III. MATERIALS AND METHODS

The materials used and methods adopted in the present study entitled **"Evaluation of the anticancer properties of *Acorus calamus* L. rhizome using *in vitro, in vivo* and *in silico* models"** is furnished below

3.1 Preliminary phytochemical studies

3.1.1 Collection and identification of the plant

A. calamus, (Fig.1) the plant used for the present study was collected from Alappuzha district of Kerala, India. Identification of the plant was done in the Department of Botany, Sanadhana Dharma College, Alappuzha. A voucher specimen (10001) is preserved as herbarium and submitted to the Department of Zoology, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore.

Fig. 1. *A. calamus*

3.1.2 Preparation of *A. calamus* rhizome powder

The plant *A. calamus* with rhizome washed thoroughly to remove soil particles and adhered debris using sterile distilled water. Fresh rhizomes used for extraction were shade dried and powdered using a mechanical grinder. Fine powder was obtained by sieving (Fig.2 and Fig.3).The powder was collected in two clean air tight containers. Powdered plant material in one container was used for determining the physico-chemical parameters and the other was used for methanolic extraction.

Fig.2. Rhizome of *A. calamus*

Fig.3. Rhizome powder of *A. calamus*

3.1.3 Soxhlet extraction

10gm of the powder was weighed using an electrical balance (Denver 210) and made in to 8 packets using Xerohaze filter paper (10 A grade SD's). The powder was subjected to sequential Soxhlet extraction (Fig.4) using different solvents like petroleum ether, chloroform, ethanol, methanol and water to get respective extracts. All the extracts were filtered and evaporated to dryness and percentage yields of the extracts were determined. From this the maximum percentage yield was noted and that extract alone was stored in a refrigerator and used for the further analysis.

Fig.4. Soxhlet apparatus used for plant extraction

3.1.4 Preliminary phytochemical screening

The extract was subjected for determination of physicochemical parameters such as moisture content, total ash value, acid insoluble ash value, water soluble ash value, alcohol soluble and water soluble extractive values. The qualitative chemical tests were carried out to find out various phytoconstituents such as alkaloids, steroids, flavonoids, terpenoids, phenolic compounds and tannins present in the methanolic extract of *A. calamus.* The procedures are given in the Appendix I.

3.2 Antitumor activity of *A. calamus*

3.2.1 Acute cytotoxicity study

Chemicals

Trypan blue, Foetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics were purchased from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol were purchased from E.Merck Ltd., Mumbai, India.

Experimental tumor cells

The DAL cell line was obtained from Amala Cancer Research Institute, Thrissur, Kerala and was propagated into transplantable tumors in the peritoneal cavity of mice. The freshly aspirated cells from the mouse peritoneum were washed with Phosphate buffer saline (PBS) under sterile conditions and their concentration was determined using a hemocytometer before transplantation. Animals were inoculated with $1x10^6$ cells / mouse.

Preparation of test solutions

For cytotoxicity studies, each weighed *A. calamus* extract were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

3.2.1.1 Determination of cell viability by Trypan blue dye exclusion technique

Principle: The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay was based on the assumption that the dead

cells will take the dye and viable cells won't (Unnikrishnan and Ramadasan Kuttan, 1988).

Procedure

Short term cytotoxicity studies were done on DAL cells by Trypan blue exclusion method. Cells were aspirated from the peritoneal cavity of tumor bearing mice and washed in PBS twice and counted using a haemocytometer. 2 X 10^5cells/ml were taken for cell cytotoxicity studies. Different concentrations of MEAC (1000, 500, 250, 125, 62.5 µg/ml) was added to the cells and then made up to 1 ml with PBS. Cells were incubated for 3 hours at 37^{o}C. After incubation, the cell death was evaluated using trypan blue exclusion method. To the cell suspension, 3 drops of trypan blue (0.5 % in PBS) was added and the cells were loaded immediately on to a haemocytometer. The number of dead cells was counted and the percentage of dead cells was calculated. Viable cells exclude the dye while non-viable cells take up the dye and appear blue in colour. The percentage growth inhibition was calculated and CTC_{50} value is generated from the dose-response curves for each cell line.

$$\% \text{ Growth inhibition} = 100 - \left(\frac{\text{Total cells} - \text{Dead cells}}{\text{Total cells}} \right) \text{ X } 100$$

3.3 Screening of *in vivo* antitumor activity of *A. calamus*

Experimental animals

Healthy Swiss albino mice, *Mus musculus* (20±5 gm) were used for the study. The animals were obtained from Amala Cancer Institute, Thrissur, Kerala and brought to the laboratory. Animals were kept in polypropylene cages with sawdust bedding and maintained in laboratory conditions. Standard pellets were given as diet and water was provided *ad libitum*. The animals were acclimatized to laboratory condition for about one week before commencement of the experiment. The experiments were performed after the approval from the institutional Animal Ethical Committee (IAEC No: KMCRET / Ph.D. / 2012 -13)

and in accordance with the recommendation for the proper care and use of the laboratory animals.

Acute toxicity study

Healthy Swiss albino mice (20±5 g), starved overnight, were divided in to five groups. Group I – IV animals were orally fed with MEAC in increasing dose levels of 0.5, 1.0, 1.5 and 2.0 g/kg body weight, while group V (untreated) served as control. The animals were observed continuously for first 2 h for any gross change in behavioral, neurological and autonomic profiles or any other symptoms of toxicity and mortality if any, and intermittently for the next 6 h and then again after 24 h, 48 h and 72 h for any lethality or death. One – tenth and one – fifth of the maximum safe dose of the extract tested for acute toxicity were selected for the *in vivo* experiment (Ghosh, 1984).

Treatment procedure

Animals were divided in to five groups (Fig.5) each comprising six animals. One group served as the control while the remaining four groups were injected with Dalton's ascites lymphoma (1 x 10^6 cells/ mouse) to induce tumor. The treatments were given intraperitoneally at 24 h after the tumor inoculation and continued for 14 consecutive days.

The designation of the animal groups and treatment details were as follows:

Group I → Normal control

Group II → DAL control

Group III → DAL + Positive control (5-Fluoro Uracil: 10 mg/kg)

Group IV → DAL + MEAC 100mg /kg

Group V → DAL+MEAC 200mg /kg

All the treatments were continued for 14 days. The body weights of all animals in all groups were noted daily. After the final dose, five animals of each group were sacrificed to study the tumor growth parameters (mean survival time, viable, nonviable cell count, tumor volume, tumor weight and tumor packed cell volume).

Blood was collected by cardiac puncture for the various hematological and biochemical analysis. Tissues like liver and kidney were removed, washed in saline water to remove the adhering blood clot if any, and fixed in 40% formalin for carrying out histological and histopathological studies. Tissues were also homogenized with 0.025M Tris-HCl buffer to carrying out various antioxidant analyses. The rest were kept with food and water *ad libitum* to check with the percentage increase in the life span of the tumor in host.

