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Antibacterial activity of plant extracts from northwestern Argentina

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Abstract

Aims: To determine the antibacterial and cytotoxic activities of aqueous and ethanolic extracts of northwestern Argentinian plants used in folk medicine. To compare the mentioned activities with those of five commercial antibiotics. To identify the compounds responsible for the antibacterial activity.

Methods and Results: Plant extracts were prepared according to traditional uses in northwestern Argentina. Antibacterial activity was assayed by agar dilution in Petri dishes and broth dilution in 96-well plates. Lethal dose 50 (LD_{50}) was determined by the *Artemia salina* assay. Phytochemical analysis was performed by sample adsorption on silica gel, thin-layer chromatography (TLC), bioautography and UV-visible spectra. The results showed that *Tripodanthus acutifolius* aqueous extracts have lower minimal inhibitory concentrations (MIC) (502 and 506 μg of extracted material (EM) per ml for infusion and decoction, respectively) than cefotaxim MIC (640 μg ml^{-1}) against *Acinetobacter freundii* (303). These data were lower than their LD_{50}. *Tripodanthus acutifolius* tincture showed lower MIC (110 μg of EM per ml) and minimal bactericidal concentration (MBC) (220 μg of EM per ml) than cefotaxim (MIC and MBC of 320 μg ml^{-1}) for *Pseudomonas aeruginosa*. This extract also showed a MIC/MBC of 110/220 μg of EM per ml, lower than oxacillin (MIC/MBC of 160/220 μg ml^{-1}) for *Staphylococcus aureus* (ATCC 25923). The cytotoxicity of all extracts were compared with that of commercial antibiotics. Rutin (3,3′,4′,5,7-pentahydroxyflavone 3-β-rhamnosilglucoside), *iso*-quercitrin (3,3′,4′,5,7-pentahydroxyflavone 3-β-glucoside) and a terpene would be partially responsible for the antibacterial activity of *T. acutifolius* infusion.

Conclusions: *Tripodanthus acutifolius* extracts had the ability to inhibit bacterial growth. The antibacterial activity differs with the applied extractive method, and it could be partially attributed to glycoflavonoids. This paper contributes to the knowledge of antibacterial capacity of plants from northwestern Argentina.

Significance and Impact of the Study: These antibacterial activities support further studies to discover new chemical structures that can contribute to alleviate or cure some illnesses.

Introduction

For thousand years, mankind has learnt about the benefits of plant use to alleviate or cure illnesses. The plant kingdom constitutes a source of new chemical compounds which may be important owing 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, and about 30% of the worldwide drug sales are based on

natural products (Grabley and Thiericke 1999). It is estimated that there are about 2 500 000 species of higher plants throughout the world, and most of them have not been examined in detail for their pharmacological activities (Jeevan Ram *et al.* 2004). However, for some decades, there has been increasing interest in plant uses and the detection of their constituents with antibacterial activity. The most important reason for the latter was that infections represent one of the main causes of illness and mortality around the world (World Health Organization 1998; Hamill *et al.* 2003), in particular, infections caused by *Enterococcus* and *Staphylococcus* species, which would be agents of many intrahospital infections (Maple *et al.* 1989; Mullingen *et al.* 1993). Furthermore, the development of drug resistance and the appearance of undesirable side effects of certain antibiotics (Davies 1994; Poole 2001; WHO publication 2001) have led to the search for new antimicrobial agents, mainly among plant extracts, in order to find new chemical structures to overcome the aforementioned disadvantages (Ordóñez *et al.* 2003; Arias *et al.* 2004). Many bacteria are able to penetrate through skin, mucosa lesions or hair follicles, producing bacterial infections. These infections occur as local purulences (e.g. oedema, ulcers, etc.), which can frequently be generalized in the whole organism. External infections have increased in the last two decades. In the past, commercial antibiotics were successful to fight them, but some pathogenic bacteria have developed resistance to these antibiotics. Consequently, natural products from higher plants are an interesting source of antibacterial agents to be applied for external use, e.g. cataplasms, compresses, gargles, and ointments.

The purpose of our research was the determination of the antibacterial activity of aqueous and ethanolic extracts of 12 northwestern Argentinian plants used in folk medicine. Their antibacterial activity and cytotoxicity were compared with those of five commercial antibiotics. Compounds that were possibly responsible for the antibacterial activity were identified from one of the extracts.

Materials and methods

Plant material

The plants were collected from the province of Tucumán, in northwestern Argentina. They 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.

Chemicals

All chemicals were of analytical grade. Acetic acid, chloroform, formic acid, 96% ethanol, ethyl acetate, methanol and NaCl were from Cicarelli Labs (Santa Fe, Argentina). Ethyl ether and sterile polystyrene 96-well plates ('U' bottom) were from Biopack (Buenos Aires, Argentina). $CaCl_2$, coumarin, glycerol, $MgCl_2$ and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), Na_2HPO_4 and NaH_2PO_4,oxacillin (sodium salt) and vancomycin were from Sigma-Aldrich (St Louis, MO, USA). Ampicillin was from Klonal Labs (Quilmes, Buenos Aires, Argentina). Cefotaxim was from Duncan Labs (Buenos Aires, Argentina). Folin-Ciocalteu reagent, imipenem, Silica Gel 60, Silica Gel 60 F_{254}plates, and preparative TLC Silica Gel 60 plates were from Merck (Darmstadt, Germany). Brain heart infusion (BHI) medium, Muller Hinton (MH) agar medium and MH broth medium were from Britania S.A. Labs (Buenos Aires, Argentina). Difco bacto agar was from Difco (Detroit, MI, USA). Rutin and *iso*-quercitrin were purchased from Indofine Chemical Company, Inc. (Belle Mead, NJ, USA). Millipore membrane filters (pore size 0·22 μm) were from Millipore Corp. (Bedford, MA, USA).

