Food Chemistry 126 (2011) 395–404

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Isolation of antibacterial components from infusion of *Caesalpinia paraguariensis* bark. A bio-guided phytochemical study

M.A. Sgariglia, J.R. Soberón, D.A. Sampietro, E.N. Quiroga, M.A. Vattuone *

Cátedra de Fitoquímica, Instituto de Estudios Vegetales "Dr. A.R. Sampietro" , Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 471, 4000 San Miguel de Tucumán, Argentina

ARTICLE INFO

Article history:
Received 30 July 2009
Received in revised form 27 September 2010
Accepted 25 October 2010

Keywords:
Caesalpinia paraguariensis
Bark infusion
Antibacterial activity
Toxicity
Bio-guided study
Ellagic acid
3-*O*-methylellagic acid

ABSTRACT

The antimicrobial activities and toxicity of infusion, decoction and tincture of *Caesalpinia paraguariensis* Burk. bark (CPBEs) were investigated to validate its traditional use as drink additive and to identify microbicidal component(s). The minimum inhibitory concentrations (MIC) of CPBEs against aerobic bacteria (Gram-negative and Gram-positive species) were determined using standardised dilution methods. The LC_{50} were determined by Brine Shrimp Test. CPBEs showed bacteriostatic and bactericidal activity against tested strains. The highest activity was observed for infusion (MIC:200 μg/mL) against *Morganella morganii*, *Erwinia carotovora*, *Bacillus* spp., *Staphylococcus spp.* and *Enterococcus spp.* The bacterial species were susceptible to CPBEs (MIC:200–1993 μg/mL) at lower concentration than sodium benzoate, a known food preservative. Two bioactive components were isolated from liophylised infusion by bio-guided chromatographic procedures; these were identified by spectrometric techniques as ellagic and 3-*O*-methylellagic acids. This study demonstrated that *C. paraguariensis* bark infusion it is safe for human consumption and a possible source of food natural preservatives.

1. Introduction

Natural sources (mainly plants) have provided products for food preservation and the primary healthcare of every known culture (Balandrin, Kinghorn, & Farnsworth, 1993). Although utilised for thousands of years as condiments, medicines, fragrances, dyes, and ornaments, scientific information associated with the growth and use of this group of plants is limited. For some decades there has been an increasing awareness that the significance of aromatic and medicinal plant studies go beyond mere anthropological curiosity.

Spoilage of foods due to the contamination with bacteria and fungi has attracted people attention for decades because it causes considerable loss worldwide. The emergence of food-borne pathogens has lately become a major public health concern. Many of these microorganisms have developed multiple drug resistance and infections were turned in one of the main causes of illness and mortality around the world (Hamill et al., 2003; World Health Organization, 1998). As many synthetic drugs with some degree of toxicity are used as food preservatives (Boberg, Taxvig, Christiansen, & Hass, 2010; Pérez Martín et al., 2010), the demand for innocuous preservatives has been growing, together with the awareness and reports on harmful effects of the use of synthetic compounds added to food preparations.

Most plants produce many compounds that are biologically active (antimicrobial, allelopathic, antioxidant and bio-regulatory properties), either as part of their normal program of growth and development or in response to pathogen attack or stress. Consequently on antimicrobial activities, mode of action, and potential uses of plant derivatives have regained momentum. Then, there arises a need to study traditionally used plants in searching of bioactive compounds with the described properties.

Caesalpinia paraguariensis (D. Parodi) Burkart (Fabaceae), popular name "Guayacán", is a tree that can attain 18 m in height and 1 m in diameter of the trunk; with a thin bark (2–3 mm of thickness) that detaches and falls every year. It is a subtropical species that spontaneously grows in the "Chaqueña" region of Northern Argentina, this tree is used as dietary supplement and for traditional treatment of a variety of ailments. Today, rural populations use to take it as infusion, adding ritidome pieces to the traditional beverage called "mate" (a drink prepared as infusion of *Ilex paraguariensis* leaves and twigs, commonly named "yerba mate"). This species is an important source of wood and forage for livestock; but it is an endangered species in the region due to uncontrolled logging (WCMC, 1996).

There is only one study on the structural elucidation of constituents of *C. paraguariensis* aerial parts (leaves and twigs) and antibacterial activity (Woldemichael, Singh, Maiese, & Timmermann,

* Corresponding author.
E-mail addresses: sampietro@tucbbs.com.ar (D.A. Sampietro), mvattuone@fbqf.unt.edu.ar (M.A. Vattuone).

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doi:10.1016/j.foodchem.2010.10.104

2003), but this study was not carried out through a bio-guided procedure, nor popular extractive forms were used.

The purposes of our study were to investigate antibacterial activity of extracts of *C. paraguariensis* bark, to compare the antibacterial potency of extracts (originals and partially purified fractions) with substances currently used for food conservation, to isolate and identify microbicidal component(s) from the bioactive fraction(s), and to assess their possible toxicity through the purification process.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade purity. NaCl was from Cicarelli Labs. (Argentina), $CaCl_2$, coumarin, glycerol, $MgCl_2$, $FeCl_3$. $6H_2O$, diphenylboryloxyethylamine (NP), Na_2HPO_4, NaH_2PO_4, CH_3COONa. $3H_2O$ (sodium acetate), sodium dodecyl sulphate (SDS), triethanolamine (TEA), bovine serum albumin (Fraction V), tannic acid, gallic acid, chlorogenic acid, caffeic acid and (+)catechin were from Sigma Aldrich (St. Louis, MO, USA). Ciprofloxacin (hydrochloride) I.V. infusions (Roemmers Labs., Argentina). Streptomycin sulphate (Rontag Labs., Argentina). Folin–Ciocalteu reagent, polyethylenglycol 4000, Silica Gel 60 F_{254} plates, cellulose 60 G plates, ellagic acid and 3-(4, 5-dimethylthiazol-2-*yl*)-2,5-diphenyltetrazolium bromide (MTT), sodium benzoate were from Merck (Germany). Difco bacto agar was purchased from Difco (Detroit, MI, USA), brain heart infusion (BHI) and Mueller Hinton (MH) from Britania Labs. (Argentina). Glacial acetic, hydrochloric and formic acids, chloroform, sulphuric ether, methanol and ethyl acetate were from Cicarelli Labs. Dimethyl sulphoxide (DMSO) was from Merck (Darmstadt, Germany). Toluene was from Biopack (Argentina). Amyl alcohol, *ter*-butyl alcohol, and methanol and acetonitrile (ACN) of HPLC grade were from Sintorgan (Argentina). Millipore membrane filters (pore size 0.22 μm) were purchased from Millipore Corp. (Bedford, MA, USA); Nylon membrane filters (pore size 0.45 μm) were purchased from Pall Corp. (Michigan, USA); sterile polystyrene 96 well microplates (Biopack, Argentina); Sephadex LH-20 (Amersham Biosciences, Sweden); Whatman N°1 chromatographic paper.

