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ANTIBACTERIAL ACTIVITY OF ORSELLINATES

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SHORT COMMUNICATION

ABSTRACT

In order to obtain new compounds with antibacterial activity, the derivatives 2,4-dihydroxy-6-methylbenzoic acid (orsellinic acid) and 2,4-dihydroxy-6-methylbenzoates (orsellinates) were obtained through alcoholyses of lecanoric acid. All these substances were tested against Gram-positive and Gram-negative bacteria by a microdilution method. *Staphylococcus aureus*, *Xanthomonas campestris* var. *vesicatoria* and *Ralstonia solanacearum* were most sensitive to *n*-propyl 2,4-dihydroxy-6-methylbenzoate, *n*-pentyl 2,4-dihydroxy-6-methylbenzoate and *n*-hexyl 2,4-dihydroxy-6-methylbenzoate with MIC (Minimal Inhibitory Concentration) values ranging from 62.5 to 7.8 $\mu g \cdot mL^{-1}$. These results showed that homologation in carbon chain may lead to compounds with more pronunced activities.

Key words: orsellinates, lecanoric acid, orsellinic acid, antibacterial activity.

Because of known problems posed for the treatment of diseases caused by antibiotic-resistant microorganisms and because of the difficulties faced by pharmaceutical laboratories in their search for new molecules with antimicrobial properties, research in this area is no longer restricted to customary sources. In fact, algae, lichens, macrofungi, and higher plants now constitute important sources for prospecting for new bioactive molecules, either by the direct use of their secondary metabolites or by employing their biosynthetic or semi-synthetically derived compounds, which are produced with the aim of attaining higher effectiveness, improved absorption, or even decreased toxicity (3,5,7). In accordance with this line of action, the present work studied the antibacterial activity of lecanoric acid, extracted from the lichen *P. tinctorum*, as well as its derivatives obtained through alcoholysis.

Parmotrema tinctorum (Nyl.) Hale was collected in the state of Mato Grosso do Sul, Brazil, in March 1999. It was identified by Dr. Mariana Fleig, and a voucher specimen (number 0488) was deposited at the Herbarium of the Department of Chemistry of Universidade Federal de Mato Grosso do Sul.

The lichen was triturated to powder (65.5 g) and exhaustively extracted with $CHCl_3$. The remaining powder was then extracted with acetone and evaporated. The residue of this acetone extraction was solubilized in ethyl ether and treated with a 5% solution of $NaHCO_3$. After treatment of the aqueous phase with a 1N solution of sulfuric acid, lecanoric acid (9.25 g) precipitated and was filtered under vacuum (1).

The derivatives 2,4-dihydroxy-6-methylbenzoic acid [2] and 2,4-dihydroxy-6-methylbenzoates [3 to 11] were prepared by reacting 200 mg of lecanoric acid [1] with 50 mL of a corresponding alcohol in a reflux system (2). After 20 hours of reaction, the alcohol was evaporated and the products 2 to 11 were separated in a silica gel chromatographic column with $CHCl_3$. Compound 2 was separated with $CHCl_3$/acetone 93:7 v/v.

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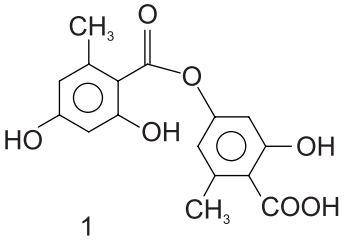
The ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) spectra were determined for all substances by using the solvent acetone-d_6. FT-IR and EI mass spectra were also obtained. Spectral data were in accordance with the literature (2,4,6).

The antibacterial activity of the main compound isolated from the lichen, and of the derivatives obtained through alcoholysis, were investigated by employing a microdilution method. The assay was carried out with two bacterial species that are pathogenic to humans — *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 — and with two plant pathogens — *Xanthomonas campestris* var. *vesicatoria* and *Ralstonia solanacearum*. Mueller-Hinton agar and broth (Difco Laboratories) were used for bacterial growth. The inoculum was an overnight culture of each bacterial species in Mueller-Hinton broth diluted in the same media to a final concentration of approximately 10^8 CFU/mL. 4 mg of each test substance were dissolved in 200 mL of dimethyl sulfoxide (DMSO) and the volume was completed to 2,000 µL with Mueller-Hinton broth. Further 1:2 serial dilutions were performed and an equal volume of Mueller-Hinton agar was added to each dilution tube to reach a final concentration within a 3.90 µg/mL to 1,000 µg/mL range. Two hundred microliters of each dilution were distributed in 96 well plates, as well as in growth and sterility controls (2 x 200 µL of a vehicle made of Mueller-Hinton broth and agar plus DMSO, without antimicrobial substance). Each testing and growth control well was inoculated with 5 µL of a bacterial suspension containing approximately 10^8 CFU/mL (5.10^5 CFU/well). All experiments were performed in duplicate and the microdilution trays were incubated at 36ºC for 18 h. Then, 20 µL of an aqueous solution (0.5 mg/mL) of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) were added to each well and the trays were again incubated at 36ºC for 30 min. Afterwards, in those wells where bacterial growth did occur, INT changed from yellow to purple. Any remaining yellow color indicated absence of growth. MIC was defined as the lowest concentration of each substance at which no color change occurred, and was expressed in µg/mL. Penicillin and tetracycline were used to assess the MIC of the reference strains. The procedures used were based on those described by Woods and Washington (10), with two modifications. First, the culture media distributed in the wells of the microdilution plate contained an agar concentration equivalent to 50% of that corresponding to normal Mueller-Hinton agar, in order to avoid the evaporation of water and thus maintain the concentration of the test substances throughout the incubation period. Second, the INT indicator was added to the media to better distinguish the presence of bacterial growth.

