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Anti-inflammatory properties of phenolic lactones isolated from *Caesalpinia paraguariensis* stem bark

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A B S T R A C T

Ethnopharmacological relevance: *Caesalpinia paraguariensis* (D. Parodi) Burkart stem bark infusion (CPBI) is traditionally used in Argentina because their "vulnerary" properties.
Aim of the study: CPBI was studied throughout bio-guided purification procedures conducted by *in vitro* biological assays in order to isolate the main bioactive compounds.
Material and methods: Anti-inflammatory activity was assessed by enzyme inhibition assays of Hyaluronidase (Hyal) and inducible Nitric Oxide Synthase (iNOS). The antioxidant properties were evaluated by DPPH free radical scavenging assay, lipid peroxidation inhibition assay on erythrocyte membranes, and a cell-based assay that included the fluorescent probe (DCFH-DA) for indicating reactive oxygen species (ROS) generation. Bioactive compounds were purified by chromatographic methods and their structures elucidated using spectroscopic methods (ESI-MS and 1D/2D-$^{1}H/^{13}C$-NMR).
Results: Four main bioactive compounds were isolated from CPBI: ellagic acid (1), 3-*O*-methylellagic acid (2), 3,3'-di-*O*-methylellagic acid (3) and 3,3'-di-*O*-methylellagic-4-β-D-xylopyranoside (4). These were bioactive at concentrations in which are present in CPBI, being compounds 2 and 3 the best enzyme inhibitors of Hyal and iNOS, reaching the 90% inhibitory concentration (IC_{90}) values ranging from 2.8 to 16.4 μM, that are better than that of the positive controls, aspirin (IC_{90}: no reached) and aminoguanidine (IC_{90}: 20.2 μM) respectively. Compounds 2 and 3 were also better scavengers for lipoperoxides than butylated hydroxytoluene (BHT), reaching the 90% effective concentration (EC_{90}) at 1.2–4.5 μg/ml, and for DPPH radical (2.5–7.3 μg/ml); moreover compounds were able to exert its scavenging action on intracellular ROS. Structural features relevant to the biological activities are discussed.
Conclusions: This work provides scientific validity to the popular usage of CPBI.

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1. Introduction

Caesalpinia paraguariensis (D. Parodi) Burkart stem bark is traditionally used, on the subtropical Chaco region, to treat several diseases (Aronson and Saravia Toledo, 1992; Schmeda-Hirschmann, 1993; Filipov, 1994; Scarpa, 2004; Sgariglia et al., 2011). This study was focused on the "vulnerary" property of bark, this term is employed to denote those plants that are externally used to heal skin disorders with an inflammatory background, such as dermatitis, eczema, wounds, swelling, bumps or bruises, chilblains, ringworm, *etc.* (Bourdy et al., 2004; Svetaz et al., 2010), as infusion or decoction preparations.

The Hyaluronidases (Hyal), which degraded hyaluronan (HA), are interesting from different points of view; on one hand the enzyme plays an important role in inflammatory processes, by acting on extracellular matrix of mammalian tissues (ECM) increasing the permeability and access thereto, which exposes them to major risk of infections. In turn, the higher permeability of tissues leads to edema and the inflammatory symptoms are intensified. Mammalian Hyaluronidases (EC 3.2.1.35) also are considered to be involved in many pathophysiological processes such as degranulation of mast cells in type I allergy, in tumor growth and metastasis (Csóka et al., 1997; Kojima et al., 2000). Bacterial Hyaluronidases, also called Hyaluronate Lyases (EC 4.2.2.1), contribute to the spread of microorganisms in tissues.

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Streptococcus pneumoniae (Gram negative) and *Staphylococcus aureus* (Gram positive), agents responsible for serious infections, are producers of these enzymes, which were considered a virulence factor (Jedrzejas, 2004; García-Suarez et al., 2006).

Only a small portion of the skin Hyal exists in the free state (Mayer, 1952). Studies on experimental acute dermatitis indicate that the levels of free Hyal increase to 40 times normal value in inflamed skin tissue, also it suggests that these large amounts of free Hyal are released from the skin cells contained within the inflamed area during the inflammation (Averbeck et al., 2007). At the same time, degradation of HA in injured tissues was observed. Here, degradation of HA is caused either enzymatically by Hyal or non-enzymatically by mechanisms such as free radical-related depolymerization (Noble, 2002). Depolymerization of HA occurs in the presence of reactive oxygen species and Maillard products, resulting in HA oligomers of different length (Uchiyama et al., 1990; Agren et al., 1997; Deguine et al., 1998; Hawkins and Davies, 1998), which so produced are highly bioactive and pro-inflammatory (Termeer et al., 2003).

Under inflammatory conditions, the high levels of HA oligomers activate the synthesis of certain inducible enzymes regulated by NF-κB (Jiang et al., 2011), among them, inducible Nitric Oxide Synthase (iNOS) (McKee et al., 1997; Lee et al., 2005). The majority of skin cells (keratinocytes, melanocytes, Langerhans cells, fibroblasts and endothelial cells) employ nitric oxide (NO) as a mediator of metabolic functions to maintain the skin homeostasis, but when iNOS levels are increased, NO generated in increased amount acts as a pro-inflammatory agent, activating the synthesis of other pro-inflammatory mediators and other ECM-degrading enzymes (Fieber et al., 2004).

The interactions in the complex inflammatory response and its relationship with the antioxidant activity, suggest the importance of regulating the activity of these enzymes as an anti-inflammatory therapeutic strategy (Serrano et al., 2009). Therefore the detection and identification of substances capable of inhibiting Hyal and iNOS activities would mean possible therapeutic options to modulate inflammation allowing proper regeneration of tissue (Botzki et al., 2004), and could be pharmacognostic chemical markers for this plant species.

In this work anti-inflammatory activity in *Caesalpinia paraguariensis* plant extract was studied through an *in vitro* experimental model that included inhibition assays of enzyme activities and oxidative systems. The isolation of bioactive substances was carried out following the above activities in successive purification steps. The most active molecules were chemically identified and their activities were comparatively analyzed.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

All chemicals were of analytical grade. Sodium chloride, phosphoric acid, trichloroacetic acid (TCA), n-butanol, iron (II) sulfate ($FeSO_4 \cdot H_2O$), aluminum chloride ($AlCl_3$), glacial acetic, hydrochloric and formic acids, chloroform, sulfuric ether, ethyl acetate were from Cicarelli Labs. (Sta. Fe, Argentina); $CaCl_2$, $FeCl_3 \cdot 6H_2O$, *diphenylboryloxyethylamine* (NP), sodium phosphates (Na_2HPO_4, NaH_2PO_4), sodium acetate ($NaCH_3COO \cdot 3H_2O$), N-(1-naphtyl)ethylendiamine, sulfanilic acid, bovine Hyaluronidase (Hyal), sodium hyaluronate, potassium tetraborate ($K_2B_4O_7$), p-dimethylaminobenzaldehyde (p-DMAB), L-arginine, butyl hydroxytoluene (BHT), HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer, flavin adenine dinucleotide (FAD), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), aminoguanidine, cholesterol and vanillin were from Sigma Aldrich (St. Louis, MO, USA); polyethylenglycol 4000, Silica Gel $60F_{254}$ plates, cellulose 60G plates, thiobarbituric acid, dimethylsulfoxide (DMSO), ellagicacid and 2,7dichlorodihydrofluorescein diacetate (DCFH-DA) were from Merck (Darmstadt, Germany); potassium phosphates (K_2HPO_4, KH_2PO_4), NaCl, ascorbic acid and toluene were from Biopack (Bs. As., Argentina); methanol of analytical grade, methanol and acetonitrile (ACN) of HPLC grade were from Sintorgan (Bs.As., Argentina); tetrahydrobiopterine (BH_4) was from Schircks Labs. (Switzerland); Nylon membrane filters (pore size 0.45 μm) were purchased from Pall Corp. (Michigan, USA); Sephadex LH-20 and dithiotreitol (DTT) were from Amersham Biosciences (Sweden); nicotinamide adenine dinucleotide phosphate (NADPH) was from Indofine Inc. (Belle Mead, NJ, USA); Whatman No. 1 chromatographic paper; inducible Nitric Oxide Synthase (iNOS) was from Cayman Chemical Co. (Michigan, USA); acetylsalicylic acid (ASA) was from Anhui BBCA LiKang Pharmaceutical Co. (China).