2.2. Plant material

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

2.3. Preparation of plant extracts

Aqueous (infusion, decoction) and alcoholic extracts (tincture) were prepared from *C. paraguariensis* ritidome coarse powder. Aqueous extracts were prepared at 10% (w/v) with distilled water, and tincture at 20% (w/v) with 96% (v/v) ethanol. Extracts were filtered through Whatman N° 1 filter paper, centrifuged at 7155 × g, at 4 °C for 10 min, and the clarified extracts were concentrated (aqueous extracts were lyophilized, tincture was dried under vacuum). Dried extracts were weighted in an analytical balance (Mettler, model H54AR). The weight of dried extracts represented the extracted material (EM) for each extract. Extracts were stored at −20 °C until use.

For antimicrobial assays, aqueous extracts and antibacterial substances were sterilized by filtration through Millipore membranes (0.22 μm pore size). For MIC determination of tincture by broth microdilution the solvent (ethanol 96%) was changed by 0.2% (v/v) aqueous DMSO.

2.4. Bioassays

2.4.1. Assays for antibacterial activity

2.4.1.1. Microorganisms. Gram-negative bacteria: *Acinetobacter freundii* (IEV303)*; *Enterobacter cloacae* (IEV302)*; *Escherichia coli* (IEV301)*; *Escherichia coli* 0157-H7 (ATCC 43895); *Klebsiella pneumoniae* (IEV310)*; *Morganella morganii* (IEV320)*; *Proteus mirabilis* (IEV304)*; *Pseudomonas aeruginosa* (IEV305)*; *Salmonella typhimurium* (IEV415); *Serratia marcescens* (IEV313)*; and Gram-positive bacteria: *Bacillus cereus* (IEV404); *Bacillus subtilis* (ATCC 6633); *Enterococcus faecalis* (IEV208)***; *Enterococcus faecium* (IEV229)***; *Listeria monocytogenes* (ATCC 35152); MRCN *Staphylococcus aureus* (IEV20)**; *Staphylococcus aureus* (IEV7)**; were used (*): resistant to Imipenem and Cefotaxime; (**): resistant to Oxacillin and Cefotaxime; (***): resistant to Ampicillin and Vancomycin (Soberón, Sgariglia, Sampietro, Quiroga, & Vattuone, 2007).

Phytopathogenic species were from Colección Española de Cultivos Tipo (CECT): *Pseudomonas syringae* (CECT 4429), *Pseudomonas corrugata* (CECT 124), *Erwinia carotovora* var. carotovora (CECT225) and *Xanthomonas campestris* (CECT 4480).

Strains from the American Type Culture Collection (ATCC): Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853); and Gram-positive bacteria: *Enterococcus faecalis* (ATCC 29212); *Staphylococcus aureus* (ATCC 29213); were included for quality control as recommended by the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards (NCCLS), 1999a). Stock bacterial cultures were made in BHI medium added with 0.3% (w/v) agar, 1.5% (v/v) glycerol, and stored at −20 °C in sterile Eppendorf tubes. *Pseudomonas* spp. was kept in sterile water supplemented with 1% (v/v) glycerol and stored at room temperature (Liao & Shollenberger, 2003).

2.4.1.2. Inoculum preparation. Stock bacterial cultures were streaked on an MH agar plate, and incubated at 37 °C for 24 h. Inocula were prepared by colony suspension from those plates in sterile saline solution (0.9% w/v) up to reach a 0.09 ± 0.01 optical density (DO) at 625 nm, (10^8 colony forming units (cfu)/mL is 0.5 McFarland scale). For the agar dilution assays suspensions of 10^7 cfu/mL (working bacterial suspensions) were prepared.

2.4.1.3. MIC and MBC determinations

2.4.1.3.1. Agar dilution assay (Macrodilution). Serial dilutions of each extract (12–3780 μg EM/mL) in sterile water (for aqueous extracts) or 96% ethanol (for tincture) were prepared. One mL of each extract dilution was incorporated to 9 mL of molten MH agar, vigorously vortexed, dispensed into Petri dishes (90 mm in diameter), and left to stand until solidification. Two microlitres of each working bacterial suspension (2×10^4 cfu) were punctually inoculated on the surface of the agar in different places. Bacterial growth controls and solvent controls were made. Plates were incubated at 37 °C for 18–24 h, and bacterial growth was determined on each plate by comparing the punctual growth zones with those in the controls.

2.4.1.3.2. Broth microdilution assay (Microdilution). Briefly: sterile polystyrene 96 well microplates were added with serial dilutions

of the plant extract (30–3780 μg EM/mL) or reference antibacterials (0.064–128 μg/mL), 50 μL of bacterial suspension (5. 10^5 cfu/mL) in MH broth medium supplemented with $CaCl_2$ (25 mg/L) and $MgCl_2$ (12.5 mg/L) sterile solutions (Andrews, 2001; National Committee for Clinical Laboratory Standards, NCCLS, 1999b, currently CLSI) in a final volume of 100 μL. Bacterial growth controls and sterility controls were performed. The control of the interference of plant extract color was made in wells containing the incubation mixture without bacterial inoculation (Soberón, Sgariglia, Sampietro, Quiroga, & Vattuone, 2007). Microplates were incubated for 18–24 h at 37 °C. Each experiment was performed per quadruplicate and repeated twice. MIC was defined as the lowest concentration of a plant extract or antibacterial substance that inhibits bacterial growth compared with control growth, in experimental conditions.

2.4.1.3.3. MBC determination. MBC values were determined taking into account the MIC values. Briefly: aliquots of 25 μL taken from wells without bacterial growth (including MIC), diluted much as was needed to achieve accounting cfu values (⩽ 200 cfu), and inoculated on MH agar plate. Aliquots of growth control wells of each bacterial species were also diluted and sub-cultured. Plates were incubated 18–24 h at 37 °C. The MBC was defined as the lowest concentration of a plant extract or antibacterial substance able to kill most of the microorganisms with 99.9 of effectiveness compared with control growth. Each experiment was performed per duplicate and repeated twice.

2.4.1.3.4. Quality control of the assays. Microdilution assays were controlled by comparison with the action of an antibiotic drug: ciprofloxacin is a synthetic antibiotic belonging to the fluoroquinolones, acts like a broad-spectrum bactericida against Gram-negative bacteria, mainly *Entherobacteriaceae* and some Gram-positive species such as *Staph. sp*. (Florez, Armijo, & Mediavilla, 2004). MIC values obtained with commercial drugs against ATCC strains were confronted with those published by Andrews (2001).
2.4.1.3.5. Bio-autography. Briefly, aliquots (25.50 and 100.00 μg of each fraction) were punctually placed on sterile TLC plates (Silica gel 60 F_{254}, 3.5 × 7 cm), also the sample sowed in others plates were developed by one-dimentional TLC with the solvent system toluene/ ethyl acetate/ methanol/ formic acid (3:4:2:1, v/v/v/v). Plates with dried sample were overlaid with a semi-solid culture medium inoculated with an overnight culture of *Staph. aureus* (ATCC 25923). The plates were incubated at 37 °C for 24 h, and then sprayed with an aqueous sterile solution of 2.5 mg/mL MTT in sterile 10 mM sodium phosphate buffer, pH 7.0, 0.15 M NaCl. The color of the tetrazolium salt solution changes from yellow to intense blue coloured formazan by dehydrogenases of living microorganisms (Homans and Fuchs, 1970; Sgariglia, Soberón, Sampietro, Quiroga, & Vattuone, 2009,chap.12). The R_fs of the inhibition areas were compared with the R_fs of the bands on the reference TLC plates (duplicate).

