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Mechanistic Studies of *in vitro* Anti-Proliferative Potential of *Arisaema intermedium* Lectin

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ABSTRACT- Aberrant glycosylation has been recognized as hallmark of cancer. Exploiting differences in glycosylation between malignant and healthy tissues offers excellent opportunities to identify sensitive and specific cancer biomarkers. Plant lectins have demonstrated the ability to specifically agglutinate malignant transformed cells. Lectins are sugar binding proteins or glycoprotein of non-immune origin which agglutinate cells or precipitate glycol-conjugates. Some lectins shown to the anti- proliferative effect on cancer cells. A wide scope of this application of lectins is that it can be used for diagnosis as well as therapeutics of cancer. The objective of the present study was to purify a lectin from tubers of *Arisaema intermedium* and evaluate *in vitro* anti-proliferative potential towards HCT-15, a human colon cancer cell line. The present study was conceived as an offshoot to the ongoing work on lectins in our laboratory. The already reported *Arisaema intermedium* (AIL) lectin was purified on asialofetuin linked amino-activated silica bead matrix. The purity of the affinity purified lectin was ascertained by SDS-PAGE, pH-8.3. The lectin activity was assessed by hemagglutination and protein concentration was determined by Lowry's method. The cytotoxicity of AIL towards HCT-15 was evaluated by MTT assay. The mechanism of anti-proliferative effect was assessed by evaluation of cell morphology, trypan blue exclusion assay, DNA fragmentation and nucleic acid content determination.

Key-words- Araceae, *Arisaema*, Asialofetuin, Antiproliferative effect, Apoptosis, Cytotoxicity Lectins, Mechanistic
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INTRODUCTION

Colon cancer refers to cancerous growth in colon, rectum or caecum. It is most common malignancy worldwide and causes large scale morbidity and mortality [1]. Colon cancer is fourth most common cancer globally with 639,000 deaths reported annually [2]. It occurs most frequently in North America, Australia, New Zealand, Japan, India and Western Europe [3-5]. There are several risk factors related to colon cancer include presence of adenomatous polyps, previous history of ovary, uterus or breast cancer, contraction of specific strains of human papilloma viral infection, *Streptococcus bovis* and inflammatory bowel disease particularly ulcerative colitis.

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In addition to that smoking, alcohol drinking, gender, ethnicity with higher risk in males than females and black than white, respectively [6,7]. As we know cancer develops due to uncontrolled division of altered cells in the body.

The escapes of regulation of these cells are, in most cases, caused due to aberrant glycosylation. The portion of these alternatively glycosylated molecules reach the blood stream. So they could be serve as early marker to enable cancer detection [8,9]. There is difference in glycosylation between malignant and healthy tissue which create excellent opportunities to identify sensitive and specific biomarker. Lectins are the natural biomolecules, which interact specifically with carbohydrates. "Lectins are protein of non immune origin that either bind to carbohydrate or sugar containing substance in a specific and reversible manner or precipitate glycoconjugates" [10]. These biomolecules can be used to differentiate malignanat stage from benign stage and benign stage from normal by studying the drgree of glycosylation in all the stages. Because the altered glycosylation induces either over expression or under expression of some glycoproteins and expression of new sachharides. The lectin molecule known to mark the expression pattern with very high specificity, so that altered changes can be recognized. Lectins are majorly

known to have inhibitory and cytotoxic effect on tumour cells by inducing programmed cell death (PCD) or apoptosis. Programmed cell death is highly regulated process that involves activation of series of molecular events. It is characterized by cell shrinkage, blebbing of plasma membrane, chromatin condensation that consistent with DNA fragmentation and mitochondrial membrane transition [11-15].

In recent studies a great number of phytolectins are reported to have anti- proliferative effect on various human cancer cell lines [16-20]. One of the phytolectin *Arisaema intermedium* lectin (AIL) has been identified and characterized in our laboratory [21-22]. Therefore the present study was designed to evaluate anti-proliferative effect of *Arisaema intermedium* lectin (AIL) on HCT-15, a human colon cancer cell line. These studies were perused further to study the mechanism of action of this lectin by employing various parameters.

