

ANTICANCER AND FREE RADICAL SCAVENGING POTENTIAL OF THE MARINE ALGICOLOUS ENDOPHYTIC FUNGUS *CLADOSPORIUM UREDINICOLA*VASAVI THIRUMALANADHUNI, LAVANYA LATHA YERRAGURAVAGARI<sup>2</sup>, VANI MATHAKALA<sup>3</sup>,  
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## ABSTRACT

**Objective:** The objective was to study the antioxidant and anticancer potential of the endophytic fungus *Cladosporium uredinicola*, isolated from the marine brown alga *Dictyota dichotoma*.

**Methods:** Anticancer effect of the endophytic fungal extract was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay using MDA-MB-231 human mammary adenocarcinoma cells as *in vitro* cancer models. 3T3-L1 pancreatic adipocytes were used as *in vitro* models for the evaluation of cytotoxic activity against normal cells using MTT assay. Free radical scavenging activity was assessed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay.

**Results:** The ethyl acetate extract of the endophytic fungus showed potent cytotoxic activity against MDA-MB-231 human breast adenocarcinoma cell lines with an inhibitory concentration ( $IC_{50}$ ) value of 373  $\mu\text{g/ml}$  and a very mild cytotoxic effect on 3T3-L1 Cells with an  $IC_{50}$  value of 2403  $\mu\text{g/ml}$ . DPPH free radical scavenging assay of the extract showed an  $IC_{50}$  value of 359  $\mu\text{g/ml}$  indicating its potential free radical scavenging activity.

**Conclusion:** The results indicated that the endophytic fungus *C. uredinicola*, isolated from the marine brown alga *D. dichotoma*, acts as a potential source for anticancer and antioxidant metabolites. Moreover, these anticancer metabolites were observed to be less toxic to the normal cells, which make them prospective therapeutic agents.

**Keywords:** Marine endophytic fungi, Free radical scavenging, Cytotoxic, MDA-MB-231, 3T3-L1 cells.

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## INTRODUCTION

Marine environment is considered to be a reservoir of pharmaceutically important bioactive compounds due to its rich biodiversity and extreme physical and chemical conditions. In recent years, a number of bioactive compounds were isolated from the marine organisms such as sponges, seaweeds, and microorganisms. Some of these compounds are under pre-clinical and clinical trials [1]. More than 10,000 secondary metabolites were reported to be isolated from marine organisms [2]. Some anticancer compounds such as “hemiasterlin” from the sponges and “elasidepsin” from molluscs are under clinical trials [3].

Marine microorganisms are more advantageous than other marine organisms since they can be cultured and manipulated in the laboratory very easily. Several marine bacteria, *Actinomycetes*, and fungi were screened for bioactive metabolites [4]. Antifungal compounds such as basilikamides A and B, anticancer compounds such as bacillistatins 1 and 2, and antioxidant compounds such as wentiquinone were reported from the marine microorganisms [2].

Among several marine microorganisms, marine endophytes are largely unexplored due to the problems associated in culturing them. Endophytes reside in the living tissues of the host without causing any apparent harmful effect. These peculiarities of lifestyle compel them to encounter host defense molecules continuously. It is that, due to this complexity, the endophytes were evolved to produce various unique metabolites, which could be exploited for pharmaceutical applications [5].

Cancer is one of the leading causes of mortality and morbidity worldwide. According to the definition given by the World Health Organization, cancer is a generic term for a large group of diseases that can affect any part of the body in which there is a rapid creation of abnormal cells that

grow beyond their usual boundaries and invade adjoining parts of the body and spread to other organs. The American Cancer Society reported cancer as the second most common cause of death next to cardiovascular diseases in the USA and Europe. Resistance to anticancer compounds and side effects of the drugs are the major problems associated with chemotherapy. In this context, there is an urgent need to develop new and safe drugs for cancer treatment. In the past 20 years, an increasing number of pharmaceutically useful compounds were reported [3].

Of the eight marine drugs approved for various purposes, five compounds, namely cytarabine, ziconotide, trabectedin, eribulin mesylate, and brentuximab, have been approved for cancer treatment [6]. With this background, the present study was carried out to evaluate the free radical scavenging and anticancer potential of the marine algicolous endophytic fungus *Cladosporium uredinicola* isolated from the marine brown alga *Dictyota dichotoma*. In this study, the antioxidant activity was screened by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, whereas cytotoxic activity on both cancer and normal cells was evaluated by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

## METHODS

## Chemicals

Ethyl acetate (EA) was obtained from SD Fine-Chem Limited, Mumbai, India. DPPH, MTT, Dulbecco's Modified Eagle's Medium (DMEM), phosphate-buffered saline, trypsin - HyClone, and ciprofloxacin (cell culture) were procured from HiMedia Laboratories, Mumbai, India.

