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BRIEF REPORTS

Lethality of cytochalasin B and other compounds isolated from fungus *Aspergillus* sp. (Trichocomaceae) endophyte of *Bauhinia guianensis* (Fabaceae)

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KEYWORDS
Endophytic fungi;
Artemia salina;
Secondary metabolites

Abstract Endophytic fungi are fungi that colonize internal tissues of plants; several biologically active compounds have been isolated from these fungi. There are few studies of compounds isolated from endophytic fungi of Amazon plants. Thus, this study aimed the isolation and structural identification of ergosterol (**1**), ergosterol peroxide (**2**), mevalonolactone (**3**), cytochalasin B (**4**) and cytochalasin H (**5**) from *Aspergillus* sp. EJC 04, an endophytic fungus from *Bauhinia guianensis*. The cytochalasin B (**4**) and the diacetate derivative of cytochalasin B (**4a**) showed high lethality in the brine shrimp assay. This is the first occurrence of cytochalasins in Amazonian endophytic fungi from *B. guianensis*.

PALABRAS CLAVE
Hongos endófitos;
Artemia salina;
Metabolitos secundarios

Letalidad de citocalasina B y otros compuestos aislados del hongo *Aspergillus* spp. (Trichocomaceae) endófito de *Bauhinia guianensis* (Fabaceae)

Resumen Los hongos endofíticos son hongos que colonizan los tejidos internos de las plantas; varios compuestos biológicamente activos se han aislado a partir de estos hongos. Existen pocos estudios de compuestos aislados de hongos endófitos de plantas amazónicas. Por lo tanto, este

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estudio tuvo como objetivo el aislamiento y la identificación estructural de ergosterol (**1**), peróxido de ergosterol (**2**), mevalonolactona (**3**), citocalasina B (**4**) y citocalasina H (**5**) a partir de *Aspergillus* spp. EJC 04, un hongo endofítico de *Bauhinia guianensis*. La citocalasina B (**4**) y el derivado diacetato de citocalasina B (**4a**) mostraron una alta letalidad en el ensayo de *Artemia salina*. Esta es la primera aparición de citocalasinas en hongos endófitos amazónica de *B. guianensis*.

The *Aspergillus* genus has more than one hundred species, and belongs to the Ascomycota division, Deuteromycotina subdivision, Hyphomycetes class, Moniliales order, Moniliaceae family. The species are widely found in nature, they can be isolated from plants, soil, air and decaying matter[13]. Several species have been described as producers of toxic metabolites[3]. The *Aspergillus* fungi have been an important source of natural products useful for exploration in medicine, agriculture and industry. Several compounds with cytotoxic activity have been isolated from endophytic fungi[1,16,19]. Compounds of the xanthones class isolated from *Aspergillus sydowii* showed immunosuppressive activities[17], and the tensyuic A acid isolated from *Aspergillus niger* showed antimicrobial activity[6]. There are reports of the isolation of cytochalasin E from an endophytic fungus that showed cytotoxicity[18]. Petersen et al.[12] isolated from the fungus *Aspergillus sclerotioniger* cytochalasins sclerotionigrin A and B that showed cytotoxic activity in vitro against lymphocytic leukemia cells. The aims of this study were the isolation and structural identification of ergosterol (**1**), ergosterol peroxide (**2**), mevalonolactone (**3**), cytochalasin B (**4**), cytochalasin H (**5**), and the derivative 7,20-diacetyl-cytochalasin B (**4a**) from the endophytic fungus *Aspergillus* sp. EJC 04 cultures, and testing the lethality of isolated compounds against *Artemia salina*.

The ^{1}H and ^{13}C NMR experiments were recorded on a NMR spectrometer (Mercury 300, Varian, Oxford, Oxfordshire, UK) with $CDCl_3$ (Cambridge®) as solvent and standard. The MS spectra were carried out in the mass spectrometer using ESI (+) ion mode (Acquity TQD, Waters, Milford, MA, USA). The specific rotation was performed on a specific rotation equipment (Nova Instruments No. 1412, Piracicaba, Brazil).

