CHAPTER III
MATERIALS AND METHODS

3.1. Research design and methodology

Healthy male albino rats weighing approximately 130 to 150 grams were used for this study. They were obtained from Small Animal Breeding Centre, Kerala Agricultural University, Mannuthy, Thrissur. The rats were grouped and housed in polypropylene cages and maintained under standard laboratory conditions (temperature 25±2°C) with dark / light cycle (14/10 hours). They were allowed free access to standard dry pellet diet and water ad libitum. All the animal experimentations were premeditated and executed in compliance with the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (743/03/abc/ CPCSEA dated 3.3.03/ Ph.D 02/ 2010- 2011).

3.2. Dosage fixation for luteolin

Luteolin concentrations viz., 0.2, 0.4, 0.6, 0.8 and 1.0 mg/kg body weight were used to determine its minimal effective optimum dosage. It was observed that luteolin treatment administered orally at a dose of 0.2 mg/kg body weight significantly (p<0.05) altered the activities of pathophysiological marker enzymes such as alkaline phosphatise (ALP), aspartate transaminase (AST) and alanine transaminase (ALT) and γ-glutamyl transferase in the liver to near normal values in DEN-induced rats. Hence, the minimal dose of 0.2 mg/kg b.wt. was fixed as the optimum dosage for subsequent studies.

3.3. Induction of hepatocellular carcinoma

Hepatocellular carcinoma (HCC) was induced intraperitonially in the Wistar albino rats with DEN in saline at a dose of 100mg/kg body weight, once a week for a period of three weeks (Rahman et al., 2003).
3.4. Experimental design

The experimental animals were divided into four groups, each group comprising six animals.

Group I : Control rats (Untreated) were fed with standard diet and water *ab libitum*

Group II : Rats were administered with luteolin alone (0.2 mg/kg b.w.t.)

Group III : Rats were induced intraperitoneally with DEN (100 mg/kg b.w.t.) once in a week for a period of six weeks

Group IV : Luteolin (0.2 mg/kg b.w.t.) was administered intraperitoneally to DEN induced group of rats after the proliferation of tumor from 6th week onwards up to 16 weeks daily.

The experiment was terminated after 16 weeks at the end of the acclimatization period and the rats were anaesthetized with sodium-pentothal after overnight fasting and euthanized by cervical dislocation. Blood and liver tissues were collected for further analysis.

3.5. Preparation of tissue homogenate

The liver tissues were excised and rinsed in ice-cold saline. Known amount of the tissue were homogenized in 0.1 M Tris–HCl buffer, pH 7.4 at 4°C, in a Potter–Elvehjem homogenizer with a Teflon pestle at 600 rpm for 3 minutes. The homogenate was centrifuged at 3000 × g for 10 minutes at 4°C using Sorvall 5 B refrigerated centrifuge. The supernatant was collected as tissue homogenate, which was used to assay various parameters.

3.6. Estimation of proteins

The protein content was estimated according to the method of Lowry *et al.* (1951). To 0.1 ml of homogenate/serum, 0.9 ml of water and 4.5 ml of alkaline copper reagent were added and kept at room temperature for 10 min. To this, 0.5 ml of Folin’s reagent (1:2) was added and the blue colour developed was read after 20 min at 640 nm. Protein content was expressed as g/dl of plasma.
3.7. Basic biochemical parameters

3.7.1. Estimation of Blood Glucose

Blood glucose level was estimated by the method of Sasaki et al., (1972). To 0.1 ml of blood, 1.9 ml of 10% TCA solution was added to precipitate proteins and then centrifuged. One ml of the supernatant was mixed with 4.0 ml of O-toluidine reagent and was kept in a boiling water bath for 15 minutes. The green color developed was read at 600 nm in a Shimadzu spectrophotometer. A series of standard glucose solutions (1 mg/ml) were also treated similarly. The values were expressed as mg of glucose/dl of whole blood.

3.7.2. Estimation of Urea

Urea was determined by the method of Natelson et al. (1951). To 0.1 ml of blood, 3.3 ml of water, 0.3 ml each of 10% sodium tungstate and 0.67 N sulphuric acid were added. The suspensions were centrifuged and to 1.0 ml of the supernatant, added 1.0 ml of water, 0.4 ml of diacetylmonoxime and 2.6 ml of 0.67 N sulphuric acid-phosphoric acid reagent. Standard urea (20 to 50 µg/ml) were also treated in a similar manner and all the tubes were heated in a boiling water bath for 30 minutes, cooled and the color developed was measured at 480 nm in a Shimadzu spectrophotometer. The values were expressed as mg of urea/dl of blood.

