Brazilian Journal of Microbiology (2021) 52:1201–1214
https://doi.org/10.1007/s42770-021-00502-6

BIOTECHNOLOGY AND INDUSTRIAL MICROBIOLOGY - RESEARCH PAPER

Chromolaena laevigata (Asteraceae) as a source of endophytic non-aflatoxigenic *Aspergillus flavus*: chemical profile in different culture conditions and biological applications

Rodolfo B. Balbinot[1] · Josiane A. M. de Oliveira[1] · Darlon I. Bernardi[1] · Andressa D. Polli[2] · Julio C. Polonio[2] · Márcia R. P. Cabral[1] · Érica B. Zanqueta[3] · Eliana H. Endo[3] · Jean E. Meneguello[4] · Rosilene F. Cardoso[4] · João L. Azevedo[5] · Benedito P. Dias Filho[3] · Tania U. Nakamura[3] · Marta R. B. do Carmo[6] · Maria H. Sarragiotto[1] · João A. Pamphile[2] · Debora C. Baldoqui[1]

Received: 6 November 2020 / Accepted: 16 April 2021 / Published online: 30 April 2021

Abstract

Endophytes are microorganisms that form symbiotic relationships with their host. These microorganisms can produce a variety of secondary metabolites, some of which have inhibitory effects on pests and pathogens or even act to promote plant growth. Due to these characteristics, these microorganisms are used as sources of biologically active substances for a wide range of biotechnological applications. Based on that, the aim of this study was to evaluate the production of metabolites of the endophytic *Aspergillus flavus* CL7 isolated from *Chromolaena laevigata*, in four different cultivation conditions, and to determine the antimicrobial, cytotoxic, antiviral, and antioxidant potential of these extracts. The multiphasic approach used to identify this strain was based on morphology and ITS gene sequence analysis. The chemical investigation of *A. flavus* using potato dextrose and minimal medium, using both stationary and agitated methods, resulted in the isolation of kojic acid, α-cyclopiazonic acid, and 20,25-dihydroxyaflavinine. Another 18 compounds in these extracts were identified by UHPLC-HRMS/MS, of which dideacetyl parasiticolide A has been described for the first time from *A. flavus*. Aflatoxins, important chemomarkers of *A. flavus*, were not detected in any of the extracts, thus indicating that the CL7 strain is non-aflatoxigenic. The biological potential of all extracts was evaluated, and the best results were observed for the extract obtained using minimal medium against *Trichophyton rubrum* and *Mycobacterium tuberculosis*.

Responsible Editor: Adalberto Pessoa

✉ Debora C. Baldoqui
dcbaldoqui@uem.br

[1] Departamento de Química, Universidade Estadual de Maringá, Av. Colombo 5790, 87020-900, Maringá, Paraná, Brazil

[2] Departamento de Biotecnologia, Genética e Biologia Celular, Universidade Estadual de Maringá, Av. Colombo 5790, 87020-900 Maringá, Paraná, Brazil

[3] Departamento de Ciências Básicas da Saúde, Universidade Estadual de Maringá, Av. Colombo 5790, 87020-900, Maringá, Paraná, Brazil

[4] Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Av. Colombo 5790, 87020-900, Maringá, Paraná, Brazil

[5] Departamento de Genética, Escola Superior de Agricultura Luiz de Queiroz – ESALQ/USP, Universidade de São Paulo, Av. Pádua Dias 11, 13418-900, Piracicaba, São Paulo, Brazil

[6] Departamento de Biologia Geral, Universidade Estadual de Ponta Grossa, Av. Carlos Cavalcanti, 4778, 84030-910, Ponta Grossa, Paraná, Brazil

Keywords Endophytic fungi · Secondary metabolites · Antimicrobial activity · Antiviral activity · Biotechnology

Introduction

Endophytic microorganisms colonize intra- and intercellular plant tissues. This colonization/association is asymptomatic, causing no damage to the host plant. Among the microorganisms capable of this association, the most common are bacteria and fungi [1]. Our research group has been studying the chemical and biological diversity of the Eupatorieae tribe [2–5]; as part of our investigation of species belonging to this tribe, we selected *Chromolaena laevigata* (basionym: *Eupatorium laevigatum*) to characterize its foliar endophytic fungi. *Chromolaena laevigata* (Lam.) RM King & H. Rob is a shrub native from Mexico to Argentina [6], widely distributed in Brazil. This species is usually found in the Cerrado, Atlantic

Forest, Amazon, and Caatinga [7] and is considered an invasive species prone to infesting pastures, crops, roadsides, and vacant lots [8]. Previous studies on the aerial parts of *C. laevigata* have shown the presence of cadinene, clerodane, steroids, and flavonoids [5, 9]. *Aspergillus flavus* was identified here as an endophytic fungus from this species.

Aspergillus is widespread in nature and includes more than 250 species. Several *Aspergillus* species are classified as pathogens of harvested fruits and seeds, especially *A. flavus* which is well known for its production of toxins such as aflatoxins and cyclopiazonic acid [10–12]. Among the various classes of secondary metabolites produced by *Aspergillus* species, aflatoxins play an important role as chemomarkers. The word "aflatoxin" is derived from the expression "*Aspergillus flavus* toxin", since *A. flavus* and *A. parasiticus*, as well as *A. nomius*, *A. bombycis*, and *A. pseudotamarii*, are the species predominantly responsible for aflatoxin contamination of agricultural crops before harvest or during storage [13]. In addition to aflatoxins, *A. flavus* can also produce a range of other compounds of different classes, such as cyclopiazonic acid, β-nitropropionic acid, aspertoxin, aflatrem, gliotoxin, aspergillic acid, 20,25-dihydroxyaflavinine, and versicolorin A [14].

There are promising possibilities in exploring the biotechnological potential of endophytic fungi to produce a multitude of known and new biologically active specialized metabolites. For example, using controlled fermentation conditions and changing the process parameters, such as the type of medium, aeration, pO_2, pCO_2, pH, temperature, agitation, sampling, and harvest points, the production of specialized metabolites can be optimized [15–17], and the variation of medium conditions has been shown to have variable effects on the production of these metabolites [16, 18, 19]. Often, the variation of culture conditions is used to optimize the yields of a specific compound, such as an active metabolite or drug produced through microorganisms [20–23].

Herein, we evaluated the influence of different cultivation conditions on the production of specialized metabolites from an *A. flavus* strain isolated from a new host, *C. laevigata*. First, the multiphasic approach, based on morphology and ITS gene phylogenetic analysis, used to identify endophytic *A. flavus* is described. Subsequently, the metabolites produced when this endophyte was submitted to four different cultivation conditions were identified using 1D and 2D NMR spectroscopic experiments, and a dereplication technique using UHPLC-HRMS/MS. In vitro antimicrobial, antiviral, and antioxidant activity was also evaluated. Three compounds were isolated, kojic acid, cyclopiazonic acid and 20,25-dihydroxyaflavinine, and 18 compounds were identified by dereplication; however, no aflatoxins were detected in any of the culture media, indicating a non-aflatoxigenic *A. flavus* strain, with biotechnological potential for use as a biocontrol tool.

Materials and methods

General experimental procedures

Chromatography separations were performed on Sephadex LH-20 (Sigma) chromatography columns (CC). Thin layer chromatography (TLC) was performed on normal-phase precoated silica gel 60G or $60GF_{254}$ plates (Merck). 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.

NMR spectra were recorded on a VARIAN Mercury Plus spectrometer operating at 300 and 75.5 MHz, and a Bruker advance III HD spectrometer operating at 500 and 125 MHz, using $CDCl_3$ and DMSO-d_6 as solvents. The UHPLC analysis was performed on a Shimadzu Nexera X2 instrument, equipped with a CBM-20A system controller, two LC-30AD pumps, a CTO-30A column oven, and a SIL-30AC autosampler. The mass spectra were recorded on a Bruker IMPACT II mass spectrometer, with an electrospray ionization source (ESI) in the positive and negative ion modes, quadrupole-time of flight (Q-TOF) analyzer, and multichannel plate (MCP) detector.

Biological material

The endophyte CL7 was previously isolated from *Chromolaena laevigata* (Lam.) R. M. King & H. Rob. (Asteraceae) leaves and was retrieved from the Collection of Endophytic and Environmental Microorganisms at the Laboratory of Microbial Biotechnology (CMEA/LBIOMIC-UEM), 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. *Chromolaena laevigata* (Lam.) R. M. King & H. Rob. (Asteraceae) 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 Drª. Marta Regina Barrotto do Carmo. A voucher specimen was deposited at the herbarium at Universidade Estadual de Ponta Grossa (HUEPG 21697); in addition, this plant was registered at SISGEN under code A6E6D08.

Microculture

For morphological analysis, strain CL7 was submitted to the microculture technique. A circular piece of filter paper was placed in a sterile Petri dish, and a sterile glass slide (26 × 76 mm) was put on the filter paper. A block of lemon-leaf agar (100 g L^{-1} crushed lemon leaves, 15 g L^{-1} agar, 5 g L^{-1} peptone, and 15 g L^{-1} glucose) was cut with a sterile scalpel

and transferred to the glass slide. Using a sterile wire needle, the fungus was then inoculated from the culture plate to the four sides of the agar block. A sterile coverslip was put over the block, and 1 mL of sterile water was put onto the filter paper. The plate cover was replaced, and it was incubated at 28 °C for 5 days. After 5 days, the slide was removed and the block was deposited on another slide containing a drop of Cotton Blue dye. The sample was analyzed using a ZEISS Axioskop 2 Plus optical microscope equipped with an Axiocam HRc camera.

