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Antimicrobial Activity of *Inga fendleriana* Extracts and Isolated Flavonoids

Luisa Pistelli[1*], Alessandra Bertoli[1], Cecilia Noccioli[1], Jeanette Mendez[2], Rosa Anna Musmanno[3], Tiziana Di Maggio[3] and Grazietta Coratza[3]

[1]*Dipartimento di Scienze Farmaceutiche*, *Università di Pisa*, *Italy*

[2]*Grupo de Productos Naturales*, *Centro de Quimica Organica*, *Escuela de Ciencias*, *Universitat Central de Venezuela*, *Venezuela*

[3]*Dipartimento di Biologia Molecolare*, *Università degli Studi di Siena*, *Italy*

luipi@farm.unipi.it

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The EtOAc and *n*-BuOH extracts of *Inga fendleriana* inhibited Gram-positive, but not Gram-negative bacteria; a narrow spectrum of activity against *Staphylococcus epidermidis* was detected. The MIC values of the extracts ranged from 125 to 850 μg/mL. Quercetin 3-methylether, myricetin 3-*O*-rhamnoside and tricetin showed antibacterial activity against the same bacterial strains with MICs in the range from 31 to 250 μg/mL. In time-kill kinetic studies, the flavonoids showed bactericidal effects at the concentrations corresponding to four times the MICs.

Keywords: *Inga fendleriana*, Fabaceae, antimicrobial activity, myricitrin, quercetin 3-methylether, tricetin.

The interest in medicinal plants has been increased during the last decades as they are potential sources of therapeutic substances. In particular, the emergence of microbial resistance to the available antibiotics [1-3] and the unexpected side effects of synthetic compounds have increased the need for new substances with antimicrobial properties. Studies of the antimicrobial activity of plant extracts and phytochemicals may lead to the discovery of new antibiotics useful to treat infectious diseases caused by resistant microorganisms. Extracts of various medicinal plants have been reported to possess antimicrobial activity [4-10] and their constituents with these properties have actively been investigated as alternatives to synthetic compounds [11]. Among these, flavonoids seem to be potent candidates because they show broad pharmacological activities and are widely distributed in many edible plants and beverages [12]. Plants belonging to the Fabaceae [13] are rich in flavonoids, some of which show defence functions against pathogenic microorganisms.

Inga fendleriana Benth. is a tropical tree restricted to the Andean rain forests in Venezuela and Bolivia, where it is known as "Guama peludo" or "Guama negro" [14]. The literature reports flavonol glycosides [15], pipecolic acid derivatives [16], and the triterpenes, betulinic acid and lupeol [17] as constituents of *Inga* species.

Many species of *Inga* have been used in folk medicine as astringents in diarrhea and dysentery, as a diuretic herb, as a lotion for arthritis and rheumatism, to treat furunculosis, and to aid in the treatment of wounds. The pulp of the fruits of some species is used for cleaning teeth and secretions from the eyelids [18]. The fruits of some species, especially *I. edulis* Mart., are edible and known as Ice Cream Beans; they are very popular in the local markets of Central and South America for their sweet flavor.

No data have been reported on the antimicrobial activity of *I. fendleriana* and so the aim of this study was to investigate the antimicrobial activity of extracts of this species and its main flavonoid constituents in order to support its traditional use.

Table 1: HPLC-UV-ESI-MS analyses of *I. fendleriana* extracts (μg/mg).

Compounds	EtOAc extract	*n*-BuOH extract
Quercetin 3-methylether	4.54	-
Myricetin 3-O-rhamnoside	85.55	2.34
Tricetin	5.88	-

Table 2: MIC values (μg/mL) of *I. fendleriana* extracts and isolated compounds.

Bacterial strains	EtOAc extract	*n*-BuOH extract	Quercetin–3 methyl ether	Myricetin-3-*O*-rhamnoside	Tricetin
Staphylococcus epidermidis 14990	500	250	62.5	62.5	31.25
Staphylococcus epidermidis SM 1	250	500	62.5	31.25	31.25
Staphylococcus aureus 25923	500	500	250	125	250
Staphylococcus epidermidis 27	500	500	125	125	250
Staphylococcus epidermidis 29	250	500	62.5	62.5	250
Staphylococcus epidermidis 30	850	500	125	125	250
Staphylococcus epidermidis 33	250	500	62.5	125	125
Staphylococcus epidermidis 34	250	500	62.5	62.5	31.25
Staphylococcus epidermidis 35	250	500	125	250	125
Staphylococcus epidermidis 36	250	500	125	250	125
Staphylococcus epidermidis 37	125	125	31.25	31.25	31.25
Staphylococcus epidermidis 38	500	850	125	250	125

From the aerial parts of *I. fendleriana*, myricitrin (**1**), quercetin 3-methylether (**2**) and tricetin (**3**) were isolated and characterized. The quantification of these compounds in the extracts was performed by LC-UV-ESI-MS analyses (Table 1). Each extract and the main isolated constituents (**1-3**) were tested for their antimicrobial activity using a range of bacterial strains.