3.7.3. Estimation of total and direct bilirubin (Autozyme Kit)

To 50 µl of serum/tissues samples, 1000 µl of total bilirubin reagent and direct bilirubin reagent and 20 µl of respective activator reagent was added. The reaction mixture were mixed well and incubated for 10 minutes at 37°C. At the same time, blank and standard solution was prepared. The absorbance of sample against reagent blank was read at 546 nm. The activity was calculated by using the formula:

\[
\text{Total bilirubin} = \frac{\text{O.D. of sample} - \text{O.D. of blank}}{\text{O.D. of standard}} \times 10
\]

\[
\text{Direct bilirubin} = \frac{\text{O.D. of sample} - \text{O.D. of blank}}{\text{O.D. of standard}} \times 7.7
\]
3.7.4. Estimation of total Albumin (Autozyme Kit)

To 0.01 ml of serum/tissue extract, 1.0ml of working solution was added and incubated the assay mixture for 1 minute at 37° C. After completion of incubation period, the absorbance was measured at 600 nm. The activity was calculated by using the formula

Total albumin in g % = Absorbance of sample/Absorbance of standard x 5.

3.8.9. Lipid profile studies

3.8.9.1. Extraction of Lipids

The lipids were extracted from liver by the method of Folch et al. (1957). The liver tissues were dried, weighed and a known weight was homogenized with 10 ml of chloroform-methanol mixture. The homogenate was filtered through Whatmann filter paper No.42 into a separating funnel. The filtrate was mixed with 0.2 ml of physiological saline and the mixture was kept overnight undisturbed. The lower phase containing the lipid was drained off into pre-weighed beakers. The upper phase was re-extracted with excess of chloroform-methanol mixture and the extracts were pooled and evaporated under vacuum at room temperature. The lipid extract was re-dissolved in 3.0 ml of chloroform-methanol (2:1) mixture and aliquots were taken for the estimation of cholesterol and phospholipids. The total lipid contents were calculated and expressed as mg/g of fresh tissue.

3.8.9.2. Estimation of Total Cholesterol

Cholesterol content was estimated by the method of Parekh and Jung (1970). About 0.1 ml of test sample was made up to 10 ml with ferric acetate-uranyl acetate reagent. 0.1 ml of the aliquot of the total lipid extract was taken and it was evaporated to dryness. The dried extract and standards were made up to 3.0 ml with ferric chloride-uranyl acetate reagent. Then 2.0 ml of sulphuric acid-ferrous sulphate reagent was added to all the tubes and the contents were mixed well. After 20 minutes, the color developed was read at 540 nm using a Shimadzu UV spectrophotometer. Total cholesterol level was expressed as mg/dl for plasma and tissue cholesterol as mg/g of fresh tissue.
3.8.9.3. Estimation of Triacylglycerides (TG)

Triacylglycerol was estimated by the method of Rice (1970). Lipids were extracted with chloroform: methanol mixture (2:1 v/v). Phospholipids present in the lipid extract were adsorbed onto silicic acid and the triacylglycerol remaining in solution was saponified with alcoholic potassium hydroxide (400 mg of potassium hydroxide was dissolved in 100 ml of 95% ethanol). The liberated glycerols were oxidized by periodate to formaldehyde and the excess oxidizing power was destroyed by reaction with 0.5M sodium arsenite. The formaldehyde formed was determined by the chromotropic colour reaction. 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 one hour and then centrifuged. The supernatant (saline-methanol phase) was discarded. The washed chloroform phase was filtered into a dry tube. 200 mg of activated silicic acid was added to chloroform phase, shaken vigorously and allowed to stand for 30 minutes. After centrifugation, 0.5 ml of the supernatant as well as tripalmitin standards was evaporated to dryness. Then to the test, standard and blank tubes, 0.5 ml of alcoholic potassium hydroxide solution were added and the mixture was saponified in a 60°C to 70°C water bath for 20 minutes. 0.5 ml of 0.2 N sulphuric acid was added and heated in a boiling water bath for 10 minutes. After cooling the tubes, 0.1 ml of sodium metaperiodate was added and allowed to stand for 10 minutes. The excess periodate was reduced by the addition of 0.1 ml of sodium arsenite. Then 5.0 ml of chromotropic acid reagent was added, mixed thoroughly and kept in a boiling water bath for 30 minutes. After cooling, 0.5 ml of thiourea solution was added. The colour developed was read at 540 nm against a blank in a Shimadzu spectrophotometer. Triacylglycerol content was expressed as mg/dl in plasma and mg/g in fresh tissue.

3.8.9.4. Cholesterol in the lipoprotein fractions
3.8.9.4.1. High Density Lipoprotein-Cholesterol (HDL-C)

HDL-Cholesterol fraction was separated by the precipitation techniques of Burstein et al. (1970) and the cholesterol content was determined by method of Parekh and Jung (1970). To 1.0 ml of serum, 0.18 ml of heparin-manganese chloride reagent was added and mixed. The solution was allowed to stand at 4°C for 30 minutes and then centrifuged in a
refrigerated centrifuge at 1800 × g for 30 minutes. The supernatant represented the HDL-C fraction. An aliquot of supernatant was used for cholesterol estimation. The values were expressed as mg/dl.

3.8.9.4.2. Very Low Density Lipoprotein-Cholesterol (VLDL-C)

VLDL-cholesterol was calculated using the following equation (Friedewald et al., 1972).

\[
\text{VLDL-C} = \text{Triglycerides}/5
\]

The values were expressed as mg/dl.

3.8.9.4.3. Low Density Lipoprotein-Cholesterol (LDL-C)

LDL-C was calculated using the following equation:

\[
\text{LDL-C} = \text{Total cholesterol} - (\text{HDL-C} + \text{VLDL-C})
\]

The values were expressed as mg/dl.

