Chapter 2

Materials and Methods
MATERIALS AND METHODS

D-PINITOL INHIBITS 7, 12-DIMETHYLBENZ(a)ANTHRACENE INDUCED MAMMARY CARCINOMA - IN VIVO STUDY

Animals

Healthy female Sprague Dawley rats at the age group of 45-48 days were used for this present investigation. They were obtained from the Central Animal House Facility, Dr.ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai, Tamilnadu, India. The animals were kept in large spacious polypropylene cages and received standardized rat pellet and water ad libitum. The animal room was well ventilated and a 12 h day and light rhythm was maintained throughout the experimental period. During the course of the study, the temperature was maintained between 27ºC to 37ºC. The maintenance and breeding of experimental animals were followed as defined by the Ministry of Social Justice and Empowerment of India 1998 (IAEC No. 01/05/2011).

Diet

The feed contained protein (21%), lipids (5%), crude fiber (4%), ash (8%), calcium (1%), Phosphorus (0.6%) and nitrogen free extract (55%).

Chemicals

7, 12-Dimethylbenz(a)anthracene (DMBA) and D-Pinitol were purchased from Sigma Chemical Company, St Louis, MO, USA. All other chemicals including solvents used were of high purity analytical grade marketed by Glaxo Laboratories, Mumbai, and Sisco Research Laboratories Pvt. Ltd, Mumbai, India.

Tumor induction

7, 12-Dimethylbenz (a) anthracene (DMBA) was used as a carcinogen for the present investigation. Mammary cancer was induced by a single dose of 20 mg/kg body weight of DMBA diluted in corn oil (1ml) given intragastrically using a gavage (Anbuselvam et al., 2007).
**Experimental design**

The rats were divided into four groups with six animals in each group and were given dose regimen as given below.

**Group I** : Animals received single dose of 1 ml of emulsion of corn oil given orally served as vehicle treated control.

**Group II** : Mammary carcinoma induced with single dose of 20mg/kg body weight of DMBA dissolved in corn oil (1ml) administered intragastrically.

**Group III** : Animals received a single dose of 20 mg of DMBA dissolved in corn oil (1 ml) administered intragastrically and from the eighth week followed by D-Pinitol (200 mg/kg body weight) given for 45 days orally.

**Group IV** : Animals received D-Pinitol alone at the concentration of 200 mg/kg body weight for 45 days orally.

**Collection of blood and tissues**

Body weight changes were recorded at weekly intervals. At the end of the experimental period, all the animals were sacrificed by cerevical dislocation. Animals were starved overnight before sacrifice. Blood was collected and the serum was separated by centrifugation. The breast and liver tissues were dissected out, washed in ice-cold saline and blotted to dryness. A 10% homogenate of the liver and breast tissues were prepared in 0.1 M Tris–HCl buffer (pH 7.4), centrifuged and the clear supernatant was used for further assays.

**Tumor weight**

Tumor weight was estimated according to the method of Geren *et al.*, (1972). The resultant solid tumor was considered to be prelate ellipsoid with one long axis and two short axis. The two short axis were measured with vernier calliper. The tumor weight was calculated by multiplying the length of the tumor with the square of the width and dividing the product by two.


\[
\text{Tumor weight (g)} = \frac{\text{Length (Cm) \times Width (Cm}^2)}{2}
\]

**ESTIMATION OF TOTAL PROTEIN**

Total protein was estimated according to the method of Lowry et al., (1951).

**Reagents**

1. **Lowry’s reagent**: 50 ml of reagent ‘A’ which consisted of 2% \( \text{Na}_2\text{CO}_3 \) in 0.1 N NaOH was mixed with 1 ml of reagent ‘B’ which consisted of 0.5% \( \text{CuSO}_4 \) in 1% sodium potassium tartarate. 50 ml of solution A was mixed with 1.0 ml of solution B just before use.

2. **Folin’s Ciocalteau reagent**: One volume of Folin's phenol reagent was diluted with two volumes of distilled water just before use.

3. **Standard**: 20 mg of BSA was dissolved in 100 ml of distilled water. 10 ml of the stock was diluted to 100 ml with distilled water to get a working standard (20 µg/ml).

**Procedure**

0.1 ml of the sample was made up to 1 ml with distilled water. To this 0.5 ml of Lowry’s reagent was added, mixed and allowed to stand at room temperature for 20 min. Later 0.5 ml of Folin’s phenol reagent was added and shaken well. The blank and standards were treated in a similar manner. The blue complex formed was measured at 640 nm after 15 min against the blank. The protein content was expressed as mg/gm wet tissue and mg/dL for serum.
ESTIMATION OF NUCLEIC ACIDS

Extraction of nucleic acids

The nucleic acids were extracted by the method of Schneider (1957). Known amount of tissues were homogenized in 5.0 ml of ice-cold distilled water using Potter-Elvehjem homogenizer with a Teflon pestle. 5 ml of 5% TCA was added to the homogenate and the mixture was kept in ice for 30 min to allow complete precipitation of proteins and nucleic acids. The mixture was centrifuged and the precipitate obtained was washed thrice with ice cold 10% TCA. Then, it was treated with 95% ethanol to remove lipids. The final precipitate was heated at 90°C for 15 min with occasional shaking, which facilitated the quantitative separation of nucleic acids from protein. The supernatant after centrifugation was used for the estimation of DNA and RNA.

Estimation of deoxy ribonucleic acid (DNA)

DNA was estimated by the method of Burton (1956).

Reagents

1. Diphenylamine reagent: 1.5 gm of diphenylamine was dissolved in 100 ml of redistilled acetic acid and 1.5 ml of concentrated H₂SO₄ was added. The reagent was stored at 4°C in dark place. Before using, 0.1 ml of 0.16% aqueous acetaldehyde was mixed with every 20 ml of the reagent.

2. Stock standard: Highly polymerized calf-thymus DNA was dissolved in 5 mM NaOH to give a concentration of 0.4 mg/ml.

3. Working standard: This was prepared by mixing 2.0 ml of the stock solution with an equal volume of 1 N perchloric acid and was heated at 70°C for 15 min.
Procedure
A known volume of the nucleic acid extract was made up to 3.0 ml with 1 N perchloric acid. This was mixed with 2.0 ml of diphenylamine reagent. A blank and standards were also carried out concurrently. This was kept in a boiling water bath for 10 min and the blue colour developed was read at 640 nm in a spectrophotometer. The DNA content was expressed as mg/gm wet tissue.

Estimation of ribonucleic acid (RNA)
RNA was estimated by the method of Rawal et al., (1977).

Reagents
1. Orcinol-ferric chloride reagent: 1 gm of orcinol was dissolved in 100ml of concentrated HCl containing 0.5 gm of ferric chloride. This reagent was prepared freshly.
2. Standard: Standard was prepared by dissolving 2.0 mg of yeast RNA in 100 ml of 5% TCA.

Procedure
Aliquots of nucleic acid extracts were made up to 2.0 ml with 5% TCA. To this, 3.0 ml of orcinol-ferric chloride reagent was added and mixed well. The tubes were heated in a boiling water bath for 20 min. A blank and standards were also treated in the similar manner. The tubes were cooled and the colour developed was measured at 640 nm in a spectrophotometer. The RNA content was expressed as mg/gm wet tissue.

ESTIMATION OF MACROMOLECULAR DAMAGES
Assay of lipid peroxidation (LPO)
Lipid peroxidation was estimated by the method of Ohkawa et al., (1979)

Reagents
1. 0.8% TBA
2. 8.5% Sodium dodecyl sulphate  
3. 20% Glacial acetic acid

**Procedure**

1.5 ml of TBA, 0.2 ml of sodium dodecyl sulphate, 1.5 ml of glacial acetic acid was added to test tubes containing 0.1 ml of samples. The test tubes were heated in water bath for 1h. The test tubes were then cooled and 1 ml of distilled water was added. The optical density was determined at 532 nm using a reagent blank. Standard malondialdehyde was also processed in a similar fashion. The results were expressed as n moles of malondialdehyde liberated/ mg protein/min.

**ANTIOXIDANT ENZYMES**

**Enzymic antioxidants**

**Superoxide dismutase (Superoxide oxidoreductase) (E.C 1.15.1.1)**

The Superoxide dismutase was estimated by the method of Marklund and Marklund (1974).

**Reagents**

1. *Tris-HCl buffer*: 0.1M, pH 8.2.
2. *Tris-HCl buffer*: 0.05 M, pH 7.4.
3. *Pyrogallol stock solution*: 25.2 mg was dissolved in 1 ml of 0.05 M Tris-HCl buffer (pH 7.4).
4. *Pyrogallol working solution*: At the time of assay, 0.5 ml was diluted to 50 ml with the 0.05 M Tris-HCl buffer (pH 7.4).
5. *Absolute ethanol*
6. *Chloroform*

**Procedure**

To a known amount of sample, 0.25 ml of ethanol and 0.15 ml of chloroform were added. After 15 min of shaking in a mechanical shaker, the
suspension was centrifuged and the supernatant obtained constituted the enzyme extract. The reaction mixture for auto-oxidation consist of 2 ml of Tris-HCl buffer (pH 8.2), 0.5 ml of 2 mM pyrogallol and 1.5 ml of distilled water. Initially the rate of auto-oxidation of pyrogallol was noted at an interval of 1 min to 3 min. The assay mixture for the enzyme contain 2 ml of the buffer, 0.5 ml of pyrogallol, aliquots of the enzyme preparation and distilled water to give a final volume of 4 ml. The rate of inhibition of pyrogallol auto-oxidation after the addition of the enzyme was noted. The enzyme activity was expressed in terms of units/mg protein/min, in which one unit corresponds to the amount of enzyme required to inhibit the auto-oxidation reaction by 50%.

**Catalase (Hydrogen Peroxide: Hydrogen Peroxide Oxidoreductase) (E.C.1.11.1.6)**

The activity of Catalase was estimated by the method of Sinha (1972).

**Reagents**

1. *Phosphate buffer*: 0.01 M, pH 7.0.
2. *Hydrogen peroxide* (*H₂O₂*): 0.2 M.
3. *Stock dichromate/acetic acid solution*: This reagent was prepared by mixing a 5% solution of potassium dichromate with glacial acetic acid with the ratio of 1:3.
4. *Working dichromate/acetic acid solution*: The stock was diluted to 1:5 distilled with water to make the working dichromate/acetic acid solution.

**Procedure**

The assay mixture contained 0.5 ml of *H₂O₂*, 1 ml of buffer and 0.4 ml of water. 0.2 ml of the diluted enzyme was added to initiate the reaction. 2 ml of the dichromate/acetic acid reagent was added after 15, 30, 45 and 60 sec of incubation. To the control tube the enzyme was added after the addition of
the acid reagent. The tubes were then heated for 10 min and color developed was read at 610 nm. The activity of catalase was arrived at from the \( \mu g \) of \( H_2O_2 \) consumed/mg protein/min.

**Glutathione peroxidase (Glutathione: Hydrogen Peroxide Oxidoreductase, GPx) (E.C 1.11.1.9)**

Gluathione peroxidase was estimated to the method of Rotruck *et al.*, (1973).

**Reagents**

1. *Sodium phosphate buffer*: 0.4M, pH 7.0.
2. *Sodium azide*: 10 mM.
3. *Hydrogen peroxide (H\( _2 \)O\( _2 \))*: 2.5 mM
4. *Reduced glutathione*: 4 mM.
5. *Disodium hydrogen phosphate*: 0.3 M
6. *EDTA*: 0.8 mM
7. *Trichloroacetic acid (TCA)*: 10% in 0.3M phosphate solution
8. *5,5-dithio bis-2-nitrobenzoic acid (DTNB)*: 0.04% in 1% sodium citrate solution.
9. *Reduced glutathione standard*: 20mg of reduced glutathione was dissolved in 100 ml of water.

**Procedure**

The reaction mixture consisted of 0.2 ml each of EDTA, sodium azide, \( H_2O_2 \), 0.4 ml of phosphate buffer and 0.1 of ml homogenate. This was incubated at 37\(^\circ\)C at different time intervals. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged at 2000 rpm. To 0.5 ml of supernatant, 4 ml of disodium hydrogen phosphate and 0.5 of ml DTNB were added and the colour developed was read at 420 nm immediately. A blank was prepared with disodium hydrogen phosphate solution and 1 ml of the DTNB reagent. Suitable aliquots of the standard were
taken and treated in the same manner. The activity of GPx was expressed as μ moles of glutathione utilized/mg protein/min.

**NON-ENZYMIC ANTIOXIDANTS**

**Reduced glutathione (GSH)**

Reduced glutathione was determined by the method of Moron et al., (1979).

**Reagents**

1. 5, 5′dithiobis (2-nitrobenzoic acid) (DTNB): 0.6 mM of DTNB was dissolved in 0.2 M phosphate buffer (pH 8.0).
2. Phosphate buffer: 0.2 M, pH 8.0.
3. Trichloroacetic acid (TCA): 5%
4. Standard GSH: 100 mg of reduced glutathione was dissolved in 100 ml distilled water. The working standard contained 100 μg/ml.

**Procedure**

Samples were precipitated with 5% TCA. The precipitate was removed by centrifugation. To an aliquot of the supernatant, 0.2 ml of DTNB and 0.5 ml of phosphate buffer were added to make a final volume of 3 ml. The absorbance was read at 512 nm against a reagent blank. A series of standards treated in a similar manner were also carried out. The amount of glutathione was expressed as μg of GSH/mg protein/min.

**Vitamin-C**

Ascorbic acid was estimated by the method of Omaye et al., (1979).

**Reagents**

1. Trichloroacetic acid (TCA): 5%
2. Sulphuric acid (H₂SO₄): 65%
3. *DNPH- Thiourea-CuSO₄ (DTC) reagent*: 3 gm of DNPH, 0.4 gm of thiourea and 0.05 gm of CuSO₄ were dissolved in 9N H₂SO₄ and made up to 100 ml with the same.

