# 4. MATERIALS AND METHOD

## 4.1. Materials

### 4.1.1. Procurement of drug and powdered part of plant

Tamoxifen was procured from Sigma Aldrich Ltd. The authenticate powder of *Butea monosperma* flowers and *Lycopersicon esculentum* fruits were procured from Amines Biotech, Vadodara. The authenticate powder of *Cassia fistula* pods was procured from Kisalaya Herbals Ltd., Indore. (Annexure 9.1)

### 4.1.2. Preparation of extracts

The powder was soaked separately with water, methanol, butanol, and ethyl acetate for 48 h at room temperature with occasional stirring. After filtration through cheese cloth, the filtrates were concentrated using either rotary evaporator (organic solvents) or on water bath (water). The extracts were stored at 4°C until use. The extracts were labeled as follows:

| Plants | Aqueous | Methanol | Butanol | Ethyl acetate |
|---|---|---|---|---|
| *Butea monosperma* (flowers) | AEBM | MEBM | BEBM | EAEBM |
| *Lycopersicon esculentum* (fruits) | AELE | MELE | BELE | EAELE |
| *Cassia fistula* (pods) | AECF | MECF | BECF | EAECF |

### 4.1.3. Chemicals and kits

Estrogen positive MCF-7, HER-2 positive MDA-MB-453 and triple negative (ER –ve, PR –ve, and HER-2 –ve) MDA-MB-231 human breast cancer cell lines and Ehrlich ascites carcinoma (EAC) were procured from National Center for Cell Science (NCCS), Pune. MCF-10A was procured from ATCC. Methylnitrosourea (MNU), Propidium iodide, diphenylamine, orcinol were procured from Sigma Aldrich. MTT (3-4,5- dimethylthiazol-2,5 biphenyl tetrazolium bromide), Dimethyl Sulphoxide (DMSO), Culture media

(Dulbecco's modified Eagle's medium), fetal bovine serum (FBS), penicillin G-streptomycin antibiotic solution, trypsin- EDTA, trichloroacetic acid, HEPES buffer, Thiobarbituric acid were procured from Himedia. Superoxide dismutase, Catalase, Reduced gluthathione was procured from Sigma. Annexin V-FITC assay kit was procured from BD sciences. The mouse VEGF ELISA kit was procured from Krishgen Biosystems, Mumbai. The mouse IL-6 and TNF-α ELISA kit were procured from RayBiotech, USA. The β- glucosidase and Caspase-9 kits were procured from Genxbio Health Sciences Pvt. Ltd, Delhi. The liver serum enzymes (AST and ALT) kits were procured from Span Diagnostic Ltd**,** India.

### 4.1.4. Cell line and cell culture

The human breast cancer cell line (MCF-7, MDA-MB-231, MDA-MB-453, MCF-10A) and mouse (EAC) cell line were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) - high glucose supplemented with 10% FBS, 1% penicillin G-streptomycin antibiotic solution. Cells were grown at 37° C in humidified incubator with 5% $CO_2$.

## 4.2. *In-vitro* cytotoxicity assay (MTT Assay)

*Day* 1: [156]

- Determination of cell count:

The tissue culture flask was trypsinized, for detachment of the cells and then the cell suspension was suitably diluted to a fixed volume. Ten µl of trypan blue was taken and in that 10 µl of cell suspension was added and vortexed gently. This resultant mixture was then added carefully in the haemocytometer chamber and observed under optical microscope (Nikon Eclipse TS100 10X). Cells in the four squares in the corner were counted and average number of cells per square was calculated, from this total cells per 1 ml was calculated

from the following formula: Total cells = Average cells per square * Dilution factor * $10^4$.

- Seeding of 96-well Plates

Cells to be assayed were seeded onto 96-well plate at a density of 5 x$10^6$ cells/ well. Cells were allowed to adhere on the inner surface of wells while plate was placed in incubator for 24 hours (37$^o$C with 5% $CO_2$).

*Day* 2:

After 24 h, cells were treated with extracts in different concentration such as 10, 100, 200, 300, 500, 1000 µg/ml & blank. The cells were incubated for 24, 48 and 72 hrs.

*Day* 3:

After incubation period, wells were washed thoroughly with PBS three to four times. After washing 20µl of freshly prepared MTT dye solution (5mg/ml) was added to each well containing cell and incubated for 4 hrs. After incubation, 200µl of DMSO was added to each well containing cells and developed color was measured at 570nm in Microplate ELISA Reader (680 XR, Biorad, India).

% Cell inhibition was calculated using following formula: [156]

$$\% \text{ cell inhibition} = [[A_{Control} - A_{Treated}] / A_{Control}] \times 100$$

$$\% \text{ cell viability} = (A_{Treated} / A_{Control}) \times 100$$ [260]

Similar MTT assay procedure was carried out for all cell lines.

## 4.3. *In-vivo* evaluation of extracts (AEBM, MEBM, EAELE, MELE, AECF and MECF) in MNU induced mammary carcinogenesis

### 4.3.1. Animals

Nulliparous Sprague Dawley female rats were obtained from Zydus Research Centre, Ahmedabad. The animals were housed in a group of 6 rats per cage under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ±

5%) and 12hrs/12hrs light-dark cycle. The animals had free access to conventional laboratory diet and distilled water *ad libitum*.

The experiment was carried out as per guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and The Prevention of Cruelty to Animals act (PCA), 1960. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC), Pharmacy Department, The Maharaja Sayajirao University of Baroda (MSU/IAEC/2014-15/1436).

