2. MATERIALS AND METHODS

2.1 PLANT MATERIAL

The leaves of Caesalpinia sappan (Family: Pontederiaceae) and Clitoria ternatea L. (CT) (Family: Berberidaceae) were collected from Nilgiri hills Ooty, Tamilnadu, India in May 2010. The plant materials were cleaned with distilled water, shade dried at room temperature and taxonomically identified and authenticated by Dr. S. Rajan, Field Botanist, Survey of Medicinal Plants and Collection Unit, Central Council for Research in Homoeopathy, Department of AYUSH, Ministry of Health and Family Welfare Govt. of India. Udhagamandalam. Voucher specimen (CAHC-01&2/2007-2008) has been retained in the Dept of Botany C. Abdul Hakeem College, Melvisharam, Tamilnadu, India. The shade dried plant materials were powdered by using electric blender.

2.1.1 Preparation of plant extracts

The powdered plant materials were extracted separately to exhaustion in a soxhlet apparatus using chloroform, ethyl acetate and ethanol solvent (Merk Chemicals, India) systems. All the extracts were filtered through a cotton plug followed by Whatman filter paper (No.1). All the solvents were completely removed by rotary vacuum evaporator. The extracts were freeze dried and stored in vacuum dedicator. The dry chloroform, ethyl acetate and ethanol extracts of CS and CT were dissolved in 0.25% DMSO in serum free culture medium at required concentrations (200, 400 and 600 μg/ml) for in vitro studies. Another set of ethanol crude extracts was taken for in vivo studies.
2.2. IDENTIFICATION OF PHYTOCOMPONENTS IN CS & CT EXTRACTS (GC-MS STUDY)

The identification of the chemical composition of ethanol extract of CT and CS was performed using a GC-MS spectrograph (Agilent 6890/Hewlett-Packard 5975) fitted with electron impact (EI) mode. The ethanol extract (2.0µl) of CT and CS was injected with a Hamilton syringe to the GC-MS manually for total ion chromato- 125 graphic analysis in split mode. In quantitative analysis, selected ion monitoring (SIM) mode was employed during the GC-MS analysis. SIM plot of the ion current resulting from very small mass range with only compounds of the selected mass were detected and plotted.

2.3. IN VITRO STUDIES

2.3.1. In vitro Hepatoprotective activity of CS and CT extract

2.3.1.1. HepG2 Cell

The human derived HepG2 cell line was obtained from NCCS Pune. Cells were maintained in RPMI -1640 medium supplemented with heat inactivated fetal calf serum (FCS 10%), penicillin (100U/ml) and streptomycin (100 µg/ml). The cells were grown in 25 cm² tissue culture flasks and maintained at 37°C in a humidified, 5% CO₂ -atmosphere through out the experiment.

2.3.1.2 Experimental design

A total of 6 groups were used for the experimental studies for cell viability including trypan blue staining and MTT assay.
**Group 1:** (DMSO control 0.25% v/v): These cells were treated with DMSO alone.

**Group 2:** (Extract alone): These cells were treated with 600μg/ml extract alone.

**Group 3:** (10 mM APAP): In this group, cells were treated with 10mM acetaminophen only (Ryu et al., 2000).

**Group 4:** (200 μg/ml extract + APAP): These cells were treated with 200 μg/ml extract combined with 10 mM APAP.

**Group 5:** (400 μg/ml extract + APAP): In this group cells were treated with 400 μg/ml extract with 10 mM APAP.

**Group 6:** (600 μg/ml extract + APAP) in this group cells were treated with 600 μg/ml extract with 10 mM APAP.

The extracts were incubated for 24 hrs for trypan blue staining and 72 hrs for MTT assay. The same kind of experimental groups were designed for three different solvent extracts (Chloroform, Ethyl acetate and Ethanol) of CS and CT.

### 2.3.1.3. Cell viability

To the monolayer cultures, 50 μl of 10mM acetaminophen was added to each well followed by the addition of 50 μl of three different solvent (chloroform, ethyl acetate and ethanol) extracts of CT and CS at different concentrations (200, 400 and 600 μg/ml) and incubated at 37°C for 24 hours. After incubation, the cell viability was determined by trypan blue dye exclusion
method. MTT assay and the minimum effective concentration and effective solvent extract of CS and CT were also determined.

