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Research Article

HPLC Quantification of Cytotoxic Compounds from *Aspergillus niger*

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A high-performance liquid chromatography method was developed and validated for the quantification of the cytotoxic compounds produced by a marine strain of *Aspergillus niger*. The fungus was grown in malt peptone dextrose (MPD), potato dextrose yeast (PDY), and mannitol peptone yeast (MnPY) media during 7, 14, 21, and 28 days, and the natural products were identified by standard compounds. The validation parameters obtained were selectivity, linearity (coefficient of correlation > 0.99), precision (relative standard deviation below 5%), and accuracy (recovery > 96).

1. Introduction

Studies on marine-derived fungi as source of natural products have shown a sharp increase in recent years, due to the discovery of new molecular structures containing a broad of pharmacological property [1–5].

As part of an investigative effort to find promising anticancer agents from marine-derived fungi [6–8], the chemical investigation of a strain of *Aspergillus niger* (BRF074), recovered from sediments of the Northeast Brazilian coast, yielded the new furan ester derivative **(1)** and the cyclopeptides malformins A **(2)** and C **(3)** [9] (Figure 1).

Malformins are a group of cyclic pentapeptides with a disulfide bond formed from two cysteine thiols. These compounds show a variety of biological activities [10, 11] such as enhanced fibrinolytic activity [12] and antimalarial and antitrypanosomal properties [13]. The distinct activity profiles of the malformins A **(2)** and C **(3)** against cancer cell lines are also well reported in the literature [14–16]. These compounds have been found to be strongly cytotoxic against the human cancer cell lines NCI-H460 (non-small-cell lung carcinoma, IC_{50} 0.07 μM), MIA Pa Ca-2 (pancreatic inducing root curvatures and malformations in plants [17, 18], antibacterial activity cancer, IC_{50} 0.05 μM), MCF-7 (breast cancer, IC_{50} 0.10 μM), and SF-268 (CNS cancer; glioma, IC_{50} 0.07 μM) with slight selectivity towards the pancreatic cancer cell line (MIA Pa Ca-2) compared with the normal human primary fibroblast cells WI-38 (IC_{50} 0.10 μM) [16]. Compound **1**, a furan ester derivative, showed cytotoxic activity against HCT-116 tumor cell line with IC_{50} 2.9 μM [9].

The production of the cytotoxic compounds **1–3** by *A. niger* (BRF 074), cultured under different growth conditions, was investigated and quantified through high-performance liquid chromatography (HPLC) method using a diode array detector (DAD).

2. Experimental

2.1. Samples, Chemicals, and Materials. The furan ester derivative **(1)** and malformins A **(2)** and C **(3)** used for the preparation of standard solutions were isolated from the marine-derived fungus *Aspergillus niger* (BRF 074) as previously described [9]. HPLC-grade acetonitrile and methanol were purchased from Tedia®. All other chemicals were of analytical-grade in the highest purity available. Water was distilled and purified using a Millipore Milli Q Plus system

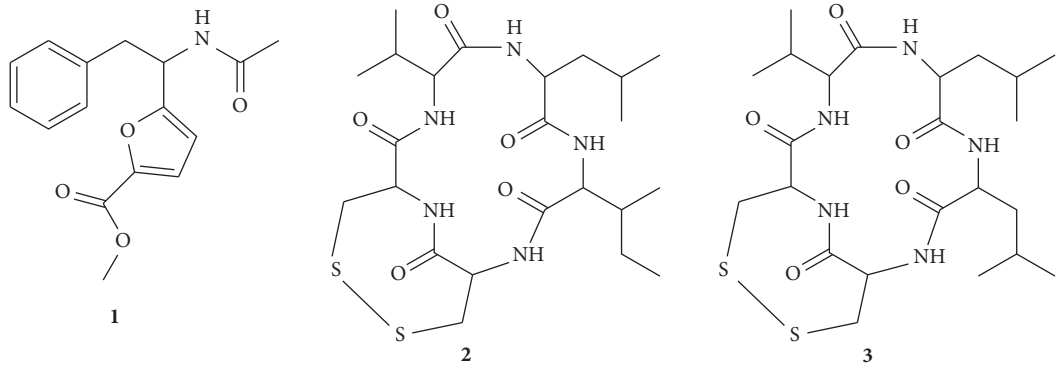

Figure 1: Cytotoxic compounds **1–3** produced by *Aspergillus niger* (BRF074).

(Bedford, USA). The nutrient media used in this work, malt peptone dextrose, MPD, potato dextrose yeast, PDY, and mannitol peptone yeast, MnPY, were obtained from Himedia® Laboratories.

