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ORIGINAL ARTICLE

Characterization of the Secondary Metabolites from Endophytic Fungi *Nodulisporium* sp. Isolated from the Medicinal Plant *Mikania laevigata* (Asteraceae) by Reversed-Phase High-Performance Liquid Chromatography Coupled with Mass Spectrometric Multistage

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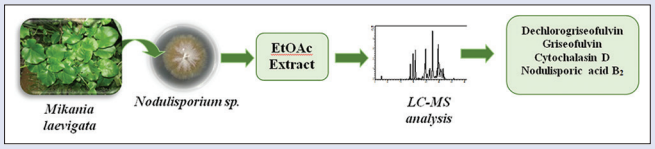
ABSTRACT

Background: The endophytic fungi are an excellent source of secondary metabolites as a natural product with antimicrobial, insecticidal, cytotoxic, antioxidant, and anticancer activities. No studies on the chemical compounds of the endophytes of *Mikania laevigata* have been described in the literature. **Objective:** The objective of the study is to investigate by reversed phase high-performance liquid chromatography coupled with mass spectrometric multistage (RP-HPLC-MS/MS) the secondary metabolites of the endophytic fungi *Nodulisporium* sp., isolated from the medicinal plant *M. laevigata* (Asteraceae). **Materials and Methods:** The mycelia biomass strains of *Nodulisporium* sp. were accumulated and separated from the aqueous medium by filtration; the filtrate was subjected to a liquid–liquid partition with ethyl acetate (EtOAc) resulting in the crude EtOAc extract. This extract was analyzed by RP-HPLC-MS/MS. **Results:** The total mass chromatogram of the EtOAc crude strain *Nodulisporium* sp. extract showed compounds eluted between 13 and 20 min. The peak 1 (Rt 13.8 min), peak 2 (Rt. 14.8 min), peak 3 (Rt. 15.7 min) and peak 4 (Rt. 19.6 min) were characterized as dechlorogriseofulvin ($C_{17}H_{18}O_6$), griseofulvin ($C_{17}H_{17}O_6Cl$), cytochalasin D ($C_{30}H_{37}NO_6$) and nodulisporic acid B2 ($C_{43}H_{57}NO_7$), respectively. **Conclusion:** The present work provides the first scientific report on constituents of endophytic fungi *Nodulisporium* sp. isolated from the *M. laevigata* (Asteraceae).

Key words: Endophytic fungi, *Mikania laevigata, Nodulisporium*, reversed-phase high-performance liquid chromatography coupled with mass spectrometric multistage, Xylariaceae

SUMMARY

- The endophytic fungi *Nodulisporium* sp. was previous isolated from the medicinal plant *Mikania laevigata* (Asteraceae).
- The crude EtOAc extract of *Nodulisporium* sp. was analyzed by RP-HPLC-MS/MS.
- The compounds dechlorogriseofulvin, griseofulvin, cytochalasin D and nodulisporic acid B2 were characterized.



Abbreviations used: EtOAc: Ethyl acetate; RP-HPLC-MS/MS: Reversed-Phase High-Performance Liquid Chromatography coupled with mass spectrometric multistage; CCMB: Culture collection of microorganisms from Bahia; Rt: Retention time.

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INTRODUCTION

Mikania laevigata (*Asteraceae*), a Brazilian medicinal plant commonly known as "guaco," is used for the treatment of respiratory disorders, such as asthma, bronchitis, and chronic lung diseases and also for coughing.[1,2] The antiulcer, anti-inflammatory, analgesic, antispasmodic, and antimicrobial activities have also been investigated for this plant.[3-5] Chemical studies on *M. laevigata* leaves report the isolation and identification of coumarin, terpenes, steroids, flavonoid glycosides, o-coumaric acid, cinnamoyl grandifloric acid, cupressenic acid, isopropiloxi-grandifloric acid, isobutiloxi-grandifloric acid, saponins, and tannins.[1,2,4,5]

The symbiotic relationship among endophytic fungi as well as plants gives endophytes the powerful capability to produce new bioactive substances. The endophytic fungi are an excellent source of secondary metabolites as a natural product with antimicrobial, insecticidal, cytotoxic, antioxidant, and anticancer activities. Thus, they have great promising applications in agriculture and medicine.[6,7]

No studies on the chemical compounds of the endophytes of *M. laevigata* have been described in the literature. Henceforth,

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previous studies conducted by our researcher group showed the isolation and identification of the endophytic fungus from *M. laevigata* and the evaluation of its extracts against four strains of *Salmonella* spp.[8,9] All the fungal extracts showed antimicrobial activity, and the microorganisms of genus *Nodulisporium*, Xylariaceae family, were the dominant group of fungi associated with this plant species. *Nodulisporium*, a group of common endophytic fungi known to produce bioactive secondary metabolites, contained antiflea, cytotoxic, antiplasmodium, antifungal, human DNA polymerase k inhibitors, and mycotoxin activity.[10]

In this work, the ethyl acetate (EtOAc) crude strain Nodulisporium sp. extract (isolated from *M. laevigata*) was chemically analyzed by reversed-phase high-performance liquid chromatography coupled with mass spectrometric multistage (RPH-PLC-MS/MS).

