Two Chitotriose-Specific Lectins Show Anti-Angiogenesis, Induces Caspase-9-Mediated Apoptosis and Early Arrest of Pancreatic Tumor Cell Cycle

Ruby Singh1☯, Laxman Nawale2☯, Dhiman Sarkar2*, C. G. Suresh1*

1 Biochemical Sciences Division, CSIR-National Chemical Laboratory, Pune, India, 2 Combichem-Bioresource Center, CSIR-National Chemical Laboratory, Pune, India

☯ These authors contributed equally to this work.
* cg.suresh@ncl.res.in (CGS); d.sarkar@ncl.res.in (DS)

Abstract

The antiproliferative activity of two chito-specific agglutinins purified from Benincasa hispida (BhL) and Datura innoxia (DiL9) of different plant family origin was investigated on various cancer cell lines. Both lectins showed chitotriose specificity, by inhibiting lectin hemagglutinating activity. On further studies, it was revealed that these agglutinins caused remarkable concentration-dependent antiproliferative effect on human pancreatic cancerous cells but not on the normal human umbilical vein endothelial cells even at higher doses determined using MTT assay. The GI50 values were approximately 8.4 μg ml⁻¹ (0.247 μM) and 142 μg ml⁻¹ (14.8 μM) for BhL and DiL9, respectively, against PANC-1 cells. The growth inhibitory effect of these lectins on pancreatic cancer cells were shown to be a consequence of lectin cell surface binding and triggering G0/G1 arrest, mitochondrial membrane depolarization, sustained increase of the intracellular calcium release and the apoptotic signal is amplified by activation of caspases executing cell death. Interestingly, these lectins also showed anti-angiogenic activity by disrupting the endothelial tubulogenesis. Therefore, we report for the first time two chito-specific lectins specifically binding to tumor glycans; they can be considered to be a class of molecules with antitumor activity against pancreatic cancer cells mediated through caspase dependent mitochondrial apoptotic pathway.

Introduction

For decades, DNA used to be the main target of anticancer drugs, but due to reoccurrence of resistance the molecular targeted therapy with molecules having specificity and selectivity such as lectins have shown better promise for the cancer treatment. These lectins are proteins that can bind saccharide containing moieties in a reversible manner using direct interaction or through water bridges and facilitated by oligomerization and post translational modifications [1]. Recently, plant lectins have attracted much attention due to their ability to trigger various
biological processes, such as cell agglutination, immunomodulation along with possessing antimicrobial and anti-viral activities. In addition to these properties, some lectins can recognize tumor associated glycans and therefore can differentiate malignant cells from normal cells based on the degree of glycosylation associated with metastasis. Due to lectin’s specificity and selectivity property, they are known to induce cytotoxicity to tumor cells [2]. Extensive research done to characterize new plant lectins has expanded lectin classification from 7 families to 12 families [3]. Among the major lectin families, legume and type 2 ribosome inactivating proteins (RIPs) lectins have received more attention due to their remarkable antineoplastic activity. For instance, a legume lectin like ConA possesses anti-tumor activity by inducing apoptosis and autophagy [4] and mistletoe lectins (MLs), a type 2 RIPs induce apoptosis via mitochondria/ death receptor pathways [5, 6]. MLs act as adjuvant agent reducing the treatment associated side effects in chemotherapy and radiotherapy in pre-clinical trials [7].

Any deregulation in the apoptosis process causes cancer, autoimmune diseases, bone marrow rejection, neuro-degenerative disorders and many such disorders. Apoptosis, a type I programmed cell death (PCD) is an evolutionary conserved process which is characterized by cytoplasmic and nucleic condensation, DNA fragmentation, membrane blebbing and phagocytosis [8]. The two major pathways of apoptosis are distinguished by the involvement or not of caspases, while mitochondria connect the different pathways as cross-talk intermediate. The caspase cascade is triggered by the depolarization of mitochondrial membrane releasing cytochrome c and calcium ions which act as crucial secondary messenger, initiates the formation of apoptosomes leading to the cell death. Therefore, the molecules blocking the tumorigenesis cascade, like plant lectins can become an attractive strategy for modulating the components of cell death machinery via apoptotic pathway [9]. Many lectins have been purified and characterized for its anti-neoplastic activity like Sclerotium rolfsii [10], leczyme [11], Rhizoctonia bataticola [12] and wheat germ agglutinin (WGA) [13]. Tumor angiogenesis also plays critical role in tumor proliferation and spreading through metastasis [14]. Based on different anti-angiogenesis approaches, new anticancer strategy has also emerged and is undergoing extensive study in Phase I–III trials [15].

Among all cancers, pancreatic cancer is the most deadly type. It is ranking fourth among the leading causes of cancer death, behind lung, colorectal, and breast cancer. The five-year survival rate is 5% for all combined patients due to difficulties in its early detection. Surgery, radiotherapy and chemotherapy treatments are available for pancreatic cancer. However, there is no improvement in the survival rate and side effects are by no means inconsequential. Research for developing safer and effective therapies is required. The present study was undertaken to investigate the anticancer properties of two chito-specific lectins, Benincasa hispida lectin purified from ashgourd fruit (BhL) and Datura innoxia lectin purified from datura seeds (DiL9). These agglutinins are structurally unrelated but bind to same sugar (chitotriose) with different affinities. Our recent studies have shown that they have strong killing effect on pancreatic cancerous cells (PANC-1, CFPAC-1 and MIA PaCa-2) in vitro at lower doses. Both the lectins induced apoptosis in these cells via caspase-dependent mitochondrial pathway and also inhibited angiogenic activity of endothelial cells.

Materials and Methods

Purification of lectins

BhL was purified from fruit extract of Benincasa hispida using chitin affinity chromatography and eluted using 0.05 M Glacial acetic acid. DiL9 was purified from Datura innoxia seeds using Q-sepharose ion exchange column, followed by Sephacryl S-200 gel filtration chromatography for achieving final homogenous lectin preparation [16]. The lectin purity was confirmed using...
12% SDS-PAGE and activity by hemagglutination assay using 3% rabbit’s erythrocyte suspension. All cell line studies were conducted using purified lectin preparations only. The lectin solutions were filter sterilized for cell line studies.