Fig.5 Experimental Setup

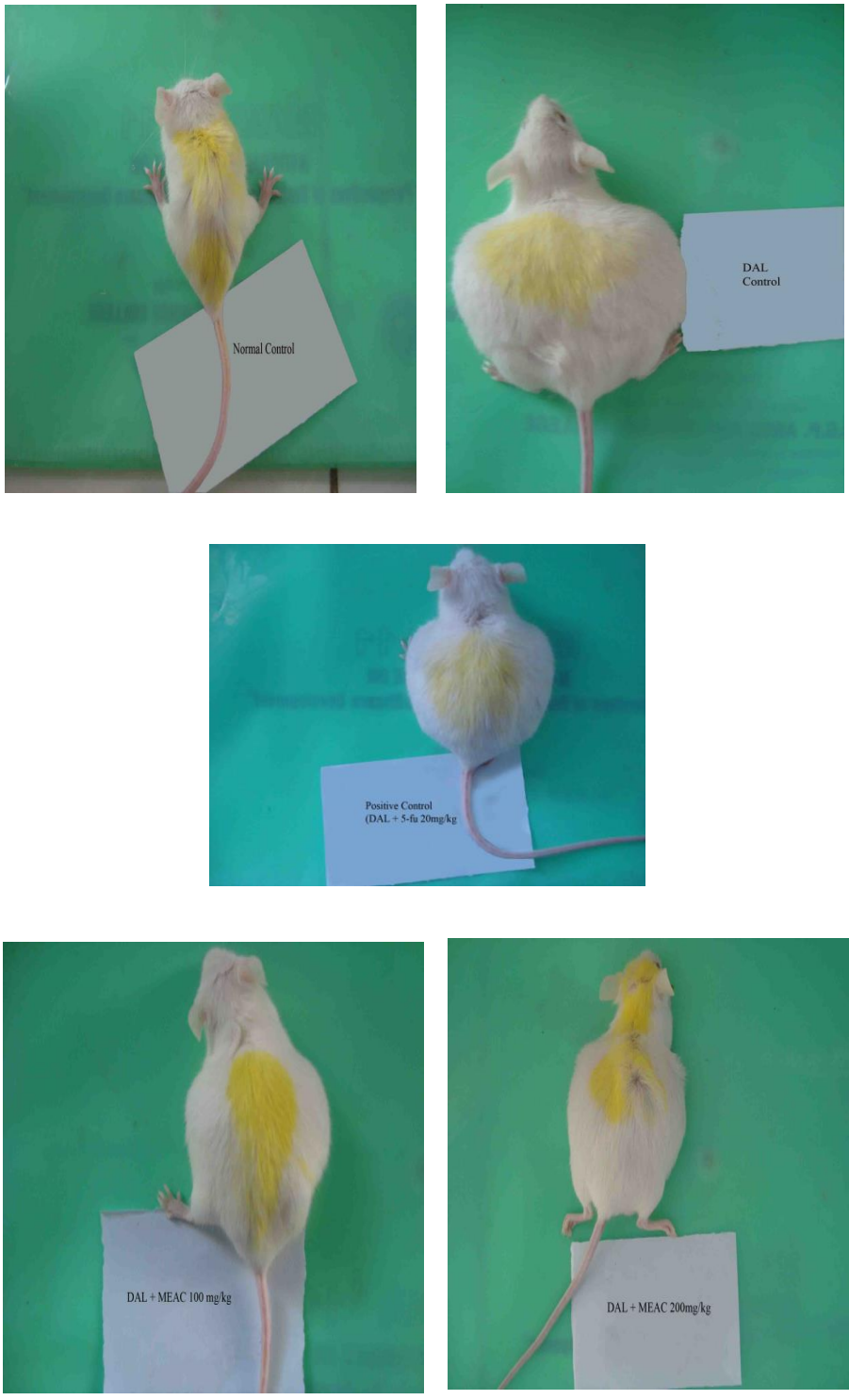

Fig.6. Experimental set up showing DAL inoculated mice and different treatment groups

3.3.1 Determination of tumor growth parameters

3.3.1.1 Mean Survival Time (MST)

After induction, every day, check all the groups for mortality and record how many days the mouse is survived. The mean survival time (MST) and percentage increase in lifespan (ILS %) was calculated by using the formula.

Mean survival time = [First Death + Last Death] / 2

3.3.1.2 Increased Life Span

ILS (%) = [(Mean survival of treated group / Mean survival of control group) – 1] x 100

3.3.1.3 Tumor volume

After 14 days treatment the mice were dissected and the ascetic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube.

3.3.1.4 Tumor PCV

3.3.1.5 Viable and non-viable cell count

Requirements: Dalton's ascites lymphoma (DAL) cells, Phosphate buffer saline solutions, Haemocytometer, Tryphan blue solution (0.4%).

After 14 days treatment animals were slightly anaesthetized with diethyl ether. From the intraperitoneal cavity of mice, take 0.2ml of cell suspension and mixed with 0.5ml of 0.4% tryphan blue, 0.3ml of normal saline or PBS and kept aside for 5 min. and not more than 15 min.

From this one drop of solution was taken on a Neubauer chamber and a cover slip is placed. This is placed on Haemocytometer and the viable and non-viable cells were counted under 10X power. Viable cells does not take colour

and these cells appear in white colour on blue back ground. Non-viable cells (dead cells) take blue colour and give dark blue shading to the cells; cell count was calculated using the formula.

Cell count = No. of cells x dilution factor x volume factor

3.3.2 Body weight analysis

All the mice were weighed for every five days, after tumor inoculation. Average gain in body weight was determined and recorded. The percentage decrease in body weight was calculated by the formula.

% Decrease in body weight = (Decrease in body weight / initial body weight) x 100

3.4. Estimation of hematological parameters

The following hematological parameters were assayed in the experimental animals

3.4.1 Enumeration of red blood cells

The total RBC count was done using a Haemocytometer. Blood collected from the experimental animals was mixed well and drawn in to an RBC pipette up to the 0.5 mark and RBC diluting fluid was taken in the same pipette up to the 11 mark. The loaded RBC pipette was kept on the palm and gently rolled to and fro in order to ensure thorough mixing of the blood and the RBC diluting fluid.

The Neubauer chamber is then loaded with the fluid blood mixture in the RBC pipette and kept aside for one minute to ensure settling of the RBC's. The RBC's were counted in all four corner squares of the Neubauer chamber using a microscope under high power objective. The cells were expressed as number of cells per cubic millimeter of blood.

3.4.2 Estimation of hemoglobin

Sahli's acid haematin method

0.1N Hydrochloric acid was added up to the lowest mark in the Hemoglobinometer using a pipette. 20 µl of the blood sample was drawn in the Sahli's pipette. Care was taken to avoid air bubbles if any in the Sahli's pipette. Excess of blood on the sides of the pipette was wiped off using clean, dry cotton and blown the blood in to the acid solution in the graduated tube. Rinsed the pipette well in the acid solution itself and allowed the mixture to stand at room temperature for two to three min. During this time, the Hemoglobin present in the blood sample is converted in to a brown coloured acid haematin solution. Diluted the acid haematin solution in the graduated tube with distilled water till its colour matches exactly with that of the permanent comparable block. The upper meniscus was noted and the value was expressed as gram /100 ml.

3.4.3 Enumeration of white blood cells

The total WBC count was done using a Haemocytometer. Blood collected from the experimental animals was mixed well and drawn in to a WBC pipetteup to the 0.5 mark and Turk's WBC diluting fluid was taken in the same pipette upto the 11 mark. The loaded WBC pipette was kept on the palm and gently rolled to and fro in order to ensure thorough mixing of the blood and the WBC diluting fluid.

The Neubauer chamber is then loaded with the fluid blood mixture in the WBC pipette and kept aside for one minute to ensure settling of the WBC's. The WBC's were counted in all four corner squares of the Neubauer chamber using a microscope under low power objective. The cells were expressed as number of cells per cubic millimeter of blood.

3.4.4 Differential leukocyte count

One drop of blood was placed at one end of a clean slide. Another slide held an angle of 45^0 above this slide was gently and steadily pushed forward so

as to get a thin smear of blood. Allowed the smear to dry and then stained with Leishman's stain. Examined the stained smear under the oil immersion objective to identify and count the different types of WBC's. The percentage of different types of WBC's was estimated by Battlement count.

