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In-vitro antifungal and anticancer potential of *Xylaria curta* fruiting body fractions against human fungal pathogen and cancer cell lines

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Abstract

In the present study, the dried fruiting bodies were extracted and fractionized by column chromatography method. Fractions were tested for anticancer and antifungal activity against human cancer cell lines and fungal strain respectively. Phytochemical analysis was performed by colour tests to characterize the putative compounds responsible for this bioactivity. Among the various fraction, fraction D was found to be the best for the bioactive principles from *Xylaria curta.* Fraction D showed a maximum inhibition zone of 22.9 mm against *Candida albicans* and also had significant cytotoxic activity of 58.5% against A-549 human Lung cancer cell lines at a concentration of 60 µg/mL. These results indicate that partially purified extract of *Xylaria curta* as source of eco friendly potent pharmaceutical new drugs for controlling human fungal pathogens and human cancer cell lines.

Key words – Ascomycete fungi – bioactivity – human cancer cell lines – MTT assay – partial purification

Introduction

The need for new and useful natural products to provide assistance and relief in all aspects of human pathological condition is ever growing and even challenging. Nature has proven and continues to be a promising source for the discovery of bioactive compounds, important for the development of new pharmaceuticals. Natural products play a dominant role in the discovery and development of drugs in the treatment of human diseases (Newman et al. 2003). Throughout the history of drug development, natural products have provided a fundamental source of drugs for fighting infection, inflammation and cancer in humans. They have been the most successful source of potential drug leads (Mishra & Tiwari 2011, Rey-Ladino J Ross 2011). Today natural products still serve a role directly as traditional medicines.

Development of new drugs opens a new innovative research interest in the field of oncology and infectious diseases. Cancer is the second leading causes of public health problem in worldwide,

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given that it is estimated that approximately 25 million people suffer one of its different manifestations and 10 million new cases are annually reported (WHO 2002) for that reason, there is an increasing demand for more effective anticancerigenous substances and therapies (Lord & Ashworth 2010). Most of the cancer treatments rely heavily on chemotherapy however, chemotherapy has limitations. Chemotherapeutic drugs lack selectivity and they can also kill normal cells. Hence, there is an urgent need for new drugs that are highly effective, possess low toxicity and have a minor environment impact. In that regard, several studies have reported cytotoxic activity against cancer cells of organic extracts of spores (Fukuzawa et al. 2008), vegetative bodies (Hu et al. 2002, Choi et al. 2009) and fruiting bodies (Takaku et al. 2001) from several species of macro fungi.

Fungi are a diverse and valuable source with an enormous chemical potential. Since the discovery of penicillin from *Penicillium notatum*, fungal organisms suddenly became a hunting ground for novel drug leads (Strobel et al. 2004). They are of major interest because only a small percentage of them have been investigated for their role in producing novel bioactive compounds and hence offer huge potential. In the unremitting search for novel bioactive compounds from the species of *Xylaria* led to abundant natural products with bioactivities. Many biologically active secondary metabolites such as anticancer and antifugal compounds have been isolated from the genus *Xylaria*, for example, cytotoxic cytochalasins (Pongcharoen et al. 2007, Rukachaisirikul et al. 2009, Zhang et al. 2010) isopimarane diterpene glycosides (Shiono et al. 2009), multiplolides (Boonphong et al. 2001) griseofulvin (Park et al. 2009). Besides that, the crude extract of *Xylaria curta* had significant bioactivity against antibacterial activity against drug resistant *Staphylococcus aureus and Pseudomonas aeruginosa* (Ramesh et al. 2012a). Therefore, the present study is aimed to work the anticancer activity of partially purified fractions of *Xylaria curta* against human cancer cell lines.

Materials & Methods

Collection and extraction of fruiting bodies

The macro fungal samples were collected from tropical evergreen forest of Courtallam Hills, Western Ghats, Tamil Nadu, India. The collected sample was identified and authenticated (Ramesh et al. 2012b). The shed dried fruiting bodies were used for extraction and preparation of samples by using cold extraction. The powdered fruiting bodies samples were extracted in ethyl acetate and evaporated under reduced pressure at approximately 40°C. The dried extracts were used for further analysis.