The tested samples were arbitrarily considered as active when MICs were 1000 μg/mL or lower for at least one bacterial strain. On this basis, only the EtOAc and *n*-BuOH extracts were active; both showed a narrow range of activity to Gram-positive strains, but no inhibitory effect against the Gram-negative ones.

The MIC values of the two extracts against the *Staphylococcus* strains are reported in Table 2. Both extracts had MICs ranging from 850 to 125 μg/mL but the MIC values of the EtOAc extract were generally slightly lower than those of the *n*-BuOH extract. All the tested pure compounds also showed antibacterial activity against the selected strains, with MIC values ranging from 31.25 to 250 μg/mL (Table 2). Their pattern of activity was similar; indeed the strains, except for *S. epidermidis* SM29, exhibited similar behavior of either sensitivity or resistance to the substances; this strain was sensitive to quercetin 3-methylether and myricitrin (MIC 62.5 μg/mL), but was resistant to tricetin (MIC 250 μg/mL). The DMSO, at the used concentrations, did not produce any inhibition of bacterial growth.

For more accurate evaluation of the antibacterial activity of the substances, time-kill assays were performed using the strains with MICs of 62.5 μg/mL or lower. In these experiments, each compound was used at concentrations corresponding to 1xMIC, 2xMIC, and 4xMIC. The results obtained with *S. epidermidis* 14990 are reported in Figure 1; the other strains showed a similar behavior.

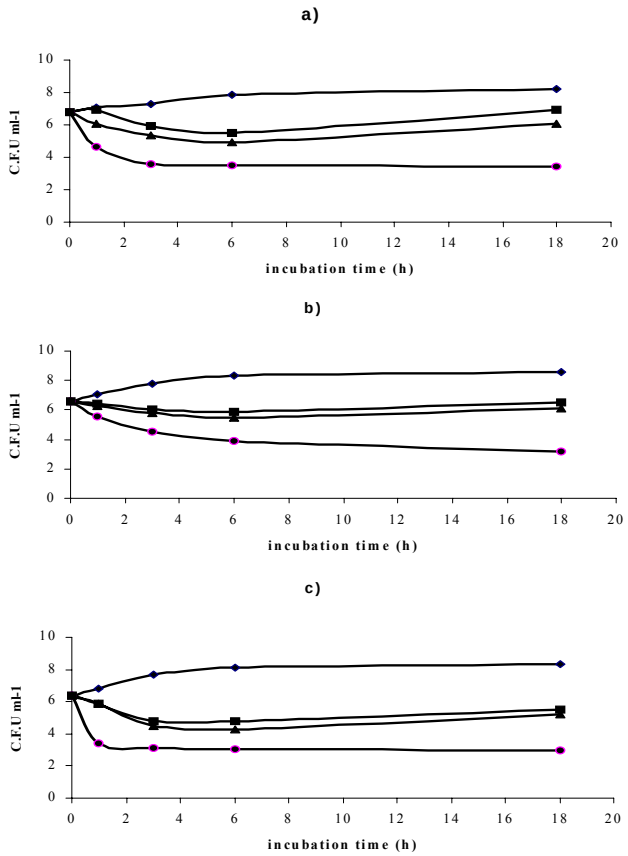

Figure 1: Killing curves of *S.epidermidis* ATCC 14990. (a) quercetin 3-methylether; (b) myricetin 3-*O*-rhamnoside; (c) tricetin. (υ) control; (ν) 1 x MIC; (σ) 2 x MIC; (λ) 4 x MIC.

All the tested compounds showed a bacteriostatic effect at 1x MIC and 2x MIC, as the bacteria remained at the inoculum density after over night incubation; a transient killing (1/2 log units) during the 6 hours exposure period was observed; this effect was followed by regrowth (to > 10^6 CFU/mL) over night This was not due to mutations: In all cases the MICs for isolates recovered after regrowth were the same as those for the initial isolates. At four times the MICs, the compounds produced a bactericidal effect that resulted in over 3 $\log_{10}$ killing by 18 h.

