

Anti-*Helicobacter pylori* effect of the antioxidant extract from *Baccharis trimera* Less. (DC)

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Abstract

One of the main risk factors for the development of stomach ulcers and cancer is infection with *Helicobacter pylori* bacteria, which is accompanied by considerable oxidative stress. Therefore, the aim of the present study was to assess the anti-*Helicobacter pylori* activity of *B. trimera* hydroalcoholic extract (HE) and aqueous (AqF), hexanic (HxF), and acetonitrile/chloroform (ACF) fractions, as well as their oxidant potentials.

A preliminary phytochemical screening was carried out. Anti-*Helicobacter pylori* activity was assessed using a microdilution assay. After exposure to the investigated samples, the bacterial morphology was analysed under a scanning electron microscope (SEM). The antioxidant activity was evaluated in hydrogen peroxide (H₂O₂), superoxide anion (O₂^{•-}), hypochlorous acid (HOCl), hydroxyl radical (HO[•]) and nitric oxide (NO[•]) assays.

The highest concentration of polyphenols was found in HE, the highest concentration of flavonoids was found in ACF, and of tannins was found in AqF. In the anti-*H. pylori* assay, the MIC₉₀ was 512 µg/mL for HE and 1024 µg/mL for ACF, which was bactericidal. The SEM showed morphological alterations such as cell lysis in the tested samples. In the O₂^{•-} inhibition assay, the EC₅₀ of AqF was 5.85 ± 0.86. In the HOCl, HO[•] radical, NO[•] and H₂O₂ scavenging assays, the best results were in ACF, with an EC₅₀ of 15.50 ± 0.80, 2.90 ± 0.48, 132.13 ± 7.38 and 66.70 ± 2.30 µg/mL, respectively. The analyses indicate that compounds present in *B. trimera*, especially in HE and ACF, are promising candidates for the prevention and treatment of diseases caused by *H. pylori*.

Key Word: *Helicobacter pylori*; antioxidant; morphological alterations; secondary metabolites

Introduction

Infection with *Helicobacter pylori* is the most common cause of chronic gastritis and peptic ulcer worldwide. *H. pylori* produces progressive damage to the gastric mucosa and has been shown to cause various diseases, including gastritis, ulcers and MALT (mucosa-associated lymphoid tissue) lymphoma of the stomach. Although more than 70% of infected individuals are asymptomatic, 10-20% of infected individuals may develop this disease at some point in life. *H. pylori* is also a relevant factor in the pathogenesis of stomach cancer [1,2]. The estimated risk of *H. pylori*-mediated stomach cancer in developing countries is approximately 75% [3].

H. pylori is a gram-negative, microaerophilic, spiral-shaped and flagellate bacterium that typically colonises the human stomach, where it may remain for decades without causing any adverse consequence. The risk of acid-peptic diseases in individuals infected with *H. pylori* is associated with characteristics (such as

virulence factors) of the involved strain and with the host's response to infection [4,5].

Some studies suggest that in the course of infection with *H. pylori*, the concentration of reactive oxygen species (ROS) increases in the gastric mucosa. The virulence factor known as *H. pylori* neutrophil activating protein (HP-NAP) activates the enzyme reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which increases neutrophil oxygen consumption, resulting in the so-called oxidative burst. The oxidative burst triggers the release of superoxide anion (O₂^{•-}) into the extracellular space or phagosomes, and other relevant reactive species may also be produced, including hydrogen peroxide (H₂O₂), hydroxyl radical (HO[•]), and hypochlorous acid (HOCl). An excess of these reactive species is associated with various pathological conditions, including gastric cancer [6].

Standard treatments for the eradication of *H. pylori* include proton pump inhibitors, bismuth, and histamine receptor H₂ antagonists combined with two antibiotics. However, problems in the adherence

and increases in the rate of resistance to standard treatments are a cause of much concern [1]

In this context, natural products stand out as alternative sources of compounds with anti-*H. pylori* and antioxidant activity. The use of plants for the treatment, cure and prevention of diseases is one of the oldest medicinal practices of humanity. In Brazil, the great biodiversity of plants used without scientific support has encouraged researchers to study the biological activities of native plants to determine their efficacy and safety.

Baccharis trimera (Less) DC, popularly known as “carqueja”, is a member of the Asteraceae family and is an erect, branchy, glabrous subshrub that reaches up to 80 cm in height. Native to South America, *B. trimera* is cultivated mainly in Brazil, Argentina, Paraguay and Uruguay. In Brazil, aerial parts of *B. trimera* are used in folk medicine in the treatment of hepatic and digestive problems [7,8]. Several studies have demonstrated the presence of flavones, flavonols, saponins and diterpenes [9]. The phenolic compounds previously identified in *B. trimera* include apigenin, 7,4'-di-*O*-methyl-apigenin, cirsimaritin, eupatorin, genkwanin, hispidulin, isoquercetin, luteolin, nepetin, quercetin, 3-*O*-methylquercetin, 5,6-dihydroxy-7,3',4'-trimethoxyflavone and rutin. In relation to terpenoids, *B. trimera* presents mainly saponins, among which echinocystic acid is the major aglycone [10]. Many biological activities, such as anti-inflammatory, analgesic, anti-hepatotoxic and muscle relaxant effects, have been ascribed to *B. trimera*. Studies have also shown that *B. trimera* is able to minimise the effects of reactive oxygen species [8,11].

B. trimera is in the Brazilian Pharmacopoeia and is also on the national list of plants of interest to SUS (Health Unic System) [12], which is composed of species with therapeutic potential. Therefore, considering that *B. trimera* is widely associated with popular use and that many studies are needed to prove its effects, the aim of the present study was to study the anti-*H. pylori* activity, to conduct screenings of phytochemicals and to evaluate their inhibitory action against oxidants of hydroalcoholic extract and fractions, thus corroborating studies that facilitate the future use of this plant for the treatment and prevention of gastrointestinal disorders.

Materials and Methods

Plant material

The present study used the aerial parts of *B. trimera*, which was provided by a pharmaceutical company, from Brazil, in the form of a plant drug, produced in compliance with the guidelines described in the ANVISA Collegiate Board of Directors Resolution nº 26/2014 [13].

