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Short communication

Inhibition of development, swarming differentiation and virulence factors in *Proteus mirabilis* by an extract of *Lithrea molleoides* and its active principle (Z,Z)-5-(trideca-4',7'-dienyl)-resorcinol

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ABSTRACT

Antibacterial activity of *Lithrea molleoides* extract against *Proteus mirabilis* has been previously reported by our group. In the present study, the compound (Z,Z)-5-(trideca-4',7'-dienyl)-resorcinol (**1**) was isolated as its responsible active principle. The effects of the compound obtained and of *L. molleoides* extract on *P. mirabilis* growth and virulence factors were evaluated.

Compound **1** showed MIC and MBC values of 4000 μg/ml. It was found that the extract, at four times the MIC, produced complete killing of the uropathogen at 2 h from the beginning of the experiment, while the alkylresorcinol, at four times the MIC, produced the same effect after 24 h. Hemolysis was adversely affected in treatments with both products at 8 μg/ml, while hemagglutination was not altered. The whole extract induced complete autoaggregation of *P. mirabilis* at 2000 μg/ml, while compound **1** at the same concentration did not show this property. Swarming motility was delayed in treatments with the extract and with **1** at 1000 and 8 μg/ml, respectively, at 8 h from the beginning of the assay. Complete inhibition of the phenomenon was still observed after 24 h when compound **1** was added at 125 μg/ml.

These findings offer the possibility of new classes of antimicrobial medicines to tackle infections caused by *P. mirabilis*.

Introduction

Proteus mirabilis is the second most common cause of urinary tract infections (UTIs), especially in individuals with complicated urinary tracts and catheterized patients (Coker et al. 2000). Its virulence, which is crucial for successful colonization and the establishment of the infection, has been related to several potential factors including hemolysin production, adherence to the uroepithelium due to fimbriae and swarming motility mediated by flagella (Rozalsky et al. 1997).

Like many other microorganisms, *P. mirabilis* has acquired resistance to many antibiotics including β-lactams (Aragon et al. 2008), aminoglycosides (Wachino et al. 2006) and fluoroquinolones (Saito et al. 2006) with a progressive increase in the number of resistant isolates over recent years. In this context, and continuing the search for bioactive compounds of plant-origin (Carpinella et al. 2005; Palacios et al. 2007), we have recently reported the antibacterial activity of ethanolic extracts derived from 51 native plants of central Argentina (Joray et al. 2011). From this screening, *Lithrea molleoides* (Vell.) Engl. (Anacardiaceae) extract showed itself to be one of the most effective with notable bacteriostatic and bactericide activity against gram-positive and negative bacteria. Its effect on *P. mirabilis* showed MIC and MBC values of 2000 and 8000 μg/ml, respectively (Joray et al. 2011). No active principle had been isolated as responsible for this effect.

The goal of this study was to isolate and identify the active ingredient able to interfere with the growth, virulence factors and swarming motility of *P. mirabilis*.

Materials and methods

Plant material

L. molleoides was collected in the Province of Córdoba, Argentina in February–March 2008. A voucher specimen has been deposited in the "Marcelino Sayago" Herbarium of the School of Agricultural Science, UCC: UCCOR 183. Plant material was extracted by 48 h maceration with ethanol. Yield of the extract, expressed as percentage weight of air-dried plant material, was 10.9%.

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Bacterial strain

The uropathogenic tetracycline, erythromycin and polymyxin-resistant *P. mirabilis* strain Pr2921 was originally isolated from a patient with symptomatic UTIs (Zunino et al. 2003, 2007). Bacterial suspensions were prepared on PBS (0.1 M, pH 7.2) from an overnight-grown organism in Nutrient Agar (NA) at 37 °C. Turbidity was spectrophotometrically adjusted to 0.5 McFarland standard.