2.3.1.4 Trypan Blue Staining

Trypan blue is one of the several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live cells do not take up dye unlike the dead cell. After 24h incubation with the extracts and acetaminophen, cells were trypsinised and resuspended in MEM.

A cell suspension containing approximately $2.5 \times 10^5$ cells/ml was prepared in MEM and 0.2 ml of cell suspension was added, and mixed thoroughly with 0.4% trypan blue. The mixture was allowed to stand for 5 minutes. The suspension was viewed in a hemocytometer and analysed for viable cells. Viable cell count was determined as per the method described previously (Freshney 2005) by using the following calculations:

\[
\text{Cells / ml} = \text{Average cell count per square} \times \text{dilution factor} \times 10^4
\]

\[
\text{Total cells} = \text{Cells / ml original volume of fluid from which cell sample was removed.}
\]

\[
\% \text{ Cell viability} = \text{Total viable (Unstained) / Total cells (stained and unstained)} \times 100
\]
2.3.1.5. MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) Assay

The inhibitory effect of different solvent extracts on HepG2 cells was determined by MTT assay (Hussain et al., 1993). The MTT test is based on the enzymatic reduction of the tetrazolium salt in MTT in viable / metabolically active cells. HepG2 monolayer cells were trypsinized, collected and the cell count was adjusted to 1×10^5 cells/ml with MEM containing 10% fetal calf serum. 0.1 ml of the diluted cell suspension (10,000 cells) was added to each well of the 96 well microtitre plates. After 24 hours, when a partial monolayer formed the supernatant was flicked off, washed once with PBS, different extracts with different concentrations (200, 400 and 600 μg) and 10 mM acetaminophen (in 0.25% DMSO prepared in serum free culture medium) was added to the cells.

The plates were incubated at 37°C for 3 days in 5% CO₂ atmosphere and the medium in the well was discarded and 50 μl of 5 mM MTT was added to each well. The plates were gently shaken and incubated for 4 hours. The supernatant was removed and 50 μl of propanol was added and gently solubilized the formazon. The absorbance was measured using microtitre plate at 540 nm. The inhibitory rate of cell growth was calculated with the following formula.

\[
\% \text{ growth inhibition} = 1 - \frac{\text{OD extract treated}}{\text{OD negative control}} \times 100
\]

The same kind of experimental groups were designed for three different solvent extracts (Chloroform, ethyl acetate and ethanol).
2.4. IN VITRO NEPHROPROTECTIVE ACTIVITY OF CS AND CT

2.4.1. Vero cells

Vero (a normal African green monkey kidney cell line) cells were obtained from the cell bank of National Center for Cell Science (NCCS), Pune, India. Cells were maintained in RPMI -1640 medium supplemented with heat inactivated fetal calf serum (FCS 10%), penicillin (100U/ml) and streptomycin (100 μg/ml). The cells were grown in 25 cm² tissue culture flasks at 37°C in a humidified, 5%, CO₂ throughout the experiment.

2.4.2. Experimental design

The groups were of the same kind as described in section 2.3.1.2, the only difference was that here Vero cell line was used for the Cytotoxicity assay.

2.4.3. Cell viability

The viability of Vero cells were measured as mentioned in chapter 2.3.1.3.

2.4.4. Trypan blue staining

The cell viability was measured as mentioned in chapter 2.3.1.4.

2.4.5 MTT assay

The inhibitory effect of different solvent extracts on Vero cells was determined by MTT assay (Hussain et al., 1993) as described in section 2.3.1.5.
2.5 ANIMALS

Adult male *Albino* rats of Wistar strain weighing around 180 to 200 gms were procured from Tamilnadu Veterinary and Animal Sciences University, Chennai. The animals were kept in polypropylene cages (Six in each cage) 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. They were fed with commercially available rat chow (Hindustan Lever Ltd., Bangalore, India) and had free access to water. The experiments were designed and conducted with the approval of the institutional animal ethics committee (No 1011/C/06/CPCSEA).

