Metabolomics: Open Access
ISSN: 2153-0769

Sadananda et al. Metabolomics 2016, 6:1
DOI: 10.4172/2153-0769.1000162

Research Article Open Access

In Vitro Apoptotic Activity of Endophytic Fungal Lectin Isolated from Endophyte, *Aspergillus flavus* of *Viscum album* on Human Breast Adenocarcinoma Cell Line (MCF-7)

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Abstract

Lectin was isolated and purified from the endophytic fungi *Aspergillus flavus* of *Viscum album* and it was a D-galactose and N-acetylgalactoseamine specific lectin capable of agglutinating A+ve blood group tested in hemeagglutination method. The lectin was confirmed a glycoprotein PAS staining on NATIVE-PAGE and it is having single band of 64 kDa protein (in SDS-PAGE) similar to plant lectin. The peptide sequences were identified from purified lectin 64 kDa protein band is shown. NVRFDLSGATSSSYK similar to Cucurbia pepo protein peptide sequence and it is a ribosome inactivating protein. Apoptotic activity was tested on human breast adenocarcinoma cancer (MCF-7) cell line and it is a time and dose dependent manner. Dose dependent inhibition of cancer cells was observed with IC50 values of 0.02 µg/ml from *Aspergillus flavus*. MCF-7 cell death elicited by the lectin extract was found to be apoptotic in nature based the indication of nucleus condensation, shrinkage of nucleus membrane; membrane bebbling and also DNA fragmentation are the hallmark of apoptosis was observed in MCF-7 cells after staining with Hoechst 33342. Caspase mediated apoptosis was observed in lectin treated MCF-7 cells by cleaving fluorogenic peptide substrates, Ac-DEVD-AMC. The caspase-7 was observed as an effector in cleaving other caspases, further inhibition of caspase with an inhibitor provide clear evidence of caspase-7 mediated apoptosis in MCF-7 treated cells.

Keywords: Apoptosis; *Aspergillus flavus*; Caspase-7; Lectin; MALDI-TOF-MS; MCF-7

Introduction

Apoptosis is a highly organized cell death process characterized by loss of plasma membrane asymmetry, condensation of nuclear chromatin, the nucleus breaks up and DNA cleavage by enzymes [1]. Apoptosis plays an important role in the maintenance of tissue homeostasis; induction of apoptosis in cancer cells is recognized as a valuable tool for cancer treatment.

The secondary metabolites from nature have played a leading role in the pharmaceutical industry for various purposes from plant source. If continuously exploiting the plants, there is a chance to affect the biodiversity, so that scientists are searching for a new source of bioactive metabolites from the nature, among them is endopohytes. Endophytic microorganisms are colonizing in the living cells of the internal tissue of plants without causing any immediate or overt negative effects [2]. Endophytes have shown as potential for having various bioactive metabolites can be used directly or indirectly as therapeutic agents to treat numerous diseases [3] and the beneficial substitutable approach to efficiently produce valuable bioactive compounds [4] within short-time at high quantity.

Lectins are carbohydrates binding non-immune origin proteins and initiates their activity by binding to the carbohydrates surface present on the cell surface and induces a variety of functions viz., signal transduction, immune defense, cytotoxicity and apoptosis. Lectins are known from the past several decades, interest has been focused because of the potential application in diagnosis, immunomodulatory, drug delivery and anticancer therapy.

Cancer progression is associated with aberrant glycosylation of cell surface molecules [5] and as carbohydrate-binding proteins; lectins were expected to bind specifically to these glycosylated cell surface molecules. Furthermore, studies have shown plant lectins affect the proliferation of cells by altering the mammalian cell function [6,7]. Major advantage of lectins is their availability in natural source; they can be expected to cause minimum side effects, when used as a drug. Plant lectins promotes proliferation of T lymphocytes [8], inhibit the proliferation [9] in different cancer cell line in induction of apoptosis. Lectins from different source have ability to inhibit cancer cell growth leads to apoptosis through various morphological and molecular changes. Lectins are found in all kinds of organism includes animals, plants, fungi, bacteria and viruses [10].

The plant *Viscum album* is commonly known for the presence of cytotoxic lectin *Viscum album* agglutinins (VAA). Three classes of VAA has been identified such has (VAA-I, II, III), among these VAA-I are potent cytotoxic lectins belongs to type II ribosome inactivating proteins exerts potent cytotoxic effects on tumor cells by triggering a mitochondria mediated apoptotic pathway by activating extracellular signal regulated kinase and remarkable generation of ROS by activation of caspase and SEK/JNK pathway [11,12]. Based on literature survey, the present work was aimed to identify lectin from endophytic fungal species of *V. album*, characterization and evaluated for apoptotic action on human breast cancer cell line (MCF-7).

