Chapter - 3

Materials and Methods
3. MATERIALS AND METHODS

3.1 Phytochemical studies

*Embelia basaal* (Roem. & Schult.) DC. is widely distributed in Kolli hills, Namakkal district of Tamil Nadu State, India. Fresh plant was collected in the month of June 2005 and authenticated (Specimen No. 1523) by Dr. M. V. Rao, Professor, Department of Plant Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India.

Macroscopical evaluation refers to evaluation of drugs by color, odor, taste, shape and fracture. It is a technique of qualitative evaluation based on the study of morphological and sensory profiles of the stem bark. The dried bark was taken for the present study [Hajra *et al.*, 1997; Ray & Esau, 1965]. The photograph of the plant *E. basaal* and its morphology are presented in Fig. 1 and Fig. 2.

3.1.1 Extraction

*Embelia basaal* stem bark was dried under shade and then powdered to get a coarse powder. Powdered bark (2 kg) was extracted with ethanol by continuous hot extraction method for 72 h by using Soxhlet apparatus [Harborne, 1983]. The ethanol extract was concentrated to a dry mass by vacuum distillation. After complete drying the extracted material was weighed and the extractive value in percentage was calculated with reference to the air dried sample [Wagner & Bladt, 1996].

The ethanol extract was subjected to qualitative chemical tests for the detection of various plant constituents like carbohydrates, glycosides, proteins and amino acids, fixed oils and fats, gums and mucilage, alkaloids, phytosterols, flavonoids, tannins and phenolic compounds, saponins, triterpenoids, etc. [Trease & Evans, 1989; Vorro *et al.*, 1981; Deb, 1998].
3.1.2 Thin layer chromatography

Chromatography is an analytical method that is widely used for the separation, isolation, identification, and quantification of components in a mixture. The selection of a solvent for application of the sample can be a critical factor in achieving reproducible chromatography with distortion free zones. In general, the application solvent should be a good solvent for the sample and should be as volatile as possible and more non-polar [Fried & Sherma, 1994]. Out of the various trials made, the mobile phase, chloroform: ethyl acetate (1: 3) was found to be effective. Silica gel was chosen as stationary phase, since it is an efficient adsorbent for the TLC separation of most of the plant extracts and plant drug extracts [Houghton et al., 1996].

3.1.3 Column chromatography

Ethanol extract of *E. basaal* stem bark (EEEB) was subjected to column chromatography for the separation of phytoconstituents.

Running of column

A column of suitable size (1 m x 1.5 inch) was chosen and packed with silica gel 60-120 mesh by adding slurry of the adsorbent in hexane. EEEB was dissolved in ethanol and mixed with silica gel (60-120 mesh) and fed to the column through a funnel. Hexane was added to the column and kept aside without disturbance for overnight for the settlement of the extract. Maximum precautions were taken to remove the air bubbles. The column was eluted with different organic solvents in the order of increasing polarity (petroleum ether, chloroform, methanol and ethanol). Fractions showing similar R$_f$ value and identification test, were pooled together and solvents evaporated to get residues.
3.2 Spectral characterization

The chemicals were purchased from Aldrich Chemical Co. Bangalore, India. Pre-coated 60 F254 silica gel aluminium backed plates (Merck, Darmstadt) were used. Visualization of spots for thin layer chromatography was performed using a UV GL-58 Mineral-Light lamp. Melting points were determined using a Gallenkamp Melting Point apparatus microscope (UK). IR Spectra were recorded using a Bruker Tensor 27 spectrometer, having a resolution of 4 cm⁻¹.

NMR spectra were recorded using a Bruker Avance-300 spectrometer (7.05 T) equipped with a 5 mm single-axis Z-gradient quattro nucleus probe, operating at 300 MHz for ¹H and at 75 MHz for ¹³C. The spectrometer was running TOPSPIN NMR system software (Version 2.0). Chemical shifts (δ) are reported in parts per million (ppm), peak positions relative to Me₄Si (0.00 ppm) for ¹H-NMR and ¹³C-NMR spectra. Abbreviations used for splitting patterns are: s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, unresolved multiplet. Mass spectra were recorded at the Department of Chemistry, University of Manchester using a Micromass PLATFORM II (ES) and Thermo Finnigan MAT95XP (Accurate mass) instrument. UV-VIS spectra were recorded in 1 mL quartz cuvettes using a Cary 4000 UV-visible spectrophotometer equipped with a Peltier-thermostatted cuvette holder.

3.2.1 Infrared spectroscopy

IR is used to probe bond vibrations and bending in molecules and to reveal the types of functional groups present in compound. Functional group region is in the range from 4000-1600 cm⁻¹ and Finger print region is from 1550-660 cm⁻¹.

3.2.2 NMR spectroscopy

NMR is an important spectroscopic method and the premier organic spectroscopy to determine the detailed chemical structure of the chemicals they were
isolating from natural products. NMR spectroscopy is used to study chemical
structure of simple molecules by one dimensional technique (1D-NMR) and the
structure of more complicated molecules by two-dimensional technique (2D-NMR).

$^1\text{H}-^1\text{H}$ COSY (COrelated Spectroscopy) experiment finds use when the homo
nuclear decoupling experiment is unsuitable, for example in complex spectra, where
selective decoupling is not possible because of resonance overlap. The COSY
experiment is a very efficient way of establishing connectivity when a large number
of coupling networks need to be identified, involving 2J, 3J and 4J, as it maps all
correlations with a single experiment and is applied in this research also.