The fungus *Aspergillus* sp. was obtained from a collection of ''Laboratório de Bioensaios e Química de Micro-organismos – LaBQuiM/UFPA''. This collection contains isolates from *Bauhinia guianensis*. The fungus was inoculated into a Petri dish containing PDA (Potato Dextrose Agar) culture medium (Himedia®) and incubated at 25 °C (BOD, Quimis®) for 8 days to reactivation. One strain is deposited with a code EJC 04. The fungus *Aspergillus* EJC 04 was identified by observing the morphology and microscopic aspects of the colony in an optical microscope and by DNA sequence through analyses of the ITS5 region.

Six Erlenmeyer flasks (1000 ml) containing 200 g of rice (Uncle Ben's®) and 75 ml of water per flasks were autoclaved for 45 min at 121 °C (autoclave Prismatec®). Small pieces of PDA containing mycelium of *Aspergillus* sp. were added to 4 Erlenmeyer flasks under sterile conditions, then, the Erlenmeyer flasks were incubated at 25 °C for 23 days, two Erlenmeyer flasks were used as control. Biomass was macerated with ethyl acetate (Tedia®) (3× 500 ml). The biomass was separated of the ethyl acetate solution by filtration. Then, the ethyl acetate extracts (10 g) were obtained after evaporation of the resulting solution in rotary evaporator at 45 °C (Quimis®). Part of the ethyl acetate extract (5.0 g) was fractionated on silica gel column using hexane/ethyl acetate (9:1, 4:1, 7:3, 1:1, 3:7), ethyl acetate/methanol (3:7, 1:1) and methanol, resulting in 9 fractions. The hexane/ethyl acetate 9:1 fraction (500 mg) was submitted on silica gel column chromatography eluted with hexane/ethyl acetate (9:1, 4:1, 7:3, 1:1, 3:7) and ethyl acetate, resulting in 92 fractions. The fractions were pooled (A1 to A8); the fraction A2 provided a white amorphous solid identified as ergosterol **1** (30 mg), and the A4 fraction provided a white amorphous solid identified as ergosterol peroxide **2** (35 mg). The hexane/ethyl acetate 8:2 fraction (600 mg) was submitted on silica gel column chromatography eluted with hexane/ethyl acetate (9:1, 4:1, 7:3, 1:1, 3:7) and ethyl acetate, resulting in 100 fractions pooled as B1 to B9, the fraction B2 afforded a yellow oil identified as mevalonolactone **3** (6 mg). The ethyl acetate fraction (2 g) was submitted on silica gel column chromatography, eluted with hexane/ethyl acetate (9:1, 4:1, 7:3, 1:1, 3:7), ethyl acetate, ethyl acetate/methanol (3:7, 1:1) and methanol, resulting in 120 fractions pooled as C1 to C12; fraction C5 afforded a white solid identified as cytochalasin B **4** (100 mg) and the fraction C7 gave a white solid identified as cytochalasin H **5** (5 mg). All compounds **1–5** were identified by NMR and MS spectrometric data.

The cytochalasin B (**4**) was acetylated; for this, 10 mg of compound **4** were removed and solubilized in 100 μl of pyridine (Tedia®), then it was added 250 μl of acetic anhydride (Tedia®) and kept at rest for 24 h at room temperature. After this period, the material was transferred to a separatory funnel, and it was added 25 ml of 5% HCl solution to remove excess pyridine, and extraction was performed with ethyl acetate (3× 15 ml). The ethyl acetate phase was further washed with distilled water (3× 25 ml) and after separation was added anhydrous sodium sulfate in the ethyl acetate phase, which after filtered was evaporated to obtain the acetylated product **4a**.