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Received December 28, 2015; **Accepted** January 11, 2016; **Published** January 14, 2016

Citation: Sadananda TS, Govindappa M, Ramachandra YL, Chandrappa CP, Umashankar T (2016) *In Vitro* Apoptotic Activity of Endophytic Fungal Lectin Isolated from Endophyte, *Aspergillus flavus of Viscum album* on Human Breast Adenocarcinoma Cell Line (MCF-7). Metabolomics 6: 162. doi:10.4172/2153-0769.1000162

Materials and Method

Collection and mass production of endophytic fungal species

The endophytic fungal species *Aspergillus flavus* was collected from stock cultures of Department of Biotechnology, Shridevi Institute of Engineering and Technology, Sira Road, Tumkur, Karnataka, India and it was isolated from stem part of *V. album*. The fungus was cultured on PDB broth for large scale cultivation and then incubated at room temperature (28 ± 2°C) for 8 days. The fungal mycelial mat and *V. album* extract was washed in distilled water on cheese cloth and homogenized in 50 ml (w/v) of 50 mM sodium phosphate buffer (pH 7.2), containing 154 mM NaCl (PBS) for 5 mins and stirred overnight at 4°C. Both the extracts were centrifuged at 9500 rpm for 30 min at 4°C separately. The resulted supernatants were filtered using cheese cloth and used for further purification [13]. The protein concentrations of lectin extracts of *V. album* and fungal endophyte were estimated using bovine serum albumin as standard [14].

Hemagglutination and hemagglutination inhibition assays

Hemagglutination tests were performed in standard microtitre plates by 2-fold serial dilution method [15]. 50 µl aliquot of the human erythrocytes (A^{+ve}, B^{+ve}, AB^{+ve}, O^{+ve}) suspension was mixed with 50 µl of serially diluted lectin of plant and fungi and agglutination was examined visually after incubation for 30 min at ~ 25°C. The unit of hemagglutination activity (HU) was expressed as the reciprocal of the highest dilution (titre) of the lectin that showed complete agglutination. To determine the sugar binding specificity of lectin was determined using different sugar including D-galactose, D-mannose, D-glucose, maltose and N-acetylgalactosamine were tested for their ability to inhibit lectin induced hemagglutination. Serial two-fold dilutions of each carbohydrate were prepared in 10 to 100 mM range and dissolved in 0.15 M NaCl solution and mixed with equal volumes of extracts containing 4 units of haemagglutination. Mixtures were incubated for 30 min at room temperature, after which a suspension of human erythrocytes (4%) was added and the whole incubated for 1 h. The low carbohydrate concentration produced complete inhibition of haemagglutination was determined.

Lectin purification

Plant and endophytic lectin extracted was purified using lactose–agarose (Sigma-Aldrich, India) column equilibrated and elute with extraction buffer at a flow rate of 3 ml/min^{-1} until the column effluent showed absorbance at 280 nm of less than 0.05 [16]. Bound proteins were eluted with 100 mM lactose in equilibration buffer. The elution was monitored at 280 nm and 3 ml fractions were collected manually and tested for haemagglutinating activity on human A^{+ve} erythrocytes. Active fractions collected was adjusted to be saturated in 60% ammonium sulfate, precipitated protein dissolved in PBS and dialyzed extensively against same buffer, aliquot, freeze-dried and stored at -30°C until use.

Molecular weight determination by SDS-PAGE

The polyacrylamide gel electrophoresis was performed [16] on 2 mm thick vertical slab gels using 5 and 12.5% stacking and running gels. These were cast in 10.2 cm × 5 cm × 0.75 mm slab gels. Endophytic fungal and *V. album* lectin was dissolved in 0.0625 M Tris-HCl (pH 6.8) containing 1% SDS, 0.1% coomassie brilliant blue (CBB) and 10% glycerol was incubated at 90°C for 5 min. The molecular markers employed were ß-galactosidase (116 kDa), fructose-6-phosphate kinase (80 kDa), bovine serum albumin (72 kDa), ovalbumin (68 kDa), glutamate (60 kDa), carbonic anhydrase (36 kDa), myoglobin (29 kDa) (Aristogene Bioscience Ltd., Bangalore). Marker and lectin samples were loaded to respective wells and electrophoresis was carried out at a constant current of 50 V for 3 h. After electrophoresis, the gel was fixed by fixing solution for 1 h and stained with Coomassie brilliant blue R-250 and destained with destaining solution. The molecular weight of the purified lectin was determined by comparing its electrophoresis mobility with the standard molecular weight marker proteins.

Periodic acid-schiff staining (PAS)

PAS staining was performed with slight modified method [17]. The protein separated on NATIVE-PAGE was soaked in 7.5% (v/v) acetic acid for 30 min and then with 0.2% (w/v) periodic acid for 1 h at 4°C. The periodic acid solution was removed and the Schiff reagent was added and incubated for 1 h at 4°C. Reddish-pink bands of stained glycoprotein would be visible. The PAS reagent was removed and the gel was soaked in 7.5% acetic acid for 1 h and subsequently stored in water and photographed.

In-gel digestion of protein spots

Protein digestion was performed as described previously [18]. Spots were excised from the stained (SDS-PAGE) gel and washed with CH_3CN:H_2O (1:1, v/v) containing 25 mM ammonium bicarbonate to remove the dye. The gel plug was dehydrated with 100% acetonitrile and was dried under vacuum and incubated overnight at 37°C with 20 µl of 10 µg/ml porcine trypsin in 20 mM ammonium bicarbonate. The resulting tryptic fragments were eluting by diffusion into CH_3CN:H_2O (1:1 v/v) and 0.5% trifluoroacetic acid. A sonic bath was used to facilitate the diffusion. The extracts were vacuum dried and the pellet was dissolved in CH_3CN:H_2O (1:1 v/v) and 0.1% trifluoroacetic acid. The extracted tryptic peptides were used for mass spectrometric analysis.