3.2.3 Mass spectrometry

Mass spectrometry provides both molecular weight and fragmentation pattern
of the compound. It relies of production of ions from a parent compound and the
subsequent characterization of the pattern that are produced.

3.3 Toxicological studies

Animals

Albino mice of Swiss strain and Albino rats of Wistar strain were used for
toxicological studies. These animals were inbreeding stock maintained in the animal
house of JKKN College of Pharmacy, Komarapalayam, Namakkal district of
Tamil Nadu, India. Female mice selected were nulliparous and non-pregnant. Female
mice weighing 25 to 30 g and rats of either sex weighing 125 to 150 g were used for
the study. Each animal, at the commencement of its dosing, was between 8 and 12
weeks old and their weight variation was within $\pm 20\%$ of the mean weight of any
previously dosed animals. The temperature in the experimental animal room was
22$^\circ\text{C}$ ($\pm 3^\circ\text{C}$) and the relative humidity was between 50-60%. These animals were fed
with pellet diet manufactured by Amrut laboratory, Animal Feed Company, Sangli,
Maharashtra and drinking water *ad libitum*. They were kept in 12 h/12 h light/dark cycle and maintained for at least 5 d prior to dosing to allow for acclimatization to the laboratory conditions. The animal experimental protocol has been approved by our Institutional Animal Ethics Committee vide reference no: 887/ac/05/CPCSEA.

### 3.3.1 Acute oral toxicity study

The acute toxicity study was carried on albino mice as per the guidelines No: 423 given by the Organization for Economic Co-operations and Development, Paris [OECD 423, 2001]. Three Albino mice were fasted over night and the test sample EEEB was given orally at a starting dose of 5 mg/kg b. wt. Animals were observed for a period of 2 h, then occasionally for 4 h for severity of any toxic signs and mortality. Since no mortality was observed, same dose was repeated with another group of animals. The procedure was repeated for doses of 50, 300 and 2000 mg/kg in separate group of animals. From the maximum dose of 2000 mg/kg, 1/10\(^{th}\) and 1/5\(^{th}\) of the values were taken as treatment dose for further studies [Paget & Barnes, 1983; Tanira *et al.*, 1988]. Behavior as well as other toxic symptoms if any was observed for 24, 48 and 72 h [Miller & Trainter, 1944]. The animals were kept under observation up to 14 d after drug administration to find out delayed mortality if any [Sharada *et al.*, 1993].

### 3.3.2 Sub-acute toxicity study

The sub-acute toxicity study was done on Wistar strain albino rats as per methods previously reported [Biswa *et al.*, 1998; Ghosh, 1984]. Albino rats of either sex were divided into 3 groups of 6 animals each (3 males and 3 females). First group served as solvent control and was given normal saline (1 mL/kg) and the other two groups were administered EEEB at the dose of 200 mg/kg and 400 mg/kg respectively, p.o. The test extracts were given once daily orally for 30 d. All the rats
were observed for any physiological and behavioral changes and mortality if any. Food and water consumption was checked daily. Body weight was recorded on 1\textsuperscript{st} and 30\textsuperscript{th} day.

Haematological parameters such as total red blood corpuscles (RBC), white blood corpuscles (WBC), differential count (DC) and haemoglobin were estimated on the thirty first day [Ghai, 1993]. All the animals were sacrificed on the thirty first day and the blood samples were collected from each rat individually into non-heparinised tubes and were allowed to coagulate. Serum was separated by centrifugation and glucose, cholesterol, urea, creatinine, proteins, albumin, total bilirubin, acid phosphatase, alkaline phosphatase, SGOT and SGPT were analyzed. Vital organs such as liver, kidney, spleen, brain, lungs and stomach were removed and the color of the organs was observed for gross pathological changes. Liver and kidney were fixed in Bouin’s fixative and processed for histopathological examination [Bancroft & Stevens, 1996]. The slides were stained with hematoxylin and eosin and observed under low and high power microscope for pathological changes if any.

**Haematological studies**

The haematological parameters like Haemoglobin, total RBC count, total WBC count and Differential count were estimated by standard procedures [Ghai, 1993].

**Biochemical studies**

[Kaplan, 1972], SGOT [Doumas & Briggs, 1969] and SGPT [Henry, 1974a] were analyzed as per the reported methods.

3.4 Pharmacological screening

3.4.1 Hepatoprotective activity

Test compounds

After performing the acute oral toxicity study, the EEEB at a p.o. dose of 200 mg/kg and 400 mg/kg b. wt. were selected for further pharmacological screening.

Animals

Albino rats of Wistar strain weighing 125-150 g of either sex were used for the study. They were housed in polypropylene cages with not more than six animals per cage and maintained under standard conditions. All the experiments were performed in the morning according to current guidelines for the care of the laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals.

Carbon tetrachloride induced hepatotoxicity

Liver damage was induced in rats by administering CCl₄ subcutaneously (s.c.) in the lower abdomen in a suspension of liquid paraffin (LP) in the ratio 1 : 2 v/v at the dose of 1 mL CCl₄/kg b. wt. of each animal. CCl₄ was administered twice a week, on every first and fourth day of all the 13 weeks [Surendra & Alpana, 2007].