Fractionation of extract

The concentrated crude ethyl acetate extract was mixed with silica gel (70-230 mesh) to prepare the extract-silica gel slurry and air dried. A glass column was packed with silica gel up to 25 cm height and washed with 200 mL of hexane. Then, the extract-silica gel slurry was loaded onto the column and eluted initially with hexane followed by different ratios of hexane ⁄ ethyl acetate mixture by increasing polarity [9:1 to 1:9 (v⁄v)] followed by the ethyl acetate and methanol mixture [9:1 to 1:9 (v⁄v)]. About 100 mL of each solvent system was used for elution and 5 mL of each fraction were collected. The presence of compounds was analyzed by thin-layer chromatography (TLC) using pre-coated silica gel plate with hexane and ethyl acetate at 2:3 ratios (v⁄v) as solvent system. Fractions showing similar spots on TLC were pooled and concentrated. The partially purified extracts were further fractionated using 230-400 mesh silica-gel column chromatography. Fractions showing a similar single spot on TLC were pooled together and concentrated. Totally seven fractions were collected and reconstituted in 5% dimethylsulphoxide (DMSO) to a final concentration of 10 µg/mL and subjected for preliminary screening against *Candida albicans* to select the active fractions. This fraction was tested for anticancer activity against human cancer cell lines.

Test microorganisms and cell lines

The human fungal pathogen of *Candida albicans* (MTCC3018) was purchased from the Microbial Type Culture Collection (Chandigarh, India). Human cancer cell lines such as MDA-MB-231 (Breast carcinoma cells), A549 (Lung carcinoma cells) and MCF-7 (Breast carcinoma cells) were obtained from ATCC (American Type Culture Collection, Rockville, DD) and were used for anticancer activity. The cell lines were grown in DMEM (Dulbecco's modified eagle's medium) supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 mg/mL) in 96-well tissue culture plates (Costar, Cambridge, MA). Cells were incubated with 0.5 mL of media at 37°C in a tissue culture incubator equilibrated with 95% air and 5% Co_2 (HERACELL 240-I).

Bioassays

Antifungal activity

A 100 µl of fungal culture/spore was spread out onto the surface of potato dextrose agar medium. Immediately, 100 µl of sample was loaded onto the well. The fungal culture was incubated at 30^{o}C for 48-72 h and the zone of inhibition was recorded around the well.

Cell viability assay

Cell viability was examined using the MTT assay. In brief, human cancer cell lines were cultured at 1×10^5 cells per well in 96-well plates containing MEM medium. After an overnight incubation, cells were treated with 1% DMSO (control) plus various concentrations (2, 10, 20 and 60 µg) of fraction D for followed by incubating the plates at 37^{o}C for 48 h. After 48 h, media was aspirated and washed with DPBS and 0.5 mL of serum free MEM was added and incubated with 5 mg/mL of MTT and incubated for 1 h at 37^{o}C. The culture medium was aspirated and 0.5 mL of DMSO was used to dissolve the formazan crystals/precipitate. Triplicate wells were analyzed at each concentration. The absorbance was measured at 562 nm using automated TECAN multimode reader. OD value was subjected to sort out percentage of viability by using the following formula (Al-Fatimi et al. 2007)

$$\text{Percentage of viability} = \frac{\text{Mean OD value of experimental sample}}{\text{Mean OD value of experimental control}} \times 100$$

Each experiment was carried out in triplicate. Similar results were obtained in all experiments. The entire cytotoxic assay was done at Chemical Biology Lab, (IICT-CSIR) Hyderabad, India.

Morphological observation

A-549 cells (35000 cells/well) were grown in 24-well plates and treated with fraction D at the concentration of 40 µg/mL and 60 µg/mL. Morphological changes of cells in both of the treated group and control group were analyzed at 8 h and 24 h under the OLYMPUS 1×71 Inverted Fluorescence microscope at 10× magnification.

Determination of bioactive components

The preliminary screening for the active components in fraction D was performed by Harborne method (Harborne 1998).

Results

Identification of macro fungus

The present macro fungus was identified *as X. curta*, based on the morphological characteristics such as size, shape, and color of the fruiting bodies, which was reinforced by the sequence of its 18S rRNA gene and submitted to NCBI (GenBank No. JF795289). The morphological characteristics were already reported in our earlier study (Ramesh et al. 2012b).

Antifungal and cytotoxic activity

The unknown potential of macro fungi as sources of novel bioactive substances is now widely recognized. Macro fungi based products either from the mycelia or from fruiting bodies is consumed in the form of capsules, tablets or extracts (Nitha et al. 2007). The preliminary antimicrobial screening revealed that the ethyl acetate extract showed an effective antimicrobial activity against both bacterial and fungal pathogens (Ramesh et al. 2012a). The bioactive assays were carried out using the DMSO solutions. DMSO was chosen as solvent after comparative toxicity assays (data not shown) and was not toxic. The in vitro antifungal activity of the seven fractions of *X. curta* against human fungal pathogen *C. albicans* is shown in Table 1.

