Chemical, biological, and pharmacological evaluation of the aqueous extract of *Ilex paraguariensis*, St. Hill. (Aquifoliaceae)

Avaliação química, biológica e farmacológica do extrato aquoso de *Ilex paraguariensis*, St. Hill. (Aquifoliaceae)

Evaluación química, biológica y farmacológica del extracto acuoso de *Ilex paraguariensis*, St. Hill. (Aquifoliaceae)

Andressa Flores Santos
ORCID: https://orcid.org/0000-0001-6496-4333
Federal University of Paraná, Brazil
E-mail: andressaflores@ufpr.br
Elberth Manfron Schiefer
ORCID: https://orcid.org/0000-0002-9270-6881
Federal University of Technology, Brazil
E-mail: elberthschiefer@alunos.utfpr.edu.br
Monica Surek
ORCID: https://orcid.org/0000-0003-4048-3046
Federal University of Paraná, Brazil
E-mail: monicasurek@ufpr.br
Cleverson Antônio Ferreira Martins
ORCID: https://orcid.org/0000-0001-5211-2061
Tuiuti University of Paraná, Brazil
E-mail: cleverson.martins@utp.br
Paulo Roberto Worfel
ORCID: https://orcid.org/0000-0002-1493-0992
Federal University of Paraná, Brazil
E-mail: p.worfel@hotmail.com
Guilherme Lanzi Sassaki
ORCID: https://orcid.org/0000-0001-7420-168X
Federal University of Paraná, Brazil
E-mail: sassaki@ufpr.br
Lauro Mera De Souza
ORCID: https://orcid.org/0000-0001-9166-2146
Pelé Pequeno Principe Research Institute, Brazil
E-mail: lauro.bioq@gmail.com
Luciana Cristina Nowacki
ORCID: https://orcid.org/0000-0001-7291-4854
Tuiuti University of Paraná, Brazil
E-mail: lunowacki@hotmail.com
Wesley Mauricio de Souza
ORCID: https://orcid.org/0000-0001-7918-7936
Federal University of Paraná, Brazil
E-mail: wesleysouza@ufpr.br

Abstract
This study aimed to quantify the total phenolic and flavonoid contents in the aqueous extract of *Ilex paraguariensis* (EIP) and investigate its antioxidant, antimicrobial, antiulcerogenic, and antidepressant properties with concomitant verification of its effects on relevant biochemical parameters using *in vivo* models. EIP has in its composition 236.28 $\pm$ 11.83 mg GAE/g of total phenolics and 44.07 $\pm$ 5.56 mg QE/g of total flavonoids, corroborating its antioxidant activity. Antimicrobial assays against gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and gram-negative bacteria (*Escherichia coli* e *Pseudomonas aeruginosa*) showed a promising activity of EIP. The EIP administered at 500 and 1000 mg/kg doses prevented the development of gastric ulcers induced in rats following immobilization at 4 °C, however, in the ethanol-induced ulcers no significant effects were observed up to a dose of 1000 mg/kg. Gastric secretion and total acidity index in pylorus-ligated rats were reduced after treatment with EIP, and the pH did not change significantly compared to the control in the tested model. Administration of EIP to mice (250, 500, and 1000 mg/kg) significantly altered the barbiturate-induced sleep time and results of the tail suspension and forced swim test. Repeated doses of EIP did not significantly alter the evaluated biochemical markers (blood

glucose, urea, creatinine, triglycerides, and total cholesterol). The results indicate that EIP may relieve gastrointestinal disorders by reducing acid secretion and decreasing immobility time in mice, suggesting an antidepressant effect. Notably, administration of multiple doses of EIP was considered preliminarily safe.
Keywords: Yerba mate; Gastroprotection; Phenolics; Biochemical markers; Antimicrobial; Antioxidants.

Resumo

Este estudo teve como objetivo quantificar os teores fenólicos e flavonóides totais no extrato aquoso de *Ilex paraguariensis* (EIP) e investigar suas propriedades antioxidantes, antimicrobianas, antiulcerogênicas e antidepressivas com verificação concomitante de seus efeitos em parâmetros bioquímicos relevantes por meio de modelos *in vivo*. O EIP possui em sua composição 236,28 ± 11,83 mg GAE/g de fenólicos totais e 44,07 ± 5,56 mg QE/g de flavonóides totais corroborando com sua atividade antioxidante. Ensaios antimicrobianos contra bactérias gram-positivas (*Staphylococcus aureus* e *Enterococcus faecalis*) e gram-negativas (*Escherichia coli* e *Pseudomonas aeruginosa*) mostraram uma promissora atividade do EIP. O EIP administrado em doses de 500 e 1000 mg/kg evitou o desenvolvimento de úlceras gástricas induzidas em ratos após imobilização a 4 °C, contudo, nas úlceras induzidas por etanol não foram observados efeitos significativos até dose de 1000 mg/kg. A secreção gástrica e o índice de acidez total em ratos com ligadura no piloro foram reduzidos após o tratamento com EIP e o pH não alterou significativamente em comparação com o controle no modelo testado. A administração de EIP em camundongos (250, 500 e 1000 mg/kg) alterou significativamente o tempo de sono induzido por barbitúricos e os resultados da suspensão da cauda e do teste de natação forçada. Doses repetidas de EIP não alteraram significativamente os marcadores bioquímicos avaliados (glicemia, uréia, creatinina, triglicerídeos e colesterol total). Os resultados indicam que EIP pode aliviar distúrbios gastrointestinais, reduzindo a secreção gástrica e diminuindo o tempo de imobilidade em camundongos, sugerindo um efeito antidepressivo. Notavelmente, a administração de doses múltiplas de EIP foi considerada preliminarmente segura.
Palavras-chave: Erva mate; Gastroproteção; Fenólicos; Marcadores bioquimicos; Atividade antimicrobiana; Antioxidantes.

Resumen

El presente trabajo tuvo como objetivo cuantificar los niveles de fenoles y flavonoides a partir del extracto acuoso de *Ilex paraguariensis* (EIP) e investigar sus propiedades antioxidantes, antimicrobianas, antiulcerogénicas y antidepressivas, pero también para analizar sus efectos bioquímicos a través de modelos *in vivo*. EIP es compuesto por 236,28 ± 11,83 mg GAE/g de fenoles totales y 44,07 ± 5,56 mg QE/g de flavonoides totales, lo que justifica su efecto antioxidante. Estos componentes presentan propriedades antimicrobianas contra las bacterias gram positivas (*Staphylococcus aureus* y *Enterococcus faecalis*) y gram negativas (*Escherichia coli* y *Pseudomonas aeruginosa*). La administración de EIP (500 y 1000 mg/kg) evitó el desarrollo de úlceras gástricas inducidas en los ratones después de la inmovilización a 4°C, mientras que, no se observaron efectos significativos hasta la dosis de 1000 mg/kg de etanol. La secreción gástrica y el índice de acidez total en los ratones con píloro ligado se redujeron considerablemente después del tratamiento con EIP y el pH no cambió en comparación con el modelo control. La administración de EIP en ratones (250, 500 y 1000 mg/kg) alteró significativamente el tiempo de sueño inducido por barbitúricos, y los resultados de la suspensión de la cola y la prueba de nado forzado. Además, las dosis repetidas de EIP no alterarón los marcadores bioquímicos, a saber, la glucemia, urea, creatinina, triglicéridos y colesterol total. Con los resultados de este trabajo indican que EIP puede aliviar las alteraciones gastrointestinales, reduciendo la secreción gástrica y disminuyendo el tiempo de inmovilidad en ratones, por esta razón puede ser un efecto antidepresivo. Del mismo modo, la administración de múltiples dosis de EIP se consideró segura.
Palabras clave: Yerba mate; Gastroprotección; Fenólicos; Marcadores bioquímicos; Antimicrobianos; Antioxidantes.

1. Introduction

Ilex paraguariensis, St. Hill. is a plant widely consumed in South American countries and particularly in Brazil (the states of Rio Grande do Sul, Santa Catarina, Paraná, and Mato Grosso do Sul). A commercialized product known as maté, yerba mate, or erva-mate from the processed leaves and branches of I. paraguariensis is used to prepare several types of beverages (de Souza et al., 2011; Heck & De Mejia, 2007).

Studies have shown that yerba mate contains many chemical compounds with nutritional, therapeutic, and pharmacological properties including phenolic compounds such as chlorogenic acid, caffeic acid, 5-caffeoylquinic acid, and Rutin (Martin et al., 2013; Bojic et al., 2013; Berté et al., 2011), methylxanthines (Reginatto et al., 1999), saponins (de Souza et al., 2011), and flavonoids (Ricco et al., 1991); amino acids, carbohydrates, vitamins, and carotenoids have also been identified (Villela, 1938; Cascon, 1995).

Ilex paraguariensis has been reported to have medicinal properties, acting as diuretics (Gonzalez et al., 1993), antirheumatic (Gosmann & Schenkel, 1989), anti-inflammatory (Schinella et al., 2000), chemopreventive (Ramirez-Mares et al., 2004), antioxidant (Gugliucci, 1996), and antimicrobials (Pressi et al., 2021). Furthermore, it is indicated as a central nervous system stimulant (Gonzalez et al., 1993), anti-depression (Lim et al., 2015), analgesic (Nowacki et al., 2021), choleretic agents and used for gastrointestinal disorders (Gorzalczany et al., 2001).

A peptic ulcer is a gastrointestinal disease caused by an imbalance between the aggressive and cytoprotective factors of the gastric mucosa (Borrelli & Izzo, 2000), its development is associated with *Helicobacter pylori* infection, the extensive use of nonsteroidal anti-inflammatory drugs, and the consumption of cigarettes and alcoholic beverages (Brunton, 1996). Worldwide, this disorder is very common, and the costs of treating this pathology and its complications reach billions of dollars (Birdane et al., 2007). Gastric protective agents are one of the most commonly administered drugs (Ferreira & Barata, 1998) and act by inhibiting the proton pump or blocking histamine receptors (Mulholland & Debas, 1987). However, these drugs can cause serious adverse reactions such as hypersensitivity, liver and gastrointestinal disorders, arrhythmia, and impotence (Chan & Leung, 2002).

