



# ANTI-PROLIFERATIVE EFFICACY OF ALSTONIA SCHOLARIS (L.) R.BR. EXTRACT ON CULTURED DAUDI CELLS: AN IN VITRO APPROACH

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## ABSTRACT

**Objective:** The aim of this study is to observe the apoptosis of *ALSTONIA SCHOLARIS (L.) R.Br.* against Daudi cells and to study its primary mechanism.

**Materials and Methods:** Antiproliferative activity of cultured Daudi cells was evaluated using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay in a dose- and time-dependent manner after treatment with the hydroalcoholic extract of *A. scholaris*. Trypan blue viability assay was also performed. Apoptosis induction in the cells post treatment was determined by DNA fragmentation assay, Agarose gel electrophoresis, and Acridine orange/Ethidium bromide dual staining. Protein isolation and analysis was carried out using the standard polyacrylamide gel electrophoresis protocols.

**Results:** The extracts inhibited the growth and proliferation of Daudi cells through induced cell death, which was dose-dependent and time-dependent. The  $IC_{50}$  value was found to be 260  $\mu\text{g/ml}$  after 72 h of treatment. The induction of DNA fragmentation and increase in a number of apoptotic cells post treatment suggest the possibility of apoptosis induction. A significant decrease in protein level was also observed.

**Conclusion:** The results raise the possibility that the hydroalcoholic extract of *A. scholaris* could be a potent chemotherapeutic agent for the treatment of various cancers. Further evaluation of its potency as a chemotherapeutic agent is imperative.

**Keywords:** Antiproliferation, apoptosis, Daudi cell line, *Alstonia scholaris*

## INTRODUCTION

Cancer is one of the major causes of death globally and it is the second leading cause of mortality after cardiovascular disease. As per the World Health Organization's report, cancer burden doubled in the last three decades worldwide and the number of new cases each year will nearly triple (to 21.4 million) by 2030. In developing countries such as India, the occurrence of variety of cancer is dramatically increasing for various reasons<sup>1</sup>. The causes of cancer include external as well as internal factors, which may act together or in a sequence to cause cancer<sup>2</sup>. Preventing and curing cancer are major world-wide issues due to economic burden on the societies and families caused by this dreadful disease.

Cancer that can cause enlarged lymph nodes is called Lymphoma. Lymphoma is a type of blood cancer that appears in the lymphatic system. According to the GLOBOCAN (IARC) data, 2012, lymphoma is one of the ten most common cancers in the world in 2012. Lymphoma is divided into Hodgkin lymphoma (HL) and Non-Hodgkin lymphoma (NHL). About 90% of lymphoma patients with Non-Hodgkin's Lymphoma and remaining are Hodgkin's Lymphoma<sup>3</sup>.

Non-Hodgkin's lymphoma occurs due to mutations in the immune system, caused by infectious agents, carcinogenic substances and history of other diseases suffered by a person<sup>4</sup>. Burkitt's Lymphoma is a type of Non-Hodgkin Lymphoma, mature neoplasm of B lymphocyte cells. Burkitt's lymphoma is a malignancy of B-lymphocytes that can be cured and is first associated with HIV.

Burkitt's lymphoma was the first human tumour to be associated with a virus (Epstein-Bar Virus) (EBV)<sup>5</sup> as well as with HIV infection<sup>6</sup>. It is one of the first tumours shown to have a chromosomal translocation that activates an oncogene<sup>7-8</sup>. Burkitt's lymphoma is the fastest growing human tumour, with a cell doubling time of 24-48 h, and was the first childhood tumour to respond to chemotherapy alone<sup>9</sup>.

Medicinal plants and their products have been valued for medicinal properties and are known to have potent phytochemical and pharmacological properties. Herbal extracts from medicinal plants are cost effective and have less side effects as well as they are the richest source of metabolites having potent biological activities. It was reported that, *Alstonia* was used to cure fevers, diarrhea and dysentery, skin disorders. The paste of the bark is used to cure chronic arthritis. The bark juice is used to cure sores, tooth pains, ulcers and rheumatism.



