ANTICANCER ACTIVITY OF COMPOUNDS ISOLATED FROM MARINE ENDOPHYTIC FUNGUS ASPERGILLUS TERREUS

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ABSTRACT

The recent increase in cancer disease requires new drugs to conflict them. The sources have been found very rare in microorganisms, those from extreme habitats and from endophytes. In this present study the biological activity of the endophytic fungus associated with marine seaweed Codium decorticatum was assessed. The cytotoxic effect was evaluated using HepG2 cancer cell line. The seaweed endophytic fungi Aspergillus terreus producing compounds (F7 and F8) were evaluated for the anti cancer potentials. The results revealed that the compounds from the Aspergillus terreus showed better activity against cancer cell line. In the present study, the isolated compounds (F8 and F7) showed low Cytotoxicity on vero cell lines and also it inhibited the growth of the cancer cells.

Keywords: Aspergillus terreus, endophytic fungus, cancer cell.

INTRODUCTION

There is a need for the new medicines, due to the resistance to available treatments in many microorganisms, specifically antifungal, anti-protozoal, anti-viral and antibacterial. [1] The natural products covers far greater area of chemical space than the synthetic compound and also the property distribution is similar to those of drugs currently in use. Approximately 50% of the drugs presently used for clinical purposes are natural product and their analog or derived molecules. Whereas regarding anticancer drugs, 63% of them fall into this category. [2, 3, 4] The emerging evidence suggest that marine natural products, especially the secondary metabolites from marine organisms are more likely to yield anticancer drugs than terrestrial sources [5-10]. The research work on natural products from marine organism started only 50 years ago. In the 50 years the microorganisms have provided key structure and compounds
that proved the potentials for industrial development. In this way marine natural products are used as in medicinal purposes. There are about 10,000 compounds isolated from the marine microorganisms and 300 patents were issued between the year of 1969 and 1999. \[11\] Natural products are formulated to generate different types of effective drugs to enhance the anticancer activities. Cancer is a dreadful disease which is characterized by irregular proliferation of the cells. As a cell progress from the normal to cancerous, so the biological essential to survive and perpetuate derives fundamental changes in cells behavior. \[12\] Cancer has become an increasing public health problem due to its high rates of morbidity and mortality. Because the conventional cancer chemotherapy has the limitation of multidrug resistance cause by over expression of integral membrane transporters, such as P-gp which can efflux intracellular anticancer drugs thus increasing drug accumulation. These multi drug resistance cells (MDR) are resistant to cytotoxic effects of various structurally and mechanically unrelated chemotherapeutic agents. Many studies related to the developing of new drugs that are efficient to overcome the MDR cells have been reported. \[13, 14, 15\]

Endophytic microorganisms are considered as an outstanding source of bioactive natural products because many of them occupied millions of unique biological niches in many unusual environments. Naturally occurring biologically active metabolites have been identified from marine derived fungi which are associated with various substrates such as sponges, mangroves, algae, crabs, seahares, tunicates as well as sediments. \[16-19\] Since early in human history, plants have served as a most important source of medicinal natural products. \[20\] Marine derived fungi are a prolific source of new bioactive secondary metabolites with diverse chemical structure that have equally interesting biological activities namely anticancer, antibacterial, antifungal and antiviral, also it is involved in the specific enzyme inhibition activities. \[21, 22, 23\] Hence the marine derived fungi have received much more attention in terms of their ability to produce a wide variety of chemical compounds with biomedical potential. \[24\]

**MATERIALS AND METHODS**

**Preparation of extracts**

A loopful of culture was inoculated in 50 ml of PDB and kept for 7 days in the shaker. After 7 days, the culture broth was centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was taken for extraction by using a separating funnel. Ethyl acetate was added separately to the collected supernatants and subjected to vigorous shaking for an hour. Then
the separating funnel was kept undisturbed for 5 minutes; the aqueous phase was collected in a beaker and subjected to concentration through evaporation in a vacuum desiccators. After evaporation, the concentrated residue was used for further studies. The compound was purified from the ethyl acetate extract by using column chromatography and the active fractions were tested for cytotoxicity assay. Different concentrations (200-1000 µg/mL) were used to test the cytotoxicity of *Aspergillus terreus* extracted compounds.

**Cell lines**

The present work was carried out with HepG2 cancer cell line and Vero normal cell lines. The cytotoxicity studies were carried out in Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Mumbai, India.

**Culturing of cells**

Cancer cells were allowed to grow as monolayer in minimal essential medium (MEM) (Himedia) with the addition of 10% FCS (Fetal calf serum), 3% glutamine and 100 U/mL each of penicillin and streptomycin at 37°C in 5% CO₂ atmosphere. Stocks were maintained in 25 cm² tissue culture flasks. After counting the cell number, cells were seeded at 1×10⁵ cells/mL in 96 well plates, cells were harvested by trypsinization.

**Study scheme**

Study was carried out by using the HepG2 and Vero cell lines to test the cytotoxicity of the two fractions (F7 and F8) from single organism at different concentrations in a double dilution scheme.

