

Artigo

Brefeldin A and Other Chemical Constituents from Endophytic Fungus *Scedosporium apiospermum*

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Brefeldina A e Outros Constituintes Químicos do Fungo Endofítico *Scedosporium apiospermum*

Resumo: Este estudo relata o isolamento de cinco compostos de *Scedosporium apiospermum* EJCP13 isolados como endofíticos de *Bauhinia guianensis*, planta típica da Amazônia. Os compostos brefeldina A (**1**), ergosterol (**2**), peróxido de ergosterol (**3**), cerevisterol (**4**) e ducitol (**5**) foram isolados por procedimentos cromatográficos e identificados por métodos espectroscópicos de RMN 1D e 2D e EM. O composto brefeldina A (**1**) apresentou boa atividade antimicrobiana e os compostos **1-5** são reportados pela primeira vez em gêneros de *Scedosporium*.

Palavras-chave: Brefeldina A; *Scedosporium*; fungo endofítico.

Abstract

This study reports the isolation of five compounds of *Scedosporium apiospermum* EJCP13 isolated as an endophytic of *Bauhinia guianensis*, a typical plant of the Amazon. The compounds brefeldin A (**1**), ergosterol (**2**), ergosterol peroxide (**3**), cerevisterol (**4**) and ducitol (**5**) were isolated by chromatographic procedures and identified by spectroscopic methods of 1D and 2D NMR and MS. The compound brefeldin A (**1**) showed a good antimicrobial activity and the compounds **1-5** are reported for the first time in *Scedosporium* genera.

Keywords: Brefeldin A; *Scedosporium*; endophytic fungus.

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Brefeldin A and Other Chemical Constituents from Endophytic Fungus *Scedosporium apiospermum*

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1. Introduction

2. Experimental

2.1. General procedures

2.2. Plant material

2.3. Microorganism

2.4. Culture of *S. apiospermum* EJCP13 in rice and isolation of chemical constituents

2.5. Antimicrobial assay

2.6. Quantification of brefeldin A by UPLC/MS in *S. apiospermum* extracts

3. Results and Discussion

3.1. Antimicrobial activity of the hydro-methanolic extract of *S. sedosporium* biomass

3.2. Isolation and identification of brefeldin A

3.3. Quantitative analysis of brefeldin A content in organic extracts

4. Conclusion

1. Introduction

Among endophytic microorganisms, there are fungi and bacteria,¹ but fungi are the most common isolates.² Zhang *et al*³ state that endophytic fungi produce secondary metabolites in greater amounts than any other class of endophytic microorganisms.

As a part of a fungal community, endophytic fungi may have one or more functional roles during their life cycles.¹ They

can host in plants growing in different environmental regions, such as the Arctic and Antarctica, geothermal soils, deserts, oceans, forests, mangroves and coastal forests, colonizing a wide range of hosts, including algae, bryophytes, sponges, pteridophytes, gymnosperms and angiosperms.⁴⁻⁵ In addition, endophytic fungi form symbiotic relationships with their hosts. These relationships may bring advantages to both the fungus and the host plant.⁶⁻⁸

The *Bauhinia guianensis* is typical of the Amazon region; it is used in folk medicine against infections. In previous work with *B. guianensis* compounds with antimicrobial activity were not found.⁹ Thus we decided to study the endophytic fungi from *B. guianensis*.

Among fungi, few papers described the genus *Scedosporium* as for the isolation of secondary metabolites. Huang *et al.*,¹⁰ studied the alkaloid production of a strain of *S. apiospermum* by inducing the production of these compounds using amino acid-enriched cultures. Kuroda *et al.*¹¹ reported the compound AS-1 83, which inhibits acyl-CoA, and Staerck *et al.*¹² reported the isolation of boydone A polyketide with anti-*Staphylococcus aureus* activity of *S. boydii*. The genus *Scedosporium* causes different infections, hence the importance of knowing more about its secondary metabolism.

In preliminary tests in our research group, we verified that the biomass extracts of *S. apiospermum* EJCP13 presented a moderate antimicrobial activity. Thus, this study describes the isolation of secondary metabolites, as well as the test of their antimicrobial activities. The compounds brefeldin A (**1**), ergosterol (**2**), ergosterol peroxide (**3**), cerevisterol (**4**) and ducitol (**5**) were isolated. Brefeldin A (**1**) showed a good antimicrobial activity and the compounds **1-5** are being reported for the first time for the genus *Scedosporium*.

2. Experimental

2.1. General Procedures

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. Plant material

Bauhinia guianensis was collected in the city of Belém-PA and a voucher specimen (n° IAN 177.179) was deposited at the Herbarium of “Empresa Brasileira de Pesquisa Agropecuária” – EMBRAPA.