MATERIALS AND METHODS

Plant material

The tubers of *Arisaema intermedium* were collected from Shimla in the month of August, 2013, Department of Molecular Biology and Biochemistry, Guru Nanak Dev University, India.

Isolation of lectin

The tubers were washed and dried. After drying, the tubers were weighed and cut into small pieces. These are mixed with 0.01 M PBS, pH 7.2 in 1:5 ratio (w/v) and homogenized in whirring blender. After blending the slurry in PBS (Phosphate buffer saline) was kept overnight at 4°C. The slurry thus prepared was filtered with surgical gauge. The filtrate was then centrifuged at 12,000 rpm in refrigerated centrifuge (CPR24, REMI) for 20 minutes and the supernatant (crude extract) was collected. It was stored at -20°C till further use.

Hemagglutination assay

2% erythrocyte suspension of rabbit was used for the determination of lectin activity by hemaglutination assay [23]. The assay was performed in 96 well microtitre plate (Tarsons). To 30 µl of lectin sample, an equal volume of 2% rabbit erythrocyte suspension was added. The plate was incubated for 30 minutes at 37°C and then kept at 4°C overnight. Positive lectin activity was determined by mesh formation, while button formation indicates absence of lectin activity. The titre of crude extract was determined by serial two fold dilution of the sample in 0.01 M PBS, pH 7.2.

Purification and determination of protein concentration

The lectin was purified using asialofetuin linked amino-activated silica beads column according to the already explained protocol [24]. 12 ml of Crude extract containing 16.4 mg of protein was loaded on the column and was re-circulated twice to ensure complete adsorption of lectin molecules. The column was washed with 0.01 M PBS, pH 7.2 for the removal of any unbound molecules. The bound lectin was eluted with 0.1 M Glycine-HCl, pH 2.5 and the fractions were immediately neutralized with 2 M Tris-HCl, pH 8.8. The column was equilibrated with 0.01 M PBS, pH 7.2. The purity of lectin preparation was determined by SDS-PAGE. The SDS-PAGE of the heat denatured lectin sample was performed according to method of Lamelli [25] using 11% (w/v) separating and 5% stacking gel. Electrophoresis was carried out at 25°C at a constant voltage of 90 V in Tris glycine buffer (electrode buffer) pH 8.3, when the tracking dye was in stacking gel and switched to 100 V when it entered the resolving gel. When the tracking dye reached approximately 1.5 cm above the bottom of the gel, the electrophoresis was switched off and the gel was stained with 0.25% Coomassive Blue for 2 h and subsequently destained by gentle agitation on a shaker until the background of bands became clear. Further protein concentration was determined by method [26].

Cell line and Cell culture

Colon cancer cell line HCT-15 (Passage No. 16) was procured from National Centre for Cell Sciences (NCCS) Pune and cells were cultured in RPMI-1640 medium containing 10% FBS. The cell cultures were maintained in CO_2 incubator with 5% CO_2 and 70-80% humidity.

Cytotoxicity assay

The cytotoxicity of AIL to colon cancer cell line HCT-15 (Colorectal adenocarcinoma) was evaluated with the help of MTT assay to produce formazon crystals [27]. For this, 100 µl of cell suspension at concentration of 8.0×10^3 was added to each well and plates were placed overnight in CO_2 incubator at above mentioned condition. The lectin was serially diluted in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), to obtain the desired concentration at a final volume of 100 µl. A negative control was set with cells cultured with RPMI-1640 medium alone. After 42 h treatment with AIL, the medium was removed and 100 µl of MTT solution in RPMI-1640 (0.5 mg/ml) was added. Plates were again incubated in the CO_2 incubator (Heraeus, USA) for 2-3 h. The ELISA plate was centrifuged (Centrifuge5804R, Eppendorf) at 2,000 rpm for 2 minutes. The medium was discarded and 100 µl of DMSO was added to each well. The intensity of colour so produced was measured at 540 nm on ELISA reader (Multiscan Ex Labsystem).

