Research Paper

Cytotoxic and Apoptotic Efficacy of Concanavalins Isolated from Canavalia ensiformis and Canavalia gladiata on Colon Carcinoma Cells

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Suvarna *et al.*: Antiproliferative effect of concanavalins *in vitro*

Concanavalin a lectin from seeds of *Canavalia* spp. is extracted and purified to study antitumor effect *in vitro*. *Canavalia ensiformis* and *Canavalia gladiata* were tested for their cytotoxicity, apoptotic potential and effect on cell cycle phases in HT-29 colon carcinoma cells. It was revealed that both lectins inhibit growth of HT-29 cells in dose dependent manner without affecting normal Human embryonic kidney 293T cells. The half maximal inhibitory concentration values were 14.86 and 10.45 µg/ml for *Canavalia ensiformis* and *Canavalia gladiata* respectively. Both lectins induced apoptotic morphology like chromatin condensation, deoxyribonucleic acid fragmentation and apoptotic bodies in HT-29 cells. Moreover, apoptosis was further confirmed in *Canavalia ensiformis* and *Canavalia gladiata* by increased proportion of cells in sub-G_0/G_1 phase. Apoptosis was induced by down regulation via tumor suppressor protein, serine/threonine kinase and extracellular signal regulated kinase 1/2 pathways as demonstrated by quantitative reverse transcription polymerase chain reaction. The above results indicated that both lectins selectively induce apoptosis in HT-29 cellss.

Key words: Concanavalin, HT-29 cells, cytotoxicity, apoptosis, cell cycle

Concanavalin (Con), a lectin from *Canavalia* spp. possess various biological functions due to its binding to cell surface receptors. *Canavalia* lectins share high similarity in nucleotide and protein homology. However, small number of positional changes in amino acids elicits diverse biological properties[1]. Con show high specificity towards mannose/glucose sugars as demonstrated by hemagglutination inhibition assay[2]. These amino acid changes and interactions influence binding of Con to cell surface receptors. *Canavalia ensiformis (*ConA) (jack bean) is the most characterized lectin with antioxidant, hemagglutinating, mitogenic, insecticidal, antimicrobial and antitumor activity[3]. ConA has been extensively investigated and reported for targeting human cancer cell death by inducing apoptosis and autophagy. Induction of apoptosis is characterized by cytotoxicity, nuclear condensation, deoxyribonucleic acid (DNA) fragmentation and membrane blebbing. Apoptotic potential of ConA in different cell lines and its possible use as anti-cancer agent is widely reported[4-6]. *Canavalia gladiata* (ConG) popularly known as sword bean shows hemagglutination, mitogenic, Natural Killer (NK) cell activity and affinity towards mannose/glucose moieties[7].

Glycans on the tumor cell surface play a key role in the tumor cell division and metastasis[8]. Cancer cells also display modified glycosylation patterns, which induce significant alterations in specific metabolic processes[9]. Lectins can reversibly interact with mono and oligosaccharide moieties with a very high affinity and specificity[10]. They can specifically target glycosylation markers present on the tumor cell surfaces, hence they can be exploited as efficient anticancer lead molecules[11]. Colorectal cancer (CRC) is the third most common frequently occurring cancer worldwide. The global incidence of CRC in 2018 was ~1.85 million with 8 80 792 deaths worldwide[12]. In this study, we evaluate the cytotoxicity and apoptotic

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efficacy of ConA and ConG on HT-29 colon carcinoma cells.

MATERIALS AND METHODS

Materials:

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), antibiotic antimycotic solution, trypsin ethylenediaminetetraacetic acid (EDTA) solution and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were procured from Himedia, India. Dimethyl sulfoxide (DMSO), acridine orange and ethidium bromide were obtained from Merck, India. Primers for Quantitative Reverse Transcription polymerase chain reaction (qRT-PCR) in lyophilized form were procured from BioServe Biotechnologies Ltd. (Hyderabad, India). Verso complementary DNA (cDNA) synthesis kit and SYBR Green master mix were from Thermo Scientific, USA.

