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Original Article

CYTOTOXIC ACTIVITY OF ENDOPHYTIC FUNGI HHPCYL03 ISOLATED FROM CYMBOPOGON FLEXUOSUS NEES EX STEUD

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ABSTRACT

Objective: Cymbopogon grass is one of common aromatic grass species used for extraction of essential oil. The endophytic fungus HHPCYL03isolated from the *Cymbopogon flexuosus*, a medicinal grass species collected from Kemmannugundi regions of Karnataka.

Methods: Secondary metabolites were extracted from fungi using organic solvent Ethyl Acetate and screened for anticancer assay against breast cancer cell (MDA-MB-231), lung cancer cell (Calu-6) and colorectal cancer cell (HCT116) lines.

Results: The extract showed the positive result against HCT116 cells.

Conclusion: The fungal endophytes certainly become a repository of good economically, socially benefitted bioactive compounds.

Keywords: Endophytic fungi, Anticancer, Inhibition, Metabolite.

INTRODUCTION

Cymbopogon grass is one of the important aromatic grass species native to India and Tropical Asia. Grass yields essential oil called "lemon grass oil" or "citronella oil" which is used as the pesticide and in preservations. Some researchers showed that lemon grass oil has antifungal and anti-cancer properties [1]. Scientists found that lemon grass caused programmed cell death in cancer cells [2]. Most of the studies conducted showed that the endophytic fungi extracts were effective against Cancer cell lines, Herpes simplex virus type 1, malaria and other diseases [3-5]. Plants and fungi engage in intimate relationships that range from harmful to beneficial activities. Endophytes are microbes often bacteria or fungi which lives within a plant at least for part of its life without causing any apparent negative effects. They reside inside the tissues of nearly all healthy plants. A wide diversity of fungi was isolated from healthy tissues of most terrestrial and aquatic plants [6]. The leaves appear to house a greater diversity of fungi than the roots. Plant tissues remain entire and functional even though endophytes are residing inside the plant. Endophytic fungi live in plants internally, either intercellular or intracellular and asymptomatically within tissues and are distinguished from mycorrhizae by lacking external hyphae or mantels [7]. Endophytes as organisms that cause symptomatic infections within plants. This definition excludes pathogenic infections and mycorrhizal fungi [8].

A wide range of plants have now been examined for endophytes, and endophytes have been found in almost all of them. Both plants and the endophytes were mutually benefitted by this endo-symbiosis. Endophytes protect their hosts from disease causing agents and adverse environmental conditions by secreting bioactive secondary metabolites [9-11]. Some specific group of endophytic fungi has a close association with grasses. These fungi are transmitted vertically (through seed) and their impact on the economic value of the host has led to study of their biology [12]. The grass endophytes, and related fungi, are considered separately from the more common general endophytes.

Endophytic filamentous fungi represent an important genetic resource for biotechnology. Now a day's fungal metabolites are the sources for the many medicinal products. Need of new and effective bioactive compounds are very much needed for mankind which is safe over synthetic compounds used as drugs. The emergence of new diseases and resistance of microorganisms over the drugs is the biggest challenge to the human beings. This made the scientists to investigate new sources of drugs. In recent days fungi have been used as a source of secondary metabolites.

The discovery of first antibiotic penicillin by Alexander Fleming in 1928 is the milestone in this field. After this several compounds were isolated. Taxol is another promising anti-cancerous drug isolated from *Taxomyces andreanae* [13]. These filamentous fungi were frequently used for the isolation of secondary metabolites and increased the list of effective drugs from fungal origin. In the current study, we have used the endophytic fungi HHPCYL03 isolated from *Cymbopogon flexuosus* grass for the extraction of secondary metabolites.

MATERIALS AND METHODS

Collection of grass

The environmental samples were collected from Kemmannugundi regions of Karnataka. Samples collected carefully along with the ball of soil to avoid damage to roots, brought in an icebox and processed within 24 hours of collection.

