Chapter – 5

ANTICANCER ACTIVITY STUDIES OF THE LEAVES OF *BISCHOFIA JAVANICA* AND *FRAXINUS FLORIBUNDA*

5.1 Introduction:

Cancer is a disease characterized by uncontrolled proliferation of cells. Tumor development is accelerated by disruption of the balance between cell proliferation and cell death, which is maintained through regulation of various signal transduction pathways. [1-2] The use of drugs in the treatment of cancer is targeting the uncontrolled proliferating cells, the specificity of the drug killing the cancer cell, without affecting from the normal cells provides the importance of the safety and efficacy of the drug. Cancer cell nuclei are the main targets for anticancer drugs, as the replication takes place via nuclei. Apoptosis (programmed cell death) is a process through which cells initiate their own death. [3] The existing chemotherapeutic agents like Vincristine, cis-platinum, cyclophosphamide, paclitaxel, 5FU etc were found to induce apoptosis in cancer cells. [4]

Anticancer screening of a drug can be performed by both *In-vivo* and *In-vitro* models. The drugs showing cytotoxicity in *In-vivo* animal model is not commonly reliable on human carcinomas. Therefore studies on animal model are not appreciable for anticancer activities in modern research. Studies on *In-vitro* models, using cell lines are more reliable in human carcinomas. However the maintenance of cancer cells requires many precautionary measures and involves high cost. In this study anticancer screening was done on human leukemia cell lines U-937, K-562 and HL-60 adopting the procedures based on various principles.

5.1.1 Cell Viability Studies:

Cell viability studies are performed with the help of inverted microscope and haemocytometer. Trypan blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that living cells do not take up certain dyes, whereas dead cells do.

5.1.2 Cytotoxicity Studies:

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide] is a dye used to form a purple colour (formazan complex) with endonuclease of living cells.[5] The intensity of the colour is directly proportional to the number of living cells. If the optical density of the drug

treated cells is lower than the control cells then it indicates the cytotoxicity of the drugs. MTT assay is an important study for the drugs entering into subsequent stage of screening procedures like DNA fragmentation assay, Morphological screening by microscopical studies, etc.

5.1.3 DNA Fragmentation Assay:

During apoptosis a series of reorganization process occur in the cells. Chromatin condensations, loss of cell volume and membrane blebbing are some of the most evident morphological changes of apoptotic cells. Although the molecular mechanisms leading to such changes are not completely known, many of them seem to proceed in parallel with biochemical events. This is the case of chromatin condensation and nuclear envelop breakdown. In fact, in parallel with them the DNA fragmentation occurs, which is a biochemical hallmark of apoptosis in the majority of cells. DNA cleavage is believed to be an endogenous Ca++- and Mg++-dependent endonuclease which is able to break double strand DNA at internucleosomal sites. Therefore, apoptotic DNA cleavage results in characteristic fragments of oligonucleosomal size (180-200 bp). Such phenomenon can be visualized by an agarose gel electrophoresis analysis.[6] This is a suitable method for qualitative determination of DNA fragmentation.

5.1.4 Fluorescence and Confocal Microscopy:

Cells undergoing apoptosis display some typical features namely, cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation. Dramatic changes occur within the nucleus during apoptotic death. It is commonly thought that the nuclear changes are due to activation of endogenous nuclease(s) which cleaves DNA into oligonucleosomal fragments. This is associated with the appearance of dense, crescent-shaped chromatin aggregates which line nuclear membrane. Later, the nucleolus disintegrates, nuclear membrane develops deep invaginations and, ultimately, the nucleus fragments into dense granular particles (apoptotic bodies). Chromatin condensation, nuclear shrinkage and formation of apoptotic bodies can easily be observed under fluorescence microscopy, after appropriate staining of nuclei with DNA-specific fluorochromes. [7] Confocal Laser Scanning Microscopy (CLSM) has recently

emerged as a technique which offers several advantages over conventional fluorescence microscopy. [8-9] The out-of-focus blur is virtually absent from confocal images, giving the capability for serial optical sectioning of intact specimens and subsequent three-dimensional reconstruction. For a precise analysis of stereo-spatial relationships of different cellular organelles, it is critical in fixation and mounting of specimens which preserve the structural architecture of the cells. The ideal fixative should penetrate the tissue quickly, act rapidly and preserve the cellular structure before the cell can react to produce structural artifacts.

5.2 Materials:

5.2.1 Human Leukemia Cell Lines U-937, K-562 and HL-60:

U-937 Myeloid leukemic cell lines, K-562 Erythro leukemic cell lines and HL-60 Acute myeloblastic leukemic cell lines were obtained from National Facility for Animal Tissue & Cell culture, Pune (India). The growth of the cell lines were maintained in the Drug Development Division, Indian Institute of Chemical Biology, Kolkata (India) in presence of RPMI-1640 supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) and gentamycin (40μg/ml), penicillin (100 units/ml) and streptomycin (10μg/ml). They were grown at 37°C in a humidified atmosphere of 5% CO2, 95% air in a CO_2 incubator.

5.2.2 Instruments, Chemicals and Drugs:

Inverted microscope (Olympus CKX41), Fluorescent microscope (Leica-USA), Confocal microscopy (Leica DM-TRB), Gel electrophoresis apparatus (Horizontal-Atto India), ELISA Plate Reader (Labsystem MS) were available in IICB, Kolkata. RPMI-1640, Fetal Bovine Serum (FBS), Gentamycin, Penicillin, Streptomycin, Trypan blue, Agarose, Tris were purchased from GIBCO-BRL. RNase A, Proteinase K, Propidium Iodide (PI),Acridine orange, Ethidiumbromide,MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide],Sodium Dodecyl Sulphate (SDS), Dimethyl sulfoxide(DMSO) and Cytarabine (Ara-C) were purchased from Sigma-Aldrich. Other reagents were of analytical grade and procured locally.

5.3 Anticancer Studies of the Leaves of *Bischofia javanica:*

5.3.1 Cell Viability Studies:

Log phase cell suspension of U-937, K-562 and HL-60 at a concentration of 10^5 / ml in RPMI-1640 (with 10% FBS) were used for the experiment in 96 well microtitre sterile plate. To each well 100µl of cell suspension was taken. The test drugs methanol extract of *Bischofia javanica* (BJ) was added at different concentration (5, 10 and 15µg/ml) and the isolated compound Friedelin-3α-acetate (FA) was added at the concentrations of 5 and 10 µg/ml against the standard drug Cytarabine (Ara-C) (20µg/ml) and the viable count was done by Trypan blue exclusion principle [10] after 24 hours of treatment. For each concentration of drugs the readings were taken in quadruplicate.

5.3.2 Cytotoxicity Studies:

Cell lines (U-937, K-562, HL-60) in exponential growth phase were washed, trypsinised and resuspended in RPMI-1640 media. Cells were kept at a concentration of 10^5 cells/well in 96 well microtitre plate .The cells were treated with different concentration of test drug, methanol extract of *Bischofia javanica* (BJ) (5, 10 and 15µg/ml) and the isolated compound, Friedelin-3α-acetate (FA) was added at the concentrations of 5 and 10 µg/ml against the standard drug Ara-C (20µg/ml), and the control which contained only the media and incubated for 24h. MTT solution [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well to make the final concentration of 400µg/ml and further incubated at 37°C in a CO_2 incubator (5% CO_2) for 3h. [11] The reaction resulted in the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a purple formazan product. The MTT-formazan product was dissolved in DMSO and estimated by measuring the absorbance at 492 nm in an ELISA plate reader. For each concentration of drugs readings were taken in quadruplicate. Initially the IC_{50} (50%Cytotoxicity) for the test drug BJ at a different concentrations (0.5,1.0, 2.5, 5,10,15,20 and 40µg/ml) was studied after 24, 48 and 72hrs of incubation with the cancer cell lines. [12] The isolated compound Friedelin-3α-acetate (FA) of BJ was also studied for IC_{50} in the above mentioned doses.

5.3.3 DNA Fragmentation Assay:

The isolation of fragmented DNA was carried out by conventional method [13] with some modifications .Cells((2×10^6/ml) were seeded in 55mm dish and treated with the test drugs of BJ and FA at a different concentrations for overnight . After harvesting, the cells were washed with PBS and pelleted by centrifugation .The cell pellets were then treated with 100µl lysis buffer (1% NP-40 in 20mM EDTA, 50mM Tris-HCl, pH 7.5) and centrifuged for 5minutes and the supernatant was collected. The extraction was repeated and to the combined supernatant SDS was added (final concentration 1%) and the mixture was treated overnight with RNaseA (5µg/ml) at 56°C followed by digestion with Protienase K (2.5 µg/ml) for 4h at 37°C.After addition of 0.5ml of 10M ammonium acetate the DNA was precipitated with 2.5ml of cold ethanol. DNA precipitate was dissolved in loading buffer (50mM Tris-HCl, pH 7.5; 10mM EDTA containing 0.25% bromophenol blue and 30% glycerol). Aliquot was loaded on 1.8% agarose prepared in 40mM Tris-HCl, pH 8.0, 10mM EDTA containing 0.5µg/ml ethidium bromide .The oligosomal DNA fragments were separated by electrophoresis at 20V for 12hrs.

5.3.4 Fluorescence Microscopy:

U-937, K-562 and HL-60 cell suspensions at a concentration of 10^5/ml was taken in a petri dish and treated with 10µg/ ml of test drug, BJ for 24 hours. To distinguish the living cells from apoptotic and dead cells , the cells were washed with PBS and stained with combination of Acridine orange (100µg/ml) : Ethidium bromide (100µg/ml) 1:1 ratio for 10 minutes and 10µl of the cell suspension was taken on a slide and images were scanned [8] using Fluorecence microscope (Model Leica, USA). Cells that exhibited reduced nuclear size, chromatin condensation and nuclear fragments were analyzed by the intense red or mixture of red-green colored fluorescence at the nuclear level. Images were obtained and rescaled identically in Adobe Photoshop6.

5.3.5 Confocal Microscopy:

U-937, K-562 and HL-60 cell suspensions at a concentration of 10^5/ml was taken in a Petri dish and treated with 10µg/ ml of test drug BJ for 24 hours. The cells were then washed with

PBS and stained with 10μg/ml of propidium iodide (PI) for 5 minutes and 10μl of the cell suspension was taken on a slide and images were scanned [9] using confocal laser scanning microscope (Model Leica DM-TRB using Leica TCS-SP2 system). Images of PI was acquired from argon /krypton laser pass filter. The diameters of the detection pen hole correspond to one airy unit of 512×512 Pixel. Images were obtained and rescaled identically in Adobe Photoshop6.