Media, chemicals and apparatus

All media were from Difco Laboratories (Detroit, MI, USA) and Oxoid Ltd. (Basingstoke, Hampshire, UK). Chemicals were purchased from Sigma–Aldrich Corporation (St Louis, MO). Silica gel grade 70–230 mesh, 60 Å, for column chromatography was used and all solvents were HPLC grade. ^{1}H and ^{13}C NMR spectra were recorded in Chloroform-*d* with Bruker AVANCE II 400 spectrometer (Bruker Corporation, Ettlingen, Germany) operated at 400 MHz for ^{1}H and at 100 MHz for the ^{13}C nucleus. MS spectra were measured with a ZAB SEQ (BeqQ) instrument (VG Analytical, Manchester). HPLC was performed on a Phenomenex Prodigy 5 μ ODS (4.6 mm i.d. × 250 mm) reversed-phase column eluting with 90% acetonitrile in water with 1% trifluoracetic acid (TFA) and UV detection at 280 nm. For electron microscopy, a Jeol JEM 1010 electron microscope was used. Light microscopy was performed in an Olympus BX61 equipped with a graticule.

Determination of MIC and MBC

MIC and MBC assays were carried out as previously described (Joray et al. 2011). Positive controls with gentamicin sulfate (potency: 550–590 μg/mg; Montreal, SA) and erythromycin (potency: 863 μg/mg; Unifarma) were simultaneously carried out.

Bioguided isolation of the antibacterial compound

L. molleoides extract was subjected to a column chromatography with hexane/Et_2O/MeOH gradient. Antibacterial fractions were re-chromatographed to finally obtain an active fraction which was further purified in radial preparative chromatography (solvent gradient CH_2Cl_2/Et_2O) furnishing a yellowish oil (yield 0.78 g/100 g of crushed plant material, 92% purity, by HPLC). This substance was identified as (Z,Z)-5-(trideca-4',7'-dienyl)-resorcinol (**1**) according to ^{1}H NMR, ^{13}C NMR and MS spectra, and to the comparison with previous reports (Chiari et al. 2010; Valcic et al. 2002).

Time kill curves

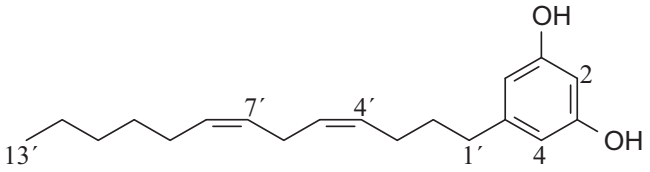
These assays were conducted in Luria Bertoni broth (LB) containing tested products according to Joray et al. (2011). An aliquot of the *P. mirabilis* suspension was added to each tube to finally reach 5×10^5 CFU/ml. Each tube was incubated at 37 °C with agitation.

Electron microscopy

For TEM, fresh cultures of bacteria grown for 24 h in the absence or presence of 8000 μg/ml of extract or compound **1** were stained with 1% uranyl acetate.

Hemagglutination and hemolysin inhibition assay

The inhibition on hemagglutination and hemolysis was determined according to Mobley and Chippendale (1990). Controls of bacterial growth with or without ethanol were run simultaneously. Negative controls with no addition of *P. mirabilis* were also performed. For the hemolysin inhibition assay, *L. molleoides* extract or compound **1** (previously dissolved in ethanol) was added to brain-heart-infusion broth, then the adjusted inoculum of the test organism was added to finally obtain 1×10^7 CFU/ml.



1

Fig. 1. Chemical structure of (Z,Z)-5-(trideca-4',7'-dienyl)-resorcinol (**1**).

For hemagglutination, LB containing the appropriate amounts of extract or **1** dissolved in ethanol was inoculated with Pr2921 inoculum reaching a final concentration of 1×10^7 CFU/ml.

Autoaggregation assay

An assay in order to determine bacterial settling kinetics over time was carried out according to Sosa and Zunino (2009).

Inhibition of swarming differentiation

The effect of the extract or **1** on swarming behavior was determined as described by Liaw et al. (2000).

Statistical analysis

The results were analyzed statistically by the Kruskal–Wallis test and Dunn's post test at 0.05 significance level using InfoStat software (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Córdoba, Argentina).

Results and discussion

After submitting complete *L. molleoides* extract to antibacterial bioguided isolation, one active compound identified as (*Z,Z*)-5-(trideca-4,7-dienyl)-resorcinol (**1**) (Fig. 1) was obtained. The chromatographic fingerprints of the extract and of the alkylresorcinol are shown in Fig. 2. This is the first time that a compound showing antibacterial properties has been isolated from *L. molleoides*.

