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Antibacterial activities of *Ligaria cuneifolia* and *Jodina rhombifolia* leaf extracts against phytopathogenic and clinical bacteria

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Six plant extracts prepared from *Ligaria cuneifolia* and *Jodina rhombifolia* were screened for their potential antimicrobial activities against phytopathogens and clinically standard reference bacterial strains. Bioautography and broth microdilution methods were used to study samples antibacterial activities against 7 bacterial strains. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of samples were attained. An antibacterial activity guided isolation and identification of active compounds was carried out for *L. cuneifolia* methanolic extract (LCME). Both methanolic and aqueous extracts from *L. cuneifolia* showed inhibitory activities against phytopathogenic bacteria, with MICs ranging from 2.5 to 156 μg mL^{-1} for LCME and 5 mg mL^{-1} for the aqueous extract. None of the three *J. rhombifolia* extracts showed significant antibacterial activities against phytopathogenic strains (MIC > 5 mg mL^{-1}), except for the aqueous extracts against *Pseudomonas syringae* (MIC = 312 μg mL^{-1}). Only LCME showed bactericidal activities against phytopathogenic strains (MBCs = 78 μg mL^{-1}). The LCME exhibited significant inhibitory activity against reference clinical strains: *Escherichia coli* (MIC = 156 μg mL^{-1}) and *Staphylococcus aureus* (MIC = 78 μg mL^{-1}, MBC = 312 μg mL^{-1}). LCME active compounds were identified as flavonol mono and diglycosides, and gallic acid. The antibacterial activity of purified compounds was also evaluated. A synergistic effect against *S. aureus* was found between gallic acid and a quercetin glycoside. Hence, anti-phytopathogenic bacteria potential compounds isolated from *L. cuneifolia* could be used as an effective source against bacterial diseases in plants.

[**Key words:** *Ligaria cuneifolia*; *Jodina rhombifolia*; Phytopathogenic bacteria; Phenolics; Isoquercitrin; Gallic acid; Synergic effect]

The plant kingdom constitutes a source of new chemical compounds which may be important due to their potential use in medicine and other applications (e.g., food and forage conservation). Plant extracts were regarded by ancient civilizations to be significant for the treatment of various ailments (1). However, for some decades there was an increasing interest in plant uses and in the detection of their constituents with antibacterial activity (2). Organisms like *Escherichia coli* and *Staphylococcus aureus* are implicated to cause severe infections in human, as they are found in multiple environmental habitats (3). *Agrobacterium tumefaciens*, *Pseudomonas corrugata*, *Erwinia carotovora*, *Pseudomonas syringae* and *Xanthomonas campestris* were reported to be severe phytopathogens, causing damage in carrot, potato, tomato, leafy greens, onion, green pepper, squash and other cucurbits. Furthermore, these phytopathogens cause disease in any plant tissue it invades (4). *Ligaria cuneifolia* and *Jodina rhombifolia* are plants species which grow in northwestern Argentina. While *L. cuneifolia* is an endemic shrub that spontaneously grows in the arid and semiarid region of Northwestern Argentina between 1800 and 2700 m on the sea level, *J. rhombifolia* is a small armed tree with rhomboid leaves that grows from 500 to 1000 m on the sea level. Both species are used in traditional medicine as antibacterial, haemostatic and vulnerary (5). There are few studies on the biological activities of *L. cuneifolia* (6–8), mainly focused on haemostatic properties of protein extracts, and much less studies on *J. rhombifolia* (9,10), mainly focused on ethnopharmacological references.

In the present study, the antimicrobial potency of dichloromethanic, ethanolic, and aqueous extracts attained from *L. cuneifolia* and *J. rhombifolia* were investigated. The antibacterial activities were determined by broth microdilution method. Five plant pathogens and two clinically important reference bacterial strains from American Type Culture Collection (ATCC) were used as test cultures. The phytochemical screening and activity guided isolation was performed for the major active extract, and the chemical structures of the active compounds were determined.

MATERIALS AND METHODS

Chemicals Analytical grade and HPLC solvents were from Sintorgan Labs (Vicente Lopez, Buenos Aires, Argentina). Sterile polystyrene 96 well plates ("U" bottom) were from Biopack (Argentina). Streptomycin sulphate salt, oxytetracycline

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hydrochloride, $CaCl_2$, $MgCl_2$, NaCl, 2-aminoethyl diphenylborate, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), Na_2HPO_4 and NaH_2PO_4 were from Sigma–Aldrich (St. Louis, MO, USA). DMSO, glycerol, Folin–Ciocalteu reagent, Silica Gel 60 F_{254} plates, triethanolamine and vanillin were from Merck (Darmstadt, Germany). Bacto agar, brain heart infusion medium (BHI), Müller Hinton agar (MHA), MH broth (MHB) mediums were from Britania S.A. Labs (Ciudad Autonoma de Buenos Aires, Buenos Aires, Argentina). Flavonoids standards (HPLC quality) were from Indofine Chemical Company Inc. (Belle Mead, NJ, USA). Membrane filters (pore size 0.22 μm) were from Pall Life Sciences (Ann Arbor, MI, USA). Sephadex LH-20 was from Amersham Biosciences (Uppsala, Sweden).

