American Journal of Current Microbiology
http://ivyunion.org/index.php/ajcmicrob/

Research Article

# Assessment of Invitro Anticancer Potential of Marine Derived Fungus Collected from South West Coast of Tamilnadu

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**Abstract**

Cancer is currently one of the most globally prevalent diseases. Furthermore, the incidence of cancer is steadily increasing, accompanying the ageing and growth of populations, as well as imbalanced life-styles and declining environmental conditions. The present work describes the anticancer activity of marine sediment derived fungus obtained during the course of sampling. The marine sediment was collected from the south west coastal area of Tamilnadu. Sampling results in the isolation of 58 fungal species belongs to 37 genera. Among the 58 fungal isolates, the frequent fungus *Aspergillus flavus* was isolated from the sediment based on frequency of distribution, while it was cultured on Potato Dextrose Agar (PDA) amended with streptomycin 150 units/ml. The second half of the study was based on the production of secondary metabolites on fermentation medium. The metabolites were concentrated using rotary vacuum evaporator. The anticancer activity of the metabolites was evaluated on human breast adenocarcinoma cell line purchased from National centre for cell sciences, Pune. Further the results revealed that the metabolite produced by the frequently isolated fungus has showed positive cell inhibitory effect on cell lines. The determined $IC_{50}$ value of the metabolite was 29.03 µg/ml. The study concludes that the marine derived organisms especially fungi is a promising agent to be exploited against the hunt for cancer drugs.

**Keywords**: marine sediment; *Aspergillus flavus*; Human breast adenocarcinoma cell line; IC50
**Peer Reviewer**: Sihua Peng, Department of Biology, Shanghai Ocean University, China
**Received**: November 20, 2015; **Accepted**: December 29, 2015; **Published:** February 14, 2016
**Competing Interests**: The authors have declared that no competing interests exist.

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# Introduction

Cancer is currently one of the most globally prevalent diseases particularly in developing country like India [1]. Cancer is considered a leading cause of death worldwide and estimates reports that, in 2030, 27 million incident cases of cancer and 17 million of deaths are expected [2]. Furthermore, the incidence of cancer is steadily increasing, accompanying the ageing and growth of populations, as well as imbalanced life-styles and declining environmental conditions. The conventional treatment procedure of cancer is commonly (partially or solely) based on chemotherapy and radiation therapy. Several forms of chemotherapeutic drugs have been developed in order to combat cancer, such as alkylating agents, which damage the DNA, antimetabolite agents, which induce cell death at S phase and inhibit enzymes responsible for RNA and DNA synthesis, compounds that interfere with enzymes involved in DNA replication, mitotic inhibitors, which inhibit the progression of mitosis and associated enzymes and corticosteroids with cytotoxic and cytostatic activity [3] however, inability of the radiation to discriminate between normal cell from cancer cells hazardous side effects highlight the importance of the search for safer, more efficient and tumour-specific anticancer drugs.

The marine environment is home to an immensely vast and complex array of species and ecosystems, most of which remain undiscovered. This does not come as a surprise, considering that the ocean covers approximately 70% of the planet's surface. This massive body of water encompasses different ecological niches, some of which are highly productive and prosperous in biodiversity, such as the sea-land interface and deep ocean thermal vent communities, others, such as the vast open ocean waters, possess limited production and are poor in biomass and diversity. Appeltans *et al*., [4] enumerate a total of approximately 226,000 described marine eukaryotic species, and estimate that one-third to two-thirds of marine species are yet to describe [4].

The attention of the research is pulled towards to the marine environment, where the broad and yet to explore biodiversity make promise of new chemical structures. In spite of this interest, the hunt for marine natural products poses several challenges which limit its expansion; large and complex molecules, enhanced costs to collect and manipulate species, difficult to culture in laboratory conditions, the lack of technological tools and innovations and also environmental concerns [5]. In the last few years, marine fungi have gained a growing interest from the scientific community as sources of bioactive compounds of biotechnological interest. This interest rises from the fact that fungi produce secondary metabolites with potential concern in pharmacological and biological studies [6].