The inhibitory effect of **1** on *P. mirabilis* was determined, showing a MIC and MBC of 4000 μg/ml. Gentamicin and erythromycin showed MIC and MBC values of 10 and 10, and 500 and > 4000 μg/ml, respectively. The results obtained agree with previous data where other 5-alkylresorcinols are described as possessing antibacterial properties (Zarnowski and Suzuki 2004; Jin and Zjawiony 2006).

In time kill assays, the *L. molleoides* extract added at four times the MIC produced a reduction of ~3.5 $\log_{10}$ in the viable cell count at the first hour from the beginning of the experiment and complete killing at 2 h (Fig. 3). This short time effect may be associated with membrane disruption (Kubo and Fujita 2001), which was further confirmed through the electron microscopy assay. Compound **1** added at four times the MIC, gave rise to a decrease of 2.2–2.9 $\log_{10}$ in the viability of the uropathogen from 1 to 5 h, respectively, producing bactericidal effect after 24 h.

TEM revealed that cells exposed to 8000 μg/ml of *L. molleoides* extract showed undefined edges, were agglutinated and were markedly smaller in size than microorganisms from the control group. Similar results were observed in treatments with the same concentration of **1**. The alterations observed in the bacte-

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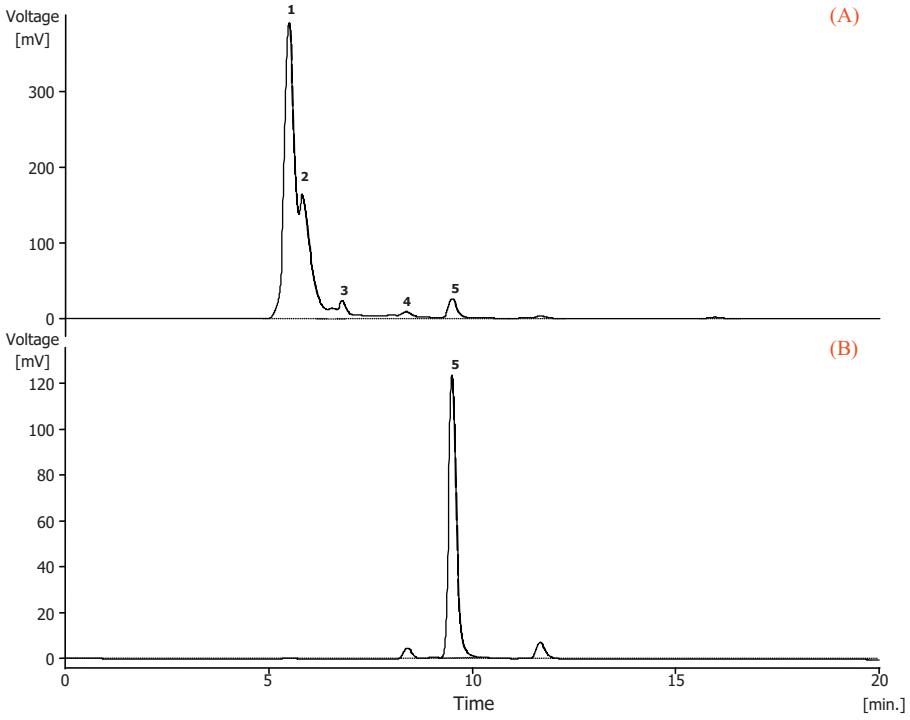

Fig. 2. Chromatographic fingerprints of *Lithrea molleoides* ethanolic extract (A) and **1** (B), obtained by HPLC. **5**: (Z,Z)-5-(trideca-4',7'-dienyl)-resorcinol.

