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Article

Antimicrobial Screening of Endophytic Fungi Isolated from the Aerial Parts of *Paepalanthus chiquitensis* (Eriocaulaceae) Led to the Isolation of Secondary Metabolites Produced by *Fusarium fujikuroi*

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The screening for antimicrobial activity of twenty five endophytic fungi isolated from the aerial parts of *Paepalanthus chiquitensis* (Eriocaulaceae) was assayed against the bacteria Gram-positive *Staphylococcus aureus*, Gram-negative *Escherichia coli* and *Salmonella setubal*, and the yeast fluconazole-resistant *Candida albicans*. The ethyl acetate extract produced by *Fusarium fujikuroi* was the most bioactive and this fungus was chosen for the chemical study, affording the isolation of an alkaloid 2-(4-butylpicolinamide) acetic acid and three known metabolites: fusaric acid, indole acetic acid and the sesterterpene terpestacin. The minimal inhibitory concentration of the extract and of fusaric acid and indole acetic acid for all the tested microorganisms had values from 125 to 1000 μg mL^{-1}.

Keywords: *Fusarium fujikuroi*, endophytic fungi, antimicrobial activity, *Paepalanthus chiquitensis*

Introduction

The *Paepalanthus chiquitensis* Herzog (synonym *Paepalanthus giganteus* Sano)[1] represents one of the 1200 species belonging to the Eriocaulaceae. *Paepalanthus* is the largest genus of this family with approximately 500 species, of which more than 400 exist only in Brazil.[2] Species of this family represent an important source of income for rural communities, especially in the Jalapão region. The coils of *Syngonanthus nitens* scapes together with buriti palm strips are used to make traditional handcrafts, acessories and other decorative items.[3]

Endophytic fungi are defined as fungi that live asymptomatically within the tissues of higher plants.[4] They are a rich source of secondary metabolites with novel structures and biomedical potential.[5] There is only one study of endophytes isolated in the Eriocaulaceae.[6] Among the endophytes that have been described so far, there are many *Fusarium* species[7] which are interesting in the production of bioactive secondary metabolites as well as a source of a variety of chemical structures.

The *Fusarium* species are able to produce mycotoxins, including the phytotoxic fusaric acid, which may be a component in the pathogenicity of some biotypes of the *Fusarium*.[7] Contrarily, the class of chemical compounds called auxin, which are phytohormones and promoters of plant growth can be produced by fungal endophytes as well, for example the *Fusarium tricinctum*.[8]

Herein, we report the antimicrobial screening used as a tool to select the endophytic fungus of *P. chiquitensis*. The investigation of the EtOAc extract resulted in the isolation and structure elucidation of four metabolites produced by *F. fujikuroi*: fusaric acid (**1**), indole acetic acid (**2**), 2-(4-butylpicolinamide) acetic acid (**3**) and the sesterterpene terpestacin (**4**). Furthermore, the antibacterial and antifungal activities of the EtOAc extract and major isolated compounds (**1**) and (**2**) have been described.

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Experimental

General experimental procedures

Infrared (IR) spectrum was obtained using a Thermo Scientific Nicolete iS5 FT-IR 17 spectrometer. Circular dichroism (CD) J-815 of Jasco. The nuclear magnetic resonance (NMR) analyses ^{1}D and ^{2}D experiments of the secondary metabolites were obtained on Bruker FOURIER 300 MHz and on Bruker AVANCE III 600 MHz (Bruker, Switzerland) spectrometers using the non-deuterated residual solvent signal as a reference. The high resolution mass spectra were recorded on a Q-TOF Bruker MaXis Impact™ mass spectrometer with a direct insertion device in the sample-injection analysis continuous flow of 3.0 μL min^{-1}. The samples were solubilized in MeOH:H_2O (1:1, v/v) and were ionized by electrospray (ESI) in negative or positive mode. The analyses of the tandem mass spectrometry (MS/MS) were performed by a Mass Spectrometer 3200 QTrap LC/MS/MS (linear ion trap quadrupole mass spectrometer), AB Sciex Instruments operating in a positive mode and turbo ion spray ionization. The analyses were carried out by direct infusion using an automatic serynge in a 10.0 μL min^{-1} flow at 0.6 ppm in MeOH:H_2O (1:1, v/v, containing 0.1% formic acid). The spectra were obtained in the following conditions: ionspray: 5500 V; gas 1:17 psi; declustering potential: 25 V; entrance potential: 10 V and collision energy: 35 V.

