

Anticancerous potential of *Rosellinia sanctae cruciana* sp- Endophytic fungi isolated from *Azadirachta indica*

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Abstract:

The increase in drug resistance among microorganisms results in occurrence of hazardous disease like Cancer, so there is requirement to look for new sources which are readily available and reliable for the treatments of such hazardous diseases. Natural products are preferred over synthetic products and one of such promising natural sources is secondary metabolites from Endophytic microbes. In our present study an attempt was made to study anticancerous potential of an endophytic Fungi-*Rosellinia sanctae – cruciana* from *Azadirachta indica*. The cytotoxic effect was studied using culture filtrate extract of chloroform :methanol (6 :3) ratio on MCF7 breast cancerous cell line using MTT assay. The extract showed potent activity against cancerous cells with IC₅₀ value of 198± 12.4 µg/ml and the viable cells were around 74.34% at 100µg/ml which decreased to 47.20% at 200 µg/ml indicating activity in a dose dependent manner while the cytotoxicity of the same extract on non cancerous 3T3 fibroblast cell lines. 68.68% cell viability was reported as 500µg/ml and induces apoptosis.

To the best of our knowledge this is the first report on *Rosellinia sanctae – cruciana* as an endophyte from *A. indica*.

Keywords :-Endophytic fungus ; *Rosellinia sanctae* ; *Azadirachta indica* ; anticancer ; apoptosis.

Cancer- an alarming disease characterized by an irregular proliferation of the cells. As a cell progress from the normal to cancerous, certain changes occur in biological behaviour as well as in the appearance of the cell. (Ashworth *et al.*, 2011) Cancer has become a major threat to human health due to its high rates of morbidity and mortality. The 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. There is need to find new sources of drugs and also develop new and promising drugs which efficient to overcome the MDR cells. (Smyth *et al.*, 1998, Ruefli *et al.*, 2002, Shi *et al.*, 2007). One such promising and unexplored sources are ENDOPHYTES.

Endophytes (bacteria and fungi) thrive within plant tissues which are steady environment and so they are more bioactive than any other microbes from plants and soil. These endophytes spend almost their whole life within host plant and do not harm host plant rather they help the host plant to survive in stress conditions as they serves as huge reservoirs of natural bioactive compounds or secondary metabolites of unique structures which have high potentials for agriculture, medicinal, industrial uses (Priti *et al.*, 2009).

The secondary metabolites or novel compounds produced by endophytes having unique structure- flavonoids, tannins, steroids, phenols etc shows high antioxidant activities (Herreara-Carrillo *et al.*, 2009) and can thus it can be said that Endophytes are promising and less explored group of microorganisms which can effectively control oxidative stresses and diseases keeping the body fit and healthy (Strobel and Daisy, 2003).

MATERIALS AND METHODS

Isolation of Endophytic fungi from leaves of *A.indica*:

Sample collection: Healthy leaves of *Azadirachta indica* (Neem) were collected from Niwai region (Rajasthan) in months of October and November, 2014. The samples were transported to lab in sterile polythene bags and processed for isolation of endophytic fungi under sterile conditions.

Isolation of Endophytic fungi: The plant material or leaves from the healthy plants were subjected to endophytic isolation soon after the collection according to the procedure given by Verma *et al.*, (2007). The healthy leaves collected were thoroughly washed with running tap water for 45 minutes to wash away all the soil particles and then air dried. All the work was performed under laminar air flow hood maintaining sterile conditions. The cleaned leaves after air

drying were surface sterilized by immersion in 75% ethanol for 1 minute, followed by 2.5% sodium hypochlorite solution for 5 minutes and then washed thrice (1 minute each time) with sterile distilled water and left for drying under sterilized condition. Both the borders of the sterilized leaf segments were cut off with the help of sterile blade and about 1 cm of the plant material (leaf segment) was subjected to endophytic isolation. The small leaf segments were placed on potato dextrose agar (PDA) media plates supplemented with antibiotic streptomycin (100 mg/ml) so as to avoid bacterial growth. Each plate was inoculated with seven leaf segments. All the plates were sealed and packed with parafilm and were incubated at 25-28°C along with control and last rinse plates. The plates were observed daily for 15-20 days for emergence of endophytes. The emerging fungal hyphal tips from the plant leaf segments were picked and transferred on PDA plates to check purity of the culture. The pure cultures were maintained on PDA slants at 4°C.

Anti-cancerous activity of PD 17 Chloroform: Methanol extract

MTT assay was performed for anticancer activity using MCF7 cancerous cell lines at Centre for biological sciences, Pondicherry as per the method given by Carrie *et al.*, (2014).

