to tannins (16.13%) due to their higher content compared to flavonoids (2.86%). Many compounds from plants have biological activity, specially anti-ulceration and antimicrobial character, such as flavonoids, tannins and terpenoids, and therefore powerful allies in the eradication of *H. pylori*. The phytochemical screening and the FT-ICR-ESI-MS identification of major components of *E. uniflora* MeOH extract evidenced the presence of tannins and flavonoids, in accordance with previously reports of the Brazilian Pharmacopeia V (2010) and several scientific studies [28,30,32].

(-) - ESI FT-ICR MS technique was applied to analysis *E. uniflora* MeOH extract and the choice for the negative ionization method for the methanolic extract was based on the fact that *E. uniflora* leaves are a rich source of phenolic compounds [54]. Although carbohydrates are less acid than phenolic compounds, they showed intense peaks on the mass spectrum (Figure S1), indicating that carbohydrates should be the major compounds of the extract.

The evidence of the ability of *H. pylori* to induce the production of reactive species by neutrophils and macrophages causing oxidative stress, led us to evaluate the capacity of the extract to capture oxidants such as O$_2^-$, HOCl and NO [18].

The results indicates the capacity of *E. uniflora* MeOH extract to capture the oxidants O$_2^-$ and HOCl compared to gallic acid, notably for HOCl scavenging capacity (84.98%, 85.69% at 50 and 100 μg/mL, respectively). This effect may be related to the presence of total phenol, tannins and flavonoids by phenol stabilization from quinone production [55]. When compared to literature data, *E. uniflora* presents better capability to capture O$_2^-$ than plants of the same family such as *Eugenia stipitata* (EC$_{50}$ 126.14 μg/mL) and *Syzygium malaccense* (EC$_{50}$ 30.49 μg/mL) [56]. However, the extract did not show significant data on NO scavenging.

Macrophages were stimulated with LPS for cellular activation and release of inflammatory mediators, including increased inducible nitric oxide synthase (iNOS) with the generation of NO and proinflammatory cytokines. In this study, the results showed the *E. uniflora* MeOH extract ability to inhibit the formation and/or to capture NO produced by LPS-stimulated macrophages. This inhibition can be due to the direct capture, inhibition of iNOS and/or modulation of transcription factors in the cascade of NO formation. In assessing the ability of NO scavenging by the chemical method, *E. uniflora* MeOH extract demonstrated inhibition about 20% at 100 μg/mL, indicating the possibility of the extract to...
inhibit iNOS in a greater extent, since 100% inhibition was demonstrated in the LPS-stimulated macrophage assay at the same concentration.

Due to the mutagenic effect of NO, its excessive production during the immune response to *H. pylori*, could be related to the development of gastric cancer [1,57]. Furthermore, the ONOO\(^-\) ions generated from NO, is far more cytotoxic than NO due to its instability and short half-life, causing DNA damage, closely related to carcinogenesis [58]. Thus, the ability to inhibit NO is an important resource in the gastric cancer prevention associated with *H. pylori*.

The immunomodulatory results from the *E. uniflora* MeOH extract presented NO, TNF-\(\alpha\) and IL-6 inhibition profile. The extract at 100 \(\mu\)g/mL demonstrated the ability to inhibit IL-6 (83.19%), TNF-\(\alpha\) (39.10%), and NO (97.12%).

The NO and cytokines inhibition by *E. uniflora* MeOH extract could be related to an anti-ulcer mechanism, since TNF-\(\alpha\) and IL-6 are programmed to recruit more inflammatory cells to consequently produce reactive species such as NO, responsible for damage to the gastric tissue.

Although the inhibition mechanism of these cytokines has not been clearly elucidated, an independent anti-inflammatory pathway not related to the common cyclooxygenase-1 (COX-1) enzyme inhibition is an interesting approach. The usual COX-1 inhibition of this enzyme leads to severe gastric damage due to the hindrance of prostaglandins production and hence their homeostatic protective function of gastric epithelium and increased mucus production, contributing to the epithelium regeneration [59].

In this context, inhibition of pro-inflammatory cytokines would act on cell infiltration and release of reactive species during the inflammatory process, reducing the chances of tissue damage and cancer development. Chronic inflammation regulation is an important role in the etiology of cancer, the anti-*H. pylori* and anti-inflammatory profile of *E. uniflora* MeOH extract could help in treating lesions caused by this bacterium to prevent the establishment of gastric cancer.

**CONCLUSION**

The MeOH extract of *E. uniflora* presented *H. pylori* bacteriostatic activity. However, other activities related to the infection were found, such as the antioxidant activity by the capture of the oxidants O\(2^-\) and HOCl and the immunomodulatory activity expressed by NO, TNF-\(\alpha\) and IL-6 inhibition. All this reported activities from *E. uniflora* MeOH extract could be related to the FT-ICR-ESI-MS identification of major components as flavonoids and tannins highlighting this vegetal specie as a valuable source in the search of new treatments for *H. pylori* infection.

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**Conflicts of Interest**
The authors declare no conflict of interest.

**REFERENCES**


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35. Dimich GS, Soares LAL. Ferreira MA, De Oliveira AGV, Carvalho MDC, Ximenes EA. Phytochemical and antibacterial investigations of the extracts and fractions from the stem bark of Hymenaea stigonocarpa Mart. ex Hayne and effect on ultrastructure of Staphylococcus aureus induced by hydroalcoholic extract. Sci World J. 2013;2013.

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