Our earlier studies have demonstrated the efficacy of *Mimosa pudica* and *Phyllanthus fraternus* plant extract as well as Kaempferol and L-mimosine compounds as potent anti-proliferative agents<sup>10-11</sup>.

## **MATERIALS AND METHODS**

### **Preparation of Plant Extract**

*A. scholaris* was collected from University campus, and the herbarium specimen was authenticated at the Botany Department. Leaves along with bark of *A. scholaris* were collected, washed and shade-dried under ambient temperature. After complete drying plant was powdered and defatted with petroleum ether (40 °C-60 °C) for 24 h at room temperature with constant shaking. A total of 50 gm of the defatted powdered material was capsulated in filter paper and kept in the thimble, 500 ml solvent (water: ethanol 70: 30) was added into the flask and continuous extraction was carried out in the Soxhlet apparatus for 72-74 h at 60 °C (till the colour in the siphon became colourless). The crude solvent collected in the flask was dried at reduced pressure and kept at 4 °C until further use<sup>12</sup>.

### **Cancer cell culture**

For cancer cell culture, Daudi cell line was obtained from the National Centre for Cell Science (NCCS), Pune. Cells were cultured in RPMI 1640 media with 10% fetal bovine serum (FBS) and antibiotic-antimycotic solution (Hi-Media, Mumbai, Maharashtra, India). Cell cultures were maintained in a CO<sub>2</sub> incubator at 5% CO<sub>2</sub> and 37 °C.

### **Cell viability assay by trypan blue dye exclusion technique**

Any compound, which is cytotoxic to cells, inhibits the cell growth proliferation and kills the cells. Trypan blue is a supravital dye, used to estimate the number of cells present in the population<sup>13</sup>. It can penetrate dead cells and give it a blue colour. This method gives a score of dead and viable cells<sup>14</sup>.

The cellular viability of the cells was measured using trypan blue exclusion assay. Sterility was maintained throughout the procedure. Briefly, 2×10<sup>6</sup> cells were seeded into 24-well plates and treated with or without (as control) hydroalcoholic extract of *A. scholaris* (12.5- 400 µg/ml) for 24, 48 and 72 h. After the incubation period, the cultures were harvested and washed twice with Phosphate Buffered Saline (PBS). The cell pellet was then resuspended with 0.5 ml PBS. Then, 20 µL of cells were mixed with equal volume of 0.4% trypan blue and was counted using a Neubauer haemocytometer by clear field microscopy. Viable and nonviable cells were counted. The percentage of cell viability was calculated using the equation shown below:

$$\% \text{ Viability} = \frac{\text{Live cell count}}{\text{Total cell count}} \times 100$$

### **3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay**

The ability of cells to survive a toxic insult is the basis of most cytotoxic assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. It is described by the modified method of Mosmann and Wilson<sup>15-16</sup>.

The assay detects the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenase to blue formazan product, which reflects the normal function of mitochondria. 2×10<sup>6</sup> viable cells/ml were plated into the 96-well cell culture plate. The hydroalcoholic extract was added with the concentrations (12.5-400 µg/ml), respectively for 24, 48 and 72 h and incubated at 37 °C. After incubation, the supernatants were removed and incubated with MTT (0.5 v/v) in RPMI 1640 without FBS and without phenol red for 4 h in a humidified atmosphere at 37 °C and 5 % CO<sub>2</sub> incubator. The absorbance (A) of the coloured solution was quantified at 540 nm wavelengths by an enzyme-linked immunoabsorbent assay reader (ELISA READER, MERCK MIOS mini). Each extract and control was assayed in triplicate in three independent experiments. Percent growth inhibition of cells exposed to treatments was calculated as follows:

$$\% \text{ Inhibition} = \left( 100 - \left( \frac{\text{Corrected mean Absorbance of sample}}{\text{Corrected mean Absorbance of control}} \right) \right) \times 100$$