Plant material Aerial parts of *L. cuneifolia* (Ruiz and Pavón) blume and *J. rhombifolia* (Hook. ex Arn) leaves were used. Plants were collected in the province of Tucumán, in Argentinian northwest, as described elsewhere (1). Plants were taxonomically identified by Dr. A. R. Sampietro (Instituto de Estudios Vegetales, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina). Voucher specimens were deposited in the herbarium of the Instituto de Estudios Vegetales for future references. Plant materials were dried in the shade in a well ventilated chamber, ground to a coarse powder and stored into closed flasks at −20°C in the dark until they were used.

Extract preparations Extracts were prepared from each plant sample by lixiviation at room temperature with solvents in an increased polarity order: dichloromethane, methanol and water. The extractions were carried out according to Farmacopea Argentina 8th edition (11) with slight modifications. Briefly, plant coarse powder (5 g) was imbibed with dichloromethane (10 mL), then loaded into a glass column (equipped with a PTFE stopcock), covered with 200 mL of solvent and leaved closed and covered at room temperature for 24 h, afterwards column was opened and extract was collected. More solvent was added till exhaust the plant material (about 1 L). The extracted material was dried at 25°C prior next solvent extraction. The procedure was repeated with each solvent. The three obtained extracts from each plant sample were named dichloromethanic (DCM), methanolic (MeOH) and water extracts. These were filtered three times through Whatman N°1 filter paper.

Extraction yield The yield of each extraction (y%) was calculated as described elsewhere (1). Briefly, DCM and MeOH extracts were dried under reduced pressure using rotary vacuum evaporator at 30°C. Water extracts were dried in a VirTis liter freezer dryer, SL model. The dried material obtained represented the extracted material (EM) for each sample. The extractions were performed at least three times to attain standard deviations. The EM (mg mL^{-1}) ± s.d. and y% obtained for *L. cuneifolia* were: DCM 87.60 ± 2.30 (y% = 5.71), MeOH 461.40 ± 0.70 (y% = 27.18) and water 83.79 ± 1.40 (y% = 14.71), while for *J. rhombifolia* were: DCM 62.20 ± 2.70 (y% = 2.35), MeOH 114.05 ± 0.50 (y % = 11.57) and water 8.76 ± 1.60 (y% = 3.59). Stock solutions from DCM and MeOH extracts were prepared in aqueous triethanolamine (0.5% v/v) to perform microbiology assays. The triethanolamine present in those samples did not affect results of biological tests, as assessed in control experiments.

Commercial antibiotics Streptomycin and oxytetracycline were used as commercial reference antibiotics, taking into account their high frequency agricultural applications (12). Stock solutions (1 mg mL^{-1}) were prepared and stored at −20°C.

Microorganisms The microorganisms used were bacterial strains from ATCC: *E. coli* ATCC 25922 (gram-negative strain) and *S. aureus* ATCC 25923 (gram-positive strain). These wild type strains are largely used and their inclusion is recommended by Clinical and Laboratory Standards Institute (CLSI) for antibacterial sensitivity tests (13). Plant pathogenic strains were: *E. carotovora* var carotovora ATCC 15713, *X. campestris* ATCC 13951, *P. syringae* ATCC 10862, *P. corrugata* ATCC 29736 and *A. tumefaciens* ATCC 15955. Strains were stored in BHI medium supplemented with 0.3 % w/v agar.

Broth microdilution and colony count assays The assays were performed to assess the bacteriostatic (broth microdilution assays) and bactericidal (colony count assay) capacity of samples (14). Broth microdilution assays were carried out in sterile polystyrene 96-well microtiter plates. DCM, MeOH and water extracts from each species were assayed at 2.5–5000 μg mL^{-1}, while commercial antibiotics were assayed at 10–1280 μg mL^{-1}. Final volumes were adjusted to 100 μL with MHB. Colony count assays were performed in sterile Petri plates with MHA according to CLSI (13). Experiments were conducted eight times for each sample concentration, and repeated twice. The arithmetic means of the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were calculated and reported (14).

Bioautography assays The antibacterial activity of samples was screened to assess their ability to inhibit the bacterial growth, as described elsewhere (15). Briefly: sterile samples aliquots (sterilized through a 0.22 μm membrane filter) containing between 50 and 500 μg of EM were punctually placed on the surface of sterile Silica Gel F_{254} thin layer chromatography (TLC) plates, and leaved till solvent evaporation. The plates were covered with 5 mL of soft MHA (sMHA − 2.2 g of MHB and 0.5 g of bacto agar per 1 L of water) containing 10^6 CFU mL^{-1} of bacterial strain and incubated at 37°C for 24 h. Afterwards the plates were covered with MTT solution (0.8% w/v MTT in sterile sodium phosphate buffer − 0.1 M − pH 7.4) and incubated at 37°C for 1 h. The presence of a yellow inhibition zone against a dark blue background indicated the antibacterial activity of the evaluated sample. The inhibition zones diameters were proportional to the antibacterial power of the assayed samples. All of the strains were used for screening purposes, and *S. aureus* ATCC 25923 was chosen for activity guided assays (14).

