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Antibacterial Activity of Extracts from Plants of Central Argentina—Isolation of an Active Principle from *Achyrocline satureioides*

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Key words
- antibacterial activity
- *Achyrocline satureioides*
- Asteraceae
- 23-methyl-6-*O*-desmethyl-auricepyrone
- plant extracts

Abstract

The great increase in bacterial infections is fueling interest in the search for antibacterial products of plant origin. Extracts obtained from 51 native and naturalized plants from central Argentina were therefore evaluated for their *in vitro* inhibitory activity on pathogenic bacteria with the aim of selecting the most active ones as new sources of effective antibiotics. The susceptibility of reference and clinical strains of *Enterococcus faecalis, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella enterica* serovar Enteritidis, and *Staphylococcus aureus* was determined. Extracts from *Achyrocline satureioides, Flourensia oolepis, Lepechinia floribunda*, and *Lithrea molleoides* were the most potent, with MIC and MBC values ranging from 0.006 to 2 and 0.012 to 10 mg/mL, respectively, on both gram-positive and negative bacteria. The antibacterial activity-guided isolation of *A. satureioides* ethanol extract showed 23-methyl-6-*O*-desmethylauricepyrone (**1**) to be the most active compound. This compound showed inhibitory effects against gram-positive bacteria with MIC and MBC values of 0.002 and 0.008 mg/mL, respectively, while on gram-negative strains, the MIC and MBC were 0.062–0.250 and 0.062–0.500 mg/mL, respectively. The strong antibacterial activity shown by the four plant extracts or the compound isolated from *A. satureioides* suggests that they could become part of the arsenal of antibacterial drugs currently used.

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Introduction

Bacterial infections are dramatically increasing every day for diverse reasons, mainly due to the development of resistance to conventional antibiotics [1], which leads to a diminution of their effectiveness. Many of the commercial antibacterial agents used today also have high production costs and many side effects [2–4]. In this situation, and given the lack of new highly effective antibiotics, numerous extracts and compounds obtained from plants are being studied for chemical and antimicrobial characterization [5].

The use of plants as medicinal agents is common in developing countries as an alternative solution to health problems, and it is now acquiring more importance in developed societies among those interested in a healthier lifestyle [6]. Although many plant families are being studied as sources of new antibiotics, the plant world is far from being totally explored and this also applies to the native flora from Argentina. Hence, in our ongoing search for compounds with the capacity to inhibit agents that cause diseases [7–10] we focused this study on new compounds from plants collected in central Argentina that may be highly effective for the control of pathogenic bacteria.

We report here the notable antibacterial activity of four plants selected among 51 native and naturalized plants which were screened against gram-positive and negative bacteria. The most potent of these plants, *Achyrocline satureioides* (Lam.) DC. (Asteraceae), was submitted to bioguided fractionation for further isolation of its active principle. Being able to obtain and subsequently use these bactericidal products substantially increases the use we can give to our rich native flora.

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Materials and Methods

Microorganisms

The effectiveness of extracts was assayed on strains of *Enterococcus faecalis* (Andrews and Horder) Scheifer and Klipper-Balz (ATCC 29212), *Escherichia coli* (Migula) Castellani and Chalmers (ATCC 25922), *Pseudomonas aeruginosa* (Schroester) Migula (ATCC 27853), and *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC 6538), as well as on clinical isolates of tetracycline, erythromycin, and polymyxin-resistant *Proteus mirabilis* (Pr2921) and ampicillin-resistant *Salmonella enterica* serovar Enteritidis (Kauffmann and Edwards) Le Minor and Popoff. These isolates were identified by conventional biochemical assays and by the commercial system API20E (bioMérieux SA). *Salmonella* serovar was determined against somatic (O) and flagellar (H) antigens according to the Kauffmann-White scheme [11]. Antimicrobial resistances were determined by disk diffusion susceptibility testing (Kirby-Bauer Method) according to CLSI, 2006 [12]. Bacterial suspensions were prepared on sterile saline from each overnight grown organism. Turbidity was spectrophotometrically adjusted to 0.5 McFarland standard. Dilution with sterile saline to give an adjusted concentration of 1.5×10^7 CFU/mL was carried out for agar dilution assay. For the time-kill curve study, suspensions of *S. aureus* were adjusted to 7.5×10^6 CFU/mL.

