In vitro studies on the growth inhibitory effects of the lectin (MLL) purified from M. alba L. leaves

4.1. INTRODUCTION

Lectins are proteins/glycoproteins of non-immune origin which are capable of reversibly binding with specific carbohydrates (Goldstein et al., 1980). The specificity of lectins towards carbohydrates have made them molecules of great interest in cancer studies as researchers have found that lectins have the ability to distinguish between malignant and non-malignant cells (Sharon and Lis, 2002). Programmed cell death (apoptosis) is the most common way that a cell maintains homeostasis and balance the number of living cells. Cancer results from uncontrolled growth of cells and the mechanism of programmed cell death is at stake in cancer cells. Lectins target various pathways in apoptosis and steer the cell to death. Mistletoe lectins (MLs), ricin, concanavalin A (ConA) and Polygonatum cyrtonema lectin (PCL) brings about programmed cell death in cancer cells by targeting the apoptotic pathways (Fu et al., 2011). The mode of action of lectin in inducing apoptosis varies in each type of cell. A few lectins induce apoptosis by down-regulating the apoptotic proteins of Bcl-2 family whereas, few others act through inhibition of telomerase activity by transcriptional down regulation of proteins. Up-regulation of Bax through p53- and p21-independent pathway is another mode of action taken up by lectins from other sources. Upstream and downstream regulation of many proteins including caspases is observed and it is important to have an idea of the mode of action of lectin in terms of apoptosis induction (Choi et al., 2004). The present chapter deals with checking the ability of mulberry leaf lectin (MLL) to induce apoptosis. Various assays were carried out to establish the mechanism behind cell death induction by MLL.
4.2. METHODS

4.2.1 CELL CULTURE

The cell lines (MCF-7 and HCT-15) were purchased from NCCS (National Centre for Cell Science), Pune, India. The cells were maintained in DMEM and RPMI 1640 respectively, supplemented with 10% fetal bovine serum (FBS), 100 mg/l streptomycin and 100 IU/ml penicillin, at 37°C in a 5% CO₂ incubator.

4.2.2. MTT ASSAY

The cytotoxicity assay (MTT assay) is based on the reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase to an insoluble purple coloured formazan product. The formazan crystals formed were solubilised with an organic solvent like isopropanol or dimethylsulphoxide and measured colorimetrically at 570 nm.

The cytotoxic effect of MLL on the cells was measured by MTT colorimetric assay, following the protocol of Scudiero et al. (1988) with slight modification. Cancer cell lines (1 × 10⁴ cells - both MCF-7 and HCT-15) in their exponential growth phase were seeded into each well of a 96-well culture plate and incubated overnight. After attachment, the cells were treated with different concentrations of purified MLL and incubated for 24 h. After incubation, 25 µl of MTT (5 mg/ml) was added to the cells and kept for 4 h at 37°C in the incubator. The supernatant was removed and the formazan crystals formed were dissolved in 100 µl DMSO and the absorbance was measured at 570 nm using a micro plate reader (BioRad). The per cent viability of the cells was calculated as,

\[
\% \text{ viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100.
\]

The minimum concentration of extract/compound required to cause 50% inhibition of cell growth (GI₅₀) was calculated. According to National Cancer Institute, concentration of any crude drug below 30 µg/ml is good enough to be considered as having cytotoxic effect (Suffness and Pezzuto, 1990).
4.2.3. MORPHOLOGICAL ANALYSIS

Generally, cells going through apoptosis show certain characteristic changes in morphology like reduction in cell volume leading to cell shrinkage, chromatin condensation, formation of cytoplasmic blebs, etc. that can be observed under microscope. Cells (1x10^4 cells/ml of both MCF - 7 and HCT - 15) were grown in 96-well plates and treated with GI_{50} concentration of MLL and cisplatin (used as positive control) for 24 h. The confluent monolayer cells were then washed with 1X phosphate buffer saline (PBS). Morphological changes in the cells after treatment with MLL and cisplatin were observed using a phase contrast microscope (Axiovert 40® , Carl Zeiss Argentina) attached with camera. Untreated cells served as control.