3.7. Enzyme linked immunosorbent assay (ELISA) of AFP and CEA

Quantitative estimation of tumor markers viz., alpha-feto protein (AFP) and carcinoembryonic antigen (CEA) was carried out by solid phase enzyme linked immunosorbent assay (ELISA) using the UBI MAGIWELL (USA) enzyme immunoassay kit (Sell and Becker 1978). The desired number of coated wells were secured in the holder. 10 uL of standards, controls or serum samples were then dispensed into appropriate wells. One well was saved for the blank, to which standards or enzyme conjugate should not be added. 50 uL of antibody solution was dispensed into each well except the blank well. 50 uL of enzyme conjugate (conjugated to horse radish Peroxidase) was dispensed in to each well except the blank well. The wells were then incubated for one hour at room temperature. After incubation mixture the wells were rinsed with working washing buffer (50 ml, 20X) five times. Then, 100 uL of Solution A (phosphate buffer solution containing hydrogen peroxide) and 100 uL Solution B (Tetramethylbenzidine solution) was added into each well including the blank well. This was then incubated for 30 minutes at room temperature, after which, the enzyme reaction was stopped by the addition of 50 uL of stop reagent and the intensity of the color measured with microreader at 450 nm.
3.8.4. Assay of Aspartate Aminotransferase (AST)

The enzyme activity was assayed by the method of King (1965a). To 1.0 ml of the buffered substrate, 0.1 ml of enzyme source was added and incubated for one hour at 37°C. At the end of the incubation period, 0.07 ml of aniline-citrate reagent was added and incubated for another 20 minutes. Then, 1.0 ml of the dinitrophenyl hydrazine reagent was added and left for 20 minutes. At the end of 20 minutes, 10 ml of 0.4N sodium hydroxide was added and the color developed was read at 540 nm in a Shimadzu spectrophotometer after 10 minutes. The standards were also treated similarly. The enzyme activity in serum was expressed as µmoles of pyruvate/hour/mg of protein and in tissues as moles of pyruvate/minute/mg of protein.

3.8.5. Assay of Alanine Aminotransferase (ALT)

The enzyme activity was assayed by the method of King (1965a). To 1.0 ml of the buffered substrate, 0.1 ml of enzyme source was added and incubated at 37°C for 30 minutes. The reaction was arrested by the addition of 1.0 ml of dinitrophenyl hydrazine and left aside for 20 minutes at room temperature. The colour developed by the addition of 10 ml of 0.4N sodium hydroxide was read at 540 nm in a Shimadzu spectrophotometer against the reagent blank. The enzyme activity in serum was expressed as µmoles of pyruvate/hour/mg of protein and in tissues as moles of pyruvate/minute/mg of protein.

3.8.6. Assay of Alkaline Phosphatase (ALP)

Alkaline phosphatase was assayed by the method as described by King (1965b). The incubation mixture of 3.0 ml contained 1.5 ml of buffer, 1.0 ml of substrate and 0.5 ml of the enzyme source. The tubes were incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1.0 ml of Folin's phenol reagent. To the control tubes, the enzymes were added after arresting the reaction. The contents were centrifuged and to 1.0 ml of the supernatant, 1.0 ml of 15 % sodium carbonate, 1.0 ml of substrate and 0.1 ml of MgCl₂ were added and incubated for 10 minutes at 37°C. The colour developed was read at 640 nm in a Shimadzu spectrophotometer against a blank. The standard solutions of phenol of varying concentrations were also treated similarly. The enzyme activity in serum and tissues were expressed as µmoles of phenol liberated/minute/mg of protein.
3.8.7. Assay of Lactate Dehydrogenase (LDH)

Lactate dehydrogenase (LDH) was assayed according to the method of King (1965c). To 1.0 ml of the buffered substrate, 0.1 ml of enzyme preparation was added and the tubes were incubated at 37°C for 15 minutes. After adding 0.2 ml of NAD+ solution, the incubation was continued for another 15 minutes. The reaction was arrested by addition of 1.0 ml of DNPH reagent and then the tubes were incubated for a further period of 15 minutes at 37°C. After the incubation period, 7.0 ml of 0.4N sodium hydroxide solution was added and the colour developed was measured at 420 nm in a Shimadzu spectrophotometer. Suitable aliquots of the standards were also analyzed by the same procedure. The activity of the enzyme was expressed as µmoles of pyruvate formed/hour/mg of protein for tissues under incubation conditions.

3.8.8. Assay of γ-Glutamyl transferase (γ-GT)

The activity of γ-glutamyl transferase (γ-GT) was estimated in serum and tissues according to the method of Rosalki and Rau (1972) with minor modifications. One ml of 0.1M Tris-HCl, 2.2 ml of glycyl glycine, 0.2 ml of homogenate was added to the incubation mixture containing 0.5 ml of the substrate and the total volume was made up to 4.0 ml with water and incubated for 30 minutes at 37°C. Then the samples were heated at 100°C for 5 minutes and centrifuged at 5000 rpm. The amount of p-nitroaniline in the supernatant was measured at 410 nm. The activity of γ-glutamyl transferase was expressed as nmoles of p-nitroaniline formed/minute/mg protein.