Thin layer chromatography (TLC) analyses were performed using a Sorbent Technologies silica gel 60. Spots on the TLC plates were visualized under UV light and by being sprayed with an anisaldehyde - H_2SO_4 reagent followed by heating at 130 °C. The chromatographic column was performed on Sephadex LH-20 (Pharmacia Biotech, Sweden).

Analytical high performace liquid chromatography (HPLC) was performed on a Jasco (Tokyo, Japan) equipped with a photodiode array (PDA) detector. The analytical column used was the Phenomenex Luna (2) RP18 (250.0 × 4.6 mm i.d.; 5 μm). Semi-preparative HPLC was performed on a Jasco (Tokyo, Japan) equipped with a MD-2010 PDA detector, using a Phenomenex Luna (2) RP18 column (250 × 10 mm i.d.; 10 μm), at a flow rate of 3.0 mL min^{-1}. The HPLC-grade acetonitrile was purchased from JT Baker (Baker Mallinckrodt, Phillipsburg, NJ, USA). HPLC-grade water was prepared with a Millipore (Bedford, MA, USA) Milli-Q purification system.

Plant material

The aerial parts of *P. chiquitensis* (capitulae, scapes, and leaves) were collected in February, 2012, in Serra do Cipó, in the Minas Gerais State, Brazil, geographical coordinates of 19°14'58.92''S, 43°31'04.40''W, and authenticated by Prof Dr Paulo Takeo Sano from Universidade de São Paulo (USP), Brazil. A voucher specimen (3402 SPF) was deposited at the Herbarium of the IB-USP.

Fungal isolation and identification

The endophytic fungi were isolated from healthy aerial parts of *P. chiquitensis* which were subjected to surface sterilization. The capitulae, scapes, and leaves were first washed with water and soap and immersed in a 70% aqueous ethanol (EtOH) for 1 min, 1% aqueous sodium hypochlorite solution for 2 min and 70% aqueous EtOH for 1 min. Finally, the vegetal material was immersed in sterile H_2O for 2 min. The sterilized material was cut into 2 × 2 cm pieces and deposited onto a Petri dish containing PDA (potato dextrose agar) and gentamicin sulfate (100 μg mL^{-1}). Single fungal strains were obtained following serial transfers on PDA plates and then deposited at the NuBBE fungi collection in Araraquara, Brazil (stored in sterile water at 25 °C).[9,10]

Fungal identification was carried out through the DNA extracted and the internal transcribed spacer (ITS) region sequence amplified. The sequence data obtained from the fungal strain was deposited into GenBank. The phylogenetic tree and the phylogenetic analyses[11] were made in MEGA 6.06.

Fungal growth and extraction

The endophytic fungi isolated from aerial parts of *P. chiquitensis* grew up on a solid culture PDA for 14 days. Small pieces of a solid medium PDA from the Petri dish containing biomass were used to inoculate each fungus into three Erlenmeyer flasks (500 mL), each containing 300 mL of liquid culture PDB (potato dextrose broth). The medium was autoclaved at 125 °C for 15 min. After cooled, the medium was inoculated with the endophyte and incubated at 25 °C in static mode for 28 days. The mycelia were separated from the liquid cultures by filtration using filter paper. The filtered were extracted with EtOAc (3 × 1/3 volume filtered). The organic layers were combined and washed with distilled H_2O (3 × 1/5 volume filtered), the remaining water was removed by the drying agent $MgSO_4$, and the solid was removed by filtration. The solvent was removed under reduced pressure yielding twenty-five EtOAc extracts. Besides, the *F. fujikuroi* grew at a large-scale into 21 Erlenmeyer flasks (500 mL), resulting in the EtOAc extract used for the isolation of the metabolites by chromatographic techniques.