4. *Standard*: Standards of ascorbic acid were made in 5% TCA in the range of 1 to 20 μg/ml

**Procedure**

Aliquots of the sample was precipitated with 5% ice cold TCA and centrifuged for 20 min at 3500 rpm. 1 ml of the supernatant was mixed with 0.2 ml of DTC and incubated for 3 h at 37°C. Then 1.5 ml of ice cold 65% H₂SO₄ was added, mixed well. The solutions were allowed to stand at room temperature for an additional 30 min. Absorbance was determined at 520 nm. The amount of Vitamin-C was expressed as mg/dL for serum and mg/gm of wet tissue.

**Vitamin-E**

Vitamin-E was estimated by the method of Desai (1984).

**Reagents**

1. *Absolute ethanol*
2. *Hexane*
3. *Bathophenthroline reagent*: 0.2% solution of 4,7-diphenyl-1-10-pheneanthroline in purified absolute ethanol.
4. *Ferric chloride reagent*: 0.001 M ferric chloride solution in purified absolute ethanol. This reagent was prepared fresh and was kept in amber colored bottle.
5. *Orthophosphoric acid reagent*: 0.001 M orthophosphoric acid solution in purified absolute ethanol.
6. *Vitamin E standard*: α-tocopherol standards in the range 1-10μg/ml of purified absolute ethanol were prepared and treated in the same manner as test samples.
**Procedure**

To 1 ml of sample, 1 ml of ethanol was added and thoroughly mixed. Then 3 ml of hexane was added, shaken rapidly and centrifuged. 2 ml of supernatant was taken and evaporated to dryness. To this 0.2 ml of bathophenanthroline was added. The assay mixture was protected from light and 0.2 ml of ferric chloride was added followed by 0.2 ml of 0-phosphoric acid. To the total volume was made up to 3 ml with ethanol. Standard \( \alpha \)-tocopherol acetate was also treated similarly. Absorbance was read at 530 nm. The amount of Vitamin E was expressed as mg/dL for serum and mg/gm of wet tissue.

**ASSAY OF LIPID PROFILE**

**Preparation of tissue lipid extract**

The tissues were washed with saline and dried between filter paper. A weighed amount of tissue (500 mg) was homogenized with 7 ml of methanol in a Potter-Elvehjem homogenizer and filtered through a Whatmann No.1 filter paper into a conical flask. The residue after filtration was scraped and homogenized in 14 ml of chloroform. The residue was once again scraped from the filter paper and ground with 10 ml of chloroform-methanol mixture (2:1 v/v) and the resulting filtrate was evaporated to dryness.

The lipids were purified by the Folch’s wash procedure (Folch *et al.*, 1951). The dried lipid residue, after evaporation was dissolved in 5 ml of chloroform-methanol mixture. The redissolved lipid extract was mixed with 1 ml of 0.1 N KCl and the contents were shaken well. The upper aqueous phase containing gangliosides and other water-soluble compounds were separated. The lower chloroform phase containing neutral and phospholipids was again washed thrice with 2 ml of Folch’s reagent (0.1 N KCl: methanol: chloroform mixed in the ratio of 10:10:1) and the upper aqueous phase was aspirated. The lower chloroform phase was made up to a known volume and aliquots were
used for the analysis of total cholesterol, free cholesterol, phospholipids, triglycerides and free fatty acids.

**Lipid analysis in plasma and tissues**

**Total cholesterol**

Total cholesterol content was estimated by the method of Parekh and Jung (1970).

**Reagents**

1. *Ferric chloride-Uranyl acetate reagent*: 500mg of ferric chloride was dissolved in 10.0 ml of distilled water, 3.0 ml of concentrated ammonia was added to it and centrifuged. The precipitate was washed several times with distilled water and dissolved in one litre of glacial acetic acid. 100mg of uranyl acetate was added to the mixture and the contents were shaken well and kept over night. The reagent was also stable for six months.

2. *Sulphuric acid-ferrous sulphate reagent*: 100mg of ferrous sulphate was dissolved in 100 ml of glacial acetic acid and 100 ml of sulphuric acid. After cooling to room temperature, the volume was made up to one litre with concentrated sulphuric acid. The reagent was stable for six months.

3. *Cholesterol standard*: 200mg of cholesterol, recrystallised from ethanol was dissolved in 100 ml of chloroform. 1 ml of stock cholesterol was diluted to 100 ml to obtain a working standard of 20µg of cholesterol/ml.

**Procedure**

0.05 ml of plasma/tissue extract was treated with 10.0 ml of ferric chloride-uranyl acetate reagent. The mixture was shaken well and allowed to stand for 5 min. After centrifugation, 3.0 ml of supernatant was taken for analysis. A set of standards containing 10-50 µg of cholesterol, were made up to 3.0 ml with ferric chloride-uranyl acetate reagent. 2 ml of sulphuric acid-
ferrous sulphate reagent was added to all the tubes and the contents were mixed well. After 20 min, the optimal density was measured at 540 nm in a spectrophotometer. Cholesterol content in plasma and tissue was expressed as mg/dL plasma and mg/gm wet tissue, respectively.

**Free cholesterol**

Free cholesterol was estimated by the method of Leffler and McDougald (1963).

**Reagents**

1. **Digitonin**: 5 mg digitonin was dissolved in 1.0 ml of 50% ethanol
2. **Isopropanol**
3. **Acetone**
4. **Ferric chloride – uranyl acetate reagent**: 500 mg of ferric chloride was dissolved in 10.0 ml of distilled water, to this 3.0 ml of concentrated ammonia was added and centrifuged. The precipitate was washed several times with distilled water and dissolved in one litre of glacial acetic acid. 100 mg of uranyl acetate was added to the mixture and the contents were shaken well and kept overnight.
5. **Ferrous sulphate – sulphuric acid reagent**: 100 mg of ferrous sulphate was dissolved in 100 ml of glacial acetic acid and 100 ml of sulphuric acid. After cooling to room temperature, the volume was made up to one litre with concentrated sulphuric acid.

**Procedure**

To 0.05 ml of dried extracts of plasma and tissue samples, 1.0 ml of isopropanol was added. The contents were mixed well and centrifuged. To the supernatant, 0.5 ml of digitonin was added, mixed well and stored at 4°C for 30 min. Then, it was centrifuged at 3000 rpm for 10 min. The supernatant was decanted off. The precipitate (digitonide of free cholesterol) was washed
twice with acetone. The free cholesterol in plasma and tissue was expressed as mg/dL plasma and mg/gm wet tissue, respectively.

**Phospholipids**

Phospholipids were estimated by the method of Rouser et al., (1970) after digesting the lipid extract with perchloric acid.

**Reagents**

1. **TCA (10%)**: 10 gm of TCA was dissolved in 100 ml of distilled water.
2. **Perchloric acid**
3. **Ammonium molybdate (3%)**: 3 gm of ammonium molybdate was dissolved in 100 ml of distilled water.
4. **Ascorbic acid (3%)**: 3 gm of ascorbic acid was dissolved in 100 ml of distilled water.
5. **Standard phosphate**: 35.1 mg of potassium dihydrogen phosphate was dissolved in 100 ml of distilled water to give a concentration of 80 μg of phosphate/ml.
6. **Working standard**: A concentration of 8 μg/ml was prepared by diluting 10 ml of the stock solution to 100 ml in a standard flask.

**Procedure**

0.1 ml sample (plasma/tissue extract) was diluted to 2.0 ml with distilled water and 2.0 ml of 10% TCA was added. The precipitated proteins were sedimented by centrifugation at 2000 rpm for 10 min. The supernatant was discarded. 1 ml of perchloric acid was added to the residue and digested on sand path till the solution turned colorless. After cooling, the solution was made up to 5.0 ml with distilled water. 0.5 ml each of ammonium molybdate and ascorbic acid were added and the mixture was kept in a boiling water bath for 6 min. The blue color developed was read at 620 nm in a spectrophotometer. Phospholipid content was expressed as mg/dL plasma and
mg/gm wet tissue after multiplication by a factor of 25 to give the phospholipids content according to Scheig and Isselbacher (1965).

**Triglycerides**

Triglyceride was estimated by the method of Rice (1970) based on the method of Van Handel (1961).

**Reagents**

1. *Chloroform : Methanol mixture 2:1 (v/v).*
2. *Sodium chloride:* Saturated in distilled water.
3. *Activated silicic acid:* Silicic acid was washed with 4 N hydrochloric acid and then with distilled water until the pH was neutral. After drying, ether was added in sufficient amount, stirred well and the supernatant was decanted. Silicic acid was then dried at 60°C and activated at 100°C overnight prior to use.
4. *Potassium hydroxide (0.4% in ethanol):* 400mg of potassium hydroxide was dissolved in 100 ml of ethanol.
5. *Sodium meta peroxide (0.1M):* 2.149 gm of sodium meta peroxide was dissolved in 100 ml of distilled water.
6. *Sodium meta arsenite (0.5M):* 6.496 gm of sodium meta arsenite was dissolved in 100 ml of distilled water.
7. *Chromotropic acid:* 1.14 gm of chromotropic acid was dissolved in 100 ml of distilled water and stored as a stock solution in a brown bottle. Before use, 10.0 ml of this solution was mixed with 45.0 ml sulphuric acid water in the ratio of 2:1 (v/v).
8. *Sulphuric acid (0.2 N):* 0.5 ml of concentrated sulphuric acid was made upto 100 ml with distilled water.
9. *Tripalmitin standard:* 100mg of tripalmitin was dissolved in 100 ml of chloroform in a 100 ml standard flak. The stock
solution was diluted to 1 in 10 with chloroform. The standard solution contained 100 µg of tripalmitin/ml.

Procedure

0.2 ml plasma/tissue extract was mixed with chloroform-methanol mixture (9.8 ml) and left a side for 30 min before centrifugation. After centrifugation, 4.0 ml of the lipid extract was added to tubes containing 8.0 ml of saturated sodium chloride and shaken vigorously. The contents were allowed to settle for an hour and then centrifuged. The supernatant saline-methanol phase was discarded. The washed chloroform phase was filtered into a dry tube. 200mg of activated silicic acid was added to chloroform phase, shaken vigorously and allowed for 30 min. After centrifugation, to 0.5 ml supernatant, as well as standard and blank, 0.5 ml of alchololic potassium hydroxide solution was added and the mixture was saponified in a water bath at 60-70°C for 20 min. 0.5 ml of 0.2 N sulphuric acid was added and kept in a boiling water bath for 10 min. After cooling, 0.1 ml of sodium meta periodate was added and allowed to stand for 10 min. The excess periodate was reduced by the addition of 0.1 ml sodium meta arsenite. 5ml chromotropic acid reagent was added, mixed thoroughly and kept in a boiling water bath for 30 min. After cooling, the color developed was read at 540 nm in a spectrophotometer. Triglyceride was expressed as mg/dL plasma and mg/gm wet tissue, respectively.

Free fatty acids

Plasma and tissue free fatty acid were estimated by the method of Horn and Menahan (1981).

Reagents

1. *Chloroform-heptane-methanol mixture, in the ratio 200:150:7 (v/v).*

2. *Copper nitrate-triethanolamine solution (Cu-TEA):* 50 ml of 0.1M copper nitrate and 50 ml of 0.2 M triethanolamine were mixed with 33.0 gm of sodium chloride.
3. **Color reagent**: 100mg of diethyl dithio carbonate was dissolved in 100 ml of n-butanol.

4. **Activated silicic acid**: Silicic acid was washed with 4 N hydrochloric acid and then with distilled water until the pH was neutral. After drying, ether was added in sufficient amount, stirred well and the supernatant was decanted. Silicic acid was then dried at 60°C and activated at 100°C overnight prior to use.

5. **Standard palmitic acid**: 20 mg of standard palmitic acid was made up to the mark in a 100 ml standard flask with distilled water. The working standard was prepared by diluting the stock from 1 to 10 ml with distilled water. The working standard contained 20 µg of palmitic acid/ml.

**Procedure**

An aliquot of 0.2 ml plasma/tissue extract were mixed with 6.0 ml of chloroform: heptane: methanol solvent and was shaken vigorously. 200mg of activated silicic acid was added, shaken and left aside for 30 min. The solution was then centrifuged and the supernatant was transferred to the tube containing 2.0 ml Cu-TEA reagent. Blank contained only the solvent; while standards with different concentrations were made up to a known volume with the solvent and then, 2.0 ml of Cu-TEA reagent was added. The contents were agitated using a mechanical shaker for 20 min. The mixture was separated into phases by centrifugation. 2ml of the upper layer was mixed with 1.0 ml of the color reagent. The color developed was read at 430 nm in a spectrophotometer. Free fatty acids concentrations in plasma and tissue was expressed as mg/dL plasma and mg/gm wet tissue, respectively.

**ASSAY OF LIPID AND LIPID METABOLIZING ENZYMES**

**Lipoprotein lipase (EC 3.1.1.3)**

The lipoprotein lipase activity was assayed by the method of Schmidt (1974).
Reagents

1. Tris-HCl buffer: 0.01 M, pH 8.5
2. Deoxycholate: 10 mM
3. Chloroform
4. Hexane
5. Olive oil emulsion
6. Modified Doles extraction mixture: Isopropanol, hexane and 2 N sulphuric acid were mixed in the ratio of 40: 10: 1 (v/v).
7. Sulphuric acid: 0.01N
8. Copper reagent: 3.25 g of copper nitrate, 6.25 g of potassium sulphate and 17 g of sodium sulphate were dissolved in 50 ml of distilled water. To this solution, 7 ml of triethanolamine and 0.3 ml of glacial acetic acid were added and the final volume was made up to 100 ml with distilled water.
9. Diethyl dithiocarbamate: 0.1 %
10. Standard: 20 mg of palmitic acid was dissolved in 100 ml of chloroform.

Procedure

0.5 ml of olive oil emulsion, 0.1 ml of deoxycholate, 0.5 ml of buffer and 0.5 ml of enzyme were incubated at 37°C for 30 minutes along with control tubes to which enzyme was not added. The reaction was arrested by keeping the tubes in a boiling water bath for one minute.

To all the tubes 2.5 ml of chloroform and 5 ml of doles mixture were added and mixed well. Then 3 ml of hexane and 2 ml of distilled water were added, shaken well and centrifuged.

