World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Available online at: http://www.wjpsonline.org/
Original Article

Cytotoxic activities of some marine endophytic fungi isolated from *Phallusia nigra*

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Received: 10-02-2021 / Revised Accepted: 23-02-2021 / Published: 27-02-2021

ABSTRACT

Tunicates have been reported to be a wealthy source of biologically active compounds. The present study aims at isolation of endophytic filamentous fungi from *Phallusia nigra*, a familiar tunicate from the Red Sea, then medium optimization for the highest yield fungus which was identified as *Aspergillus niger* (Trichocomaceae) by Internal Transcribe Sequences (ITS) technique. Secondary metabolites of *A. niger* were extracted from solid fermentation medium. Different concentrations of the extract were evaluated for their potential cytotoxic activity on Hepatoblasotoma (HepG2), Colorectal carcinoma (HCT-116) and Mammary gland (MCF-7) cell lines by using tetrazolium bromide (MTT) assay. The extract showed strong cytotoxic activity on HCT-116 and MCF-7 cell lines with IC_{50} 12.01 and 15.53 *µg/ml*, respectively and moderate activity on HepG2 cell line with IC_{50} 26.33 *µg/ml*. This inhibitory activity on cells growth could be probably due to the sensitivity of the cell lines to the bioactive metabolites in the extract.

Key words: *Phallusia nigra, Aspergillus niger*, methanolic extract, cytotoxicity, HePG2, HCT-116 and MCF-7 cell lines.

INTRODUCTION

Recently, marine fungi have received increased attention as a source of extraordinary diverse secondary metabolites of clinically important compounds [1, 2]. Fungi live in composite ecosystems and had to compete with multifarious organisms, such as algae, bacteria, other fungi, small metazoans and protozoans. So, fungi must fight for survival, they have probably acquired the capability to produce secondary metabolites which may inhibit the growth of their competitors. Accordingly, it is not surprising that a number of important drugs have been included in fungal

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How to Cite this Article: Ghada Mahmoud Abdelwahab, Tarek A. Abdelaziz, Mohamed Farid I. Lahloub, Ashraf Taha Khalil. Cytotoxic activities of some marine endophytic fungi isolated from *Phallusia nigra*. World J Pharm Sci 2021; 9(3): 144-149.

secondary metabolites such as the antibiotic penicillin [3] and the immunosuppressant cyclosporine [4]. Even though a noteworthy number of marine fungal natural products are already known, the potential for further exploration is still wide open.

Oncology poses many complications owing to some issues related to drug efficacy and dangerous side effects for normal cells. Seeking novel drugs is still a priority goal for chemotherapy, due to the fast resistance developed to numerous chemotherapeutic drugs. Thus, an increasing demand for lead antitumor drugs active against untreatable tumors, which have lower side effects, and/or with better therapeutic efficiency [5].

Considering this as the main target, isolation of some filamentous fungi from *Phallusia nigra* was done as cytotoxic activity of its methanolic extract has been reported [6]. Among several isolates, *Aspergillus niger* (Trichocomaceae) was chosen for our study as it has the highest yield of secondary metabolites and also, was a known source for various cytotoxic compounds, which induced apoptosis in various kinds of cancer cells [7-9. The metabolites of funal mycelia were extracted from solid fermentation media using methanol. Different concentrations of the extract were evaluated for their potential cytotoxic activity on HePG2, HCT-116 and MCF-7 cell lines.

MATERIALS AND METHODS

General experimental procedures: Light microscope and stereomicroscope were used for determination of the morphological structures and colonial features of the fungi. The RPMI-1640 medium, tetrazolium bromide (MTT) and DMSO (sigma co., St. Louis, USA) while Fetal Bovine serum (GIBCO, UK) was used for MTT assay. Doxorubicin was the standard anticancer drug used for comparison. The colorimetric assay (MTT) is measured at absorbance 570 nm by a plate reader (EXL 800, USA). Inverted microscope (Labomed, Germany) was used to observe and photograph the cells.

Fungal material: The Red Sea tunicate was collected by SCUBA diving at 5 m depth, in June 2017, from Hurghada, Egypt. Identification was done by the National Institute of Oceanography and Fisheries (NIOF), Hurghada, Egypt. The specimen was rinsed three times with sterile sea water then it was translocated in a cooler bag previously filled with sterile sea water to the laboratory to be processed instantly.

Culture conditions and fungi isolation: *Phallusia nigra* was rinsed triple with sterile seawater and superficially sanitized with 70% ethanol for 90 seconds then, cut into tiny pieces (1 x 1 cm) aseptically, by using a sterile dissection razor and cultivated on Potato Dextrose Agar (PDA) medium (*Oxoid* Ltd, Basingstoke, Hants, UK) Petri plates dissolved in 50% aged sea water and contained 250 mg/L Amoxycillin to inhibit bacterial growth. These plates were incubated at 25 °C for 10 days in the dark until satisfactory growth of the fungus. Repeated re-inoculations on saline PDA Petri plates were done to obtain pure strains of isolated fungi. Pure cultures were streaked on PDA slants and maintained for identification and further studies [10].