Percentage proliferation was calculated by the formula:

$$\text{Percentage proliferation} = \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

DNA fragmentation and Nucleic acid content (NAC) analysis

In DNA fragmentation, apoptosis was assessed by electrophoresis of extracted genomic DNA from treated HCT-15 cell lines and in second assay the number of cells in both the control and treated cell samples were estimated on the basis of their nucleic acid content. Cells were reseeded at concentration of 2.0×10^5 cells/ml in 6-well plates and exposed to different concentration of AIL (100 µg/ml, 50 µg/ml, 25 µg/ml) in for 24 and 48 h. Then, cells were harvested for DNA isolation**.** Cell suspensions of 2 ml were centrifuged (Centrifuge 5804R, Eppendorf) for 10 minutes at 13,000 rpm. The supernatant was discarded and white pellet was dissolved in 300 µl of WBC's lysis buffer by vortexing it briefly. After that 25 µl of 10% SDS was added in the tubes. The centrifuged tubes were then incubated for 30 minutes at 56°C and then allowed to cool at room temperature. After cooling, 150 µl of Ammonium acetate was added to the tubes and vortexing was done. Then, the tubes were centrifuged at 13,000 rpm for 10 minutes. Supernatants were collected into fresh tubes and 95% ethanol was added and then spooling was done to precipitate the DNA and centrifuged at 13,000 rpm for 10 minutes. Supernatant was discarded and the pellet was washed with 70% ethanol twice. The pellet was dried at 60°C on the dry bath for 5 minutes. The dried pellet was resupended in 50 µl of TE buffer and incubated at 65°C for 10 minutes to dissolve DNA. The gradation in nucleic acid content was assessed by taking absorbance at 260 nm in treated cells compared to controls on spectrophotometer (Bio Spectrophotometer, Eppendorf) and by electrophoresis using 1.5% agarose gel at 50 V for 2 h. The gel was photographed using alpha imager mini.

Trypan blue exclusion assay

Effect of AIL on viability of HCT-15 cell line was determined with the help of trypan blue exclusion assay. For this, the cells were plated in the 96-well plate at the concentration of 50,000 cells/ml. At 70% confluency cells were treated with various concentration of AIL (100 µg/ml, 50 µg/ml, 25 µg/ml) for 24 h and non treated cells were used as control. The AIL diluted RPMI-1640 was then discarded and 40 µl of non- trypsinized Hank's was added for two minutes. The concentration of trypsin diluted with the addition of 100 µl of RPMI-1640 so that enzymatic activity can stop. Then the cells were agitated properly and transferred into fresh 15 ml vial centrifugation was done at 1,500 rpm for 5 minutes. After centrifugation, the supernatant was discarded and cells were resuspended in 50 µl of RPMI-1640. Cell suspension of about 10 µl was diluted with 10 µl of RPMI-1640. This diluted cell suspension then mixed with trypan blue at (0.4%) in ratio 1:1. This mixture was kept on neubaur's hemocytometerand covered with coverslip and observed under microscope at 40X.

The numbers of viable cells per ml of volume were calculated by cell viability formula:

$$\text{Percentage viability} = \frac{\text{No. of viable cell counted}}{\text{Total cells counted}} \text{ X } 100$$

Evaluation of Cell morphology

Effect of AIL on morphology of HCT-15 cell line was observed with the help of phase contrast microscope (Magnus, Olympus). The cells were treated with various concentrations of AIL in time and dose dependent manner. The cells were plated in the 6- well plate at concentration of 1.0×10^5 cells/ml and non treated cells were used as control. After achieving the 70% confluency, cells were treated with AIL at different concentration i.e. 100 µg/ml, 50 µg/ml, 25 µg/ml for 24 and 48 h. RPMI-1640 added in the cells after rinsing in Non-trypsinized Hank's solution and photographed using digital camera attached to the microscope.

RESULTS AND DISCUSSION

Apoptosis is main mechanism of cell death caused by lectins as reported in literature [28,14-15]. An important marker of cell death is the disruption of mitochondrial membrane accessed by MTT assay. The present study reports the anti-proliferative potential of AIL on HCT-15 induced by apoptosis. As shown in table 1, the MTT based assay demonstrated that with increasing concentration of AIL, i.e. from 12.5 µg/ml to 100 µg/ml, the percentage of growth inhibition also increased. At the highest dose tested i.e. 100 µg/ml the percentage growth inhibition was 53% after 42 h of AIL exposure. Thus, the detailed analysis of the results clearly indicated that AIL caused significant growth inhibition of HCT-15 cells in dose dependent manner as shown in Fig. 1. Cytotoxicity is potent to induce includes disruption of cytoplasmic and mitochondrial membrane and permeabilize them [29]. These findings are in consonance with the previous reports which showed that lectins inhibit growth of cancer cells in culture conditions as well as in animal models [30-34].