2.2. Fungal Strain. The fungal strain of *A. niger* (BRF 074) was recovered from sediments collected by autonomous diving in the vicinities of Pecém's offshore port terminal (3°32′2″ S; 38°47′58″ W), state of Ceará, Northeast of Brazil, and its isolation and identification was performed as previously reported [8, 9].

2.3. Cultivation of A. niger (BRF 074) and Extract Preparation. The strain of *A. niger* (BRF 074) was previously grown in potato dextrose agar (PDA) in reconstructed sea water. Agar pieces with 5 mm diameter of 7-day-old cultures were used as precultures and transferred to a 250 mL Erlenmeyer flask with 100 mL of the nutrient medium: malt peptone dextrose, MPD (malt, 20.0 g L^{-1}; peptone, 2.0 g L^{-1}; dextrose, 20.0 g L^{-1}), potato dextrose yeast, PDY (potato dextrose yeast, 24.0 g L^{-1}; yeast, 3.0 g L^{-1}), or mannitol peptone yeast, MnPY (mannitol, 4.0 g L^{-1}; peptone, 2.0 g L^{-1}; yeast, 2.0 g L^{-1}).

The fungal strain was grown under static conditions at room temperature (28°C) during 7, 14, 21, and 28 days. The culture broths were separated from mycelium by filtration and submitted to partition with EtOAc (3 × 30 mL) to afford the crude extracts (MPD, PDY, and MnPY) after solvent distillation.

2.4. Quantitative High-Performance Liquid Chromatography Analysis (HPLC Analysis). Samples were filtered through a 0.45 μm Millex-HV PVDF membrane (Millipore, New Bedford, MA, USA). HPLC analyses were performed on a Shimadzu chromatographer equipped with a ternary pump (Shimadzu LC-20AT) and DAD detector (Diode Assay Detector) (Shimadzu SPD-M20A, Kyoto, Japan) and carried out on an analytical column (Phenomenex® ODS 100 A 250 mm × 4.60 mm, 5 μm) preceded by an C18 guard column (2.0 cm × 4.0 mm; 5 μm), also from Phenomenex. LC solutions software (version 1.25) was used for data processing. Chromatographic conditions were gradient using acetonitrile and water as mobile phase, initially consisting of acetonitrile/water (2 : 8 v/v) and increasing up to acetonitrile/water (8 : 2 v/v) in 30 minutes at a flow rate of 1 mL·min^{-1}. The mobile phase was prepared daily and degassed by sonication before use. The column temperature was 25°C and the injection volume was 20 μL. The UV spectra were monitored over a range of 450 to 200 nm, while the chromatograms were recorded at 273 nm to detect the furan ester derivative **(1)**, and to detect malformins A **(2)** and C **(3)** the PAD detector was set at λ = 203 nm.

Methanol was used to prepare all standard solutions and extracts. The standard solutions were used in six different concentrations, as follows: 12.5, 25.0, 100.0, 150.0, 250.0, and 500.0 μg·mL^{-1}, and the concentration of the solution extracts was 2,000 μg·mL^{-1}.

2.5. Analytical Method Validation. The validation of the analytical method was performed in the HPLC-DAD system and the validated parameters evaluated were specificity, linearity, accuracy, precision (repeatability and intermediate precision), limit of quantification (LOQ), and limit of detection (LOD), which are in accordance with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines [19].

The linearity was determined by the correlation coefficients of the analytical curves, which were built by analyzing the working solutions at six different concentrations. The method linearity for the three compounds was tested through linear least-square regression analysis based on calibration curves constructed by using 12.5, 25.0, 100.0, 150.0, 250.0, and 500.0 μg·mL^{-1} solutions (Graphpad Prism® 5.03 software).

The limits of detection (LOD) and quantification (LOQ) were calculated based on the standard deviation and slope of the regression curves.

The precision was evaluated in two steps: repeatability and intermediate precision. The repeatability was determined by injecting in triplicate, on the same day, standard solutions at three different concentrations (25.0, 100.0, and 500.0 μg mL^{-1}). Intermediate precision was estimated by analyzing, in triplicate, the same solutions employed in the repeatability test on three consecutive days. The results obtained were expressed in terms of relative standard deviation (RSD).

Table 1: Parameters of the analytical curves utilized for quantification of the bioactive compounds **1–3** produced by *A. niger* (BRF 074).