Plant source and lectin isolation:

Seeds of *C. ensiformis* and *C. gladiata* were collected from Vittal (12°45'55.9"N, 75°05'56.4"E) and Adyanadka (12°69'54.2"N, 75°10'77.5"E) region of Dakshina Kannada District, Karnataka, India. Samples were authenticated by a taxonomist and further used for purification of Con using salting out technique[13]. Crude extract was prepared by homogenising seed cotyledon powder (10 %) in 0.15 M Sodium chloride (NaCl). Filtered extract was subjected to ammonium sulphate precipitation at different saturation levels (0-20 %, 20-40 %, 40-60 %, 60-80 %, 80-100 %). Protein precipitate was dialysed against distilled water, dried and dissolved in Phosphate-buffered saline (PBS) to perform cytotoxicity assays. Lectin concentration was assessed by Lowry's method[14] and specific activity was determined by hemagglutination assay which is a simple and sensitive method for quantification of lectins[15].

Ethical clearance:

The hemagglutination assay was performed in human Red blood cells (RBCs). Peripheral blood was collected from healthy volunteer with the approval from institutional ethical committee (YUEC protocol no2018/052).

Cell lines and cell culture:

Human colon carcinoma HT-29 and normal Human embryonic kidney 293T cell (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 (10 000 U penicillin, 10 mg streptomycin and 25 µg amphotericin B). The cultures were maintained at 37° in an incubator with 5 % CO_2 and humidified atmosphere. Cells were subcultured upon attaining 70 % confluence and used for various experiments.

Cytotoxicity assay:

MTT assay[16] was used to determine the effects of different ConA and ConG concentrations on HT-29 and HEK293T cell viability. 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 was added and incubated for 24 h at 37°. Untreated cells were used as negative control. The MTT reagent (1 mg/ml) was added and further incubated for 4 h at 37°. 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 and Germany). Cell viability was determined using the formula (Absorbance in control well–Absorbance in test well/Absorbance in control well)×100. Dose response curve was plotted and the half maximal inhibitory concentration (IC_{50}) value was calculated.

Cell apoptosis assay:

Acridine Orange/Ethidium Bromide (AO/EB) dual staining was used for the morphological changes in HT-29 cells treated with different concentrations of ConA and ConG[17]. The HT-29 cells (2×10^4 cells/well) were seeded into 12-well plate and treated with IC_{50}, ½ IC_{50} and ¼ IC_{50} of ConA and ConG in triplicates and incubated at 37° 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° for 15 min. The stained cells were washed with PBS and observed under fluorescence cell imager (ZOE, Bio-Rad, USA) with Blue Excitation-2A (B-2A) filter at 20X magnification.

Cell cycle distribution analysis:

The HT-29 cells (2×10^4 cells/well) were seeded into 12-well plate and treated with concentrations of IC_{50} and ½ IC_{50} ConA and ConG at 37° for 24 h. Untreated cells were used as control. Cells were harvested and suspended in PBS containing Ribonuclease (RNase) A (0.1 mg/ml) and propidium iodide (5 µg/ml). Cell cycle

distribution was analyzed by a flow cytometer (Guava EasyCyte, Merck Millipore, USA) and different cell cycle phases were analyzed by Flowing software v2.5.1[18].

Cell signaling gene expression:

Quantitative reverse transcriptase PCR was used to quantify relative gene expression of ConG treated HT-29 cells. The genes examined for expression are tumor suppressor protein (p53), serine/threonine kinase (AKT1) and extracellular signal-regulated kinase (ERK) 1/2 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° in 5 % CO_2. The cells were treated with IC_{50}, ½ IC_{50} and ¼ IC_{50} concentration of ConG for 24 h. Untreated cells were used as control. The cells were harvested and total Ribonucleic acid (RNA) was isolated for each treatment group by modified Trizol extraction method[19] and quantified using Nano spectrophotometer (Titertek-Berthold, Germany). For each treatment group, approximately 500 ng of RNA was used to synthesize cDNA (Agilent Sure Cycler 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° for 3 min and amplification of 40 cycles (95° for 15 s for denaturation, 57.5° for 1 min for annealing and 65° for 5 s for extension). The expression of the genes was normalized to β-actin and average fold-change for each gene and treatment was determined compared to untreated cells using $2^{-\Delta\Delta Ct}$ method[20]. A value of $2^{-\Delta\Delta Ct}$ <1 compared to control reflects decrease in gene expression.

