Cytotoxic Potential from the Extracellular Filtrate from *Aspergillus niveus* Cultured in YPD Medium against Human Tumor Cells

Thaís Barboni Alves, Gabriela Molinari Roberto, Maria Sol Brassesco, and Luis Henrique Souza Guimarães

**ABSTRACT**

Different fungal species, especially from the genus *Aspergillus*, have been reported as producers of small molecules, including proteins, with biological activity and a better understanding of their sources, structure, function, and toxicity is essential for their biotechnological applications. According to this, our aim was to evaluate the cytotoxic activity of the extracellular filtrate produced by *A. niveus*. The crude filtrate obtained in YPD medium containing 18 kDa protein, after cultivation for 120 h, was selected for cytotoxic assay, assessed by Giemsa staining, against different human tumor cell lines. Crude filtrate inhibited (from 27% to 50%) the ONS (medulloblastoma), HT144T (melanoma), HOS (osteosarcoma), T98G (glioblastoma) human tumor cell lines and MRC-5 (fibroblasts) human normal cells, at 20 µg/mL for 72 h treatment. According to this, the 18 kDa protein band and the fractions obtained after DEAE-Cellulose procedure were evaluated through mass spectrometry (MS/MS) analysis, revealing the presence of peptides with similarity to the alpha-sarcin, mitogillin and AspF1 ribotoxins described for other *Aspergillus* species. In conclusion, the *A. niveus* extracellular filtrate containing ribotoxin-like proteins reduced, *in vitro*, the growth of human tumor cell lines indicating their biotechnological potential, indicating a possible future application in the elaboration of immunotoxins.

**Keywords:** *Aspergillus*, anticancer activity, ribotoxin-like protein.

I. INTRODUCTION

Filamentous fungi are found in different environments establishing diverse ecological relations with other organisms as predation, defense and mutualism [1]. In this context, a myriad of molecules is produced allowing the fungal survival under different conditions, as proteins, pigments, enzymes and toxins, and the biotechnological potential of these molecules have been revealed. The secondary metabolites, characterized as low molecular molecules, from different fungal strains have been exploited with different purposes as the control of the tumor cells growth [2], [3]. A worldwide increase about 70% in the numbers of cases of cancer is estimated for the year 2030 [4]. In this context, the search by new alternative molecules with cytotoxic activity against tumor cells is essential to overcome this issue.

The anticancer bioactivity has been reported for fungal extracts as from *Ganoderma sinense*, *Fomitopsis officinalis*, *Polyporus melanonpus*, *Taiwanofungus camphorates* and mainly from *Fomitopsis pinicola*. For the *G. sinense* extract, the antiproliferative activity on tumor cells is attributed to the triterpenoids, sterols and nucleosides. Triterpenes and sterols were also observed in the *F. pinicola* extract and heterogalactan as well [4]. Other chemicals as polyketides, alkaloids, peptides and lactones produced by fungi also present potential as chemotherapeutics [5]. Low molecular weight preparation from the fungus *Cerrena unicolor*, with inhibitory activity against human colon cancer cells, was reported by Matuszewksa et al. [6].

Some fungal strains are able to produce small proteins as ribotoxins (150 amino acids), a family of ribonucleases (RNases) [7]. The biological role of ribotoxins is not clear yet, but recently, antibacterial, antiviral, and antifungal activities were reported for the ageritin from the basidiomycete *Agrocybe aegerita*, indicating an important role in the mechanism of defense [8]. The ageritin also inhibited the cell proliferation of several CNS model cell lines [9].

These proteins cleave single phosphodiester bonds in the GAGA tetra loop, one of the most significant structural features of the highly conserved sarcin/ricin loop (SRL) of large ribosomal RNA (rRNA) molecules in all living organisms [1], [10]. The action of ribotoxins on rRNA inhibits protein biosynthesis leading to cell death by apoptosis [11]. Moreover, ribotoxins, such as alpha-sarcin, can also interact with acid phospholipid-containing membranes, causing vesicles aggregation, intermixing of
phospholipids and leakage of intravesicular aqueous contents [12], [13]. These combined actions of ribotoxins can explain their cytotoxicity [14]-[16].