Checkerboard dilution test The antibacterial effects that resulted from combining the more active antimicrobial purified compounds were assessed by checkerboard dilution tests on 96-well plates (16). Serial dilutions of compounds were mixed in MHB. Samples concentrations tested ranged from 2.0 to 250 μg/mL. Inocula preparation and size were the same as described for broth microdilution assay. The MICs for combinatory wells (C-MICs) were determined after 24 h of incubation at 37°C. The fractional inhibitory concentration (FIC) index was determined by the following formula:

$$\text{FIC index} = \text{FIC}_A + \text{FIC}_B = \text{MIC}_A/\text{C-MIC}_A + \text{MIC}_B/\text{C-MIC}_B \quad (1)$$

where MIC_A is the concentration of drug A, C-MIC_A is its MIC in the combinatory well, and FIC_A is the FIC of compound A, while MIC_B, C-MIC_B, and FIC_B are defined in the same fashion for compound B. The FIC index obtained was interpreted as follows: <0.5, synergy; 0.5–0.75, partial synergy; 0.76–1.0, additive effect; >1.0–4.0, indifference; and >4.0, antagonism (16). Experiments were repeated at least three times.

Photometric assays The assays were performed to differentiate bacteriolytic (cell lysis) from non bacteriolytic effects of samples, as described elsewhere (17). Briefly, bacteria cultures (5×10^6 CFU mL^{-1}) were prepared in sterile polystyrene 96-well microtiter plates containing samples at 20× MIC (14). All of the strains were used. Final volumes were adjusted to 200 μL with MHB. Plates were covered, carefully mixed and incubated at 37°C for 24 h. Optical densities (OD) were measured in a Bio-Rad 550 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) using a 620 nm emission filter at 0, 1, 2, 4, 6, 12 and 24 h of incubation. Growth controls replaced samples with samples solvents, samples colour wells replaced bacterial inoculums with MHB (this avoided the interference of the samples colour in the results), and sterility controls only included MHB. Experiments were conducted eight times for each sample concentration, and repeated twice. The arithmetic means of the OD lectures were corrected for each substance (by subtraction of colour wells OD from samples OD values for each incubation time) and percent OD values (*OD%*) were calculated as

$$OD\% = ((ODt - ODo)/ODo) \times 100 \quad (2)$$

where *ODt* was the corrected OD value for each incubation time, and *ODo* was the corrected OD value at 0 h of incubation. *OD%* against incubation time was plotted for each substance.

Statistical analysis Data were analysed by either Student's *t* test or one way ANOVA, considering a probability level lower than 0.05 as statistically significant.

Activity guided fractionation of LCME extract An antibacterial activity guided fractionation of was conducted as follows: an aliquot of LCME containing 500 mg of EM was chromatographed (methanol as mobile phase) on Sephadex LH-20 (210 mL bed volume). The 160 aliquots (3 mL each one) resulting from column elution were analysed by TLC, joined according to their chemical composition into six groups (L1–L6), evaporated under reduced pressure (at 45°C) to yield EM residues which were dissolved in methanol for further experiments. Aliquots were taken from L1 to L6 groups for bioautography, TLC and HPLC experiments.

HPLC experiments L3, L4 and L5 groups were selected for HPLC analysis. The analytical experiments were performed on a Gilson HPLC (Villiers Le Bel, Val d'Oise, France) using an Luna C18 column (5 μm, 250 × 4.6 mm ID) from Phenomenex (Torrance, CA, USA), an IB-SIL RP 18 precolumn (5 μm, 30 × 4.6 mm ID, Phenomenex), a 118 UV–Vis detector from Gilson, and a Rheodyne injector fitted with a 50 μL loop. A gradient elution was performed with solvent A: 0.5% (v/v) formic acid in water and solvent B: 0.5% formic acid in water:acetonitrile (50:50 v/v). The gradient was: 0% B to 39.8% B, 12 min; 39.8% B to 40.8% B, 13 min; 40.8% B to 41.2% B, 5 min; 41.2% B to 41.4% B, 5 min; 41.4% B to 41.8% B, 2 min; 41.8% B to 97.5% B, 8 min; 97.5% B to 100% B, 12 min; 100% B, 8 min. Re-equilibration was done at 100% B, 10 min; 100 to 0% B, 10 min; 0% B, 10 min. Compounds were detected at 254 nm at a flow rate of 0.5 mL min^{-1}. The retention times (*Rts*) were registered. The detected compounds were collected, dried by lyophilization and then dissolved in methanol for further experiments. Multiple injections (50 μg of EM at a time) were carried out to obtain adequate quantity of material. The semipreparative experiments employed a Luna C18 column (5 μm, 250 × 10 mm ID) from Phenomenex and detector employed on analytical conditions. A Rheodyne injector fitted with a 500 μL loop was used. A gradient elution was performed with the solvents A and B employed on analytical experiments. The gradient was: 0% B to 42% B, 8 min; 42% B to 49% B, 21 min; 49% B to 100% B, 6 min; 100% B, 6 min. Re-equilibration was done at 100% B, 10 min; 100 to 0% B, 8 min; 0% B, 6 min. Compounds were detected at 254 nm at a flow rate of 3 mL min^{-1}. The detected compounds were collected, dried by lyophilization and dissolved in methanol for further experiments. Multiple injections (≈5 mg of EM at a time) were carried out to obtain adequate quantity of material. The purity of all the isolated compounds was verified by analytical HPLC experiments.

