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Antimicrobial phenylpropanoids from the Argentinean highland plant *Parastrephia lucida* (Meyen) Cabrera

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ABSTRACT

Ethnopharmacological relevance: The Argentinean highland plant *Parastrephia lucida* (Meyen) Cabrera is used in traditional medicine as an antiseptic and anti-inflammatory crude drug.
Aim of the study: To relate the antimicrobial effect of the crude drug with the constituents of the active fractions and traditional use.
Materials and methods: Assay-guided isolation of the methanol (MeOH) plant extract was carried out using bacteria and yeasts as target organisms. Both ATCC and local strains were included in the study. The antimicrobial fractions and compounds were detected by bioautographic assays. Minimum inhibitory concentrations (MIC) of each extract and fraction were determined and compared with reference antibiotics. Fractions were analyzed by HPLC–DAD, GC–MS, ^{1}H NMR and ^{13}C NMR.
Results: From the MeOH extract of the plant, assay-guided isolation of the antimicrobial constituents led to 12 phenylpropanoids and two simple phenolics. Most of the compounds occurring in the active fractions were *E*-caffeoyl or *E*-cinnamoyl esters including prenyl and phenethyl derivatives. The MIC values of the most active fractions ranged between 12.5 and 200 μg/mL against reference strains and local isolates of *Staphylococcus aureus* and *Enterococcus faecalis*.
Conclusions: The antimicrobial effect found in the crude drug was associated with mixtures of phenylpropanoids, including prenyl and phenethyl esters of caffeic and cinnamic acids. The results support at least in part the traditional use of the plant as local antiseptic.

1. Introduction

Human beings living in the Andes highlands (Puna) selected the available natural resources for medicine, building, forage, firewood and as cultural elements in spiritual activities (Villagrán et al., 2003; Toursarkissian, 1980). In the traditional medicine of the Andes highlands, *Parastrephia lucida* is used to relive toothache, by applications of leaves, for bone fractures and bruises and as vulnerary (Villagrán et al., 2003). It is also used as firewood and to fed llamas and alpacas (Ayma et al., 1995). The genus *Parastrephia* (Asteraceae) comprises five species, namely *P. lucida*, *P. lephidophylla*, *P. phyllicaeformis*, *P. quadrangularis* and *P. teretiuscula* (Cabrera, 1978). *P. lucida* Meyen Cabrera, known under the common name *romero*, *tola*, *chachakoa*, *tola de rio*, *tola de agua*, is abundant in the Argentinean highlands, an environment characterized by high altitude (3000–4200 m over sea level), high exposure to ultraviolet radiation, low oxygen concentration, high daily temperature variations and mean annual rainfall of 100–200 mm or even less. Plants from these ecosystems have particular adaptations to environmental stress, including the biosynthesis of secondary metabolites with relevant pharmacological activities (Kleier and Lambrinos, 2005).

The biological activities of alcoholic and aqueous extracts of *P. lucida* reported so far include anti-inflammatory (Alberto et al., 2009), acaricide (Ayma et al., 1995), antioxidant (Zampini et al., 2008), antibacterial (Zampini et al., 2009). However, little is known on the identity of its chemical constituents. Phytochemical studies on other *Parastrephia* species afforded isofraxidin; 4-hydroxy-3-methoxypropiophenone; *p*-hydroxyacetophenone; methoxytremetone and tremetone in *P. lepidophylla* (Bohlmann et al., 1979) while 5,7-dihydroxy-3,8,3′,4′-tetramethoxyflavone; umbelliferone, [1-cinnamoyl-oxy-ethyl] benzol; 4-[1′-methoxy-ethyl]-phenol; 1, 1′-bis-[4-ethylphenol]-ether were identified in *P. quadrangularis* (Loyola et al., 1985). Scopoletin and *p*-coumaroyl-oxy

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tremetone occurs in both *Parastrephia* species (Bohlmann et al., 1979; Loyola et al., 1985; Barboza et al., 2009).

Studies on plants with antimicrobial activity and assay-guided isolation can lead to naturally occurring compounds from different structural types and skeletons. The findings can open opportunities to disclose the mechanisms involved in microbial growth inhibition, either separately or associated with synthetic conventional antimicrobials. Phenolics are a family of plant-derived compounds with potentially exploitable effects, including direct antibacterial activity, synergism with antibiotics, and suppression of bacterial virulence (Ríos and Recio, 2005; Cushnie and Lamb, 2011). These compounds can also ameliorate infection by interfering with aspects of bacterial pathogenesis, for example inhibition of quorum sensing, inhibition of enzymes (sortase, urease), neutralization of bacterial toxins, inhibition of virulence factors secretion, etc. (Cushnie and Lamb, 2011).

Some of the most problematic multidrug resistance (MDR) microorganisms currently found in hospitals include *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* bearing extended-spectrum β-lactamases, vancomycin resistant enterococci, methicillin and vancomycin-resistant *Staphylococcus aureus* (MRSA and VRSA, respectively). Some bacteria like methicillin-resistant *S. aureus* couple MDR with exceptional virulence capabilities (Miller et al., 2005; Alekshun and Levy, 2007). The majority of the mycoses-related deaths were associated with *Candida*, *Aspergillus*, and *Cryptococcus* sp. infection. More than 90% of invasive infections due to *Candida* spp. are attributed to the species: *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* (Pfaller and Diekema, 2007).