3 ml of supernatant was taken and 3 ml of 0.01 N sulphuric acid was added, mixed well and centrifuged. To 2 ml of the supernatant 2 ml of chloroform and 2 ml of copper reagent were added. The tubes were shaken well. For this 2 ml of aliquot was taken and 0.5 ml of diethyl dithiocarbamate
was added. The colour developed was read at 430 nm using a colorimeter. Aliquots of standards were also treated similarly.

Lipoprotein lipase activity was expressed as nmoles of free fatty acids liberated/hr/mg protein under incubation conditions.

**Lecithin: Cholesterol acyl transferase (LCAT) (EC 2.3.1.43)**

LCAT activity was assayed by the method of Legraud *et al.*, (1979) with the modifications of Hitz *et al.*, (1983).

**Reagents**

1. Dextran sulphate in saline : 0.2%
2. Isopropanol
3. Acetone
4. Digitonin: 5 mg dissolved in 1 ml of 50% ethanol
5. Reagents for cholesterol were prepared as described by Parekh and Jung (1970)
6. Substrate: A pool of control plasma was warmed at 56°C for 30 minutes to inactivate the lecithin: Cholesterol acyl transferase present in the plasma. The plasma was then incubated at 4°C for 15 minutes with 0.2% dextran sulphate (1 part of dextran sulphate was added to 20 parts of plasma). This was followed by centrifugation for 10 minutes at 1750 x g, the supernatant rich in HDL, was used as the substrate for the enzyme assay.

**Procedure**

The incubation mixture contained 0.6 ml of the substrate and 0.6 ml of enzyme, 0.4 ml of mixture was immediately transferred to a tube containing isopropanol (1 ml) to arrest the reaction. This gives the cholesterol present at the beginning of the experiment. Incubation at 37°C was continued for 90 minutes when another 0.4 ml was pipetted out and the reaction was arrested. Incubation was further continued for the next 90 minutes and the reaction was arrested. After centrifugation the clear supernatant was
quantitatively transferred to another set of tubes. To this was added, 2 ml acetone and 1 ml digitonin. It was left aside for an hour and then centrifuged and was processed as in total cholesterol estimation, for the estimation of free cholesterol content at zero time, 90 minutes and 180 minutes were estimated as described by Parekh and Jung (1970).

LCAT activity was expressed as nmoles of cholesterol esterified/hr/mg protein under incubation conditions.

**Cholesterol ester synthetase (EC 2.3.1.6)**

The enzyme activity was assayed by the method of Kothari *et al.*, (1970)

**Reagents**

1. Acetate buffer: 0.1 M, pH 4.0
2. Acetone ethanol mixture: 1 : 1 (v/v)
3. Acetone ether mixture: 1 :2 (v/v)
4. Glacial acetic acid: 10%
5. Digitonin
6. Substrate: 35.4 mg of cholesterol and 63.87 mg of oleic acid were dissolved in 1.8 ml of chloroform. To this 103.6 mg of sodium taurocholate and 31.1 mg of ammonium chloride in 7.5 ml of acetate buffer was added and homogenized.

**Procedure**

The incubation mixture contained 0.5 ml acetate buffer, 0.2 ml substrate and 0.2 ml enzyme preparation. Incubation was carried out at 37°C for 6 hours with occasional shaking. The reaction was arrested by addition of 5 ml acetone-ethanol mixture.

The precipitated proteins were sedimented after centrifugation. One ml digitonin was added to the supernatant, followed by 2 drops of acetic acid. The contents were mixed well, securely closed and kept in a dark chamber for 16 hours. The cholesterol digitonide which was sedimented after centrifugation, was washed twice with acetone-ether mixture and finally with
dry ether. The cholesterol content was estimated as described by Parekh and Jung (1970).

Cholesterol ester synthetase activity was expressed as nmoles of cholesterol etherified/hr/mg protein under incubation conditions.

**Cholesterol ester hydrolase (EC3.1.1.13)**

The enzyme activity was assayed by the method of Kothari et al.,(1970) with slight modification by Kritchevsaky and Kothari (1973).

**Reagents**

1. Acetate buffer: 0.1M, pH 4.0
2. Acetone-ethanol mixture: 1:1 (v/v)
3. Acetone-ether mixture: 1:2 (v/v)
4. Glacial acetic acid: 10%
5. Digitonin
6. Substrate: 15.54 mg of cholesterol acetate and 41.7 mg of lecithin were dissolved in 1.5 ml of chloroform. To this 30.12 mg of sodium taurocholate in 15 ml of acetate buffer was added and homogenized.

**Procedure**

0.3 ml acetate buffer was added to 0.4 ml of substrate and 0.2 ml of enzyme and mixed well. This was incubated at 37°C for 6 hours with occasional shaking. The reaction was stopped by the addition of 5 ml of acetone-ethanol mixture. The precipitated proteins were sedimented by centrifugation at 2000 x g.

The free cholesterol oleate was precipitated by the addition of 1 ml digitonin followed by the addition of 2 drops of glacial acetic acid. The contents were mixed well. The tubes were securely closed and kept in a dark chamber for 16 hours. The precipitated cholesterol digitonide was sedimented by centrifugation at 1000xg for 15 minutes and the upper phase was carefully decanted and discarded. The precipitate was washed twice with acetone-ether mixture and finally with dry ether.
Ether was evaporated off and the tubes were heated in a sand bath at 110°C to 115°C for 30 minutes. The precipitate was then dissolved in 3 ml of ferric chloride-uranyl acetate reagent and cholesterol content was estimated.

Cholesterol ester hydrolase activity was expressed as nmoles of cholesterol liberated/ hr/mg protein under incubation conditions.

ESTIMATION OF GLYCOPROTEINS

The samples were precipitated and hydrolyzed and then the protein bound hexose, hexosamine and sialic acid was estimated from the hydrolysate. To 0.05 ml of sample, 2.0 ml of ethanol was added and centrifuged. The supernatant was decanted. The precipitate was hydrolyzed with acid to liberate protein bound carbohydrates according to the method of Tettamanti et al., (1983) and estimated for hexose, hexosamine and sialic acid. Similarly, a known amount of delipidised residues of tissues, prepared according to the methods of Floch et al., (1951).

Acid hydrolysis for estimation of hexose, hexosamine and sialic acid

To the ethanol precipitate, 2.0 ml of 4 N hydrochloric acid was added and the mixture was refluxed at 100°C for 4h in a test tube with suitable marble lids. The hydrolysate was neutralized with sodium hydroxide. Aliquots of the neutralized samples were taken for analysis.

Estimation of hexose

Hexose was estimated by the method of Niebes (1972).

Reagents

1. Orcinol-sulphuric acid reagent

   Reagent A: sulphuric acid - water mixture (3:2 v/v)
   Reagent B: 1.6 M of orcinol in 100 ml of distilled water
   Reagents A and B were mixed in the ratio of 15:2 (v/v) just before use.
2. *Standard hexose solution:* 50 mg of galactose and 50 mg of mannose were dissolved in 100 ml distilled water. This solution was diluted to 1:10 proportion, which gave a concentration of 100 µg/ml.

**Procedure**

To 0.5 ml of neutralized sample, 7.0 ml of orcinol reagent was added very slowly, by placing the tubes in ice water bath. The contents were mixed well and the tubes were heated at 80°C for 15 min and cooled. Then the color developed in dark, and this was read at 540 nm using a spectrophotometer. Standard solution containing 25-100 µg of hexose and blank containing 0.5 ml of distilled water instead of neutralized sample was also treated in a similar manner. The hexose content was expressed as mg/dL for serum and mg/gm of defatted tissue sample.

**Estimation of hexosamine**

Hexosamine was estimated by the method of Wagner (1979).

**Reagents**

1. *Acetyl acetone reagent 3.5%*

   *Reagent A:* Trisodium phosphate, 1 M – 3.8 gm of trisodium phosphate was dissolved in 100 ml of distilled water.

   *Reagent B:* Potassium tetraborate, 0.5 M - 1.9 gm of borate was dissolved in 10 ml of distilled water.

   5ml of acetyl acetone was added to the mixture containing reagent A and B in the ratio of 98: 2 (v/v).

2. *Ehrlich's reagent:* 320 mg of p-dimethylaminobenzaldehyde was dissolved in 21 ml of isopropanol and 3.0 ml of concentrated HCl was added to it.

3. *Standard:* 10 mg of galactosamine was dissolved in 100 ml of distilled water with the concentration of 100 µg/ml.
**Procedure**

0.5 ml of the neutralized sample was made up to 1.0 ml with distilled water. Standard galactosamine (in the range of 10-40 µg) was also made up to 1.0 ml. Blank comprised of 1.0 ml distilled water, 0.6 ml of acetyl acetone reagent was added to all the tubes and heated in a boiling water bath for 30 min. After cooling, 2.0 ml of Ehrlich's reagent was added and the contents were shaken well. The pink color developed was read at 540 nm against a blank using spectrophotometer. Hexosamine content was expressed as mg/dL for serum and mg/gm of defatted tissue sample.

**Estimation of sialic acid**

Sialic acid was determined by the method of Warren (1959).

**Reagents**

1. *Periodate (0.25 M)*: 53.4 mg of sodium periodate was dissolved in 100 ml of 0.1 N H₂SO₄.
2. *Sodium arsenite (4%)*: 4 gm of sodium arsenite was dissolved in 100 ml of 0.5 N HCl
3. *Thiobarbituric acid (0.1M)*: 144 mg of TBA was dissolved in 10 ml of distilled water. The pH was adjusted to 9 with 6 N NaOH and the reagent was prepared fresh.
4. *Acidified butanol*: 95ml of n-butanol was mixed with 5.0 ml conc. HCl.
5. *Standard*: 10mg of N-acetyl neuraminic acid was dissolved in 100 ml of distilled water, which has a concentration of 100 µg/ml.

**Procedure**

To 0.5 ml of neutralized sample, 0.25 ml of periodate was added and incubated at 37°C for 30 min. After incubation, the reaction was arrested by the addition of 0.25 ml of arsenite. The tubes were shaken well and 2.0 ml of TBA was added and the tubes were heated in a boiling water bath for 6 min. After cooling, 5.0 ml of acidified butanol was added and the butanol
phase was separated after shaking well. The absorbance was read at 540 nm against a blank treated similarly using a spectrophotometer. Standard solutions containing 10-50 µg of N-acetyl neuraminic acid were also treated similarly. The values were expressed as mg/dL for serum and mg/gm of defatted tissue sample.

**ESTIMATION OF MEMBRANE BOUND ADENOSINE TRIPHOSPHATASES (E.C 3.6.1.3)**

Adenosine triphosphatases catalyse the conversion of adenosine triphosphate in to adenosine diphosphate. During the conversion, one molecule of inorganic phosphate is liberated. The inorganic phosphate is estimated according to the method of Fiske and Subbarow (1925). The proteins were precipitated with trichloroacetic acid. The free filtrate reacts with acid molybdate to form phospho molybdic acid which is reduced by the addition of 1-amino 2-naphthol-4-sulphonic acid (ANSA) to produce blue colour. The intensity of the color is proportional to the amount of inorganic phosphate present in the sample.

**Preparation of hemolysate and isolation of erythrocyte membrane**

Preparation of hemolysate and isolation of erythrocyte membrane was carried out by the method of Dodge et al., (1963) with a change in buffer, according to Quist (1980). Blood collected with EDTA was centrifuged at 2000 rpm for 20 min at 4°C. The packed cells were washed with isotonic saline to remove the buffy coat. An aliquot of packed cells were then washed three times with isotonic Tris-HCl (0.3 M, pH 7.4) buffer. An aliquot of 1.0 ml washed cells were lysed using 9.0 ml of hypotonic Tris-HCl buffer (0.015 M, pH 7.2). The lysed cells were centrifuged for 30 min at 15,000 rpm. The pellet was repeatedly washed with hypotonic Tris-HCl buffer until a clear pale pink or colorless supernatant was obtained. The resulting erythrocyte membrane pellet was suspended in 0.01 M Tris-HCl buffer, pH 7.4 for subsequent analysis.
Preparation of homogenate

Tissues were blotted, weighed accurately and placed in chilled 0.1 mol/l Tris–HCl buffer, pH 7.4. The samples were homogenized using a Potter-Elvehjem homogenizer filled with Teflon pestle to produce 10% homogenates and used for determining the biochemical parameters such as membrane bound ATPases described below.

Protein concentrations of the tissue homogenates were determined by the standard method of Lowry et al., (1951) using bovine serum albumin as the standard. Inorganic phosphate was estimated by the method of Fiske and Subbarow (1925).

Estimation of Na\(^+\) K\(^+\) - ATPase (E.C 3.6.1.3)

Na\(^+\) K\(^+\) - ATPase was assayed according to the method of Bonting (1970).

Reagents

1. *Tris-HCl buffer (184 mM, pH 7.5)*: 2.23 gm of Tris was dissolved in 100 ml of deionised water and pH was adjusted to 7.5 with 1N HCl.
2. *Magnesium sulphate (50 mM)*: 369.9 mg of MgSO\(_4\) was dissolved in 25 ml of deionised water.
3. *Potassium chloride (50 mM)*: 93.2 mg of KCl was dissolved in 25 ml of deionised water.
4. *Sodium chloride (600 mM)*: 876.6 mg of NaCl was dissolved in 25 ml of deionised water.
5. *EDTA (1 mM)*: 3.72 mg of EDTA was dissolved in 10 ml of deionised water.
6. *ATP (40 mM)*: 44.56 mg of ATP was dissolved in 2.0 ml of deionised water.
7. *Sodium bisulphate (15%)*: 15 gm of anhydrous sodium bisulphate was dissolved and made upto 100ml with distilled water.
8. **Sodium sulphite (20%):** 20gm of sodium sulphite was dissolved and made upto 100ml with distilled water.

9. **1-amino 2-napthol 4-sulphonic acid (ANSA):** 500mg of ANSA was dissolved in a solution containing 195 ml of 15% sodium bisulphate and 5 ml of 20% sodium sulphite and stored in a brown bottle.