Plant material

Plants were collected in the hills of Córdoba Province, Argentina, from November 2006 to December 2008. Voucher specimens have been deposited in the "Marcelino Sayago" Herbarium of the School of Agricultural Science, Catholic University of Córdoba and were authenticated by the botanist Gustavo Ruiz.

Plants were selected according to their availability, accessibility, and especially the lack of scientific information about their activity and/or chemical pattern.

Crushed aerial plant material of each plant was extracted by 48 h maceration with ethanol. The yields of each extract, obtained after solvent removal and expressed as percentage of weight of air-dried crushed plant material, are shown in **Table 1** and in Supporting Information.

Chemicals, equipment and reagents

^{1}H- and ^{13}C-NMR spectra were recorded with a Bruker AVANCE II 400 spectrometer (Bruker Corporation). Chemical shifts (parts per million) are relative to internal tetramethylsilane used as a reference. MS spectra were measured with a ZAB SEQ (BeqQ) instrument (VG Analytical). HPLC was performed on a Shimadzu LC-10 AS (Shimadzu Corp.), equipped with a Phenomenex Prodigy 5 µ ODS (4.6 mm i.d. × 250 mm) reversed-phase column. The mobile phase was 90% acetonitrile in water with 1% trifluoracetic acid (TFA) as the mobile phase and UV detection at 280 nm.

All media were from Oxoid, Ltd. (Basingstoke). Analytical and preparative TLC was performed on silica gel 60 F_{254} plates purchased from Merck and Analtech, respectively. Silica gel grade 70–230 mesh, 60 Å, for column chromatography was purchased from Sigma-Aldrich Corporation.

Determination of MICs and MBCs

MICs were carried out by the agar dilution test according to CLSI, 2006 [12] with some modifications in order to adapt the technique to the measurement of activity of plant products. Briefly, agar dilution bioassays were conducted by adding the plate count agar medium (PCA) to the suitable amount of each extract, fraction, or the pure compound previously dissolved in hexane or ethanol as appropriate. The final concentration of solvent was 2% (no adverse effects were observed at this concentration). After solidification of the mixture in the respective plates, 1–2 µL of each bacteria suspension were placed on the agar surface. At least two replicates were used for each treatment. Plates were incubated in ambient air at 37 °C for 24 h. For reading, visual observations were carried out. Nitro blue tetrazolium (Sigma-Aldrich Corporation) solution in saline phosphate buffer (PBS) was applied on each spot for confirmation. Plates containing only the culture medium, with or without the addition of the dissolution solvent, were used as controls for each bacteria studied. Positive controls with commercial gentamicin sulfate (potency: 550–590 µg/mg; Montreal S.A) or erythromycin (potency: 863 µg/mg; Unifarma) were simultaneously carried out. MIC was defined as the lowest concentration that completely inhibits growth of the microorganism.

The bactericidal effect was further studied from each concentration of product showing growth inhibition. For this study, 0.5 mm portions of agar, coincident with the spot in which the inoculum had been placed and showing negative growth at 24 h, were cut and transferred to brain-heart infusion broth (BHI). Tubes were incubated in ambient air at 37 °C for five days. At the end of this period, the MBC values, defined as the lowest concentration with absence of turbidity, were recorded.