Reagent Preparation

MTT Solution

MTT was dissolved in Dulbecco's Phosphate Buffered Saline, pH=7.4 (DPBS) to 5 mg/ml. This MTT solution was filter-sterilized through a 0.2 µm filter into a sterile, light protected container. The MTT solution was stored at 4°C for frequent use or at -20°C for long term storage and was protected from light.

Solubilization Solution

All the work was done in a ventilated fume hood and selection of appropriate solvent resistant container was done. Firstly, 40% (vol/vol) dimethylformamide (DMF) was prepared in 2% (vol/vol) glacial acetic acid. To this 16% (wt/vol) sodium dodecyl sulfate (SDS) was added and dissolved, at pH = 4.7. The solution was stored at room temperature to avoid precipitation of SDS. If a precipitate forms, warm to 37°C and mix to solubilise SDS.

b. MTT Assay Protocol

All the cells and test compounds were prepared in 96-well plates containing a final volume of 100 µl/well. Incubation for desired period of exposure was carried out and 10 µl MTT solution was added per well to achieve a final concentration of 0.45 mg/ml. Incubation was done for 1 to 4 hours at 37°C. To this, 100 µl Solubilization solutions were added to dissolve formazan crystals to each well and were mixed thoroughly for complete solubilisation. Absorbance was taken at 570 nm.

Dual AO/ EB fluorescent staining protocol:

This dual staining was carried to check apoptotic or necrotic nature of the cells. The cells were treated with test compound at IC50 concentration and incubated for 24 hours in CO2 incubator at 37°C. The cells were removed by trypsinization and collected by centrifugation including the non adherent cells. The cell pellet was resuspended in medium and cell suspensions (25 µl) were transferred to glass slides. Dual fluorescent staining solution (1 µl) containing 100 µg/ml AO and 100 µg/ml EB (AO/EB, Sigma) was added to each suspension and then covered with a cover slip. The morphology of apoptotic cells was examined and counts the cells within 20 min using a fluorescent microscope.

Acridine orange is a vital dye and will stain both live and dead cells. Ethidium bromide will stain only cells that have lost membrane integrity. Live cells will appear uniformly green. Early apoptotic cells will stain green and contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells will also incorporate ethidium bromide and therefore stain orange, but, in contrast to necrotic cells, the late apoptotic cells will show condensed and often fragmented nuclei. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin.

TOXICITY STUDY:

The toxicity studies were carried out in 3T3 fibroblast cell lines using MTT assay at Centre for biological sciences, Pondicherry.

Results and Discussion

Antioxidant potential of the chloroform: methanol (6:3) culture filtrate extract of *Rosellinia sanctae – cruciana* have been reported in our previous studies hence, the extract of *Rosellinia sanctae – cruciana* was further tested for its anticancerous potential. The natural products having anticancer property are preferred over the synthetic compounds having market value around 63% among all drugs available (Cragg *et al.*, 2009). The existing anticancer drugs have a limited selectivity and are highly toxic. The upcoming studies suggests that natural products isolated from endophytes, especially the secondary metabolites are more likely to yield anticancer drugs (Hong *et al.*, 2009). The antioxidant plays a preventive or curative role against cancer by removing reactive oxygen species (Sgambato *et al.*, 2001) The anticancerous activity of various endophytic fungi has been reported. Wu *et al.*, (2015) reported anticancer activity of fifteen endophytic fungus against human carcinoma cell lines, LU-1 (lung), PC-3 (prostate), and MCF-7 (breast cancer cell line). The sample was noncytotoxic to all the cancer

cell lines tested at a concentration of 50 µg/ml. The ethyl acetate extracts of all endophytic fungi were noncytotoxic to the cancer cell lines examined except for the leaf endophytes *Stemphylium solani*, *Leptosphaerulina australis* and *Xylaria sp.* Significant antitumor activity of ethyl acetate extract of endophyte *Phomopsis liquidambari* against HL-60 leukemia, MCF-7 breast and COLO 205 colon cell lines was reported by Qian *et al.*, (2014). Lakshmi and Selvi (2013) reported anticancer activity of fungus isolated from leaves of *Barringtonia acutangul.* The anticancer assay was carried out on Human Colon Cancer cell lines HT29. In the present study the anticancer activity of chloroform and methanol fraction of *Rosellinia sanctae-cruciana* exhibiting antioxidant potential was tested on breast cancer cells MCF-7. Cisplatin was used as a standard. *Rosellinia sanctae-cruciana* extract resulted in a decrease in cellular viability of cancer cells in a dose dependent manner with IC₅₀ value of 198 ± 12.4 µg/ml (Fig. 1). The IC₅₀ value for cisplatin treated cells was 12.5 ± 1.65 µg/ml (Fig.2). The variation between the positive drug and sample may be due to the use of pure drug as compared to crude extract. Overall in MCF-7 breast cancer cell lines, the viable cells were around 74.34% at 100 µg/ml which decreased to 47.20% at 200 µg/ml (Fig.19). This indicates the extract is toxic at higher concentrations.