**Procedure**

The incubation mixture contained 1.0 ml of buffer, 0.2 ml each of magnesium sulphate, potassium chloride, sodium chloride, EDTA, ATP and the test samples. The mixture was incubated at 37°C for 15 min. The reaction was arrested by the addition of 1.0 ml of TCA, mixed well and centrifuged and the supernatant was used for the estimation of inorganic phosphate by the method of Fiske and Subbarow (1925). The control without enzyme was also incubated and after arresting with TCA, the enzyme was added. The enzyme activity was expressed as µ moles of inorganic phosphate liberated/mg protein/min.

**Estimation of Ca\(^{2+}\) - ATPase (E.C 3.6.1.3)**

Ca\(^{2+}\)- ATPase was estimated as described by the method of Hjerten and Pan (1983).

**Reagents**

1. **Tris-HCl buffer (125 mM, pH 8.0):** 1.514 gm of Tris was dissolved in 100 ml of deionised water and the pH was adjusted to 8.0 with 1N HCl.

2. **Calcium chloride (50 mM):** 273.6 mg of calcium chloride was dissolved in 25 ml of deionised water.

3. **ATP (10 mM):** 33 mg of ATP was dissolved in 6.0 ml of deionised water.

4. **Sodium bisulphate (15%):** 15gm of anhydrous sodium bisulphate was dissolved and made upto 100ml with distilled water.
5. **Sodium sulphite (20%)**: 20gm of sodium sulphite was dissolved and made up to 100ml with distilled water.

6. **1-amino 2-napthol 4-sulphonic acid (ANSA)**: 500mg of ANSA was dissolved in a solution containing 195 ml of 15% sodium bisulphate and 5 ml of 20% sodium sulphite and stored in brown bottle.

**Procedure**

The incubation mixture contained 0.1 ml each of buffer, calcium chloride, ATP and test samples. The mixture was incubated at 37°C for 15 min. The reaction was arrested by the addition of 1.0 ml 10% TCA. The color developed was read at 620nm after 20 min against the reagent blank using a spectrophotometer. The amount of inorganic phosphate liberated was estimated by the method of Fiske and Subbarow (1925). The enzyme activity was expressed as µ moles of inorganic phosphate liberated/mg protein/min.

**Estimation of Mg²⁺- ATPase (E.C 3.6.1.3)**

Mg²⁺- ATPase was assayed by the method of Ohnishi *et al.* (1962).

**Reagents**

1. **Tris-HCl buffer (374 mM, pH 7.6)**: 4.536 gm of Tris was dissolved in 100 ml of deionised water and the pH was adjusted to 7.6 with 1N HCl.

2. **Magnesium chloride (50 mM)**: 50.8 mg of MgCl₂ was dissolved in 5.0 ml of deionised water.

3. **ATP (10 mM)**: 33mg of ATP was dissolved in 6.0 ml of deionised water.

4. **Sodium bisulphate (15%)**: 15gm of anhydrous sodium bisulphate was dissolved and made up to 100ml with distilled water.

5. **Sodium sulphite (20%)**: 20gm of sodium sulphite was dissolved and made up to 100ml with distilled water.
6. *1-amino 2-napthol 4-sulphonic acid* (ANS): 500mg of ANSA was dissolved in a solution containing 195 ml of 15% sodium bisulphate and 5 ml of 20% sodium sulphite and stored in brown bottle.

**Procedure**

The incubation mixture contained 0.1 ml each of buffer, magnesium chloride, ATP and test samples incubated at 37°C for 15 min. The reaction was arrested by the addition of 1.0 ml 10% TCA and centrifuged. The color developed was read at 620nm after 20 min against the reagent blank using a spectrophotometer. The liberated inorganic phosphate was estimated by the method of Fiske and Subbarow (1925). The enzyme activity was expressed as µ moles of inorganic phosphate liberated/mg protein/min.

**LYSOSOMAL ENZYMES**

**β-D- galactosidase (EC: 3.2.1.23)**

The activity of β-D- galactosidase was estimated by the method of Kawai and Anno (1971).

**Reagents**

1. *Citrate buffer (0.1 M, pH 4.2)*: 1.04 g of citric acid and 1.48 g of sodium citrate were dissolved in 100 ml of water.
2. *Glycine buffer (0.2 M, pH 10.7)*: This was prepared by mixing equal volumes of 0.2 M glycine, 0.125 M sodium carbonate, and 0.1 M sodium chloride solution.
3. *P-Nitrophenyl- β-D-galactoside*: 1 mg of the substrate was dissolved in 1 ml of distilled water.
4. *Standard*: 5 mg of p-nitrophenol was dissolved in 100 ml of distilled water.

**Procedure**

The mixture contained 2.0 ml of 0.1 M citric acid sodium buffer, 0.1 ml of substrate and 0.1 ml of homogenate was incubated for 1 hour at 37°C. The
reaction was terminated by the addition of 3.0 ml of glycine buffer. The reaction mixture was centrifuged for 5 minutes at 2000 rpm and absorbance of the released p-nitrophenol in the supernatant was measured at 400nm. A standard p-nitrophenol was carried out simultanesouly. The β-D- galactosidase was expressed as n moles of p-nitrophenol liberated/ mg protein/h.

**β-D- glucuronidase (EC: 3.2.1.31)**

β-D- glucuronidase activity was estimated by the method of Delvin and Gianetoo (1970).

**Reagents**

1. Acetate buffer (0.1M, pH 4.5): 0.49 ml of 0.1 M sodium acetate was mixed with 51 ml of 0.1 M acetic acid.

2. Glycine buffer (0.2M, pH 10.7): This was prepared by mixing equal volumes of 0.2 M glycine, 0.125 M sodium carbonate, and 0.1 M sodium chloride solution.

3. p-Nitrophenyl- β-D- glucuronide: 1 mg of the substrate was dissolved in 1ml of distilled water.

4. Standard: 5 mg of p-nitrophenol was dissolved in 100 ml of distilled water.

**Procedure**

0.1 ml of substrate, 0.1 ml of acetate buffer, 0.1 ml of sample was incubated at 37° C for 1 h. The reaction was arrested by the addition of 3.3 ml of glycine buffer. Standards were also run simultanesously along with water blank. The colour developed was read at 420 nm. The enzyme activity was expressed as μ moles of p-nitrophenol liberated/mg protein/h.

**N-acetyl- β-D- glucosaminidase (EC: 3.2.1.30)**

β- N-acetyl- D- glucosaminidase was assayed by the method of Marhun (1976).

**Reagents**

1. Substrate: 3.42 mg of 4-nitrophenyl-N-acetyl glucosaminidase was dissolved in 1 ml of citrate buffer.
2. *Citrate acid-Sodium citrate buffer (0.1 M, pH 4.2):* 46 ml of 0.1 M sodium citrate solution was mixed with 54 ml of 0.1 M citric acid solution.

3. *Glycine buffer (0.2 M, pH 10.7):* Glycine buffer was prepared by mixing equal volumes of 0.2 M glycine, 0.125 M sodium carbonate and 0.1 M sodium chloride.

4. *Standard:* 5 mg of p-nitrophenol was dissolved in 100 ml of distilled water.

**Procedure**

To 0.2 ml of sample, 0.1 ml of buffered substrate was added and incubated at 37°C for 40 min. At the end of the incubation period, the reaction was arrested by the addition of 2.2 ml of 0.2 M glycine buffer and the colour developed was read at 420 nm. The enzyme activity was expressed as μ mole of p-nitrophenol liberated/mg protein/h.

**Cathepsin-D (EC: 3.4.23.5)**

Cathepsin-D activity was estimated by the method of Sapolsky *et al.*, (1973).

**Reagents**

1. *Sodium acetate buffer (0.1 M, pH 3.6):* 92.5 ml of 0.1 M acetic acid was mixed with 7.5 ml of 0.1 M sodium acetate solution.

2. *Substrate 1.5%:* 1.5 g of hemoglobin was dissolved in 100 ml of sodium acetate buffer.

3. 5% TCA

4. Folin’s phenol reagent.

5. 5% NaOH

6. Alkaline copper reagent (Lowry’s reagent).

7. *Standard:* A solution of tyrosine in the concentration of 10 mg/100 ml was prepared with 0.1 N HCl.
Procedure

0.9 ml of buffered substrate was mixed with 0.1 ml of sample and incubated for 2 h at 37ºC. The reaction was arrested with 1 ml of 5% TCA and the samples were centrifuged for 10 min. To the control tubes the enzyme preparation was added after the addition of TCA. To 1 ml of supernatant 1 ml of 5% sodium hydroxide and 4.5 ml of alkaline copper reagent were added. After 20 min, 0.5 ml of Folin’s phenol reagent was added and the colour developed was read at 640 nm after 10 min. The standards were treated similarly. Enzyme activity was expressed as µ mole of tyrosine release/mg of protein/h.

ASSAY OF BIOTRANSFORMATION ENZYMES

Preparation of liver microsomes

The liver microsomes were separated according to the method of Boyd and Burka (1978) with slight modification by Kamath and Narayanan (1972).

Reagents

1. Homogenizing buffer (Tris-HCl buffer (50 mM, pH 7.4) containing 0.15 M KCl): 605.5 mg of Tris and 1.15 gm of KCl were dissolved in 100 ml of water and the pH was adjusted to 7.4 with HCl.
2. Calcium chloride (8mM): 117.6 mg of calcium chloride was dissolved in 100 ml of distilled water.

Sample preparation

2.5 gm of tissue was homogenized with 10ml of distilled water in Potter-Elvehjem homogenizer fitted with Teflon pestle at 4º C at uniform speed with 5 return strokes to give a concentration of 25% homogenate. Then the tubes were covered with parafilm and mixed gently by inversion. Then the homogenate was centrifuged for post mitochondrial supernatant at 10,000 rpm for 30 min at 2-4º C. The floating lipid layer on the top of the supernatant was
discarded. Calcium chloride was added to the post mitochondrial supernatant and centrifuged at 15,000 rpm for 10-15 min. The pellet was resuspended in buffer, homogenized and made up to a known volume. The microsomal suspension was used for the analysis of biotransformation enzymes.

**Microsomal protein**

The protein content of microsomes in the tissues was estimated according to the method of Lowry *et al.*, (1951). The protein content was expressed as mg/microsomes in gm wet tissue.

**Assay of phase I hepatic drug metabolizing enzymes**

**Estimation of microsomal Cytochrome P**\(_{450}\)

Cytochrome P\(_{450}\) was estimated by the method of Omura and Sato (1964).

**Reagents**

1. *Phosphate buffer (0.2 M pH 7.4):*
   Solution A: 1.36gm of potassium dihydrogen phosphate was dissolved in 25ml of distilled water,
   Solution B: 0.2N NaOH

2. *Sodium dithionate*

3. *Carbon monoxide gas:* Carbon monoxide can be generated freshly by the action of concentrated sulphuric acid on formic acid and purified by passing through KOH-dithionite solution (Since CO is a highly poisonous gas, necessary precaution should be followed during the preparation).

**Procedure**

To the mixture containing 1.0 ml of buffer and 0.1 ml of tissue homogenate 2 gm of solid sodium dithionite was added. The cuvette containing the sample and blank (containing buffer) were scanned between 450 to 490 nm
attains a clear base line. Then carbon monoxide was gently bubbled approximately for 1 min and the samples were read from 450 to 490 nm. The difference in absorption spectrum between 450 nm and 490 nm can then be used for the calculation of cytochrome P\textsubscript{450} activity. The level of cytochrome P\textsubscript{450} was expressed as n moles/mg microsomal protein based on molar extinction coefficient 91 cm\textsuperscript{2}/mole for absorbance at 450 to 490 nm.

**Estimation of microsomal Cytochrome b\textsubscript{5}**

The amount of Cytochrome b\textsubscript{5} was measured by the method of Omura and Sato (1964).

**Reagents**

1. *Phosphate buffer, (0.2 M, pH 7.4):* 2.88 mg of disodium hydrogen phosphate and 456 mg of sodium dihydrogen phosphate were dissolved in 100 ml of distilled water.

2. *NADH (2\%) (w/v):* 200 mg of NADH was dissolved in 1 ml of distilled water.

**Procedure**

The mixture containing 0.1 ml of buffer and 0.5 ml of enzyme was read in a spectrophotometer at 450 nm and 590 nm against a buffer blank. After the addition of 0.025 ml of NADH again the absorbance was read at 450 and 590 nm. The difference in the absorption spectrum which is the cytochrome b\textsubscript{5} content and result was expressed as n moles/mg microsomal protein/min.

**Assay of NADPH - Cytochrome C (P\textsubscript{450}) reductase (E.C 1.6.2.4)**

The activity of NADPH-cytochrome c(P\textsubscript{450}) reductase was assayed by the method of Phillips and Langdon (1962).

**Reagents**

1. *Phosphate buffer (0.3 M, 7.6):* 5.11gm of disodium hydrogen phosphate and 514.7mg of sodium dihydrogen phosphate were dissolved in 100ml of distilled water.
2. **NADPH (0.045 mM)**: 0.35mg of NADPH was dissolved in 10ml of distilled water.

3. **Potassium cyanide (1 mM)**: 3.292mg of KCN was dissolved in 10ml of distilled water.

4. **Cytochrome c (0.05 mM)**: 0.006mg of cytochrome c was dissolved in 100ml of distilled water.

**Procedure**

The assay mixture containing 2.5 ml of buffer, 0.2 ml of potassium cyanide and 0.1 ml of cytochrome C was mixed gently. After 3 minutes, 0.1 ml of NADPH was added and the change in OD was recorded at an interval of 30 seconds for 3 minutes at 550 nm UV spectrophotometer. The activity of NADPH-cytochrome P$_{450}$ reductase was expressed as n moles of cytochrome C oxidized/mg protein/min.

**ASSAY OF PHASE II BIOTRANSFORMATION ENZYMES**

**Glutathione-S-Transferase (EC 2.5.1.18)**

Glutathione S-transferase was assayed by the method of Habig et al., (1974).