Due to the great potential of these small proteins, a better understanding of their sources, structure, function and toxicity are essential for their use in different biotechnological applications, such as antitumor agents [1], [15], [17], [18]. Considering these aspects and the unexplored fungal biodiversity, the search for new species able to produce small proteins with biotechnological potential is an important challenge.

The Aspergillus nives is well known for its high potential to produce enzymes with industrial applications such as β-fructofuranosidases, amylase, xylanase, inulinases, β-glucosidas, endoglucanases and pectinases [19]-[21]. On the other hand, the potential of A. nives extract to control the tumor cell proliferation was not previously reported. Here, we describe, for the first time, the cytotoxic effect of the A. nives extracellular extract on different tumor cell lines and its protein characterization.

II. MATERIAL AND METHODS

A. Fungal Strain and Culture Conditions

The filamentous fungus A. nives was grown on PDA (potato dextrose agar) slants for 5 days. The culture was carried out in 250 mL Erlenmeyer flasks containing 50 mL of different media: YPD [22], Minimal medium [23], Czapeck [24], Adams [25], SR [26], Khanna [27] and M5 [28]. The media were autoclaved at 120 °C, 1.5 atm for 30 min and inoculated with a spore suspension (10⁵ spores/mL) obtained by addition of distilled water and scraping of the slants of A. nives. The cultures were maintained at 30 °C under orbital agitation (100 rpm) for 120 h.

B. Crude Filtrate and Protein Quantification

After cultivation, the cultures were harvested by vacuum filtration through 3-layers of cheesecloth and the crude filtrates obtained were dialyzed against distilled water at 4 °C for 24 h. The mycelia were discharged.

Protein quantification was determined according to the Bradford method using bovine serum albumin (BSA) as standard [29].

C. SDS-PAGE Electrophoresis

Samples from the crude filtrate from each culture condition were submitted to denaturing electrophoresis (12% SDS-PAGE) according to the Laemmli method [30]. The lyophilized samples were then suspended in 40 mM Tris/HCl buffer pH 6.8 (containing 8% glycerol, 2% SDS, 0.1 % bromophenol blue and 200 mM dithiothreitol), heated at 96 °C for 5 min and loaded on the gel. The electrophoresis was performed using a power source adjusted to 120 V and 40 mA for 60 min. As molecular weight markers the Precision Plus ™ Kaleidoscope (Bio-Rad) from 10 to 250 kDa, or the Molecular Weight Marker from 14 to 66 kDa (Sigma-Aldrich) were used. The gel was stained with Coomassie Brilliant Blue R250.

D. Cytotoxic Assessment

The ONS (medulloblastoma) and HT144T (melanoma) human cell lines were grown on RPMI medium and the HOS (osteosarcoma), T98-G (glioblastoma) and MRC-5 (normal fibroblasts) cell lines were grown on HAM F10 medium (Gibco BRL, Life Technologies®). Both media were supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin.

The cell lines were seeded in 96-multiwell plates at a density of 2×10³ cells/well. After 24 h of incubation at 37 °C in a humidified 5% CO₂ incubator, the cells were treated with crude filtrate obtained from cultivation of A. nives in YPD medium for 120 h. Four doses (2.5, 5, 10 and 20 μg/mL) were used for 24, 48 and 72 h of incubation at 37 °C in a humidified 5% CO₂ incubator. Untreated controls received equal volume of RPMI or HAM F10 media according to each cell line analyzed.

After each treatment period, inhibition of the cell proliferation was determined by Giemsa staining [31], with modifications. The cells were washed with PBS buffer and fixed using absolute methanol for 15 min. Thereafter, the methanol was removed and the plates were maintained at room temperature for evaporation of the methanol residue. The fixed cells were stained with 1% Giemsa solution, washed three times with PBS and destained using a methanol:acetic acid solution (1:3, v/v). The absorbance value for each well was determined at 655 nm using the iMark Microplate Absorbance Reader (BioRad).

All experiments were performed in triplicate for each extract concentration and independently repeated three times. Values are shown as mean ± SD. Two-way ANOVA followed by the appropriate post-hoc test (Bonferroni) was used to check for significant differences between groups (differences between doses or times). Differences were considered significant at p <0.05.