MALDI-TOF-MS

The dried fungal and plant lectin extract was dissolved in 10 µl of 0.1% trifluoroacetic acid (TFA), purified by Zip Tip C18 tips prior to MS analysis. The elute was mixed with 10 mg ml^{-1} of 2,5-dihydroxycinnamic acid (DHB) in 50% acetonitrile and 0.1% TFA for spotting onto sample plate and dried for MALDI-TOF-MS analysis. MALDI-TOF-MS analysis was performed with the 4700 proteomics analyzer (Applied Biosystems, Framingham, MA, USA) equipped with an ND:YAG laser (355 nm) having a 3-7 ns pulse and a 200 Hz firing rate. MALDI-TOF-MS analyses were obtained in the reflectron positive ion mode. The accelerated voltage of the ion source for MS and MS/MS analyses set at 20 and 8 kV respectively peaks were detected using the internal algorithm of the 4000 series software with the signal-to-noise ratio parameter set at 30 for MS, analysis was done at IISc, Bangalore, India.

Protein identification

Peptide Mass Fingerprinting (PMF) and peptide fragments were examined with proteomics analyzer. For each sample spectra generated was used for protein identification. Protein identification was performed by searching for other green plant, using Swiss-Prot protein sequence database, using the MASCOT search engine [19]. The following parameters were applied during database searching enzyme trypsin, allowed missed cleavages 4, fixed modifications carbidomethyl (C), variable modifications oxidation (m), peptide tolerance 100 ppm MS/MS tolerance 0.5 Da, peptide charge +1, instrument MALDI-TOF-MS. A protein was considered if it had hit with a random match

probability lower than 0.005. SCAN PROSITE tool was employed for the similar identification of conserved patterns, sequence obtained from MASCOT analysis, was employed for pattern identification.

Cancer cell line

Human breast adenocarcinoma (MCF-7) cells were obtained from the National Center for Cell Science (Pune, India) and maintained in laboratory (Sri Raghavendra Biotechnologies, Bangalore, Karnataka, India). Cells were routinely grown in Eagle's Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 1% 1-glutamine and 80 µg/ml gentamycin sulfate. Cells were cultured in 75 cm^2 flasks with loosened caps and incubated in a 5% CO_2 in humidified air at 37°C.

MTT Assay

The cytotoxicity of endophytic fungal lectin on MCF-7 cancer cell lines was determined by MTT assay [17]. Cells (1×10^5/well) were plated in 100 µl media/well in 96 well plates. Endophytic fungi *A. flavus* lectin sample in 10 fold serial dilutions in PBS in the following concentrations (20 µg, 2.0 µg, 0.2 µg, 0.02 µg, 0.002 µg, 0.0002 µg/well), positive control (doxorubicin) 0.26 µg/well, vehicle control 10% PBS, media control and plates were incubated at 37°C, 5% CO_2 in triplicates for 24 h and 48 h were set for each concentration. After incubation entire media was removed and 100 µl of DMSO was added to dissolve the formazan crystals and again incubated for 20 min at 37°C, 5% CO_2. Transfer the entire DMSO from each well to the cuvette and absorbance was then recorded at 545 nm using the microplate spectrophotometer system (Biotek ELx 800, USA). Each treatment was completed in triplicate (each experiment repeated at three times) and IC_{50} (concentration of drug that inhibits cell growth by 50%) values were determined from the concentration vs. percent viability curve. Three steps were repeated for 48h in triplicates. The percent viability was calculated as follows

$$\text{Percent viability} = \frac{\text{Test absorbance} - \text{Blank absorbance}}{\text{Control absorbance} - \text{Blank absorbance}} \times 100$$

DNA fragmentation assay

The lectin extract from the endophytic fungi *A. flavus* treated to MCF-7 were seeded in a culture plate at a 1×10^5 cells/well. The cells were treated with lectin at a concentration of 0.02 µg/ml from *A. flavus*. Cells were incubated for 24 to 48 h. DMSO, phosphate buffered saline (pH 7.2) treated cells were considered as control. At the end of treatment, adherent cells were harvested by trypsinization and pooled with suspended dead cells. Then cells were centrifuged at 2000 rpm for 5 min at room temperature, washed with HBSS twice. The smear of the cells was made on the clean slide and fixed with 4% paraformaldehyde and washed the slide with normal saline. Cells were stained with Hoechst 33342 for 10 min at 37°C. At the end of staining cells were washed with ice cold HBSS and cell were covered with a cover slip and observed under inverted fluorescent microscope equipped with 350 nm excitation source and emission filter of 450 nm wavelength [18]. Morphological characteristics of apoptosis such as condensed nuclei, fragmented nuclei and membrane blebbing were observed in both 20X and 40X magnifications.