Statistical analysis:

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

RESULTS AND DISCUSSION

Crude lectin extract from *C. ensiformis* and *C. gladiata* seed cotyledon 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 fractions. The specific activity evaluated using total protein and hemagglutination unit was found to be higher in +40-60 % precipitated fraction. ConA showed 2.19 -fold increase in specific activity with 44.44 % recovery compared to crude extract, whereas 2.14-fold increase in specific activity with 45.87 % recovery was observed with ConG. Desalting of 40-60 % precipitated fraction was performed by dialysis in distilled water. The purity of final product was 1.86 and 2.43-fold higher than crude extract in ConA and ConG respectively (Table 2).

Both ConA and ConG induced HT-29 cell death in a dose-dependent manner and potent inhibitory effects were observed upto 100 µg/ml with significant ($p<0.001$) percentage viability at different concentrations (fig. 1). The IC_{50} values determined from the concentration response curve was 10.45 µg/ml for ConG and 14.86 µg/ml for ConA respectively. However, both the lectins did not show significant toxicity in normal HEK293T cell line (unpublished data). The results indicate ConG has a more potent growth inhibitory activity towards HT-29 cells than ConA.

Notable apoptotic morphological changes such as chromatin condensation, DNA fragmentation and apoptotic bodies were evident in ConA and ConG treated HT-29 cells (fig. 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 selectively induced HT-29 apoptosis.

TABLE 1: PRIMER SEQUENCES FOR qRT-PCR

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

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

TABLE 2: PURIFICATION OF ConA AND ConG BY AMMONIUM SULFATE PRECIPITATION

Lectin	Fractions		Total hemagglutination unit (HU)[a]	Total protein (mg)[b]	Specific activity (HU/mg)[c]	Purification fold[d]	Recovery of activity (%)[e]
Con A	Crude extract		6912000	6667.83	1036.62	1	100
	Ammonium sulfate precipitation	0-20 %	139200	147.43	944.17	0.91	2.01
		20-40 %	182000	248.12	733.53	0.71	2.63
		40-60 %	3072000	1355.03	2267.11	2.19	44.44
		60-80 %	1104000	1409.05	783.51	0.76	15.97
		80-100 %	37000	166.04	222.84	0.21	0.54
	Dialysed 40-60 % fraction		2368000	1225.70	1931.96	1.86	34.26
Con G	Crude extract		55808000	6251.14	8927.65	1	100
	Ammonium sulfate precipitation	0-20 %	332800	56.23	5919.00	0.66	0.60
		20-40 %	960000	442.55	2169.26	0.24	1.72
		40-60 %	25600000	1337.47	19140.56	2.14	45.87
		60-80 %	12544000	1270.14	9876.04	1.11	22.48
		80-100 %	230400	61.23	3762.87	0.42	0.41
	Dialysed 40-60 % fraction		20480000	944.13	21692	2.43	36.70

[a]HU (hemagglutination unit) is the reciprocal of highest dilution of protein showing complete agglutination of human erythrocytes, [b]Protein estimated by Lowry's method, [c]Specific activity is the ratio between total hemagglutination unit and total protein, [d]Purification fold is the fold increase in specific activity compared to crude extract, [e]Recovery of activity (%) is the measure of agglutination activity retained compared to crude extract.

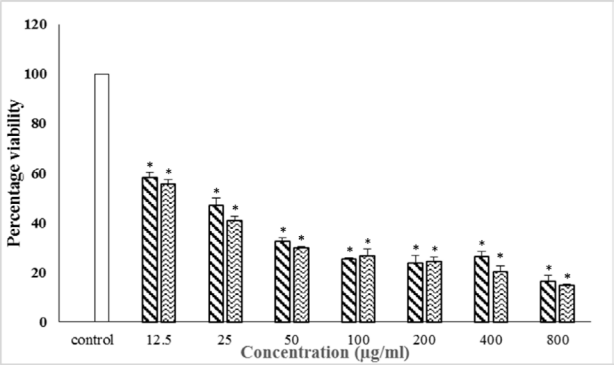

Fig. 1: Effect of concanavalins on the proliferation of HT-29 cells

Cell viability of HT-29 cells was determined by MTT assay after 24 h treatment with different concentrations of ConA and ConG. 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)

Flow cytometry data demonstrated that the treatment of HT-29 cells with ConA and ConG resulted in a significant increase in sub-G_0/G_1 phase indicating apoptosis with cells having fractional DNA content and cell shrinkage. HT-29 cells treated with ConA and ConG showed dose-dependent effect with IC_{50} concentration showing higher apoptotic effect than ½ IC_{50} and control group (fig. 3).