The aim of this work was to identify the antimicrobial agents from the aerial parts of *P. lucida*. A bio-guided isolation and identification was carried out using human multi-resistant Gram positive and Gram negative pathogenic bacteria and *Candida* species. In this study, the effect of the extracts and fractions was assessed on American Type Culture Collection (ATCC) strains and clinical isolates of a local hospital.

2. Materials and methods

2.1. Plant material

The aerial parts of *P. lucida* (Meyen) Cabrera were collected from January to February 2006 at 3600 m.o.s.l in Antofagasta de la Sierra, Provincia de Catamarca, Puna de Atacama. A voucher specimen no. 607923/LIL, was deposited in the Herbarium of "Fundación Miguel Lillo", Tucumán, Argentina and the plant was authenticated by Lic. Soledad Cuello. The samples were dried at 40 °C. The parts used were leaves and stems (aerial parts), according to the traditional use.

2.2. Reagents

Gallic acid (GA), 3-(4, 5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), diphenylboric acid-ß-ethylamino ester (NP), KOH were supplied by Sigma-Aldrich (Missouri, USA). Analytical grade MeOH was from Merck Argentina (Buenos Aires, Argentina). HPLC grade organic solvents were from Sintorgan Argentina (Buenos Aires, Argentina) and water was Milli Q Plus quality. For column chromatography, silica gel 60 (0.040–0.063 mm, 230–400 mesh, Merck, Darmstadt, Germany) was used. Analytical TLC was carried out using pre-coated plates (Kieselgel 60 F254, 0.2 mm, Merck, Germany).

2.3. Extraction and isolation of bioactive fractions

Dried and powdered plant material was macerated in methanol (MeOH) (250 g of dry plant material/L) for 7 days with gentle shaking or stirring (40 cycles/min) at room temperature. The methanol extract (MeOH) was filtered through Whatman no. 4 filter paper and concentrated under reduced pressure at 40 °C to obtain 75 g of a crude MeOH extract, which was stored at 4 °C until use. A liquid–liquid partition of the crude MeOH extract was performed with dichloromethane (DCM) and water to obtain two phases. The aqueous phase (AQ) was lyophilized. The DCM phase (33 g) was taken to dryness and permeated in a chromatographic column (80 cm length × 4 cm internal diameter) using Sephadex LH-20 gel as support and eluting with methanol (Henke, 1998). Some 85 drops/tube were collected and combined into seven fractions A to G, based on TLC profiles revealed with NP reagent. Fraction C (82.5 mg/g DCM fraction) was chromatographed on silica gel (column length 30 cm, internal diameter 2.5 cm) eluting with the following gradient of increasing polarity: $CHCl_3$, $CHCl_3$:EtOAc, EtOAc and EtOAc:MeOH. Fractions of 10 ml each were collected and combined into 19 pools (C I–C XIX based on TLC analysis (SiO_2, $CHCl_3$:MeOH 7:3 v/v). The fractionation is summarized in Fig. 1.

2.4. Phytochemical screening

2.4.1. Thin layer chromatography

The crude MeOH, AQ, DCM extracts and DCM fractions from the Sephadex column were analyzed by TLC. The solvent mixtures used were EtOAc:acetic acid:water (10:0.5:0.5 v/v/v) and

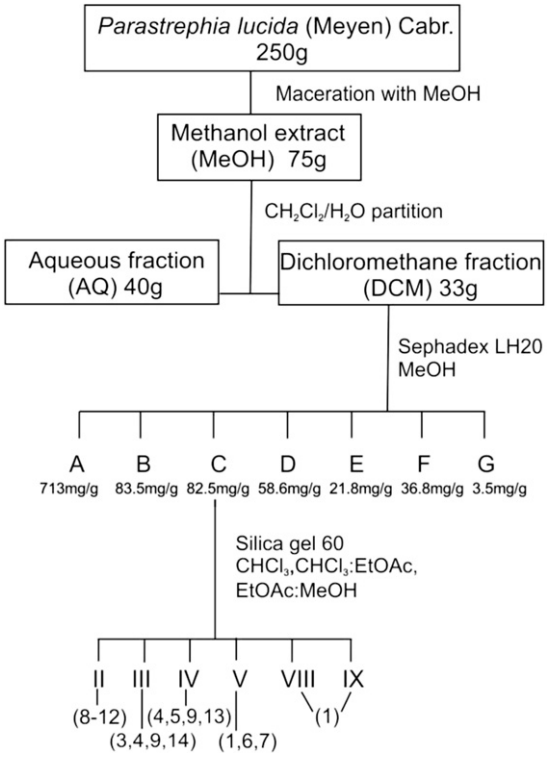


Fig. 1. Bioguided fractionation of antimicrobial compounds from *Parastrephia lucida* aerial parts. Compounds: Cinnamic acid (1), caffeic acid methyl ester (3), ferulic acid methyl ester (4), ferulic acid ethyl ester (5), 3-(4-hydroxy-phenyl)-propionic acid methyl ester (6), 3-(3,4-dihydroxy-phenyl)-propionic acid methyl ester (7), isoferulic acid prenyl ester (8), ferulic acid prenyl ester (9), methyl ferulic acid prenyl ester (10), ferulic acid 2-phenethyl alcohol (11), dimethylcaffeic acid 2-phenethyl ester (12), vanillin (13) and 4-hydroxy-3-methoxy benzoic acid methyl ester (**14**).