Fractionation and isolation

The EtOAc extract (680 mg) obtained from a large-scale culture of *F. fujikuroi* was dissolved in 100% MeOH (3 mL) and twice centrifuged for 8 min at 3500 rpm. The combined supernatants were fractionated on a Sephadex LH-20 (Pharmacia) column (85.0 × 2.5 cm) and eluted with 100% MeOH affording 78 fractions (10 mL each) which were analyzed by silica gel TLC eluted with ($CHCl_3$/MeOH/*n*-PrOH/H_2O, 5:6:1:4, v/v/v/v, organic phase). The spots were visualized under UV light at 254 nm and anisaldehyde-H_2SO_4.

The fractions of the Sephadex LH-20 Fr A (74 mg), Fr B (144 mg) and E (124 mg) were separated by semi-preparative HPLC-PDA Jasco PU 2086 series pump systems equipped with a Jasco MD-2010 PDA detector and Chromnav software, using a Rheodyne 500 µL manual injector loop, at a flow rate of 3.0 mL min^{-1}. The mobile phase consisted of H_2O (eluent A) and acetonitrile (eluent B), both containing 0.01% TFA (trifluoracetic acid). The isocratic mode was used to purify the fraction Fr. E (38% B for 30 min, λ 270 nm) yielding the pure compound (**2**) (Rt = 21.93 min, 24.0 mg). The gradient mode was used to purify the fraction Fr. A (10-100% B for 30 min, λ 270 nm) affording the compound (**1**) (Rt = 15.93 min, 12.0 mg); the fraction B (38-100% B for 30 min, λ 270 nm) yielding the compounds (**3**) (Rt = 26.80 min, 1.0 mg) and (**4**) (Rt = 35.80 min, 3.0 mg).

All the substances isolated were analyzed by analytical HPLC performed on a Jasco (Tokyo, Japan) equipped with a PU-2089 quaternary solvent pump with degasser, a MD-2010 DAD detector, and a Rheodyne AS-2055 sample injector with a 100 µL sample loop. The analytical column was a Luna (2) RP18 (Phenomenex, Luna (2), 250.0 × 4.6 mm i.d.; 5 µm) equipped with a Phenomenex security guard column (4.0 × 3.0 mm i.d.). The composition of mobile phases was H_2O (eluent A) and ACN (eluent B), both solvents containing 0.01% TFA (exploratory gradient run, 60 min, λ 270 nm).

Antibacterial activity and minimum bactericidal concentration (MBC)

The evaluation of the antibacterial activity and the minimal inhibitory concentration (MIC) was determined by the broth microdilution method, as described in the M7-A6 reference guideline of the Clinical and Laboratory Standards Institute.[12] The biological activity was evaluated against *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923) and *S. setubal* (ATCC 19196). They were incubated in Muller-Hinton broth (MHB) for 24 h at 37 °C. These inoculums were standardized at 1.0×10^8 CFU mL^{-1} (corresponding to 0.5 McFarland standards) by adjusting the optical density to 0.10-0.15 at 620 nm.

The assay was carried out in 96-well microplates containing 80 µL of MHB. The extracts and the substances **1** and **2** were dissolved in DMSO:H_2O (2:8, v/v) to initial concentration of 2000 µg mL^{-1}. A two-fold serial dilution was made in order to obtain concentration ranges of 7.8-1000 µg mL^{-1}. As positive control ampicillin was used and DMSO:H_2O (2:8, v/v) as negative control. The plates were incubated at 37 °C for 24 h. The assay was displayed in triplicate. The MIC of the samples was detected after the addition of 30 µL of the resazurin solution (100 µg mL^{-1}), incubated at 37 °C for 2 h. The growth of bacteria changes the blue dye resazurin into a pink color. The pink color indicates positive growth, whereas the blue indicates growth inhibition.

The MIC was defined as the lowest sample concentration which prevented this change and exhibited inhibition of microorganism growth. For the determination of minimum bactericidal concentration (MBC), a portion from each well that showed antibacterial activity was plated on Muller-Hinton agar and incubated at 37 °C for 24 h. The lowest concentration that showed no bacteria growth in the subcultures was used as the MBC.[13]

Antifungal activity and minimum fungicidal concentration (MFC)

The evaluation of the antifungal activity and the MIC were determined by the broth microdilution method, as described in the M27-A3 reference guideline of the Clinical and Laboratory Standards Institute,[14] with modifications.[15] The biological activity was evaluated against the fluconazole-resistant *C. albicans* (ATCC 10231). The yeast strain was incubated in 100 µL of RPMI 1640 (adjusted to pH 7.0 with 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer, 0.165 mol L^{-1}) for 48 h at 37 °C. The inoculum of yeast was standardized at 5.0×10^6 CFU mL^{-1} (corresponding to 0.5 McFarland standards) by adjusting the optical density to 0.12-0.15 at 530 nm.

