



In vitro Anticancer Activity of Marine Fungus *Penicillium cyaneum*

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Abstract	Article Info
<p>Cancer is one of the most life-threatening diseases worldwide. Over the last few years, several metabolites produced by marine fungi have displayed potent anticancer effects. In the present study the anticancer activity of marine fungus <i>Penicillium cyaneum</i> against HepG 2 cells (human liver carcinoma cells) was assessed by <i>in vitro</i> cytotoxicity assay. The results revealed that <i>P. cyaneum</i> ethyl acetate extract showed potent cytotoxic effects in HepG 2 cells, with cell viability (29.5%) observed at 1000µg/mL. The IC₅₀ of ethyl acetate extract of <i>P. cyaneum</i> was 242.24 µg/mL.</p>	<p>Accepted: 08 April 2017 Available Online: 20 April 2017</p> <hr/> <p>Keywords</p> <p>Cancer, marine fungus, HepG 2, cytotoxicity.</p>

Introduction

Cancer is a generic term for a large group of diseases characterized by the growth of abnormal cells beyond their usual boundaries that can then invade adjoining parts of the body and/or spread to other organs. Cancer can affect almost any part of the body and has many anatomic and molecular subtypes that each requiring specific management strategies. Cancer is the second leading cause of death globally and accounted for 8.8 million deaths in 2015. Lung, prostate, colorectal, stomach and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, cervix and stomach cancer are the most common among women (WHO, 2017). In India, around 2.5 million of people living with the disease, 556 400 national cancer deaths occurred in 2010. 395 400 (71%) cancer deaths occurred in people aged 30–69 years (200 100 men and 195 300 women). Every year, 7 lakhs new cancer patients registered. Cancers of oral cavity and lungs in males and cervix and breast in females account for over 50% of all cancer deaths in India (Nandakumar, 2009; Dikshit *et al.*,

2012). The biggest challenges in the field of cancer biology are to discover an anticancer drug which has the ability to kill the cancer cells without any side effects to the surrounding cells. The recent and systematic chemical characterization of fungi from the marine environment has provided a total number that currently exceeds 1000 new natural products (Rateb and Ebel, 2011; Gomes *et al.*, 2015). The marine fungus is having the ability to produce chemically unique bioactive molecules, which is supported by the identification of new anticancer metabolites through the application of classical screening and isolation techniques. Therefore, in the present study we designed to study the anticancer activity of marine fungus against HepG 2 cells (human liver carcinoma cells).

Materials and Methods

Isolation and identification of mangrove fungus

Dilution plating technique described by Waksman (1922) was used to isolate the mycoflora from soils. The fungus

P. cyaneum was isolated from mangrove soil of Karankadu, Ramanathapuram Dt, Tami Nadu, India. The identification was done by using standard manuals such as Manual of Penicillia (Raper and Thom, 1949) and Manual of Soil fungi (Gillman, 1957).

Preparation ethyl acetate extract from *P. cyaneum*

The crude ethyl acetate extract from *P. cyaneum* was prepared following the methodology described by Joel and Bhimba (2012).

Fungal broth culture

In order to obtain secondary metabolites the pure culture of *P. cyaneum* was grown in potato dextrose broth culture medium at 28°C for 3 days. After that a preinoculum was prepared by introducing small fragments (1cm square) of the growth culture into 250ml Erlenmeyer flasks containing potato dextrose broth and cultivated on a rotary shaker at 200rpm, 28° C for 5 days. Then the mycelium and the filtrate were separately subjected to solvent extraction.

Extraction of mycelia

The fresh mycelium of *P. cyaneum* was washed three times with water (distilled water: sea water 1:1) to remove adherent filtrate, and then plotted between folds of whatman filter paper no 1. The plotted mycelium was crushed using mortar and pestle with ethyl acetate and subjected to sonication (Labsonic, Sartorius, Germany) for 3 -4 hours to obtain intracellular metabolites. Centrifuged at 2000 -2500 rpm for 5 mins and the supernatant was used for further studies.