E. Chromatographic Procedure

The crude filtrate (200 mL) obtained from the culture of A. nives using YPD medium was loaded in DEAE-Cellulose (Diethylaminoethyl cellulose, 2×13 cm) chromatographic column equilibrated with 10 mM Tris/HCl buffer, pH 7 and eluted using a linear gradient of NaCl (0-1.5 M) in the same buffer. Fractions of 3 mL were collected at a flow rate of 1 mL/min. The protein content for each fraction was monitored at 280 nm.

F. Protein Identification by Mass Spectrometry

The low molecular weight protein (18 kDa) observed in SDS-PAGE for the crude filtrate was cut from the gel and incubated in 1 mL of wash solution (50% 0.1M ammonium bicarbonate solution, pH 8.0 plus 50% acetonitrile), at room temperature for 24 h. After incubation, the solution was completely removed and 250 μL of acetonitrile were added for 2 h, at room temperature, for spot dehydration. The acetonitrile was then removed, and the dried spot transferred to a new microtube to be submitted to the digestion protocol using trypsin.

The two protein pools obtained from DEAE-Cellulose were precipitated with 20% TCA (Trifluoracetic acid) and also submitted to the digestion protocol using trypsin. For each sample the digestion by trypsin was done using 0.5 μg
of trypsin plus 17 µL of 0.1 M ammonium bicarbonate buffer pH 8.0. After digestion, the tryptic peptides were loaded to reverse phase resin Poros 50 R2 (PerSeptive Biosystems). The purified peptides were hydrated in 6 µL of matrix solution [5 mg/mL a-cyan-4-hidroxicaminic acid in 50% acetonitrile and 0.1% trifluoroacetic acid (v/v)] and 2 µL of each sample were applied to the plate of MALDI-TOF/TOF (Axima performance, Shimadzu). The MS/MS obtained for each digested protein were analyzed using the MASCOT software (Matrix Science) and the SwissProt database. The peptides alignment was done using the Clustal Omega software.

III. RESULTS AND DISCUSSION

A. Protein Profile of the Extracellular Extracts

The diversity of the proteins found in the fungal secretome depends on the culture conditions used during the fermentation. In general, 40-60% of this secretome comprises small secreted proteins with less than 300 amino acids [32]. The 12% SDS-PAGE profile obtained for the secreted proteins by the fungus A. niveus cultured for 120 h in different media, revealed the presence of small proteins and differences at the level of gene expression (Fig. 1). Some protein bands are similar among the media such as the 18 kDa protein band present in the crude filtrate obtained from the M5, Czapek and YPD media. A reduced number of protein bands can be observed for the profile obtained for the YPD medium, especially the highly expressed 18 kDa protein band. In spite of this 18 kDa protein in the Czapek and M5 medium, high molecular weight proteins were also detected. Considering the potential of small proteins with molecular weight minor than 20 kDa, this study focused in the investigation on the cytotoxic activity of the YPD extract containing 18 kDa protein produced by A. niveus.

![12% SDS-PAGE of crude filtrates obtained by the cultivation of A. niveus in different culture media for 120 h. 1- M5; 2- Adams; 3- Minimal medium; 4- Khatna; 5- SR; 6- Czapek; 7- YPD and M- Molecular Weight Marker (Precision Plus™ Kaleidoscope (Bio-Rad) from 10 to 250 kDa).](image)