$CHCl_3$:MeOH (7:3 v/v). The plates were developed, dried and sprayed with 1% methanolic NP and visualized under UV light at 254 and 365 nm. Methanolic KOH was used for coumarin and Dragendorff's reagent for alkaloid detection (Wagner et al., 1984).

2.4.2. Determination of total phenolic and nonflavonoid phenolic content

Total phenolic content of the samples was determined using the Folin–Ciocalteau method (Singleton et al., 1999). Results are expressed in μg gallic acid equivalents (GAE) per milligram of dry weight (μg GAE/mg DW). Non-flavonoid phenols were measured by determination of total phenol content remaining after precipitation of the flavonoids with acidic formaldehyde according to Zoecklein et al. (1990). Results are expressed in μg GAE/mg DW.

2.5. Antimicrobial assays

2.5.1. Microorganisms

The reference microorganisms used were: *S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *E. coli* ATCC 35218 and ATCC 25922, and *K. pneumoniae* ATCC 700603. Clinical isolates of antibiotic multi-resistant *S. aureus* ($n=2$), *E. faecalis* ($n=2$), *E. coli* ($n=1$), *K. pneumonia* ($n=1$), *Proteus mirabilis* ($n=1$) and *P. aeruginosa* ($n=1$) were obtained from Hospital Dr. Nicolás Avellaneda, San Miguel de Tucumán, Tucumán, Argentina (Zampini et al., 2009). The yeasts *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parasilopsis* and *C. guilliermondii* were obtained from clinical samples of hospital del Niño Jesús, San Miguel de Tucumán, Tucumán, Argentina.

Bacterial strains were identified by the biochemical profiles according to Murray et al. (1999). All organisms were maintained in brain–heart infusion (BHI medium, Britania) containing 30% (v/v) glycerol at −20 °C. *Candida* strains were maintained at 4 °C in Sabouraud dextrose agar (Merck). For assays, individual colonies were isolated and suspended in 5 ml of 0.9% NaCl solution. The inocula were prepared by adjusting the turbidity of the suspension to match the 0.5 McFarland standards and diluted in cation-adjusted Müeller–Hinton broth (CAMHB) for bacteria and Sabouraud dextrose broth (SDB) for yeasts, in order to achieve adequate inocula in each case.

2.5.2. Bioautographic assays

The crude MeOH, AQ and DCM extracts, fractions A–G and subfractions obtained from fraction C (I to XIX) were seeded on silica gel in dot or developed by TLC using $CHCl_3$:MeOH (7:3) as the mobile phase. Then, the plates were covered with 2 mL of soft medium (BHI with 0.6% agar) containing 10^5 colony forming units (CFU) of different microorganisms. Methicillin resistant *S. aureus* (F7) and *P. mirabilis* (F304) were used as model strains. The plates were incubated at 37 °C for 16–20 h. Then, the plates were sprayed with a 2.5 mg/mL MTT solution in PBS (10 mM sodium phosphate buffer, pH 7, with 0.15 M NaCl). The yellow-colored growth inhibition areas were compared with the R_f of the related spots on the TLC plate observed under UV light and revealed with NP reagent.

2.5.3. Minimal inhibitory concentration determination

Minimum Inhibitory Concentration (MIC) of each extract and fraction was determined by the broth microdilution technique according to the guidelines of the Clinical and Laboratory Standard Institute for yeasts (NCCLS, 2002) and bacteria (NCCLS, 2006). The method was performed in sterile 96-well microplates. Samples were dissolved in 1% DMSO. The concentrations tested were 12.5, 25, 50, 100, 200 and 400 μg/mL. The inoculum (100 μL) containing 5×10^5 CFU/mL was added to each well. A number of wells were reserved in each plate for sterility control (no inocula added), inocula viability (no extract added), and solvent effect (DMSO). Plates were aerobically incubated at 37 °C for bacteria and 30 °C for yeasts for 20 h and 40 h, respectively. After incubation, microbial growth was indicated by the presence of a pellet on the well bottom. MIC was defined as the lowest concentration of extract without macroscopically visible growth. As reference, the following antibiotics were used: Ampicillin and Oxacillin for bacteria and Nystatin for *Candida* species.

2.6. Compound identification

2.6.1. HPLC analysis

The HPLC system used to analyze the DCM fractions consisted of a Waters 1525 Binary HPLC Pump system with a 1500 Series Column Heater, a manual injection valve with a 20 μL loop (Rheodyne Inc., Cotati, CA) and a Waters 2998 photodiode array detector (PDA). The column was a XBridgeTM C18 column (150 mm × 4.6 mm, 5 μm; Waters corporation, Milford, MA). The gradient solvent system used consisting of solvent A (9% acetic acid in water) and solvent B (methanol). The conditions were as follows: 25–45% B from 0 to 10 min and kept at 45% B from 10 to 20 min, 45–70% B from 20 to 40 min, 70–75% B from 40 to 50 min, 75–100% B from 50 to 55 min and kept at 100% B from 55 to 65 min. The flow rate was set at 0.8 mL/min. Spectra were run from 220 to 600 nm.