Extraction of the filtrate

The filtrate of *P. cyaneum* was extracted several times with ethyl acetate (v/v) in a separating funnel. The extracts from both mycelia and filtrate were evaporated under vaccum at 50° C till dryness. The obtained solid material was dissolved in ethyl acetate to form the crude extract and tested for cytotoxicity assay.

Cell culture of cancer cells and Cytotoxicity assay

Cell culture

HepG 2 cells obtained from NCCS (National Centre For Cell Science, Pune) were cultured in Rose well Park Memorial Institute medium (RPMI), supplemented with

10% fetal bovine serum, penicillin/streptomycin (250 U/mL), gentamycin (100µg/mL) and amphotericin B (1mg/mL). All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. Cells were allowed to grow to confluence over 24 h before use.

Cytotoxicity assay

Cytotoxicity was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mosmann, 1983). Cells (1×10^5 /well) were plated in 24-well plates and incubated in 37°C with 5% CO₂ condition. After the cell reaches the confluence, the various concentrations (15.6, 31.2, 62.5, 125, 250, 500 and 1000 µg/ml) of the ethyl acetate extract of *P. cyaneum* were added and incubated for 24hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or MEM without serum. 100µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells. The absorbance at 570nm was measured with UV-Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC₅₀) was determined graphically. The morphological changes of the HepG 2 cells were microscopically observed.

The % cell viability was calculated using the following formula:

$$\% \text{ cell viability} = \frac{A_{570} \text{ of treated cells}}{A_{570} \text{ of control cells}} \times 100$$

Graphs are plotted using the % of Cell Viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments.

Results and Discussion

In the present study, *P. cyaneum* was isolated and identified from mangrove soils of Karankadu, Ramanathapuram Dt. Macro and micro morphology of isolated *P. cyaneum* was shown in Figure 1.

Scientific Classification of *P. cyaneum*

Class	:	Eurotiomycetes
Order	:	Eurotiales
Family	:	Trichocomaceae

Genus : Penicillium
 Species : *cyaneum*
 Synonymous : *Citromyces cyaneus*

Cytotoxicity of *P. cyaneum* against HepG2 cell lines

The results of cytotoxicity of *P. cyaneum* against HepG2 cell lines were illustrated in Table 1 and Figure 1. The ethyl acetate extract of *P. cyaneum* showed significant cytotoxicity in the HepG 2 cells (human liver carcinoma cells). The results are in harmony with the findings of Joel and Bhimba (2012). They reported the considerable cytotoxic effect of the crude extracts of *Pestalotiopsis microspora* VB5 at various concentrations against Hep 2 cell lines. Recently a study by Suja *et al.*, (2014) who accounted *Aspergillus terreus* showed better activity against HepG2 cell line. Bioactive compounds such as sargassamide, halimide and avrainvillamide isolated from a marine fungus have shown selective inhibition against cancer cell lines, and shown *in vivo* activity in preclinical models (P-388 lymphocytic leukemia) (Bhadury *et al.*, 2006).

Macroscopic Observation of *P. cyaneum*

Colony morphology on PDA medium growing restrictedly, consisting of a close-textured basal felt, radically furrowed, with central areas commonly raised, surface appearing velvety, gray-green, re-verse colorless.

Microscopic Observation of *P. cyaneum*

Conidiophores arising branches from ascending aerial hyphae, with walls smooth, irregularly branched, penicilli monovercillate, small, usually consisting of closely crowded verticils of 5 to 8 parallel sterigmata, conidia elliptical, with ends often more or less pointed, smooth-walled.