Identification of fungi: Fungal identification methods were based on the morphological features of the fungal colonies and spores characteristics [11, 12]. Currently, the PCR primers are an extra-accurate way for fungal identification. This method gained broad acceptance with fungal Internal Transcribe Sequences (ITS) were "ITS1" and "ITS4" that amplify the highly changeable ITS1 and ITS2 sequences nearby the 5.8 S-coding sequence and located between the ribosomal operon Small SubUnit-coding sequence (SSU) and the ribosomal operon Large SubUnit-coding sequence (LSU). DNA Extraction then PCR amplification of wide range of fungal targets work well for analyzing the isolated DNA from individual organisms [13].

Cultivation and Extraction of Fungal Culture: Each pure strain was inoculated on five PDA plates and kept at 25 °C for 7 days. Until sufficient growth of the colonies was observed, the five plates were homogenized then transferred to 1 liter flask contained 500 ml methanol and left overnight at 25 °C. The mycelial mass was filtered through Whatmann No.1 filter paper. The mixture was dried using a rotary evaporator at 40 °C. The obtained solid material was weighed forming the crude extract.

Optimization for culture medium conditions for the highest weight crude extract (*Aspergillus niger*) was done. Three PDA plates of *A. niger* were transferred as seed into three pre-autoclaved 1L flask; the first containing 80 g wheat, the second containing 80 g bran, the last one containing 80 g rice in 110 ml 50% aged sea water. The fermentation flasks were incubated for 21 days at room temperature in the dark.

Extraction of fungus secondary metabolites was done by methanol (3 x 1L) using sonication at 50 $^{\circ}$C for 15 minutes for each flask. The mixture was filtered and evaporated using rotary vacuum evaporator till dryness at 50 $^{\circ}$C, and then the crude extract was weighed.

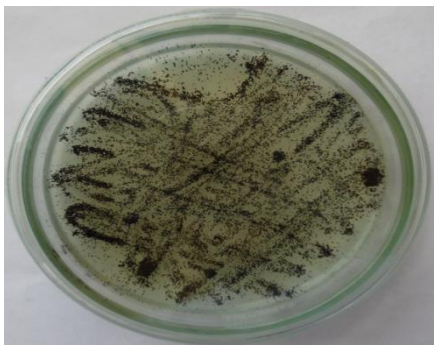
Cell lines: Mammary gland (MCF-7), Hepatocellular carcinoma (HEPG-2) and Colorectal carcinoma (HCT-116). The three cell lines were purchased from ATCC via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt.

MTT assay: The cytotoxicity of the *A. niger* crude extract was determined using MTT colorimetric assay. Conversion of the yellow tetrazolium bromide (MTT) to a purple formazan derivative by the mitochondrial succinate dehydrogenase which present in viable cells is the clue of this colorimetric assay. Cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum. Penicillin 100 µg/ml and streptomycin 100 µg/ml were added at 37 °C in a 5% Co_2 incubator. Each cell line was seeded in a 96-well plate at a density of 1.0 x10^4 cells/ well at 37 °C for 48 h under 5% Co_2. After incubation the cells were treated with varying concentrations of the crude extract and incubated again for 24 h., then 20 µl of MTT solution at 5mg/ml was added to each well and further incubation for 4 h in a dark place. Adding 100 µl of dimethyl sulfoxide (DMSO) into each well was done to dissolve the formed purple formazan crystals. The absorbance was recorded at 570 nm using enzyme‑linked immunosorbent assay (ELISA) by a microplate reader. The results of this assay were used to obtain the half maximal inhibitory concentration (IC_{50}) values. The cells were observed under an inverted microscope and photographed. The experiment was conducted in triplicate and the averages of three independent experiments represent the values [14, 15]. The relative cell viability was calculated as percent by the following formula:

$$\% \, Cell \, viability = \frac{Mean \, absorbance \, in \, test \, well}{Mean \, absorbance \, in \, control \, well} \times 100$$

