CHAPTER-3

MATERIAL AND METHODS

3.1. Plant material

The fresh whole plant of *Combretum albidum* (Family: Combretaceae) and *Root of Salacia fruticosa* (Family: Hippocrateaceae) were collected from Tirunelveli and Kanyakumari district of Tamilnadu, India, in the month of February. These plants were identified and authenticated by Dr. V. Chelladurai, Research Officer (Retired), Botany Central Council for Research in Ayurveda and Sidha(C.C.R.A.S.), Govt. of India. The voucher specimen (KPCP3 and KPCP4/2014), was deposited for future reference in the Dept. of Pharmaceutical chemistry laboratory, Kamalakshi Pandurangan college of Pharmacy, Tiruvannamalai, Tamilnadu, India. The plant materials were washed with water and dried at room temperatures under shade, made into a coarse powder with the mechanical grinder, passed through 40 mesh sieves and stored in closed containers for further use.

3.2. Extraction of plants materials

3.2.1. Extraction for Phytochemical analysis and animal activity

The dried, coarsely powdered whole plant materials of *Combretum albidum* (500g) and roots of *Salacia fruticosa* (500g) were extracted separately to exhaustion in a Soxhlet apparatus with ethanol [90%v/v -Merk Chemicals, India] at 60°C. Then all the solvent were completely recovered on the ethanol extract of *Combretum albidum* (EECA) and *Salacia fruticosa* (EESF) under reduced pressure by a rotary vacuum evaporator. The concentrated extract were dried on a water bath and preserved in vacuum desiccator.
3.2.2. Extraction for GC-MS/MS analysis

About 25 g powder of TM was soaked in 30 ml of ethanol overnight and then filtered through filter paper. The filtrates was used for investigation of phytochemicals by GC-MS/MS analysis.

3.3. Chemicals, Reagents and Drugs

Silymarin and Paracetamol were obtained from Micro labs, Tamilnadu, India. ALT, AST, ALP, Bilirubin and Total Protein kits were procured from Span Diagnostics, Surat, India. Thiobarbituric acid (TBA), nitro blue tetrazolium chloride (NBT), Phenazine methosulphate was purchased from Central Drug House, New Delhi, India and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), carbon tetrachloride purchased from SICCO Research Laboratory, Mumbai, India. All other chemicals reagents and solvent were of analytical grade and commercially available.

3.4. PHYTOCHEMICAL ANALYSIS

3.4.1. Preliminary Qualitative phytochemical screening

The concentrated ethanol extract of *Combretum albidum* (EECA) and *Salacia fruticosa* (EESF) were subjected to various colour reactions to identify the nature of the phytoconstituents.

3.4.1.1. Test for Alkaloids

Mayer’s test

To a few ml of plant sample extract, two drops of Mayer’s reagent are added along the sides of test tube. Appearance of white creamy precipitate indicates the presence of alkaloids. [Evans, 1997]
**Wagner’s test**

A few drops of Wagner’s reagent are added to few ml of plant extract along the sides of test tube. A reddish-Brown precipitate confirms the test as positive. [Wagner, 1993].

**Dragendroff’s test**

To the extract, Dragendroff’s reagent was added. The mixture was heated and observed for the formation of reddish orange color precipitate which indicated the presence of alkaloids. [Evans, 1997]

3.4.1.2. Test for Amino acids

The extract (100 mg) is dissolved in 10 ml of distilled water and filtered through Whatmann No. 1 filter paper and the filtrate is subjected to test for Amino acids.

**Ninhydrin test**

Two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) are added to 2 ml of aqueous filtrate. Appearance of purple colour indicates the presence of amino acids.[Yasuma, 1953]

3.4.1.3. Test for Carbohydrates

**Molish’s test**

To 2 ml of plant sample extract, two drops of alcoholic solution of α-naphthol are added. The mixture is shaken well and few drops of concentrated sulphuric acid is added slowly along the sides of test tube. A violet ring indicates the presence of carbohydrates.
**Benedict’s test**

To 0.5 ml of filtrate, 0.5 ml of Benedict’s reagent is added. The mixture is heated on a boiling water bath for 2 minutes. A characteristic coloured precipitate indicates the presence of sugar.

**3.4.1.4. Test for Fixed oils and Fats**

**Spot test**

A small quantity of extract is pressed between two filter papers. Oil stain on the paper indicates the presence of fixed oils.