Another important disease, the depression is a psychiatric illness with a high prevalence in humans, associated with 21% of the worldwide population (Schechter et al., 2005). It is well known that the pathophysiology of depression involves the dysfunction of monoamine neurotransmitter circuits in the central nervous system (Nemeroff & Owens, 2002). Previous studies have demonstrated that an infusion of *I. paraguariensis* can improve memory in rats treated with haloperidol and this effect was related to an indirect modulation of oxidative stress (Colpo et al., 2007).

Oxidative stress occurs when there is an excess of free radicals in the intrinsic and physiological protective system of each cell, contributing to a constant production of free radicals from aerobic metabolism (Valduga et al., 2016). Antioxidants inhibit or quench free radical reactions and delay or inhibit cellular damage. Although antioxidant defenses differ from species to species, the presence of antioxidants is universal. Antioxidants exist in both enzymatic and non-enzymatic forms in the intracellular and extracellular environments (Nimse & Pal, 2015).

Due to the wide variety of chemical compounds present in *Ilex paraguariensis* and its various medicinal applications, there has been an interest in studying its protective effects on the gastrointestinal tract and the depression related mainly to the generation of free radicals (Borrelli & Izzo, 2000). When the excessive generation of free radicals, highly toxic and reactive substances to healthy cells overcomes the body's natural antioxidant capacity, the body becomes more susceptible to developing pathologies such as diabetes, depression, cardiovascular events, autoimmune and degenerative diseases, between several others.

The present study aimed to quantify the total phenolic and flavonoid contents, investigate the antiulcerogenic and antidepressant properties of *Ilex paraguariensis* extract (EIP), determine its antioxidant capacity and antimicrobial properties, and evaluate the biochemical markers using in vivo models by the repetitive use of doses of yerba mate.

2. Methodology

2.1 Chemicals

The following drugs were used in this study: ranitidine hydrochloride, imipramine hydrochloride, caffeine (all from Teuto, Brazil), and sodium thiopental (Cristália, Brazil). Sulfuric acid (98%), Folin-Ciocalteu's phenol reagent (2 N), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid (97.5% purity), ascorbic acid (99%), and quercetin (99%) were purchased from Sigma-Aldrich (St, Louis, MO, USA). Sodium carbonate and aluminum chloride hexahydrate were obtained from Synth (Diadema, SP, Brazil) and ammonium molybdate tetrahydrate from J. T. Baker (Radnor Township, Pennsylvania, USA). Milli-Q water was obtained from Millipore (Bedford, MA, USA). Other solvents were purchased from Merck (Kenilworth, New

Jersey, USA).

2.2 Plant material, extract preparation, and chemical analysis

Leaves, petioles, and branches of *I. paraguariensis* (Aquifoliaceae, 3 kg) were donated by the food industry Leão Junior®, Curitiba, PR, Brazil in February 2007. A voucher specimen (136.A) was deposited at the Herbarium Prof. Carlos Stellfeld of the Pharmacognosy Laboratory of the Federal University of Paraná (Curitiba, PR, Brazil). The extract of *Ilex paraguariensis* was prepared as described by Nowacki et al. (2021). Briefly, 50 g of ground yerba mate was refluxed in a Soxhlet extractor with 160 mL of distilled water for 6 h. The extract was then concentrated using a rotary evaporator at 60 °C, frozen, and freeze-dried to obtain a 33% yield (m/v) of the aqueous extract of *I. paraguariensis* (EIP). The doses used in this study were chosen according to previous toxicity studies (Nowacki et al., 2021). The project was registered in the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGEN) under protocol No. A6878C0. Chromatographic and spectrometric analyses using UPLC-PDA-HRMS along with chemical identification of the EIP have been previously published (Nowacki et al., 2021).

2.2.1 Determination of total phenolic content

Total phenolic content was determined by the Folin-Ciocalteu method with modifications (Woisky and Salatino, 2009). Using a 96-well microplate, 100 µL of Folin-Ciocalteu reagent (10% v/v), 20 µL of EIP (500 µg/mL) or gallic acid dilution in ethanol (500, 400, 300, 200, 100, 75, 50, 25, 10, and 5 µg/mL), and 80 µL of sodium carbonate solution (7.5% m/v) were added. The plate was left to rest in the dark for 60 min, and the absorbance was measured on a Multiskan FC microplate reader (Thermo Scientific, USA) at 690 nm. Concentrations of total phenolics were determined from the standard curve of gallic acid ($y = 0.0043x + 0.0337$; $R^2 = 0.99$) and expressed in mg GAE/g of extract (GAE, gallic acid equivalents). The assay was performed in triplicate.

2.2.2 Determination of total flavonoid content

Total flavonoid content was determined according to Devequi-Nunes et al. (2018) using dilutions of quercetin in ethanol (250, 125, 93.75, 62.5, 46.88, 31.25, 23.44, 15.62, and 7.81 µg/mL) to create a standard curve. Using a 96-well microplate, 100 µL of EIP (500 µg/mL) or quercetin dilution and 100 µL of aluminum chloride in methanol (2.0% m/v) were added. The plate was left to rest in the dark for 60 min and the absorbance was measured using a Multiskan FC microplate reader (Thermo Scientific, USA) at 414 nm. The concentration of total flavonoids was determined using the standard curve of quercetin ($y = 0.065x + 0.0447$, $R^2 = 0.99$) and expressed in mg QE/g of extract (QE, quercetin equivalents). The assay was performed in triplicate.

2.3 Antioxidant capacity

2.3.1 DPPH assay

The antioxidant capacity of the EIP was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to the methodology described by Mensor et al. (2001). Six EIP concentrations (500, 250, 125, 62.5, 31.25, and 15.62 µg/mL) were evaluated in triplicate. Each dilution (2.5 mL) was transferred to a tube containing 1.0 mL of ethanolic solution of DPPH (0.3 mM). After 30 min of incubation in the dark at room temperature, the reduction of the free radical DPPH was measured by reading the absorbance using a spectrophotometer WUV-M51, Weblabor (Mogi das Cruzes, Brazil) at 517 nm. Ethanol and EIP were used as blanks, and DPPH solution with ethanol was used as a control. Equation 1 was used to calculate the capacity to sequestrate radicals, expressed as a percentage of the radical oxidation inhibition. Ascorbic acid (40, 20, 10, and 5 µg/mL)

was used as the standard. The IC_{50} value was calculated using the equation (y = -0.7433x + 85,014; R^2 = 0.9829) based on the concentrations and respective percentages of radical DPPH sequestration. Data are reported from three independent experiments performed in triplicate.

$$Eq.\ 1 - IC\% = 100 - \{[(Abs\ sample - Abs\ blank) \times 100]/Abs\ control\}$$

2.3.2 Phosphomolybdenum assay (total antioxidant capacity)

The total antioxidant capacity assay of EIP was carried out using the phosphomolybdenum method (Prieto et al., 1999). A 0.3 mL aliquot of the sample solution (200 µg/mL) was shaken with 3 mL of reagent solution (3 mol/L sulfuric acid, 0.1 M sodium phosphate, and 0.03 mol/L ammonium molybdate tetrahydrate). The test tubes were covered with plastic film and incubated in a water bath at 95 °C for 90 min. After the samples were cooled, the absorbance of the mixture was measured at 695 nm. Ascorbic acid was used as a standard. The antioxidant capacity was estimated using Equation 2:

$$Eq.\ 2 - AAR\% = (Abs\ sample - Abs\ blank)/\ Abs\ ascorbic\ acid - Abs\ blank) \times 100.$$

2.4 Antimicrobial assay

Antimicrobial activity was first assessed using the broth microdilution technique according to the Clinical and Laboratory Standards Institute (CLSI, 2012). The EIP was serially diluted (1000, 500, 250, 125, 62.5, 31.25, and 15.62 µg/mL) with Mueller Hinton II broth to a final volume of 100 µL. The bacterial inoculum was prepared in 0.85% saline with a turbidity equivalent to the McFarland 0.5 scale (~1.5 ×10^8 CFU/mL). This suspension was diluted 1:20 (~5 ×10^5 CFU/mL), and 10 µL was added to each well. The antimicrobial agent gentamicin (512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 µg/mL) was used as a positive control. As a negative control, the microorganisms were treated with 1% DMSO. The plates were incubated for 18 h at 35 ±1 °C. For subsequent determination of the MIC, the extract concentration that caused the total inhibition of bacterial growth when observed with the naked eye was expressed as microgram per milliliter (µg/mL). The minimum bactericidal concentration (MBC) was considered as the lowest concentration at which no growth of colonies on the culture medium (cystine lactose electrolyte deficient, CLED) surface was observed after seeding of 10 µL of MIC and two above concentrations. To evaluate the antimicrobial activity, *Staphylococcus aureus* (ATCC 6358), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* (ATCC 25922) were used.

2.5 Animals

For the *in vivo* experiments, female rats (*Rattus norvegicus*, Wistar, 150 - 220 g) and male Swiss albino mice (25 - 35 g) from the Immunobiological Research and Production Center (Piraquara, PR, Brazil) were used. The animals were maintained under controlled temperature and lighting conditions with access to water and food *ad libitum*. In experiments on the gastrointestinal tract, the animals were starved for 12 - 18 h with access to only 5% glucose water. The experiments were performed with the approval of the Animal Ethics Committee of the Tuiuti University of Paraná under protocol n°. 005/10P/CEUA, and following the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised in 1985).