### **Agarose Gel Electrophoresis**

DNA fragmentation was assessed by Agarose Gel Electrophoresis of extracted genomic DNA from Daudi cells<sup>17</sup>. 2 × 10<sup>6</sup> cells/ml were taken and treated with the extract at its IC<sub>50</sub> concentration. After 72 h the culture were harvested and washed twice with PBS. The cell pellet was then resuspended with DNA-Xpress reagent (Hi Media, Mumbai, India), homogenized and centrifuged for 10 min at 13,000 rpm. The upper aqueous phase was transferred to another tube and 1 ml ethanol (100 %) was added. The sample was mixed by inverting the tube several times, allowed to stand for 5 mins (25 °C) and centrifuged at 8500 rpm for 4 min. The supernatant was discarded; 1 ml of ethanol was added and mixed by inverting the tube several times. Allowed to stand for 3-4

min and DNA became visible. DNA was washed twice with 1 ml 95% ethanol and dissolved in 8mM NaOH. Electrophoresis was carried out on a 2% agarose gel. The gel was stained with 1µg/ml ethidium bromide (EB) and photographed using gel documentation system.

**Acridine orange/Ethidium bromide fluorescence staining**

Analysis of changes in cell morphology was evaluated using acridine orange/ethidium bromide fluorescence staining according to the method described by Jimenez *et al.*, with slight modification<sup>18</sup>. Briefly 2×10<sup>6</sup> cells were plated in to 24 well cell culture plates and incubated for 72 h with or without (as control) hydroalcoholic extract of *A. scholaris*. The cells were removed from the plate in 1 ml microfuge in PBS pH 7.4. To each sample 1 µl of AO/EB was added just prior to microscope examination. 10 µl of cell suspension was placed on microscope slide and observed under a fluorescence microscope under 40 X Objective.

**Protein estimation**

For protein estimation, cells after dosing with an IC<sub>50</sub> concentration of the extract for 72 h, were then removed and washed with PBS twice. PBS was discarded and cells were suspended in 500 µl RIPA Buffer (G-Biosciences, St. Louis, USA) and kept on ice for 20 min. Cells centrifuged for 15 min at 14000 rpm at 4 °C. The supernatant was taken into another tube for further analysis.

The concentration of protein was determined by Lowry’s method using the Folin-Ciocalteu reagent, with crystalline Bovine Serum Albumin as the standard<sup>19</sup>.

**Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis**

Polyacrylamide gel electrophoresis (PAGE) was performed for protein separation. Cells were treated with the extract at IC<sub>50</sub> concentration for 72 h and protein was extracted from the cells by the above-mentioned protocol. The protein sample was prepared by mixing sample obtained, with sample buffer containing glycerol, 20 % Sodium dodecyl sulfate (SDS), Bromophenol blue dye, 1 M Tris-Cl and β- Mercaptoethanol. 20 µl of samples were loaded into the wells after the polymerization of gel and were run on the electrophoresis unit. The gels were fixed and then stained 0.25 % Coomassie brilliant Blue. After overnight staining, the gels were destained and then photographed and analyzed.

**Statistical Analysis**

Each parameter was performed in triplicate, and the results were expressed as mean ± standard error. The data was statistically analyzed using Student’s *t*-test and the values of *p* < 0.05 were considered statistically significant.