4.2.4. ACRIDINE ORANGE/ETHIDIUM BROMIDE (AO/EB) STAINING

The cells are generally stained with fluorescent dyes like acridine orange and ethidium bromide to evaluate the nuclear morphology of apoptotic cells. Acridine orange is a vital dye that will stain both live and dead cells whereas ethidium bromide will stain only those cells that have lost their membrane integrity. After staining, the cells are variously colored by the fluorescent dye - green represent viable cells, yellow represent early apoptotic cells and reddish or orange represents cells in late stage of apoptosis. Morphological features like chromatin condensation, membrane blebbing, fragmented nuclei, etc. are clearly visible after staining, which helps in the further identification of apoptotic cells.

AO/EB staining was performed to check the level of apoptosis according to the protocol of Ariffin et al. (2009) with slight modification. MCF - 7 and HCT - 15 cells were treated with GI_{50} concentration of MLL and cisplatin for 48 h. After the incubation period, the cells were washed with PBS and trypsinised. Untreated cells served as control. 25 µl of cell suspension (1x10^4 cells) was incubated with 1 µl of acridine orange/ethidium bromide (one part each of 100 µg/ml acridine orange and 100 µg/ml ethidium bromide in 1X PBS) prior to microscopy. 10 µl aliquot of the gently mixed cell suspension was placed on microscope slide covered with cover slip and examined under fluorescent microscope (Olympus 1X 71) connected with a digital imaging system.
4.2.5. DNA FRAGMENTATION (DAPI STAINING)

DAPI (4',6-diamidino-2-phenylindole) is a fluorescent dye that stains dsDNA by binding with the minor groove of DNA at regions that are rich in AT (Reddy et al., 1999; Farahat et al., 2010). The morphology of the nucleus that had undergone apoptosis on treatment with MLL was visualized by staining the cells with DAPI according to the protocol of Chazotte (2011) with slight modification. MCF - 7 and HCT - 15 cells were treated with MLL and cisplatin (GI_{50} concentration) for 48 h. Untreated cells served as control. After the incubation period, the cells were washed with PBS and fixed with 4% PFA (Para Formaldehyde - 60 µl/well) for 10 min. The cells were washed thrice with 1X PBS for 5 min each. The cells were finally stained with 50 µl of DAPI (1:2000 dilutions with 1X PBS from the 5 mg/ml stock solution) solution for 5 min. The solution containing DAPI was removed and the cells were hydrated with 50 µl of 1X PBS and analyzed using Fluorescent microscope (Olympus 1X 71).

4.2.6. ANNEXIN V STAINING

Under normal physiological situations, the inner leaflet of plasma membrane (PM) facing the cytosol is predominantly occupied by aminophospholipids like phosphatidylserine (PS), a negatively charged phospholipid. On the other hand, under circumstances where the cell is pathologically unfit, apoptosis is initiated and PS leaks out to the outer membrane of the PM where it is asymmetrically distributed (Zhang et al., 1997). As a consequence of the translocation of PS to the extra-cellular membrane, the cells become vulnerable to phagocytosis. The translocation of PS from inner to outer leaflet of PM is an initial but widespread event in apoptosis. PS can be detected by fluorescently labelled annexin V in a calcium-dependent manner (Vermes et al., 1995).

Annexin V belongs to a family of calcium-dependent phospholipid-binding proteins (Annexins - of approx. 36 kDa) that have a high affinity to PS in the presence of physiological concentrations of calcium (Ca^{2+}). During apoptosis, permeabilization of plasma membrane allows Annexin V to enter cells and bind with PS present on the PM. When labelled with a suitable fluorescent dye like AnnCy3 or 6- carboxyfluorescein diacetate (6-CFDA), Annexin V can serve as a sensitive probe for the exposed PS determination. In the present study, Annexin V Cy3™ (APO–AC, Sigma) was used.
according to the manufacturer’s instructions to measure the apoptosis induction by MLL on MCF-7 and HCT-15 cells. The kit contained two fluorescent labels to measure cell viability and apoptosis. Non-fluorescent 6-CFDA is hydrolyzed to a green fluorescent compound, 6-carboxyfluorescein (6-CF), by esterase present in the live cells. Annexin-Cy3.18 (AnnCy3) binds to PS present on the outer leaflet of the plasma membrane of cells. Live cells are labelled only with 6-CF and appear as green while the cells in the early stage of apoptosis are labelled with both AnnCy3 (stained red) and 6-CF (stained green).