2.5. Toxicity assay

Artemia salina L. assay was used for toxicity determination of plant extracts and synthetic drugs (Meyer et al., 1982). Briefly, brine shrimp eggs hatch by incubation in 3.8% NaCl at 25 °C under a lamp (2000 Lux). Ten larvae were placed in vials containing sea water and increasing concentrations of plant extracts (between 25 and 4800 μg of EM/mL) or commercial antibiotics (between 50 and 1000 μg/mL) in a final volume of 5 mL. Ethanolic or methanolic extracts were dried and the residue solubilised in 0.2% (v/v) DMSO in water. Controls were made in vials containing 5 mL of sea water and a maximum of 250 μL of 0.2% (v/v) DMSO, and without it for aqueous extracts. These vials were exposed to the same light conditions and controlled temperature (20–30 °C). After 24 h, survivors were counted and the 50% lethal concentration (LC_{50}) values estimated using the Probit statistical method by Finney computer program.

2.6. Bio-guided fractionation of infusion components

2.6.1. Fractionation by extraction 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 of increasing polarity (ethyl ether, chloroform, and methanol). Three main fractions were obtained, dried, weighted and then dissolved in 100% methanol. Each fraction was analysed by TLC (Silica gel 60 F_{254}) and tested for antibacterial activity by bio-autography.

2.6.2. Sephadex LH-20 column chromatography (CC)

100 mg of methanolic fraction (MF) was loaded on a column (66 x 2.0 cm) packed with Sephadex LH-20 (particle size: 27–160 μm) and eluted with 100% methanol. Fractions (4 mL each) were collected, their absorbance read at 280 nm (Wettasinghe, Shahidi, & Amarowicz, 2002), and analysed by TLC (Silica gel 60 F_{254}). Twelve (I-XII) pools were made. Their solvent was evaporated under vacuum at 40 °C. Fractions were analysed by TLC and bio-autography.

2.6.3. Isolation of compound(s) by liquid chromatography (LC)

The bioactive fractions pool (IX-XI: antibacterial fractions, ABF) from Sephadex LH-20 CC was analysed by reverse phase high pressure liquid chromatography (RP-HPLC) on a gradient HPLC 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 x 4,6 mm) *Phenomenex* column (Torrance, California, USA) at 25 °C was used to separate components. The elution gradient was performed with solvent A (1% v/v formic acid in water) and solvent B (1% v/v formic acid in a mixture water-acetonitrile 1:1 v/v): 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 0.7 mL/min. The retention times were registered. The fractions corresponding to each peak were collected, dried by lyophilisation and then dissolved in methanol or DMSO for further experiments.

In order to obtain sufficient quantity of material, multiple injections of ABF were carried out employing a semi-preparative *IB-SIL* 5 C18 column (5 μm, 250 × 10 mm ID) from *Phenomenex*, and a *Rheodyne* injector fitted with a 500-μl loop. Adjusting the flow rate at 2.8 mL/min the chromatogram viewed at 254 nm was reproducible. Fractions corresponding to peaks were collected, dried by lyophlization and dissolved in DMSO. The purity of all the isolated compounds was verified by analytical HPLC experiments.

2.7. Chemical analysis

2.7.1. Phenolic content determination

Total phenolic content of bark extracts was determined by the Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventos, 1999) using coumarin (0–50 μg/mL) as standard. Results were expressed as quantity equivalent of coumarin.

2.7.2. Determination of tannin content

Tannin content of infusion and methanolic fractions (1 mg EM/mL) was evaluated according to the spectrophotometric method by Hagerman and Butler (1978) with modifications. The standard curve was carried out with tannic acid (standard solution 1 mg/mL). Absorbance at 510 nm was read in a Beckman DU 650 Spectrophotometer. The average A_{510} of triplicate samples of SDS-TEA solution (1% (w/v) and 5% (v/v), respectively) plus ferric

Table 1
MIC and MBC values of *Caesalpinia paraguariensis* bark extracts (CPBEs) and commercial preservatives against several bacterial species.

Mircoorganisms	Infusion		Decoction		Tincture		Ciprofloxacin		Na Benzoate		Streptomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	μg *CPBE*/mL						μg/ml					
Gram-negative												
Morganella morganii (IEV 320)*	200	1500	249	997	410	810	/	/	/	/	/	/
Escherichia coli (IEV 301)*	1500	1500	1993	–	3250	3250	/	/	5120	10240	/	/
Enterobacter cloacae (IEV 302)*	1500	1500	997	997	810	3250	/	/	/	/	/	/
Acinetobacter freundii (IEV 303)*	1500	1500	997	997	200	810	/	/	/	/	/	/
Proteus mirabilis (IEV 304)*	1500	1500	997	997	410	1620	/	/	2560	2560	/	/
Pseudomonas aeruginosa (IEV 305)*	1500	1500	1993	–	–	–	/	/	5120	5120	/	/
Klebshiella pneumoniae (IEV 310)*	3000	3000	1993	1993	1620	1620	/	/	/	/	/	/
Serratia marcescens (IEV 313)*	1500	1500	997	997	3250	3250	/	/	/	/	/	/
Salmonella typhimurium (IEV 415)	3000	3000	1993	1993	1620	1620	/	/	>10240	/	/	/
Pseudomonas aeruginosa (ATCC 27853)	800	800	–	–	3250	3250	0.25	0.25	5120	5120	/	/
Escherichia coli (ATCC 25922)	3000	–	1993	–	1620	1620	0.012	0.12	5120	5120	/	/
Escherichia coli 0157-H7 (ATCC 43895)	3000	–	1993	–	1620	1620	0.012	0.12	10240	10240	/	/
Erwinia carotovora (CECT 225)	200	200	249	997	410	810	/	/	/	/	2	8
Pseudomonas syringae (CECT 4429)	200	200	249	249	410	410	/	/	/	/	1	16
Xantomonas campestris (CECT 95)	200	200	249	997	410	410	/	/	/	/	1	16
Pseudomonas corrugata (CECT 124)	800	1500	249	997	410	–	/	/	/	/	2	8
Agrobacterium tumefaciens (CECT 472)	400	1500	249	997	1620	1620	/	/	/	/	1	4
Gram-positive												
Bacillus subtilis (ATCC6633)	1500	1500	997	997	1620	1620	0.03	0.12	10240	10240	/	/
Bacillus cereus (IEV404)	200	400	249	997	410	810	32	64	>10240	/	/	/
Staphylococcus aureus (IEV 20)**	800	1500	997	-	810	1620	0.06	0.5	>10240	/	/	/
Staphilococcus aureus (IEV 7)**	200	400	249	997	410	810	0.12	0.5	>10240	/	/	/
Staphylococcus aureus (ATCC 29213)	200	800	249	249	410	410	0.25	2	10240	10240	/	/
Staphylococcus aureus (ATCC 25923)	200	800	249	1993	410	1620	0.5	0.5	5120	5120	/	/
Enterococcus faecali (IEV 208)***	1500	1500	997	997	810	810	2	2	/	/	/	/
Enterococcus faecium (IEV 229)***	1500	1500	997	997	1620	3250	0.25	2	/	/	/	/
Enterococcus faecali (ATCC 29212)	1500	1500	997	997	810	3250	1	2	5120	5120	/	/
Listeria monocytogenes (ATCC 35152)	1500	1500	997	997	810	3250	1	2	5120	5120	/	/

References: MIC is minimal inhibitory concentration; MBC is minimal bactericidal concentration; (–): inactive at the highest tested concentration of CPBE used, (/): non determinate.

chloride reagent (0.01 M $FeCl_3$ in 0.01 M HCl) was subtracted from the A_{510} of each sample to correct for background absorbance.