Marine microbes, particularly fungi, have recently been utilized as a new source of novel bioactive secondary metabolites [7, 8]. As evidenced by various research articles suggesting that the marine derived fungi could be a source of plethora of bioactive compounds and on the basis of the availability of less number of articles on the identification of lead molecules towards cancer, the present work is designed to isolate compounds with anticancer activity.

# Materials and Methods

### Collection of marine sediment

Sediment samples were collected from different coastal lines located along the south west coast of Tamilnadu, Kanyakumari (Dt). The samples were collected by Hand core pushing technique and transferred to a sample carrier (purchased under UGC – SERO, Hyderabad Minor research Grant – Proposal number 231) and transferred to the laboratory in 24 hrs of duration.

### Analysis of Mycoflora

Dilution plating technique described by [9] was used to isolate the fungi from sediment sample. Marine Sediment samplesweredilute (1:1,000, 1:10,000 and 1:100,000) in sterilized seawater followed by 200 µL of diluted sample was inoculated in Petri dishes containing

Potato Dextrose Agar (Himedia, Mumbai) culture medium. The inoculated plates were incubated in a dust free cubourd at the room temperature (26±2 ℃) for 7 days. After the incubation, the development of fungal colonies were observed. The fungal cultures were then transferred; subcultured and pure cultures were maintained. The semi-permanent slides were prepared using lacto phenol cotton blue staining method [10].

**Identification**

The slides were observed under microscope (Olympus cx22) under (400X) and identified with the help of keys given by [11-17] and following the taxonomic arrangement proposed in the 6th edition of Ainsworth and Bisby's Dictionary of the Fungi [18].

**Production of secondary metabolites**

Once all the fungus has been identified, a small tuft from the frequent fungi was separated from the well grown culture and aseptically transfers it in to a conical flask containing potato dextrose broth (Himedia, Mumbai) prepared with sterilized sea water. It was incubated in a controlled environment for6 – 7 days at 30±2 ℃. The change in the color of the medium indicated the production of secondary metabolites.

**Extraction of Secondary metabolites**

The production medium was gently filtered using whatmann No 1 filter paper and the filtrate was transferred into separating funnel mixed with ethyl acetate (a highly polar solvent) in a ratio of 1:1. The mixture was vigorously shaken at regular intervals (30 minutes) continuously for 8 hours. Later it was mounted in a stand and left the setup for overnight without disturbance. Next day, the organic portion was alone separated and concentrated using rotary vacuum evaporator.

**Separating Bioactive compounds**

**Thin layer chromatography**

TLC is used to separate the compound present in the crude extract. The separation of the compound also depends on the usage of the specific solvent system. The drug with the concentration of 0.5 mg/mL was plotted on the TLC plate and dried. It was then run with different solvent ratio the spots were identified both in the UV light and in the iodine chamber. The $R_f$ value was calculated using the formula:

$R_f$ value=Distance travelled by the solute / Distance travelled by the solvent

***Invitro* cytotoxicity assay**

**Methodology**

The human breast adenocarcinoma cell line (MCF7) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at $37^0$ C, 5% $CO_2$, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

**Cell treatment procedure**

The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted by tryphan blue exclusion assay using a hemocytometer. The cell suspension was diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. one hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of

10,000 cells/well and incubated to allow for cell attachment at $37^0$C, 5% $CO_2$, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the metabolites. They were initially dispersed in phosphate buffered saline (PBS) and diluted to twice the desired final maximum test concentration with serum free medium. Additional four, 2 fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample (metabolite) dilutions were added to the appropriate wells already containing 100 µl of medium, resulted the required final sample concentrations. Following metabolites addition the plates were incubated for an additional 48 h at $37^0$ C, 5% $CO_2$, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

**MTT assay**

3-[4,5-dimethylthiazol-2-yl] 2,5- diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

After 48h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at $37^0$C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

% Cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % Cell inhibition and Log concentration and $IC_{50}$ was determined using GraphPad Prism software.