5.4 Anticancer Studies of the Leaves of *Fraxinus floribunda:*

5.4.1 Cell Viability Studies:

Log phase cell suspension of U-937, K-562 and HL-60 at a concentration of 10^5 / ml in RPMI-1640 (with 10% FBS) were used for the experiment in 96 well microtitre sterile plate. To each well 100μl of cell suspension was taken. The test drugs methanol extract of *Fraxinus floribunda*(FF) was added at different concentration (5,10 and15 μg/ml) and the isolated compound β-amyrin was added at the concentrations of 5 and 10 μg/ml against the standard drug Ara-C (20μg/ml) and the viable count was done by Trypan blue exclusion principle [10] after 24 hours of treatment.

5.4.2 Cytotoxicity Studies:

Cell lines in exponential growth phase were washed, trypsinised and resuspended in RPMI-1640 media. Cells were kept at a concentration of 10^5 cells/well in 96 well microtitre plate. The cells were treated with different concentration of test drug, methanol extract of *Fraxinus floribunda* (FF) (5, 10 and 15μg/ml) and the isolated compound, β-amyrin was added at the concentrations of 5 and 10 μg/ml against the standard drug Ara-C (20μg/ml) and the control which contained only the media and incubated for 24h. MTT solution [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well to make the final concentration of 400μg/ml and further incubated at 37°C in a CO_2 incubator (5% CO_2) for 3h. [11] The reaction resulted in the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a purple formazan product. The MTT-formazan product was dissolved in DMSO and estimated by measuring the absorbance at 492 nm in an ELISA plate reader. Initially the IC_{50} (50%Cytotoxicity) for the test drug, FF at a different concentrations (0.5, 1.0,

2.5, 5,10,15,20 and 40μg/ml) was studied after 24, 48 and 72hrs of incubation with the cancer cell lines. [12] The isolated compound, β-amyrin was also studied for IC_{50} in different doses.

5.5 Statistical Analysis:

Student's "t" test was done in every observation. [14] Statistical significance was done with the control, $P<0.05$ was considered as significant.

5.6 Results of Anticancer Activities of the Leaves of *Bischofia javanica:*

5.6.1 Cell Viability Studies:

Methanol extract of leaves of *Bischofia javanica* (BJ) shown to have significant inhibition on cell growth of all the used leukemia cell lines, U-937, K-562 and HL-60 in dose dependent manner which is represented in the **Tables 5.1, 5.2, 5.3** and **Figures 5.1, 5.2, 5.3.** The isolated compound of the leaves of *Bischofia javanica,* Friedelin-3α-acetate at the concentration of 5 and 10μg/ml shown to have significant inhibition on cell growth of all the cell lines at 48 and 72 hrs of incubation $P<0.001$, but in the case of cell line K-562 it did not show significant reduction in cell growth at 24 hrs of incubation (**Table** and **Figure 5.2**). The highest inhibition on cell growth (96.20%) was obtained at 72 hrs incubation of U-937 cell line treated with BJ-15 μg/ml **(Table 5.1)**. The reference drug Cytarabine (Ara-C) shown to have significant reduction in viable cells in all the cell lines used. Cell viability study revealed that the percentage inhibition on cell growth in K-562 cell line was relatively lower compared to U-937 and HL-60 cell lines in time and dose dependent manner.

5.6.2 Cytotoxicity Studies:

Cytotoxicity studies revealed that BJ and Cytarabine have significant cytotoxicity at the level $P<0.001$ at 72 hrs of incubation of all the three cell lines and depicted in **Tables and Figures 5.4, 5.5 and 5.6.** In U-937 cell line BJ at 15μg/ml and FA at 10μg/ml provided cytotoxicity by 69.33 and 44.00% at 72 hrs of incubation. In K-562 cell lines the drugs shown to have decreased cytotoxicity compared to U-937 and HL-60. The highest cytotoxicity (77.41%) was obtained with BJ at 15 μg/ml in HL-60 cell line at 72 hrs of incubation **(Table 5.6)**. The IC_{50} values of BJ and FA were represented in the **Table 5.7**. It revealed that the dose required to

kill 50% of HL-60 cancer cells by BJ and FA are 3.5 and 3.7μg/ml at 72 hrs respectively which are very low and it was maximum in case of K-562 cell lines (12.9 and 20.0μg/ml).

5.6.3 DNA Fragmentation Assay:

In U937, K-562 and HL-60 cell lines the test drugs shown to have ladder formation by fragmenting the DNA **(Figure 5.7)**.DNA fragmentation is the hallmark of apoptosis. It is due to cleavage of nuclear DNA at internucleosomal linking sites yielding DNA fragments in multiples of 180 bp, which upon electrophoresis yields a ladder pattern. The test drugs and standard drug did not show ladder formation prominently in the K-562 cell line **(Figure 5.7)**. Friedelin-3α-acetate also did not produce significant ladder formation in U-937 and K-562 cell lines but it was very much observed in HL-60 cell line.

5.6.4 Fluorescence Microscopy:

The cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation of cancer cell lines U-937, K-562 and HL-60 shown by the drug BJ at 10μg/ml is depicted in the **Figures 5.8, 5.9 and 5.10** respectively. Acridine orange is a metachromatic dye differentially stains the nucleic acids in double-stranded and single-stranded nucleoproteins. It emits red fluorescence when interacts with DNA. Chromatin condensation is an early event of apoptosis and the condensed chromatin is much more sensitive to DNA denaturation than normal chromatin. It was observed by staining of nuclei of different cell line in red or mixture of red with green colour. Emission of red fluorescence from the different cell lines after drug treatment revealed the apoptosis pathway of drug action.

5.6.5 Confocal Microscopy:

The nuclear membrane blebbing, chromatin condensation and nuclear fragmentation of cancer cell lines, the predictive phenomenon of apoptosis is depicted in the **Figures 5.11, 5.12 and 5.13** after staining with propidium iodide. Moreover the untreated cell lines were clearly differentiated in the figures from BJ 10μg/ml treated cell lines of U-937, K-562 and HL-60. The appearance of dense, crescent-shaped chromatin aggregates which line nuclear membrane,

development of deep invaginations in the nuclear membrane and the fragmentation of nucleus into dense granular particles (apoptotic bodies) are depicted in the **Figures 5.11-5.13.**

5.7 Results of Anticancer Activities of the Leaves of *Fraxinius floribunda:*

5.7.1 Cell Viability Studies:

Methanol extract of leaves of *Fraxinus floribunda* (FF) shown to have no significant effect of inhibition on cell growth in the cell lines of U-937, K-562 and HL-60 as depicted in the **Tables 5.8, 5.9, 5.10 and Figures 5.14, 5.15, 5.16.** In U-937 cell line FF shown to have 37.16% and 40.00% inhibition on cell growth in 48 and 72 hrs of incubation. β-amyrin, the isolated compound from FF have significant inhibition ($P<0.01$) on cell growth at 10μg/ml at 72hrs **(Table 5.8).** FF and β-amyrin did not show any activity at significant level in K-562 cell line **(Table 5.9).** Though the test drugs, FF and β-amyrin showed dose dependent activity, the level of activity is very low in terms of inhibition of the proliferating cell growth, as compared to the standard drug Cytarabine (86.59%).

5.7.2 Cytotoxicity Studies:

MTT assay revealed the feed back of cytotoxicity which was very similar to the results of cell viability studies **(Tables 5.11, 5.12, 5.13 and Figures 5.17, 5.18 and 5.19).** The percentage range of cytotoxicty offered by the test drug, FF on U-937 cell line was 3.77 at the concentration of 5μg/ml in 24hrs of incubation and 19.11at 15μg/ml in 72hrs of incubation **(Table 5.11).** β-amyrin at the concentration of 10μg/ml in HL-60 cell line shown to have significant cytotoxicity in 24, 48 and 72 hrs of incubation **(Table 5.13).** However it failed to show any cytotoxicity in U-937 and K-562 cell line. The IC_{50} value of methanol extract of *F.floribunda* and the isolated compound β-amyrin on K-562 cell line in 24 hrs incubation found to be 72.1μg/ml and 116.1μg/ml respectively **(Table 5.14).** On basis of the results of the cell viability and cytotoxicity studies which were not much encouraging, the methanol extract of the leaves of *F.floribunda* and β-amyrin were not subjected to the further anticancer studies.

Table 5.1 Cell viability study of leaves of *Bischofia javanica* on human leukemia cell line U-937

Treatment (µg/ml)	Mean Cells(x4x10^4) ± SEM (% Inhibition of cell growth)		
	24 hrs	48hrs	72 hrs
Control	24.25±1.44	37.75±2.17	53±4.34
BJ-5	9.25±1.11*** (65.97)	8.25±0.85*** (78.14)	6.75±0.48*** (87.2)
BJ -10	6.5±0.65*** (73.19)	5.25±0.48*** (86.66)	4.25±0.63*** (91.98)
BJ -15	4.00±0.41*** (83.50)	2.25±0.75*** (94.03)	2.00±0.71*** (96.22)
Friedelin-3α-acetate-5	17.00±1.71* (29.89)	15.5±2.65*** (58.94)	10.25±1.71*** (80.66)
Friedelin-3α-acetate -10	15.5±1.29** (36.08)	14.5±1.71*** (60.92)	8.5±0.29*** (83.96)
Cytarabine-20	7.25±0.95*** (70.10)	5.00±0.91*** (86.75)	3.00±0.41***(94.33)

Each value represents quadruplicate samples of Mean± SEM, Statistical significance test with control was done *P<0.05,**P< 0.01,***P<0.001, BJ-5, BJ-10 and BJ-15 - Methanol extract of *Bischofia javanica* 5,10 and 15µg/ml

Table 5.2 Cell viability study of leaves of *Bischofia javanica* on human leukemia cell line K-562

Treatment (µg/ml)	Mean Cells(x4x10^4) ± SEM (% Inhibition of cell growth)		
	24 hrs	48hrs	72 hrs
Control	22.75±0.91	32.25±0.48	48.25±2.94
BJ-5	14.00±0.71*** (38.46)	13.75±0.85*** (57.36)	11.25±0.85*** (76.68)
BJ -10	11.25±0.85*** (50.54)	7.75±0.48*** (75.96)	7.00±1.29*** (85.49)
BJ -15	8.00±0.71*** (64.83)	4.75±0.85*** (85.27)	4.25±0.65*** (91.19)
Friedelin-3α-acetate-5	18.5±1.71 (18.68)	16.5±1.71*** (48.83)	15.25±2.29*** (68.39)
Friedelin-3α-acetate -10	14.0±1.29** (38.46)	11.25±1.11*** (65.11)	9.75±0.85*** (79.79)
Cytarabine-20	10.25±0.85*** (54.94)	7.75±0.85*** (75.95)	5.00±0.91*** (89.63)