The assay was carried out in 96-well microplates containing 100 µL of RPMI 1640. The extracts and the substances **1** and **2** were dissolved in 20% DMSO and water to initial concentration of 2000 µg mL^{-1}. Then, a two-fold serial dilution was made in order to obtain concentration ranges of 7.8-1000 µg mL^{-1}. As positive controls were used fluconazole and amphotericin B and the DMSO:H_2O (2:8, v/v) as negative control. The plates were incubated at 37 °C for 48 h. The assay was displayed in triplicate. The MIC of the samples was detected after the addition

of 20 µL of triphenyl-tetrazolium chloride (TTC) solution (0.02 g mL^{-1}), and after that incubated at 37 °C for 2 h. Yeast growth changes the colorless TTC to a red color.

MIC was defined as the lowest sample concentration that prevented this change and exhibited inhibition of microorganism growth. For the determination of minimal fungicidal concentration (MFC), a portion from each well that showed antifungical activty was plated on Sabouroud agar and incubated at 37 °C for 48 h. The lowest concentration that demonstrate no yeast growth in the subcultures was used as the MFC.[13]

Results and Discussion

The screening for antimicrobial activity was used as a bioassay-guided strategy to select the most active among the twenty-five EtOAc extracts prepared from endophytic fungi isolated from the aerial parts of *P. chiquitensis*. The MIC was evaluated against four human pathogenic microorganisms: *S. aureus*, *E. coli*, *S. setubal* and *C. albica*ns (Table 1).

Overall, the EtOAc extracts demonstrated antimicrobial activity with an MIC value of 1000 µg mL^{-1} or above this concentration (data not displayed). However, only one EtOAc showed moderate activity against all the four tested microorganisms. The MIC values were 500 µg mL^{-1} for *E. coli* and *S. setubal*, 250 µg mL^{-1} for *S. aureus* and 1000 µg mL^{-1} for the yeast fluconazole-resistant *C. albicans*.

The literature does not provide a consensus in terms of the MIC values obtained for natural products.[16] These authors considered the MIC for plants extract with values lower than 500 µg mL^{-1} as being potent inhibitors, MIC between 600 and 1500 µg mL^{-1} to be moderate inhibitors and MIC above 1600 µg mL^{-1} to be weak inhibitors. In another literature,[17] a satisfactory MIC values equal to or less than 1000 µg mL^{-1} was established. However, Ramos *et al*.[18] considered the MIC with values below the concentration of 1000 µg mL^{-1} as being representative when investigating the antifungal potential of the methanolic extract of the scapes from *S. nitens* against ATCC and clinical trains of *Candida krusei*.

Even though the major compounds **1** and **2** do not show significant biological activities, there are currently a few articles available to describe their antimicrobial activities. Among these interesting works, we can cite one with fusaric acid **1**, in which the antimycobacterial activity of copper ion complex and cadmium ion complex of (**1**) against *M. tuberculosis* H37Rv strain is shown, and the MIC value was 10 µg mL^{-1}.[19] Besides this, in another work, the indole acetic acid complexes were described by Punitha *et al*.[20] in which the antimicrobial activity against the strains of *S. aureus* and *Aspergillum niger* (*A. niger*) is displayed using the agar well diffusion technique. On this, it was found that the metal complexes of IAA (indole acetic acid) are more active than the free ligand against both the bacterial and fungal organisms tested.

The fungal strain isolated from *P. chiquitensis* was identified and the ITS sequence showed similarity of 99.0% with the *Fusarium fujikuroi* (KJ000432.1). The phylogenetic tree is shown in Figure 1, as well as the comparison among closely related fungal strains with the *F. fujikuroi*.