3.4.5 Estimation of Packed Cell Volume (PCV)

Estimation of PCV was done by using semi auto analyzer (Photometer 5010 v_{5+}) using standard enzymatic kits procured from Piramal Healthcare limited, Lab Diagnostic Division, Mumbai, India.

3.5. Estimation of biochemical parameters

Various biochemical parameters like alkaline phosphatase (ALP), serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminases (SGPT), total protein (TP), creatine, urea, uric acid, Total cholesterol, Triglycerides and high density lipoprotein (HDL) were estimated using semi – auto analyser (Photometer 5010, Germany) with kits procured from Primal health care limited (Lab diagnostic division, Mumbai).

3.5.1 Estimation of alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) and hydrolytic enzyme act optimally at an alkaline pH. They are present in blood in numerous distinct forms which originate mainly from bone and liver.

p-Nitro phenyl phosphate +water $\xrightarrow{\text{ALP}}$ Phosphate + *p*-Nitro phenol

Kinetic photometric test, according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)

Reagents

Reagent 1: 2-Amino-2-methyl-1-propanol (pH 10.4) - 0.35 mol/l, Magnesium sulphate -2.0 mmol/l, Zinc sulphate - 1.0mmol/l and HEDTA-2.0 mmol/l.

Reagent 2: p-Nitrophenylphosphate -16.0mmol/l

Assay method

1. Taken 1000 µl of reagent-1 in a 5 ml test tube
2. To this added 250 µl of reagent-2 and mixed well
3. Add 20 µl of serum and mix well and take reading immediately using a photometer.

Principle: Under alkaline condition, colorless p-nitrophenol is converted to 4-nitrophenoxide, which develops a very intense yellow color. Its intensity is proportional to the activity of alkaline phosphatase in the sample.

P-Nitro-phenylphosphate + H_2O $\longrightarrow$ ALP > p-Nitrophenol + Phosphate

3.5.2 Estimation of serum glutamate oxalo acetate transaminase (SGOT)

Reagents of the serum glutamate oxalo acetate transaminase (SGOT) in the kit are:

Reagent 1: TRIS p^H 7.8 - 80mmol/l, L-Aspartate -240mmol /l, MDH (Malate dehydrogenase) - ≥ 600U/ l, LDH (Lactate dehydrogenase) - ≥ 600U/l

Reagent 2: 2- oxaloglutarate - 12mmol, NADH – 0.18 mmol, Good buffer p^H 9.6-0.7 mmol/l, Pyridoxal - 5- Phosphate – 0.09mmol/l

Method: Optimized UV test according to the International Federation of Clinical Chemistry (IFCC) and laboratory medicine. 800µl of the reagent 1 mixed with 200µl of the reagent 2 in a 5ml test tube and to this add 100 µl of serum. Mixed well and took the reading immediately.

3.5.3 Estimation of serum glutamate pyruvate transaminase (SGPT) (Moss *et al.*, 1987)

Principle

L-Alanine +2-oxoglutarate $\xleftrightarrow{\text{ALAT}}$ L-Glutamate + Pyruvate

Pyruvate + NADH + H^+ $\xleftrightarrow{\text{LDH}}$ D-Lactate + NAD^+

Addition of pyridoxal-5-phosphate (P-5-P) stabilizes the transaminases and avoids falsely low values in samples containing insufficient endogenous P-5-P.

Reagents

Reagent 1: TRIS pH 7.5 - 100 mmol/l, L- Alanine - 500 mmol/l, LDH (lactate dehydrogenase) - ≥ 1200 U/l.

Reagent 2: 2-Oxoglutarate - 15 mmol/l, NADH - 0.18 mmol, Pyridoxal-5-phosphate FS, Good buffer pH 9.6 - 0.7 mmol/l, Pyridoxal-5-phosphate - 0.9 mmol/l.

Method: Kinetic UV test, according to the International Federation of clinical chemistry (IFCC) and laboratory medicine. Mix 800 µl of the first reagent with 200 µl of the second reagent in a 5ml test tube and add 100 µl of the serum in to this. Mixed it well and took the reading immediately.

3.6 Estimation of serum protein and lipid

3.6.1 Estimation of total protein

Principle: Procedure described by Lowry *et al*. (1951) was used for protein estimation. The method is based on the biuret reaction, formation of a protein-copper complex and reduction of phosphomolybdo tungstate reagent (Folin-ciocalteu phenol reagent) by tyrosine and tryptophan residues of protein to form a coloured product.

Reagents: Solution A: 1 ml $CuSO_4$ $5H_2O$ (1%) + 1 ml sodium potassium tartarate (2%) + 98 ml 2% Na_2CO_3 in 0.1N NaOH. Solution B: Folin Ciocalteu reagent and distilled water mixed in 1:1 ratio just before use.

Procedure: 0.01 ml of tissue homogenate (2.5%) was diluted to 1.2 ml and mixed with 6 ml of solution A. The mixture was incubated at room temperature for 10 min and adds 0.3 ml solution B was added, mixed immediately and kept at room temperature for 30 min. Optical density was taken at 750 nm. The amount of protein was calculated from the standard curve of Bovine Serum Albumin (BSA).

Reagent A: 2% Sodium carbonate in 0.1 N Sodium hydroxide

Reagent B: 0.5 % Copper sulphate in 1% Sodium tartarate

Reagent C: This is a mixture of Reagent A and B. It is prepared freshly by adding 50ml of Reagent A with 1 ml of Reagent B.

Standard: 0.1% Bovine Serum Albumin.

Folin's reagent: 50% dilution

10% Trichloroacetic acid (protein precipitant)

For the estimation of protein, 100mg of tissue and 1ml of serum was precipitated with 10% trichloroacetic acid. This was centrifuged at 5000 rpm for 15 min. The precipitant thus obtained was used for protein estimation. The precipitate was again dissolved in 1ml of 1N Sodium hydroxide. This was treated as sample. 1ml of this sample of tissue and serum was taken and to it 5ml of Reagent C was added and left for 10 min. After 10 min. this was mixed with dilute Folin's reagent and left for 20 min. Then the sample was read in a Spectrophotometer at 720 nm.

3.6.2 Estimation of urea (Burtis *et al.,* 1999)

Urea is the nitrogen-containing end product of protein catabolism. States associated with elevated levels of urea in blood are referred to as hyperuremia or azotemia.

Estimation of urea was carried out by Urease-GLDH (Glutamate dehydrogenase): enzymatic UV test

Principle

$$Urea + 2H_2O \xrightarrow{Urease} 2NH_4 + 2HCO_3$$

$$2\text{- Oxoglutarate} + NH_4^+ + NADH \xrightarrow{GLDH} \text{L- Glutamate} + NAD^+ + H_2O$$

Reagents

Reagent 1: TRIS pH 7.8 - 120 mmol/l, 2-Oxoglutarate - 7 mmol/l, ADP - 0.6 mmol/l, Urease - ≥ 6 KU/l and GLDH - ≥ 1 KU/l.

Reagent 2: NADH - 0.25 mmol.

Reagent 3: Standard - 40 mg/dl

Procedure

1000 µl of reagent 1 and 250 µl of reagent 2 was taken in a 5 ml test tube. To this added 10 µl of serum

Mixed well and immediately read the test sample at 340 nm Hg, optical path 1 cm against reagent blank (2-point kinetic) and noted down the values.

Urea is the nitrogen-containing end product of protein catabolism. States associated with elevated levels of urea in blood are referred to as hyper uremia or azotemia.