Measurement of caspase-7 activity

MCF-7 cells were treated with 0.02 µg/ml of *A. flavus* incubated for 48 h. After incubation, the cells were collected and suspended with cell lysis buffer, incubated the cells on ice for 10 min and centrifuged at 10,000 rpm for 5 min, the supernatant obtained was treated with 100 µl of 4 mM fluorogenic peptide substrates, Ac-DEVD-AMC (caspase 7) and incubated at 37°C for 3.5 h. The amount of pNA released was measured at 405 nm using an ELISA microplate reader. Similarly, cells treated with DMSO and phosphate buffered saline (pH 7.2) used as control,

0.26 µg of doxorubicin (positive control) were used. The caspase activity was calculated as a ratio of the absorbance of treated cells to untreated cells. The action of caspase-7 in the extract was studied by pre-incubated with inhibitor in cell lysate sample for 10 min at room temperature before adding caspase-7 substrate solution [19].

Results

Mass production and isolation of lectin from plant and endophytes

From the extract, the protein concentration was high in *V. album* of 2.4 mg/ml and the *A. flavus* yielded 1.4 mg/ml.

Hemaglutination and hemagglutination inhibition assays

Crude protein extracts of *V. album* and endophytic fungi were found to contain a hemagglutinating protein. Among the different blood types (A, B, O, AB), lectin from the plant and endophytic fungi agglutinated only with A^{+ve} blood group. Absence of agglutination observed as red button in the bottom of the ELISA plate. This could be explained by differences in glycosylation of the surface protein of RBC tested.

Carbohydrate specificity of lectin was performed in the presence of different sugars and results are depicted. D-galactose and N-acetylgalactosamine were found to be highly effective for inhibiting agglutination for *V. album* lectin at 25 mM, same type of agglutination inhibition was observed for lectin from *A. flavus* at 25 mM and 10 mM. It confirms that lectin from *V. album* and endophytes, *A. flavus* was D-galactose and N- acetylgalactosamine specific (Figure 1 and Table 1).

Characterized the purified the lectins of endophytic fungi and plant in SDS-PAGE showed singal band have molecular weights of 64 kDa which is similar to plant (Figure 2A). The endophytic fungal lectin stained purplish pink with Schiff's reagent in NATIVE-PAGE and it confirms that it is a glycoprotein (Figure 2B). The lectin band of 64 kDa was digested with trypsin and peptide fingerprints were identified in MALDI-TOF-MS. The *Aspergillus flavus* lectin showed 2 peptides with maximum intensities (Figure 3). The peptide patterns were subjected to database analysis using mascot with NCBI and Swiss-Prot sequence database (http://www.matrixscience.com). The endophytic fungi, *Aspergillus flavus* was identified as ribosome-inactivating protein was similar to *Cucurbita pepo* (Figure 4).

MTT assay

Cytotoxic and growth inhibitory effect of purified endophytic fungal lectin extract (D-galactose and N-acetyl-galactoseamine) from *A. flavus* on human cancer cells (MCF-7) were studied by MTT assay. The MCF-7 cell viability was assayed after exposure to various concentrations of *A. flavus* lectin for 24 to 48 h. Results of apoptotic activity observed in a concentration dependent inhibitory effect on the growth of MCF-7 cells. In *A. flavus* lectin concentration at 0.0002 µg, 0.002 µg, 0.02 µg, 0.2 µg 2.0,20 µg/ml showed decreased cell viability percentage and increased the cell death percentage was compared with vehicle control after 24 and 48 h of treatment. It indicates that concentration dependent inhibitory effect on the growth was observed on the cancer cell from endophytic fungal lectin. The minimum

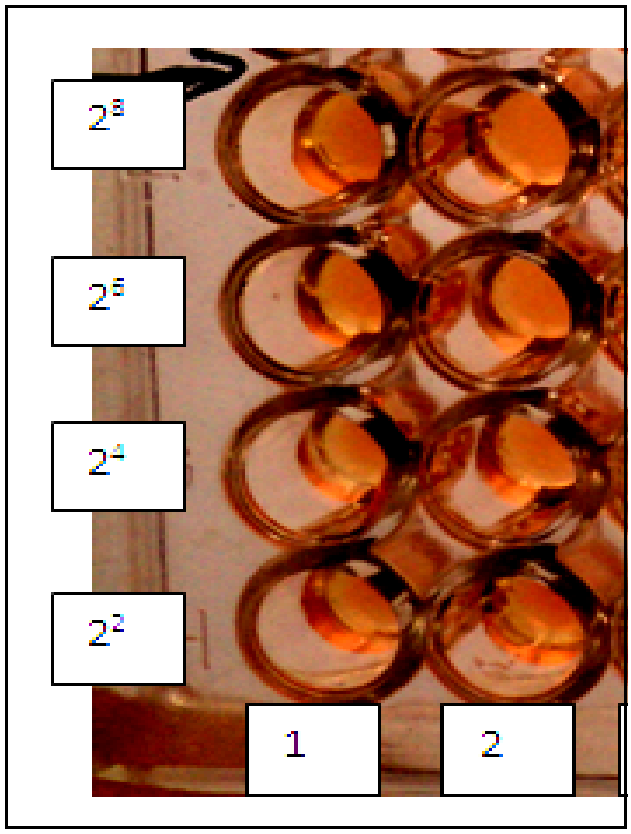

Figure 1: Hemagglutination activity of plant and endophytic fungal species lectin in A^{+ve} blood group at different concentrations, 1: *Viscum album,* 2: *Aspergillus flavus.*