Cell lines and culture conditions

The effect of lectins on cell growth was determined in a primary human umbilical vein endothelial cells (HUVECs), a mouse fibroblast cell line (L929; Passage No. 40), and in a panel of human tumor cells including lung adenocarcinoma (A549; Passage No. 37), acute monocytic leukemia cell line (THP-1; Passage No. 16) and pancreatic adenocarcinoma (PANC-1; Passage No. 29), Human pancreatic ductal adenocarcinoma cell line (CFPAC-1; Passage No.25), Human pancreatic epithelial carcinoma cell line (MIA PaCa-2; Passage No.19) and cervix adenocarcinoma (HeLa) obtained from the European Collection of Cell Cultures (ECCC, Salisbury, UK). HUVECs were maintained in M200 Media supplemented with 50X LVES (Gibco, Invitrogen); THP-1 was maintained in RPMI 1640; L929, A-549, PANC-1, CFPAC-1 and MIA PaCa-2 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM). HeLa and macrophages were cultured in Eagle’s Minimum Essential Medium (EMEM). All media used were supplemented with 10% fetal bovine serum (FBS; Gibco) and the cells were maintained at 37°C and 5% CO₂ in a humidified atmosphere.

Cell growth inhibition assay

The in vitro cyto-toxic effects of lectins were determined by using reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) assay to produce formazan crystals [17]. An aliquot of 100 μl of each sub-confluent cell lines (cell density: 1×10⁵ cells ml⁻¹) were seeded in 96-well flat bottom microtitre plate. The plates were incubated at 37°C in an atmosphere of 5% CO₂ and 95% relative humidity within a CO₂ incubator. After 24 h of incubation, the cells were treated with serial dilutions of lectins (BhL and DiL9, 0.05 – 5.0 mg ml⁻¹) and Carboplatin (1 mg ml⁻¹, Sigma Chemicals, USA, positive control). At the end of the incubation, the cells were washed thrice with phosphate buffered saline (PBS) followed by the addition of 10 μl of MTT solution (5 mg ml⁻¹, Sigma Chemicals, USA) in each well. After 4 h of incubation at 37°C, the formazan product was solubilized by the addition of 200 μl of acidified isopropanol and absorbance was measured on a SPECTRAmx PLUS 384 plate reader ( Molecular Devices Inc, USA), at 570 nm. Percentage of cell viability was calculated with respect to untreated cells considered as 100% grown. All experiments were performed in triplicates, and the quantitative measurement was expressed as the average ± standard deviation.

Microscopic observation of cell morphology

Acridine orange (AO) staining. Staining of cells with Acridine orange (AO) was performed to study cell death pattern induced by lectins after 24 h of incubation. Pancreatic cancer cells were selected for further studies based on the MTT assay (Table 1), where both the lectins (BhL and DiL9) showed high inhibitory effect on cell growth at lower doses. Exponentially growing PANC-1 cells were seeded in a 96 well flat bottom plate and treated with lectins, BhL and DiL9 at their GI₅₀ values (50% growth inhibitory concentration), 0.247 and 14.8 μM, respectively for 24 h. The untreated cells were taken as control. The cells were collected by centrifugation and washed with PBS. Apoptotic nuclear morphology was visualized after fixing the cells with 3.7% paraformaldehyde and stained with AO (8.5 μg ml⁻¹, Sigma Chemicals, USA) for 20 min in the dark. The cells were visualized under EVOS FL Cell Imaging System (Life technologies) using filter sets, 470 nm excitation, and 525 nm emission.
Annexin V-FITC Apoptosis Assay. Pancreatic cancer cells were treated with lectins at their GI\textsubscript{50} values (as mentioned before) for 2, 24, 48 and 72 h. After incubation, the cells were harvested and resuspended sequentially in binding buffer (0.01 HEPES, pH 7.4, containing 140 mM NaCl and 25 mM CaCl\textsubscript{2}) containing Annexin V- Fluorescein isothiocyanate (FITC, 3 \mu g ml\textsuperscript{-1}, Sigma Chemicals, USA), 4',6-Diamidino-2-phenylindole (DAPI, 1 \mu M ml\textsuperscript{-1}, Sigma Chemicals, USA) and Propium iodide (PI, 10 \mu gm l\textsuperscript{-1}, Sigma Chemicals, USA) [18]. To determine the proportion of apoptotic and necrotic cells, the number of cells positive for Annexin V-FITC and PI were analyzed using a laser-scanning confocal microscope (LSCM), magnification 20X (Olympus FV1000) and 3D multichannel-image processing was done using Thermo Scientific High Content Screening (HCS) Studio 2.0 Cell Analysis Software.

Cell Cycle Analysis
PANC-1 cells were treated with lectins at their GI\textsubscript{50} values for 6, 12, 18 and 24 h, respectively. Cell cycle and sub G\textsubscript{0}/G\textsubscript{1} distribution of cells were determined by staining with DAPI binding to DNA. The DNA content was measured using LSCM at 386 nm and the data was analyzed using HCS as described before for determining the cells in different phases of cell cycle [19].

Detection of caspase activity
Activation of caspase-8, -9 and -3 in lectin stimulated pancreatic cancer cells was measured by caspase fluorimetric assay kit, according to the instructions given by the kit manufacturers. Apo Alert Caspase Luminescent Assay Kit (Promega, USA) and EnzChek Caspase-3 Assay Kit (Molecular probes, USA) were used for measuring caspase -8/-9 and -3 activity, respectively. Briefly, the cancer cells were treated with lectins and incubated for indicated time periods. The cells were lysed with lysis buffer at 4°C for 10 min, centrifuged and supernatant was collected. Supernatant samples (50 \mu l) were mixed with equal volumes of 2X reaction buffer and specific substrate conjugate (Z-DEVD-AMC) for caspase-3, acetyl-Ile-Glu-Thr-Asp p-nitroaniline

**Table 1. Inhibitory effect of Bh\textsubscript{L} and Di\textsubscript{L9} on different cell lines.**

| Cell name | Bh\textsubscript{L} | Di\textsubscript{L9} | bCarboplatin |
|-----------|------------------|------------------|--------------|
|           | GI\textsubscript{50} | GI\textsubscript{90} | GI\textsubscript{50} | GI\textsubscript{90} | GI\textsubscript{50} | GI\textsubscript{90} |
| HUVEC     | >130             | >130             | >520         | >520         | >10          | >10          |
| L929      | >130             | >130             | >520         | >520         | >10          | >10          |
| THP-1     | >208             | >208             | >1000        | >1000        | 0.1374±0.53  | 5.8140±0.22  |
| A549      | 29.32±0.23       | 200.54±2.08      | 344.04±0.56  | >520         | 0.0035±0.71  | 0.0706±0.60  |
| HeLa      | 34.09±0.15       | 168.45±0.55      | 146.01±0.35  | >520         | 0.0048±0.36  | 0.075±0.56   |
| PANC-1    | 8.39±0.49        | 84.98±0.34       | 141.93±0.65  | 468.16±0.32  | 0.851±0.96   | 5.715±0.19   |
| CFPAC-1   | 11.32±0.45       | 172.81±0.25      | 86.49±0.15   | 913.32±0.35  | 0.968±0.46   | 8.76±0.36    |
| MIA PaCa-2| 13.99±0.4        | 186.13±0.5       | 67.92±0.45   | 943.13±0.5   | 0.376±0.36   | 5.0±0.66     |
| Macrophage| 58.52±1.32       | >208             | >1000        | >1000        | >10          | >10          |

\[a\] Growth Inhibition (GI): GI\textsubscript{50} /GI\textsubscript{90} (concentration which resulted in 50% /90% decrease in cell viability). Expressed in \mu gm ml\textsuperscript{-1}.

\[b\] Standard anticancer drug and positive control.

