Revista Virtual de Química
ISSN 1984-6835

Artigo

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Rev. Virtual Quim., **2019**, *11* (3), 596-604. Data de publicação na Web: 15 de abril de 2019

http://rvq.sbq.org.br

Atividade Antibacteriana de Austidiol Isolado de *Mycoleptodiscus indicus* Contra *Xanthomonas axonopodis* pv. *passiflorae*

Resumo: Doenças de plantas causadas por bactérias resultam em perdas graves para a agricultura. O estudo químico de fungos endofíticos representa uma área em potencial no desenvolvimento de pesticidas biológicos. Assim, o presente trabalho teve como objetivo verificar a atividade antibacteriana do extrato do fungo endofítico *Mycoleptodiscus indicus* NF12 de *Morinda citrifolia* contra *Xanthomonas axonopodis* pv. *passiflorae*, bactéria causadora da mancha bacteriana do maracujazeiro, bem como, determinar qual composto é responsável pela atividade observada. O composto austidiol (**1**) foi isolado através de métodos cromatográficos do extrato metanólico da biomassa do fungo endofítico *M. indicus* NF12 e apresentou atividade bacteriostática em todas as concentrações testadas sobre cinco isolados de *X. axonopodis* pv. *passiflorae.*

Palavras-chave: Fungos endofíticos; austidiol; *Xanthomonas axonopodis* pv. *passiflorae*.

Abstract

Plant diseases caused by bacteria result in severe losses to agriculture. The chemical study of endophytic fungi represents a potential area in the development of biological pesticides. Thus, the present work aimed to verify the antibacterial activity of the extract of the endophytic fungus *Mycoleptodiscus indicus* NF12 from *Morinda citrifolia* against *Xanthomonas axonopodis* pv. *passiflorae*, a bacterium that causes bacterial blight of passion fruit, as well as to determine which compound is responsible for the observed activity. The compound austdiol (**1**) was isolated by chromatographic methods from the methanolic biomass extract of the endophytic fungus *M. indicus* NF12 and showed bacteriostatic activity at all concentrations tested on five strains of *X. axonopodis* pv. *passiflorae.*

Keywords: Endophytic fungi; austdiol; *Xanthomonas axonopodis* pv. *passiflorae*.

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DOI:10.21577/1984-6835.20190045

Volume 11, Número 3 Maio-Junho 2019

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Antibacterial Activity of Austdiol Isolated from *Mycoleptodiscus indicus* Against *Xanthomonas axonopodis* pv. *passiflorae*

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Recebido em 18 de abril de 2018. Aceito para publicação em 28 de março de 2019

1. Introduction

Bacterial blight is caused by *Xanthomonas axonopodis* pv. *passiflorae* (Pereira) Gonçalves & Rosato and represents one of the main diseases of passion fruit. It was first observed in Brazil, in 1968, in the region of Araraquara, state of São Paulo,[1] and occurs in all localities where passion fruit is grown, becoming more severe in hotter and more humid regions.[2] The disease can be found in orchards of the species *Passiflora edulis* f. *flavicarpa*, *P. alata*, *P. cincinata*, *P. nitida*, *P. quadrangularis*, *P. amethystina*, *P. setacea* and *P. edulis* f. *edulis*.[3] *Xanthomonas axonopodis* pv. *passiflorae* is a gram-negative, strict aerobic bacterium, with mucoid, convex, bright, yellow-colored colonies.[4]

Bacterial blight, also known as oily spot, bacterial boll rot or bacteriosis, when affecting passion fruit, presents typical symptoms distinct from other diseases of passion fruit. In the leaves, the pathogen produces small

water-soaked and translucent lesions, which necrosis, assuming a reddish-brown color, and may also form a chlorotic halo around the spot (Figure 1). Whereas in the fruits, the spots are large, initially greenish and oily, then brown, generally circular and well delimited.[5]

Figure 1. Symptom of bacterial blight in a leaf of passion fruit, caused by *Xanthomonas axonopodis* pv. *passiflorae*

Endophytic fungi have been studied in relation to their ability to act as inhibitors of phytopathogenic microorganisms, with the perspective of applying them as biocontrol agents, an alternative to chemical control. Their use can occur directly, in which they are applied alive and act as antagonists; or indirectly, through their metabolites.[6,7]

In previous work, endophytic fungi of *Morinda citrifolia* were studied and the extracts of some of these fungi showed good antibacterial activity against *X. axonopodis* pv. *passiflorae*.[8] Thus, the present study aimed to verify the antibacterial activity of the biomass extract of the endophytic fungus *Mycoleptodiscus indicus* NF12 from *M. citrifolia*, as well as to determine which compound is responsible by activity observed.