### 4.3.2. Dose fixation

The literature revealed that the selected extracts were non-toxic. The *Butea monosperma* flowers, *Lycopersicon esculentum* fruits and *Cassia fistula* pods were safe up to 6000 mg/kg[222], 5000mg/kg[223] and 6600mg/kg.[220] The doses (100, 200 and 400mg/kg) was fixed on basis of toxicity data and the anticancer studies.[28]

### 4.3.3. MNU induced mammary carcinogenesis

Mammary cancer in rats was induced by a single intraperitoneal injection of 50mg/kg body weight of MNU, dissolved in 0.9% saline adjusted with acetic acid (pH=4).[149, 150, 157, 159, 160]

The rats were randomly divided into twenty-two experimental groups of six animals each. Group I served as normal control and received saline. All groups (II-XXII) except normal control animals received MNU. Group II served as model control and received MNU (50 mg/kg, i.p). Group III served as vehicle control and received sesame oil as per the body weight. Group IV served as standard group and received Tamoxifen (1mg/kg b.w; s.c).[261] Groups V-XXII served as test group. Groups V-VII received MEBM (100 mg/kg, 200 mg/kg and 400 mg/kg respectively; p.o). Groups VIII-X received AEBM (100 mg/kg, 200 mg/kg and 400 mg/kg respectively; p.o). Groups XI-XIII received EAELE

(100 mg/kg, 200 mg/kg and 400 mg/kg respectively; p.o). Groups XIV-XVI received MELE (100 mg/kg, 200 mg/kg and 400 mg/kg respectively; p.o). Groups XVII-XIX received AECF (100 mg/kg, 200 mg/kg and 400 mg/kg respectively; p.o). Groups XX-XXII received MECF (100 mg/kg, 200 mg/kg and 400 mg/kg respectively; p.o). The treatment was given for 100 days from the day of MNU injection.

During experimental period, the rats were palpated for tumors every two weeks. Body weight (weekly) and food intake (daily) were measured. At the end of experimental period, blood was withdrawn from retro-orbital plexus from each animal and total blood count was performed. Animals were euthanized humanely for assessing different parameters.

### 4.4.1 Growth rate

Body weights were measured daily. The growth rate was measured with the formula: [262]

$$\textbf{Growth Rate} = \left(\frac{\text{Final Body Weight}}{\text{Initial Body weight}}\right)^{\left(\frac{1}{n-1}\right)} - 1$$

n=number of time periods

### 4.4.2 Feed consumption efficiency

The food consumption was measured daily and feed consumption efficiency was measured by formula: [262]

$$\textbf{Feed Consumption Efficiency} = \left(\frac{\text{Weekly Body weight gain}}{\text{Weekly Food Consumption}}\right) \times 100$$

### 4.4.3 Tumor parameters

Tumor parameters involved tumor incidence (number of rats with tumor per total number of rats), number of tumors, tumor multiplicity (average number of tumors per rat), weight, volume (volume = $(\pi/6) \times d1^2 \times d2$; $d1$ is the shortest

diameter, and *d*2 is the longest diameter), and latency period (lag time between MNU injection and first tumor appearance) [263-265]

### 4.4.4 Estrogen and Progesterone receptor expression studies by immunohistochemistry

For identification of the presence of estrogen (ER) and progesterone receptor (PR), standard immunohistochemical procedures were followed in breast tumor samples of rats.[266, 267] Briefly, tumor sections were rehydrated in PBS and endogenous peroxidase activity was quenched with 0.5% hydrogen peroxide. Tissue sections were blocked with 1.5% normal goat serum solution followed by incubation overnight at 4°C in primary antibody, at 1 Ag/mL, against human ER (rabbit polyclonal ER) or human PR (rabbit polyclonal PR). These antibodies are known to cross-react with rat ER and PR. Sections were incubated with a biotinylated secondary antibody at 1 Ag/mL (goat anti-rabbit IgG) and labeled with a streptavidin-peroxidase. Color development was detected using, 3V-diaminobenzidine as substrate. Tissues were counterstained with Harris hematoxylin, dehydrated in a series of 95% and 100% ethanol, and cleared in xylene, and coverslips were mounted. Positively stained cells were identified by darkly stained nuclei, whereas those stained with a nonspecific rabbit IgG remained blue and stained only with hematoxylin. The procedure was performed at Deshpande Laboratories, Bhopal.

### 4.4.5 Nucleic acid contents (DNA and RNA)

The nucleic acids were extracted by the method of Schneider.[218] In the breast tissue homogenates 5 mL of 5% TCA was added to allow complete precipitation of proteins and nucleic acids. The reaction mixture was centrifuged and the precipitate was heated at 90°C for 15 min with occasional shaking, which facilitated the quantitative separation of nucleic acids from protein.

DNA was estimated by the method of Burton.[219] In this reaction 3 mL of above sample was mixed with 2 mL of diphenylamine reagent and this was kept in water bath for 10 min to form blue color substance, which was read at 595 nm using spectrometer. The DNA content was expressed as mg/g wet tissue.

The level of RNA was estimated by the method of Rawal et al.[219, 268] In this reaction, 3 mL of orcinol-ferric chloride reagent was added to 2 mL of sample. The tubes were heated in water bath for 20 min. After that, the tubes were cooled and the color developed was measured at 595 nm using spectrometer. The RNA content was expressed as mg/g wet tissue.

### 4.4.6 Antioxidant enzyme status

**a)** Tissue protein[269]

Reagents:

**1.** Sodium hydroxide (0.1M): 4g of sodium hydroxide was dissolved in 400 ml of distilled water and the final volume was made up to 1000 ml with distilled water.