3.6.3 Estimation of uric acid (Burtis *et al.,* 1999)

Method

Enzymatic photometric test using TOOS (N-ethyl-N-(hydroxyl-3-sulfopropyl)-m-toluidin)

Principle

$$\text{Uric acid} + H_2O + O_2 \xrightarrow{\text{URICASE}} \text{Allantoin} + CO_2 + H_2O_2$$

$$\text{TOOS} + \text{4-Aminoantipyrine} + 2H_2O_2 \xrightarrow{\text{POD}} \text{Indamine} + 3H_2O$$

Reagents

Reagent 1: Phosphate buffer pH 7.0 - 100 mmol/l, TOOS - 1 mmol/l and Ascorbate oxidase - ≥ 1 KU/l.

Reagent 2: Phospahte buffer pH 7.0 - 100 mmol/l, 4-amino antipyrine - 0.3 mmol/l, $K_4[Fe(CN)_6]$ - 10 µ mol/l, Peroxidase - ≥ 1 KU/l and Uricase - ≥ 50 U/l.

Procedure:

800 µl of Reagent-1 was taken in a 2ml centrifuge tube. To this, 20 µl of serum was added. Mixed well and incubated at 30^{o}C for 5 min. Then added 200 µl of Reagent 2.Mixed well, incubated for 5 min at 37^{o}C. and noted the values.

3.6.4 Estimation of creatinine (Moss *et al*., 1975)

Principle

Creatinine levels were estimated by modified Jaffe Method. Creatinine forms a colored complex with picrate in alkaline medium. This rate of formation is measured.

Reagent 1: Standard creatinine (2 mg/100 ml)

Reagent 2: Picric acid solution

Reagent 3: Sodium hydroxide solution

Procedure

500 µl of reagent -2 and 500 µl of reagent -3 was taken in a 5ml test tube. To this added 100 µl of serum. Mixed well and immediately read the test sample at Hg 492 nm, 1cm light path and noted down the values.

3.6.5 Total cholesterol (TC) (Deeg and Ziegenhorn, 1983)

Principle

Determination of cholesterol is done after enzymatic hydrolysis and oxidation. The colorimetric indicator is quinoneimine, which is generated from 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase (Trinder's reaction).

Cholesterol ester + H_2O $\xrightarrow{CHE}$ Cholesterol + Fatty acid

Cholesterol + O_2 $\xrightarrow{CHO}$ Cholesterol-3-one +H_2O_2

$2H_2O_2$ + 4- Amino antipyrine + Phenol $\xrightarrow{POD}$ Quinonelimine + 4 H_2O_2

Method:

CHOD-PAP: Enzymatic photometric test

Reagents: **O**ds buffer (pH 6.7) - 50 mmol/ l, Phenol - 5 mmol/l, 4-aminoantipyrine - 0.3 mmol/l, Cholesterol estrase - > 200 U/l, Cholesterol oxidase - > 100 U/l, Peroxidase - 3 KU/l, Standard - (5.2 mmol/l)

Assay procedure: 1 ml (1000 µl) of reagent-1 is taken in a 5 ml test tube. Added 0.01 ml (10 µl) of serum mixed well and incubated at 37°C for 5 min. Read the test sample.

3.6.6 HDL cholesterol

Principle: Chylomicrons, VLDL and LDL are precipitated by adding Phosphotungstic acid and magnesium ions to the sample. Centrifugation leaves only the HDL in the supernatant. The cholesterol content in it is determined enzymatically.

Method: Phosphotungstic acid precipitation method.

Reagents: Phosphotungstic acid - 0.55 mmol/l and Magnesium chloride - 25 mmol/l.

Assay procedure:

A. Preparation of supernatant for the HDL-CHL estimation

Added 200 µl of serum to the 500 µl of HDL-Cholesterol precipitating reagent (from HDL kit) in 1.5 ml centrifuge tube and mixed well. Centrifuged the solution at 4000 rpm for 10 min.

B. Preparation of test sample for the estimation of HDL-Cholesterol

1000 µl of reagent-1 (from cholesterol kit) was taken in a 5 ml test tube. 100 µl of supernatant from above centrifuged solution was added. Mixed well and incubated at 37°C for 15 min. Read the test sample.

3.6.7 Estimation of triglycerides (Cole *et al.*, 1997)

Triglyceride was estimated by using Wako method and the modifications by McGowan *et al*. (1983) by using a standard kit obtained from ERBA diagnostics, Manheim Ltd.

Principle

Determination of triglycerides (TG) alters enzymatic splitting with lipoprotein lipase. Indicator is quinoneimine which is generated from 4-

aminoantipyrine and 4- chlorophenol by hydrogen peroxidase under the catalytic action of peroxidase.

Triglycerides $\xrightarrow{LPL}$ Glycerol + fatty acid

Glycerol + ATP $\xrightarrow{GK}$ Glycerol-3-phosphate+ ADP

Glycerol-3-phosphate +O_2 $\xrightarrow{GPO}$ Dihydroxyacetone phosphate + H_2O_2

$2H_2O_2$ + 4- Amino antipyrine + 4- chlorophenol $\xrightarrow{POD}$ Quinonelimine + HCl + $4H_2O_2$

Kit Contents were as follows:

Reagent 1 - triglycerides DES reagent

Lipoprotein Lipase, Glycerol kinase, Glycerol-3-PhosphateOxidase, Peroxidase, 4-aminoantipyrine and ATP, 3-5 D H B S and buffer (pH 7.0) Mg2+, GPO.

Reagent 2 - triglycerides standard (200 mg/dl)

Preparation of the working reagent: Dissolved content of one bottle of reagent with the amount of AQUA- 4 indicated on the label. Swirled to dissolve, allowed to stand for 10 min. at room temperature.

Colorimetric enzymatic test using glycerol-3-phosphate-oxidase (GPO):

Reagents: 4-chloroPhenol - 4 mmol/l, ATP - 2 mmol/l, Mg^{2+} - 15 mmol/l, Glycerokinase - > 0.4 Kμ/l, Peroxidase - > 2 Kμ/l, Lipoprotein lipase - > 4 Kμ/l, 4-aminoantipyrine - 0.5 mmol/l, Glcerol-3-phosphate- oxidase - > 1.5Kμ/l, Standard - (2.3 mmol/l).

Assay procedure

a. 1 ml (1000 µl) of reagent-1 is taken in a 5 ml test tube.
b. Added 0.01 ml (10 µl) of serum.

c. Mixed well and incubated at 37°C for 15 min.
d. Read the test sample.

3.7 Estimation of antioxidant parameters

Preparation of tissue homogenate

The liver samples were weighed and 10% tissue homogenate was prepared with 0.025 M Tris –HCl buffer, pH 7.5. After centrifugation at 10,000 x g for 10 min the clear supernatant was used to measure Thiobarbituric Acid Reactive Substances (TBARS). For the estimation of non-enzymic and enzymic antioxidants, tissue was minced and homogenized (10% w/v) in 0.1 M phosphate buffer (pH 7.0) and centrifuged for 10 min. and the resulting supernatant was used for enzyme assays.

3.7.1 Enzymatic antioxidant activity

3.7.1.1 Estimation of superoxide dismutase (SOD)

Reagents

Adrenaline**,** Carbonate buffer (pH 10.2), 0.1Mm EDTA

The activity of superoxide dismutase (SOD) was assayed by the method of Kakkar *et al*. (1984) based on the oxidation of epinephrine adrenochrome transition by enzyme. The post-mitochondrial suspension (PMS) of mice liver (0.5 ml) was diluted with distilled water (0.5).To this chilled ethanol (0.25 ml) and chloroform (0.15 ml) was added. The mixture was shaken for 1 min. and centrifuged at 2000 x g for 10 min. The PMS (0.5 ml) was added with PBS buffer (pH 7.2; 1.5 ml).The reaction initiated by the addition of epinephrine (0.4 ml) and change in optical density (O.D., min-1) was measured at 470 nm. SOD activity was expressed as U/mg of tissue. Change in O.D (min-1) at 50% inhibition to adrenochrome transition by the enzyme was taken as one enzyme unit.