After incubating with MLL and cisplatin for 24 h, the cells (1x10^6) were washed twice with PBS and re-suspended in 1 ml PBS. 50 µl each of the sample and control cells were added to a microscope slide and excess sample was removed by blotting with filter paper and left undisturbed for 10 min at room temperature. 50 µl of the staining solution (AnnCy3 and 6-CFDA) was added and incubated for 10 min at room temperature. The cells were washed with 50 µl of 1X binding buffer to remove excess label from the cells. Finally, 35 µl of 1X binding buffer was added to the sample and the results were observed using a fluorescence microscope and photographed. Care was taken to use the correct filter and light source depending on the label.

4.2.7. CASPASE 3 ASSAY

Caspases are important mediators of apoptotic cell death. Caspase 3 is one of the critical and most actively involved proteins in the caspase cascade of apoptotic machinery. It is responsible for the proteolysis/hydrolysis of a large number of substrates in the cascade (Cohen, 1997).

Principle of the assay:

The hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroaniline (Ac-DEVD-pNA) by caspase 3 to acetyl-Asp-Glu-Val-Asp and p-nitroaniline (pNA) moiety is the basis of the assay. pNA has a high absorbance at 407 nm. The concentration of the pNA released from the substrate can be calculated from the absorbance values at 407 nm or from a calibration curve prepared with defined pNA solutions. The yellow color produced by the pNA solution is a direct measure of the amount of caspase present in the sample. In the present study, caspase activity was measured in the cell lysate by
means of a spectrophotometric assay kit (Caspase 3 Assay Kit, Colorimetric, Sigma-Aldrich, USA), following manufacturer’s instructions. Apoptosis was induced by adding GI₅₀ concentration of lectin and cisplatin in HCT - 15 cells (10⁶ cells) and incubated overnight. A sample of non induced cells was reserved for use as control.

**Preparation of cell lysate:**

After incubation in the CO₂ incubator, the cells were pelleted by centrifugation (600xg for 5 min at 4°C). The supernatants were removed and pellets were washed with 1 ml PBS, centrifuged and pellet suspended in 1X lysis buffer (100 µl/10⁶ cells). The cells were incubated on ice for 15-20 min and the lysed cells were centrifuged at 16000 to 20000xg for 10-15 min at 4°C. The supernatants were then transferred to new tubes. The lysates were used immediately and remaining samples were frozen in liquid nitrogen and stored as aliquots in −80°C. Protein was estimated by BCA method using the protein determination kit.

**Assay procedure:**

5 µl of cell lysate or caspase 3 positive control was placed in the appropriate wells and 1X assay buffer was added to each of the wells. Caspase 3 inhibitor was added to the appropriate wells and the reaction was started by adding 10 µl of caspase 3 substrate to each well and mixed gently by shaking. Care was taken to avoid formation of bubbles in the wells. The plate was covered and incubated over night at 37°C. Color developed was read at 407 nm. The results were calculated using a p-nitroaniline calibration curve.

**Plotting standard curve:**

A series of p-nitroaniline solutions (ranging from 3.125 to 100 µM) were prepared by diluting the p-nitroaniline stock solution in 1X assay buffer. 100 µl of each dilution was added to respective wells, with 100 µl of assay buffer used as a blank. The absorbance was read at 405 nm. A calibration curve of the absorbance values versus the concentrations of the p-nitroaniline solutions was plotted. The caspase 3 activity was expressed as micromoles of pNA released per min per mg of cell protein.
4.2.8. EFFECT OF LECTIN ON DIFFERENT PHASES IN THE CELL CYCLE OF CANCER CELLS (FACS ANALYSIS)

The possibility that anticancer agents induced apoptosis via cell cycle arrest cannot be ruled out. Several reports state that cell cycle arrest occurs as part of or after apoptosis induced by anticancer drugs (Lyu et al., 2001). Normal cells follow a definite path/cycle of events and this helps in maintaining the system in equilibrium. Any disorder or disease condition will disrupt this order and the variations are reflected in cell cycle also. Accordingly, cell cycle analysis is a good means to measure and validate the extent of damage that has happened to the cell. Determining the cellular DNA content as well as the identification of the cell distribution during various phases of the cell cycle is possible by cell cycle analysis using flow cytometry (Nunez, 2001). There are four distinct phases of proliferating cell populace that could be distinguished by flow cytometry methods – the G0/G1, S, G2 and M phases.