2. Experimental

2.1. General procedures

Chromatogram and UV spectrum were obtained from Analytical HPLC Waters e2695 equipped with DAD Waters 2998. ESIMS data were acquired in positive and negative ion mode using a Waters Acquity TQD instrument. 1D and 2D NMR spectra were recorded on a Varian Mercury 300, using solvent signal as reference. The chemical shifts are given in delta (δ) values and the coupling constants (*J*) in Hertz (Hz).

2.2. Microorganism

The endophytic fungus *Mycoleptodiscus indicus*, code NF12, was obtained from a collection of the Laboratory of Plant Pathology of the Embrapa Amazônia Oriental, Belém-PA, that contains the endophytic fungi isolated from *Morinda citrifolia* (IAN 188703).

2.3. Culture of *Mycoleptodiscus indicus* NF 12 in rice and chemical constituent isolation

Two Erlenmeyer flasks (125 mL) containing 30 g rice (Tio João®) and 15 mL distilled water per flask were autoclaved for 45 min at 121 °C. Small cubes of PDA medium containing mycelium of *M. indicus* NF 12 were added in 01 Erlenmeyer flask under sterile condition, another flask was used as control. After 15 days of growth at 25 °C the biomass obtained was macerated with methanol. The methanol solution was evaporated under reduced pressure, producing a yellowish residue of the methanolic extract NF12 (103.2 mg). The methanolic extract NF12 was fractionated on a sephadex column using methanol as the mobile phase, from which 16 fractions were obtained, which after analysis by TLC the fractions were pooled from A1 to A6. The fraction A5 was fractionated on ODS column, using water/methanol 20 to 100 % as mobile phase, obtaining 47 fractions, which after analysis by TLC the fractions were pooled from B1 to B10 and of fraction B4 the compound **1** was isolated (5 mg).

2.4. Antibacterial assay with the methanolic extract NF12 and compound 1

With the methanolic extract NF12 and compound **1**, the antibacterial assay was performed at the Laboratory of Plant Pathology of the Embrapa Amazônia Oriental through of the dilution method as recommended by the Subcommittee on Antifungal Susceptibility Testing of the Clinical and Laboratory Standards Institute (CLSI), with modifications.[9] The assay was performed in 96-well ELISA plates using liquid culture medium 523[10] and 5 strains of *X. axonopodis* pv. *passiflorae* from the municipalities of Igarapé-Açu (PA 1), Castanhal (PA 4.3), Maracanã (PA 5.2), São Francisco do Pará (PA 18) and Tomé-Açu (PA 20), all in the state of Pará, in the concentrations of 1×10^8 CFU mL^{-1}. The methanolic extract NF12 and compound **1** were tested at concentrations of 0; 3.125; 6.25, 12.5; 25; 50 and 100 µg mL^{-1}. The experimental design was randomized with 3 replicates and the Elisa plates were incubated for 24 h at 28 °C. The evaluation was performed by adding 10 µL of 2 % TTC (2,3,5-triphenyltetrazolium chloride) into each well of the plate. The wells which did not show red color, extract and compound **1**, were reinoculated in Petri dishes containing solid culture medium 523 and incubated for 48 h at 28 °C. Where bacterial growth occurred, it was indicated that the extract and/or compound had bacteriostatic effect at this concentration and where there was no bacterial growth, the extract and/or compound presented bactericidal effect.

3. Results and Discussion

3.1. Antibacterial assay with the methanolic extract NF12 and compound 1

The methanolic extract NF12 presented bacteriostatic activity at almost all concentrations on the growth of all strains of *X. axonopodis* pv. *passiflorae* (Table 1), showing very promising antibacterial activity. Analytical HPLC analysis of the methanolic extract NF12 showed the presence of a major compound at retention time 12.4 min and maximum absorption at 257 and 377 nm, which is suggestive of a compound of the class of azaphilones (Figure 2). The methanolic extract NF12 was fractionated by chromatographic methods and the compound **1** was isolated, which had its activity tested against the strains of *X. axonopodis* pv. *passiflorae*, showing to be active at all concentrations tested (Table 1), which showed that compound **1** is responsible for the activity verified in the methanolic extract NF12.