Compound	Linearity range (μg mL^{-1})	Regression equation[a]	Correlation factor (r^2)
Furan ester **(1)**	12.5–500.0	$y = 11717.0x - 136817.5$	0.9984
Malformin A **(2)**	12.5–500.0	$y = 26362.2x - 246504.3$	0.9996
Malformin C **(3)**	12.5–500.0	$y = 26160.0x - 246504.3$	0.9991

[a]Six data points ($n = 3$).

Table 2: Repeatability and intermediate precision data of the bioactive compounds **1–3** produced by *A. niger* (BRF 074).

Compound	Repeatability ($n = 3$)				Intermediate precision ($n = 3$)			
	Concentration (μg mL^{-1})	Measured values (μg mL^{-1})	Mean (μg mL^{-1})	RSD (%)	Concentration (μg mL^{-1})	Measured values (μg mL^{-1})	Mean (μg mL^{-1})	RSD (%)
Furan ester **(1)**	50.0	54.2892 54.3437 54.2872	54.3067	0.06	50.0	50.1338 50.2537 50.0912	50.1595	0.17
	250.0	233.8591 234.3952 234.1043	234.1195	0.11	250.0	245.7478 245.7127 245.7350	245.7319	0.01
	500.0	509.2980 509.2856 509.2549	509.2795	0.01	500.0	500.9200 500.8662 501.3840	501.0567	0.06
Malformin A **(2)**	50.0	51.7388 51.7246 51.6044	51.6893	0.14	50.0	49.3617 49.4094 49.4188	49.3967	0.06
	250.0	246.2154 246.0825 246.1936	246.1638	0.03	250.0	239.8196 240.0045 240.0410	239.9550	0.05
	500.0	503.6637 502.9370 503.2363	503.2790	0.07	500.0	505.5801 505.6485 505.8089	505.6792	0.02
Malformin C **(3)**	50.0	52.8589 52.7992 52.8364	52.8315	0.06	50.0	51.1270 51.0972 50.9613	51.0618	0.17
	250.0	241.1501 241.3867 241.4114	241.3161	0.06	250.0	231.0606 231.2140 231.3173	231.1973	0.06
	500.0	505.8446 506.9641 505.1826	505.9971	0.18	500.0	510.3529 510.2716 510.1154	510.2467	0.02

The accuracy was determined from recovery tests with solutions of the standard compounds having low, medium, and high concentration levels, and the recoveries of the tree standard compounds were calculated from the corresponding calibration curve according to the following equation:

$$\text{Recovery (\%)} = \left(\frac{\text{amount found}}{\text{amount added}}\right) \times 100. \quad (1)$$

The test concentrations used were 140.0, 186.0, and 233.0 μg mL^{-1} for the furan ester derivative **(1)**; 70.0, 92.0, and 115.0 μg mL^{-1} for malformin A **(2);** and 101.0, 134.0, and 166.0 μg mL^{-1} for malformin C **(3)**. All the samples were analyzed in quintuplicate.

Accuracy was expressed as the percentage of deviation between the measured value and the reference value.

The selectivity of the method was evaluated by analyzing sterile medium (blank) and fungal culture broth under the conditions previously established.

2.6. Quantification and Validation Procedures. To ensure the linearity, calibration curves of the three compounds were obtained. Standard solutions of the furan ester derivative **(1)** and malformins A **(2)** and C **(3)** were prepared at a concentration range of 12.5 to 500.0 μg mL^{-1}. All the curves showed a linear response with $r^2 > 0.998$ in the selected range for each sample (Table 1).

The furan ester derivative **(1)** showed LOD of 46.0 μg mL^{-1} and LOQ of 67.9 μg mL^{-1}. Malformin A **(2)** showed LOD of 22.7 μg mL^{-1} and LOQ of 33.6 μg mL^{-1}, while malformin C **(3)** showed LOD of 34.7 μg mL^{-1} and LOQ of 51.3 μg mL^{-1}.

The precision was estimated by measuring repeatability (intraday assay) and intermediate precision (interday assay), both in triplicate. All the values of relative standard deviation (RSD) in the repeatability and intermediate precision estimates were below 5% (Table 2), which did not exceed the limits recommended in the literature [19, 20].

Table 3: Accuracy data of the bioactive compounds **1**–**3** produced by *A. niger* (BRF 074).