Analytical HPLC experiments were also performed using the Gilson HPLC with a Luna NH_2 column (5 μm, 250 × 4.6 mm ID, Phenomenex), a Refraction Index detector from Gilson, and a Rheodyne injector fitted with a 50 μL loop. An isocratic elution was performed with acetonitrile:water (80:20 v/v) as mobile phase, at a flow rate of 0.9 mL min^{-1} for 35 min. Samples injected were the sugar moieties attain after hydrolysis of purified compounds and authentic standard drugs. Co-injection techniques were also carried out. The retention times (*Rt*s) were registered.

Hydrolysis The compounds purified by HPLC were separately dried under reduced pressure (at 45°C) and then hydrolysed by standard procedures (18). The procedure yielded ethyl acetate extracts containing aglycone moieties (EAE), and aqueous extracts containing sugar moieties (AE), which were separately analysed as described elsewhere (18).

One-dimensional silica gel TLC LCME, L2 to L5, compounds purified by HPLC and corresponding EAEs were loaded onto analytical TLC plates, and developed using: ethyl acetate–formic acid–acetic acid–water (100:11:11:27 v/v/v/v) named F1, or toluene–ethyl acetate–formic acid (50:40:10 v/v/v) named F2, as the mobile phases. After drying, bands were located by viewing under short (254 nm) and long (365 nm) UV radiation (UV Lamp Model UV 5L-58 Mineralight Lamp). The following sprays were used to locate the bands on the TLC: NP/PEG for flavonoids and phenolic acids, $FeCl_3$ for phenolic compounds, Vainillin/H_2SO_4 reagent for terpenes, and Lieberman-Bouchard reagent for steroidal nuclei (19). Standard compounds were used as positive reagent controls: gallic acid, chlorogenic acid, caffeic acid, rutin, kaempferol and quercetin with phenolic sprays, cholesterol with Vanillin – H_2SO_4 and Lieberman–Bouchard.

Two-dimensional cellulose TLC LCME, L2 to L4, compounds purified by HPLC and corresponding EAEs were analysed by ascending two-dimensional cellulose chromatography (6.5 × 6.5 cm plates) with tertbutyl alcohol–acetic acid–water (3:1:1 v/v/v), in the first direction and 15% (v/v) aqueous acetic acid in the second (20). After drying the plates were viewed under UV lamp at 254 and 365 nm and after spraying with NP/PEG reagent. Standard compounds were used as positive reagent controls: gallic acid, chlorogenic acid, caffeic acid, quercetin and kaempferol.

Paper chromatography The sugar moieties present in AEs were preliminary identified by paper chromatography as described elsewhere (21). Briefly: AEs were loaded onto Whatman no. 1 paper and eluted with *n*-butanol–pyridine–water (6:4:3 v/v/v). After drying, the papers were sprayed with silver nitrate–acetone reagent and then NaOH 0.5 N in ethanol (22). Reducing sugars produced dense black spots. Standard sugars were used as positive reagent controls: D-glucose, D-galactose and L-rhamnose.

UV–visible spectroscopy Compounds purified by HPLC and corresponding EAEs were dissolved in methanol. UV–VIS spectra were scanned between 200 and 500 nm on a Beckman DU 650 spectrophotometer. UV–VIS spectra of standard drugs (0.1 mg mL^{-1}) were also made when necessary, in the same experimental conditions. Reactives for identification of flavonoids were employed and UV spectra obtained were compared with those of standard drugs and bibliographic data (23).

Polarimetric assays Sugar moieties present in AEs were dissolved in water. Optical rotations were measured in a 0.1 dm cell at 25°C with a Horiba Sepa-300 polarimeter (Horiba Ltd., Kyoto, Japan) at 589 nm, and the $(\alpha)_D^{25}$ values were calculated. The results were compared with literature data and analytical grade standards (24).

Mass spectrometry Compounds purified by HPLC and corresponding EAEs were analysed by high resolution mass spectrometry (HRMS) employing a direct sample introduction technique on a Thermo Finnigan Polaris Q ion trap mass spectrometer (Austin, TX, USA). Negative ion chemical ionization mode was selected for the assays with flavonoid samples, using isobutane as a reagent gas to generate the molecular ion $M^{-\cdot}$ and typical fingerprints. Electronic energy was 90 eV at 200°C. The heating was between 50°C and 40°C at 200°C min^{-1}. The mass spectrometer was scanned over a *m*/*z* range of 100–630 Da. Electron ionization (EI) mode at 70 eV was selected for the assays with gallic acid. The data processing was performed using Xcalibur 1.3 software (Austin, TX, USA).

RESULTS

Extraction and purification The y%s were higher by using polar than nonpolar solvents, especially MeOH. Methanol extracted the highest quantity of material (EM), and y% than the other solvents. LCME showed the highest y% and EM among all samples.

