



Chemical profiling and cytotoxic activity of the endophytic fungus *Epicoccum sorghinum* isolated from *Disynaphia filifolia*

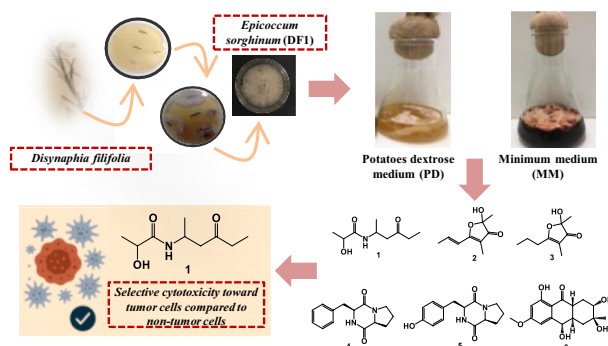
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Abstract

Endophytic fungi are recognized as important sources of bioactive specialized metabolites. In this study, six compounds were isolated from cultures of endophytic fungus *Epicoccum sorghinum* (DF1) associated with *Disynaphia filifolia*, including a new aliphatic amide (**1**) identified as 2-hydroxy-*N*-(4'-oxohexan-2'-yl)-propanamide and other known compounds 2,3-dihydro-2-hydroxy-2,4-dimethyl-5-*trans*-propenylfuran-3-one (**2**), 2,3-dihydro-2-hydroxy-2,4-dimethyl-5-propylfuran-3-one (**3**), cyclo-*L*-Pro-*L*-Phe (**4**), cyclo-*L*-Pro-*L*-Tyr (**5**) and tetrahydroaltersolanol B (**6**). The structures were established by spectroscopic analysis and comparison with related compounds reported in the literature. The cytotoxic activities of the isolated compounds were evaluated against human prostate carcinoma (PC-3), cervical carcinoma (HeLa), and non-tumorigenic keratinocyte (HaCaT) cell lines. Among the tested compounds, cyclo-*L*-Pro-*L*-Phe showed the most pronounced cytotoxic effects against PC-3 and HeLa cells. The new amide derivative (**1**) exhibited weak activity toward PC-3 cells and no detectable activity against HeLa cells, while presenting low cytotoxicity toward HaCaT cells. The remaining compounds displayed weak to moderate cytotoxic activity, with PC-3 cells being the most sensitive overall. These findings contribute to the chemical characterization of metabolites produced by *E. sorghinum*, isolated for the first time from *D. filifolia*, and highlight its potential as a source of structurally diverse compounds with biological activity to be explored.

Graphical Abstract



Keywords Asteraceae · Endophytic fungi · Cytotoxicity · Specialized metabolites · Tumor cells

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Introduction

Endophytic microorganisms inhabit the internal tissues of plants without causing disease and are increasingly recognized for their biotechnological and therapeutic potential [1, 2]. Among these, endophytic fungi are recognized as producers of structurally diverse specialized metabolites with a wide range of biological activities [3, 4]. Consequently, the isolation and characterization of metabolites from endophytic fungi represents a promising alternative source for the discovery of novel bioactive molecules of pharmaceutical interest [3, 5].

The genus *Epicoccum* (Didymellaceae) comprises filamentous fungi widely distributed in the environment, including soil, vegetation, and marine ecosystems. Species of this genus may occur as endophytes or plant pathogens and are frequently isolated from asymptomatic plant tissues [6–9]. Approximately 18 species of *Epicoccum* have been taxonomically recognized, with *Epicoccum nigrum* being the most extensively studied due to its ecological relevance and widespread occurrence [10].

Species of *Epicoccum* are known for producing a variety of bioactive specialized metabolites, including polyketides, diterpenes, and carotenoids, which display antimicrobial, antioxidant, antiviral and antiproliferative effects [8, 11, 12]. Additionally, non-toxicogenic *Epicoccum* strains are promising sources of natural pigments for food industry applications, particularly carotenoids and polyketides like anthraquinones, naphthoquinones and azaphilones [13, 14].

Epicoccum sorghinum, formerly classified as *Phoma sorghina*, is widely recognized as one of the primary fungal species associated with sorghum grains [15]. Chemical studies involving endophytic strains of this species have reported the isolation of diphenyl esters [16], phenolic compounds [17], and bioactive exopolysaccharides (EPS) [18], indicating that its specialized metabolite profile is still underexplored.

Our research group has focused on the chemical investigation of endophytic fungi associated with Asteraceae species from the Campos Gerais region of Paraná, Brazil, an ecotone between the Atlantic Forest and Cerrado biomes [19]. In this context, *Epicoccum sorghinum* was isolated as an endophyte from *Disynaphia filifolia*, a native species whose aerial parts have previously been reported to contain tremetone as the major chemical constituent, along with other benzofuran derivatives, terpenes, phenols, and flavonoids [20].

