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Cytotoxicity of Ethyl Acetate Extract of Endophytic *Aspergillus fumigatus* on A549 Cell Lines

D. Prabavathy* and C. Valli Nachiyar

Department of Biotechnology, Faculty of Bio and Chemical Enginnering,
Sathyabama University, Rajiv Gandhi Salai, Jeppiaar Nagar, Chennai - 600119, India.

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Endophytes are mutualistic symbionts of healthy plants known for production of wide range of bioactive compounds with a broad spectrum of activity. In the present study, endophytic *Aspergillus fumigatus* was isolated from *Justicia beddomei*. The bioactive compounds were extracted with ethyl acetate by solvent extraction procedure. Fraction IV obtained by partial purification with a mixture of hexane and ethyl acetate in silica gel column demonstrated cytotoxicity on A549 and L6 cell lines. The IC 50 was determined to be 8.37 μg/ml and 110.37 μg/ml respectively by MTT assay. The dual staining and Hoechst staining indicated the induction of apoptosis in the tumor cells. DNA fragmentation further confirmed the DNA of tumor cells as the molecular target of the fraction for cytotoxicity. Further structural characterization studies of the fraction are necessary to reveal the structure –function relationship of the compounds isolated.

Key words: Endophytic *Aspergillus sp*, MTT assay, A549 cells, Apoptosis, DNA fragmentation.

Cancer poses a severe threat and challenge to the mankind since time immemorial. Cancer is a disease characterized by unregulated cell proliferation, leading to spread of abnormal cells and uncontrolled tissue growth. Cancer is also the foremost cause of mortality worldwide[1]. The treatment for cancer remains elusive in many cases due to lack of specificity and availability of drugs[2]. Many endophytes are now known for their anticancer metabolites. It began with the discovery of taxol producing endophytes, the most potent anticancer drug, from the culture of the endophytic fungi, *Taxomyces andreanae* isolated from *Taxus brevifolia* tree, and endophytic fungus *Pestalotiopsis microspora* from medicinal plants *Taxus wallichiana* and bald cypress *Taxodium distichum*[3]. Also, other common endophytic genera such as *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Fusarium*, and *Mucor* spp. have been reported as producers of taxol[4-8].

Endophytes are defined as "microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects"[9]. Endophytes are chemical synthesizers inside the plants[10]. They play a key role as selection systems for production of bioactive substances with low toxicity toward higher organisms[11]. Extensive studies on the endophytic fungi of medicinal plants are reported from diverse environmental conditions demonstrating the diverse bioactivity of the secondary metabolites produced by them[12-14].

Justicia beddomei (Family: Acanthaceae) is a glabrous shrub widely distributed in Southern parts of India. The analgesic, diuretic, antimicrobial

* To whom all correspondence should be addressed.
Tel.: 044-24503145;
E-mail: prabagrp@gmail.com

activity of various extracts of the leaves of *Justicia beddomei* has been demonstrated by Srinivas *et al* 2007[15]. The present investigation was carried out to isolate and elucidate the mechanism of cytotoxicity of the ethyl acetate extract of the endophytic fungi on A549 and L6 cell lines.

MATERIALS AND METHOD

Isolation and Identification of endophytic fungi

Leaves of healthy and mature plants (*Justicia beddomei*) were chosen for sampling. The surface sterilization was done according to the method described by Petrini, 1986 [16]. About 4-5 segments are placed per petridish of potato dextrose agar (PDA) supplemented with chloramphenicol 50µg\ml of medium to check the growth of bacteria. The plates are incubated for about 4-6 weeks at 27ºC in dark. The fungal colonies that arose were sub cultured repeatedly on PDA plates until pure culture was obtained. The morphological characterization of the isolated fungi includes both macroscopic and microscopic observation of the fungal structures. The hyphal and reproductive structures morphology was observed by Lactophenol cotton blue wet mount. The DNA from the endophytic fungi was extracted and the ITS regions were amplified with the universal ITS primers. The amplified product was sequenced and aligned with the library sequences available at Genbank by BLASTN program. The sequence was submitted to the GenBank under Accession Number KC545969. The sequences are aligned using ClustalW2 program employing the neighbor-joining algorithm to establish the phylogeny. The isolated species had a maximum of 99% similarity to *Aspergillus fumigatus*.