Antibacterial activity Samples were punctually placed and assayed by direct bioautography as the first stage for the antibacterial activity screening. Polar extracts (e.g., MeOH and water extracts) showed antibacterial activity against the tested strains. There were significant differences between inhibition zones diameters generated by LCME against most bacterial strains assayed and than those generated by the other extracts ($p < 0.05$). As DCMs showed no antibacterial activities, these samples were not further considered for microdilution and colony count assays. Results are showed in Table 1.

As MeOH and water extracts showed antibacterial activity against a wide range of strains by bioautography, they were selected for further studies. Broth microdilution and colony count assays were carried out in order to obtain MIC and MBC values for the selected extracts and commercial antibiotics (Table 2).

LCME exhibited the highest antibacterial activity, because this sample inhibited the bacterial growth of all the tested strains. LCME MIC values ranged from 2.5 to 19.5 μg mL^{-1} against phytopathogenic strains. MICs and MBCs for LCME were, in general, lower than those of commercial antibiotics, except for streptomycin against *E. carotovora* and *E. coli*, which MICs were lower than LCME. *L. cuneifolia* water and *J. rhombifolia* MeOH and water extracts showed antibacterial activity by bioautography assays (Table 1), though these extracts did not showed antibacterial activity bellow 5 mg of EM mL^{-1} against almost all assayed strains, the exception was *P. syringae* which was inhibited by *J. rhombifolia* MeOH at 312 μg of EM mL^{-1}.

According to the results from bioautographic, broth microdilution and colony count assays, LCME was considered a good inhibitor for the phytopathogenic and clinical pathogenic bacteria (Tables 1 and 2). Consequently, LCME was selected for further studies. LCME was analysed by photometric assay against all strains at 20× MICs. There were not significant decrease in the bacterial growth after incubation for 24 h ($p > 0.05$), that was interpreted as non bacteriolytic action (data not shown).

Activity guided fractionation of LCME extract LCME was selected to determine the source of the antibacterial activity. A complete flow chart of purification and identification procedure is shown in Fig. 1. Column chromatography of LCME on Sephadex LH-20 yielded eluents which were pooled into six groups (L1–L6) based on TLC and bioautographic analysis. Groups L3, L4 and L5 showed antibacterial activity against *St. aureus* determined by bioautography assay (data not shown). HPLC analysis of L3 and L5 yielded five compounds preliminary identified as flavonoid glycosides, and L4 yielded a phenolic acid substance.

TABLE 1. Antibacterial activity screening of *L. cuneifolia*, *J. rhombifolia* extracts and commercial antibiotics by direct bioautography.

Sample	Zone of inhibition (mm diameter)						
	X. campestris	*A. tumefaciens*	*P. corrugata*	*P. syringae*	*E. carotovora*	*E. coli*	*S. aureus*
L. cuneifolia – DCM	(–)	(–)	(–)	(–)	(–)	(–)	(–)
L. cuneifolia – MeOH	6 ± 0.2	6 ± 0.2	7.5 ± 0.3	9 ± 0.1	6 ± 0.1	4.5 ± 0.0	5.2 ± 0.0
L. cuneifolia – water	3 ± 0.2	3 ± 0.2	3.2 ± 0.2	3.4 ± 0.2	3.3 ± 0.2	3 ± 0.0	3 ± 0.0
J. rhombifolia – DCM	(–)	(–)	(–)	(–)	(–)	(–)	(–)
J. rhombifolia – MeOH	2.7 ± 0.2	3 ± 0.1	2.8 ± 0.1	4 ± 0.0	3 ± 0.2	3 ± 0.1	3 ± 0.1
J. rhombifolia – water	2.5 ± 0.1	3 ± 0.1	2.6 ± 0.2	3 ± 0.1	2.5 ± 0.1	2 ± 0.2	2.2 ± 0.1
Streptomycin	4 ± 0.1	4 ± 0.1	4 ± 0.1	4 ± 0.1	6 ± 0.1	6 ± 0.0	5.5 ± 0.0
Oxytetracycline	5.5 ± 0.2	5.5 ± 0.3	4 ± 0.1	5.5 ± 0.2	5.5 ± 0.1	(–)	4 ± 0.0

±: mean standard deviation of triplicates; quantity of samples: 500 μg; positive controls (streptomycin and oxytetracycline): 50 μg; solvent controls (triethanolamine and water) did not produce inhibition (data not shown).

TABLE 2. MIC/MBC values (μg of EM mL^{-1}) for MeOH and water samples and commercial antibiotics (μg mL^{-1}) determined by broth microdilution and colony count assays.

Sample	MIC/MBC						
	X. campestris	*A. tumefaciens*	*P. corrugata*	*P. syringae*	*E. carotovora*	*E. coli*	*S. aureus*
L. cuneifolia – MeOH	19.5/NBE	19.5/78	9.8/78	2.5/78	19.5/78	156/NBE	78/312
L. cuneifolia – water	>5000/NBE	>5000/NBE	>5000/NBE	>5000/NBE	>5000/NBE	>5000/NBE	>5000/NBE
J. rhombifolia – MeOH	>5000/NBE	>5000/NBE	>5000/NBE	312/NBE	>5000/NBE	>5000/NBE	>5000/NBE
J. rhombifolia – water	>5000/NBE	>5000/NBE	>5000/NBE	>5000/NBE	>5000/NBE	>5000/NBE	>5000/NBE
Streptomycin	100/NBE	100/NBE	100/NBE	100/NBE	10/160	100/NBE	160/NBE
Oxytetracycline	160/640	160/160	320/640	160/320	160/160	NIE/NBE	320/640

NIE: non-inhibitory effect. NBE: non-bactericidal effect.