Extract preparations

Three extracts were prepared from each plant: infusions, decoctions (aqueous extracts) and tinctures (alcoholic extracts). The extractions were carried out according to Farmacopea Nacional Argentina (1978), with slight modifications. Briefly, infusions were prepared by adding boiling distilled water to plant coarse powder (5 g per 50 ml), and leaving it covered at room temperature for 20 min. Decoctions were prepared by boiling plant coarse powder with distilled water (5 g per 50 ml) for 20 min, and then left at room temperature. Tinctures were prepared in sealed flasks by adding plant coarse powder with 96% ethanol (10 g per 100 ml), stirring (40 cycles per minute) at 37 °C for 7 days. Extracts so obtained were filtered through Whatman No. 1 filter paper.

Phenolic content determination

The total phenolic content was determined by the Folin–Ciocalteu method (Singleton *et al.* 1999) using coumarin as standard.

Extraction yield

The yield of each extraction was calculated according to Farmacopea Nacional Argentina (1978) with slight modi-

fications. Briefly, aqueous extracts were dried in a Virtis Liter freezer dryer, SL model (Virtis, Gardiner, NY, USA). Tinctures were dried under reduced pressure using rotary vacuum evaporator at 30 °C. The dried material obtained represented the extracted material (EM) for each extract.

Commercial antibiotics

Taking into account their properties, action mode and their high prescription frequency to combat the microbial agents in northwestern Argentina, five commercial antibiotics (ampicillin, cefotaxim, imipenem, oxacillin sodium salt and vancomycin) were selected. Ampicillin is a potent antibiotic clinically used for the treatment of a broad range of bacterial infections (Mandell *et al.* 1990). Cefotaxim is a third-generation cephalosporin used in nosocomial gram-negative infections (Adu and Armour 1995). Imipenen is a β-lactamic antibiotic from carbapenem group, especially used in gram-negative and multiorganism infections (Klastersky 2003). Oxacillin is a β-lactamic antibiotic used against gram-positive strains, such as *Staphylococcus aureus* (Kristiansen *et al.* 2003), and vancomycin is a safe, effective antibiotic for a variety of serious gram-positive infections (Lundstrom and Sobel 2000). Stock solutions (1 mg ml^{-1}) were prepared and stored at −20 °C.

Micro-organisms

The micro-organisms used included eight gram-negative bacteria: *Escherichia coli* (301), *Enterobacter cloacae* (302), *Acinetobacter freundii* (303), *Proteus mirabilis* (304), *Pseudomonas aeruginosa* (305), *Klebsiella pneumoniae* (310), *Serratia marcescens* (313), and *Morganella morganii* (320); four gram-positive bacteria: *Staph. aureus* (F7), *Staph. aureus* coagulase-negative methicillin-sensible (F20), *Enterococcus faecali* (F208) and *Enterococcus faecium* (F229), all of them isolated and classified by Dr Norma Cudmani from cutaneous wounds at Hospital 'Nicolás Avellaneda', SIPROSA, Tucumán, Argentina. The figures in parentheses indicate the numbers of the isolates. The assays also included reference strains from American Type Culture Collection (ATCC): *Ent. faecali* (ATCC 29212), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *Staph. aureus* (ATCC 25923) and *Staph. aureus* (ATCC 29213), as recommended by National Committee for Clinical Laboratory Standards (NCCLS 1999a); Yu *et al.* 2004. The strains were stored in BHI medium supplemented with 0·3% w/v agar and 1·5% v/v glycerol at −20 °C. *Pseudomonas aeruginosa* strains were stored in sterile water (Liao and Shollenberger 2003) supplemented with 1% v/v glycerol at room temperature.

Inoculum preparation

Stock bacterium cultures were maintained at room temperature for 2 h. Each strain was streaked on MH agar plate, and incubated at 37 °C for 24 h. The inoculum was prepared by emulsifying a minimum of three colonies from those plates in sterile 0·9% NaCl (w/v) till 10^8 colony forming units (CFU) per ml (0·5 McFarland scale) are formed. For the agar dilution assays, the suspensions were diluted with sterile 0·9% NaCl (w/v) until they reach 10^7 CFU per ml (working bacterial suspensions). The use of a laminar flux equipment assured the sterile conditions of the procedures.

Sterilization

Plant extracts and commercial antibiotics were filtered through a 0·22-μm membrane filter before use.

MIC and MBC determinations

Agar dilution assay (macrodilution)
The assay was carried out in MH agar (Thornsberry and McDougal 1983), according to Cooper *et al.* (2002), with slight modifications. Briefly, serial dilutions of each extract (30–325 000 μg of EM per ml) in sterile water (for aqueous extracts) or in 96% ethanol (for tinctures) were prepared. Commercial antibiotics were diluted (10–6400 μg ml^{-1}) in sterile water. One millilitre of extract or commercial antibiotic dilution was incorporated in 9 ml of molten MH agar, vigorously vortexed, dispensed into Petri dishes (90-mm diameter), and left to stand until solidification. Two microlitres of each working bacterial suspension were punctually placed on the solid medium surface at different places. Bacterial growth controls were made without extract addition, using MH agar medium and 1 ml of 96% ethanol (solvent controls for the tinctures) or 1 ml of sterile water (controls for aqueous extracts). The plates were incubated at 37 °C for 24 h, and the bacterial growth was determined on each plate by comparing the punctual growth zones with those in the controls. The absence of growth was interpreted as antibacterial activity.