2.1.2. Plant material

Stem bark (ritidome) from *Caesalpinia paraguariensis* (D. Parodi) Burkart, Family Fabaceae, was collected in Las Breñas, Province of Chaco, in the North of Argentina. The tree was taxonomically classified by the biologist Dr. Graciela Ponessa from the Instituto "Miguel Lillo", Tucumán, Argentina. Voucher specimens were deposited in the Herbarium of the same Institute for future reference (LIL 462833). Bark was dried in a well-ventilated room, and stored in the dark until use.

2.2. Procedures

2.2.1. Preparation of plant extract

Infusion was prepared from *C. paraguariensis* stem bark coarse powder at 10% (w/v) with distilled water. Extract was filtered through Whatman No. 1 filter paper, centrifuged at $7155 \times g$ at 4 °C for 10 min, and the clarified extract was lyophilized in a Liter freezer dryer, SL model (Virtis, Gardiner, NY, USA). Dried extract was weighted in an analytical balance (Mettler, model H54AR) and represents the extracted material (EM). For enzyme assays, organic fractions were dried under vacuum at 30 °C, these and anti-inflammatory drugs were dissolved in the corresponding reaction buffer or DMSO immediately before use.

2.2.2. Bio-assay guided fractionation

2.2.2.1. Fractionation with solvents of increasing polarity. Lyophilized infusion was packet in a column of 30×3 cm (10.8 g) and sequentially extracted with 300 mL of solvents ethyl ether, chloroform, and methanol. Three main fractions were obtained, dried, weighted and then dissolved in 100% methanol. Each fraction was analyzed by TLC (Silica gel $60F_{254}$) and tested for Hyal inhibitory activity and DPPH staining on TLC (Fig. 1A).

2.2.2.2. Fractionation by Sephadex LH-20 column chromatography (CC). Methanol fraction (MF) 100 mg was loaded on a column (66×2.0 cm) packed with Sephadex LH-20 (particle size: 27–160 μm) and eluted with 100% methanol. Fractions (4 mL each) were collected and analyzed by TLC and revealed under 365 nm UV lamp with and without adding NP spray-reagent. Twelve (I–XII) pools were made (Fig. 1B). Each fraction was dried by vacuum distillation at 40 °C, and then weighted; these were properly prepared according to the test (Hyal inhibitory activity, DPPH staining on TLC or lipid peroxidation inhibitory activity) to determine their respective bioactivities.

2.2.2.3. One-dimensional silica gel TLC. Fractions (5–10 μg) were loaded onto analytical TLC plates, and developed using $PhCH_3$/

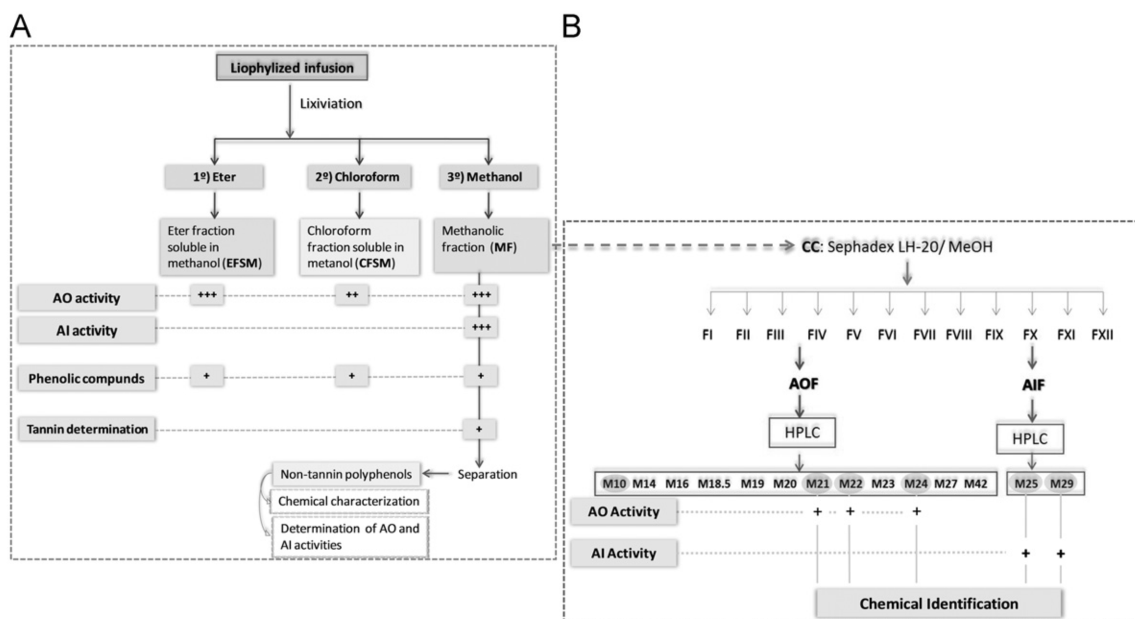


Fig. 1. This scheme summarized the bio-guided purification process; A: fractionation of *C. paraguariensis* bark infusion by the lixiviation method with solvents of different polarity (Soberón et al., 2010; Sgariglia et al., 2011), results of antioxidant and anti-inflammatory tests for fractions, additional proofs. B: fractionation by column chromatography, detection of most bioactive fractions in order to isolate the bioactive compounds, results of antioxidant and anti-inflammatory tests for purified compounds, structural elucidation of bioactive compounds.

EtOAc/MeOH/HCOOH (3:4:2:1) as the mobile phase (M1); other system used was Cellulose TLC plates, developed with *n*-BuOH/HOAc/H_2O (4:1:5) as mobile phase (BAW) (Sgariglia et al., 2010). After drying, spots were located by viewing under short (254 nm) and long (365 nm) UV lamps. The following sprays were used to locate the bands on the TLC: NP-PEG, $FeCl_3$ for phenolic compounds, $AlCl_3$ for flavonoids with hydroxyl groups in *-orto* position, Vainillin-H_2SO_4 reagent for terpenoids and phenylpropane derivatives, Dragendorff reagent for alcaloids, and Lieberman-Bouchard reagent for steroidal nuclei. All reagents were prepared according to Wagner et al. (1996).

2.2.3. Isolation of component(s) by liquid chromatography (LC)

Bioactive fractions pools (III–IV and IX–XI, see Fig. 1B) from Sephadex LH-20 CC were analyzed by RP-HPLC on a gradient HPLC in a Gilson system (Villiers Le Bel, France) equipped with 118 UV–vis detector at 254 nm, Rheodyne injector fitted with loop at 20 μL. An *IB-SIL* C18 (5 μm, 250 × 4.6 mm ID) *Phenomenex column* (Torrance, California, USA) at 25 °C was used to separate components. The IX–XI AIF pool was chromatographed applying an elution gradient performed with solvent A (1% HCOOH aq.) and solvent B (1% HCOOH aq. in H_2O-ACN 1:1): $t=0$ min, 0% B; $t=35$ min, 100% B; $t=45$ min 100% B. Chromatographic peaks were detected at 254 nm at a flow rate of 0.65 ml/min. The III–IV AOF pool was eluted applying an elution gradient performed with solvent A (1% HCOOH aq.) and solvent B (1% HCOOH aq. in MeOH-ACN 6:4): $t=0$ min, 0% B; $t=1$ min, 10% B; $t=32$ min, 90% B; $t=33$ min, 100% B; $t=45$ min, 100% B. Chromatographic peaks were detected at 350 nm at a flow rate of 0.7 ml/min. The fractions corresponding to each peak were properly treated to be analyzed by TLC.