*Primary cells: 1HUVECs- Human Umbilical Vein Endothelial Cells

*Cell Line from mouse origin: 2L929- areolar and adipose tissue fibroblast Cells

*Human cancer cell lines: 3THP-1 from acute monocytic leukemia, 4A549 from lung adenocarcinoma, 5HeLa from cervix adenocarcinoma, 6PANC-1 from pancreas carcinoma, 7CFPAC-1 from pancreatic ductal adenocarcinoma, 8MIA PaCa-2 from pancreatic epithelial carcinoma

*Macrophage-PMA (phorbol myristate acetate)-differentiated human THP-1 macrophages.

**Article:** Activity of Chitotriose-Specific Lectins against Pancreatic Tumor Cell

**DOI:** 10.1371/journal.pone.0146110.t001

Annexin V-FITC Apoptosis Assay. Pancreatic cancer cells were treated with lectins at their GI\textsubscript{50} values (as mentioned before) for 2, 24, 48 and 72 h. After incubation, the cells were harvested and resuspended sequentially in binding buffer (0.01 HEPES, pH 7.4, containing 140 mM NaCl and 25 mM CaCl\textsubscript{2}) containing Annexin V- Fluorescein isothiocyanate (FITC, 3 \mu g ml\textsuperscript{-1}, Sigma Chemicals, USA), 4',6-Diamidino-2-phenylindole (DAPI, 1\mu M ml\textsuperscript{-1}, Sigma Chemicals, USA) and Propium iodide (PI, 10 \mu gm ml\textsuperscript{-1}, Sigma Chemicals, USA) [18]. To determine the proportion of apoptotic and necrotic cells, the number of cells positive for Annexin V-FITC and PI were analyzed using a laser-scanning confocal microscope (LSCM), magnification 20X (Olympus FV1000) and 3D multichannel-image processing was done using Thermo Scientific High Content Screening (HCS) Studio 2.0 Cell Analysis Software.

Cell Cycle Analysis
PANC-1 cells were treated with lectins at their GI\textsubscript{50} values for 6, 12, 18 and 24 h, respectively. Cell cycle and sub G\textsubscript{0}/G\textsubscript{1} distribution of cells were determined by staining with DAPI binding to DNA. The DNA content was measured using LSCM at 386 nm and the data was analyzed using HCS as described before for determining the cells in different phases of cell cycle [19].

Detection of caspase activity
Activation of caspase-8, -9 and -3 in lectin stimulated pancreatic cancer cells was measured by caspase fluorimetric assay kit, according to the instructions given by the kit manufacturers. Apo Alert Caspase Luminescent Assay Kit (Promega, USA) and EnzChek Caspase-3 Assay Kit (Molecular probes, USA) were used for measuring caspase -8/-9 and -3 activity, respectively. Briefly, the cancer cells were treated with lectins and incubated for indicated time periods. The cells were lysed with lysis buffer at 4°C for 10 min, centrifuged and supernatant was collected. Supernatant samples (50 \mu l) were mixed with equal volumes of 2X reaction buffer and specific substrate conjugate (Z-DEVD-AMC) for caspase-3, acetyl-Ile-Glu-Thr-Asp p-nitroaniline
(Ac-IETD-pNA) for caspase-8 and acetyl-Leu-Glu-His-Asp-p-nitroaniline (Ac-LEHD-p-NA) for caspase-9 provided by the kit manufacturers. 50 μl of the cell lysis buffer was taken as no-enzyme control to determine the background fluorescence of the substrate. After additions of substrates, the plates were incubated at 37°C for 1 h during which, the caspases cleave the substrates to release p-nitroaniline (p-NA). The role of caspase activation during apoptosis process was also studied by using caspase inhibitors. The fold increase in the activity of caspases was calculated by measuring the fluorescence intensities of the resulting product using a plate reader (VarioskanFlash, using SkanIt Software 2.4.5 RE, Thermo Scientific.) with 496/520-nm filters for caspase-3, 400/500-nm filters for caspase-8 and 380/460-nm filters for caspase-9.

Measurement of cytoplasmic calcium release concentration \([\text{Ca}^{2+}]_i\)

A fluorescent Ca\(^{2+}\) binding indicator, Fluo-4 acetoxymethyl ester (Fluo-4/AM, 4 μmol l\(^{-1}\), Invitrogen) was used to measure cytoplasmic \([\text{Ca}^{2+}]\) change by LSCM. In brief, the PANC-1 cells were treated with lectins at their GI\(_{50}\) values and incubated for 4, 8 and 12 h, respectively. After incubation, the plate was centrifuged at 200x g for 5 min and incubated with Fluo-4/AM for 30 min at 37°C. The cells were washed with cold PBS and fixed with 3.7% paraformaldehyde at 37°C for 15 min. The fixed cells were stained with DAPI (1 μM ml\(^{-1}\)) and changes in level of calcium released were quantified by measuring the fluorescence intensity at a detection spectrum of 488 nm.

Determination of Mitochondrial membrane potential (Δψ\(_m\))

After incubating the PANC-1, CFPAC-1 and MIA PaCa-2 cells treated with lectins for 4, 8 and 12 h, the cells were centrifuged and resuspended in 100 μl of DMEM loaded with Mito Tracker Red (0.1 μmol l\(^{-1}\), Invitrogen) incubated for 15 min at 37°C. Later, the cells were washed twice with PBS, fixed with 3.7% paraformaldehyde and the nuclei were stained with DAPI (1 μM ml\(^{-1}\)). The images were acquired using a LSCM and analysed through HCS as already described. Alterations in the Δψ\(_m\) of lectin treated cells was quantified by the uptake of fluorochrome MitoTracker Red dye, a cationic cell permeant fluorochrome that passively diffuses through plasma membrane of viable cells and readily get sequestered by mitochondria with active Δψ\(_m\) and have no cytotoxic effects [20]. A decrease in red fluorescence intensity was considered as an indication of mitochondrial membrane dysfunction.