Despite the recognized chemical diversity and biological relevance of endophytic fungi, microorganisms associated with *Disynaphia* species remain unexplored. Herein, we report the chemical characterization of metabolites produced by the endophytic fungus *Epicoccum sorghinum*

DF1 isolated from *Disynaphia filifolia*, including the identification and structural elucidation of a previously undescribed aliphatic amide. In addition, the cytotoxic activities of isolated compounds were evaluated against two tumor cell lines and one non-tumoral cell line, contributing to the understanding of the chemical diversity and biological potential of endophytic fungi associated with this native plant species.

Materials and methods

Biological material

The endophyte DF1 was previously isolated from *Disynaphia filifolia* (Hassl.) R. M. King & H. Rob (Asteraceae) aerial parts and was retrieved from the Collection of Endophytic and Environmental Microorganisms at the Laboratory of Microbial Biotechnology (CMEA/LBIOMICUEM), Universidade Estadual de Maringá, Brazil. This endophyte was registered at the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under code AEE079A-17. *Disynaphia filifolia* is a native specimen, it was collected at Ponta Grossa city, Paraná State, Brazil (25° 05' 16" S, 50° 05' 43" W) in March 2016 and identified by Dr^a. Marta Regina Barrotto do Carmo. A voucher specimen was deposited at the herbarium at Universidade Estadual de Ponta Grossa (HUPG 22451). In addition, this plant was registered at SISGEN under code A6E6D08.

DNA extraction, amplification and molecular identification

The identification of endophytic fungi was based on molecular and morphological analysis. The molecular identification was carried out using the internal transcript spacer regions (ITS), and partial sequence for beta-tubulin gene (TUB). The ITS region was amplified using primers ITS1 (5'-TCCG TAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTT ATTGATATGC-3') [21]. For the TUB gene, primers T1 (5'-AACATGCGTGAGATTGTAAGT-3') and Bt2b (5'-ACCC TCAGTGTAGTGACCCTTGGC-3') were employed [22]. The amplification products were purified using a combination of two enzymes: Shrimp Alkaline Phosphatase (SAP) and Exonuclease I (EXO). The samples were sequenced by ACTGene Análises Moleculares LTDA (Ludwigbiotec). Fungal sequences were submitted to GenBank and compared with type sequences using BLAST. Multi-locus alignments were performed with MAFFT [23] in Geneious Prime v.2019.1.1. The best-fit evolutionary model was selected via MrModelTest v.2.3 [24]. Bayesian inference was conducted

using MrBayes v.2.2.4 [25] (MCMC, 1 million generations; $SD < 0.01$), with posterior probabilities indicated at the nodes. The resulting phylogenetic tree was visualized and edited in FigTree v.1.4.2 [26].

The ITS and TUB DNA sequences were deposited in GenBank under accession numbers PX097382 and PX101957.

Fermentation and extraction

Axenic maintenance of the endophytic DF1 strain was cultured and maintained using potato dextrose broth (PDB) (Acumedia®) and potato dextrose agar (PDA) (Acumedia®). The DF1 strain was subjected to two different cultivation conditions, using PDB and minimal medium (MM), at pH 6.8. The study hypothesis guiding this choice was that differences in nutrient composition, comparing a complex medium (PDB) with a chemically defined medium (MM), could modulate the expression of specialized metabolic pathways, potentially leading to qualitative variations in the metabolite profile.

The MM was prepared in the following concentrations: NaNO_3 60.00 g L⁻¹, KH_2PO_4 15.00 g L⁻¹, KCl 5.00 g L⁻¹, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 5.00 g L⁻¹, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01 g L⁻¹, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01 g L⁻¹, $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01 g L⁻¹. At the time of use, the MM was diluted 1:10 with distilled water and 10.00 g L⁻¹ *D*-glucose was added [19].

Submerged cultivation of the endophytic was carried out to produce specialized metabolites. Three 10 mm diameter agar discs from the fungal strain grown on PDA were inoculated into 20 Erlenmeyer flasks (250 mL), each containing 100 mL of medium, totaling 2.0 L of culture volume. The pH of both media was adjusted to 6.8. Cultivation was carried out at 28 °C for 21 days under stationary conditions [27]. At the end of the incubation period, the mycelium was separated from the broth by vacuum filtration using a filter membrane, Büchner funnel and Kitasato. The resulting culture broth was extracted with ethyl acetate (3 × 150 mL). The organic phase was concentrated using a rotary evaporator (Tecnal TE-210) at 37 °C, yielding 80 mg and 55 mg of PDB and MM extracts, respectively. These values correspond to approximate yields of 40 mg L⁻¹ for PDB and 27.5 mg L⁻¹ for MM.