The RP-HPLC conditions were adjusted for semi-preparative column [*IB-SIL* C18 (5 μm, 250 × 10 mm ID) *Phenomenex*] using a 500 μl Rheodyne loop; adjusting flow rate at 3.2 ml/min (III–IV pool) and 2.8 ml/min (IX–XI pool), *RT* were reproducible. Fractions corresponding to peaks were collected, dried by lyophilization, and were weighted for identification purposes and/or to test in bioassays.

2.2.4. Methods for chemical identification

2.2.4.1. UV–visible spectroscopy. UV spectrophotometric analyses were achieved in methanol on a Becknam DU 650 spectrophotometer by scanning between 200 and 500 nm.

2.2.4.2. Liquid chromatography–mass spectrometry (LC–MS). Bioactive purified compounds isolated by RP-HPLC were analyzed in a LC system (*Agilent* 1100 Series HPLC with 100-position auto-sampler), with diode array detector (DAD) coupled to a QSTAR (*Applied Biosystems*) with Q-TOF hybrid mass spectrometer, with ESI ion source conducted in both positive and negative ion modes. This equip employed a binary pump system and a C18 (5 μm, 150 × 4.6 mm ID) *Agilent* column. For M21, M22 and M24 compounds the mobile phases consisted of 0.1% HCOOH aq. (phase A) and 0.1% HCOOH in MeOH (phase B) run at 0.7 ml/min, with a gradient elution program ($t=0$ min, 0% B; $t=1$ min, 10% B; $t=22$ min, 90% B; $t=35$ min, 100% B; $t=38$ min, 100% B). For M25 and M29 compounds the mobile phase consisted of 0.1% HCOOH aq. (phase A) and 0.1% HCOOH in ACN/H_2O (1:1) (phase B) run at 0.7 ml/min, with a gradient elution program ($t=0$ min, 0% B; $t=35$ min, 100% B; $t=38$ min, 100% B). 40 μl of samples at 0.5 mg/ml concentration were injected in each experiment. The mass spectrometer was scanned over an m/z range of 50–2000 Da.

2.2.4.3. Mass spectrometry (MS). The purified substances were analyzed by mass spectrometry with ESI ion source employing a direct sample introduction technique by an outfitted integrated syringe pump on a QSTAR Elite, which is a high performance quadrupole time-of-flight (QqTOF) mass spectrometer, conducted in both positive and negative ion modes. The system analyzed a mass range of m/z 6000 Da, resolution at 8000 FWHM and accuracy at 5–10 ppm. The mass spectrometer was scanned over an m/z range of 50–1500 Da in each experiment. It includes a Turbo IonSpray probe with a flow rate range from 5 μL to 1000 μL. ESI source was affected by spray voltage of 4.5/−4.5 (M25) and

5.5/−5.5 (M29) kV, and the capillary was not heated, maintaining at room temperature. The software package includes Analyst® QS 1.1 whereby was calculated the elemental composition for each analyzed sample.

2.2.4.4. Nuclear magnetic resonance (NMR). **M21**, **M22**, **M29** and **M21a** (a: acetylated) samples were recorded on a Bruker DRX-500 MHz spectrometer (500.13 MHz) equipped with a QNP inverse probe at 5 mm $^1H/X$, HP Workstation 2000 computer, variable temperature unit and (X, Y, Z) gradient unit. **M24** was recorded in a Bruker Avance (300 MHz) spectrophotometer equipped with a broadband probe (BBO) at 5 mm. Isolated samples by RP-HPLC were dissolved in deuterated dimethylsulfoxide (DMSO-d_6) as solvent, except M24 which was dissolved in MeOH-d_4; **M21a** dissolved in $CDCl_3$. Experimental conditions: all samples were analyzed at 25 °C. **M21** was analyzed recording mono-dimensional (^1H RMN, ^{13}C RMN) and bi-dimensional (^1H–^1H COSY, ^1H–^{13}C HSQC, ^1H–^{13}C HMBC, NOESY, ROESY) spectra. **M21a** was acetylated (Markham, 1982) and analyzed by ^1H RMN. **M24** was analyzed by ^1H RMN, ^{13}C RMN, ^1H–^1H COSY, ^1H–^{13}C HSQC techniques. **M25** was analyzed by ^1H RMN, ^{13}C RMN spectra. **M22** and **M29** were analyzed by ^1H RMN.

2.2.5. Hyaluronidase activity inhibition assay

The inhibitory activity of plant samples on Hyal was performed by colorimetric method based on Morgan–Elson reaction, modified by Reissig et al. (1955). Briefly, 10 μl of enzyme containing 20 UI in 10 mM potassium phosphate buffer, pH 6 were added to 200 μl of reaction (0.2 M sodium acetate, pH 4.0) and activated with 0,1 M $CaCl_2$ at 37 °C for 15 min. Extract, fractions and/or isolated compounds (1–500 μg/ml) dissolved in DMSO (up to 3% v/v) were added, also was tested ASA standard (1–500 μg/ml); after 10 min at room temperature, the reaction was started with addition of 100 μl of 1.08 mg/ml sodium hyaluronate at 37 °C, 60 min; then 50 μl of 0.8 M potassium tetraborate pH 10, was added and stoped the reaction at 100 °C for 3 min. The released N-acetyl glucosamine reacts with Ehrlich's reagent (1.5 ml), and the formed chromogen was measured at 585 nm. Controls as R_C: reaction control; B: reaction blank; E_C: enzyme control; S_C: substrate control; $Solvent_C$, were included. Percent inhibition calculations of were made according to: (%) Inhibition= $[(A-B)-(C-D)]/(A-B)\times 100$, where A: absorbance of enzymatic reaction without samples (R_C), B: absorbance of reaction blank (without enzyme), C: absorbance of reaction mix with samples assayed and D: absorbance of dilution control for samples. Percent inhibition values were plotted as function of sample concentration (μg/ml). Half maximal inhibitory concentration (IC_{50}) was determined for each tested sample. IC_{50} or IC_{90} represents the concentrations of sample capable of producing 50% or 90% inhibition of enzyme activity (significance level was ≥30% inhibition, $p=0.01$).

2.2.6. inducible Nitric Oxide Synthase (iNOS) activity inhibition assay

This assay was conducted according to Durak et al. (2001) using the commercial *i*NOS (EC 1.14.13.39) enzyme, taking into account considerations raised by Ignarro et al. (1993) and Tarpey et al. (2004a). NO_2^- was detected as reaction product using discontinuous spectroscopic methods, where the formation of diazo-derivative generated by the Griess reaction is measured at 540 nm. Samples dissolved in DMSO (up to 3% v/v) were assayed between 10 and 500 μg/ml according to Kobuchi et al. (1999). Calculations and criteria were those considered in Section 2.2.5.

2.2.7. DPPH radical-antioxidant assay

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity was performed in polystyrene 96-well plates according to Ley and Bertram (2003), quercetin and BHT were used as reference substances. The DPPH staining method (Brem et al., 2004) was performed for samples over TLC plates during bio-guided purification.