The anti-proliferative effect of AIL further assessed by decrease in nucleic assay content of treated HCT-15 cells in dose dependent and time dependent manner. The decrease of DNA content in gradation showing that as the dose concentration increases there is reduction in DNA content.

Table 1: Cytotoxic effect of *A. intermedium* lectin on HCT-15 cell line

Conc. of AIL (µg/ml)	0 (Control)	12.5	25	50	100
Percentage proliferation ±SD	100	87.9±1.1	80.91±0.6	92.33±0.3	47.23±0.5

Cell were employed at a concentration of 8.0×10^3 cells/ml

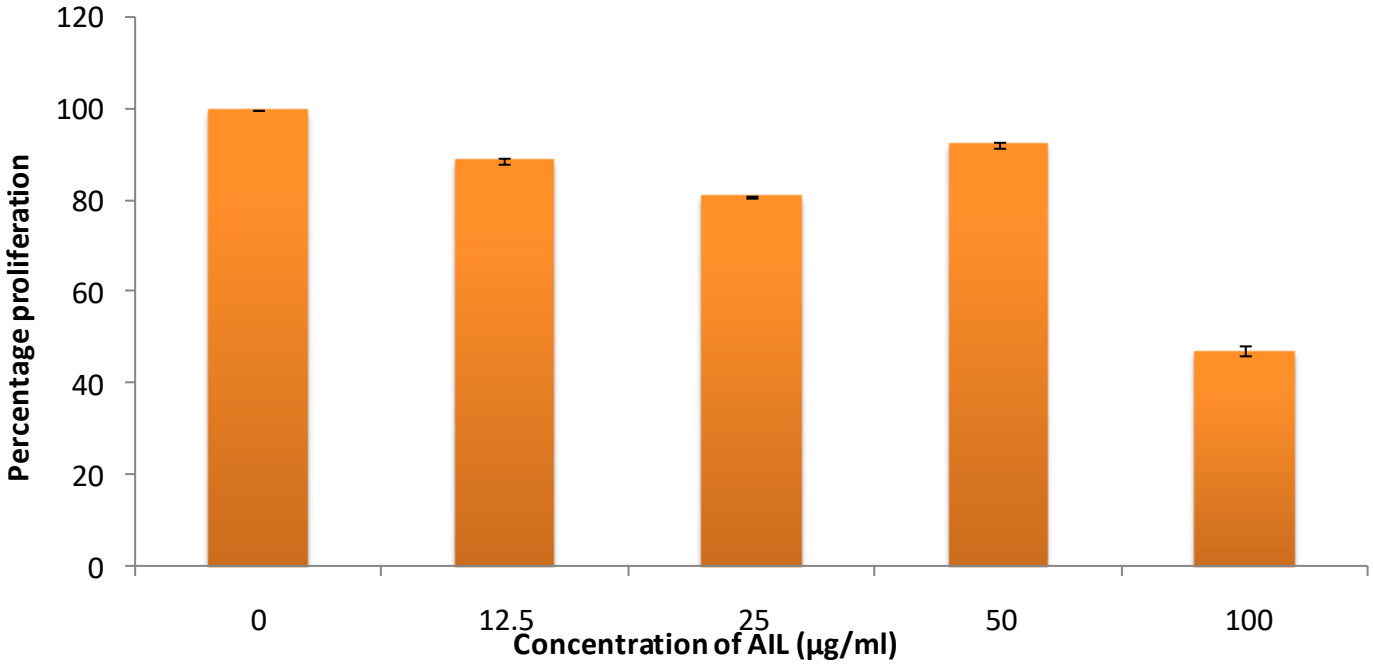

Fig. 1: Cytotoxic effect of *A. intermedium* lectin on HCT-15 cell line: *In vitro* anti-proliferative potential of AIL towards HCT-15 cell line was evaluated by MTT assay. Bars represent the mean±SD of percentage proliferation

The nucleic acid content gradation was accessed at dose concentration 25µg/ml, 50µg/ml and 100µg/ml for 24 and 48 h. The assay was performed in duplicates. A consistent reduction in DNA content was shown after 48 h treatment of AIL, data given in Table 2. This also supports the MTT result that showed that cytotoxic effect of lectin observed around 48 h of treatment. The nucleic acid content reduced to 51% at 50 µg/ml AIL concentration further reduced to 35% at 100 µg/ml (Fig. 2). The significant growth inhibition activity of AIL lead us to investigate the role of AIL in induction of apoptosis.