MATERIALS AND METHODS

Collection of plant material and isolation of endophytic fungus

The strains of *Nodulisporium* sp. were isolated from the leaves of *M. laevigata*, collected in a floral Garden located in the State University of Santa Cruz, Ilheus, state of Bahia, Brazil, in September 2009. Three plants were selected for the withdrawal of healthy leaves. From each plant, five leaves were collected, totaling a sample of 15 leaves which were immediately subjected to endophytic fungi isolation. Both the isolation and identification of these fungi are described in a previous study of the same research group.[8] These fungi were deposited as *Nodulisporium* sp. Culture Collection of Microorganisms from Bahia (CCMB) 562 (Genbank n° JNO51356), at the CCMB, in the Department of Biology, State University of Feira de Santana.

Cultivation and preparation of the fungal strain

The endophytic fungi *Nodulisporium* sp. grown on Malt Extract Agar at 28°C for 5 days was inoculated into 500-ml Erlenmeyer flasks containing 300-ml Malt Extract Broth at room temperature for 4 weeks. The mycelia biomass accumulated in the Erlenmeyer was separated from the aqueous medium by filtration, and the filtrate was subjected to a liquid–liquid partition with EtOAc (3 mL × 200 mL). The EtOAc fraction was evaporated resulting in the crude EtOAc extract.

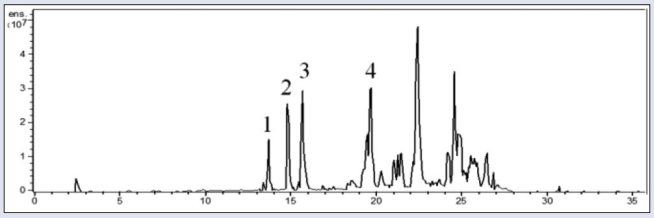

Figure 1: Total mass chromatogram of EtOAc extract of mycelium of *Nodulisporium sp.*: (1) dechlorogriseofulvin, (2) griseofulvin, (3) cytochalasin D, and (4) nodulisporic acid B_2

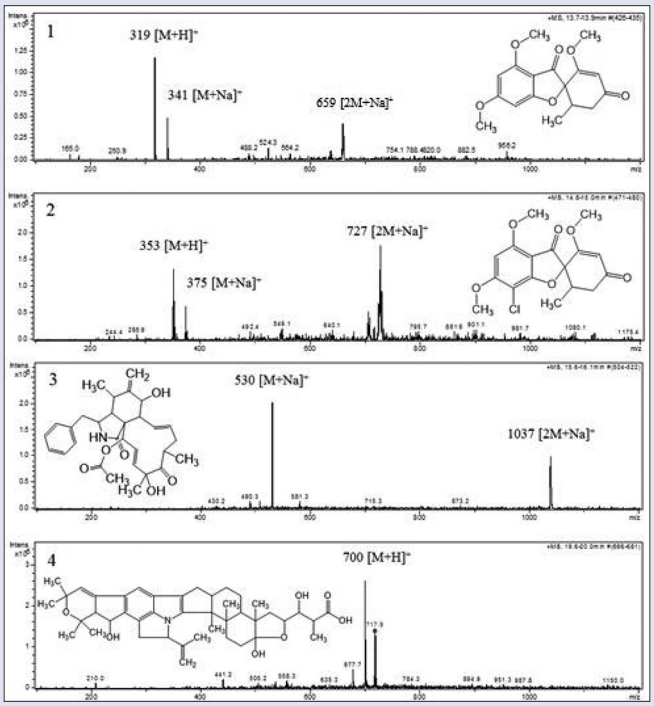

Figure 2: MS spectra of peaks 1, 2, 3, and 4 of ethyl acetate extract of mycelium from *Nodulisporium* sp.