Animal groups

Forty two rats were divided into seven groups of six animals each as follows:

1. Group I animals served as control and received oral administration of normal saline at the dose of 1 mL/kg b. wt., once a day for a duration of 13 weeks [Rajesh et al., 2009].
2. Group II and III animals received EEEB stem bark at a dose of 200 mg/kg and 400 mg/kg b. wt. orally respectively in the form of aqueous solution of 1 mL once in a day for 13 weeks respectively [Biswadev et al., 2002].

3. Group IV animals received s.c. administration of CCl₄ twice a week for a total of 13 weeks.

4. Group V animals were the silymarin (the known hepatoprotective compound) treated group, and received the same at a p.o. dose of 100 mg/kg b. wt. along with CCl₄ [Mishra & Sureshkumar, 2008].

5. Group VI and VII animals were the EEEB stem bark treated and received the same at a p.o. dose of 200 mg/kg and 400 mg/kg b. wt. respectively along with CCl₄.

Assessment of hepatoprotective activity

Replenishing a known quantity of fresh food daily at 8.00 a.m. and thereby measuring the food intake of the previous day and carried out measurement of daily food consumption. Body weight of rats was recorded weekly to assess percentage of weight gain of each animal. Animals were kept starved overnight on the last day. On the next day, after recording the weight of each animal, they were euthanized by decapitation under ether anesthesia, by making an incision on jugular vein; blood was collected in sterile centrifuge tubes and allowed to clot. The liver was dissected out immediately, rinsed with ice cold phosphate buffer and homogenized with 5% formalin solution. The liver homogenate was used for antioxidant studies and a part of the tissue was processed for histological examination.

Serum was separated from the collected blood by centrifugation and subjected to various biochemical estimations like serum glutamate oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase
(SALP), serum gamma glutamyl transpeptidase (SGGTP), sugar, total bilirubin, total protein, triglyceride, total cholesterol, high density lipoprotein-cholesterol (HDL-cholesterol), very low density lipoprotein-cholesterol (VLDL-cholesterol) and low density lipoprotein-cholesterol (LDL-cholesterol) to determine the functional state of the liver. Immunological parameters like tumor necrosis factor - alpha and interleukin-6 were also estimated. With the liver homogenate the antioxidant studies like estimation of superoxide dismutase, catalase, glutathione peroxidase, lipid peroxidation, and glutathione transferase were carried out.

Liver slices fixed for 12 h in Bouine’s solution were processed for paraffin embedding following standard micro techniques. Liver section of 5 µm stained with alum hematoxylin and eosin was observed microscopically for histopathological changes.

3.4.1.1 Estimation of biochemical parameters

The estimation protocol of the below mentioned parameters are given in Appendix.

**Serum glutamic oxaloacetic transaminase (SGOT)**

The SGOT level was estimated by Doumas and Briggs method [Doumas & Briggs, 1969]. SGOT also known as serum aspartate aminotransferase (AST) is a tissue enzyme that catalyzes the exchange of amino and keto groups between alpha amino acids and alpha-keto acids. SGOT is widely distributed in tissue principally cardiac, hepatic, muscle and kidney. Injury to these tissues results in the release of the SGOT enzyme to general circulation. Following a myocardial infarction, serum levels of SGOT are elevated and reach a peak 48 to 60 h after onset. Hepatobiliary diseases, such as cirrhosis, metastatic carcinoma, and viral hepatitis also will increase SGOT levels [Henry, 1974a].
**Serum glutamate pyruvate transaminase (SGPT)**

The enzyme glutamate pyruvate transaminase is widely reported in a variety of tissue sources. The major source of SGPT is of hepatic origin and has led to the application of SGPT determinations to the study of hepatic diseases. Elevated SGPT levels are found in hepatitis, cirrhosis, and obstructive jaundice. Levels of SGPT are only slightly elevated in patients following a myocardial infarction. The SGPT level was estimated by Winsten method [Henry, 1974b].

**Serum alkaline phosphatase (SALP)**

Distributed in almost every tissue of the body, SALP levels are of interest in the diagnosis of hepatobiliary disorder and bone disease [Kochmar & Moss, 1976]. Most of the ALP in normal adult’s serum is from the liver or biliary tract [Karmen et al., 1955]. Normal alkaline phosphatase levels are age-dependent and are elevated during periods of active bone growth. Moderate elevations of ALP (not involving the liver or bone) may be attributed to Hodgins’ disease, congestive heart failure, and abdominal bacterial infections. The SALP level was estimated by Roy method [Kaplan, 1972].

**Gamma glutamyl transferase peptidase (GGTP)**

Gamma glutamyl transferase (g-GT) is one of a large group of enzymes known as peptidases. g-GT catalyzes the transfer of gamma glutamyl groups from peptides or peptide like compounds to an acceptor peptide molecule. Although renal tissue has the highest level of g-GT, the major source of the enzyme present in serum is of hepatic origin. Elevated levels of g-GT are found in association with hepatobiliary and pancreatic disorders; alcoholics and heavy drinkers, in myocardial disorders, and in diabetics [Tietz, 1976b]. A synthetic substrate gamma glutamyl-p-nitroanilide (GGPNA) has been used more extensively for assessing g-GT. Glynicylglycine was
introduced as an activator and Szasz adapted this activator and the GGPNA to a 30°C kinetic photometric method [Szasz, 1969]. Rosalki adapted optimal concentrations of the activator and GGPNA substrate to a 37°C colorimetric endpoint method [Rosalki et al., 1970]. The procedure has been optimized according to Szasz and Rosalki method and is performed as either an endpoint or kinetic procedure.