**RESULTS**

**Cell Viability Assay**

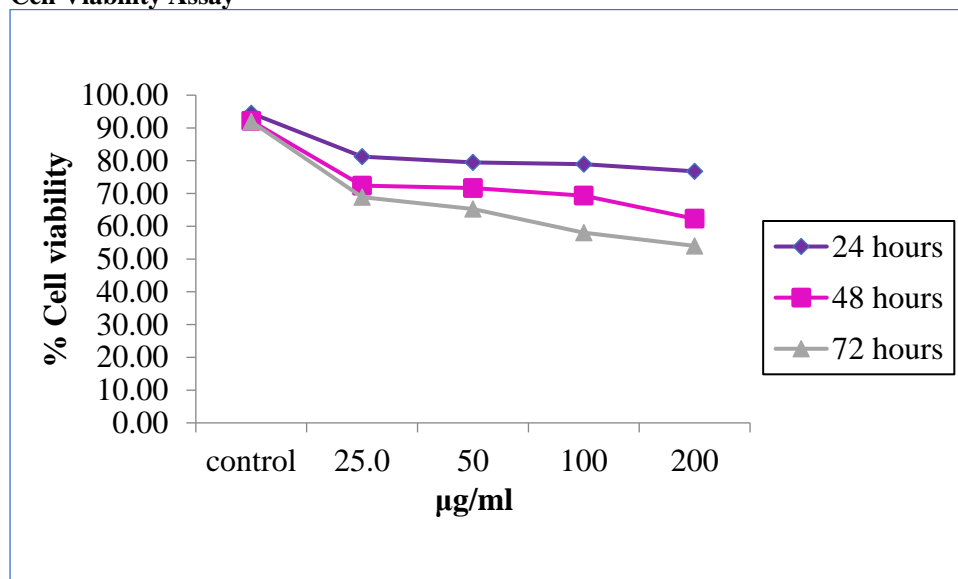


Fig. 1: Showing the effect of *Alstonia scholaris* extract on the percentage viability of Daudi cells. Values are mean ± S.E for the three individual experiments. \*\*= *p* < 0.005, \*\*\*= *p* < 0.001

We evaluated the cell viability assay by trypan blue dye exclusion technique (Fig. 1). This assay showed that there was a highly significant ( $p < 0.001$ ) decrease in viability with an increase in time and concentration in extract treated Daudi cells as compared to untreated controlled cells (Fig. 1).

**3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay**

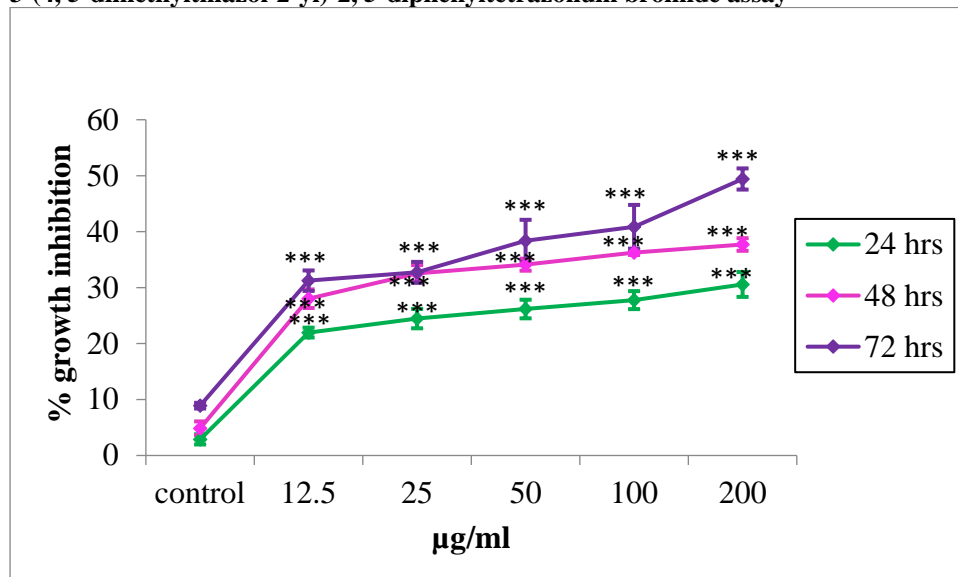


Fig. 2: Showing the effect of *Alstonia scholaris* extract on the percentage decrease of proliferation in Daudi cells. Values are mean  $\pm$  S.E for the three individual experiments. \*\*\*=  $p < 0.001$ .