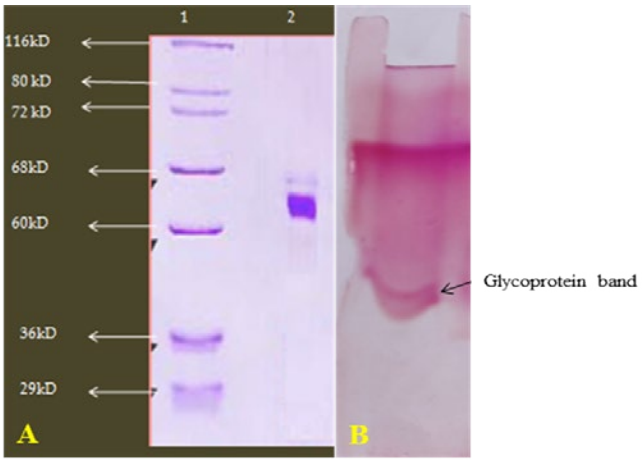

Figure 2: A SDS-PAGE profiles of endophytic and plant lectin, 1: Molecular weight marker, 2: *Aspergillus flavus*, B. NATIVE-PAGE glycoprotein staining of endophytic lectin showing glycoprotein band.

inhibitory concentration of *A. flavus* lectin IC_{50} values observed was 0.02 µg/ml. Even at low concentration of lectin (20 µg/ml) 21.23 ± 0.1 and 78.67 ± 0.2 of percent cell viability and cell death was observed after 24 h of incubation. After 48 h incubation, 6.1 ± 0.01 and 93.9 ± 0.01 of percent cell viability and death was observed in respectively (Table 2) (Figures 5 and 6). In compared with positive control doxorubicin showed 15.1 % cell viability and 98.2% cell death was observed. It confirms that concentration dependent inhibitory effect on the growth and death of cancer cells was observed.

DNA fragmentation assay

The MCF-7 cell lines were treated with lectin extract of *A. flavus* incubated for 24 and 48 h and after incubation of cells was treated with fluorescent dye Hoechst 33342. A hallmarks property of apoptosis such as condensed nuclei, DNA fragmentation and shrinkage was observed in lectin treated MFC-7 cells. Apoptotic phenotype of extract treated cells was compared with doxorubicin treated cells. These results confirm that endophytic fungal lectin of *A. flavus* induced apoptosis in human breast cancer cells MCF-7 showing fragmentation of DNA (Figure 7).

Caspase-7 activity assay

The caspase-7 activity was measured calorimetrically using pNA-conjugated substrates in lectin treated cancer cells by incubating 48 h. Caspase-7 is the main downstream effecter of caspase that are present in the most of the cell types and plays an important role in inducing apoptosis, by cleaving cellular substrate. We compared its activity in treated and untreated cells, with standard pNA curve (Figure 8), amounts of pNA released in treated sample was recorded. The results showed that caspase-7 was significantly activated in treated cancer cells about 1.84 units of enzyme activity were observed in *A. flavus* lectin. The activity was blocked in both treated samples by adding broad range of caspase inhibitor DEVD-CHO; it suggests that the apoptosis signaling induced by lectin extract was in a caspase-dependent pathway. In the presence of inhibitor in the lectin sample of *A. flavus* did not show caspase-7 activation. This shows that caspase-7 was main component involved in the apoptosis of MCF-7 cell lines (Figure 8).

Discussion

Viscum album agglutinin (VAA) is a type of lectin which is one of the major ingredients of the plant. There are three types of lectin have been reported (VAA-I, VAA-II, VAA-III), among these VAA-I shown anticancer properties and they are type-2 ribosome inactivating protein (RIP) as its of D-galactose and N-acetyl-galactosamine specific lectin, VAA-I have a molecular weight of 64 kDa [20]. High amounts of protein concentration were observed in the extract of *V. album* and endophytic fungi, *A. flavus* identified as lectin using a hemagglutination assay at different concentration on human A^{+ve} blood group and it was blood group specific. Blood group specificity was mainly due to the presence of specific carbohydrates surface of erythrocytes. The lectins have able to agglutinate erythrocytes. Similarly, *Erythrina variegate* lectin specially agglutinates the human erythrocytes A, B, AB and O group [21]. Carbohydrate specificity of lectins from *V. album* and *A. flavus* was D galactose and N-acetyl galactosamine specific. Our results are confirmatory with our previous report [22,23]. It has been reported that *V. album* lectin (VAA-I) was D-galactose and N-acetyl-galactosamine specific lectin belongs to a ribosomal inactivating protein [24].

The activity of caspase (cysteine dependent aspartate-specific proteinase) known as important biochemical feature in apoptotic signaling. The mechanism of apoptosis induced by the endophytic fungal lectin in MCF-7 cell lines was studied using colorimetric assay. Caspase is the main effector molecule for apoptosis in the most of cell types in inactive states and plays an important role in apoptosis by cleaving the cellular substrates. Cells undergoing apoptosis were found to have an elevation of cytochrome-c in the cytosol with a corresponding decrease in the mitochondria [25]. After the release of

Source of Lectin	Sugar	Sugar inhibitory concentrations	Titer
Viscum album	D-galactose	25 mM	2^6
Aspergillus flavus	D-galactose and N-acetyl-galactosamine	25 mM	2^6

*Each experiment was repeated thrice

Table 1: Carbohydrate specificity of *V. album* and its endophytic lectin.