## Medium for culturing of endophytic fungi

A novel medium by name “MGM medium” (patented) was formulated for culturing of endophytic fungi [7].

### Fungal culture

The endophytic fungus isolated from marine alga *D. dichotoma* was coded as marine brown algal endophytic fungus (MBEF) and it was identified as *C. uredinicola*, by 28 S ribosomal DNA sequence analysis. The sequence was deposited at the GenBank under the accession number MG719993.

### Cell lines

MDA-MB-231 Cells and 3T3-L1 cells were procured from National Center for Cell Sciences, Pune, India.

### Extraction of the metabolites

A 15-day-old culture broth of *C. uredinicola* was filtered using Whatman NO.1 filter paper, and the filtrate was extracted with an equal volume of EA. The organic phase was collected and dried in a rotary evaporator [8]. The EA extract was stored at 4°C for further analysis and designated as MBEF-EA extract.

### Free radical scavenging activity

Antioxidant activity of the crude extract was detected by DPPH free radical scavenging activity assay [9,10]. A solution of 1 ml of 0.1 mM DPPH solution was added to different concentrations of the extract (10–500 µg/ml) and incubated at room temperature in the dark for 20 min, and the intensity of the color was read at 517 nm using ultraviolet spectrophotometer.

The inhibitory percentage of DPPH was measured using the following formula:

$$\text{Inhibitory\% of DPPH} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

### Cytotoxic activity of EA extract

#### Cell culture

MDA-MB-231 estrogen receptor-negative human breast adenocarcinoma cell lines and 3T3-L1 murine preadipocytes were routinely maintained in DMEM, supplemented with 2 mmol/l glutamine, 10% fetal bovine serum, and 10 µg/ml of ciprofloxacin in a 5% CO<sub>2</sub> incubator at 37°C.

The cytotoxic activity of the extract was analyzed using MTT [11,12]. The MDA-MB-231 cells (1×10<sup>4</sup>/well) and 3T3-L1 cells were seeded in 96 well plates and incubated for 24 h at 37°C in 5% CO<sub>2</sub> and then treated with different concentrations of the EA extract ranging from 100 to 500 µg/ml of the extract dissolved in 0.1% of dimethyl sulfoxide (DMSO). After 24 h exposure, 0.02 ml of MTT (5 mg/ml in phosphate buffer saline) was added to each well and incubation was extended for another 4 h. The MTT solution was removed by aspiration and 0.15 ml of DMSO was added to each well to solubilize the formazan crystals, and absorbance was read in microplate reader at 570 nm.

The percentage proliferation for each treatment was calculated using the following formula:

$$\text{Cell proliferation(\%)} = \frac{\text{Mean OD value of the test}}{\text{Mean OD value of the control}} \times 100$$

## RESULTS

### Free radical scavenging activity of EA extract

The DPPH free radical scavenging activity of the extract is depicted in Table 1. The extract showed significant activity in dose-dependent manner with a mean inhibitory concentration value of 359 µg/ml.

### Cytotoxic activity

The EA extract showed selective toxicity against cancer cells (Fig. 1). A potent cytotoxic activity of the EA extract of endophytic fungus against MDA-MB-231 human breast adenocarcinoma cell lines with an IC<sub>50</sub> value of 373 µg/ml (Fig. 2) was observed. The extract was found to induce the visible symptoms of apoptosis, namely retraction, rounding, and granulation, in MDA-MB-231 cell lines (Fig. 3). There was a very mild cytotoxic effect of the extract observed on 3T3-L1 cells with an IC<sub>50</sub> value of 2403 µg/ml (Fig. 4). Visible symptoms of apoptosis were induced by the extract at very higher concentrations only (Fig. 5).