# Results

The preliminary isolation and morphological characterization procedure has led us to isolate 58 isolates belonging to 37 genera and on the basis of frequency of distribution, the fastidious fungi was identified as *Aspergillus flavus* (Fig 1a and 1b). The percentage (%) of distribution was estimated and given in Table 1. The production of secondary metabolites and its characterization by TLC revealed the presence of prominent compound (Fig 2a and 2b). The crude metabolite was concentrated and different dilutions 6.25µg, 12.5µg, 25µg, 50µg and 100µg were prepared (Table 2a). The result has clearly showed that, as the concentration of the metabolite increases with increase in absorbance. No visible agglomerate was seen in the control as it does contain the metabolite. Simultaneously, the percentage of cell inhibition was also calculated. Remarkable changes were obtained when the concentration reaches 50 and 100µg/ml and between % Cell inhibition and Log concentration and $IC_{50}$ was determined using GraphPad Prism software the $IC_{50}$ were found to be 29.03 µg/ml (Table 2b and Fig 3).

**Table 1** List of taxonomic group of fungi and its individual contribution

| S.No | Taxonomical group of fungi | Individual contribution (%) |
|---|---|---|
| 1 | Ascomycota | 48 |
| 2 | Mucoromycotina | 2 |
| 3 | Mitosporic fungi | 5 |
| 4 | Anamorphic fungi | 2 |
| 5 | Hyphomycetes | 1 |

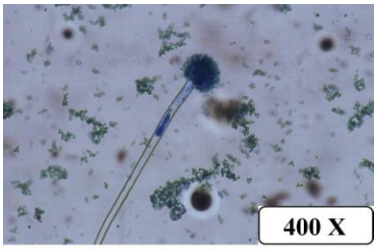
**Table 2a** Concentration of the metabolites against the absorbance

| **Conc** | **6.25 µg** | **12.5 µg** | **25 µg** | **50 µg** | **100 µg** | **Cont** |
|---|---|---|---|---|---|---|
| ABS | 0.535 | 0.521 | 0.446 | 0 | 0 | 0.548 |
| | 0.537 | 0.509 | 0.478 | 0 | 0 | 0.544 |
| | 0.55 | 0.51 | 0.469 | 0 | 0 | 0.538 |
| **Avg** | **0.540667** | **0.513333** | **0.464333** | **0** | **0** | **0.543333** |

**Table 2b** Concentration of the metabolite (µg/ml) and % of cell inhibition

| **Conc (µg/ml)** | **% Cell Inhibition** | | | | |
|---|---|---|---|---|---|
| 6.25 | 0.490798 | | **IC 50** | **29.03 µg/ml** | |
| 12.5 | 5.521472 | | | | |
| 25 | 14.53988 | | **R ²** | **0.9971** | |
| 50 | 100 | | | | |
| 100 | 100 | | | | |

Fig 1a. Plate image of *Aspergillus flavus* on potato dextrose agar




**Aspergillus flavus**

Fig1b. Microscopic observation of *Aspergillus flavus* [400X]

Fig 2a Production of secondary metabolites on Potato dextrose broth by *Aspergillus flavus*

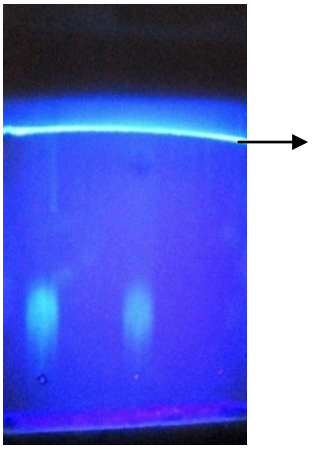
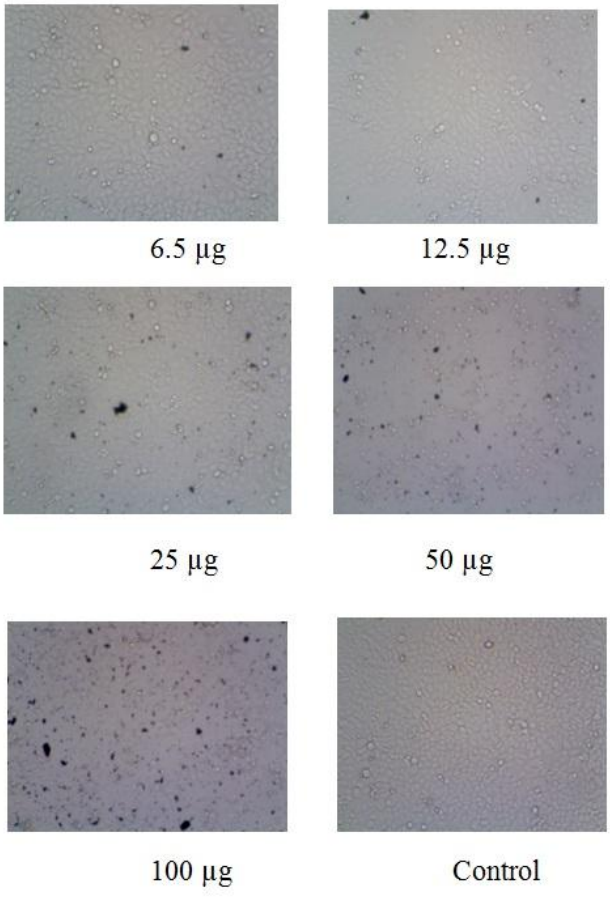


