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Article

Aromatic Polyketides and Macrolides from *Microsphaeropsis arundinis*

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The endophytic fungus *Microsphaeropsis arundinis* was isolated from the capitula of the tropical plant *Paepalanthus planifolius* (Eriocaulaceae). The fungus was cultivated in seven different media, and the ethyl acetate extracts were tested against tumor cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay. The culture was scaled up using parboiled rice medium, and the crude extract was purified by classical chromatographic analyses, leading to the isolation of seven compounds. The seven compounds isolated from the culture media included a new aromatic polyketide (**1**) and other known metabolites. The structures of all the compounds were elucidated by 1D and 2D nuclear magnetic resonance (NMR) and high resolution mass spectrometry (HRMS) analyses. An evaluation of the cytotoxicity of all the isolated compounds showed that these compounds exhibit less activity in comparison with the extract cultured in parboiled rice.

Keywords: aromatic polyketides, macrolides, *Microsphaeropsis arundinis*, *Paepalanthus planifolius*

Introduction

Endophytic fungi are known to be ubiquitous in the plant kingdom and represent an interesting source of metabolites with biological potential which have not been explored to date.[1] The fungi often colonize internal organs of plants and tend to produce bioactive secondary metabolites in their symbiotic association. These metabolites are found to help plants in their growth, development and adaptation.[2] According to some authors, there are some criteria involving the selection of host plants for the isolation of endophytic fungi. According to Strobel and Daisy,[3] from the chemical and biological point of view, the plant is required to be capable of adapting easily to the environment and must grow in regions of high biodiversity or be endemic. The Eriocaulaceae species are found to be endowed with these properties; these species grow in the Espinhaço range, a Brazilian mountain chain that extends from Minas Gerais to Bahia.[4]

Eriocaulaceae species are a family of herbaceous monocots, which are typically present in tropical regions. *Paepalanthus* is the largest genus in this family of 380 species known to be heavily concentrated in Brazilian Campos Rupestres, where most of them are found to be endemic.[4] The *Paepalanthus* genera are composed of different plant species; these include the *Paepalanthus planifolius* (Bong.) Körn, which has already been reported in the literature. It is noteworthy that the authors of the present work have previously described the presence of naphytopyranones in the *Paepalanthus planifolius* plant species. The naphytopyranones have been shown to possess biological potential; these include planifolin and planifoliusin A. Planifolin is found to exhibit cytotoxic activity, while planifoliusin A is endowed with antimicrobial activity.[5,6]

By virtue of the biological properties of the compounds present in this plant species, an endophytic fungus from *Paepalanthus planifolius* (host plant) was selected for chemical and biological studies. *Microsphaeropsis arundinis* is related to the class of Coelomycetes fungi. According to the literature, this fungus is associated with rare infections of the skin and tissues of immunocompromised hosts.[7] Studies in the literature have reported the presence of sesquiterpenes (endowed with antifungal activity),[8] preussomerins (endowed with cytotoxic potential),[9]

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diketopiperazines (endowed with antibiotic activity),[10] and benzophenone dimers (endowed with antibacterial potential)[11] in the *Microsphaeropsis* genus.

Considering the properties of the compounds present in the *Microsphaeropsis* genus, cultivating the endophyte in different media and screening the extracts for the presence of biological activity is a good way to evaluate the metabolic production of *Microsphaeropsis arundinis* in search of biologically active compounds. In the present work, the endophytic fungus *M. arundinis* was cultivated in different media, and the crude extracts were tested against tumor cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay in order to select the most active extract. Based on the results, a culture of the strain was scaled up using parboiled rice medium. By chromatographic purification, compounds showing the metabolic production of the strain were isolated.

The fermentation of the strain along with the isolation and identification of the compounds are discussed in this work.

Results and Discussion

The endophytic fungus *M. arundinis* was cultivated in seven different culture media and the ethyl acetate extracts were tested against tumor cell lines using MTT assay (Table 1).[12] Afterwards, a culture of the strain was scaled up using parboiled rice medium. This culture was chosen because it presented the lowest half-maximal inhibitory concentration (IC_{50}) value against murine breast adenocarcinoma (LM3).