2.6.2. GC–MS analysis

Equipment: Perkin Elmer Turbo Mass. Column: fused silica capillary column, SP-2330 (Supelco), 30 m × 0.25 μm. Carrier: He, split flow 50.0 mL/min, initial set point: 20.0 PSIG. Oven program: total run time: 66 min, initial temperature: 100 °C, initial hold: 1.00 min, Ramp: 10.0 °C/min to 250 °C, hold for 50.00 min. Injection volumen: 1 μL.

2.6.3. NMR analysis

^{1}HNMR spectra were recorded at 400 MHz while the ^{13}C NMR spectra were measured at 100 MHz on a Bruker Avance spectrometer (Bruker, Rheinstetten, Germany). Chemical shifts are given in δ (ppm) with tetramethylsilane as the internal standard.

2.7. Results and discussion

2.7.1. Assay-guided isolation

In the present work, a bio-guided identification of the antimicrobial agents from the aerial parts of *P. lucida* was carried out. The whole MeOH extract was fractionated giving the DCM and AQ extracts. The DCM extract with higher antimicrobial activity (Table 1) was permeated on Sephadex LH-20 to afford seven active fractions (A-G), which were evaluated for their phytochemical composition (Figs. 2 and 3) and antimicrobial activity (Table 1, Fig. 4I). TLC analysis showed the presence of phenolic compounds. After spraying with the NP reagent (Fig. 2), color suggests that fractions A to D could be phenolic acids or coumarins (blue spots) while flavonoids occurs in fractions E to G (orange yellow and red orange spots). Quantitative assays indicate that nonflavonoids are the main compounds in fractions C and D (Fig. 3).

Fraction C, containing nonflavonoid phenolics was selected for bio-guided identification of constituents due to the higher yield (8.3%) compared with other active fractions (Fig. 1, Fig. 3)

The fraction C (2.5 g) was chromatographed on silica gel affording 19 subgroups as follows: I (32.8 mg), II (19.43 mg), III (299.2 mg), IV (134.0 mg), V (142.4 mg), VI (50.0 mg), VII (223.0 mg), VIII (67.7 mg), IX (32. 6 mg), X (144.2 mg), XI (167.6 mg), XII (129.6 mg), XIII

(7.8 mg), XIV (8.1 mg), XV (385.6 mg), XVI (10.2 mg), XVII (54.6 mg), XVIII (24.3 mg), XIX (17.7 mg). The subfractions were assessed at 15 and 50 μg against the methicillin resistant *S. aureus* F7 strain by dot and developed bioauthography (Fig. 4). At 50 μg, 15 of 19 subfractions showed antimicrobial activity but at 15 μg only 4 subfractions (II–V) resulted inhibitory (Fig. 4II and III). The MIC values of those showing activity were determined against *S. aureus*, *E. faecalis* and *C. albicans* (Table 2). Eleven out of 15 subfractions showed some effect on reference or antibiotic-resistant strains of *S. aureus* and *E. faecalis* (Table 2). The more active subfractions were II to V with MIC values between 12.5 and 200 μg/mL. The most susceptible microorganism (MIC values of 12.5 to 50 μg/mL) was *E. faecalis*, a pathogen frequently isolated in bacteremia, endocarditis, urinary tract infection, and with capacity for high-level biofilm formation. The MIC values for subfractions II, IV, V, XI, XIII, XIV were lower than the reference compound Ampicillin. Subfractions VIII and IX were more active on *S. aureus* (50–100 μg/mL). All fractions were weakly active on *Candida* species with MIC values of 200 μg/mL, being less to the reference drug Nystatin. Gram negative bacteria were no inhibited by C subfractions (Table 2).

Table 1
Antimicrobial activity (MIC values, μg/mL) of MeOH, dichloromethane (DCM) and aqueous (AQ) extracts and DCM fractions (C-G) of *Parastrephia lucida*.

Strains	MeOH	DCM	AQ	C	D	E	F	G
Bacteria								
Staphylococcus aureus								
F7 (MRSA)	R	200	R	200	100	200	100	100
F22 (MRSCN)	R	100	400	200	100	200	100	100
ATCC 29213	R	R	400	200	100	200	100	100
Enterococcus faecalis								
F208	R	R	R	400	400	400	100	100
F226	R	200	R	400	200	200	100	100
ATCC 29212	R	200	400	200	100	200	100	100
Fungi								
Candida albicans	400	200	400	100	100	100	100	100
Candida tropicalis	400	100	400	100	100	100	100	100
Candida glabrata	400	400	400	200	100	100	100	100
Candida parasilopsis	400	100	400	100	100	100	100	100
Candida guilliermondii	400	100	400	100	100	100	100	100

R: resistant until 400 μg/mL, Oxacillin (MIC > 16 μg/mL) for *Staphylococcus aureus* F7 and F22, Ampicillin (MIC ≥ 64 μg/mL) for *Enterococcus faecalis* F208 and F226 and Nystatin (MIC ≥ 300 μg/mL) for *Candida* strains. Methicillin resistant *Staphylococcus* coagulase negative (MRSCN), methicillin resistant *Staphylococcus aureus* (MRSA).