B. Cytotoxic Assessment

The crude filtrate obtained in YPD medium was used to verify their toxicity against the human tumor cell lines ONS (medulloblastoma), HT144T (melanoma), HOS (osteosarcoma), T98-G (glioblastoma) and normal cell line MRC-5 (fibroblasts). As can be observed in the Fig. 2, for the crude filtrate the highest inhibition of the cell proliferation (27 to 43%) was observed using 20 µg/mL at different incubation times, especially for 72 h, showing its capacity to inhibit the proliferation of tumor cell lines (HOS, HT144T, ONS and T98-G). The most pronounced effect was observed on medulloblastoma and melanoma cell lines. In general, the inhibition of HOS, ONS76, HT144T and T98G tumor cell lines was dose-dependent. For fibroblasts cell line (MRC-5), the crude filtrate showed higher inhibitory effect on cell growth after 24 h of incubation, though after longer periods (48 h and 72 h of incubation) such effect was milder and dose-independent. The water extracts from F. pinicola, G. sinense, F. officinalis, P. melanopus and T. camphorates inhibited the proliferation of different tumor cell lines. In this context, the best inhibitory activity was observed using 50 µg/mL. F. officinalis extract on colon cancer cells (HCT-116) for 24h treatment [4]. The ethanol extract from Fusarium oxyporum inhibited the human cervical cancer (HeLa) cells with IC50 of 33.35 µg/mL for 48 h treatment [33]. Table 1 summarizes the highest percentages of inhibition of tumor and normal cell lines promoted by the YPD crude filtrate. Considering the cytotoxic effect on tumor cell lines observed, the 18 kDa protein band was excised from the gel, digested with trypsin and analyzed through mass spectrometry.

| Sample | Cell line | Highest Inhibition (%) | Concentration of sample (µg/mL) | Incubation period (h) |
|--------|-----------|------------------------|---------------------------------|----------------------|
| HOS    | 36 ± 7.3  | 20                     | 72                              |
| Crude  | HT144T    | 43 ± 3.5               | 20                              | 72                   |
| filtrate| ONS       | 43 ± 4.9               | 20                              | 72                   |
| (YPD)  | T98-G     | 27 ± 10                | 20                              | 24-72                |
| MRC-5  | 40 ± 12.5 | 20                     | 24                              |

C. Protein Characterization of the Filtrate

According to the analysis of the MS/MS profiles, the tryptic peptides obtained from 18 kDa protein presented similarity with Asp f1 allergen from A. fumigatus allowing its identification as a ribotoxin-like protein (Table 2). The production of ribotoxins by different fungal species has been reported [8], but this is the first description of the production of a ribotoxin-like protein by A. niveus.

The DEAE-Cellulose chromatographic profile of the crude filtrate obtained from the cultivation of A. niveus using YPD shows two protein peaks (Fig. 2). The first one (FI) did not interact with the resin while the second interacted and it was eluted with 300 mM NaCl. Both fractions, FI and FII, were digested by trypsin and analyzed using mass spectrometry. The two tryptic peptides from the FI fraction showed similarity with the allergen Asp f15 and with the mitogillin from Aspergillus fumigatus, while the tryptic peptides from the FII fraction showed similarity with the ribonucleases alpha-sarcin and mitogillin from Aspergillus fumigatus and Aspergillus giganteus, respectively (Table 2), confirming the production of the ribotoxin-like proteins by A. niveus. Interesting, any other peptide differing from that found in the ribotoxins was observed in the MS/MS profile, indicating the purity of the fractions.

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TABLE 2: MASS SPECTROMETRY (MS/MS) ANALYSIS OF THE PROTEINS PRESENT IN CRUDE FILTRATE OBTAINED FROM THE CULTURE OF A. NIVEUS IN YPD MEDIUM AND IN THE FI AND FII FRACTIONS OBTAINED FROM DEAE-CELLULOSE

| Protein name | Species | Nominal mass | Protein score | Cover |
|--------------|---------|--------------|---------------|-------|
| 18 kDa band  | Asp f1 allergen | A. fumigatus | 14026 | 98 | 36% |
|              | DDHYLLEFPTPDHGHDYK  |                |              |      |
|              | VFCGIYAHER           |                |              |      |
| FI Allergen as f 15 | A. fumigatus | 16162 | 60 | 7% |
| Mitogillin   | A. fumigatus | 19815 | 73 | 10% |
| FII A Mitogillin | A. fumigatus | 19811 | 135 | 26% |
| B Alpha-sarcin | A. giganteus | 19940 | 122 | 10% |

Fig. 2. Inhibition of the proliferation of the cell lines treated with crude filtrates obtained from A. niveus maintained in YPD medium for 120 h at 30ºC, at 2.5, 5, 10 and 20 µg/mL, for 24, 48 and 72 h. The cell lines used were: T98G (glioblastoma), HOS (osteosarcoma), HT144T (melanoma), ONS (medulloblastoma) and MRC5 (normal fibroblasts). Untreated cells represent the control. Asterisks indicates a statistically significant difference between the treated and control group.