The large scale cultivation of *F. fujikuroi* afforded the EtOAc extract used for the fractionation and isolation of the secondary metabolites. The major secondary metabolites (**1**) showed MIC of 250 µg mL^{-1} for all the microorganisms tested, while the compound **2** displayed MIC values of 500 µg mL^{-1} for *S. setubal*, 250 µg mL^{-1} for *E. coli* and *S. aureus*. The best MIC was for the fluconazole-resistant *C. albicans* (125 µg mL^{-1}). In addition, the results for the MBC and MFC for the EtOAc extract showed bactericidal and fungicidal activities (1000 µg mL^{-1}). The compound **2** showed MBC of 500 µg mL^{-1} for *E. coli* and 1000 µg mL^{-1} for *S. setubal*.

Table 1. Antimicrobial activity of the EtOAc extract and of the major compounds **1** and **2**

Sample	MIC (MBC)[a] / (µg mL^{-1})		MIC (MFC)[a] / (µg mL^{-1})	
	E.coli	*S. setubal*	*S. aureus*	*C. albicans*
F. fujikuroi extract	500 (1000)	500 (1000)	250 (1000)	1000 (1000)
1	250 ([a])	250 ([a])	250 ([a])	250 ([a])
2	250 (500)	500 (1000)	250 ([a])	125 (1000)
Ampicillin	6.25	12.5	0.15	NA
Amphoterecin B	NA	NA	NA	8.0
Fluconazole	NA	NA	NA	R

[a]Minimal inhibitory concentration (MIC) > 1000.0 µg mL^{-1}. MBC: minimum bactericidal concentration; MFC: minimum fungicidal concentration; NA: not applicable; R: resistant.

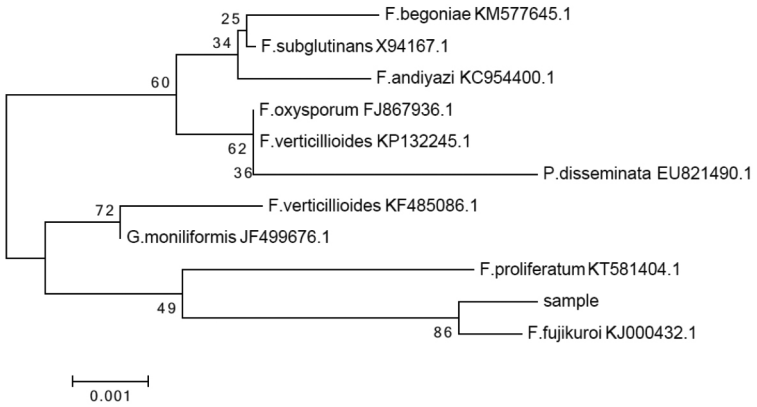

Figure 1. Phylogenetic tree of the fungus FZ04 based on the15.8S ribosomal RNA gene.

The compound **1** showed a value higher than 1000 μg mL^{-1}, indicating then bacteriostatic and fungistatic behavior of this compound for all strains tested.

Figure 2 presents the chemical structures of the isolated compounds **1**,[21] **2**,[22,23] and **4**[24] by chromatographic methods and the HPLC-PDA chromatogram of the EtOAc extract of *F. fujikuroi*.

Compound **3** was obtained as an amorphous powder. The UV spectra showed the λ_{max} in 232 and 272 nm, suggesting the presence of a pyridine ring. The ESI-QTOF-HRMS analysis exhibited an ion at *m/z* 237.1239 $[M + H]^+$ (calcd. 237.1237) evidencing the molecular formula $C_{12}H_{16}N_2O_3$. The ESI-QTrap-MS/MS spectrum showed fragmentation ions at *m/z* 191 $[M - 46 + H]^+$, *m/z* 180 $[M - 57 + H]^+$, *m/z* 162 $[M - 57 - 18 + H]^+$, *m/z* 134 $[M - 57 - 18 - 28 + H]^+$. The fragmentation scheme proposed to the compound **3** is shown in Figure 3.

The ^{1}H NMR spectrum showed three hydrogen aromatic signals at δ 8.37 (brs, H-3), 8.05 (brs, H-6) and 7.60 (brs, H-5). It is also evident the signals of methyl group at δ 0.92 (t, H-10). Furthermore, four methylene hydrogens were observed at δ 4.09 (H-4), 2.65 (H-7), 1.59 (H-8) and 1.34 (H-9). A broad singlet was observed at δ 8.50 (N-1) as well, suggesting the presence of a hydrogen bonded with nitrogen (Table 2). In the heteronuclear single quantum correlation (HSQC)-^{15}N-^{1}H NMR was confirmed the nitrogen value at δ 100.