2.2.8. Lipid peroxidation inhibition assay

This experiment was conducted on erythrocyte membranes by the thiobarbituric acid method (Kang et al., 2003), with minor modifications. Briefly, erythrocyte membranes were obtained according to Teppa-Garrán et al. (2004) and were labeled "ghosts". Reaction was initiated by addition of 0.05 ml of 100 μM $FeSO_4\cdot H_2O$ to a mixture of: 0.35 ml of 0.1 M sodium phosphate buffer pH=7.4, 0.05 ml of 0.1 mM ascorbic acid, 0.05 ml of ghost suspension (7.5 mg protein/ml) determined by Lowry et al. (1951), and a maximum of 0.05 ml of the sample solution; reaction mixture was incubated at 37 °C for 60 min. The reaction was stopped by addition of 0.5 ml of 2.8% TCA and 0.5 ml of 0.5% thiobarbituric acid, and heated at 100 °C for 30 min. Then, were centrifuged at 3500 × *g* for 10 min; supernatants were assessed by measuring absorbance at 532 nm of thiobarbituric acid reactive products (malondialdehyde). Lipid peroxidation inhibitory activity was calculated as follows: $[1-(T-B)/(C-B)]\times 100(\%)$, in which *T*, *C*, and *B* are the absorbance values at 532 nm of the sample treatment, the control (without the sample), and the zero time control, respectively.

2.2.9. Antioxidant cell-based assay

The intracellular ROS scavenging levels were determined by a bacterial cell-based assay, which uses a fluorescent probe (DCFH-DA: 2,7dichlorodihydrofluorescein diacetate) Tarpey et al. (2004a); Gomez et al. (2005). This assay was performed according to Girard-Lalancette et al. (2009) adapted to bacterial cell culture, briefly: *Escherichia coli* (ATCC 25922) was cultured aerobically in M9-glycerol–tryptone medium, overnight until an $A_{560}=0.5$. After centrifugation the culture medium was discarded and bacterial cells were washed 2–3 times with 50 mM PBS buffer (0.02 M PO_4^{3-} with 0.15 M NaCl) pH=7.0, next were re-suspended in PBS until an $A_{560}=0.5$. Bacterial suspension was sonicated (30 min), treated with 10 μM DCFH-DA solution and incubated 1 h at 37 °C, then was centrifuged and the pellet was re-suspended in PBS. To assess antioxidant activity, aliquots of 50 μl (5×10^6 CFU) were incubated (1 h) with 50 μl of growing concentrations of pure compounds (10, 50, 100 or 250 μg/ml, dissolved in 3% DMSO aq.), next were washed and re-suspended 2–3 times with PBS, and treated with 1 mM tert-butylhydroperoxide (*t*-BuOOH). Fluorescence was measured immediately after t-BuOOH administration and also 90 min later on the automated plate reader (Kary Eclipse Spectrophotometer) using an excitation wavelength of 490 nm and an emission wavelength of 519 nm. IC_{50} are the mean ± standard deviations of three determinations.

2.3. Statistical calculations

The results were expressed as mean ± standard error of means (S.E.M) of four determinations at each concentration for each sample. The IC_{50} values were calculated using Microsoft Excel. Statistical significance was calculated for a significance level ≥30% inhibition by one-way analysis of variance (ANOVA), followed by Turkey's test ($p=0.01$).

3. Results and Discussion

3.1. Bio-assay guided purification

The purpose of isolating anti-inflammatory and antioxidant substances from *C. paraguariensis* bark infusion through bio-assay guided purification was the chemical identification of the main substances responsible for the evidenced activities in original extract, which is popularly used by its vulnerary effects. After each purification step, the most active fractions were selected to continue the isolation of active compounds (Fig. 1A, B). In intermediate purification steps, precipitable tannin concentration was determined and Hyal inhibition tests were performed with fractions without tannin (Fig. 1A) in order to investigate whether the observed inhibition of the original extract and methanol fraction (MF) was due to substances other than tannins (known protein precipitants). The presence of Hyal inhibitory activity in fractions without precipitable tannin (Sgariglia et al., 2006) justified the continuation of the bio-assay guided purification of extract components. Fig. 1 B shows the selected fractions after each purification step. The purification of more active analytes isolated was conducted by RP-HPLC-(DAD).

3.2. Identification of bioactive compounds

Ellagic acid, chemical name 2,3,7,8-tetrahydroxy[1]benzopyrano[5,4,3-*cde*][1]benzopyran-5,10-dione **(M25)** was isolated as a pale yellow–brown amorphous powder (10 mg), purified through semi-preparative RP-HPLC, and dried by liophylization. This component was quantified, its concentration in CPBI was 14.07 μg/ml, and its yield was 0.135 percent of compound/liophylized CPBI (% w/w). R*f* 0.45–0.63 (M1), 0.20–0.32 (BAW), were close to those reported by Seikel and Hillis (1970), Nawwar and Souleman (1984). UV λ_{max} (MeOH) (ε): 255.5 (39790), 358 (9182), these data were approximated to those published by Bhargava et al. (1968) and Nawwar et al. (1982); ESI-MS data in Table 1. ^{1}H-NMR, an only signal for 2H at 7.51 ppm, this datum was according to the literature (Nawwar et al., 1984, 1994).

3,3'-di-*O*-methylellagic-4-*O*-β-D-xylopyranoside (M21). Pale yellow–brown amorphous powder (10.0 mg) was isolated and purified through semi-preparative RP-HPLC, and dried by liophylization. This component was quantified, its concentration in CPBI was 10.42 μg/ml, and its yield was 0.100 (% w/w). R*f* 0.51 (M1). $[\alpha]_{D(DMSO)}^{27^{\circ}C}$ +5.90. UV λ_{max} (MeOH) (ε): 244.9 (21323), 266 sh, 285 sh, 368.5 (3.376). ESI-MS, Table 1. ^{1}H-NMR, Table 3; ^{13}C-NMR, Table 2; the signal assignment for $C_{1'}$ (111.80 ppm) was verified in 2D ^{1}H–^{13}C HMBC spectrum (Fig. 5), where this carbon correlates with con $H_{5'}$ at three bounds (^3J); signal assignment for $H_{4''}$, $H_{2''}$, $H_{5a''}$ and $H_{3''}$ were confirmed by 2D ^{1}H–^{13}C HSQC spectrum (not shown), where the correlation between ^{1}H and ^{13}C identify corresponding signals by *cross-peaks*, despite the overlapping band of H_2O; moreover allowed to define assignments for C_1, $C_{1'}$, C_5, $C_{5'}$, C_6 y $C_{6'}$; C_5 (112.35) and $C_{5'}$ (112.31); ^{1}H–^{13}C HMBC *cross-peak* allowed to confirm assignment of carbons. The cross-peak ROESY correlating to $H_{1''}$ (anomeric) with H_5 (aglycone) confirms that sugar binds to the aglycone in C_4 (Fig. 5A/B); experimental spectral data were consistent, and these coincided with the data published by Khac et al. (1990).

***Per*-acetylated 3,3'-di-*O*-methylellagic-4-*O*-β-D-xylopyranoside (M21a).** Pale amorphous powder (1.6 mg) was analyzed by ^{1}H-RMN ($CDCl_3$) in order to confirm sugar signals (Table 3; Fig. 6).

3,3'-di-*O*-methylellagic acid (M24). Pale yellow–brown amorphous powder (5.7 mg) was isolated and purified through semi-preparative RP-HPLC, and dried by liophylization. This component was quantified, its concentration in CPBI was 8.02 μg/ml, and its yield was 0.077 (% w/w). R*f* 0.73 (M1). UV λ_{max} (MeOH) (ε): 246.9 (30811), 288 sh, 361 sh, 375.7 (9360). ESI-MS, Table 1. ^{1}H-NMR, Table 3; ^{13}C-NMR, Table 2; the fact of being a symmetric molecule kept consistent with the low number of signals observed in NMR spectra, the axis of symmetry of the molecule passes through C_2 and $C_{2'}$ (Fig. 6), this allows the substituents present on C_3 and $C_{3'}$ were equivalent. Our experimental spectral data were consistent among themselves, and correspondence was found with data published by Nawwar et al. (1982), Khac et al. (1990) and Khallouki et al. (2007). The proposed molecule (Fig. 6) presents

Table 1
HPLC-ESI(±)-MS^n Analysis of bioactive analytes isolated by RP-HPLC from *C. paraguariensis* stem bark infusion.