2.6 ACUTE TOXICITY STUDIES (SAFETY EVALUATION) OF CS & CT EXTRACT

The safety study was carried out using Organisation for Economic Co-Operation (OECD) guide lines No. 423. Three male *albino* rats of the same age group and weight were given the maximum dose of CS and CT extracts of 2000 mg/kg body weight orally. The animals were observed for 1h continuously and then hourly for 4 h, and finally after every 24 h up to 15 days for any mortality or gross behavioral changes (Chandan *et al.*, 2007).

2.7 IN VIVO HEPATOTOXICITY STUDY

2.7.1 Experimental induction of hepatotoxicity
The rats were treated with single dose of acetaminophen (APAP) 750 mg/kg body weight for seven days.

2.7.1.1 Experimental protocol

Animals were divided into five groups of six animals each.

**Group 1:** Normal rats.

**Group 2:** Rats treated with acetaminophen (APAP) at 750 mg/kg body weight for seven days orally were kept as toxin control.

**Group 3:** APAP induced rats were administrated ethanolic leaf extract of CS (250 mg/kg body wt.) orally by using intra gastric tubes for 7 days.

**Group 4:** APAP induced rats were administrated ethanolic leaf extract of CS (500mg/kg body wt.) orally by using intra gastric tubes for 7 days.

**Group 5:** Rats were fed with standard drug silymarin 25 mg/kg plus APAP daily for seven days.

**Group 6:** APAP induced rat + 250 mg CT extract/kg body weight.

**Group 7:** APAP induced rat + 500 mg CT extract/kg body weight.

The extract/silymarin was administered by oral gavages 1 h before APAP administration (Deepak *et al*., 2007).
The same kind of experimental groups were designed for ethanolic extract of CT.

2.7.2 Preparation of tissue homogenate

Known amount of hepatic tissue was homogenized in Tris-HCl buffer and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was collected and stored at -80°C for assay of the marker enzymes and antioxidant studies.

2.7.3 Estimation of biochemical parameters

After the experimental period, the rats in the different groups were sacrificed by decapitation. The blood was collected from the animals and centrifuged. The serum samples were collected in separate containers for biochemical estimations. Liver tissues were also collected in ice cold container for various biochemical estimations.

2.7.3.1 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.

AST
L-Asp + α-KG $\rightarrow$ Oxaloacetate + L-Glu

ALT

L-Ala + α-KG $\rightarrow$ Pyruvate + L-Glu

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.

**2.7.3.2 Estimation of serum alkaline phosphatase (ALP)**

Alkaline phosphatase was assayed by the method of King and Armstrong (1934) 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.

**2.7.3.3 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.

2.7.3.4 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 nmol/100g tissue.

2.7.4 Lipid peroxidation and antioxidant status

2.7.4.1 Estimation of thiobarbituric acid reactive substance (TBARS)

TBARS was estimated by thiobarbituric acid assay by the method of Nichans and Samuelson (1968). In this method malondialdehyde and other thiobarbituric acid reactive substances (TBARS) were measured by their reactivity with thiobarbituric acid in the acidic condition to generate a pink coloured chromophore, which was read at 535 nm.

Reagents

1. Tris - HCl buffer - 0.025M, pH 7.5
2. 15% w/v trichloro acetic acid (TCA)
3. 0.375% w/v thiobarbituric acid (TBA)

4. 0.25N hydrochloric acid (HCl)

5. TCA-TBA-HCl - reagent (1:1:1)

6. Stock standard: 4.8 mmoles of 1,1’,3,3’-tetramethoxy propane was prepared for preparation of stock solution.

7. Working standard: Stock solution was diluted to get a concentration of 48 nmol/ml.

Procedure

Tissue homogenate was prepared in Tris-HCl buffer. 1.0 ml of the tissue, homogenate was combined with 2.0 ml of TCA-TBA-HCl reagent and mixed thoroughly. The solution was heated for 15 minutes in boiling water bath. The flocculent precipitate was removed by centrifugation at 1000g for 10 minutes. The absorbance of the sample was read at 535 nm against a blank that contained no tissue homogenate.