2.7.2. Compound identification

The active subfractions II–V, VIII and IX were analyzed by GC–MS and NMR. The GC–MS profiles were determined and compared with the same samples after methylation (diazomethane in diethyl ether, 20 min). The comparison allowed to disclose if the carboxylic acid function of compounds occurring in the fractions was free or esterified. The GC–MS fingerprint of subfraction II did not showed relevant changes after methylation. The main compound of the fraction was in agreement with ferulic acid prenyl ester (compound **9**) (Fig. 5). Several minor constituents

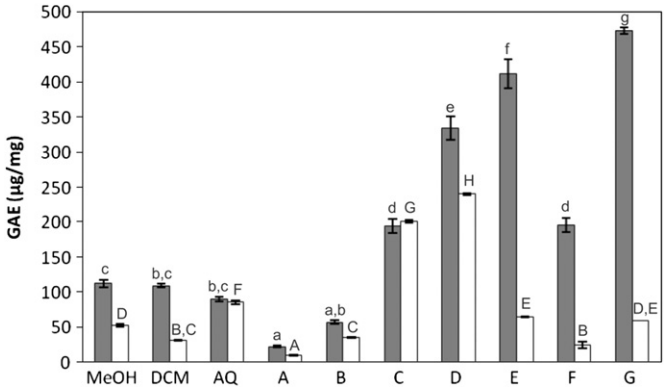


Fig. 3. Total phenolics (■) and nonflavonoids (□) content of MeOH, DCM and AQ extracts and DCM fractions (A–G) of *Parastrephia lucida*. Values are presented as gallic acid equivalents (GAE, μg/mg). Data are means ± SD. Values with the same letter are not significantly different (Tukey's HSD, $p \leq 0.05$). $N=4$.

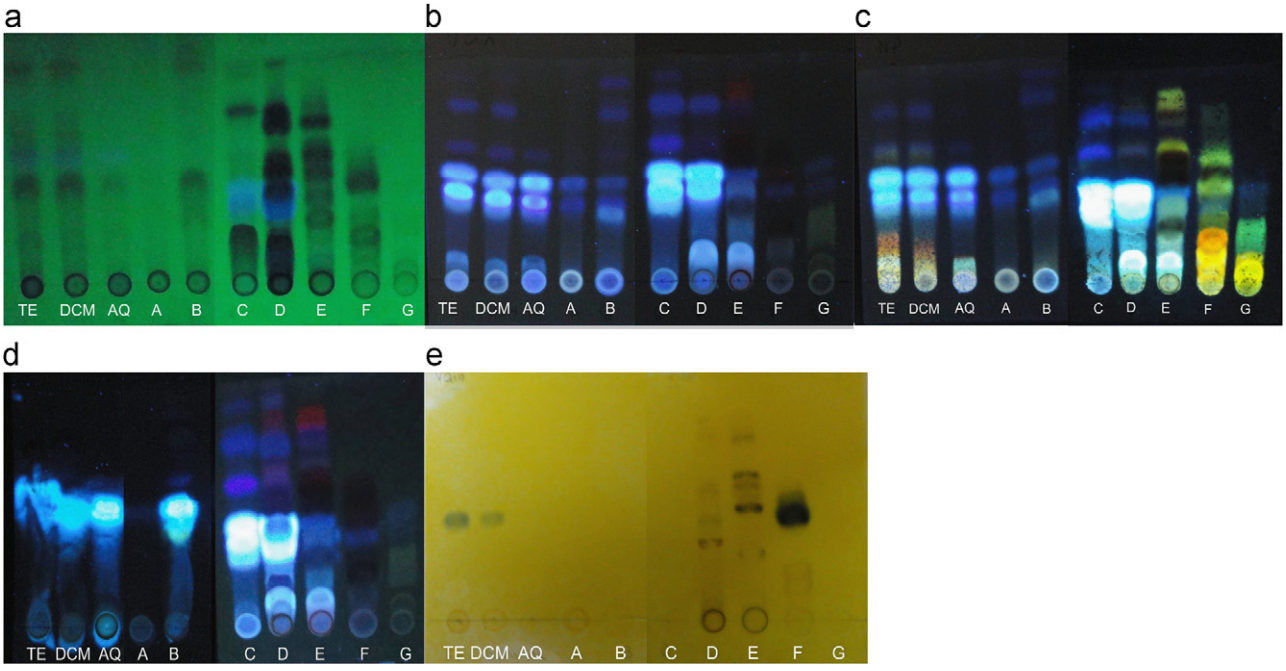


Fig. 2. Phytochemical profile of total methanol (MeOH), dichloromethane (DCM), aqueous (AQ) extracts and DCM fractions (A–G) of *Parastrephia lucida* (TLC, Silica gel, $CHCl_3$:MeOH 7:3 v/v; 50 μg/spot). Detection: **a**: UV 254 nm; **b**: UV 365 nm; **c:** Natural products reagent; **d**: KOH, UV 365 nm; **e**: Dragendorff's reagent.