**Glucose**

Glucose is the major carbohydrate present in the peripheral blood. The oxidation of glucose is the major source of cellular energy in the body. Glucose determinations are run primarily to aid in the diagnosis and treatment of diabetes mellitus. Elevated glucose levels are mainly associated with insulinemia or insulin-induced hypoglycemia [Cooper, 1973]. A number of secondary factors also can contribute to elevated blood glucose levels. These include pancreatitis, pituitary or thyroid dysfunction, renal failure and liver disease [Henry, 1979]. An enzymatic approach for glucose determination involves hexokinase coupled with glucose-6-phosphate dehydrogenase [Barthelmai & Czek, 1962]. A revision of this approach is proposed by the U.S. Center for Disease Control as the reference method for glucose and forms the basis of the reagent for glucose.

**Total bilirubin**

Bilirubin is a metabolite of the heme portion of heme proteins, mainly haemoglobin. Normally it is excreted into the intestine and bile from the liver. The site of the catabolism of haemoglobin is the reticulo endothelial system (RES). Bilirubin is then released into the blood stream where it binds tightly to albumin and is transported to the liver. Upon uptake by the liver, bilirubin is conjugated with glucuronic acid to form bilirubin mono and diglucuronide which are water soluble metabolites. The metabolites are then excreted in the bile [Tietz, 1976a]. Elevation
of total serum bilirubin may occur due to a) excessive hemolysis or destruction of the red blood cells e.g. hemolytic disease of the newborn, b) liver diseases e.g. hepatitis and cirrhosis and c) obstruction of the biliary tract e.g. gallstones. The method proposed by Walters was employed for the estimation of total bilirubin [Walters & Gerarde, 1970].

**Protein**

Through osmotic pressure, serum protein is involved in the maintenance of normal distribution of water between blood and tissues. Low protein is primarily caused by malnutrition, impaired synthesis, loss (as by hemorrhage), or excessive protein catabolism. Elevated protein levels are caused mainly by dehydration. The estimation of protein was done by Gornall method [Gornall et al., 1949]. The present method for quantitative determination of total protein in serum is based on the method proposed by the American Association for Clinical Chemistry (AACC) [Doumas et al., 1981] and National Committee for Clinical Laboratory Standards (NCCLS) [NCCLS, 1979].

**Triglycerides**

Triglycerides are esters of fatty acids and are hydrolyzed to glycerol and free fatty acids. Triglyceride determinations when performed in conjunction with other lipid assays are useful in the diagnosis of primary and secondary hyperlipoproteinemia. They are also of interest in following the course of diabetes mellitus, nephrosis, biliary obstruction and various metabolic abnormalities due to endocrine disturbances. Standard methods for the measurement of triglyceride concentrations have involved either enzymatic or alkaline hydrolysis for the liberation of glycerol. This formulation makes use of the enzymatic hydrolysis and quantification since it is specific and not subject to interference by phospholipids [Searcy, 1969]. The method
proposed by Fosatti was employed for the estimation of triglycerides [Fosatti & Prencipe, 1982].

**Total cholesterol**

Cholesterol is a fatty substance found in blood, bile and brain tissue; it serves as a precursor to bile acids, steroids and vitamin D. The determination of serum cholesterol is a major aid in the diagnosis and classification of lipemias [Beaumont *et al.*, 1972]. Other conditions such as hepatic thyroid diseases influence cholesterol levels [Holvey, 1972]. The method proposed by Allain was employed for the estimation of total cholesterol [Allain *et al.*, 1974].

**HDL Cholesterol**

The concentration of total cholesterol in serum has been associated with metabolic, infectious and coronary heart diseases. In the plasma, cholesterol is transported by three lipoproteins: high density lipoprotein (HDL-Cholesterol); low density lipoprotein (LDL Cholesterol) and very low density lipoprotein (VLDL-Cholesterol) [Warnick & Albers, 1978]. Castelli and his co-workers have indicated that an inverse relationship exists between serum HDL-cholesterol and the risk of coronary heart disease. The measurement of total and HDL cholesterol and triglycerides provides valuable information for the prediction of coronary heart disease and for lipoprotein phenotyping [Castelli *et al.*, 1977; Gordon *et al.*, 1977]. The method proposed by Izzo was employed for the estimation of HDL Cholesterol [Izzo *et al.*, 1981].

**LDL Cholesterol**

LDL-cholesterol forms a precipitate by the action of the precipitating reagent heparin on the serum [Lopez-virellu *et al.*, 1977]. The supernatant contains VLDL and HDL-cholesterol which are measured by the use of the cholesterol CHOD/PAP
method. LDL-cholesterol is equal to the difference between total cholesterol and cholesterol in the supernatant [Assmann, 1979].

3.4.1.2 Estimation of immunological parameters

Tumor necrosis factor alpha (TNF-α)

Introduction

TNF-α is a potent cytokine with a myriad of innate immune anti-tumor properties. TNF-α has a critical role in the bone and cartilage damage associated with rheumatoid arthritis [Taylor, 2001]. TNF-α may be involved in the pathogenesis and/or progression of gestational diabetes mellitus [Coughlan et al., 2001]. TNF-α is expressed in myocardium during compensated pressure-overload hypertrophy and contributes to post-ischemic myocardial dysfunction [Stamm et al., 2001; Ohta et al., 2001]. The serum levels of TNF-α were also significantly elevated in active WG (Wegener's Granulomatosis), in the late stages of HIV-associated disease and in the spinal cord of arthritic patients [Nanki et al., 2001; Caso et al., 2001].