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done .*P<0.01,***P<0.001, BJ-5,10,15 - Methanol extract of *Bischofia javanica* 5,10,15µg/ml

Table 5.3 Cell viability study of leaves of *Bischofia javanica* on human leukemia cell line HL-60

Treatment (µg/ml)	Mean Cells(x4x10^4) ± SEM (% Inhibition of cell growth)		
	24 hrs	48hrs	72 hrs
Control	36.75±1.11	51.75±2.14	65.5±2.99
BJ-5	18.00±0.41*** (51.02)	13.5±0.65*** (73.91)	10.5±0.65*** (83.96)
BJ -10	12.75±1.25*** (65.3)	9.25±0.95*** (82.12)	8.5±0.65*** (87.02)
BJ -15	9.25±0.48*** (74.82)	6.25±0.85*** (87.92)	4.5±0.65*** (93.12)
Friedelin-3α-acetate-5	19.00±2.08*** (48.29)	17.75±0.85*** (65.7)	15.00±1.47*** (77.09)
Friedelin-3α-acetate -10	16.00±0.91*** (56.46)	13.00±0.91*** (74.87)	11.25±1.32*** (82.82)
Cytarabine-20	9.5±1.04*** (74.14)	8.75±1.11*** (83.09)	6.75±0.85*** (89.69)

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done ***P<0.001, BJ-5,10,15 - Methanol extract of *Bischofia javanica* 5,10,15µg/ml

Table 5.4 Cytotoxicity study of leaves of *Bischofia javanica* on human leukemia cell line U-937

Treatment (µg/ml)	Mean OD at 492 nm ± SEM (% Cytotoxicity)		
	24 hrs	48hrs	72 hrs
Control	0.67±0.04	0.72±0.06	0.75±0.03
BJ-5	0.46±0.06* (31.34)	0.39±0.04** (45.83)	0.34±0.02*** (54.66)
BJ -10	0.39±0.04** (41.79)	0.32±0.03*** (55.55)	0.28±0.01*** (62.66)
BJ -15	0.32±0.05*** (52.23)	0.25±0.06*** (65.27)	0.23±0.02*** (69.33)
Friedelin-3α-acetate-5	0.59±0.04 (11.94)	0.56±0.05 (22.22)	0.51±0.03 (32.00)
Friedelin-3α-acetate -10	0.54±0.01* (19.4)	0.48±0.02* (33.33)	0.42±0.03* (44.00)
Cytarabine-20	0.33±0.05** (67.00)	0.27±0.04*** (62.5)	0.25±0.05*** (66.66)

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done.*P<0.05,*P<0.01,***P<0.001, BJ-5,10,15-Methanol extract of *Bischofia javanica* 5,10,15µg/ml

Table 5.5 Cytotoxicity study of leaves of *Bischofia javanica* on human leukemia cell line K- 562

Treatment (µg/ml)	Mean OD at 492 nm ± SEM (% Cytotoxicity)		
	24 hrs	48hrs	72 hrs
Control	0.53±0.02	0.57±0.03	0.61±0.04
BJ-5	0.51±0.02 (3.7)	0.47±0.01 (17.54)	0.44±0.06 (27.86)
BJ -10	0.46±0.01 (13.2)	0.43±0.04 (24.56)	0.31±0.05* (49.18)
BJ -15	0.44±0.03 (16.98)	0.27±0.02* (52.63)	0.25±0.02** (59.01)
Friedelin-3α-acetate-5	0.50±0.04 (5.61)	0.48±0.01 (15.78)	0.45±0.04 (26.22)
Friedelin-3α-acetate -10	0.48±0.05 (9.42)	0.47±0.03 (17.54)	0.43±0.05 (29.56)
Cytarabine-20	0.39±0.04 (26.41)	0.29±0.05* (49.12)	0.26±0.05** (57.37)

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done.*P<0.05,*P<0.01, BJ-5,10,15-Methanol extract of *Bischofia javanica* 5,10,15µg/ml

Table 5.6 Cytotoxicity study of leaves of *Bischofia javanica* on human leukemia cell line HL-60

Treatment (µg/ml)	Mean OD at 492 nm ± SEM (% Cytotoxicity)		
	24 hrs	48hrs	72 hrs
Control	0.527±0.03	0.57±0.02	0.62±0.02
BJ-5	0.32±0.02* (39.62)	0.22±0.01** (61.4)	0.18±0.04*** (70.96)
BJ -10	0.28±0.03* (47.16)	0.18±0.04** (68.42)	0.17±0.05*** (72.58)
BJ -15	0.20±0.06** (62.26)	0.16±0.05*** (71.92)	0.14±0.02*** (77.41)
Friedelin-3α-acetate-5	0.36±0.04 (32.07)	0.28±0.03** (50.87)	0.24±0.03***(61.29)
Friedelin-3α-acetate -10	0.29±0.02* (45.28)	0.26±0.01** (54.38)	0.19±0.02*** (69.35)
Cytarabine-20	0.24±0.04* (54.71)	0.18±0.03** (68.42)	0.16±0.01*** (74.19)

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done.*P<0.05,*P<0.01,***P<0.001, BJ-5,10,15-Methanol extract of *Bischofia javanica* 5,10,15µg/ml

Table 5.7 IC_{50} values of the leaves of *B.javanica* on different leukemia cell lines by Cytotoxicity studies

Cell lines	IC_{50} (µg/ml) of BJ and Friedelin -3α-acetate (FA)					
	24hrs		48hrs		72hrs	
	BJ	FA	BJ	FA	BJ	FA
U937	14.2	32.1	8.8	26.4	4.1	12.2
K562	29.5	58.7	17.3	38.1	12.9	20.0
HL60	11.5	11.1	4.2	4.9	3.5	3.7

BJ-Methanol extract of leaves of *Bischofia javanica,* FA-Friedelin-3α-acetate

Table 5.8 Cell viability study of leaves of *Fraxinus floribunda* on human leukemia cell line U-937

Treatment (µg/ml)	Mean Cells(x4x10^4) ± SEM (% Inhibition of cell growth)		
	24 hrs	48hrs	72 hrs
Control	22.21±6.36	38.75±3.26	55±6.34
FF-5	20.25±4.12 (8.82)	32.5±4.80 (16.12)	40.55±3.48 (26.27)
FF -10	19.35±3.45 (12.87)	30.25±5.47 (21.93)	36.50±8.64 (28.63)
FF -15	19.25±6.41 (13.32)	24.35±4.75* (37.16)	33.00±7.71* (40.00)
β-amyrin -5	20.35±5.73 (8.37)	32.50±5.71 (16.12)	38.25±4.82 (30.45)
β-amyrin -10	18.50±2.29 (16.70)	28.5±3.71 (26.45)	22.5±4.37** (59.09)
Cytarabine-20	8.25±1.93*** (62.85)	6.25±2.10*** (83.87)	3.50±2.48*** (93.63)

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done.*P<0.05,***P<0.001, FF-5,10,15-Methanol extract of *Fraxinus floribunda* 5,10,15µg/ml.

Table 5.9 Cell viability study of leaves of *Fraxinus floribunda* on human leukemia cell line K-562

Treatment (µg/ml)	Mean Cells($x4x10^4$) ± SEM (% Inhibition of cell growth)		
	24 hrs	**48hrs**	**72 hrs**
Control	28.25±1.81	37.35±2.38	48.50±3.24
FF-5	26.50±2.72 (6.19)	33.25±1.83 (10.97)	42.50±2.35 (12.37)
FF -10	25.50±3.82 (9.73)	31.75±2.38 (14.99)	37.00±2.29 (23.71)
FF -15	23.00±0.94 (18.58)	28.50±4.87 (23.69)	34.25±2.65 (29.38)
β -amyrin -5	24.5±3.72 (13.27)	30.75±1.71 (17.67)	39.25±2.29 (19.07)
β -amyrin -10	23.25±2.29 (17.69)	29.00±5.11 (22.35)	36.75±4.87 (24.22)
Cytarabine-20	11.50±1.82*** (59.29)	7.50±1.25*** (79.91)	6.50±4.23*** (86.59)

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done.***P<0.001, FF-5,10,15 - Methanol extract of *Fraxinus floribunda* 5,10,15µg/ml.

Table 5.10 Cell viability study of leaves of *Fraxinus floribunda* on human leukemia cell line HL-60

Treatment (µg/ml)	Mean Cells(x4x10^4) ± SEM (% Inhibition of cell growth)		
	24 hrs	48hrs	72 hrs
Control	32.50±1.61	55.25±2.54	67.5±3.99
FF-5	29.00±2.41 (10.76)	48.5±3.65 (12.21)	58.5±1.55 (13.33)
FF -10	27.75±1.45 (14.61)	46.25±2.97 (16.28)	52.5±3.15 (22.22)
FF -15	27.00±3.42 (16.92)	41.25±1.54 (25.33)	49.75±4.65 (26.29)
β -amyrin -5	30.00±2.04 (7.69)	47.75±1.95 (13.57)	49.00±1.86 (27.4)
β-amyrin -10	26.00±4.91 (20.00)	33.25±2.92* (39.81)	35.25±1.23* (47.77)
Cytarabine-20	8.25±1.04*** (74.61)	6.75±1.11*** (87.78)	4.25±1.71*** (93.7)

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done.*P<0.05,***P<0.001, FF-5,10,15-Methanol extract of *Fraxinus floribunda* 5,10,15µg/ml.

Table 5.11 Cytotoxicity study of leaves of *Fraxinus floribunda* on human leukemia cell line U-937.

Treatment (µg/ml)	Mean OD at 492 nm ± SEM (% Cytotoxicity)		
	24 hrs	**48hrs**	**72 hrs**
Control	0.53±0.01	0.62±0.04	0.68±0.03
FF-5	0.51±0.03 (3.77)	0.59±0.02 (4.83)	0.64±0.02 (5.88)
FF -10	0.49±0.04 (7.54)	0.55±0.03 (11.29)	0.57±0.01 (16.17)
FF -15	0.46±0.01 (13.20)	0.52±0.04 (16.12)	0.55±0.03 (19.11)
β - amyrin -5	0.51±0.04 (3.77)	0.56±0.03 (9.67)	0.61±0.03 (10.29)
β - amyrin -10	0.50±0.01 (5.66)	0.51±0.02 (17.74)	0.54±0.04 (20.58)
Cytarabine-20	0.30±0.02** (43.39)	0.25±0.04*** (59.67)	0.21±0.01*** (69.11)

Each value represents means of quadruplicate determinations ± SEM .Statistical significance test with control was done .**P<0.01,***P<0.001, FF-5,10,15-Methanol extract of *Fraxinus floribunda* 5,10,15µg/ml.

