Research article

Cytotoxic and apoptotic activity of *Canavalia* seed extract in HT-29 human colon carcinoma cellsGeetha Suvarna¹, Ashwini Prabhu², Katheeja Muhseena N.², Bhagya B. Sharma¹¹Centre for Environmental Studies, ²Yenepoya Research Centre, Yenepoya (Deemed to be University), Mangalore-575018, Karnataka, India

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Corresponding author: Bhagya B. Sharma. Email: bagyabs@gmail.com

ABSTRACT

Introduction and Aim: Concanavalin the lectin from *Canavalia* spp. share high structural similarity among the same taxon and exhibit wide range of biological functions. The antiproliferative activity of lectins ConA (*C. ensiformis*), ConC (*C. cathartica*), ConG (*C. gladiata*) and ConM (*C. rosea*) were studied in human colon carcinoma.

Materials and Methods: Human colon carcinoma HT-29 cells and human normal kidney HEK293T cells were examined. The cytotoxicity was evaluated by MTT assay. Cell apoptosis was analyzed by AO-EB (acridine orange-ethidium bromide dual staining) method. Cell cycle was investigated by flow cytometry (RNase-propidium iodide-based flow cytometry). The expression of cell signaling genes was analyzed by qRT-PCR.

Results: All concanavalins significantly inhibited the proliferation of HT-29 cells in dose-dependent manner (IC₅₀ values were ConG-10.45, ConA-14.86, ConC-33.34 and ConM-48.98 µg/mL respectively). ConA and ConG induced apoptotic morphology in HT-29 cells without affecting HEK293T cells. Similarly, both lectins increased the sub-G₀/G₁ proportions in HT-29 cells dose-dependently. ConG showed down-regulation of AKT1, ERK1/2 and mutant p53 in HT-29 cells.

Conclusion: Concanavalin extract is shown to possess cytotoxic effect through inducing apoptosis, especially causing cell cycle arrest at G₀/G₁ phase in HT-29 cells.

Keywords: *Canavalia* seed extracts; HT-29 cell lines; cytotoxicity; apoptosis; cell cycle.

INTRODUCTION

Concanavalin (Con), a lectin from *Canavalia* spp. exhibit various biological functions due to its sugar binding property in reversible manner. They are homo-tetramers with each monomer consisting of a carbohydrate recognition domain (CRD), two metal binding sites and a hydrophobic site (1). *Canavalia* lectins share high similarity in nucleotide and protein homology. However, few positional changes in amino acids residues results in differential biological functions (2). Concanavalin show high specificity towards mannose/glucose sugars as demonstrated by hemagglutination inhibition assay (3). These amino acid changes and interactions influence binding of concanavalin to cell surface receptors. The concanavalin extracted from *C. ensiformis* (ConA) is the widely studied lectin exhibiting various functions like hemagglutination, mitogenicity, antitumor, insecticidal and antimicrobial activity (see 3). ConA has been reported to induce cell death in different human cancer cell lines through apoptosis and autophagy. Induction of apoptosis is characterized by cytotoxicity, nuclear condensation, DNA fragmentation and membrane blebbing. Apoptotic efficacy of ConA is reported in different cell lines (4-6), while concanavalins from *C. gladiata* (ConG), *C.*

cathartica (ConC) and *C. rosea* (ConM) are less explored (1, 3).

Glycans present on the tumor cell surface play an integral role in the cancer cell proliferation. A modified glycosylation patterns is observed on the cancer cells, which results in substantial change in specific metabolic processes (7). Lectins are cell recognition molecules which can interact with carbohydrate moieties with high affinity and specificity. Due to their selective specificity towards altered glycans present on the tumor cell surfaces, they can be exploited as efficient anticancer lead molecules and cancer biomarker (7). According to global cancer statistics (2018), colorectal cancer (CRC) is the most commonly diagnosed cancer with incidence rate of ~1.85 million and 880,792 deaths (8). The present study evaluated the cytotoxicity and apoptotic efficacy of semi-purified concanavalin extracts on human colon carcinoma HT-29 cells.