Bioguided isolation of the active principle from *Achyrocline satureioides*

The ethanol extract from *A. satureioides* (8.2 g) was chromatographed using vacuum liquid chromatography on silica gel (60 µm, 70–230 mesh: 11 × 17 cm) with hexane-ethyl ether-methanol gradient. Those fractions showing antibacterial activity were further purified in successive column chromatographies on silica gel (60 µm, 220–440 mesh: 1.5 × 40 or 2.5 × 50 cm), finally obtaining fractions F1–F4. Of these, F-3 (25 mg), eluted with chloroform/acetonitrile 80 : 20, showed bactericidal activity at 2 mg/mL. In order to purify the active principle, this fraction was submitted to preparative TLC, yielding one compound (97% purity, by HPLC) which was identified as 23-methyl-6-*O*-desmeth-

Table 1 Native plants from the central region of Argentina.

Plant species	Family	Common name	Extract yield (%)	Status	Voucher: UCCOR number
Achyrocline satureioides (Lam.) DC.	Asteraceae	marcela hembra	4.5	Native	140
Flourensia oolepis S.L. Blake	Asteraceae	chilca	23.0	Native	135
Lepechinia floribunda (Benth.) Epling	Lamiaceae	salvia blanca	3.7	Native	195
Lithrea molleoides (Vell.) Engl.	Anacardiaceae	molle de beber	7.2	Native	183

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ylauricepyrone (**1**) (**Fig. 1**) [13] (yield 0.30 g/100 g of crushed plant material, by HPLC).

Time-kill curve assay

This assay was performed in duplicate in 4 mL of Mueller-Hinton broth containing ethanol solutions (final concentration of ethanol: 1–2%) of extracts from *A. satureioides, Flourensia oolepis, Lepechinia floribunda*, and *Lithrea molleoides*, or pure compound, in an amount sufficient to reach final concentrations ranging from MIC to 4 × MIC. Each tube was then inoculated with 200 µL of *S. aureus* suspension. Controls contained only ethanol. The suspensions were exhaustively mixed and samples of each were removed, 100-fold diluted and plated onto PCA medium. Remaining suspensions were then incubated at 37 °C with shaking, and samples of each were taken at 1, 2, 3, or 24 h. The number of viable cells was determined at these times after plating onto PCA medium aliquots of undiluted and 10-fold serial dilutions of each sample. Plates were then incubated for 24 h under the same conditions and CFU were counted, providing a lower limit of detection of 10^2 CFU/mL.

Statistical analysis

InfoStat software (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Córdoba, Argentina) was used.

Supporting information

Information about the 51 plants studied (family, common name, extract yield, status, use in traditional medicine, and voucher number) together with their antibacterial activity (MICs and MBCs) are available as Supporting Information. Chemical data of the antibacterial compound 23-methyl-6-*O*-desmethylauricepyrone are also provided as Supporting Information.

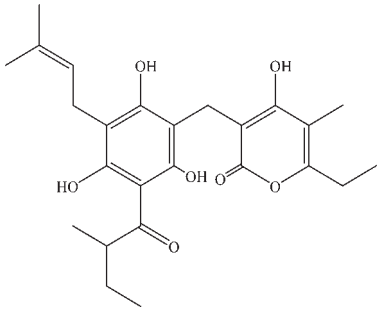

Fig. 1 Chemical structure of 23-methyl-6-*O*-desmethylauricepyrone (**1**).

Results and Discussion

When looking for new drugs for the control of pathogenic bacteria, plants offer a wide array of possibilities. Native and naturalized plants from the central region of Argentina showed themselves to be highly promising. After screening the 51 extracts obtained from the selected plants (see Supporting Information), those from *A. satureioides, F. oolepis, L. floribunda*, and *L. molleoides* showed high effectiveness in inhibiting all gram-positive and gram-negative bacteria tested, with MICs ranging from 0.006 – 2 mg/mL (**Table 2**). According to Fabry et al., 1998 [14], those extracts showing MIC values below 8 mg/mL are considered to possess some antimicrobial activity, and thus these extracts have potential for controlling diseases caused by tested bacteria.