2.3. Microorganism

Scedosporium apiospermum was obtained from a collection of the Laboratório de Bioensaios e Química de Micro-organismos (LaBQuiM), Faculdade de Química - Universidade Federal do Pará. This collection contains isolates from *Bauhinia guianensis*. One strain is deposited in the LaBQuiM with the code EJCP13.

2.4. Culture of *S. apiospermum* EJCP13 in rice and isolation of chemical constituents

Initially, the fungus *S. apiospermum* EJCP13 was cultivated on a small scale to test the antimicrobial activity of the extract. 10 g of rice were added into a 100 mL Erlenmeyer flask containing 7 mL of distilled water, and autoclaved for 45 min at 121 °C. The hydro-methanolic extract (1.2 g) was obtained at 28 days of culture. After the test of antimicrobial activity of the hydro-methanolic extract, the cultivation was carried out in an amplified scale to isolate the active compound. Twenty Erlenmeyer flasks (1,000 mL) containing 200 g rice (Tio João[®]) and 100 mL distilled water per flask were autoclaved for 45 min at 121 °C. Small cubes of PDA medium containing mycelium of *S. apiospermum* EJCP13 were added in 18 Erlenmeyer flasks under sterile condition. Two flasks (with rice only) were used as control. After 28 days of growth at 25 °C the biomass obtained was macerated with hexane, ethyl acetate and methanol sequentially in polarity gradient. The all solvents solutions were evaporated under reduced pressure, the ethyl acetate producing a yellowish residue (37.7 g). Ten grams of the

ethyl acetate extract after successive fractionations on silica gel chromatography column eluted with hexane, ethyl acetate and methanol in polarity gradient and were obtained the compounds 1–5.

2.5 Antimicrobial assay

Susceptibility of the microorganisms to the test extract and compound was determined by the microbroth dilution assay according Pinheiro et al (2017)¹³. The assays were carried out with *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633) and *Salmonella typhimurium* (ATCC14028). Bioactivity was recorded as absence of red coloration in the wells after addition of 10 µL of 2,3,5-triphenyltetrazolium chloride. Penicillin, vancomycin and tetracycline (25 µg/mL each) were used as positive controls; the cultivation medium (MHB only) was used as negative control.

2.6. Quantification of brefeldin A by UPLC/MS in *S. apiospermum* extracts

For the preparation of a standard solution, 1.00 mg of brefeldin A was solubilized in 999 µL of HPLC grade methanol, giving a stock solution at the concentration of 1,000 µg/mL. From the stock solution, a 10 µL aliquot was withdrawn, and 495 µL of water and 495 µL of methanol were added to a solution of 10 µg/mL. From this stock solution, dilutions were made to obtain solutions at concentrations of 1.0 µg/mL, 0.8 µg/mL, 0.4 µg/mL, 0.2 µg/mL, 0.1 µg/mL and 0.05 µg/mL. Each solution was injected into the chromatographic system in triplicate. The quantification of brefeldin A was carried out from the ethyl acetate, hexane and methanolic extracts of the biomass of the endophytic fungus *S. apiospermum*. Thus, a calibration curve was plotted by varying the

concentration of brefeldin A, and extract concentrations were fixed at 10 µg/mL.

The calibration curve was constructed on the mass spectrometer using a 50 mm x 2.1 mm (1.7 µm pore diameter) Acquity UPLC BEH® C18 analytical column, water and methanol with the addition of 0.1 % TFA as the mobile phase in gradient mode from 0 to 1.4 min water/methanol at 30 %, 1.5 to 2.0 min water/methanol at 99 %, and finally from 2.1 to 3.5 min water/methanol at 30 %, returning to the initial condition. The flow was 0.4 mL/min, and the solvent used to dissolve the extracts was methanol.

3. Results and Discussion

3.1. Antimicrobial activity of the hydro-methanolic extract of *S. sedosporium* biomass

For *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis* and *Salmonella typhimurium*, assays were carried out with the hydro-methanolic extract of the fungal biomass of the fungus *S. sedosporium*. The hydro-methanolic extract presented a moderate activity (Table 1). Thus, we decided to cultivate the fungus on an enlarged scale and isolate the constituent responsible for the activity.