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

2.7.4.2 Estimation of superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was estimated by the method of Kakkar et al., (1984).

The assay of SOD was based on the formation of NADH - phenazine methosulphate - nitroblue tetrazolium formation. The reaction was initiated by addition of NADH. After incubation for 90 seconds, the reaction was stopped by
the addition of glacial acetic acid. The colour formed at the end of the reaction was extracted into butanol layer and measured at 560 nm.

Reagents

1. Sodium pyrophosphate buffer - 0.02M (pH 8.3)
2. Phenazine methosulphate (PMS) - 186 µmol
3. Nitroblue tetrazolium (NBT) - 300 µmol
4. NADH - 780 µmol
5. Glacial acetic acid
6. n - butanol
7. Chloroform
8. Ethanol

Procedure

0.5 ml of tissue homogenate (Using 0.02M phosphate buffer, pH 8.3) was diluted to 1 ml with distilled water. Then 2.5 ml of ethanol and 1.5 ml of chloroform (all reagents chilled) were added. The mixture was shaken for 1 minute at 4°C and then centrifuged. The enzyme activity in the supernatant was determined.

The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazine methosulphate, 0.3 ml of nitroblue tetrazolium, appropriately diluted enzyme preparation and water in a total volume of 3.0 ml. The reaction was initiated by the addition 0.2 ml of NADH. After
incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1ml of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged and the colour intensity of the chromogen in butanol layer was measured at 560 nm against butanol blank. The system devoid of enzyme served as control. One unit of enzyme activity is defined as the enzyme reaction, which gave 50% inhibition of NBT reduction in one minute under the assay condition and expressed as specific activity in units/mg protein.

2.7.4.3 Estimation of Catalase (CAT)

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

Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate when heated in the presence of H₂O₂. Chromic acetate was formed.

The catalase preparation was allowed to split H₂O₂ for different periods of time. The reaction was stopped at different time intervals by the addition of dichromate / acetic acid mixture and the remaining H₂O₂ as chromic acetate was determined at 620 nm.
Reagents

1. Phosphate buffer - 0.01M, (pH - 7)
2. Hydrogen peroxide - 0.2 M
3. Potassium dichromate - 5%
4. Dichromate acetic acid reagent: 1:3 ratio of potassium dichromate was mixed with glacial acetic acid
5. Standard hydrogen peroxide - 2µmol/ml

Procedure

0.9 ml of phosphate buffer, 0.1 ml of tissue homogenate (homogenized in 0.01M phosphate buffer, pH - 7) and 0.4 ml of hydrogen peroxide were added to test tubes. After 30 and 60 seconds, 2 ml of dichromate acetic acid reagent was added. The tubes were kept in boiling water bath for 10 minutes and the colour developed was read at 620 nm. Standards in the range of 2 to 19 µmol were taken and preceded as the test.

The activity was expressed as µmol of hydrogen peroxide consumed/minute/mg protein.

2.7.4.4 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.
Reagents

1. Tris buffer - 0.4M (pH 7)
2. Sodium azide solution - 10 mM
3. Trichloro acetic acid - 10%
4. EDTA - 0.4 mM
5. H$_2$O$_2$ solution - 0.2 mM
6. Glutathione - 2 mM

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 H$_2$O$_2$ 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.

2.7.4.5 Estimation of glutathione-S-transferase (GST)

Activity of glutathione-S-transferase was measured in tissue homogenate by following the increase in absorbance at 340 nm using 1-chloro-2,4-dinitro benzene as substrate (Habig et al., 1974).
2GSH + H₂O₂ → GSSG + 2H₂O

Reagents

1. Phosphate buffer - 0.3M (pH 6.5)
2. Reduced glutathione - 30 mM
3. 1-chloro-2,4-dinitrobenzene (CDNB) - 30 mM was prepared in ethanol

Procedure

The reaction mixture contained 1.0ml of phosphate buffer, 0.1 ml of CDNB, 0.1 ml of tissue homogenate (Homogenized by using phosphate buffer - 0.3 M, pH 6.5 and 0.7 ml of distilled water). The reaction mixture was incubated at 37°C for 5 minutes, and then the reaction was initiated by the addition of 0.1 ml of 30 mM glutathione. The absorbance change was read at 340 nm for 5 minutes. Reaction mixture without the enzyme was used as the blank.