The activity of *A. satureioides* on *E. faecalis* was noteworthy, with a MIC value (MIC = 0.006 mg/mL) in the same order of magnitude as that of gentamicin (MIC = 0.008 mg/mL), erythromycin (MIC = 0.001 mg/mL), vancomycin (MIC = 0.004 mg/mL) [15], or chloramphenicol (MIC = 0.004–0.016 mg/mL) [16]. The same level of effectiveness was observed on *S. aureus*, and in this case the MIC (MIC = 0.006 mg/mL) compares very well with that of gentamicin (MIC = 0.008 mg/mL) or erythromycin (MIC = 0.001 mg/mL) but was lower than that of actinonin (MIC = 0.032 mg/mL) [17].

A. satureioides also showed the strongest bactericidal effect on both *E. faecalis* and *S. aureus*, with MBCs of 0.050 and 0.012 mg/mL, respectively, while *L. molleoides* showed the same effect with an MBC of 0.050 mg/mL on both strains. The activity of these plants is comparable to that of both tested commercial antibiotics, which have MBCs ranging from 0.010–0.060 mg/mL (**Table 2**).

A. satureioides, F. oolepis, L. floribunda, and *L. molleoides* also showed inhibitory activity against *E. coli* and *P. aeruginosa* (MIC = 0.250–2 mg/mL), with the first plant being the most effective, with MICs of 0.500 and 0.250 mg/mL, respectively. All four plants demonstrated effectiveness on a resistant isolate of *P. mirabilis*. Bactericidal effect on these gram-negative strains showed MBC values ranging from 1 to 10 mg/mL (**Table 2**).

There is very little information about natural compounds of plant origin exhibiting inhibition on the growth of the food-borne pathogen *Salmonella enterica*, but the four active extracts showed bacteriostatic and bactericide inhibition on this bacterium at a concentration of up to 2 mg/mL.

Results of time-kill assays are shown in **Fig. 2 a–d**. At MIC value, extracts of *A. satureioides* and *L. molleoides*, each of which pre-

Table 2 Antibacterial activity of the most potent extracts obtained from native plants from the central region of Argentina and of 23-methyl-6-*O*-desmethylauricepyrone (**1**).

Species/Compound	MIC/MBC (mg/mL)					
	Escherichia coli	*Pseudomonas aeruginosa*	*Proteus mirabilis*	*Salmonella enterica*	*Enterococcus faecalis*	*Staphylococcus aureus*
Achyrocline satureioides	0.500/2	0.250/1	1/4	1/1	0.006/0.050	0.006/0.012
Flourensia oolepis	1/1	2/4	0.500/1	1/1	0.250/0.500	0.100/1
Lepechinia floribunda	1/2	1/1	0.250/1	2/2	0.250/0.500	0.250/0.500
Lithrea molleoides	2/10	1/1	2/8	2/2	0.050/0.050	0.050/0.050
23-Methyl-6-*O*-desmethylauricepyrone (**1**)	0.250/0.500	0.062/0.062	0.125/0.250	–	0.002/0.008	0.002/0.008
Erythromycin	0.125/4	0.062/0.500	0.500/> 4	–	0.001/0.030	0.001/0.060
Gentamicin	0.004/0.008	0.004/0.004	0.010/0.010	0.001/0.010	0.008/0.010	0.008/0.010