**Saponification test**

A few drops of 0.5 N alcoholic potassium hydroxide solution is added to a small quantity of extract along with a drop of phenolphthalein. The mixture is heated on a water bath for 2 hours. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats. [Kokate, 1999]

**3.4.1.5. Test for Glycosides**

For 50 mg of extract is hydrolysed with concentrated hydrochloric acid for 2 hours on a water bath, filtered and the hydrolysate is subjected to the following tests. [Evans, 1997]

**Borntrager’s test**

To 2 ml of filtered hydrolysate, 3 ml of chloroform is added and shaken, chloroform layer is separated and 10% ammonia solution is added to it. Pink colour indicates presence of glycosides

**Legal’s test**

50 mg of extract is dissolved in pyridine, sodium nitroprusside solution is added and made alkaline using 10% NaOH. Presence of glycoside is indicated by pink colour.
3.4.1.6. Test for Phenolic compounds and Tannins

**Ferric Chloride test**

The extract (50 mg) is dissolved in 5 ml of distilled water. To this few drops of neutral 5% ferric chloride solution are added. A dark green colour indicates the presence of phenolic compound/Tannins. [Mace, 1963]

**Gelatin test**

The extract (50 mg) is dissolved in 5 ml of distilled water and 2 ml of 1% solution of Gelatin containing 10% NaCl is added to it. White precipitate indicates the presence of phenolic compounds. [Evans, 1997].

**Lead acetate test**

The extract (50 mg) is dissolved in of distilled water and to this 3 ml of 10% lead acetate solution is added. A bulky white precipitate indicates the presence of phenolic compounds/Tannins.

3.4.1.7. Test for flavones and flavonoids

**Alkaline reagent test**

An aqueous solution of the extract is treated with 10% ammonium hydroxide solution. Yellow fluorescence indicates the presence of flavonoids.

**Magnesium and Hydrochloric acid reduction (Schinoda’s) test**

The extract (50 mg) is dissolved in 5 ml of alcohol and few fragments of magnesium ribbon and concentrated hydrochloric acid (drop wise) are added. If any pink to crimson colour develops, presence of flavonol glucosides is inferred. [Harborne, 1998].
3.4.1.8. Test for phytosterols

**Libermann-Burchard’s test**

The extract (50 mg) is dissolved in of 2 ml acetic anhydride. To this, 1 or 2 drops of concentrated sulphuric acid are added slowly along the sides of the test tube. An array of colour change shows the presence of phytosterols (steroids and terpenes [Finar, 1986]

**Salkowski Test**

1 ml of concentrated sulphuric acid was added to 10 mg of extract dissolved in 1 ml of chloroform. A reddish-blue color exhibited by chloroform laye and green fluorescence by the acid layer suggested the presence of triterpenoids

3.4.1.9. Test for Proteins

The extract (100 mg) is dissolved in 10 ml of distilled water and filtered through Whatmann No. 1 filter paper and the filtrate is subjected to test for proteins.

**Millon’s test**

To 2 ml of filtrate few drops of Millon”s reagent are added. A white precipitate indicates the presence of proteins. [ Rasch, 1960].

**Biuret test**

2 ml of filtrate is treated with 1 drop of 2% copper sulphate solution. To this 1 ml of ethanol (95%) is added, followed by excess of potassium hydroxide pellets. Pink colour ethanolic layer indicates the presence of protein. [ Gahan, 1984].

3.4.1.10. Test for Saponins

The extract (50 mg) is diluted with distilled water and made up to 20 ml. The suspension is shaken in a graduated cylinder for 15 minutes. A two cm layer of foam indicates the presence of saponins. [Kokate, 1999]
3.4.1.11. Test for gum and Mucilages

The extract (100 mg) is dissolved in 10 ml of distilled water and to this 2 ml of absolute alcohol is added with constant stirring. White or cloudy precipitate indicates the presence of Gums and Mucilages. [Whistler, 1993]

3.4.2. GC-MS/MS analysis of Phytochemicals in CA and SF

The GC-MS/MS is a Scion 436-GC Bruker version coupled with a Triple quadruple mass spectrophotometer with fused silica capillary column BR-5MS (5%Diphenyl/ninety five% Dimethyl polysiloxane) and length: 30m; internal diameter: 0.25 mm; Thickness: 0.25 μm. Helium gas (99.999%) have become used as the provider gas at a consistent go with the flow rate of 1ml/min and an injection volume of 2 μl was employed (split ratio of 10:1). The column oven temperature application was as follows: 80 °C maintain for 2 min, upto 160 °C at the rate of 20 °C/min-No hold, up to 280 °C on the charge of 5°C/min-No maintain, as much as 300°C on the charge of 20 °C/min-10 min hold, Injector temperature 280 °C and total GC running time b became 41 min. This closing increase has become to clean the column from any residues.