RESULTS AND DISCUSSION

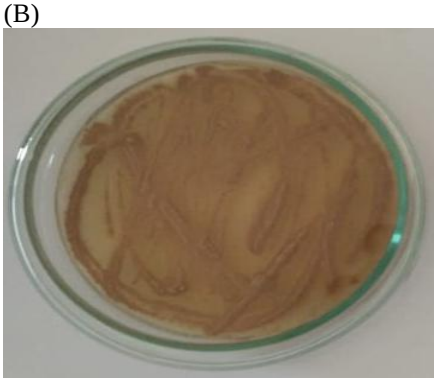
Identification of fungi: *Trichoderma longibrachiatum*, *Aspergillus ochraceus*, *Aspergillus terreus and Aspergillus sydowii* were morphologically characterized (Fig. 1) from the features of conidiophores at the Mycological Center, Faculty of Science, Assiut University, Egypt (pure cultures deposited with AUMC no. 13026, 13955, 13956, 13957, respectively). *Aspergillus niger* was identified by PCR using the universal fungal primers Internal Transcript Spacer regions (ITS1 and ITS4) (GenBank accession No.LC582533). Isolated pure fungi voucher strains are deposited at the biological laboratory of the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Egypt.



(A)

(B)

(C)



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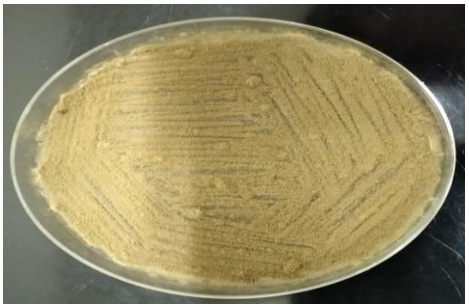

(E)

Figure (1): Pure strains of the isolated fungi. (A) *Aspergillus niger; (B) Trichoderma longibrachiatum*; (C) *Aspergillus ochraceus; (D) Aspergillus terreus and (E) Aspergillus sydowii***.**

Culture medium optimization for the highest yield fungus: Preliminary quantitative analysis of the crude extract of the pure isolated fungi was performed for identification of the highest yield-fungi to be used in further studies. The crude extract of each strain was weighed after dryness to yield 0.63, 0.54, 0.52 and 0.46 mg for *Trichoderma longibrachiatum*, *Aspergillus ochraceus, Aspergillus terreus and Aspergillus sydowii, respectively while Aspergillus niger yielded 1.23 gm. So,* the black-colored fungus; *Aspergillus niger;* was chosen for the Culture medium optimization and the cytotoxic study. Further quantitative analysis was done for the three extracts which was cultivated on different media. The mat of each media was weighed yielding 0.5, 0.95 and 1.2 gm for rice, bran and wheat media.

MTT assay: Among the isolates, *A. niger* was selected for the study as it was an established source of various cytotoxic secondary metabolites on different cell lines [7-9]. Extraction of the metabolites from the fungal mycelia was by using methanol. The percent relative viability of cells treated with the methanolic extract of *A. niger* (Fig. 2, 3), and (Table 1) showed that the inhibition was dose-dependent during a concentration range from 1.56 to 100 µg/ml. This extract showed the highest apoptotic activity on cultured HCT-116 cells with IC_{50} 12.01±1.1 as compared with doxorubicin (Table 2). The IC_{50} values is fell within the criteria of the cytotoxic activity for the crude extracts, as recognized by the American National Cancer Institute (NCI) is $IC_{50} \leq 30$ µg/ml [16] and the highly inhibiting activity means $IC_{50} \leq 20$ µg/ml [17], thus *A. niger* is considered a candidate for promising anticancer drugs.

Table 1: Cytotoxic activity IC_{50} (µg/ml) of *A. niger* extract on the experimented cell lines.

Compounds	*In vitro* Cytotoxicity IC_{50} (µg/ml)		
	HePG2	HCT-116	MCF-7
Doxorubicin	4.50±0.2	5.23±0.3	4.17±0.2
Aspergillus niger extract	26.33±1.8	12.01±1.1	15.53±1.2

Table 2: The relative viability % of *A. niger* extract at different concentrations (ug/ml) on the tested cell lines.

Conc.(µg/ml)	HePG-2	HCT-116	MCF-7
100	24.3	13.9	21.1
50	37.1	21.3	23.7
25	49.2	32.7	37.2
12.5	61.5	41.8	52.8
6.25	78.6	67.2	65.0
3.125	97.1	79.0	86.9
1.56	100	98.1	100