Broth dilution assay in 96-well plates (microdilution)
The assay was carried out in MH broth supplemented with $CaCl_2$ and $MgCl_2$(12·5 mg l^{-1} of Ca^{+2} and Mg^{+2}), as recommended by the National Committee for Clinical Laboratory Standards (NCCLS 1999b). The assay was performed according to Hammer *et al.* (1999), with slight modifications. Briefly, serial dilutions of plant extracts (3–32 500 μg of EM per ml) or commercial antibiotics (1–640 μg ml^{-1}) were prepared in sterile polystyrene 96-

well plates. The final concentration of each strain suspension was adjusted to 5×10^5 CFU ml^{-1} with 50 μl of bacterial suspension in supplemented MH broth, in a final volume of 100 μl. For tinctures, bacterial growth controls were made by replacing ethanolic extracts with the same volume of 96% ethanol (in order to eliminate the possible antibacterial effect of the solvent). For aqueous extracts and commercial antibiotics, bacterial growth controls were made without adding plant extracts or commercial antibiotics (those were replaced with the same volume of water). Sterility controls were prepared by using MH broth medium alone. Extract colour wells included the incubation mixture without bacterial inoculum (this avoided the interference of the plant extract colour in the results). The plates were covered with a sterile plate sealer, carefully mixed and incubated at 37 °C for 24 h. Bacterial growth was indicated by the turbidity. The absence of bacterial growth was interpreted as antibacterial activity. The MIC value was taken as the lowest concentration of the test agent that caused complete inhibition (100%) of bacterial growth (Shin *et al.* 1998) in experimental conditions. To determine the MBC, 25 μl of broth aliquots were taken from each well and incubated in MH agar at 37 °C for 24 h. Bacterial viability controls included aliquots taken from growth control wells. MBC was defined as the lowest concentration of assayed samples which produced 99·9% reduction in CFU ml^{-1} as compared with the control (Bosio *et al.* 2000).

Cell cytotoxicity assay

Artemia salina assay was used for cytotoxicity determination of plant extracts and commercial antibiotics, according to Meyer *et al.* (1982). Briefly, brine shrimp eggs were placed in sea water (3·8% NaCl, w/v, in distilled water) and incubated at 25 °C opposite a lamp (100 W, 2000 Lux). Eggs hatch and mature within 24 h, providing a large number of larvae (nauplii). Ten larvae were placed in vials containing 5 ml of sea water and increasing concentrations of plant extracts or commercial antibiotics. The dose ranges used for each extract or commercial antibiotics were chosen by using the MIC and MBC values as reference with the purpose of including them in the assay. Serial dilutions were prepared in order to reach the chosen concentrations in the final volume (5 ml). Controls were made in vials containing 5 ml of sea water and a maximum of 50 μl of 96% ethanol (for ethanolic extracts) and without ethanol for both aqueous extracts and commercial antibiotics. Living nauplii were counted after 16 h at 25 °C (2000 Lux). The results were expressed as micrograms of EM per millilitre for plant extracts and μg ml^{-1} for commercial antibiotics.

Statistical analysis

Macrodilution assays were performed in triplicate and repeated twice. Microdilution assays were conducted eight times for each plant extract or commercial antibiotic concentration, and repeated twice. The arithmetic means of the MIC and MBC were calculated and reported (Hili *et al.* 1997). Cell cytotoxicity assays were carried out in triplicate, and repeated twice for each plant extract or commercial antibiotic concentration. The results were evaluated with Finney computer program to determine the lethal dose 50 (LD_{50}) values (Finney 1971), expressed as micrograms of EM per millilitre for plant extracts and as μg ml^{-1} for commercial antibiotics.

Bioactivity-guided fractionation of *Tripodanthus acutifolius* infusion

A bioactivity-guided fractionation of *T. acutifolius* infusion was conducted as follows: lyophilized infusion (200 g) was successively extracted using ethyl ether (1 l), ethyl acetate (1 l) and methanol (1 l) in an order of increasing solvent polarity. The solvents were evaporated to dryness under reduced pressure using a rotary vacuum evaporator. The dried residues were redissolved in methanol to 10 mg $ml^{-1}$for ethyl ether and ethyl acetate extracts, and 500 mg $ml^{-1}$for the methanol extract. Bioautographic analysis showed that the methanol extract exhibited high antibacterial activity. Subsequently, only the methanol extract was chosen for further isolation of antibacterial compounds. Six millilitres of the methanol extract (3 g) were adsorbed to 175 g of Silica Gel 60 ($62 \times 3{\cdot}3$-cm column, particle size 0·2–0·5 mm). The column was eluted with 85 : 15 v/v (375 ml), 75 : 25 v/v (375 ml), 65 : 35 v/v (375 ml) and 0 : 100 v/v (375 ml) chloroform–methanol mixtures (in an order of increasing polarity) to obtain 100 fractions of 15 ml. Fractions from 1–25, 26–30, 31–42, 43–48, 49–69, 70–89 and 90–100, which showed similar composition and antibacterial activity were separately pooled, and referred to as pool 1, 2, 3, 4 and 5, respectively.

Isolation and purification of active substances in the bioactive fraction

Twenty-five milligrams of pool 3 (49–69 fractions, eluted with 65 : 35-v/v chloroform : methanol) was further fractionated by means of preparative TLC Silica Gel 60 plates (20×20 cm) using ethyl acetate–methanol–water (100 : 13·5 : 10 v/v) as the mobile phase. After developing the plates, the positions of different compounds were determined by fluorescence under UV light (254 and 366 nm, UV Lamp Model UV 5L-58 Mineralight

Lamp). Compounds were removed by scraping off the silica and washing five times with methanol (Ouellette 1992). The purity of the compounds was tested by qualitative TLC (as described later) using different mobile phases.