The mass spectrometer became operated within the positive electron ionization (EI) mode with ionization energy of 70eV. The solvent delay was 0-3.0 min. A scan interval of 0.5 seconds and fragments from m/z 50 to 500 Da was programmed. The inlet temperature was set at 280 °C, source temperature 250 °C. The relative percentage amount of every component became calculated by way of comparing its average height area to the total areas. Software program adopted to handle mass spectra and chromatograms was MS Work station 11. The NIST Version 2.0 library database of National Institute Standard and Technology (NIST) having more than 62,000 patterns become used for identifying the chemical components. The
The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials have been ascertained [Srinivasan et al., 2015]. The GC-MS/MS was performed by Food Safety and Quality Testing Laboratory, Institute of crop processing technology, Thanjavur.

3.5. Animals

Studies have been carried out the usage of Wister albino male rats (180-200g), bought from Indian Veterinary Preventive Medicine (IVPM), Ranipet, Tamilnadu, India. The animals had been grouped and housed in polyacrylic cages (38 x 23 x 10 cm) with not greater than six animals per cage and maintained at an ambient temperature of 25±2°C and 55-65% relative humidity. A 12±1hr light and dark schedule was maintained in the animal house till the animals were acclimatized to the laboratory conditions. The animals were fed with general pellet diet furnished by using the Poultry Research Station, Nandhanam, India, and sparkling water ad libitum. All animal studies were approved by Institutional Animal Ethical Committee (IAEC) in accordance with the guidelines of CPCSEA [Ref.no.745/RE/PO/S/2003/CPCSEA/KPVP/03]

3.6. Acute Toxicity Study of CA and SF Extract

An acute oral toxicity study was performed as per OECD-423 guidelines (acute toxic class method), Albino rats (n=6) of single-sex, same age group and weight were selected for the study. The animals were administered with a single oral dose of 2000mg/kg body weight of ethanolic extract of CA and SF, respectively to overnight fasted rats and the food was withheld for a further 3-4 hours after administration of the drug. Animals were observed individually at least once during the first 30 mins after dosing, periodically during the first 24 hours (with special
attention during first 4 hours) and daily there after for a period of 14 days. Once daily cage side observation included changes in skin, far eyes, mucous membrane (nasal) and behavior pattern. Attention should be given to salivation, diarrhea, sleep and coma, etc. [Chandan et al., 2007; OECD, 2000]

3.7. *IN VIVO* HEPATO protective STUDY

3.7.1. Experimental design

**CCL₄-induced hepatotoxicity**  [Shukla et al., 1997]

After acclimatization, the rats were divided into 7 groups of 6 rats each.

**Group I:**

Served as Normal control which received liquid paraffin 2ml/kg body weight (b. w), intraperitoneal (I. P).

**Group II to V:**

Were administered with CCl₄ in liquid paraffin (1:2) in the dose 1 ml/kg body weight I. P, once in every 72 h for 16 days (1,4, 7, 10, 13, 16 d).

**Group III and IV:**

Were administrated with ethanolic extract of *Combretum albidum* at the dose of 250 mg/kg and 500 mg/kg body weight orally once in every 24 h for 16 days respectively.

**Group V and VI:**

Were administrated with ethanolic extract of *Salacia fruticosa* at the dose of 250 mg/kg and 500 mg/kg body weight orally once in every 24 h for 16 days respectively.

**Group-VII:**

Was administrated with reference drug Sillymarin at the dose of 25 mg/kg body weight orally once in every 24 h for 16 days.
3.7.2. Estimation of biochemical parameters

The biochemical parameters were determined after 24 h fasting of the last dose. Blood was obtained from all animals by puncturing retro orbital plexus. The blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm at 30 °C for 15 min and used for the estimation of various biochemical parameters.

3.7.2.1. Estimation of Aspartate transaminase (AST) and Alanine transaminase (ALT)

Activities of AST and ALT were assayed by the method of Reitman and Frankel (1957). AST catalyses the transfer of an amino acid from L-aspartate (L-Asp) to alpha-ketoglutarate (α-KG) to yield oxaloacetate and L-glutamate (L-Glu). ALT catalyses the transfer of an amino acid from L-alanine (L-Ala) to alpha-ketoglutarate to yield pyruvate and L-glutamate. The liberated oxaloacetate and pyruvate react with 2,4-dinitrophenyl hydrazine to form 2,4-dinitrophenyl hydrazone, which was read at 540 nm.

\[
\begin{align*}
L\text{-Asp} + \alpha\text{-KG} & \rightarrow \text{Oxaloacetate} + L\text{-Glu} \\
L\text{-Ala} + \alpha\text{-KG} & \rightarrow \text{Pyruvate} + L\text{-Glu}
\end{align*}
\]

Reagents

1. Phosphate buffer - 0.1M, pH 7.5
2. Substrate
a) Aspartate transaminase: 0.3g of dl - aspartic acid and 50mg of α - keto-glutaric acid were dissolved in 20ml of phosphate buffer. The pH was adjusted to 7.5 with 1N sodium hydroxide and made up to 100 ml with phosphate buffer.

b) Alanine transaminase: 5.0g of dl - alanine and 20mg of α-keto-glutarate acid were dissolved in 20 ml of phosphate buffer. The pH was adjusted to 7.5 with 1N sodium hydroxide and made up to 100ml with phosphate buffer.