In order to isolate the active compounds, the extract was scaled up (3.0 g) and purified using the semi-preparative high performance liquid chromatography coupled to a photodiode array detector (HPLC-PDA) and performance liquid chromatography coupled to a refractive index detector (HPLC-RI) techniques. Based on this approach, seven compounds were successfully isolated (Figure 1). The isolated compounds included one aromatic polyketide, named (*R*)-1'-(2,5-dihydroxyphenyl)-1'-oxobutan-3'-yl acetate (**1**); this compound has not been described in the literature. The known compounds isolated included one polyketide, named (*R*)-1-(2,5-dihydroxyphenyl)-3-hydroxybutanone (**2**)[13] (this compound has recently been described as natural product) and another compound, named 1-(2,5-dihydroxyphenyl)-2-buten-1-one (**3**) (this has already been associated with *M. arundinis)*.[14] The present study also reports the isolation of macrolides - named modiolide D (**4**)[13] and modiolide E (**5**),[13] derived from *Paraconiothyrium* sp. and recently described in the literature. The other compounds isolated are modiolide A (**6**) (this has been previously reported in the literature),[15] which is related to marine-derived fungus *Paraphaeosphaeria* sp., and chromanone, named (*R*)-6-hydroxy-2-methyl-4-chromanone (**7**).[14]

Compound **1** was isolated as an optically active, yellow amorphous solid with high resolution mass spectrometry (HRMS) signal consistent with the molecular formula $C_{12}H_{14}O_5$; this is related to the presence of the peak $[M + Na]^+$. The analysis of ^1H nuclear magnetic resonance (NMR) spectrum (Figure S11, Supplementary Information (SI) section) showed the presence of aromatic hydrogens in H-3 (δ_H 6.80, *J* 8.9), H-4 (δ_H 7.02, *J* 8.9 and 2.9) and H-6 (δ_H 7.23, *J* 2.9), which resulted in a trisubstituted ring. The ^{13}C NMR data (Figure S12, SI section) indicated the presence of eight sp^2 carbons, including a carbonyl group in C-1', an ester group in C-1'' (δ_C 172.3), a methylene group in C-2' (δ_C 45.3), and six aromatic carbons. Furthermore, four sp^3 carbons were also found to be present; these included two methyl groups in C-4' (δ_C 20.3) and C-2'' (δ_C 21.1) with different multiplicity.

Table 1. Cytotoxic assays with the fungal extracts of *M. arundinis* cultivated in different media tested against three tumor cell lines

Fungal culture media	$IC_{50} \pm SD$ / (μg mL^{-1})		
	LM3	LP07	MCF-7
Potato dextrose broth	108.15 ± 1.36	266.12 ± 2.36	170.10 ± 5.56
Yeast malt (YM) broth	82.47 ± 7.67	793.84 ± 19.58	89.99 ± 23.20
Malt extract	63.21 ± 2.39	93.66 ± 6.33	66.60 ± 2.54
Nutrient broth	496.90 ± 9.24	> 1000	428.96 ± 34.40
Czapek-Dox broth	444.88 ± 52.37	> 1000	559.65 ± 39.72
Rice	39.91 ± 9.03	155.88 ± 3.28	51.93 ± 0.60
Corn	118.66 ± 7.62	180.37 ± 3.69	96.03 ± 14.38
Cisplatin (reference compound)	9.09 ± 1.11	1.30 ± 0.12	5.88 ± 1.38

IC_{50}: half-maximal inhibitory concentration; SD: standard deviation.