## DISCUSSION

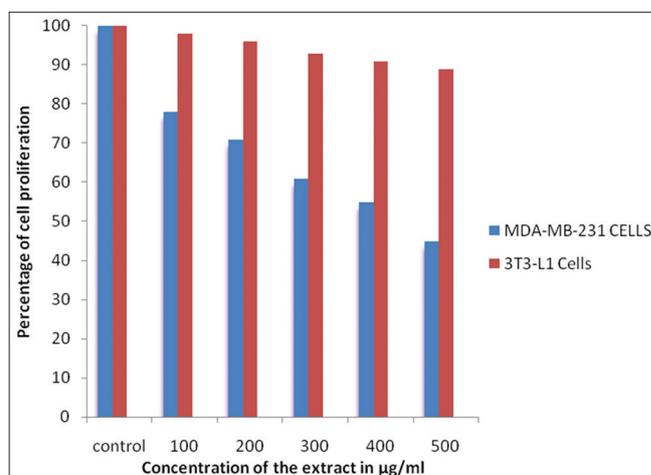
India has a coastal line of 7516.6 km [13] holding several biomes and ecosystems. In the process of ongoing search for new bioactive compounds, marine environment is being explored in the recent years. The microbiota appears to be a promising source for novel pharmaceutical compounds as the microorganisms can be grown easily under laboratory conditions [14]. Despite this fact, majority of the marine microorganisms remained unexplored. Marine endophytes are among such microorganisms hidden in their hosts. Endophytes are universally found in the plants [15]. The salient properties of endophytes project them as prospective candidates for the extraction of novel metabolites with various biological activities [16].

In the present work, an endophytic fungus isolated from the marine brown alga *D. dichotoma* was subcultured and the culture broth was extracted with EA. As per the literature, EA seems to be the common

**Table 1: DPPH free radical scavenging activity of MBEF-EA extract**

Concentration of the extract (µg/ml)	Inhibition percentage of DPPH	IC <sub>50</sub> value (µg/ml)
100	16±0.1	312.5
200	26±0.2	384.6
300	43±0.1	348.8
400	57±0.1	350.9
500	63±0.2	396.9

The data represent mean±SEM of three independent experiments. MBEF-EA: Marine brown algal endophytic fungus-ethyl acetate, DPPH: 2, 2-diphenyl-1-picrylhydrazyl, IC<sub>50</sub>: Inhibitory concentration, SEM: Standard error of the mean



**Fig. 1: Differential cytotoxic effect of the marine brown algal endophytic fungus-ethyl acetate extract on normal adipocytes (3T3-L1 cells) and breast cancer cells (MDA-MB-231 cells). Each bar represents the data of triplicate determinant, and there is a significant difference on comparison with control (p<0.01)**

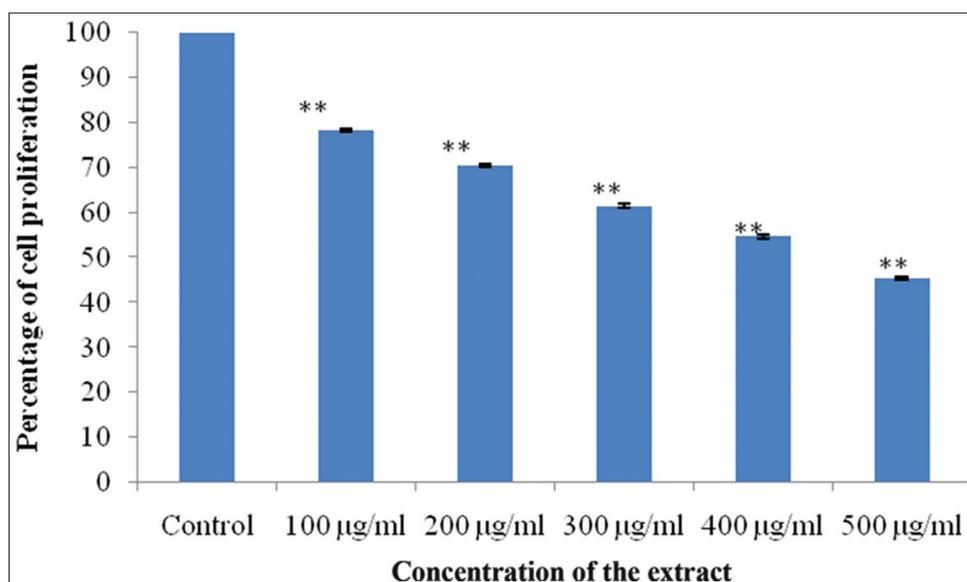


Fig. 2: Effect of marine brown algal endophytic fungus-ethyl acetate extract on the viability of MDA-MB-231 cells. The data are the average of three independent experiments. \*Indicates  $p < 0.05$ , \*\*indicates  $p < 0.01$