3.7.1.2 Estimation of catalase (CAT) activity

Reagents

Dichromate/acetic acid reagent (5% solution of potassium dichromate in acetic acid at 1:3 ratios), 0.01 M Phosphate buffer, pH 7.0 and 0.2 M Hydrogen peroxide.

Catalase (CAT) was estimated by the method of Sinha (1972). The reaction mixture (1.5 ml) contained 1.0 ml of 0.01 M phosphate buffer (pH 7.0), 0.1 ml of tissue homogenate and 0.4 ml of 2 M H_2O_2. The reaction was stopped by the addition of 2.0ml dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio).Then the absorbance was measured at 530 nm. CAT activity was expressed as µM of H_2O_2 consumed/min/mg protein.

3.7.1.3 Estimation of glutathione peroxidase (GPx)

Reagents

0.32 M phosphate buffer, pH 7.0, 0.8 mM EDTA, 10 mM sodium azide, 3 mM reduced glutathione, 2.5 mM H_2O_2, 10% TCA, 0.3 M disodium hydrogen phosphate, DTNB solution (40 mg of DTNB in 100 ml of 1% sodium citrate)

Procedure: Glutathione peroxidase (GPx) was measured by the method described by Rotruck *et al*. (1973). Briefly, the reaction mixture contained 0.2 ml 0.4 M phosphate buffer (Ph 7.0), 0.1 ml 10 mM sodium azide, 0.2 ml tissue homogenized in 0.4 M phosphate buffer pH 7.0, 0.2 ml tissue homogenized in 0.4 M phosphate buffer, pH 7.0, 0.2 ml reduced glutathione and 0.1 ml 0.2 mM Hydrogen peroxide. The contents were incubated for 10 min at 37°C, 0.4 ml 10% TCA was added to stop the reaction and centrifuged at 3200 x g for 20 min.The supernatant was assayed for glutathione content using Ellman's reagent (19.8 mg 5, 5'-dithiobisnitrobenzoic acid (DTNB) in 100 ml 0.1% sodium nitrate). The activities were expressed as µg of GSH consumed/ min/mg protein.

3.7.2 Non enzymatic antioxidant activity

3.7.2.1 Estimation of reduced glutathione (GSH)

Reagents: 10% TCA, 0.6 mM 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) in 0.2 M sodium phosphate and 0.2 M Phosphate buffer, pH 8.0.

GSH was measured by the method of Ellman (1959). The Post Mitochondrial Suspension(PMS) of rat liver (720 µl) and 5% TCA were mixed to precipitate the protein content of the supernatant. After centrifugation at 10,000 x g for 5 min, the supernatant was taken. DTNB (5,5'-dithio-bis (2-nitrobenzoic acid), Ellman's reagent was added to it and the absorbance was measured at 412 nm. A standard graph was drawn using different concentration of standard GSH solution.GSH contents were calculated in the PMS of rat liver.

3.7.2.2 Estimation of lipid peroxidation (LPx).

Reagents: Thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA

Lipid peroxidation in liver was estimated calorimetrically by measuring thiobarbituric acid reactive substances (TBARS) using the method of Fraga *et al*. (1988). In brief, 0.1 ml of tissue homogenate was treated with 2 ml of TBA-trichloroacetic acid – HCl reagent (0.37% TBA, 0.25 M HCl and 15% TCA, 1:1:1 ratio), placed for 15 min in a water bath and then cooled and centrifuged at 3500 x g for 10 min. at room temperature, the absorbance of clear supernatant was measured at 535 nm against a reference blank. Values were expressed as Mm/100 g tissue.

3.7.2.3 Estimation of vitamin C and E

Estimation of Vitamin C and E was done by using semi auto analyzer (Photometer 5010 v_{5+}) using standard enzymatic kits procured from Piramal Healthcare limited, Lab Diagnostic Division, Mumbai, India.

3.8 Histopathological analysis.

Histopathology is the microscopical study of tissues for pathological alterations. This involves collection of morbid tissues from biopsy or necropsy, fixation, preparation of sections, staining and microscopical examination.

Collection of materials: Thin pieces of 3 to 5 mm thickness are collected from tissues showing gross morbid changes along with normal tissue.

Fixation: Keeping the tissue in fixative (10% Formalin) for 24 - 48 hours at room temperature

Haematoxylin and Eosin method of staining: Deparaffinised the section by xylol 5 to 10 min. and removed xylol by absolute alcohol, then washed in tap water. Stained with haematoxylin for 3-4 min. and washed in tap water. Allowed the sections in tap water 5-10 min. and washed in tap water. Counterstained with 0.5% until section appears light pink (15 to 30 sec.), then washed in tap water. Blot and dehydrate in alcohol. Clear with xylol (15 to 30 sec.). Mount in Canada balsam or DPx mountant. Keep slide dry and remove air bubbles.

3.9 *In vitro* anticancer activity (MTT assay)

Cell lines: The human breast cancer cell lines (MCF -7) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagle Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37^{o} C, 5% CO_2, 95% air and 100% relatively humidity maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell Treatment Procedure

The mono layer cells were detached with trypsin – ethylene amine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a haemocytometer and diluted with medium containing 5% FBS

to give final density of 1 x 10^5 cells / ml. 100 microliters per well of cell suspension were seeded in to 96 – well plates at plating density of 10000 cells per well and incubated to allow for cell attachment at 37º C, 5% CO_2, 95º air and 100% relatively humidity. After 24 hour the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and diluted to twice the desired final maximum test concentration with serum free medium. Additional four, two fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulted the required final sample concentrations. Following plant extract addition, the plates were incubated for an additional 48 hour at 37ºC , 5% CO_2, 95º air and 100% relatively humidity. The medium without samples were served as control and triplicate was maintained for all concentrations.

MTT assay

3-[4, 5 –dimethylthiazol-2-yl] 2, 5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate- dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan was directly proportional to the number of viable cells. After 48hr of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37ºCfor 4 hour. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using microplate reader. The percentage cell inhibition was determined using the following formula.

% cell inhibition = 100 – Abs (sample) / Abs (control) x 100

3.10 Cytokinesis block micronucleus assay (CBMN)

3.10.1 Cytotoxicity study of MEAC in cultured human lymphocyte

In order to determine the concentration of MEAC which would allow the evaluation of the DNA damage without affecting the cell cycle or inducing cell death, cellular viability tests were performed using a concentration response

curve before carrying out the micronucleus assay. The cytotoxicity of MEAC on human lymphocytes was assayed using the Trypan blue exclusion method after incubation of cells with samples of *A. calamus* at the concentrations of 10, 20, 40, 60 ,80 and 100 µg/ml for 24 h. Viable cells were determined based on the ability of cells to exclude the dye.

Protocol: Collected fresh blood by venepuncture and transferred to heparinised vacutainers. Isolated lymphocytes on lymphoprep (pharmacia) gradients as follows: Added 2 ml of lymphoprep to a 10 ml centrifuge tube and carefully overlay 4 ml of diluted blood sample and centrifuged at 1000 rpm for 10 min. Drawn off the lymphocyte layer using a sterile Pasteur pipette and transferred to a 10 ml tube. Suspended the cell pellet in RPMI 1640 medium and centrifuged for 10 min. The supernatant was removed and repeated the step. Culture the lymphocytes in sterile bottles using RPMI 1640 medium containing 15 % foetal calf serum.