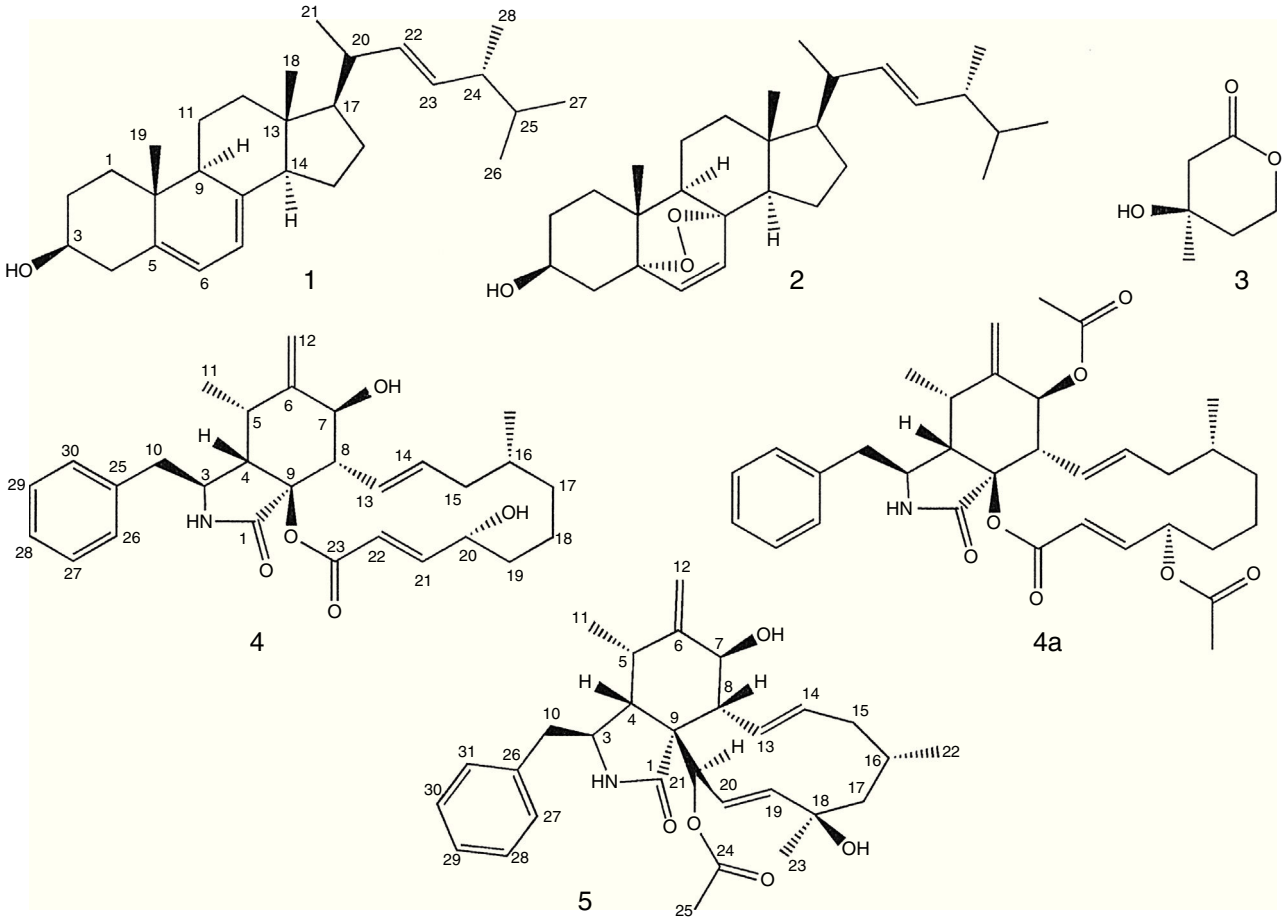

Figure 1 Compounds isolated from *Aspergillus* sp. EJC 04.