DNA extraction, amplification, and phylogenetic analysis

The genomic DNA was extracted using a PowerSoil® DNA isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), with 200 mg of mycelia initially added to the tubes, before then following the manufacturer's instructions.

Amplification of the ribosomal DNA region, ITS1-5.8S-ITS2, was performed using primers V9G (5′-TTAC GTCCCTGCCCTTTGTA-3′) and ITS4 (5′-TCCC CGCTTATTGATATGC-3′) described by White et al. [24]. The PCR technique was performed under the following conditions: 50 ng of DNA, 1× PCR buffer (50 mM KCl, 200 mM Tris-HCl, pH 8.4), 1.5 U of Taq polymerase (Invitrogen, Carlsbad, CA, USA), 0.25 μM of each primer, 0.2 mM dNTP (Amersham Bioscience, Freiburg, Germany), and 3 mM $MgCl_2$ in a final volume of 25 μL. Amplification was performed on a TC1000-G thermocycler (DLAB®) using the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 50 s, and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 5 min.

PCR products were purified using the enzyme alkaline phosphatase (shrimp; Sigma-Aldrich®) and exonuclease I (*E. coli*; BioLabs®). The extracted DNA was then subjected to quantification on 1% agarose gel. Amplification products were sequenced by ACTGene molecular analysis using an ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequencing results were evaluated using BioEdit Sequence Alignment Editor v. 7.2.2. The results were compared with other sequences deposited at the NCBI (National Center for Biotechnology Information) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Identification was based on the best percentage identity, followed by phylogenetic analysis.

Alignment and phylogenetic analysis were conducted using Geneious prime software (v. 2020.0.4). Based on the BLAST similarity to the CL7 sequence of the *A. flavus* clade, sequences of nearby species were rescued for phylogenetic reconstruction [12]. The gene sequences were aligned using MAFFT v. 1.4.0 [25]. For phylogenetic analysis based on maximum likelihood and Bayesian inference, MrModelTest v. 2.3 was used to choose the best evolutionary model [26]. The phylogenetic tree was constructed using MrBayes v. 2.2.4 [27], taking into consideration the parameters generated by MrModelTest, with a Markov chain Monte Carlo (MCMC) simulation which lasted until the average standard deviation of the split frequencies was below 0.01. The Bayesian probability was demonstrated on the nodes between each individual. The tree was edited with FigTree v. 1.4.2 and Adobe Illustrator v. 23.0.3 [28].

The DNA sequence was deposited in GenBank under accession number MN955851.1.

Culture conditions and isolation

Axenic maintenance of the endophytic CL7 strain was performed on potato dextrose broth (PD) (HiMedia M403), potato dextrose agar (HiMedia M096), and minimal medium (MM), at pH 6.8. 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}, $MgSO_4{\cdot}7H_2O$ 5.00 g L^{-1}, $FeSO_4{\cdot}7H_2O$ 0.01 g L^{-1}, $ZnSO_4{\cdot}7H_2O$ 0.01 g L^{-1}, $CuSO_4{\cdot}7H_2O$ 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.

The CL7 strain was subjected to four different axenic cultivation conditions, using PD and MM and stationary (PD-S; MM-S) and agitated methods (PD-A; MM-A). The strain was cultivated in three 1-L Erlenmeyer flasks, each containing 500 mL of medium. A spore solution was prepared at a concentration of 10^7 spores mL^{-1} according to Uldahl et al. with modifications. Briefly, spores were harvested when the cultures were fully sporulated, which was achieved after 10 days of incubation. The spores were released by flooding the Petri dishes with 5 mL sterile distilled water containing 0.1% (*v*/*v*) of tween 80 and the gently rubbing the culture with a sterile L-shaped spreader, a hemocytometer slide was used to count spore concentration [29]. Subsequently, 500 μL of this solution was added to each culture flask. After inoculation, the flasks were stored in a growth greenhouse at 28 °C for 21 days for the stationary cultures and in a shaker culture incubator (Nova Instruments NI1712) at 28 °C at 110 rpm for 21 days for the agitated cultures.

At the end of the incubation period, the mycelium was separated from the broth by vacuum filtration using a filter membrane, Büchner funnel and Kitassato. The mycelium was stored in a drying oven at 55 °C for 5 days to determine the dry weight of the mycelium for each cultivation condition. After filtration, the broths (approximately 500 mL) were extracted with ethyl acetate (3 × 150 mL). The solvent present was evaporated using a rotary evaporator (Tecnal TE-210) at 37 °C and the resulting extract was submitted to column chromatography, NMR, and UHPLC-HRMS/MS analysis.

PD-S crude extract yielded compound **1** (491.7 mg), with no purification step. Part of the PD-A crude extract (60.0 mg)

was subjected to a CC (Φ = 1.00 cm × *h* = 25.0 cm) in Sephadex LH-20 using methanol as the mobile phase, to give subfractions PD-A1 to PD-A18. Compound **1** (8.0 mg) was reisolated from subfraction PD-A6, and subfraction PD-A15 afforded compound **2** (16.0 mg).

An aliquot of MM-S (28.0 mg) was subjected to a CC (Φ = 1.00 cm × *h* = 25.0 cm) in Sephadex LH-20, using methanol as the mobile phase, to give subfractions MM-S1 to MM-S15. Subfraction MM-S6 yielded compound **3** (5.1 mg), and subfraction MM-S10 afforded compound **2** (3.5 mg), previously isolated from PD-A15. MM-A extract was not obtained in an amount sufficient for chromatographic purification.

UHPLC-HRMS/MS

The samples were prepared in MeOH (1.0 mg mL^{-1}) and chromatographic separations were performed using UHPLC on a Symmetry C18 column (75 × 2.0 mm i.d.; 1.6 μm Shim-pack XR-ODS III), maintained at a temperature of 40 °C. The mobile phase used in both the positive and negative ionization modes consisted of H_2O (solvent A) and CH_3CN (solvent B), with the addition of 0.1% formic acid when analyzed in positive ionization mode. The gradient program was as follows: initial 0–1 min, using elution A–B (95 : 5, *v*/*v*), 1–3 min (30 : 70 *v*/*v*), 3–12 min (5 : 95 *v*/*v*), and kept at 95% B for 16 min at a flow rate of 0.2 mL min^{-1}. The injection volume was 3 μL. High-resolution mass spectrometry (HRMS) analysis was carried out in a Q-TOF mass spectrometer with an electrospray ionization interface. The capillary voltage was operated in positive and negative ionization modes, set at 4500 V, using sodium formate (10 μM) as calibrant. The dry gas parameters were set to 8 L min^{-1} at 200 °C with a nebulization gas pressure of 4 bar. Collision-induced dissociation (CID) fragmentation was performed using argon (Ar) collision gas and collision energy of 20–45 eV. Spectra data of the investigated compounds were collected from *m*/*z* 50–1300 with a resolution of 50,000 at an acquisition rate of 5 spectrums per second. The ions of interest were selected by auto MS/MS scan fragmentation. The data processing software used was DataAnalysis 4.3 (Bruker). To identify the compounds, a library of already identified compounds isolated from the genera *Aspergillus* and *Chromolaena* was made, with annotations based on the values of mass error and fragmentation profile/characteristic fragment ions for the compounds. The mass error value was calculated using the following equation: Error (ppm) = $[(M_{calc} - M_{exp})/M_{calc}] \times 10^6$, where M_{calc} is the exact theoretical mass and M_{exp} is the exact experimental mass obtained. Only molecular formulas with ≤ 10 ppm of error were considered in this study.

Antimicrobial activity

Antimicrobial assays were performed by the microdilution method in sterile flat-bottom microplates, according to CLSI [30–32]. The bacteria *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6623, and *Staphylococcus aureus* ATCC 25923 were cultivated in Mueller–Hinton broth (MHB) (Difco) at 37 °C. The yeasts *Candida albicans* ATCC 10231 and *C. parapsilosis* ATCC 22019 were cultivated in Sabouraud dextrose broth (SDB) (Difco) at 37 °C. And the dermatophyte fungi *Trichophyton rubrum* ATCC 28289 and *Microsporum gypseum* ATCC 14683 were cultivated in SDB (Difco) at 28 °C.

Each well contained appropriate test samples, culture medium and approximately 10^5 cells mL^{-1} for bacteria, 10^3 cells mL^{-1} for yeasts, and 10^4 spores mL^{-1} for dermatophyte fungi. Serial three-fold dilutions of each extract were done in a microdilution plate (96 wells) containing 100 μL of culture medium. Next, the inoculum was added to each well. The microplates were incubated at 37 °C for 24 h for bacteria and 48 h for yeast and at 28 °C for 72 h for dermatophyte fungi. The MIC was defined as the lowest concentration which resulted in inhibition of visual growth. Minimum bactericidal and fungicidal concentrations (MBC and MFC) were determined by subculturing 10 μL of the culture from each negative well and from the positive control.

Anti-*M. tuberculosis* activity was performed using a resazurin microtiter assay plate (REMA) [33]. Firstly, crude extract PD-S, PD-A, MM-S, and MM-A stock solutions in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and two-fold serial dilutions were prepared in supplemented Middlebrook 7H9 to obtain final concentrations ranging from 31.25 to 1000 μg mL^{-1}. Then, 100 μL of each standardized inoculum was added to each well of the microplate containing the crude extract dilutions. The microplates were covered with their lids and incubated at 35 °C, in a normal atmosphere, for 7 days. After this period, 30 μL of fresh 0.01% resazurin solution (Acros, Morris Plains, NJ, USA) was added to each well and the microplates were re-incubated at 35 °C for an additional 24 h before visual reading was carried out. A color change from blue to pink indicated mycobacterial growth and the MIC was interpreted as the lowest crude extract concentration that prevented the color change from blue to pink. The medium, drug sterility, and bacterial growth with and without 2.5% (*v*/*v*) DMSO controls were included in all tests.