Table 5.12 Cytotoxicity study of leaves of *Fraxinus floribunda* on human leukemia cell line K-562

Treatment (µg/ml)	Mean OD at 492 nm ± SEM (% Cytotoxicity)		
	24 hrs	48hrs	72 hrs
Control	0.48±0.01	0.55±0.02	0.59±0.03
FF-5	0.47±0.02 (2.08)	0.53±0.03 (3.63)	0.55±0.04 (6.77)
FF-10	0.43±0.01 (10.41)	0.47±0.05 (14.54)	0.50±0.04 (15.25)
FF-15	0.43±0.03 (10.41)	0.41±0.02 (25.45)	0.43±0.02 (27.11)
β –amyrin -5	0.45±0.03 (6.25)	0.48±0.01 (12.72)	0.46±0.02 (22.03)
β-amyrin -10	0.44±0.04 (8.33)	0.47±0.07 (14.54)	0.44±0.06 (25.42)
Cytarabine-20	0.28±0.02* (41.66)	0.24±0.05** (56.36)	0.22±0.05** (62.71)

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done .*P<0.05,**P<0.01, FF-5,10,15-Methanol extract of *Fraxinus floribunda* 5,10,15µg/ml.

Table 5.13 Cytotoxicity study of leaves of *Fraxinus floribunda* on human leukemia cell line HL-60

Treatment (µg/ml)	Mean OD at 492 nm ± SEM (% Cytotoxicity)		
	24 hrs	**48hrs**	**72 hrs**
Control	0.47±0.03	0.58±0.01	0.65±0.01
FF-5	0.44±0.02 (6.38)	0.51±0.02 (12.06)	0.55±0.02 (15.38)
FF -10	0.42±0.01 (10.63)	0.48±0.04 (17.24)	0.51±0.05 (21.53)
FF -15	0.38±0.02 (19.14)	0.45±0.01 (22.41)	0.48±0.02 (26.15)
β –amyrin -5	0.40±0.03 (14.89)	0.46±0.03 (20.68)	0.44±0.03 (32.30)
β-amyrin -10	0.31±0.01* (34.04)	0.36±0.01* (37.93)	0.39±0.02* (40.00)
Cytarabine-20	0.21±0.04** (55.31)	0.17±0.03** (70.68)	0.13±0.04*** (80.00)

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done.*P<0.05,**P<0.01,***P<0.001, FF-5,10,15-Methanol extract of *Fraxinus floribunda* 5,10,15µg/ml.

Table 5.14 IC_{50} values of the leaves of *Fraxinus floribunda* on different leukemia cell lines by Cytotoxicity studies

Cell lines	IC_{50} (µg/ml) of FF and β-amyrin					
	24hrs		48hrs		72hrs	
	FF	β-amyrin	FF	β-amyrin	FF	β-amyrin
U-937	67.3	82.16	39.4	42.6	32.6	29.3
K-562	72.1	116.1	51.3	89.3	38.7	63.0
HL-60	57.5	28.2	34.1	22.1	29.4	15.1

FF-Methanol extract of leaves of *Fraxinus floribunda*

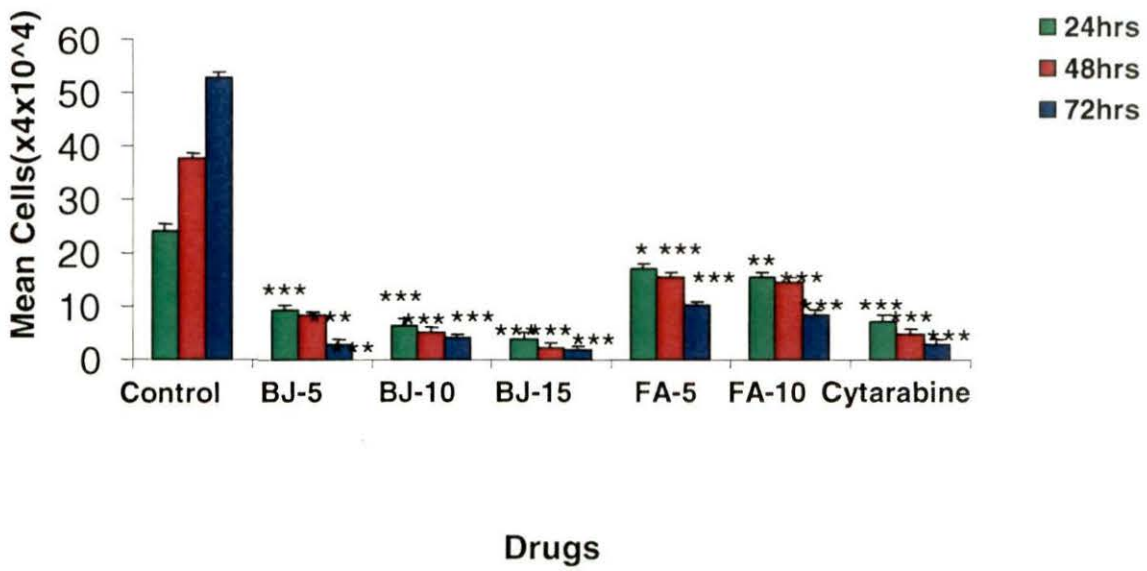


Figure 5.1 Cell viability study of leaves of *Bischofia javanica* on human leukemia cell line U-937

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done.*P<0.05,*P<0.01,***P<0.001, BJ-5,10,15-Methanol extract of *Bischofia javanica* 5,10,15µg/ml,FA-5,10-Friedelin-3α-acetate 5,10 µg/ml

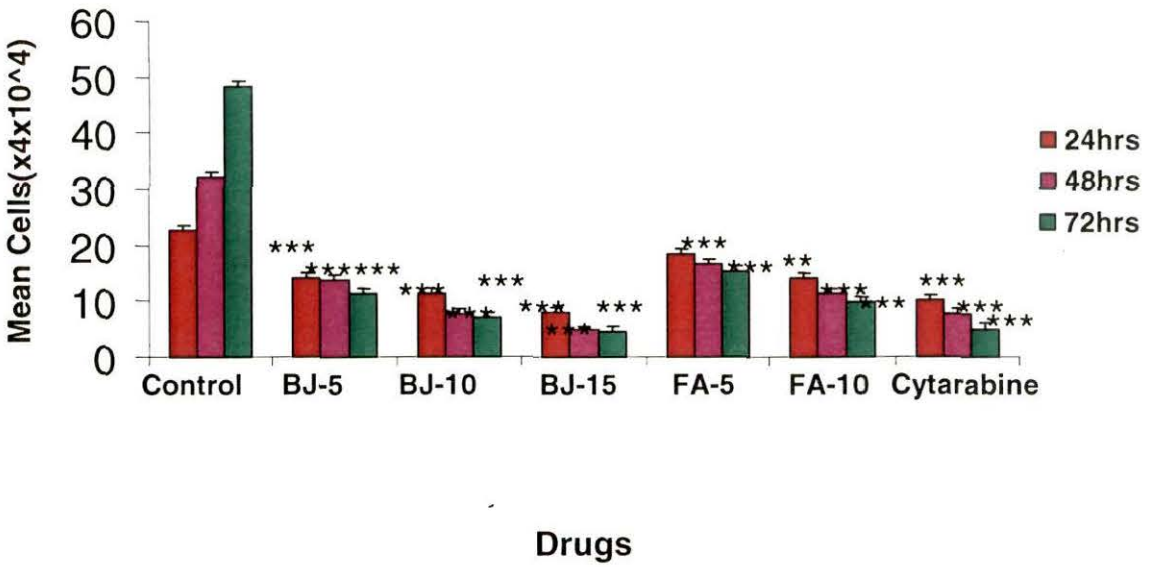


Figure 5.2 Cell viability study of leaves of *Bischofia javanica* on human leukemia cell line K-562

Each value represents means of quadruplicate determinations ± SEM .Statistical significance test with control was done .**P<0.01,***P<0.001, BJ-5,10,15-Methanol extract of *Bischofia javanica* 5,10,15μg/ml,FA-5,10-Friedelin-3α-acetate 5,10 μg/ml

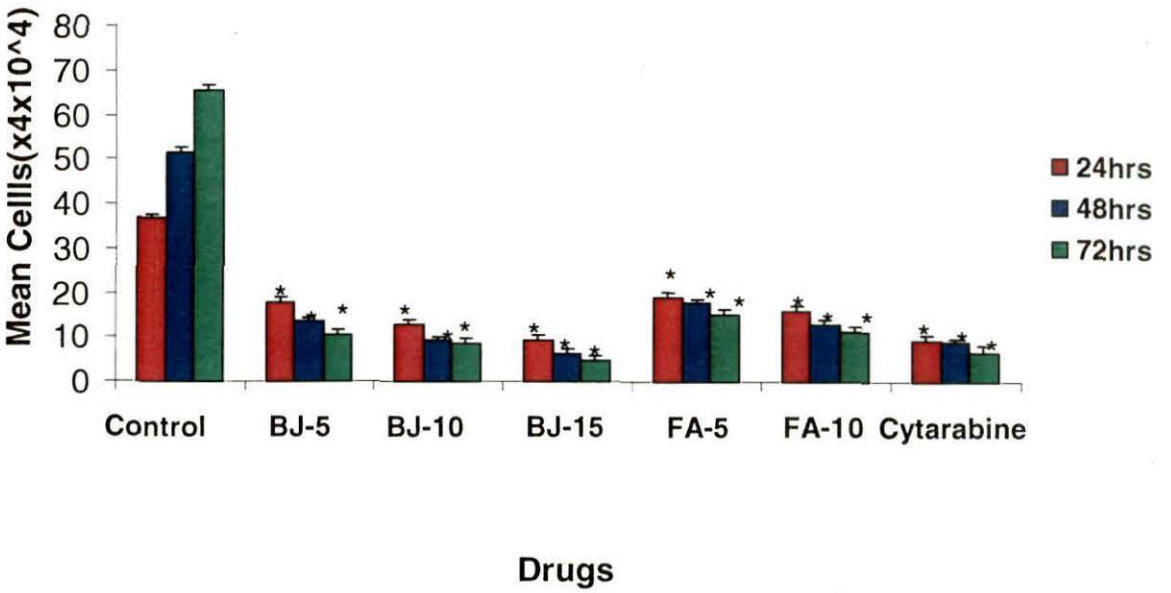


Figure 5.3 Cell viability study of leaves of *Bischofia javanica* on human leukemia cell line HL-60