Fig: 2b presence of prominent band indicates the production of metabolites



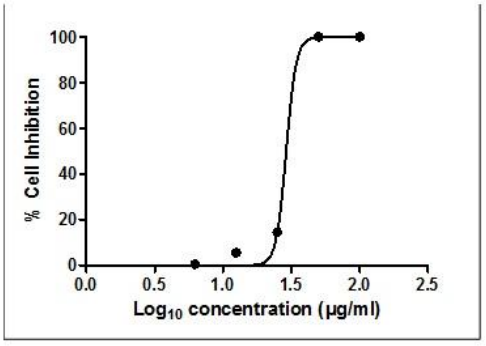




Fig 3b Non linear graph showing the relativity between concentration of the metabolite and % ofcell inhibition

Fig 3a Cell inhibitory pattern of different concentration of metabolites on HeLa cell lines

# Discussion

Distribution of filamentous fungi in the present study has shown a higher diversity. Of the 58 isolates belonged to 37 genera comprising 48% Ascomycota, 2% Mucoromycotina, 2% Anamorphic fungi, 1% Hyphomycetes and 5% Mitosporic fungi (Table 1). However, the present study matches the findings of several investigators who found Ascomycetes fungi as the major contributor to the filamentous higher marine fungi [16 , 19, 20].

According to a report submitted by Mathan *et al.*, [21], that 200mg of the compound from marine sediment derived *Aspergillus protuberus sp*1 was required to evaluate the anticancer activity in albino mice, but in the present study the compound produced by *Aspergillus flavus* was required in least concentrations ranging from 6.25µg to 100 µg. It indicates that the *Aspergillus flavus* has potent inhibitory nature against cancer cell lines.

Another study conducted by Bhimba, *et al.*, [22], reported that 125 µg/mL of the compound from *Irpex hydnoides* - endophytic fungi was required to achieve 50% cell viability inhibition but in the present study revealed that about 29.03 µg/ml concentration was able to bring effective cell inhibition. This result has a promising output towards the identification of targets and possible exploitation of sediment derived fungi against cancer.

According to a research conducted by Abo-Kadoum *et al.*, [23], reported they had evaluated the crude extract of *Penicillium aurantiogriseum* against hepatic cellular carcinoma (HEPG2). The cell line was treated with serial concentrations of 6.25, 12.5, 25, 50 and 100 µg/ml as the dilution procedure adopted in the present investigation. The result has revealed that the maximum concentration 100 µg/ml killed 82.76 % of the viable cells, while the minimum concentration 6.25 % killed 52.54% of the viable cells. In the present investigation, under 100 µg/ml, all the viable cells were completely inhibited and least inhibition was observed in 6.25 µg/ml is about 0.490798. The results are entirely differing from the available reports on drugs against cancer research.

# Conclusion

The results of present study revealed that *Aspergillus flavus* is a potent producer of safe bioactive compounds which can be used as an anticancer compound. Further studies are required to separate, identify and purify these compounds and determine the right compound has the biological activity to use in biomedical application.

# Acknowledgement

The authors are very much thankful to UGC –SERO, Hyderabad for financial assistanceship for carrying out this research work in a spectacular manner and also we are thankful to THE MANAGEMENT and Dr.V.Pandiyarajan, PRINCIPAL of Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi, Tamilnadu, India.

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