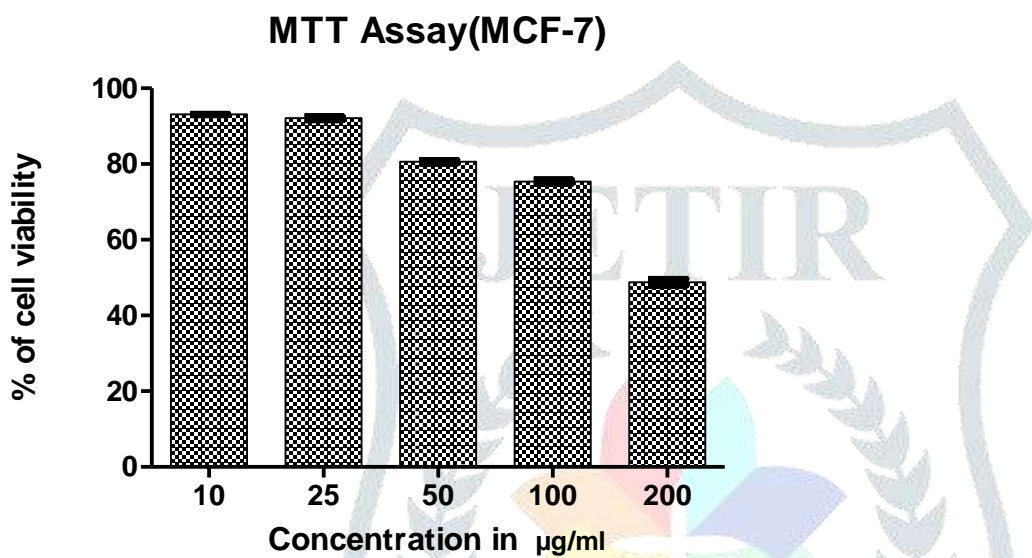


Fig. 1: Percent cell viability of test sample against MCF7 breast cancer cell line.

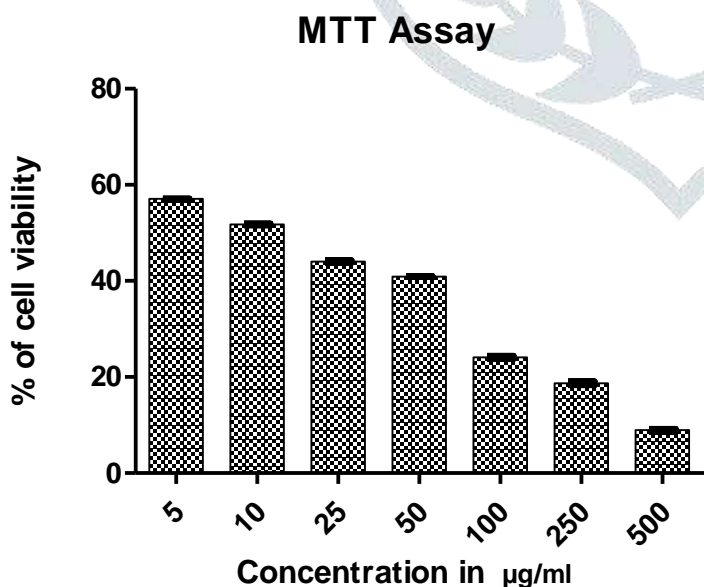


Fig 2: Percent cell viability of standard (Cisplatin) on MCF7 breast cancer cell line.

The cytotoxicity of *Rosellinia sanctae – cruciana* extract was determined on non cancerous 3T3 fibroblast cell lines. 68.68% cell viability was recorded at 500µg/ml (Fig.3).