3.12. Glycoprotein components
3.12.1. Separation of Tissue and Plasma Glycoprotein Components

The plasma glycoproteins were precipitated with alcohol. To 0.1 ml of plasma, added 2.0 ml of alcohol and centrifuged. The supernatant was decanted. The precipitate and defatted tissues were hydrolysed with acid to liberate protein bound hexose, sialic acid and fucose.

3.12.2. Hydrolysis of Samples for Glycoprotein Estimation

The precipitate was washed thrice with 5% TCA according to the method of Glossman and Naville (1971). Both the alcoholic precipitate and a known amount of defatted tissue were hydrolysed with 1.0 ml of 2N HCl and 1% phosphotungstic acid at 100°C for four hours. The
hydrolysed material was neutralized with sodium hydroxide. Aliquots of these neutralized samples were used for the analysis of hexose and fucose. For sialic acid estimation, the samples were hydrolysed with 0.1 N H₂SO₄ for 60 to 90 minutes at 90°C. Thus hydrolysed sample was used for the analysis of sialic acid.

3.12.3. Estimation of Hexose

Hexose was estimated by the method of Niebes (1972). 0.5 ml of the neutralized sample was made up to 1.0 ml with water. 2.0 ml of orcinol-sulphuric acid reagent was added very slowly to the tubes in ice-cold condition. Then, the tubes were heated at 80°C for 15 minutes, cooled and the color intensity was read at 540 nm after 20 minutes. Standard hexose in the range of 20 to 100 μg/ml and blank were treated in the same manner. The values were expressed as mg/g of defatted tissues and mg/dl in plasma.

3.12.4. Estimation of Fucose

Fucose was estimated by the method of Dische and Shettle (1948). Two tubes each containing 0.1 ml of sample was taken. 5.0 ml of 95% ethanol was added, mixed well and then centrifuged. The precipitate was dissolved in 1.0 ml of 0.1 N NaOH. 1.0 ml of distilled water served as the blank. A series of standards in 1.0 ml volume were also set up along with the test. All the tubes were kept in ice-cold condition and 4.5 ml of H₂SO₄-water mixture was added. The tubes were kept in a boiling water bath for 3 minutes and cooled. 0.1 ml of cysteine reagent was added to all the tubes except control and kept for one hour at room temperature. The color developed was read at 396 nm and 430 nm against the blank. The fucose content was calculated from the differences in the readings obtained at 396 nm and 430 nm and then subtracting the values obtained without cysteine. The values are expressed as mg/dl for plasma and mg/g for tissues.

3.12.5. Estimation of Sialic Acid

Sialic acid was estimated by the method of Warren (1959). To 0.2 ml of the hydrolysed sample, 0.2 ml sodium metaperiodate reagent was added and kept at 37°C for 20 minutes. Then 0.2 ml of sodium arsenite was added and shaken well. After adding 3.0 ml of thiobarbituric acid, the tubes were heated in a boiling water bath for 15 minutes. The tubes were cooled and 5.0 ml of
acidified butanol was added. The absorbance of the pink butanolic phase, after centrifugation was read at 540 nm. Standards and blank were processed in the same manner. The values were expressed as mg/g of defatted tissues and mg/dl in plasma.

3.9. Assay of antioxidant enzymes

3.9.1. Assay of Superoxide Dismutase

Superoxide dismutase was assayed according to the method of Misra and Fridovich (1972). 0.1 ml of tissue homogenate was added to the tubes containing 0.75 ml of ethanol and 0.15 ml of chloroform under chilled condition and centrifuged. To 0.5 ml of supernatant, 0.5 ml of 0.6 mM EDTA solution and 1.0 ml of 0.1 M carbonate-bicarbonate buffer (pH 10.2) were added. The reaction was initiated by the addition of 0.5 ml of 1.8 mM epinephrine and the increase in absorbance at 30 second interval for 3 minutes was measured at 480 nm in a Shimadzu UV spectrophotometer. One unit of superoxide dismutase activity is the amount of protein required for 50% of inhibition of epinephrine autoxidation/minute.

3.9.2. Assay of Catalase

Catalase was assayed according to the method of Takahara et al. (1960). To 1.2 ml of 0.01 mM phosphate buffer (pH 7.0), 0.5 ml of tissue homogenate was added. The enzyme reaction was started by the addition of 1.0 ml of 0.2 mM hydrogen peroxide solution. The decrease in absorbance was measured at 240 nm for every 30 seconds up to 3 minutes. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide. The enzyme activity was expressed as μmoles of hydrogen peroxide decomposed/minute/mg of protein.