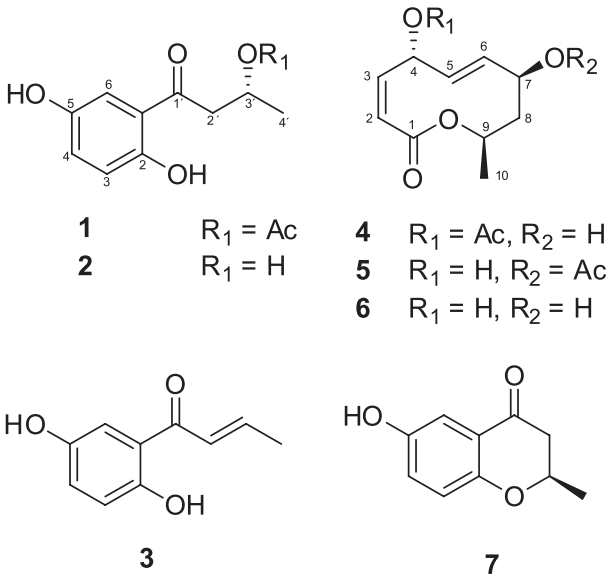

Figure 1. Compounds isolated from *Microsphaeropsis arundinis*.

The bidimensional NMR analyses (Figures S13-S14, SI section) showed that the heteronuclear single-quantum correlation (HSQC) spectra contained diastereotopic hydrogens H-2'a (δ_H 3.16) and H-2'b (δ_H 3.40) in the methylene group (δ_C 45.3). Heteronuclear multiple bond correlation (HMBC) data were found to be useful for the structure determination through the correlation of H-6 (δ_H 7.23), H-2' (δ_H 3.16 and 3.40) and H-3' (δ_H 7.23) with the carbonyl group in C-1'. The hydrogens related to the methyl group H-2'' (δ_H 1.97) only showed correlation with C-1''; these hydrogens were represented as a simplet, indicating their position at the extremity of the ester group. The ^{1}H and ^{13}C NMR data can be found in Table 2; and relevant bidimensional correlations are presented in Figure 2.

Other polyketides have also been isolated in this work. These compounds present the same trisubstituted aromatic ring found in the previous compound but they do not possess the acetyl group in C-3'. The acetyl group is substituted by a hydroxyl group in compound **2** and one alkene in compound **3** (Figures S17-S28, SI section). Based on the comparison of the optical rotation data obtained in this work with those reported in the literature,[13] the absolute configuration of compound **2** in C-3' was defined as *R*.

The configuration of C-3' in compound **1** was determined based on the comparison of the ECD data of compound **2**. Analyses of ECD spectra of compounds **1** and **2** displayed similar cotton effects (CE), a negative CE at 330 nm and a positive CE at 380 nm due to n-π^* transitions (Figure S16, SI section). In view of that, the absolute configuration of C-3' in compound **1** was assigned as *R*; the compound is a new natural product named (*R*)-1'-(2,5-dihydroxyphenyl)-1'-oxobutan-3'-yl acetate.

Table 2. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for compound **1** (in CD_3OD)

No.	**1**	
	δ_C / ppm, type	δ_H / ppm, mult. (*J* / Hz)
1	120.7, C	–
2	156.6, C	–
3	119.7, CH	6.80 d (8.9)
4	126.0, CH	7.02 dd (8.9, 2.9)
5	150.7, C	–
6	115.7, CH	7.23 d (2.9)
1'	204.5, C	–
2'a	45.3, CH_2	3.16 dd (16.7, 5.0)
2'b	45.3, CH_2	3.40 dd (16.7, 7.6)
3'	68.6, CH	5.43 dqd (7.6, 6.3, 5.0)
4'	20.3, CH_3	1.34 d (6.3)
1''	172.3, C	–
2''	21.1, CH_3	1.97 s

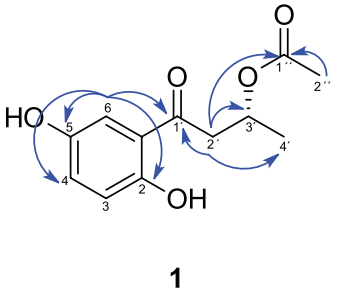

Figure 2. Selected HMBC (↷) correlations for compound **1**.

Another class of metabolites isolated from *M. arundinis* rice extract was ten-membered macrolides named modiolides. With the aid of HRMS, NMR and nuclear Overhauser effect spectroscopy (NOESY) 1D data, the structures of compounds **4**-**6** were confirmed as modiolide D (**4**),[13] modiolide E (**5**)[13] and modiolide A (**6**).[15]

Based on the HRMS, NMR, electronic circular dichroism (ECD) and optical rotation data, the structure of compound **7** was confirmed as (*R*)-6-hydroxy-2-methyl-chromanone.[16]

The compounds obtained in the present work are different from flavonoids and naphthopyranones - which are found in the host plant (*P. planifolius*). In addition, the compounds reported here also differ from chromanones obtained from other endophytic fungi of the host;[17] this shows that the cultivation of the fungus in different culture media contributed to the diversity of the metabolic production.