MATERIALS AND METHODS

Chemicals

Antibiotic-antimycotic solution, Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA solution and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were bought from Himedia, India. Acridine orange,

Dimethyl sulfoxide (DMSO) and ethidium bromide were acquired from Merck, India. Primers for qRT-PCR in lyophilized form were procured from BioServe Biotechnologies Ltd. (Hyderabad, India). Verso cDNA synthesis kit and SYBR® Green master mix were from Thermo Scientific, USA.

Cell culture

Human colon carcinoma HT-29 and normal HEK293T cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) comprising 10% fetal bovine serum and 1% antibiotic-antimycotic solution (10000 U penicillin, 10 mg streptomycin and 25 µg amphotericin B). The cultures were maintained at 37°C in an incubator with 5% CO₂ and humidified atmosphere. Cells were subcultured upon attaining 70% confluence and used for various experiments.

Canavalia seed collection

Seeds of *C. cathartica*, *C. ensiformis*, *C. gladiata* and *C. rosea* were collected from Kallapu (12°49'47.3"N, 74°51'35.2"E), Vittal (12°45'55.9"N, 75°05'56.4"E), Adyanadka (12°69'54.2"N, 75°10'77.5"E) and Tannirbavi (12°88'67.7"N, 74°81'51.7"E) region of Dakshina Kannada District, Karnataka, India. All plant samples were authenticated by a taxonomist and voucher specimens deposited at the herbarium.

Fractional precipitation of the concanavalin with ammonium sulfate

Crude extract was prepared by homogenizing seed cotyledon powder (10%) in 0.15 M NaCl and subjected to salting-out using ammonium sulfate (9). The extract was precipitated by ammonium sulfate at different saturation levels (0-20%, 20-40%, 40-60%, 60-80%, 80-100%). Protein concentration was assessed by Lowry's method (10) and specific activity was determined by hemagglutination assay which is a simple and sensitive method for quantification of lectins (11). Protein precipitate with high specific activity was dialysed against distilled water, dried and dissolved in phosphate buffered saline. The resultant semi-purified concanavalin extracts were used to perform cytotoxicity assays.

Ethical clearance

The hemagglutination assay was performed in human erythrocytes. Peripheral blood (~ 3 mL) was collected from healthy volunteer with the approval from institutional ethical committee (YUEC protocol no2018/052).

MTT assay

The cell viability was assessed by MTT assay (12). HT-29 cells were seeded into 96-well plates at 5×10^3 cells/well. The different concentrations (12.5, 25, 50, 100, 200, 400 and 800 µg/mL) of concanavalin extract was added and incubated for 24 h at 37°C.

Untreated cells were used as negative control. The MTT reagent (1 mg/mL) was added and further incubated for 4 h at 37°C. The MTT formazan crystals formed were solubilized in 100 µL of DMSO and absorbance was recorded at 570 nm in multimode microplate reader (FluoSTAR Omega, BMG Labtech, Germany). Percentage inhibition was determined using the formula,

Percentage growth inhibition = [(Mean absorbance in control well – Mean absorbance in test well)] / Mean absorbance in control well × 100.

Dose response curve was plotted and IC₅₀ value was calculated.

Detection of Apoptosis

AO-EB dual staining

Acridine Orange/Ethidium Bromide (AO/EB) dual staining was applied for determining the morphological changes in cells (13). The HT-29 cells were seeded in 12-well plate at a density of 2×10^4 cells/well and incubated with IC₅₀, ½ IC₅₀ and ¼ IC₅₀ concentrations of ConA and ConG extracts in triplicates at 37°C for 24 h. Untreated cells were used as control. The cells were fixed with chilled methanol for 30 min and stained with AO (2 µg/mL) and EB (2 µg/mL) at 37°C for 15 min. The stained cells were washed with PBS, observed, and photographed under fluorescence cell imager (ZOE, Bio-Rad, USA) with B-2A filter at 20 X magnification. The apoptotic cells were identified by cellular morphology and fluorescence emission. The cells emitting green fluorescence with intact nuclear structure were considered as viable cells, whereas early apoptotic cells fluoresce yellow-green and late apoptotic cells fluoresce orange red with condensed chromatin.