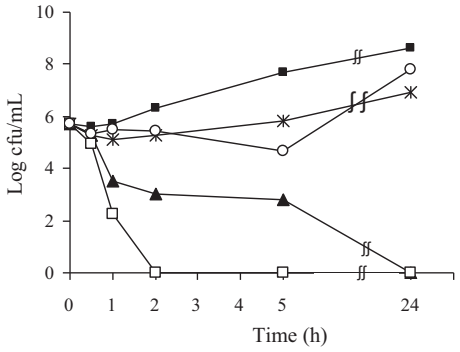

Fig. 3. Time kill curves of *Proteus mirabilis* Pr2921 in: ethanol control suspensions (filled squares) and in treatments with: extract at four times the MIC (open squares), extract at two times the MIC (open circles), (Z,Z)-5-(trideca-4',7'-dienyl)-resorcinol at four times the MIC (filled triangles) and (Z,Z)-5-(trideca-4',7'-dienyl)-resorcinol at two times the MIC (asterisks).

rial membrane could be associated to the amphipathic structure of **1**. Hydrophilic groups, such as OH present in the resorcinol moiety, associate with the membrane phospholipids through hydrogen bonds, thereby creating disorder in the lipid bilayer and in membrane-bound respiratory enzymes (Bitkov et al. 1992; Kozubek et al. 1988; Kubo et al. 2009). In addition, the hydrophobic hydrocarbon chain disrupts hydrogen bonding in the lipid-protein interface (Kubo et al. 2009).

The minimum concentration of extract or **1** in which hemolysis was inhibited was 8 μg/ml. Since hemolysin has been shown to be correlated with the ability of bacteria to invade cells (Peerbooms et al. 1984), its inhibition suggests that the products tested are promising therapeutic agents for preventing cell invasion.

L. molleoides extract and **1** were not able to inhibit hemagglutination at the maximum concentrations tested where these products did not provoke hemolysis *per se* (62 and 250 μg/ml, respectively).

Autoaggregation assays showed a significant increase after 1 h in autoaggregation of Pr2921 in treatments with 1000 and 2000 μg/ml of the extract in comparison to control ($p < 0.05$). According to these results, complete extract increases the autoaggregation capacity of Pr2921, but this property was not due to the effect of the isolated alkylresorcinol, since treatments with **1** did not affect *P. mirabilis* autoaggregation.

When the Pr2921 culture was supplemented with the extract at 1000 μg/ml, a delay in swarming migration was observed with the bacteria lying in the original colony place for the first 8 h (Table 1). Compound **1**, however, showed a noticeable inhibition of swarming. A complete delay in swarming motility was observed up to 8 h from the beginning, even at 8 μg/ml (Table 1). The activity of **1** was higher than that observed in the other plant-origin com-

Table 1
Swarming inhibition of *Proteus mirabilis* by *Lithrea molleoides* extract and (Z,Z)-5-(trideca-4',7'-dienyl)-resorcinol (**1**).

Time (h)	Distance (mm)[a]										
	Extract (μg/ml)				Compound **1** (μg/ml)					Control	Control EtOH
	1000	500	250	125	125	62	31	16	8		
6	–	1.0 ± 0	1.7 ± 0.1	2.0 ± 0	–	–	–	–	–	1.7 ± 0.1	1.5 ± 0
8	–	2.0 ± 0	6.0 ± 0	8.0 ± 0	–	–	–	–	–	2.0 ± 0	2.0 ± 0
24	7 ± 1.0	6.0 ± 0	9.0 ± 0	8.0 ± 0	–	1.0 ± 0	3.0 ± 0	3.0 ± 0	6.0 ± 0	10.3 ± 0.3	9.0 ± 0

[a] Data represent the mean ± standard error of the parameter evaluated. Control ETOH: control ethanol; –: no growth.

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pound, resveratrol, which showed complete blocking of swarming at 60 μg/ml at 8 h (Wang et al. 2006). After 24 h, total inhibition of the phenomena was observed at 125 μg/ml, while at 62 and 16 μg/ml, 90 and 70% inhibition was still observed.

Even when compound **1** showed promising inhibitory activity against *P. mirabilis* development and its virulence factors, its effectiveness could be enhanced through chemical modifications. Different studies carried out with other 5-alkylresorcinols indicated that the antibacterial activity increases when the number of double bonds in the side chain increases (Jin and Zjawiony 2006; Zjawiony 2009). Compound **1** could be thus considered as a lead for further chemical structure optimization in order to have an umbrella of new molecules which show inhibitory properties against *P. mirabilis*.

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