**In vitro Cytotoxicity Screening**

Vero cells were harvested by trypsinization and 1×10⁵ cells/mL suspension was added to the 96 well microtitre plates. Plates were incubated for 48 hrs in COD incubator at 37°C. Following the incubation, the plates were microscopically examined for confluent monolayer, turbidity and toxicity. Then the growth medium was removed and the cell monolayer was washed once with MEM without FCS and removed. To the washed cell sheet, 1ml of medium (without FCS) containing defined concentration of fraction 7 and 8 of *Aspergillus terreus* was added in tetrads in the designated wells. Control well was added with 1ml of MEM medium alone. The cells in the microtitre plates were viewed microscopically after the immediate administration of the fractions for their cytotoxicity. Then plates were incubated for 24 hrs to
check the cytotoxicity properties of the active fractions. The morphological changes of the vero cells were microscopically observed.

Anticancer activity of compound

SRB assay was used to study the anticancer activity.\textsuperscript{[26]} The sulfordamine B (SRB) assay is used for cell density determination, based on the measurement of cellular protein content. The compounds were tested for their activity on HepG2 cell line. The different concentrations of active fractions (F7 and F8) were 1000 µg/mL, 800 µg/mL, 600 µg/mL, 400 µg/mL, 200 µg/mL and 100 µg/mL. For this assay the cells were plated into 96 well microtitre plates in 100 µL of medium and allowed to attach for 24 hrs. The compounds were solubilised in DMSO then serial dilutions were prepared in medium and added to the cells. Total cellular protein was used as an indicator of cell number and was measured at 0 and 48 hrs after sample addition. Cells were fixed by addition of 50 µL of 50% TCA for 30 min at 4°C, rinsed 5 times in running water then air dried before staining with 50 µL 0.4% SRB in 1% acetic acid for 30 mins. The SRB was then solubilised in 10 mM Tris (100 mL) and plates were read on a Wallac victor plate reader. Growth inhibition of 50% (GI\textsubscript{50}) and total growth inhibition (TGI) were determined by comparing the sample treated values to those of a vehicle adriamycin (ADR).

LC\textsubscript{50} is a concentration of drug causing cell kill at 50%, GI\textsubscript{50} is a concentration of drug causing inhibition of cell growth at 50%, and TGI is concentration of drug causing total inhibition of cell growth. IG\textsubscript{50} value < 1µmolar or < 10 µg/mL is considered to demonstrate activity in case of pure compounds.

\[ \text{GI}_{50} = \frac{50}{(T_i-T_z)/(C-T_z)} \times 100 = 50 \]
\[ \text{TGI} = \text{Total growth inhibition is calculated by using the following formula Ti} = T_z \]
\[ \text{LC}_{50} = \text{It is calculated from } [(T_i-T_z)/T_z] \times 100 = -50. \]

RESULTS

Cytotoxicity

In the present study, the active fractions of purified compounds were tested against cancer cells (Fig. 1). Based on the potentials of the \textit{Aspergillus terreus}, it was used against vero normal cell line for their cytotoxicity activity. The active fraction F8 showed toxicity against cancer cells whereas in normal vero cells no such changes were observed. The cytotoxicity assay was assessed by the morphological characteristics of the cells such as rounding of cells,
shrinkage, aggregation, cell death etc., and it was observed through phase contrast microscope.

**Anticancer activity**

*In vitro* anticancer test was performed by SRB method against the HepG2 cells. Cells were cultured for 24hrs in the presence of different concentrations of active fractions and percentage of cell viability, was evaluated using the sulforadamine B (SRB), adriamycin was used as a reference standard. The 50% growth inhibition (GI\textsubscript{50}), total growth inhibition (TGI), and concentration of drug causing lethality to 50% of the cells (LC\textsubscript{50}) was calculated and it was shown that the fraction 7 and 8 showed LC\textsubscript{50} and TGI values more than 80. But for GI\textsubscript{50}, fraction 7 and 8 showed > 10 and <10 respectively. Comparatively F8 is considered to be active than F7. But LC\textsubscript{50}, TGI and GI\textsubscript{50} values of standard drug adriamycin was 51.9, 14.4 and <10 respectively. (Fig. 2) (Tab. 1) (Plate. 1).

![Cytotoxicity of active fractions](image)

**Fig. 1. Cytotoxicity of active fractions of Aspergillus terreus**

![Control vs Treated](image)

**Plate. 1. Cancer cell line HepG2 treated with active fractions of Aspergillus terreus.**
Table: 1. Treatment on HepG2 Cancer cell line

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LC50</th>
<th>TGI</th>
<th>GI50</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADR</td>
<td>51.9</td>
<td>14.4</td>
<td>&lt;10</td>
</tr>
<tr>
<td>F8</td>
<td>&gt;80</td>
<td>&gt;80</td>
<td>&lt;10</td>
</tr>
<tr>
<td>F7</td>
<td>&gt;80</td>
<td>&gt;80</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

**Fig. 2. Anti-cancer activity of active fractions against HepG2 Cell line**

**DISCUSSION**

Marine microorganisms are known to be rich sources of novel bioactive compounds; natural products have provided the most important successes in the chemotherapy of cancer. Most of the major anticancer compounds are obtained from natural products which includes plants and microorganisms. [27] Earlier reports showed that the secondary metabolites from fungi provided an important group of new biological agents and having low toxicity on normal cells. Further, the secondary metabolites are low molecular weight compounds, exhibiting a potential anticancer activity. It is speculated that biological activity of the fungi extracts is associated with the endogenous environment of the plant. [28] It is observed that the existing anticancer drugs have a limited selectivity and are highly toxic.