Flow cytometric assay

Effect on cell division was assessed by flow cytometric assay. The HT-29 cells (2×10^4 cells/well) were seeded into 12-well plate and incubated with IC₅₀ and ½ IC₅₀ concentrations of extracts of ConA and ConG at 37°C for 24 h. Untreated cells were used as control. Cells were harvested and suspended in staining solution containing RNase A (0.1 mg/mL) and propidium iodide (5 µg/mL). Stained cells were analyzed for cellular DNA (Deoxyribonucleic acid) content by flow cytometer (Guava EasyCyte, Merck Millipore, USA). The data was analyzed by flowing software v2.5.1 (14) and the proportion of cells in three major phases of cell cycle (G₀/G₁, S and G₂/M) and apoptotic cells with fractional DNA content (sub-G₀/G₁ phase) was determined.

qRT-PCR

Relative gene expression of genes influencing cell cycle was quantified by quantitative reverse transcription polymerase chain reaction (qRT-PCR)

method. The genes examined for their expression are p53, AKT1 and ERK1/2 involved in cell signaling regulation and proliferation. A total of 2×10^4 HT-29 cells were plated in a 60 mm culture dish and incubated for 24 h at 37°C in 5% CO₂. The cells were treated with IC₅₀, ½ IC₅₀ and ¼ IC₅₀ concentration of ConG extract for 24 h. Untreated cells were used as control. The total RNA was isolated from treated cells and control by modified Trizol extraction method (15) and quantified in a Nano-spectrophotometer (Titertek-Berthold, Germany). For each treatment group, approximately 500 ng of RNA was used to synthesize cDNA (Agilent Sure Cyclor 8800) using Verso cDNA synthesis kit (Thermo Scientific) as per manufacturer's instructions. The

primer sequence used is shown in Table 1. The qRT-PCR experiment was performed using SYBR® Green master mix in a real-time thermal cycler (Bio-Rad CFX96, Singapore) with following conditions: initial denaturation at 95°C for 3 min and amplification of 40 cycles (95°C for 15 s for denaturation, 57.5°C for 1 min for annealing and 65°C for 5 s for extension). The $2^{-\Delta\Delta C_t}$ method was used to determine the effect of the experimental treatment and to calculate relative changes in mRNA expression of target gene normalized to β -actin and average fold-change for each gene was determined compared to untreated cells (16). The fold change value less than 1 implies decrease in gene expression.

Table 1: Primer sequences for qRT-PCR

Gene	Forward primer	Reverse primer
AKT1	TGGACTACCTGCACTCGGAGAA	GTGCCGCAAAAGGTCTTCATGG
ERK1	TGGCAAGCACTACCTGGATCAG	GCAGAGACTGTAGGTAGTTTCGG
ERK2	ACACCAACCTCTCGTACATCGG	TGGCAGTAGGTCTGGTGCTCAA
p53	CCTCAGCATCTTATCCGAGTGG	TGGATGGTGGTACAGTCAGAGC
β-actin	CACCATGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT

AKT is serine/threonine kinase, ERK is extracellular signal-regulated kinase, p53 is tumor suppressor protein, β -actin is internal control.

Statistical analysis

The data were analyzed using Statistical Package for Social Sciences (SPSS) software version 23.0. The data are expressed as mean $\pm$ SD from three replicates per treatment. Treatment groups were compared to control and analysed by one-way ANOVA followed by Tuckey's post-hoc test. The level of significance was set at $p < 0.05$.