3.2. Isolation and identification of brefeldin A

The Compound 1 was isolated from hexanic and AcOEt extracts of the biomass of the endophytic fungus *S. apiospermum*, and its structure was determined by NMR and MS. The ESIMS mass spectrum, in the positive mode, showed an ion peak in m/z at 281 $[M+H]^+$. This information, together with RMN ¹H and ¹³C data, helped to confirm the molecular formula C₁₆H₂₄O₄ for the compound 1. The structural determination was also

based on a comparison of RMN data from the literature.¹⁴

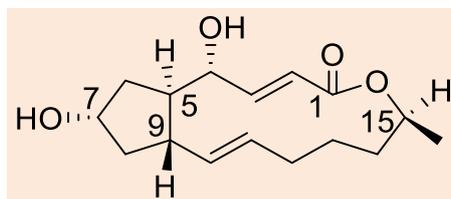


Figure 1. Structure of Compound 1

The RMN spectrum of ^1H showed signals for the compound **1** in δ_{H} at 4.02 (*m*, H-4), δ_{H} 4.20 (*quint.*, $J = 5.2$ Hz, H-7) and δ_{H} 4.80 (*m*, H-15), characteristic of carbinolic hydrogens. It also showed signals in δ_{H} 5.81 (*dd*, $J = 15.6$ and 2.1 Hz, H-2), δ_{H} 7.44 (*dd*, $J = 15.6$ and 3.0 Hz, H-3), δ_{H} 5.26 (*dd*, $J = 15.0$ and 9.6 Hz, H-10) and δ_{H} 5.74 (*ddd*, 15.0, 10.1, 5.1 Hz, H-11) characteristic of olefinic hydrogens; H-3 has a signal at δ_{H} 7.44 due to the carbonyl conjugated double bond, also the signals to hydrogens H-6 (2.03 *m* and 1.83 *m*) and H-8 (2.16 *m* and 1.43 *m*) were observed. In the analysis of the RMN ^{13}C spectrum, signals are observed referring to 16 carbon atoms. The carbon sign at δ_{C} 168.4 is characteristic of lactonic carbonyl group. The carbon signals at δ_{C} 155.1, δ_{C} 138.1, δ_{C} 131.4 and δ_{C} 117.8 were assigned to C-3, C-10 and C-11 and C-2 olefinic carbons, respectively, while the signals at δ_{C} 76.6, δ_{C} 73.2 and δ_{C} 73.0 were assigned to the carbinolic carbons at C-4, C-15 and C-7, respectively. Still the signals to carbons C-6 (41.8) and C-8 (44.0) were observed to pentacyclic moiety.

The analyses of HMBC and HSQC correlations allowed determining a macrocyclic ring in **1** containing an α,β -unsaturated lactone group, on the macrocycle. Through the COSY experiment, a correlation was found between H-5 and H-9 with a coupling constant of 9.6 Hz, suggesting that H-5 and H-9 are related *trans*. Still following HMBC correlations for H-5 and H-9, it allowed us to propose that these hydrogens

are part of a pentacyclic ring fused to the macrocycle. Thus, the compound **1** can be identified as brefeldin A. The geometry of the double bonds were inferred as being *E* based on the characteristic values of the coupling constants. The substance brefeldin A, previously isolated from the endophytic fungus *Aspergillus clavatus*, has antifungal and antiviral activity and a higher cytotoxic activity than that of taxol.¹⁴

The compound **1** was tested against the bacteria *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis* and *S. tiphymuruim* to confirm if it was responsible for the activity verified in the hydro-methanolic extract. It presented an antimicrobial activity against the bacteria tested (Table 1). The compound **1** showed a better activity at the lowest concentration tested against *S. tiphymuruim*, which causes gastroenteritis in humans and animals.

There are few studies on the secondary metabolism of fungi of the genus *Scedosporium*, hence the importance of knowing the compounds produced by this important genus of fungus. In this study, besides brefeldin A (**1**), we isolated the compounds ergosterol (**2**), ergosterol peroxide (**3**), cerevisterol (**4**) and ducitol (**5**), which are commonly isolated by fungi. Compounds **1-5** are being reported for the first time for the genus *Scedosporium*. All compounds were isolated by chromatographic procedures and identified by spectral methods of 1D and 2D NMR and MS.¹⁵⁻¹⁶

Table 1. Determination of the antibacterial activity ($\mu\text{g/mL}$) of the MeOH extract of *S. apiospermum* and Brefeldin A against Gram positive and Gram negative bacteria standard strains.

Sample	Inhibitory Concentration ($\mu\text{g/mL}$)				
Hydro-methanolic extract	156.25 (-)	156.25 (-)	625 (-)	2,500 (-)	NP
Brefeldin A (1)	62.5 (-)	(+)	62.5 (-)	(+)	7.81 (-)
Bacterium	<i>Bs</i>	<i>Sa</i>	<i>Ec</i>	<i>Pa</i>	<i>St</i>

Legend: *Bacillus subtilis* (Bs); *Staphylococcus aureus* (Sa); *Escherichia coli* (Ec); *Pseudomonas aeruginosa* (Pa); *Salmonella typhimurium* (St); without activity (+); Bacteriostatic (-); bactericidal (=); not performed (NP).

3.3. Quantitative analysis of brefeldin A content in organic extracts

The compound **1**, brefeldin A, showed a good antimicrobial activity and was the majority secondary metabolite in extracts of *S. apiospermum*. Thus, we decided to quantify this substance in crude extracts to verify the brefeldin A content in the extracts.