Researchers in the molecular and cellular biology are regularly identifying novel potential targets, which are specific or selective for cancer cells. [29] The effect of the extracts of endophytic fungi isolated from mangrove plants on the cancer cells as well as their effects on topoisomerase was reported. [30] A new Topo I isomerase inhibitor, (+)-3, 3, 7, 7, 8, 8-hexahydroxy-5, 5-dimethylbianthraquinone was recently isolated from mangrove endophytic fungi. [31] The cytotoxic effects of the compound obtained from *Aspergillus candidus* on carcinoma cell lines were assayed using 3-(4, 5-Dimethythiazol-2-yl)-2, 5-
diphenyltetrazolium bromide (MTT). Similarly the compound isolated from the *Aspergillus terreus* showed better activity against cancer cell line. The compound had antitumor activities against HEp-2 and HEpG2 cancerous cells with IC$_{50}$ of 7 µg/mL. Also it was reported that none of the examined dilutions showed cytotoxicity to normal cells. In the present study 50% growth inhibition (GI$_{50}$), total growth inhibition (TGI), and concentration of drug causing lethality to 50% of the cells (LC$_{50}$) with <10, >10, >80, >80, and >80 respectively. And the standard ADR with <10, 14.4, 51.9 respectively.

Anticancer activity of 14 anthracenedione derivatives separated from the secondary metabolites of the mangrove endophytic fungi *Halorosellinia* sp and *Guignardia* sp was reported. The compound displayed strong cytotoxicity with IC$_{50}$ values of 31.7 and 3.21 µM to KB and KBv200 cells, respectively. Furthermore it was demonstrate that mechanism involved in the apoptosis induced by compound is probably related to mitochondrial dysfunction. Another researcher screened 87 marine products from mangrove fungi in the South China Sea for anticancer activity by MTT assay. 14% of the compounds exhibited a potent activity against cancer *in vitro*. Further the structure activity analysis indicated that the hydroxyl group was important for their cytotoxic activity and that bulky functional groups such as phenyl rings could result in a loss of biological activity.

The cytotoxic activity of methanolic extracts of *Artocarpus heterophyllus* plant by various *in vitro* cytotoxic assays like MTT and SRB against different cell lines like HEK293, A549, HeLa and MCF-7. The IC$_{50}$ values of methanolic extract of *Artocarpus heterophyllus* were to be found 35.26 µg/ml and 35.27 µg/ml against A549 cell line by MTT and SRB assay methods respectively whereas this extract was found to be non toxic to normal cells (HEK293). Further, it proved that the methanolic extract exhibited significant anti cancer potential with no toxicity on normal cell line. In recent years endophytic microorganisms have emerged as a promising source of new drugs. Endophytic microorganisms are symbiotically associated which causes no discernible manifestation of disease. Endophytes have been recognized as a rich source of bioactive secondary metabolites such as namely naphthalene, cryptocandin, pseudomycin, β lactamn etc.,

Main difficulty in the battle against cancer is the limited specificity of current chemical treatments; thus to address this problem, increasingly more researchers are seeking to isolate and screen natural compounds that can effectively target cancer cells, without causing undesirable adverse side effects on normal cells. A number of anticancer agents have been
isolated as natural compounds from plants, animals and microorganisms.\textsuperscript{[43]} Example of such agents currently used in the treatment of different forms of cancers are vincristine, vinblastine, taxol, and bleomycin.\textsuperscript{[44]} With nearly a two million recognized living species that exhibits enormous chemical diversity, nature offers a potentially huge source of new therapeutic compounds. For many organisms atleast a fraction of this chemical diversity seems to be the result of evolutionary selection associated with defense or predatory mechanisms.\textsuperscript{[45]} Biochemical injuries make cancer cells more vulnerable than normal ones to pro-apoptotic events such as mitochondrial membrane permeabilization and release of apoptosis inducing factor and ultimately, more exposed to the action of pharmacological agents.\textsuperscript{[46]} In the present study, the compounds of F8 and F7 showed low cytotoxicity on vero cell lines and also it inhibit the growth of the cancer cells.

CONCLUSION
From the results obtained from SRB assay method, by comparison of the GI 50 values and linearity of the activity. The compound (F8) from ethyl acetate extract of \textit{Aspergillus terrus} showed excellent cytotoxicity against the cell line but had a less activity was recorded in compound F7 on HepG2 cancerous cell line respectively. The GI50 value found in compound F8 on HepG2 cancer cell line was <10 and the results were compared with the standard ADR respectively. It proves that the compounds from \textit{Aspergillus terreus} (ethyl acetate extract) had potential cytotoxicity against cancer. But non toxic to the normal cells.

REFERENCES