Anti-angiogenesis activity of lectins

To evaluate the anti-angiogenic activity of lectins, the three-dimensional tubular vessel formation by HUVECs, a widely used human endothelial cell line was used for the in vitro assay. 96-well culture plates were coated with Matrigel which was then allowed to solidify at 37°C for 1 h. HUVECs were washed, suspended in appropriate media, and added to Matrigel-coated wells (2.5 x 10\(^4\) cells per well), treated with the known pro-angiogenic compound, Vascular Endothelial Growth Factor (VEGF, Angiogenesis Starter Kit, Life technologies) and incubated to promote angiogenic tube formation. Cells were subsequently treated with lectins (Blh: 0.247 and DiL9: 14.8 μM) and incubated for 12 h at 37°C in a 5% CO\(_2\) humidified atmosphere. Suramin (5 μg ml\(^{-1}\)/4.0 μM, Sigma Chemicals, USA), a known anti-angiogenic agent was used as positive control. After 24 h of lectin treatment, the tubes were stained with rhodamine conjugated phalloidin which labels F-actin enabling the entire tube to be identified and the nuclei was stained with DAPI, enabling an estimate of the number of nuclei per tube. Automated imaging was done on Thermo Scientific Cellomics ArrayScan HCS Reader.
Results

Cytotoxic effect of lectins on primary and human cancer cells

In this study, we investigated the anticancer activity of two lectins, BhL (34 kDa, homodimer) and DiL9 (9 kDa, monomer), purified from Cucurbitaceae and Solanaceae family, respectively. The lectin purification profile is shown in the S1 Fig. Both these lectins have chitotriose (N-acetylglucosamine oligomer) specificity, thermostable (upto 80°C) and stable at extreme pH range but widely vary in their structure as evidenced from biophysical studies. However, these lectins exhibit different affinity ($K_a$, association constant) for the same chito sugar, with BhL being more effective than DiL9.

The antiproliferative effect of BhL and DiL9 on human primary cells and four cancer cell lines were evaluated by MTT assay. Fig 1A and 1B represents the percentage cytotoxicity shown by various cell lines on lectin treatment in a concentration-dependent manner after 48 h of exposure. However, the effect on cell viability was more pronounced in cultures treated with BhL as per the MTT assay. Lectins showed no significant cytotoxicity towards HUVECs and L929 cells (<30% growth inhibition) even at higher concentration, demonstrating selectivity for tumor cells. After 48 h, GI50 of BhL on the viability of PMA (phorbol myristate acetate)-differentiated human THP-1 macrophages was 58.52 μg ml⁻¹ (1.7 μM) whereas DiL9 had nil effect. This might be due to differences in affinity of these lectins towards the cell surface glycans. 50% of cell growth cessation was achieved in the case of A549, HeLa and PANC-1 cells, but the lowest values of GI50 (BhL: 8.4 μg ml⁻¹/0.247 μM; DiL9: 142 μg ml⁻¹/14.8 μM) and GI90 (BhL: 85 μg ml⁻¹/2.5 mM; DiL9: 468 μg ml⁻¹/48.75 μM) were observed in the case of PANC-1 cells by both the lectins as shown in Table 1. The discrepancy in lectin cytotoxicity may be caused by the divergence of glycoprotein expression on different cell lines. Carboptatin (GI50: 0.85 μg ml⁻¹/2.28 μM) exhibited potent cytotoxicity against all cell lines, as expected. To confirm lectin’s antiproliferative activity against human pancreatic cancer cell line, two different cancer cells were also tested, namely CFPAC-1 and MIA PaCa-2 of the same origin (S2 Fig). On comparing the GI50 and GI90 values among these three pancreatic cancer cells (Table 1), we found that both the lectins had comparatively higher inhibitory effect on PANC-1 cells.
The effect of these lectins on cancer cells was also estimated in the presence of 100% serum, to simulate the in vivo environments. For this, the lectins were pre-incubated with serum for 24 h and anti-proliferative activity was checked with MTT assay as described previously. 20% of growth inhibition was observed at higher concentration 1mg ml\(^{-1}\) (30 \(\mu\)M) of BhL, whereas the effect of DiL9 was negligible (S3 Fig). This could be due to the binding of glycoproteins to the glycan binding regions of the lectins, thus inhibiting its activity [16].

Lectin induced apoptosis in PANC-1 cells

To understand the mechanism involved in the cytotoxicity of chito-specific lectins, we first investigated the cell cycle arrest and the lectin-mediated morphological changes in PANC-1 cells. The distribution of cells in different stages of cell cycle was evaluated by exposing the PANC-1 cells to lectins (BhL: 0.247 and DiL9: 14.8 \(\mu\)M) for 24 h followed by DAPI staining and HCS analysis. The untreated cells exhibited all the three phases \(G_0/G_1\), \(S\) and \(G_2/M\) of the cell cycle. Carboplatin treatment showed \(S\) phase arrest whereas lectin treated cells showed \(G_0/G_1\) arrest with total of 87% populations till 24 h. The effect of both the lectins on PANC-1 cells appeared similar and time-independent, arresting the cells at \(G_0/G_1\) phase, with a parallel decrease of the cell population in the \(S\) and \(G_2/M\) phase to 8 and 2%, respectively (Fig 2).

Observing changes in the cell morphology is the standard method for recognizing the apoptosis process. Lectin treated cells showed complete disintegration of nuclei and they transformed into small condensed apoptotic bodies observed using AO staining (Fig 3A). The apoptotic potential of lectin was quantified using Annexin V-FITC/PI staining, and also differentiates between viable, apoptotic and necrotic cells. There has been a time-dependent increase in Annexin V-FITC positive cells from 12% at 24 h to approximately 20% at 48 h and 50% at 72 h for both the lectins, indicating that these two lectins induce apoptosis in PANC-1 cells. The graph (Fig 3B) depicts the percentage of apoptotic cells (Annexin V-FITC positive) after BhL and DiL9 exposure over a period of time. Absence of PI-negative cells even after 72 h of incubation following lectin treatment suggests that major cause of chito-specific lectin induced PANC-1 cell death is apoptosis activation and not necrocytosis (Fig 3C). Similar results were obtained also in the case of CFPAC-1 and MIA PaCa-2 cell lines on incubating with lectins for 72 h (S4 and S5 Figs).