General experimental procedures of metabolites isolation and identification

Chromatography separations were performed on silica gel (200–300 mesh; Qingdao Marine Chemical Plant Branch., China) or Sephadex LH-20 (100–200 mesh; Beijing Solarbio Technology Co., Ltd., China) chromatography columns (CC). Plates precoated with silica gel 60G or silica gel F254 (Rushan, Shandong Sun Desiccant Co., Ltd.) were used for

thin layer chromatography (TLC). Visualization of the compounds on TLC was accomplished by UV irradiation at 254 and 366 nm and/or by spraying with H_2SO_4 /anisaldehyde/acetic acid/methanol (5:0.5:10:85 mL) solution followed by heating at 150 °C or Dragendorff's solution. A Shimadzu LC-20AR HPLC was also used for analysis and isolation. For analysis, a Supelcosil LC-18 (25 cm × 4.6 mm, 5 µm) was used. The isolation was achieved on an Agilent semi-preparative Supelcosil LC-18 column (250 × 20 mm, 15 µm). UHPLC-ESI/MS data were acquired on a Shimadzu Nexera X2 instrument system coupled with a Bruker IMPACT II mass spectrometer system equipped with an Electrospray ionization (ESI) source, in the positive and negative ion modes, quadrupole-time of flight (Q-Tof) analyzer, and multichannel plate detector. Optical rotations were acquired on a JASCO P-1010 polarimeter. NMR spectra were recorded on a Bruker Avance III HD spectrometer operating at 300 or 500 MHz (¹H) and 75.5 or 125 MHz (¹³C). 75.5 MHz, using methanol-*d*₄, DMSO-*d*₆ and chloroform-*d* as solvents.

Isolation and purification of compounds

The MM extract (55.0 mg) was fractionated using a silica gel column (flash chromatography), eluting with chloroform:methanol (100:0, 98:02, 95:05, 90:10, 85:15, 80:20, 30:70 and 50:50 v/v) gradient system to give eight fractions (DF1-MM-1 to DF1-MM-8). Subfraction DF1-MM-6 yielded compound **1** (4.3 mg). Subfraction DF1-MM-3 (7.0 mg) afforded the mixture of compounds **2** and **3** (7.0 mg).

PD extract (80.0 mg), which previously showed a TLC profile enriched in relatively polar compounds with strong retention on silica gel, was initially subjected to column chromatography in Sephadex LH-20 using methanol–water as the mobile phase in decreasing polarity gradient system (25:75, 50:50, 100:0), to give subfractions DF1-PD-1 to DF1-PD-12. Compound **2** (4.7 mg), previously isolated from the MM extract and identified based on its similar TLC profile, was reisolated from subfraction DF1-PD-2. The DF1-PD-3 subfraction was subjected to silica gel CC (flash chromatography) eluting with a gradient system hexane:ethyl acetate (50:50, 60:50, 0:100 v/v) to produce seven subfractions (DF1-PD-3–1 to DF1-PD-3–7). Subfractions DF1-PD-3–2 and DF1-PD-3–4 afforded the isolated compounds **4** (2.5 mg) and **5** (3.1 mg), respectively. The subfraction DF1-PD-5 (19.0 mg) was purified using semi-preparative reverse-phase HPLC equipped with a Shim-pack PREP-ODS C18 column (250 mm × 20 mm; 15 µm) using isocratic elution with MeOH:H₂O (50:50 v/v) for 30 min, flow rate: 12 mL/min, to obtain compound **6** (2.4 mg, *t*_R = 11.2 min).

2-hydroxy-*N*-(4'-oxohexan-2'-yl)-propanamide (**1**): Yellow gum; $[\alpha]_D^{24} + 45.5$ (c 0.11, MeOH); ¹H (300 MHz, CDCl₃) and ¹³C (75.5 MHz, CDCl₃) NMR data, see Table 1;

Table 1 ^1H and ^{13}C NMR data of compound 1

Compound 1		
Position	δ_{H} (ppm, J =Hz) ^a	δ_{C} (ppm) ^a
1	1.40 (3H, d, 6.6)	21.4
2	4.17 (1H, q, 6.8)	68.3
3	-	173.7
1'	1.24 (3H, d, 6.73)	20.3
2'	4.32 (1H, m)	42.0
3'	2.65 (2H, dd, 5.2; 12.7)	47.4
4'	-	210.7
5'	2.45 (2H, m)	36.7
6'	1.04 (3H, t, 7.35)	7.6
NH	6.88 (1H, brs)	-

^a(δ -ppm; 300 and 75.5 MHz; recorded in CDCl_3)

HR-ESI-MS calculated for $\text{C}_9\text{H}_{17}\text{NO}_3$ $[\text{M}+\text{H}]^+$: 188.1281, found: 188.1275.