RESULTS

Concanavalin precipitate

Crude extract from *Canavalia* seeds were purified by using ammonium sulfate precipitation at different

saturation levels (0-20%, 20-40%, 40-60%, 60-80%, 80-100%). The protein concentration was quantified and hemagglutination activity was determined for each fraction. The specific activity evaluated using total protein and hemagglutination unit was found to be higher in 40-60% precipitated fraction. The ConA, ConC, ConG and ConM fraction respectively showed 2.19, 1.40, 2.14 and 1.40-fold increase in specific activity compared to crude extract. Desalting of 40-60% precipitated fraction was performed by dialysis in distilled water. The purity of final product was 1.86 (ConA), 1.62 (ConC), 2.43 (ConG) and 1.50-fold higher than crude extract (Table 2).

Table 2: Total protein concentration and specific activity of concanavalin extract

Concanavalin	Fractions	Total protein (mg) ^a	Specific activity (HU/mg) ^b	Purification fold ^c	Recovery of activity (%) ^d
ConA	Crude extract	6667.83	1036.62	1.00	100.00
	40-60% Ammonium sulphate precipitate	1355.03	2267.11	2.19	44.44
	Dialysate	1225.70	1931.96	1.86	34.26
ConC	Crude extract	7831.23	7159.03	1.00	100.00
	40-60% Ammonium sulphate precipitate	2603.40	10029.95	1.40	46.58
	Dialysate	1859.56	11564.01	1.62	38.36
ConG	Crude extract	6251.14	8927.65	1.00	100.00
	40-60% Ammonium sulphate precipitate	1337.47	19140.56	2.14	45.87
	Dialysate	944.13	21692.00	2.43	36.70
ConM	Crude extract	7705.76	7242.37	1.00	100.00
	40-60% Ammonium sulphate precipitate	2518.41	10165.16	1.40	45.87
	Dialysate	1884.59	10867.07	1.50	36.70

^aProtein estimated by Lowry's method, ^bSpecific activity is hemagglutination unit per mg of protein, ^cPurification fold is the fold- increase in specific activity compared to crude extract, ^dRecovery of activity (%) is the measure of agglutination activity retained compared to crude extract.

Cytotoxic effects

All concanavalins have cytotoxic effects in HT-29 cells. Potent inhibitory effects were observed in a dose-dependent manner up to 100 µg/mL with significant ($p < 0.001$) percentage viability at different concentrations (figure 1). The IC_{50} values determined from the concentration-response curve was in the following order – ConG (10.45 µg/mL) > ConA (14.86 µg/mL) > ConC (33.34 µg/mL) > ConM

(48.98 µg/mL). Higher cytotoxicity was seen in ConA and ConG compared to ConC and ConM. However, the extracts did not show significant toxicity in normal HEK293T cell line (17). The results indicate ConA and ConG extracts have more potent growth inhibitory activity towards HT-29 cells than other concanavalins and hence were further evaluated for their apoptotic efficacy.

Fig. 1: Effect of concanavalins on the proliferation of HT-29 cells

Cell viability of HT-29 cells was determined by MTT assay after 24 h treatment with different concentrations of lectin extracts.

Data are presented as the Mean±SD of three replicates. Percentage viability in treatment group statistically differed from control group (* $p < 0.001$; One - Way ANOVA)

Observation of cellular morphology

The results of AO-EB staining show notable apoptotic morphological changes such as chromatin condensation, DNA fragmentation and apoptotic bodies (figure 2). AO-EB differential staining demonstrated late apoptotic cells with orange fluorescence at higher concentrations and early

apoptotic cells displayed yellowish green fluorescence at lower concentrations. However, ConA and ConG did not result in apoptotic morphology in control group. The results indicated that both ConA and ConG extract selectively induced HT-29 apoptosis.