Table 2: Nucleic acid content measured at different concentrations of AIL for 48 h

Concentration of AIL (µg/ml)	A_{260} Mean ± SD	Concentration (ng/µl) Mean ±SD
0 (Control)	0.319± 0.11 (100%)	2007.75 ±711.98 (100%)
25	0.165± 0.053 (51.72%)	1165.82±185.68 (58.02%)
50	0.103± 0.007 (32.28%)	1038.37±73.75 (51.17%)
100	0.079 ± 0.006 (24.76%)	703.06±77.187 (35.01%)

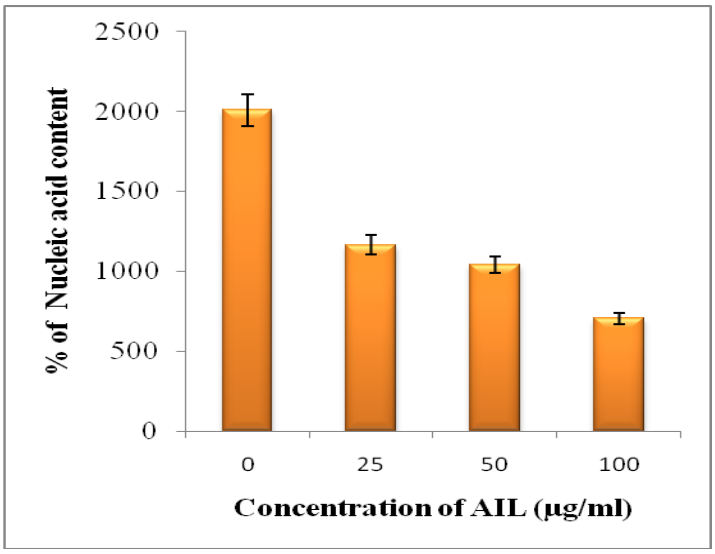

Fig. 2: Nucleic acid content analysis: *in vitro* anti-proliferation was assessed on the basis of nucleic acid content of the treated cells with AIL for 48 h

It is well documented that DNA fragmentation is a secondary consequence of apoptosis and is one of the most common technique for confirmation of apoptosis [35]. Apoptosis typically involves intra-nucleosomal chromatin cleavage by endonucleases leading to DNA fragmentation as expected for apoptotic cells. Endonuclease involved a potential indication of DNA fragmentation that occurs after the release of enzymes from cytoplasmic membrane, an event that potentially occurs only after the final lytic event in the apoptotic sequence. The efficient induction of apoptosis was observed at all the three dosage 25 µg/ml, 50 µg/ml and 100 µg/ml for 48 h. As shown in (Fig. 3). The amount of oligonucleosomal-sized fragments in HCT-15 cells treated with AIL were increased with increase in concentration of AIL from 25 µg/ml to 100 µg/ml. The results obtained are in agreement with the effect of Con A and Con Br on HCT-15, A 375 and leukemic cell lines [36-37]. Trypan blue exclusion assay was performed to check the viability percentage (%) of colon cancer cell line HCT-15 against AIL at series of concentration. The result found a gradual decrease in cells viability with increase in AIL concentration. The percentage viability was reduced to 45% at 50 µg/ml and at AIL concentration of 100 µg/ml, it was further decreased to 32% (Table 3).