2.6 Antiulcerogenic activity

2.6.1 Ethanol-induced gastric lesions

The rats were divided into five groups (n = 6/group) and treated orally with saline (5 mL/kg), ranitidine (75 mg/kg), and EIP (250, 500, and 1000 mg/kg). After 30 min of treatment, gastric lesions were induced in each animal by oral administration of 5 mL/kg of 70% ethanol according to the method described by Robert et al. (1979). Then, 1 h after ethanol administration, the animals were euthanized by cervical dislocation, and their stomachs were removed and opened along the small curvature. The total index of the gastric lesions of each animal was calculated according to the method described by Zhou et al. (2020).

2.6.2 Stress-induced gastric lesions

Five groups of rats (n = 6/group) were orally administered the vehicle (saline, 5 mL/kg), ranitidine (75 mg/kg), and EIP (250, 500, and 1000 mg/kg). After 60 min, the animals were anesthetized with isoflurane to immobilize them in the appropriate containment tubes. The containers were placed in a cold chamber at 4 °C for 3 h, followed by euthanization by cervical dislocation (Senay and Levine, 1967); their stomachs were then removed and opened along the lower curvature. The mucosa was washed with distilled water and stretched to examine the gastric lesions using a magnifying glass. The total gastric injury index was determined using the method described by Zhou et al. (2020).

2.6.3 The antisecretory activity of gastric acid

Five groups of rats (n = 6/group) were sedated with isoflurane, placed in the supine position, incisions were made 2 cm below the xiphoid process, the stomach was located, and then the pylorus was connected using a cordon line. The groups of animals were intraduodenally administered saline (5 mL/kg), ranitidine (75 mg/kg), and EIP (250, 500, and 1000 mg/kg). The abdominal wall and skin were then sutured. Four hours after the surgery, the animals were euthanized by cervical dislocation, and their stomachs were removed after clamping the esophagus to avoid loss of secreted material. Then, the stomachs were washed externally with distilled water, dried using gauze, and kept in a Petri dish. Each stomach was opened along the small curvature to collect the gastric residue, and the volume was measured. In this procedure, the mucosa was washed with 3 mL distilled water, collected in test tubes, centrifuged (252 x g for 30 min), and the supernatant was collected for the analysis of parameters of acid secretion (pH and total acidity) according to Shay et al. (1945) and Baggio et al. (2002). Gastric volume and pH were determined directly in graded tubes and by pH meter, respectively. The total acidity (mEq [H^+]/mL) was determined by simple titration with sodium hydroxide (NaOH) solution (0.1 N), using phenolphthalein as an indicator (Domer, 1971).

2.7 Antidepressant properties

2.7.1 Thiopental-induced sleep duration

Swiss male mice (n = 6/group) were treated orally with saline (0.1 mL/10 g), imipramine (20 mg/kg), caffeine (30 mg/kg), or EIP (250, 500, and 1000 mg/kg) for 7 days followed by the thiopental-induced sleep test. One hour after the last treatment, sodium thiopental (60 mg/kg) was administered via intraperitoneal (*i.p.*) injection, and the return of the animals' voluntary movements (hypnosis time) was measured as an index of sleep duration (Sousa et al., 2004; Khan et al., 2016).

2.7.2 Forced swim test (FST)

The forced swim test was conducted according to the method originally described by Porsolt et al. (1977). Mice were individually placed in a cylindrical glass container (10 × 25 cm) containing 19 cm of water (25 ± 1°C). Each session lasted for 6 min with the first 2 min considered as the "learning time," and then the immobility time during the last 4 min was recorded.

Swiss male mice (n = 6/group) were treated orally for 7 days with saline (0.1 mL/10 g), imipramine (20 mg/kg), caffeine (30 mg/kg), or EIP (250, 500, and 1000 mg/kg), and 1 h after the last treatment, all groups were subjected to the forced swimming test (Campos et al., 2004). A decrease in the duration of immobility is indicative of an antidepressant-like effect (Yankelevitch-Yahav et al., 2015).

2.7.3 Tail suspension test (TST)

Swiss male mice (n = 6/group) were treated orally for 14 days with saline (0.1 mL/10 g), imipramine (20 mg/kg), caffeine (30 mg/kg), or EIP (250, 500, and 1000 mg/kg); 1 h after the last treatment, the mice were suspended approximately 50 cm from the bench using 1 cm of adhesive tape placed before the end of the tail. The time the animals remained immobile during the 6-min period was monitored (Steru et al., 1985; Campos et al., 2004; Liu et al., 2019).

2.8 Analysis of biochemical parameters

Biochemical parameters were determined in triplicate as described by Nowacki et al. (2021). Briefly, animals were treated with saline (control), while the EIP-treated group received doses of 250, 500, and 1000 mg/kg. The treatments were administered orally at a standard volume of 10 mL/kg over 30 consecutive days. The blood samples were collected in tubes, centrifuged, and the serum were refrigerated until analysis by TP-Analyzer Plus® equipment (Thermo-Plate). The biochemical parameters of interest (glucose, creatinine, urea, triglycerides, and total cholesterol) were analyzed at the Tuiuti University of Paraná following the manufacturer's instructions of the kits used (Labtest, Lagoa Santa – MG, Brazil).

2.9 Statistical analysis

Results are expressed as the mean ± standard deviation. Statistical differences were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test and $P < 0.05$ was considered significant. The analysis was performed using OriginLab (2016) software with the Python language program. Pearson correlation analysis was performed to compare treatments and controls using the Python language program.

3. Results and Discussion

3.1 Chemical analysis

Total phenolic and flavonoid contents in EIP were 236.28 ± 11.83 mg GAE/g and 44.07 ± 5.56 mg QE/g of extract, respectively. These results are in agreement with those of Martin et al. (2013) who showed a phenolic content of 193.9 g GAE/kg in 60% ethanolic extract. Although close, the values found in the present work and in the literature for phenolic and flavonoids compounds are not the same, this is due to the fact that extraction conditions affect the content of these compounds in mate tea extracts, such as solvent polarity and extraction method (Bisognin et al., 2018; Grujic et al., 2012), as well as the influence of different geographic locations and different growing conditions (Nakamura et al., 2013). In a previous work, LC-MS analysis of EIP revealed the presence of a complex mixture containing isomers of chlorogenic and dicaffeoylquinic acids, flavonoid glycosides, and saponins where at least 25 compounds have been identified in the extract (Nowacki et al., 2021). These compounds have an anti-inflammatory effect by modulating several metabolic pathways, and also have the ability to maintain redox balance, minimizing the effects of oxidative stress at the cellular level. Concerning the gastric mucosa, these compounds present antisecretory and cytoprotective activity (Cola-Miranda et al., 2006, Liang & Kitts., 2015).

3.2 Antioxidant Capacity

In evaluating the scavenging capacity of DPPH radicals, EIP presented IC_{50} = 47.11 µg/mL, being inferior to ascorbic acid (IC_{50} = 13.38 µg/mL), a substance recognized for its antioxidant action and the ability to protect cells against free radical damage, including protection of biomembranes (Beyer., 1994, Pehlivan, 2017). Anesini et al. (2012) demonstrated that the aqueous extracts obtained from green mate and from commercial mate (*Ilex paraguariensis*) presented scavenging activity on the free radical DPPH. Additionally, caffeoyl derivatives (caffeic and chlorogenic acids) and the flavonoid rutin presented a DPPH scavenging effect and preventive action on lipid peroxidation in a concentration dependent manner. Among these compounds, chlorogenic acid was the most potent, however, caffeine did not present any scavenging activity on DPPH.

The AAR% of EIP was equal to 48.95 ± 2.70% relative to ascorbic acid in the phosphomolybdenum assay. Phytochemical analyses demonstrated a large content of phenolic substances in EIP. Many phenolic substances are important antioxidants as they have a favorable carbon skeleton for the stabilization of free radicals (Gõni et al., 1996). Indeed, the relationship between phenolic content and antioxidant activity has been widely described in the literature for different plants (Heck and Mejia, 2007; Bastos et al., 2007).

3.3 Antimicrobial activity

Assays performed with EIP showed promising activity against gram-positive and gram-negative human pathogens such as *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli,* and *Pseudomonas aeruginosa*. For the gentamicin standard, the MICs were 0.12, 6.66, 0.50, and 1.66 µg/mL, respectively, and are in agreement with results by Kidsley et al. (2018) and Tam et al. (2006). For all bacteria evaluated, the MIC and MBC were 1000 µg/mL, except for *Enterococcus faecalis* (MIC = 833 µg/mL). The activity of the aqueous extracts of yerba mate were shown against *Staphylococcus aureus* (standard and clinical strains), *Klebsiella pneumoniae* (standard strains) and *Acinetobacter baumanii* (standard and clinical strains) with Minimum Inhibitory Concentrations (MICs) of 1.88 - 3.75 mg/mL, 1.88 mg/mL and 2.5 - 10 mg/mL, respectively (Fayad et al., 2020). Costa et al. (2017) demonstrated that the hydroethanolic extract of yerba mate exhibited antimicrobial activity *in vitro* against *E. coli* and *P. aeruginosa* at a concentration of 400 µg/mL, inhibiting the growth of these bacteria. Furthermore, caffeic acid together with flavonoids has activity against bacteria, fungi, and viruses. *Staphylococcus aureus* was found to be the more sensitive to dialyzed aqueous extracts of *Ilex paraguariensis* than *Escherichia coli* O157:H7. Minimum bactericidal concentrations were determined to be approximately 150 to 800 µg/mL and 25 to 50 µg/mL against *E. coli* O157:H7 and *S. aureus*, respectively (Burris et al., 2011).