Table.1 Cytotoxicity of *P. cyaneum* against HepG2 cell lines

S. No.	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.09	29.5
2	500	1:1	0.13	38.2
3	250	1:2	0.17	51.6
4	125	1:4	0.21	59.5
5	62.5	1:8	0.29	63.5
6	31.2	1:16	0.35	71.0
7	15.6	1:32	0.41	85.1
8	Cell control	-	0.59	100

Fig.1 Macro and Microscopic view of *P. cyaneum*

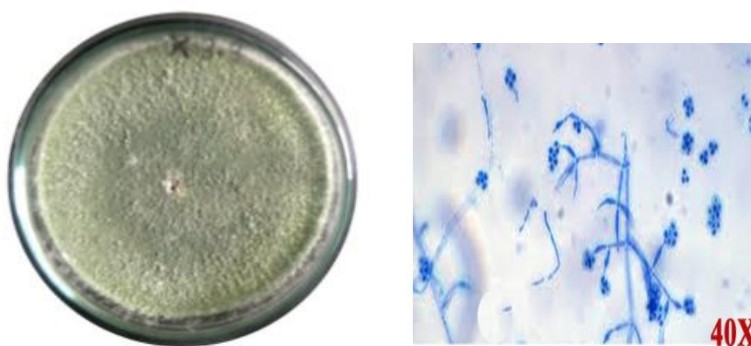


Fig.2 Cytotoxicity of *P. cyaneum* against HepG2 cell lines

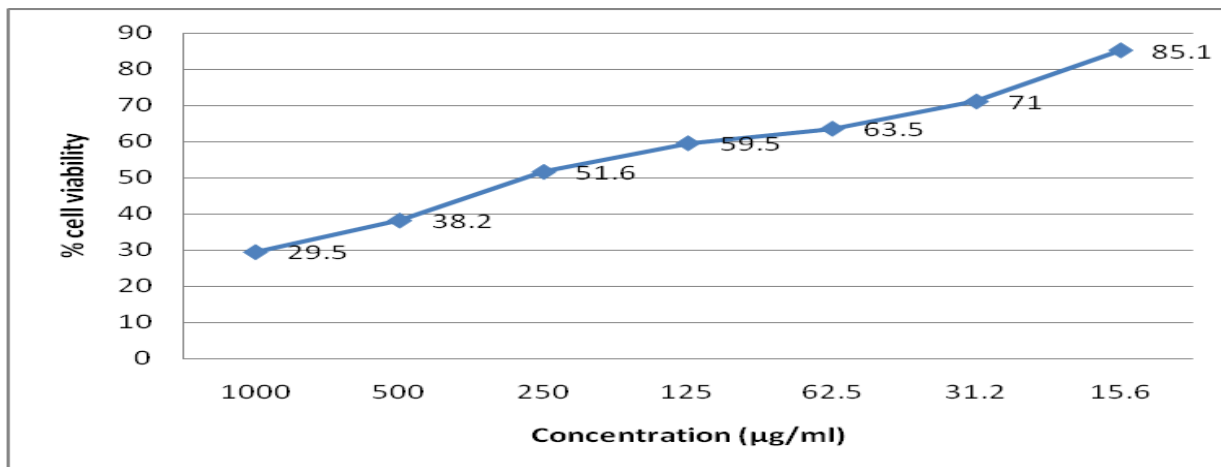
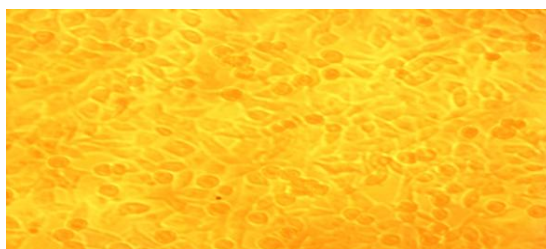
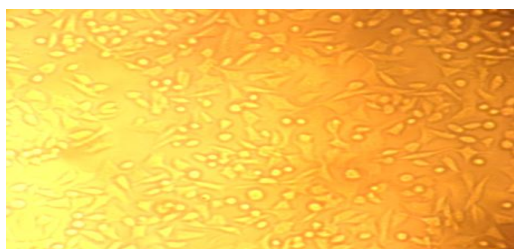