The results from qRT-PCR analysis of ConG treated HT-29 cells on selected cell signaling genes indicate decreased expression than untreated cells in dose-dependent manner (fig. 4). At IC_{50} concentration, the expression of mutant p53 gene in HT-29 cells was reduced with a fold change of 0.28 compared to control. However, ConG showed predominant effect in ERK2 expression with 0.33-fold reduction compared to other cell signaling genes.

Plant lectins are extensively studied for antitumor activity that induce cytotoxicity, apoptosis and inhibit the growth of cancer cells[4,5]. ConA can induce mitochondrial apoptosis, mitochondrial autophagy, increased Reactive oxygen species (ROS) production, increased caspase-3, -8 and -9 activity, up regulation of pro apoptotic proteins (Bax, Bid), down regulation of anti-apoptotic proteins (B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra-large (Bcl-XL)), down-regulation of cell cycle proteins (cyclin dependent kinases (CDK) 1, CDK2)[4-6]. Colorectal cancer progression is associated with deregulation of signaling pathways promoting proliferation and inducing metastasis[21].

In this study ConA (14.86 µg/ml) and ConG (10.45 µg/ml) was found to be the most effective dose to cause 50 % cell death in HT-29 colon cancer cells. ConA is reported to induce cell death in leukemic (human T lymphoblast (MOLT-4) and human leukaemic cells (HL)-60), melanoma (A375), breast cancer (MCF7), cervical cancer (HeLa) cells and no toxicity in normal cells[4-6,22]. ConG showed inhibition of mouse lymphocytic leukemia cell line (L1210 leukemia) and murine tumor (B16 melanoma) cell lines[7,23]. Apoptosis in HT-29 cells is in a dose-dependent manner and ConG was more