3. DNPH: 200 mg of 2, 4 dinitrophenyl hydrazine (DNPH) was dissolved in hot 1N hydrochloric acid and made up to 100ml with the same.

4. Aniline- citrate reagent: 50g of citric acid was dissolved in 50ml of distilled water and to this was added an equal volume of redistilled aniline.

5. Sodium hydroxide - 0.4N

6. Standard pyruvate solution: 12.5mg of sodium pyruvate was dissolved in 100ml of phosphate buffer.

Procedure

One ml of substrate was incubated for few minutes at 37°C, then 0.2 ml of serum was added and incubated for one hour in the case of AST and 30 minutes for ALT, then 2 drops of aniline citrate reagent were added to both test and control. To the control, serum was added after incubation.

The reaction was arrested using 1.0 ml of DNPH solution and the tubes were kept at room temperature for 20 minutes. 1.0 ml of 0.4N sodium hydroxide was added to all the tubes. Sets of standards were also treated in similar manner. The colour developed was read at 540 nm.
Activities of aspartate transaminase and alanine transaminase were expressed as IU/L/min/mg protein.

3.7.2.2. Estimation of serum alkaline phosphatase (ALP)

Alkaline phosphatase was assayed by the method of King (1965) using disodium phenyl phosphate as the substrate. Disodium phenyl phosphate is hydrolysed by alkaline phosphatase with the liberation of phenol, which reacts under alkaline condition with Folin phenol reagent to form blue color, which was estimated colorimetrically at 680 nm.

**Reagents**

1. Bicarbonate buffer - 0.1 m, (pH -10)
2. Substrate - 0.01m - phenyl phosphate di-sodium salt solution.
3. Folin - Phenol reagent
4. Sodium carbonate - 10%
5. Standard phenol solution -5µg/ml

**Procedure**

An incubation mixture containing 150 µmoles of bicarbonate buffer and 10µmoles substrate in 2.9 ml - distilled water was pre - incubated at 37°C for 10 minutes. 0.2 ml of serum was added to this and incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1.0ml of Folin - phenol reagent. The suspension was centrifuged and 2.0ml of 10% sodium carbonate was added to the supernatant. The solution was incubated at 37°C for 10 minutes. The standard phenol solution (2.5 µg - 10 µg) was also treated with Folin - phenol reagent and sodium carbonate. The blue colour developed was read at 680 nm.

The enzyme activity was expressed as KA units / dl.
3.7.2.3. Estimation of serum bilirubin

Serum bilirubin was estimated by the method of Malloy and Evelyn (1937). Serum bilirubin was estimated by Vanden Bergh reaction. It was based on the formation of purple colored azobilirubin, which reacts with diazotized sulphanilic acid.

Reagents

1. Absolute methanol.
2. Hydrochloric acid - 1.5%
3. Diazo reagent:
   Solution - A: 1.0g of sulphanilic acid was dissolved in 15 ml of concentrated hydrochloric acid and made up to 1L with distilled water.
   Solution - B: 0.5g of sodium nitrate was dissolved in water and made up to 100 ml.
   Freshly prepared before use by adding 0.3 ml of solution-B to 10 ml of solution - A
4. Standard bilirubin solution: 10mg/100ml choloroform.

Procedure

0.2 ml of serum was diluted to 2.0 ml with distilled water in two tubes marked as test and blank. The test and blank were taken and 0.5 ml of the diazo reagent and 0.5 ml of 1.5% hydrochloric acid were added. Finally to both tubes 2.5 ml of methanol was added and the tubes were kept at room temperature for 30 minutes. The colour developed was read at 540nm. For a standard curve, one in five dilution of stock standard in methanol was made to obtain a solution containing 2.0 mg/100 ml. The amount of serum bilirubin was expressed as mg/dl.
3.7.2.4. Estimation of protein

Protein in the enzyme extract was determined after trichloro acetic acid precipitation by the method of Lowry et al. (1951).

The -CONH group in the protein molecule reacts with the copper sulphate in alkaline medium to give a blue colour, which was read at 680 nm.