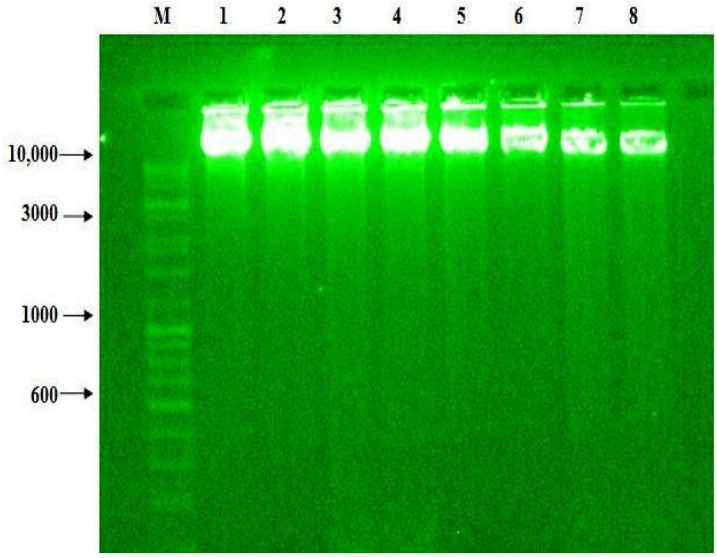

Fig. 3: AIL induced DNA fragmentation of genomic DNA: DNA fragmentation was studied in HCT-15 cell line after treatment with different concentration 25, 50 and 100 (µg/ml) of AIL in duplicates for 48 h.
A prominent fragmentation was observed at all doses after 4

Table 3: Sample distribution shown in Lanes

Lane	M	1, 2	3, 4	5, 6	7, 8
Legend (µg/ml)	Marker	0 (Control)	25	50	100

Table 4: Cell viability (%) at different concentrations of *A. intermedium* lectin

S. No.	Dose conc. (µg/ml)	Viability %
1	0(control)	87.50 (100)*
2	1.56	87.50 (100)*
3	3.12	80.00 (91.36)*
4	6.25	78.57 (89.73)*
5	12.50	75.00 (85.65)*
6	25	69.23 (79.06)*
7	50	40.00 (45.68)*
8	100	28.57 (32.65)*

*** The values in bracket indicate percentage of viability taking viability of control as 100%**

The cancerous cells were further studied for the morphological changes induced by AIL. As evident from Fig. 4 (A-G), the AIL treatment produced significant morphological changes in HCT-15 cells in culture. Under normal conditions (control), these cells are adherent and show epithelial like morphology. These cells tend to cluster together and have characteristic pavement like appearance (Fig.4 A). After treatment with 25 µg/ml of AIL for 24 h, although there was no visible change in morphology, the cells started becoming non-adherent. Exposure of cells to 100 µg/ml concentration of AIL for 24 h resulted in complete change in morphology and cells became nearly non-adherent. Furthermore, the extracellular matrix was also found to disappear (Fig. 4 B-D). With increase in exposure time of AIL i.e. from 24 h to 48 h, cells started to loss adherence to cells substratum at 25 µg/ml dose of AIL as observed after 24 h treatment at same dose. Along with this some cell membrane damage was also seen, which was shown to increased with increase in concentration of AIL. At the highest concentration tested i.e. 100 µg/ml. There was appreciable damage of cellular membrane as well as loss of extracellular matrix (Fig.4 E-G). In nut shell, the lectin treatment resulted in lose of integrity, adherence and extracellular matrix along with cell membrane disruption. These events may disrupt the cellular physiology thus leading to cell death. This type of cell morphology disruption of HCT-15 cells has earlier been observed with treatment of various other compounds [38-40].