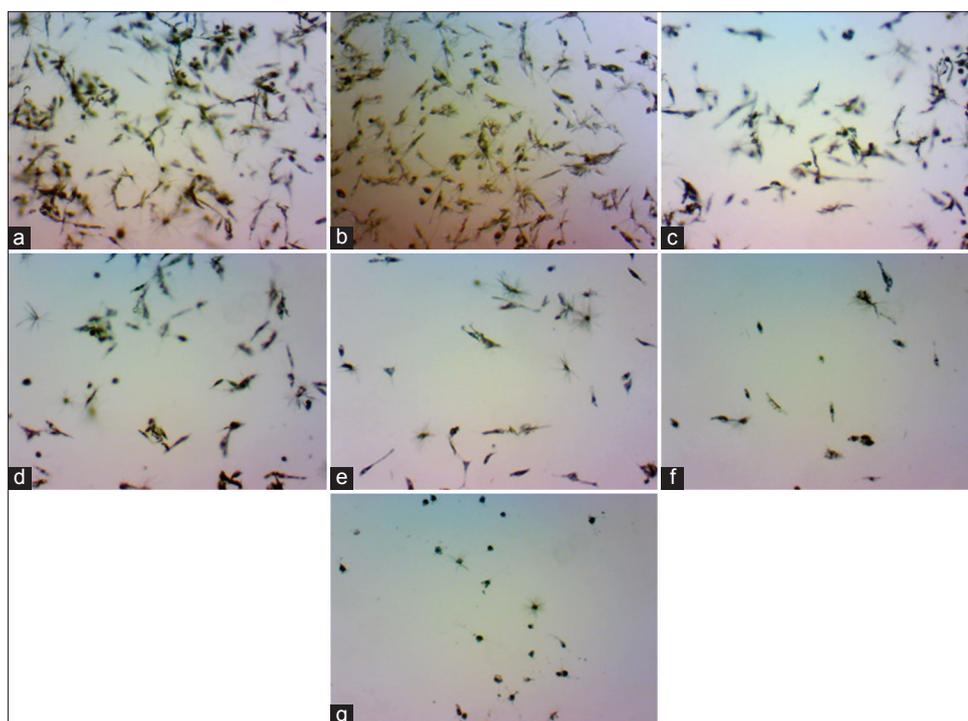


Fig. 3: Photomicrograph of inverted microscope depicting the cytotoxic effect of marine brown algal endophytic fungus-ethyl acetate (MBEF-EA) extract on MDA-MB-231 cells (a) Control (untreated cells), (b) dimethyl sulfoxide (vehicle control), (c) 100 µg/ml, (d) 200 µg/ml, (e) 300 µg/ml, (f) 400 µg/ml, (g) 500 µg/ml of MBEF-EA

solvent used for screening bioactive metabolites of endophytic fungi [17]. From the results of the antioxidant activity, the extract was found to have free radical scavenging activity. The DPPH radical scavenging activity was reported with *Wardomyces anomalus*, inhabitant of *Enteromorpha* sps, and fungus *Epicoccum* sps. of marine alga *Fucus vesiculosus* [18]. From the results of the MTT assay, it can be concluded that the organic extract of the isolated endophytic fungus was found to have cytotoxic effect on cancer cells without much affecting the normal cells. *Penicillium chrysogenum*, an endophyte isolated from the marine red alga *Laurencia*, was found to show cytotoxic activity against human liver carcinoma cell

line [19]. Similarly, norditerpenoid purified from *Aspergillus wentii*, an endophyte of *Sargassum* spp., demonstrated cytotoxicity against tumor cell lines [20]. Further analysis of the EA extract may reveal the bioactive principles responsible for the antioxidant and selective cytotoxic properties of the endophytic fungal extract.

#### CONCLUSION

From the results of the present work, it can be concluded that the endophytic fungus *C. uredinicola* isolated from the marine brown alga *D. dichotoma* was found to be a source of bioactive metabolites and worth exploring further.