Reagents

1. Alkaline copper reagent:
   Solution A: 2% sodium carbonate in 0.1N sodium hydroxide.
   Solution B: 0.5% copper sulphate in water.
   Solution C: 1% sodium potassium tartarate in distilled water.
   50 ml of solution A was mixed with 0.5 ml of solution B and 1.0 ml of solution C just before use.

2. Folin’s phenol reagent:
   The reagent was diluted in the ratio of 1:2 with double distilled water just before use.

3. Standard bovine serum albumin (BSA):
   100 mg of BSA was dissolved in 100 ml of 0.1N sodium hydroxide in a standard flask. Small quantities of alkali could be added for complete dissolution of BSA. 10.0 ml of the stock was diluted to 100 ml to get a working standard containing 100 µg/ml.

Procedure

0.5 ml of tissue homogenate was mixed with 0.5 ml of 10% TCA and centrifuged for 10 minutes. The precipitate was dissolved in 1.0 ml of 0.1N sodium
hydroxide. From this an aliquot was taken and to this 5.0 ml of alkaline copper reagent was added, allowed to stand at room temperature for 10 minutes, 0.5 ml Folin’s reagent was added to all tubes and the blue colour developed read after 20 minutes at 680 nm. A standard curve was obtained using bovine serum albumin and was used to determine the tissue protein level for enzyme activity. The protein levels were expressed as mg/dl tissue.

3.7.3. Preparation of liver tissue homogenate

After the collection of blood samples, the rats were anesthetized with diethyl ether and acrificed by cervical dislocation for separation of the liver. The dissected liver tissue were washed with normal saline (0.9%) and divided into two parts. A part of liver tissue was homogenated in ice-cold phosphate buffer (5%), and then centrifuged at 1000 rpm for 10 min followed by centrifugation of the supernatant at 12000 rpm for 15 min to get the mitochondrial fractions. These fractions were used for the estimations of tissue antioxidant properties.

3.7.4. Lipid peroxidation and antioxidant Study

3.7.4.1. Estimation of thiobarbituric acid reactive substance (TBARS)

The level of lipid peroxidation in the tissue was measured as TBARS according to the method of Ohkawa et al. (1979).

**Principle**  This method was used to measure spectrophotometrically the color produced by the reaction of TBA with malondialdehyde (MDA). The MDA-TBA adduct formed during the reaction in acidic medium was extracted to the organic layer and the absorbance was measured at 532 nm.

**Procedure**

4 ml of reaction mixture containing 0.4 ml of the tissue homogenate, 1.5 ml of 0.8 % TBA, 1.5 ml of acetic acid (20 %, pH 3.5) and distilled water was
kept for 1 h in a boiling water bath at 95°C. After 1 hr, the reaction mixture was removed from the water bath, cooled and added 1 ml of distilled water. 5 ml of butanol: pyridine mixture (15:1) was added to the reaction tube, mixed well and centrifuged at 3000 rpm for 10 min. Absorbance of the clear supernatant was measured at 532 nm against a blank that contained no tissue homogenate.

TBARS in tissue were expressed as nmol/100 g tissue.

3.7.4.2. Estimation of Reduced glutathione (GSH):

Reduced glutathione (GSH) level was estimated by the method of Sedlak and Lindsay (1968).

**Principle**

The assay is based on the reaction of GSH with DTNB that gives a yellow colored compound, namely, 2-Nitro-5-thiobenzoic acid.

**Procedure**

Tissue homogenate was added to distilled water and 50% TCA solution to precipitate the protein, mixed and centrifuged at 3000×g for 10 min. The resulting supernatant so obtained after centrifugation was then taken for GSH estimation. To the supernatant, 0.4 M Tris buffer solution (pH 8.9) and DTNB was added and then the intensity of the color was read at 412 nm. The GSH content in the samples was calculated using a molar extinction coefficient of 14,150 M$^{-1}$cm$^{-1}$ and expressed as nanomoles/mg protein.

3.7.4.3. Estimation of Super oxide dismutase

The activity of superoxide dismutase (SOD) was measured according to Misra and Fridovich (1972).
Principle

This activity is based on the ability of SOD to inhibit the autoxidation of epinephrine at alkaline pH. An indirect method of inhibiting auto-oxidation of epinephrine to its adrenochrome was used to assay SOD activities.

Procedure

Auto-oxidation of epinephrine was initiated by adding a 0.1ml of tissue homogenate was added to the tube containing 0.75 ml ethanol and 0.15 ml chloroform (chilled in ice) and centrifuged. To 0.5 ml of supernatant, added 0.5 ml of EDTA solution and 1 ml of buffer. The reaction was initiated by the addition of 0.5 ml of epinephrine and the increase in absorbance at 480 nm was measured in an UV-Visible Spectrophotometer (Systronics UV-Visible Spectrophotometer 117, India). The enzyme activity was expressed as 50% inhibition of epinephrine auto oxidation/min.