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done.*P<0.001, BJ-5, 10, 15-Methanol extract of *Bischofia javanica* 5,10,15μg/ml,FA-5,10-Friedelin-3α-acetate 5,10 μg/ml

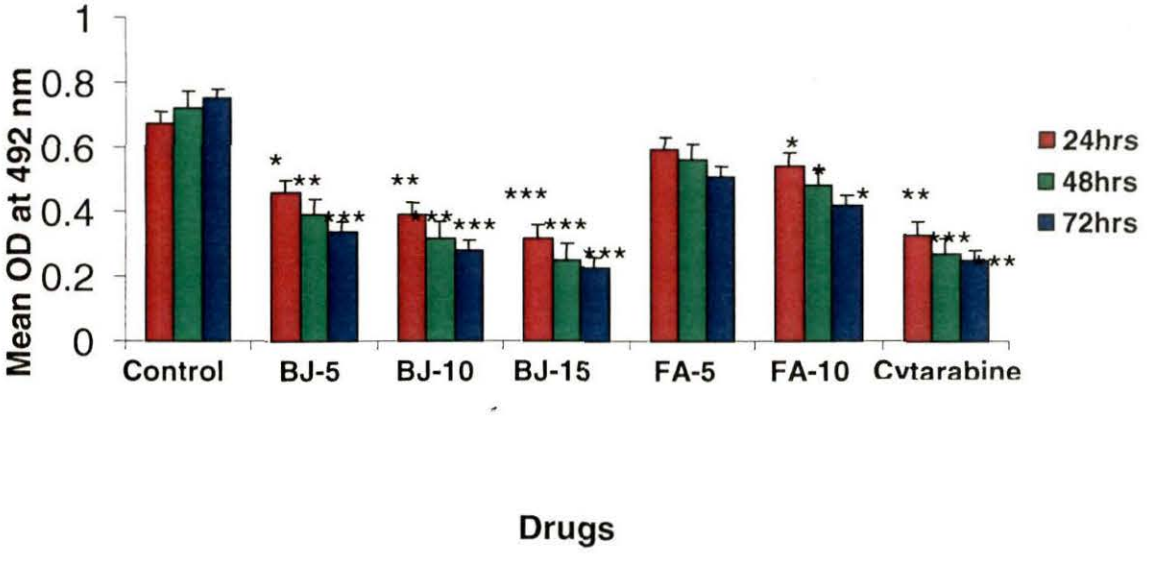


Figure 5.4 Cytotoxicity study of leaves of *Bischofia javanica* on human leukemia cell line U-937

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done .*P<0.05,**P<0.01,***P<0.001, BJ-5,10,15-Methanol extract of *Bischofia javanica* 5,10,15µg/ml,FA-5,10-Friedelin-3α-acetate 5,10 µg/ml

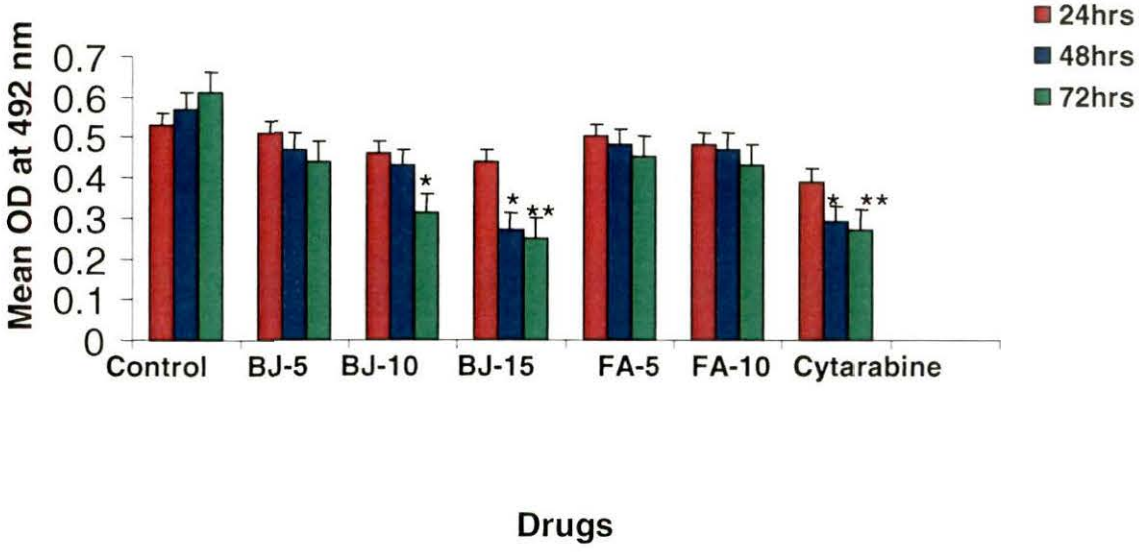


Figure 5.5 Cytotoxicity study of leaves of *Bischofia javanica* on human leukemia cell line K-562

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done .*P<0.05,**P<0.01,***P<0.001, BJ-5,10,15-Methanol extract of *Bischofia javanica* 5,10,15μg/ml,FA-5,10-Friedelin-3α-acetate 5,10 μg/ml

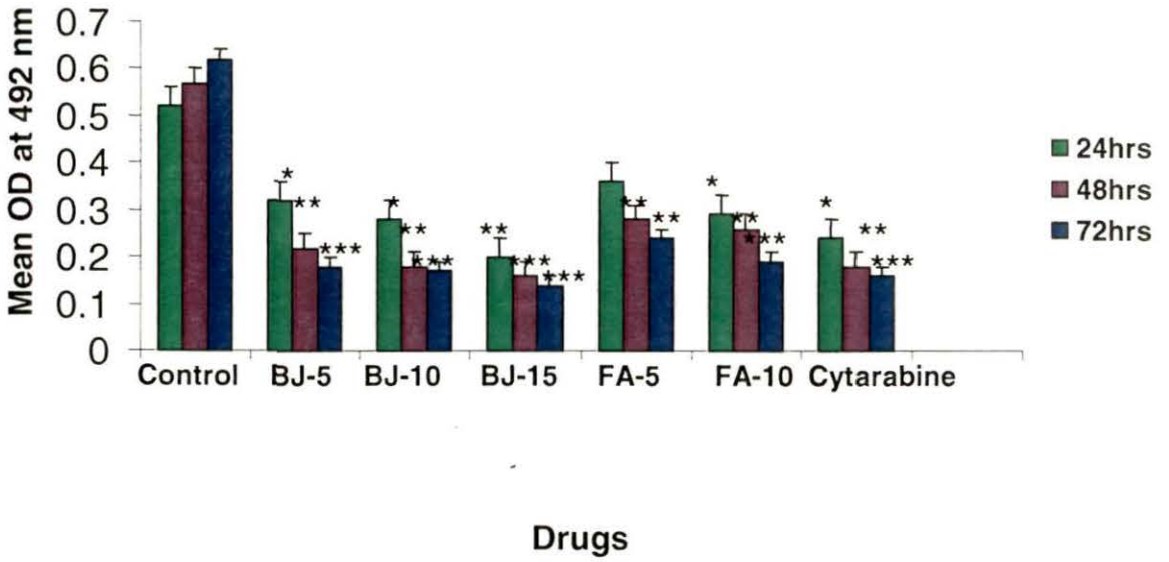


Figure 5.6 Cytotoxicity study of leaves of *Bischofia javanica* on human leukemia cell line HL-60

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done .*P<0.05,**P<0.01,***P<0.001, BJ-5,10,15-Methanol extract of *Bischofia javanica* 5,10,15µg/ml,FA-5,10-Friedelin-3α-acetate 5,10 µg/ml

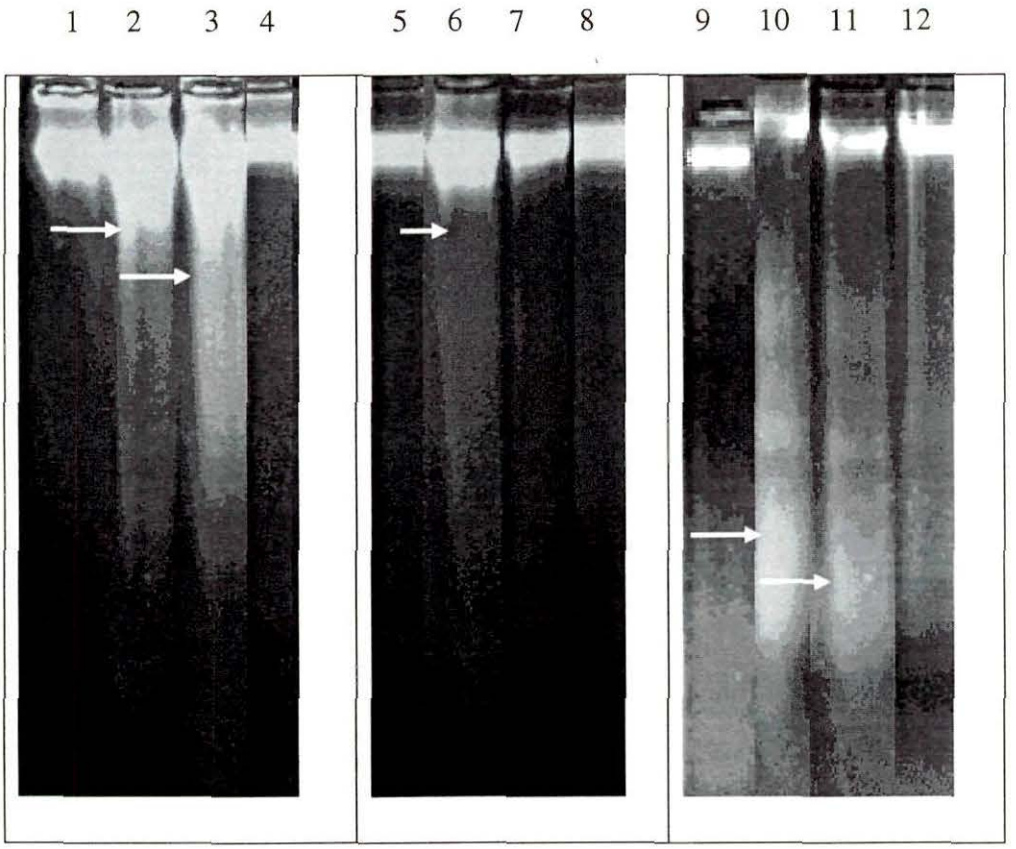
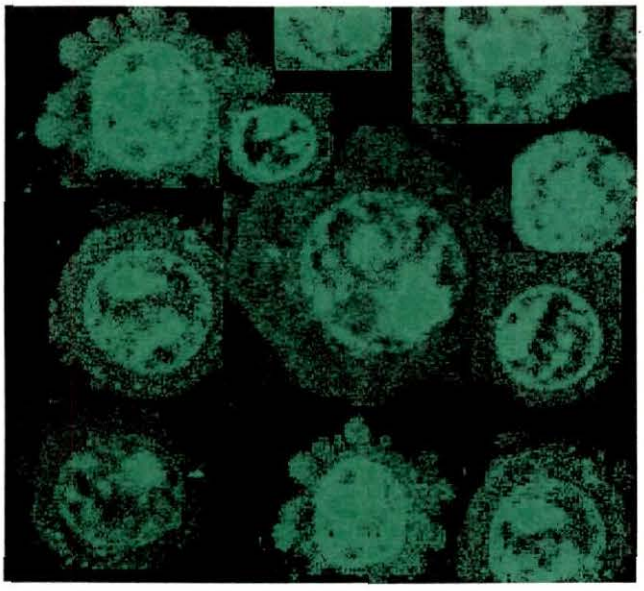
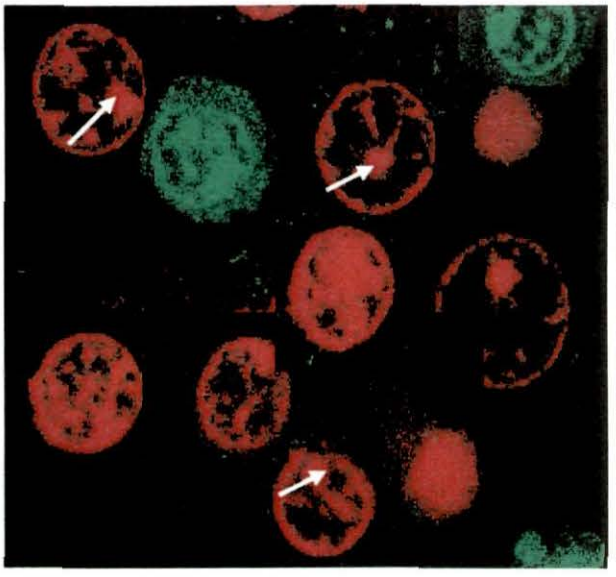