Antioxidant activity

The antioxidant activity of the crude extracts PD-S, PD-A, MM-S, and MM-A was evaluated using three different methodologies: DPPH, ABTS, and evaluation of phenolic compounds. To perform the tests, the samples were solubilized

at a concentration of 1 mg mL^{-1} in distilled water with 5% methanol [34].

To evaluate the antioxidant activity of crude extracts of metabolites by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical method, the methodology of Ma et al. was used [35]; 25 μL of the samples and 2 mL of 6.25×10^{-5} mol L^{-1} DPPH solution was added into light-protected glass tubes kept in the dark for 30 min. Absorbance was measured at 517 nm, and the results were expressed as millimoles of Trolox per milligram of sample (mmol Trolox mg $sample^{-1}$).

The ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) method was performed according to the method described by Rajurkar and Hande [36]. Prior to the test, a solution of the $ABTS^{+}$ radical containing 5 mL of ABTS solution (7 mM) and 88 μL of potassium persulfate (140 mM) was prepared, remaining in reaction for 16 h in the dark. After this period, 3 mL of the $ABTS^{+}$ solution was transferred into tubes protected from the light; 30 μL of the extract from each sample was added and the solution was kept in the dark for 6 min. Absorbance was measured at 734 nm, and the results were expressed as millimoles of Trolox per milligram of sample (mmol Trolox mg $sample^{-1}$).

The total phenolic content was determined using the Folin–Ciocalteu method as described by Singleton and Rossi, with modifications [37]. One aliquot (125 μL) of each sample was mixed with 125 μL of the Folin–Ciocalteu reagent (1 : 1 *v/v* distilled water) and 2250 μL of 3.79 M sodium carbonate solution. This mixture was kept in the dark for 30 min. Absorbance was measured at 725 nm, and the results were expressed as micrograms of gallic acid equivalent per milligram of extract (μg GAE mg $sample^{-1}$).

Cytotoxic and antiviral activity

The cytotoxicity of the crude extracts PD-S, PD-A, MM-S, and MM-A from fungus *A. flavus* CL7 at concentrations from 31.25 to 1000 μg mL^{-1} was evaluated on a monolayer of Vero cells (CCL-81, American Type Culture Collection) prepared in 96-well plates at an initial density of 2.5×10^{5} cells mL^{-1} and incubated at 37 °C for 72 h in a humid oven with 5% CO_2. To determine antiviral activity, the cells were pre-infected with HSV-1 $TCID_{50}$ (KOS strain) and incubated for 1 h at 37 °C for adsorption and viral penetration, and subsequently treated with different concentrations of the crude extracts (3.125–100 μg mL^{-1}) for 72 h at 37 °C. In both cases, cell viability was determined by the MTT method, and the CC_{50} (cytotoxic concentration for 50% of cells) and EC_{50} (effective concentration that protects 50% of viral infection) were calculated by linear regression analysis [38]. Acyclovir (Sigma-Aldrich, 99% purity) was used as a positive control for anti-HSV-1 activity. All concentrations were tested in triplicate in at least three independent experiments and the results were expressed as mean ± standard deviation.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8.0.1. The differences among treatments in each experiment were compared using one-way analysis of variance (ANOVA) followed by Tukey's test. In all cases, the threshold for significance was 5%.

Results

Multiphasic identification of CL7

A multiphasic approach was used to identify strain CL7, based on morphology and phylogenetic analysis. The ITS sequence of strain CL7 showed higher values of identity with *A. flavus* species by BLAST analysis in GenBank (100% identity). Therefore, phylogenetic analysis was conducted with the aim of confirming the identification at species level, considering the *A. flavus* clade according to Frisvad et al. [12]. The results show that strain CL7 has greater phylogenetic proximity to *A. flavus* and *A. oryzae* with 100% Bayesian probability (Fig. 1a).

The macro- and microstructures corroborate this identification (Fig. 1b–d). The colonies are a yellowish-green color, with conidiophores and large sclerotia characteristic of these species. In addition, it may be observed in subsequent sections that the chemical compounds produced by these strains match the literature data. Thus, to the best of our knowledge, this is the first time that *A. flavus* is described as an endophyte from the Eupatorieae tribe.

Culture conditions

Observing the macroscopic characteristics of *A. flavus* (CL7) cultured in different conditions (Fig. 2), it was possible to notice the formation of a mycelium with an intense yellow color, and the presence of a large amount of exudate when the PD-S methodology was used (Fig. 2a). On the other hand, when *A. flavus* CL7 was cultivated in MM using the stationary method (MM-S), the mycelium of the fungus started to present a predominantly dark green color, and the amount of exudate on the surface was reduced (Fig. 2c). In the agitated cultures, with both PD and MM media, the fungus developed in the form of pellets, very similarly in both media (Fig. 2b and d).

The greatest dry biomass production was obtained when MM-S conditions were used (3.407 ± 0.088 g), followed by PD-A and PD-S that had statistically the same production, 2.601 ± 0.186 g, and 2.589 ± 0.035 g, respectively. On the other hand, the highest production of total metabolites dissolved in ethyl acetate was obtained using PD-S methodology,

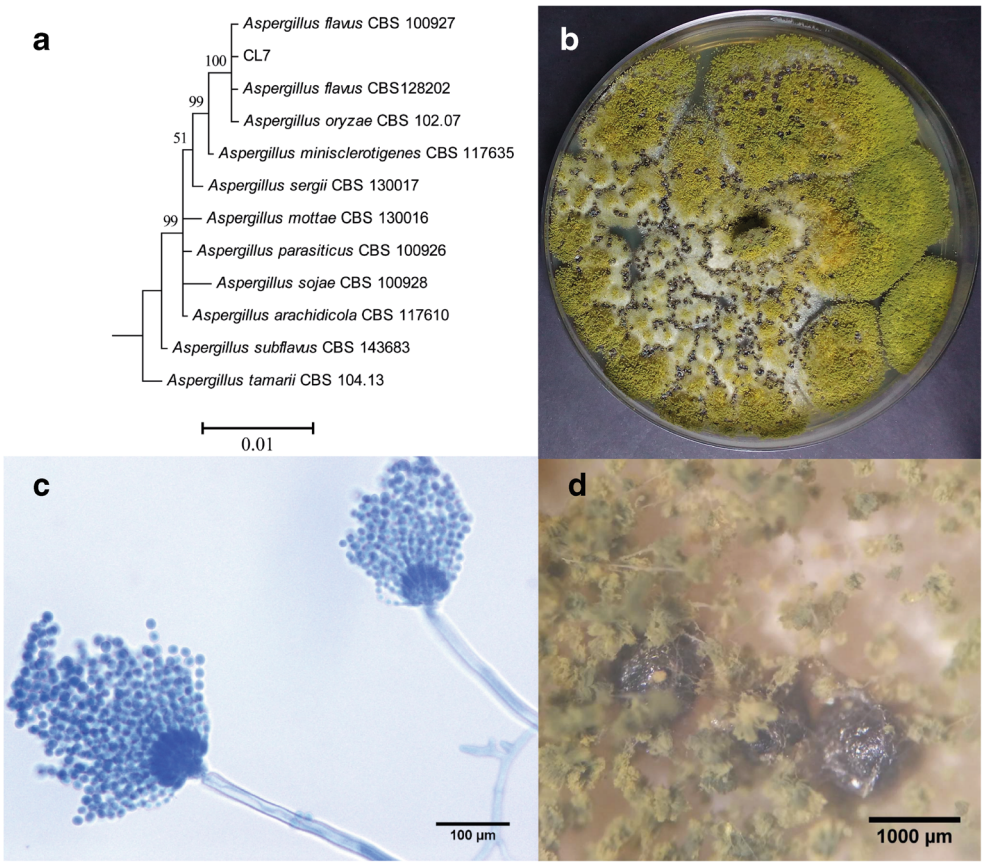

Fig. 1 Phylogenetic analysis and macro- and micromorphological structures of *Aspergillus flavus* CL7. **a** Cladogram obtained from a Bayesian inference based on the alignment of ITS gene. Bayesian probability was demonstrated at the nodes between each organism. The strain *Aspergillus tamarii* CBS 104.13 was used as external group; **b** 7-day-old culture colony on potato dextrose agar medium; **c** Conidiophores and conidia; **d** large sclerotia

163.8 ± 20.7 g of metabolite extract, and only 11.8 ± 2.0 g was obtained using MM-S (Table S1).

Chemical constituents

Chemical investigation of the endophytic fungus *A. flavus* CL7, associated with the leaves of *C. laevigata*, yielded three compounds, identified by comparison of their spectroscopic 1D and 2D NMR data with those reported in the literature.

Compound **1** was identified as kojic acid [39], and it was obtained from the culture of *A. flavus* CL7 grown on PD medium using the stationary and agitated methods (Table S2; Figs. S1–S5). Compound **2** was isolated from the *A. flavus* CL7 culture grown on PD medium under agitation and basal MM using the stationary method, and it was identified as cyclopiazonic acid (**2**) (Table S3; Figs. S6–S17) [40]. Compound **3**, 20,25-dihydroxyaflavinine, was isolated only from culture on basal MM using the stationary method (Table S4; Figs. S18–S32) [41].