Preparation of the extract and fractions

The hydroalcoholic extract (HE) was obtained using a maceration technique at 10% w/v with 70% alcohol. The resultant solution was filtered, subjected to evaporation at 50°C under reduced pressure in a rotary evaporator and subsequently lyophilised in a LIOTOP

L101 lyophiliser (São Paulo - BRAZIL). The dry residue obtained was fractionated using hexane, acetonitrile/chloroform, and water, which yielded hexanic (HxF), acetonitrile/chloroform (ACF) and aqueous (AqF) fractions, respectively.

Screening phytochemical

The qualitative phytochemical characterization of the extracts and fractions was based on the classic identification tests of the following compounds: flavonoids, saponins, cardiac glycosides, anthraquinones, tannins, alkaloids, polyphenols, terpenes and steroids. For these metabolites, there are specific reactions that allow the verification of colour change and the formation of foam, fluorescence or precipitate.

Determination of total polyphenol content (TPC) and tannins totals (TTC)

The total polyphenol content (TPC) was determined using the methods described by Neves *et al.*, (2009) [14], with adaptations. First, 125 µL of Folin Ciocalteu reagent diluted to 10% v/v in distilled water and 25 µL of sample from the initial solution (1 mg/mL diluted 1:3 in distilled water) were added to a microplate. The solution was allowed to stand for 3 to 8 minutes. Next, 100 µL of 4% sodium carbonate was added. The microplate was allowed to stand protected from light for approximately 2 h. The absorbance at 750 nm was read using a microplate reader iMark®, BioRad (Washington, USA). Gallic acid was used as the standard.

To quantify the tannins, the method described by Dimech *et al.*, (2013) [15] was used, with adaptations. The initial solution (1 mg/mL) was diluted in water at the same proportion that was indicated previously (1:3). After the addition of 100 mg of casein, the solution was stirred for 1 hour, and then filtration was conducted. The filtered sample was the content of polyphenols (NACP) not adsorbed to casein. To determine the NACP, the same method that was used to quantify TPC was carried out. The difference between TPC and NACP was the total tannin content (TTC). Gallic acid (GA) was used to perform the calibration curve, and the results were expressed in mgEGA/g.

Determination of the total flavonoid content (TFC)

The total flavonoid content (TFC) was determined according to Perdigo (2012) [16]. A 25 mg sample was diluted in 50 mL of methanol. Then, a 1 mL aliquot of that solution was added to 1 mL of methanol. Next, 0.6 mL of glacial acetic acid, 10 mL of pyridine-water solution (20:80) and 2.5 mL of 6.5% aluminium chloride-methanol solution were added to the mixture. Distilled water was added to achieve a total volume of 25 mL. Readings were performed 30 minutes later with a UV-2000A spectrophotometer (Instrutherm, São Paulo – BRASIL) at 420 nm. Rutin (RU) was used as the standard. The results were expressed in mgERU/g.

Assessment of anti-*H. pylori* activity

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The anti-*H. pylori* activity was evaluated by determining the minimum inhibitory concentration (MIC) using the broth microdilution technique according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [17]. Microplate wells were filled with 100 μ L of liquid culture medium (Brain Heart Infusion supplemented with 10% foetal bovine serum) containing various concentrations of extract and fractions (32 μ g/mL to 1,024 μ g/mL), onto which an equal volume of *H. pylori* (ATCC 43504) suspension (with approximately 10^6 - 10^7 bacteria/mL) was added. Spectrophotometric readings were performed at 620 nm using a microplate reader, iMark®, BioRad (Washington, USA). The microplate was incubated in a 10% CO₂ atmosphere at 36-37°C for 72 h. The plate was homogenised, and a new reading was conducted at the same wavelength. The tests were performed in triplicate, repeated at least three times, and included a positive control (absence of extracts) and negative controls (culture medium added with various concentrations of extract and tested substances - colour control). MIC was graphically defined as the lowest concentration of antibacterial substance that promoted an abrupt decline (90%) in absorbance; sub-MIC was defined as the concentration immediately below the concentration that induced 90% bacterial growth inhibition. Amoxicillin and metronidazole were used as standard antibiotics. The CBM was defined as the lowest concentration of extract that inhibited colony formation on Columbia Agar plates. In this test, the concentration that did not achieve growth in microplates was plated on BHI agar plate Columbia containing 5% sheep blood (incubated at 37 °C under an atmosphere containing 10% CO₂ for 72 h).

Morphological analysis of *H. Pylori*

The morphological analysis of *H. pylori* following exposure to MIC and sub-inhibitory (sub-MIC) was performed using a scanning electron microscope (SEM). The culture medium containing bacteria that was exposed to the extract was separated in aliquots and centrifuged at 4,000 rpm for five minutes. The supernatant was discarded, and 1 mL of 0.1 M cacodylate buffer, pH 7.2, was added; next, the sample was centrifuged again. The cell pellet was collected and placed in 200 μ L of 0.1 M cacodylate buffer. An aliquot of the sample was placed at the centre of a slide, allowed to dry and then fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 30 minutes. The sample was dehydrated with alcohol, allowed to dry, metallised, and subjected to reading under SEM JEOL®, JSM-6610LV (Tokyo, JAPAN) with an accelerating voltage of 10 kV.

Urease inhibition assay

Urease inhibition activity was determined based on the production of ammonia catalysed by the enzyme urease, according to the method described by Tanaka *et al.*, (2003) [18]. The reaction microplate contained a mixture of 25 μ L of urease 4 UI (Sigma Jack Bean urease type III) and 25 μ L of the sample at varying concentrations and was incubated at room temperature for two hours. Next, 25 μ L of phenol red (0.02%) and 200 μ L of urea (50 mM) in 100 mM phosphate buffer (pH 6.8) were added to the microplate. After 20 minutes, the mixture absorbance was read at 540 nm using a microplate reader, iMark®, BioRad (Washington, USA). Boric acid was used as the standard positive control for urease inhibition.

Antioxidant assays

In the antioxidant assays, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX), which is widely employed as an antioxidant control, was used. The results were expressed as the percentage of inhibition of reactive species formation and the half maximal effective concentration (EC₅₀), i.e., the concentration that inhibits 50% of the biological oxidants in solution.