Figure 5.7 DNA-Fragmentation assay of the leaves of *Bischofia javanica* on human leukemia cell lines U937, K562 and HL-60

Lanes 1, 2, 3 and 4 were for U-937 Control, Cytarabine 20μg/ml, *B.javanica* 10μg/ml and Fiedeline-3α-acetate 10μg/ml treated respectively. Lanes 5, 6, 7 and 8 were for K-562 Control, Cytarabine 20μg/ml, *B.javanica* 10μg/ml and Fiedeline-3α-acetate 10μg/ml treated respectively. Lanes 9,10,11,12 were for HL-60 Control, Cytarabine 20μg/ml, *B.javanica* 10μg/ml and Fiedeline-3α-acetate 10μg/ml treated respectively. Arrow marks showing the fragmentation of DNA after drug treatment with ladder formation.



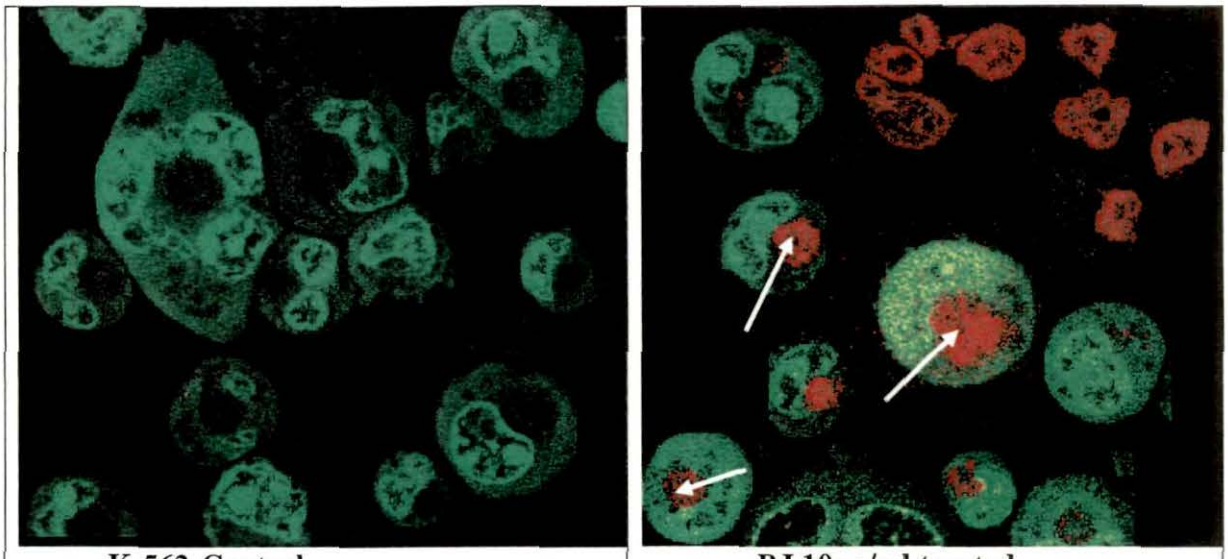
U-937-Control



BJ 10μg/ml treated

Figure 5.8 Fluorescent microscopic study of the leaves of *Bischofia javanica* on human leukemia cell line U937

In BJ 10μg/ml treated cells, arrow marks revealing the apoptosis of cells by chromatin condensation and nuclear blebbing with intact cell membrane.



K-562-Control | BJ 10μg/ml treated

Figure 5.9 Fluorescence microscopic studies of the leaves of *Bischofia javanica* on human leukemia cell line K-562

In BJ 10μg/ml treated cells, arrow marks revealing the apoptosis of cells by chromatin condensation and nuclear blebbing with intact cell membrane.

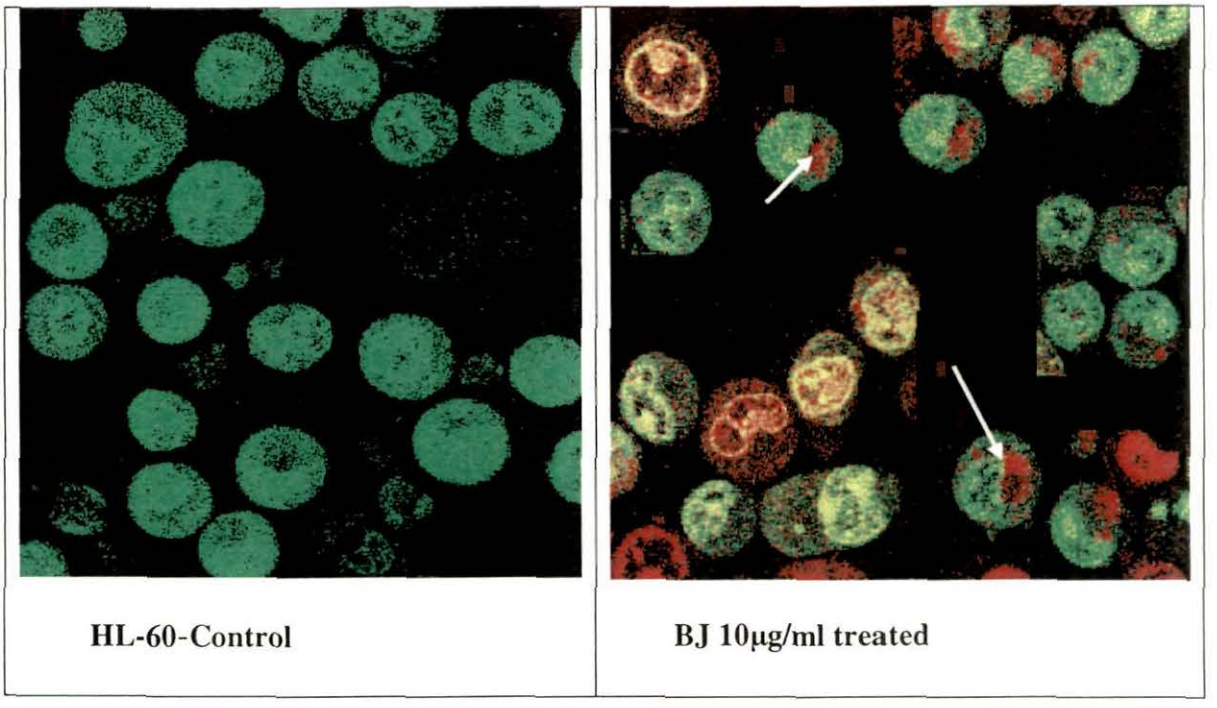


Figure 5.10 Fluorescence microscopic study of the leaves of *Bischofia javanica* on human leukemia cell line HL-60

In BJ 10µg/ml treated cells, arrow marks revealing the apoptosis of cells by chromatin condensation and nuclear blebbing with intact cell membrane.