To provide comprehensive coverage of the *A. flavus* metabolome, fungal extracts PD-S, PD-A, MM-S, and MM-A were analyzed in both positive and negative ion modes by UHPLC-HRMS/MS. This strategy allowed the identification of 21 compounds (Table 1; Table S5).

Antimicrobial assay

The antimicrobial activity of crude extracts obtained from the different cultures of *A. flavus* CL7 was evaluated against four pathogenic bacterial strains, two Gram-negative (*P. aeruginosa* and *E. coli*) and two Gram-positive (*B. subtilis* and *S. aureus*), and four fungal strains (*Candida albicans*, *C. parapsilosis*, *T. rubrum*, and *M. gypseum*).

The crude extracts were inactive against the Gram-negative bacteria; however, they were active against the Gram-positive bacteria, with the exception of PD-S which was inactive against all microorganisms evaluated. The best results were observed for the extract obtained using MM against the dermatophyte *T. rubrum* (Table 2).

The crude extracts were also evaluated against *M. tuberculosis* H37Rv, using the REMA technique. As observed for the other microorganisms, PD-S was the least active extract, and the best result was observed for MM-S, with an MIC of 62.5 μg mL^{-1}.

Antioxidant assay

The antioxidant potential of *A. flavus* CL7 extracts, obtained in different culture media, was determined using DPPH and

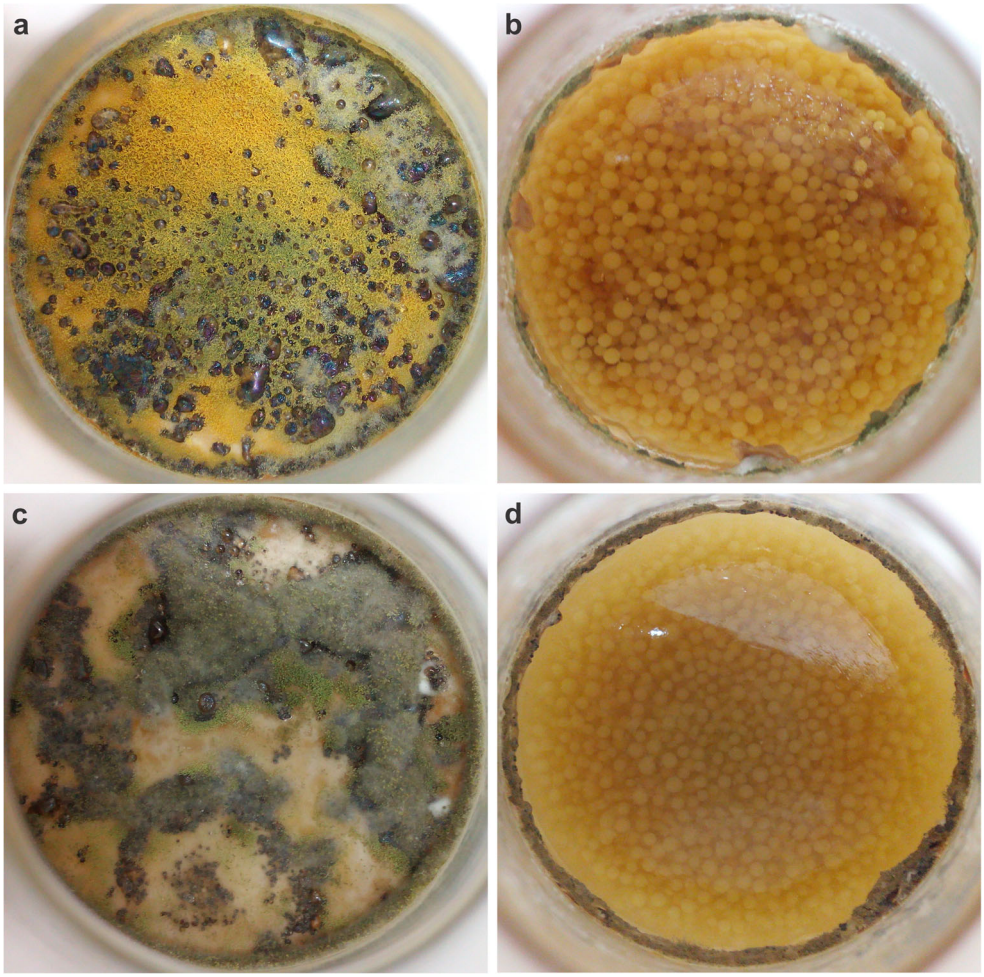

Fig. 2 Micrograph of the mycelium of the endophytic fungus Aspergillus flavus CL7 under different conditions after 21 days of cultivation. a) Potato Dextrose Stationary (PD-S); b) Potato Dextrose Agitated (PD-A); c) Minimum Medium Stationary (MM-S); d) Minimum Medium Agitated (MM-A)

ABTS methods, together with the total phenolic content (TPC) (Table 3). Crude extracts PD-S (93.85 μg GAE mg sample^{-1}) and PD-A (84.22 μg GAE mg sample^{-1}) presented the highest levels of phenols and, consequently, they showed potent antioxidant activity; PD-S exhibited higher scavenging potential for the ABTS method (2.29 mmol Trolox mg sample^{-1}; 98.97%) and PD-A exhibited higher scavenging potential for the DPPH method (0.23 mmol Trolox mg sample^{-1}; 8.85%).

Cytotoxic and antiviral assays

The evaluation of cytotoxicity showed that among all crude extracts tested, PD-S was the least toxic to the cells. However, all crude extracts were inactive, at the concentrations evaluated, for cells infected with HSV-1 $TCID_{50}$ (KOS strain) (Table 4).

Discussion

As mentioned before, our research group has been studying species from the Eupatorieae tribe, especially from the genus *Eupatorium* sensu lato; based on the fact that there are few reports on endophytic fungi in species of this genus, *Chromolaena laevigata* (basionym: *Eupatorium laevigatum*) was selected for this study [2–5].

An endophytic fungus from the genus *Phomopsis* has been described from *Chromolaena arnottiana* (basionym: *Eupatorium arnottianum*) [42]. Six morphologically different endophytes have been found to inhabit the leaves of *Ageratina adenophora* (basionym: *Eupatorium adenophorum*); four morphotypes are close to *Alternaria*, *Cladosporium*, *Pestalotiopsis*, and *Didymella*, while two morphotypes are close to unidentified fungi [43]. Three endophytic fungi have been identified from *Eupatorium buniifolium*; they were identified as *Fusarium solani*, *Alternaria alternata*, and *Neofusicoccum* sp. [44].

The endophytic fungus isolated from *Chromolaena laevigata* in this work was identified as *Aspergillus flavus*. Endophytic *A. flavus* has been previously isolated from many plant species, for example, from *Paspalum maritimum* Trin. [45], *Aegle marmelos* [46, 47], *Cephalotaxus fortunei* [48], *Tylophora ovata* [49], *Catharanthus roseus*, *Annona squamosa*, and *Curcuma xanthorrhiza* [50]. So, this is the

Table 1 Specialized metabolites identified of *Aspergillus flavus* CL7 extracts by UHPLC-HRMS/MS

Compounds	Extracts			
	PD-S	PD-A	MM-S	MM-A
α-Cyclopiazonic acid (**2**)	+	+	+	+
2-Oxo-cyclopiazonic acid	+	+	+	+
Kojic acid (**1**)	+	+	+	
Phomaligin A	+	+	+	
Asparasone A	+		+	+
Aspergillic acid	+		+	+
Aspyrone	+	+		
20,25-dihydroxyaflavinine (**3**)	+		+	
Itaconic acid	+		+	
Paspaline	+		+	
Hydroxysydonic acid		+		+
Terrein		+		+
14-Deacetyl parasiticolide A	+			
Dideacetyl parasiticolide A	+			
Spinulosin	+			
Citreoisocoumarin		+		
Gregatin B		+		
Podophyllotoxin		+		
Flufuran			+	
Speradine F			+	
α-Cyclopiazonic acid imine				+

The identification of the compounds was done by comparison of their exact masses and fragmentation patterners to reported literature (see Online Resource 1, Table S5)

PD-S potato dextrose stationary, *PD-A* potato dextrose agitated, *MM-S* minimum medium stationary, *MM-A* minimum medium agitated

second study about endophytes from *Chromolaena* species, and the first time that endophytic *Aspergillus* has been described from *Eupatorium* sensu lato.

Characterization of the fungus as *Aspergillus flavus* was based on BLAST analysis in GenBank; however, the characteristics of the macro- and microstructure were essential for confirming the species. Many studies have indicated that *A. oryzae* is the domesticated form of *A. flavus* and can be distinguished from the wild type due to many factors, among them the presence of larger and more smooth conidia, more floccose aerial mycelia and weaker sporulation, the absence of sclerotia, no production of aspergillic acid, and a lack of aflatoxin production [12, 51–53]. According to Frisvad et al., *A. oryzae* is isolated from fermented products, while strains from other environments, even if they are not aflatoxin producers, are identified as *A. flavus* [12]. Therefore, based on the phylogenetic analysis performed on the type of host, on the macroscopic characteristics of the culture together with the presence of large sclerotia and the profile of identified secondary metabolites, strain CL7 was confirmed as being the species *A. flavus*.