In vitro cytotoxic activity

The cytotoxic potential of compounds 1, 2, 4, 5, 6 and mixture of 2+3 were measured using the MTT colorimetric assay [28]. Cell viability was determined for the tumor cell lines HeLa (cervical adenocarcinoma, ATCC® CCL-2), PC-3 (prostate adenocarcinoma, ATCC® CRL-1435) and non-tumor HaCaT (immortalized human keratinocyte, CLS 300493). PC-3 cells were cultured in RPMI-1640 (pH 7.6), and HeLa and HaCaT cells were cultured in DMEM medium (pH 7.2). Additionally, the media were supplemented with *L*-glutamine and FBS at 10%, and incubated at 37 °C under a 5% CO_2 atmosphere. The cells were plated at a density of 2.5×10^5 cells mL^{-1} (equivalent to 2.5×10^4 cells per well in 96-well plates, 100 μL /well). After 24 h, the cells were treated with increased concentrations (1.0 to 200 $\mu\text{g mL}^{-1}$) of isolated compounds for 48 h. All stock solutions were prepared at 20 mg mL^{-1} in DMSO and diluted into culture medium to reach the working concentrations used in the assays. The final DMSO concentration in all experimental conditions did not exceed 1% (v/v). Doxorubicin was used as a reference drug treatment. After the treatment, media were carefully removed. Cells were washed twice with PBS (pH 7.2), subsequently 50 μL of MTT solution (2 mg mL^{-1} in PBS) was added to each well. The plate was incubated at 37 °C for 4 h. Then, the supernatant was removed, the formazan crystals were solubilized in DMSO and the absorbance reading was performed at 570 nm in a plate spectrophotometer (Power Wave XS, BioTek). Half-maximal inhibitory concentration (IC_{50}) was determined as the concentration capable of reducing 50% of the optical density of the treated cells compared to the control, analysis performed by means of non-linear regression. The results were expressed as the mean $\pm$ standard deviation (SD) of at least three independent experiments.

In-silico absorption, distribution, metabolism, excretion, and toxicity (ADMET) prediction

Due to its structural novelty, the pharmacokinetic properties of undescribed compound 1, including absorption, distribution, metabolism, excretion and toxicity (ADMET), were predicted using the SWISSADME web server (<http://www.swissadme.ch/>) [29]. Key pharmaceutical parameters assessed included molecular weight, lipophilicity (Log P), solubility (Log S), blood–brain barrier (BBB) permeability, gastrointestinal (GI) absorption/oral bioavailability, and compliance with Lipinski's Rule of Five (ROF). Additionally, CYP450 enzymes inhibition potential was evaluated through predictions generated by the SWISSADME web tool. The SMILES were used as an input.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism version 8.0.1 software (GraphPad, San Diego, CA, USA). Data normality was assessed using the Shapiro–Wilk test prior to statistical comparisons. As all datasets satisfied normal distribution criteria ($p > 0.05$), one-way analysis of variance (ANOVA) followed by Tukey's test was used to determine if there were statistically significant differences between treatments within each experiment. A 95% significance level ($p < 0.05$) was applied throughout the study.

Results and discussion

Identification of the fungal strain DF1

Morphological characteristics of strain DF1 on a PDA plate were observed after 21 days of growth at 28 °C and a phylogenetic tree was established based on ITS sequences (Fig. 1). When cultivated on PDA, it forms dense and fluffy mycelia with cottony and velvety textures, displaying a gray color and producing a reddish-brown pigment on the underside of the Petri dish. The ITS1-5.8 s-ITS2 gene of the endophytic fungus isolated from *D. filifolia* were sequenced and submitted to BLASTN in GenBank. The results indicate several species of 'phoma-like' belonging to the genus *Epicoccum* as the closest matches. Among the species analyzed, *Epicoccum sorghinum* strains, particularly CBS 627.68, exhibited 100% identity in the ITS region and 97% identity in the TUB gene. Phylogenetic analysis revealed that strain DF1 clusters closely with *E. sorghinum*, forming a well-supported clade with a bootstrap value of 98%.