**2.** Lowry C reagent: (a) Copper sulphate in 1% sodium potassium tartarate (1% w/v) 0.5g of copper sulphate was dissolved in 1% sodium potassium tartarate (Prepared by dissolving lgm of sodium potassium tartarate in 100 ml of distilled water), (b) Sodium carbonate in 0.1M sodium hydroxide (2% w/v) 2g of sodium carbonate was dissolved in 100 ml of 0.1M sodium hydroxide. 2ml of solution (a) was mixed with 100 ml of solution (b) just before use.

**3.** Standard Protein (Bovine serum albumin): 20mg of bovine serum albumin was dissolved in 80 ml of distilled water and few drops of sodium hydroxide were added to aid complete dissolution of bovine serum albumin and to avoid frothing. Final volume was made up to 100 ml with distilled water and stored overnight in a refrigerator.

**4.** Folin's phenol reagent: Folin's phenol reagent was diluted with distilled water in the ratio of 1:2. (i.e. 1ml of Folin's phenol reagent was mixed with 2ml of distilled water).

Procedure**:**

Diluted breast tissue aliquots (0.1ml) were taken in test tubes. To this, 0.8ml of 0.1 M sodium hydroxide and 5ml of Lowry C reagent was added and the solution was allowed to stand for 15 minutes. Then 0.5ml of Folin's phenol reagent was added and the contents were mixed well on a vortex mixer. Colour developed was measured at 640nm against reagent blank containing distilled water instead of sample. Different concentrations (40-200µg) of standard protein (Bovine serum albumin) were taken and processed as above for standard graph. The values were expressed as mg of protein/ g of wet tissue (mg/g).

**b)** Malondialdehyde (MDA)[268, 270]

Reagents:

1. Thiobarbituric acid (0.67% w/v): 0.67gm of thiobarbituric acid was dissolved in 50 ml of hot distilled water and the final volume was made up to 100 ml with hot distilled water.

2. Trichloroacetic acid (10% w/v):10gms of trichloroacetic acid was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.

3. Standard Malondialdehyde stock solution (50mM): A standard malondialdehyde stock solution was prepared by mixing 25 µl of 1, 1, 3, 3-tetraethoxypropane up to 100 ml with distilled water. 1.0 ml of this stock solution was diluted up to 10 ml to get solution containing 23µg of malondialdehyde/ml. One ml of this stock solution was diluted up to 100 ml to get a working standard solution containing 23ng of malondialdehyde/ml.

Procedure:

Two ml of the tissue homogenate (supernatant) was added to 2.0 ml of freshly prepared 10% w/v trichloroacetic acid (TCA) and the mixture was allowed to stand in an ice bath for 15 minutes. After 15 minutes, the precipitate was separated by centrifugation and 2.0 ml of clear supernatant solution was mixed with 2.0 ml of freshly prepared thiobarbituric acid (TBA).

The resulting solution was heated in a boiling water bath for 10 minutes. It was then immediately cooled in an ice bath for 5 minutes. The colour developed was measured at 532nm (UV-1700, Shimadzu, Japan) against reagent blank. Different concentrations (0-23nM) of standard malondialdehyde were taken and processed as above for standard graph. The values were expressed as nM of MDA/mg protein.

**c)** Reduced glutathione (GSH) [268, 271]

Reagents:

1. Trichloroacetic acid (20% w/v): 20gms of trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was made up to 100 ml with distilled water.

2. Phosphate Buffer (0.2M, pH 8.0): 0.2M sodium phosphate was prepared by dissolving 30.2gms sodium phosphate in 600 ml of distilled water, the pH was adjusted to 8.0 with 0.2M sodium hydroxide solution and the final volume was adjusted up to 1000 ml with distilled water.

3. DTNB reagent (0.6mM): 60mg of 5, 5'-dithiobis (2-nitro benzoic acid) was dissolved in 50 ml of buffer and the final volume was adjusted to 100 ml with buffer.

4. Standard Glutathione: l0mg of reduced glutathione was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.

Procedure:

Equal volumes of tissue homogenate (supernatant) and 20% TCA were mixed. The precipitated fraction was centrifuged and to 0.25ml of supernatant, 2ml of DTNB reagent was added. The final volume was made up to 3ml with phosphate buffer. The colour developed was read at 412nm (UV-1700, Shimadzu, Japan) against reagent blank. Different concentrations (10-50µg) of standard glutathione were taken and processed as above for standard graph. The amount of reduced glutathione was expressed as µg of GSH/mg protein.

**d)** Superoxide dismutase (SOD) [268, 272]

Reagents:

1. Carbonate Buffer (0.05M, pH 10.2):16.8gms of sodium bicarbonate and 22g of sodium carbonate was dissolved in 500 ml of distilled water and the volume was made up to 1000 ml with distilled water.
2. Ethylene diamine tetra acetic acid (EDTA) solution (0.49M):1.82g of EDTA was dissolved in 200 ml of distilled water and the volume was made up to 1000 ml with distilled water.
3. Hydrochloric acid (0.1N):8.5ml of concentrated hydrochloric acid was mixed with 500 ml of distilled water and the volume was made up to 1000 ml with distilled water.
4. Epinephrine solution (3mM): 0.99gm epinephrine bitartarate was dissolved in 100 ml of 0.1N hydrochloric acid and the volume was adjusted to 1000 ml with 0.1N hydrochloric acid.
5. Superoxide Dismutase (SOD) standard (100 U/L): lmg (1000 U/mg) of SOD from bovine liver was dissolved in 100 ml of carbonate buffer.