Macroscopic differences in cultures were observed when the nutrients in the media were changed, and also when the cultures were agitated (Fig. 2). A culture medium must have a source of carbon and nitrogen, in addition to mineral salts and some other nutrients, and the constitution of the medium directly influences the growth of the mycelium and the production of bioactive metabolites [54–56]. When PD was used as the medium, greater production of spores was observed compared to culture in MM. The highest dry biomass was observed for the MM-S condition; however, it provided the smallest amount of metabolite extract. Although cultivation in PD produced a similar dry biomass using agitated and stationary conditions, production of the metabolite extract was 240% higher when the stationary method was used.

The effect of culture conditions on the production of specialized metabolites by the endophytic fungus *A. flavus* CL7 was also investigated in this work. First, the metabolite extracts were submitted to a CC, and three metabolites were isolated. Kojic acid (**1**) was the major compound found in PD-S extract, and it was identified directly in this extract, without purification. This compound is widely described from *Aspergillus* genus, having already been found for *A. aflatoxiformans*, *A. alliaceus*, *A. arachidicola*, *A. aspearensis*, *A. austwickii*, *A. bombycis*, *A. bertholletius*, *A. caelatus*, *A. cerealis*, *A. flavus*, *A. flocculus*, *A. hancockii*, *A. lanosus*, *A. leporis*, *A. luteovirescens*, *A. minisclerotigenes*, *A. mottae*, *A. neoalliaceus*, *A. nomius*, *A. novoparasiticus*, *A. oryzae*, *A. parasiticus*, *A. parviscierotigenus*, *A. pseudocaelatus*, *A. pseudonomius*, *A. pseudotamarii*, *A. sergii*, *A. sojae*, *A. subflavus*, *A. tamarii*, *A. transmontanensis*, and *A. vandermerwei* [12, 57, 58]. Kojic acid has also been identified as a major compound from *A. flavus* associated with *Catharanthus roseus*, *Annona squamosa*, and *Curcuma xanthorrhiza* [50].

Cyclopiazonic acid (**2**), an indole tetramic acid, was described for the first time from *A. flavus* in 1977 [59]. However, this metabolite is briefly described from endophytic *Aspergillus*, being reported from the marine-derived fungus *A. flavus* [60–62]. For plant-derived fungus, this metabolite was described from *A. flavus* isolated from *Triticum aestivum*, and from *A. versicolor* isolated from *Paris polyphylla* [63, 64].

Compound **3**, 20,25-dihydroxyaflavinine, is frequently described from the sclerotia of *A. flavus*; however, this is the first time that this compound has been isolated from an endophytic *A. flavus* strain [41, 65–68].

Other compounds were putatively identified using the dereplication technique by UHPLC-HRMS/MS. In PD, 18 compounds were identified, and 15 compounds were detected in MM. Only cyclopiazonic acid and its derivative 2-oxo-

Table 2 Antimicrobial activity of *Aspergillus flavus* CL7 extracts (MIC, MBC, or MFC)

Microorganisms	MIC/MBC or MFC (μg mL^{-1})				
	Extracts				Ref. antibiotics
	PD-S	PD-A	MM-S	MM-A	
Pseudomonas aeruginosa	> 1000	> 1000	> 1000	> 1000	3.125[a]
Escherichia coli	> 1000	> 1000	> 1000	> 1000	1[a]
Bacillus subtilis	1000/> 1000	125/500	250/500	125/250	0.2[b]
Staphylococcus aureus	1000/> 1000	250/500	500/1000	250/> 1000	0.02[c]
Candida albicans	1000/> 1000	250/500	> 1000	250/> 1000	3.9[d]
Candida parapsilosis	> 1000	500/> 1000	> 1000	500/> 1000	3.9[d]
Trichophyton rubrum	> 1000	500/> 1000	31.2/> 1000	31.2/1000	2.5[d]
Microsporum gypseum	> 1000	125/125	125/1000	250/1000	2.5[d]
Mycobacterium tuberculosis	250	125	62.5	125	0.125[e]

The highest concentration tested was 1000 μg mL^{-1}. Values expressed as > 1000 μg mL^{-1} indicate that there was no inhibition of growth of the microorganisms at the concentrations tested. Tests were performed in triplicate

Data from three independent experiments

PD-S potato dextrose stationary, *PD-A* potato dextrose agitated, *MM-S* minimum medium stationary, *MM-A* minimum medium agitated

[a] Tetracycline

[b] Vancomycin

[c] Penicillin

[d] Nystatin

[e] Rifampicin

cyclopiazonic acid were produced in all culture media evaluated. Comparing the metabolites produced when the culture of *A. flavus* was grown in PD using the stationary method, with those produced in the same medium but under agitation, considerable variation was observed: only five substances were common between them (aspyrone, α-cyclopiazonic acid, 2-oxo-cyclopiazonic acid, kojic acid, and phomaligin A). For culture in MM, the number was even lower: only four compounds were common (asparasone A, aspergillic acid, α-cyclopiazonic acid, and 2-oxo-cyclopiazonic acid).

The principal variation was observed when the agitation method was applied. It was also possible to verify that some compounds were produced in both culture media; however, a variation in the metabolites produced was observed when the culture was carried out under agitation or without agitation. Itaconic acid, paspaline, and 20,25-dihydroxyaflavinine were detected from both culture media when they were kept without agitation. On the other hand, hydroxysydonic acid and terrein were identified when the media were kept under agitation. These results corroborate the variable effects of medium

Table 3 Antioxidant activity by DPPH, ABTS, and total phenolic compounds of *Aspergillus flavus* CL7 extracts

Extracts	DPPH		ABTS		Total phenolic compounds	
	mmol trolox/mg	% activity	mmol trolox/mg	% activity	μg GAE/mg	% activity
PD-S	0.07 ± 0.00a	2.16 ± 0.10a	2.29 ± 0.01a	98.97 ± 0.22a	93.85 ± 16.27a	13.71 ± 2.38a
PD-A	0.23 ± 0.01b	8.85 ± 0.34b	0.95 ± 0.03b	41.64 ± 1.24b	84.22 ± 17.32a	12.71 ± 2.61ac
MM-S	0.21 ± 0.01b	8.15 ± 0.49b	0.67 ± 0.07c	29.57 ± 2.92c	49.78 ± 6.67b	9.09 ± 1.22ac
MM-A	0.05 ± 0.01a	1.29 ± 0.23c	0.41 ± 0.04d	18.28 ± 1.90d	8.67 ± 2.22c	3.46 ± 0.89bd

Data expressed as mean ± standard deviation of three independent experiments. In the same raw, the values marked with the same lowercase letter are similar ($p > 0.05$), whereas those marked with different ones are significantly different ($p < 0.05$)

PD-S potato dextrose stationary, *PD-A* potato dextrose agitated, *MM-S* minimum medium stationary, *MM-A* minimum medium agitated, *GAE* gallic acid equivalent

Table 4 Cytotoxicity and antiviral activity of *Aspergillus flavus* CL7 extracts by the MTT method

Extracts	CC_{50} (μg mL^{-1})	EC_{50} (μg mL^{-1})
PD-S	> 1000	> 100
PD-A	60.17 ± 7.02a	> 100
MM-S	40.17 ± 2.75b	> 100
MM-A	21.08 ± 1.38c	> 100
Acyclovir	> 1000	0.1115 ± 0.005

The highest concentration tested was 1000 μg mL^{-1} for cytotoxicity assay and 100 μg mL^{-1} for antiviral activity. Tests were performed in triplicate

Data expressed as mean ± standard deviation of three independent experiments. In the same raw, the values marked with the same lowercase letter are similar ($p > 0.05$), whereas those marked with different ones are significant

PD-S potato dextrose stationary, *PD-A* potato dextrose agitated, *MM-S* minimum medium stationary, *MM-A* minimum medium agitated, CC_{50} cytotoxic concentration for 50% of cells, EC_{50} effective concentration that protects 50% of viral infection

conditions on the production of specialized fungal metabolites [18, 19].

The major compounds produced by *A. flavus* are mycotoxins, especially aflatoxins and cyclopiazonic acid derivatives, and it has been demonstrated that the ability to produce these toxins can vary considerably [69–71]. It was previously reported that the culture of *A. flavus* in PD enhances the formation of aflatoxins as compared to CZPY medium, and it was also shown that cyclopiazonic acid is produced in liquid static culture in much greater concentrations than aflatoxins [69–72]. However, in our work, only cyclopiazonic acid was isolated, even using PD under agitation, and aflatoxins were not found.

The results from the chemical investigation of *A. flavus* have revealed constituents that agree with the identification, classification, and chemotaxonomy of this fungal strain. However, no aflatoxins were identified, which are chemomarkers of this species; so, to provide comprehensive coverage of the *A. flavus* metabolome, all extracts were submitted for analysis by UHPLC-HRMS/MS. Table 1 shows the secondary metabolites identified, which corroborate the classification of our strain as *A. flavus*. Only compounds already described from this species were identified, except for dideacetyl parasiticolide A, which is described here for the first time in this species [12, 73–76]. However, aflatoxins were not detected in these extracts which were obtained by different methods. The absence of these chemomarkers allows us to indicate strain CL7 as a non-aflatoxigenic *A. flavus*.