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

2.7.5 Liver mitochondrial studies

2.7.5.1 Isolation of Mitochondria

The mitochondria of liver were isolated by the method of Johnson and Lardy (1967).
1. 0.005M Tris-HCl buffer containing 0.25 M Sucrose, pH 7.4.

**Procedure**

A 10% (w/v) homogenate was prepared in 0.05 M Tris-HCl buffer containing 0.25M sucrose and centrifuged at 600×g for 10 minutes. The supernatant fraction was decanted and centrifuged at 15,000×g for 5 minutes. The resultant mitochondrial pellet was then washed and resuspended in the same buffer.

The purity of mitochondrial fraction was assessed by measuring the activity of succinate dehydrogenase.

**2.7.6 Antioxidant system**

**2.7.6.1 Assay of superoxide dismutase**

The mitochondrial superoxide dismutase activity was assayed by the method of Kakkar *et al.* (1984) as described in section 2.7.4.2.

**2.7.6.2 Assay of glutathione peroxidase (GPx)**

The mitochondrial glutathione peroxidase activity was assayed by the method of Rotruck *et al.* (1973) as described in section 2.7.4.4. The enzyme activity was expressed as μmoles of glutathione oxidized/min/mg protein.

**2.7.6.3 Assay of Glutathione-S-transferase**

The mitochondrial Glutathione-S-transferase activity was assayed by the method of Habig *et al.* (1974) as described in section 2.7.4.5.
2.7.7 TCA cycle enzymes

2.7.7.1 Assay of Isocitrate dehydrogenase (ICDH)

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

Reagents

1. Tris-HCl : 0.1 M, pH 7.5
2. Trisodium isocitrate : 0.1 M in 0.15 M NaCl
3. Manganese chloride : 0.015 M
4. NADP⁺ : 0.001 M
5. DNPH : 0.001 M in 1 N HCl
6. EDTA : 0.005 M
7. NaOH : 0.4 N
8. α-ketoglutarate : 15 mg in 50 ml of 0.1 M Tris-HCl, pH 7.4

Procedure

To 0.1 ml of Tris-HCl, 0.2 ml of trisodium isocitrate, 0.3 ml of manganese chloride, 0.2 ml of mitochondrial suspension and 0.2 ml of NADP⁺ (0.2 ml of saline for control) were added. After 60 minutes of incubation, 1 ml of DNPH was added followed by 0.5 ml of EDTA and kept at room temperature for 20 minutes. Then 10 ml of NaOH was added and the colour developed was read at 420 nm in a Systronic UV spectrophotometer. A standard containing α-ketoglutarate was run simultaneously.
The isocitrate dehydrogenase activity was expressed as nmoles of α-ketoglutarate formed/h/mg protein.

2.7.7.2 Assay of α-ketoglutarate dehydrogenase (α-KGDH)

The activity of α-ketoglutarate dehydrogenase was assayed by the method of Reed and Mukherjee (1969).

Reagents

1. Potassium phosphate buffer : 1 M, pH 6.0
2. α-ketoglutarate : 0.5 M
3. Thiamine pyrophosphate : 0.002 M
4. Magnesium chloride : 0.003 M
5. Potassium ferricyanide : 0.25 M
6. Potassium ferrocyanide : 0.01%
7. TCA : 10%
8. Duponol : 4%
9. Ferric ammonium sulphate - duponol reagent: 1.7 g of ferric ammonium sulphate was dissolved in 10 ml of water and this mixture was filtered. Then 20 ml of a solution containing 1.5 g of duponol was added to the filtrate. To this solution, 27 ml of 85% α-phosphoric acid was added and made upto 140 ml with water.