Compound	Accuracy ($n = 5$)			
	Concentration (μg mL^{-1})	Measured values (μg mL^{-1})	Mean (μg mL^{-1})	Recovery (%)
Furan ester **(1)**	140.0	134.1553 133.9125 134.1571 134.2570 133.8316	134.0627	95.8
	186.0	178.5342 178.6454 178.5064 178.4201 178.6202	178.5453	95.9
	232.0	224.0426 223.6944 223.9213 224.0262 224.1322	223.9634	96.4
Malformin A **(2)**	70.0	72.7681 73.0678 73.0619 72.9432 72.9750	72.9632	104.2
	92.0	98.0389 97.8777 97.9863 97.9622 97.7580	97.9246	106.4
	115.0	117.8192 117.5671 117.6607 117.7728 117.5518	117.6743	102.3
Malformin C **(3)**	101.0	102.5096 102.4978 102.7050 102.5853 102.6210	102.5837	101.5
	134.0	130.4046 130.5980 130.5686 130.6417 130.2936	130.5013	97.4
	166.0	160.7364 160.7337 160.5222 160.4871 160.6094	160.6178	96.8

The accuracy of the method was evaluated by recovery experiments with the standard solutions at three different concentration levels. The recovery values ranged from 95.8 to 106.4% (Table 3) and were also in line with ICH parameters.

2.7. Method Application: Growth Behavior of Aspergillus niger (BRF 074). The method developed showed being efficient for the quantitative analyses of three bioactive compounds produced by *A. niger* (BRF 074) in three fermentative mediums: malt peptone dextrose (MPD), potato dextrose yeast (PDY), and mannitol peptone yeast (MnPY).

These analyses were carried out after inoculation of the mycelia obtained from seed medium into MPD, PDY, and MnPY. In order to evaluate the kinetic production of the compounds, different periods of incubation were analyzed