Drug Concentration (µg)	Percent Viability		Percent Cell Death	
	24 h	48 h	24 h	48 h
20	21.23 ± 0.1	6.1 ± 0.01	78.67 ± 0.2	93.9 ± 0.01
2	27.43 ± 0.1	9.0 ± 0.02	72.57 ± 0.1	91 ± 0.01
0.2	36.28 ± 0.2	10.24 ± 0.02	63.72 ± 0.1	89.76 ± 0.02
0.02	45.13 ± 0.1	11.88 ± 0.01	54.87 ± 0.3	88.2 ± 0.01
0.002	82.30 ± 0.1	64.34 ± 0.01	17.7 ± 0.2	35.66 ± 0.01
0.0002	87.61 ± 0.2	76.63 ± 0.01	12.39 ± 0.2	23.37 ± 0.01
Vehicle control	98.05 ± 0.2	90.16 ± 0.01	1.95 ± 0.1	9.84 ± 0.01
Doxorubicin	10.12 ± 0.1	08.14 ± 0.1	89.82 ± 0.1	91.86 ± 0.1
IC_{50}	**0.02 µg**		**0.02 µg**	

Table 2: Effect of *A. flavus* lectin on a MCF-7 cells viability and cell death after 24 and 48 h ± mean of triplicates.

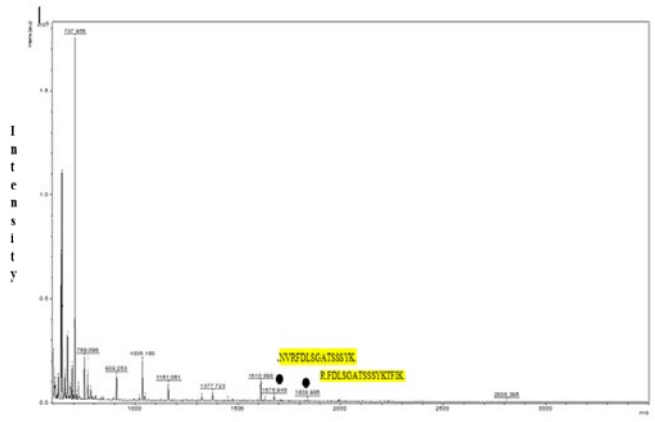

Figure 3: MALDI-TOF mass spectra of the tryptic digest of the SDS-PAGE gel slices of *Aspergillus flavus* lectin. Labeled peaks (black circles (●) correspond to the matched with peptides of ribosome inactivating protein of *Cucurbita pepo*.

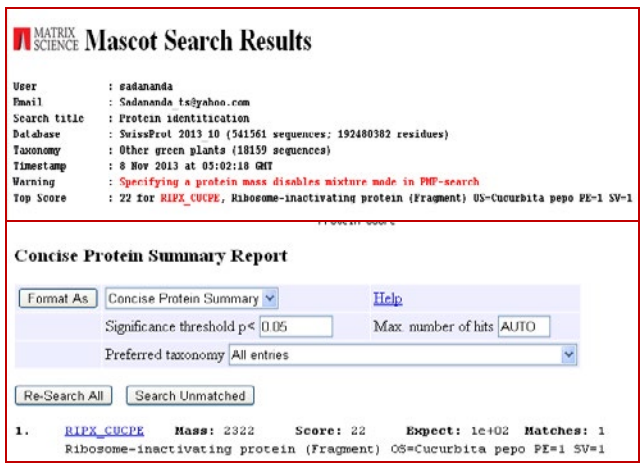

Figure 4: MALDI-TOF-MS data analysis of *A. flavus* lectin in Mascot analysis.

mitochondrial cytochrome c the cysteine protease 32 kDa proenzyme CPP32, a caspase is activated by proteolytic cleavage into an active heterodimer [26]. Activated caspase is responsible for the proteolytic degradation of poly (ADP-ribose) polymerase, which occurs at the onset of apoptosis.

The results of the experiment showed that the involvement of caspase-7. There are two pathways of apoptosis viz., extrinsic and intrinsic. Each needs specific signals to activate the molecular events and each pathway activates its own caspase which in turn activates the executioner caspase-7 is common to both extrinsic and intrinsic death pathways. Results obtained from the study showed that apoptosis pathway involved in MCF-7 cells might be through extrinsic pathway. The results were supported that the caspase-7 inhibitor DEVD-CHO when added it inhibits the action of caspase-7 that results decreased generation of pNA a chromogenic substrate, it confirms the presence of caspase-7 dependent programmed cell death. The results were similar to earlier work on lectin from *Musca domestica* and *Antrodia camphorata* activates the caspase 3, 7 and mitochondrial dependent apoptosis [11]. The acacetin-induced apoptosis in human breast cancer MCF-7 cells is mediates by caspase activation cascades (caspase 7, 8, 9), ROS generation, mitochondria-mediated cell death signaling [27]. The results shows that D-galactose and N-acetyl-galactosamine specific lectin from endophytic fungi (*A. flavus*) was capable of inducing cytotoxicity at a minimum concentration of human breast cancer cell lines (MCF-7) and induces the apoptosis through caspase-7 dependent manner.