Identification of purified compounds Preliminary experiments were carried out through one dimensional TLC on Silica Gel F_{256} and two-dimensional TLC on cellulose. The *Rfs* of isolated compounds were recorded and compared with those of bibliographic data and commercial available standards after staining with NP-PEG reagent (14,19,20). As the attained *Rfs* and the spot colours matched with those of authentic standard and bibliographic data, these evidences allowed the identification of rutin (quercetin-3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside) and nicotiflorin (quercetin-3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside) as main L2 constituents, whereas isoquercitrin (quercetin-3-*O*-β-D-glucopyranoside), hyperoside (quercetin-3-*O*-β-D-galactopyranoside) and quercitrin (quercetin-3-*O*-α-L-rhamnopyranoside) from L5. Acid hydrolysis experiments supported these evidences, since quercetin was found in EAEs from rutin, isoquercitrin, quercitrin and hyperoside, while nicotiflorin yielded kaempferol (the aglycone moiety) from EAE, both quercetin and kaempferol were identified by TLC, analytical HPLC and MS, and their spectra fingerprints were compared with those of authentic standards.

Analytical HPLC experiments were also carried out with purified compounds and the corresponding aglycones plus authentic HPLC quality standards, which resulted in co-elutions, thus supporting the preliminary identifications. Spectroscopic methods allowed us to unequivocally complete the chemical identification of active compounds. MS spectra of isoquercitrin gave the molecular ion at *m*/*z* (relative intensity %) 464 (81.4), and ions due to the aglycone at *m*/*z* 302 (100) and sugar moiety, while hyperoside gave the molecular ion at *m*/*z* 464 (79.6), and ions due to the aglycone at *m*/*z* 302 (100) and sugar moieties. These two quercitrin-3-*O*-monoglycosides generated typical *O*-glycosidic bond cleavage sugar ions at *m*/*z* 161 (5.8), 143 (13.0) and 126 (4.4) for isoquercitrin and at *m*/*z* 161 (0.8), 143 (1.7) and 126 (0.6) for hyperoside, which were attributed to the successive elimination of water from those sugar moieties. The peaks at *m*/*z* 143 were relevant for identification of the hexose moieties (25). Quercitrin gave the molecular ion at *m*/*z* 448 (37.8), and ions at *m*/*z* 302 (100) due to the aglycone, and at *m*/*z* 153 (0.4), 145 (1.4) and 127 (9.1), due to the elimination of water from rhamnose, used as diagnose of this deoxyhexose. Nicotiflorin and rutin, the two flavonoid *O*-diglycosides, also gave typical MS spectra, as nicotiflorin gave ions at *m*/*z* 594 (15.8) corresponding to the $M^{-\bullet}$, 448 (3.6) as the $M^{-\bullet}$ minus terminal rhamnose, 286 (100) due to the aglycone, sugar moiety ions at *m*/*z* 290 (15.0) due to elimination of water from the disaccharide (rutinose = 6-rhamnosylglucose), 163 (28.1), 145 (12.4), 143 (1.7), 127 (1.8) and 126

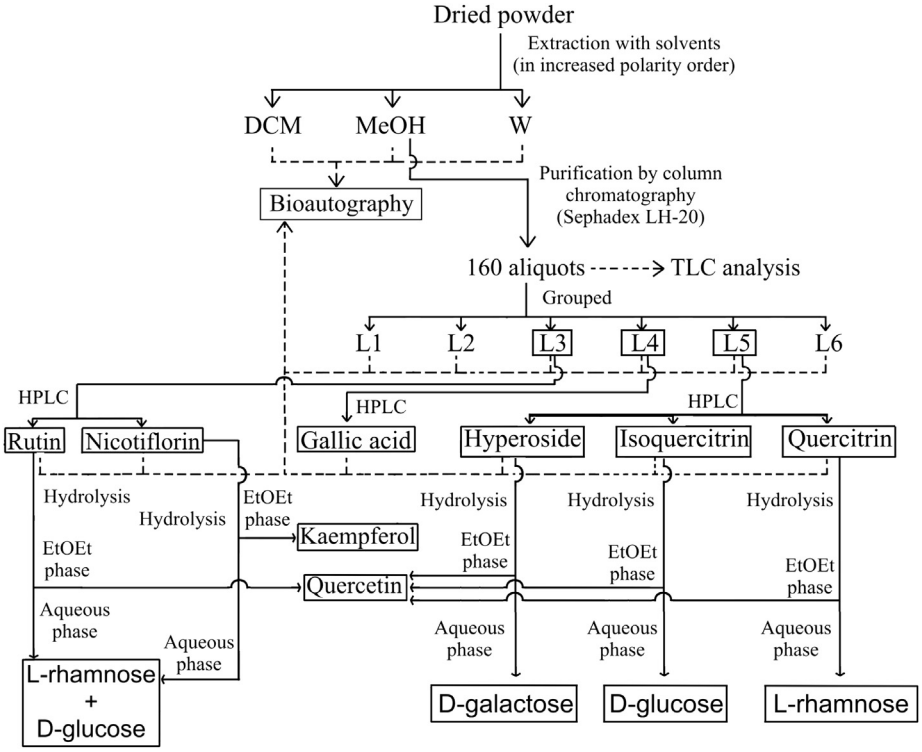

FIG. 1. Flow chart of the total purification and identification procedure. Full lines: purification steps. Dotted lines: antibacterial activity experiments.