Procedure

To 0.15 ml of phosphate buffer, 0.1 ml each of thiamine pyrophosphate, magnesium sulphate, α-ketoglutarate and potassium ferricyanide
were added. The total volume was made up to 1.2 ml with water and 0.2 ml of mitochondrial suspension was added and incubated at 30°C for 30 minutes. Then 1 ml of TCA was added and centrifuged. 0.2 ml of mitochondrial suspension was added to the control after the addition of TCA. To the supernatant, 0.1 ml of potassium ferricyanide, 1 ml of duponol and 0.5 ml of ferric ammonium sulphate-duponol reagent were added and then incubated at 25°C for 30 minutes. The colour developed was measured at 540 nm in a Systronic UV spectrophotometer.

The activity of α-ketoglutarate dehydrogenase was expressed as nmoles of ferrocyanide formed/h/mg protein.

2.7.7.3 Assay of Succinate dehydrogenase (SDH)

The activity of succinate dehydrogenase was assayed according to the method of Slater and Bonner (1952).

Reagents

1. Phosphate buffer : 0.3 M, pH 7.6
2. EDTA : 0.03 M
3. Potassium cyanide : 0.03M
4. Sodium succinate : 0.4 M
5. Bovine serum albumin: 3%
6. Potassium ferricyanide: 0.075 M

Procedure

The reaction mixture containing 1 ml of phosphate buffer, 0.1 ml of EDTA, 0.1 ml of BSA, 0.3 ml of sodium succinate and 0.2 ml of potassium
ferricyanide were made up to 2.8 ml with double distilled water. The reaction was started by the addition of 0.2 ml of mitochondrial suspension. The change in OD was recorded at an interval of 15 seconds for 5 minutes at 420 nm in a Systronic UV spectrophotometer.

The succinate dehydrogenase activity was expressed as nmoles of succinate oxidized/min/mg protein.

2.7.7.4 Assay of Malate dehydrogenase (MDH)

The activity of malate dehydrogenase was assayed by the method of Mehler et al. (1948).
Reagents

1. Potassium phosphate buffer : 0.25 M, pH 7.4
2. Oxaloacetate : 0.0076 M
3. NADH : 0.005 M

Procedure

The reaction mixture contained 0.75 ml of phosphate buffer, 0.15 ml of NADH and 0.75 ml of oxaloacetate. The reaction was carried out at 25°C and was started by the addition of 0.2 ml of mitochondrial suspension. The control tubes contained all reagents except NADH. The change in OD at 340 nm was measured for 2 minutes at an interval of 15 seconds in a Systronic UV spectrophotometer.

The activity of malate dehydrogenase was expressed as nmoles of NADH oxidized/min/mg protein.

2.7.8 Respiratory marker enzymes

2.7.8.1 Assay of NADH dehydrogenase

The activity of NADH dehydrogenase was assayed according to the method of Minakami et al. (1962).

Reagents

1. Phosphate buffer : 0.1 M, pH 7.4
2. NADH : 0.1%
3. Potassium ferricyanide : 0.03 M
Procedure

The reaction mixture contained 1.0 ml of phosphate buffer, 0.1 ml of potassium ferricyanide, 0.1 ml of NADH and 0.2 ml of mitochondrial suspension. The total volume was made upto 3 ml with water. NADH was added just before the addition of the enzyme. A control was also treated similarly without NADH. The change in OD was measured at 420 nm as a function of time for 3 minutes at an interval of 15 seconds in a Systronic UV spectrophotometer.

The activity of NADH dehydrogenase was expressed as nmoles of NADH oxidized/min/mg protein.

2.7.8.2 Assay of Cytochrome C oxidase (Cyt C oxidase)

Cytochrome c oxidase activity was assayed by the method of Pearl et al. (1963).

Reagents

1. Phosphate buffer : 0.03 M, pH 7.4
2. Cytochrome C : 0.01%
3. N-phenyl-p-phenylene diamine : 0.2% solution

Procedure

The reaction mixture contained 1.0 ml of phosphate buffer, 0.2 ml of 0.2% N-phenyl-p-phenylene diamine, 0.1 ml of 0.01% cytochrome C and 0.5 ml of water. The sample was incubated at 25°C for 5 minutes. 0.2 ml of the enzyme
preparation was added and the change in OD was recorded at 550 nm for 5 minutes at an interval of 15 seconds each in a Systronic UV spectrophotometer. A control containing all reagents except cytochrome C was also processed in the same manner.