Activation of intrinsic apoptotic pathway

To analyze the details of lectin-induced apoptosis in PANC-1, CFPAC-1 and MIA PaCa-2 cells, the effect of BhL and DiL9 on activation of initiator caspases-8 and -9 involved in apoptotic pathway was evaluated. The time course profiles showed a drastic difference in the caspase-8 and -9 activities. Caspase-8 activity was not detected even after 36 h of lectin treatment (Fig 4A) whereas caspase-9 activity increased significantly over a period of time when compared with untreated cells (Fig 4B). To confirm this, we analyzed the caspase-8 and -9 activation patterns under the presence of specific caspase inhibitors. Caspase-8/9 inhibitor was added to the cell cultures according to the manufacturer’s instructions to completely inhibit the enzyme activity. In the presence of caspase-9 inhibitor, induction of apoptosis was prevented. On the other hand, pretreatment with caspase-8 inhibitor could not abolish cell death. The results clearly established that only caspase-9 was activated by both the lectins. A time-dependent increase in caspase-3 proteolytic activity was also observed by BhL and DiL9 treatment with 5-fold and 3-fold increase, respectively, after 48 h of incubation when compared with untreated cells (Fig 4C). Pretreatment of caspase-3 inhibitor, Ac-DEVD-CHO also prevented lectin-induced cell apoptosis. Caspase-9 activity was detected after 3 h followed that by caspase-3 activity at 12 h, implying activation of caspase-9 prior to caspase-3. This activity profile was
found similar to carboplatin response [21, 22]. The aforementioned findings indicate that both
the lectins, BhL and DiL9 induced caspase-dependent apoptosis in a similar manner where cas-
pase-9 is strongly activated. These observations on the effect of lectins on PANC-1 were found
to be similar on CFPAC-1 and MIA PaCa-2 cell lines also (Fig 4).

Perturbation of mitochondrial membrane potential (Δψₘ)

In the intrinsic pathway of apoptosis, caspase-9 is an initiator enzyme which gets activated
by upstream pro-apoptotic signal molecules like cytochrome c and Ca²⁺, released to trigger
the cascade of cell death. Increase in the cytoplasmic level of calcium ions is known to sec-
ondarily alter mitochondrial homeostasis. The effect of lectins in modulating apoptosis by
elevating the [Ca²⁺]ᵢ in PANC-1 cells was studied using calcium sensitive dye (Fluo-4 AM).
The number of cells releasing calcium increased significantly in a time-dependent manner,
measured as 93.6% (BhL), 96.2% (DiL9) and 95.8% (Carboplatin), while the control showed

![Diagram of cell cycle changes](image.png)

**Fig 2. Effect of BhL and DiL9 on different phases of cell cycle.** The progressive cell cycle changes were observed with DAPI staining after 6, 12, 18 and 24 h on lectin (BhL: 0.247 and DiL9: 14.8 μM) treatment and analyzed using HCS software. The darkened numbers indicate the percentage of cells arrested in different phases of cell cycle. The data is mean ± SD of three independent experiments.

doi:10.1371/journal.pone.0146110.g002
only 0.9% even after incubating for 12 h (Fig 5A and 5B). Slow accumulation of $[\text{Ca}^{2+}]_i$ in mitochondria leads to its overloading and forms a transition pore, disrupting the $\Delta \psi_m$. To evaluate the integrity of mitochondrial membranes, the lectin-treated PANC-1 cells were stained with Mitotracker dye. Treatment with the lectins resulted in the decrease of fluorescent intensity of Mitotracker dye staining indicative of loss of $\Delta \psi_m$ in a time-dependent manner from 8 h post-lectin-treatment with further decline occurring at 12 h (Fig 6A and 6B). Hence, these results indicate that BhL and DiL9 induced cytotoxicity is through caspase-9 dependent apoptosis in which mitochondrial perturbation occurs as upstream events. The observations were similar in the case of other pancreatic cell lines CFPAC-1 and MIA PaCa-2, thus showing mitochondrial dysfunction on treatment with quantity equivalent to GI$_{50}$ values of lectins (S6 Fig).

**Lectins showing anti-angiogenesis activity**

To study the response of adding lectins BhL and DiL9 on anti-angiogenesis activity, the HUVECs were treated with lectins. When plated with matrigel, these endothelial cells underwent rapid reorganization, ceased proliferation and formed capillary like tubular structures in

**Fig 3. BhL and DiL9-induced apoptosis in PANC-1 cells.** (A) Acridine orange staining of PANC-1 cells after incubation with lectins (BhL: 0.247 and DiL9: 14.8 $\mu$M) for 24 h. The arrows indicate apoptotic bodies formed inside the lectin treated cells. Annexin V-FITC/PI staining of lectin stimulated PANC-1 cells incubated for indicated time. (B) The graph represents percentage of cells undergoing apoptosis, mean value ± SD of three independent experiments. (C) The overlay represents the cells that have undergone apoptosis (Annexin V-FITC positive, green) or necrosis (PI positive cells, red) after 72 h of incubation. The analysis was carried out using HCS 2.0 Cell Analysis Software.

doi:10.1371/journal.pone.0146110.g003
the presence of large vessel endothelial supplement (LVES) and VEGF and in its absence the endothelial cells tend to proliferate. Initially, we have shown that both the lectins don’t inhibit the growth of HUVECs as evaluated by MTT assay but surprisingly they could inhibit endothelial tubulogenesis in vitro (Fig 7). Both the lectins interrupted in the tube formation in a similar manner, like suramin inhibiting angiogenesis.

**Discussions**

Many anticancer agents have been derived from natural sources, including plants, animals, microbes and marine organisms [23]. Due to the property of selectivity and specificity, lectins...
have gained more attention from researchers in identification of cancer and degree of metastasis [2]. In this study, we have investigated the anticancer activity of two lectins, BhL and DiL9, purified from different families of plant sources. Both these lectins showed chito-oligosaccharide specificity, specifically distinct affinity for chitotriose, but widely vary in their structures. As a result they showed similar response against human neoplastic cell lines but differ in terms of dose-response to activity. Even though many lectins have been purified and reported to have wide range of applications, there are only few studies that refer to their anti-cancer potential or reporting on their underlying mechanism of cytotoxicity. Our study showed that the lectins BhL and DiL9 displayed cytotoxicity against all human cancer cell lines, with minimum GI50 values for pancreatic cancer cells (PANC-1). BhL is found to be more active compared to DiL9. Both the lectins showed hemagglutination inhibition activity when incubated with mucin, where mucin-like glycans are commonly over-expressed on 90% of tumors [24]. This clearly shows that the binding of the lectins to the glycoproteins/receptor present on tumor cells appear to be the necessary step for action. Considering example of mucin glycoproteins (MUC1), it has been reported that each pancreatic cancer cell line expresses a unique pattern of MUC1 glycoforms [25]. So binding of these complex lectins to PANC-1, CFPAC-1 and MIA PaCa-2 selectively could provide more accurate analyses of glycans present on these tumor cells and could provide more insight for the treatment of pancreatic cancer effectively. For normal cell lines like HUVECs and L929, BhL and DiL9 were not cytotoxic even at higher concentrations (> 200 μg ml⁻¹), implying its exclusive specificity for cancerous cells.