Polyphenolic constituents, flavonoids, tannins, and simple phenolic esters such as quercetin, rutin, and apigenin can have important antimicrobial activities (Basile et al., 2000). A study by Girolometto et al. (2009) demonstrated that the inhibitory capacity to inhibit bacteria depends on the way extracts are produced, suggesting that volatile phytochemicals may be lost during the processes, resulting in solutions with greater or lesser content of these compounds, indicating that they are responsible for their antimicrobial activity. Additionally, the climate, type of cultivation, management, and origin of the progeny can directly affect the chemical composition of yerba mate. The composition is related to the antimicrobial capacity of the species since polyphenols and other compounds are responsible for this biological effect (Ferrera et al., 2016).
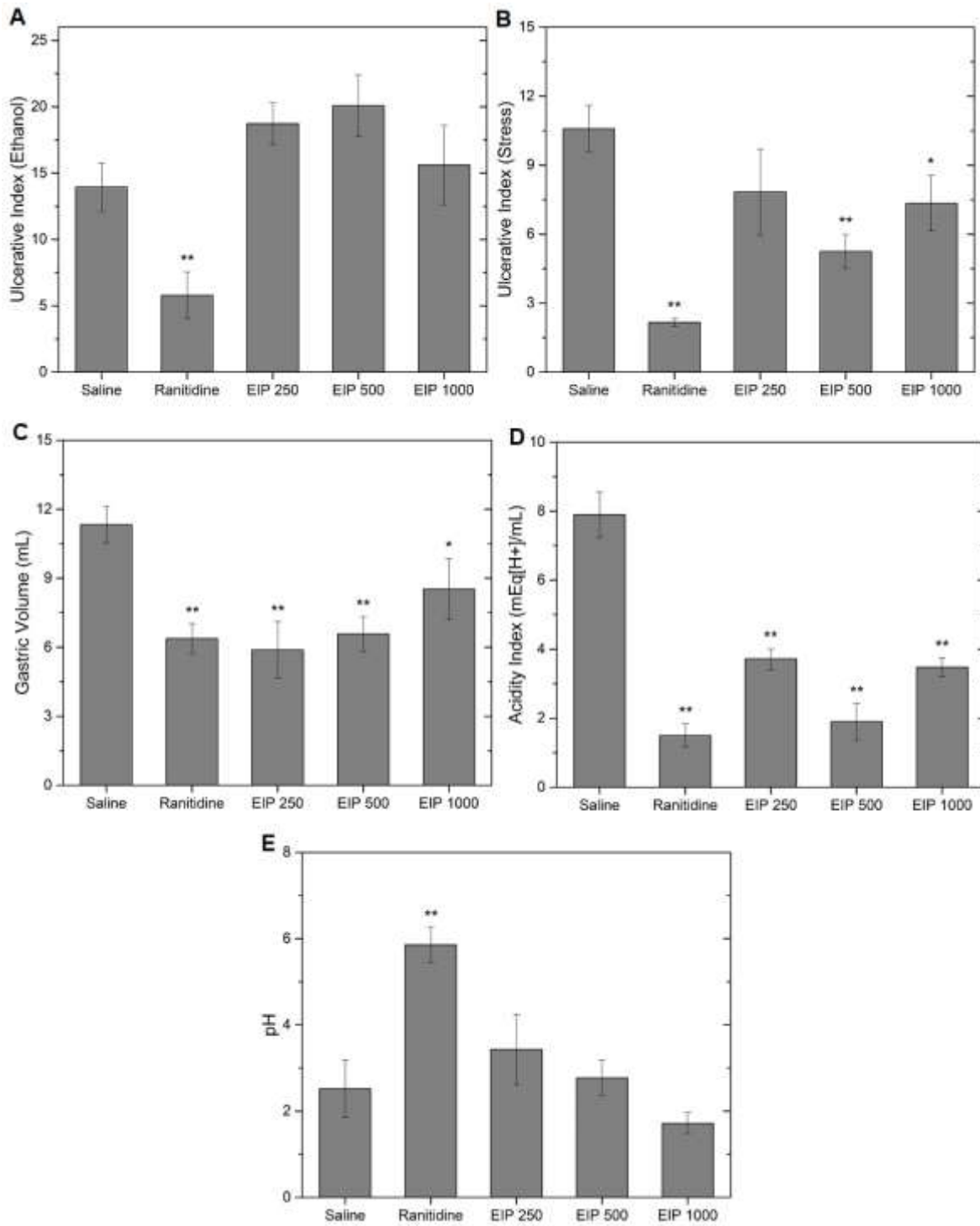
3.4 Antiulcerogenic activity

Alcohol consumption and stress are the inducers most frequently involved in human gastric pathologies (Ash & Schild, 1996). Ethanol induces gastric lesions by depressing the gastric defense mechanisms leading to effects such as reduced mucus production, gastric mucosal blood flow, bicarbonate secretion, endogenous glutathione, and prostaglandin levels (Kinoshita et al., 1995). Furthermore, ethanol increases histamine release, calcium influx, leukotriene production, and free

radical generation (Glavin & Szabo, 1992; Mendonça, 2020). Robert et al. (1979) reported that ethanol-induced ulcerations are not inhibited by substances that interfere with acid secretion but are mainly inhibited by agents that increase mucosal defense factors such as prostaglandins.

Animal models are widely used for research related to gastrointestinal tract disorders and the induction of gastric lesions by stress and ethanol in animals provides important indications about the ability of compounds to reverse the injuries caused, thus indicating possible new treatments (Baggio et al., 2002; Glavin & Szabo, 1992).

In the ethanol-induced lesion experiments, the mucosa was unprotected at all doses of EIP (250, 500, and 1000 mg/kg). The lesions were characterized by the presence of hyperemic and hemorrhagic foci, indicating the impairment of blood flow by the injurious agent (Szabo et al., 1992; Oates & Hakkinen, 1988). The results of the antiulcerogenic activity test showed that after induction of ulcerative lesions with 70% ethanol, there was no significant reduction in gastric lesions with EIP, whereas ranitidine (75 mg/kg) showed a $58 \pm 13\%$ reduction ($P < 0.01$, Figure 1 A). EIP did not protect the mucosa, suggesting that there was no cytoprotection up to the highest dose tested (1000 mg/kg). Considering the ulcers induced by stress, EIP reduced the gastric lesions to $50 \pm 13\%$ ($P < 0.01$) at 500 mg/kg and $31 \pm 13\%$ ($P < 0.05$) at 1000 mg/kg (Figure 1B). The gastric volume was reduced to $48 \pm 11\%$, $42 \pm 8\%$ (both $P < 0.01$), and $25 \pm 13\%$ ($P < 0.05$) at EIP doses of 250, 500, and 1000 mg/kg, respectively (Figure 1C). The acidity index decreased after treatment with all doses of EIP ($P < 0.01$), and 500 mg/kg showed the highest reduction of $76 \pm 7\%$ compared to saline treatment (Figure 1D). The pH of the gastric residue did not show any statistically significant difference between the control group (saline) and the EIP-treated groups, while the 250 mg/kg-treated group showed the highest pH values (Figure 1E).

Figure 1. Analysis of the effect of EIP extract (250, 500, and 1000 mg/kg) on ethanol-induced or stress-induced gastric lesions, and the interference on gastric volume, acidity index and pH of the gastrointestinal mucosa in rats submitted to pyloric ligation (n = 6). **(A)** Effect of EIP extract on ethanol-induced gastric lesions, **(B)** Effect of EIP extract on stress-induced, **(C)** Effect of EIP extract on gastric volume, **(D)** Effect of EIP extract on acidity index, and **(E)** Effect of EIP extract on pH of the gastrointestinal mucosa.

Results are means ± standard deviation. *P < 0.05 and **P < 0.01 compared to the saline treatment, using analysis of variance (ANOVA) followed by Dunnett's test. Source: Authors.