Data related to studies reported in the literature show that compounds **2** and **3** have anti-inflammatory activity.[13] However, no data have been reported regarding

their cytotoxic activity. By virtue of that, the isolated compounds were tested against adenocarcinoma cell lines, which included murine breast (LM3), murine lung (LP07) and human breast (MCF-7). The compounds showed relatively weaker activity in comparison with the positive control (doxorubicin). The best activities were found in compounds **2** and **3**, which recorded IC_{50} values against LM3 of 36.83 ± 4.86 μg mL^{-1} and MCF-7 of 33.95 ± 3.62 μg mL^{-1}, respectively (Table S1, SI section).

Biosynthetically, these compounds were obtained via the acetate pathway. The condensation of units of acetyl-CoA and malonyl-CoA led to the generation of a reactive poly-β-keto chain.[18] The polyketides presented here were derived from phloroacetophenone. After the production of the poly-β-keto chain, reduction and oxidation reactions led to the generation of compound **2**, while cyclization reaction yielded compound **7**. Acetylation and dehydration reactions led to the production of compounds **1** and **3**, respectively (Figure S63, SI section).[18] For the production of the ten-membered macrolides, units of acetyl-CoA and malonyl-CoA were condensed. The condensation of units of acetyl-CoA and malonyl-CoA yielded a series of reactions, including dehydrations, oxidation and acetylation, which gave rise to the production of compounds **4**-**6** (Figure S64, SI section).[18]

Conclusions

The findings of this work show that the endophytic fungus *Microsphaeropsis arundinis* is incapable of producing naphytopyranones - which represent the major class of metabolites from the host plant *P. planifolius* and are responsible for the relevant cytotoxic activity. Nonetheless, the endophyte (*Microsphaeropsis arundinis)* is found to produce polyketides and macrolides, which are normally found in the *Microsphaeropsis* genus and in other endophytes known to be characterized by anti-inflammatory activity. The present study evaluated the isolated compounds in cytotoxic assays in order to compare their activity in the host environment. The compounds exhibited weak cytotoxicity against murine breast (LM3), murine lung (LP07) and human breast (MCF-7) adenocarcinoma cell lines.