2.7.3. Thin Layer Chromatography (TLC)

2.7.3.1. One-dimensional Silica gel TLC. Methanol fractions were loaded onto analytical TLC plates, and developed using toluene/ ethyl acetate/ methanol/ formic acid (3:4:2:1, v/v/v/v) as the mobile phase. After drying, bands were located by viewing under short (254 nm) and long (365 nm) UV radiation. 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 alcaloids, and Lieberman-Bouchard reagent for steroidal nuclei. All reagents were prepared according to Markham (1982) and Wagner, Blat, and Zgainski (1996). Standard compounds were used as positive reagent controls: (+) catechin, gallic acid and quercetin with phenolic sprays, quinine with Dragendorff reagent, cholesterol with Lieberman-Bouchard.

Table 2
Lethal concentration (LC_{50}) values of CPBEs, purified fractions and antibacterial drugs (μg/mL) determined by brine shrimp cytotoxicity assay.

Antibacterial substances	LC_{50} (μg/mL)	MIC (μg/mL)	LC_{50}/MIC
Infusion	2570.4 ± 0.5	200 ± 0.50	2.85
Decoction	1880.6 ± 0.9	249 ± 0.40	7.55
Tincture	<162.0 ± 0.5	410 ± 0.50	0.40
Methanolic fraction (MF)	966.1 ± 0.5	75.0 ± 0.10	9.30
Antibacterial fractions (ABF)	1992.3 ± 0.5	50.0 ± 0.05	9.70
Ellagic acid	>1000.0 ± 0.5	8.0 ± 0.05	125.00
3-*O*-metyl ellagic acid	>1000.0 ± 0.5	32.0 ± 0.05	31.25
Streptomycin	>1000.0 ± 0.5	0.25 ± 0.05	4000.00
Ciprofloxacin	>1000.0 ± 0.5	0.50 ± 0.04	2000.00
Sodium benzoate	<50.0 ± 0.3	5120.00 ± 0.30	<0.01

References: > or <: above or below concentrations values which were not tested. The MIC values correspond to suceptibility test for *Staph. aureus* ATCC 25923.

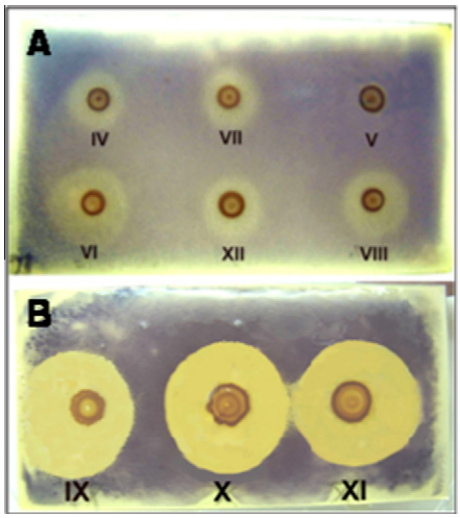


Fig. 1. Antibacterial activity of fractions (I-XII) analysed by dot-blot bio-autography. Fractions (25 μg of each) were punctually sowed on the plate and dried. Plates were overlaid with a semi-solid culture medium inoculated with an overnight culture of *Staphylococcus aureus* (ATCC 25923). The plate was incubated at 37 °C for 24 h, and then sprayed with a sterile solution of MTT. Bacterial growth inhibition is observed as pale zones (yellow) on a coloured background (blue) of formazan. **A:** Fractions IV–VIII and XII. **B:** Fractions IX–XI. (For interpretation of references to colors in this figure legend, the reader is referred to see the web version of this article.)

2.7.3.2. Two-dimensional cellulose TLC. Antibacterial fractions (ABF) from Sephadex LH-20 were analysed by ascending two-dimensional cellulose chromatography (6.5 × 6.5 cm plates) with *ter*-buthyl alcohol/acetic acid/water, 3:1:1 (v/v/v) TAW) in the first direction and 15% (v/v) aqueous acetic acid in the second. After drying the plate was viewed under UV lamp at 254 and 365 nm and after spraying with NP/PEG reagent. Standard compounds were used as positive reagent controls: *(+)* catequin, gallic acid, ellagic acid, chlorogenic acid, caffeic acid and kaempferol.

2.7.4. UV–Visible Spectroscopy

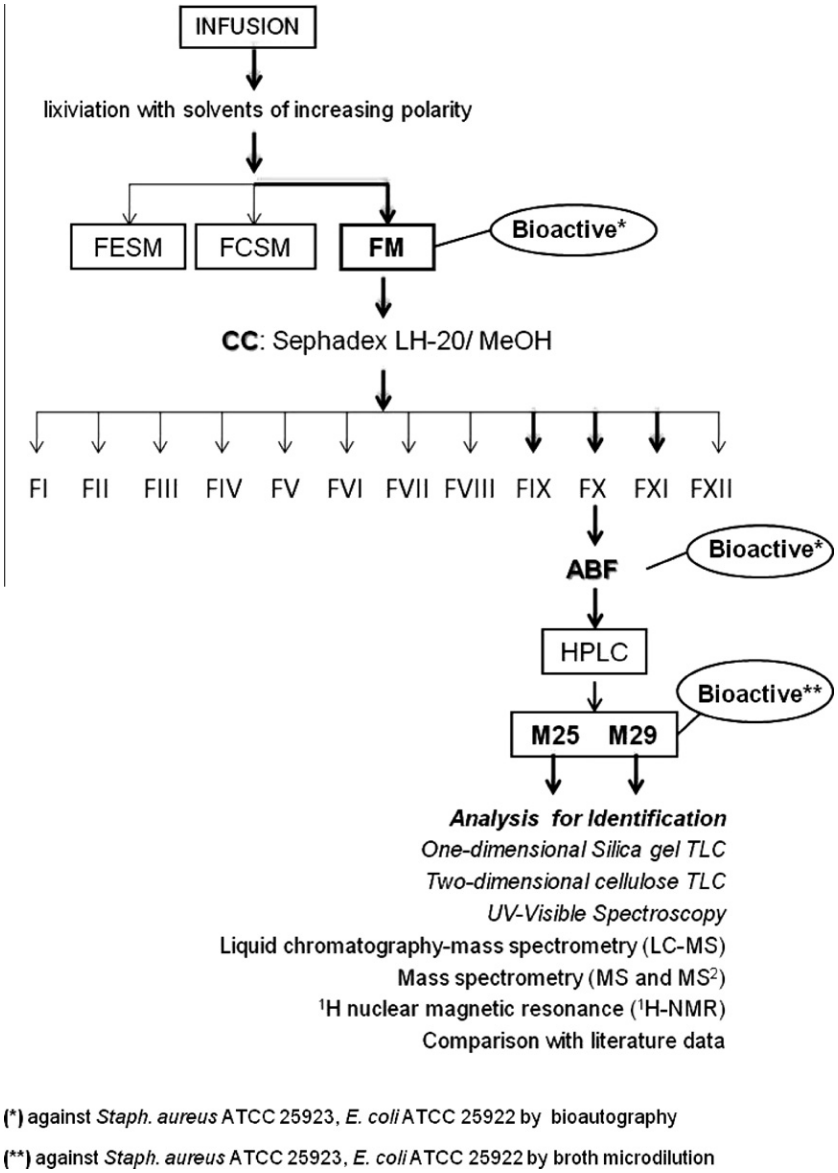
Bioactive components from ABF were scraped from TLC plates and extracted with 100% methanol. UV–VIS spectra were scanning between 200 and 500 nm on a Becknam DU 650 spectrophotometer. UV–VIS spectra of standard drugs (0.1 mg/mL) also made when necessary, in the same experimental conditions.