Reagents used

TNF-α microplate:

Coated with a murine monoclonal antibody against TNF-α sealing tapes.

TNF-α standard:

Recombinant rat TNF-α in a buffered protein base (2 ng, lyophilized).

Biotinylated TNF-α antibody (100 x):

A 100-fold biotinylated polyclonal antibody against TNF-α (90 µL).

Streptavidin-peroxidase conjugate:

A 100-fold concentrates (90 µL).
**MIX diluent concentrate (10 x):**

A 10-fold buffered protein base (30 mL).

**Wash buffer concentrate (10 x):**

A 10-fold concentrated buffered surfactant (2 x 30 mL).

**Chromogen substrate:**

A ready-to-use stabilized peroxidase chromogen substrate tetramethyl benzidine (8 mL).

**Stop solution:**

Hydrochloric acid (0.5 N, 12 mL) to stop the chromogen substrate reaction.

Microplate reader capable of measuring absorbance at 450 nm.

Pipettes (1-20 µL, 20-200 µL, and multiple channels).

De-ionized or distilled reagent grade water.

**Standard curve**

The rat TNF-α standard (2 ng) was reconstituted with 2 mL of MIX diluent to generate a 1 ng/mL of solution. The standard was allowed to stand for 10 min with gentle agitation prior to making dilutions. Triplicate standard points were prepared by serially diluting the TNF-α standard solution 1:4 with MIX diluent to produce 0.25, 0.0625, 0.0156 and 0.0039 ng/mL. MIX diluent served as the zero standard (0 ng/mL).

<table>
<thead>
<tr>
<th>Standard point</th>
<th>Dilution</th>
<th>TNF-α (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Standard (1 ng/mL)</td>
<td>1.0000</td>
</tr>
<tr>
<td>P2</td>
<td>1 part P1 + 3 part MIX diluent</td>
<td>0.2500</td>
</tr>
<tr>
<td>P3</td>
<td>1 part P2 + 3 part MIX diluent</td>
<td>0.0625</td>
</tr>
<tr>
<td>P4</td>
<td>1 part P3 + 3 part MIX diluent</td>
<td>0.0156</td>
</tr>
<tr>
<td>P5</td>
<td>1 part P4 + 3 part MIX diluent</td>
<td>0.0039</td>
</tr>
<tr>
<td>P6</td>
<td>MIX diluents</td>
<td>0.0000</td>
</tr>
</tbody>
</table>
MIX diluent concentrate (10 x):

The concentrate was diluted in the ratio of 1:10 with reagent grade water.

Biotinylated TNF-α antibody (100 x):

The antibody was spun briefly and diluted the desired amount of the antibody 1:100 with MIX diluent.

Wash buffer concentrate (10 x):

The concentrate was diluted in the ratio of 1:10 with reagent grade water.

SP Conjugate (100 x):

The conjugate was spun briefly and diluted the desired amount of the conjugate 1:100 with MIX diluent.

Procedure

1. All reagents, working standards and samples were prepared as instructed, and maintained at room temperature before use. The assay was performed at room temperature (20-30°C). Excess microplate strips were removed from the plate frame and returned them immediately to the foil pouch with desiccant inside.

2. Added 50 µL of standard or sample per well. The wells were covered and incubated for two hours. The timer was started after the last sample addition. It was washed five times with 200 µL of Wash buffer. Inverted the plate and decanted the contents, and hit it 4-5 times on absorbent paper towel to completely remove liquid at each step.

3. Biotinylated TNF-α antibody (50 µL) was added to each well and incubated for 2 h. It was washed five times with 200 µL of Wash buffer as above. Streptavidin-Peroxidase conjugate (50 µL) was added to every well and incubated for 30 min. Turned on the microplate reader and set up the program in advance. Again it was washed five times with 200 µL of Wash buffer as above. Chromogen substrate
(50 µL) was added to all the wells and incubated for approximately 10 min till the optimal blue color density developed. Gently tapped the plate to ensure thorough mixing and break the bubbles in the well with pipette tip.

4. Upon addition of Stop Solution (50 µL) to each well, the color changed from blue to yellow. With a microplate reader absorbance was noted at a wavelength of 450 nm immediately.

Data analysis

The mean value of the triplicate readings for each standard and sample was calculated. A standard curve was generated by plotting a graph using the standard concentrations on the X-axis and the corresponding mean absorbance on the Y-axis. The best-fit line was determined by regression analysis of the linear portion of the curve. The unknown sample concentration was determined from the standard curve and by multiplying the value by the dilution factor.

Interleukin-6

Introduction

Interleukin-6 (IL-6) is a cytokine that was discovered as a B-cell differentiation factor. IL-6 is not only an antibody-producing system, but its physiological activities, such as induction of acute-phase protein synthesis in hepatocytes, and growth stimulation based on its synergistic effect with IL-3, etc., have attracted great interest. Associations between IL-6 and pathology have been steadily demonstrated. For example, the growth factor in several diseases has been reported to be IL-6, and myeloma cells themselves have been reported to synthesize IL-6 and express IL-6 receptors. In addition, IL-6 has been suggested to contribute to a variety of inflammatory diseases and autoimmune diseases. The procedure was
followed as per the instructions given in the leaflet of Rat IL-6 assay kit supplied by Immuno Biological Laboratories Co., Limited, Japan.