Regarding the possible applications of secondary metabolite extracts obtained by different methods of culturing the endophyte CL7, the antimicrobial activity of extracts was evaluated. Despite the extracts showing higher MIC than the reference antibiotics, some of them showed promising values. According to Holetz et al., extracts with an MIC below 100 μg mL^{-1} present good antimicrobial activity; between 100 and 500 μg mL^{-1} indicates moderate antimicrobial activity and from 500 to 1000 μg mL^{-1} indicates weak antimicrobial activity; above 1000 μg mL^{-1}, the extract may be considered inactive [77]. In this context, PD-S extract did not present antimicrobial activity against the microorganisms tested, since the MIC was greater than 1000 μg mL^{-1}. On the other hand, the PD-A extract, which used the same culture medium but was cultured on a shaker, showed an improvement in antimicrobial activity, with moderate activity against *C. albicans*, *T. rubrum*, *S. aureus*, *B. subtilis*, and *M. gypseum*. In addition, for the dermatophyte fungus *M. gypseum*, it was observed that the microorganism died at the same MIC (MIC = MFC). This activity may be attributed to the podophyllotoxin detected in this extract, which is known to have antimicrobial properties [78].

The best results were observed for the extract obtained using MM against the dermatophyte *T. rubrum*, with an MIC of 31.2 μg mL^{-1}. This may be related to the presence of aspergillic acid in this extract, which has been described as an antibiotic and antifungal agent that is derived from certain *A. flavus* strains [79]. Although this metabolite was also detected in PD-S, and this extract did not show antimicrobial activity, this could be related to the concentration of aspergillic acid in the extract as the major compound in the PD-S extract is kojic acid.

In evaluating anti-tuberculosis activity, Tosun et al. considered inactive those extracts that could not prevent the growth of *M. tuberculosis* up to a concentration of 200 μg mL^{-1} and, according to Gu et al., an MIC of ≤ 128 μg mL^{-1} is defined as active against *M. tuberculosis*. By these measures, only PD-S extract showed no significant activity, and MM-S extract was the most potent inhibitor of *M. tuberculosis* proliferation [80, 81].

Among the extracts evaluated, PD-S was the least toxic against Vero cells, since its CC_{50} was higher than the highest concentration tested (1000 μg mL^{-1}). On the other hand, the extracts PD-A, MM-S, and MM-A were highly cytotoxic to Vero cells, with CC_{50} of 60.17, 40.17, and 21.08 μg mL^{-1}, respectively. The high toxicity of extracts grown in MM and those grown on PD under shaking conditions is probably related to the compounds isolated from these fractions, such as α-cyclopiazonic acid and 20,25-dihydroxyaflavinine [82]. Regarding antiviral activity, the extracts did not show a significant inhibition capacity for HSV-1 virus (KOS strain), since the EC_{50} were all higher than 100 μg mL^{-1}, whereas for the standard drug (acyclovir), the value determined was 0.11 μg mL^{-1}.

The extract of the metabolites of the endophyte *A. flavus* CL7 obtained by steady-state culture in PD presented high antioxidant potential by the ABTS method, with almost 99%

antioxidant activity. This result was probably due to the major presence of kojic acid (**1**), an important antioxidant used by the cosmetics industry [83].

In summary, we can highlight the identification of a non-aflatoxigenic strain of *A. flavus* from a new host *C. laevigata*. Dereplication analysis by UHPLC-HRMS/MS enabled the identification of 21 compounds, one of which, dideacetyl parasiticolide A, is being reported for the first time in this species. This work also corroborates the direct influence of culture medium on the production of specialized metabolites. In addition, the biological activity of the different extracts was evaluated; the extract obtained from stationary culture on MM proved to be the most active in general, mainly due to the MIC obtained against *T. rubrum* and *M. tuberculosis*, two pathogenic microorganisms that affect the population and that are becoming more and more resistant to conventional therapies. Thus, we describe a strain of *A. flavus* with biotechnological potential for use as a biocontrol tool, as a source of biomolecules active against other pathogenic microorganisms and as a promising producer of kojic acid, an important metabolite used in the food and beauty industries.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42770-021-00502-6.

Acknowledgements We are grateful to Concelho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for fellowship (D.I. Bernardi, J.A. Pamphile, and M.H. Sarragiotto), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (J.A.M. Oliveira), and Fundação Araucária (R.B. Balbinot). This study was funded by the CAPES and Instituto Nacional de Ciência e Tecnologia Biodiversidade e Produtos Naturais–INCTBioNat (CNPq 465637/2014-0). This work also was supported by the Complexo de Central de Apoio à Pesquisa (COMCAP) from Universidade Estadual de Maringá (UEM).

Author contribution RBB, JAP, and DCB conceived and designed research. RBB, JAMO, DIB, ADP, and JCP conducted experiments. MRBC, MHS, MRPC, and JAP contributed new reagents or analytical tools. EBZ, EHE, JEM, RFC, BDPF, and TUN realized biological activities. RBB, JAMO, and JCP analyzed data. RBB, JAMO, DIB, ADP, JCP, and DCB wrote the manuscript. All authors read and approved the manuscript.

Funding This work was financially sponsored by Concelho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)-Finance Code 001, Fundação Araucária and Instituto Nacional de Ciência e Tecnologia Biodiversidade e Produtos Naturais–INCTBioNat (CNPq 465637/2014-0).

Declarations

Conflict of interest The authors declare no competing interests.

References

1. Tan RX, Zou WX (2001) Endophytes: a rich source of functional metabolites. Nat Prod Rep 18(4):448–459. https://doi.org/10.1039/b100918o
2. Bernardi DI, Moreno BP, Oliveira JAMD, Carmo MRBD, Sarragiotto MH, Baldoqui DC (2016) Chemotaxonomic implications of the absence of sesquiterpene lactones in *Grazielia multifida* (DC.) R.M.King&H.Rob. (Asteraceae). Biochem Syst Ecol 69:15–17. https://doi.org/10.1016/j.bse.2016.08.006
3. Oliveira JAMD, Bernardi DI, Balbinot RB, Avíncola ADS, Pilau E, Carmo MRB, Sarragiotto MH, Baldoqui DC (2017) Chemotaxonomic value of flavonoids in *Chromolaena congesta* (Asteraceae). Biochem Syst Ecol 70:7–13. https://doi.org/10.1016/j.bse.2016.10.013
4. Balbinot RB, Oliveira JAMD, Bernardi DI, Melo UZ, Zanqueta EB, Endo EH, Ribeiro FM, Volpato H, Figueiredo MC, Back DF, Basso EA, Ruiz ALTG, Carvalho JED, Foglio MA, Prado Filho BD, Nakamura TU, Nakamura CV, Carmo MRBD, Sarragiotto MH, Baldoqui DC (2019) Structural characterization and biological evaluation of 18-nor-ent-labdane diterpenoids from *Grazielia gaudichaudeana* (DC.) R. M. King & H. Rob. (Asteraceae). Chem Biodivers 16(5):e1800644. https://doi.org/10.1002/cbdv.201800644
5. Oliveira JAMD, Bernardi DI, Balbinot RB, Cabral MRP, Zanqueta EB, Endo EH, Prado Filho BD, Nakamura TU, Figueiredo MC, Ruiz ALTG, Foglio MA, Carmo MRB, Sarragiotto MH, Baldoqui DC (2020) New cadinene-sesquiterpene from the aerial parts of *Chromolaena laevigata* (Lam.) R. M. King & H. Rob (*Asteraceae*) and biological activities. Nat Prod Res. https://doi.org/10.1080/14786419.2020.1747456
6. King RM, Robinson H (1970) Studies in the Eupatorieae (Compositae). XXIX The genus *Chromolaena*. Phytologia 20:196–209. https://doi.org/10.5962/bhl.part.7118
7. Oliveira CT (2015) *Chromolaena* in: Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB16058 (Accessed: 5 January 2020)
8. Murakami C, Lago JHG, Perazzo FF, Ferreira KS, Lima MEL, Moreno PRH, Young MCM (2013) Chemical composition and antimicrobial activity of essential oils from *Chromolaena laevigata* during flowering and fruiting stages. Chem Biodivers 10(4):621–627. https://doi.org/10.1002/cbdv.201200025
9. Misra LN, Jakupovic J, Bohlmann F, Schmeda-Hirschmann G (1985) Isodaucane derivatives, norsesquiterpenes and clerodanes from *Chromolaena laevigata*. Tetrahedron 41:5353–5356. https://doi.org/10.1016/S0040-4020(01)96789-4
10. Amare MG, Keller NP (2014) Molecular mechanisms of *Aspergillus flavus* secondary metabolism and development. Fungal Genet Biol 66:11–18. https://doi.org/10.1016/j.fgb.2014.02.008
11. Taniwaki MH, Pitt JI, Megan N (2018) *Aspergillus* species and mycotoxins: occurrence and importance in major food commodities. Curr Opin Food Sci 23:38–43. https://doi.org/10.1016/j.cofs.2018.05.008
12. Frisvad JC, Hubka V, Ezekiel CN, Hong SB, Nováková A, Chen AJ, Arzanlou M, Larsen TO, Sklenár F, Mahakarnchanakul W, Samson RA, Houbraken J (2019) Taxonomy of *Aspergillus* section *Flavi* and their production of aflatoxins, ochratoxins and other mycotoxins. Stud Mycol 93:1–63. https://doi.org/10.1016/j.simyco.2018.06.001
13. Yu J, Whitelaw CA, Nierman WC, Bhatnagar D, Cleveland TE (2004) *Aspergillus flavus* expressed sequence tags for identification of genes with putative roles in aflatoxin contamination of crops. FEMS Microbiol Lett 237:333–340. https://doi.org/10.1016/j.femsle.2004.06.054
14. Hedayati MT, Pasqualotto AC, Warn PA, Bowyer P, Denning DW (2007) *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. Microbiology 153:1677–1692. https://doi.org/10.1099/mic.0.2007/007641-0