The ^{1}H-^{1}H COSY (correlation spectroscopy) spectrum showed correlation between the aromatic hydrogens at δ 8.05 (H-6) and at 7.60 (H-5). The spectrum showed the coupling of methylene hydrogens of the aliphatic side-chain at δ (2.65 ↔ 1.59 ↔ 1.34 ↔ 0.92) and the H-4' at δ 4.09 and the hydrogen bonded with nitrogen H-3' at δ 8.50.

The ^{13}C and HSQC NMR data of **3** confirmed the presence of 12 carbons corresponding to 5 aromatic carbons, including two quaternary sp^2 carbons (C-2, C-4), four methylene (C-4', C-7, C-8, C-9), one nitrogen bonded to hydrogen (N-3') and two carbonyl groups (C-2', C-5').

The heteronuclear multiple bond correlations (HMBC) from H-7 to C-3 and C-5 connected the aliphatic side chain to the pyridine ring C-4.

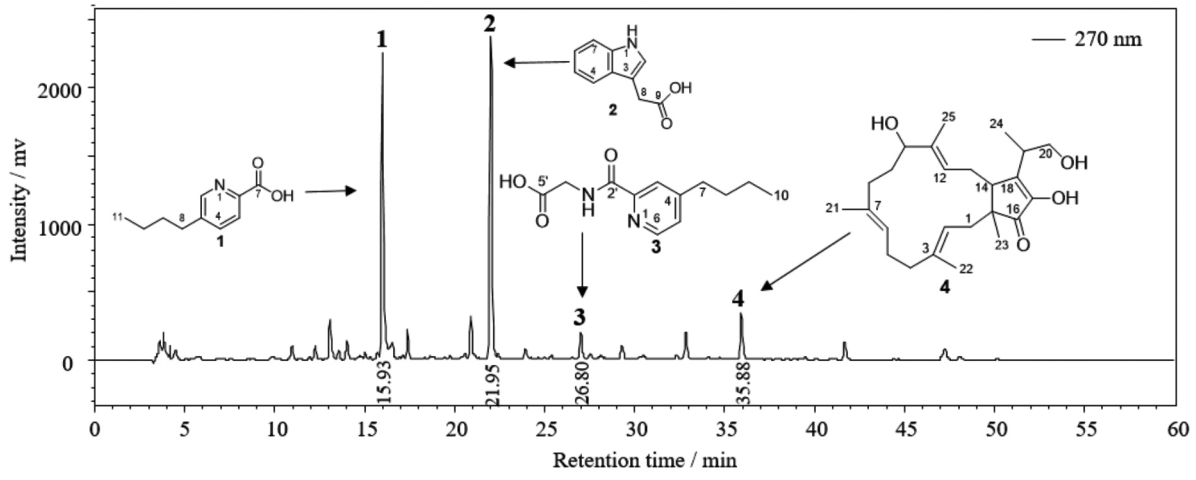

Figure 2. HPLC-PDA chromatogram recorded at 270 nm of the EtOAc extract of the *F. jujikuroi* and related isolated metabolites **1**-**4**.