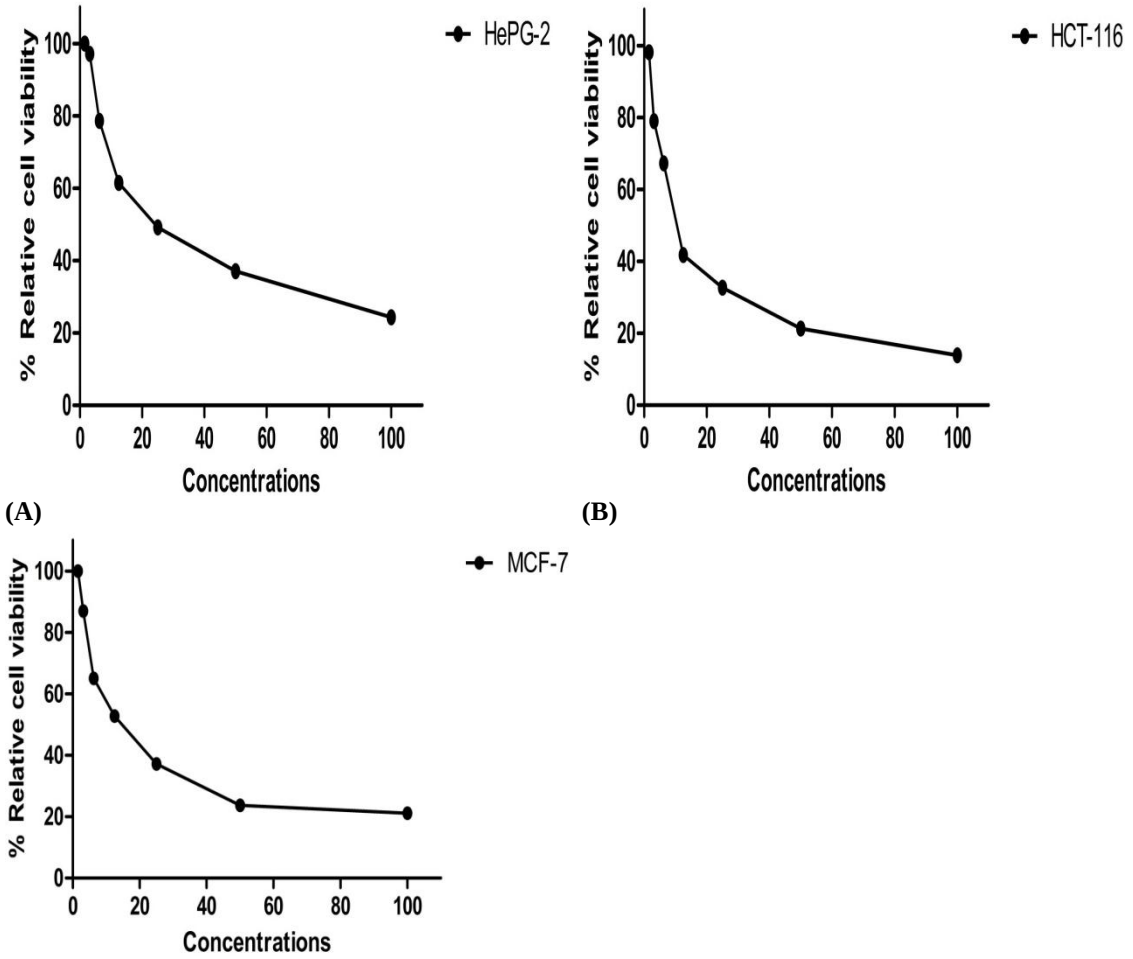

Figure (2): Growth inhibitory effect of *A. niger* methanolic extract on the three cell lines; (A) Hepatoblasotoma (HepG2), (B) Colorectal carcinoma (HCT-116) and (C) and Mammary gland (MCF-7).

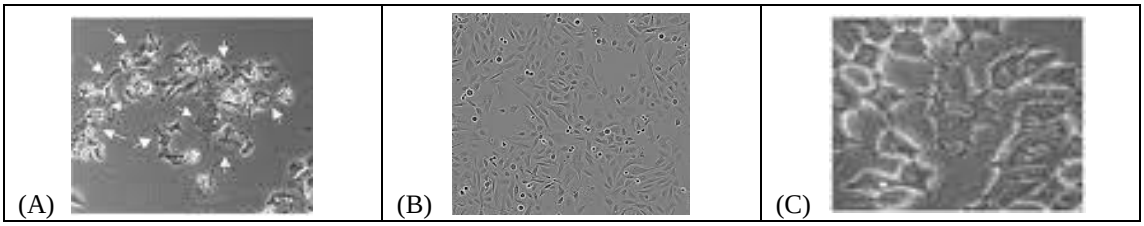

Figure (3): Morphological observation of (A) Hepatoblasotoma HepG2 cells, (B) Colorectal carcinoma (HCT-116) cells and Mammary gland (MCF-7) cells treated with *Aspergillus niger* extract.

CONCLUSION

The findings of this study will contribute to increase the chance for natural control of various tumor cells. *In vivo* assay may be requisite to corroborate our results. These data forecast the potential of this strain to be used as candidate for the production of anticancer agents with least side effects. Further studies can be carried out on optimization of other *A. niger* fermentation parameters as PH, temperature, pressure and salinity. Bioassay-guided fractionation of this potentially anticancer extract for the isolation and structural elucidation of the active compounds is required to analyze the exact mechanism and possible industrial exploitation.

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