2.7.5. Liquid chromatography–mass spectrometry (LC–MS)

Bioactive purified compounds isolated by RP-HPLC were analysed in a LC system is comprised of an Agilent 1100 Series HPLC with 100-position auto-sampler, diode array detector (DAD) coupled to a QSTAR (Applied Biosystems) with Q-TOF hybrid mass spectrometer, with electrospray (ESI) ion source conducted in both positive and negative ion modes. HPLC employed a binary pump system and a C18 (5 μm, 150 x 4.6 mm) *Agilent* column. Mobile phases consisted of 0.1% formic acid in water (phase A) and 0.1% formic acid en methanol (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). 40 μL of samples at 0.5 mg/mL concentration were injected in each experiment. To obtain the UV absorption spectrum for each HPLC peak, this was sampled at the start, maximum and the end of the HPLC peak.

2.7.6. Mass spectrometry (MS)

The purified substances were analysed by mass spectrometry with ESI ion source employing a direct sample introduction technique by an outfitted integrated syringe pump on a QSTAR Elite is a high performance quadrupole time-of-flight (QqTOF) mass spectrometer, conducted in both positive and negative ion modes to obtain the accurate mass of analytes. The system analysed a



Scheme 1. Bio-guided purification scheme for the isolation of ellagic acid and 3-*O*-ellagic acid from the infusion (aqueous extract) of the bark of *C. paraguariensis*.

mass range of m/z 6000 Da, resolution at 8000 FWHM and accuracy at 5–10 ppm. It includes a Turbo Ion Spray probe with a flow rate range from 5 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, and was maintained at room temperature. The software package includes Analyst® QS 1.1. The mass spectrometer was scanned over an *m/z* range of 50–1500 in experiment by direct sample introduction, and 50–2000 in experiment LC–MS.

2.7.7. ^{1}H nuclear magnetic resonance ($_1$H-NMR)

NMR spectra were recorded on a Bruker DRX-500 MHz spectrometer (500.13 MHz) equipped with a QNP inverse prove at 5 mm ^{1}H/X. About 6 mg of the each sample dissolved in DMSO-d_6 was used for recording the spectra at 25 °C.

2.8. Statistical analysis

Data were expressed as mean ± standard deviation (SD) of four determinations. Lethality assays were evaluated with Finney computer program to determine the LD_{50} values with 95% confidence intervals. A copy of this program for an IBM PC is available from Dr. J.L. McLoughlin, Department of Medicinal Chemistry and Pharmacology, School of Pharmacy, Purdue University, West Lafayette, IN, USA.

3. Results and Discussion

3.1. Extracts preparations from C. paraguariensis bark

The choice of extraction conditions is a critical step for research of natural bioactive compounds (Tura & Robards, 2002). Consequently, in this study the extractive methodologies were selected according to ethnographical studies of peoples and communities of Northwestern Argentina (Scarpa, 2004). Three extractive forms (decoction, infusion and tincture) were assayed to select the best solvent and experimental conditions. Samples (40 g of stem bark powder) extracted with boiling distilled water (infusion) showed the best recovery rate (104.25 ± 4.10 mg CPBI/g of stem bark). The yields of decoction and tincture extractions were 92.25 ± 2.10 and 31.51 ± 1.50 mg CPBD and CPBT/g of stem bark, respectively.

3.2. Antibacterial activity of C. paraguariensis bark extracts (CPBEs)

Macrodilution of CPBEs in solid medium showed that the three extracts (infusion, decoction and tincture) were active against tested bacterial species in concentrations ranging from 200 up to 3250 μg of CPBE/mL of culture medium. These results guided the selection of the concentration range to test the MIC determinations by broth microdilution. The ATCC strains were used to compare MIC values for an antibiotic (ciprofloxacin) versus the reference values published by Andrews (2001). The MIC values obtained with ciprofloxacin were consistent with the reference values or differ in a dilution value, supporting the experimental conditions employed to obtain the MIC and MBC values with CPBEs. Sodium benzoate and streptomycin were used to compare the values of MIC with those obtained with plant extracts. CPBI showed MIC/MBC values more effective than sodium benzoate (SB) in the assay conditions for the assay (pH 7.2–7.4) against local bacterial isolates (IEV strains), at concentrations between 200 and 1500 μg/mL, while SB was effective from 2560 up to 5120 μg/mL (Table 1). The values of MIC/MBC obtained for CPBD and CPBT against the same species were comparable with those of CPBI.

Phytopathogenic species (*Erw. carotovora, X. Campestris, Ps. corrugata* and *Ps. syringae*) were also sensitive to the CPBEs at concentrations between 200 and 1620 μg/mL (Table 1). Streptomycin MIC/MBC values were quantitatively better than CPBEs; but streptomycin is an antibiotic used in agriculture but its application is currently restricted and was banned in some countries (Mc Manus, Stockwell, Sundin, & Jones, 2002). Moreover, the increase in number of resistant pathogenic species is an emergency because of the risk of homologous recombination with human pathogenic bacteria.

The tested bacterial species are of ample incidence in the Province of Tucumán, Argentina (Casellas et al., 2003). Many of these species may be present in foods as contaminants, and have an elevated prevalence as infective agents in the human body. *B. cereus*, a spore-forming bacteria that cause special problems for the food industry, were sensitive to CPBEs, *S. typhimurium,* usually responsible of food poisoning, was also sensitive to plant extracts. Therefore, the finding of new compounds effective against infections caused by antibiotic-resistant bacteria is very important for food conservation and health care.

3.3. Cell toxicity

The toxicity of CPBEs was evaluated according the values of LC_{50} and LC_{50}/MIC rates in the *Brine shrimp* assay. These values were compared with those of reference substances. LC_{50} values indicated that CPBI has minor toxicity than CPBD, while CPBT shows the highest toxicity (Table 2). According to the LC_{50}/MIC rate for CPBI (12.85), LC_{50} exceed ten times the MIC value for tested species, being much larger than the observed for SB, for which the inhibitory concentration coincides with the MIC value. Otherwise, streptomycin was safe at bioactive concentration. It is worth to take into account that this technique is appropriated to determine toxicity of chemical and natural products (Meyer et al., 1982) and shows good correlation with LC_{50} values reported in *in vivo* assays with mice (Lagarto Parra, Silva Yhebra, Guerra Sardiñas & Iglesias, 2001), constituting a useful tool for predicting toxicity in plant extracts.