To develop lectin-based anti-cancer treatment it is important to understand the underlying mechanism involved during human tumor cell apoptosis induced by lectins [4]. The proposed

Fig 5. Increase in intracellular [Ca²⁺]i release of lectin stimulated PANC-1 cells. PANC-1 cells were treated with lectins (BhL: 0.247 and DiL9: 14.8 μM) for 4, 8 and 12 h, stained with Fluo-4/AM (4μM, green) and DAPI (blue). (A) Depicts the percentage of cells releasing calcium over a period of time. (B) Represents the overlay of confocal microscopy images of fluorescence intensity of cells bound with Fluo-4/AM (green) releasing calcium after 12 h of incubation. The analysis was carried out using LSCM, Magnification 20X (scale, 100 μm).

doi:10.1371/journal.pone.0146110.g005
underlying mechanism of these lectin-induced (BhL and Dil9) pancreatic cell death is depicted in Fig 8. In order to understand the mechanism of lectin’s antiproliferative effect on tumor cells, morphological changes, activation of caspases, release of calcium ions and mitochondrial membrane depolarization studies were conducted. As mentioned before, PCD, a process that eliminates improperly developed cells, has become the plausible strategy for cancer therapy. The agents targeting apoptotic pathway, specifically in tumor cells, have potential to be useful for antitumor therapy [26]. Shown in this investigation is that BhL and Dil9 induce apoptosis strongly against PANC-1 cells than in normal cells, by which they would fit into the category of such anticancer agents.

The morphological analysis by Annexin V-FITC/PI staining demonstrated that the lectin treated cells predominantly undergo apoptosis and not necrosis. When a cell undergoes apoptosis, phosphatidylserine (PS) which usually present on the inner side of the membrane gets exposed on the surface, acting as a key marker for apoptotic bodies to get recognized by phagocytic cells [27]. It is also widely known that the antitumor or DNA damaging agents induce cell death by arresting the cell cycle. In normal cells, the cell cycle checkpoints ensure damage repair, whereas in malignant cells the apoptosis eliminates them. Our study demonstrated that PANC-1 cells treated with BhL and Dil9 showed cell death by arresting the cells at G0/G1 phase of the cell cycle.

Among the caspases that belong to cysteine proteases family, caspase-3 plays an important role in causing apoptosis after its activation by stimulation from cell death receptors (extrinsic) or mitochondria (intrinsic) [28, 29]. For caspases to get activated, the upstream proapoptotic signal molecules like cytochrome c and Ca2+ should be released to trigger the cascade of cell death. Thus, mitochondrial membrane potential and release of calcium ions was also evaluated. After 12 h incubation of lectin treated PANC-1 cells, a significant increase in the number of
Fig 7. Inhibition of angiogenesis by BhL and DiL9. HUVECs were plated on Matrigel (±LVES+VEGF) precoated 96-well plates as control. The cells were treated with lectins. The tubes were stained with rhodamine conjugated phalloidin (red) and nuclei with DAPI (blue). Anti-angiogenic activity is determined by the breakage in the tubule formation. Cell imaging was done on Cellomics’ ArrayScan HCS Reader.

doi:10.1371/journal.pone.0146110.g007
cells releasing Ca\(^{2+}\) was observed with severely disrupting mitochondrial membrane potential resulting in the release of cytochrome c which formed apoptosome complex and activated caspase-9. This further led to the activation of caspase-3 leading to final cell death. Hence, these results indicate that cytotoxicity of BhL and DiL9 is induced through caspase-9 dependent apoptosis in which mitochondrial perturbation occurs as upstream events. Unlike previously reported lectins such as ConA and WGA [30–32], BhL, among the two lectins studied here, is more potent because of its ability to induce cell death at very low concentrations and activating early intrinsic apoptotic cascade.

Another important factor for the spread of tumor is neovascularization. Tumor-induced angiogenesis is an integral part of maintaining tumor growth and progression by providing the necessary blood supply and also allowing metastatic cells to go into circulation [33]. Angiogenesis inhibitors can block any of the steps in the angiogenic cascade, including endothelial cells proliferation and attachment to the extracellular matrix proteins, migration and invasion through the matrix to form a thin tube meshwork [34]. Thus, angiogenesis also becomes an interesting target for developing anti tumor therapies. Currently, in many laboratories natural and synthetic inhibitors of angiogenesis are studied extensively [35, 36]. For instance, many inhibitors are peptides derived from snake venom like disintegrins [37] and lebectins [38]. So, to evaluate the anti-angiogenic activity of BhL and DiL9, HUVEC tube formation assay was used. It is a well established and simple in vitro angiogenesis assay based on the ability of endothelial cells to form three-dimensional capillary-like tubular structures that form on matrigel composed of growth factor-reduced basement membrane extracts. Here, both the lectins efficiently inhibited the tubulogenesis process without affecting the viability of confluent HUVECs, also confirmed by MTT assay. So far as we know, there are no reports of chito-specific lectin possessing anti-angiogenic activity at such a low lectin concentration. Viscum...
album extracts inhibits angiogenesis by inducing apoptosis in endothelial cells [39] and ConA targets anti-angiogenesis pathway at 25 μg ml⁻¹ [40, 41] whereas BhL at 8 μg ml⁻¹ (247 μM) shows much more effective response. This effect of lectins might be due to blocking of the co-receptor binding site of growth factors on endothelial cells, inhibiting its adherence to the matrigel, and thus disrupting the tube formation by endothelial cells for angiogenesis. Therapies for brain tumors are the most risky, where these lectins as anti-angiogenic agents can be the hope for such treatments. According to our present results, we suggest that BhL and Dil9 lectins could be used with high efficiency for the inhibition of the brain angiogenic process without any side effects and warrants further investigations like in vivo studies.

Previously, using mistletoe lectins many researchers have conducted in vivo experiments on different animal models and had reported reduction in tumor size and growth when injected intratumorally [42]. Mostly, these in vivo investigations on the ability of lectins to inhibit cancer cell proliferation in animal models have given inconsistent results due to factors such as heterogeneity of tested animal models, difference in route of administration and sample size. During further investigations, when carrying out clinical trials, many limitations have been reported such as registration of small number of patients and lack of proper control group. Taking into consideration the applications of our reported lectins being non-toxic to normal cells, oral or intratumoral administration might be a promising alternative therapy for pancreatic cancer patients. Further qualitative in vivo and clinical trials evaluating the effect of lectins on pancreatic cancer patients must be carried out addressing safety parameters, standard dosage, and appropriate endpoint measures. These studies should take into account the sample size of patients, dosage limit, time of administration and methodological design to prevent failures encountered during the trial of previously tried lectins.

Conclusions

In summary, we report for the first time that two chito-oligosaccharide specific lectins, BhL and Dil9 belonging to different family origin, possess remarkable antiproliferative activity. Among these two lectins, BhL gave much more effective cytotoxicity response than Dil9. Both lectins induced apoptosis in human pancreatic cancer cells in a similar manner. The underlying apoptotic mechanism was through the intrinsic pathway causing depolarization of mitochondrial membrane potential and increase in intracellular calcium leading to activation of executioner caspases (caspases-9 and -3). Interestingly, both the lectins also inhibited endothelial tubulogenesis. Our results would open exploration of plant dietary lectins combining them with slow-release and point-insert biomaterials as potential novel candidates for pharmaceutical exploitation. Hence, the successful therapy will be based on the selective elimination of the abnormal cells without disturbing the function of the normal cells.