Lymphocytes stimulate to divide with PHA and incubated for 72 hrs at 37℃; 44 hour after PHA stimulation, added cytochalasin B to the cultures to give a final concentration of 4.5 µg / ml. 28 h after addition of cytochalasin –B, transferred the whole content in to a sterile centrifuge tube and centrifuged for 10 min.; removed the supernatant and shaken the pellet in a cyclomixer. Added 10 ml of 0.075 M KCl solution to the cell button and kept at 37℃ for 10 min. After this, added 2 drops of freshly prepared fixative (Methanol: acetic acid) in the ratio 3:1. Again centrifuged at 1000 rpm for 10 min. Removed the supernatant and mixed the cell button in a cyclomixer and added 10 ml of freshly prepared fixative and centrifuged at 1000 rpm for 10 min. Repeated this process until the supernatant becomes clear and the cell button becomes white. From the cell button, prepare cell suspension.

Preparation of slides

The cell suspension (7-8 drops) was dropped on pre cleaned, labeled and chilled slides from a particular height. The slides were flamed gently on spirit lamp, blown gently on the material and air dried. Stained the slides with 10-20%

Giemsa stain solution and allowed to remain for 10 min. After 10 min. the excess stain was washed off with running water; slides were air dried. The slides were examined at 100 X magnification. The number of MN in number less than 1000 binucleated cells was scored and the distribution of MN among binucleated cells was recorded.

Criteria for identifying binucleated cytokinesis block cells

The cytokinesis-blocked cells scored for micronuclei frequency have to satisfy following criteria: Cells should have 2 nuclei of approximately equal size, The 2 nuclei may be attached by a fine nucleoplasmic bridge, The 2 nuclei may overlap slightly or touch each other at the edges, and Cells should not contain more than 6 micronuclei.

Criteria for identifying micronuclei

Micronuclei are morphologically identical to, but smaller than normal nuclei. They have the following characteristic: Size of the micronuclei should be less than the 1/6th of the main nuclei, they are non refractile, they are not linked to the main nuclei via a nucleoplasmic bridge, and Micronuclei may sometimes overlap the boundaries of the main nuclei (Fenech, 1985).

3.10.2 DNA repair proficiency by bleomycin induced chromosome sensitivity analysis.

Mutagen (bleomycin) induced chromosome sensitivity analysis was performed as described by Hsu *et al.* (1989). The cells were cultured for determining the mutagen sensitivity. The culture medium was RPMI 1640, supplemented with 15% fetal bovine serum, 10µg/ml of phytohaemagglutinin, 100 U/ml of benzyl penicillin, 25µg/ml of Amphotericin B and 100µg/ml streptomycin (Fig.6). Bleomycin treatment was given 6 h before harvesting to induce chromosomal breakage. At the end of 70th hour, the cultures were treated with colchicine (0.04 µg/ml) to accumulate mitosis before harvesting. Then the culture was incubated further for 2 h at 37^{0}C.

For mutagen sensitivity, the slides were stained with Giemsa. Chromosomal lesions such as breaks, gaps, acentric fragments, double minute ring chromosomes, dicentrics chromosome, etc. were also scored. The frequency of chromatid breaks were considered as a measure of an individual's DNA repair capacity.

Sample collection: 2-3 ml of peripheral blood was collected aseptically by venipuncture in a sodium heparinized vaccutainer from a healthy donor.

Preparation of tissue culture medium

RPMI 1640 Medium

1) 10.4g of RPMI media powder was weighed and dissolved in 1000ml of triple distilled water at room temperature with constant, gentle stirring until the medium was completely dissolved. The container was rinsed with tissue culture grade water to remove all traces of powder and added to the above solution.

2) Adjusted the pH to 0.2-0.3, using 1N Hydrochloric acid or Sodium hydroxide since the pH tends to rise during filtration.

3) Aseptically added sterile supplements (100µg/ml Benzyl penicillin, 100units /ml of Streptomycin, 25µg/ml of Amphotericin B).

4) The medium was sterilized immediately by using a sterile membrane filter with a porosity of 0.2 micron.

5) The medium was observed for 3 days at room temperature for any contamination.

6) Stored at 2-6^{0}C till use.

Preparation of reagents

Phytohaemagglutinin (PHA-M : 5mg/stock):- Dissolved 5mg stock of PHA-M in 5 ml of distilled water

Bleomycin:- Dissolved 15 U of Bleomycin in 1ml sterile distilled water

Colchicine (stock -1gm):- Dissolved 1 mg of stock colchicine (98%) in distilled water to obtain the desired quantity of 0.04µg/ml concentration.

Hypotonic KCl solution (0.075M):- Dissolved 5.6g of KCl (99.9%) in 1000ml of distilled water.

Fixative: Methanol and Glacial acetic acid in the ratio 3:1(v/v). Mixed three parts of methanol (99.8%) and one part of glacial acetic acid (99.7%).

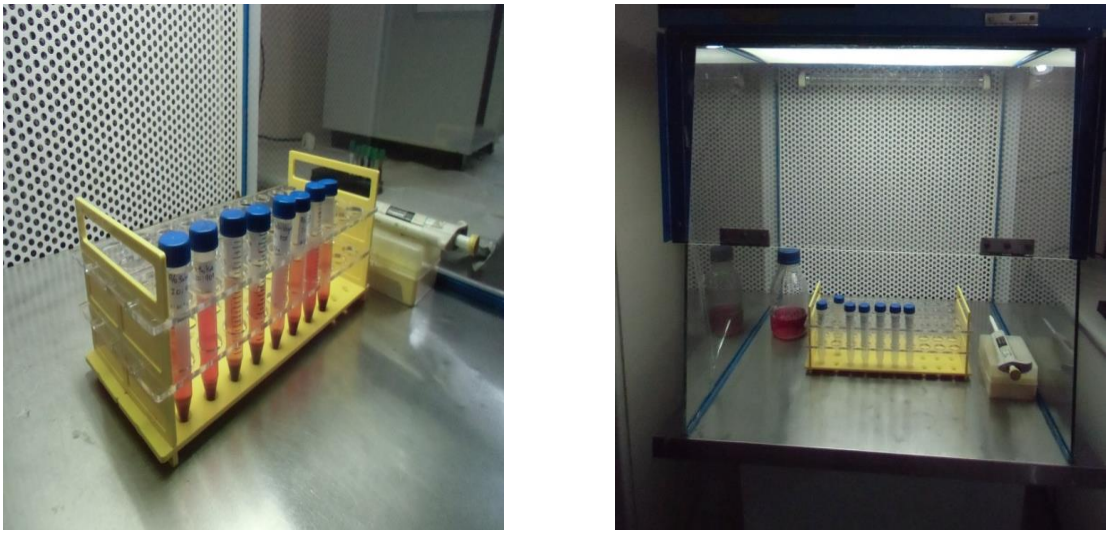

Fig.7. Experimental setup for human peripheral blood lymphocyte culture.

Procedure:

The procedure was proposed by Hsu *et al.,* 1987. Sterile blood sample was collected by venipuncture and transferred to a vaccutainer containing sodium or lithium heparin as anticoagulant. Added 5-6 drops of sample to a culture bottle containing 10ml RPMI 1640 medium supplemented with 20% fetal bovine serum. 10µg/ml of phytohaemagglutinin was added to the medium. The plant extracts were added as given in the table. Incubated for 72 hours at 37^0C. At 66^{th} hour, Bleomycin (0.03units/ml) was added to the cultures and incubated. At the 70^{th} hour, one drop of colchicine (0.04µg/ml) was added to the culture for arresting the cell division at metaphase and incubation continued.