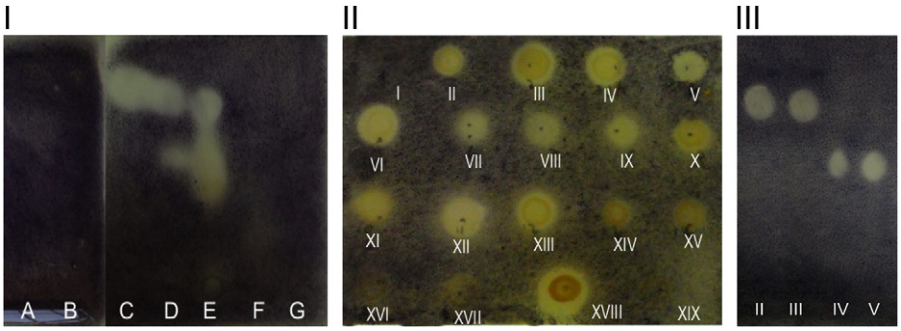


Fig. 4. Bioautographic assays against *Staphylococcus aureus* F7 of *Parastrephia lucida* DCM fractions (Fig. 4I, 50 ug/spot) and subfractions obtained from fraction C (Fig. 4II and III, 50 and 15 ug/spot, respectively). Plates were seeded in dot (II) or developed (I and III) using as solvent system $CHCl_3$:MeOH (7:3). Then, the plates were covered with 2 mL of soft medium (BHI with 0.6% agar) containing 10^5 colony forming units (CFU) of different microorganisms.

Table 2
Antimicrobial activity (MIC values, μg/mL) of subfractions C from *Parastrephia lucida*.

Fraction	*Staphylococcus aureus*		*Enterococcus faecalis*	
	F7	ATCC 29213	F208	ATCC 29212
II	200	50	25	12.5
III	200	200	25	12.5
IV	200	100	25	12.5
V	200	100	50	25
VIII	100	50	100	200
IX	100	50	100	200
XI	200	50	200	50
XIII	200	50	200	50
XIV	100	50	200	50
XV	100	200	200	200
XVIII	100	200	200	200

Oxacillin (MIC > 16 μg/mL) for *Staphylococcus* strains, Ampicillin (MIC ≥ 64 μg/mL) for *Enterococcus* strains and Nystatin (MIC ≥ 300 μg/mL) for *Candida* strains.

(compounds **8**, **10**, **11** and **12**) were identified by the mass fragmentation pattern who showed characteristic loss of 68 and 104 amu, compatible with a isoprenyl (compounds **8**, **10**) and phenethyl (compounds **11**, **12**) units. The placement of the units in the aromatic ring follows from the comparison with other phenylpropanoids and examination of the NMR spectra. HMBC correlations showed that the prenyl unit of compound **9** is esterified to the ferulic acid unit as shown by clear interaction of the signal at δ 4.72 with the C at δ 167.75. The spectrum also showed a minor constituent differing from compound **9** in the number of methoxy groups (two instead of one methoxy group) and was in agreement with methyl ferulic acid prenyl ester (compound **10**): ^{1}H NMR (400 MHz, $CDCl_3$, δ-values, J in Hz): 7.05 br s (1H; H-2); 6.91 d (J=8.4 Hz) (1H; H-5); 7.12 dd (J=8.4 and 1.6 Hz) (1H; H-6); 7.65 d (J=16 Hz) (1H; H-7); 6.35 d (J=16 Hz) (1H; H-8); 4.66 d (J=7.2 Hz) (2H; H-1); 5.40 t q (J=7.2 and 1.2 Hz) (1H; H-2); 1.74 br s (3H; H-4); 1.78 br s (3H; H-5); 3.95 s (3H; OCH_3); 3.94 s (3H; OCH_3). The mass spectrum of compounds **11** and **12** showed a similar fragmentation pattern and the loss of 104 amu, compatible with a phenetyl unit. The compound **11** was in agreement with phenethyl ferulate and compound **12** differs from **11** by the pressence of an additional CH_3 unit. While the molecular formula of compound **12** was the same as that required for 1-methyl-2-phenylethyl isoferulate described as a constituent of the Umbelliferae *Notopterygium* spp. by Kozawa et al. (1983), the fragmentation pattern indicate that the phenethylalcohol should be placed as an ester and an additional CH_3 group in the aromatic ring. The MS spectral data are presented in Table 4. The subfraction III showed a main compound (**9**) and minor constituents with Rt=7.96, 11.75 and 12.03 min, identified by comparison with database as 4-hydroxy-3-methoxy benzoic acid methyl ester (compound **14**) (Rt=7.96), ferulic acid methyl ester (Rt=11.75) (compound **4**), and caffeic acid methyl ester (Rt=12.03 min) (compound **3**), respectively.