Table 1 Antifungal activity of fractions of *Xylaria curta* against *Candida albicans.*

Fractions used	Zone of inhibition (mm)
A	14.2±0.22
B	11.1±0.12
C	10.1±0.34
D	22.9±0.01
E	10.4±0.21
F	12.5±0.16
G	11.0±0.19

Results are mean ±S.D. of triplicate experiments.

Results showed that seven fractions exhibited various levels of antifungal effect against C. *albicans*. Among the various fractions, the fraction D showed maximum antifungal activity of 22.2 mm against *C. albicans*, whereas the significant activity of 14.2 mm was observed in fraction A. All the other fractions showed moderate antifungal activity.

Breast cancer is the most common cause of cancer death in women and the most frequently diagnosed cancer among women in 140 of 184 countries worldwide. It now represents one in four of all cancers in women (WHO 2013)). A major clinical problem is the breast tumors, which are initially responsive to both hormonal and chemotherapeutic approaches, generally progress to more aggressive forms that are poorly responsive to either category of agents (Vani et al. 2006). Although chemically synthesized chemotherapeutic agents demonstrated activity in the metastatic breast cancer setting (Tripathy 2007), some of these chemotherapeutic drugs have undesirable toxic side effects. Therefore, the identification of natural antiproliferative and non-toxic agents is of particular interest and a unique of macro fungus as natural complexes demonstrated significant anticancer activities.

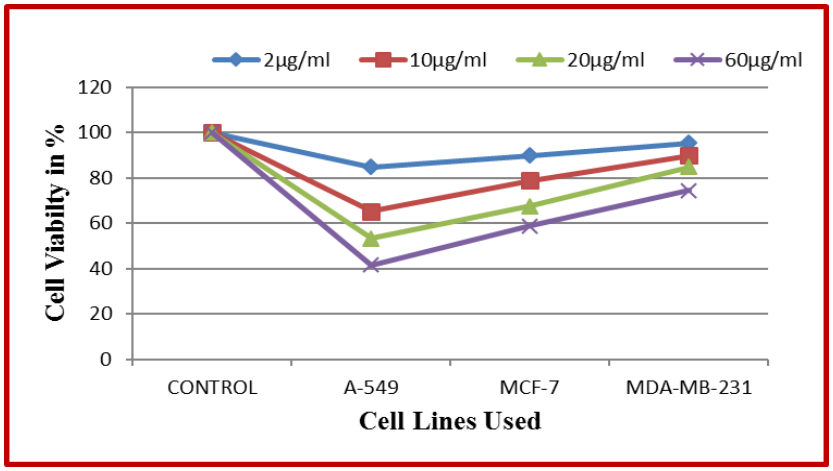


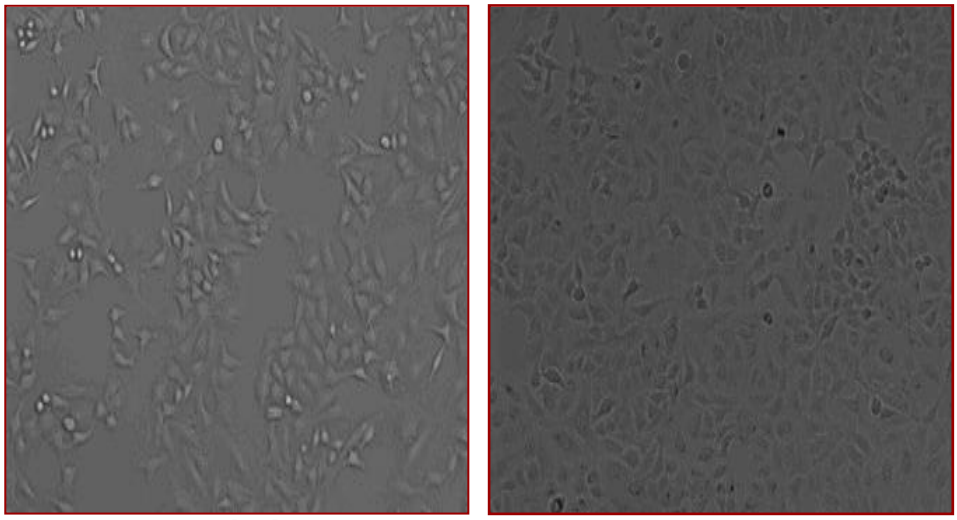
Fig. 1 – Cell viability of fraction D of *Xylaria curta* against human cancer cells lines.