Sample	*RT* (min)	UV (nm)	ESI	BP (*m/z*); ID	MS^{b,c}(*m/z*)^a	MS^2 (*m/z*)^a	MW (amu)	Compound
M21	23.81	247.96; 290 sh; 367.87	(+)	463; $[M+H]^+$	$[M+H]^+$ **463.0859** $[M+Na]^+$ 485.0688 $[2M+Na]^+$ 947.1448	463; 385; 331; $[M-Xyl+2H]^+$	462	$C_{21}H_{18}O_{12}$ with 13 unsaturations
M22	23.72	250; 370	(+)	**317**; $[M+H]^+$ 334; $[M+NH_4]^+$ 339; $[M+Na]^+$ 650; $[2M+NH_4]^+$	ESI (−) $[M-H]^-$; **315.9945** $[M-Me]^-$; 300.9970	NA	316	$C_{15}H_8O_8$ with 12 unsaturations
M24	25.83	247.96; 291 sh; 375.73	(+)	**331**; $[M+H]^+$ 348; $[M+NH_4]^+$ 353; $[M+Na]^+$ 378;$[2M+NH_4]^+$	ESI (−) $[M-H]^-$; 329.0311 $[M-Me]^-$; 315.0114 $[2M-H]^-$; 659.0693	NA	330	$C_{16}H_{10}O_8$ with 12 unsaturations
M25	19.87	253.7;367.8	(−) (+)	**301**; $[M-H]^-$ 303; $[M+H]^+$ 325; $[M+Na]^+$	ESI (−) $[M-H]^-$; **301.1480**	301; 257; 229	302	$C_{14}H_6O_8$ with 12 unsaturations
M29	25.52	250; 374 sh;368	(−) (+)	**315**; $[M-H]^-$ 631; $[2M-H]^-$ **317**; $[M+H]^+$ 339; $[M+Na]^+$	ESI (−) $[M-H]^-$; **315.0143**	NA	316	$C_{15}H_8O_8$ with 12 unsaturations

References: HPLC conditions were detailed in Materials and methods.
[a] Ions in boldface indicate the most intense production, which was chosen for MS^2 in the MS/MS spectrum; (NA) Not acquired; (BP) base peak;
[b] Direct sample introduction.
[c] Error is in the fourth decimal cipher.

a structural isomer, with the substituents (di-*O*-methyl) at positions C_4 and $C_{4'}$, our NMR and UV experimental data were consistent with those reported by Sato (1987) for the isomer substituted at C_3 and $C_{3'}$.

3-*O*-methylellagic acid (M22 and M29). Each compound was separately analyzed, and described like a pale amorphous powder (2.5 and 3.3 mg, respectively) isolated and purified through semi-preparative RP-HPLC, and dried by liophylization. M22 was quantified, its concentration in CPBI was 4.17 μg/ml, and its yield was 0.040 (% w/w); M29 was quantified, its concentration in CPBI was 4.58 μg/ml, and its yield was 0.044 (% w/w). R*f* 0.8 (M1). UV λ_{max} (MeOH) (ε): 249.9 (25650), 346 sh, 367.2 (8400). ESI-MS, Table 1. ^{1}H-NMR, Table 3; ^{13}C-NMR, Table 2; δ_C of methoxyl carbon (–O$\underline{C}H_3$) at 61.25 ppm was typical of C_3 substitution instead of C_4, since for the substitution on C_4, the measured δ_C should be close to 57 ppm (Sato, 1987). Our experimental data were consistent with those reported by Tanaka et al. (1998), Khallouki et al. (2007) and Da Silva et al. (2008).

Gallic acid (3,4,5-trihydroxy-benzoic acid) (M10). Was the eluate collected at *RT* 9.7 min from III–IV pool (AOF) chromatographed by RP-HPLC. The corresponding fraction was analyzed by TLC co-spotting (on silicagel/M1) and by co-elution (on the RP-HPLC chromatographic on AOF experimental conditions) with a standard drug of gallic acid, this developed to closest in R*f* value (0.68) revealed with NP/PEG under 365 nm UV lamp, and co-eluted with M10 at *RT* 9.7 ± 0.2 min (*N*=5). UV λ_{max} (aquous solution) 225 nm (ε=26500), 260 nm (ε=21200).

Table 2
125.76 MHz **^{13}C-NMR data** for compounds in DMSO-*d6* [δ_C (ppm)[b]].

C	M21	M22	M24[a]	M29
1 (C=C̲=C, Arom. 4th)	114.83	112.55	112.23	112.55
2 (O=C–O–C̲=C)	142.15	142.15	141.58	142.15
3 (=C̲–O–CH$_3$)	142.37	141.12	141.05	141.12
4 (=C̲–O–Sugar)	151.62	152.62	152.51	152.50
5 (H–C̲=C, Aromatic)	112.35	112.25	111.36	112.21
6 (O=C=C̲=C, Arom. 4th)	113.31	113.31	112.34	113.28
7 (O–C̲=O, lactonic)	158.96	159.56	159.29	159.16
1′(C=C̲=C, Arom. 4th)	111.80	112.90	112.23	112.90
2′(O=C–O–C̲=C)	141.43	137.20	141.58	137.20
3′(=C̲–O–CH$_3$)	140.78	140.43	141.05	140.43
4′(=C̲–OH, phenolic)	151.62	149.12	152.51	149.05
5′(H–C̲=C, Aromatic)	112.31	111.31	111.36	111.31
6′(O=C=C̲=C, Arom. 4th)	112.25	108.95	112.34	108.95
7′ (O–C̲=O, lactonic)	158.91	159.45	159.29	159.38
3 –OC̲H$_3$	62.11	61.25	61.13	61.15
3′–OC̲H$_3$	61.41		61.13	
Sugar:				
1" (anomeric)	102.26			
2" (C–HC̲–OH)	73.52			
3" (C–HC̲–OH)	76.60			
4" (C–HC̲–OH)	69.72			
5" (O–C̲H$_2$–C$_{Sp3}$)	66.27			

References:
[a] 75.14 MHz ^{13}C-NMR for M24.
[b] With TMS (tetramethylsilane) as internal standard.

3.3. Free radical scavenging activity

Free radical scavenging activity evaluated by DPPH assay showed the scavenging potency of isolated compounds on bases of 50% Scavenging Concentration (SC_{50}): ellagic acid (1.5 ± 0.15) > gallic acid (2.5 ± 0.12) > 3-*O*-methylellagic acid (2.8 ± 0.15) > 3,3′-*O*-dimethylellagic acid (5.10 ± 1.05) > 3,3′-*O*-dimethylellagic-4-β-D-xylopyranoside (7.50 ± 0.10). CPBI and compounds isolated reached 90% of DPPH scavenging activity (Fig. 2A) at concentrations lower than 10 μg/ml. All isolated compounds exhibited lipid peroxidation inhibitory activity (LPIA) on erythrocyte membranes, the most effective were the 3,3′-*O*-dimethylellagic derivative (0.25 ± 0.02), followed by 3-*O*-methylellagic acid (0.50 ± 0.05) and ellagic acid (1.30 ± 0.07); glycosylated derivative was less active than these ones (15.00 ± 0.50) Fig. 2B. Fractions and non-glycosylated ellagic acid derivatives reached the $CIPL_{90}$ at concentrations lower than 15 μg/ml. To compare scavenging activities of isolated compounds with BHT, a known antioxidant widely used in the food and pharmaceutical industry, it was observed that the latter is lesser active than ellagic acid derivatives.