Hypochlorous acid (HOCl) scavenging assay

This method was based on the oxidation of 5-thio-2-nitrobenzoic acid (TNB) by HOCl, resulting in the formation of 5-5'-dithio-bis (2-nitrobenzoic) acid (DTNB). In this assay, the modified method described by Ching *et al.*, (1994) [19] was used. One hundred microliters of HOCl (25 μ M) were incubated with 100 μ L of extract at varying concentrations (3.125 to 100 μ g/mL). A volume of 300 μ L of phosphate buffer (50 mM), pH 6.6, was added, and after five minutes, 500 μ L of TNB (50 μ M) were subsequently added. The TNB solution was prepared with DTNB (1 mM), ethylenediaminetetraacetic acid (EDTA) (5 mM) and sodium borohydride (NaBH₄) (20 mM) to a final volume of 50 mL. The HOCl solution was adjusted to pH 12 using 10% NaOH. Next, the final desired concentration of HOCl (25 μ M) and TNB (50 μ M) solution was achieved based on the molar extinction coefficient. The reaction was read using a microplate reader, iMark®, BioRad (Washington, USA).

Superoxide anion (O₂^{•-}) scavenging assay

The method described by Suzumura *et al.*, (1999) [20] was used, with some modifications. In this method, O₂^{•-} radical reduces nitroblue tetrazolium (NBT) to formazan at pH 7.4 and room temperature. Antioxidant molecules react with O₂^{•-}, inhibiting the formation of formazan. One hundred microliters of the sample at varying concentrations (3.125 to 100 μ g/mL), 25 μ L of phenazine

methosulphate (PMS) (5 μ M), 100 μ L of NBT (45 μ M), and 750 μ L of $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer (50 mM), pH 7.4 were added into a 24-well microplate. After two minutes, 25 μ L of reduced nicotinamide adenine dinucleotide (NADH) (0.125 mM) was added. The reading was performed at 540 nm using a microplate reader, iMark®, BioRad (Washington, USA).

Hydrogen peroxide (H_2O_2) assay

In this assay, peroxidase breaks H_2O_2 down, yielding an intermediate product that is able to oxidise phenol red and transform phenol red into a yellow compound that becomes purplish red in alkaline pH and is quantified at 610 nm [21]. The reaction solution contained 20 μ L of extract at varying concentrations (3.125 to 100 μ g/mL), 40 μ L of H_2O_2 (2 mM), 16 μ L of a solution containing NaCl (140 mM), phenol red (0.1 mg/mL), dextrose (5.5 mM) in potassium phosphate buffer (10 mM), pH 7, and 20 μ L of peroxidase (8.5 U/mL). Phosphate buffer, pH 7, was added to achieve a final reaction volume of 200 μ L. After ten minutes, the reaction was terminated through the addition of 20 μ L of 1N NaOH. The oxidised phenol red purple complex was quantified at 610 nm using iMark Absorbance Reader (Bio-Rad, Washington, USA).

Hydroxyl radical (HO^\bullet) scavenging assay

In the hydroxyl radical assay, the method described by Halliwell *et al.*, (1987) [22] was used, with some modifications. The reaction contained 200 μ L of the sample at varying concentrations (3.125 to 100 μ g/mL), 100 μ L of FeCl_3 (50 μ M), 100 μ L of EDTA (100 μ M), 100 μ L of ascorbate (100 μ M), 100 μ L of H_2O_2 (1 mM), 100 μ L of deoxyribose (2.8 mM), and 300 μ L of $\text{KH}_2\text{PO}_4/\text{KOH}$ buffer 10 mM, pH 7.4 (final volume: 1 mL). The reaction was incubated at 37°C for one hour; then, 500 μ L of 1% thiobarbituric acid prepared in 50 mM NaOH and 2.8% trichloroacetic acid were added, and the reaction was incubated at 100°C for 5 minutes. HO^\bullet formation was determined using a microplate reader, iMark®, BioRad (Washington, USA) at 540 nm.

Nitric Oxide scavenging assay (NO^\bullet)

This assay was based on the methodology presented by Marcocci *et al.*, (1994) [23], with adaptations. Initially, sodium nitroprusside

(SNP) (1.25 mM) in phosphate buffer pH 7.0 (0.1 M) was prepared. After obtaining this solution, 50 μ L of the solution was incubated with 50 μ L of sample, to be tested at different concentrations (25, 50, 100, 200, 400, 800 μ g/mL) for 1 hr at room temperature. After the incubation, 100 μ L of Griess reagent (1% w/v sulphanilamide, 0.1% w/v naphthylethylenediamine, 2.5% v/v orthophosphoric acid) were added, and the reaction mixture was read at 540 nm in a microplate reader, iMark®, BioRad (Washington, USA).

Statistical analysis

Statistical analysis was performed using two-way ANOVA. Differences were considered to be significant when $p < 0.05$. Tukey's test was used as the *post hoc* test. EC_{50} was calculated using non-linear regression. Pearson's correlation was used to investigate the association between TPC and TFC. The statistical analysis was performed using GraphPad Prism 6.0.

Results and Discussion

Eradication of *H. pylori* from infected individuals is a strategy to treat gastrointestinal diseases such as ulcers and cancer, thus reducing disease complications [24]. These gastrointestinal disorders are intimately related with excessive production of oxygen or nitrogen reactive species [6]. According to Ding *et al.* (2007) [25], *H. pylori* infection also increases the production of ROS in infected gastric epithelial cells, resulting in apoptosis. Thus, compounds that are able to minimise the damage caused by oxidative stress and have anti-*H. pylori* activity are crucial for treating infections with this bacterium. In the search for such compounds, *B. trimera* HE, ACF, AqF, and HxF were tested in the present study.

The yield of HE extract obtained by maceration was 10.83%. Following fractionation, using 15 g of HE, the total yield was 80.6%, corresponding to 59.4% AqF, 19.4% ACF, and 1.8% HxF. The preliminary phytochemical characterisation of the *B. trimera* extract and fractions indicated the presence of polyphenols, coumarins, and terpenes/steroids in HxF and the presence of flavonoids, polyphenols, tannins, coumarins, and terpenes in HE, AqF, and ACF. The results of polyphenol, tannin and flavonoid content are presented in Table 1. The phytochemical screening of HxF did not detect the presence of flavonoids.

Table 1: Total flavonoid and polyphenol content of *B. trimera* HE and fractions (AqF, ACF, and HxF).