Procedure:

0.5ml of tissue homogenate was diluted with 0.5ml of distilled water, to which 0.25ml of ice-cold ethanol and 0.15ml of ice-cold chloroform was added. The

mixture was mixed well using cyclo mixer for 5 minutes and centrifuged at 2500 rpm. To 0.5ml of supernatant, 1.5ml of carbonate buffer and 0.5ml of EDTA solution were added. The reaction was initiated by the addition of 0.4ml of epinephrine and the change in optical density/minute was measured at 480nm (UV-1700, Shimadzu, Japan) against reagent blank. SOD activity was expressed as units/mg protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme is taken as the enzyme unit. Calibration curve was prepared by using 10-125 units of SOD.

**e)** Catalase (CAT) [268, 273]

Reagents:

1. Phosphate Buffer (50mmol/l, pH 7.0): (a) 6.81g of potassium dihydxogen orthophosphate ($KH_2PO_4$) was dissolved in distilled water and made up to 1000 ml. (b) 8.90g of disodium hydrogen orthophosphate (Na2HPO4) was dissolved in distilled water and made up to 1000 ml. The solutions (a) and (b) were mixed in the proportion of 1:1.5 (v/v).

2. Hydrogen Peroxide (30 mmol/1): 0.34ml of 30% hydrogen peroxide was diluted with phosphate buffer to 100 ml. This solution was prepared fresh each day.

3. Catalase standard (65,000 U/mg protein; lmg protein/ml): Crystalline beef liver catalase suspension was centrifuged to isolate the crystals of the enzyme that was dissolved in 0.01M phosphate buffer (pH 7.0) to give a final concentration of lmg protein/ml. Before assay, it was diluted with distilled water to obtain 1000 U/ml.

Procedure:

To 2ml of diluted sample 1ml of hydrogen peroxide (30 mmol/1) was added to initiate the reaction. The blank was prepared by mixing 2ml of diluted sample (similar dilution) with 1ml of phosphate buffer (50mmol/l; pH 7.0). The

dilution should be such that the initial absorbance should be approximately 0.500. The decrease in absorbance was measured at 240nm (UV-1700, Shimadzu, Japan). Catalase activity was expressed as µmoles of $H_2O_2$ consumed/min/mg protein.

**4.4. *In-vitro* assay of angiogenesis (Chick Choriollantoic Membrane Assay)**

On day 0, the fertile chick eggs were placed in a fan-assisted egg incubator at 37$^{o}$C. After acclimatization for three days, the eggs were removed, swabbed with 70% IPA and a small window was made on the shell at the pointed end of the egg. The eggs were returned to the incubator (37–37.5 $^{o}$C) till 8th day. On 8th day, a 5mm X 5 mm filter paper disks loaded with extracts (AEBM, MEBM, EAELE, MELE, AECF and MECF) was placed on CAM. For selection of dose, five concentrations were screened ranging from 10 to 50 µg/ml per filter paper disk. Eggs were sealed and were returned to the incubator for a further 48 hr. At the end of the experiment, the eggs were opened and CAM was photographed. The radius of the zone of inhibition of blood vessel growth was visually assessed from the center of each disk to the furthest contiguous area in which tertiary blood vessels were absent. The experiment was repeated with lowest inhibitory concentration. [274-276]

**4.5. *In-vitro* assay of apoptosis (Annexin V FITC/PI binding assay)**

Apoptosis was studied using Annexin V-FITC/ propidium iodide (PI) double staining and analyzed by flow cytometry.[277] The assay was performed according to the manufacturer's instructions. Briefly, the adhered MCF-7 cells were incubated with extracts (AEBM, MEBM, EAELE, MELE, AECF and MECF) (Concentration: $IC_{50}$ obtained in MTT assay on MCF-7 cell line at 24 hours) for 24 h. After 24 h, the cells were removed and centrifuged at 311 ×g for 4 min. The supernatant was removed, and the cells were incubated with 500 µl of binding buffer (0.01 M Hepes/NaOH, pH 7.4, containing 0.14 M NaCl

and 2.5 mM $CaCl_2$). The suspensions were transferred to tubes and centrifuged at 311 ×g for 6 min. The cells were resuspended in 50 µl of binding buffer with 3 µl of annexin V/FITC and 5 µl of PI (50 µg/ml). The cells were incubated at room temperature for 30 min with the addition of 300 µl of binding buffer and analyzed in a flow cytometer (FACS Diva Version 6.1.3)

### 4.6. *In-vitro* assay of cell motility (Scratch Motility Assay)

MCF-7 cells ($3.5 \times 10^5$ cells/well) were seeded in a 6-well plate and grown till confluent. The cell monolayer was then scratched with a sterile pipette tip vertically, washed thrice with PBS and incubated with media containing extracts (AEBM, MEBM, EAELE, MELE, AECF and MECF) (Concentration: $IC_{50}$ obtained in MTT assay on MCF-7 cell line at 24 hours). The cells in the denuded area was photographed with camera attached to inverted microscope (Nikon Eclipse TS100) and counted at 0- and 24- h incubation. The experiment was performed in triplicates.[278]

The percentage inhibition was calculated as follows:

$$\text{Percent inhibition}=100-\left(\left(\frac{no.of\ cells\ in\ denuded\ area\ of\ sample}{no.of\ cells\ in\ denuded\ area\ of\ control}\right) X\ 100\right)$$

### 4.7. *In-vivo* evaluation of extracts (MEBM, EAELE and AECF) in EAC induced solid mammary tumors

#### 4.7.1. Animals

Nulliparous BALB-C female mice were obtained from Torrent Research Centre, Ahmedabad. The animals were housed in a group of 6 mice per cage under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12hrs/12hrs light-dark cycle. The animals had free access to conventional laboratory diet and distilled water *ad libitum*.