Different bioactivities of derivatives of the same molecule were attributed to certain structural characteristics; Cai et al. (2006) suggested that both the number of hydroxyls in the molecule as the position thereof, are essential requirements. In the DPPH assay is important that the hydroxyls were in ortho position to obtain significant increase in activity. Heim et al. (2002) demonstrated the influence of methoxy groups (–OCH$_3$) as substituents on scavenging activity. Also was observed that carbonyl *group conjugated* with a

Table 3
500.12 MHz **^{1}H-NMR** data for compounds in DMSO-*d6* [δ_H (ppm)[c], *J*(Hz)].

H	M21	M21a[a]	M22	M24[b]	M25	M29
5 (H̲–C=C, Aromatic)	7.77 (s)		7.53 (s, 1H)	7.48 (s, 2H)	7.51 (s, 2H)	7.51 (s, 1H)
5′(H̲–C=C, Aromatic)	7.55 (s)		7.49 (s, 1H)	7.48 (s, 2H)	7.51 (s, 2H)	7.48 (s, 1H)
3 –OCH̲$_3$	4.08 (s, 3H)		4.08 (s, 3H)	4.02 (s, 6H)		4.06 (s, 3H)
3′–OCH̲$_3$	4.06 (s, 3H)			4.02 (s, 6H)		
CH$_3$C=O (acetyl)		2.15 (s, 3H)				
		2.17 (s, 3H)				
		2.18 (s, 3H)				
CH̲$_3$(C=O)–O-C$_{4'}$ (acetyl)		2.44 (s, 3H)				
Sugar (Xylose):						
H-1" (anomeric)	5.17 (d, 1H, *J* $_{7.25\ Hz}$)	5.40 (d, 1H, *J* $_{5,2\ Hz}$)				
H-2" (C–H̲C–OH)	3.39	5.32 (m, 2H)				
H-3" (C–H̲C–OH)	3.33	5.32 (m, 2H)				
H-4" (C–H̲C–OH)	3.41	5.09 (m, 1H)				
H-5" a (O–CH̲$_2$–C$_{Sp3}$)	3.38	3.69 (m, 1H)				
H-5" b (O–CH̲2–C$_{Sp3}$)	3.83 (dd, 1H, *J* $_{4,4/10,4\ Hz}$)	4.33 (m, 1H)				

References:
[a] M21a compound was dissolved in DCCl$_3$.
[b] 300 MHz ^{1}H-NMR for M24.
[c] With TMS (tetramethylsilane) as internal standard.

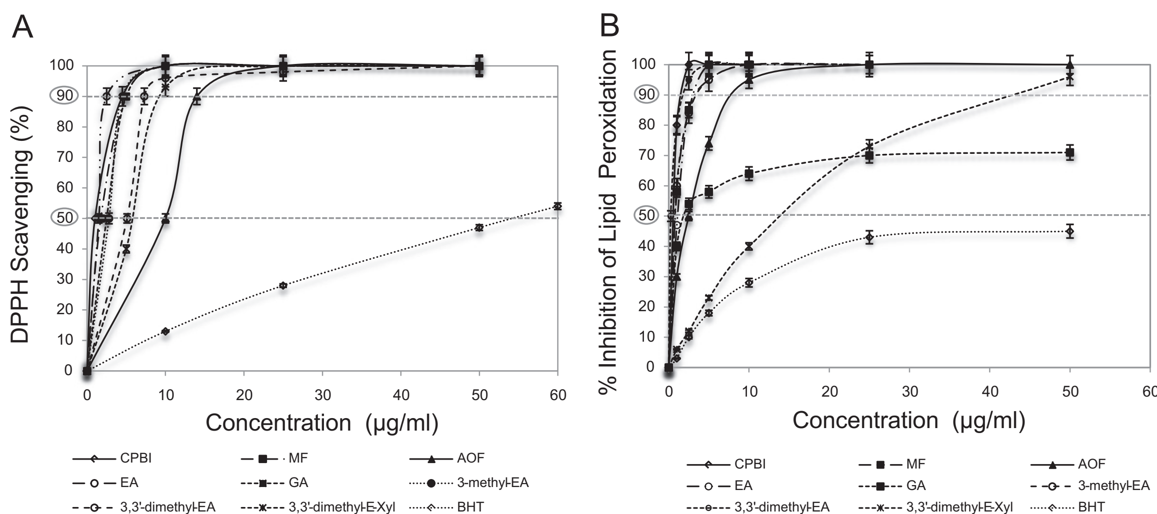


Fig. 2. Concentration-Response (Antiradical activities of samples) Curves; BHT: butyl hydroxytoluene CPBI: *C. paraguariensis bark infusion* bark infusion; MF: methanol fraction; AOF: antioxidant fraction; EA: ellagic acid; GA: gallic acid; 3,3'-dimethyl-EA: M24; 3-methyl-EA: M22/M29; 3,3′-dimethyl-E-Xyl: M21.

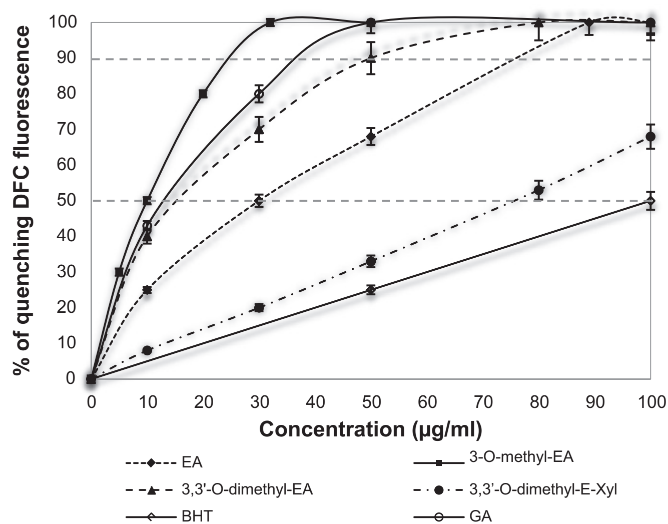


Fig. 3. Effect of isolated compounds and a standard substance on quenching DFC florescence (oxidized product of DCFH). ROS were induced adding *t*-BuOOH (1 mM) on sonicated cells, and after 90 min, the differences in fluorescence due to DFC was measured by addition of 200 µl of PBS; these were significantly different from fluorescence of DCF without tested compounds ($p < 0.05$).

double bond would be an important structural element for the antiradical power of certain phenolic compounds.

According to these guidelines, antioxidant behavior observed for isolated compounds, suggested that increasing the degree of substitution in ellagic acid derivatives decreased the anti-DPPH activity and increased the inhibitory capacity of lipid peroxidation on erythrocyte membranes; the results of both tests indicate that glycosylation decreased the antiradical power (Fig. 2A/B).