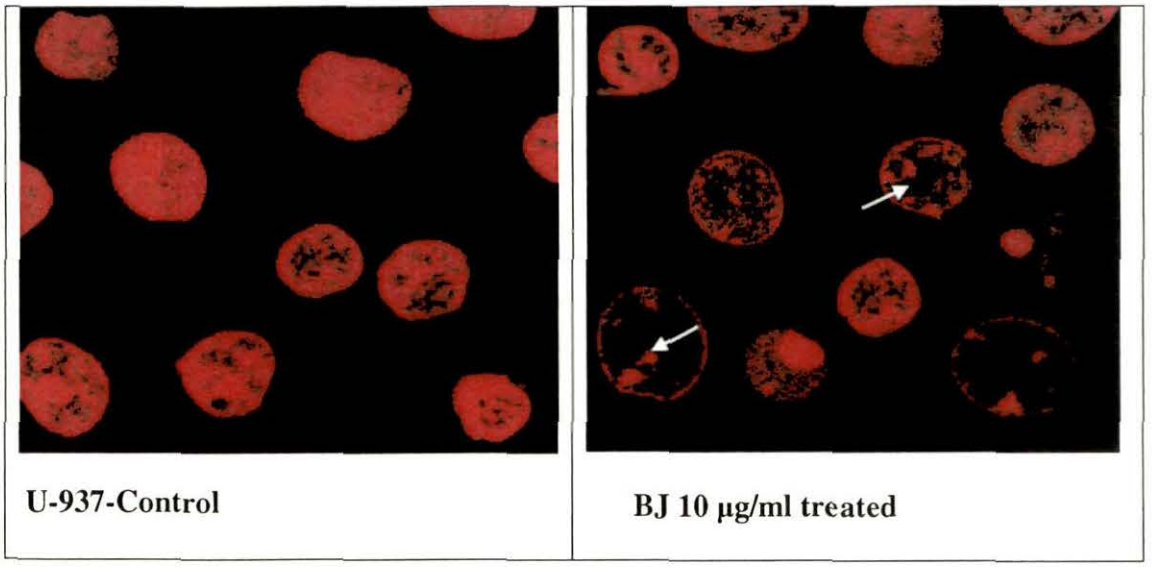
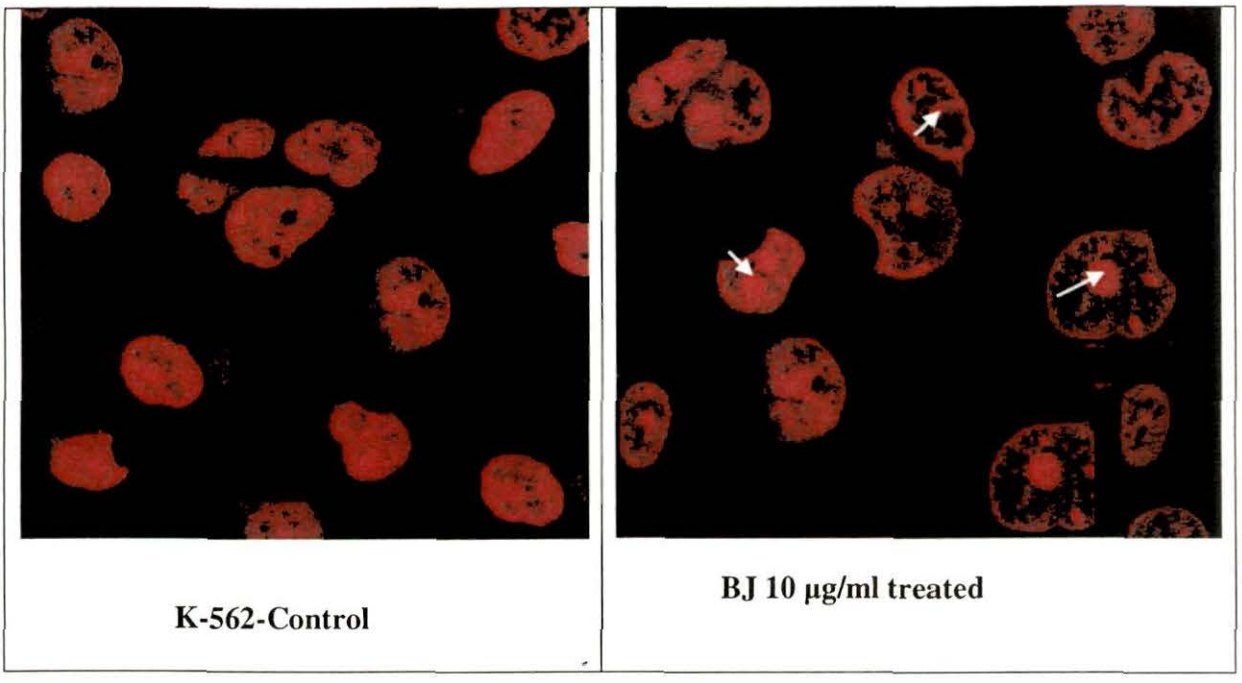


Figure 5.11 Confocal microscopic studies of the leaves of *Bischofia javanica* on human leukemia cell line U-937

In BJ 10µg/ml treated cells, arrow marks revealing the apoptosis of cells by chromatin condensation and nuclear blebbing with intact cell membrane.



K-562-Control

BJ 10 µg/ml treated

Figure 5.12 Confocal microscopic studies of the leaves of *Bischofia javanica* on human leukemia cell line K-562

In BJ 10µg/ml treated cells, arrow marks revealing the apoptosis of cells by chromatin condensation and nuclear blebbing with intact cell membrane.

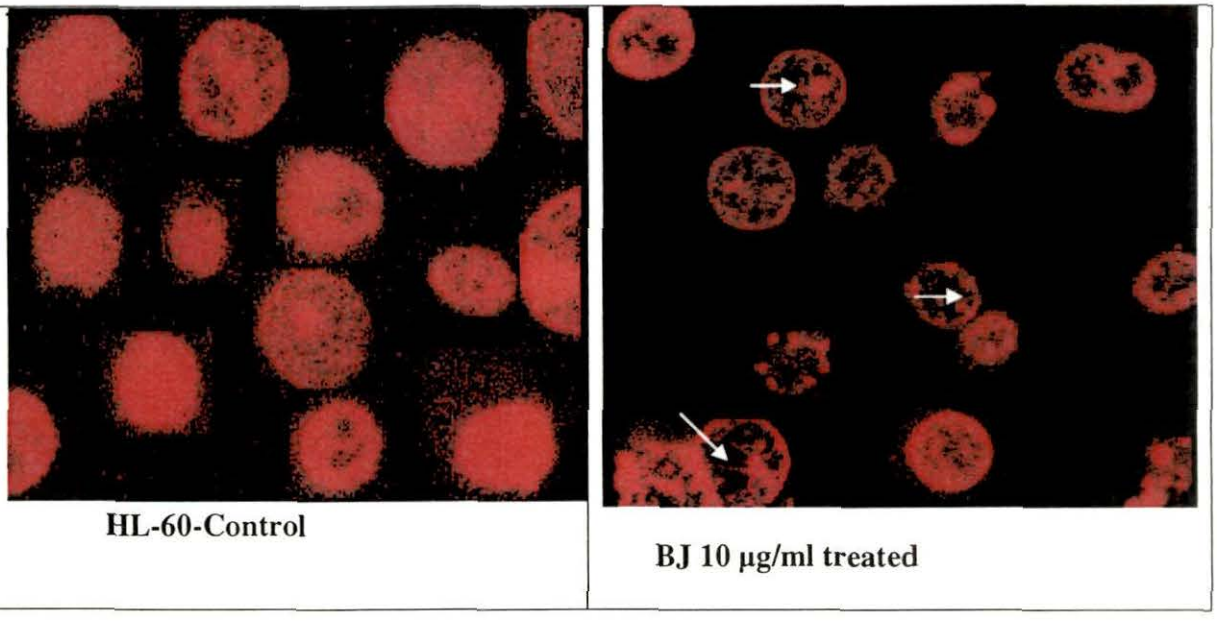


Figure 5.13 Confocal microscopic studies of the leaves of *Bischofia javanica* on human leukemia cell line HL-60

In BJ 10µg/ml treated cells, arrow marks revealing the apoptosis of cells by chromatin condensation and nuclear blebbing with intact cell membrane.

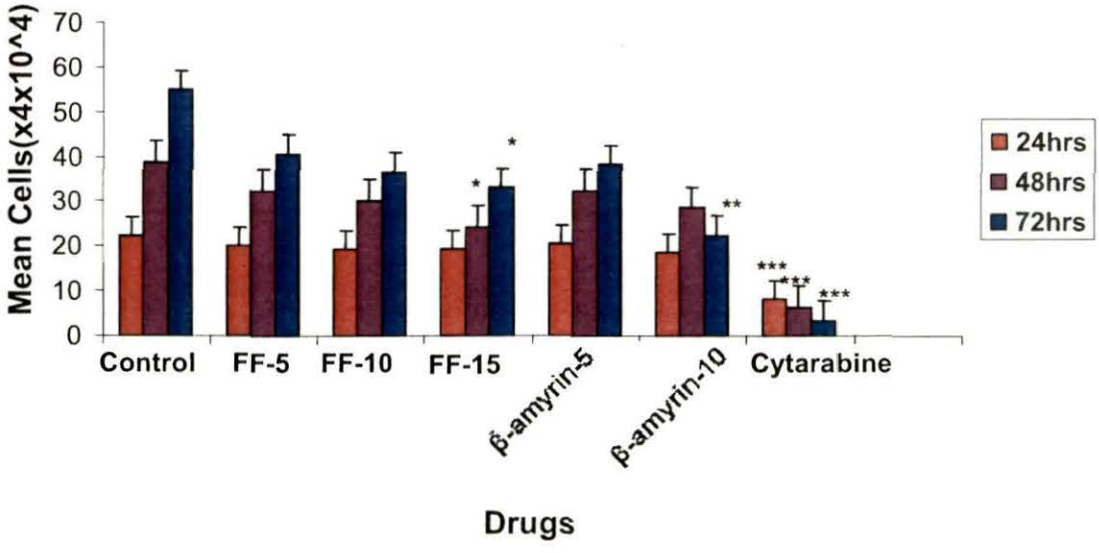


Figure 5.14 Cell viability study of leaves of *Fraxinus floribunda* on human leukemia cell line U-937

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done .*P<0.05,**P<0.01,***P<0.001, FF-5,10,15-Methanol extract of *Fraxinus floribunda* 5,10,15µg/ml

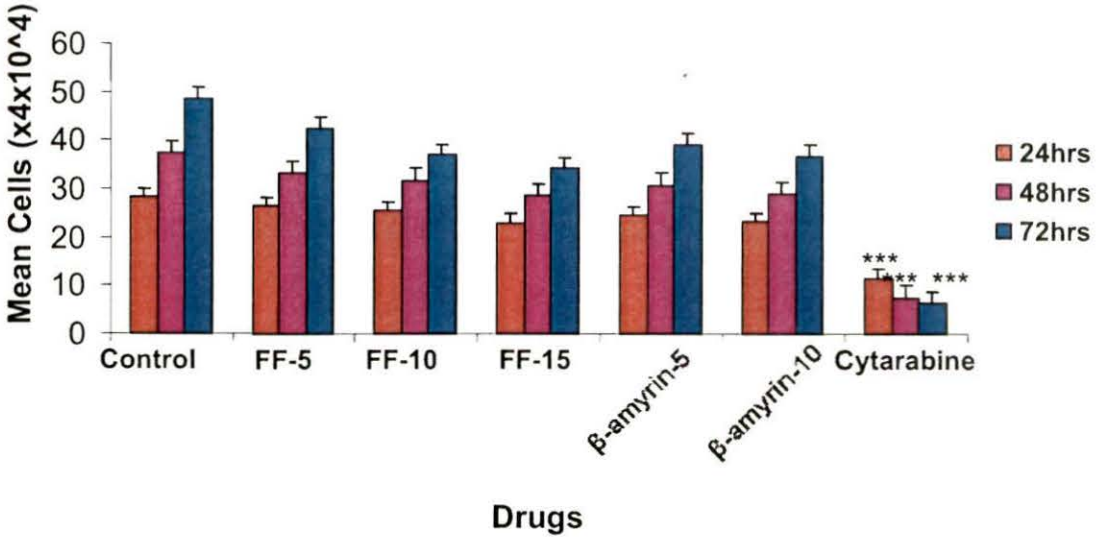


Figure 5.15 Cell viability study of leaves of *Fraxinus floribunda* on human leukemia cell line K-562