Immobilization and cold are stressful stimuli that induce lesions in the gastric mucosa and changes in the CNS. These changes lead to gastric hyperfunctioning through the vagus nerve, mediated by the release of thyrotropin-releasing hormone (Takeuchi et al., 1999). In addition, these changes promote increased gastric secretion through the production of various substances, reduction of local blood flow, and induction of lipid peroxidation, causing an increase in the generation of reactive oxygen species (Sairam et al., 2002). EIP protects the gastric mucosa from stress-induced injuries at a dose of 500 mg/kg, suggesting that it may prevent the reduction of local blood flow and increase motility, and its effects are related to the reduction of gastric mucosal ischemia and oxidative injuries (Arakchaa et al, 2019; Hersey & Sachs, 1995; Paré & Glavin, 1992).
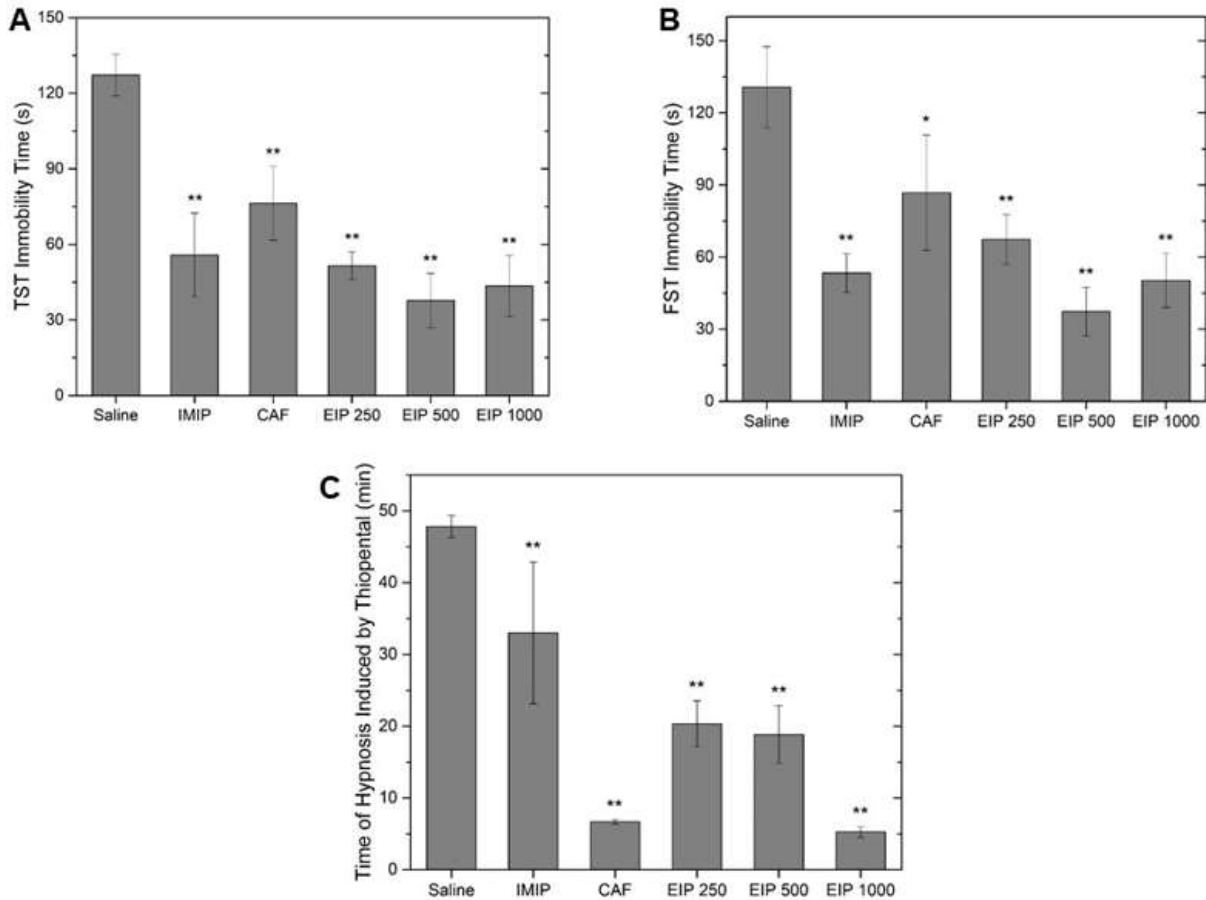
The antiulcerogenic properties of this plant species are probably attributable to flavonoids (e.g. rutin, quercetin-hexoside, and kaempferol-rutinoside) and phenolic acids (e.g. neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid) (Nowacki et al, 2021) that are present in the plant under study, since these compounds show to be active against gastric lesions with effects mediated by an increase in protective factors or antioxidant activity, mainly against free radicals (Akinci, 2017; Gonzalez & Di Stasi., 2002; Aguwa & Lawal., 1988).

Our results demonstrate that EIP prevented gastric lesions induced by stress, reduced the gastric volume at all doses and total acidity, and maintained the pH relative to the control. Considering that H_2 receptor blockers (such as ranitidine) and proton pump blockers (omeprazole) are the most widely used agents for the suppression of gastric secretion, it could be hypothesized that the constituents of the EIP extract inhibit one or more pathways that promote ulcerative lesions. Another possible mechanism involves the antioxidant activity attributed to flavonoids since free radicals are an important factor in the formation of ulcerative and erosive lesions in the gastrointestinal tract (Elshazly et al., 2018; Borrelli & Izzo, 2000).

3.5 Antidepressant properties

Treatment with EIP significantly reduced the immobility time of mice in both the TST and FST at all tested doses (Figure 2 A-B) compared to saline treatment. Imipramine (20 mg/kg) and caffeine (30 mg/kg) also reduced the immobility time. Reductions of $60 \pm 7\%$, $70 \pm 21\%$, and $66 \pm 19\%$ were observed in the TST and $48 \pm 10\%$, $71 \pm 21\%$, and $61 \pm 16\%$ in the FST with EIP at doses of 250, 500, and 1000 mg/kg, respectively. Furthermore, administration of EIP at all doses and caffeine reduced the thiopental-induced sleep time. Imipramine also reduced the barbiturate-induced sleep time compared to that in the control group by $18 \pm 23\%$ ($P < 0.01$, Figure 2 C).

Figure 2. (**A**) Effect of *Ilex paraguarienses* extract (EIP) in the tail suspension test (TST), (**B**) forced swim test (FST), and (**C**) thiopental-induced sleep time in mice.

Values are means ± standard deviation (n = 6). *$P < 0.05$ and **$P < 0.01$ compared to the saline treatment, using analysis of variance (ANOVA) followed by Dunnett's test. Saline = control, IMIP = imipramine, CAF = caffeine, EIP 250, 500, and 1000 = *Ilex paraguarienses* extract 250, 500, and 1000 mg/kg doses, respectively. Source: Authors.

The findings of this study showed that all EIP doses reduced the mice immobility time in both the FST and TST; this is usually indicative of a potential antidepressant effect. Imipramine used as a positive control significantly reduced the parameters measured in both tests. Caffeine also reduced the immobility time but cannot be considered a potential antidepressant based on this result as it was used as a false-positive control because of its known motor stimulant effects (Silva, 2010). In a previous study of the open field test, Nowacki et al. (2021) demonstrated an increase in the spontaneous movement of mice by 250 mg/kg of aqueous crude extract of *Ilex paraguariensis* (63.8%) and 30 mg/kg of the caffeine (69.9%) when compared to the control group treated with saline. Caffeine is classically used to assess drugs that alter the spontaneous movement of animals (Carlini, 1973). This observation suggests that the decreased immobility time noted in FST and TST was not due to an antidepressant effect but was caused by motor stimulation. Based on this observation, EIP could be considered to have shown an antidepressant effect only at doses below 250 mg/kg. These findings corroborate those of Reis (2017) who demonstrated that *I. paraguariensis* infusions ranging from 50 to 200 mg/kg did not produce any significant change in exploratory activity after 7 days of treatment.

Imipramine, a classic tricyclic antidepressant, has been reported to reduce the immobility time of mice without altering the number of lines crossed in the open field test at 20 mg/kg, while some studies have reported a reduction in spontaneous movement in mice at doses > 30 mg/kg (Abbasi-Maleki et al., 2020; Nielsen et al, 2004). Furthermore, the reduction of immobility time by motor excitatory drugs is usually associated with motor stimulation as observed with caffeine

in the present study. Caffeine, also a component of *Ilex* extracts (Nowacki et al., 2021), could explain this effect observed with doses of EIP (Nehlig et al., 1992). Caffeine acts on the CNS stimulating alertness and motor activity (Silva, 2010) that probably mediates the stimulating properties of mate on the motor activity of the animals. Many effects of caffeine are believed to be mediated via competitive antagonism of adenosine receptors (Prediger et al., 2008). In another study, Ludka et al. (2016) suggested that methylxanthines are, at least in part, responsible for the antidepressant-like effect of the *Ilex paraguariensis* hydroalcoholic extract, but it does not have neuroprotective properties.

The results of the thiopental-induced sleep time also corroborate the stimulatory potential of the EIP because barbiturate sleep reduction is an established animal model for investigating CNS-stimulating agents (Zhang et al., 2014). *Ilex paraguariensis* has previously been shown to affect the CNS. Milioli et al. (2007) reported the antiparkinsonian effect of 250 and 500 mg/kg of a hydroalcoholic extract, probably mediated by antioxidant and adenosine A2A antagonist activities. Another study showed short- and long-term modulation of learning and memory in rats treated with a hydroalcoholic extract (Prediger et al., 2008). Rats treated orally for 4 weeks with an aqueous extract of *I. paraguariensis* showed an antidepressant effect with no anxiolytic activity. The same study also reported no alteration in the number of open field crossings but a reduction in the number of rearing was observed (Reis et al., 2014). Nardi et al. (2016) suggested that the effect of *I. paraguariensis* infusion could partly be due to modulation of the noradrenergic pathways that could be caused by flavonoids or other components of the EIP (Meinhart et al., 2010). The results suggest that the aqueous extract of *Ilex paraguariensis* (EIP) may be an alternative for use in therapies considering its chemical constituents.

3.6 Analysis of biochemical parameters

All biochemical markers evaluated in this study were unchanged from the control values after long-term oral administration of EIP (250, 500, and 1000 mg/kg). The results are summarized in Table 1.

Table 1. Evaluation of biochemical parameters of animals treated with extract of *Ilex paraguarienses* (EIP) for 30 consecutive days.