Supporting Information

S1 Fig. Purity check of lectins on 12% SDS-PAGE. (A) BhL. Lane 1: chitin affinity column fraction; 2- loaded on Sephacryl S-200 column; 3-Protein molecular marker; 4-6- Pure BhL. (B) Lane 2: crude extract (homogenate); 3–60% saturation ammonium sulfate-precipitated fraction; 4- dialysed ammonium sulfate fraction; 5-unbound fraction from Q-sepharose column; 6-Biorad broad range protein molecular weight marker; 7- Pure Dil9 after Sephacryl S-200 column. (TIF)

S2 Fig. Anti-proliferative activity of BhL and Dil9 on CFPAC-1 and MIA PaCa-2 cells: The cancer cell lines were treated with serial dilutions of lectins and incubated for 48 h. The
growth inhibition (%) was measured by MTT assay by considering untreated cells as 100%. (A) Effect of BhL and (B) DiL9 treatment on above mentioned cell lines. The values presented in the graph are the mean ± SD of two independent experiments done in triplicates.

(TIF)

S3 Fig. Effect of serum incubated lectin on cancer cells: Both the lectins (BhL and DiL9) were incubated with 100% serum for 24 h. MTT assay was carried out to determine the effect of serum incubated lectin on pancreatic cancer cell lines (PANC-1, CFPAC-1 and MIA PaCa-2). The values depicted in the graph are the mean ± SD of three independent experiments done.

(TIF)

S4 Fig. Apoptotic effects of BhL and DiL9 on CFPAC-1 cells. The human pancreatic CFPAC-1 cells were incubated with or without lectins (BhL and DiL9, GI50 conc) for 72 h. The cells were stained with DAPI, Annexin V-FITC and PI. The overlay represents the cells that have undergone apoptosis (Annexin V-FITC positive, green) or necrosis (PI positive cells, red). The analysis was carried out using HCS 2.0 Cell Analysis Software.

(TIF)

S5 Fig. Apoptotic effects of BhL and DiL9 on MIA PaCa-2 cells. The human pancreatic MIA PaCa-2 cells were incubated with or without lectins (BhL and DiL9, GI50 conc) for 72 h. The cells were stained with DAPI, Annexin V-FITC and PI. The overlay represents the cells that have undergone apoptosis (Annexin V-FITC positive, green) or necrosis (PI positive cells, red). The analysis was carried out using HCS 2.0 Cell Analysis Software.

(TIF)

S6 Fig. Effect of lectins on MMP of CFPAC-1 and MIA PaCa-2 cells: Lectin treated cells were incubated for 12 h. The mitochondria were red with Mito Tracker Red (0.1 μmol l⁻¹) and nuclei were stained with DAPI (1 μmol l⁻¹) for 15 min at 37°C. Decrease in red intensity indicates loss in MMP. The images have been recorded by LSCM, Magnification 20X objective (scale, 100 μm).

(TIF)

Acknowledgments
RS received Senior Research Fellowship of CSIR, New Delhi, India. RS is a registered PhD student of AcSIR and DS & CGS are faculty of AcSIR.

Author Contributions
Conceived and designed the experiments: RS LN DS CGS. Performed the experiments: RS LN. Analyzed the data: RS LN DS CGS. Contributed reagents/materials/analysis tools: DS. Wrote the paper: RS LN DS CGS.