– Not determined

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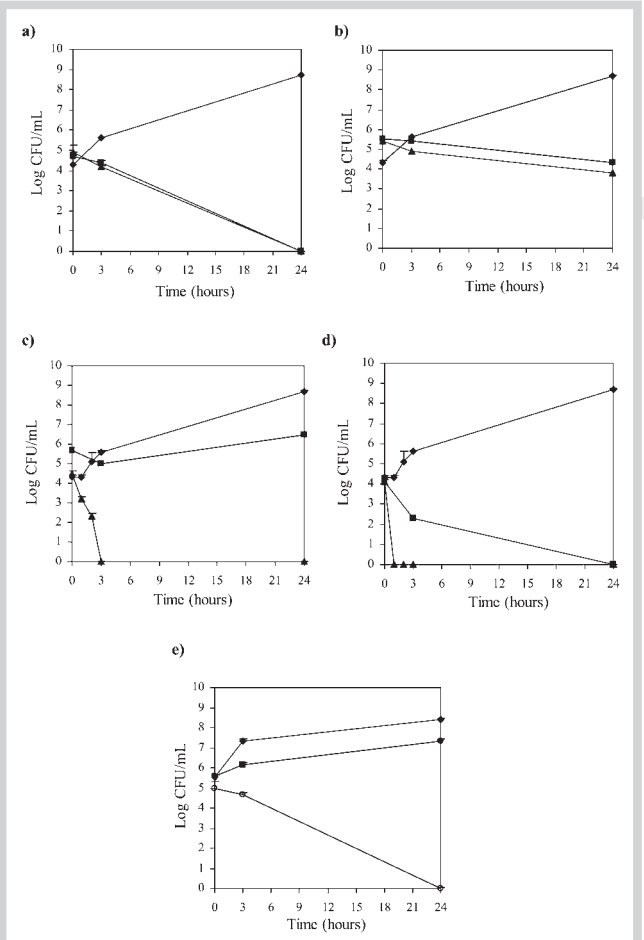

Fig. 2 Bactericidal effect of **a** *Achyrocline satureioides*, **b** *Flourensia oolepis*, **c** *Lepechinia floribunda*, **d** *Lithrea molleoides*, and **e** 23-methyl-6-*O*-desmethylauricepyrone (**1**) against *S. aureus*. Growth control (filled diamond); plant extracts or compound **1** at MIC value (filled square); plant extracts at 3 × MIC (filled triangle), and compound **1** at 4 × MIC (open circle) added to Mueller-Hinton broth and incubated at 37 °C with agitation. Each symbol indicates the means (± SE) for at least two replicates on separate occasions.

sented very similar or identical MICs and MBCs in the agar dilution test, produced complete lethality at 24 h. The latter plant showed a marked decrease (~ 2 $\log_{10}$) in cell growth at only 3 h. Extract of *A. satureioides*, applied at 3 × MIC (0.018 mg/mL), showed complete lethality at 24 h, while *L. floribunda*, also applied at 3 × MIC (0.750 mg/mL), reached bactericidal effect at 3 h after an immediate decrease of ~ 1–2 $\log_{10}$ in bacterial viability. The same tendency was observed for *L. molleoides*, which showed complete bacterial killing at only one hour at 0.150 mg/mL (3 × MIC).

Our results showed both cocci tested to be the least resistant, while gram-negative bacteria proved to be the most resistant in agreement with other studies carried out with other plant extracts [18,19].

As far as we know, this is the first time that the antibacterial effect of *Lepechinia floribunda* has been reported, while the antibacterial activity of *A. satureioides, F. oolepis*, and *L. molleoides* were reported once by Gutkind et al. (1981) [20], Palacios et al. (2007) [10], and Penna et al. (2001) [21], respectively. No active principle has been shown to be responsible for the above-mentioned effect in any of these plants.

Given these results, *A. satureioides* was the first plant selected to be submitted to antibacterial-guided isolation. From this process, a prenylated α-pyrone-phloroglucinol identified as 23-methyl-6-*O*-desmethylauricepyrone (**1**) was obtained as the most active substance.

Antibacterial inhibitory activity of compound **1** is presented in ▶ **Table 2**. Both gram-positive bacteria were very sensitive to compound **1** with MIC and MBC values of 0.002 and 0.008 mg/mL, respectively. Bacteriostatic activity was also observed against all gram-negative strains tested, with MICs ranging from 0.062 to 0.250 mg/mL, while its bactericidal effect showed MBC values of 0.062–0.500 mg/mL. The decrease in the MICs and MBCs by 1.5– to 16-fold reflects the higher activity of the purified compound compared with that of the complete extract.