3.7.4.4. Estimation of Catalase

The Catalase activity was measured by the method of Takahara et al.

Principle

Decomposition of hydrogen peroxide by catalase was measured in terms of decreasing the absorption with time. The enzyme activity could be arrived at from this decrease.

Procedure

To 1.2 ml of phosphate buffer, 0.2 ml of the tissue homogenate was added and the enzyme reaction was started by the addition of 1.0 ml of H2O2 solution. The decrease in absorbance was measured at 240 nm at 30 sec intervals for 3 min. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide.
3.7.4.5. Estimation of Glutathione S-transferase (GST)

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

**Principle** The enzyme activity was measured by following the increase of absorbance at 340 nm of CDNB - GSH conjugate generated as a result of GST catalysis between glutathione and 1- chloro-2, 4-dinitrobenzene (CDNB). GST CDNB + GSH • CDNB - GSH (conjugate product)

**Procedure**

In 0.1 ml of cytosol fraction (supernatant) (10%w/v in 0.15 M KCl), 2.7 ml of 1.0 mM glutathione solution (prepared in 0.2 M Phosphate buffer) and 0.2 ml CDNB (1.0 mM) substrate prepared in acetone were mixed. The change in absorbance at 340 nm was recorded at room temperature after 15 seconds each for 3 minutes against a blank containing 0.1 ml DDW in place of supernatant. Protein content in enzyme source was also determined.

The enzyme activity was expressed as µmoles of CDNB - GSH conjugate formed per minute/mg protein.

3.7.4.6. Estimation of glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) was estimated by the method of Rotruck et al. (1973). A known amount of enzyme preparation was allowed to react with H₂O₂ in the presence of GSH for a specified time period. The remaining GSH content was measured.

**Procedure**

To 0.2 ml Tris buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide and 0.5 ml of tissue homogenate (Tris buffer - 0.4M, pH 7) were added. To the mixture, 0.2
ml of GSH followed by 0.1 ml of H₂O₂ was added. The contents were mixed well and incubated at 37°C for 10 minutes, along with a control containing all reagents except tissue homogenate. After 10 minutes, the reaction was arrested by the addition of 0.5 ml of 10% TCA. The tubes were centrifuged and the supernatants were assayed for GSH by the method of Ellman.

The activity was expressed as mg of GHS consumed/minute/mg protein.

3.8. *INVIVO NEPHROPROTECTIVE STUDY*

3.8.1 Experimental design

**APAP induced Nephrotoxicity**

After acclimatization, the Rats were divided randomly into six groups (I–VI) of six rats.

**Group I**: Rats served as untreated control and was fed orally with normal saline 5ml/kg body weight daily for 7 days.

**Group II**: Rats (APAP only) were similarly treated as Group I.

**Groups III and IV** rats were treated with EECA 250 mg/kg and EECA 500 mg/kg for 7 days, respectively.

**Groups V and VI** rats were treated with EESF 250 mg/kg and EESF 500 mg/kg for 7 days, respectively.

On the 7th day, acetaminophen suspension was given by oral route, in a dose of 750 mg/kg body weight to all the groups of rats except the rats in Groups I.

3.8.1.1. **Biochemical parameters assessed for renal function**

After 48 h, animals had been sacrificed with the aid of chloroform anaesthesia. Blood samples had been collected by using cardiac puncher, the usage of 21 gauges (21 G) needles mounted on a 5ml syringe (Hindustan Syringes and clinical devices Ltd, Faridabad, India) and divided into two parts. The blood was once
centrifuged for 10min at 5000 rpm. The acquired clear sera had been stored at −20°C for subsequent estimation of urea (UR), creatinine (CR) and uric acid (UA) levels utilizing colorimetric assay kits, Bayer (Seamon) as per the producer's direction.

3.8.2.1. Estimation of urea

Urea was determined in the blood by the method of Natelson et al. (1951).

Reagents

1. Sodium tungstate solution (10%)
2. Sulphuric acid (0.67 N)
3. Diacetyl monoxime (DAM) reagent: 2g of DAM was dissolved in 100 ml of 2% acetic acid.
4. Sulphuric acid-phosphoric acid mixture: 25 ml of concentrated sulphuric acid was mixed with 75 ml of 85% O-phosphoric acid and 70 ml distilled water.