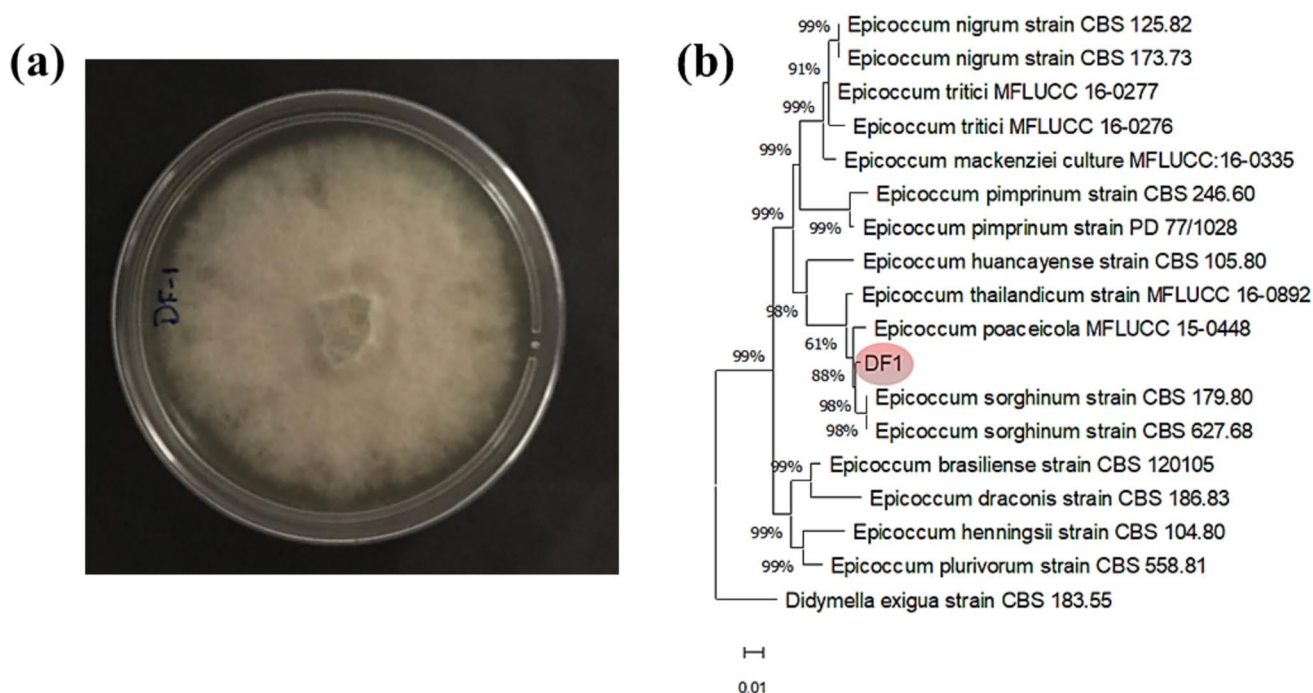
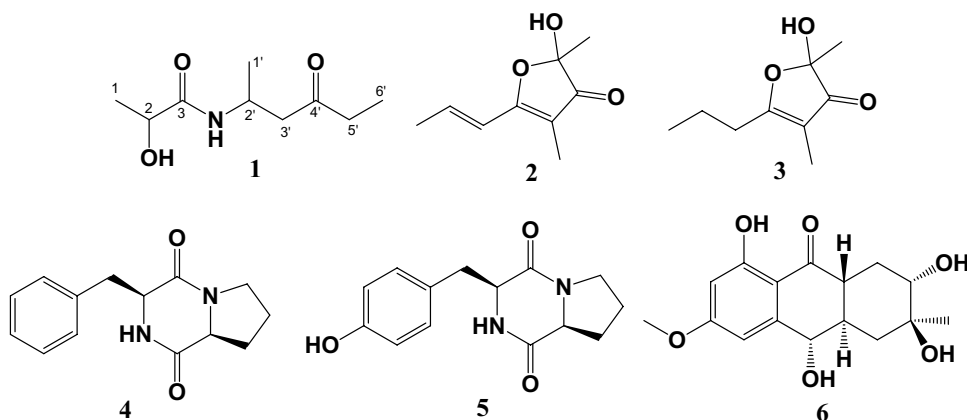


Fig. 1 Identifying the species of strain DF1. (a): Conidiation morphology after 21 days of culture on a PDA plate at 28 °C (b): Phylogenetic consensus tree based on ITS1-5.8S-ITS2 gene sequences calculated

by Bayesian inference. Bayesian probability was demonstrated at the nodes between each organism. The strain *Didymella exigua* CBS 183.55 was used as external group.

Fig. 2 Chemical structures of compounds 1–6 isolated from *Epicoccum sorghinum*



Purification and structure elucidation of compounds from *Epicoccum sorghinum*

From the ethyl acetate extract of a culture of the endophytic fungus *E. sorghinum* DF1, a undescribed compound denominated 2-hydroxy-N-(4'-oxohexan-2'-yl)-propanamide (1) together with five known compounds, including two furanones 2,3-dihydro-2-hydroxy-2,4-dimethyl-5-trans-propenylfuran-3-one (2) [30–32], 2,3-dihydro-2-hydroxy-2,4-dimethyl-5-propylfuran-3-one (3) [30], two diketopiperazines cyclo-L-Pro-L-Phe (4) [33, 34], cyclo-L-Pro-L-Tyr (5) [34], and a polyketide, tetrahydroaltersolanol B (6) [35, 36], were isolated (Fig. 2). The chemical structures were identified by NMR and ESI-MS/MS data and by

comparison with the literature. Compound 1 was isolated exclusively from fermentation in minimal medium (MM). Compounds 4, 5 and 6 were obtained only in potato dextrose medium (BD).

Compound 1 was obtained as an optically active yellow gum with a specific rotation of $[\alpha]_D^{24} + 45.5$ (c 0.11, MeOH), had a molecular formula $C_9H_{17}NO_3$ as defined according to the HR-ESI-MS peak at m/z 188.1275 $[M+H]^+$ (calcd. for $C_9H_{17}NO_3$, 188.1281) (Fig. S12).