Reagents used

Pre-coated plate:

Anti-rat IL-6 Rabbit IgG Affinity Purify 96 Well x 1.

Labeled antibody concentrate:

(30 x) HRP conjugated Anti-Rat IL-6 Rabbit IgG Fab’ Affinity Purify 0.4 mL x 1.

Standard:

Recombinant Rat IL-6 - 0.5 mL x 2.

EIA buffer:

1% BSA, 0.05 % Tween 20 in PBS 30 mL x 1.

Solution for labeled antibody:

1% BSA, 0.05 % Tween 20 in PBS 12 mL x 1.

Chromogen:

TMB solution 15 mL x 1.

Stop solution:

1N H$_2$SO$_4$ 12 mL x 1.

8 Wash buffer Conc.:

(40 x) 0.05% Tween20 in phosphate buffer 50 mL x 1.

Plate reader (450 nm), micropipette and tip, graduated cylinder and beaker, de-ionized water, refrigerator (as 4°C), graph paper (log/log), paper towel, tube for dilution of standard washing bottle for pre-coated plate, disposable test tube for “2, Labeled antibody Conc.” and “6, Chromogen”.
Preparation of wash buffer:

“8, Wash buffer Conc.” is a concentrated (40 x) buffer. The temperature of “8, Wash buffer Conc.” was adjusted to room temperature and then, mixed it gently and completely before use. 50 mL of “8, Wash buffer Conc.” was diluted with 1,950 mL of de-ionized water and mixed it. This was the wash buffer used. This prepared wash buffer was stored in refrigerator and used within 2 weeks after dilution.

Preparation of labeled antibody:

“2, Labeled antibody Conc.” is a concentrated (30 x). “2, Labeled antibody Conc.” was diluted with “5, Solution for Labeled antibody” in 30 times according to required quantity into a disposable test tube. This resulting solution was used as labeled antibody.

Preparation of standard:

To the vial of “3, Standard” added 0.5 mL of de-ionized water and was mixed gently and completely. This was Rat IL-6 standard solution of 1,500 AU/mL.

Dilution of standard:

Prepared 8 tubes for dilution of “3, Standard” and added 230 µL each of “4, EIA buffer” into the tube.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Dilution (AU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube-1</td>
<td>750.00</td>
</tr>
<tr>
<td>Tube-2</td>
<td>375.00</td>
</tr>
<tr>
<td>Tube-3</td>
<td>187.50</td>
</tr>
<tr>
<td>Tube-4</td>
<td>93.75</td>
</tr>
<tr>
<td>Tube-5</td>
<td>46.88</td>
</tr>
<tr>
<td>Tube-6</td>
<td>23.44</td>
</tr>
<tr>
<td>Tube-7</td>
<td>11.72</td>
</tr>
<tr>
<td>Tube-8</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Standard solution of 230 µL was added into the tube-1 and mixed it gently. 230 µL of tube-1 mixture was then added into tube-2. The standard solution was diluted two times in series to set up 7 points of diluted standards between 750 AU/mL and 11.72 AU/mL. Tube-8 was the test sample blank as 0 AU/mL.

**Dilution of test sample:**

Test sample was diluted with “4, EIA buffer” when needed.

**Procedure**

All reagents were brought to room temperature approximately 30 min before use. Maximum precautions were taken to ensure that there was no change in quality of the reagents. Standard curve was prepared simultaneously with the measurement of test samples.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Test sample</th>
<th>Standard</th>
<th>Test sample (Blank)</th>
<th>Reagent Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test sample</td>
<td>Diluted standard (Tube 1-7)</td>
<td>EIA buffer (Tube-8)</td>
<td>EIA buffer 100 µL</td>
</tr>
<tr>
<td></td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
<td></td>
</tr>
</tbody>
</table>

- Incubation overnight at 4°C with plate lid
- Washing 7 times
- **Labeled antibody** 100 µL 100 µL 100 µL ----
- Incubation for 30 min at 4°C with plate lid
- Washing 9 times
- **Chromogen** 100 µL 100 µL 100 µL 100 µL
- Incubation for 30 min at room temperature (shielded)
- **Stop solution** 100 µL 100 µL 100 µL 100 µL
- Read the plate at 450 nm against a Reagent blank within 30 min.
1. 100 μL each of “4, EIA buffer” was transferred into the reagent blank wells.

2. 100 μL each of test sample blank (tube-8), test sample and dilutions of standard (tube-1-7) was transferred into the appropriate wells.

3. The pre-coated plates were incubated overnight at 4°C after covering it with plate lid. Each well of the pre-coated plate was washed vigorously with wash buffer using the washing bottle. Each well was then filled with wash buffer and left the pre-coated plate lay for 15~30 s. Wash buffer was then removed completely from the pre-coated plate by snapping. More than 7 times this procedure was repeated and then removed the remaining liquid from all wells completely by snapping the pre-coated plate on to paper towel.

4. Labeled antibody solution 100 μL was transferred into the wells of test samples, diluted standard and test sample blank. The pre-coated plate was incubated for 30 min at 4°C after covering it with plate lid and then washed the pre-coated plate 9 times.

5. “6, Chromogen” was taken the required quantity into a disposable test tube. 100 μL was then transferred from the test tube into the wells.

6. The pre-coated plate was incubated for 30 min at room temperature in the dark. The liquid was then turned blue by the addition of “6, Chromogen”.