Fig. 2: Morphologic observations of HT-29 cells treated with ConA and ConG

Control cells (A); Cells treated with ConA (IC_{50} (B), $\frac{1}{2} IC_{50}$ (C), $\frac{1}{4} IC_{50}$ (D)) and ConG (IC_{50} (E), $\frac{1}{2} IC_{50}$ (F), $\frac{1}{4} IC_{50}$ (G)). Images of cells stained with acridine orange-ethidium bromide was captured by overlapping green and red channels with a scale bar of 100 μm . Labels: V-viable cell, EA-early apoptosis, LA-late apoptosis, CC-chromatin condensation, NF-nuclear fragmentation, AB-apoptotic body

Cell cycle analysis

As demonstrated in figure 3, the extracts of ConA and ConG (Figure 3) markedly increased HT-29 cell sub- G_0/G_1 proportions in a dose-dependent manner,

indicating that both extracts induced apoptosis in HT-29 cells. Moreover, both lectins decreased the proliferation phases of cell cycle in G_0/G_1 , S and G_2/M .

Fig. 3: Effect of ConA and ConG on cell cycle distribution in HT-29 cells

Histograms of the DNA content of each phase of the cell cycle in untreated HT-29 cells (A); Cells treated with ConA (IC_{50} (B), $\frac{1}{2} IC_{50}$ (C)) and ConG (IC_{50} (D), $\frac{1}{2} IC_{50}$ (E)).

Analysis of apoptosis-related genes

The qRT-PCR analysis shows relative mRNA decrease in expression of AKT1, ERK1/2 and mutant

p53 genes. The data demonstrated that treatment of HT-29 cells with ConG resulted in down-regulation of these genes in a dose-dependent manner (figure 4).

Fig. 4: Effect of ConG on expression of apoptosis-related genes in HT-29 cells

Fold change in gene expression was determined by qRT-PCR. Results are represented as Mean $\pm$ SD of three replicates

DISCUSSION

Legume lectins are extensively studied for antitumor activity that induce cytotoxicity, apoptosis and inhibit the growth of cancer cells (4, 5). Concanavalins from *Canavalia* spp. is a member of legume lectin family and share high genetic similarity (1, 2). This similarity is replicated in primary protein structure with conserved carbohydrate recognition domain (CRD), metal binding site and hydrophobic site (1). However, variation in configuration of these functional sites was observed in quaternary structures of concanavalins (2).

ConA is the first concanavalin to be isolated and is widely studied for its structure and function. However, there are few reports on biological activities of other concanavalins. These homologous proteins are shown to exhibit distinct biological activities despite high similarity in their structural configuration (1-3). ConA is reported to exhibit more specificity towards D-mannose as per reports (1, 3). ConC, ConG and ConM also showed similar specificity but difference was observed in binding affinity (unpublished data) which might influence its functional properties. Lectins induce apoptosis by binding to glycoproteins on the cell surface (7). The sugar affinity is seen to have positive correlation for antiproliferative activity in ConA (6). In this study, ConA, ConC, ConG and ConM were isolated from four accessions of *Canavalia* spp. and studied for their antiproliferative activity in human colon carcinoma HT-29 cells.

Various methods of concanavalin purification have been reported mainly affinity chromatography using different columns (1). In the present study, a modified protocol (9) for extraction of concanavalin from seeds of *Canavalia* by salting-out technique is

followed. The presence of concanavalin in the extract was assessed by hemagglutination assay (11). The lectin extracts with higher specific activity was selected for antiproliferative activity analysis.

The concanavalins showed significant cytotoxic effect with ConA and ConG being more toxic than ConC and ConM. This distinct activity exhibited by these homologous proteins may be due to variation in volume and configuration of CRD and pH-dependent oligomerization state (1, 2). In addition, these lectins did not cause any toxicity in normal kidney HEK293T cells (17). Such difference in binding affinity is due to altered glycosylation pattern on the cancer cell surface (7). Breast cancer cell lines are shown to over-express high mannose glycans compared to normal epithelial cell line (18). The selective toxicity displayed by mannose-specific concanavalins in the present study supports this observation. ConA is reported to induce cell death in leukemic (MOLT-4 and HL-60), melanoma (A375), breast cancer (MCF7) and cervical cancer (HeLa) cells (4-6, 19). ConG showed inhibition of L1210 leukemia and B16 melanoma cell lines (20, 21).