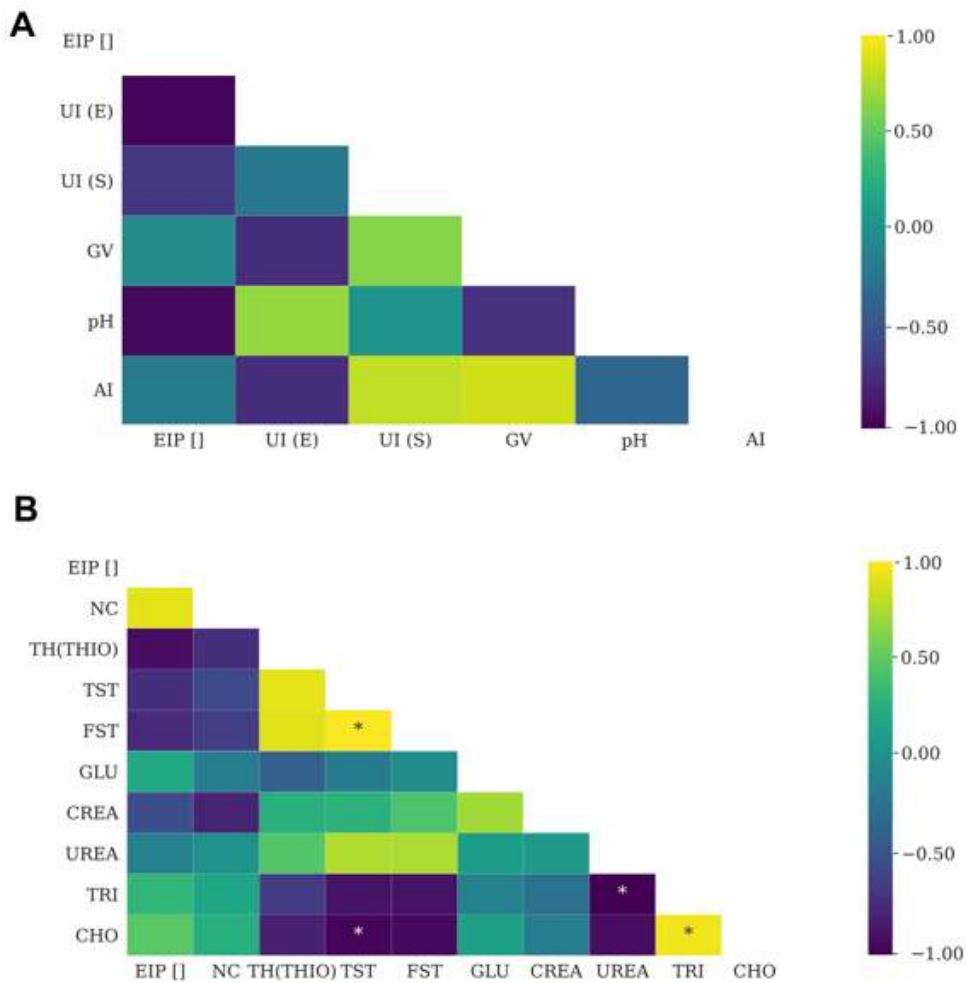| Biochemical markers	Saline 0.9%	EIP 250 mg/kg	EIP 500 mg/kg	EIP 1000 mg/kg
Glucose (mg/dL)	93.20 ± 2.52	99.60 ± 3.77	89.60 ± 4.89	98.00 ± 3.78
Creatinine (mg/dL)	0.40 ± 0.01	0.43 ± 0.02	0.36 ± 0.02	0.38 ± 0.04
Urea(mg/dL)	60.38 ± 3.34	53.45 ± 4.54	52.32 ± 5.45	57.98 ± 3.54
Triglycerides (mg/dL)	87.00 ± 18.93	120.00 ± 28.93	129.40 ± 49.26	106.80 ± 26.85
Cholesterol (mg/dL)	73.10 ± 7.50	88.80 ± 10.46	90.30 ± 36.59	78.20 ± 25.91

*$P < 0.05$ compared to the saline treatment, using analysis of variance (ANOVA) followed by Dunnett's post-hoc test (n = 6). Source: Authors.

A previous study by Nowacki et al. (2021) also demonstrated that the aqueous extract of *I. paraguariensis* administered orally (125 - 1000 mg/kg) did not change liver damage biomarkers such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The levels of serum biochemical parameters that are commonly used as biomarkers of liver or kidney function as well as serum glucose were not altered, indicating that consumption of EIP did not change the general metabolism of the rats. Similar results were reported by de Andrade et al. (2012) and Stein et al. (2005), indicating that yerba mate extract did not cause preliminary damage to rat tissues.

3.7 Pearson correlation analysis

The Pearson correlation analysis of the results presented in Figure 1 indicates that there was no correlation between EIP, ulcerative index, gastric volume or pH (Figure 3 A). However, when the same analysis was applied to the values in Figure 2 and Table 1, significant correlations were observed between the TST and thiopental-induced sleep time ($P < 0.05$), TST and FST ($P < 0.01$), and FST and thiopental-induced sleep time ($P < 0.05$, Figure 3 B). As shown in Figure 3 B, doses of EIP were directly correlated with the number of crossings ($R > 0.6$) and negatively correlated with thiopental-induced sleep time ($R < 0.6$).

Figure 3. Pearson correlation analysis of results shown in (**A**) Figure 1 and (**B**) Figure 2 and Table 1.

*$P < 0.05$ and **$P < 0.01$. EIP [ ] = concentration of extract of *Ilex paraguarienses*; UI (E) and UI (S) = ulcerative index ethanol and stress, respectively; GV = gastric volume; pH = hydrogenionic potential; AI = acidity index; NC = number of crossings; TH (THIO) = time of hypnosis induced by thiopental; TST = tail suspension test; FST = forced swim test; GLU = glucose; CREA = creatinine; UREA = urea; TRI = triglycerides; CHO = cholesterol. Source: Authors.

Although the correlations with concentrations of EIP were not significant, they may indicate that the higher the concentration of EIP, the lower the ulcerative index, the shorter the thiopental-induced sleep time, and the higher the number of crossings. Thus, these observations indicate that EIP has gastroprotective effects when induced by *stress* and antidepressant effects at doses below 250 mg/kg.

4. Conclusion

The results demonstrated that EIP is rich in phenolic compounds and presented antiulcerogenic effects induced by stress, supporting its widespread use in the treatment of digestive problems. EIP suggests an antidepressant effect at doses lower than 250 mg/kg. EIP also showed a promising antimicrobial activity against gram-positive and gram-negative bacteria. Repeated doses of EIP did not significantly alter the evaluated biochemical parameters, suggesting that it could be considered a potentially safe therapeutic drug candidate. Although our biochemical approach is interesting from a clinical point of view, further studies investigating the underlying mechanism of the gastric-protection action and histopathological effects of EIP as well as studies of the cellular and tissue morphological changes are necessary. The data obtained in the *in vivo* model in the present study have led us to suggest that the EIP exhibited possible gastric protection and antidepressant effects at certain doses.

Acknowledgments

References

Abbasi-Maleki, S., Kadkhoda, Z., & Taghizad-Farid, R. (2020). The antidepressant-like effects of Origanum majorana essential oil on mice through monoaminergic modulation using the forced swimming test. *Journal of Traditional and Complementary Medicine,* 10 (4), 327-335.

Aguwa, C., & Lawal A. (1988). Pharmacologic studies on the active principles of Calliandra portoricensis leaf extracts. *Journal of Ethnopharmacology*, 22(1), 63–71.

Akıncı, A., Esrefoglu, M., Taşlıdere, E., & Ateş, B. (2017). Petroselinum Crispum is effective in reducing stress-induced gastric oxidative damage. *Balkan Megdical Journal,* 34(1), 53-59.

Anesini, C., Turner, S., Cogoi, L., & Filip, R. (2012). Study of the participation of caffeine and polyphenols on the overall antioxidant activity of mate (Ilex paraguariensis). *LWT – Food Science and Technology*, 45(2), 299-304.

Arakchaa, K. D., Salchak, S. M., Razuvaeva, Y. G., Toropova, A. A., Smirnova, I. N., Nikolaev, S. M., Merzlikin, A. V., & Ponomarenko, G. N. (2019). The gastroprotective action of acidic mineral water from the Azhyg-Sug source on the stress-induced injuries to the stomach of white rats. Voprosy Kurortologii, Fizioterapii*, i Lechebnoi Fizicheskoi Kultury*, 96(2), 54-60.

Ash, A. S. F., & Schild, H. O. (1966). Receptors mediating some actions of histamine. *British Journal of Pharmacology and Chemotherapy*, 27(2), 427–439.

Baggio, C. H., Freitas, C. S., Nhaducue, P. F., Rieck, L., & Marques, M. C. A. (2002). Action of crude aqueous extract of leaves of Achillea millefolium L. (Compositae) on gastrointestinal tract. *Revista Brasileira de Farmacognosia,* 12(1), 31-33.

Basile, A., Sorbo, S., Giordano, S., Ricciardi, L., Ferrara, S., Montesano, D., Cobianchi, R. C., Vuotto, M. L., & Ferrara, L. (2000). Antibacterial and allelopathic activity of extract from Castanea sativa leaves. *Fitoterapia,* 71, 110-116.

Bastos, D. H. M., Saldanha, L. A., Catharino, R. R., Sawaya, A., Cunha, I. B., Carvalho, P. O., & Eberlin, M. N. (2007). Phenolic antioxidants identified by ESI-MS from Yerba-mate (Ilex paraguariensis) and green tea (Camelia sinensis) extracts. *Molecules*, 12(3), 423-432.

Berté, K. A. S., Beux, M. R., Spada, P. K. W. D. S., Salvador, M., & Hoffmann-Ribani, R. (2011). Chemical composition and antioxidant activity of yerba-mate (Ilex paraguariensis A.St.-Hill., Aquifoliaceae) extract as obtained by spray drying. *Journal of Agricultural and Food Chemistry,* 59(10), 5523-5527.

Beyer, R. E. (1994). The role of ascorbate in antioxidant protection of biomembranes: interaction with vitamin E and coenzyme Q. *Journal of Bioenergetics and Biomembranes,* 26(4), 349-358.

Birdane, F. M., Cemek, M., Birdane, Y. O., Gülçin, I., & Büyukokuroglu, M. E. (2007). Beneficial effects of Foeniculum vulgare on ethanol-induced acute gastric mucosal injury in rats. *World Journal of Gastroenterology,* 13(4), 607-611.

Bisognin, D. A., da Luz, L. V., Lencina, K. H., Santos, C. O., & Sautter, C. K. (2019). Contents of total phenolics and flavonoids in and antioxidant activity of Ilex paraguariensis leaves. *Pesquisa Agropecuaria Brasileira* 54, 1-8.

Bojic, M., Haas, V. S., Ɫaric, D., & Malel, Ž. (2013). Determination of flavonoids, phenolic ccids, and xanthines in mate tea (Ilex paraguariensis St.-Hill.). *Journal of Analytical Methods in Chemistry,* 2013, 1-6.

Borrelli, F., & Izzo, A. A. (2000). The plant kingdom as a source of anti-ulcer remedies. *Phytotherapy Research*, 14, 581-591.