Extraction of bioactive metabolites

A block of actively growing fungal endophyte was cultivated on the Potato Dextrose Broth (PDB).The cultures were filtered through sterile mesh cloth to remove mycelia mats. Fungal metabolites of the filtrate and biomass were extracted thrice by solvent extraction procedure with ethyl acetate as the organic solvent. The purification of the crude extract was carried out by gradient elution with a mixture of hexane and ethyl acetate (100%hexane, 75hexane:25 ethyl acetate, 50:50, 25 hexane: 75 ethyl acetate, 100% ethyl acetate) on a silica column. The fractions obtained were further used for cytotoxicity assays.

Cell proliferation assay (MTT assay)

The Human lung Adenocarcinoma cell lines (A549) were purchased from National Centre for Cell sciences (NCCS) Pune. Proliferation of A549 cells was assessed by MTT assay[17]. Cells were plated in 96-well plate at a concentration of 5×10^4 cells/well. After 24h of cells incubation, the DMEM medium was replaced with 100µl partially purified extract containing medium at different concentrations (1 – 1000µg/ well) and incubated for 24h. Untreated cells served as control and received only 0.1% DMSO in which the fraction was prepared. At the end of treatment period, media from control and extract -treated cells was discarded and 50µl of MTT (5mg/ml PBS) was added to each well. Cells were then incubated for 4h at 37°C in CO_2 incubator. MTT was then discarded and the coloured crystals of produced formazan were dissolved in 200µl of DMSO. Spectro-photometrical absorbance of the purple blue formazan dye was measured using an ELISA reader (BIORAD) at 570nm. Optical density of each sample was compared with control optical density and graphs were plotted to determine the IC50.

Dual Staining

Ethidium bromide and acridine orange staining was carried out by the method described by Gohel *et al*., 1999[18]. A549 cells were plated at a density of 1×10^4 in 48-well plates. Equal volumes of cells from control and extract treated were mixed with 100µl of dye mixture (1:1) of ethidium bromide and acridine orange) and viewed immediately under Nikon inverted fluorescence microscope (Ti series) at 10x magnification.

Hoechst 33342 Staining

Hoechst staining was carried out by the method of Yamakawa *et al.,* 2008 [19]. Nuclear morphology of apoptotic cells with condensed/ fragmented nuclei was examined by fluorescence microscopy and at least 1×10^3cells were counted for assessing apoptotic cell death.

DNA fragmentation assay

DNA extraction and agarose gel electrophoresis were performed on control A549 cells and extract treated cells to analyse the effect of the extract on the DNA.

RESULTS AND DISCUSSION

A549 cells are widely studied as models of not only lung cancer but also as human primary alveolar epithelial cells *in vitro.* Distinct morphological changes were observed in A549 cells treated with various concentrations (1 - 1000µg) of extract when subjected to MTT assay. The treatment of L6 cells and A549 cells with the extract resulted in significant dose dependent reduction in cell growth after 24h of incubation. The IC_{50} value was found to be 8.37µg\ml for A549 cells whereas it was 110.37 µg\ml for L6 cells (Table 1). The IC_{50} value of 8.37µg\ml against A549 cells and a significantly reduced cytotoxicity on L6 cells indicates the specificity of the components in the extract on cancer cell lines. Similar findings have been reported in the ethyl acetate extracts of cultures grown in liquid Czapek media and on solid rice media of the fungal endophyte *Fusarium oxysporum* SS46 isolated from the medicinal plant *Smallanthus sonchifolius*. Hexane extract of *F.oxysporum* SS50 grown on solid rice media also afforded a mixture of compounds that displayed cytotoxic activity against different cancer cell lines[20]. Ruma *et al.,* 2013 [21] demonstrated the cytotoxicity of *Aspergillus fumigatus* isolated from *Garcinia sp* on the Hela cell lines with an IC 50 value of 88.54µg\ml.