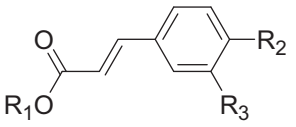
The subfraction IV showed two main compounds at Rt= 11.75 min and 6.51 min, identified as ferulic acid methyl ester (compound **4)** and vanillin (compound **13**), respectively. Minor compounds found in the fraction were ferulic acid ethyl ester (compound **5**) and ferulic acid prenyl ester (compound **9**).

The GC–MS trace of fraction V shows a main compound with Rt=7.28 min, m/z 148 identified as cinnamic acid (compound **1**) by its mass spectrum and minor compounds with Rt=8.75 min and Rt=8.86 min, identical to 3-(4-hydroxy-phenyl)-propionic acid methyl ester (compound **6**) and 3-(3,4-dihydroxy-phenyl)-propionic acid methyl ester (compound **7**), respectively. After methylation, the GC trace showed a main product with Rt=6.47 min, identical to cinnamic acid methyl ester (compound **2**). Subfractions VIII and IX showed in GC–MS a broad peak as a main constituent with Rt=7.17–7.27 min. The molecular ion peak and fragmentation pattern were consistent with cinnamic acid (compound **1**), the identity was confirmed after methylation who afforded cinnamic acid methyl ester (compound **2**). Twelve phenylpropanoids were identified by spectroscopic and spectrometric means (Fig. 5 and Table 3).

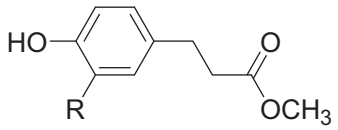
3. Discussion

In a previous study Zampini et al. (2009), reported antibacterial activity of *P. lucida* ethanol extract. However, the identity of the active constituents remained unknown. Assay-guided isolation led to active antimicrobial mixtures. The subfraction II, which was more active as antimicrobial than other C subfractions, contains ferulic acid prenyl ester (compound **9**) and methyl ferulic acid prenyl ester (compound **10**), which confer to the molecule a strong affinity to biological membranes (Sharma, 2011). Furthermore, the ferulic acid 2-phenethyl alcohol (compound **11**) and dimethylcaffeic acid 2-phenethyl ester (compound **12**), were also present.

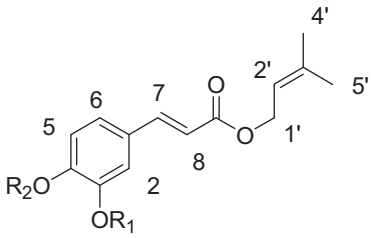
Several biological and pharmacological properties: antioxidant, anti-inflammatory, anti-carcinogenic, antiviral, and inmunomodulatory activities were demonstrated to caffeic acid phenethyl ester (Dang et al., 2010). The activity of this compound was found to be well-correlated to lipophilicity and the presence of α, β unsaturated carbonyl which can be considered as a highly reactive Michael acceptor (Hoda et al., 2010). The compound **9,** ferulic acid prenyl ester, was previously reported from *Populus*



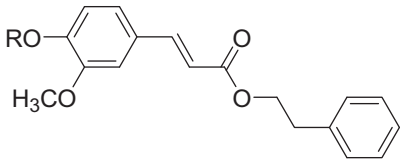
Compound	R_1	R_2	R_3
1 Cinnamic acid	H	H	H
2	CH_3	H	H
3	CH_3	OH	OH
4	CH_3	OH	OCH_3
5	C_2H_5	OH	OCH_3



Compound	R
6	H
7	OH



	R_1	R_2
8	H	CH_3
9	CH_3	H
10	CH_3	CH_3



	R
11	H
12	CH_3

Fig. 5. Structure of *Parastrephia lucida* constituents occurring in the bioactive fractions.

Table 3
Identification of *Parastrephia lucida* antimicrobial compounds by GC–MS analysis.