3.9.3. Assay of Glutathione Peroxidase (GPx)

The activity of glutathione peroxidase was assayed by the method of Rotruck et al. (1973). The reaction mixture consisting of 0.2 ml of 0.8 mM EDTA, 0.1 ml of 10 mM sodium azide, 0.1 ml of 2.5mM H₂O₂, 0.2 ml of GSH, 0.4 ml of 0.4 mM phosphate buffer (pH 7.0) and 0.2 ml of homogenate was incubated at 37°C for 10 minutes. The reaction was arrested by the addition of 0.5 ml of 10% TCA and the tubes were centrifuged at 2000 rpm. To the supernatant, 3.0 ml of 0.3M disodium hydrogen phosphate and 1.0 ml of DTNB were added and the color developed was
read at 420 nm immediately. The activity of GPx was expressed as \( \mu \)moles of glutathione oxidized/minute/mg of protein.

### 3.9.4. Assay of Glutathione-S-transferase (GST)

Glutathione-S-transferase was assayed by the method of Habig et al. (1974). The reaction mixture containing 1.0 ml of 0.3 mM phosphate buffer (pH 6.5), 0.1 ml of 30mM CDNB and 0.1 ml of tissue homogenate was made up to 2.5 ml with water. The reaction mixture was pre-incubated at 37\(^\circ\)C for 5 minutes. 0.1 ml of 30 mM GSH was added and the change in O.D. was measured at 340 nm for 3 minutes at 30 seconds interval. Activity of glutathione S-transferase was expressed as nmoles of CDNB conjugate formed/minute/mg of protein.

### 3.10. Estimation of non-enzymatic antioxidants

#### 3.10.1. Ascorbic acid

The ascorbic acid content was determined by the method of Omaye et al. (1979). To 0.5 ml of test sample, 0.5 ml of water and 1.0 ml of 5% TCA were added, mixed thoroughly and centrifuged. To 1.0 ml of the supernatant, 0.2 ml of DTC reagent was added and incubated at 37\(^\circ\)C for 3 hours. Then 1.5 ml of 65% sulphuric acid was added, mixed well and the solution was allowed to stand at room temperature for another 30 minutes. The colour developed was read at 520 nm. The level of ascorbic acid in plasma was expressed as mg/dl and in tissues as \( \mu \)g/mg of protein.

#### 3.10.2. \( \alpha \)-Tocopherol

\( \alpha \)-Tocopherol content was estimated by the method of Desai (1984). To 1.0 ml of test sample, added 1.0 ml of ethanol and mixed thoroughly. To this mixture, 3.0 ml of petroleum ether was added, shaken rapidly and centrifuged. 2.0 ml of supernatant was taken and evaporated to dryness. To this, 0.2 ml of bathophenanthroline reagent was added. Tubes containing \( \alpha \)-tocopherol standards were also treated exactly in the same way as the test samples. Care was taken to reduce unnecessary exposure to direct light. Then added 0.2 ml of 0.001M ferric chloride reagent and vortex mixed. After one minute, 0.2 ml of 0.001M orthophosphoric acid reagent was added and mixed thoroughly and the total volume of all the tubes was made up to 3.0 ml with ethanol. The
absorbance was read at 536 nm against the reagent blank containing ethanol. The level of α-tocopherol in plasma was expressed as mg/dl and in tissues as µg/mg of protein.

3.10.3. Estimation of total reduced glutathione

Total reduced glutathione was determined by the method of Sedlak and Lindsay (1968) modified according to the method of Moron et al. (1979). 0.1 ml of the test sample was precipitated with 5% TCA. The precipitate was removed by centrifugation. To 2.0 ml of the supernatant, added 2.0 ml of 0.6 mg of DTNB in 0.2 M phosphate buffer (pH 6.5). The absorbance was read at 412 nm against a blank containing TCA instead of sample. A series of standards treated in a similar manner were also run to determine the glutathione content. The amount of glutathione was expressed as mg/dl for plasma and mg/100 g of tissues.

3.10.4. Assay of lipid peroxidation (LPO)

Malondialdehyde contents were estimated according to the method of Buge and Aust (1978). To 1.0 ml of the sample, 2.0 ml of TCA-TBA-HCl reagent was added and mixed thoroughly. The solution was heated for 15 minutes in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 g for 10 minutes. The absorbance was determined at 535 nm against a blank that contains all the reagents except the sample. The results were expressed as nmoles of MDA formed/minute/mg protein using an extinction coefficient of the chromophore 1.56 x 10^5 M cm and expressed as nmoles of MDA formed/minute/mg protein.

3.11. Membrane bound phosphatases

3.11.1. Assay of Sodium potassium (Na⁺-K⁺) ATPase

Na⁺-K⁺ ATPase was assayed by the method of Bonting (1970). The incubation mixture contained 1.0 ml of Tris-HCl buffer (90 mM, pH 7.5), 0.2 ml each of 50 mM magnesium sulphate, 50 mM potassium chloride, 600 mM sodium chloride, 1 mM EDTA, 40 mM ATP and the homogenate. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1.0 ml of 10% TCA, mixed well and centrifuged. The phosphorus content of the supernatant was estimated according to Fiske and Subbarow (1925) method. The enzyme activity
was expressed as \( \mu \)moles of phosphate liberated/minute/mg of protein under incubation conditions.