The *A. salina* lethality test was performed following the Meyer et al.[11] method adapted, preparing a solution of sea salt at a concentration of 30 g/l. The pH was adjusted between 8.0 and 9.0 using 0.1 mol/l NaOH solution. This solution was used for hatching of *A. salina* and for preparing the other dilutions. The eggs were placed to hatch in saline solution for 48 h with constant aeration at 25 °C. About ten *A. salina* larvae were transferred to tubes containing saline solution and samples to be tested, with the following concentrations: 50 μg/ml, 25 μg/ml, 10 μg/ml, 5 μg/ml and 1 μg/ml, a tube containing only *A. salina* was used as control. The assay was performed in triplicate, and the counting of the dead and live animals carried out after 24 h. This is the first report of isolation of cytochalasins of the endophytic fungi from *B. guianensis* and of its lethality against *A. salina* (Table 1, Fig. 1).

Table 1 Lethality of the compounds isolated from *Aspergillus* sp. EJC 04 against *Artemia salina*

Compound	LC_{50} (μg/ml)
2	≫250
SD	±1.11
3	≫250
SD	±0.01
4	24.9
SD	±2.85
4a	22.8
SD	±3.31
5	69.1
SD	±2.24

SD, standard deviation.

The compound **4** was identified through analysis of the spectroscopic data of 1D and 2D NMR and MS-ESI (+), and compared with literature data[7]. The molecular formula $C_{29}H_{37}NO_5$ was deducted from ESI mass spectrum *m/z* 480 $[M+H]^+$. The signals observed in the ^1H NMR spectrum in 7.17 (*m*), 7.30 (*m*) and 7.24 (*m*) indicate the existence of a monosubstituted aromatic ring in **4**, attributed to hydrogens H-27/31, H-28/30 and H-29. The signals at δ 5.94; 5.46; 7.05; and 5.90 were assigned to the hydrogens of the double bonds $\Delta^{13,14}$ and $\Delta^{21,22}$, respectively; the coupling constant (15.5 Hz) between H-13/H-14 and H-21/H-22 indicates that both double bonds have the *E* geometry. Signs at δ 0.89 (*d*, 6.6) and δ 1.10 (*d*, 6.6) were assigned for the methyl CH_3-11 and CH_3-24. The signals of carbinolic hydrogens at δ 3.90 and 4.51 were assigned to H-7 and H-20 hydrogens. In the ^{13}C NMR spectrum were observed 28 signals; 6 in the aromatic region, 6 for olefinic carbon, 7 sp^3 CH carbons, 3 carbinolic carbons, 4 CH_2 carbons, and 2 carbonyl carbons. In the HSQC spectrum, H-10 is correlated with C-10 (δ

41.7). Following HMBC correlations to H-10, one can find the aromatic ring by correlations with C-25, C-26 and C-30. Yet, HMBC correlations to H-10 and H-4 with the carbonyl carbon C-1 (δ 171.2) allowed proposing a γ-lactam ring. The correlations observed in the COSY spectrum between H-7 and H-8, H-13 with H-8 and H-14, along with the HMBC correlations Me-24 to C-15 (δ 44.2) and C-17 (δ 34.9), and carbinolic hydrogen H-7 with the carbons of the double bond C-21 (δ 152.1) and C-22 (δ 119.3), plus HMBC correlations of H-21 with C-20 (δ 69.0) and carbon carbonyl C-23 (δ 164.5) allowed proposing the macrocyclic ring. Thus, **4** was identified as cytochalasin B and the specific rotation calculated was $[\alpha]^D$: +143 (c 1.0, acetone). The relative configuration to compound **4** was determined by comparison of the chemical shifts values ^{1}H and ^{13}C NMR, coupling constants and comparison with the literature data[10].