**Reagents**

1. **Phosphate buffer**: 0.3 M, pH 6.5
2. **1, chloro-2, 4-dinitrobenzene (CDNB)**: 30 mM
3. **Reduced glutathione (GSH)**: 30 mM

**Procedure**

To 1.0 ml of phosphate buffer, 0.1 ml of CDNB, 1.7 ml of water and 0.1 ml of enzyme source was added. After 5 minutes of incubation at 37°C, 0.1 ml of GSH was added and the change in optical density was measured immediately for 3 minutes. A complete assay mixture without enzyme was used as control. Optical density was measured at 340 nm in a spectrophotometer. The enzyme activity is expressed as n moles of CDNB utilized/mg microsomal protein/min.
UDP-Glucuronyl Transferase

The UDP-glucuronyl transferase was estimated by the method of Issalbacher et al., (1962) modified by Hollman and Touster (1962).

Reagents

1. **Tris-HCl buffer 1M pH 7.4**: 12.11 gm of Tris was dissolved in 100 ml of distilled water and pH was adjusted to 7.4 with 0.1N HCl.
2. **Triton X-100 (0.25% (w/v))**: 250 mg of Triton X-100 was dissolved in 100 ml of distilled water.
3. **Magnesium chloride 50mM**: 47.5 mg of MgCl$_2$ was dissolved in 10 ml of distilled water.
4. **P-Nitrophenol 5mM**: 0.84 mg of p-Nitrophenol was dissolved in 1 ml of distilled water.
5. **UDP-glucuronic acid 30mM**: 19.38 mg of UDP-glucuronic acid was dissolved in 1 ml of distilled water.
6. **TCA**: 5%
7. **NaOH**: 2M.

Procedure

The incubation mixture containing 0.5 ml buffer, 0.2 ml Triton X-100, 0.05 ml MgCl$_2$, 0.05 ml p-nitrophenol, 0.18 ml water and 0.1 ml enzyme was incubated at 37°C for 2 minutes. Then 0.1 ml of UDP-glucuronic acid was added. Then 0.1 ml aliquot of this mixture was arrested at 0, 10 and 15 minutes with TCA and centrifuged. To 1 ml of the supernatant 0.25 ml of NaOH was added and read at 450 nm using a spectrophotometer. The enzyme activity is expressed as n moles/mg microsomal protein/min.
ENZYME IMMUNOASSAYS

Tumor markers

Estimation of Carcinoembryonic antigen (CEA)

The UBI MAGIWEL CEA Quantitative CM-201 is a solid phase enzyme linked immunsorbent assay kit. This test provides quantitative measurement of carcinoembryonic antigen (CEA) in serum

Materials provided

1. Microwell Strips: Anti-CEA antibodies coated wells, 8 x 12 strips, 96 wells
2. Sample Diluent or Zero Standard (11 ml)
3. Enzyme Conjugate (11 ml): Anti-CEA antibody conjugated to horseradish peroxidase
6. Reference Standard Set (0.75 ml each vial): Calibrated to 1.5, 3, 6, 15 and 30 ng/ml in the sample diluent.
7. Stop Solution: 2 N HCl.
8. Well Holder: For securing individual wells.
9. Microwell Reader
10. Pipetor with tips for 25 and 100 µl

Procedure

The collected blood was allowed to clot and serum was separated. All the reagents and samples were brought to room temperature and mixed. Then 25µl of serum sample controls and standards were dispensed into the assigned wells. Then 100µl of enzyme conjugate was dispensed into each well and again incubated for 60 minutes at room temperature. 100µl of solution A and 100µl of solution B were added into each well and incubated for 30 minutes at room temperature. The reaction was stopped by the addition
of 2N HCl to each well and optical density at 450 nm was read with microwell reader. The CEA content was expressed as ng/ml.

**Estimation of CA 15-3**

The UBI MAGIWEL CA 15-3 Quantitative CM-701 is a solid phase enzyme linked immunsorbent assay kit. This test provides quantitative measurement of in serum CA 15-3

**Materials provided**

1. Microwell Strips: Anti-CA 15-3 antibodies coated wells, 8 x 12 strips, 96 wells
2. Sample Diluent or Zero Standard (11 ml)
3. Enzyme Conjugate (11 ml): Anti-CA 15-3 antibody conjugated to horseradish peroxidase
4. Solution A (11 ml): containing H$_2$O$_2$
5. Solution B (11 ml): Tetramethylbenzidine.
6. Reference Standard Set (0.75 ml each vial): Calibrated to 1.5, 3, 6, 15 and 30 ng/ml in the sample diluent.
7. Stop Solution: 2 N HCl.
8. Well Holder: For securing individual wells.
9. Microwell Reader
10. Pipetor with tips for 25 and 100 µl

**Procedure**

The collected blood was allowed to clot and serum was separated. All the reagents and samples were brought to room temperature and mixed. Then 25µl of serum sample controls and standards were dispensed into the assigned wells. Then 100µl of enzyme conjugate was dispensed into each well and again incubated for 60 minutes at room temperature. 100µl of solution A and 100µl of solution B were added into each well and incubated for 30 minutes at room temperature. The reaction was stopped by the addition of 2N HCl to each well and optical density at 450 nm was read with microwell reader. The CA-15-3 content was expressed as ng/ml.
INFLAMMATORY MARKERS

Determination of cytokines

The levels of cytokines such as Interleukin (IL)-1β, Interleukin (IL)-6, Interleukin(IL)-8 and Tumor Necrosis Factor-α (TNF-α) in serum were determined by using specific ELISA kits (Biosource, California, US). The analyses were performed according to instructions of the manufacturer’s. Standard plots were constructed by using standard cytokines and the concentrations for unknown samples were calculated from the standard plot.

HORMONAL ASSAY

Estimation of 17β-Estrodiol and Progesterone

The hormones such as 17β-estrodiol and progesterone levels were determined by using specific ELISA kits (DiaSorin Inc.Stilwater,USA). Both the tests were done following the procedure mentioned in the commercial kit. The test values above the standard curve were retested with appropriate dilutions.

DNA FRAGMENTATION

Isolation of DNA

Isolation of total DNA or mDNA is based on the standard phenol chloroform method. About 25mg of breast tissue or mitochondrial pellet (5mg/ml) was homogenized or resuspended, respectively in 1.0ml of ice cold TE buffer at 4°C. The homogenate was subjected to RNAse (10µg/ml) digestion for 1h at 37°C followed by proteinase k (100µg/ml) digestion for 3 hour at 50°C water bath. The contents were cleared off by centrifugation at 12,000 rpm for 5 min. To 0.2ml of the supernatant 10µl of 20% SDS, 0.25 ml each of Tris- saturated phenol and chloroform: isoamyl alcohol (24:1) were added and kept in ice for 5 min to aid complete precipitation of protein.
The contents were centrifuged at 12,000 rpm for 15 min and the supernatant was carefully removed into a fresh tube. To this sodium acetate was added to give a final concentration of 0.3 M followed by the addition of two volumes of ice cold isopropanol and mixed gently by repeated inversion of the tubes. DNA precipitation was enhanced by storing at -20°C. After overnight incubation DNA was pelleted out by centrifugation at 15,000 rpm for 20 min. The DNA pellet was washed twice with 70%. Ice cold ethanol and allowed to air dry to remove ethanol. The pellet was resuspended 50-100µl of the TE buffer and stored at -80°C. All centrifugation steps were carried out at 4°C unless otherwise stated.

**Determination of DNA concentration and purity**

The concentration and the degree of purity of plasmid DNA was determined based on the Beer-Lambert law by measuring the absorbance at 260nm and 280nm.

$$A_{260} = \varepsilon_{260} C l$$ and $$A_{260} \times 50 = \mu g/\mu l$$ (Where l=km)

where $$A_{260}$$ is the absorbance at 260nm, $$\varepsilon_{260}$$ is the molar absorption coefficient, C is the molar concentration and l is the optical path length (usually 1cm). For a protein free and RNA free solution the ratio of $$A_{260} / A_{280}$$ should be 1.5-2. Any protein contamination present in the preparation would decrease the ratio <1.5 where as RNA contamination would increase the ratio >2. DNA preparations showing the $$A_{260} / A_{280}$$ ratio of 1.5-2.0 were used for analysis of DNA fragmentation in an agarose gel electrophoresis.

**Agarose gel electrophoresis of DNA**

The DNA fragmentation was analyzed by agarose gel electrophoresis. Concentration of agarose used for analysis is inversely proportional to the size of the DNA of interest, that is the larger the DNA the lowest the concentration of agarose.

*Tris Borate-EDTA (TBE) buffer (5X)*

54gm Tris base

27.5gm Boric acid
20ml of 0.5M EDTA (pH 8.0)

The ingredients were made up to one liter with distilled water and stored at room temperature.

Gel loading buffer (6x)
0.25% (w/v) bromophenol blue
30% glycerol in 1X TBE

Procedure

The required amount of agarose was taken in 1X TBE buffer and melted by boiling in a microwave oven. The agarose solution was poured into a cassette and allowed to solidify. The agarose gel was immersed in chamber with TBE buffer and DNA sample in gel loading buffer was loaded in the gel. The electrophoresis was carried out at 100V after that the gel was stained in Ethidium bromide (EtBr) bath (0.5µg/ml). Binding of EtBr by intercalation of its planar group between the stacked bases of the DNA increases its fluorescent yield compared to that of the dye in free solution. During ultraviolet irradiation, DNA absorbs at 254nm and transmits energy to the dye and the bound dye itself absorbs radiation at 302nm as well as 366nm. As a result of both cases, the energy is reemitted at 590nm in the red orange region of the visible spectrum, which was used to visualize DNA under a UV transilluminator. The gel was photographed using a gel documentation system.

PROTEIN EXPRESSIONS OF p53, CASPASE-3, Bax, Bcl-2 AND NF-κB IN BREAST TISSUES OF CONTROL AND EXPERIMENTAL ANIMALS.

Preparation of homogenate

Breast tissues were blotted, weighed accurately and placed in chilled RIPA buffer, pH 7.4. The samples were homogenized using a homogenizer. 10% homogenate was used for determining the protein expression as described below.
Quantification of protein

The protein concentrations in breast of experimental animals were estimated by BioRad protein assay kit (Catalog # 223-9950). The western blotting procedure was followed by Fido et al., (1995).

Reagents

1. *Radio Immuno Precipitation Buffer (RIPA):* (150mM NaCl, 50 mM Tris, 1mM EDTA, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, 1mM β-glycerophosphate, 1mM Sodium orthovanadate and 1mM Sodium fluoride, pH 7.4).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>302 mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>438 mg</td>
</tr>
<tr>
<td>EDTA</td>
<td>186 mg</td>
</tr>
<tr>
<td>Na-deoxycholate</td>
<td>25 mg</td>
</tr>
<tr>
<td>SDS</td>
<td>50 mg</td>
</tr>
<tr>
<td>NP-40</td>
<td>500 ml</td>
</tr>
<tr>
<td>β-glycerophosphate</td>
<td>10.8 mg</td>
</tr>
<tr>
<td>Sodium orthovanadate</td>
<td>9.2 mg</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>2.0 mg</td>
</tr>
</tbody>
</table>

All these chemicals were dissolved in 40 ml of Milli Q water, pH was adjusted to 7.4 with 1N HCl and then the volume was made up to 50 ml.

2. *Protease inhibitor (PI):* Protease inhibitor cocktail was purchased from Pierce Biotechnology Inc, USA. PI was used 1:100 as per the manufacturer’s instruction (For 1ml, 10µl PI).

3. *Running gel buffer (1.5 M Tris, pH 8.8):* 45.375 gm of Tris was dissolved in 200 ml of Milli Q water and pH was adjusted with HCl and the volume was made up to 250 ml.
4. **Stacking gel buffer (0.5 M Tris, pH 6.8):** 15 gm of Tris was dissolved in 200 ml of Milli Q water and pH was adjusted with HCl and the volume was made up to 250 ml.

5. **SDS 10%:** 10 gm of SDS was dissolved in 100 ml of Milli Q water and stored at room temperature.

6. **Acrylamide 30%:** 29 gm of Acrylamide (29%) and 1 gm of N, N’ Bis-acrylamide (1%) were dissolved in 100 ml of Milli Q water.

7. **Ammonium Persulfate 10%:** 100 mg of Ammonium persulfate was dissolved in 1ml of Milli Q water.

8. **10X SDS Electrophoresis buffer (2.5 M Tris, 1.92 M Glycine and 1% SDS):** 30 gm of Tris, 144 gm of Glycine and 10 gm of SDS were dissolved in 800 ml of Milli Q water, volume was made up to one litre.

9. **1X SDS Electrophoresis buffer:** 50 ml 10X SDS electrophoresis buffer was made up to 500 ml with Milli Q water.

10. **10X Transfer buffer (250 mM Tris and 1.92 M Glycine):** 30.3 gm of Tris and 144 gm of Glycine were dissolved in 800 ml of Milli Q water and the volume was made up to one litre.

11. **1X Transfer buffer with 20% methanol:** 100 ml of 10X transfer buffer was mixed with 700 ml of Milli Q water and 200 ml of methanol were mixed and kept cold until used.

12. **2X Sample buffer with reducing agent (125 mM Tris-HCl (pH 6.8), 4 % SDS, 20% glycerol, 10 % 2-mercaptoethanol and 0.004 % bromophenol blue):** 2.5 ml of 0.5 M Tris-HCl (pH 6.8), 4 ml of 10% SDS, 2 ml of glycerol, 1 ml of 2-Mercaptoethanol and 0.4 mg bromophenol blue were mixed well and the volume was made up to 10 ml with Milli Q water and stored at -20°C.
13. **10X Phosphate buffered Saline (0.1 M Sodium phosphate)**: 13 gm of NaH$_2$PO$_4$ was dissolved in Milli Q water, the pH was adjusted to 7.2 with NaOH, the volume was made up to one liter and stored at room temperature.

14. **Blocking Buffer 5% (PBS with 10 % glycerol, 5 % milk powder and 0.2 % Tween-20)**: 50 ml of 10X PBS, 50 ml of Glycerol, 4.5 gm of NaCl, 25 gm of non fat dry milk powder, 1 ml of Tween-20 were dissolved in 400 ml of Milli Q water. All these were taken in a beaker and stirred well until dissolved. The volume was made up to 500 ml with milli Q water.