Isolation of endophytic fungi

Thoroughly washed plant materials were separated into root, stem, and leaves. They were surface sterilized by immersing the samples in 70 per cent Ethyl Alcohol for 3 min followed by immersion in 3 per cent Sodium hypochlorite for 1 minute and again washed twice with distilled water. The excess water content was removed by placing inside the laminar airflow and blowing the air. The efficacy of the surface sterilization was confirmed by inoculating the surface sterilized water collected from the last wash of the sample in a nutrient medium. The absence of growth of any fungi on the media confirms efficient surface sterilization of the segments. Surface sterilized small pieces were cut into 0.3-0.5 cm segments. Four hundred segments each samples (root, stem and leaf) inoculated in Potato Dextrose Agar (PDA) medium (PDA, Hi Media Laboratories, Mumbai, India) by gently pressing on it which is supplemented with Amoxillin (250 mg L^{-1}) to inhibit the growth of bacteria. The inoculated Petri plates were wrapped with Petri seal, and incubated at lab temperature. After 3 days of inoculation, the plates were observed daily for growth of fungi from cut ends of segments up to one month. The fungi that were grown very fast were discarded and slow growing was retained for further study. The emerged fungal endophytes were transferred to new Petri plates containing Potato Dextrose Agar Medium and pure cultured. The endophyte was identified by using standard manuals [14, 15].

Extraction of secondary metabolites

The mycelia of different endo-symbiotic fungi were purified on Potato Dextrose Agar medium. Potato Dextrose Broth was used for production of secondary metabolites by the fermentation method. The pure culture of endo-symbiotic fungi was inoculated to 1000 ml conical flasks containing 500 ml of broth medium on a rotary shaker for 15-22 days at 25 °C. The culture filtrate was filtered through a Watmann filter paper and the filtrate was extracted with the same volume of Ethyl Acetate for twice and the extract was air dried for further analysis.

MTT assay

The MTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenases. The MTT method is simple, accurate and yields reproducible results. The key component is (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTT, is a water soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells. Sensitivity of Breast cancer cells (MDA-MB-231), colorectal cancer cells (HCT-116) and lung cancer (Calu-6) cells to crude extracts of *C. lunata* were determined individually by the MTT colorimetric assay. About 50,000 cells/well of MDA-MB-231, HCT-116 and Calu-6 cells were seeded in a 96 well plates and incubated for 24 hrs at 37ºC in 5 % CO_2 incubator. Test samples from 0-320µg/ml (2 fold variations) concentration in DMEM media without PBS (Phosphate buffer solution) was made and incubated for 24 hr. About 8850 µg/well of MTT was added to all the wells and incubated for 3-4 hours. After incubation with MTT reagent the reagent was then discarded and 100 µl of DMSO was added to each well to dissolve the formazan crystals. The optical density (OD) was recorded at 590 nm in a microplate reader.

$$\text{Ig IC50absolute=IgClower=} \frac{\text{50\%-IPlower}}{\text{IPhigher-IPlower}}$$

Where C_{lower}= concentration of tested preparation, which resulted in<50% inhibition of proliferation;

C_{higher} = concentration of tested preparation, which resulted in>50% inhibition of proliferation; IP_{lower}= inhibition of proliferation (%) calculated for C_{lower}; IP_{higher} = inhibition of proliferation (%) calculated for C_{higher}.

RESULTS

Screening of ethyl acetate, extract of HHPCYL03resulted in good anticancer activity against Calu-6 cell lines when compared to MDA-MB-231, HCT-116 cells. The percent of cancer cell inhibition were found to be concentration dependent (fig. 1), which is comparable to results obtained from the extract of endophytic fungi *Cephalotheca faveolata* on HCT-116 cells [16]. The per cent of inhibition was increased from 6.11±1.00 percent to 65.60±1.21 percent in case of Breast cancer cell lines, 3.00±2.12 percent to 52.11±1.01per cent in case of colorectal cancer cells whereas in case of Lung cancer cells it is increased from 5.76±0.73 per cent to 76.43±0.36 percent with the increase in the concentration of the extract (table 1). The minimum concentration used was 5µg/ml and the highest concentration was 320 µg/ml. Following results were obtained when anticancer activities against three cell lines were studied.