7. 100 μL of “7, Stop solution” was transferred into the wells and mixed the liquid by tapping the side of pre-coated plate, and the liquid was turned yellow by addition of “7, Stop solution”. The plate reader was run and the absorbance was measured at 450 nm against a reagent blank.

**Calculation of test result**

Absorbance of test sample blank was subtracted from all data, including standards and unknown samples before plotting and plotted the subtracted absorbance
of the standards against the standard concentration on log-log graph paper. The best smooth curve was drawn through these points to construct a standard curve. The concentration of unknown samples was determined from the standard curve.

<table>
<thead>
<tr>
<th>Concentration [AU/mL]</th>
<th>Absorbance [450 nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>750.00</td>
<td>1.849</td>
</tr>
<tr>
<td>375.00</td>
<td>1.065</td>
</tr>
<tr>
<td>187.50</td>
<td>0.584</td>
</tr>
<tr>
<td>93.75</td>
<td>0.305</td>
</tr>
<tr>
<td>46.88</td>
<td>0.159</td>
</tr>
<tr>
<td>23.44</td>
<td>0.084</td>
</tr>
<tr>
<td>11.72</td>
<td>0.052</td>
</tr>
<tr>
<td>0.00</td>
<td>0.014</td>
</tr>
</tbody>
</table>

3.4.1.3 Histopathological studies

Liver of all the animals were preserved in buffered neutral formalin. Sections were cut and processed as follows:

**Preparation of tissues**

**Fixation:**

This is a process of killing and hardening. The first phase of fixation is rapid killing of the tissue. The second phase, the hardening. Tissue was placed in the fixative immediately upon removal from the body. Blocks were cut thin enough so that the fixing fluid could penetrate the tissue in a reasonably short time. Buffered neutral formalin, which was used as the fixative for our studies, has the following composition:
Formaldehyde 37-40% - 100 mL, Distilled water - 900 mL, Sodium phosphate monobasic - 4 g, and Sodium phosphate dibasic - 6.5 g.

**Processing of tissues (Dehydrating, clearing and embedding):**

Embedding in paraffin is accomplished most rapidly and gives a best result when thin sections are of soft tissues. Since paraffin is not miscible with water, the tissue was dehydrated and then cleared in a solution that is miscible with paraffin. The schedule for paraffin processing is as follows:

- Alcohol 80% 1-2 h, alcohol 95% 1-2 h each, alcohol absolute 1-2 h each, xylene-2 changes 1-2 h each, melted paraffin-3 changes 1-2 h each. The processed tissue was then embedded in paraffin in shallow tin pans with slightly slopping slides ranging from 1x2 inches and ¾ inch in depth. The pan was warmed gently with a Bunsen burner and filled with filtered and melted paraffin. Each piece of tissue was placed in position in the pan with the appropriate string tag (carrying the group and individual number of the animal). These pans were left undisturbed so that paraffin would get cool and hardened. The paraffin, when hardened throughout, contracted from the sides of the pan. The mass was then lifted out and cut into blocks of appropriate size.

**Preparation and cutting of sections:**

The paraffin block was secured at the appropriate position in the microtome and was aligned with the knife. A piece of cotton in a dish of tap water and an ice cube were kept in a petridish beside the microtome. To facilitate sectioning, the wet cotton was applied to the surface of the block after rough cutting. Sections of about 5 µ were cut after chilling the paraffin block with the ice cube. The section ribbons were collected in a water bath and unfurled gently with a fine tip camel’s hairbrush. Since the results produced by histological techniques depend greatly upon the knife
used to cut the sections, it was important that the knife be very sharp and without nicks.

**Attaching sections to slides:**

The glass slides on which tissue sections were to be mounted were smeared with albumin and were marked with the identifying number of the animal. The slides were taken below the floating section; the section was placed in the center of the slide and was left to dry and to be stained subsequently.

**Staining:**

The sections were stained using hematoxylin-eosin stain. Composition of hematoxylin solution was as follows:

Hematoxylin crystals - 5 g, Alcohol absolute - 50 g, Ammonium or potassium alum - 100 g, Mercuric oxide (red) - 5 g, and Distilled water - 1000 mL.

Composition of eosin solution (1%) was as follows:

Eosin Y, water soluble - 1 g, distilled water, dissolved and added alcohol, 95%. The schedule followed for staining was:

- Xylene - 3 changes 30 min each, xylene + alcohol 30 min, alcohol 50% 30 min, alcohol 70% 30 min, alcohol absolute 30 min, distilled water 20 min, hematoxylin 10 min, running tap water 1 h, acid alcohol 3-10 quick dips, alcohol 50% 10 min, alcohol 95% 10 min, eosin 45 s - 1 min, alcohol absolute (2 changes) 5 min each, xylene + alcohol 5 min, xylene (3 changes) 10 min each. The stained sections were finally mounted in DPX mountant.

**3.4.2 Antioxidant assays**

After the collection of blood samples, the rats were sacrificed. Then their liver was excised, rinsed in ice-cold normal saline followed by cold 0.15 M Tris (hydroxymethyl aminomethane) HCl (pH 7.4), blotted dry, and weighed. Homogenate (10% w/v) was prepared in 1.15% potassium chloride. Trichloro acetic
acid (TCA) was added to precipitate proteins, centrifuged and the supernatant was used for the estimation of glutathione. The remaining homogenate was centrifuged at 2000 rpm for 15 min at 40°C. The supernatant thus obtained was used for the estimation of superoxide dismutase and catalase.