(3.3). Rutin gave the $M^{-\bullet}$ at *m/z* 610 (4.0), and ions at *m/z* 464 (0.6) for the $M^{-\bullet}$ minus terminal rhamnose, 302 (100) for the aglycone, and identical sugar moiety ions than nicotiflorin at *m/z*: 290 (33.7), 179 (0.6), 163 (21.7), 145 (15.1), 143 (4.7), 127 (0.8) and 126 (27.3). Ions due to the disaccharide moieties in these two flavonoid *O*-diglycosides were confirmed by the presence of ions at *m/z* 290 (25). MS fingerprints matched with those of commercial standards. UV–Vis spectroscopy data complemented the identification regarding the sugar position in the aglicones, whereas rutin, nicotiflorin, isoquercitrin, hyperoside and quercitrin showed typical band I between 328 and 357 nm, a characteristic signal for 3-*O*-glycosylated flavonols (23). Chemical shifts of band I and band II after addition of reagents for flavonoids identification (23) provided valuable information which complemented MS data, which can be found elsewhere (14).

Acid hydrolysis of isolated flavonoids yielded AEs containing sugar moieties, which were preliminary identified by paper chromatography as D-galactose (the sugar moiety of hyperoside), D-glucose (the sugar moiety of isoquercitrin), L-rhamnose (the sugar moiety of quercitrin), while equimolar quantities of D-glucose and L-rhamnose were obtained from both rutin and nicotiflorin aqueous extracts. The *Rf* of each spot matched with that of authentic standards and bibliographic data (14). Analytical HPLC experiments carried out with sugar moieties plus commercial standards resulted in co-elutions, i.e., one signal on the chromatogram, which gave supporting information on the true identity of those sugars. Polarimetric assays performed with isolated sugars generated typical $(\alpha)_D^{25}$ values for D-glucose (+52.5°), D-galactose (+80.2°), L-rhamnose (+8.9°) and D-glucose + L-rhamnose (+30.8°). Chromatographic collected evidences (i.e., paper chromatography and HPLC) and spectroscopic techniques (i.e., MS and polarimetry) unequivocally confirm the identity of those compounds (14,20,25,26).

A compound isolated from L5 was identified as gallic acid (3,4,5-trihydroxybenzoic acid). The purified sample showed a signal at 272 nm in UV–Vis, an $Rf = 0.36$ (light blue spot after NP/PEG stain) in Silica Gel F_{256} TLC (F2 as the mobile phase). These results were also attained with authentic gallic acid standard. Analytical HPLC experiment of purified sample plus gallic acid HPLC quality standard resulted in co-elution. The MS signals were at *m/z* 170 ($M^{+\bullet}$), *m/z* 126 ($C_6H_6O_3^{+}$ resulted from CO_2 loss from $M^{+\bullet}$), *m/z* 108 ($C_6H_4O_2^{+}$ resulted from H_2O loss from $C_6H_6O_3^{+}$), and *m/z* 44 (CO_2^{+}). The purified sample MS fingerprint matched that of gallic acid standard and bibliographic data (27).

Antibacterial activity of purified compounds The purified phenolic compounds were assayed for they antibacterial activity against all described strains by direct bioautography. Quercetin monoglycosides (i.e., isoquercitrin, hyperoside and quercitrin) showed better antibacterial skills than diglycosides (rutin and nicotiflorin) against the tested strains. There were significant differences between inhibition zones diameters generated by quercetin monoglycosides and gallic acid than those generated by diglycosides against most bacterial strains assayed ($p < 0.05$). Results are shown in Table 3.

As isoquercitrin and gallic acid showed better antibacterial skills than the other isolated compounds, they were analysed by broth microdilution combinatory assay against *St. aureus* ATCC 25923. The MICs attained for isoquercitrin and gallic acid against this strain were 100 μg/mL and 125 μg/mL respectively. This assay showed that 10 μg/mL of isoquercitrin and 15.1 μg/mL of gallic acid combination was enough to reach the MIC, so the $FIC_{isoquercitrin}$ was 0.1 and $FIC_{gallic\ acid}$ was 0.12, thus the FIC index attained was 0.21, interpreted as synergistic effect between these two compounds against the assayed strain.