The experiment was carried out as per guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and The Prevention of Cruelty to Animals act (PCA), 1960. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC), Pharmacy Department, The Maharaja Sayajirao University of Baroda (MSU/IAEC/2016-17/1639).

### 4.7.2. Dose fixation

The literature revealed that the selected extracts were non-toxic. The *Butea monosperma* flowers, *Lycopersicon esculentum* fruits and *Cassia fistula* pods were safe up to 6000 mg/kg[222], 5000mg/kg[223] and 6600mg/kg.[220] The dose of 400 mg/kg was fixed on basis of toxicity data and the anticancer studies.[224]

### 4.7.3. EAC induced solid mammary tumors

The design of the prevention studies described above did not lend itself to study the mechanism of action of extracts at the tumor cell level. However, to address mechanistic issues, solid tumors were induced by injecting Ehrlich ascites carcinoma (EAC) (0.2 ml of 2 X $10^6$) sub-cutaneously at the abdomen region in BALB/C female mice.[279, 280] From the two potent extracts screened *in-vivo* in MNU induced mammary carcinogenesis and in *in-vitro* mechanistic studies, MEBM, EAELE and AECF were found to be most potent. So, in EAC induced solid mammary tumor model, above mentioned extracts were screened.

The animals were randomly divided into six groups, each of six animals. Group I served as normal control and received distilled water. All groups (II-VI) except normal control were injected EAC (0.2 ml of 2 X $10^6$) sub-cutaneously. Group II served as model control. Group III served as standard control and received Tamoxifen (5µg/ml; s.c)[281, 282]. Group IV- VI were test group and received orally MEBM 400mg/kg, EAELE 400 mg/kg and AECF 400 mg/kg

respectively. The treatment began after 5 days of injecting EAC for period of 30 days.

During experimental period, the mice were palpated for tumors every two weeks and body weights were measured. After experimental period, not more than 0.5 ml blood was withdrawn from retro-orbital plexus from each animal and total blood count, inflammatory markers and liver markers were performed. Animals were euthanized humanely for assessing tumor parameters (weight, volume, and tumor incidence, % increase in life span, mean survival time), organ weight, VEGF, p53, Caspase-9, lysosomal enzymes and histopathology (Liver, Kidneys).

### 4.7.4. Body weight

Body weights were measured daily in grams.

### 4.7.5. Tumor parameters[283, 284]

Tumor parameters involved tumor weight, volume (volume = (4/3) $\pi r^2$; r is mean of r1 and r2 which are two independent radii of tumor mass), % reduction in tumor volume, mean survival time and % increase in life span. [278, 279]

% reduction in tumor volume was calculated by following equation

$$\frac{Tumor\ volume\ of\ control\ on\ 30th\ day - Tumor\ volume\ of\ treated\ on\ 30th\ day}{Tumor\ volume\ of\ control\ on\ 30th\ day} \times 100$$

Mean survival time was calculated by following equation

$$\frac{Day\ of\ first\ death + Day\ of\ Last\ death}{2}$$

% Increase in life span was calculated by following equation

$$\left(\frac{Mean\ survival\ time\ of\ treated\ group}{Mean\ survival\ time\ of\ control\ group} - 1\right) \times 100$$

### 4.7.6. Liver weight

At the end of the experiment, animal were euthanized humanely and liver was excised. Liver weights were measured in grams.

### 4.7.7. Liver enzymes

Procedure:

The liver enzymes[285] were measured with diagnostic kits as per manufacturer's protocol. Reagents were reconstituted and allowed to attain room temperature.

| Pipette into tube marked | Blank | Standard | Test | Control |
|---|---|---|---|---|
| Reagent 1 | 0.25 | 0.25 | 0.25 | 0.25 |
| Serum | - | - | 0.05 | - |
| Standard | - | 0.05 | - | - |
| Mix well and incubate at 37$^{o}$ c for 30 minutes | | | | |
| Reagent 2 | 0.25 | 0.25 | 0.25 | 0.25 |
| Deionised water | 0.05 | - | - | - |
| Serum | - | - | - | 0.05 |
| Mix well and allowed to stand room temperature (15-30°C) for 20 minutes | | | | |
| Solution 1 | 2.5 | 2.5 | 2.5 | 2.5 |

Blank, standard, test and control samples were prepared by mixing the reagents listed in the above table and then OD was read against purified water in a colorimeter using UV spectrophotometer (UV-1700, Shimadzu, Japan) at 505 nm, within 15 minutes.

Calculation:

**AST or ALT activity (in IU/L) =** (Absorbance of Test/Absorbance of Standard) × Concentration of Standard

### 4.7.8. Blood count

At the end of the experiment, the blood was withdrawn form retro-orbital plexus. It was analyzed by automatic analyzer. (BC-2800Vet Auto Hematology Analyzer)

### 4.7.9. Cytokines levels

The cytokine levels were measured with ELISA kits as per manufacturer's protocol

- TNF-α measurement

Reagent preparation:

1. Wash Buffer (1X):

   Dilution: Wash Buffer (1X) was made by adding 5ml of Wash Buffer (20X) to 95ml of distilled water (DI). This was the working solution.

2. Assay Diluent (1X):

   Dilution: Assay Diluent (1X) was made by adding 1ml of Assay Diluent (5X) to 4ml of DI water. This was the working solution.