According to the characteristic signals of the ^1H NMR spectrum (Table 1, Fig. S2-S3) revealed the presence of one oxymethinic proton δ_H 4.17 (1H, q, $J=6.8$ Hz, H-2), one methinic δ_H 4.32 (1H, m, H-2'), two methylene [δ_H 2.65 (2H, dd, $J=5.2$ Hz; 12.7 Hz, H-3') and δ_H 2.45 (2H, m,

H-5'), and three methyl groups [δ_{H} 1.40 (3H, d, $J=6.6$ Hz, H-1), δ_{H} 1.24 (3H, d, $J=6.7$ Hz, H-1'), and δ_{H} 1.04 (3H, t, $J=7.4$ Hz, H-6')]. The ^{13}C NMR data (Table 1; Fig. S4) and the HSQC spectrum (Fig. S8-S9) of **1** displayed 9 signals that could be assigned as two carbonyl carbons at δ_{C} 210.7 (C-4') and 173.7 (C-3) characteristic of the ketone and amide groups, respectively. In addition to two methynic carbon at δ_{C} 42.0 (C-2') and 68.3 (C-2), two methylene at δ_{C} 36.7 (C-5') and 47.4 (C-3'), as well as three methyl carbon at δ_{C} 21.4 (C-1), 20.3 (C-1') and 7.6 (C-6'). A broad singlet at δ_{H} 6.88 (NH), with no corresponding correlations in the HSQC spectrum (Fig. S8-S9), was attributed to hydrogen bonded to the nitrogen atom (NH) of the structure. This assignment was confirmed by the correlation observed in the COSY spectrum (Fig. S5-S7) between the signal at δ_{H} 4.32 (H-2') and the signal at 6.88 (NH). Additionally, correlations were observed between the signals δ_{H} 4.17 (H-2) and δ_{H} 1.40 (H-1); δ_{H} 2.45 (H-5') and δ_{H} 1.04 (H-6'); and the signal at δ_{H} 4.32 with the signals at δ_{H} 1.24 (H-1') and δ_{H} 2.65 (H-3').

The spin coupling system of H-2 (δ_{H} 4.17 1H, q, $J=6.8$ Hz), along with the HMBC correlations (Fig. 3, Fig. S10-S11) of H-2 with C-1 (δ_{C} 21.4) and C-3 (δ_{C} 173.7), confirmed that the lactamide moiety was linked to the structure. In addition to the correlations between the signals at δ_{H} 2.65 (H-3') with the carbons δ_{C} 42.0 (C-2'), 20.3 (C-1') and 210.7 (C-4'), and the terminal methyl group at δ_{H} 1.04 (H-6') with the carbons δ_{C} 36.7 (C-5') and 210.7 (C-4').

On this basis, compound **1** was identified as 2-hydroxy-*N*-(4'-oxohexan-2'-yl)-propanamide. Due to the limited amount of material, the absolute configuration could not be established. Although amide-containing metabolites have been reported from microbial sources [37–40], the compound **1** contains two stereogenic centers (C-2 and C-2'), and no closely related analogues with established absolute configurations were identified to support a reliable configurational assignment based on biosynthetic or spectroscopic comparison. In similar simple aliphatic amide systems, stereochemical outcomes may vary depending on the producing organism and biosynthetic pathway. If future studies enable the isolation of compound **1** in larger amounts, advanced optical stereochemical techniques could be employed to establish its absolute configuration. To the best of our knowledge, this study represents the first chemical characterization of this compound.

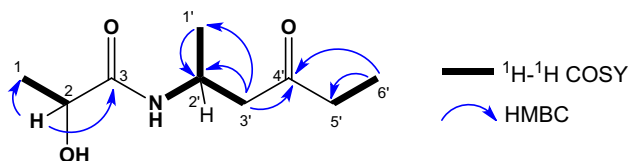


Fig. 3 Key ^1H – ^1H COSY and HMBC correlations of compound **1**

Compound **2** was previously isolated from the fungus *Stemphylium radicinum* [30], and has subsequently been identified in the endophytic fungus *Mollisia nigrescens*, associated with the leaves and stems of *Vaccinium angustifolium* [32]. It has also been reported in an endophytic *Penicillium* sp. isolated from *Taxus brevifolia* [31], and in the marine-derived fungus *Ascochyta salicorniae* from the green alga *Ulva* sp. [41]. The compound **3** was originally described by Grove (1971) [30] as the product of a chemical reduction of 2,3-dihydro-2-hydroxy-2,4-dimethyl-5-*trans*-propenylfuran-3-one (**2**). To the best of our knowledge, this is the first report of compound **3** being isolated as a natural product. Furthermore, this study provides the first report of the occurrence of these metabolites within the genus *Epicoccum*. Diketopiperazines **4** and **5** were previously isolated from *Epicoccum nigrum* strain M13 by Qader et al. (2021) [37]. However, this is the first report of these compounds in the species *E. sorghinum*. These cyclic dipeptides represent a prominent class of specialized metabolites that are widely produced by diverse fungal genera. Tetrahydroaltersolanol B (**6**) was previously isolated from the fungi *Alternaria solani* [35] and *Stemphylium botryosum* [42]. However, as far as we know, it is the first time to report of this compound within the genus *Epicoccum*.