Procedure

To 0.1 ml of blood, 3.3 ml of water was added and mixed with 0.3 ml of 10% sodium tungstate and 0.3 ml of 0.67 N sulphuric acid. The suspension was centrifuged and to 1.0 ml of the supernatant, 1.0 ml of water, 0.4 ml of DAM reagent and 2.6 ml of sulphuric acid - phosphoric acid reagent were added in the above order and kept in boiling water for 30 minutes. It was cooled and the colour developed was measured at 480 nm in a Shimadzu UV spectrophotometer. Aliquots of standard urea were also treated in a similar manner. The values were expressed as mg/dl blood.

3.8.2.2. Estimation of creatinine

Creatinine was estimated according to the method of Broad and Sirota (1948) using Jaffes reaction.
Reagents

1. Saturated picric acid
2. Sodium hydroxide (0.75 N)
3. Sulphuric acid (2/3 N)
4. Sodium tungstate (10 %)
5. Stock standard creatinine: 100 mg of creatinine was dissolved and made up to 100 ml in 0.1 N hydrochloric acid. Working standard was prepared by appropriate dilution of the stock solution.

Procedure

A protein free filtrate was prepared by precipitating 1.0 ml of serum with 8.0 ml of water, 0.5 ml of 2/3 N sulphuric acid and 0.5 ml of 40% sodium tungstate. After centrifugation, 5.0 ml of the clear filtrate was taken and 1.5 ml of saturated picric acid solution and 1.5 ml of 0.75 N sodium hydroxide were added. The colour intensity was measured at 460 nm after 15 minutes in a UV spectrophotometer. Standard and blank were also processed similarly.

3.8.2.3. Estimation of Uric acid

Uric acid was estimated in the serum according to the method of Caraway (1963).

Reagents

1. Sodium carbonate (20%)
2. Colouring reagent: 50g of molybdate free sodium tungstate was dissolved in 400 ml of distilled water followed by the addition of 40 ml phosphoric acid and refluxed for 2 hours. A drop of bromine was added, cooled and diluted to 500 ml with water.
3. Standard uric acid: 100 mg of uric acid was dissolved in 150 ml of water containing 60 mg of lithium carbonate by heating at 60°C. The solution was cooled at room temperature and 2 ml of formaldehyde (400 ml/l), diluted to about 500 ml and slightly acidified with sulphuric acid was added.

4. Working standard: 1.0 ml of the stock standard and 2.0 ml of 300 g/l BSA were diluted to 10 ml with water. The working standard was prepared freshly. Albumin was added to account for the positive error induced by a co-precipitation of uric acid and proteins.

**Procedure**

5.4 ml of diluted tungstic acid was added to 0.6 ml of plasma. The contents were mixed and centrifuged. To 3 ml of supernatant in the test tubes, standard and water (as blank) were taken. 0.6 ml of sodium carbonate and 0.6 ml of phosphotungstic acid reagent were added, mixed and placed in a water bath at 25°C for 10 minutes. The blue colour developed was read at 700 nm in a Shimadzu UV spectrophotometer.

Uric acid values were expressed as mg/dl of serum.

**3.8.3 Hematological study**

After 48 h, animals were sacrificed by chloroform anaesthesia. Blood samples were collected by cardiac puncture under diethyl ether anesthesia, using 21 gauge (21 G) needles mounted on a 5 ml syringe (Hindustan Syringes and Medical Devices Ltd., Faridabad, India.) and divided into portions. A portion of blood was mixed into ethylene diamine tetra-acetic acid (EDTA) - coated sample bottles to analyze hematological parameters of complete blood count like hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular
hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet concentration (PLC). These parameters were analyzed using automatic hematological system (Sysmex Hematology - Coagulation system, Model MO-1000 I, Trans Asia, Japan).

3.8.4 Preparation of Renal tissue homogenate

The kidneys have been eliminated and dissected free from the encircling fats and connective tissue. Each kidney used to be longitudinally sectioned, and renal cortex was separated and kept at −8°C. Along these lines, the renal cortex used to be homogenized in ice-cold potassium phosphate buffer (0.05 M, pH 7.4). The renal cortex homogenates had been centrifuged at 5000 rpm for 10 min at 4°C. The ensuing supernatant was used for the determination of malondialdehyde (MDA) content, reduced glutathione (GSH) and antioxidant enzyme like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activity using colorimetric assay.

3.8.4.1 Estimation of MDA

Measurement of MDA content was according to the method of Zhang using 500 ml

**Principle** This method was used to measure spectrophotometrically the color produced by the reaction of TBA with malondialdehyde (MDA). The MDA-TBA adduct formed during the reaction in acidic medium was extracted to the organic layer and the absorbance was measured at 532 nm.