15. **Tris Buffered Saline (TBS)**: (20 mM, Tris 500 mM NaCl, pH 7.5): 1.21 gm of Tris and 14.62 gm of NaCl were dissolved in 400 ml with Milli Q water and pH was adjusted to 7.5 with HCl and made up to 500 ml.

16. **T-TBS (0.2 % Tween-20 in TBS)**: 0.2 ml of Tween-20 was dissolved in 100 ml of TBS and stored in refrigerator.

17. **Prestained SDS-PAGE standard**: Protein marker purchased from Bio Rad, Laboratories, USA.

**Preparation of gel**

**Running gel (10 %)**: The following volumes of solutions were taken for one slab gel.

- Acrylamide (30 %) 3.3 ml
- Running gel buffer 2.5 ml
- D.D H$_2$O 4.0 ml
- SDS (10%) 0.1 ml
- APS (10%) 0.1 ml
- TEMED 0.007 ml

**Stacking gel (5%)**: The following volumes of solutions were taken for one slab gel.
Acrylamide (30 %)  0.5 ml
Stacking gel buffer   0.38 ml
D.D H2O  2.1 ml
SDS (10%)  0.03 ml
APS (10%)  0.03 ml
TEMED  0.003 ml

Equal amount of total protein was mixed with 2X sample buffer and boiled for 5 min. The protein was separated on 10% SDS-PAGE and electrotransfered in to a Polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA). The blots were blocked with 5% blocking buffer for 4 h. After blocking, membrane was incubated with respective rabbit monoclonal antibodies p53, Bcl-2, caspase-3, Bax and NF-κB (Santa Cruz Biotechnology, CA) in 1:1000 dilution overnight at 4°C. Then the membranes were washed thrice with T-TBS for 10 minutes each, followed by horseradish peroxidase conjugated secondary antibody (1:10,000 dilutions) incubation for 45 min at room temperature. Finally, signals were visualized using Enhanced Chemiluminescent System (Pierce Biotechnology Inc, USA) and the signals were captured by Chemi Doc XRS system (Bio Rad, USA) and the intensity of the bands were quantified by Quantity One software (Bio Rad, USA). Immunoblot for β-actin was used as an internal control for equal loading in the gel.

Isolation of total RNA

Total RNA was isolated from the frozen tissues using TRI reagent (TRIZOL) by the method of Chomczynski and Sacchi (1987). The total RNA obtained is free from protein and DNA contamination.

Reagents

1. TRI reagent: Phenol, guanidium isothiocyanate, urea, detergents, buffering agents and stabilizers,
2. Chloroform (molecular grade),
3. Isopropanol (molecular grade),
4. 75% ethanol, Diethylpyrocarbonate (DEPC) water – 0.2% (or) sterile water

Procedure

To 100 mg tissue, 1 ml of TRI reagent was added and homogenized well. Following homogenization, the homogenate was stored for 5 min at 4ºC to permit complete dissociation of nucleoprotein complexes. To this, 0.2 ml of chloroform/ml of RNA reagent was added, shaken vigorously for 15 sec and centrifuged at 10,000 rpm for 15 min at 4ºC. After centrifugation, the upper aqueous phase containing RNA were separated. The volume of the aqueous phase was about 40-50% of the total volume of the homogenate.

The aqueous phase was carefully transferred to a fresh eppendorf micro centrifuge tube without disturbing the interphase. Equal volume of isopropanol was added to the eppendorf tube and then centrifuged at 10000 rpm for 10 min at 4ºC. RNA got precipitated and formed as a white pellet at the bottom of the tube. The supernatant was removed and the pellet was washed twice with 75% ethanol. Subsequently, the eppendorf tube was vortexed and centrifuged at 6500 rpm for 15 min at 4ºC. At the end of the experiment, the pellet was dried under vaccum for 5 min. Care was taken not to allow the RNA pellet to dry completely, as it will greatly decrease its solubility. The RNA pellet was then dissolved in 30µl of 0.2% sterile water and placed in a water bath at 60ºC for 10 min to ensure complete solubility of RNA. The RNA sample was subsequently vortexed for 10 min and stored at -80ºC.

Quantification of RNA

Diluted RNA sample was quantified spectrophotometrically by measuring the absorbance (A) at 260 nm. An absorbance of 1 OD is
equivalent to RNA concentration of 40µg/ml. Therefore, the yield can be calculated by multiplying the absorbance at 260 nm with dilution factor and 40µg. By determining the absorbance of the sample at 260 nm and 280 nm, to assess the purity of the RNA. This is a reflection of the protein contamination in the sample. A ratio of absorbance at 260/280nm is generally considered as good quality RNA (>1.8) (Fourney et al., 1988).

**Reverse Transcription (RT)**

**First strand DNA synthesis**

The RT Kit was purchased from INVITROGEN

**Reagents**

1. First Strand buffer(5x): (250mM Tris-HCl (pH8.3), 75mM KCl, 15mM MgCl₂
2. dNTP (2.5µM each dNTP)
3. OligodT (10µM): Stock (2µg/µl): 147.1µg of oligodT was dissolved in 73.55µl of RNase free water. Working solution: 3µl of stock was diluted with 37µl of sterile water
4. Superscript III Reverse Transcriptase (200U/µl): This was isolated from an Escherichia coli strain carrying the Reverse Transcriptase gene from M-MuLV (Moloney Murine Leukemia Virus).
5. Template (RNA-1.5µg for 20µl reaction)
6. RNase free water

**Procedure**

Total reaction of 20µl was performed as follows
Components Volume (µl)

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OligodT(10µM)</td>
<td>5</td>
</tr>
<tr>
<td>dNTP(10µM)</td>
<td>1</td>
</tr>
<tr>
<td>RNATemplate (1µg)</td>
<td>Varies</td>
</tr>
<tr>
<td>RNase free water</td>
<td>Varies</td>
</tr>
</tbody>
</table>

The mixture was heated at 65°C for 5 minutes and incubates on ice for 1 minute, and the content was collected and centrifuged. To the supernatant the following reagents were added.

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Strand buffer (5x)</td>
<td>4</td>
</tr>
<tr>
<td>DTT (0.1M)</td>
<td>1</td>
</tr>
<tr>
<td>Super Script III Reverse Transcriptase (200U/µl)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**Total reaction** 20

For each step the components were mixed gently and spun briefly and incubated at three different temperatures and time duration in the thermal cycler at (25°C for 5 minutes, 50°C for 45 minutes, 70°C for 15 minutes) and finally maintained at 4°C for 5 min. After the reaction, samples were stored at -20°C or proceed to the PCR.

**Polymerase chain reaction**

**Reagents**

The kit was purchased from KAPATaq ReadyMix DNA Polymerase.

**Master mix components (2X)**

- KAPATaq DNA Polymerase (Isolated from the Thermophilic bacterium *Thermus Aquaticus* purified from recombinanant *Escherichia coli*).
- Buffer (Mg²⁺ and 0.4mM each dNTP)
  - Forward primer (6µM)
  - Reverse primer (6µM)
  - cDNA- Template
  - RNase and DNase free Water
**PRIMERS DETAIL**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Gene bank no</th>
<th>Amplified product</th>
<th>Annealing temperature and number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>Sense: 5'- AGCGACTACAGTTAGGGGT -3'</td>
<td>NM030989.3</td>
<td>122</td>
<td>55°C, 35</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'- TCTCCTGACTCAGAGGGAGC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF-κB</td>
<td>Sense: 5'- AGCACAACACCACCTCCAG-3'</td>
<td>NM017059.1</td>
<td>280</td>
<td>55°C, 35</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'- TTATCAACCGTAGGCTCGGC -3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspases-3</td>
<td>Sense: 5'- GTTCATCCAGTCACCTTTGTGCT-3'</td>
<td>NM012922.2</td>
<td>310</td>
<td>55°C, 35</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'- GCAGGCAGTGTTATGTTCA -3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense: 5'- GGCGCTCTGCTTGCTCCCTCCCT-3'</td>
<td>NM017008.3</td>
<td>453</td>
<td>55°C, 35</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'- GGCATCAGCGGAAGGGGC -3'</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Procedure**

Total reaction of 25µl was performed in 0.25 ml of PCR vial

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Master mix (2µM)</td>
<td>12.5</td>
</tr>
<tr>
<td>Gene of interest</td>
<td>Forward primer</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
</tr>
<tr>
<td>Internal control</td>
<td>Forward primer</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
</tr>
<tr>
<td>RT sample</td>
<td>1.5</td>
</tr>
<tr>
<td>Sterile Water</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total Reaction</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>
For each step the reaction mixture was mixed gently and spinned briefly. After spinned the mixture was incubated in the thermocycler as per the following reactions:

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>94</td>
<td>2 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>Varies</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>4 min</td>
</tr>
</tbody>
</table>

Amplified product was analyzed by agarose gel electrophoresis with ethidium bromide staining.

**Agarose gel electrophoresis**

Agarose gel electrophoresis is an effective method for the identification of purified DNA molecules (Sambrook *et al.*, 1989).

**Reagents**

1)  *TBE buffer 1X*: (Tris, Boric acid, EDTA) (pH 8.0): 3.7g Tris, 1.92g Boric acid and 0.2604g EDTA were dissolved in 350ml of autoclaved RNAse free water and the pH was adjusted to 8.0.

2)  *1% Ethidium bromide*: 10 mg in 1 ml of RNAse and DNAse free water

3)  *2% Agarose*: 2.0 gm in 100 ml of 1X TBE buffer

4)  *Gel loading dye*

   - 0.1 M EDTA - 2ml
   - 0.01 M Tris HCl - 0.1ml
   - 0.25% Bromophenol blue - 25mg
   - 50% Glycerol - 5ml
2.9ml of autoclaved DNAse and RNAse free water was added to make the final volume of 10ml.

**Procedure**

2.5g of agarose (electrophoresis grade) was added to 130ml of 1X TBE buffer (2%). It was then melted on a microwave oven and 2µl of 1% Ethidium bromide (EtBr) was added, evenly mixed and cooled to 4ºC. It was then poured into a sealed gel-casting platform and comb was inserted ensuring that there were no bubbles. The gel was then allowed to harden. After 15 min, the comb was removed taking care that the sample wells were not teared. The platform was then placed into the tanks and electrophoresis buffer was added until it covered the gel, making sure that no air was trapped within the wells.

5µl of cDNA was then taken from each reaction tube, mixed with 2µl 6 X gels loading dye and then loaded to each well. A 100 bp molecular weight marker DNA was simultaneously loaded in the first lane. The power supply was turned on and the current adjusted to 100V. The gel was run till the dye reached the end of the gel, then the gel containing cDNA was visualized with the help of fluorescent imager (Bio-Rad, USA). The Band intensity was quantified by Quantity One Software. The band intensification for each enzyme mRNA was normalized with that of the internal control GAPDH using Quantity One Software.

**TISSUE PROCESSING FOR HISTOLOGICAL STUDIES**

The histopathological studies in the breast and liver tissues were performed as detailed by Bancroft and Cook (1994).

The formalin fixed tissues were washed and subjected to dehydration in ascending grades of alcohol. The tissues were blocked in paraffin was, sectioned into ribbion and fixed on glass slides. After staining and destaining
with hematoxylin and eosin. They were permanently mounted with DPX mount for histopathological studies.

**Reagents**

1. *Phosphate buffered formalin (0.1 M pH 7.4):* 420 ml of 0.1 M disodium hydrogen phosphate was mixed with 0.1 M potassium dihydrogen orthophosphate to attain the pH 7.4 and 10% (v/v) formalin was prepared using this phosphate buffer.

2. *Hematoxylin stain:* 1 g of hematoxylin stain was dissolved in 10 ml of absolute alcohol and mixed with the solution containing 20 g of potassium alum, previously dissolved in hot distilled water. After bringing the mixture to boiling point, 0.5 g of mercuric oxide was added. This was cooled rapidly under tap water then filtered and left to 10 to 15 days.

3. *Eosin stain (0.5%):* 500 mg of eosin yellow powder was dissolved in 100 ml of distilled water and stored.

4. Absolute alcohol
5. 30, 50, 70 and 90% alcohol
6. Xylene
7. Paraffin wax
8. Egg albumin

Immediately after sacrifice, the animals were autopsied. Their breast and liver were rapidly excised and approximately 5 to 10 mm\(^3\) of tissue section were prepared with a razor blade and fixed in formal saline then washed thoroughly in running water. Then the fixed tissue was dehydrated in an increasing order of alcohol (30, 50, 70, 90 and 100%) for 30 min. It was then transferred to xylene for clearing and subjecting to cold infiltration at room temperature in a mixture containing paraffin wax dissolved in xylene for 30 min.

The tissue was then hot infiltrated in an oven at 58 to 60\(^\circ\)C in molten paraffin wax for 1 h. The hot infiltrated tissue was removed and blocked quickly.
using metal blocks. The solidified blocks were trimmed to small size and sectioned using microtome to get ribbons of 8 to 10 micron thickness. The ribbed sections were placed on glass microscopic slides coated with egg albumin. The microscopic slides were then exposed to the decreasing order of alcohol (100, 90, 70, 50 and 30%) for 5 min each. The sections were then dipped in hematoxylin stain for 5 min and washed in running tap water for 3 to 5 min. After dried, the tissues were exposed to 0.5% eosin stain for 5 min and washed again in running tap water to remove the excess stain. The slides were dried and dehydrated in an increasing order of alcohol (30, 70 and 90) for few seconds. After dried, the slides were cleared in xylene twice for 10 min. The tissue sections were then permanently mounted with DPX mountant. The permanently mounted sections of tissues samples were observed under light microscope for histological evaluations.

**IMMUNOFLUORESCENCE ANALYSIS**

Immunofluorescence analysis was performed following the methods used by Bancroft and Gamble (2002).