Using MTT assay, the effect of the hydroalcoholic extract of *A. scholaris* on the proliferation of Daudi cell line was studied after 24, 48 and 72 h of incubation. As shown in Fig. 2, the treatment of Daudi cells with the extract lead to inhibition in the cell proliferation as concluded by the  $IC_{50}$  value 260  $\mu\text{g/ml}$ . This revealed a moderate anti proliferative activity of the extract against Daudi cells. Increasing the concentrations of the extract in the culture medium of Daudi cells resulted increase in the percentage of inhibition of proliferation with increased time duration as compared to untreated control cells ( $p < 0.001$ ).

**Agarose gel electrophoresis**

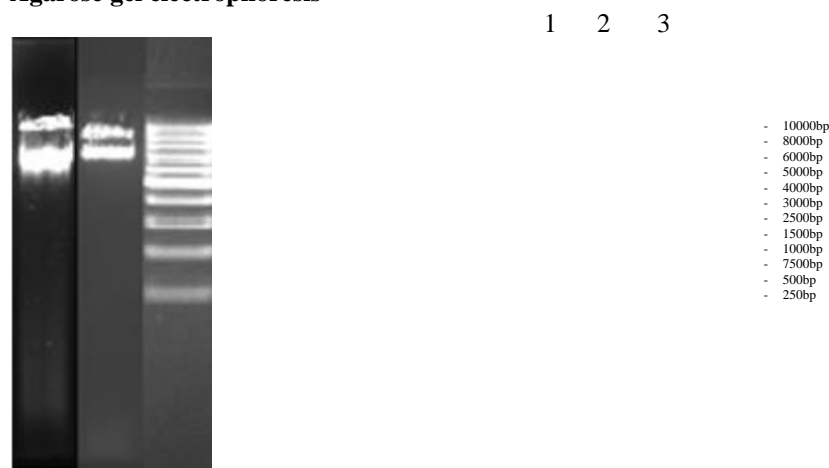


Fig. 3: Showing DNA pattern of treated and untreated Daudi cells at 72 h after maximum dosing, along with marker on agarose gel electrophoresis.

Lane 1 – Showing effect of *Alstonia scholaris* extract on Daudi cell DNA

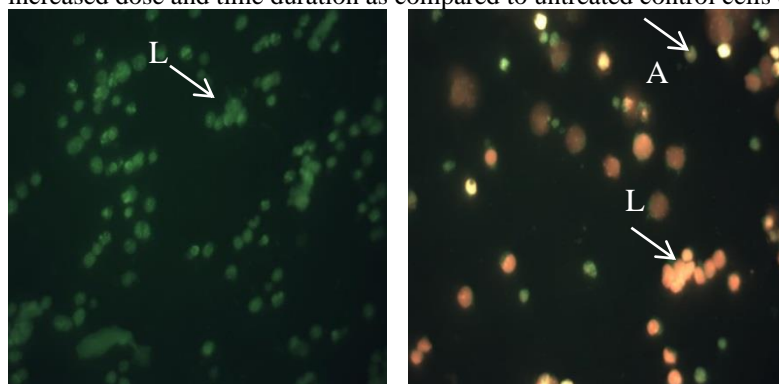
Lane 2 – Untreated control Daudi cell DNA

Lane 3 – DNA ladder

Cleavage of chromosomal DNA into oligonucleosomal size fragments is an integral part of apoptosis. The conventional agarose gel electrophoresis was performed on the Daudi cells treated at  $IC_{50}$  dose concentration of hydroalcoholic extract for 72 h. The results showed that DNA cleavage produced no ladder pattern for the hydroalcoholic extract-treated cells (Fig. 3). The DNA may be intact, or no DNA fragmentation was detected.

**Acridine orange/ethidium bromide double staining**

The control and treated Daudi cells were stained with AO/EB dual stain where the live cells took up green colour and the apoptotic cells/nonproliferative cells were seen as yellowish orange. The result obtained depicted that there was a significant increase ( $p < 0.001$ ) in number of apoptotic cells/non-proliferative cells with increased dose and time duration as compared to untreated control cells (Fig. 4).