Secondary metabolites	Extract	Fractions		
	HE	AqF	ACF	HxF
Flavonoids (mg/RE)*	87.4 \pm 2	56.4 \pm 5.9	95.5 \pm 3.8	-
Polyphenols (mg/GAE)**	177.3 \pm 11.5	162.2 \pm 15.3	138.1 \pm 0.2	16.15 \pm 0.67
Tannins (mg/GAE)**	80.6 \pm 3.3	102.4 \pm 0.2	45.2 \pm 1.7	-

HE: Hydroalcoholic Extract; AqF: Aqueous Fraction; HxF: Hexanic Fraction; ACF: Acetonitrile/Chloroform Fraction

*mg GAE: milligrams of gallic acid equivalent **mg RE: milligrams of rutin equivalent milligrams

The anti-*H. pylori* activity of *B. trimera* HE and fractions is depicted in Figure 1. The MIC values were 512 µg/mL for HE, 1024 µg/mL for ACF, and over 1024 µg/mL for HxF and AqF. The data relative to the minimum bactericidal concentration (MBC) show that ACF had bactericidal capacity against *H. pylori* at 1024 µg/mL. The sub-MIC of HE was 256 µg/mL, and the sub-MIC of ACF was 512 µg/mL. AqF and HxF did not achieve MIC₉₀. The urease inhibition assay was performed to evaluate various mechanisms of anti-*H. pylori* activity. No samples were able to achieve 50% inhibition of activity at the tested concentrations. ACF achieved 36.24±5.59% inhibition at its highest concentration (1024 µg/mL). At their highest concentrations, HE and AqF achieved 26.51±2.30% and 25.76±1.09% inhibition, respectively.

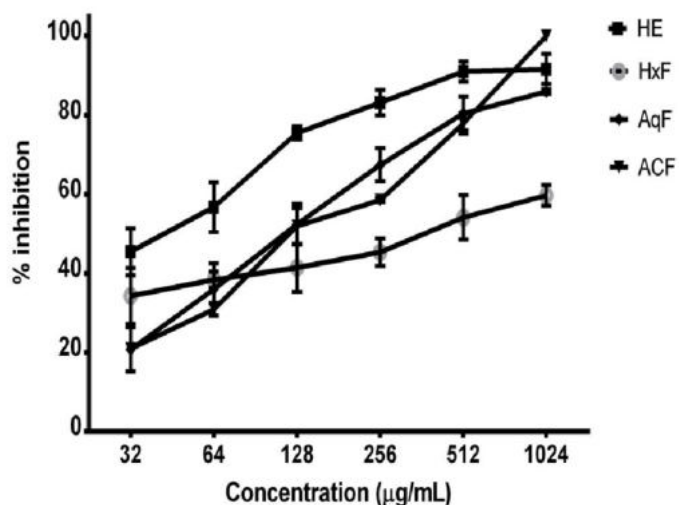


Figure 1: *H. pylori* growth inhibition by *B. trimera* HE and fractions AqF (Aqueous Fraction) and ACF (Acetonitrile/Chloroform Fraction).

Based on this result, scanning electron microscopy was performed to identify morphological alterations in the bacterial cells, as presented in Figure 2. The Figures 2A and 2B correspond to the negative control, without treatment, showing the most common form found in microaerophilic cultures, i.e., normal bacillus with intact outer membrane without changes, and Figures 2C and 2D represent bacteria treated with HE at 256 µg/mL (sub-MIC) and 512 µg/mL (MIC), respectively. Inspection under SEM revealed structural abnormalities compared with the control, mainly consisting of bubble formation in the bacterial wall. The bacteria treated with ACF at 512 µg/mL and 1024 µg/mL are presented in Figures 2E and 2F, respectively, displaying more severe morphological abnormalities, including cell lysis and narrowing of the bacteria, very different characteristics when compared to the

control. Another important finding was the presence of filamentous cells in ACF 1024 µg/mL, which is not a common bacterial form, as shown in Figure 2G.

According to Curtis *et al.*, (1979) [26], cefuroxime was able to block cell division by binding to penicillin-binding protein 3 (PBP3), resulting in filamentation of the bacterial cells. Similar studies were conducted by Deloney & Schiller (1999) [27] using aztreonam, and they also detected filamentous bacteria. Although some tests have not been made exclusively with *H. Pylori*, we also observed filamentation after exposure to ACF at 1024 µg/mL, suggesting a probable mechanism of action and the need for further studies to elucidate the effects of the tested sample. The results obtained in the present study showed that all the tested samples induced more than 50% inhibition of bacterial growth, however, only HE and ACF attained MIC₉₀. The presence of terpenes and the absence of flavonoids and tannins account for the lower capacity of HxF to inhibit bacterial growth compared with the other samples. The slightly lower amount of flavonoids in AqF might explain the inability to attain MIC₉₀, whereas the higher flavonoid concentration in ACF, in addition to the presence of terpenes and tannins, may reflect its bactericidal capacity. According to Dzoyem *et al.*, (2013) [28], flavonoids can damage the bacterial membrane, causing cell death. By contrast, the study by Jasmine *et al.*, (2011) [29] showed that *Elephantopus scaber* extract has antibacterial activity mediated by membrane destruction, which is attributed to the presence of terpenes. Tannins are also described as having antimicrobial activity, with the suggested mechanisms of action related to enzymatic inhibition by complexation, inhibition of oxidative phosphorylation, and deprivation of substrates essential for microbial survival [30].

In antioxidant assays, only the HE, ACF and AqF samples were tested because HxF did not exhibit activity in the preliminary antioxidant screening against synthetic radicals such as DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)], with 3.62% and 5.40% inhibition, respectively (data not presented). The results of EC₅₀ regarding the analysed samples are shown in Table 2.