**Reagents**

1. 0.3% H$_2$O$_2$ in methanol.
2. 10x phosphate buffered saline (PBS): Working solution 1xPBS: 80 g of NaCl, 2 g of KCl, 11.5 g of Na$_2$HPO$_4$ and 2 g of KH$_2$PO$_4$, were dissolved in 600 ml of distilled water, pH was adjusted to 7.6 and the final volume was made up to 11ml with distilled water, filtered and autoclaved.
3. 10% BSA in 1x PBS
4. Primary antibodies (Santa Cruz Biotechnology, CA)
   i. Caspase-3 : anti- caspase 3 goat polyclonal IgG
   ii. Bcl-2 : anti-bcl 2 goat polyclonal IgG
   iii. p53 : anti p53 rabbit polyclonal IgG
iv. NF-κB : anti-NF-kB rabbit polyclonal IgG

5. Secondary antibodies (GeNei, Bangalore)

i. Caspase-3 : Rabbit anti-goat IgG conjugated
   Tetramethyl Rhodamine
   Isothiocyanate (TRITC)

ii. Bcl-2 : Rabbit anti-goat IgG conjugated
   TRITC

iii. p53 : Goat anti-rabbit IgG conjugated
   Fluorescein Isothiocyanate (FITC)

iv. NF-κB : Goat anti-rabbit IgG conjugated
   FITC

Procedure

Preparation of sections

Breast tissue sections of 5µm thickness were taken using microtome from paraffin-embedded tissue blocks, placed on slides and then incubated at 37°C for overnight. Then the sections were deparaffinized in xylene, using three changes of fresh xylene, for 10 min each and further dehydrated gradually through descending grades of alcohol (95, 90, 70, 50, 30%), for 5 min each. Finally, the sections were hydrated in distilled water. In order to quench endogenous peroxidase activity and were incubated in 0.3% H₂O₂ in methanol for 30 min and washed in distilled water for 5 min.

Immunoreactivity

The tissue sections were rinsed with phosphate buffered saline (PBS) and incubated with 3% BSA in PBS, for 30 min in a humid chamber, to suppress non-specific binding of antibody. Then, the tissue sections were washed with 1x PBS thrice and incubated with primary antibodies at 1:500 dilution in 1x PBS, overnight at 4°C in a humid chamber. Again rinsed with three times of 1x PBS. Then it was dried and incubated with secondary antibodies at 1:500 dilution in 1x PBS, for 90 min at room temperature in
humid chamber. Rinsed again with 1x PBS thrice. Then Counter stain with 4,6- diamidino-2-phenylindole (DAPI). Wash with PBS. Then the sections were mounted with glycerol (90 % in PBS) and placed upon with coverslip. Finally sections were observed under epifluorescence microscope (Nikon Corporation, Tokyo, Japan) with appropriate filters and photographed.

TRANSMISSION ELECTRON MICROSCOPIC STUDIES (TEM)

Ultra structural changes of the breast tissues were investigated by transmission electron microscopic studies as described by Robinson and Gray (1996).

Briefly, the glutaraldehyde fixed breast tissues were washed in buffer and post fixed in osmium tetraxide and subjected to dehydration in increasing order of alcohol. The tissues were then embedded in epoxy resin and ultra thin sections were cut using ultra microtome, these sections were then stained with uranyl acetate and lead citrate and examined under TEM.

Reagents

1. Sodium cacodylase buffer (pH 7.2): 10.6 g of sodium cacodylase was dissolved in 125 ml of distilled water and the pH was adjusted to 7.2 using HCl.

2. Glutaraldehyde (2.5%): 2.50 g of sodium cacodylase was dissolved in 44 ml of distilled water, to which 6 ml of glutaraldehyde was added and the pH was adjusted to 7.2 using HCl.

3. Osmium tetraoxide (OsO₄): 50 mg of Osmium tetraoxide was dissolved in 50 ml of sodium cacodylase buffer.

4. EMBED 812 resin for infiltration: It was prepared by mixing 4 ml of resin, 3 ml of dodecenyl succinic anhydride (DDSA) and 1.5 ml of Nadic methyl anhydride (NMA).

5. EMBED 812 resin for embedding: It was prepared by mixing 4 ml of resin, 3 ml of DDSA, 1.5 ml of Nadic methyl
anhydride (NMA) and 6 drops of the catalyst 2,4,6-tri
demethylaminoethyl phenol.

6. **Toluidine blue O solution (TBO):** 1 g of sodium tetra borate
   was dissolved completely in 120 ml of distilled water to
   which 0.2 g of pyramin Y and 1 g of toluidine blue was added
   and filtered before use.

7. **Uranyl acetate:** 20 ml of filtrate 50% ethanol was added to
   saturated solution of uranyl acetate and centrifuged for 2 min.
   The excess uranyl acetate was allowed to settle and the clear
   supernatant was used for staining.

8. **Lead citrate:** 400 mg of Lead citrate was dissolved in 100 ml
   of 1 N NaOH and was filtered to remove debris. This filtrate
   solution was used for staining the sections.

9. **Sodium hydroxide (1N NaOH):** 4 g of sodium hydroxide
    pellets were dissolved in 100 ml of distilled water.

**Procedure**

Approximately 1mm³ of fresh breast tissue, was fixed immediately
in glutaraldehyde (primary fixatives) for 4 to 6 h at 4ºC. Then the tissue was
rinsed in buffer wash twice for 15 min each and post fixed in 1% buffered
osmium tetraoxide (secondary fixative) for 2 h at 4ºC. Subsequently, the
tissue was washed thoroughly in buffer (for washing) twice for 15 min each,
to remove excess osmium tetraoxide. Then, the tissue was dehydrated
gradually in increasing concentration of ethyl alcohol (30, 50, 70, 80, and 90)
each for 10 min and finally with 100% alcohol twice. The alcohol was cleared
using propylene oxide then infiltrated with propylene oxide Epon 812
EMBED resin mixture (electron microscopy sciences, USA) at increasing
concentration (25, 50, 75 and 100% resin) each for 2 h at room temperature
using slow speed rotary shaker.

The infiltrate tissue was then embedded in epoxy resin (Epon 812
resin mixture) for 48 h at 60ºC. The blocks thus obtained, were then trimmed
and sectioned using ultramicrotome (Leica ultracut or ultramicrotome) with a diamond knife. Initially, the semi-thin sections were stained with freshly filtered Toluidine blue O solution (TBO) and were screened under high microscopy (binocular microscopy) for observing any histopathological changes. After the areas were chosen from the semi thin sections and ultra thin sections were cut and on copper grids and were stained in uranyl acetate and lead citrate and screened in JEOL JEM 100 S transmission electron microscopy at 80 Kv. The images of ultra thin sections of TEM were photographed for subsequent evaluation.

ANTICANCER EFFECT OF D-PINITOL ON HUMAN MAMMARY CARCINOMA CELL LINES (MCF-7 & MDA-MB-231) - IN VITRO STUDY

Chemicals

Acrylamide, bis-acrylamide, ammonium per sulfate and TEMED were purchased from Bio Rad Chemicals, USA. Dulbecco’s Modified Eagles medium (DMEM) F-12 HAM nutrient medium, Sodium bicarbonate, trypsin-EDTA were purchased from Biochrome, Germany, antibiotic/antimycotic solution and Fetal Bovine Serum (FBS) were purchased from GIBCO, Invitrogen, USA. All other chemicals including solvents were of high purity and of analytical grade marketed by Glaxo Laboratories, Mumbai and Sisco Research Laboratories Pvt, Ltd, Mumbai, India. All antibodies used in this study were purchased from Cell Signaling Technologies, USA.

Cell culture reagents

1. *Dulbecco’s Modified Eagle’s Medium (DMEM)* F-12 HAM nutrient medium: 15.6 gm DMEM and 1.5 gm sodium bicarbonate were dissolved in 900 ml of sterile Milli Q water. To this solution, 10 ml of antibiotic-antimycotic solution were added, pH was adjusted to 7.4 using NaOH and final volume
was made up to one litre with Milli Q water. Then the medium was sterile filtered (using 0.22 micron), and stored at 4°C.

2. **Growth medium (DMEM F-12 HAM with 10% FBS):** 100 ml of growth medium was prepared by adding 10 ml FBS in 90 ml (DMEM) F-12 HAM and stored in a sterile container at 4°C.

3. **Phosphate Buffer Saline (PBS pH 7.4):** 0.63 gm of sodium phosphate monobasic (NaH$_2$PO$_4$) and 4.5 gm of sodium chloride (NaCl) were dissolved in 500 ml of sterile Milli Q water and pH was adjusted to 7.4 with NaOH, sterile filtered (0.22 micron) and stored in refrigerator.

4. **Hanks Balanced Salt Solution (HBSS):** 9.8 gm of HBSS and 350 mg of sodium bicarbonate were dissolved in 900 ml of sterile Milli Q water. To this solution, 5 ml of Antibiotic-antimycotic solution were added and final volume was made up to one litre with Milli Q water. Then the medium was sterile filtered (using 0.22 micron) and stored at 4°C.

**Cell culture**

Human MCF-7 and MDA-MB-231 cells were procured from the National Centre for Cell Sciences (NCCS), Pune. The cells were grown in T-75 culture flasks containing DMEM F-12 HAM supplemented with 10% FBS.

**Passaging the cells**

Upon reaching confluence, the medium from T-75 culture flask was aspirated, rinsed with 2 ml of HBSS and aspirated quickly, then 5ml of trypsin-EDTA solution was added and kept at room temperature (in the laminar hood) for 40 seconds. Then trypsin-EDTA solution was aspirated quickly and the flask was kept at 37°C in CO$_2$ incubator for 3 min and tapped gently over the flask for complete detachment of cells from the surface of the flask. The cells were then gently resuspended in fresh growth medium and
transferred to sterile T-75 flasks and the volume of the medium was made up to 10 ml with growth medium.

**CELL VIABILITY ASSAY**

3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) method was performed as described by Cardile et al., (2004).

The viability of the cells was assessed by (MTT) assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Cells were seeded in 96-well microplates (1 ×10^4 cells/well in 180µl medium) and routinely cultured in a humidified incubator at 37°C in 5% CO₂ for 24 h. D-Pinitol were added in serial concentrations such as 20, 40, 60, 80, 100 and 120µM and reincubated for 24h. Then the medium was discarded and 30µl of MTT dye solution (0.5mg/ml in PBS) was added to every well and re-incubated for 4 h. After removing un-transformed MTT reagent, 100 µl of DMSO was added to dissolve the formed formazan crystals. Amount of formazan was determined by measuring the absorbance at 540 nm using an ELISA plate reader.

**ASSAY OF LACTATE DEHYDROGENASE (LDH)**

The activity of Lactate dehydrogenase was assayed by the method of King (1965).

**Reagents**

1. Glycine buffer: 0.1 M, pH 7.4
2. Buffered substrate: 4 gm of lithium lactate was dissolved in 75 ml 0.1 N NaOH and was made upto 200 ml with glycine buffer.
3. NAD⁺ (5 mg/ml)
4. Dinitro phenyl hydrazine (DNPH) 0.02% in 1N HCl
5. NaOH: 0.4 N
6. Stock Standard Trisodium pyruvate: 11 mg of trisodium pyruvate was dissolved in 100 ml of buffered substrate.

7. NADH solution (1 μM/L): 8.5 mg of NADH was dissolved in 10 ml of buffered substrate.

**Procedure**

100 µl of sample from the growth medium of experimental cultures, 1.0 ml of buffered substrate, 0.1 ml of medium, 0.2 ml of distilled water and 0.2 ml of NAD⁺ were added and incubated at 37°C for 15 min. Then 1 ml of DNPH was added and again incubated for another 15 min at 37°C. Then 0.5 ml of NaOH was added and the color developed was read at 440 nm within 60 seconds. For the standard curve, the test tubes labeled S₁ to S₅ were taken with a graded volume of trisodium pyruvate from 0.1 ml to 0.6 ml with the concentration ranging from 11 μg to 66 μg and all the above reagents were added except for the test sample NAD⁺ and were incubated for 15 min and then 0.5ml of NaOH was added and the optical density was read at 440 nm. The activity of LDH was expressed as μ mole of pyruvate liberated/mg protein/min.

**ESTIMATION OF GLUTATHIONE (GSH)**

Reduced glutathione was determined by the method of Moron et al., (1979).

**Reagents**

1. 5,5’-dithio bis 2-nitrobenzoic acid (0.6 mM), in 0.2 M phosphate buffer, pH 8.0.
2. Phosphate buffer: 0.2 M, pH 8.0
3. 5% TCA solution
4. Standard GSH: 100 mg of reduced glutathione was dissolved in 100 ml of distilled water. The working standard contained 100 μg/ml.
Procedure

5% TCA was added to MCF-7 and MDA-MB-231 cell lines (1×10^6 cells). The precipitate was removed by centrifugation. To an aliquot of the supernatant 2 ml of DTNB was added to make a final volume to 3 ml. The absorbance was read at 412 nm against a blank containing TCA instead of sample. Aliquots of the standard solution were treated similarly. The amount of glutathione was expressed as n moles/1×10^6 cells.

LIGHT MICROSCOPIC OBSERVATION

Light microscopic examination of the cells was performed to observe the morphological changes after the treatment with D-Pinitol for 24 h. The MCF-7 and MDA-MB-231 cells were grown in 35 mm sterile petriplates and treated with D-Pinitol at the concentration of 40, 60µM (MCF-7 cells), 80, 100µM (MDA-MB-231 cells) and control. Then cells morphological changes were observed under inverted microscope (Nikon, Japan).

FLUORESCENT MICROSCOPIC STUDIES

Nuclear staining with DAPI

The MCF-7 and MDA-MB-231 cells were grown in 35 mm sterile petriplates and treated with D-Pinitol at the concentration of 40, 60µM (MCF-7 cells), 80, 100µM (MDA-MB-231 cells) and control. Cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with 4, 6-diamidino-2-phenylindole (DAPI) solution for 10 min at room temperature. The cells were washed two more times with PBS and analyzed via a fluorescent microscope excitation at 346 nm and emission at 454 nm.
Mitochondrial membrane potential

MCF-7 and MDA MB-231 cells (1000–2000 cells/sample) were grown on 12 mm × 12 mm cover slips placed in each well of a six well plate and D-Pinitol was exposed in subconfluent stage for 24 hours. After 24 hours the coverslip containing cells were washed with PBS and fixed with methanol for 10 minutes at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes at room temperature and incubated with 5 µg/ml of rhodamine-123 for 30 minutes at 37°C. This dye is taken up by the mitochondria forms aggregates and exhibits intense red fluorescence. However, once mitochondrial membrane potential (Δψm) is lost, rhodamine cannot accumulate in the mitochondria and stays in the cytosol. The stained cells were observed under fluorescent microscope (Nikon Eclipse-80i, Japan) with an excitation at 488 nm and emission at 525 nm wavelengths.