Brunton, L. L. (1996). Fármacos para controle da acidez gástrica e tratamento de úlceras pépticas. In: Goodman & Gilman as bases farmacológicas da terapêutica, (9a ed.), *McGraw-Hill,* 663-674.

Burris, K. P., Davidson, P. M., Stewart Jr, C. N., & Harte, F. M. (2011). Antimicrobial activity of Yerba Mate (Ilex paraguariensis) aqueous extracts against Escherichia coli O157:H7 and Staphylococcus aureus. *Journal of Food Science*, 76(6), 456-462.

Campos, M. M., Fernandes, E. S., Ferreira, J., Bortolanza, L. B., Santos, A., & Calixto, J. B. (2004). Pharmacological and neurochemical evidence for antidepressant-like effects of the herbal product catuama. *Pharmacology Biochemistry and Behavior,* 78, 757–764.

Carlini, E. A. (1973). *Farmacologia pratica sem aparelhagem*. Ed. Sarvier, São Paulo, Brazil.

Cascon, C. S. (1995). *Amino acids in Ilex paraguariensis.* Boletin del Instituto de Quimica Argentina, 38, 7–15.

Chan, F. K., & Leung, W. (2002). Peptic-ulcer disease. *The Lancet*, 360, 933–941.

Clinical and Laboratory Standards Institute – CLSI (2012). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, sixth ed. NCCLS document, Pennsylvania, USA.

Cola-Miranda, M., Barbastefano, V., Hiruma-lima, C. A., Calvo, T. R., Vilegas, W., & Brito, A. R. M. S. (2006). Antiulcerogenic activity of Indigofera truxillensis Kunth. *Biota Neotropica,* 6(3), 1-13.

Colpo, G., Trevisol, F., Teixeira, A. M., Fachinetto, R., Pereira, R. P., Athayde, M. L., Rocha, J. B. T., & Burger, M. E. (2007). Ilex paraguariensis has antioxidant potential and attenuates haloperidol-induced orofacial dyskinesia and memory dysfunction in rats. *Neurotoxicity Research,* 12, 171-180.

Costa, D. E. M., Racanicci, A. M. C., & Santana, A. P. (2017). Atividade antimicrobiana da erva-mate (Ilex paraguariensis) contra microrganismos isolados da carne de frango. *Ciência Animal Brasileira*, 18, 1–7.

de Andrade, F., de Albuquerque, C. A. C., Maraschin, M., & da Silva, E. L. (2012). Safety assessment of yerba mate (Ilex paraguariensis) dried extract: Results of acute and 90 days subchronic toxicity studies in rats and rabbits. *Food and Chemical Toxicology,* 50, 328–334.

de Souza, L.M., Dartora, N., Scoparo, C. T., Cipriani, T. R., Gorin, P. A., Iacomini, M., & Sassaki, G. L. (2011). Comprehensive analysis of maté (Ilex paraguariensis) compounds: Development of chemical strategies for matesaponin analysis by mass spectrometry. *Journal of Chromatography A*, 1218, 7307–7315.

Devequi-Nunes, D., Machado, B. A. S., Barreto, G. A., Silva, J. R., da Silva, D. F., da Rocha, J. L. C., Brandão, H. N., Borges, V. M., & Umsza-Guez, M. A. (2018). Chemical characterization and biological activity of six different extracts of propolis through conventional methods and supercritical extraction. *PLoS One,* 13, 1-20.

Domer, F. R. (1971). *Animal experiments in pharmacological analysis.* Thomas, Illinois, USA.

Elshazly, S. M., Abd El Motteleb, D. M., & Ibrahim, I. A. A. E. (2018). Hesperidin protects against stress induced gastric ulcer through regulation of peroxisome proliferator activator receptor gamma in diabetic rats. *Chemico-Biological Interactions,* 291, 153-161.

Ferreira, S. H., & Barata, L. E. S. (1998). Medicamentos a partir de plantas medicinais no Brasil. Academia Brasileira de Ciências, Rio de Janeiro, Brazil.

Ferrera, T. S., Heldwein, A. B., Santos, C. O., Somavilla, J., & Sautter, C. K. (2016). Substâncias fenólicas, flavonoides e capacidade antioxidante em erveiras sob diferentes coberturas do solo e sombreamento. *Revista Brasileira de Plantas Medicinais,* 18, 588 – 596.

Girolometto, G., Avancini, C. A. M., Carvalho, H. H. C., & Wiest, J. M. (2009). Atividade antibacteriana de extratos de erva mate (Ilex paraguariensis A.St.-Hill.). *Revista Brasileira de Plantas Medicinais,* 11, 49 – 55.

Glavin, G. B., & Szabo, S. (1992). Experimental gastric mucosal injury: laboratory models reveal mechanisms of pathogenesis and new therapeutic strategies. *The FASEB Journal*, 6, 825– 831.

Gõni, M. A., & Eglinton, T. I. (1996). Stable carbon isotopic analyses of lignin-derived CuO oxidation products by isotope ratio monitoring-gas chromatography-mass spectrometry (irm-GC-MS). *Organic Geochemistry,* 24, 601-615.

Gonzalez, A., Ferreira, F., Vazquez, A., Moyna, P., & Alonsi Paz, E. (1993). Biological screening of Uruguayan medicinal plants. *Journal of Ethnopharmacology*, 39, 217-220.

Gonzalez, F. G., & Di Stasi, L. C. D. (2002). Anti-ulcerogenic and analgesic activities of the leaves of Wilbrandia ebracteata in mice. *Phytomedicine,* 9, 125–134.

Gorzalczany, S., Filip, R., del Alonso, M. R., Miño, J., Ferraro, G. E., & Acevedo, C. (2001). Choleretic effect and intestinal propulsion of 'mate' (Ilex paraguariensis) and its substitutes or adulterants. *Journal of Ethnopharmacology,* 75, 291–294.

Gosmann, G., & Schenkell, E. P. (1989). A new saponin from mate, Ilex paraguariensis. *Journal of natural Products*, 52(6), 1367-1370.

Grujic, N., Lepojevic, Z., Srdjenovic, B., Vladic, J., & Sudji, J. (2012). Effects of different extraction methods and conditions on the phenolic composition of mate tea extracts. *Molecules,* 17, 2518-2528.

Gugliuci, A. (1996). Antioxidant effects of Ilex paraguariensis: induction of decreased oxidability of human LDL in vivo. *Biochemical and Biophysical Research Communications,* 224, 338-344.

Heck, C. I., & De Mejia, E. G. (2007). Yerba mate tea (Ilex paraguariensis): A comprehensive review on chemistry, health implications, and technological considerations. *Journal of food science*, 72, 132-151.

Hersey, S. J., & Sachs, G. (1995). Gastric acid secretion. *Physiological Reviews*, 75, 155–189.

Khan, A. W., Khan, A., & Ahmed, T. (2016). Anticonvulsant, anxiolytic, and sedative activities of Verbena officinalis. *Frontiers in Pharmacology,* 7, 1-8.

Kidsley, A. K., Abraham, S., Bell, J. M., O'Dea, M., Laird, T. J., Jordan, D., Mitchell, P., McDevitt, C. A., & Trott, D. J. (2018). Antimicrobial susceptibility of Escherichia coli and Salmonella spp. isolates from healthy pigs in Australia: Results of a pilot national Survey. *Frontiers in Microbiology*, 9, 1-11.

Kinoshita, M., Noto, T., & Tamaki, H. (1995). Effect of a combination of ecabet sodium and cimetidine on experimentally induced gastric lesions and gastric mucosal resistance to ulcerogenic agents in rats. *Biological & Pharmaceutical Bulletin*, 18, 223–226.

Liang, N., & Kitts, D. (2015). Role of chlorogenic acids in controlling oxidative and inflammatory stress conditions. *Nutrients*, 8(1), 16-26.

Lim, D. W., Kim, J. G., Han, T., Jung, S. K., Lim, E. Y., Han, D., & Kim, Y. T. (2015). Analgesic effect of Ilex paraguariensis extract on postoperative and neuropathic pain in rats. *Biological and Pharmaceutical Bulletin*, 38, 1573-1579.

Liu, L., Dong, Y., Shan, X., Li, L., Xia, B., & Wang, H. (2019). Anti-depressive effectiveness of baicalin in vitro and in vivo. *Molecules,* 24, 1-8.

Ludka, F. K., Tandler, L. F., Kuminek, G., Olescowicz, G., Jacobsen, J., & Simone, M. (2016). Ilex paraguariensis hydroalcoholic extract exerts antidepressant-like and neuroprotective effects: involvement of the NMDA receptor and the L-arginine-NO pathway. *Behavioural Pharacology,* 27(4), 484-392.

Martin, J. G. P., Porto, E., Alencar, S. M., Glória, E. M., Corrêa, C. B., & Cabral, I. S. R. (2013). Antimicrobial activity of yerba mate (Ilex paraguariensis St. Hill.) against food pathogens. *Revista Argentina de Microbiologia*, 45, 93-98.

Meinhart, A. D., Bizzotto, C. S., Ballus, C. A., Rybka, A. C. P., Sobrinho, M. R., Cerro-Quintana, R. S., Teixeira-Filho, J., & Godoy, H. T. (2010). Methylxanthines and phenolics content extracted during the consumption of mate Ilex paraguariensis St. Hill.) beverages. *Journal of Agricultural and Food Chemistry*, 58, 2188–2193.

Mendonça, M. A. A., Ribeiro, A. R. S., & Lima, A. K. (2020). Red propolis and its dyslipidemic regulator formononetin: Evaluation of antioxidant activity and gastroprotective effects in rat model of gastric ulcer. *Nutrients,* 12, 1-13.