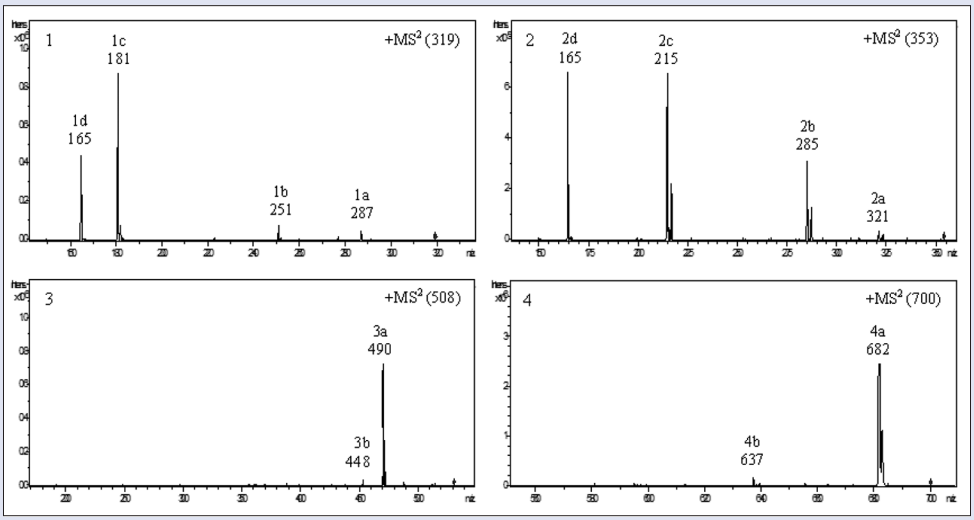

Figure 3: (1) MS^2 analysis of MS 319 (peak 1); (2) MS^2 analysis of MS 353 (peak 2); (3) MS^2 analysis of MS 508 (peak 3); and MS^2 analysis of MS 700 (peak 4)

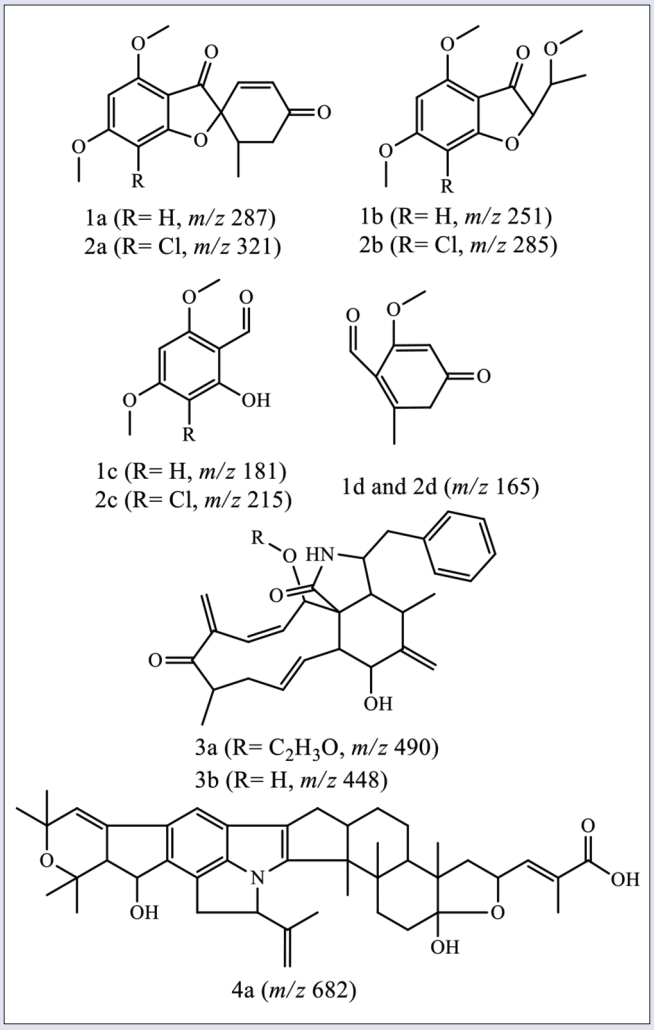

Figure 4: Dechlorogriseofulvin (1a-1d), griseofulvin (2a-2d), cytochalasin D (3a-3b), and nodulisporic acid B_2 (4a) fragments

Reversed-phase high-performance liquid chromatography coupled with mass spectrometric multistage analysis

The crude EtOAc extract of mycelium was analyzed by RP-HLC-MS/MS in positive ion mode using an Esquire 3000-plus mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a CBM-20A controller, LC-20AD pump, SIL-20AC autosampler, and SPD-20A detector. A Phenomenex Luna C-18 (2) column (250 mm × 4.6 mm, 5 µm) was used. The mobile phase consisted of water containing 0.05% phosphoric acid (A) and methanol (B) at a flow rate of 1.0 mL/min using the following gradients: 0–20 min: A (75%–0%) and B (25%–100%), 20–24 min: A/B: 0/100%, 24–25 min: A (0%–75%) and B (100%–25%), and 25–35 min: A/B: 0/100%. The detection was done on a DAD detector set at 340 nm. The mobile phase was prepared daily, filtered through a 0.45-mm membrane filter (Millipore), and sonicated before use.