DISCUSSION

Phytopathogenic bacterial infections are among agricultural practices concerns, given that some strains are responsible of great sickness and looses in appropriate climatic conditions. *Pseudomonas*, *Xanthomonas*, *Erwinia* and *Agrobacterium* are among the main phytopathogenic bacterial genus. *E. carotovora* is responsible of the soft rot disease in potato stems and tubers (28), *P. syringae* cause decrease in the yield through foliar necrosis and fruit blemish in tomato plants (*Lycopersicon esculentum* Mill.) (29), *X. campestris* cause leaf scald in *Saccharum officinarum* (30), only for citing some well known sickness. Streptomycin and oxytetracycline are often used in agricultural applications to prevent or treat the invasive development of phytopathogens, such as *Pseudomonas* spp., *A. tumefaciens*, and *X. campestris* (12). The excessive application of such agrochemicals to prevent or treat phytopathogens derived diseases have been related to cause bacterial resistance, environmental damages and human health complications (31). Another concern is the economic impact derived from the field application of those substances.

Among the six evaluated plant extracts, LCME generated six phenolic compounds which were isolated and identified. Flavonoids from plant sources are well known to possess antibacterial activity (32). The isolated flavonoids were completely identified as quercetin and kaempferol glycosides. The presence of quercetin glycosides is in accordance with previous findings in this species, where an acidic hydrolysis was performed to attain quercetin enriched extracts chemically characterized through chromatographic methods (6). The presence of a kaempferol glycoside is first described here, giving an updating of other authors results, who claimed quercetin as the only one flavonol present in *L. cuneifolia* (8). Betulin and betulinic acid were previously isolated and unequivocally identified from *L. cuneifolia* DCM extract (8). These substances were not found in MeOH and water extracts in our experiments. Given the lack of antibacterial activity of *L. cuneifolia* DCM extract (which contains these substances), we conclude that these compounds do not contribute to the antibacterial activity. The five isolated flavonoids had been previously referred as antibacterial substances (14,33), but they were not previously detected or characterized in *L. cuneifolia*. Bactericidal activity is highly desirable because it diminishes the complications derived from a bacteriostatic action, such as strain resistance to toxic side effects. Our results suggest that LCME compounds act with bactericidal effects

TABLE 3. Antibacterial activity of isolated compounds and commercial antibiotics by direct bioautography.

Sample	Zone of inhibition (mm diameter)						
	X. campestris	*A. tumefaciens*	*P. corrugata*	*P. syringae*	*E. carotovora*	*E. coli*	*S. aureus*
Rutin	0.5 ± 0.0	0.5 ± 0.1	0.7 ± 0.1	0.9 ± 0.0	0.6 ± 0.0	(−)	0.6 ± 0.0
Nicotiflorin	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	(−)	0.6 ± 0.0
Gallic acid	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.4 ± 0.0	1.3 ± 0.1	(−)	0.7 ± 0.0
Hyperoside	1.5 ± 0.0	1.0 ± 0.0	0.8 ± 0.0	1.9 ± 0.0	1.2 ± 0.0	0.4 ± 0.0	0.9 ± 0.1
Isoquercitrin	1.7 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	2.0 ± 0.0	1.2 ± 0.1	0.4 ± 0.0	1.0 ± 0.0
Quercitrin	1.5 ± 0.1	0.9 ± 0.1	0.7 ± 0.0	1.8 ± 0.1	1.1 ± 0.0	0.4 ± 0.0	1.0 ± 0.0

±: mean standard deviation of triplicates; quantity of samples: 50 μg.

against 5 out 7 assayed strains. LCME exerted no bacteriolytic action against the assayed strains according to the growth curves (17). It had been suggested that hydrophobic section of phenolic antibacterial compounds are key structures in cell lysis actions (14). As isolated compounds are mainly polar substances (glycosides), the lack of lytic action would be partially justified. Phenolic compounds with vicinal hydroxyl groups (as catechol) possess antimicrobial activity (34). Alkyl or high polar substituents are known to confer less antibacterial potency than that due to simple phenolic compounds (34). All these facts lead us to conclude that the polarity increase by glycosylation due to the bulky sugar moiety could be the reason of the absence of bacteriolytic effects. Quercetin and its glycosides (e.g., isoquercitrin) had been described to cause an increase in permeability of the inner bacterial membrane and a dissipation of the membrane potential (35), thus affecting the ATP synthesis, which could play a key role in the antibacterial activity. Gallic acid has been suggested to act as a proline analogue which affects proline oxidation via bacterial proline dehydrogenase, as the amino acid proline could overcome the inhibitory effect of this compound against *S. aureus* (36). Synergistic effects could be explained based on multi-target mechanisms of action (37). As flavonoids (e.g., isoquercitrin) and phenolic acids (e.g., gallic acid) may individually act over different bacterial targets, this might explain the synergistic effect observed between isoquercitrin and gallic acid against *S. aureus*. This antibacterial mode of action could represent an important antimicrobial defense on *L. cuneifolia* invading bacterial pathogens.

L. cuneifolia is a perennial shrub insensible to phytopathogenic attack (38). The antibacterial activity exhibited by all of the LCME isolated compounds justifies, at least partially this observation. The determination of antimicrobial activity of LCME and some of the responsible compounds could be more informative for the future use in controlling phytopathogenic bacteria and also in clinical treatment as a source of natural antimicrobials. The biodegradation of natural compounds after field application is another benefit which deals the environmental bioaccumulation troubles derived from some commercial antibiotics currently used.

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