In conclusion the EtOAc and *n*-BuOH extracts from *I. fendleriana* show a narrow spectrum of antibacterial activity against *Staphylococcus* strains. The isolated flavonoids (quercetin 3-methylether, myricitrin and tricetin) are also active against the same strains. The MICs of the extracts are lower than those expected from just the flavonoids tested. The results suggest that these substances may contribute to the antimicrobial activity of the two extracts, but they are not the only ones responsible.

This is the first report on the antibacterial activity of tricetin and apparently this substance has the largest spectrum of activity. It inhibited at 31.2 μg/mL four bacterial strains, while myricitrin and quercetin 3-methylether inhibited two and one, respectively.

The *I. fendleriana* extracts and their isolates, although not active against Gram-negative organisms, displayed an inhibitory effect *in vitro* against clinical isolated *Staphylococci*, which confirms the traditional use of some species of *Inga* to treat forunclosis and to clean teeth and the eyelids.

The antimicrobial properties of the extracts and flavonoids from *I. fendleriana* against clinical isolates of *Staphylococcus epidermidis* could suggest their use to sanitize medical devices, since this bacterial species, actually not considered pathogenic, is responsible for some infections caused by the use of contaminated medical instruments.

Experimental

Plant material: *Inga fendleriana* Benth. aerial parts were collected in the National Park Henri Pittier (Maracay, Venezuela) in January 1999. A voucher specimen was authenticated and deposited (MY Cardoso *et al.* 2667).

Extraction and isolation: Dried and powered *I. fendleriana* aerial parts (600 g) were extracted in a Soxhlet apparatus with light petroleum, chloroform and methanol. The extracts were concentrated under reduced pressure at 40°C, to obtain the residues E (4.29 g), C (8.16 g) and M (27.94 g). The methanolic extract was partitioned with EtOAc and then with *n*-BuOH to obtain the corresponding residues A (4.7 g) and B (7.1 g). Myricitrin (47 mg), quercetin 3-methylether (8 mg) and tricetin (20 mg) were obtained as pure compounds from the EtOAc residue by comparison of their NMR spectroscopic data with those reported in the literature [19-22] or by direct comparison with authentic samples. NMR spectra were measured on a Bruker AC-200 spectrometer, using TMS as internal standard.

Chemicals: Myricetin 3-*O*-rhamnoside, quercetin 3-methylether, and tricetin were included in a home-made data base of natural compounds isolated and characterised by NMR and MS techniques in our laboratory. The standard compounds contained small quantities of impurities, which, although low in amount, could easily be detected by HPLC-PDA-MS. Therefore, all these compounds were analysed and purified by HPLC in the same gradient conditions used for the analyses of the samples in order to guarantee their purity (>98%) before using as reference material. Acetonitrile, acetic acid and methanol were HPLC grade [Baker (Netherlands)]. HPLC-water was purified by a Milli-Q Plus system (Millipore Milford, MA, USA).

Sample preparation for HPLC analyses: Three samples of each extract of *I. fendleriana* were dissolved in methanol (20 mg/mL) and filtered through a cartridge-type sample filtration unit with a polytetrafluoroethylene filter (PTFE, 0.45 μm, 25 mm) before HPLC injections. Injected volume of the extracts: 20 μL.

HPLC-PDA-ESI-MS analyses: The HPLC system consisted of a Surveyor Thermofinnigan liquid chromatography pump equipped with an analytical Lichrosorb RP-18 column (250 x 4.6 mm i.d., 5 μm, Merck), a Thermofinnigan Photodiode Array Detector and a LCQ Advantage mass detector.

The analyses were carried out with a linear gradient using water with 0.1% HCOOH (solvent A), CH_3CN (solvent B) with 0.1% HCOOH at flow rate of 0.7 mL/min in the following conditions: from 15:85 v/v (B-A) to 30:70 (B-A) in 25 min for 15 min, then to 70:30 v/v (B-A) in 25 min for 15 min and then conditioning to the initial condition (15:85 v/v B-A) for 10 min. The total analytical run time was 65 min for each sample.

The spectral data from the PDA detector were collected during the whole run in the range 210-700 nm and the peaks were detected at 260 nm. An aliquot (20 μL) of each sample was analyzed in triplicate.

In order to confirm the presence of the analytes in the EtOAc and *n*-BuOH extracts, LC-ESI-MS analyses

Table 3: The linear regression equations, HPLC-UV and correlation coefficients for the analyzed compounds in *Inga fendleriana* extracts.