3.4. Fractionation

3.4.1. Fractionation of lyophilized infusion

The ample spectrum of antibacterial action, the high extraction yield, the absence of toxicity evidenced by the LC_{50} and LC_{50}/ MIC

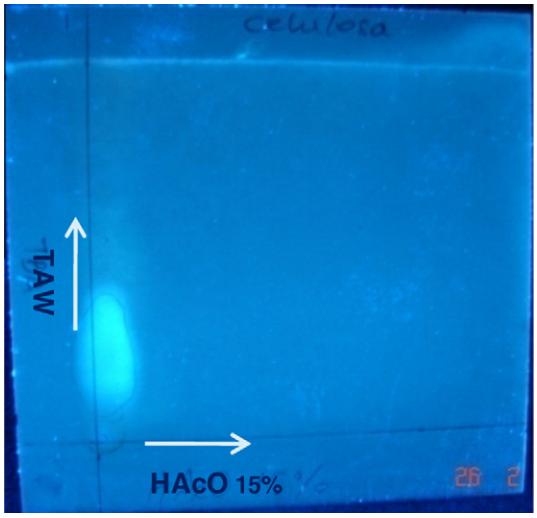


Fig. 2. Two-dimensional cellulose TLC of the M25. An aliquot of ABF was developed first in one direction with TAW as solvent system, and then in the other direction with 15% (v/v) acetic acid. The NP/PEG revealed a brilliant light spot with green edges under UV lamp of 365 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

parameters, and the traditional usage of the stem bark of the *C. paraguariensis* impelled us to select CPBI to perform a bio-guided purification of antibacterial component/s.

Lyophilized CPBI was extracted by lixiviation with solvents of increasing polarity (ethyl ether, chloroform and methanol) giving three fractions: ethyl ether fraction (EF), chloroform fraction (CF) and methanol fraction (MF) with an yield of 0.25 ± 0.02; 0.04 ± 0.01 and 72.70 ± 3.5% (w/w), respectively. Dried fractions were suspended in 100% methanol. Bio-autographic tests showed antibacterial activity in EF and MF while CF was inactive (not shown). Both active fractions presented a high content of phenolic compounds (13.87 ± 0.05 and 2.42 ± 0.05 mg/mL, expressed as equivalent amounts of coumarin for MF and EF, respectively). TLC analysis of MF and EF revealed with different reagents showed the presence of phenolic compounds and the absence of alkaloids and catequins. According to the profiles obtained with TLC (Fig. 1), EF contains phenolics and/or phenylpropanoid derivatives ($FeCl_3$, V/S, NP/PEG and UV_{365nm} light). MF showed a complex composition with high phenolic content (NP/PEG and UV_{365nm} light).

3.4.2. Tannins analysis

The origin of the extracted material (bark), extraction methodology (infusion), and the observed biocide activity, suggested the presence of tannins in the MF. This was investigated by the protein precipitation method (Hagerman & Butler, 1978), which allowed to detect, quantify (MF: 0.91 ± 0.01 μg/mL, expressed as equivalent amounts of tannic acid standard; regression factor was F = 439 ± 10) and separate them from non-tannin phenolics. Tannins were separated from the BSA soluble fraction of MF and dissolved in SDS-TEA. Both fractions were tested by dot-blot bio-autography. The antibacterial activity was present in the BSA soluble fraction. Consequently, the contribution of tannins (recognised microbicidals) to the biocide activity of MF seems to be negligible.

3.4.3. Fractionation by Sephadex LH-20

The components from MF (the most bioactive fraction) were separated by column chromatography on Sephadex LH-20 with 100% methanol (100%) as eluent. Sephadex LH-20 is recognised as a fast and successful fractionation medium, since silica gel or

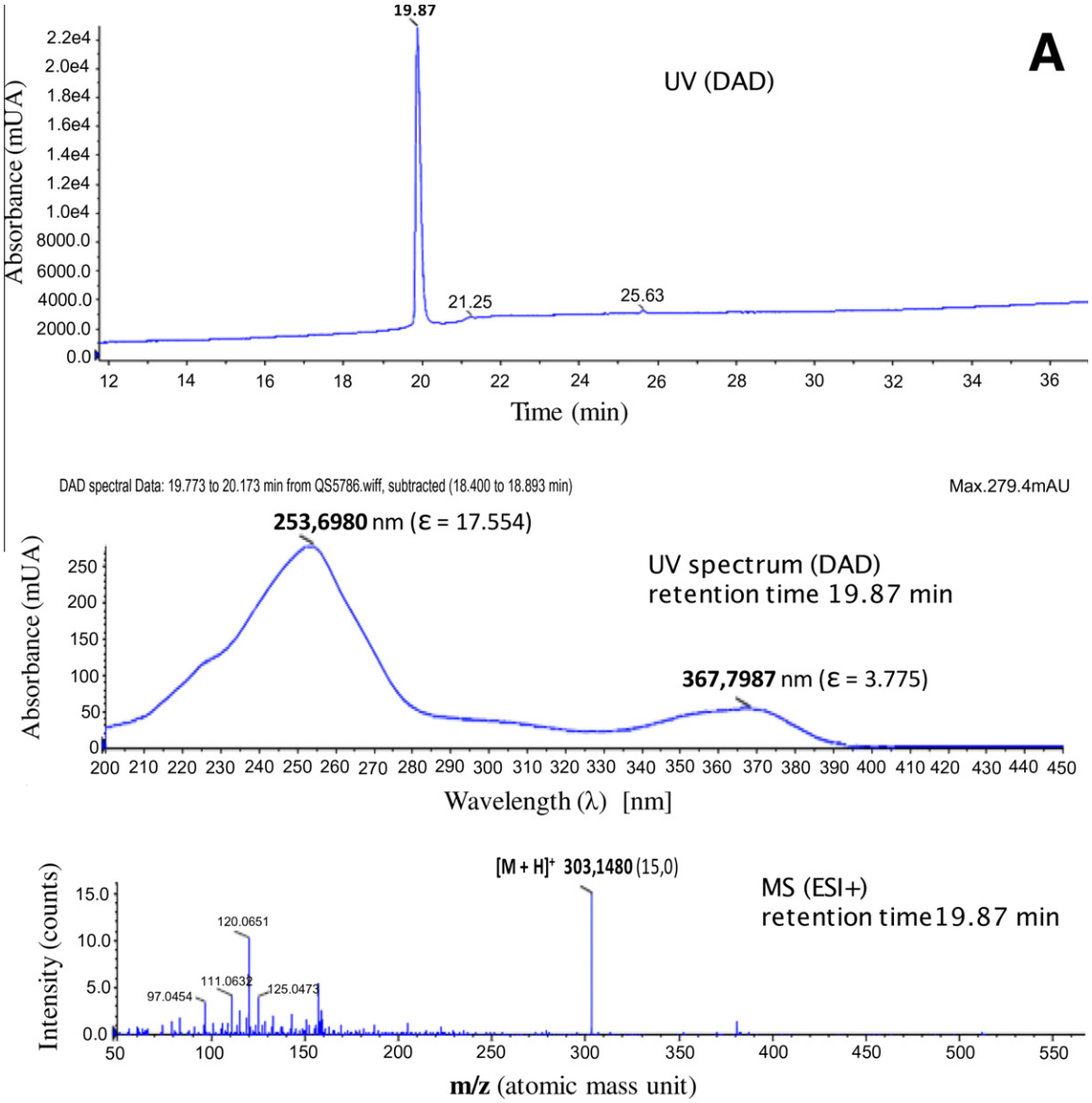


Fig. 3A. UV (DAD) and MS (ESI +) spectra from chromatographic peak at 19.87 retention time of the M25 (ellagic acid). Chromatographic conditions used were described in 2.7.5.