Mensor, L. L., Menezes, F. S., Leitão, G. G., Reis, A. S., dos Santos, T. C., Coube, C. S., & Leitão, S. G. (2001). Screening of brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phytotherapy Research*, 15, 127–130.

Milioli, E. M., Cologni, P., Santos, C. C., Marcos, T. D., Yunes, V. M., Fernandes, M. S., Schoenfelder, T., & Costa-Campos, L. (2007). Effect of acute administration of hydroalcohol extract of Ilex paraguariensis St. Hill. (Aquifoliaceae) in animal models of parkinson's disease. *Phytotherapy Research*, 21, 771–776.

Mulholland, M. W., & Debas, H. T. (1987). Recent advances in the treatment of duodenal ulcer disease. A surgical perspective. *The Western Journal of Medicine*, 147, 301–308.

Nakamura, T., Silva, F. S., da Silva, D. X., de Souza, M. W., & Moya, H. D. (2013). Determinação da atividade antioxidante e do teor total de polifenol em amostras de chá de ervas comercializadas em sachets. *ABCS Helath Science,* 38, 8-16.

Nardi, G., de Carvalho, E., de Oliveira, S., Nardi, V., Gelinski, T., Bortoluzzi, M., & Maraschin, M. (2016). Ilex paraguariensis promotes orofacial pain relief after formalin injection: Involvement of noradrenergic pathway. *Pharmacognosy Research,* 8, 31-37.

Nehlig, A., Daval, J. L., & Debry, G. (1992). Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. *Brain Research Reviews,* 17, 139–170.

Nemeroff, C. B., & Owens, M. J. (2002). Treatment of mood disorders. *Nature Neuroscience*, 5, 1068–1070.

Nielsen, D. M., Carey, G. J., & Gold, L. H. (2004). Antidepressant-like activity of corticotropin-releasing factor type-1 receptor antagonists in mice. *European Journal of Pharmacology,* 499(1-2), 135–146.

Nimse, S. B., & Pal, D. (2015). Free radicals, natural antioxidants, and their reaction mechanisms. *Royal Society of Chemistry Advances*, 5, 27986–28006.

Nowacki, L. C., Stechman-Neto, J., Schiefer, E. M., Santos, A. F., Stinghen, A. E. M., Sassaki, G. L., de Souza, L. M., Cristoff, K. E., & Souza, W. M. (2021). Ilex paraguariensis extract as an alternative to pain medications. *Acta Pharmaceutical,* 71(3), 383-398.

Oates, P. J., & Hakkinen, J. P. (1988). Studies on the mechanism of ethanol-induced gastric damage in rats. *Gastroenterology,* 94, 10–21.

Paré, W. P., & Glavin, G. B. (1993). Animal models of stress in pharmacology. *Techniques in the Behavioral and Neural Sciences*, 10, 413–441.

Pehlivan, F. E. (2017). *Vitamin C: An antioxidant agent.* InTech, 1-13.

Porsolt, R. D., Bertin, A., & Jalfre, M. (1977). Behavioral despair in mice: a primary screening test for antidepressants. *Archives Internationales de Pharmacodynamie et de Therapie,* 229(2), 327–336.

Prediger, R. D., Fernandes, S., Rial, D., Wopereis, S., Pereira, V. S., Bosse, T. S., Silva, C. B. D., Carradore, R. S., Machado, M. S., Cechinel-Filho, V., & Costa-Campos, L. (2008). Effects of acute administration of the hydroalcoholic extract of mate tea leaves (Ilex paraguariensis) in animal models of learning and memory. *Journal of Ethnopharmacology,* 120, 465–473.

Pressi, C., Wietholter, P., & Flores, R. A. (2021). Antibacterial and antifungal action of yerba mate extract and evaluation of toxicity in Artemia salina. *Brazilian Applied Science Review,* 5, 241-256.

Prieto, P., Pineda, M., & Aguilar, M. (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Analytical biochemistry*, 269, 337-41.

Ramirez-Mares, M. V., Chandra, S., & de Mejia, E. G. (2004). In vitro chemopreventive activity of Camellia sinensis, Ilex paraguariensis and Ardisia compressa tea extracts and selected polyphenols. *Mutation Research,* 554(1-2), 53–65.

Reginatto, F., Athayde, M., Gosmann, G., & Schenkel, E. (1999). Methylxanthines accumulation in Ilex species - caffeine and theobromine in erva-mate (Ilex paraguariensis) and other ilex species. *Journal of the Brazilian Chemical Society,* 10(6), 443-446.

Reis, E. M., Schreiner Neto, F. W., Cattani, V. B., Peroza, L. R., Busanello, A., Leal, C. Q., Boligon, A. A., Lehmen, T. F., Libardoni M., Athayde, A. L., & Fachinetto, R. (2014). Antidepressant-like effect of Ilex paraguariensis in Rats. *Biomed Research International*, 2014, 1-9.

Ricco, R. A., Wagner, M. L., & Gurni, A. A. (1991). Estudio comparative de flavonoids en seis especies austrosudamericanas del gênero Ilex. *Acta Farmaceutica Bonaerense,* 10, 29-35.

Robert, A., Nezamis, J. E., Lancaster, C., & Hanchar, A. J. (1979). Cytoprotection by prostaglandins in rats. Prevention of gastric necrosis produced by alcohol, HCl, NaOH, hypertonic NaCl, and thermal injury. *Gastroenterology*, 77, 433–443.

Sairam, K., Rao, C., Babu, M., Kumar, K., Agrawal, V., & Goel, R. K. (2002). Antiulcerogenic effect of methanolic extract of Emblica officinalis: an experimental study. *Journal of Ethnopharmacology,* 82, 1–9.

Schechter, L. E., Ring, R. H., Beyer, C. E., Hughes, Z. A., Khawaja, X., Malberg, J. E., & Rosenzweig-Lipson, S. (2005). Innovative approaches for the development of antidepressant drugs: current and future strategies. *The Journal of American Society for Experimental Neuro* Therapeutics, 2, 590-611.

Schinella, G., Troiani, G., Dávila, V., de Buschiazzo, P., & Tournier, H. (2000). Antioxidant effects of an aqueous extract of Ilex paraguariensis. *Biochemical and Biophysical Research Communications,* 269, 357–360.

Senay, E.C., & Levine, R.J. (1967). Synergism between gold and restraint for rapid production of stress ulcers in rats. *Experimental Biology and Medicine,* 124, 1221–1223.

Shay, H., Komarov, S., Fels, S., Meranze, D., Gruenstein, M., & Siplet, H. (1945). A simple method for the uniform production of gastric ulceration in the rat. *Gastroenterology,* 5, 43–61.

Silva, P. (2010). *Farmacologia. Guanabara koogan,* Brazil.

Sousa, F., Melo, C., Monteiro, A., Lima, V., Gutierrez, S., Pereira, B., Barbosa-Filho, J., Vasconcelos, S., Fonteles, M., & Viana, G. (2004). Antianxiety and antidepressant effects of riparin III from Aniba riparia (nees) Mez (Lauraceae) in mice. *Pharmacology Biochemistry and Behavior*, 78(1), 27– 33.

Stein, F. L. P., Schmidt, B., Furlong, E. B., Soares, L. A. S., Soares, M. C. F., Vaz, M. R. C., & Baisch, A. L. M. (2005). Vascular responses to extractable fractions of ilex paraguariensis in rats fed standard and high-cholesterol diets. *Biological Research For Nursing*, 7(2), 146–156.

Steru, L., Chermat, R., Thierry, B., & Simon, P. (1985). The tail suspension test: A new method for screening antidepressants in mice. *Psychopharmacology,* 85, 367–370.

Szabo, S., Nagy, L., & Plebani, M. (1992). Glutathione, protein sulfhydryls and cysteine proteases in gastric mucosal injury and protection. *Clinica Chimica Acta*, 206(1-2), 95–105.

Takeuchi, Y., Matsushita, H., Kawano, H., Sakai, H., Yoshimoto, K., & Sawada, T. (1999). TRH increases cerebrospinal fluid concentration of kynurenine. *NeuroReport,* 10(17), 3601–3603.

Tam, V. H., Kabbara, S., Vo, G., Schilling, A. N., & Coyle, E. A. (2006). Comparative pharmacodynamics of gentamicin against Staphylococcus aureus and Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy*, 50(8), 2026-2031.

Valduga, A. T., Gonçalves, I. L., Borges, A. C. P., Mielniczki-Pereira, A. A., & Picolo, A. P. (2016). Cytotoxic/antioxidante activity and sensorial acceptance of yerbamate development by oxidation process. *Acta Scientiarum,* 38(1), 115 – 121.

Villela, G. G. (1938). Determination of vitamin B1 in yerba mate´ by the Schopfer-Jung method. Comptes Rendus des Seances de la Societe´ de Biologie et de ses *Filiales,* 129, 987–989.

Woisky, R. G., & Salatino, A. (1998). Analysis of propolis: Some parameters and procedures for chemical quality control. *Journal of Apicultural Research,* 37(2), 99–105.

Yankelevitch-yahav, R., Franko, M., Huly, A., & Doron, R. (2015). The forced swim test as a model of depressive-like behavior. J*ournal of Visualized Experiments*, 97, 1-13.

Zhang, C., Zhao, X., Mao, X., Liu, A., Liu, Z., Li, X., Bi, K., & Jia, Y. (2014). Pharmacological evaluation of sedative and hypnotic effects of schizandrin through the modification of pentobarbital induced sleep behaviors in mice. *European Journal of Pharmacology,* 744, 157–163.

Zhou, D., Yang, Q., Tian, T., Chang, Y., LI, Y., Duan, L., Li, H., & Wang, S. (2020). Gastroprotective effect of gallic acid against ethanol-induced gastric ulcer in rats: involvement of the nrf2/ho-1 signaling and anti-apoptosis role. *Biomedicine & Pharmacotherapy,* 126, 1-10.