HE and ACF achieved more than 50% inhibition of HOCl with an EC₅₀ of 17.58 ± 2.79 µg/mL and 15.50 ± 0.80 µg/mL, respectively. AqF failed to induce 50% inhibition, with its highest concentration attaining only approximately 40% inhibition. HE and ACF achieved 53% and 75% inhibition, respectively, at their highest concentrations. The antioxidant standard achieved 56% inhibition at 100 µg/mL, with an EC₅₀ of 16.02 ± 1.78 µg/mL. The statistical analysis showed the superior performance of some of the tested samples at some concentrations compared with TROLOX (Figure 3A) and a strong negative correlation between EC₅₀ and TFC ($r = -0.9824$), which demonstrates the

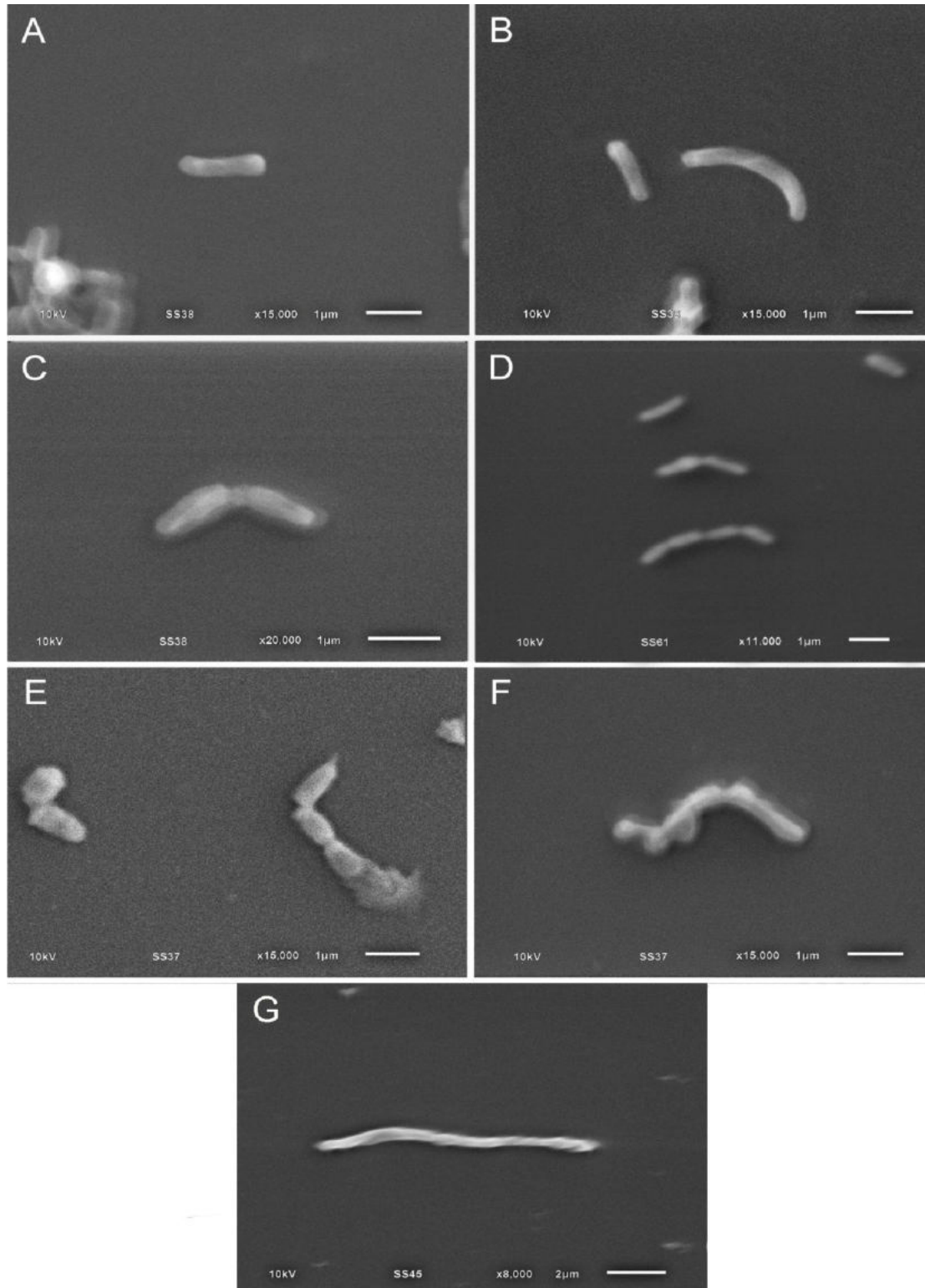


Figure 2: Scan microscopy electronic (SEM) of *H. pylori* in the absence of samples (Figures A and B) and after exposure to *B. trimera* Hydroalcoholic extract (HE) (Figures C and D) and the Acetonitrile/Chloroform fraction (ACF) (Figures E, F and G).



relevance of flavonoids. Therefore, the lower percentage of flavonoids seems to account for the poorer performance of AqF in this assay.

The results of the $O_2^{\bullet-}$ inhibition assay are presented in Figure 1B and Table 2. HE, AqF, and ACF achieved more than 50% inhibition, with EC_{50} values of $9.52 \pm 1.48 \mu\text{g/mL}$, $5.85 \pm 0.86 \mu\text{g/mL}$, and $52 \pm 4.73 \mu\text{g/mL}$, respectively. The antioxidant

standard, TROLOX achieved more than 50% inhibition at $100 \mu\text{g/mL}$, with an EC_{50} of $88.81 \pm 2.92 \mu\text{g/mL}$, and the statistical analysis demonstrated that the performance of the tested samples was superior. TT/TPC and EC_{50} exhibited a strong negative correlation in the $O_2^{\bullet-}$ scavenging assay ($r=-0.9473/-0.8917$), which indicates that tannins and others phenolic compounds were also important in the findings.

Table 2: EC_{50} results \pm standard deviation ($\mu\text{g/mL}$) for assays of antioxidant activity from hydroalcoholic extract (EH) of *B. trimera* and its fractions, aqueous (AqF) and acetonitrile / chloroform (ACF). The antioxidant Trolox was used as control.

Samples	EC_{50} of Antioxidant Assays				
	HOCl	$O_2^{\bullet-}$	H_2O_2	HO^{\bullet}	NO^{\bullet}
HE	17.58 ± 2.79	9.52 ± 1.48	>100	4.27 ± 1.01	167.5 ± 3.8
AqF	>100	5.85 ± 0.86	>100	4.06 ± 0.90	183.5 ± 8.65
ACF	15.50 ± 0.80	52.00 ± 4.73	66.70 ± 2.30	2.90 ± 0.48	132.13 ± 7.8
TROLOX	16.02 ± 1.78	88.81 ± 2.92	26.88 ± 0.34	4.59 ± 0.45	39.22 ± 5.03

Figure 3C presents the results of the hydrogen peroxide assay. ACF at $100 \mu\text{g/mL}$ scavenged over 50% of H_2O_2 , with 57% inhibition and EC_{50} of $66.70 \pm 2.30 \mu\text{g/mL}$. HE and AqF achieved 44% and 31% inhibition at $100 \mu\text{g/mL}$, respectively. The antioxidant standard exhibited an EC_{50} of $26.88 \pm 0.4 \mu\text{g/mL}$. The EC_{50} of the analysed samples are shown in Table 2. A strong negative correlation was found between TFC and the EC_{50} values ($r=-0.9458$). Notably, the fraction with the highest flavonoid concentration was the only fraction that achieved more than 50% inhibition of hydrogen peroxide, which suggests that the antioxidant activity of flavonoids is highly relevant, as reported by other studies in the literature [31].