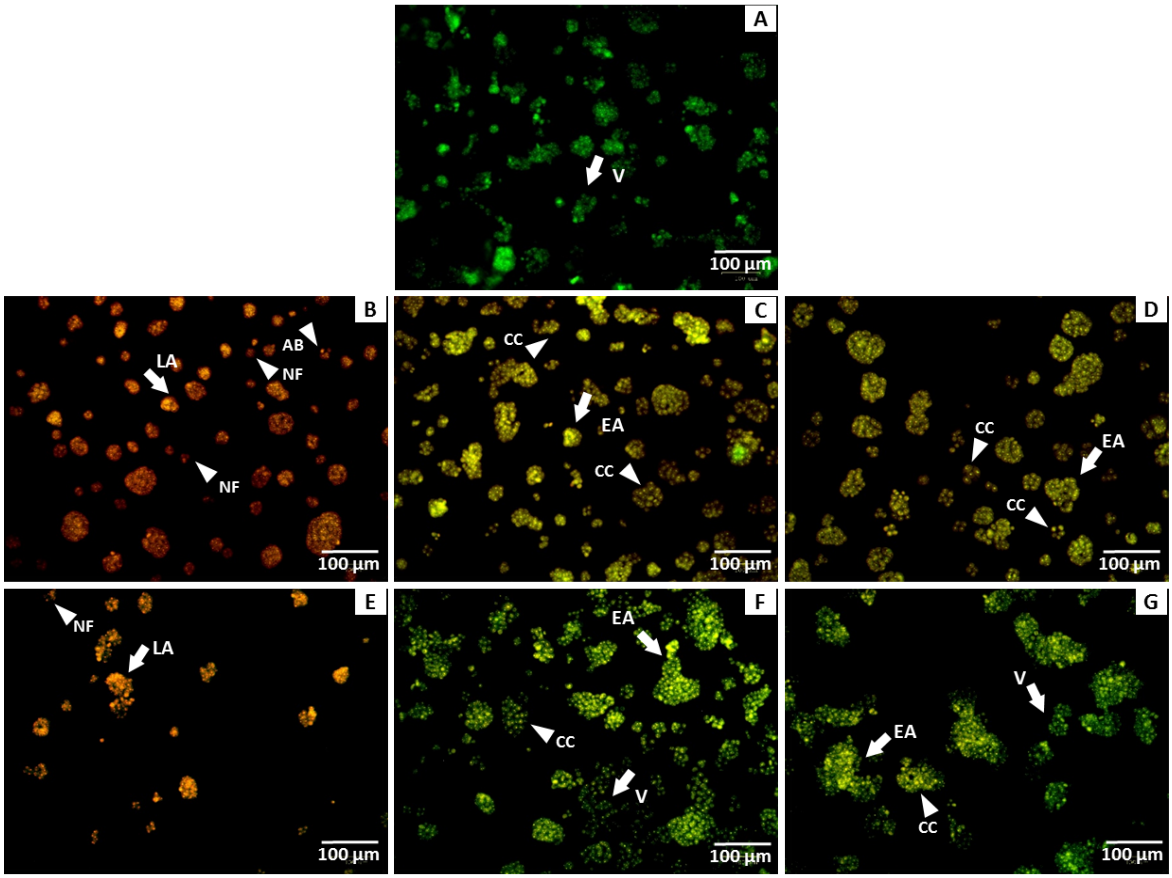

Fig. 2: Morphologic observations of ConA and ConG treated HT-29 cells
Control cells (A); Cells treated with ConA- IC_{50} (B), ½ IC_{50} (C), ¼ IC_{50} (D); Cells treated with ConG- IC_{50} (E), ½ IC_{50} (F), ¼ IC_{50} (G). Images of cells stained with acridine orange-ethidium bromide was captured by 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

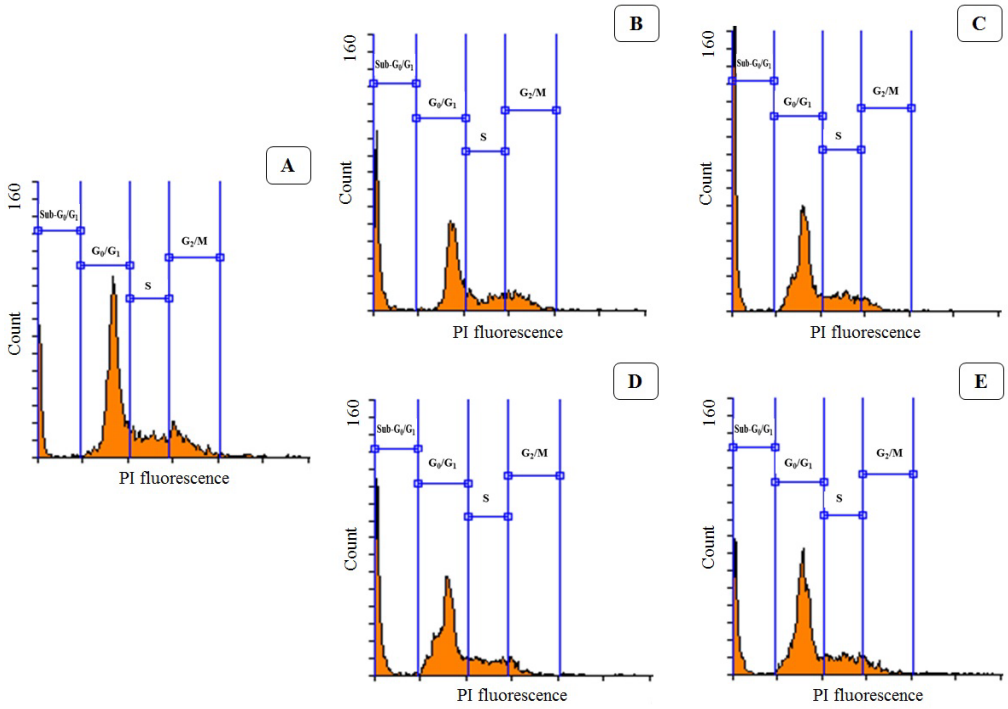

Fig. 3: Effect of ConA and ConG on cell cycle distribution in HT-29 cells
Histograms of the DNA content of each phase of the cell cycle in untreated HT-29 cells (A); cells treated with IC_{50} (B) and ½ IC_{50} (C) of ConA; cells treated with IC_{50} (D) and ½ IC_{50} (E) of ConG.