Control    260 μg/ml

Fig. 4: Apoptotic changes of Daudi cells detected with acridine orange/ ethidium bromide staining and visualized under fluorescence microscope (40X) L- Live cells and A- apoptotic cells/ non-proliferative cells.

**Protein estimation**

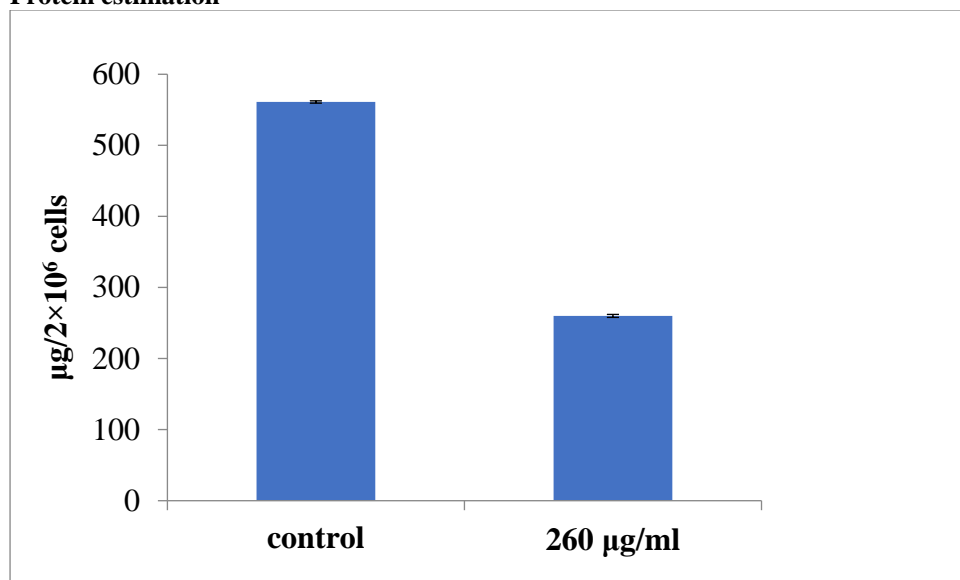


Fig. 5: Showing alterations in levels of total proteins after treatment with *Alstonia scholaris* extract compared with untreated control Daudi cells. Values are mean ± S.E for the three individual experiments. \*\*\*=  $p < 0.001$  To further confirm the inhibitory effect of hydroalcoholic extract at cellular level, Daudi cells were treated with an  $IC_{50}$  dose concentration of extract followed by protein estimation. Cells treated with the extracts showed a significant decrease in the protein levels as compared to untreated control Daudi cells (Fig. 5).

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis**

1    2    3

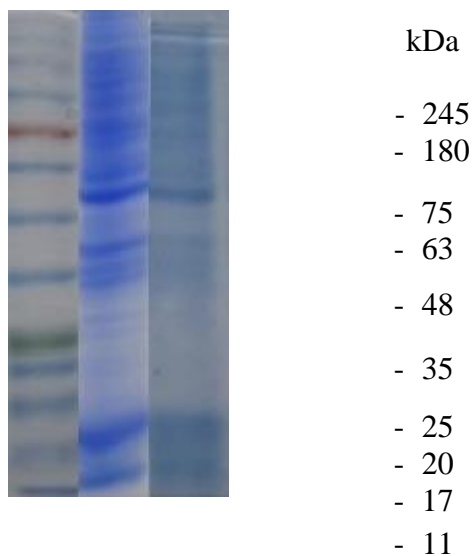


Fig. 6: Showing separated protein pattern of treated and untreated Daudi cells at 72 h after maximum dosing, along with marker on Polyacrylamide gel electrophoresis.