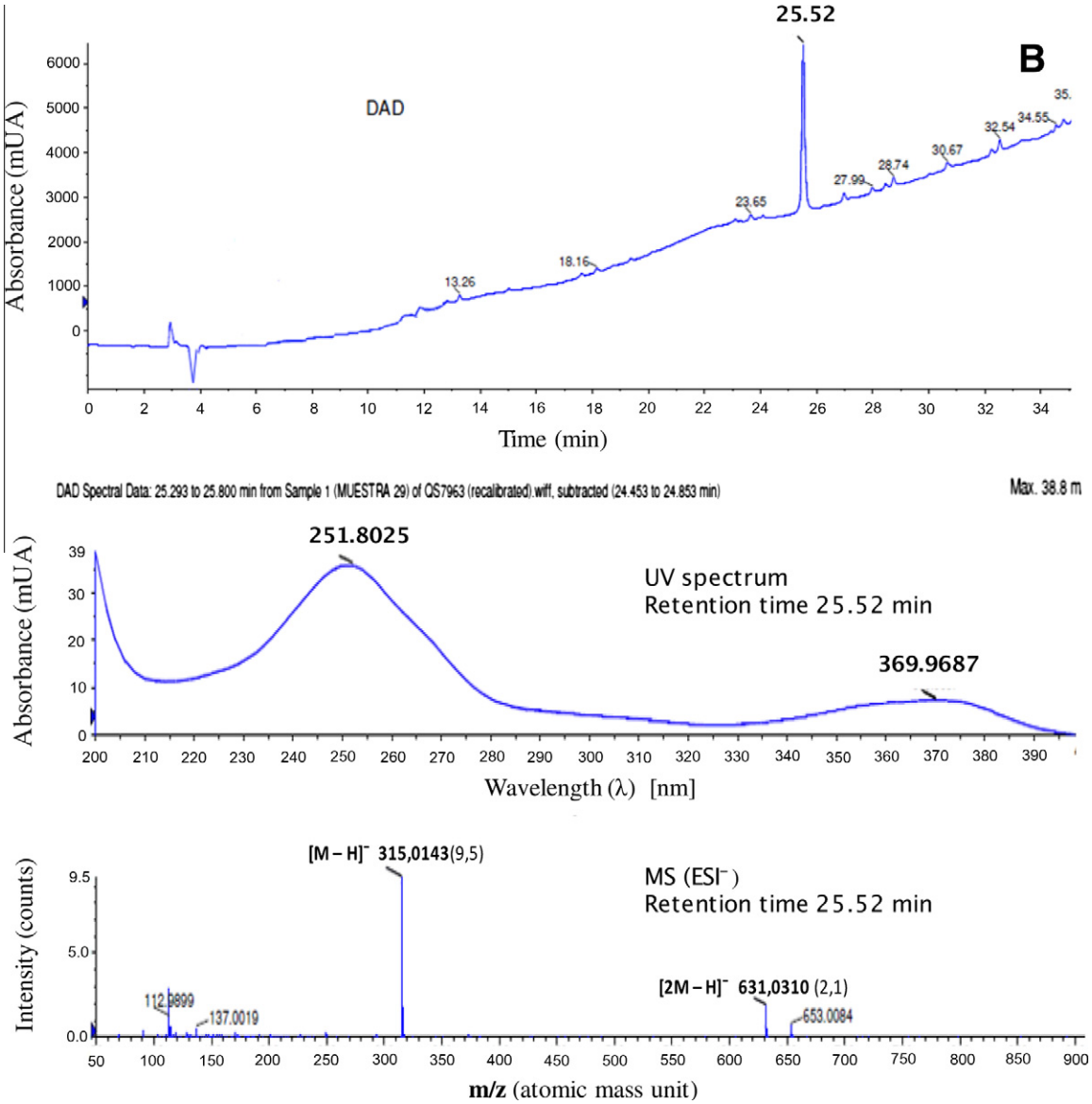


Fig. 3B. UV (DAD) and MS (ESI +) spectra from chromatographic peak at 19.87 retention time of the M29 (3-*O*-methylellagic acid). Chromatographic conditions used were described in 2.7.5.

Table 3
MIC and MBC values of *Caesalpinia paraguariensis* components and preservatives determined by broth microdilution and cell viability assay.

Microorganisms	MIC/MBC (μg/mL)			
	Ellagic acid	3-*O*-metyl ellagic acid	Sodium benzoate	Streptomycin
Gram-negative				
Morganella morganii (IEV 320)*	32/32	64/64	>10240	/
Proteus mirabilis (IEV 304)*	8/16	8/8	2560/2560	/
Salmonella typhimurium (IEV 415)	16/16	16/32	>10240	/
Escherichia coli (ATCC 25922)	16/16	32/32	>10240	/
Erwinia carotovora (CECT 225)	16/32	32/32	/	2/8
Xanthomonas campestris (CECT 95)	8/8	8/16	/	1/16
Gram-positive				
Bacillus cereus (IEV 404)	16/16	16/32	>10240	/
Staphylococcus aureus (ATCC 25923)	8/8	64/64	5120/5120	/

References: > or <: above or below concentrations values which were not tested. MIC is minimal inhibitory concentration; MBC is minimal bactericidal concentration; (–): inactive at the highest tested concentration of sample or substance used; (/): non determinate.

other similar phases are not appropriate for polar substances (Wettasinghe et al., 2002). Twelve fractions were separated on the basis of the TLC profile analysis (one-dimensional silica gel 60 F_{254}). Several fractions were bioactive by dot-blot bio-autography (Fig. 1) and all fractions revealed presence of phenolic compounds when were observed under 254 nm UV lamp, and by staining with NP/PEG spray reagent under 365 nm UV lamp.

3.5. Analysis and identification of bioactive components

Fractions IX, X and XI showed the highest bioactivity and the same TLC profiles (Scheme 1). They were pooled and labeled antibacterial fraction (ABF, Fig. 1). A brilliant white spot with green edges at R_f: 0.27–0.45, and a dark pink spot at R_f 0.8 were observed on TLC using NP/PEG and 365 nm UV lamp. The change of colour of spots at R_f: 0.27–0.45 and 0.80 sprayed with $AlCl_3$ suggested the complex formation among neighboring phenolic hydroxyl groups (not shown).