Conclusion

The lectin was isolated from endophytic fungi *Aspergillus flavus* from *V.album* was a D-galactose and N-acetyl-galactosamine when interact with A^{+ve} blood and purified the lectin. It shows molecular weight of 64 kDa and taken purplish color in PAS stain it indicates it is a glycoprotein. The MALDI-TOF-MS result confirms that the endophytic fungal lectin is ribosome inactivating protein from its peptide sequence and it is similar to *Cucurbita pepo* ribosome inactivating protein. In low concentration of lectins induces apoptosis by shows membrane blebbing, DNA fragmentation and caspase-7 activity. Further research is needed to identify exact roles of endophytic lectins in apoptosis. The

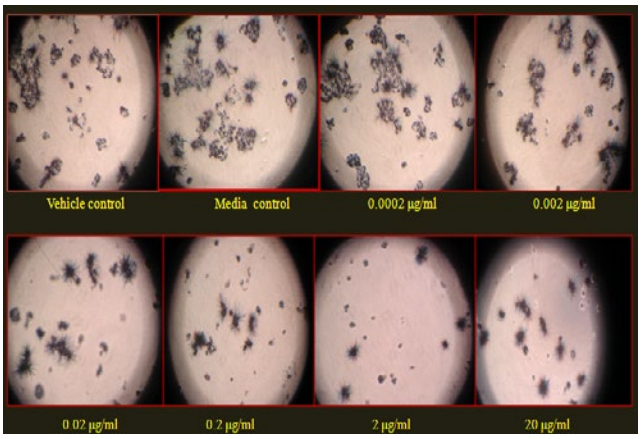

Figure 5: Effect of *A. flavus* lectin extract on MCF-7 cancer cell line at different concentration.

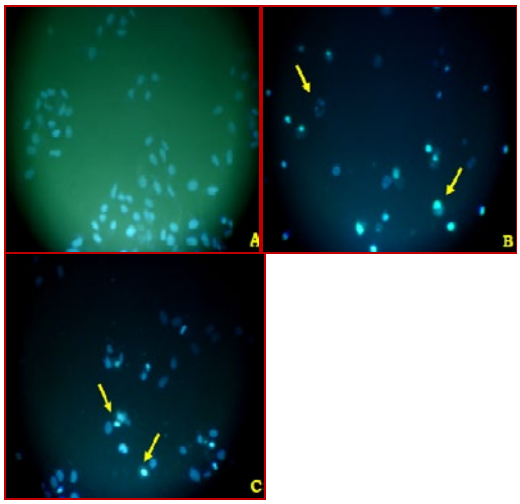

Figure 6: Hoechst 33342 assays for the detection of apoptosis (*A. flavus* lectin sample).
A: Control sample, B: 24 h Treated sample, C: 48 h Treated sample
The arrow indicates the condensed fragmented nuclei and plasma membrane blabbing.

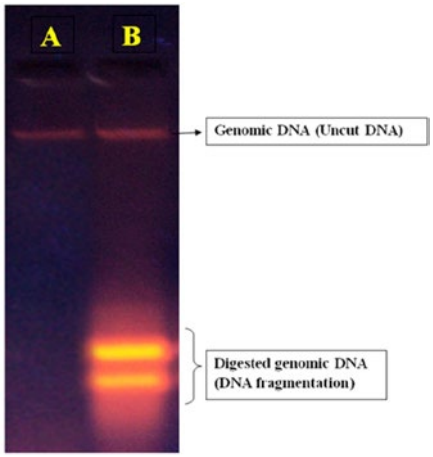

Figure 7: DNA fragmentation assay, A) untreated cancer cells DNA and B) Endophytic lectin treated MCF-7 cancer cells DNA after 24 h of treatment.

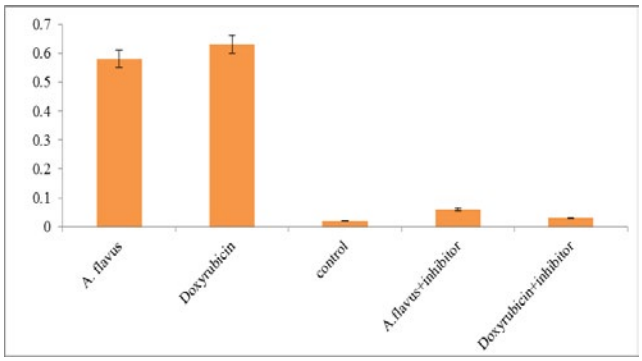

Figure 8: Colorimetric assay of caspase-3 activity of lectin on cancer cell MCF-7. Normal and treated with lectin extract at 0.02 µg/ml (*A. flavus*) at 37°C for 48 h, DEVD-CHO is a Caspase-7 inhibitor. The results shown are the representatives of three independent experiments. The treated samples showed significantly increased in caspase activity compared to those untreated (control) and caspase inhibitor (P<0.05).

endophytic fungi, *Aspergillus flavus* can be used to derive their lectins within short time to produce high amount for various purposes.

Acknowledgement

We thank Dr. MR Hulinaykar, Managing Trustee, Sri Shridevi Charitable Trust (R.), Dr. MN Eshwarappa Principal, SIET, Tumkur, Karnataka, India and Dr. Josthna Rao, Chairman of Sri Raghavendra Biotechnologies, Bangalore, India for encouragement and suggestions during the research.