Table 1. Effect of the methanolic extract NF12 and compound **1** from the endophytic fungus *Mycoleptodiscus indicus* NF12 on the growth of different strains of *Xanthomonas axonopodis* pv. *passiflorae*

Strains of *X. axonopodis* pv. *passiflorae*	Samples	Concentrations (µg mL^{-1})						
		0	3.125	6.25	12.5	25	50	100
PA 1	NF12	+	-	-	-	-	-	-
	C1	+	-	-	-	-	-	-
PA 4.3	NF12	+	+	-	-	-	-	-
	C1	+	-	-	-	-	-	-
PA 5.2	NF12	+	+	-	-	-	-	-
	C1	+	-	-	-	-	-	-
PA 18	NF12	+	-	-	-	-	-	-
	C1	+	-	-	-	-	-	-
PA 20	NF12	+	-	-	-	-	-	-
	C1	+	-	-	-	-	-	-

"+" without action; "-" bacteriostatic; "NF12" methanolic extract NF12; "C1" compound 1

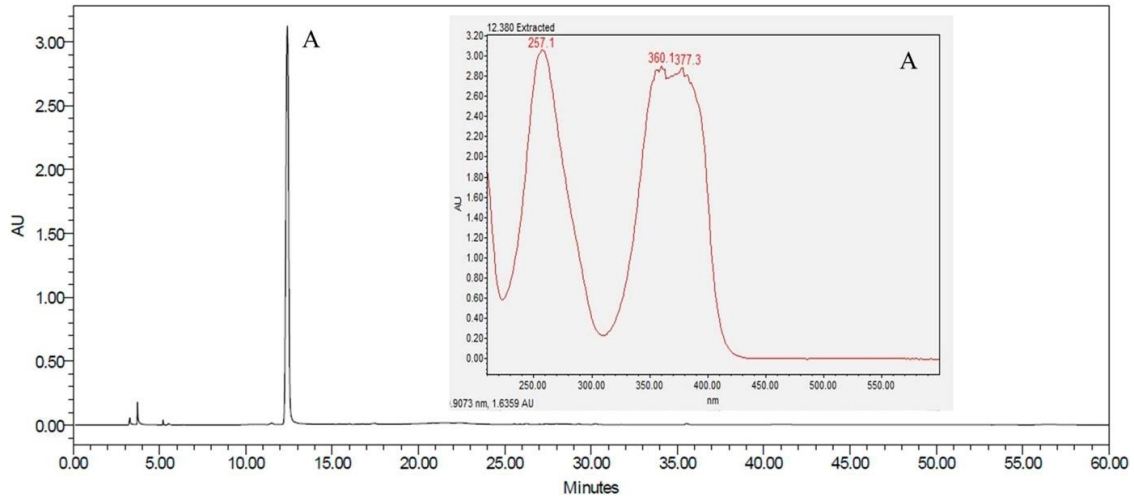

Figure 2. Chromatogram obtained by HPLC-DAD for the extract NF12, λ = 254 nm. A - Band with t_R of 12.4 min presents UV spectrum with λ_{max} of 257 and 377 nm

3.2. Structural elucidation of compound 1

The compound **1** (Figure 3) was isolated as a yellow methanol-soluble solid from fraction B4 of the methanolic extract of the endophytic fungus *M. indicus* NF12. Its structure was elucidated based on its NMR and MS data and comparison with literature data.

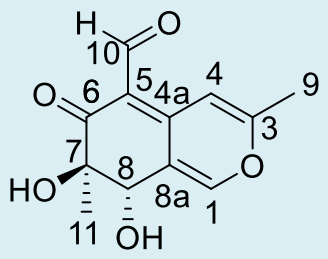

Figure 3. Structure of compound **1**

In the ESI(+) mass spectrum of compound **1**, ions of *m/z* 237 $[M+H]^+$ and *m/z* 259 $[M+Na]^+$ were observed, which, together with the NMR data, confirmed the molecular formula $C_{12}H_{12}O_5$. In the ^1H NMR spectrum, a singlet signal was observed at δ_H 1.11 (3H, *s*, H-11) relative to methyl hydrogens linked to carbinolic carbon, in addition to a singlet signal at δ_H 2.45 (3H, *s*, H-9) relative to methyl hydrogens linked to sp^2 carbon. Moreover, there was a singlet signal at δ_H 4.58 (*s*, H-8), attributed to oxymethinic hydrogen, a doublet signal at δ_H 8.15 (1H, *d*, H-1, *J* = 1.5 Hz), relative to olefinic hydrogen next to the heteroatom and a singlet signal at δ_H 8.32 (1H, *s*, H-4), which refers to olefinic hydrogen next to the methyl-linked carbon. Finally, a singlet signal was observed at δ_H 10.0 (1H, *s*, CHO-10), characteristic of aldehyde hydrogen. In the ^{13}C NMR spectrum, 12 carbon signals were observed, 2 being attributed to methyl carbons (δ_C 18.6, C-11; and δ_C 20.3, C-9), 4 attributed to non-hydrogenated carbons (δ_C 111.5, C-5; δ_C 125.5, C-8a; δ_C 153.3, C-4a; and δ_C 168.5. C-3), 2 attributed to carbinolic carbons (δ_C 72.4, C-7; and δ_C 74.5, C-8), 2 to olefinic carbons (δ_C 108.4, C-4; and δ_C 155.5, C-1), 1 attributed to aldehyde carbonyl carbon (δ_C 191.3, C-10) and 1 attributed to ketone carbonyl carbon (δ_C 198.2, C-6).