Phytochemical screening

Silica Gel 60 F_{254}plates (20 × 20 cm) (Camm *et al.* 1975) with the solvent system: ethyl acetate–formic acid–acetic acid–water (100 : 11 : 11 : 27 v/v), suitable as a screening system for the TLC investigation of flavonoid glycosides (Wagner *et al.* 1984) and chloroform–methanol (85 : 15 v/v) were used for the fractionation of infusion and column fraction components by ascendent TLC. The separated compounds were observed under UV light (254 and 366 nm). The group of chemicals to which these compounds belonged were identified by using standard staining procedures with the following reagents: modified Dragendorff's reagent for alkaloids, ferric chloride and natural product reagent (1% methanolic 2-aminoethyl diphenylborate) for total phenolic compounds, vanillin/sulfuric acid for steroids and terpenes and blood reagent for saponins (Krebs *et al.* 1969; Wagner *et al.* 1984).

Bioautographic analysis

The assay was performed according to Quiroga *et al.* (2004), with slight modifications. Briefly, the analysis was made on Silica Gel 60 plates by TLC of the extracts in different solvents (Homans and Fuchs 1970). After the plates (6 × 8 cm) were dried under sterile conditions, they were covered with 2 ml of soft medium (BHI added with 0·6% agar) containing 10^5 CFU $ml^{-1}$of *Staph. aureus* (ATCC 25923) or *P. aeruginosa* (F305), and incubated at 37 °C for 16–20 h. Subsequently, the plates were covered with 5 ml of 2·5 mg ml^{-1} of MTT in sodium phosphate buffer (10 mmol l^{-1}, pH 7), with 0·15 mol l^{-1} NaCl. The plates were incubated in the dark for nearly 1 h (or until colour development). Bacterial growth inhibition appeared as yellow areas on a dark background. Their positions were compared with those of the spots revealed with other reagents.

Identification of active compounds

Active compounds were identified by qualitative TLC using commercially available standards. UV spectra were obtained for purified compounds on a Beckman DU 650 spectrophotometer. UV spectra were compared with those of standard drugs and bibliographic data (Mabry *et al.* 1970).

Results

The detailed list of the 12 plant species, belonging to 10 families, used in this work is shown in Table 1 (Gupta 1995; Alonso 1998; Lahitte *et al.* 1998). We used three different kinds of extractive forms of plant aerial parts (infusions, decoctions and tinctures). Consequently, a total of 36 extracts were tested against 10 gram-negative and seven gram-positive bacteria. The yield of the extractions was higher for the aqueous, mainly decoction, than for the ethanolic extracts. Water extracted the highest quantity of material (EM), and the highest yield of phenolic compound extraction was obtained by decoction (Table 2). Macrodilution was the first stage for the antibacterial activity screening of plant extracts (Fig. 1). All the tinctures (but *Tropaeolum majus*) showed antibacterial activity against the tested strains. *Leonurus sibiricus*, *T. acutifolius*, *Psittacanthus cuneifolius* and *Jodina rhombifolia* tinctures showed the highest activity (Table 3). Only *L. sibiricus*, *T. acutifolius*, *P. cuneifolius* and *J. rhombifolia* aqueous extracts (infusions and decoctions) showed an inhibitory effect with this method at the assayed concentrations. As these species showed antibacterial activity against a wide range of strains, they were selected for further studies. Decoctions showed lower antibacterial activity than infusions in nearly 90% of the cases (Table 4).

Microdilution assays were carried out in order to obtain MIC and MBC values for the selected plant extracts and commercial antibiotics (Tables 3–5). *Tripodanthus acutifolius* extracts exhibited the highest antibacterial activity, because they inhibited the bacterial growth of every tested strain. MIC and MBC for plant extracts are, in general, higher than those of commercial antibiotics, though some of them are lower, e.g. *T. acutifolius* infusion and decoction MIC values against *A. freundii* are lower than cefotaxim MIC value for the same strain (note that cefotaxim is a wide spectrum β-lactamic antibiotic). It is clear that *T. acutifolius* infusion MIC against *P. aeruginosa* (ATCC 27853), *Staph. aureus* (F7), *Staph. aureus* (ATCC 29213) and *Staph. aureus* (ATCC 25923) are lower than LD_{50} obtained by *A. salina* assay (Table 6). This extract also presented lower MBC than LD_{50},e.g. against *Staph. aureus* (F7) and *Staph. aureus* (ATCC 25923). *Tripodanthus acutifolius* decoction showed lower MIC than LD_{50}, e.g. MIC against *Staph. aureus* (F7), *Staph. aureus* (F20), *Staph. aureus* (ATCC 29213), *Staph. aureus* (ATCC 25923) and *A. freundii*. Furthermore, *P. cuneifolius* aqueous extracts showed lower antibacterial activity than *T. acutifolius* aqueous extracts; the former were not active against some strains, and their MIC were nearly always higher than those of *T. acutifolius*. Moreover, *L. sibiricus* and *J. rhombifolia* aqueous extracts showed a restricted antibacterial activity range.