Rt (min)	*m/z*	Fragmentation pattern	Molecular formula	Identity
6.46	162	162 (91), 131 (59), 103 (58), 77 (100)	$C_{10}H_{10}O_2$	Methyl cinnamate **2**
6.51	152	152 (85), 151 (100), 137 (6), 123 (19), 109 (22), 81 (34), 65 (13), 53 (21), 52 (21), 51 (21)	$C_8H_8O_3$	Vanillin **13**
7.28	148	148 (80), 147 (100), 131 (23), 103, (63), 77 (60), 51 (53)	$C_9H_8O_2$	Cinnamic acid **1**
7.96	182	182 (45), 151 (100), 123 (20), 84 (18), 49 (30)	$C_9H_{10}O_4$	4-hydroxy-3-methoxy benzoic acid methyl ester **14**
8.75	180	180 (18), 149 (3), 165 (1), 120 (37), 107 (100), 91 (10), 77 (17)	$C_{10}H_{12}O_3$	3-(4-Hydroxy-phenyl)-propionic acid methyl ester **6**
8.86	196	196 (21), 147 ( ), 131 (100), 103 (69), 77 (55), 51 (46)	$C_{10}H_{12}O_4$	3-(3,4-Dihydroxy-phenyl)-propionic acid methyl ester **7**
11.75	208	208 (100), 177 (80), 145 (62), 117 (26), 89 (33), 77 (25), 51 (31)	$C_{11}H_{12}O_4$	ferulic acid methyl ester **4**
12.03	194	194 (100), 179 (25), 161 (8), 133 (32), 77 (32), 51 (28)	$C_{10}H_{10}O_4$	Caffeic acid methyl ester **3**
12.61	222	222 (100), 194 (21), 177 (88), 150 (81), 145 (76), 134 (26), 117 (32), 105 (20), 89 (44), 77 (31), 51 (36).	$C_{12}H_{14}O_4$	ferulic acid ethyl ester **5**
14.28	262	262 (15), 194 (100), 177 (24), 77 (19), 67 (56), 41 (77)	$C_{15}H_{18}O_4$	isoferulic acid prenyl ester **8**
16.15	262	262 (18), 247 (3), 217 (6), 194 (100), 177 (25), 145 (17), 69 (36)	$C_{15}H_{18}O_4$	ferulic acid prenyl ester **9**
16.37	276	276 (28), 261 (4), 245 (3), 231 (5), 208 (100), 193 (32), 164 (17), 77 (28), 67 (46), 41 (77)	$C_{16}H_{20}O_4$	methyl ferulic acid prenyl ester **10**
20.10	298	298 (15), 194 (66), 177 (20), 150 (29), 105 (100), 77 (47), 51 (28)	$C_{18}H_{18}O_4$	ferulic acid 2-phenethyl alcohol **11**
20.58	312	312 (23), 208 (63), 191 (21), 164 (34), 105 (100), 77 (65), 51 (42)	$C_{19}H_{20}O_4$	dimethylcaffeic acid 2-phenethyl ester **12**

violascens while ferulic acid 2-phenetyl alcohol (compound **11**) was isolated from *Notopterygium incisum* and *Pteronia* spp. (Dictionary of Natural Products on DVD, 2011; Zdero et al., 1990). The preparation of prenyl and phenylethyl caffeates was reported by Hashimoto et al. (1988) and Wollenweber et al. (1987). The synthesis of the compounds **11**–**12** was described by Burke et al. (1995). Merkl et al. (2010) reported antibacterial and antifungal activities for the compound **9** and demonstrated that the antimicrobial effect of phenolic acid derivatives increases with the increasing length of the alkyl chain. Cinnamic acid esters with a phenylalkyl and alcohol have been isolated from the Boraginaceae *Onosma heterophylla* (Mellidis et al., 1993). Phenethyl and cinnamoyl caffeate have been described as propolis constituents as well as caffeic acid, *p*-coumaric and cinnamic acid (Kumazawa et al., 2002, 2004; Hashimoto et al., 1988; Wollenweber et al., 1987). 3,4-dihydroxy-5-prenylcinnamic acid has been reported as an antioxidant constituent of Brazilian propolis (Hayashi et al., 1999) while 3-dimethyallylferulic acid and 2,4-dihydroxy-3-dimethylallyl-cinnamic acid were reported as constituent of *Baccharis lineris* by Brown (1994). While the MS

of the compound described by Brown (1994) present a molecular ion peak at *m*/*z* 262 (95) and fragments at 245 (20) and 207 (100), the compound **9** (ferulic acid prenyl ester), isolated from *Parastrephia*, shows in GC–MS a less intense molecular ion peak at m/z 262 (18), and a base peak at *m*/*z* 194 (100), suggesting a different substitution pattern. Ferulic acid methyl ester (compound **4**) was reported to have antitumour activity against Sarcoma 180 as well as antimicrobial activity (Nam et al., 2001). Vanillin, found as main constituent of subfraction IV, was assessed for antifungal activity against *C. albicans* by Boonchird and Flegel (1982) and antibacterial activity against Gram positive and negative bacteria by Mourtzinos et al. (2009). The MIC values reported against *C. albicans* (1,250 μg/mL) and bacteria (2.28–11.4 mg/mL) were much higher than those found in our work, therefore, vanillin should in fact be considered devoid of activity. According to literature, a pure compound has prospects as antimicrobial agent if the MIC is lower than 50 μg/mL (Ríos and Recio, 2005). The effect found in the subfraction IV suggests synergism with other constituents. Cinnamic acids have been found to display better antifungal effects than coumaric acids (Kim et al., 2004). *Trans*-cinnamic acid was found to increase the activity of various antibiotic drugs against *Mycobacterium avium*. Clinically they are not used due to toxicity problems but they can assist the action of various anti-tuberculosis drugs (Chen et al., 2011).

4. Conclusions

From the MeOH extract of *P. lucida* aerial parts, 12 phenylpropanoids and 2 simple phenolics were identified from the active fractions of the crude drug. The bio-guided fractionation led to identification of the main antimicrobial constituents as phenylpropanoid esters bearing a prenyl or phenethylalcohol. The antimicrobial activity found should be regarded as strong (for *E. faecalis*) and moderate (for *S. aureus* and *C. albicans*) according to Ríos and Recio (2005), this explain, at least in part, the traditional use of the species in the Andes highlands medicine. Further studies with the pure constituents should be undertaken to disclose the contribution of the single compounds in the activity of the complex antimicrobial mixture.

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