15. Couto SR, Sanromán MA (2006) Application of solid-state fermentation to food industry – a review. J Food Eng 76(3):291–302. https://doi.org/10.1016/j.jfoodeng.2005.05.022
16. Managurumi U, Vijayalakshmi M, Poda S, Ganduri VSRK, Badu RS (2016) Optimization of culture conditions by response surface methodology and unstructured kinetic modeling for bioactive metabolite production by *Nocardiopsis litoralis* VSM-8. 3 Biotechnol 6:219. https://doi.org/10.1007/s13205-016-0535-2
17. Guo F, Li X, Zhao J, Li G, Gao P, Han X (2019) Optimizing culture conditions by statistical approach to enhance production of pectinase from *Bacillus* sp. Y1. Biomed Res Int 2019:8146948. https://doi.org/10.1155/2019/8146948
18. Bode HB, Bethe B, Hofs R, Zeeck A (2002) Big effects from small changes: possible ways to explore nature's chemical diversity. ChemBioChem 3:619–627. https://doi.org/10.1002/1439-7633(20020703)3:7<619::AID-CBIC619>3.0.CO;2-9
19. Miao L, Kwong TFN, Qian PY (2006) Effect of culture conditions on mycelial growth, antibacterial activity, and metabolite profiles of the marine-derived fungus *Arthrinium c.f. saccharicola*. Appl Microbiol Biotechnol 72:1063–1073. https://doi.org/10.1007/s00253-006-0376-8
20. Xu P, Ding Z-Y, Qian Z, Zhao CX, Zhang KC (2008) Improved production od mycelial biomass and ganoderic acid by submerged culture of *Ganoderma lucidum* SB97 using complex media. Enzym Microb Technol 42:325–331. https://doi.org/10.3390/molecules171112575
21. Dinarvand M, Rezaee M, Foroughi M (2017) Optimizing culture conditions for production of intra e extracellular inulinase and invertase from *Aspergillus niger* ATCC 20611 by response surface methodology (RSM). Braz J Microbiol 48:427–441. https://doi.org/10.1016/j.bjm.2016.10.026
22. Pu X, Qu X, Chen F, Bao J, Zhang G, Luo Y (2013) Camptothecin-producing endophytic fungus *Trichoderma atroviride* LY357: isolation, identification, and fermentation conditions optimization for camptothecin production. Appl Microbiol Biotechnol 97:9365–9375. https://doi.org/10.1007/s00253-013-5163-8
23. Tangerina MMP, Furtado LC, Leite VMB, Bauermeister A, Velasco-Alzate K, Jimenez PC, Garrido LM, Padilla G, Lopes NP, Costa-Lotufo LV, Ferreira MJP (2020) Metabolic study of marine *Streptomyces* sp.: secondary metabolites and the production of potential anticancer compounds. PLoS One 15(12):e0244385. https://doi.org/10.1371/journal.pone.0244385
24. White TJ, Bruns TD, Lee S, Taylor JW (1990) PCR protocols: a guide to methods and applications. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Academic Press, San Diego, pp 315–322
25. Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinform 9(4):286–298. https://doi.org/10.1093/bib/bbn013
26. Nylander JAA (2004) *Mrmodeltest* version 2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala. https://github.com/nylander/MrModeltest2/releases/tag/v2.3. Accessed 30 Mar 2019
27. Ronquist F, Teslenko M, Mark PVD, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61(3):539–542. https://doi.org/10.1093/sysbio/sys029
28. Rambaut A (2009) *FigTree*. Tree figure drawing tool. http://tree.bio.ed.ac.uk/software/figtree. Accessed 30 Mar 2019
29. Uldahl SA, Knutsen G (2009) Spore swelling and germination as a bioassay for the rapid screening of crude biological extracts for antifungal activity. J Microbiol Methods 79:82–88. https://doi.org/10.1016/j.mimet.2009.08.007
30. Clinical Laboratory and Standards Institute (CLSI) (2017) Reference method for broth dilution antifungal susceptibility testing of yeast. 4th ed. CLSI standard M27, Wayne, PA, Clinical and Laboratory Standards Institute. https://clsi.org/media/1897/m27ed4_sample.pdf. Accessed 30 Jan 2020
31. Clinical Laboratory and Standards Institute (CLSI) (2017) Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. 3rd ed. CLSI standard M38. Wayne, PA, Clinical and Laboratory Standards Institute. https://clsi.org/media/1894/m38ed3_sample.pdf. Accessed 30 Jan 2020
32. Clinical Laboratory and Standards Institute (CLSI) (2018) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 11th ed. CLSI standard M07. Wayne, PA, Clinical and Laboratory Standards Institute. https://clsi.org/media/1928/m07ed11_sample.pdf. Accessed 30 Jan 2020
33. Palomino JC, Martin A, Camacho M, Guerra H, Swings J, Portaels F (2002) Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 46:2720–2722. https://doi.org/10.1128/aac.46.8.2720-2722.2002
34. Polli AD, Ribeiro MAS, Garcia A, Polonio JC, Santos CM, Silva AA, Orlandelli RC, Castro JC, Abreu-Filho BA, Cabral MRP, Sarragiotto MH, Pamphile JA, Azevedo JL (2020) Secondary metabolites of *Curvularia* sp. G6-32, an endophyte of *Sapindus saponaria*, with antioxidant and anticholinesterasic properties. Nat Prod Res. https://doi.org/10.1080/14786419.2020.1739681
35. Ma X, Wu H, Liu L, Yao Q, Wang S, Zhan R, Xing S, Zhou Y (2011) Polyphenolic compounds and antioxidant properties in mango fruits. Sci Hortic 129:102–107. https://doi.org/10.1016/j.scienta.2011.03.015
36. Rajurkar NS, Hande SM (2011) Estimation of phytochemical content and antioxidant activity of some selected traditional Indian medicinal plants. Indian J Pharm Sci 73(2):146–151. https://doi.org/10.4103/0250-474X.91574
37. Singleton VL, Rossi JAJR (1965) Colorimetry of total phenolics whit phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic 16(3):144–158 https://www.ajevonline.org/content/16/3/144.short
38. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63. https://doi.org/10.1016/0022-1759(83)90303-4
39. DellaGreca M, Tommaso GD, Salvatore MM, Nicoletti R, Becchimanzi A, Iuliano M, Andolfi A (2019) The issue of misidentification of kojic acid with Flufuran in *Aspergillus flavus*. Molecules 24:1709. https://doi.org/10.3390/molecules24091709
40. Kwon Y, Park S, Shin J, Oh DC (2014) Application of 13C-labeling and 13C-13C COSY NMR experiments in the structure determination of a microbial natural product. Arch Pharm Res 37(8):967–971. https://doi.org/10.1007/s12272-013-0254-8
41. Gloer JB, Tepaske MR, Sima JS (1988) Antiinsectan aflavinine derivatives from the sclerotia of *Aspergillus flavus*. J Organomet Chem 53:5457–5460. https://doi.org/10.1021/jo00258a011
42. Meister J, Weber D, Martino V, Sterner O, Anke T (2007) Phomopsidone, a novel depsidone from an endophyte of the medicinal plant *Eupatorium arnottianum*. Z Naturforsch C J Biosci 62:11–15. https://doi.org/10.1515/znc-2007-1-202
43. Jiang H, Shi YT, Zhou ZX, Yang C, Chen YJ, Chen LM, Yang MZ, Zhang HB (2011) Leaf chemistry and co-occurring species interactions affecting the endophytic fungal composition of *Eupatorium adenophorum*. Ann Microbiol 61:655–662. https://doi.org/10.1007/s13213-010-0186-1
44. Cecati FM, Magallanes-Noguera C, Tonn CE, Ardanaz CE, Kurina-Sanz M (2018) Ecofriendly chemical diversification of *Eupatorium bunnifolium* essential oil by endophytic fungi.