The analysis of ABF by RP-HPLC detected two peaks on the chromatogram, one major peak at retention time 25 min, and other at 29 min. The respective fractions were collected and called M25 and M29, respectively (Scheme 1). Semi-preparative RP-HPLC allowed the isolation of 10.52 mg M25 and 4.41 mg of M29 from 100 mg of ABF. Both components were active against *Staph. aureus* ATCC 25923 by bio-autography and broth microdilution. Both bioactive compounds were chemically analysed by chromatographic and spectrometric techniques, which allowed their identification:

Ellagic acid (M25): The UV spectrum showed absorption *λmáx* (MeOH) (nm) (*ε*): 255.5 (39,790), 358.0 (9182). One peak at retention time 19.87 min was detected by RP-HPLC, and the DAD allowed to obtain an UV spectrum: *λmáx* (mobile phase) (*ε*) at 253.698 (17,554) and 367.798 (3775) (Fig. 3A). The LC–MS (ESI) generated from fraction corresponding to peak at 19.87 a signal at *m/z* 303.148 from $[M + H]^+$ (Fig. 3A). Then, M25 was analysed by direct introduction (MS-ESI±) and gave following signals: ESI (-): $[M - H]^-$ *m/z* 301.14; ESI (+): $[M + H]^+$ *m/z* 303.14; $[M + Na]^+$ *m/z* 325.22. The MS^2 experiment from $[M - H]^-$ at *m/z* 301.1480 u generated the ion of 257.229 atomic mass unit (u). Accurate mass of the compound was 302.1480 u. The% molecular composition was: $C_{14}H_6O_8$ with 12 insaturations. 500.12 MHz RMN-^{1}H in DMSO-*d*6 δH (ppm): $H_{5,5'}$ 7.51 (s, 2H). On two-dimensional chromatography a dark spot was observed under UV lamp of 365 nm at R*f* (TAW): 0.27–0.45, R*f* (acetic acid): 0; the same changed to brilliant white with green edges after spraying with NP/PEG and UV observation (Fig. 2). The yield of ellagic acid in CPBI was 0.135% (w/w EM) and 141 μg/g *C. paraguariensis* stem bark.

3-*O*-metilellagic acid (M29): The UV spectrum showed absorption *λmáx* (MeOH) (nm) (*ε*): 250.2 nm (25,600), 347.0 sh, 368.0 nm (8500). One peak at retention time 25.52 min was detected by RP-HPLC, and the DAD allowed to obtain an UV spectrum: *λmáx* (mobile phase) (*ε*) at 251.80 (10,007) and 369.97 (2630) (Fig. 3B). The LC–MS (ESI) generated from fraction corresponding to peak at 25.52 a signal at *m/z* 315.0143 from $[M - H]^-$, and other of minor intensity a *m/z* 631.0310 corresponding to ion $[2\,M - H]^-$ (Fig. 3B). Then, M29 was analysed by direct introduction (MS-ESI±) and gave following signals: ESI (+): $[M + H]^+$ *m/z* 317.0289; $[M - H]^-$ m/z 315.0142; $[M - Me]^-$ *m/z* 300.9970. Accurate mass of the compound was: 316.0141 u. The% molecular composition was: $C_{15}H_8O_8$ with 12 insaturations. 500.12 MHz RMN-^{1}H in DMSO-*d*6 δH (ppm): H_5 7.51 (s, 1H), $H_{5'}$ 7.48 (s, 1H), 3-OCH_3 4.06 (s, 3H). On two-dimensional chromatography a dark spot was observed under UV lamp of 365 nm at R*f* (TAW): 0.55–0.63, R*f* (acetic acid): 0; the same changed to brilliant white with yellow edges after spraying with NP/PEG and UV observation. The yield of 3-*O*-metilellagic acid in CPBI was 0.044% (w/w ME) and 46 μg/g *C. paraguariensis* stem bark.

3.6. Cell toxicity of purified bioactive compounds

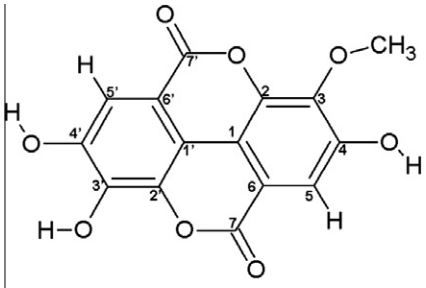
LC_{50} and LC_{50}/MIC rates of purified bioactive fractions and isolated compounds determined by *Brine Shrimp Tests* were compared with those corresponding to reference substances (Table 2). The rate LC_{50}/MIC increased during the purification (Table 2), suggesting that the pure compounds are even less toxic than the purified fractions from CPBI. The isolated compounds (ellagic and 3-*O*-metilellagic acids) showed better toxicity/bioactivity rate than sodium benzoate, the substance used as reference for control of biological food contamination.

Several species of the genus *Caesalpinia* are being used traditionally for a wide variety of properties; only some of them are known with anti-infective properties: *C. benthamiana, C. bonduc*, *C. mimosoidea*, *C. paraguariensis* and *C. pulcherrima*. *C. paraguariensis* is the only one native species from Argentina (Zuloaga & Morrone, 1999).

Only two studies were performed on the antibacterial constituents of *C. paraguariensis*. One of them analysed the organic extracts from leaves and twigs (Woldemichael et al., 2003). In the other, antibacterial activity was not found in analysed the pod (fruits) extracts (Salvat, Antonnacci, Fortunato, Suarez, & Godoy, 2001).

Other species of the genus were subjected to phytochemical investigation. Flavonoids were isolated from organic extracts of aerial parts of *C. pulcherrima* (L.) Swartz., which exhibited antibacterial activity (Srinivas et al., 2003). In *C. mimosoides* Lamk., gallic acid was found as constituent responsible of antibacterial activity (Chanwitheesuk, Teerawutgulrag, Kilburn, & Rakariyatham, 2005).

This study represents the first report on antibacterial activity and phytochemical analysis of *C. paraguariensis* bark. Ellagic and 3-*O*-methylellagic acids are reported as bioactive molecules responsible for the antibacterial activity of CPBI (the most popular



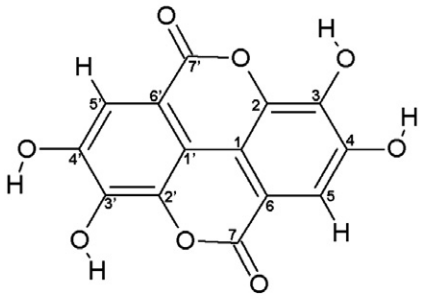


Fig. 4. Chemical structures of the isolated bioactive molecules. Note the multiple ionization potential of the phenolic hydrogens.

used preparation). The isolated polyphenols were more effective biocides than sodium benzoate against some food isolates of resistant Gram-positive and Gram-negative bacteria (Table 3). Furthermore, due to the multiple possibilities of ionisation (Fig. 4) the isolated molecules could be advantageous in terms of effectiveness in an ample pH range (Hasegawa et al., 2003). The absence of toxicity in CPBI and the isolated compounds demonstrates their safety for use as drink additive and as food preservative. Then, *C. paraguariensis* could be used as a promising source of antibacterial agents in the development of antibacterial substances, mainly for Gram-negative bacteria that are the most difficult to fight. Studies for the characterisation of other phenolic compounds, to elucidate the mechanism underlying biocide properties and the synergism among these compounds, are being carried out currently.

Acknowledgements

The research was partially supported by the Secretaría de Ciencia y Técnica of the Universidad Nacional de Tucumán (UNT), the Agencia Nacional de Ciencia y Tecnología and the Consejo Nacional de Investigaciones Científicas y Técnicas CONICET. ARGENTINA. MAS and JRS are fellowship holders from CONICET.

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