APPENDICES

APPENDICES

APPENDIX-I

CULTURING CHICK EMBRYO FIBROBLASTS

(http://homepage.gas.edu/~cellab/chapts/chapts12/eX1210.html)

The primary cells from chick embryo were established in culture flask using Dulbecco's minimal essential medium supplemented with 10 per cent Fetal Bovine Serum (FBS) and penicillin and streptomycin from 100X stock to final concentration of 1X.

REAGENTS

- Complete medium

  DMEM (PAA) containing 10 per cent fetal bovine serum (PAA) and 1X concentration of penicillin and streptomycin.

- Phosphate buffered saline

  KCl-40mg, KH_2PO_4-57.5mg and NaCl-800mg were dissolved in 100ml of water.

- Trypsin-EDTA

  0.25 per cent trypsin 1mM EDTA.2Na -1X

PROCEDURE

A fertilized egg (8-11 days old) was swabbed with 70 per cent ethanol, the top of the egg was carefully punctured with a sterile scissor point and a circle of the shell was cut out carefully, the underlying membrane (the choriallantosis) was carefully cut with another sterile scissor and removed.

The embryo was gently transferred with a sterile metal hook or a bent glass rod into a 100mm petridish containing sterile PBS. The embryo was washed several times with PBS and transferred into a fresh petriplate to completely remove the yolk and blood. The embryo was then placed in a

washed several times with PBS and transferred into a fresh petriplate to completely remove the yolk and blood. The embryo was then placed in a fresh dish with PBS. Using two sterile forceps, the head, limbs and viscera were removed. Care was taken to remove the entire limb by pulling at the proximal end. The remaining tissue was transferred to a fresh petriplate with PBS. The embryo was minced into fine pieces with a bent scissors, the fine pieces were transferred into a flask containing PBS and allowed to settle and the PBS was aspirated out with a sterile pipette. Two ml of trypsin- EDTA was added and the solution was stirred gently for 15-20 minutes at 37°C.

The larger tissue pieces were allowed to settle and the supernatant was decanted into an equal volume of medium with serum. This was then centrifuged at 1000rpm for 10minutes in a standard clinical centrifuge and the supernatant was discarded.

The pellet was resuspended in fresh DMEM containing 10per cent FBS and antibiotics. From this 20µl of the culture was taken to determine cell count and the viability by trypan blue exclusion in a hemocytometer. Then cells were seeded in 25cm^2 sterile disposable plastic culture flasks containing complete medium to a final count of 10^5 live cells/ml. These flasks were incubated at 37°C in 5 per cent CO_2 and 95 per cent humidity till a monolayer formed.

These cells were then trypsinized and passaged and seeded in well plate for further assay.

APPENDIX-II

VIABILITY TEST [SULPHORHODAMINE ASSAY]

(Skehan *et al.*, 1990)

Sulphorhodamine B (SRB) is a bright pink amino xanthene dye with two sulphonic groups. Under mildly acidic conditions, SRB binds to basic amino acids in the protein of TCA fixed cells to provide a sensitive index of cellular protein content which is directly proportional to cell viability. This assay provides a sensitive measure of drug induced cytotoxicity and is well suited to high volumes of automated drug screening.

REAGENTS

- Complete medium: DMEM, 10 per cent FBS and antibiotics
- Phosphate buffered saline (PBS)
- 40 per cent TCA
- Sulphorhodamine B (SRB) 0.4 per cent in 1 per cent TCA
- 1 per cent Acetic acid
- 10mM Tris (P^{H}10.5)

PROCEDURE

The cells after treatment were washed with PBS. An aliquot of 350µl of ice cold 40 per cent TCA was layered on top of the treated cells and incubated at 4°C for one hour after which they were washed 5 times with 200µl of ice cold PBS. The PBS was removed, SRB (350 µl) was added to each well and left in contact with the cells for 30minutes at room temperature, after which they were washed four times with 350 µl portions of 1 per cent acetic acid to remove the unbound dye, then 10mM Tris (350 µl) was added to each well to stabilize the protein bound dye. They were shaken gently for

20minutes, then the Tris layer in each group was transferred to new 96 well plate and the absorbance was read in microtitre plate reader (Anthos 2020, Austria) at 492nm. The cell survival was measured as the per cent absorbance compared to the control (untreated cells).

APPENDIX-III

CYTOTOXICITY TEST [MTT DYE REDUCTION ASSAY]

(Igarashi and Miyazawa, 2001)

Live cells convert MTT into its formazan derivative, the number of surviving cells can be determined by the amount of MTT formazan produced, which is measured in a microtiter plate reader.

REAGENTS

- Complete medium: DMEM, 10 per cent FBS and antibiotics
- Phosphate buffered saline
- MTT-3mg/ml in PBS
- Isopropanol in 0.04 M HCl (acid- propanol)

PROCEDURE

The treated cells were washed with PBS and incubated with 50 µl of MTT at 37°C for three hours. After incubation 200 µl of PBS was added to all the samples. The liquid was then carefully aspirated, then 200 µl of acid propanol was added and left overnight in the darks, the absorbance was read at 650nm in a microtitre plate reader. The optical density of the untreated control was fixed as 100 per cent viability and the per cent viability of the cells in the other treatment groups was calculated relative to it.

APPENDIX-IV

OBSERVATION OF APOPTOTIC MORPHOLOGICAL CHANGES [GIEMSA STAINING]

(Chih *et al.*, 2001)

Apoptosis is characterized by various prominent morphological changes in cells like shrinkage, membrane blebbing and the formation of apoptotic bodies. The changes can be visualized under the phase contrast microscope after staining with giemsa.