Fig. 3. Purification of ribotoxins by ion-exchange chromatography (DEAE-Cellulose). The crude filtrate obtained in the fermentation of A. niveus in YPD medium (120 h), was loaded onto DEAE-Celullose resin. Two protein fractions were obtained: the FI fraction did not interact with the resin and the FII was afterwards eluted with 300 mM NaCl.

The main peptides (with highest score; 98-135) obtained from the 18 kDa protein and from the fraction II were aligned with the ribotoxins alpha-sarcin, restrictocin and Asp f1 (Fig. 4). It was observed that these tryptic peptides presented relation with different parts of secondary structure of the alpha-sarcin, as the amino acid residues involved in the structures of the loops 1, 2 and 3; the amino acids in the β region; with cysteine residues (C²) involved in the disulfide bonds; and with amino acid residues in the catalytic center (Y, E and H). The presence of loops positively charged in the ribotoxins has been mentioned as important to their citotoxic activity [16].

Both, allergen Asp f1 and alpha-sarcin show high similarity with the amino acid sequence of mitogillin [9], [17], [34], [35]. The members of the mitogillin family of cytotoxic ribonucleases show high specificity for the RNA present in the large subunit of the ribosome, which is essential for their activity, inhibiting the protein synthesis [11]. According to this, our results indicate that the identified ribotoxin-like proteins are from the mitogillin family.

Ribotoxins also have been identified and characterized from other fungal genus. Different Penicillium species were found to have ribotoxin gene (sar gene). Among these, four species (P. resedanum, P. spinulosum, P. aculeatum, and P. chermesinum) secreted ribotoxins that act on ribosomes [36]. The entomopathogenic fungi as Hirsutella thompsonii and Metarhizium anisopliae also produce ribotoxins, as well as, the basidiomycetes Agrocybe aegerita and Pleurotus.
ostreatus that produce ageritin and ostreatin, respectively [9], [35], [37].

According to this, the cytotoxic effects observed for the crude extract from A. niveus against tumor cell lines can be explained by the presence of ribotoxin-like proteins. In spite of the action of fungal ribotoxins as inhibitor of the tumor cell proliferation, their unpecific cytotoxicity against normal cells, as observed for the treatment of fibroblasts MRC-5, has limited their potential for clinical applications.1

In spite of the inhibition of normal cells by ribotoxins, the interest for these molecules has increased in the last years because the real possibility of elaboration of immunotoxins. Immunotoxins are chimeric proteins composed of a toxic moiety linked to an antibody domain, which directs the action of the toxic domain, promoting the death of the targeted cells [38]. Carreras-Sangrà et al. [15] reported the elaboration of an immunotoxin using alpha-sarcin with high specific toxicity against GPA33-positive tumor cell lines and reduced cytotoxicity against normal cells, suggesting that this chimeric protein is a good immunotherapeutic candidate against GPA33-positive colon carcinomas [15]. The high cytotoxicity effects presented by A. niveus ribotoxin-like proteins on some tumor cell lines, makes them promising molecules that can be used for different biotechnological applications.

IV. CONCLUSION

In conclusion, the extracellular extract containing ribotoxin-like proteins produced by the filamentous fungus A. niveus was able to inhibit the proliferation of some human tumor cell lines, especially medulloblastoma and melanoma. Further studies are important for a better understanding on the activities of these ribotoxin-like proteins and their biotechnological potential.

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Figure 4. Alignment of the peptides obtained from the 18 kDa protein band and from Fraction II with the ribotoxins alpha-sarcin, restrictocin and Asp f1. The (*) indicates the conserved aminoacids among all sequences; (C) indicates the conserved cysteine residues responsible by the disulfide bonds; (.) indicates amino acid residues in the catalytic center. The elements from the alpha-sarcin secondary structure are represented above the sequences.
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