Table 1 Classification and popular medicinal properties of plants from northwestern Argentina

Family	Botanical name	Vernacular names	Folk uses	Used parts
Apiaceae	*Ammi majus* L.	Apio. Berro cima. Cicuta negra	Stomach problems, stimulant, carminative	Seeds
Apocinaceae	*Vinca minor* L.	Pervinca.	Arterial hypotensor	Whole plant
Asteraceae	*Solidago chilensis* Meyen	Vara de oro, vara de San Juan.	To relieve articular pains. Diuretic, expectorant, vulnerary	Aerial parts, roots
Laminaceae	Leonurus sibiricus L.	Cola de león, Leonuro	Febrifuge	Herbaceous parts
	Schkuhria pinnata Kuntze ex Thell.	Canchalagua.	Digestive diseases	Whole plant
Loranthaceae	*Psittacanthus cuneifolius* (Ruiz & Pav.) Blume	Liga, liguilla	To cure fractures	Aerial parts
	Tripodanthus acutifoliu (Ruiz & Pav.) Van Tieghem	Corpo	To relieve nasal congestions	Leaves
Passifloraceae	*Passiflora edulis* Sims	Maracuyá.	To settle edgy nerves, also for colic, diarrhoea, dysentery and insomnia	Leaves
Rhamnaceae	*Colletia spinosissima* J. Gmelin	Baba de tigre, quina del campo	Antiperiodic, astringent, febrifuge	Aerial parts
Santalaceae	*Jodina rhombifolia* Hook. et Arn.	Sombra de toro	Digestive, antiasthmatic, antidysenteric	Leaves
Solanaceae	*Nicotiana glauca* Graham	Palán-palán		Aerial parts
Tropaeolaceae	*Tropaeolum majus* L.	Taco de reina.	Prevents baldness, demulcent, vulnerary. Antiscorbutic, analgesic	Whole plant

Table 2 Yield of the extraction processes expressed as percentage of extracted material (EM) and of phenolic compounds

Plant extract	Extracted material (%, w/w)*	Phenolic compounds (%, w/w)*
Jodina rhombifolia		
Decoction	26·4 ± 1·8	4·0 ± 0·2
Infusion	30·8 ± 1·2	3·8 ± 0·2
Tincture	12·5 ± 0·9	2·2 ± 0·1
Leonurus sibiricus		
Decoction	24·0 ± 0·9	2·4 ± 0·1
Infusion	24·0 ± 1·3	1·8 ± 0·1
Tincture	7·2 ± 0·9	0·2 ± 0·01
Psittacanthus cuneifolius		
Decoction	46·6 ± 2·4	8·8 ± 0·5
Infusion	52·0 ± 2·1	5·8 ± 0·3
Tincture	15·0 ± 0·7	2·4 ± 0·1
Tripodanthus acutifolius		
Decoction	55·5 ± 3·2	18·7 ± 0·8
Infusion	47·6 ± 4·0	12·9 ± 0·9
Tincture	30·5 ± 2·5	3·6 ± 0·2

*EM and phenolic compounds are calculated as grams of material and grams of phenolic compounds extracted from 100 g of a dried plant sample. EM is expressed in grams and phenolic compounds as quantity equivalent to coumarin.

Antibacterial activity evaluation of the three extractive forms showed that tinctures were the most effective ones. *Tripodanthus acutifolius* tincture showed lower MIC and MBC than cefotaxim against *A. freundii* and *P. aeruginosa* (ATCC 27853), than oxacillin against *Staph. aureus* (ATCC 25923), and than ampicillin against both *Ent. faecali* strains. *Psittacanthus cuneifolius* tincture also showed significant effects against *A. freundii* (lower MIC than cefotaxim) and *Staph. aureus* (ATCC 25923) (lower MIC than oxacillin), and lower MBC than imipenem and cefotaxim against *P. aeruginosa* (305). *Leonurus sibiricus* and *J. rhombifolia* tinctures were also more active against *A. freundii* than cefotaxim, although these tinctures showed a quite restricted antibacterial spectrum.

The cytotoxicity assays showed that the LD_{50} obtained for *T. acutifolius* aqueous extracts was higher than LD_{50} for commercial antibiotics, such as cefotaxim, imipenem, oxacillin and vancomycin. *Psittacanthus cuneifolius* aqueous extracts and *J. rhombifolia* decoction LD_{50} were similar or higher than those of cefotaxim and oxacillin. *Leonurus sibiricus* aqueous extracts showed no cytotoxicity in the assayed concentrations (Table 6). Note that high LD_{50} means low cytotoxicity. These results give evidence of low cytotoxicity for those plant extracts that have shown antibacterial activity. This low toxicity of infusions and decoctions over tinctures indicates that the cytotoxic effect and the extract compositions depend on the used extraction method.