The cells (1x10^6) treated with MLL and cisplatin (GI_{50} concentration) were trypsinized, washed in PBS and centrifuged at 200xg for 5 min. The cell pellet was re-suspended in PBS and fixed in cold (-20°C) ethanol (70% v/v) on ice. The sample was incubated on ice overnight at 4°C. The cells were later centrifuged and ethanol was decanted. The cells were resuspended in PBS and centrifuged at 200xg for 5 min. The cell pellet was resuspended and mixed with propidium iodide mixture (100 µg/ml RNase A and 50 µg/ml propidium iodide in PBS) and incubated at 37°C for 30 min in dark. The cells were then analyzed by flow cytometry (BD FACS Calibur, USA) to check the distribution of DNA in different phases of cell cycle. Approximately 10,000 cells were used for the generation of cell cycle distribution analysis using flow cytometry.

4.3. STATISTICAL ANALYSIS

Results were expressed as the average of three independent experiments ± standard deviation (SD). Data were analyzed by one-way ANOVA using SPSS statistical analysis programme and differences were considered as statistically significant at p < 0.05.
4.4. RESULTS

4.4.1. GROWTH INHIBITORY STUDIES

The effect of MLL on the growth of MCF - 7 and HCT - 15 cells were studied by treating the cells with different concentrations of MLL (0-35 µg/ml) for 24 h. The viability of cells was checked by MTT assay. A concentration dependent effect on the growth inhibition of cells was observed. The concentration of MLL required for 50% inhibition of cells (GI\textsubscript{50}) was calculated as 8.5 µg/ml for MCF - 7 and 16 µg/ml for HCT - 15 cells (Figure 4.1). The GI\textsubscript{50} of the standard drug cisplatin was also checked (Figure 4.2). It was found that 2 µg/ml of cisplatin was required to bring down the viability of MCF - 7 to 50% where as only 1 µg/ml was needed in the case of HCT - 15.
Figure 4.1. Effect of MLL on growth of MCF - 7 and HCT - 15

MCF - 7 and HCT - 15 cells were treated with different concentrations of MLL for 24 h and the percentage of viable cells was calculated by MTT assay by comparing with control. Data represented are values ± SD of three independent experiments.

MCF - 7
$GI_{50} = 8.5 \mu g/ml$

HCT - 15
$GI_{50} = 16 \mu g/ml$
4.4.2. MORPHOLOGICAL ANALYSIS

The morphology of the cells treated with MLL was observed using phase contrast microscope (Figure 4.3). Highly conserved changes including detachment from the surface, cell shrinkage, membrane blebbing, apoptotic vacuole formation, etc. were observed in cells treated with MLL. Untreated cells were normal in morphology with well spread, flattened, polygonal and confluent in appearance, with normal rate of proliferation. The results were comparable to the morphological changes associated with the positive control, cisplatin.
4.4.3. ACRIDINE ORANGE/ETHIDIUM BROMIDE STAINING

The apoptosis inducing ability of MLL was tested by AO/EB staining. Acridine orange is a vital dye that stains both live and dead cells whereas ethidium bromide is taken up only by those cells that have lost their membrane integrity. MCF - 7 and HCT - 15 cells were treated with GI\textsubscript{50} concentration of MLL for 24 h and stained with the solution containing acridine orange and ethidium bromide. Viable cells appeared green because of the incorporation of acridine orange and cells in the later stages of apoptosis appeared orange due to co-staining with acridine orange and ethidium bromide, due to membrane leakage (Figure 4.4). The results were compared with cisplatin treated cells.
4.4.4. NUCLEAR FRAGMENTATION BY DAPI STAINING

The later stages of apoptosis involve changes in the nuclear morphology such as chromatin condensation and DNA fragmentation. DAPI was used to stain the cells to analyze the nuclear morphological changes associated with MLL treatment. DAPI can bind to the minor groove of the adenine-thymine regions of DNA and emit light at 488 nm, observed as blue fluorescence. Morphological changes such as nuclear fragmentation and chromatin condensation associated with the apoptotic cells were visible on treatment with MLL and cisplatin (Figure 4.5). Control cells were uniformly stained with DAPI and appeared as round with normal morphology, without any fragmentation.