Experimental

General experimental procedures

The compounds were structurally identified by NMR and HRMS analysis. Monodimensional ^{1}H NMR (600 MHz), ^{13}C NMR (150 MHz) and two-dimensional HMBC, HSQC, correlation spectroscopy (COSY), NOESY 1D and distortionless enhancement by polarization transfer (DEPT-135) experiments were performed using Bruker Avance III spectrometer. Chemical shifts (δ) were referenced to CD_3OD at 3.31 for ^{1}H and 49.0 for ^{13}C. Mass spectra were measured with Q-TOF Bruker MaXis Impact using methanol as the eluent. Optical rotation values were measured using PerkinElmer 341-LC polarimeter at the sodium D line (λ = 589 nm). Electronic circular dichroism (ECD) spectra of compounds **1** and **2** were obtained from a Jasco CD-2095 Plus chiral detector using cells of 25 mm path length, and were recorded from the range of 220-420 nm with 0.1 nm data pitch. The ECD spectra of compound **7** were obtained from a spectropolarimeter Jasco J-815 model using cells of 1 cm path length. The scans were recorded from 200-400 nm with 0.2 nm data pitch and 3 accumulations. All the extracts produced by the strain were analyzed through their chromatographic profile in a HPLC system (LC-2000, Jasco), which consisted of a quaternary pump system (PU-2086, Jasco) and automatic injector, coupled to a photodiode array detector (MD-2018) and an electronic circular dichroism chiral detector (CD-2095 Plus). The fingerprint analysis (Figures S2-S8, SI section) was carried out with the aid of an analytical reverse phase (RP) column (Eurospher II 100-5 C_{18} 250.0 × 4.6 mm internal diameter (i.d.), 5 μm, Knauer) using a gradient elution ranging from H_2O:MeOH (95:5 v/v) to H_2O:MeOH (0:100 v/v) in 60 min under a flow of 1.0 mL min^{-1}. All the chromatograms presented in the figures were recorded at 254 nm. HPLC analysis and purification of compounds **1**-**3** and **7** were performed using a HPLC system (LC-2000, Jasco), which consisted of a binary pump system (PU-2086, Jasco) and manual injector, coupled to a photodiode array detector (MD-2010, Jasco). The separation of the compounds was carried out with the aid of a semipreparative RP column (Luna C_{18} 250.0 × 10.0 mm i.d., 5 μm, Phenomenex) using a gradient elution ranging from H_2O:MeOH (57:43 v/v) to H_2O:MeOH (44:56 v/v) in 30 min under a flow of 4.0 mL min^{-1}. The purification of non-chromophoric compounds (**4-6**) was carried out using a HPLC system (Azura, Knauer), which consisted of a binary isocratic pump (P 6.1L, Knauer) and an assistant system (ASM 2.1, Knauer), coupled to a refractive index detector (Smartline RI Detector 2300, Knauer). The purification was performed using a semipreparative RP column (Luna C_{18} 250.0 × 10.0 mm i.d., 5 μm, Phenomenex) through an isocratic methodology with H_2O:MeOH (55:45 v/v) and 4.0 mL min^{-1} flow. The software programs: ChromNav, ClarityChrom and Spectra Manager, were used to control the system, data collection,

and processing. All solvents used for performing the HPLC analyses were purchased from Sigma-Aldrich and Tedia.

Plant material

Paepalanthus planifolius (Bong.) Körn (Eriocaulaceae) capitula were collected in Serra do Cipó, Minas Gerais, Brazil (19º13'21.64" S, 43º30'04.06" W) in October 2013; they were identified by Prof Paulo Takeo Sano. A voucher specimen (Sano 4979) of the plant material has been deposited at the Institute of Biosciences, University of São Paulo (USP), Brazil.

Fungal material and identification

The isolation of the endophytic fungi from the host plant *P. planifolius* was performed in line with reports in the literature.[17] The company named Genotyping Biotecnologia, located in Botucatu, São Paulo, Brazil, identified the selected strain as *Microsphaeropsis arundinis* through automatic sequencing with the aid of capillary electrophoresis equipment ABI 3500 Genetic Analyzer (Applied Biosystems). Subsequently, the alignment of the generated nucleotide sequences was conducted through comparison with reference sequences in internal transcribed spacer region deposited in GenBank (Acess No. KJ774055.1). The sequencing data are presented in Figure S1, SI section. The fungal material was preserved in slants. The strain of the endophyte was deposited at the Núcleo de Bioensaios, Biossíntese e Ecofisiologia de Produtos Naturais (NuBBE) fungi collection in Araraquara, Brazil (stored in sterile water at 25 °C).

Fermentation of the strain

The endophytic fungus *M. arundinis* was cultivated in five different liquid media, which included potato dextrose broth (Acumedia), yeast malt (YM) media (Acumedia), malt extract (Acumedia), nutrient broth (Acumedia) and Czapek-Dox broth (Kasir), and in solid media: parboiled rice and corn. For the experiments in liquid media, three Erlenmeyer flasks (500 mL) were used, each containing 300 mL of the liquid medium in water. For the experiments in solid media, 90 g of the solid medium were used for the culture. The flasks were previously autoclaved at 121 ºC for 20 min. After cooling, the endophyte was incubated at 25 ºC in static mode for 28 days for the liquid media and for 21 days for the solid media. For the flasks containing the endophyte incubated in liquid media, vacuum filtration was used to separate the medium from the mycelial biomass. Thereafter, a liquid-liquid partition of the aqueous media with ethyl acetate and the subsequent evaporation of the solvent yielded the EtOAc extracts used in subsequent steps.