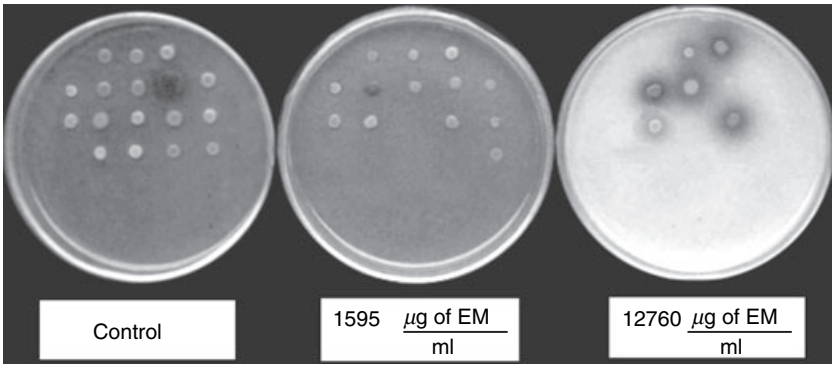

Figure 1 Antibacterial activity assayed by macrodilution in a solid medium [Muller Hinton (MH)]. It was added with 1595 and 12 760 μg of extracted material (EM) per millilitre of *Psittacanthus cuneifolius* infusion or sterile water as a control. In the medium added with 1595 μg, the growth of *Escherichia coli* (301), *Enterobacter cloacae* (302), *Acinetobacter freundii* (303), *Proteus mirabilis* (304), *Pseudomonas aeruginosa* (305), *Klebsiella pneumoniae* (310), *Serratia marcescens* (313), *Morganella morganii* (320), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), *Staphylococcus aureus* (F20), *Enterococcus faecali* (F208), *Enterococcus faecium* (F229) was observed, whereas in the medium added with 12760 μg of EM per millilitre, only the growth of *E. coli* (301), *Enterobacter cloacae* (302), *K. pneumoniae* (310), *S. marcescens* (313), *E. coli* (ATCC 25922), *Staph. aureus* (F20) was observed as compared with the control.

Table 3 Minimal inhibitory concentration (MIC)/minimal bactericidal concentration (MBC) values (micrograms of extracted material per millilitre) for plant tinctures from northwestern Argentina, determined by microdilution assay

	Jodina rhombifolia	*Leonurus sibiricus*	*Psittacanthus cuneifolius*	*Tripodanthus acutifolius*
Gram-negative bacteria				
Acinetobacter freundii (303)	180/719	216/863	101/406	110/440
Enterobacter cloacae (302)	1438/NBE*	863/863	812/1623	881/1762
Escherichia coli (301)	NIE†/NBE	NIE/NBE	1623/1623	1762/1762
E. coli (ATCC 25922)	719/NBE	NIE/NBE	1623/1623	881/881
Klebsiella pneumoniae (310)	NIE/NBE	863/NBE	812/812	881/1762
Morganella morganii (320)	NIE/NBE	NIE/NBE	406/812	110/220
Proteus mirabilis (304)	180/1438	216/432	203/406	220/440
Pseudomonas aeruginosa (305)	NIE/NBE	108/432	406/406	440/440
P. aeruginosa (ATCC 27853)	NIE/NBE	216/432	203/203	110/220
Serratia marcescens (313)	360/1438	863/NBE	101/812	440/881
Gram-positive bacteria				
Enterococcus faecali (ATCC 29212)	NIE/NBE	863/NBE	406/NBE	440/440
Ent. faecali (F208)	NIE/NBE	863/NBE	812/NBE	440/440
Enterococcus faecium (F229)	NIE/NBE	863/NBE	812/NBE	881/881
Staphylococcus aureus (ATCC 25923)	NIE/NBE	863/NBE	101/101	110/220
Staph. aureus (ATCC 29213)	NIE/NBE	863/NBE	203/203	110/220
Staph. aureus (F20)	1438/NBE	432/863	812/812	881/881
Staph. aureus (F7)	NIE/NBE	863/NBE	101/101	110/220

*NBE, no bactericidal effect.
†NIE, noninhibitory effect.

Tripodanthus acutifolius infusion was selected to determine the source of the antibacterial activity. Three extracts were obtained by extracting dried infusion with solvents of increasing polarity. Bioautographic assays showed that methanol extract possessed high antibacterial activity; therefore, this extract was targeted for isolation of antibacterial compounds. Silica gel column fractions were pooled together based on TLC and bioautographic analysis. Pool 3 was chromatographed on preparative TLC Silica Gel 60 plate. Three of the active compounds were isolated and two of them identified as rutin (3,3′,4′,5,7-pentahydroxyflavone 3-β-rhamnosylglucoside) and *iso*-quercitrin (3,3′,4′,5,7-pentahydroxyflavone 3-β-glucoside) by comparison of their R_f(0·35 and 0·6, respectively) in ethyl acetate–formic acid–acetic acid–water (100 : 11 : 11 : 27 v/v) solvent system with the commercially available standards

Table 4 Minimal inhibitory concentration (MIC)/minimal bactericidal concentration (MBC) values (micrograms of extracted material per millilitre) for infusions and decoctions from northwestern Argentinian plants, determined by microdilution assay

	Jodina rhombifolia		Leonurus sibiricus		Psittacanthus cuneifolius		Tripodanthus acutifolius	
	Decoction	Infusion	Decoction	Infusion	Decoction	Infusion	Decoction	Infusion
Gram-negative bacteria								
Acinetobacter freundii (303)	NIE*/NBE†	NIE/NBE	NIE/NBE	NIE/NBE	4855/NBE	6380/NBE	506/8102	502/2010
Enterobacter cloacae (302)	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	8102/8102	4019/4019
Escherichia coli (301)	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	8102/32 407	4019/16 076
E. coli (ATCC 25922)	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	2025/32 407	4019/8038
Klebsiella pneumoniae (310)	NIE/NBE	NIE/NBE	NIE/NBE	6857/NBE	NIE/NBE	NIE/NBE	8102/16 204	2010/8038
Morganella morganii (320)	NIE/NBE	NIE/NBE	11 163/NBE	NIE/NBE	4855/9709	3190/6380	2025/16 204	1005/8038
Proteus mirabilis (304)	NIE/NBE	NIE/NBE	11 163/NBE	NIE/NBE	4855/NBE	6380/NBE	2025/8102	1005/2010
Pseudomonas aeruginosa (305)	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	9709/19 418	12 760/12 760	4051/8102	2010/2010
P. aeruginosa (ATCC 27853)	NIE/NBE	NIE/NBE	11 163/NBE	NIE/NBE	4855/9709	6380/6380	1013/8102	502/2010
Serratia marcescens (313)	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	4051/4051	2010/8038
Gram-positive bacteria								
Enterococcus faecali (ATCC 29212)	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	1214/NBE	1595/NBE	4051/16 204	4019/4019
Ent. faecali (F208)	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	9709/NBE	6380/NBE	2025/8102	4019/4019
Enterococcus faecium (F229)	NIE/NBE	NIE/NBE	NIE/NBE	NIE/NBE	9709/NBE	6380/NBE	2025/8102	4019/4019
Staphylococcus aureus (ATCC 25923)	11 234/NBE	NIE/NBE	NIE/NBE	NIE/NBE	607/4855	NIE/NBE	506/4051	502/502
Staph. aureus (ATCC 29213)	NIE/NBE	NIE/NBE	11 163/NBE	NIE/NBE	607/4855	1595/6380	506/4051	1005/4019
Staph. aureus (F20)	NIE/NBE	NIE/NBE	NIE/NBE	6857/NBE	NIE/NBE	NIE/NBE	1013/4051	4019/8038
Staph. aureus (F7)	11 234/NBE	NIE/NBE	11 163/NBE	NIE/NBE	1214/2427	1595/1595	1013/4051	126/1005