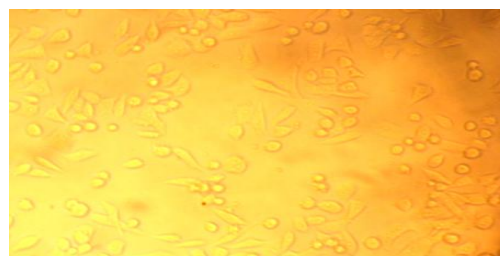
Fig.3 Morphology of HepG2 cells treated with ethyl acetate extract of *P. cyaneum*



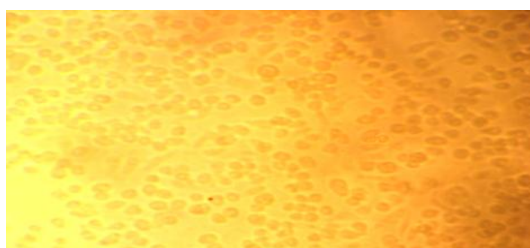
Normal HEP G 2Cell line



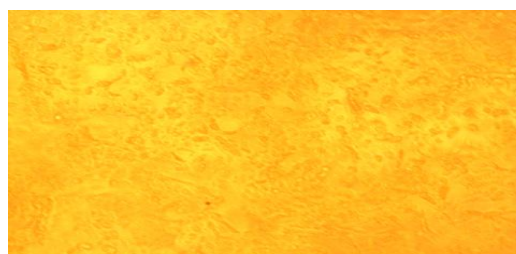
Toxicity -62.5µg/ml



Toxicity -125µg/ml



Toxicity -250µg/ml



Toxicity -1000µg/ml

In the present investigation, *P. cyaneum* ethyl acetate extract showed potent cytotoxic effects in HepG 2 cells, with cell viability (29.5%) observed at 1000µg/mL. The IC₅₀ of ethyl acetate extract of *P. cyaneum* was 242.24 µg/mL. Similar findings have been reported for marine-derived fungi *Neosartorya paulistensis* and *Neosartorya siamensis* showed decrease the number of viable cells, with an IC50 lower than 200 mg/mL in HCT116, A375 and HepG2 cells by Ramos *et al.*, (2015).

In the same way, anticancer activity of various marine fungi such as *Clonostachys* sp. ESNA-A009 against LN-caP (prostate cancer), SK-BR3 (breast cancer), HT29 (colon cancer), and HELA (cervix cancer) (Cruz *et al.*, 2006), *Acremonium* sp. against HCT-116 cells (Boot *et al.*, 2007), *Eurotium cristatum* against MCF-7 (breast adenocarcinoma), NCI-H460 (lung cancer), and A375-C5 (melanoma) (Almeida *et al.*, 2010), *Xylaria psidii* against bladder carcinoma cell line 5637 (ATCC HTB-9) (Tarman *et al.*, 2011), *Neosartorya siamensis* against HepG2, HCT116 and A375 cancer cell lines (Prata-Sena *et al.*, 2014) and *Penicillium oxalicum* against BGC823 gastric cancer and MOLT4 acute lymphoblastic leukemia cell lines (Bao *et al.*, 2014) have been reported.

The cytotoxicity effect of ethyl acetate extract of *P. cyaneum* was also confirmed by a decrease of cell density and also by structural alterations, such as rounded and detached cells as observed in a phase contrast microscope (Figure 3). Similarly Suja *et al.*, (2014) reported the morphological characteristics of the HpG2 cells treated with active fractions of *Aspergillus terreus* such as rounding of cells, shrinkage, aggregation, cell death etc., and it was observed through phase contrast microscope. To the best of our knowledge this is the first report on the *in vitro* anticancer activity of mangrove derived fungi *P. cyaneum*.

Conclusion

The present study shows that the mangrove isolate *P. cyaneum* can be a good source for alternative therapy of cancer cells, however further investigations are required to determine the anticancer compounds in the marine fungus.

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