**Procedure**

TCA-TBA basic mixture containing 101.25 g chloroacetic acid (TCA) and 2.5 g thiobarbituric acid (TBA). 1 ml enzyme extract was injected in 4 ml TCA-TBA basic mixture. Then the reaction mixture was incubated in boiling water for 20
minutes. After cooling, the reaction mixture was centrifuged at 4000 × g for 10 minutes. The absorbance of supernatant liquid was measured at 532 nm and 600 nm.

3.8.4.2 Estimation of Reduced glutathione (GSH):

Reduced glutathione (GSH) level was estimated by the method of Sedlak and Lindsay (1968). As described in section 3.7.4.2.

3.8.4.3 Estimation of Super oxide dismutase

The activity of superoxide dismutase (SOD) was measured according to Misra and Fridovich (1972) as described in section 3.7.4.3.

3.8.4.4 Estimation of Catalase

The Catalase activity was measured by the method of Takahara et al 1960 as described in section 3.7.4.4

3.8.4.5 Estimation of glutathione peroxidase (GPx)

Glutathione peroxidase activity was determined according to the method of Hafemann et al. (1974).

Principle

The activity of GPx was determined by measuring the decrease in GSH content after incubating the sample in the presence of H2O2 and NaN3.

H2O2 + 2 GSH 2H2O + 2 GSSG

Procedure

Tissue homogenate (approximately 0.5 mg protein) was incubated with 0.1 ml of 5mM GSH, 0.1 ml of 1. 25 mM H2O2, 0.1 ml of 25 mM NaN3 and phosphate buffer (0.05 mM, pH 7) in a total volume of 2.5 ml at 37°C for 10 min. The reaction was stopped by adding 2 ml of 1.65 % HPO32- and the reaction mixture was centrifuged at 1500 rpm for 10 min. 2 ml of the supernatant was mixed with 2 ml 0.4 M Na2HPO4 and 1 ml of 1mM DTNB. The absorbance of the yellow colored complex
was measured at 412 nm after incubation for 10 min at 37°C against distilled water. A sample without the tissue homogenate processed in the same way was kept as nonenzymatic reaction.

3.9. HISTOPATHOLOGICAL STUDIES

3.9.1. Histopathology of CCl₄ induced liver injury

After the collection of blood samples the rats were sacrificed and their livers were excised, rinsed in ice-cold normal saline and processed separately for histological observation. Initially the materials were fixed at 10% buffered neutral formalin solution for 48 hours and then with bovine solution for 6 hours. Paraffin sections were taken at 5mm thickness processed in alcohol-xylene series and was stained with alum hematoxylin and eosin. The sections were examined under photomicroscope for histopathological changes, necrosis, steatosis and fatty changes of hepatic cells.[Valeer et al., 2003]

Processing of tissue and staining

For cutting sections of different tissues in paraffin, dehydration and cleaning of the tissues were carried out at room temperature. The tissues were first washed in two changes of 70% alcohol for one hour each, dehydrated for two hours, further dehydrated for one hour each in two changes of 80% alcohol, graded twice in 95% alcohol and in absolute alcohol and cleared through a mixture of absolute alcohol. To remove the alcohol, chloroform was preferred over xylene as it did not cause the tissue to become hard and brittle. The tissues, after clearing, were left in a mixture of chloroform and paraffin wax (approximately 1:1) at room temperature over night. Before embedding, the tissues were impregnated in three changes of paraffin wax with cerasin of 58 to 60°C melting point for one hour each. Transverse sections of 5 µm thickness were cut using a manual rotator microtome. After deparaffinising in
xylene, the sections were hydrated through graded series of alcohol up to 70% and stained with Harris Alum Hematoxylin and counterstained with 1% alcoholic eosin (Preece, 1972). Applying the routine procedure, stained sections were dehydrated through the graded series of alcohol and mounted with glass cover slip in DPX through xylene.

**Light microscopy and Photomicrography**

The histology sections were studied using Carl Zeiss binocular compound microscope. Photomicrographs were taken with camera (Canon) attached to Carl Zeiss microscope with projection eyepiece and objectives 45X.

### 3.9.2. Histopathology of APAP induced Kidney damage

Pieces of a kidney segment from each group have been fixed right away with 10% neutral formalin for a duration of at least 24 h, dehydrated in graded (50–100%) alcohol, embedded (e in paraffin, reduce into 4–5 μm thick sections and stained with hematoxylin–eosin. The sections had been evaluated for the pathological symptoms of nephrotoxicity including necrosis, fatty infiltration, fibrosis, lymphocyte infiltration, etc. as described in section 3.9.1.

### 3.10. Statistical analysis

The investigations of animal experimental results were expressed as mean ± SD of six animals from each group. One-way ANOVA followed by Dunnetts tests have used to analyze the data by Graph pad prism software. P < 0.05 was considered statistically significant.