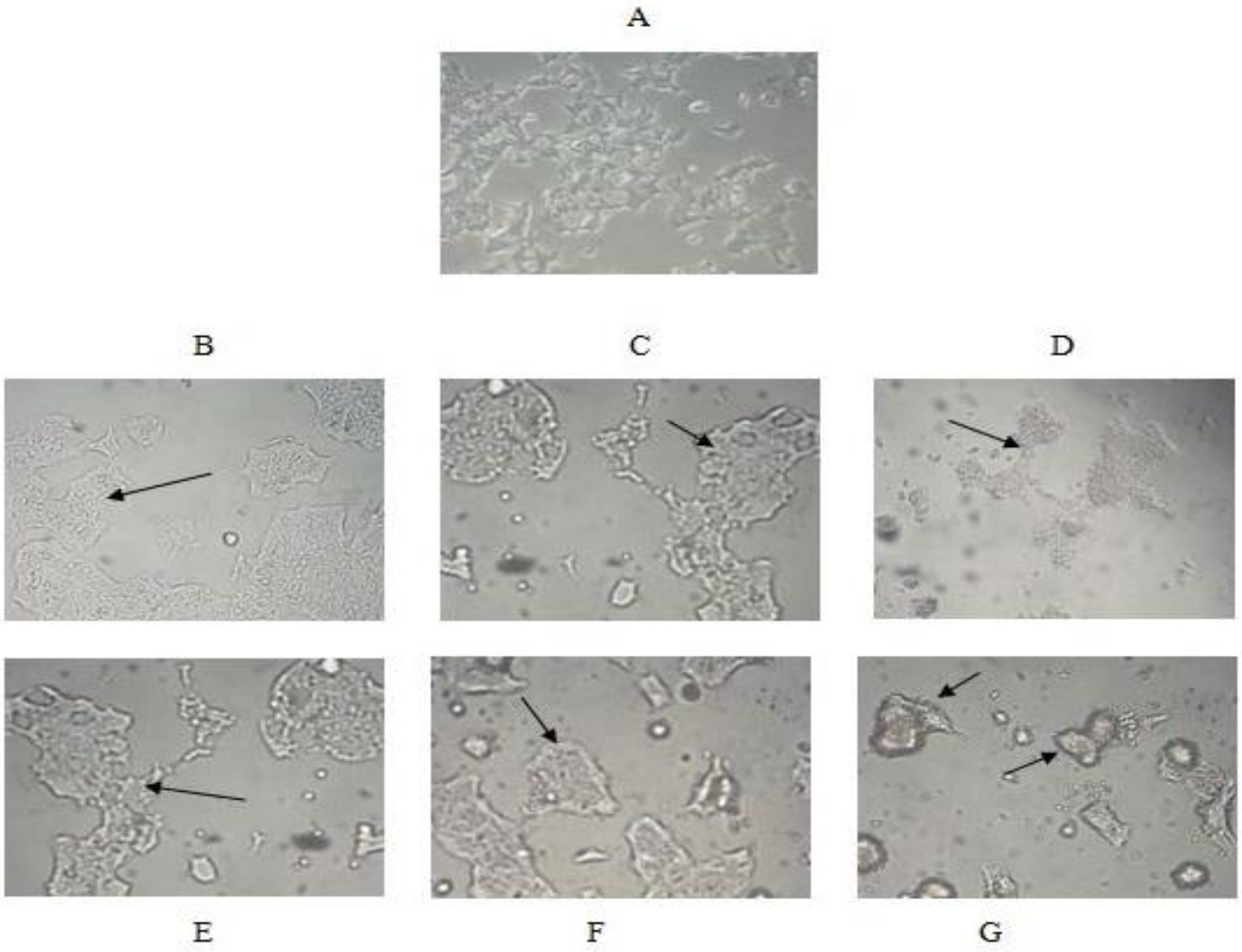

Fig. 4: Morphological analysis:
Fig A. Represents normal HCT-15 cell line (Control)
The cells were treated with AIL at 25 µg/ml, 50 µg/ml and 100 µg/ml concentrations for 24 (B-D) and 48 hour (E-G)

CONCLUSIONS

In conclusion, this was a preliminary work to evaluate *in vitro* anti-proliferative potential of AIL towards HCT-15 cell line. AIL turned out to be cytotoxic for these cells in culture. The results revealed that AIL reduces cell viability and disrupt cellular morphology. DNA fragmentation supports that AIL cause apoptosis of these cells, as DNA fragmentation is considered as secondary consequence of apoptosis. Further studies can be designed to explore the anti-proliferative mechanism at molecular and genetic level. Furthermore, the effect of AIL on cellular morphology revealed that AIL interacted specifically with HCT-15 cells. This finding can be further explored to evaluate AIL as cancer cell biomarker as well as drug delivery agent. Because of their binding specificity, they may act as recognition molecules in the biological system and can be used in diverse fields of biomedical sciences. Furthermore, their property of carbohydrates specificity can also be utilized for target drug delivery by linking lectin to drug molecule.

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