In the HO^{\bullet} radical inhibition assay, all of the tested samples achieved more than 50% inhibition starting at $6.25 \mu\text{g/mL}$, similar to the control (Figure 3D). TROLOX exhibited an EC_{50} of $4.59 \pm 0.45 \mu\text{g/mL}$, and HE, AqF, and ACF exhibited EC_{50} values of $4.27 \pm 1.01 \mu\text{g/mL}$, $4.06 \pm 0.90 \mu\text{g/mL}$, and $2.90 \pm 0.48 \mu\text{g/mL}$, respectively (Table 2). A significant correlation was not found between TPC/TFC and the EC_{50} values obtained in the HO^{\bullet} scavenging test. All the samples tested in the assay contained tannins, and some studies have shown that the presence of tannins

is crucial for the antioxidant role [30]. In the study performed by Gülçin *et al.*, (2010) [32], tannic acid, a hydrolysable tannin, exhibited strong metal chelating activity and formed complexes, such as with Fe^{2+} , which plays an important role in HO^{\bullet} radical generation. Similar studies to ours, such as Vieira *et al.*, (2011) [33], achieved low EC_{50} values for ethanol extracts of *Baccharis sp* against the radical HO^{\bullet} . Among these plants, we highlight *B. trimera* with its EC_{50} of $15 \pm 3 \mu\text{g/mL}$, and *B. articulata*, *B. spicata*, *B. usterii*, which obtained an EC_{50} of 17 ± 1 , 18 ± 2 and $12 \pm 1 \mu\text{g/mL}$, respectively.

In the NO^{\bullet} inhibition, the HE, AqF and ACF at concentrations of $200 \mu\text{g/mL}$ exhibited percentages of inhibition higher than 50%. TROLOX inhibited over 50% inhibition at a concentration of $50 \mu\text{g/mL}$. The HE, ACF and AqF showed EC_{50} of 167.5 ± 3.8 , 132.13 ± 7.38 , and $183.5 \pm 8.65 \mu\text{g/mL}$, respectively (Table 2). The ACF had the highest percentage of inhibition at higher concentrations, reaching 91.19% inhibition at $800 \mu\text{g/mL}$. TROLOX in the same concentration reached 72.47% inhibition, and the HE and AqF reached 79.95 and 82.34% inhibition, respectively (Figure 4). A strong negative

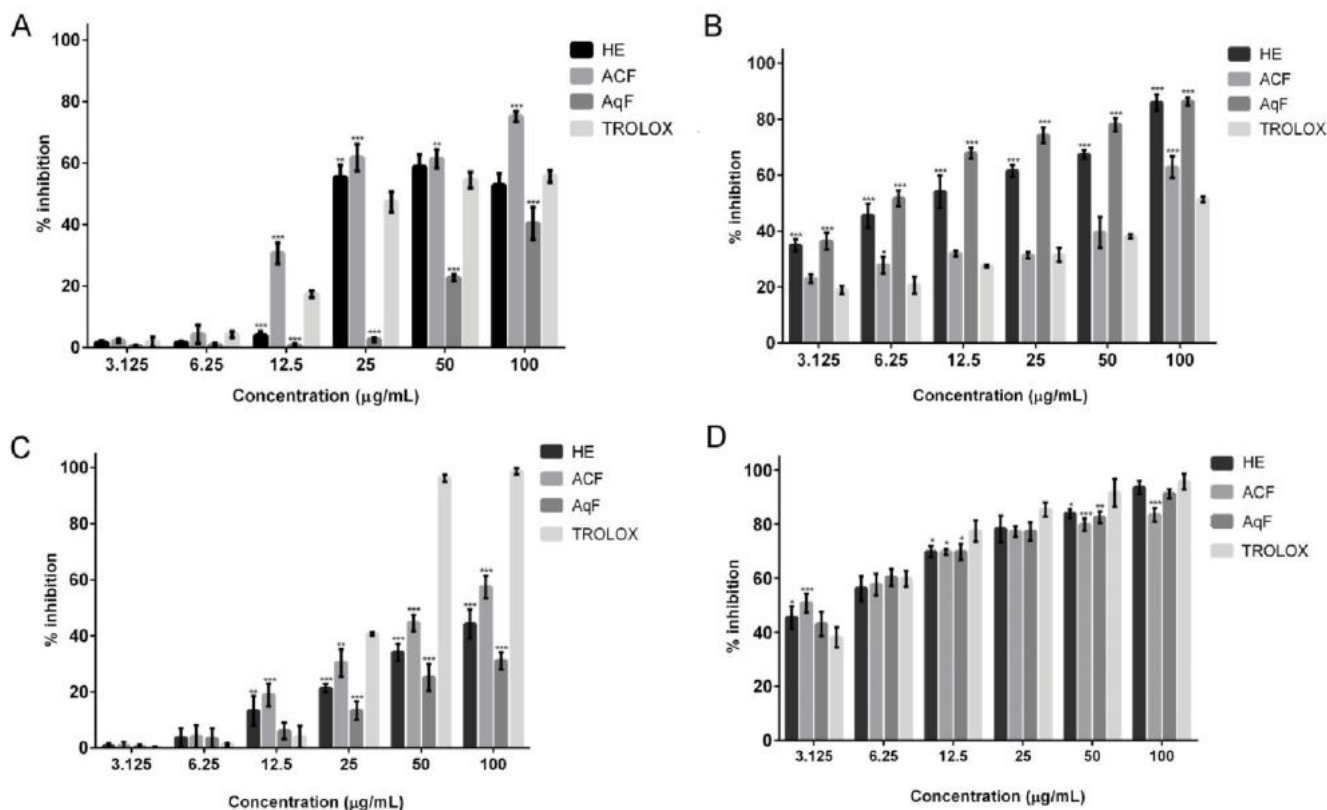


Figure 3: Percent inhibition induced by *B. trimera* hydroalcoholic extract (HE) and fractions AqF (Aqueous Fraction) and ACF (Acetonitrile/ Chloroform Fraction) in the HOCl scavenging test (1A), in the O₂^{•-} scavenging test (1B), in the H₂O₂ scavenging test (1C) and in the HO[•] scavenging test (1D). TROLOX was used as the antioxidant control. * Significant difference compared with TROLOX, $p < 0.01$; *** Significant difference compared with TROLOX, $p < 0.001$.

correlation between EC₅₀ and TFC was observed ($r = -0.8301$). These results attest to the important antioxidant activity of flavonoids present in this fraction. Khan *et al.*, (2009) [34] identified

that quercetin was able to protect the protein from potential damage arising from the production of NO[•], which reinforces the likely antioxidant role of the flavonoids.