After two hour's incubation, transferred the whole contents into a sterile centrifuge tube, and centrifuged at 1000 rpm for 10 min. removed the supernatant, shaken the pellets using a cyclomixer. 10ml of 0.075M pre-warmed KCl solution was added to the cell button and kept at 37^0C for 20 min. 2 drops of freshly prepared fixative (Methanol: Acetic acid) was added in the ratio 3:1. Again centrifuged at 1000rpm for 10 min. Removed the supernatant, mixed the cell button in a cyclomixer and 10ml of freshly prepared fixative was added to it. Kept in a refrigerator for at least 30 min for proper fixation and centrifuged at 1000rpm for 10 min. The supernatant was discarded and again shaken the pellets in a cyclomixer 10ml of freshly prepared fixative was added and centrifuged at 1000rpm for 10 min. repeated the process until the supernatant become clear and the cell button became white. Then cell suspension was prepared using the fixative.

Preparation of slides: Cleaned, labeled and chilled slides were taken. The cell suspension (7-8 drops) was dropped on to those slides from a particular height. The slides were heated gently on spirit lamp, blown gently on the material, and air dried. The slides were stained with 10-20% Giemsa solution and allowed to remain for ten min. After ten min., washed off the excess stain with running water and air-dried. The stained slides were observed under microscope for chromatid breaks, gaps, dicentrics, etc. A minimum of 50 metaphases were scored and the mean break per cell value (b/c value) was calculated for each sample. The frequency of chromatid breaks were considered as a measure of an individual's DNA repair capacity.

For chromosome sensitivity analysis, the mean b/c value was calculated using the formula: $\text{Mean break per cell value} = \frac{\frac{number\ of\ breaks\ counted}{number\ of\ metaphases\ counted} X46}{100}$

The frequency of chromatid breaks were expressed as b/c for comparison. Any individual expression < 0.8 b/c was considered hyposensitive, 0.8-1.0 was considered sensitive and those >1.0b/c was considered hypersensitive. A minimum of 50 metaphases per culture were scored and data were analyzed.

3.11 Chromatographic analysis

Chromatographic analysis was carried out using thermo GC-Trace Ultra Ver: 5.0 GC-MS (Model Thermo MS DSQ II gas chromatograph). A fused-DB35-MS Capillary standard Non-polar Column Dimension (30mts, ID: 0.25 mm, FILM: 0.25µm) was used. The GC temperature program was as follows: initial temperature was 100 °C, held for 1 min, increased to 130 °C at a rate of 2 °C/min, then to 200 °C at a rate of 3 °C/min, and finally to 280 °C at a rate of 6 °C/min and held for 10 min. The split ratio was 1:12, injection temperature was 250 °C, transfer line temperature was 270 °C, and the mass spectrometer was operated at 70 eV in run time 29 min.

3.12 *In silico* assays

3.12.1 Molecular docking study

Protein Data Bank: The structures of the target proteins structures subjected for the molecular docking study were obtained from the PDB (Protein Data Bank). The PDB is a repository for the 3-D structural data of large biological molecules, such as proteins and nucleic acids. The PDB archive contains information about experimentally-determined structures of proteins, nucleic acids, and complex assemblies. As a member of the wwPDB (Worldwide Protein Data Bank), the RCSB (The Research Collaboratory for Structural Bioinformatics) PDB curates and annotates PDB data according to agree upon standards. The structures of BRCA1 [PDB ID: 3KOH], BRCA2 [PDB ID: 1IYJ], PTEN [PDB ID: 1D5R], ATM [PDB ID: 4HDO], HER2 [PDB ID: IN8Z], CHEK2 [PDB ID: 2CN5], ERBb2 [PDB ID: 1S78] proteins were retrieved from PDB.

Chemsketch

CD/Chemsketch freeware is a drawing package that allows to draw chemical structures including organics, organometallics, polymers, and Markush structures. It also includes features such as calculation of molecular properties (e.g., molecular weight, density, molar refractivity etc.), 2D and 3D structure

cleaning and viewing, functionality for naming structures (fewer than 50 atoms and 3 rings), and prediction of log P.

Schrodinger Maestro environment to access all modules

Maestro is Schrödinger's powerful, united, multi-platform graphical user interface (GUI). It is designed to simplify modeling tasks, such as molecule building and data analysis, and also to facilitate the set up and submission of jobs to Schrödinger's computational programs. The main Maestro features include a project-based data management facility, a scripting language for automating large or repetitive tasks, a wide range of useful display options, a comprehensive molecular builder, and surfacing and entry plotting facilities.

Maestro is the graphical user interface for nearly all of the products that Schrödinger distributes: CombiGlide, ConfGen, Desmond, Epik, Glide, Impact, Jaguar, Liaison, LigPrep, Macro-Model, MCPRO+, Phase, Prime, PrimeX, QikProp, QSite, SiteMap, Strike, and WaterMap. It contains tools for building, displaying, and manipulating chemical structures; for organizing, loading, and storing these structures and associated data; and for setting up, monitoring, and visualizing the results of calculations on these structures.

3.12.2 Preparation of protein and ligand

Retrieved structures were subjected to removal of water up to 5A distances, conversation of selenomethionine into methionine, selection of monomer to interact, assigning bond order in both polar and non-polar amino acids, assigning lone pair electron atom, created disulfide bridges, filled side chains as the norms of crystalline studied protein may have missing side chains, filled loop within the active regions in the protein and addition of hydrogen bond in the hydrophobic and hydrophilic amino acids. These parameters processed from Schrodinger Suite 2013-Protein Preparation Wizard and were made viable to interact with derived group. pH of 14 isolated compounds was between 7.0 to +/- 2.0 and retained chirality and original binding state of the compound.

Grid generation

Grid files represent physical properties of a volume of the receptor (specifically the active site) that are searched when attempting to dock a ligand. The complex for this exercise is actually in two files, one containing the receptor and one containing the ligand. The prepared protein is displayed in the Workspace. The protein structure is displayed in ribbon representation. The Import panel is displayed. From the Files of type menu, Maestro is chosen. Import Options dialog box opens. The receptor structures used for grid generation were taken from the Workspace, so we excluded the ligand atoms from consideration as part of the receptor. From the Applications menu in the main window, selected Glide > Receptor Grid Generation.

The Receptor Grid Generation panel opened with the Receptor tab displayed. In the Define receptor section, ensure that Pick to identify ligand and Show markers were selected, and that, in the option menu, Molecule is chosen. In the Workspace, pick an atom in the ligand molecule. Dark green markers appear on the ligand. In the Van der Waals radii scaling section, scaling factor default value of 1.00 (no scaling) was chosen. Now that the ligand was excluded, the volume for which grids would be calculated. The entire complex was shown with several types of markers. The enclosing box is shown in purple. The center of the enclosing box is marked by green coordinate axes. The purple enclosing box represents the volume of the protein for which grids was calculated. And made the enclosing box as small as is consistent with the shape and character of the protein's active site and with the ligands to expect to dock.

The receptor grid can be set up and generated from the Receptor Grid Generation panel. The options in each tab of this panel allows to define the receptor structure by excluding any co-crystallized ligand that may be present, determine the position and size of the active site as it will be represented by receptor grids, set up Glide constraints, and set up flexible hydroxyl groups. Ligand docking jobs cannot be performed until the receptor grids have been

generated. Receptor grid generation requires a “prepared” structure: an all-atom structure with appropriate bond orders and formal charges.

Importing the prepared protein

The complex for this exercise is actually in two files, one containing the receptor and one containing the ligands.