### 3.11.2. Assay of Calcium (\( \mathrm{Ca}^{2+} \)) ATPase

The activity of \( \mathrm{Ca}^{2+} \)-ATPase was assayed according to the method of Hjerten and Pan (1983). The incubation mixture contained 0.1 ml each of 125 mM Tris-HCl buffer (pH 8.0), 50 mM calcium chloride, 10 mM ATP and homogenate. After incubation at 37\(^\circ\)C for 15 minutes, the reaction was arrested by the addition of 1.0 ml TCA. The amount of phosphorus liberated was estimated according to the method of Fiske and Subbarow (1925). The enzyme activity was expressed as \( \mu \)moles of phosphate liberated/minute/mg of protein under incubation conditions.

### 3.11.3. Assay of \( \mathrm{Mg}^{2+} \)-ATPase

The activity of \( \mathrm{Mg}^{2+} \)-ATPase was assayed according to the method of Ohnishi \textit{et al.} (1982). The incubation mixture contained 0.1 ml each of 375 mM Tris-HCl buffer (pH 7.6), 25 mM magnesium chloride, 10 mM ATP and the homogenate. The reaction mixture was incubated at 37\(^\circ\)C for 15 minutes. The reaction was arrested by the addition of 1.0 ml 10\% TCA. The liberated phosphorus was estimated according to the method of Fiske and Subbarow (1925). The enzyme activity was expressed as \( \mu \)moles of phosphate liberated/minute/mg of protein under incubation conditions.

### 3.13. Lysosomal enzyme studies

#### 3.13.1. Separation of Lysosomes

Liver lysosomal enzymes were separated according to the method of Wattiaux \textit{et al.} (1978). Fresh liver tissues were homogenised in 0.25 M sucrose solution. The homogenate was filtered and centrifuged at 3000 g for 10 minutes in a refrigerated Beckman J2-21 centrifuge. The pellet was removed and re-homogenised and resuspended as before. The supernatants were combined and centrifuged again at 15000 g for 20 minutes. The lysosome pellet obtained was suspended in 1.15\% KCl, homogenised and used for the estimation of enzymes.
3.13.2. Assay of β-N-Acetyl Glucosaminidase

The activity of β-N-Acetyl glucosaminidase was determined by the procedure of Moore and Morris (1982). The reaction mixture contained 0.5 ml of 60 mM sodium citrate and 0.1M Na₂HPO₄ in H₂O, citrate phosphate buffer (pH 4.5), 0.5 ml of freshly prepared substrate and 0.12 ml of enzyme solution. The mixture was incubated at 37°C for one hour. The reaction was stopped by the addition of 3.0 ml of 0.2M glycine-NaOH buffer (pH 11.7). Mixed well and centrifuged. The supernatant was transferred into the spectrophotometric cuvette and the absorbance of p-nitrophenol was measured at 410 nm. The activity of β-D-N-acetyl glucosaminidase was expressed as μmoles of p-nitrophenol liberated/hour/100 mg of protein.

3.13.3. Assay of Cathepsin D

Cathepsin D activity was determined by the method of Sapolsky et al. (1973). The incubation mixture contained 0.8 ml of 0.2 mM sodium formate buffer (pH 3.5), 1.0 ml of substrate and 0.2 ml of enzyme homogenate. The tubes were incubated at 37°C for two hours. The enzyme reaction was arrested by the addition of 2.0 ml of 10% TCA. The control tubes received the enzyme after arresting the reaction. After 30 minutes, the tubes were centrifuged at 3000 rpm for 15 minutes. 2.5 ml of sodium carbonate in 4% solution in 0.1 M NaOH was added to the supernatant and mixed well. Then 0.5 ml of Folin’s phenol reagent was added and the contents were immediately mixed and the blue color developed was read at 670 nm in Shimadzu UV spectrophotometer. Standards containing aliquots of tyrosine and blank containing water were also treated in a similar manner. The activity of cathepsin-D was expressed as μmoles of tyrosine released/hour/mg of protein.

3.14.1. Isolation of mitochondria

The mitochondrion of liver tissue was isolated according to the method of Johnson and Lardy (1967). A 10% (w/v) homogenate was prepared in 0.05M Tris-HCl buffer (pH 7.4) containing 0.25M sucrose and centrifuged at 1800 rpm for 10 minutes. The supernatant fraction was decanted and centrifuged at 15000 rpm for 5 minutes. The resultant mitochondrial pellet was then washed and resuspended in the same buffer and stored at -20º C for analysis.

3.14.2. Assay of isocitrate dehydrogenase

Isocitrate dehydrogenase (ICDH) was assayed according to the method of King (1965d). To 0.1 ml of 0.1 M Tris-HCl (pH 7.5), 0.2 ml of 0.1 M tri-sodium isocitrate, 0.3 ml of 0.015 M MgCl₂, 0.2 ml of mitochondrial suspension and 0.2 ml of 0.001 M NADP⁺ (0.2 ml of distilled water for control) were added. After incubation for one hour, 1 ml of 0.001 M DNPH was added followed by 0.5 ml of 0.005 M EDTA and kept at 37º C for 20 minutes. Then 10 ml of 0.4 N NaOH was added and the colour developed was read at 390 nm. A standard containing α-ketoglutarate was run simultaneously. The enzyme activity is expressed as nmoles of α-ketoglutarate liberated/minute/mg protein.