Lane 1- Protein Marker

Lane 2- Untreated control Daudi cell protein

Lane 3- Showing effect of *Alstonia scholaris* extract on Daudi cell protein

The results of SDS PAGE had shown clear dark bands of proteins having an approximate molecular weight of 200, 180, 75, 63, 50, 45, 35, 15 and 11 kDa in control Daudi cells. One prominent band between 63 and 48 kDa and two bands between 17 and 11 kDa were observed in the gel pattern after cells were treated with IC<sub>50</sub> concentration for 72 h with the extract of *Alstonia* (Fig. 6).

## DISCUSSION

Medicinal Plants are considered as rich sources of phytoconstituents which can be used in drug development. These biologically active molecules are used for treatments of varieties of disease. *A. scholaris* is a well-known herbal medicinal plant throughout the world. Many studies have reported the pharmacological efficacies and benefits of *A. scholaris*.

Trypan Blue staining was used to observed cell viability. It was verified the influence of the plant extracts in mediating cell death. As reported, cell survival was lowered in a time and dose dependent manner. Hence, it could be hypothesized that decrease in cell viability could be attributed to *A. scholaris* extract/compounds induced cell death as confirmed by cell viability trypan blue assay. It was reported that triterpenoids and sterols isolates from leaf extract of *A. scholaris* showed anti-proliferative activity against non-small-cell lung carcinoma cells<sup>20</sup>. Other *in vitro* study results showed that the hexane extract of *A. scholaris* stem bark had cytotoxic and antioxidant properties on Dalton's lymphoma ascetic (DLA) cells<sup>21</sup>. As observed in another study, a combination of alkaloids and triterpenes of *A. scholaris* leaves enhances immunomodulatory activity in C57BL/C mice *in vivo* and induces apoptosis in the A549 cell line *in vitro*<sup>22</sup>. Our *in vitro* data indicated that treatment of Daudi cells with *Alstonia* extract resulted in inhibition of proliferation of Daudi cells. Any decline in the number of metabolically active proliferating cells might mean that the proliferation pathway itself was halted (mitotic arrest), or that fraction of the cells went through a death pathway<sup>23</sup>. Hence, it could be postulated that the reduction in cell proliferation, as registered by the MTT assay could be attributed to *A. scholaris* extract induced cell death.

The changes that occurred in apoptotic cells/non-proliferative cells were perceived through AO/EB staining and this helped in assuming that the cell death observed was not due to necrosis, but may be due to apoptosis. Bagheri *et al.* have shown induction of cell specific apoptosis in DLA cells *in vitro*. In our study, when cells stained with AO/EB, it was observed that there was higher percentage of cells with orange-red nuclei with condensed chromatin which possibly indicate apoptotic cells<sup>21</sup>.

To further determine the effect of *A. scholaris* extract at cellular level, Daudi cells were treated with IC<sub>50</sub> dose concentration of extract followed by SDS PAGE analysis of its cell lysates. Administration of the *A. scholaris* extract brought about a significant decrease in the total protein content indicated loss of protein as depicted on the scanned gels by loss of protein bands.

Feng *et al.* have shown positive result with various extract of *A. scholaris* against A549 cell line. Their findings indicated that alkaloids or triterpenes of *A. scholaris* leaves might have significant synergistic effect to



induce the apoptosis of A549 cells by regulating pro-casp8 and caspase-8 levels<sup>22</sup>. Our *in vitro* data indicate that treatment of Daudi cells with *Alstonia* extract resulted in to significant inhibition of proliferation could be due to suspension of multiple signaling pathway as well as protein-protein interaction or with phytoconstituents of the extract.

## CONCLUSION

The findings of our present investigation indicate that the hydroalcoholic extract of *A. scholaris* extract showed positive effects against Daudi cells. The results reveal that *A. scholaris* has an antiproliferative effect by inducing apoptotic cell death. The mode of action of the phytocomponents present in this plant is still unclear and understanding the detailed mechanism will help in improving useful information for their possible application in cancer prevention and therapy.

## ACKNOWLEDGEMENT

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