According to reports by Gibbons, 2004 [22] and Drewes and van Vuuren, 2008 [23], those compounds with MICs of less than 0.064 mg/mL are accepted as notable bacterial inhibitors while those with activity at concentrations below 0.010 mg/mL are considered "clinically significant". The level of activity shown by compound **1** thus qualifies it as an interesting lead in antibacterial drug discovery. The inhibition showed by compound **1** is also encouraging, considering that few plant-derived chemicals are capable of inhibiting gram-negative bacteria, especially *Pseudomonas* [24,25]. Compound **1** demonstrated promising inhibitory activity against the bacterial strains tested, being at par with and in some cases exceeding the commercial antibiotics used as references. MICs of the phloroglucinol are at the same order of magnitude as those obtained for erythromycin against all the strains studied, while its MBCs were lower than those of the macrolide (▶ **Table 2**). The activity of compound **1** on both cocci was similar to the activity level of gentamicin while the commercial antibiotic was more effective than compound **1** on gram-negative strains. The lower activity displayed by compound **1** on gram-negative bacteria in comparison to gram-positive is as expected. Although many compounds of plant origin exert strong activity against gram-positive bacteria [26–28], gram-negatives appear to be more resistant [19,28–31]. This is probably because of the more complex structure and hydrophilic nature of their cell walls [28]. In time-kill assays (▶ **Fig. 2e**), compound **1**, at a concentration corresponding to 4 × MIC (0.008 mg/mL), produced complete killing of *S. aureus* at 24 h, confirming the MBC value determined by the agar dilution test (▶ **Table 2**).

The presence of compound **1** has been already reported in other Asteraceae, *Helichrysum odoratissimum* [13] and also in *A. satureioides* [32,33]. To the best of our knowledge, no bioactivity has been attributed to compound **1,** except that described in this work and thus, this is the first time that the compound responsible for the antibacterial action of the plant has been identified.

The activity of compound **1** is not surprising since other phloroglucinols linked by a methylene bridge to filicinic acid or syncarpyl moieties are known as effective antibacterial compounds [27,34–37]. Besides, phloroglucinols linked by a methylene bridge to α-pyrones have also been reported as antibacterial chemicals [38], but there is very little available information about these. In this sense, the mixture of homoarenol and arenol isolated from *Helichrysum arenarium* (L.) Moench [39] or *Helichrysum stoechas* (L.) Moench [38] showed antibacterial activity against *S. aureus* with an MIC of 0.025 mg/mL [38], which is higher than that observed for compound **1** against this bacteria (MIC = 0.002 mg/mL). On the other hand, the mixture showed lower effectiveness than that exerted by compound **1** against *P. aeruginosa* with an MIC > 0.100 mg/mL [38].

In comparison to another well-known phloroglucinol, hyperforin, isolated from *Hypericum perforatum* (St. John's wort) [40], compound **1** showed the same level of activity on gram-positive bacteria (MIC hyperforin = 0.001 mg/mL) [41], but, in contrast to compound **1**, hyperforin showed no activity on gram-negative strains [41].

Another known phloroglucinol derivative, rhodomyrtone, isolated from *Rhodomyrtus tomentosa*, displayed similar anti-gram-positive activity as compound **1** with noteworthy MICs and MBCs ranging from 0.0004–0.0016 and from 0.0004–0.012 mg/mL, respectively [27].

This study shows that *A. satureioides, F. oolepis, L. floribunda*, and *L. molleoides* as well as the compound 23-methyl-6-*O*-desmethylauricepyrone (**1**) isolated from the first mentioned plant, present very strong antibacterial activity, validating the use of these products as antibiotic agents.

Since oral administration of *A. satureioides* in rats showed no signs of toxicity [42], and it has been extensively used as a native medicinal herb for the treatment of various disorders [43,44], there are good expectations that *A. satureioides* extract or compound **1** may be used as phytotherapeutic drugs for bacterial infections. The obtained phloroglucinol could be directly used in therapeutic preparations or as leading molecule for the synthesis or semisynthesis of analogs. This scenario offers us the possibility of using these products, derived from plants native and naturalized to Argentina, as antibacterial agents.

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