Experiments were initially carried out on product ions to determine the Multiple Reaction Monitoring (MRM) channels for the

compound, from a fragmentation study, for a further quantification on a mass spectrometer. The MRM channels used for the quantification of brefeldin A were 281.4 > 245.2, 281.4 > 263.2 and 281.4 > 199.2.

The calibration curve was plotted using the values of areas and concentrations generated from the integration of brefeldin A bands into AcOEt, hexane and MeOH extracts. Figure 2 shows the integration band generated by brefeldin A from the AcOEt extract, where the chromatographic peak was identified with a retention time of 0.56 minutes.

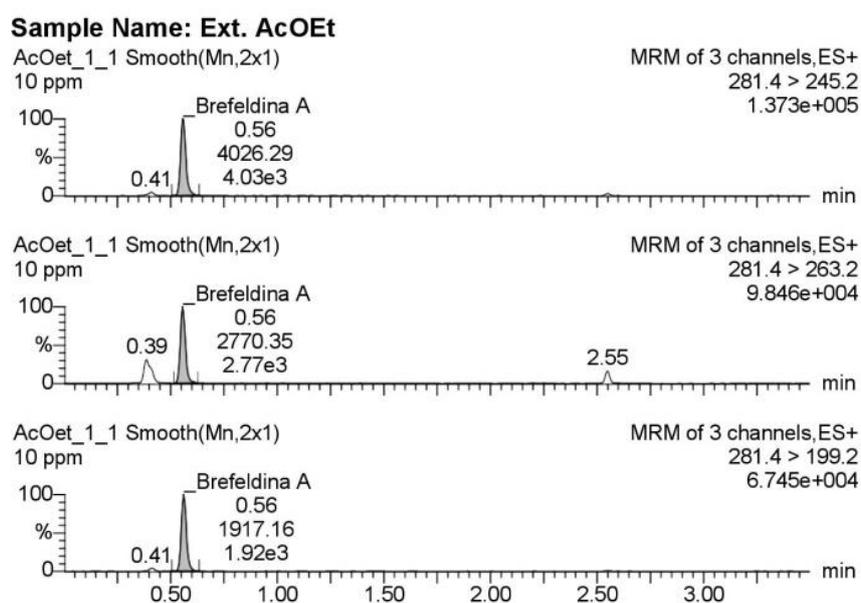


Figure 2. Integration band for the substance brefeldin A of the extract AcOEt

From the data obtained, the calibration curve of brefeldin A was plotted with the following equation: $A = 9.51505C + 648.924$, where C is the concentration of brefeldin A,

and the correlation coefficient was $R^2 = 0.9981$ (Figure 3). For the tracing of the calibration curve, the injections were performed in triplicate.

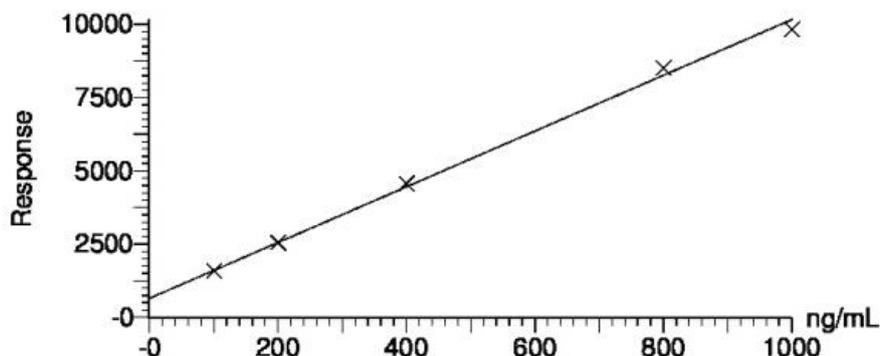


Figure 3. Brefeldin A Calibration Curve (50-1,000 ng.mL⁻¹)

By interpolation of the absorption of the analyzed extracts with the calibration curve, it was possible to determine the brefeldin A content in nanogram (ng) per milliliter (mL) of extract. The AcOEt extract is the richest in brefeldin A, with a content of 354.9 ng/mL of the compound **1**, followed by the hexane extract, with a content of 2.4 ng/mL, and brefeldin A was not detected in the methanolic extract. The extract ethyl acetate showed the highest yield with 3.55 % in brefeldin A.

4. Conclusion

The chemical study of extracts of the fungus *S. sedosporium* isolated as endophytic from *B. guianensis* showed, through quantification by UPLC/MS, that it has as major compound Brefeldin A, a substance that has a wide biological activity. In this work, brefeldin A (**1**) showed a good antimicrobial activity. Compounds **1-5** are being reported for the first time for the genus *Scedosporium*.

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