3.14.3. Assay of succinate dehydrogenase

Succinate dehydrogenase (SDH) was assayed by the method of Slater and Bonner (1952). The reaction mixture containing 1.0 ml of 0.3 M phosphate buffer (pH 7.4), 0.1 ml of 0.03 M EDTA, 0.1 ml of 3% BSA, 0.3 ml of 0.4 M sodium succinate, 0.2 ml of 0.075 M potassium ferricyanide and made up to 2.8 ml with distilled water. The reaction was started by the addition of 0.2 ml of mitochondrial suspension. The change in O.D. was recorded at 15 second interval for 5 minutes at 420 nm. The activity of SDH is expressed as nmoles of succinate oxidized/minute/mg protein.

3.14.4. Assay of Malate dehydrogenase (MDH)

Malate dehydrogenase (MDH) was assayed by the method of Mehler et al. (1948). The reaction mixture contained 75µM of phosphate buffer (pH 7.4), 0.15µM of NADH and 0.76 µM of oxaloacetate in a total volume of 3.0ml. The reaction was carried at 25º C and was started by
the addition of enzyme preparation. The control tubes contained all reagents except NADH. The change in OD at 340nm was measured for two minutes at interval of 15 seconds. The activity of the enzyme was expressed as micromoles of NADH oxidized/minute/mg protein.

3.15. DNA fragmentation analysis

DNA fragmentation analysis was carried out by the method of Wu et al. (2002). 100 mg of tissue from control and experimental groups of rats were weighed and homogenized with 1.0 ml saline-EDTA reagent to get 10% tissue homogenate. 300 µl of the homogenate from all the groups were mixed with 300 µl of Tris-saturated phenol and 300 µl of chloroform-isoamyl alcohol mixture. To this content, 25 µl of SDS was added. The contents were mixed thoroughly and centrifuged at 11000 rpm for 15 minutes. The resultant aqueous phase was collected. 9.0 µl of NaCl and two volumes of 100% ethanol (twice the volume of aqueous phase) were added. The contents were mixed and centrifuged at 12000 rpm for 5 minutes. The pellet fraction containing DNA was dissolved in TE buffer. The DNA was detected on a 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized by UV light.

3.16.1. Mast cell staining

Histochemical analyses of mast cells were carried out by the method of Ranieri et al. (2002). Briefly, 5.0 µm thickness tissue sections were dewaxed in xylene and rehydrated through decreasing concentrations of ethanol to distilled water. The sections were stained with toluidine blue for two minutes and washed with distilled water followed by staining with light green SF for 30 seconds and washed using distilled water and dehydrated in increasing concentrations through alcohol series, xylene and mounted using DPX. High power objective field (40X) was chosen for counting total number of mast cells at ten different fields per slide.

3.16.2. Argyrophilic nucleolar organizing regions (AgNOR’s) staining

AgNOR’s staining was carried out by the method of Ranieri et al. (2002). Liver sections of 5.0 µm, obtained from each paraffin block were stained by the one step silver colloid method. Briefly, slides were kept in oven for 30 minutes at 56ºC, dewaxed in xylene and rehydrated through decreasing concentrations of ethanol to distilled water. The AgNOR’s solution was
freshly prepared by dissolving 2% gelatin in 1% aqueous formic acid. This solution was mixed with twice its volume of 50% aqueous silver nitrate solution. Liver tissue sections were exposed to the staining solution for 40 minutes at room temperature in the dark. The slides were washed in distilled water for 10 minutes, rehydrated through graded alcohol, cleared in xylene, and mounted using DPX. The number of AgNOR’s per crypt cell nuclei was counted on one-step silver stained sections. The measurement was performed on 25 crypts per rat. Usages of 25 different areas in the sections were chosen in order to determine the homogeneous AgNOR’s quantification throughout all groups. AgNOR’s were visualized as distinct silver positive black dots and clusters. AgNOR’s were counted on AgNOR’s stained sections under a microscope at a magnification of 40x. The results are expressed in terms of number of AgNOR’s/nuclei in each group.

3.16. Histological studies

Histological studies were carried out by the procedure of Kleiner et al. (2005). The liver tissue samples were fixed in 10% buffered formalin and specimens were dehydrated in absolute ethanol (2 to 3 minutes in each reagent) and embedded in paraffin. Three-micrometer-thick sections were placed on slides, deparaffinised in xylene and stained with hematoxylin and eosin (H and E). The slides were then visualized under light microscope (Nikon XDS-1B; Nikon, Port Charlotte, FL, USA).

3.17. Ultrastructural studies

Ultrastructural studies using Transmission Electron Microscopy was conducted as per the procedure of McLean et al. (2003). The liver tissues were perfused with 35 to 50 ml of 0.5% glutaraldehyde. Then the liver tissue from control and experimental groups of rats were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 18 hours. Post fixation was done using 2% osmium tetroxide in 10mM sodium phosphate buffer (pH 7.4) and left over night. Then sections were dehydrated using series of ethanol solutions. The tissue was embedded in a mixture of 1,2-epoxy propane and Epon (Epikote resin) (1:1). The tissue was then hardened using Dodecyl Succinic Anhydride (DDSA) and Methyl Nadic anhydride (MNA). A diamine catalyst N-benzyl-N diethylamine was used for hardening. The specimen was kept in a block holder and placed in hot air oven at 60ºC for 48 hours. Ultrathin sections were cut, stained with uranyl
acetate and lead nitrate and collected on mesh grids coated with a thin Formvar film, and viewed in a Philips EM201C transmission electron microscope.