Apoptosis is a cellular mechanism which gets rid of damaged cells and is important for the survival of multicellular organisms. It is characterized by chromatin condensation, DNA fragmentation and formation of apoptotic bodies (13). The observation of these morphological characteristics in AO-EB stained HT-29 cells confirms apoptotic efficacy of ConA and ConG extracts. Supporting this data, ConA is shown to induce mitochondrial apoptosis, mitochondrial autophagy, increased ROS production, increased caspase-3, -8 and -9 activity, up-regulation of pro-apoptotic proteins (Bax, Bid), down-regulation

of anti-apoptotic proteins (Bcl-2 and Bcl-XL), down-regulation of cell cycle proteins (CDK1, CDK2; 4-6).

Further, the cells in different cell cycle phases was measured to evaluate the effect of concanavalins on regulation of cell cycle dynamics. The present study showed accumulation of sub-G₀/G₁ proportion with decrease in cell proportion at proliferation phases (G₀/G₁, S and G₂/M). The sub-G₀/G₁ peak in flow cytometric data is indicative of DNA fragmentation, which is one of the hallmarks of apoptosis (22). Supporting the present data, ConA and ConBr induced accumulation of sub-G₀/G₁ proportion in human leukemic MOLT-4 and HL-60 cells (5). The sub-G₀/G₁ proportion increased in dose-dependent manner in ConA treated MCF-7 cells (4) while in HeLa cells, time-dependent increase was observed (19). In this study, we report ConG induces apoptosis in human colon carcinoma HT-29 cells.

Colorectal cancer progression is associated with deregulation of signaling pathways promoting proliferation and inducing metastasis (23). The AKT, ERK, p53 and other signaling pathways are critical for cellular functions and are commonly deregulated in cancers (23, 24). The p53 is a major tumor suppressor protein, which plays an important role in cell survival under genotoxic stress. The p53 gene is mutated (mutant p53) in approximately 50% of human cancer cell types including HT-29 cells. In other malignant cells, functional inactivation of wild type p53 is observed (24). The downregulation of mutant p53 and up-regulation of wild-type p53 is related to suppression malignant properties of cancer cells (24). The serine/threonine kinase AKT (or also known as protein kinase B) regulates different cellular functions such as proliferation, apoptosis, cell migration and glucose uptake. Among the three isoforms (AKT1, AKT2 and AKT3), AKT1 plays a critical role in cancer physiology specifically cell invasion and migration in different cancer types (23). The ERK (extracellular signal-regulated kinase) is a member of mitogen activated protein kinase family (MAPK) and are crucial for several physiological processes. Hence, downregulating the expression of ERK by intermediate molecules is considered to be a promising option in achieving efficient cancer therapy (23). ConA is shown to inhibit AKT expression in p53-null cells (25). The ConA induced apoptosis via down-regulation of ERK proteins in MCF-7 cells (4). ConBr decreased phosphorylation of ERK1/2 and AKT in C6 glioma cells (26). In this study, extract of ConG induced apoptosis via down-regulation of p53, AKT1 and ERK1/2 in HT-29 cells which is comparable to the results of other concanavalins.

CONCLUSION

This study demonstrates that semi-purified concanavalin induces cell death and apoptosis in colon carcinoma HT-29 cells. ConA and ConG

increased sub-G₀/G₁ proportions inducing apoptotic cell death. The apoptotic cell death induced by ConG extract is by down-regulation of p53, AKT1 and ERK1/2 pathways. From the results, it is noteworthy that ConG was more effective than other lectins. The limitation of the current study is that a crude extract of lectin was used for the analysis. Further studies with purified lectins would help understand the structure-function relationship in concanavalins.

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CONFLICT OF INTEREST

Authors declare that they have no conflicts of interest.

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