1. Click the import structures toolbar button.

2. From the Files of type menu, Maestro is chosen.

3. If the options are not displayed, click Options.

4. Ensure that Import all structures, Replace Workspace, and Fit to screen following import are all selected.

5. From the Include in Workspace option menu, ensure that First Imported Structure is chosen.

6. Navigate to the structures directory and select the file 1fjs_prep_recep.mae.gz.

7. Click Open.

The prepared protein is displayed in the Workspace. The protein structure is displayed in ribbon representation. The structure includes solvent molecules (glycerine) and ions (Ca^{2+} and Cl^{-}), but does not include the ligand.

8. Next import the ligand structure file by clicking the Import structures toolbar button.

9. From the Files of type menu, ensure that Maestro is chosen.

10. If the options are not displayed, click Options.

11. Deselect Replace Workspace and Fit to screen following import.

12. Select the file prep_lig.mae.gz.

13. Click Open.

The prepared ligand is displayed in the Workspace in tube representation.

Ligand docking

Glide ligand docking jobs require a set of previously calculated receptor grids and one or more ligand structures. Preparation of the ligands before docking is strongly recommended. If a correct Lewis structure cannot be generated for a ligand, it is skipped by the docking job. Glide also automatically skips ligands containing unparametrized elements, such as arsenic, or atom types not supported by the OPLS force fields, such as explicit lone pair “atoms.”

The ligand docking panel

To open the Ligand Docking panel, choose Ligand Docking from the Glide submenu of the Applications menu. The Ligand Docking panel has six tabs:

- Settings
- Ligands
- Core
- Constraints
- Similarity
- Output

These tabs are described in the following sections of this chapter. We use the options in these tabs to specify settings for the ligand docking job. When we have completed our setup, the following buttons allow us to process our job:

- Start—Open the Ligand Docking - Start dialog box to start the job

- Write—Write the input files to disk without starting the job
- Reset—Discard settings and restore the default settings in all tab

Specifying a set of grid files and basic options

Docking job, and set the basic docking options.

1. Click the Clear workspace toolbar button.
2. From the Applications menu, choose Glide > Ligand Docking.
3. In the Receptor grid section, click the Browse button.
4. Navigate to the tutorial/grids directory, choose factorXa_grid.zip, and click open.
5. In the Docking section, we adopted rapid screening we can use the HTVS (high throughput virtual screening) option.
6. Under Options, ensure that Dock flexibly and Sample Ring Conformations are selected, and Penalize non-planar conformation is chosen from the Amide bonds option menu.

Examining Glide data: Glide results are examined with an emphasis on visual rather than numerical appraisal. The first set of exercises use the Project Table to display the results of the SP Glide docking job, examine individual ligand poses and their contacts with the input receptor structure.

The second set of exercises uses the Glide XP Visualizer panel to display information on the terms in the Glide XP scoring function that contribute to the ligand binding.

Importing and selecting pose data

Select the entry group protein_sp_pv1.

1. Click the Import structures button on the main toolbar.

The Import panel opens, with the contents of the glide directory displayed. This is the directory where our docking results were written.

2. Ensure that Maestro is chosen from the Files of type menu.

3. Ensure that Import all structures, Replace Workspace, and Fit to screen following import are all selected.

4. In the Import panel, select the file factorXa_sp_pv.maegz and click Open.

The receptor and the ligands are imported as an entry group named factorXa_sp_pv.maegz. The receptor is displayed in the Workspace.

5. Open using the Project Table panel.

3.12.3 Molecular docking of target protein with ligand

The ligand was docked with the target protein using Glide module of Schrödinger suite. Glide searches for favorable interactions between one or more ligand molecules and a receptor molecule, usually a protein. Each ligand must be a single molecule, while the receptor may include more than one molecule, e.g., a protein and a cofactor. Glide can be run in rigid or flexible docking modes; the latter automatically generates conformations for each input ligand. The combination of position and orientation of a ligand relative to the receptor, along with its conformation in flexible docking, is referred as a ligand pose. The ligand poses that Glide generates pass through a series of hierarchical filters that evaluate the ligand's interaction with the receptor. The initial filters test the spatial fit of the ligand to the defined active site, and examine the complementarity of ligand-receptor interactions using a grid-based method patterned after the empirical Chemscore function.

Poses that pass these initial screens enter the final stage of the algorithm, which involves evaluation and minimization of a grid approximation to the OPLS-AA non-bonded ligand-receptor interaction energy. Final scoring is then carried out on the energy-minimized poses. By default, Schrödinger's proprietary Glidescore multi-ligand scoring function is used to score the poses. If Glidescore was selected as the scoring function, a composite Emodel score is then used to rank the poses of each ligand and to select the poses to be reported to the user.

Emodel combines Glidescore, the non-bonded interaction energy, and, for flexible docking, the excess internal energy of the generated ligand conformation.

Glide uses a hierarchical series of filters to search for possible locations of the ligand in the active-site region of the receptor. The shape and properties of the receptor are represented on a grid by several different sets of fields that provide progressively more accurate scoring of the ligand poses. Conformational flexibility is handled in Glide by an extensive conformational search, augmented by a heuristic screen that rapidly eliminates unsuitable conformations, such as conformations that have long-range internal hydrogen bonds. Each rotamer group is attached to the core by a rotatable bond, but does not contain additional rotatable bonds. The core is what remains when each terminus of the ligand is severed at the “last” rotatable bond. Carbon and nitrogen end groups terminated with hydrogen (—CH_3, —NH_2, —NH_3^+) are not considered rotatable because their conformational variation is of little significance.

3.12.4 Molecular simulation of protein-ligand complex- Schrodinger suite (MacroModel)

Schrödinger suite is a commercial tool which integrates many Computational Biology and Proteomics applications. MacroModel is a force field based Molecular Modelling program with applicability to a wide range of chemical systems. MacroModel provides this study with multiple advanced methods to aid the understanding of protein structure, energetic, and dynamics. A large selection of force fields is available in MacroModel, including the latest technical advances introduced into OPLS_2005, a force field that Schrödinger is actively developing. Numerous minimization methods are available, enabling geometry optimizations for a broad selection of structural classes. A wide range of method is available for conformational searching, allowing efficient sampling of the potential energy surface for low-energy structures, for systems ranging from small molecules to entire proteins. Solvation effects can be accounted for using the efficient continuum solvation models employed by MacroModel.

Additional advanced features include molecular dynamics simulations, free energy perturbation simulations, and pure and mixed methods for ensemble sampling.

Methodology and parameterization for molecular simulation of complex:

- The structure was minimized to a low gradient using PRCG method with 500 maximum iterations and at 0.05 convergence threshold.
- The potential energy calculation was performed using OPLS_2005 force field in water solvent.
- The charges were taken from OPLS_2005 and extended cutoff selected for Van der Waals and electrostatic calculation.
- No constraints were maintained for atoms, bonds, angle or dihedral.
- Molecular dynamics and none SHAKE was selected.
- Total trajectories were set to 10 samples.
- Equilibration time was done for 1 Pico second and total simulation period up to 100 Picoseconds at 300 K temperature.

This state of observation will give protein structure stability in the TIP3P (water) environment. Post observation will give the proteins root mean square value of each trajectories in super imposed manner and the relational difference of each complex will be notified to analyze the protein structure stability.

3.12.5 Statistical analysis

The *in vivo* antitumor activity of the MEAC was determined by applying ANOVA followed by Dunnett's test; The Inhibitory Concentration (IC_{50}) was determined by plotting a nonlinear regression graph between % cell inhibition and Log_{10} concentration using Graph Pad Prism software and the chromosome sensitivity analysis was done by student's t- test.