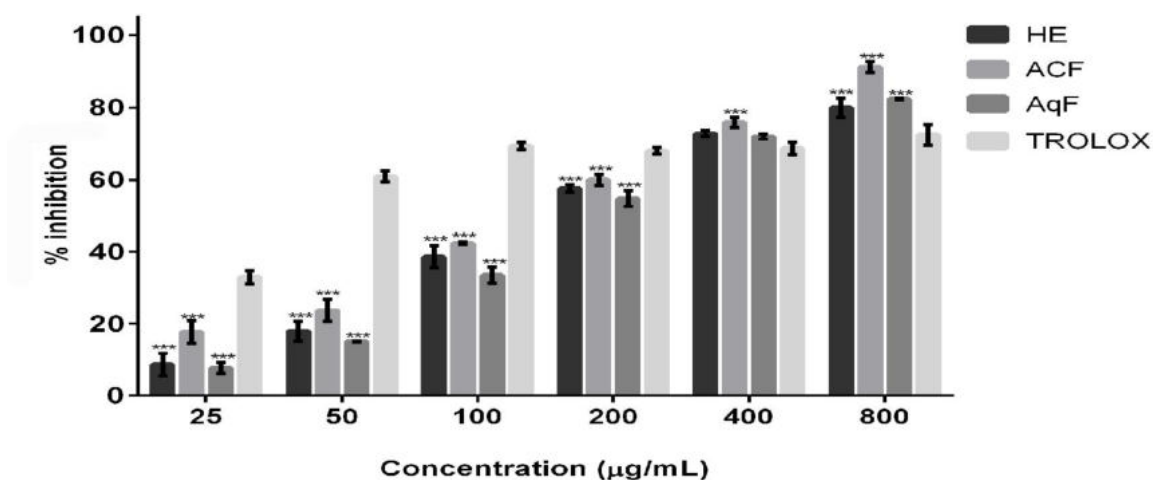


Figure 4: Percent inhibition induced by *B. trimera* hydroalcoholic extract (HE) and fractions AqF (Aqueous Fraction) and ACF (Acetonitrile/Chloroform Fraction) in the NO[•] scavenging test. TROLOX was used as the antioxidant control. * Significant difference compared with TROLOX, $p < 0.01$; ** Significant difference compared with TROLOX, $p < 0.01$; *** Significant difference compared with TROLOX, $p < 0.001$.

Phenolic compounds such as flavonoids and tannins have important antioxidant activity due to the presence of hydroxyls. Phenols may inhibit oxidant activity through several mechanisms, including direct scavenging of reactive species, enzymatic inhibition, and metal chelation, among others [35]. The hydrogen-donating ability, which might account for their antioxidant activity, appears intimately related to the number and position of free hydroxyls in their structure [30].

Conclusion

B. trimera extract and fractions exhibited considerable antioxidant activity and significant anti-*H. pylori* activity, particularly ACF, because of its bactericidal capacity. The *in vitro* results indicate that HE and the ACF and AqF fractions contain natural compounds with antioxidant and anti-*H. pylori* properties, which means that they are candidates for *in vivo* testing as well as targets of isolation studies. In the present study, the antioxidant capacity was attributed to quantitative and qualitative differences in the phenolic compounds (especially flavonoids) that were present in the extract and fractions. The anti-*H. pylori* activity may be attributed to flavonoids, terpenes, and tannins. Nevertheless, further studies are necessary to identify the possible compounds responsible for such activities and to elucidate the possible mechanisms of their anti-*H. pylori* activity.

References

- [1]. Vitor JMB, Vale FF. Alternative therapies for *Helicobacter pylori*: probiotics and phytomedicine. *FEMS Immunol Med Microbiol*. 2011;63:153-164.
- [2]. Sugano K, Tack J, Kuipers EJ, Graham DY, El-Omar EM, Miura S, Hamura K, Asaka M, Uemura N, Malfertheiner P. Kyoto global consensus report on *Helicobacter pylori* gastritis. *Gut*. 2015;0:1-15.
- [3]. Polk DB, Peek RM. *Helicobacter pylori*: gastric cancer and beyond. *Nat Rev Cancer*. 2010;10:403-414.
- [4]. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet*. 1984;1:1311-1314.
- [5]. Ladeira MSP, Salvadori DMF, Rodrigues MAM. Biopathology of *Helicobacter pylori*. *J Bras Patol Med Lab*. 2003;39:335-342.
- [6]. Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiol Rev*. 2014;94:329-354.
- [7]. Verdi LG, Brighente IMC, Pizzolatti MG. Gênero *Baccharis* (Asteraceae): Aspectos químicos, econômicos e biológicos. *Quim Nova*. 2005;28:85-94.
- [8]. Abad MJ, Bermejo P. *Baccharis* (Compositae): a review update. *ARKIVOC*. 2007;7:76-96.
- [9]. Campos FR, Bressan J, Jasinski VCG, Zuccolotto T, Silva LE da, Cerqueira LB. *Baccharis* (Asteraceae): Chemical Constituents and Biological Activities. *Chem Biodivers*. 2016;13:1-17.
- [10]. Simões-Pires CA, Queiroz EF, Henriques AT, Hostettmann K. Isolation and on-line identification of anti-oxidant compounds from three *Baccharis* species by HPLC-UV-MS / MS with post-column derivatisation. *Phytochem Anal*. 2005;16:307-314.
- [11]. Oliveira CB, Comunello LN, Lunardelli A, Amaral RH, Pires MGS, da Silva GL, Manfredini V, Vargas CR, Gnoatto SCB, Oliveira JR, Gosmann G. Phenolic enriched extract of *Baccharis trimera* presents anti-inflammatory and antioxidant activities. *Molecules*. 2012;17:1113-1123.
- [12]. BRASIL. RENISUS - Relação nacional de plantas medicinais de interesse ao SUS. Ministério da Saúde. 2009. Disponível em: <http://portalsaude.saude.gov.br/images/pdf/2014/maio/07/renisus.pdf>. Acesso em: 12 fev. 2016.
- [13]. BRASIL. Agência Nacional de Vigilância Sanitária. Resolução de Diretoria Colegiada (RDC) nº 26 de 13.05.2014. Dispõe sobre o registro de medicamentos fitoterápicos e o registro