Table 1: Percentage inhibition of HHPCYL03 extract on different cancer cell lines

Conc. µg/ml	% of cell inhibition		
	MDA-MB-231	HCT-116	Calu-6
5	6.11±1.00	3.00±2.12	5.76±0.73
10	16.74±0.57	16.61±0.75	7.36±0.24
20	23.43±1.09	28.34±0.91	8.90±0.81
40	31.16±0.99	48.47±0.79	27.52±1.41
80	44.27±0.48	49.44±0.16	47.84±1.09
160	57.03±0.63	50.10±0.51	67.36±0.93
320	65.60±1.21	52.11±1.01	76.43±0.36

Table 01 shows variation in the cell inhibition rate by fungal extracts at various concentrations. In case of all the three cell lines inhibition percentage was increased with increase in concentration of the test extract. This indicates the dependence on the concentration gradient.

Table 2: MTT assay values

Log (inhibitor) vs. response	HHPCYL03		
	MDA-MB-231	HCT116	Calu-6
Bottom	77.02±0.54	55.75±0.68	103.3±0.73
Top	3.434±0.63	-36.54±0.79	-3.417±0.09
$LOGIC_{50}$	1.784±0.146	0.842±0.124	1.973±0.234
IC_{50}	60.84±0.95	6.951±0.71	94.02±0.91

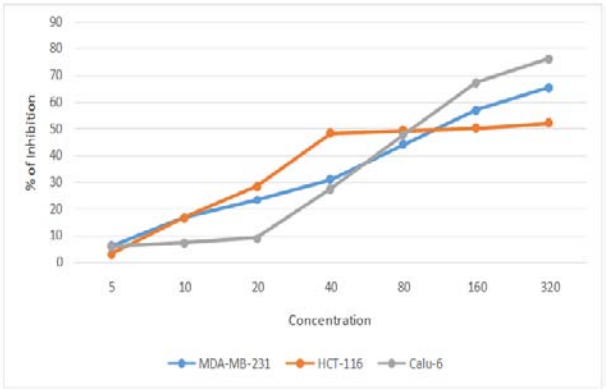

Fig. 1: Effect of concentration of HHPCYL03 extract on three cancer cell lines

DISCUSSION

The per cent of inhibition in our study is lower compared to an earlier study, where the extracts inhibited the proliferation of HeLa cells in a dose-dependent manner [17]. The cytotoxicity of HeLa cell lines using MTT assay showed an IC_{50} of 92.2±0.23 and 88.54±1.23 µg/ml for the extracts of *Fusarium* sp. and *A. fumigatus*. The extract showed significant result at 320 µg/ml against Calu-6 cells. The cytotoxic effect of fungal endophyte isolated from *Barringtonia acutangula* was tested by the MTT assay, which showed the effect of its secondary metabolites on the cell viability in HT29, human colon cancer cell line [18]. Fungi-derived natural products have been an excellent source of pharmaceuticals as well. Antibacterial penicillins, cholesterol-lowering lovastatin, antifungal echinocandin B, and immunosuppressive cyclosporin A, serve to illustrate the importance of investigating fungal sources for new medicines [19].

Extract of HHPCYL03 treatment showed the minimum level of growth inhibition on MDA-MB-231, HCT-116 and Calu-6 cell lines,

the IC_{50} values are shown in the table 1. The extract has shown significant dose dependant growth inhibition of HCT-116 cells compared to MDA-MB-231 and Calu-6 cell lines. Most of the bioactive metabolites isolated from endophytic fungi were the potential source of anticancer activity [20-22]. This was evident that bioactive compound produced by endophytes could be substitute approaches for finding of novel anticancer drugs [23, 24]. These results suggest that crude extract of HHPCYL03 has more cytotoxic effect on HCT-116 cells with the IC_{50} value ~ 7.0 as shown in the table 2 and could be a potential candidate against colorectal cancer.

CONCLUSION

In our study, the extract from fungi HHPCYL03 showed the positive result against colorectal cancer cells. Nowadays cancer is the major problem to mankind more and more novel anticancer drug discovery is the only way to combat the deadly cancer. In this regard more and more bioactive anticancer compounds have to be isolated from fungal sources. The fungal endophytes certainly become a repository of good economically, socially benefitted bioactive compounds.

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CONFLICT OF INTERESTS

Declared None

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