Process Biochem 64:93–102. https://doi.org/10.1016/j.procbio.2017.09.028
45. Corrêa MJC, Nunes FM, Bitencourt HR, Borges FC, Guilhon GMSP, Arruda MSP, Marinho AMR, Santos AS, Alves CN, Brasil DSB, Santos LS (2011) Biotransformation of chalcones by endophytic fungus *Aspergillus flavus* isolated from *Paspalum maritimum* Trin. J Braz Chem Soc 22(7):1333–1338. https://doi.org/10.1590/S0103-50532011000700019
46. Patil RH, Patil MP, Maheshwari VL (2014) Isolation and HPTLC Densitometric analysis of rutin in the broth extract of endophytic *Aspergillus flavus* from *Aegle marmelos*. J Biol Active Prod Nat 4(5-6):371–376. https://doi.org/10.1080/22311866.2014.961099
47. Patil RH, Patil MP, Maheshwari VL (2015) Biological activities and identification of bioactive metabolite from endophytic *Aspergillus flavus* L7 isolated from *Aegle marmelos*. Curr Microbiol 71:39–48. https://doi.org/10.1007/s00284-015-0805-y
48. Ma YM, Ma CC, Li T, Wang J (2015) A new furan derivative from an endophytic *Aspergillus flavus* of *Cephalotaxus fortunei*. Nat Prod Res 30(1):79–84. https://doi.org/10.1080/14786419.2015.1038262
49. Liu Z, Zhao JY, Sun SF, Li Y, Qu J, Liu HT, Liu YB (2019) Sesquiterpenes from an endophytic *Aspergillus flavus*. J Nat Prod 82:1063–1071. https://doi.org/10.1021/acs.jnatprod.8b01084
50. Ola ARB, Metboki G, Lay CS, Sugi Y, Rozari PD, Darmakusuma D, Hakin EH (2019) Single production of kojic acid by *Aspergillus flavus* and the revision of flufuran. Molecules 24:4200. https://doi.org/10.3390/molecules24224200
51. Wicklow DT (1984) Adaptation in wild and domesticated yellow-green aspergilli. In: Kurata H, Ueno Y (eds) Toxigenic fungi their toxins and health hazards, Developments in Food Science, 7. Kodansha, Tokyo, pp 78–86
52. Hunter AJ, Jin B, Kelly JM (2011) Independent duplication of alpha-amylase in different strains of *Aspergillus oryzae*. Fungal Genet Biol 48:438–444. https://doi.org/10.1016/j.fgb.2011.01.006
53. Gibbons JG, Salichos L, Slot JC, Rinker DC, McGary KL, King JG, Klich MA, Tabb DL, McDonald WH, Rokas A (2012) The evolutionary imprint of domestication on genome variation and function of the filamentous fungus *Aspergillus oryzae*. Curr Biol 22:1403–1409. https://doi.org/10.1016/j.cub.2012.05.033
54. Soper RS, Ward MG (1981) Production, formulation and application off fungi for insect control. In: Papavizas CG (ed) Biological control in crop production. Allanheld & Osmun, New York, pp 161–180
55. Alves SB (1998) Fungos entomopatôgenicos. In: Alves SB (ed) Controle microbiano de insetos. FESALQ, Piracicaba, pp 289–380
56. Bhattacharyya PN, Jha DK (2011) Optimization of cultural conditions affecting growth and improved bioactive metabolite production by a subsurface *Aspergillus* strain TSF 146. Int J Appl Biol Pharm 2(4):133–143
57. Varga J, Frisvald JC, Samson RA (2011) Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. Stud Mycol 69:57–80. https://doi.org/10.3114/sim.2011.69.05
58. Tawfike AF, Romli M, Clements C, Abbott G, Young L, Schumacher M, Diederich M, Farag M, Edrada-Ebel R (2019) Isolation of anticancer and anti-trypanosome secondary metabolites from the endophytic fungus *Aspergillus flocculus* via bioactivity guided isolation and MS based metabolomics. J Chromatogr B Anal Technol Biomed Life Sci 1106:71–83. https://doi.org/10.1016/j.jchromb.2018.12.032
59. Luk KC, Kobbe B, Towsend JM (1977) Production of cyclopiazonic acid by *Aspergillus flavus* Link. Appl Environ Microbiol 33(1):211–212. https://doi.org/10.1128/AEM.33.1.211-212.1977
60. Lin AQ, Du L, Fang YC, Wang FZ, Zhu TJ, Gu QQ, Zhu WM (2009) iso-α-Cyclopiazonic acid, a new natural product isolated from the marine-derived fungus *Aspergillus flavus* C-F-3. Chem Nat Compd 45(5):677–680. https://doi.org/10.1007/s10600-009-9433-8
61. Sun K, Li Y, Guo L, Wang Y, Liu P, Zhu K (2014) Indole diterpenoids and isocoumarin from the fungus, *Aspergillus flavus*, isolated from the prawn, *Penaeus vannamei*. Mar Drugs 12(7):3970–3981. https://doi.org/10.3390/md12073970
62. Ma X, Peng J, Wu G, Zhu T, Li G, Gu Q, Li D (2015) Speradines B-D, oxygenated cyclopiazonic acid alkaloids from the sponge-derived fungus *Aspergillus flavus* MXH-X104. Tetrahedron 71(21):3522–3527. https://doi.org/10.1016/j.tet.2015.03.050
63. Tancinova D, Felsociaová S, Dovicová M, Masková Z, Labuda R, Javoreková S (2010) Endogenous contamination of wheat by species of genera *Aspergillus* and *Penicillium*. Ecol Chem Eng A 17(1):135–142
64. Zhou M, Miao MM, Du G, Li XN, Shang SZ, Zhao W, Liu ZH, Yang GY, Che CT, Hu QF, Gao XM (2014) Aspergillines A-E, highly oxygenated hexacyclic indole-tetrahydrofuran-tetramic acid derivatives from *Aspergillus versicolor*. Org Lett 16(19):5016–5019. https://doi.org/10.1021/ol502307u
65. Cole RJ, Dorner JW, Springer JP, Coz RH (1981) Indole metabolites from a strain of *Aspergillus flavus*. J Agric Food Chem 29(2):293–295. https://doi.org/10.1021/jf00104a019
66. Wicklow DT, Down PF, Tepaske MR, Gloer JB (1988) Sclerotial metabolites of *Aspergillus flavus* toxic to a detritivorous maize insect (*Carpophilus hemipterus*, Nitidulidae). Trans Br Mycol Soc 91(3):433–438. https://doi.org/10.1016/S0007-1536(88)80119-0
67. Nozawa K, Sekita S, Harada M, Udagawa SI, Kawai KI (1989) Isolation and structures of two new indoloditerpenes related to aflavinine from a microsclerotium-producing strain of *Aspergillus flavus*. Chem Pharm Bull 37(3):626–630. https://doi.org/10.1248/cpb.37.626
68. Chang PK, Scharfenstein LL, Li RW, Arroyo-manzanares N, de Saeger S, Diana DMJ (2017) *Aspergillus flavus* aswA, a gene homolog of *Aspergillus nidulans* oefC, regulates sclerotial development and biosynthesis of sclerotium-associated secondary metabolites. Fungal Genet Biol 104:29–37. https://doi.org/10.1016/j.fgb.2017.04.006
69. Gqaleni N, Smith JE, Lacey J (1996) Co-production of aflatoxins and cyclopiazonic acid in isolates of *Aspergillus flavus*. Food Addit Contam 13(6):677–685. https://doi.org/10.1080/02652039609374453
70. Gqaleni N, Smith JE, Lacey J, Gettinby G (1997) Effects of temperature, water activity, and incubation time on production of aflatoxins and cyclopiazonic acid by an isolate of *Aspergillus flavus* in surface agar culture. Appl Environ Microbiol 63(3):1048–1053. https://doi.org/10.1016/j.ram.2015.10.001
71. El-Shanawany AA, Mostafa ME, Barakat A (2005) Fungal populations and mycotoxins in silage in Assiut and Sohag governorates in Egypt, with a special reference to characteristic Aspergilli toxins. Mycopathologia 159:281–289. https://doi.org/10.1007/s11046-004-5494-1
72. Mostafa ME, Barakat A, El-Shanawany AA (2005) Relationship between aflatoxins synthesis and *Aspergillus flavus* development. Afr J Mycol Biotechnol 13(2):35–51
73. Hamasaki T, Kuwano H, Isono K, Hatsuda Y, Fukuyama K, Tsukihara T, Katsube Y (1975) A New Metabolite, Parasiticolide A, from *Aspergillus parasiticus*. Agric Biol Chem 39(3):749–751. https://doi.org/10.1080/00021369.1975.10861681
74. Rank C, Klejnstrup ML, Petersen LM, Kildgaard S, Frisvad JC, Gotfredsen CH, Larsen TO (2012) Comparative chemistry of *Aspergillus oryzae* (RIB40) and *A. flavus* (NRRL 3357). Metabolites 2:39–56. https://doi.org/10.3390/metabo2010039
75. Shinohara Y, Kawatani M, Futamura Y, Osada H, Koyama Y (2016) Na overproduction of astellolides induced by genetic disruption of chromatin-remodeling factors in Aspergillus oryzae. J Antibiot 69:4–8. https://doi.org/10.1038/ja.2015.73

76. Shinohara Y, Takahashi S, Osada H, Koyama Y (2016) Identification of a novel sesquiterpene biosynthetic machinery involved in astellolide biosynthesis. Sci Rep 6:32865. https://doi.org/10.1038/srep32865
77. Holetz FB, Pessini GL, Sanches NR, Cortez DA, Nakamura CV (2002) Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. Mem Inst Oswaldo Cruz 97(7):1027–1031. https://doi.org/10.1590/s0074-02762002000700017
78. Gordaliza M, García PA, Corral MD, Castro MA, Gómez-Zurita MA (2004) Podophyllotoxin: distribution, source, applications and new cytotoxic derivatives. Toxicon 44:441–459. https://doi.org/10.1016/j.toxicon.2004.05.008
79. Dutcher J (1958) Aspergillic acid: an antibiotic substance produced by *Aspergillus flavus*. J Biol Chem 232:785–796 https://www.jbc.org/content/232/2/785.long
80. Tosun F, Akyüz KC, Sener B, Vural M, Palittapongarnpim P (2004) Antimycobacterial screening of some Turkish plants. J Ethnopharmacol 95:273–275. https://doi.org/10.1016/j.jep.2004.07.011
81. Gu JQ, Wang Y, Franzblau SG, Montenegro G, Yang D, Timmermann BN (2004) Antitubercular constituents of *Valeriana laxiflora*. Planta Med 70:509–514. https://doi.org/10.1055/s-2004-827149
82. Geiser DM, Dorne JW, Horn BW, Taylor JW (2000) The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus flavus*. Fungal Genet Biol 31:169–179. https://doi.org/10.1006/fgbi.2000.1215
83. Saeedi M, Eslamifar M, Khezri K (2019) Kojic acid applications on cosmetic and pharmaceutical preparations. Biomed Pharmacother 110:582–593. https://doi.org/10.1016/j.biopha.2018.12.006