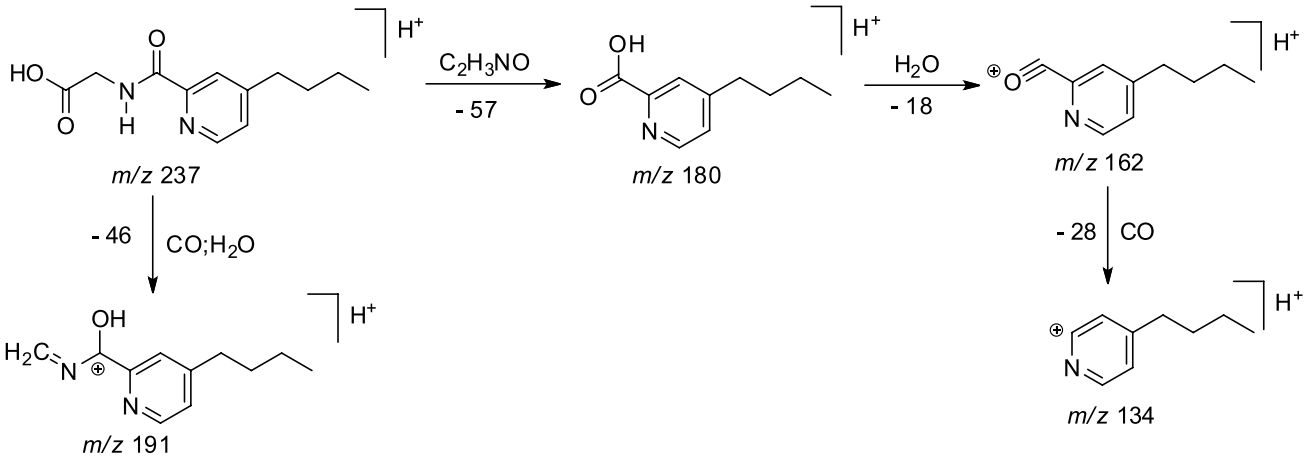

Figure 3. The fragmentation scheme proposed to the compound **3** by ESI-QTrap-MS/MS.

Table 2. ^{1}H and ^{13}C NMR data of compound (**3**) in $CDCl_3$ (δ in ppm at 600 and 150 MHz, respectively)

Position	3	
	δ_H (multiplicity, *J* in Hz)	δ_C
N-1	–	–
2	–	141.8 (Cq)
3	8.37 (brs)	148.7 (CH)
4	–	147.0 (Cq)
5	7.60 (brs)	137.2 (CH)
6	8.05 (brs)	122.3 (CH)
7	2.65 (brs)	33.2 (CH_2)
8	1.59 (brs)	33.1 (CH_2)
9	1.34 (brs)	22.3 (CH_2)
10	0.92 (t, 7.2)	14.0 (CH_3)
2'	–	166.5 (Cq)
N-3'	8.50 (brs)	–
4'	4.09 (s)	41.9 (CH_2)
5'	–	165.5 (Cq)

δ: chemical shift; *J*: coupling constant.

The fusaric acid (**1**) is biosynthesized from acetate units and aspartate, which is a derivative of the picolinic acid (2-pyridine carboxylic acid).[25] The glycine (amino acetic acid) has the molecular formula NH_2CH_2COOH and is the simplest amino acid, optically inactive and exist as a zwitterion in solution.[26]

The chemical structure of the compound **3** can be the combined product of the junction of the 4-butyl-picolinic acid with the glycine resulting in its acidic and basic caractheristics present.

The IR spectrum of the compound **3** displayed the stretching vibrations of carboxylic anion, ranging from 1545-1362 cm^{-1} and 1407-1362 cm^{-1}. It exists as a dipolar ion in which carboxyl group is present as a carboxylate ion. Because this compound does not show cotton effect in the analysis by circular dichroism (CD), we suggested that the chemical structure of the glicine is bonded to 2-hydroxyoxazolidinone. Finally, the data confirmed that **3** is the 2-(4-butylpicolinamide) acetic acid (Figure 4).

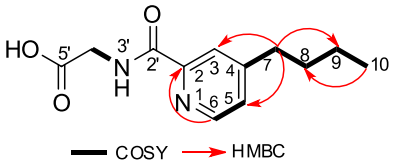

Figure 4. ^{1}H-^{1}H COSY and key HMBC correlations of **3**.

Conclusions

The screening for antimicrobial activity against four human microbial pathogenic strains led to the isolation and identification of four secondary metabolites. The alkaloid 2-(4-butylpicolinamide) acetic acid (**3**) is reported for the first time in the literature. These findings also showed the isolation and determination of the known compounds fusaric acid (**1**) and the auxin indole acetic acid (**2**). The other known compound isolated was a sesterterpene known as terpestacin (**4**). The EtOAc extract and the compounds **1** and **2** displayed moderate antimicrobial activity for all the bacterial strains evaluated. Furthermore, the compound **2** showed activity against the fluconazole-resistant *C. albicans*. This is the first ever report of endophytic fungi isolated from *P. chiquitensis* (Eriocaulaceae) and their antimicrobial activity.

Supplementary Information

Supplementary information (^{1}H and ^{13}C NMR and MS spectra for the isolated compounds **1**-**4**) is available free of charge at http://jbcs.sbq.org.br as PDF file.

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