References

1. Cotter TG, Madden SD (2008) Cell death in brain development and degeneration: control of caspase expression may be key. Mol Neurobiol 37: 1-6.
2. Bacon CW, White JF (2000) Microbial endophytes. Marcel Dekker. New York.
3. Strobel G, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiology and Molecular Biology Reviews 67: 491-502.
4. Gunatilaka AL (2006) Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity and implications of their occurrence. J Nat Prod 9: 509-506.
5. Hakomori S (1996) Tumor malignancy defined by aberrant glycosylation and sphingo (glycol) lipid metabolism. Cancer Research 56: 5309-5318.
6. Batterbury M, Tebbs CA, Rhodes JM, Grierson I (2002) *Agaricus bisporus* (edible mushroom lectin) inhibits ocular fibroblast proliferation and collagen lattice contraction. Exp Eye Res 74: 361-370.
7. Kelsall A, FitzGerald AJ, Howard CV, Evans RC, et al. (2002) Dietary lectins can stimulate pancreatic growth in the rat. Int J Exp Pathol 83: 203-208.
8. Lyu SY, Choi SH, Park WB (2002) Korean mistletoe lectin-induced apoptosis in hepatocarcinoma cells is associated with inhibition of telomerase via mitochondrial controlled pathway independent of p53. Arch Pharm Res 25: 93-101.
9. Huang LH, Yan QJ, Kopparapu NK, Jiang ZQ, Sun Y (2012) *Astragalus membranaceus* lectin (AML) induces caspase-dependent apoptosis in human leukemia cells. Cell Prolif 45: 15-21.
10. Wang HX, Liu WK, Ng TB, Ooi VEC, Chang ST (1996) The immunomodulatory and antitumor activities of lectins from the mushroom *Tricholoma mongolicum*. Immunopharmacol 31: 205-211.
11. Olsnes SF, Stirpe KS, Sandvi K, Pihl SA (1982) Isolation and characterization of viscumin, a toxic lectin from *V. album* L. (mistletoe). J Biol Chem 257: 13263-13270.
12. Kim WH, Park WB, Gao B, Jung MH (2004) Critical role of reactive oxygen species and mitochondrial membrane potential in Korean mistletoe lectin-induced apoptosis in human hepatocarcinoma cells. Mol Pharmacol 66: 1383-1396.
13. Cao X, Huo Z, Lu M, Mao D, Zhao Q, et al. (2010) Purification of lectin from larvae of the fly *Musca domestica* and *in vitro* antitumor activity in MCF-7 cells. J Insect Science 10: 164-169.

14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.

15. Moreira RA, Oliveira JTA (1983) Lectins from the genus Artocarpus. Biologia Plantarum 25:343-348.

16. Luciano SP, Celso SN, Taian MO, Tales RM, et al. (2008) Purification and molecular cloning of a new galactose-specific lectin from *Bauhinia variegata* seeds. J Biosci 33: 355-363.

17. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63.

18. Sandhu LC, Warters RL, Dethlefsen LA (1985) Fluorescence studies of Hoechst 33342 with supercoiled and relaxed plasmid pBR322 DNA. Cytometry 3: 191-194.

19. Strobel G, Daisy B (2003) Bioprospecting for microbial endophytes and their natural product. Microbiol Mol Biol Rev 67: 491-502.

20. Franz H, Ziska P, Kindt A (1981) Isolation and properties of three lectins from mistletoe (*V. album* L.). Biochem J 195: 481-484.

21. Lee AN, Jung HY, Song TJ, An HS, Kim YH, et al. (2012) Korean mistletoe (*V. album coloratum*) extract improves endurance capacity in mice by stimulating mitochondrial activity. J Med Food 15: 621-628.

22. Sadananda TS, Govindappa M, Ramachandra YL (2013) Isolation and characterization of D-galactose, N-acetylgalactosamine, fructose, maltose specific lectin from endophytes of *Viscum album*. Asian J Biomed Pharm Sci 3: 11-20.

23. Sadananda TS, Govindappa M, Dutt GV, Bhat B, et al. (2014) Isolation and characterization of antiviral and ribosome inactivating protein from the endophytic fungi *Alternaria* sp from *Viscum album* using MADLI-TOF-MS and their antibacterial activity. Drug Invent Today 6: 105-112.

24. Fischer J, Hajto T, Hostanska K, Lentzen H (2013) Investigations of cellular parameters to establish the response of a biomodulator: galactoside-specific lectin from *V. album* plant extract. Phytomed 3: 129-137.

25. Gali K, Ramakrishnan G, Kothai R, Jaykar B (2011) *In vitro* anti-cancer activity of methanolic extract of leaves of *Argemone mexicana* Linn. Int J Pharm Tech Res 3: 1329-1333.

26. Yang HL, Chen CS, Chang WH, Lu FJ, Lai YC, et al. (2006) Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by *Antrodia camphorate*. Cancer Lett 231: 215-227.

27. Shim HY, Park JH, Paik HD, Nah SY, Kim DS, et al. (2007) Acacetin-induced apoptosis of human breast cancer MCF-7 cells involves caspase cascade, mitochondria-mediated death signaling and SAPK/JNK1/2-c-Jun activation. Mol Cells 24: 95-104.