**Superoxide dismutase**

Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide radical ($O_2^-$) into hydrogen peroxide ($H_2O_2$) and elemental oxygen ($O_2$) and as such provides an important defense against the toxicity of the superoxide radical [Zhao et al., 2001]. In fact, over expression of SOD protects murine fibro sarcoma cells from apoptosis and promotes cell differentiation [Fridovich, 1989]. SOD also inhibits adriamycin-induced apoptosis in murine peritoneal macrophages. In the assay, superoxide ions ($O_2^-$), generated by xanthine oxidase (XOD) conversion of xanthine to uric acid and hydrogen peroxide, converts NBT to NBT-diformazan, which absorbs light at 560 nm. SOD reduces the superoxide ion concentration and thereby lowers the rate of NBT-diformazan formation [Beyer & Fridovich, 1987]. The extent of reduction in the appearance of NBT-diformazan is a measure of SOD activity present in an experimental sample. The assay is free of interference by other catalytic activities and is ideal for assaying SOD in mammalian cell lysates. Additionally, this system is not greatly disturbed by trace metals. Each assay requires approximately 5 min and after a simple calculation, the percent inhibition of the formation of NBT-diformazan by SOD is converted to the relative activity of the sample [Sutherland & Learmonth, 1997; Nebot et al., 1993].
Catalase

Catalase is an antioxidant enzyme that catalyses the decomposition of hydrogen peroxide ($H_2O_2$) to water and oxygen. Catalase is ubiquitously expressed in mammalian and non-mammalian aerobic cells containing the cytochrome system. The enzyme has been isolated from various sources, including bacteria and plant cells [Deisseroth & Dounce, 1970; Sebastian et al., 1997]. Catalase activity varies greatly from tissue to tissue. Highest activity is seen in liver and kidney, while lowest activity is seen in connective tissue. In eukaryotic cells, catalase in concentrated in organelles called peroxisomes [Marcel & Franz, 1999].

The production of hydrogen peroxide in eukaryotic cells is an end product result of various oxidase and superoxide dismutase reactions. Accumulation of $H_2O_2$ can result in cellular damage through oxidation of proteins, DNA and lipids thus resulting in cell death and mutagenesis. $H_2O_2$ role in oxidative stress related diseases have been widely studied. The method described in AMDCC (Animal Models of Diabetic Complications Consortium) protocols was followed [Jingxiang et al., 1999].

Glutathione peroxidase

Glutathione peroxidase catalyzes the reduction of hydroperoxides, including hydrogen peroxide, by reduced glutathione and functions to protect the cell from oxidative damage [Ursini et al., 1985]. With the exception of phospholipid-hydroperoxide glutathione peroxidase, a monomer, all of the glutathione peroxidase enzymes are tetramers of four identical subunits. Each subunit contains a
selenocysteine in the active site that participates directly in the two-electron reduction of the peroxide substrate. The enzyme uses glutathione as the ultimate electron donor to regenerate the reduced form of the selenocysteine [Paglia & Valentine, 1967]. The method described by Forstrom was followed here [Forstrom et al., 1978].

**Lipid peroxidation**

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues [Bull & Marnett, 1985]. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds [Esterbauer et al., 1991]. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) upon decomposition. The measurement of MDA and HAE has been used as an indicator of lipid peroxidation [Botsoglou et al., 1994]. This method is designed to assay either MDA alone (in hydrochloric acid) or MDA in combination with HAE (in methane sulphonic acid) [Carbonneau et al., 1991; Liu et al., 1997].

This assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1), with MDA and HAE at 45°C. One molecule of either MDA or HAE reacts with 2 molecules of Reagent R1 to yield a stable chromophore with maximal absorbance at 586 nm [Mattson et al., 2002].

\[
\begin{align*}
\text{H} & \quad \text{O} \\
R & \quad \text{Ph} \\
\text{N} & \quad \text{Ph} \\
\end{align*}
\]

MDA: \( R = \text{OH} \) \hspace{2cm} \text{Max} = 586 \text{ nm}

4-hydroxyalkenal: \( R = \text{hydroxyl alkyl} \)
Glutathione transferase

Glutathione S-transferase (GSTs) is ubiquitous multifunctional enzyme, which play a key role in cellular detoxification. The enzyme protects cells against toxicants by conjugating them to glutathione thereby neutralizing their electrophilic sites, and rendering the products more water-soluble [Boyland & Chasseaud, 1969]. The glutathione conjugates are metabolized further to mercapturic acid and then excreted. Based on their sequence homology, substrate specificity and immunological cross-reactivity, GSTs have been grouped in to species-independent classes of isozymes [Mannervik, 1985].

\[
\text{G-SH} + \text{PNBC} \xrightarrow{\text{Glutathione S-Transferase}} \text{G-PNBC Conjugate}
\]

Where,

G-SH - Glutathione, reduced form.
PNBC - p-nitrobenzyl chloride.
G-PNBC conjugate - Glutathione p-nitrobenzyl chloride conjugate.

3.5 Statistical analysis

Experimental results were grouped according to the treatment, and the arithmetic average was calculated for each group from the values for each individual with that group. This average was expressed as the mean ± the standard error of the mean (SEM) for six determinations. Experimental data were analyzed statistically by one-way analysis of variance (ANOVA). Tukey’s multiple comparison tests was used to determine significant differences between means. The p value corresponding to the test statistic value was reported to denote the degree of significance. PRISM Instat software was used for statistical analysis.