Authors' contributions

Rita de Cassia Ribeiro Goncalves, as project leader, drafted the manuscript together with Otalibio Castiglioni Nunes. Rita de Cassia Ribeiro Gonçalves and Rodrigo Rezende Kitagawa guided the realisation of anti *H. pylori* trials and antioxidant assays, analysed and interpreted data and drafted the manuscript. Claudia Masrouah Jamal guided the extraction and preparation of the fractions. Otalibio Castiglioni Nunes performed all the techniques with the guidance of other researchers who drafted the article. All the co-authors reviewed and discussed the results of the research.

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Conflicts of interests

The authors declare no conflicts of interests.

- e a notificação de produtos tradicionais fitoterápicos. Ministério da Saúde. 2014.
- [14]. Neves LC, Alencar SM de, Carpes ST. Determinação da atividade antioxidante e do teor de compostos fenólicos e flavonoides totais em amostras de pólen apícola de *Apis mellifera*. Braz J Food Technol. 2009;107-110.
- [15]. Dimech GS, Soares LAL, Ferreira MA, Oliveira AGV de, Carvalho M da C, 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;2013:1-8.
- [16]. Perdigão TL: Avaliação morfofisiológica, fitoquímica e mutagênica de *Passiflora edulis* Sims f. *flavicarpa* Derg exposta a diferentes concentrações de alumínio. Dissertação de Mestrado. Universidade Federal do Espírito Santo, Centro de Ciências Humanas e Naturais; 2012.
- [17]. Clinical and Laboratory Standards Institute (CLSI): Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. CLSI document M07- A6. 2003;23:1-49.
- [18]. Tanaka T, Kawase M, Tani S. Urease inhibitory activity of simple β -unsaturated ketones. Life Sci. 2003;73:2985-2990.
- [19]. Ching T-L, Jong J, Bast A. A method for screening hypochlorous acid scavengers by inhibition of the oxidation of 5-Thio-2-Nitrobenzoic Acid: Application to anti-asthmatic drugs. Anal Biochem. 1994;218:377-381.
- [20]. Suzumura K, Yasuhara M, Narita H. Superoxide anion scavenging properties of fluvastatin and its metabolites. Chem Pharm Bull. 1999;47:1477-1480.
- [21]. [Pick E, Keisari Y. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J Immunol Methods. 1980;38:161-170.
- [22]. Halliwell B, Gutteridge JMC, Aruoma OI. The deoxyribose method: A simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. Anal Biochem. 1987;165:215-219.
- [23]. Marcocci L, Maguire JJ, Droy-Lefaix MT, Packer L. The nitric oxide-scavenging properties of *ginkgo biloba* extract EGb 761. Biochem Biophys Res Commun. 1994;201:748-755.
- [24]. Barkun A, Leontiadis G. Systematic review of the symptom burden, quality of life impairment and costs associated with peptic ulcer disease. Am J Med. 2010;123:358-366.
- [25]. Ding S-Z, Minohara Y, Fan XJ, Wang J, Reyes VE, Patel J, Dirden-Kramer B, Boldogh I, Ernst PB, Crowe SE. *Helicobacter pylori* infection induces oxidative stress and programmed cell death in human gastric epithelial cells. Infect Immun. 2007;75:4030-4039.
- [26]. Curtis NAC, Orr D, Ross GW, Boulton MG. Competition of β -Lactam antibiotics for the penicillin-binding proteins of *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella aerogenes*, *Proteus rettgeri*, and *Escherichia coli*. Comparasion with antibacterial activity and effects upon bacterial morphology. Antimicrob Agents Chemother. 1979;16:325-328.
- [27]. DeLoney CR, Schiller NL. Competition of various beta-lactam antibiotics for the major penicillin-binding proteins of *Helicobacter pylori*. antibacterial activity and effects on bacterial morphology. Antimicrob Agents Chemother. 1999;43:2702-2709.
- [28]. Dzoyem JP, Hamamoto H, Ngameni B, Ngadjui BT, Sekimizu K. Antimicrobial action mechanism of flavonoids from *Dorstenia* species. Drug Discov Ther. 2013;7:66-72.
- [29]. Jasmine R, Selvakumar BN, Daisy P. Investigating the mechanism of action of terpenoids and the effect of interfering substances on an indian medicinal plant extract demonstrating antibacterial activity. Int J Pharm Stud Res. 2011;2:19-24.
- [30]. Shahidi F, Ambigaipalan P. Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects - A review. J Funct Foods. 2015;18:820-897.
- [31]. Wang Y, Wang X. Binding, stability, and antioxidant activity of quercetin with soy protein isolate particles. Food Chem. 2015;188:24-29.
- [32]. Gülçin , Huyut Z, Elmasta M, Aboul-Enein HY. Radical scavenging and antioxidant activity of tannic acid. Arab J Chem. 2010;3:43-53.
- [33]. Vieira TO, Seifriz I, Charão CCT, Oliveira SQ, Creczynski-Pasa TB. Antioxidant effects of crude extracts from *Baccharis* species: inhibition of myeloperoxidase activity, protection against lipid peroxidation, and action as oxidative species scavenger. Rev Bras Farmacogn. 2011;21:601-607.
- [34]. Khan MS, Priyadarshini M, Bano B. Preventive effect of curcumin and quercetin against nitric oxide mediated modification of goat lung cystatin. J Agric Food Chem. 2009;57:6055-6059.
- [35]. Bendary E, Francis RR, Ali HMG, Sarwat MI, El Hady S. Antioxidant and structure-activity relationships (SARs) of some phenolic and anilines compounds. Ann Agric Sci. 2013;58:173-181.