The molecular formula $C_{30}H_{39}NO_5$ for compound **5** was deduced through ESI mass spectrum *m/z* 494 $[M+H]^+$. The signal sets shown in ^{1}H and ^{13}C NMR spectra, bi-dimensional NMR data and comparison with the literature data[7], led to characterize **5** as cytochalasin H, similar to cytochalasin B (**4**). The main difference observed in the ^{1}H NMR spectrum of **5** with respect to **4** is the existence of a signal at δ 2.23 (*s*) assigned to the methyl CH_3-25 of the acetate group. The specific rotation calculated to **5** was $[\alpha]^D$: +16 (c 1.0, CH_2Cl_2). The relative configuration to compound **5** was determined by comparison of the chemical shifts values ^{1}H and ^{13}C NMR, coupling constants and comparison with the literature data[10].

Since the compound **4** was obtained in large quantities and showed good lethality with regard to *A. saline*, it was decided to obtain its acetylated derivative to test the relation structure-activity. The product derivatization of cytochalasin B was identified by ^{1}H and ^{13}C NMR and MS. The ^{1}H NMR spectrum of 7,20-diacetyl-cytochalasin B (**4a**) showed similarity to **4**, where the main differences observed in the ^{1}H NMR spectrum were the presence of two signals singlets at δ 1.96 and δ 2.11 assigned for the methyl of acetate groups CH_3-32 and CH_3-34. The molecular formula $C_{33}H_{41}NO_7$ was deducted through ESI mass spectrum *m/z* 564 $[M+H]^+$, in the mass spectra were also observed *m/z* 504 [M-60] common for the loss of an acetate group and *m/z* 444 [M-120] compatible with the loss of a second acetate group, which helped to confirm the demethylation in **4a**.

The compounds ergosterol (**1**), ergosterol peroxide (**2**) and (R)-(−)-mevalonolactone (**3**) were identified by ^{1}H and ^{13}C NMR, the compounds have been previously isolated from *Aspergillus* sp. EJC 08, endophyte from *B. guianensis*, together with the benzophenone monomethylsulochrin. Mevalonolactone and monomethylsulochrin showed good antimicrobial activity[14].

The lethal concentration LC_{50} ranged from 22.8 μg/ml to $\ll$250 μg/ml. Considering the Amarante et al.[2] criterion, cytochalasins H (**5**) showed to lethal, while cytochalasin B (**4**) and 7,20-diacetyl-cytochalasin B (**4a**) showed high lethality (Table 1). The lethality values were compared with those obtained by Ferraz et al.[5] for lapachol (52.5 μg/ml), where cytochalasin H (**5**) showed lethality near to the presented by lapachol, while the compounds cytochalasin B (**4**) and 7.20-diacetyl-cytochalasin B (**4a**) were two times more active. However, not was observed significant difference between the activity of cytochalasin B (**4**) and its derivative 7.20-diacetyl-cytochalasin B (**4a**) no being possible establish a relation structure-activity to the compounds.

The study of *Aspergillus* sp. EJC 04 led to the isolation of bioactive compounds, which is in agreement with previous studies with strains of *Aspergillus* species. For example, from sponge-derived fungus *Aspergillus versicolor* were isolated xanthones and anthraquinones compounds exhibited significant cytotoxicity against five human solid tumor cell lines and antibacterial activity against several clinically isolated gram-positive strains[8]. The compounds asperfumoid and asperfumin isolated from *Aspergillus fumigatus* showed antifungal activity[9]. From *Aspergillus terreus* were obtained isocoumarins that exhibited potent antioxidant activity[4]. The compound benzylazaphilone, aspergilone A, was isolated from the culture broth of a marine-derived fungus *Aspergillus* sp. exhibited in vitro selective cytotoxicity and showed potent antifouling activity[15].

Thus, this work contributed to the chemical study of endophytic fungi from Amazon plants and led to the isolation of substances that present high lethality against *A. saline*. Furthermore, this is the first report of isolation of the cytochalasins of endophytic fungi from *B. guianensis* and their lethality against *A. saline*.

Ethical responsibilities

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

The authors declare that they have no conflicts of interest.

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