3.4. Intracellular ROS scavenging activity

In our cell-based assay, the DCFH-DA fluorescent probe was used to detect intracellular ROS induced by *t*-BuOOH (oxiding agent), evaluated on the sonicated *Escherichia coli* (ATCC *25922*) cells. Fig. 3 shows that all compounds isolated from CPBI inhibit ROS induced by t-BuOOH in a dose-dependant manner. In order to compare the antioxidant activities of tested compounds, concentrations inhibiting DCFH oxidation by 50% (IC_{50}) were calculated for each. Regression coefficients (R_2) calculated for each logarithmic curve was superior to 0.95. The observed results (Fig. 3) suggested that the assayed compounds were able to cross the cell membrane and exert intracellular ROS scavenging activity, their antioxidant potentials (IC_{50}) are classified into order decreasing: 3-*O*-methylellagic acid (10.00 ± 0.89) > gallic acid (12.00 ± 0.92) > 3,3′-*O*-dimethylellagic acid (15.00 ± 1.15) > ellagic acid (25.00 ± 1.05) > 3,3′-*O*-dimethylellagic-4-β-D-xylopyranoside (75.00 ± 1.70); all these were more active than BHT. The highest scavenging activity observed for 3-*O*-methylellagic and 3,3′-*O*-dimethylellagic acid could be due to that DCFH are also located within the lipid bilayer, and these compounds having marked lipophilic properties also could act as scavengers in nearness to cell membrane, where are generated, mainly on ROS such as superoxide and H_2O_2 (Afri et al., 2004).

3.5. Anti-inflammatory properties based on enzyme inhibition

3.5.1. Hyaluronidase inhibitory activity

Ellagic acid derivatives isolated from *C. paraguariensis* stem bark infusion, assayed individually inhibited Hyal activity (Fig. 4B). The structure-activity analysis of phenolics from others plant species, already assayed for Hyal inhibitory capacity, could help explain *a priori* the different inhibitory activities evidenced by compounds isolated from CPBI. Polyphenols are a group of potential inhibitors of Hyal, and they would act on this by interaction with the tertiary structure of the enzymatic protein (Meyer et al. 1960). Kuppusami et al., 1990; Kuppusami and Das 1991 established that the inhibition is of competitive type, and that aglycones of phenolic compounds are more potent inhibitors than their respective glycosides; moreover, the presence of free phenolic hydroxyl and carbonyl groups would be important for inhibitory activity. The results found in our study for ellagic acid and derivatives corroborated the observations of Kuppusami and Das (1991), note that ellagic acid and non-glycosylated compounds reached the 90% inhibitory activity (Fig. 4 B. EA:12.6 ± 0.3 µM; 3-*O*-methyl-EA:12.6 ± 0.1 µM; 3,3'-*O*-dimethyl-EA:25.7 ± 0.5 µM). All ellagic derivatives isolated reached the IC_{50} at concentrations lower than 22 µM. Note also that the inhibitory activity of these was more intense than that observed when testing the original extract (CPBI) and purified fraction (AIF). Aspirin (ASA), standard substance used as reference, reached only 30% inhibition with the highest concentration assayed (100.0 ± 0.5 µM). Noteworthy that isolated compounds

show Hyal inhibitory activity at concentrations which were quantified (data described in Section 3.2) in the original extract (CPBI), so these might be the molecules responsible for observed activity in CPBI. Moreover, the μM bioactive concentrations found in our determinations would be promising as inhibitors of pharmacological interest.

3.5.2. *inducible Nitric Oxide Synthase (iNOS) inhibitory activity*

Isolated compounds inhibited the activity of *i*NOS at μM concentrations (Fig. 4A) comparable to aminoguanidine, a specific inhibitor of the inducible isoform (Corbett and Mc Daniel, 1996). Based on the information described in the literature, the structural requirements for phenolic molecules which inhibit to *i*NOS were analyzed (Matsuda et al., 2003). Fig. 4A shows that such structural requirements are also applicable to ellagic acid derivatives isolated in our work; the non-glycosylated compound showed higher inhibitory activity on iNOS, and the methylation of phenolic hydroxyl groups increased the inhibitory activity in concentration about 10 μM. The order of inhibitory potency for each ellagic acid derivative was: 3-*O*-methylellagic > ellagic acid > 3,3'di-*O*-methylellagic » 3,3'-*O*-dimethylellagic-4-β-D-xylopyranoside, showed that glycosylation decreased inhibitory activity in an order > 100 μM. Matsuda et al. (2003) suggested that the presence of phenolic hydroxyl groups in ortho position (catechol) markedly favors the inhibitory activity respect to trihydroxylated molecules. This would explain the low inhibition of 3,3′di-*O*-methylellagic with respect to 3-*O*-methylellagic and ellagic acids, and the fact that gallic acid does not reach IC_{50} at concentrations greater than 100 μM (Fig. 4A).

4. Conclusions

The results of our experiments suggested that *Caesalpinia paraguariensis* stem bark infusion has vulnerary properties, and allow proposing that ellagic acid and their mono and di-methylated derivatives would be the main contributors with lipoperoxidation inhibitory activity, without avoiding the possible adjuvant role of other components of the "phytocomplex"

Inhibitory activity of components from *C. paraguariensis* stem bark infusion on Hyal and iNOS

	Enzyme inhibitory activities							
	Hyaluronidase (Hyal)				Inducible Nitric Oxide Synthase (iNOS)			
	IC_{50}		IC_{90}		IC_{50}		IC_{90}	
Samples	(μg/ml)	(μM)	(μg/ml)	(μM)	(μg/ml)	(μM)	(μg/ml)	(μM)
CPBI	14.7±1.5	-	62.4±0.5	-	-		-	
MF	13.5±2.3	-	24.3±2.1	-	-		-	
AIF	11.1±1.5	-	20.0±0.5	-	-		-	
Ellagic acid	1.5±0.1	4.9±0.1	3.8±0.3	12.6±0.3	1.1±0.2	3.6±0.2	2.1±0.2	6.9±0.2
Gallic acid	50.0±1.5	294.0±1.5	NR		NR		NR	
3-*O*-methyl-EA	2.3±0.1	7.2±0.1	4.0±0.1	12.6±0.1	0.5±0.1	1.5±0.1	0.9±0.1	2.8±0.1
3,3'-*O*-dimethyl-EA	5.4±0.3	16.4±0.3	8.5±0.5	25.7±0.5	1.5±0.3	4.5±0.3	3.5±0.5	10.6±0.5
3,3'-*O*-dimethyl-E-Xyl	10.0±0.5	21.6±0.5	NR		13.0±1.2	28.1±1.2	38.0±1.5	82.2±1.5
ASA	-	100.0±0.5	NR		-	-	-	-
Aminoguanidine	-	-	-	-	0.5±0.1	19.0±0.6	1.5±0.1	20.2±0.6