3. Detection Antibody (50 µl)

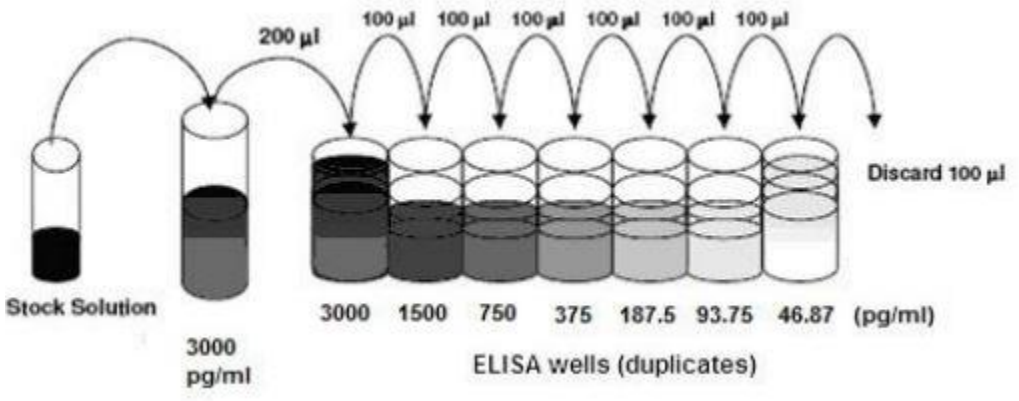
   Dilution: 24µl of Detection Antibody solution was added to 5796 µl of Assay Diluent (1X) to make final volume to 6 ml.

4. Concentrated Streptavidin-HRP 50µl

   Dilution: 6µl of Streptavidin-HRP solution was added to 5994µl of Assay Diluent (1X) to make final volume to 6 ml.

5. Standard (20 µl)

   Standard solution of 3000pg/ml was prepared and aliquots were made as shown below.




Assay procedure:

1. All reagents were brought to room temperature prior to use.
2. Hundred µl/well of standards/samples were added to wells. The plate was sealed and incubated for 2 hours at room temperature (18-25 °C).
3. The plate was washed 4 times with wash buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper.
4. Hundred µl of diluted Detection Antibody solution was added to each well, and incubated for 1 hour at room temperature (18-25 °C).
5. The plate was washed 4 times with wash buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper.
6. Hundred µl of diluted Streptavidin-HRP solution was added to each well, and incubated for 30 minutes at room temperature (18-25 °C).
7. The plate was washed 4 times with wash buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper.
8. Hundred µl of TMB Substrate solution was added and incubated in the dark for 15 minutes.
9. The reaction was stopped by adding 100µl of Stop Solution to each well.

**10.** The absorbance was recorded at 450 nm (ELISA microplate reader 680 XR, Biorad, India) within 30 minutes of stopping reaction

**11.** The concentration was calculated by plotting standard curve.

- IL-6 measurement

Reagent preparation:

1. Wash Buffer (1X):

   Dilution: Wash Buffer (1X) was made by adding 5ml of Wash Buffer (20X) to 95ml of distilled water. This was the working solution.

2. Assay Diluent (1X):

   Dilution: Assay Diluent (1X) was made by adding 1ml of Assay Diluent (5X) to 4ml of distilled water. This was the working solution.

3. Detection Antibody (50 µl)

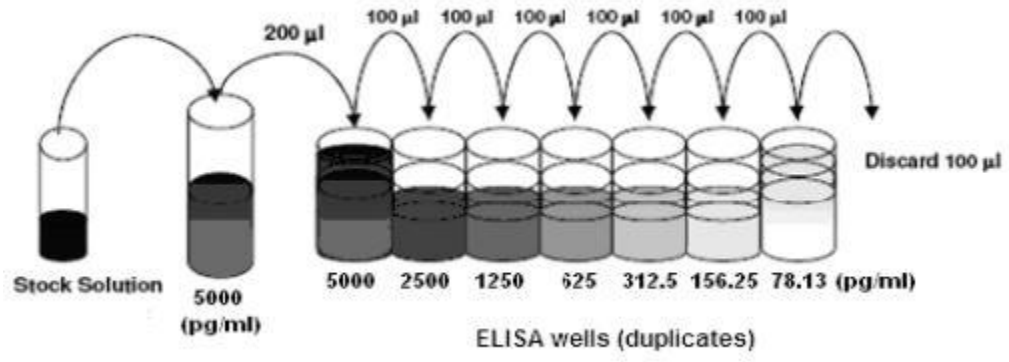
   Dilution: 12.5 µl of Detection Antibody solution was added to 4987.5 µl of Assay Diluent (1X) to make final volume to 6 ml.

4. Concentrated Streptavidin-HRP 50µl

   Dilution: 50µl of Streptavidin-HRP solution was added to 9950µl of Assay Diluent (1X) to make final volume to 10 ml.

5. Standard (20 µl)

   Standard solution of 5000pg/ml was prepared and aliquots were made as shown below.



Assay procedure:

1. All reagents were brought to room temperature prior to use.
2. Hundred µl/well of standards/samples were added to wells. The plate was sealed and incubated for 2 hours at room temperature (18-25 °C).
3. The plate was washed 4 times with wash buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper.
4. Hundred µl of diluted Detection Antibody solution was added to each well, and incubated for 1 hour at room temperature (18-25 °C).
5. The plate was washed 4 times with wash buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper.
6. Hundred µl of diluted Streptavidin-HRP solution was added to each well, and incubated for 30 minutes at room temperature (18-25 °C).
7. The plate was washed 4 times with wash buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper.
8. Hundred µl of TMB Substrate solution was added and incubated in the dark for 15 minutes.
9. The reaction was stopped by adding 100µl of Stop Solution to each well.
10. The absorbance was recorded at 450 nm within 30 minutes of stopping reaction
11. The concentration was calculated by plotting standard curve.