Substances obtained from the lichen and derivatives obtained through alcoholysis: From the extract of *P. tinctorum*, lecanoric acid [1], the main substance, was isolated with a yield of 14.13%.



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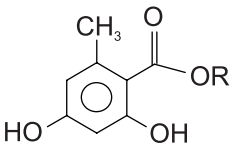

2-11

The following substances were obtained from the alcoholyses of lecanoric acid (compound 1). Substitutions on R positions were as follow:

2- R = -H – 2,4-dihydroxy-6-methylbenzoic acid
3- R = -CH_3 – methyl 2,4-dihydroxy-6-methylbenzoate
4- R = -CH_2CH_3 – ethyl 2,4-dihydroxy-6-methylbenzoate
5- R = -$(CH_2)_2CH_3$ – *n*-propyl 2,4-dihydroxy-6-methylbenzoate
6- R = -$(CH_2)_3CH_3$ – *n*-butyl 2,4-dihydroxy-6-methylbenzoate
7- R = -$(CH_2)_4CH_3$ – *n*-pentyl 2,4-dihydroxy-6-methylbenzoate
8- R = -$(CH_2)_5CH_3$ – *n*-hexyl 2,4-dihydroxy-6-methylbenzoate
9- R = -$CH(CH_3)_2$ – *iso*-propyl 2,4-dihydroxy-6-methylbenzoate
10- R = -$CH(CH_3)(CH_2CH_3)$ – *sec*-butyl 2,4-dihydroxy-6-methylbenzoate
11- R = -$C(CH_3)_3$ – *terc*-butyl 2,4-dihydroxy-6-methylbenzoate

Antibacterial activity: The results of the antibacterial activity of compounds 1 to 11 against four bacterial species are shown in Table 1. All microorganisms tested were resistant to lecanoric acid [1] and to the compounds 2,4-dihydroxy-6-methylbenzoic acid (compound 2) and *terc*-butyl 2,4-dihydroxy-6-methylbenzoate (compound 11). Of the four bacterial species studied, three were more sensitive to *n*-pentyl 2,4-dihydroxy-6-methylbenzoate (compound 7) and *n*-hexyl 2,4-dihydroxy-6-methylbenzoate (compound 8), which have the longest side chains. This phenomenon corresponds to increased lipophilicity of the molecule, wich permits penetration into cell membranes (9). The MIC values of these two compounds are equivalent to those described in the literature for a great number of commercial antibiotics used in clinical treatments (8,10,11). *E. coli* was not sensitive to the substances tested.

This study revealed that 2-4-dihydroxy-6-methyl benzoates obtained from lecanoric acid are promising antibacterial agents, mainly those with the longest side carbon chains. Results also showed that homologation or even ramification in carbon chains may lead to compounds with more pronounced activities.

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Table 1. MIC values (μg.mL^{-1}) of lecanoric acid (1), 2,4-dihydroxy-6-methylbenzoic acid (2), and 2,4-dihydroxy-6-methylbenzoates (3 to 11) against four bacterial species.

Compounds	Bacteria			
	Staphylococcus aureus	*Escherichia coli*	*Xanthomonas campestris vesicatoria*	*Ralstonia solanacearum*
1 Lecanoric acid	>1000	>1000	>1000	>1000
2 (R = -H)	>1000	>1000	>1000	>1000
3 (R = -CH_3)	500	500	500	500
4 (R = -CH_2CH_3)	500	>1000	750	250
5 (R = -$(CH_2)_2CH_3$)	62.5	>1000	62.5	31.25
6 (R = -$(CH_2)_3CH_3$)	62.5	>1000	>1000	>1000
7 (R = -$(CH_2)_4CH_3$)	15.63	>1000	46.87	15.63
8 (R = -$(CH_2)_5CH_3$)	7.8	>1000	31.25	7.8
9 (R = -$CH(CH_3)_2$)	125	>1000	>1000	>1000
10 (R = -$CH(CH_3)(CH_2CH_3)$)	250	>1000	62.5	62.5
11 (R = -$C(CH_3)_3$)	>1000	>1000	>1000	1000

The values correspond to minimal concentration (μg.mL^{-1}) of the compounds that inhibit the bacterial growth;
> 1000 : The compounds might inhibit the bacterial growth at concentration >1000 μg.mL^{-1}.

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RESUMO

Atividade antibacterina de orselinatos

Para obter novos compostos com atividade antibacteriana, foram preparados os derivados 2,4-diidróxi-6-metilbenzoatos (orselinatos) e o ácido 2,4-diidróxi-6-metilbenzóico (ácido orselínico) através de reações de alcoólise do ácido lecanórico. Todas as substâncias foram testadas contra bactérias Gram-positivas e Gram-negativas pelo método da microdiluição. *Staphylococcus aureus*, *Xanthomonas campestris* var. *vesicatoria* and *Ralstonia solanacearum* foram mais sensíveis ao 2,4-diidróxi-6-metilbenzoato de *n*-propila, 2,4-diidróxi-6-metilbenzoato de *n*-pentila e 2,4-diidróxi-6-metilbenzoato de *n*-hexila com valores de CIM (Concentração Inibitória Mínima) variando de 62,5 a 7,8 μg.mL^{-1}. Esses resultados mostraram que a homologação da cadeia carbônica pode conduzir a compostos mais ativos.

Palavras-chave: orselinatos, ácido lecanórico, ácido orselínico, atividade antibacteriana.

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