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done .*P<0.05,**P<0.01,***P<0.001, FF-5,10,15-Methanol extract of *Fraxinus floribunda* 5,10,15µg/ml

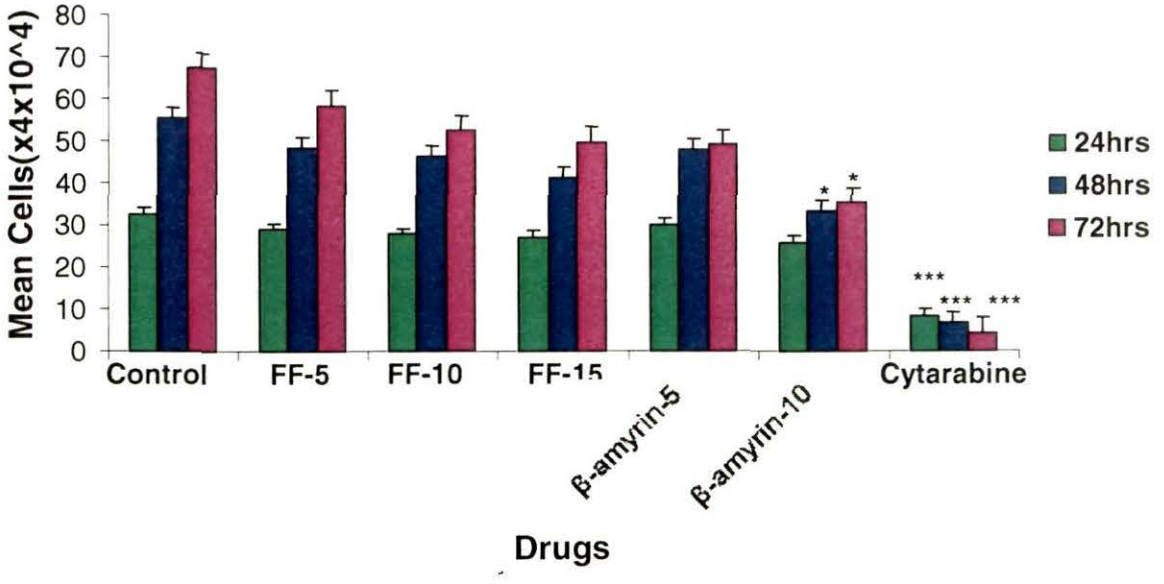


Figure 5.16 Cell viability study of leaves of *Fraxinus floribunda* on human leukemia cell line HL-60

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done .*P<0.05,***P<0.001, FF-5,10,15-Methanol extract of *Fraxinus floribunda* 5,10,15μg/ml

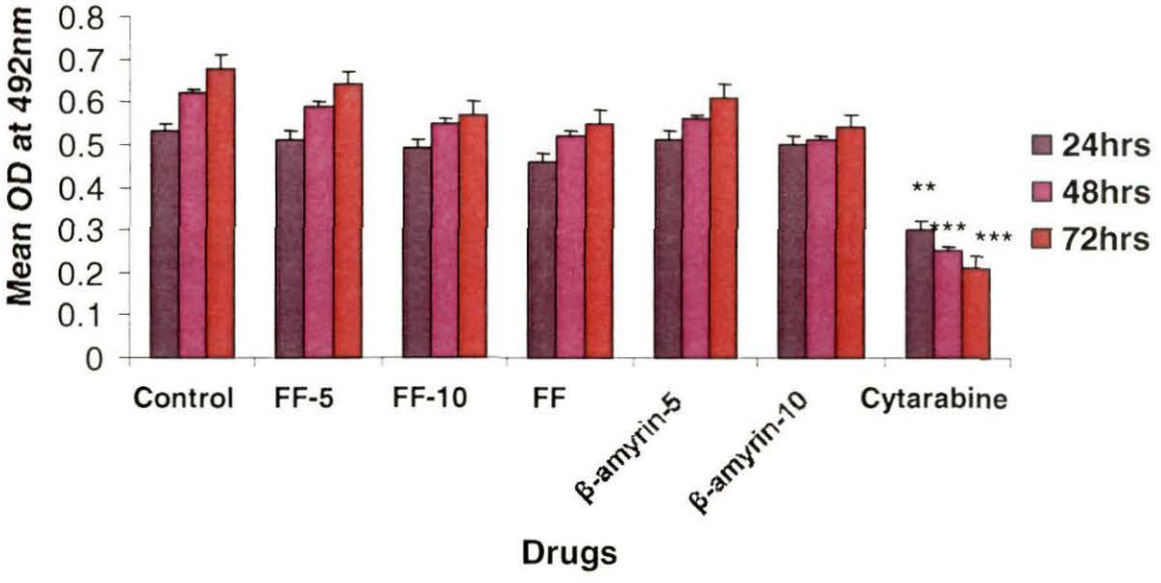


Figure 5.17 Cytotoxicity study of leaves of *Fraxinus floribunda* on human leukemia cell line U-937

Each value represents means of quadruplicate determinations ± SEM. Statistical significance test with control was done .*P<0.05,**P<0.01,***P<0.001, FF-5,10,15-Methanol extract of *Fraxinus floribunda* 5,10,15µg/ml

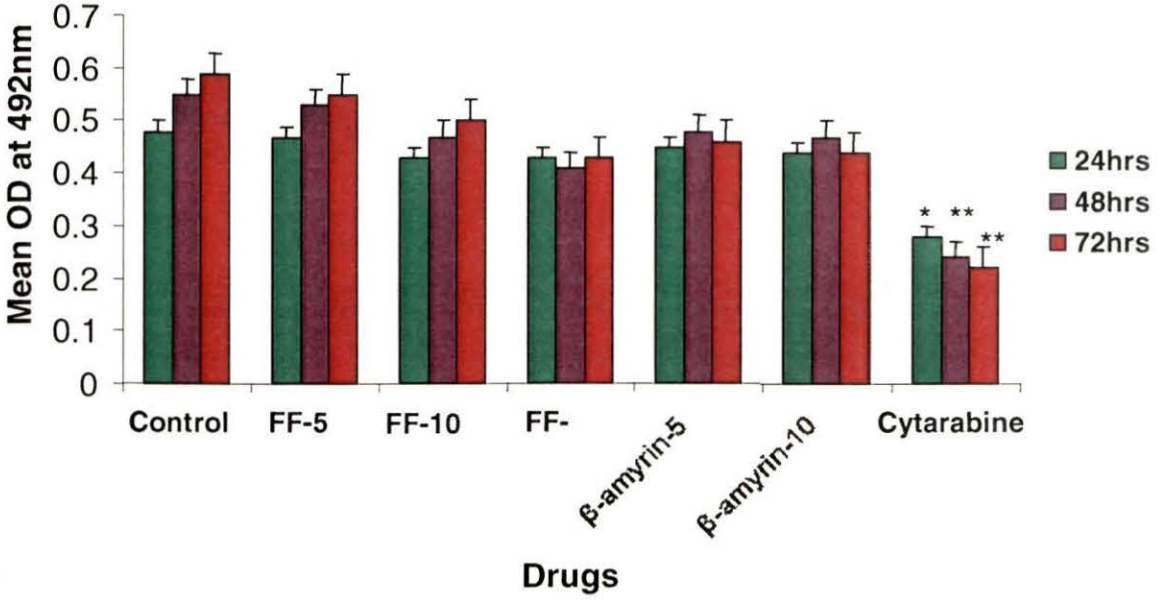


Figure 5.18 Cytotoxicity study of leaves of *Fraxinus floribunda* on human leukemia cell line K-562

Each value represents means of quadruplicate determinations ± SEM .Statistical significance test with control was done .*P<0.05,**P<0.01,***P<0.001, FF-5,10,15-Methanol extract of *Fraxinus floribunda* 5,10,15µg/ml.

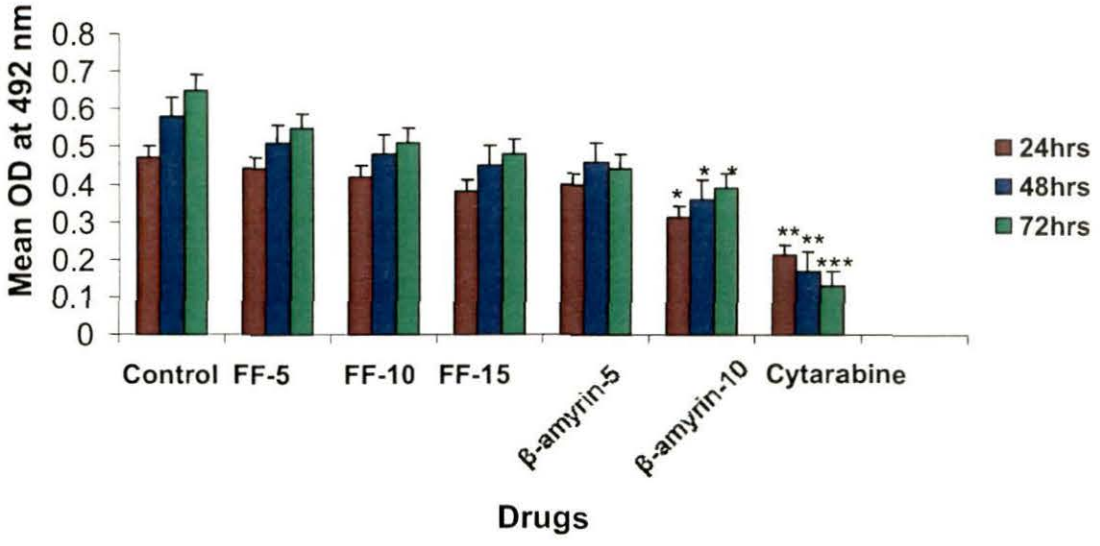


Figure 5.19 Cytotoxicity study of leaves of *Fraxinus floribunda* on human leukemia cell line HL-60

Each value represents means of quadruplicate determinations ± SEM .Statistical significance test with control was done .*P<0.05,**P<0.01,***P<0.001, FF-5,10,15-Methanol extract of *Fraxinus floribunda* 5,10,15µg/ml.

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