### 4.7.10. Lysosomal enzyme: β-glucosidase

The lysosomal enzyme (β-glucosidase) was measured with ELISA kits as per manufacturer's protocol

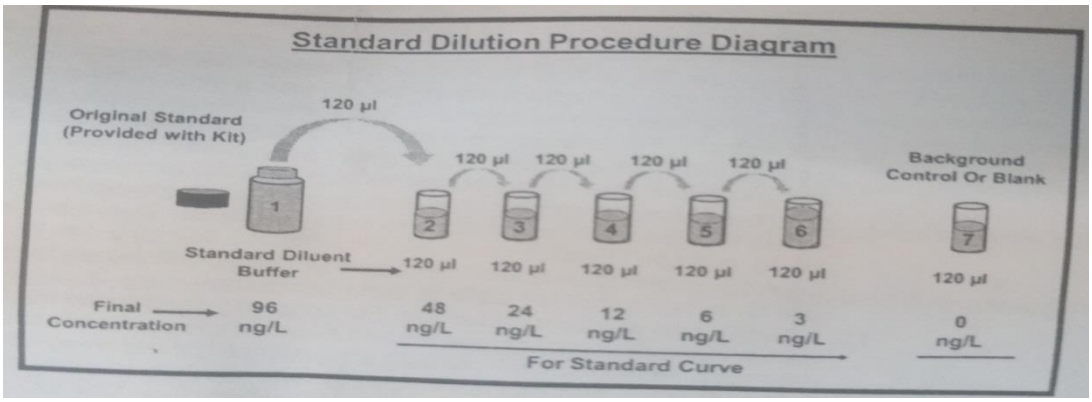
Reagent preparation:

1. Wash Buffer (1X):

   Dilution: Wash Buffer (30X) was made by adding 20 ml of Wash Buffer to 600 ml of distilled water. This was the working solution.

2. Standard (20 µl)

   Standard solution of 96ng/ml was prepared and aliquots were made as shown below.




Assay procedure:

1. All reagents were brought to room temperature prior to use.
2. Fifty µl/well of Standards and 50µl/well of streotomycin-HRP were added to wells. 40µl/well of samples, 10µl/well of B-GLU antibody and 50µl/well of streotomycin-HRP were added to wells The plate was sealed and incubated for 1 hour at room temperature (18-25 °C).
3. The plate was washed 4 times with wash buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper.
4. Fifty µl of chromogen A and 50µl of chromogen B were added and incubated in dark for 30 minutes at Room Temperature (18-25 °C).
5. The plate was washed 4 times with wash buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper.
6. The reaction was stopped by adding 50µl of Stop Solution to each well.
7. The absorbance was recorded at 450 nm (ELISA microplate reader 680 XR, Biorad, India) within 10 minutes of stopping reaction.
8. The concentration was calculated by plotting standard curve.

### 4.7.11. Vascular Endothelial Growth Factor (VEGF)

The VEGF expressions was measured with ELISA kits as per manufacturer's protocol

Reagent preparation:

1. Wash Buffer (1X):

   Dilution: Wash Buffer (1X) was made by adding 5ml of Wash Buffer (20X) to 95ml of distilled water. This was the working solution.

2. Assay Diluent (1X):

   Dilution: Assay Diluent (1X) was made by adding 1ml of Assay Diluent (5X) to 4ml of distilled water. This was the working solution.

3. Detection Antibody (50 µl)

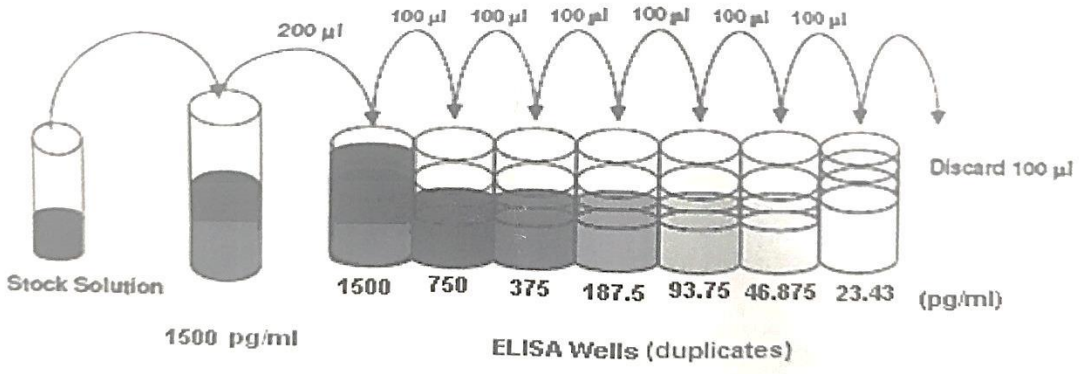
   Dilution: 12.5 µl of Detection Antibody solution was added to 4987.5 µl of Assay Diluent (1X) to make final volume to 6 ml.

4. Concentrated Streptavidin-HRP 50µl

   Dilution: 50µl of Streptavidin-HRP solution was added to 9950µl of Assay Diluent (1X) to make final volume to 10 ml.

5. Standard

   Standard solution of 1500pg/ml was prepared and aliquots were made as shown below.