REAGENTS

- Complete medium: DMEM, 10 per cent FBS and antibiotics
- Phosphate buffered saline
- Liquid giemsa stain (1:1) diluted in PBS

PROCEDURE

Diluted giemsa stain (10 µl) was added to the coverslip and spread by placing another coverslip over it. The cells were observed under phase constrast microscope (Nikon, Japan) at 400X magnification for morphological changes. The apoptotic ratio was calculated using the formula

Error!

$$\text{Apoptotic ratio} = \frac{\text{Number of apoptotic cells}}{\text{Number of normal cells}}$$

APPENDIX-V

NUCLEAR CHANGES DURING APOPTOSIS (ETHIDIUM BROMIDE STAINING)

(Modified Mercille and Massie, 1994)

Ethidium bromide intercalates between the two strands of DNA, thus staining it. The nuclear changes of DNA fragmentation and condensation and chromatin gathering at the peripheri of the nuclear membrane can be visualized and studied after staining with EtBr.

REAGENTS

- Complete medium: DMEM, 10 per cent FBS and antibiotics
- Phosphate buffered saline
- Ethidium bromide (5 μg/ml in PBS)

PROCEDURE

The treated cells were incubated for 5 minutes with 10 μl of EtBr and spread by placing a coverslip over it. The apoptotic cells were scored by counting the cells with condensed chromatin and fragmented nuclei under fluorescent microscope (Nikon, Japan) using UV2A fiter at 400X magnification.

The apoptotic ratio was calculated as

$$\text{Apoptotic ratio} = \frac{\text{Number of apoptotic cells}}{\text{Number of normal cells}}$$

APPENDIX-VI

PROPIDIUM IODIDE STAINING

(Sarker *et al.*, 2000)

Propium Iodide (PI) is a fluorescent molecule that intercalates nucleic acids and can be used to visualize the nuclear changes such as DNA fragmentation and condensation in cells undergoing apoptosis.

REAGENTS

- Complete medium: DMEM, 10 per cent FBS and antibiotics
- Phosphate buffered saline
- Acetone: methanol (1:1)
- Propidium iodide (PI)- 5µg/ml in PBS

PROCEDURE

After the treatment, the cells were washed with PBS thoroughly and were permeabilized with 50µl of acetone methanol (1:1) mixture at -20.C for 10 minutes. Then, 10µl of PI was added, spread by placing of a coverslip and incubated at 37°C for 30 minutes in the dark.

Apoptotic cells were detected using the green filter of the fluorescence microscope (Motic, HongKong) at 400X magnification. The apoptotic ratio was calculated by the formula mentioned below

$$\text{Apoptotic ratio} = \frac{\text{Number of apoptotic cells}}{\text{Number of normal cells}}$$

APPENDIX-VII

DAPI STAINING

(Rashmi *et al.*, 2003)

DAPI (4'-6-diamino-2-phenyl indole) forms fluorescent complexes with double stranded DNA. Because of this property, DAPI is a useful tool to distinguish apoptotic cells from normal cells.

REAGENTS

- Complete medium: DMEM, 10 per cent FBS and antibiotics
- Etoposide
- Phosphate buffered saline
- 3 per cent paraformaldehyde in PBS
- 0.2 per cent Triton X-100 in PBS
- DAPI (4'-6-Diamidino-2-phenyl indole)-1μg/ ml in PBS

PROCEDURE

After removing the medium and serum, the treated cells were fixed with 3 per cent paraformaldehyde (50μl) for 10 minutes at room temperature, permeabilized with 0.2 per cent Triton X-100(50 μl l) for 10minutes at room temperature. Then they were incubated for 3 minutes with 10 μl of DAPI, after placing a coverslip over the cells to enable uniform spreading of DAPI.

The apoptotic cells were scored by counting the cells with condensed chromatin and fragmented nuclei under fluorescent microscope (Motic, HongKong) using DAPI filter at 400X magnification. The apoptotic ratio was calculated as given earlier.

APPENDIX-VIII

DETECTION OF DNA FRAGMENTATION (Yin *et al.*, 1994)

DNA fragmentations that occurs during apoptosis breaks the chromosomal DNA to smaller fragments with 3' overhang. These fragments, when run on an agarose gel, form a typical laddering pattern after electrophoresis.

REAGENTS

- Phosphate buffered saline (prepared as given in APPENDIX I)
- Lysis buffer [10mMTris-HCl, 10mM EDTA and 0.2 per cent Triton X-100 (pH 4-5)]
- Buffer saturated phenol
- Chloroform: Isoamyl alcohol (24:1) prepared fresh
- 4M NaCl
- Ice cold 70 per cent ethanol
- RNase A (0.6 mg/ml)
- 2 per cent agarose (NMP)
- 1X TAE buffer (20mM Tris, 0.006 per cent glacial acetic acid)
- TE buffer
- Ethidium bromide

PROCEDURE

The cells were treated with etoposide and or plant extract as explained in methodology. The tubes were centrifuged after incubation and washed twice with cold PBS. The cell pellet was lysed in 1ml lysis buffer. After 10 minutes on ice, the lysate was centrifuged at 13,000g for 10 minutes at 4°C. The nucleic acid in the supernatant was extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous phase was brought to 4M NaCl and

the nucleic acids were precipitated with two volumes of ice cold ethanol at -20°C overnight. The pellet was rinsed with ice cold 70 per cent ethanol, air-dried and dissolved in 20µl of TE buffer.

After digestion of RNA with RNase A (0.6mg/ml) at 37°C for 30 minutes, the samples were electrophoresed in a 2per cent agarose gel with TAE buffer. The DNA was visualized with Etbr stain under UV transilluminator and documented using Alpha Digidoc gel documentation system (Alpha innotech, U.K).