RESULTS AND DISCUSSION

Figure 1 shows the total mass chromatogram of the EtOAc crude strain *Nodulisporium* sp. extract. This chromatogram showed the natural compounds eluted between 13 and 20 min. The peaks after 22 min were characterized as culture contamination and were not considered in this study. Figures 2 and 3 show MS and MS^2 spectra of peaks 1, 2, 3, and 4 of EtOAc extract of mycelium from *Nodulisporium* sp. The peak with retention time (Rt) at 13.8 min was characterized as dechlorogriseofulvin ($C_{17}H_{18}O_6$, molecular ion at 318). This compound showed protonated ion at *m/z* 319 $[M+H]^+$ and sodium adducts at *m/z* 341 $[M+Na]^+$ and 659 $[2M+Na]^+$. The MS^2 of the protonated ion showed the following fragments: 287 $[M\text{-}OCH_3+H]^+$ (1a), 251 $[M\text{-}C_4H_3O+H]^+$ (1b), 181 $[M\text{-}C_8H_9O_2+H]^+$ (1c), and 165 $[M\text{-}C_8H_9O_3+H]^+$ (1d). This fragmentation pathway was in accordance with literature for the same compound [Figure 4].[11]

The compound 2 (Rt. 14.8 min) showed protonated ion at *m/z* 353 $[M+H]^+$ and sodium adducts at m/z 375 $[M+Na]^+$ and 727 $[2M+Na]^+$. The MS^2 analysis showed fragmentation profile at m/z 321 $[M\text{-}OCH_3+H]^+$ (2a), 285 $[M\text{-}C_4H_3O+H]^+$ (2b), 215 $[M\text{-}C_8H_9O_2+H]^+$ (2c), and 165 $[M\text{-}C_8H_9O_3Cl+H]^+$ (2d) [Figure 4]. Thus, the compound 2 was characterized as griseofulvin ($C_{17}H_{17}O_6Cl$).[12] Griseofulvin is a fungistatic agent used for the treatment of dermatophytes. It is a known metabolic product of several species of fungus. It is deposited in keratin precursor cells, which become resistant to the invasion of dermatophytes. Dechlorogriseofulvin is formed by the degradation of griseofulvin.[13]

The compound 3 (Rt. 15.7 min) was characterized as cytochalasin D ($C_{30}H_{37}NO_6$). This compound showed protonated ion at m/z 508 $[M+H]^+$ and sodium adducts at m/z 530 $[M+Na]^+$. The MS^2 fragmentation profile of protonated ion showed loss of 18 Da (H_2O) and 60 Da (C_2H_2O and H_2O) correspondent to *m/z* 490 (3a) and 448 (3b), respectively [Figure 4].[14,15] The cytochalasin member is well known as mycotoxins widely distributed in various fungi. Its potent inhibition of the actin polymerization affects a wide range of cellular events. Thus, cytochalasins are important biochemical tools for studying fundamental cellular processes.[14,15]

The compound 4 (Rt. 19.6 min) showed protonated ion at m/z 700 $[M+H]^+$. A search for fungus metabolite with the same mass of compound 4 suggests its characterization as nodulisporic acid B_2. The MS^2 of protonated ion showed losses of 18 (H_2O) and 45 (COOH); ions *m/z* 682 (4a) and 637 (4b), respectively [Figure 4]. Nodulisporic acids are indole diterpenes that exhibit potent insecticidal properties.[16]

CONCLUSION

This study demonstrated that RP-HPLC-MS/MS is a suitable tool for characterization of different metabolites compounds for the endophytic fungi, it was possible to characterize four metabolites: dechlorogriseofulvin, griseofulvin, cytochalasin D, and nodulisporic acid B_2. The present work provides the first scientific report on phytoconstituents of endophytic fungi *Nodulisporium* sp. isolated from the *the M. laevigata* (Asteraceae) and these fungi proved to be a source of bioactive metabolites.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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