3.18. Statistical analysis

The values were expressed as mean ± SD for six rats in each group. All data were analysed with SPSS 10.0 student software. Hypothesis testing method included one way analysis of variance (ANOVA) followed by post hoc testing performed with least significant difference (LSD) test. Values of p < 0.05 were considered as significant.

3.19. Protein Data Bank

The Protein Data bank originally developed and housed at the Brookhaven National Laboratories, is now managed and maintained by the Research Collaboratory for Structural Bioinformatics (RCSB). RCSB is a collaborative effort involving scientists at the San Diego Supercomputing Center, Rutgers University, and the National Institute of Standards and Technology. The collection contains all publicly available three-dimensional structures of proteins, nucleic acids, carbohydrates and a variety of other complexes experimentally determined by X-ray crystallographers and NMR spectroscopists. The World Wide Web site of the Protein Data Bank at the RCSB offers a number of services for submitting and retrieving three dimensional structure data (http://www.rcsb.org/pdb/). From the RCSB the HCC Receptors (Pac1, VEGF, VIPF and Cox-2) were downloaded and used for the recepting study through docking programmers.

3.20. Pubchem

Pubchem is a free database of chemical structures of small organic molecules and information on their biological activities are hosted in NCBI web server. PubChem is organized as three linked databases within the NCBI's Entrez information retrieval system. These are PubChem Substance, PubChem Compound, and PubChem BioAssay. PubChem also provides a fast chemical structure similarity search tool. Links from PubChem's chemical structure records to other Entrez databases provide information on biological properties. These include links to PubMed scientific literature and NCBI's protein 3D structure resource. Links to PubChem's
bioassay database present the results of biological screening. Links to depositor web sites provide further information. A PubChem FTP site, Power User Gateway (PUG), Standardization service, and Deposition Gateway are also available. Three dimensional structures of the ligand molecules luteolin with structure data file (SDF) format was mined from the pubchem database. It was then converted to Protein data bank format by using molecular convertor programme.

3.21. Molecular docking

Binding of a small molecule (ligand) with a large molecule (protein) is called docking. Docking is the process by which two molecules fit together in 3D space. The objective of computational docking is to determine how two molecules will interact which will aid the interaction studies in bio-molecules. Molecular docking is often employed to aid in determining how a particular drug lead will interact to form a binding pocket. The molecular docking was performed by offline and online software’s. The offline docking software molegro was used for this study. Patch dock was used for the online docking.

3.21.1. Patch dock

Patch dock algorithm (Duhovny et al., 2002) is inspired by object recognition and image segmentation techniques that are used in computer vision. Given two molecules, their surfaces are divided into patches according to the surface shape. All possible patches concave, convex or flat surface patches which can be visually seen are detected using segmentation algorithm. The patches are then filtered, so that only patches with hot spot residues are retained. Once the patches are identified, they are superimposed using shape matching algorithm. Shape matching algorithm uses hybrid of the geometric hashing and pose clustering matching techniques to match the patches detected by segmentation algorithm. Concave patches are matched with convex patches and flat patches with any type of patches to obtain complexes. All the candidate possible complexes are examined. HCC Receptors (PAC1,VEGF,VIPF and COX-2 ) and ligand molecule - luteolin were uploaded in PDB format in Patch dock server, an automatic server for molecular docking. Clustering RMSD is chosen as 4.0 Å. e.mail addresses to retrieve the result was given. Complex type was chosen as enzyme-inhibitor type. Then the docking job was submitted to the Patch dock server.
Same steps are repeated for the ligand molecule IPP. Another docking job was submitted to the Patch dock server. Results are obtained through the e-mail address provided and the docked complex structures of HCC Receptors and Luteolin PDB are downloaded.

3.21.1.2. The metaPocket 2.0 algorithm

There are three steps in metaPocket 2.0 procedure: Calling based methods, Meta-pocket site generation and Mapping binding residues. In the first step, the given protein structure will be sent to 8 predictors of LIGSITECS, PASS, Q-SiteFinder, SURFNET, Fpocket, GHECOM, ConCavity and POCASA to identify pocket sites on its surface, all the predictors are called in parallel to save running time. In the second step, the pocket sites identified by these element predictors have different ranking scoring functions. So, it was hard to compare and evaluate the predicted pocket sites directly. To make the ranking scores comparable, a z-score was calculated separately for each pocket site in different predictors. Afterwards, only the top three pocket sites in each predictor are taken into further consideration. Therefore, there was a total of 24 pocket sites. Then the pocket sites had been clustered according to their spatial similarity and all the final clusters were ranked by the total z-score values of them. The final pocket sites are the mass center of the final clusters. The purpose of the third step was to identify functional residues around the identified meta-pocket site which could be the potential ligand binding sites on protein surface.