Cytotoxic activity

The compounds **1**, **2**, **4**, **5**, **6** and mixture of **2**+**3** were tested against tumoral cells of human prostate carcinoma (PC-3) and immortalized cervical carcinoma cells (HeLa). Cytotoxicity against a non-tumor immortalized human keratinocyte cells (HaCat) was also evaluated. Compound **3** was not isolated in pure form and, therefore, could not be subjected to cytotoxic evaluation due to insufficient available mass. The results obtained are presented in Table 2.

Among the isolated compounds, cyclo-*L*-Pro-*L*-Phe (**4**) showed the highest cytotoxic activity, with IC_{50} values of 37.91 $\mu\text{g/mL}$ against PC-3 and 24.65 $\mu\text{g/mL}$ against HeLa cells. Although these values indicate measurable inhibitory effect, the activity may be considered moderate when compared with highly potent antitumor agents. Diketopiperazines (DKPs), a class of cyclic dipeptides, have been reported to exert antitumor effects by modulation of cell proliferation and apoptosis pathways [43–45]. Previous studies have reported that diketopiperazines isolated from the ethyl acetate extract of the cell-free filtrate of *Exiguobacterium acetylicum* exhibited anticancer potential against colorectal cancer HT-29 cells in vitro. Among them, cyclo-*L*-Pro-*L*-Phe demonstrated promising cytotoxic effects, supporting the potential biological relevance of proline-containing DKPs as antitumor agents [46].

Table 2 Cytotoxicity of compounds isolated from *E. sorghinum* against different cell lines

Compounds	Cell lines/IC ₅₀ ±SD (µg/mL)			Selective index (SI)	
	HeLa*	PC-3*	HaCat**	HeLa	PC-3
1	> 200	110.58±5.6 ^a	135.49±3.9 ^a	ND	1.23
2	156.11±19.5 ^a	62.94±1.4 ^b	47.34±5.7 ^b	0.30	0.75
2+3	168.84±15.2 ^a	99.51±8.2 ^{ad}	70.48±4.4 ^c	0.42	0.71
4	24.65±3.0 ^b	37.91±1.8 ^c	39.44±0.8 ^b	1.60	1.04
5	167.12±6.9 ^a	105.31±8.0 ^a	46.37±4.9 ^b	0.28	0.44
6	> 200	91.24±9.6 ^d	67.67±1.8 ^c	ND	0.74
Doxorubicin	1.91±0.17 ^c	1.89±0.11 ^c	5.32±0.58 ^d	2.79	2.82

Data expressed as mean±standard deviation of three independent experiments. One-way analysis of variance.

(ANOVA) tests were applied, with significant differences between means identified by Tukey's post hoc test. In the same column, the values marked with the same lowercase letter are similar ($p>0.05$), whereas the values with different lowercase letter are significantly different ($p<0.05$). IC₅₀: Half-maximal inhibitory concentration (50%); SD: Standard deviation (n=3); *: tumor cell lines; **: non-tumor cell lines; HeLa: immortalized cervical carcinoma cells; PC-3: human prostate carcinoma; HaCat: immortalized human keratinocyte cells (non-tumor); (SI (Selective index)=IC₅₀ non-tumor cells/IC₅₀ tumor cells); ND: not determined.

The new compound 2-hydroxy-*N*-(4'-oxohexan-2'-yl)-propanamide (**1**) exhibited weak cytotoxicity against PC-3 cells (IC₅₀=110.58 µg/mL) and no significant activity against HeLa cells at the highest concentration tested (200 µg/mL). These results indicate that compound **1** does not act as a potent cytotoxic agent under the experimental conditions employed. The furanone 2,3-dihydro-2-hydroxy-2,4-dimethyl-5-trans-propenylfuran-3-one (**2**) demonstrated moderate activity against the PC-3 (62.94 µg/mL) and weak activity against the HeLa cells (156.11 µg/mL). However, when compounds **2** and **3** were tested in mixture, a decrease in biological activity was observed, which was lower than the activity of compound **2** when tested individually. The remaining isolated compounds (**5** and **6**) exhibited weak to moderate cytotoxic effects. Tetrahydroaltersolanol B (**6**), previously isolated from *Stemphylium globuliferum*, has been reported as inactive against human chronic myeloid leukemia (K562) and human lung (A549) cancer cell lines when compared to altersolanol A. Structural modifications, including the reduction of carbonyl groups and the removal of hydroxyl substituents, appear to markedly reduce biological activity [47].

It is important to emphasize that the cytotoxic effects observed in this study were determined exclusively through in vitro viability assays, reflecting direct pharmacodynamic interactions of the compounds with tumour cells under controlled experimental conditions. No experiments were conducted to investigate specific molecular targets or signaling pathways. Moreover, pharmacokinetic parameters such as absorption, distribution, metabolism, and clearance were not experimentally evaluated, as these require in vivo models. The ADMET analysis performed for compound **1** represents a theoretical in silico prediction and should not be interpreted as experimentally validated pharmacokinetic data. Therefore, the present findings should be considered exploratory and restricted to cellular-level pharmacodynamic effects.