DNA FRAGMENTATION

DNA fragmentation was performed followed by the method of Chen et al., (1997).

Reagents

1. Lysis buffer consisted of 10 mM Tris-HCl, 10 mM EDTA, and 0.5% SDS and the pH was adjusted to 7.5.
2. Tris EDTA (TE) buffer: 10 mM of Tris was mixed with 1 mM of EDTA and the pH was adjusted to 8.0
3. 3 M Sodium acetate, pH 5.2

The MCF-7 and MDA-MB-231 cells were plated in 60 mm culture dish at a density of 5×10^5 cells and treated with D-Pinitol at the concentration of 40,60µM (MCF-7 cells), 80,100µM (MDA-MB-231 cells) for 24 h. The cells attached at the bottom were scrapped off and collected together with unattached cells by centrifugation at 1500 rpm for 5 min at 4°C. The DNA was prepared from the pelleted cells. The cells were lysed with lysis buffer
and extracted with 2ml of phenol (neutralized with TE buffer, pH 7.5) followed by extraction with 1 ml of chloroform/isoamyl alcohol (24:1). The aqueous supernatant was precipitated with 2.5 volumes of ice-cold ethanol with 10% volume of sodium acetate at -20ºC for overnight. After centrifugation at 13,000 rpm for 10 min the pellets were air dried and resuspended with 50µl of TE buffer. DNA was separated by using 1.8% agarose gel containing 0.5 µg/ml of ethidium bromide and electrophoresed. After electrophoresis, the gel was photographed under UV light.

WESTERN BLOTTING ANALYSIS

MCF-7 and MDA-MB-231 cells were plated in 100 mm culture petriplates at a concentration of 1×10^6 cells/plate and grown in a growth medium. After 24 h, the cells (70-80% confluent) were washed twice with serum free DMEM/F-12 HAM and cells were allowed to undergo starvation by incubating in 5 ml of serum-free fresh medium for overnight. After overnight starvation, MCF-7 and MDA-MB-231 were treated with D-Pinitol for 24 h.

At the end of treatment, cells were washed twice with ice-cold PBS and lysed with 200 µl of ice-cold lysis buffer with protease inhibitor. Samples were collected into an ice-cold 1.5 ml tube and centrifuged at 12,000 rpm for 10 min at 4ºC. The supernatants were collected in new cold tubes and protein concentrations of supernatants were determined.

**Quantification of protein**

The protein concentration in MCF-7 and MDA-MB-231 cell lines were estimated by BioRad protein assay kit (Catalog # 223-9950).

**Reagents**

1. Protein Assay Dye Reagent
   
   Dye reagent was prepared by diluting 1 part of Dye Reagent Concentrate with 4 parts of Milli Q water.

2. Protein standard-BSA
1 mg of Bovine Serum Albumin (BSA) was weighed and dissolved in 1ml of Milli Q water. This can be stored at -20°C for two weeks until needed.

**Preparation of diluent**

The diluent was prepared by adding 1 ml of Radio Immuno Precipitation Buffer (RIPA) to 4 ml of Milli Q water.

**Procedure**

The protein standards were prepared ranging from 50-500 μg/ml using a BSA standard. The samples were diluted by adding 45 μl of diluent to 5 μl of sample. 5 μl of the protein standards were added to separate wells in the 96 well plates. To the blank wells, 5 μl of buffer was added. To each well being used, 100 μl of the BioRad diluted reagent was added and mixed on a shaker for approximately 30 seconds. The samples were incubated at room temperature for 5 min and the absorbance was measured at 595 nm. The net absorbance versus the protein concentration of standard was plotted. The protein concentration of the unknown sample(s) was then determined by comparing the net A<sub>595</sub> values against the standard curve.

**Western blot analysis of p53, Bcl-2, Bax and NF-κB**

p53, Bcl-2, Bax and NF-κB expressions in MCF-7 and MDA-MB-231 cell was assessed by Western blotting method (Fido et al., 1995).

**Reagents**

1. **Radio Immuno Precipitation Buffer (RIPA):** (150mM NaCl, 50 mM Tris, 1mM EDTA, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, 1mM β glycerophosphate, 1mM Sodium orthovanadate and 1mM Sodium fluoride, pH 7.4).

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>302 mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>438 mg</td>
</tr>
<tr>
<td>EDTA</td>
<td>186 mg</td>
</tr>
<tr>
<td>Na-deoxycholate</td>
<td>25 mg</td>
</tr>
</tbody>
</table>
SDS 50 mg
NP-40 500 ml
β glycerophosphate 10.8 mg
Sodium orthovanadate 9.2 mg
Sodium fluoride 2.0 mg

All these chemicals were dissolved in 40 ml of Milli Q water, pH was adjusted to 7.4 with 1N HCl and then the volume was made up to 50 ml.

2. **Protease inhibitor (PI):** Protease inhibitor cocktail was purchased from Pierce Biotechnology Inc, USA. PI was used 1:100 as per the manufacturer’s instruction (For 1ml, 10μl PI).

3. **Running gel buffer (1.5 M Tris, pH 8.8):** 45.375 gm of Tris was dissolved in 200 ml of Milli Q water and pH was adjusted with HCl and the volume was made up to 250 ml.

4. **Stacking gel buffer (0.5 M Tris, pH 6.8):** 15 gm of Tris was dissolved in 200 ml of Milli Q water and pH was adjusted with HCl and the volume was made up to 250 ml.

5. **SDS 10%:** 10 gm of SDS was dissolved in 100 ml of Milli Q water and stored at room temperature.

6. **Acrylamide 30%:** 29 gm of Acrylamide (29%) and 1 gm of N, N’ Bis-acrylamide (1%) were dissolved in 100 ml of Milli Q water.

7. **Ammonium Persulfate 10%:** 100 mg of Ammonium persulfate was dissolved in 1ml of Milli Q water.

8. **10X SDS Electrophoresis buffer (2.5 M Tris, 1.92 M Glycine and 1 % SDS):** 30 gm of Tris, 144 gm of Glycine and 10 gm of SDS were dissolved in 800 ml of Milli Q water, volume was made up to one litre.
9. **1X SDS Electrophoresis buffer**: 50 ml 10X SDS electrophoresis buffer was made up to 500 ml with Milli Q water.

10. **10X Transfer buffer (250 mM Tris and 1.92 M Glycine)**: 30.3 gm of Tris and 144 gm of Glycine were dissolved in 800 ml of Milli Q water and the volume was made up to one litre.

11. **1X Transfer buffer with 20% methanol**: 100 ml of 10X transfer buffer was mixed with 700 ml of Milli Q water and 200 ml of methanol were mixed and kept cold until used.

12. **2X Sample buffer with reducing agent (125 mM Tris-HCl (pH 6.8), 4 % SDS, 20% glycerol, 10 % 2-mercaptoethanol and 0.004 % bromophenol blue)**: 2.5 ml of 0.5 M Tris-HCl (pH 6.8), 4 ml of 10% SDS, 2 ml of glycerol, 1 ml of 2-Mercaptoethanol and 0.4 mg bromophenol blue were mixed well and the volume was made up to 10 ml with Milli Q water and stored at -20°C.

13. **10X Phosphate buffered Saline (0.1 M Sodium phosphate)**: 13 gm of NaH₂PO₄ was dissolved in Milli Q water, the pH was adjusted to 7.2 with NaOH, the volume was made up to one liter and stored at room temperature.

14. **Blocking Buffer 5% (PBS with 10 % glycerol, 5 % milk powder and 0.2 % Tween-20)**: 50 ml of 10X PBS, 50 ml of Glycerol, 4.5 gm of NaCl, 25 gm of non fat dry milk powder, 1 ml of Tween-20 were dissolved in 400 ml of Milli Q water. All these were taken in a beaker and stirred well until dissolved. The volume was made up to 500 ml with milli Q water.

15. **Tris Buffered Saline (TBS)**: (20 mM, Tris 500 mM NaCl, pH 7.5): 1.21 gm of Tris and 14.62 gm of NaCl were dissolved in
400 ml with Milli Q water and pH was adjusted to 7.5 with HCl and made up to 500 ml.

16. T-TBS (0.2 % Tween-20 in TBS): 0.2 ml of Tween-20 was dissolved in 100 ml of TBS and stored in refrigerator.

17. Prestained SDS-PAGE standard: Protein marker purchased from Bio Rad, Laboratories, USA.

**Preparation of gel**

**Running gel (10 %):** The following volumes of solutions were taken for one slab gel.

- Acrylamide (30 %): 3.3 ml
- Running gel buffer: 2.5 ml
- D.D H₂O: 4.0 ml
- SDS (10%): 0.1 ml
- APS (10%): 0.1 ml
- TEMED: 0.007 ml

**Stacking gel (5%):** The following volumes of solutions were taken for one slab gel.

- Acrylamide (30 %): 0.5 ml
- Stacking gel buffer: 0.38 ml
- D.D H₂O: 2.1 ml
- SDS (10%): 0.03 ml
- APS (10%): 0.03 ml
- TEMED: 0.003 ml

**Procedure**

Equal amount of total protein was mixed with 2X sample buffer and boiled for 5 min. The protein was separated on 10% SDS-PAGE and electrotransfered in to a Polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA). The blots were blocked with 5% blocking buffer for 4 h. After blocking, membrane was incubated with respective rabbit monoclonal antibodies p53, Bcl-2, Bax and NF-κB in 1:1000 dilution overnight at 4°C.
Then the membranes were washed thrice with T-TBS for 10 minutes each, followed by horseradish peroxidase conjugated secondary antibody (1:10,000 dilutions) incubation for 45 min at room temperature. Finally, signals were visualized using Enhanced Chemiluminescent System (Pierce Biotechnology Inc, USA) and the signals were captured by Chemi Doc XRS system (Bio Rad, USA) and the intensity of the bands were quantified by Quantity One software (Bio Rad, USA). Immunoblot for β actin was used as an internal control for equal loading in the gel.

**DNA fragmentation**

DNA extraction and agarose gel electrophoresis were performed by the method of Chaudhary et al., (2001).

**Reagents**

1. *Lysis buffer (TE buffer) (pH 8):* 12.1 gm of Tris and 7.4 gm of EDTA were dissolved in 1 litre of double distilled water. The pH was adjusted to 8.0 and to this, 8 gm of sodium dodecyl sulfate (SDS) was added.

2. *Phosphate Buffered Saline (PBS) (pH 7.4):* 0.63 gm of disodium hydrogen phosphate (Na₂HPO₄), 17 gm of sodium dihydrogen phosphate (NaH₂PO₄) and 4.5 gm of sodium chloride (NaCl) were dissolved in 500 ml of sterile distilled water. The pH was then adjusted to 7.4, filtered and then stored in a sterile container.

3. *Proteinase K:* 50 µg of proteinase K was dissolved in 1 ml of double distilled water and stored at 4°C.


5. *Absolute alcohol:* The absolute alcohol used was of AR grade.

6. *Trisodium acetate (3 M):* 2.46 gm of trisodium acetate was dissolved in 10 ml of double distilled water.
6. *Ethanol (70%)*: 70 ml of ethanol was made up to 100 ml with double distilled water.

7. *TBE buffer*: 10.8 gm of Tris, 5.5 gm of boric acid and 0.93 gm of EDTA were dissolved in 100 ml of sterile double distilled water. The pH was then adjusted to 8.3.

8. *Agarose (1.2%)*: 1.2 gm of agarose was dissolved in 100 ml of double distilled water.

9. *Ethidium bromide*: 5 mg of ethidium bromide was dissolved in 1 ml of double distilled water.

**Procedure**

1x10^6 cells were plated 100 mm petriplates with DMEM containing 10% FBS. The cells were incubated for 24 hours under 5% CO₂ and 95% air at 37°C. Then, medium was removed and the control plates received 0.01% DMSO containing medium and treatment plates received 40,60µM (MCF-7) and 80,100µM(MDA-MB-231) of D-Pinitol containing medium.

After 24 h of treatment, the DNA was extracted from the cell lysate as follows. The cells were washed with PBS and centrifuged at 1500 rpm for 5 min to collect the cell pellet, which was resuspended in 0.5 ml of lysis buffer, transferred to an eppendorf tube and incubated for 1 h at 37°C. To this, 4 µl of proteinase K was added and the tubes were incubated at 50°C for 3 h. To each tube, 0.5 ml of phenol:chloroform:isoamyl alcohol (25:24:1) were added, mixed and centrifuged at 13,000 rpm for 1 min to separate the DNA containing upper aqueous phase. Phenol-chloroform extraction was repeated twice, followed by chloroform extraction alone. To the resulting aqueous phase, 2 volume of ice-cold absolute ethanol and 1/10th volume of 3 M sodium acetate were added and incubated for 30 min on ice to precipitate DNA. DNA was pelleted by centrifuging at 15,000 rpm for 20 min at 4°C, the supernatant was aspirated and the pellet was washed with 1 ml of 70% ethanol.
After repeating the above centrifugation step and removing the last traces of the supernatant fraction, the pellet was allowed to dry at room temperature for approximately 30 min and resuspended in 50 µl of TE buffer. The DNA was quantified by UV-visible spectroscopy and 10 µg of DNA was electrophoresed in a 1.2% agarose gel containing ethidium bromide in a gel tank containing TBE buffer for 2 h under 90 V. Then the gel was examined under UV light and photographed.

STATISTICAL ANALYSIS OF DATA

The results were expressed using One-way Analysis of Variance (ANOVA) and Turkey’s Multiple Comparison Test was done to evaluate the significance of difference of means of various treatment groups, using SPSS statistical package (version: 10). The values are presented as Mean ± S.D and p - value less than 0.05 were taken as statistically significant.