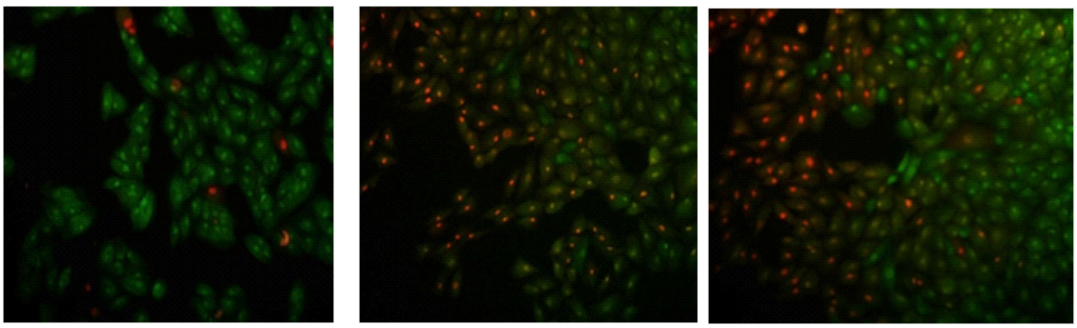
Acridine Orange/Ethidium Bromide staining (AO/EB) was evaluated the type of cell death induced by the endophytic fungal extract in A549 cells. AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA), and EB is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. Thus, live cells have a normal green nucleus, whereas the early apoptotic cells are bright green nucleus with condensed or fragmented chromatin and the late apoptotic cells display condensed and fragmented orange (Figure 1).The number of viable cells decreased tremendously with increasing concentration of extract. The percentage of apoptotic cells after treatment with 8µg/ml was found to be 41%. It drastically increased to 79% with a concentration of 16µg/ml of extract.

Table 1. Dose dependent inhibition of cell proliferation by the ethyl acetate extract of endophytic *Aspergillus fumigatus* on A549 and L6 cell lines

Concentration of extract (µg/ml)	% inhibition of cell growth	
	A549	L6
1	35.89±0.50	2.84±0.85
3	46.74±1.03	7.16±0.94
10	52.52±0.95	14.04±2.24
30	56.60±0.50	18.14±0.51
100	71.78±0.25	20.59±0.17
300	86.06±0.33	28.15±0.38
1000	96.53±0.08	35.68±0.53

Values are expressed in terms of mean ± SEM

Apoptosis was further confirmed by analyzing the nuclear morphology of extract-treated A549 cells. Nuclear morphology was evaluated with membrane-permeable stain Hoechst 33258. The treated cells contained more apoptotic nuclei when compared to untreated cells. There was characteristic nuclear fragmentation of nuclei in treated A549 cells whereas the untreated control



(a) (b) (c)

Fig. 1. Ethidium bromide/acridine orange staining of A549 cells treated with the endophytic fungal extract (a) untreated control ,(b) cells treated with 8µg/ml of extract, (c)) cells treated with 16µg/ml of extract