References
1. Vijayan M, Chandra N (1999) Lectins. Curr Opin Struct Biol; Dec; 9(6):707–14. PMID: 10607664
2. De Mejia EG, Prisecaru VI (2005). Lectins as bioactive plant proteins: a potential in cancer treatment. Crit Rev Food Sci. 45:42–445.
3. Van Damme EJM NL, Peumans WJ (2008) Plant Lectins. In:Kader JC, Delseny M (eds) Advances in botanical research. 48( Elsevier Ltd, San Diego):107–209.
4. Liu B, Bian HJ, Bao JK (2010) Plant lectins: potential antineoplastic drugs from bench to clinic. Cancer Lett. Jan 1; 287(1):1–12. doi: 10.1016/j.canlet.2009.06.013 PMID: 19487079
5. Kang TB, Song SK, Yoon TJ, YooYC, Lee KH, Her E, et al. (2007) Isolation and characterization of two Korean mistletoe lectins. J Biochem Mol Biol. Nov 30; 40(6):959–65. PMID: 18047792
6. 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. Feb; 25(1):93–101. PMID: 11885700
7. Valentinier U, Fabian S, Schumacher U, Leatham AJ (2003) The influence of dietary lectins on the cell proliferation of human breast cancer cells in vitro. Anticancer Res. Mar-Apr; 23(2B):1197–206. PMID: 12820371
8. Hengartner MO (2000) The biochemistry of apoptosis. Nature. Oct 12; 407(6805):770–6. PMID: 11048727
9. Pusztai A, Bardocz S, Ewen SW (2008) Uses of plant lectins in bioscience and biomedicine. Front Biosci. 13:1130–40. PMID: 17981618
10. Savanur MA, Eligar SM, Pujari R, Chen C, Mahajan P, Borges A, et al. (2014) Sclerotium rolfsii lectin induces stronger inhibition of proliferation in human breast cancer cells than normal human mammary epithelial cells by induction of cell apoptosis. PLoS One. 9(11):e110107. doi: 10.1371/journal.pone.0110107 PMID: 25364905
11. Tatsuta T, Hosono M, Sugawara S, Kariya Y, Ogawa Y, Hakomori S, et al. Sialic acid-binding lectin (lec-
12. Pujari R, Eligar SM, Kumar N, Barkeer S, Reddy V, Swamy BM, et al. (2013) Rhizoctonia bataticola lec-
13. Aub JC, Sanford BH, Wang LH (1965) Reactions of normal and leukemic cell surfaces to a wheat germ agglutinin. Proc Natl Acad Sci U S A. Aug; 54(2):400–2. PMID: 5217427
14. Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med. Jan; 1 (1):27–31. PMID: 7584949
15. Folkman J, Browder T, Palmblad J (2001) Angiogenesis research: guidelines for translation to clinical application. Thromb Haemost. Jul; 86(1):23–33. PMID: 11487011
16. Singh R, Suresh CG (2015) Purification and Characterization of a Small Chito-specific Lectin from Datura innoxia Seeds Possessing Anti-microbial Properties. International Journal of Biochemistry Research & Review. 9(2):1–17.
17. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. Dec 16; 65(1–2):55–63. PMID: 6606682
18. Rieger AM, Nelson Kl Fau — Konowalchuk JD, Konowalchuk Jd Fau — Barreda DR, Barreda DR (2011) Modified annexin V/propiodium iodide apoptosis assay for accurate assessment of cell death. LID - doi: 10.3791/1902. PMID: 19590723
19. Roukos V, Pegasoro G, Voss TC, Misteli T (2015) Cell cycle staging of individual cells by fluorescence microscopy. Nat Protoc. Feb; 10(2):334–48. doi: 10.1038/nprot.2015.016 PMID: 25633629
20. Haugland RP (1996) Molecular Probes: Handbook of Fluorescent Probes and Research Chemicals, 6th Ed., Molecular Probes, Inc., Eugene, OR.: 286–7.
21. Han S-J, Ahn T-K, Choi H-S, Shin J-N, Pya S, Kim T-H (2009) TRAIL-induced cell death and caspase-8 activation are inhibited by cisplatin but not carboplatin. Journal of Gynecologic Oncology. 20(2):113–6. doi: 10.3802/jgo.2009.20.2.113 PMID: 19590723
22. Lin H, Sue Y-M, Chou Y, Cheng C-F, Chang C-C, Li H-F, et al. (2010) Activation of a nuclear factor of activated T-lymphocyte-3 (NFAT3) by oxidative stress in carboplatin-mediated renal apoptosis. British Journal of Pharmacology. 161(7):1661–76. doi: 10.1111/j.1476-5381.2010.00989.x PMID: 20718735
23. Cagg GM, Newman DJ (2005) Plants as a source of anti-cancer agents. J Ethnopharmacol. Aug 22; 100(1–2):72–9. PMID: 1609521
24. Yu LG (2007) The oncofetal Thomsen-Friedenreich carbohydrate antigen in cancer progression. Glycoconj J. Nov; 24(8):411–20. PMID: 17457671
25. McCarter C, Kletter D, Tang H, Partyka K, Ma Y, Singh S, et al. (2015) Prediction of Glycan Motifs Using Quantitative Analysis of Multi-lectin Binding: Motifs on MUC1 Produced by Cultured Pancreatic Cancer Cells. Proteomics Clinical applications. 7(0):632–41.
26. Reed JC (2003) Apoptosis-targeted therapies for cancer. Cancer Cell. Jan; 3(1):17–22.
27. Shiratsuchi A, Osada S, Kanazawa S, Nakayoshi Y (1998) Essential role of phosphatidyserine externalization in apoptosing cell phagocytosis by macrophages. Biochem Biophys Res Commun. May 19; 246(2):549–55. PMID: 9610400
28. Riedl SJ, Shi Y (2004) Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol. Nov; 5(11):897–907. PMID: 15520809
29. Lavrik IN, Golks A, Krammer PH (2005) Caspases: pharmacological manipulation of cell death. J Clin Invest. Oct; 115(10):2665–72. PMID: 16200200
30. Chang CP, Yang MC, Liu HS, Lin YS, Lei HY (2007) Concanavalin A induces autophagy in hepatoma cells and has a therapeutic effect in a murine in situ hepatoma model. Hepatology. Feb; 45(2):286–96. PMID: 17256764
31. Suen YK, Fung KP, Choy YM, Lee CY, Chan CW, Kong SK (2000) Concanavalin A induced apoptosis in murine macrophage PUs 1.8 cells through clustering of mitochondria and release of cytochrome c. Apoptosis. Oct; 5(4):369–77. PMID: 11227218
32. Gastman B, Wang K, Han J, Zhu ZY, Huang X, Wang GQ, et al. (2004) A novel apoptotic pathway as defined by lectin cellular initiation. Biochem Biophys Res Commun. Mar 26; 316(1):263–71. PMID: 15003540
33. Belotti D, Vergani V, Drudis T, Borsotti P, Pitelli MR, Viale G, et al. (1996) The microtubule-affecting drug paclitaxel has antiangiogenic activity. Clin Cancer Res. Nov; 2(11):1843–9. PMID: 9816139
34. Folkman J (1992) The role of angiogenesis in tumor growth. Semin Cancer Biol. Apr; 3(2):65–71. PMID: 1378311
35. Minamiguchi K, Kumagai H, Masuda T, Kawada M, Ishizuka M, Takeuchi T (2001) Thiolutin, an inhibitor of HUVEC adhesion to vitronectin, reduces paxillin in HUVECs and suppresses tumor cell-induced angiogenesis. Int J Cancer. Aug 1; 93(3):307–16. PMID: 11433393
36. Mohan R, Sivak J, Ashton P, Russo LA, Pham BQ, Kasahara N, et al. (2000) Curcuminoids inhibit the angiogenic response stimulated by fibroblast growth factor-2, including expression of matrix metalloproteinase gelatinase B. J Biol Chem. Apr 7; 275(14):10405–12. PMID: 10744729
37. McLane MA, Sanchez EE, Wong A, Paquette-Straub C, Perez JC (2004) Disintegrins. Curr Drug Targets Cardiovasc Haematol Disord. Dec; 4(4):327–55. PMID: 15578957
38. Pilorget A, Conesa M, Sarray S, Michaud-Levesque J, Daoud S, Kim KS, et al. (2007) Lebectin, a Macrovipera lebetina venom-derived C-type lectin, inhibits angiogenesis both in vitro and in vivo. J Cell Physiol. May; 211(2):307–15. PMID: 17323838
39. Duong Van Huyen JP, Bayry J, Delignat S, Gaston AT, Michel O, Bruneval P, et al. (2002) Induction of apoptosis of endothelial cells by Viscum album: a role for anti-tumoral properties of mistletoe lectins. Mol Med. Oct; 8(10):600–6. PMID: 12477970
40. Liu B, Li CY, Bian HJ, Min MW, Chen LF, Bao JK (2009) Antiproliferative activity and apoptosis-inducing mechanism of Concanavalin A on human melanoma A375 cells. Arch Biochem Biophys. Feb; 482 (1–2):1–6. doi: 10.1016/j.abb.2008.12.003 PMID: 19111670
41. Li WW, Yu JY, Xu HL, Bao JK (2011) Concanavalin A: a potential anti-neoplastic agent targeting apoptosis, autophagy and anti-angiogenesis for cancer therapeutics. Biochem Biophys Res Commun. Oct 22; 414(2):282–6. doi: 10.1016/j.bbrc.2011.09.072 PMID: 21951850
42. Marvibaigi M, Supriyanto E, Amini N, Abdul Majid FA, Jaganathan SK (2014) Preclinical and clinical effects of mistletoe against breast cancer. Biomed Res Int. 2014:785479. doi: 10.1155/2014/785479 PMID: 25136622