Compound	*Concentration Range*	*Regression equations*
Myricetin 3-*O*-rhamnoside	5.6–112 μg mL^{-1}	y= 0.99906+9.2428E-5x(*r*=0.9998)
Quercetin 3-methylether	2.4–120 μg mL^{-1}	y=-6.296E-3+2.5567E-5x(*r*=0.9997)
Tricetin	41.5-860 μg mL^{-1}	y= 0.77755+5.944E-5x(*r*=0.9998)

were performed using the same chromatographic conditions and these specific ESI values: sheath gas flow-rate 72 psi, auxiliary gas flow 10 psi, capillary voltage –16 V and capillary temperature 200°C. Full scan spectra from m/z 200 to 700 in the positive ion mode were obtained.

The identification of each constituent was carried out by the comparison of the peaks in the extracts with the retention time, UV and MS spectra of the authentic samples previously injected in the same chromatographic condition.

Calibration curves for the reference compounds (1-3): The dosage of the analysed constituents (**1-3**) was performed by the external standard method, using 6 levels of concentration for each compound; the respective linear regression equations and their correlation coefficients for the marker compounds are shown in Table 3. An aliquot (20 μL) of each standard compound was analyzed in triplicate under the same conditions used for the analyses of the sample extracts by HPLC-PDA-ESI-MS. The standard solutions for the authentic samples (**1-3**) were prepared in methanol.

Bacteria and media: *Escherichia coli* 11303, 15597, 25922, 25404, 23739, 12435 and 15669 were obtained from the American Type Culture Collection; Strain MS 1 was a clinical isolate; and strain O21 was obtained from the Istituto Sieroterapico Milanese. *Salmonella typhimurium* 15277, 13311 and 19585 were purchased from the American Type Culture Collection, and *S. schwarzengrund* and *S. infantis* were clinical isolates. *Pseudomonas aeruginosa* 27853 and 10145 were obtained from the American Type Culture Collection, whereas strains 153, 769 and 776 were clinical isolates. *Staphylococcus epidemidis* 14990 came from the American Type Culture Collection and strain SM1 was a clinical isolate. *S. aureus* was obtained from the American Type Culture Collection. *S. epidermidis* strains 27, 29, 30, 33-38 were clinical isolates.

The used media were: Nutrient Agar (Difco) for maintenance of the strains, Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) (Difco) supplemented with 25 mg of Ca^{2+}/L, 12.5 mg of Mg^{2+}/L, and 2% NaCl for antimicrobial assays [23-24].

Antibacterial assays: The extracts and the pure compounds were dissolved in dimethylsulfoxide (DMSO) to give a concentration of 20 mg/mL and were sterilized by filtration through a Millipore filter (0.2 μm). Further dilutions of the stock solutions were prepared in sterile water.

Minimum inhibitory concentration: MICs were determined by the agar dilution method, as follows: the MHA medium was poured into the plates and allowed to solidify, then a strip of medium in the shape of a ditch was removed. Melted Mueller Hinton Agar containing appropriate dilutions of each stock solution was used to fill the gap. The final concentrations used for the extracts ranged from 1000 to 31.25 μg/mL, while the final concentrations of the pure substances ranged from 500 to 7.8 μg/mL. Table 2 reported the MICs (μg/mL) of the tested samples. Controls were carried out using DMSO (5% v/v) in order to assay toxicity due to the solvent. The inoculum suspensions were prepared using overnight broth cultures of the test organisms which were diluted to achive a turbidity corresponding to 0.5 McFarland (approximately 1 x 1 x 10^8 CFU/mL). Using a calibrated loop, 2 μL of each inoculum was streaked onto the plate perpendicularly to the strip so that each streak crossed it. The plates were incubated for 48 h at 37°C. The lowest concentration at which no visible growth occurred on the strip was defined as the MIC. All experiments were repeated twice with three triplicates.

Time-kill assays: Time-kill kinetic studies were performed in MHB, with an inoculum of 5 x 10^6 CFU/mL, in a final volume of 10 mL. The substances were used at final concentrations corresponding to 1 x MIC, 2 x MIC, 4 x MIC. The bacterial suspensions were incubated at 37°C for 18 h. Bacterial growth was followed by taking samples (100 μL) from the cultures at 0, 1, 3, 6, and 18 h. The samples were ten-fold serially diluted with peptone saline; 10 μL of the

samples (diluted and undiluted) were spread on Mueller Hinton Agar plates (4 plates per dilution) and the Colony Forming Units were counted after incubation at 37°C for 48 h. A 99.9% reduction of the original inoculum was interpreted as indicative of a bactericidal effect. The controls of bacterial growth and the DMSO inhibitory effect were respectively inoculated in MHB alone and inoculated MHB with DMSO (2.5 % v/v).

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