PC-3 cells were overall more sensitive to the tested compounds than HeLa cells. Although compound **1** showed limited inhibitory activity against tumor cells, it displayed low cytotoxicity toward non-tumor human keratinocyte cells (HaCaT), with an IC₅₀ value of 135.49 µg/mL, suggesting a relatively low degree of general cytotoxicity. This profile may be advantageous for future investigations focusing on alternative biological targets or structural optimization. The other isolated compounds showed moderate cytotoxicity against HaCaT in the range of 39.4 µg/mL to 70.5 µg/mL.

The comparatively higher sensitivity observed in PC-3 cells may be partially attributed to intrinsic biological differences between tumor cell lines. PC-3 cells present distinct membrane lipid composition and metabolic characteristics, which can influence permeability and intracellular accumulation of moderately lipophilic compounds [48, 49]. In addition, differences in mitochondrial function and redox regulation reported in prostate cancer cells may affect susceptibility to bioactive metabolites [46]. These explanations remain speculative and warrant further investigation to clarify the basis of this differential sensitivity.

Physicochemical, pharmacokinetic and pharmacological parameters play a significant role in the discovery of novel drug candidates as many invented drugs fail in the development process. Therefore, an in silico ADMET evaluation of the undescribed compound **1** was performed using SwissADME [29] web tools (Table S1). Structurally, the molecule presented a number of hydrogen bond acceptors and hydrogen bond donors less than ten and five, respectively. In addition, the number of rotatable bonds in each molecule was less than ten, values that fall within the acceptable limits defined by Veber and Egan for compounds with good oral bioavailability and permeability (Topological Polar Surface Area, TPSA=66.4 Å². The molecule showed high gastrointestinal absorption, very good aqueous solubility, with estimated solubility up to 57.8 mg/mL (ESOL Log S=-0.51), and moderate lipophilicity (consensus Log P=0.48),

indicating favorable oral bioavailability. This is consistent with the prediction of high gastrointestinal (GI) absorption, while the compound is not expected to cross the blood–brain barrier (BBB), which may be desirable depending on the therapeutic target. Although the compound presents favorable predicted pharmacokinetic properties, these characteristics do not necessarily correlate with in vitro cytotoxic potency. The relatively low lipophilicity and absence of structural motifs commonly associated with cytotoxic agents may partially explain its limited cytotoxic effects in vitro.

In terms of metabolism, compound **1** is predicted not to inhibit any of the major cytochrome P450 enzymes (CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4), minimizing the risk of metabolic interactions. No structural alerts (PAINS or Brenk) were identified, and the compound complies with Lipinski drug-likeness rule, suggesting a low risk of nonspecific toxicity, which is consistent with the reduced cytotoxicity, observed in HaCaT cells. Its synthetic accessibility score (2.22) indicates that the molecule is both synthetically tractable and suitable for medicinal chemistry optimization. Therefore, although compound **1** does not emerge as a strong cytotoxic lead, its ADMET profile supports its suitability as a chemically stable and biologically safe scaffold, potentially amenable to further structural modification or exploration of alternative biological activities.

Conclusion

This study expands the chemical knowledge of the endophytic fungus *Epicoccum sorghinum* associated with *Disynaphia filifolia*, leading to the identification of six metabolites, including a previously undescribed aliphatic amide derivative, identified as 2-hydroxy-*N*-(4'-oxohexan-2'-yl)-propanamide. The biological evaluation demonstrated distinct cytotoxic profiles among the isolated compounds. The diketopiperazine cyclo-(*L*-Pro-*L*-Phe) exhibited the most pronounced cytotoxic effects, whereas the new amide showed low cytotoxicity toward non-tumor HaCaT cells and favorable in silico pharmacokinetic properties, suggesting its potential for further structural optimization or for the investigation of alternative biological activities. To the best of our knowledge, this is the first report describing an endophytic fungus isolated from a *Disynaphia* species. These findings expand the known chemical diversity of the genus *Epicoccum* and reinforce the potential of endophytic fungi associated with native plant species as a valuable source of structurally novel natural products.

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Authors' contributions Debora Cristina Baldoqui, Maria Helena Saragiotto, and João Alencar Pamphile conceived the study, developed the methodology, and supervised the manuscript. Anderson Valdney Gomes Ramos, Jesieli Beraldo Borrazzo, and Drielli Rhiane Peres Colhado Areas were responsible for the cultivation, fermentation, and maintenance of the endophytic microorganism. Andressa Domingos Polli and Julio Cesar Polonio performed the molecular identification of the endophyte. Anderson Valdney Gomes Ramos, Nathalia da Silva Malaco, and Francielli Alana Pereira Valeze carried out the phytochemical studies, identified and characterized the isolated compounds, and performed ESI–MS/MS analyses. Camila Botin Francisco and Ernani Abicht Basso were responsible for the analysis using NMR techniques. Rodolfo Bento Balbinot and Celso Vataru Nakamura conducted the biological assays. The manuscript was written by Anderson Valdney Gomes Ramos and Debora Cristina Baldoqui. All authors discussed the results, critically revised the manuscript, and approved the final version.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

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