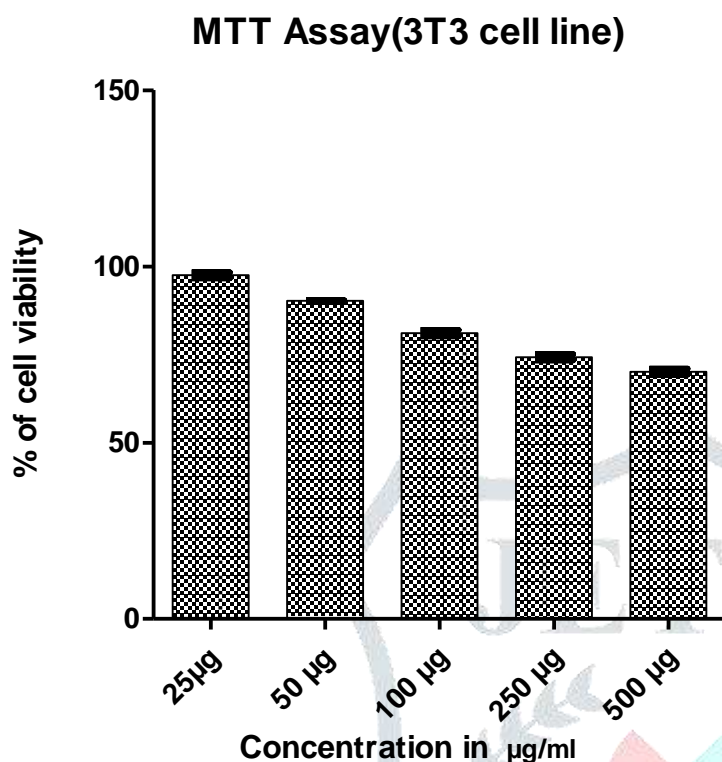


Fig.3: Percent cell viability on 3T3 fibroblast cell line.

Defects along apoptotic pathways play important role in initiation of cancer. The cells in which DNA or other components are damaged irreversibly undergo apoptotic cell death under normal condition. It is a self destructive metabolism genetically encoding cell death signal (Hooper *et al.*, 1999). Cancer cells evade apoptosis by various ways. Anticancer agents play a curative role in a damaged system caused by ROS. The apoptosis signalling system is triggered by anticancer agents in cancer cells disturbing the proliferation (Bold *et al.*, 1997). Ethyl acetate extracts of *Fusarium sp.* isolated from *Datura metel* was able to induce cytotoxicity and apoptosis in human cancer cell lines, in particular against cervical cancer cells HeLa (Kuriakose *et al.*, 2014). Acridine orange is a vital dye and will stain both live and dead cells. Ethidium bromide stains only cells that have lost membrane integrity. Live cells will appear uniformly green. Early apoptotic cells will stain green and contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells will also absorb ethidium bromide and therefore stain orange, but in contrast to necrotic cells, the late apoptotic cells will show condensed and often fragmented nuclei. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin. The presence of intact live cells in control wells were observed as green colour fluorescence, while the wells treated with test compound showed apoptotic cells as reddish orange colour fluorescence (Fig. 4).

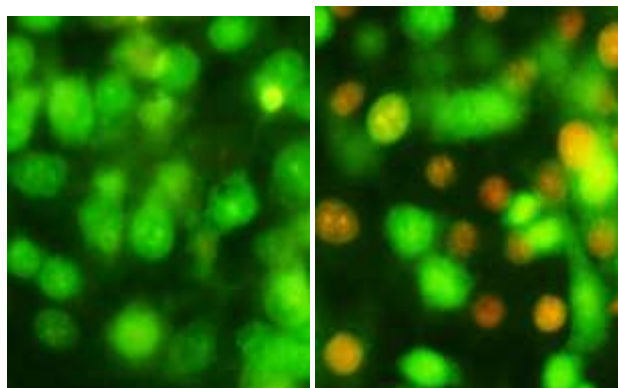


Fig. 4: Dual AO/EB fluorescent staining (MCF-7)

In the present study, the fungus *Rosellinia sanctae cruciana* endophytic to the host plant *A.indica* possessed in vitro cytotoxic activity by prohibiting the growth of cancerous cells and inducing apoptosis. It was found to be toxic on non cancerous 3T3 fibroblast cell line at 500µg/ml. Apoptosis pathway is responsible both for tumour development and progression along with treatment in cancer therapies. Most anticancer drugs currently follow apoptotic signalling pathways to trigger cancer cell death. Metabolites exhibiting this activity may be evaluated for their anticancer activity (Wang, 2011).

Several bioactive compounds from endophytes have been identified exhibiting anticancer effects (Stierle *et al.*, 1993; Eyeberger *et al.*, 2006; Kumar *et al.*, 2013). Our study suggests that this chloroform: methanol extract of *Rosellinia sanctae cruciana* might prove to be a potential source of anticancer lead molecule. Further work is needed to isolate and characterize the bioactive components for possible anticancer lead molecule.

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