\[ MCF - 7 \text{ and } HCT - 15 \text{ cells, after treatment with MLL and cisplatin, were stained with acridine orange/ethidium bromide. Live cells were observed as green, whereas the apoptotic cells appeared as orange-red due to co-staining with ethidium bromide as a result of loss of membrane integrity (Magnification 40x)} \]
4.4.5. ANNEXIN V STAINING

HCT - 15 and MCF - 7 cells were treated with MLL and cisplatin for 24 h, and annexin V staining was carried out according to the protocol described in methods. Live cells, as in control, were stained green and apoptotic cells were stained orange-red (Figure 4.6). The percentage of annexin V stained cells were calculated by counting the number of cells stained in different fields under microscope. Annexin V positive cells were found to be 46% for MCF - 7 and 15% for HCT - 15 under MLL treated condition and 41% and 35% for MCF - 7 and HCT - 15 respectively, under cisplatin treated condition (Figure 4.7).
MCF-7 and HCT-15 cells were treated with MLL and cisplatin for 48 h and stained with annexin V and observed under fluorescent microscope (Magnification 40x).

The number of annexin V positive cells (from a total number of 500 cells) was counted in different fields under microscope and the percentage was calculated. Values given are average of three independent experiments ± SD (*p < 0.05 when compared with control).
4.4.6. CASPASE 3 ASSAY

Caspase 3 activity of MLL and cisplatin treated HCT - 15 cells were measured along with control using the assay kit. Amount of caspase 3 released was calculated from the standard curve of pNA and expressed as micromoles of pNA released per minute per ml of cell lysate (Figure 4.8). A significant increase in the amount of caspase 3 in the cell lysates of MLL treated cells (83 micromoles of pNA per min per ml cell lysate) was observed compared to control (2.5 micromoles of pNA per min per ml cell lysate). Cisplatin was used as the positive control, where the caspase 3 release was estimated to be 150 micromoles of pNA per min per ml cell lysate. MCF - 7 cells do not express caspase 3 gene.

![Figure 4.8. Effect of MLL on caspase 3 Activity](image)

*Caspase 3 activity was determined in the lysates of control and treated cells using colorimetric assay kit. Values presented were the mean ± SD of three independent experiments (*p < 0.05 when compared with control)*
4.4.7. CELL CYCLE ANALYSIS BY FLOW CYTOMETRY

The cells treated with MLL and cisplatin were harvested and processed as described in methods for cell cycle analysis. Results indicated that in both MCF-7 and HCT-15 cells, MLL and cisplatin treatment led to massive decrease in the number of cells in the G0–G1 phase (Figure 4.9 and Figure 4.10 respectively). An increase in the number of cells in G2-M and sub G0–G1 phases was observed, indicating cell cycle arrest at G2-M phase and subsequent apoptosis. 41% of MCF-7 cells was found to be in sub G0–G1 phase when treated with MLL and cisplatin. Sub G0–G1 value of 12% and 33% were observed in HCT-15 cells treated with MLL and cisplatin, respectively.

Figure 4.9. Cell cycle analysis and percentage of cells in each phase of the cell cycle of MCF-7 cells treated with MLL and cisplatin

MCF-7 cells treated with MLL and cisplatin for 24 h were analyzed by flow cytometry for the distribution of cells in different phases of cell cycle. Also, the percentage of cells (from a total of 10,000 cells) in each phase was analyzed after staining with propidium iodide (represented as the bar diagram). Data presented are mean ± SD of three independent experiment ( * p < 0.05 when compared with cells in the sub G0-G1 phase of untreated control)
4.5. DISCUSSION