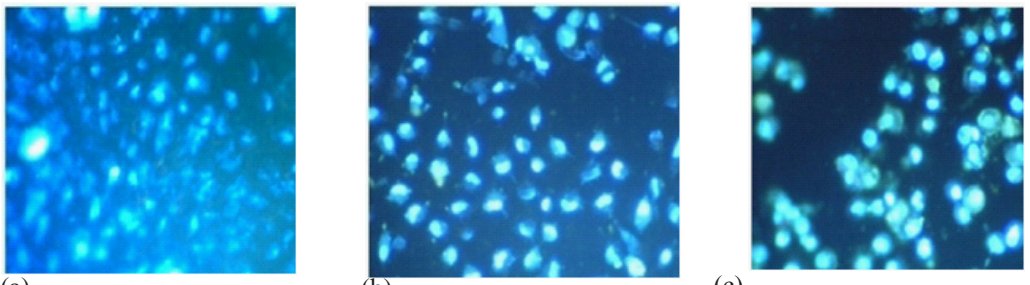


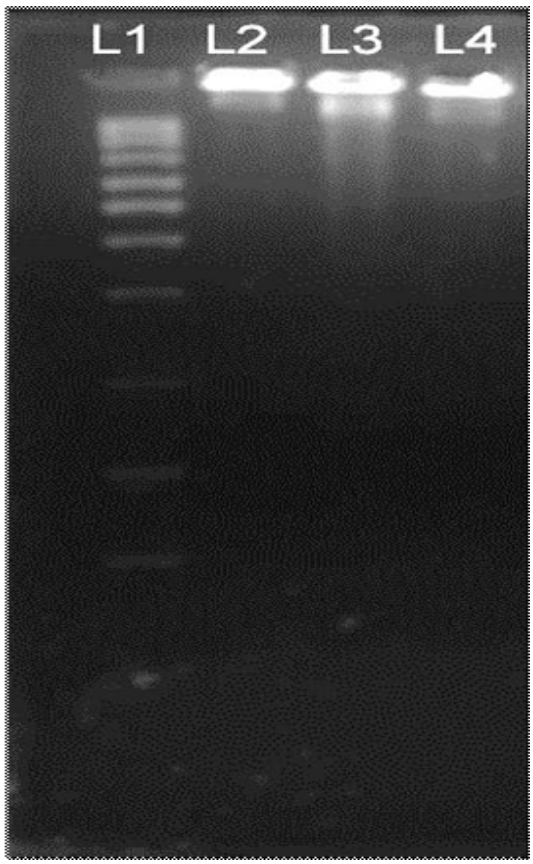
Fig. 2. Hoechst staining of A549 cells treated with the endophytic fungal extract (a) untreated control ,(b) cells treated with 8µg/ml of extract, (c)) cells treated with 16µg/ml of extract

cells did not show any nuclear fragmentation. The apoptotic cells displayed characteristic features of reduced size, intense blue fluorescence of condensed nuclear chromatin and formation of membrane blebs (Figure 2). The percentage of apoptotic nuclei after treatment with 8µg/ml was found to be 48%. These findings demonstrate the induction of apoptosis in A549 cell lines by the endophytic fungal extract consistent with the results of AO/EB dual staining.

Apoptotic DNA fragmentation is a key feature of apoptosis and is characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into internucleosomal fragments of roughly 180 base pairs (bp) and multiples thereof (360, 540 etc.). This effect can be used to detect apoptosis via the DNA laddering assay[22]. Figure 3 shows the nuclear fragmentation of the A549 cells treated with the extract and further evidences the apoptosis.

As it is well known that morphological changes are the basis of functional changes; in turn, functional changes would affect the morphological structure. The morphological changes evidenced by the staining studies indicate the induction of apoptosis. The best strategy of an anticancer agent is the induction of apoptosis in tumor cells[23].

The cytotoxicity of the ethyl acetate extract could be directly attributed to the components present in the extract. An individual compound or the synergistic action of these compounds would have resulted in the cytotoxicity of the extracts on the cell lines. In our previous studies we have reported the phytochemical constituents of the ethyl acetate extract of the endophyte. The extract was found to contain alkaloids, anthraquinones, flavonoids, phenols which could have resulted in the observed cytotoxicity[24]. It was interesting to observe the specific cytotoxic potential of the extract on the cancerous cell line such as A549 than the normal cell line L6.



L 1 – 1500bp Ladder; L2 – Control;
L3 – 8µg/ml; L4 – 16µg/ml

Fig. 3. Gel photograph showing the effect of partially purified extract on DNA of A549 cells

CONCLUSION

Endophytes are known for production of novel secondary metabolites with a broad spectrum of activity. Our present study established the cytotoxicity of endophytic *Aspergillus fumigatus* of *Justicia beddomei*. A significantly reduced toxicity to non cancerous cell line and a specific increased cytotoxicity to cancer cell line make the compound an interesting lead molecule for development of anticancer therapeutic. Future studies, the focus would be on the structural elucidation of the bioactive compounds which would aid the pharmacokinetic studies.

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