*NIE, noninhibitory effect.
†NBE, no bactericidal effect.

Table 5 Minimal inhibitory concentration (MIC)/minimal bactericidal concentration (MBC) values (μg ml^{-1}) of commercial antibiotics, determined by microdilution assay

	Ampicillin	Cefotaxim	Imipenem	Oxacillin	Vancomycin
Gram-negative bacteria					
Acinetobacter freundii (303)		640/>640	16/32		
Enterobacter cloacae (302)		>640/>640	16/32		
Escherichia coli (301)		>640/>640	8/16		
E. coli (ATCC 25922)		32/32	2/4		
Klebsiella pneumoniae (310)		>640/>640	8/16		
Morganella morganii (320)		10/20	160/320		
Proteus mirabilis (304)		320/320	16/32		
Pseudomonas aeruginosa (305)		>640/>640	320/640		
P. aeruginosa (ATCC 27853)		320/320	40/80		
Serratia marcescens (313)		16/32	32/64		
Gram-positive bacteria					
Enterococcus faecali (ATCC 29212)	>640/>640				40/160
Ent. faecali (F208)	>640/>640				20/160
Enterococcus faecium (F229)	>640/>640				5/10
Staphylococcus aureus (ATCC 25923)		40/40		160/320	
Staph. aureus (ATCC 29213)		16/32		8/16	
Staph. aureus (F20)		80/>640		>640/>640	
Staph. aureus (F7)		20/40		4/8	

(Waterman and Mole 1994). UV spectra were also obtained. UV spectrum for rutin (methanol as solvent) showed characteristic 'peaks' at 259 and 359 nm, and 'shoulders' at 266 and 299 nm. UV spectrum for *iso*-quercitrin (methanol as solvent) showed characteristic 'peaks' at 257 and 356 nm, a 'valley' at 283 nm and a shoulder at 290 nm (Fig. 2a,b). The third compound was a terpene, whose structure is under study.

Table 6 Lethal dose (LD_{50}) values of plant extracts (micrograms of extracted material per millilitre) and commercial antibiotics ($\mu g\ ml^{-1}$) determined by *Artemia salina* cytotoxicity assay

Antibacterial substances	Extractive forms	LD_{50}
Jodina rhombifolia	Decoction	816
	Infusion	418
	Tincture	52
Leonurus sibiricus	Decoction	>1120
	Infusion	>680
	Tincture	32
Psittacanthus cuneifolius	Decoction	650
	Infusion	836
	Tincture	112
Tripodanthus acutifolius	Decoction	1052
	Infusion	1200
	Tincture	112
Ampicillin		>2560
Cefotaxim		676
Imipenem		1003
Oxacillin		776
Vancomycin		>160

Lethality assays were evaluated by Finney computer statistical program to determine the LD_{50} values and 95% confidence intervals. A copy of this program for an IBM PC is available from Dr. J.L. McLaughlin, Department of Medicinal Chemistry and Pharmacology, School of Pharmacy, Purdue University, West Lafayette, Indiana 47907, USA.

Discussion

Interviews with the native people from northwestern Argentina indicated that extracts from the selected plants are employed as vulnerary remedies. Three plant extractive procedures were selected according to the traditional practices in northwestern Argentina. The purpose of using the traditional extractive methodology was to verify the information of the antibacterial applications of the chosen plants. The antibacterial screen indicated that 42% of the extracts presented activity. This reinforces the concept that ethnobotanic investigation on plants traditionally used in folk medicine is an important tool to reveal positive responses to *in vitro* screens. *Psittacanthus cuneifolius* and *T. acutifolius* aqueous extracts (infusions, in particular) appear to be the most promising candidates for further work on isolation and identification of active compound(s), not only for the activity against a wide range of bacterial species, but also because they show activity at lower concentrations than LD_{50}. The fact that infusions were more active than decoctions in 90% of the evaluated cases (Table 4), reinforces the premise that not only the employed solvent is important, but also the extraction procedure. The prolonged exposition of the plant material to the heat (boiling water) reached during the decoction preparations could lead to the destruction or partial inactivation of active compounds, even if a high extraction yield is obtained. The similarity between antibacterial effects of *T. acutifolius* and *P. cuneifolius* could be attributed to their botanical relationship, as both of them belong to the Loranthaceae family. Consequently, it is expected that they could share some similar properties. *Staphylococcus aureus* is among the most commonly