The cytotoxic effect of fraction D on three cancer cells lines was investigated by MTT assay. The tested cancer cell lines showed a good response to the effect of fraction D shown in figure 1. Fraction D displayed the strongest cytotoxicity of 58.5% at a concentration of 60 µg/mL against A-549 but normal cell was not clearly inhibited. In contrast, 60 µg/mL fraction D had moderate cytotoxicity of 41.3% and 25.6 % against MCF-7 and MDA-MB-231 human cancer cell lines respectively. But 20 and 10 µg/mL of fraction D had reasonable inhibitory effect on various types of human cancer cell lines.

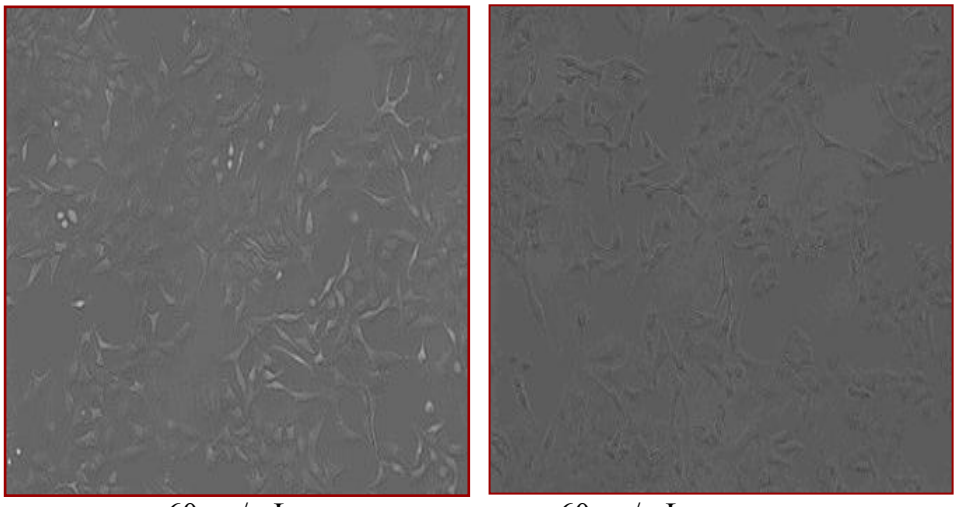
The morphological effects were also studied in 24-well plate method and the changes were observed by using Inverted Fluorescence microscope at the magnification of 10×. The growth of the A-549 cells was markedly inhibited by fraction D at 40 µg after 8 h treatment. At 60 µg, most of the cancer cells growth was arrested during cell division and the cell nuclei became condensed and segmented after 24 h treatment which was the indication of apoptosis (Fig. 2).

Preliminary nature of the active components

The preliminary chemical analysis of fraction D of *X. curta* showed the presence of active compounds such as flavonoids, ascorbic acids and terpenoids may be the reason for the action of fraction D.



Control 8 h Control 24 h



60 µg/mL 60 µg/mL

Fig. 2 – Morphological changes of human lung carcinoma cell line (A-549) by the action of fraction D of *Xylaria curta.*

Discussion

Regarding antifungal activity, the fraction D showed maximum antifungal activity of 22.2 mm against *C. albicans.* In contrast, this given result is better when compare to crude extract of *Xylaria curta* according to earlier report. Based on the significant antifungal activity, the fraction D was only taken for further anticancer studies. One of the largest causes of mortality in worldwide is cancer. Increasing interest and research on fungal medicine have revealed its importance in treating many diseases including cancer. Cancer metastasis, which consists of uncontrolled growth and invasive behavior of cancer cells, is one of the major medical problems in breast cancer patients (Punglia et al. 2007).

From the above anticancer results, the percentage of cytotoxic activity was progressively increased with increasing gradient concentration. At 10 µg/mL of fraction D, 34.9% of cytotoxicity was estimated against A-549 cancer cells. When the concentration of the fraction was increased to 20 & 60 µg/mL, the cytotoxicity further increased to 46.7% & 58.5% respectively. In addition that among three kind of cancer cell lines, A-549 human Lung cancer cell lines exhibited significant cytotoxic activity of 58.5% against at a concentration of 60 µg/mL fraction D. This result indicated that the anticancer activities depending on the concentration of extract of *Xylaria curta*. Similarly, Shiono et al [15] reported the cytotoxicity of the isopimarane diterpene glycosides extracted from the fruiting bodies of the ascomycete *Xylaria polymorpha*. IC_{50} value of the compound ranged from 71 to 607 µM.

Therefore, the present results clearly revealed that the macro fungal species of *Xylaria curta* grasp the significant bioactive compounds that can proficiently inhibit the growth of human fungal and cancer cell lines. Therefore, these studies should be extended to economically and eco friendly important untapped bioactive compounds for the invention of pharmaceutical drugs.

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