Applications of lectins in cancer diagnosis and treatment have been reported in many studies (Gorelik et al., 2001; Thies et al., 2007). They are capable of binding to the cell membrane receptors causing cytotoxicity and apoptosis (Lyu et al., 2002; Huang et al., 2012). According to a recent report by Rambaruth et al. (2012), *Helix pomatia* agglutinin (HPA) differentially recognized altered cell surface glycosylation on cancer cells under different metastatic conditions leading to the identification of specific biomarkers to aggressive metastatic phenotypes. Two lectins, wheat germ agglutinin (WGA) and *Ulex europaeus* agglutinin (UEA) were used as mediators for targeted drug
delivery in human 5637 bladder cancer cells (Plattner et al., 2008). The present study was aimed at elucidating the cell death inducing activity of a lectin purified from *M. alba* leaves. Plants belonging to the genus *Morus* are well known for various active constituents that are found to be effective as hepatoprotective, antiinflammatory and antiproliferative agents (Kikuchi et al., 2010; Dat et al., 2010; Naowaratwattana et al., 2010; Kapche et al., 2011; Tan et al., 2012). Studies revealed that mulberry fruit extract (*Morus fructus*) induced human glioma cell death in vitro through ROS-dependent mitochondrial pathway and inhibited tumor growth in vivo by means of reduction of tumor cell proliferation and induction of apoptosis (Jeong et al., 2010). Antiproliferative effect of methanol extract (Chon et al., 2009), flavanoids (Kikuchi et al., 2010), polyphenols (Naowaratwattana et al., 2010) and prenylated flavanoids (Dat et al., 2010; Kimura et al., 2010) from *M. alba* L. has been studied earlier. Anticancer properties of lectins purified from Morus species were not yet studied and the present study is the first report on the apoptosis inducing activity of a purified lectin from *M. Alba* L. Concentration dependent study on the effects of MLL on the proliferation of MCF-7 and HCT-15 cells showed significant growth inhibition. The GI<sub>50</sub> value of mulberry leaf lectin for MCF-7 and HCT-15 cells was calculated as 8.5 µg/ml for MCF-7 cells and 16 µg/ml for HCT-15 cells (Deepa and Priya, 2012).

Even though this is the first report on the anti-proliferative effect of mulberry lectin, lectins from other plant species have been already reported for their anti-proliferative effects in various cancer cells (Kaur et al., 2011; Zhang et al., 2009). The antiproliferative effect of GPL from *Gonatanthus pumilus* D. Don was evaluated against cancer cell lines and it was found that the lectin produced 50% inhibition at a concentration of 44 µg/ml against HCT-15 and 64 µg/ml against HT-29. The same effect was not observed for MCF-7, Hep-G2 and PC-3 cell lines even at a concentration of 100 µg/ml. The variation in the concentration required to bring about 50% inhibition of cells in different cell lines may be due to the presence of varying glycoconjugates present on the surface of the cells (Dhuna et al., 2007).

Untreated cells (MCF-7 and HCT – 15) exhibited normal proliferation; the cells were flat and well-spread. However, on treatment with MLL, the morphology of the cells changed significantly as revealed by the phase contrast images. The changes observed
were comparable to cisplatin treated cells. Cells treated with MLL showed cell shrinkage, membrane breakage and few cells were found detached and floating in the medium. Pouterin, a cytotoxic lectin-like protein isolated from the seeds of *Pouteria torta* also induced similar morphological changes in liver and colon cancer cells (Boleti *et al.*, 2008). Jurkat cells on treatment with Korean mistletoe lectin (KML-C) caused apparent changes in the appearance of the cells which are characteristics of apoptotic cells. Chromatin condensation, formation of apoptotic bodies, *etc.* were visible. The cells exhibited fragmented DNA in a dose dependent manner (Park *et al.*, 2001).

Acridine orange is taken up by both live and dead cells. The stain intercalates into DNA and thus the cells will have bright green color. Ethidium bromide is taken up only by non viable or dead cells as the plasma membrane is disintegrated revealing the intercalated dye. Accordingly, nucleus of the dead cells (MLL treated) appeared reddish orange (the ethidium overwhelms the acridine) due to membrane breakage indicating that MLL could induce apoptosis. Ho *et al.* (2009) reported that considerable amount of cell death was induced by vanillin in human colorectal cancer cell line HT-29 as observed by acridine orange-ethidium bromide staining.

Nuclear fragmentation, the prominent feature of apoptotic cells, was detected by DAPI staining and the morphological changes of nucleus were characteristic of apoptosis. The results of the present study was in concurrence with that of the anti-proliferative and apoptosis inducing effect of a lectin from *Astragalus mongholicus* (AMML) on human tumor cell lines as reported by Yan *et al.* (2009). They accounted that the control cells were normal in their morphology and nuclei were round while AMML-treated cells revealed disintegration of nuclear body and chromatin condensation at the periphery of nuclear membrane.