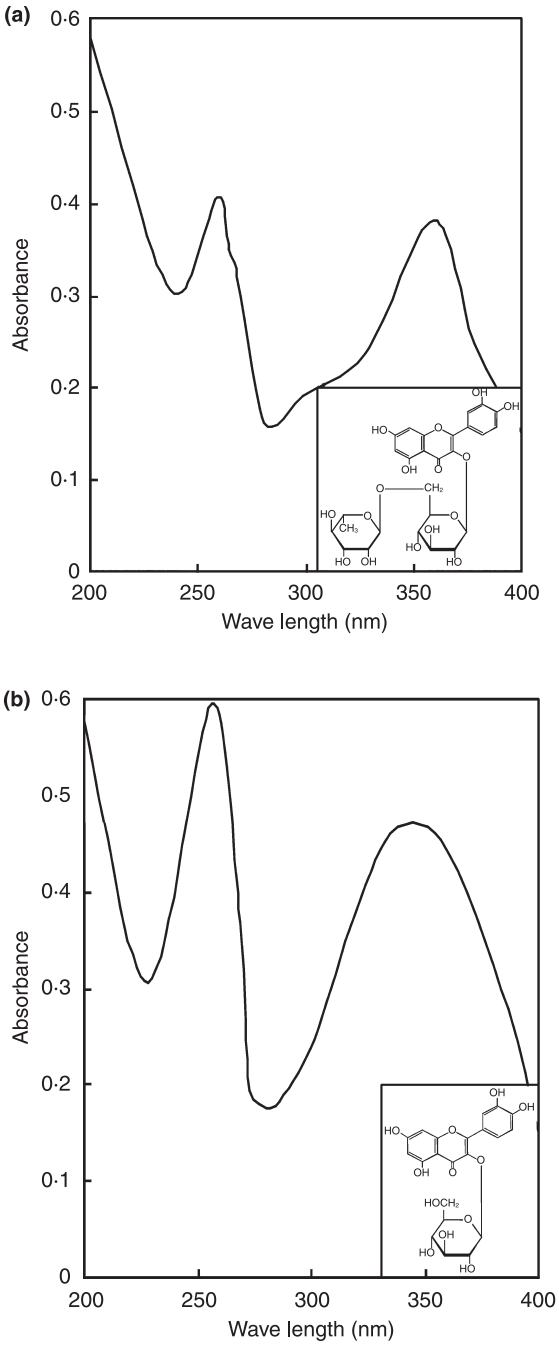

Figure 2 Ultraviolet spectra for the isolated flavonoids (solvent: methanol). (a) Rutin; (b) *iso*-quercitrin.

known pathogens that cause infectious disorders (Jones *et al.* 2003). As *T. acutifolius* and *P. cuneifolius* extracts inhibited *Staph. aureus* growth, the use of these plant extracts might be justified in the treatment of skin disorders. As shown in Tables 3 and 4, there are many coincidences between MIC and MBC values for some single strains, contrary to the general belief that bactericidal concentration should be higher than inhibitory concentration. This effect could be attributed to synergic effects exerted by compounds which act on different bacterial target structures, making the MIC and MBC values to be close or quite similar.

Ethnomedical information revealed that *T. acutifolius* extracts are used as a remedy for nasal congestions. However, there are no reports in the literature on the antimicrobial property of *T. acutifolius* extracts from leaves. The antibacterial activity of *T. acutifolius* (*Phrygilanthus acutifolius*) flowers was studied by Daud *et al.* (2005). They found antibacterial activity in ethanolic but not aqueous extracts. Consequently, the present study extends the knowledge of *T. acutifolius* utilization. Much attention should also be paid to the remaining extracts, as the inhibition of a single microbial species might reveal specific antibacterial properties when active compounds are isolated and purified. Care should be taken in the extraction procedure, as the solvent systems and extract preparation could modify the final results. Hence, the most appropriate extraction methodology is that in which the extract is the same as the one used in folk medicine or phytotherapy (Ríos and Recio 2005). Different extract compositions are expected as various extraction methodologies are used. This justifies the comparative analysis that was carried out among the extracts and commercial antibiotics. MIC and MBC of pure 'active' compound(s) are expected to decrease and the LD_{50} is expected to increase.

Three active compounds were isolated from *T. acutifolius* infusion. They were glycosylated flavones (rutin and *iso*-quercitrin) and a compound of terpenoid nature. Flavonoids have a characteristic UV spectrum; hence, this can be used as a valid identification tool (Andersen and Markham 2006). Phenolic compounds, especially flavonoids, have been reported to possess antimicrobial activity (Tereschuck *et al.* 1997). Rutin and *iso*-quercitrin have been shown to possess antibacterial activity against gram-positive and gram-negative strains (Hidalgo Báez *et al.* 1998; Basile *et al.* 2000). As flavonoids are known to be synthesized by plants in response to microbial infection (Dixon *et al.* 1983), it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of micro-organisms. Their activity is attributed probably to their ability to complex with extracellular and soluble proteins and with bacterial cell walls, or disrupt microbial membranes (Cowan 1999). Glycosylation increases the polarity of the flavonoids (necessary for storage in plant cell vacuoles), and thereby water solubility. As the infusion is an aqueous extract, glycosylated products were expected. Antibacterial activity of *T. acutifolius* infusion could be partially attributed to these compounds, and to the still unidentified terpene. Further studies are needed in order to complete the identification process.

These plant extracts may be effective as topical treatments in emergency situations when commercial antibiotics are not available. This does not imply that the results of the assays are conclusive; they are a first step in a long process towards the examination and elucidation of other useful compounds within the listed plants.

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