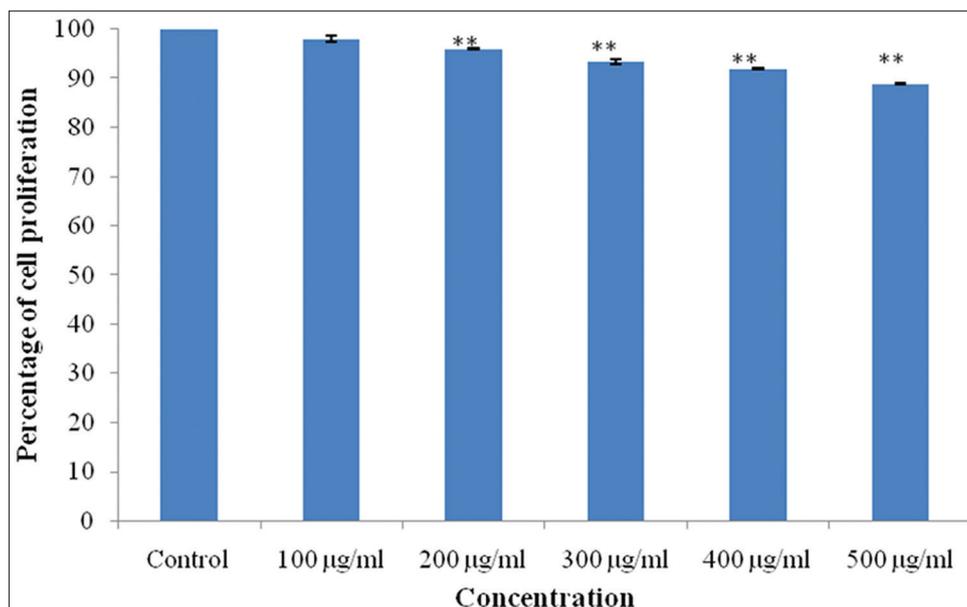


Fig. 4: Cytotoxic activity of the marine brown algal endophytic fungus-ethyl acetate against normal adipocytes 3T3-L1 cell line. The data represent the mean of three independent experiments. \* $p < 0.05$ ; \*\*indicates  $p < 0.01$

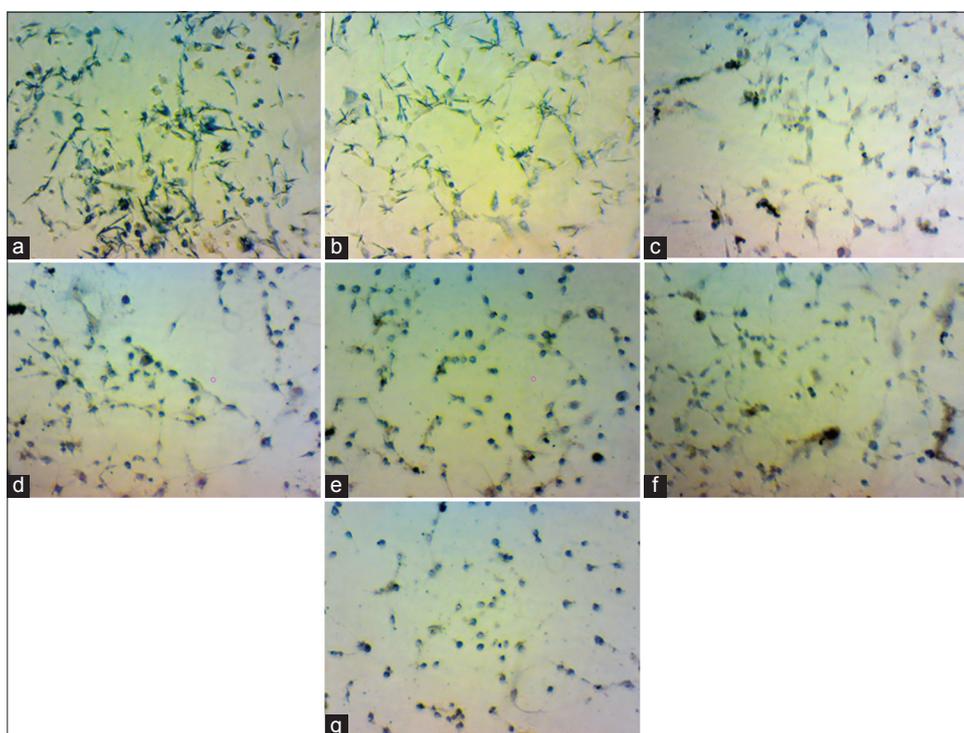


Fig. 5: Photomicrographs of optical-inverted microscopy demonstrating effect of marine brown algal endophytic fungus-ethyl acetate (MBEF-EA) extract on 3T3-L1 cells (a) control (untreated cells), (b) dimethyl sulfoxide (vehicle control), (c) 100 µg/ml, (d) 200 µg/ml, (e) 300 µg/ml, (f) 400 µg/ml, (g) 500 µg/ml of MBEF-EA extract

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#### AUTHORS' CONTRIBUTIONS

V.T and U.D were involved in design, planning, execution, data analysis, and manuscript preparation. L.Y and V.M were involved in cell culture work and also contributed to manuscript preparation.

#### CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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