HMBC correlations of H-8, along with H-4 and Me-11 with carbons C-4a, C-5 and C-6 have evidenced α,β-unsaturated carbonyl group. Following the HMBC correlations for Me-9 with carbons C-3 and C-4, along with the HMBC correlations of H-1 with carbons C-3 and C-8a, a pyran-quinone group was observed. This information showed that compound **1** belongs to the class of azophilones. Finally, the observed HMBC correlation of H-10 with carbons C-5 and C-4a made it possible to locate the localization of the aldehyde group at C-5.

Data regarding ^1H and ^{13}C NMR were compared with the literature (Table 2) and it was concluded that the compound **1** is (*7R,8S*)-7,8-dihydro-7,8-dihydroxy-3,7-dimethyl-6-oxo-6H-2-benzopyran-5-carboxaldehyde, known as austdiol.[11]

Austdiol is an azaphilone isolated for the first time from *Aspergillus ustus*, a contaminant found in foodstuffs.[11] Azaphilones are a structurally diverse class of secondary metabolites of fungi (polyketide derivatives), known pigments with a pyran-quinone structure containing a highly oxygenated bicyclic nucleus and a quaternary chiral center.[12-14]

Other works present the isolation of austdiol from endophytic fungi, in which it was also isolated from the species *M. indicus*, obtained from leaves of *Borreria verticillata*.[15] Furthermore, austdiol was isolated from the endophytic fungus coded as DgCr22.1b, from the root bark of *Duguetia stelechantha*,[16] and from the endophytic fungus coded as CRI7 from *Tiliacora triandra*.[17]

More than 170 different azaphilones occur in fungi belonging to 23 genera of 13 different families. There have been reports of several biological activities for this class of substances, such as: antimicrobial, antifungal, antioxidant, antiviral, cytotoxic, nematicidal and anti-inflammatory activity.[18]

Table 2. Data regarding NMR ^{1}H and ^{13}C (CD_3OD, 300 MHz) of compound **1**

	^{1}H	^{13}C
1	8.15 (*d*)	155.5
3	-	168.5
4	8.32 (*s*)	108.4
4ª	-	153.3
5	-	111.5
6	-	198.2
7	-	72.4
8	4.58 (*s*)	74.5
8ª	-	125.5
9-CH_3	2.45(*s*)	20.3
10-CH0	10 (*s*)	191.3
11-CH_3	1.11 (*s*)	18.6

Is describe in the literature phytotoxic activity to some azaphilones.[19-20] This information corroborate with the tendency of natural phytotoxins have been considered hit compounds to development the new herbicides in substitution of the chemical synthetics herbicides.[21] For example of azaphilones obtained from endophytic fungi with phytotoxic activity able to be cited the azaphilones chaetomugilin D and chaetomugilin J isolated from *Chaetomium globasum*, a endophytic from leaves of the *Amaranthus viridis*.[22]

For the azaphilone austdiol isolated in the present work was described cytotoxic activity against tumor cells and leishmanicidal activity on *Leishmania major* and *L. dovani*.[15] However, there are no studies on its activity against agricultural pests such as the bacterial blight of passion fruit, this being the first report of this important activity for the austdiol, which can open the possibility of production of a new antibacterial agent for the control of *X. axonopodis* pv. *passiflorae*.

4. Conclusion

The methanolic extract of the biomass of the endophytic fungus *M. indicus* NF12 showed good antibacterial activity against *X. axonopodis* pv. *passiflorae*, a bacterium that causes bacterial blight of passion fruit. The chemical study of the methanolic extract of *M. indicus* NF12 led to the isolation by chromatographic methods of the compound austdiol (**1**) as the responsible for the observed activity, being active at all concentrations tested. This is the first report of antibacterial activity of the compound austdiol against *X. axonopodis* pv. *passiflorae*, demonstrating that the chemical study of endophytic fungi is a potential area in the development of new biological pesticides.

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