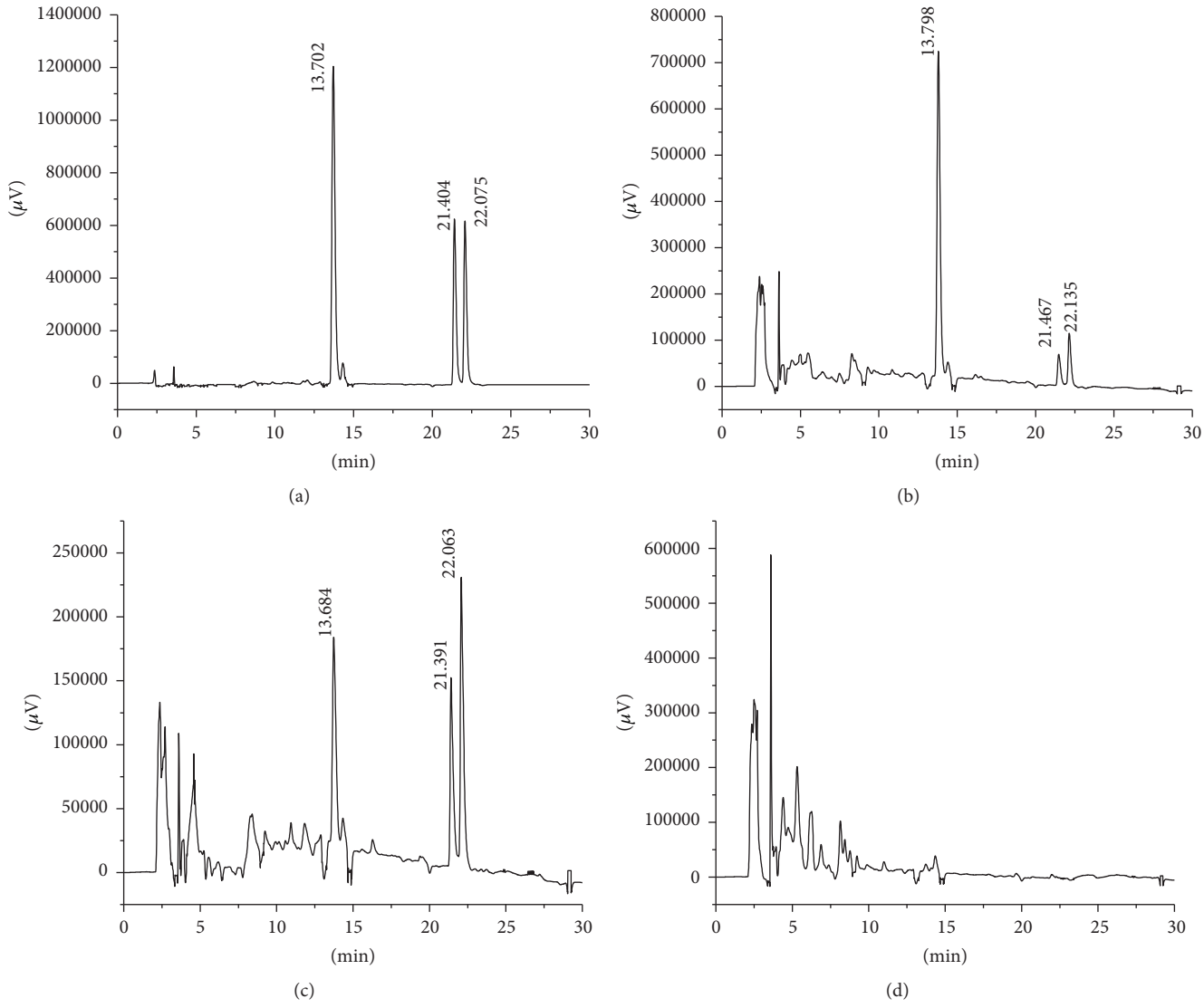

Figure 2: HPLC chromatograms of the (a) standard compounds **1–3**, (b) extract obtained from *A. niger* (BRF 074) cultured during 28 days in MPD medium, (c) extract obtained from *A. niger* (BRF 074) cultured during 28 days in PDY medium, and (d) extract obtained from *A. niger* (BRF 074) cultured during 28 days in MnPY medium. Chromatography conditions: reversed-phase column ODS (250 × 4.6 mm, 5 μm); gradient elution going from H_2O/MeCN 80 : 20 to H_2O/MeCN 20 : 80 in 30 minutes; flow rate of 1.0 mL min^{-1}; sample concentration of 2.0 μg mL^{-1}.

(7, 14, 21, and 28 days), and their concentrations in each period were obtained in triplicate ($n = 3$). The contents of the three cytotoxic compounds in the crude extracts, MPD and PDY, are shown in Table 4.

3. Results and Discussion

The focus of the study was to quantify the amount of the cytotoxic metabolites, furan ester derivative **(1),** and malformins A **(2)** and C **(3)**, produced by the marine-derived strain of *A. niger* (BRF 074) grown in varied nutrient medium and time of cultivation.

The validation method was carried out simultaneously for the bioactive compounds, and the retention time was found to be 13.7 min for **1** and 21.4 min and 22.1 min for **2** and **3**, respectively. Although the analysis showed a relative long run time (30 min), the secondary metabolites could be analyzed with good separation and baseline resolution between all peaks (Figure 2).

The chromatographic analysis of the organic extracts (MPD, PDY, and MnPY) revealed a similar HPLC profile for MPD and PDY and suggested that the bioactive compounds were produced by *A. niger* in both culture media. No significative amounts of the analyzed compounds **1–3** were found in the extract from the MnPY medium.

In our analysis, the highest content (274.0 μg mL^{-1}) of **1** was found when using MPD as culture medium, during a growth period of 28 days. In this same condition, the contents

Table 4: Contents of the bioactive compounds **1**–**3** in MPD and PDY extracts produced by *A. niger* (BRF 074). Concentration of the extracts = 2,000 μg mL^{-1}.

Growth time (days)	Furan ester **(1)** (Rt = 13.7 min)	Malformin A **(2)** (Rt = 21.4 min)	Malformin C **(3)** (Rt = 22.1 min)
	MPD extracts		
07	14.7	37.5	60.1
14	17.0	26.5	34.9
21	92.3	46.2	67.5
28	274.0	86.3	125.9
	PDY extracts		
07	13.8	37.4	50.1
14	24.8	112.7	166.7
21	44.4	179.0	233.8
28	74.3	163.1	235.4

Concentration expressed as μg mL^{-1} (n = 3).

of **2** and **3** were 86.3 μg mL^{-1} and 125.9 μg mL^{-1}, respectively. On the other hand, the best condition for the production of **2** and **3** was achieved when using PDY as culture medium, within 21 and 28 days of growth. Within 21 days of growth, the concentrations of **2** and **3** were 179.0 and 233.8 μg mL^{-1}, respectively, while the concentration of **1** was 44.4 μg mL^{-1}. With 28 days of fermentation, also in the PDY medium, the concentrations of **2** and **3** did not change considerably when compared with 21 days of growth, and their respective contents were 163.1 and 235.4 μg mL^{-1}. However, the concentration of **1** increased almost twice (74.3 μg mL^{-1}) when comparing the periods of 28 and 21 days of fermentation.

The developed chromatographic method herein described proved to be efficient in the quantitative analyses of the cytotoxic compounds **1**–**3** in the extracts from *A. niger* cultured under different conditions, as confirmed by its validated analytical parameters by HPLC-PDA system. Considering these aspects, the analytical quantitative method can be a useful and important tool for monitoring the production of the analyzed cytotoxic compounds by *A. niger*.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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