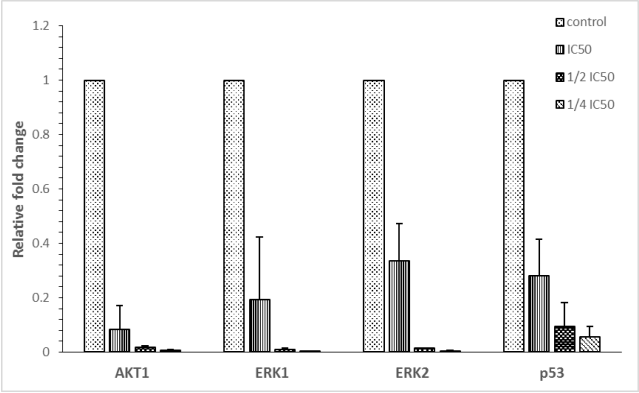

Fig. 4: Effect of ConG on expression of cell signalling genes in HT-29 cells

Fold change in gene expression was determined by qRT-PCR. Results are represented as mean±SD of three replicates.

effective compared to ConA. Similarly, ConA triggered apoptotic response in leukemic cell lines (MOLT-4 and HL-60) with no cytotoxic effect in normal peripheral blood lymphocytes[5]. ConA also showed apoptosis in murine macrophage Mus musculus lymphoid tumor (PU5-1.8) cells by mitochondrial clustering and release of cytochrome c[24].

ConA and ConG promoted apoptosis are featured by distinct apoptotic morphology including chromatin condensation, DNA fragmentation and formation of apoptotic bodies. Besides, regulation of cell cycle dynamics was evaluated by measuring cell number in different cell cycle phases. Both ConA and ConG increased sub-G_0/G_1 proportions compared to control indicating apoptosis. Deregulation of apoptotic process causes cell proliferation which can be studied in numerous molecular signaling pathways. The PI3K/AKT/mTOR and Raf/MEK/ERK signaling pathways are critical for cellular functions and are commonly deregulated in cancers[21]. ConG induced apoptosis via p53, AKT1 and ERK1/2 down-regulation in HT-29 cells compared to control. The p53 is a major tumor suppressor protein which is mutated in approximately 50 % of human malignancies or found to be inactivated by cellular and viral proteins. Down-regulation of p53 causes genomic instability resulting in apoptosis[25]. The serine/threonine kinase AKT (or also known as protein kinase B) regulates various cellular functions like proliferation, apoptosis, cell migration and glucose uptake. It consists of three isoforms (AKT1, AKT2 and AKT3) of which AKT1 is believed to be critical in cancer physiology specifically cell invasion and migration in case of thyroid cancer, colon cancer and melanoma[26]. The ERK belongs to mitogen activated protein kinase family (MAPK) and plays a crucial role in several physiological processes including cell proliferation, migration and apoptosis. Hence, down-regulating the expression of ERK by intermediate molecules is considered to be a promising option in achieving efficient cancer therapy[27]. Some studies have targeted these molecules to study apoptotic mechanism induced by ConA. ConA is shown to inhibit AKT expression in p53-null cells[28]. The ConA induced apoptosis via down regulation of ERK, p53 and JNK[4].

By conclusion, we report both ConA and ConG induce cell death and selectively induce apoptosis in HT-29 colon carcinoma cells. ConG induced apoptosis via down-regulation of p53, AKT1 and ERK1/2 pathways. Also, both lectins increased sub-G_0/G_1 proportions inducing apoptotic cell death. From the results, it is noteworthy that antiproliferative effects of ConG is higher than ConA and can be considered as an anti-cancer compound.

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Conflicts of interest:

Authors declare that they have no conflicts of interest.

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