Assay procedure:

1. All reagents were brought to room temperature prior to use.
2. Hundred µl/well of standards/samples were added to wells. The plate was sealed and incubated for 2 hours at room temperature (18-25 °C).
3. The plate was washed 4 times with wash buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper.
4. Hundred µl of diluted Detection Antibody solution was added to each well, and incubated for 2 hours at room temperature (18-25 °C).
5. The plate was washed 4 times with wash buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper.
6. Hundred µl of diluted Streptavidin-HRP solution was added to each well, and incubated for 30 minutes at room temperature (18-25 °C).
7. The plate was washed 4 times with wash buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper.
8. Hundred µl of TMB Substrate solution was added and incubated in the dark for 15 minutes.
9. The reaction was stopped by adding 100µl of Stop Solution to each well.
10. The absorbance was recorded at 450 nm (ELISA microplate reader 680 XR, Biorad, India) within 30 minutes of stopping reaction
11. The concentration was calculated by plotting standard curve.

### 4.7.12. Immunohistochemistry of p53 Gene

The tumor sections were rehydrated in PBS and endogenous peroxidase activity was quenched with 0.5% hydrogen peroxide. Tissue sections were blocked with 1.5% normal goat serum solution followed by incubation overnight at 4°C in primary antibody, at 1 Ag/mL. Sections were incubated with a biotinylated secondary antibody at 1 Ag/mL (goat anti-rabbit IgG) and labeled with a streptavidin-peroxidase. Color development was detected using, 3V-

diaminobenzidine as substrate. Tissues were counterstained with Harris hematoxylin, dehydrated in a series of 95% and 100% ethanol, and cleared in xylene, and coverslips were mounted. Positively stained cells were identified by darkly stained nuclei, whereas those stained with a nonspecific rabbit IgG remained blue and stained only with hematoxylin. The work was performed as Deshpande Laboratories, Bhopal.

### 4.7.13. Caspase-9

The Caspase-9 expressions was measured with ELISA kits as per manufacturer's protocol

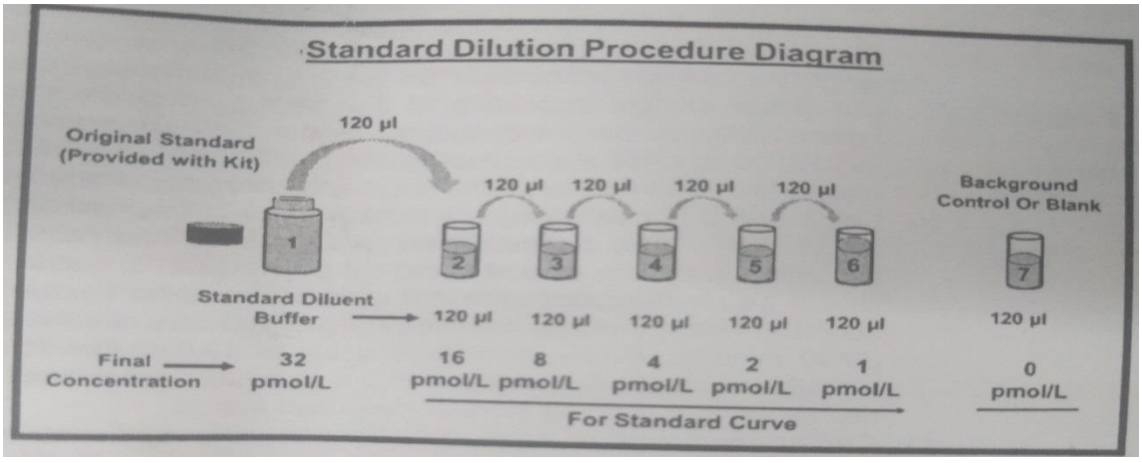
Reagent preparation:

1. Wash Buffer (1X):

   Dilution: Wash Buffer (30X) was made by adding 20 ml of Wash Buffer to 600 ml of distilled water. This was the working solution.

2. Standard

   Standard solution of 32 pmol/ml was prepared and aliquots were made as shown below.




Assay procedure:

**1.** All reagents were brought to room temperature prior to use.

2. Fifty µl/well of Standards and 50µl/well of streotomycin-HRP were added to wells. 40µl/well of samples, 10µl/well of B-GLU antibody and 50µl/well of streotomycin-HRP were added to wells The plate was sealed and incubated for 1 hour at room temperature (18-25 °C).
3. The plate was washed 4 times with wash buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper.
4. Fifty µl of chromogen A and 50µl of chromogen B were added and incubated in dark for 30 minutes at room temperature (18-25 °C).
5. The plate was washed 4 times with wash buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper.
6. The reaction was stopped by adding 50µl of Stop Solution to each well.
7. The absorbance was recorded at 450 nm (ELISA microplate reader 680 XR, Biorad, India) within 10 minutes of stopping reaction.
8. The concentration was calculated by plotting standard curve.

### 4.7.14. Histopathology

At the end of the experiment, animal were euthanized humanely and organs (liver and kidney) were excised and blotted free of blood and tissue fluids and preserved in 10% v/v formal saline solution. The specimens were given for further processing for sectioning, staining and mounting. Briefly, the tissues were washed thoroughly in repeated changes of 70% alcohol and then dehydrated in ascending grades of alcohol (70-100%). After dehydration, the tissues were cleaned in xylene and embedded in paraffin wax. Sections of 5p thickness were cut on a microtome and taken on glass slides coated with albumin. The sections were deparaffinated in xylene and downgraded through 100, 90, 70, 50, 30% alcohol and then finally in water. The sections were stained with haematoxylin and eosin.

### 4.8. Statistical Analysis

The results are expressed as mean ± SEM. Statistical difference between the means of the various groups were analyzed by using one way or two way Analysis of Variance (ANOVA) followed by Bonferroni's post hoc test with P value < 0.05. The regression analysis was performed to evaluate linearity between the doses with $r^2$ value 0.98 or above.