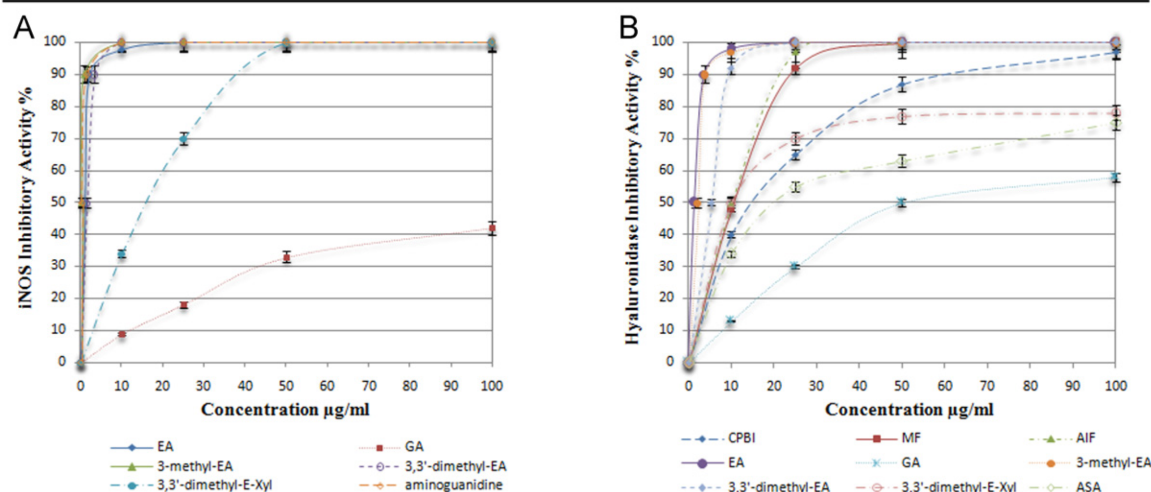
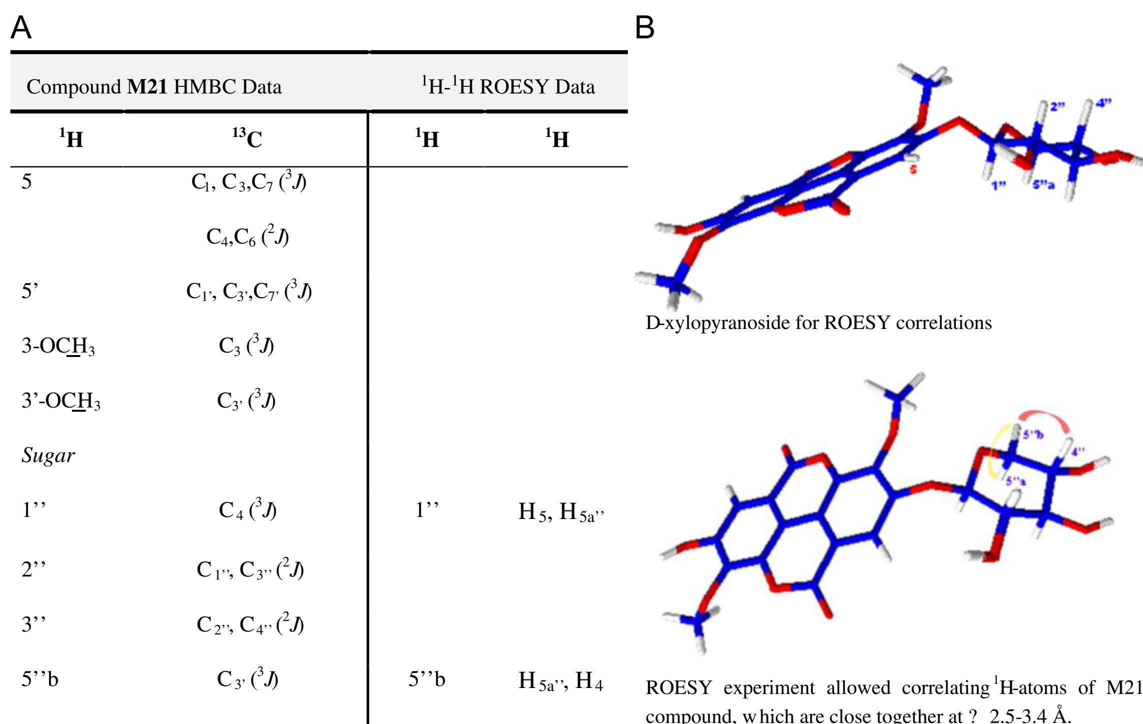


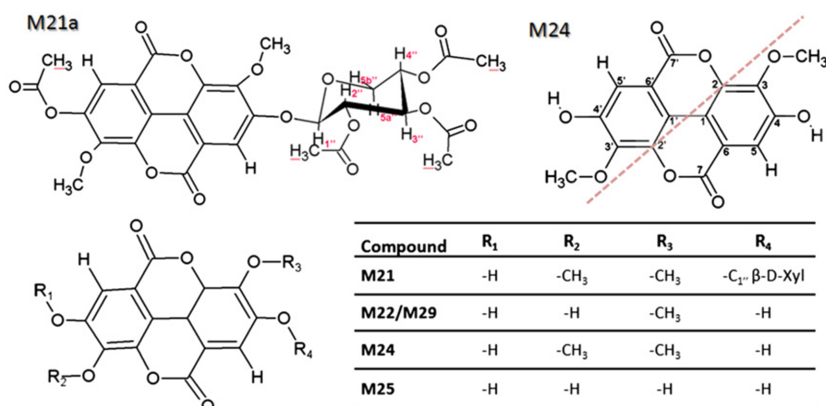
Fig. 4. Concentration-response (enzyme inhibitory activities) curves; IC: inhibitory concentration; IC50 (50%) and IC90 (90%) expressed as μg/ml and μM; MF: methanolic fraction; AIF: anti-inflammatory fraction; CPBI: *C. paraguariensis* bark infusion; EA: ellagic acid; E-Xyl: ellagic-4 β-xylopyranoside; ASA: acethylsalicylic acid; (-): not determined; NR: not reached. The significance level was ≥30% inhibition (ρ=0.01).



A

Compound **M21** HMBC Data		^{1}H-^{1}H ROESY Data	
^{1}H	^{13}C	^{1}H	^{1}H
5	C_1, C_3,C_7 (^3J)		
	C_4,C_6 (^2J)		
5'	$C_{1'}$, $C_{3'}$,$C_{7'}$ (^3J)		
3-OC<u>H</u>$_3$	C_3 (^3J)		
3'-OC<u>H</u>$_3$	$C_{3'}$ (^3J)		
Sugar			
1''	C_4 (^3J)	1''	H_5, $H_{5a''}$
2''	$C_{1''}$, $C_{3''}$ (^2J)		
3''	$C_{2''}$, $C_{4''}$ (^2J)		
5''b	$C_{3'}$ (^3J)	5''b	$H_{5a''}$, H_4

Fig. 5.



Compound	R1	R2	R3	R4
M21	-H	-CH3	-CH3	-C1'' β-D-Xyl
M22/M29	-H	-H	-CH3	-H
M24	-H	-CH3	-CH3	-H
M25	-H	-H	-H	-H

Fig. 6.

(Harnischfeger, 2000). The isolated compounds were able of intracellular ROS scavenging, this being an interesting property, to project their possible effects in more complex biological models. Moreover, ellagic acid and its derivatives were promising inhibitors on inflammatory enzymes with potential to regulate inflammatory processes, particularly in the skin, caused by diverse external agents. However, other studies should be conducted to observe the mechanism of inhibition exerted on the enzymes involved in this work.

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Glossary

AIF: pool of anti-inflammatory fraction;
AOF: pool of antioxidant fraction;
ATCC: American Type Culture Collection;
BAW: mobile phase for TLC, *n*-BuOH/HOAc/H_2O (4:1:5);
CC: chromatography in column;

DCF: oxydized DCF;
DCFH: DCF;
DCFH-DA: 2,7dichlorodihydrofluorescein diacetate;
ECM: extracellular matrix of mammalian;
EM: extracted material;
ESI: electro-spray ionization is a source in order to obtain ions;
HA: hyaluronan or hyaluronic acid;
HMBC: heteronuclear multiple-bond correlation, is a 2D NMR spectrum;
HSQC: heteronuclear single-quantum correlation, is a 2D NMR spectrum;
Hyal: hyaluronidase;
ID: internal diameter;
iNOS: inducible nitric oxide synthase;
LPIA: lipid peroxidation inhibitory activity;
M1: mobile phase for TLC, $PhCH_3$/EtOAc/MeOH/HCOOH (3:4:2:1);
MF: methanolic fraction;
NO: nitric oxide;
PBS: phosphate buffer saline;
Q-TOF: quadrupole-time of flight analyzer of mass spectrometry;
R*f*: front relation, TLC parameter to indentify spots;
ROESY: Rotating-frame Overhauser Spectroscopy, is a 2D NMR spectrum;
ROS: reactive oxygen species;
RP-HPLC: reverse phase high pressure liquid chromatography;
RT: retention time, HPLC parameter to identify peaks;
Rx: reaction;
t-BuOOH: *tert*-butylhydroperoxide;
TLC: thin layer chromatography;
UV–vis: ultraviolet–visible wavelength range;
λ_{max}: maximal wavelength.