Annexin V preferentially binds to phosphatidyl serine (PS) on the outer membrane of cells undergoing apoptosis and fluorescently labeled annexin V can be used to detect cell surface PS in a calcium dependent manner (Zhang *et al.*, 1997). Positive staining of annexin V on mulberry leaf lectin and cisplatin treated cells indicated an early onset of apoptosis. The apoptosis induction by lectins purified from *Phaseolus vulgaris* and *Viscum album* on MCF - 7, HepG2 and murine thymocytes were also studied by annexin V staining (Chan *et al.*, 2012; Hajtó *et al.*, 2003).
Caspases are an important class of proteins involved actively in apoptotic regulation; they cleave a vast array of proteins. To be precise, they are a group of aspartic acid specific cysteine proteases, synthesized aszymogens and involved in the initiation and execution of apoptosis once they get activated by proteolytic cleavage pathways. Although a variety of them are involved, caspase 3 is the best studied and the one playing central role in all the pathways leading to apoptosis (Lam and Ng, 2011). Studies on the mechanism of apoptosis induction by mistletoe lectin showed the existence of a p53/p21 independent pathway. The results indicated that both p53-positive (SK–Hep-1) and p53-negative (Hep 3B) cells underwent apoptosis by down-regulation of Bcl-2 and up-regulation of Bax functioning upstream of caspase 3 (Lyu et al., 2002). Recombinant mistletoe lectin induced p53 independent apoptosis in cancer cells (Hostanska, et al., 2003). *Polygonatum cyrtonema* lectin induced apoptosis in murine sarcoma cells in a caspase dependent manner via blocking of Ras–Raf and PI3K-Akt signaling pathways (Liu et al., 2010). *Polygonatum odoratum* lectin induced apoptosis in murine L929 by increasing the levels of FasL and FADD proteins and resulted in caspase 8 activation. There was also mitochondrial membrane damage and cytochrome c release leading to the activation of caspase 9 and caspase 3 (Liu et al., 2009). Concanavalin A was shown to induce apoptosis in p53 negative cell lines by activating the p53 family member p73 (Amin et al., 2007). Mese et al. (2000) suggested that caspase 3 could mediate apoptosis and that the induction of apoptosis through caspase 3 activation could be an effective treatment approach on malignant cancers. The present study on cell death induction by mulberry leaf lectin also indicates the involvement of caspase 3 in the induction of apoptosis.

Dysfunction of any step in the regulatory cell cycle cascade will cause abnormal cell proliferation resulting in cancer (Sulic et al., 2005). Understanding the mechanisms underlying the alterations in cell cycle progression is essential to study the conditions that lead to cancer. PI staining of DNA in cancer cell lines and its detection by flow cytometry is a widely accepted means to determine the percentage of cells in each phase of the cell cycle. Cell cycle distribution of K-562 cells treated with different concentrations of curcumin as examined by FACS analysis showed a dose-dependent increase in the sub-G1 peak indicating induction of apoptosis. DNA content histogram
analysis obtained from PI stained cells showed an increasing trend in sub-G1 peak with a progressive loss of the normal G0/G1 peak (Chakraborty et al., 2006). MLL treated cells also showed a similar pattern of cell cycle arrest.

Further studies are required to understand and establish the detailed molecular mechanism behind MLL induced apoptosis, before exploring the potential therapeutic applications of MLL.

4.6. CONCLUSION

MLL induced significant growth arrest in MCF - 7 and HCT - 15 with a GI\textsubscript{50} concentration of 8.5 µg/ml and 16 µg/ml respectively. Morphological analysis and AO/EB staining suggested apoptosis as the mechanism of growth arrest in MLL treated cells. MLL induced significant nuclear fragmentation and annexin V positive staining which further confirmed the apoptosis induction by MLL. Caspase 3 activity in HCT - 15 cells on treatment with MLL was significantly high indicating caspase mediated apoptosis. MLL induced cell cycle arrest at G2-M phase. Nevertheless, these mechanisms deserve to be studied further to ascertain the biochemical pathway of MLL-induced apoptosis in mammalian cancer cells.