The flasks containing the endophyte incubated in solid media were filtered in order to remove the mycelial biomass; and portions of ethyl acetate were added to the solid content. The EtOAc extracts were obtained in the same way as obtained via the liquid media. The EtOAc extracts were dried, resuspended in acetonitrile (ACN), and partitioned with hexane (HEX) to remove apolar compounds from the media. The ACN extracts were used for analyses in subsequent steps. The extracts were tested to evaluate their cytotoxic potential; and the most active of these extracts was cultivated in large scale using 40 Erlenmeyer flasks.

Isolation of the metabolites

The extract from the culture in parboiled rice (3.0 g) obtained from large scale culture was solubilized in H_2O:MeOH (1:1 v/v) using ultrasound. The extract was then purified by the semipreparative HPLC-PDA technique; this led to the isolation of the compounds identified as **1** (2.5 mg, t_R = 16.7 min), **2** (26 mg, t_R = 8.9 min), **3** (4.2 mg, t_R = 18.9 min) and **7** (5.9 mg, t_R = 12.6 min). See chromatographic conditions in general experimental procedures.

The compounds without chromophore group were also isolated by the HPLC-RI technique and identified as the macrolides **4** (9.7 mg, t_R = 10.9 min), **5** (2.7 mg, t_R = 21.8 min), and **6** (5.4 mg, t_R = 28.8 min). See chromatographic conditions in general experimental procedures.

The chromatograms obtained in the separation process are presented along with the retention time of each compound in Figures S9-S10 (SI section).

Cytotoxic assay

The cytotoxic activity of the extracts and the isolated compounds **1**-**7** was evaluated against adenocarcinoma cell lines (LM3, LP07 and MCF-7) with cells seeded at 5×10^4 cells mL^{-1} in Roswell Park Memorial Institute (RPMI) 1640-C with 10% fetal bovine serum (FBS) and 1% antibiotic to obtain the cell solution. A concentration of 100 µL of this solution was poured into a 96-well plate. After 24 h of incubation, 100 µL of the extracts (1000-7.8 µg mL^{-1}) and isolated compounds (250-1.95 µg mL^{-1}) were added to the content and incubated at 37 ºC in an atmosphere of 10% CO_2. After 24h, the cell growth and viability were measured using MTT assay as previously reported in the literature.[12] The analyses were carried out with number of

replicates (n) = 3. Cytotoxicity was calculated as follows: cell inhibition (%) = [Absorbance$_{540–620}$ (control) – Absorbance$_{540–620}$ (treated)]/Absorbance$_{540–620}$ (control) × 100 %. Dose-response regressions were used to calculate the IC_{50} values with 95 % confidence interval.

Spectral data for compound **1**

(*R*)-1'-(2,5-Dihydroxyphenyl)-1'-oxobutan-3'-yl acetate (**1**)

Yellow, amorphous solid; $[\alpha]_D^{26}$ +2.0 (*c* 0.2, MeOH); UV (MeOH) λ_{max} / nm 223, 259, 361; ^{1}H NMR (600 MHz, CD_3OD) δ 1.34 (d, 3H, *J* 6.3, CH_3), 1.97 (s, 3H, CH_3), 3.16 (dd, 1H, *J* 5.0, 16.7, CH_2), 3.40 (dd, 1H, *J* 7.6, 16.7, CH_2), 5.43 (dqd, 1H, *J* 5.0, 6.3, 7.6, CH), 6.80 (d, 1H, *J* 8.9, CH), 7.02 (dd, 1H, *J* 2.9, 8.9, CH), 7.23 (d, 1H, *J* 2.9, CH); ^{13}C NMR (150 MHz, CD_3OD) δ 20.3, 21.1, 45.3, 68.6, 115.7, 119.7, 120.7, 126.0, 150.7, 156.6, 172.3, 150.7; (+)-QTOF HRMS *m/z*, calculated for $C_{12}H_{14}O_5$ $[M + Na]^+$: 261.0733, found: 261.0734 (mass error: 0.38 ppm).

Supplementary Information

Supplementary information (physical data, ^{1}H NMR, ^{13}C NMR, bidimensional NMR and MS spectra for compounds **1**-**7**) is available free of charge at http://jbcs.sbq.org.br as a PDF file.

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