The activity of cytochrome C oxidase was expressed as change in OD/min/mg protein.

2.7.9 Microsomal enzyme studies

2.7.9.1 Isolation of microsomes

Microsomes were isolated by the method of Klein et al. (1983).

Reagents

1. Tris-KCl buffer : 0.02 M containing 1.15% KCl, pH 7.4
2. Phosphate buffer : 0.1M, pH 7.4

Procedure

The liver was homogenized with 4 volumes of medium containing Tris-KCl buffer in a glass homogenizer. The homogenate was centrifuged at 9,000 × g for 20 minutes in a refrigerated centrifuge and the supernatant was decanted and recentrifuged at 1,05,000 × g for 60 minutes. The microsomal pellet was recentrifuged in Tris-KCl buffer and resedimented two times as above. The washed microsomal pellet was finally resuspended in phosphate buffer (4 mg of protein/ml) and used for the analysis.

2.7.9.2 Assay of heme oxygenase
The activity of heme oxygenase was determined by the method of Tenhumen et al. (1969).

**Reagents**

1. Phosphate buffer: 0.1 M, pH 7.4
2. Hemin: 0.51 mM
3. NADPH: 5.4 mM

**Procedure**

The mixture contained 2.7 mg of phosphate buffer, 0.1 ml of hemin, NADPH and microsomal suspension. In the reference cuvette, phosphate buffer replaced NADPH. The formation of bilirubin was determined from the increase in OD at 468 nm.

The activity of heme oxygenase was expressed as μmoles of bilirubin formed/min/mg protein. The amount of bilirubin was calculated from the millimolar extinction coefficient of bilirubin, 27.2.
2.7.10 Lysosomal enzyme studies

2.7.10.1 Isolation of Lysosomes

Lysosomal fraction was isolated by the method of Wattiauz (1977).

Reagents

1. Sucrose solution : 0.25M
2. Potassium chloride : 1.15%

Procedure

Fresh liver tissue was homogenized in 0.25M sucrose solution. The homogenate was filtered and centrifuged at 3,000 × g for 10 minutes in a refrigerated centrifuge. The pellet was removed and re-homogenised and resuspended as before. The supernatants were combined and centrifuged again at 15,000 × g for 20 minutes. The lysosomal pellet obtained was suspended in 1.15% KCl, homogenized and used for the estimation of enzymes.

2.7.10.2 Estimation of β-D-Glucuronidase

The activity of β-D-Glucuronidase was determined by the method of Kawai and Anno (1971).
Reagents

1. 0.5M NaOH
2. Sodium acetate buffer : 0.1M, pH 5.0
3. P-nitrophenyl β-D-glucuronide : 2 mM
4. Glycine- NaOH buffer : 0.2 M, pH 11.7 containing 2 M SDS

Procedure

0.2 ml of the enzyme source was added to 0.5 ml of incubated buffer containing 2 mM substrate and incubated at 37°C for 2 hours. The substrate p-nitrophenyl β-D-glucuronide was dissolved in 0.1M acetate buffer. At the end of the incubation period, the reaction was stopped by the addition of 4.0 ml of 0.2 M glycine-NaOH buffer and the contents were centrifuged. To the aliquots of supernatants, 0.5M NaOH was added and the absorbance was measured at 410 nm in a UV spectrophotometer.

The enzyme activity was expressed as μmoles of p-nitrophenol liberated/mg protein.

2.7.10.3 Estimation of β-D-galactosidase

The activity of β-D-galactosidase was determined by the method of Conchie et al. (1957).
Reagents

1. Na$_2$HPO$_4$ : 0.2 M
2. Citric acid : 0.1M
3. Glycine-NaOH buffer : 0.4M, pH 10.4
4. Standard : p-nitrophenol
5. Substrate : 5 mM p-nitrophenyl β-D-galactoside

Procedure

The incubation mixture contained 2.0 ml of each of 0.2M Na$_2$HPO$_4$, 0.1 M citric acid buffer, 0.5ml of 5 mM p-nitrophenyl β-D-galactoside and 0.5ml of enzyme source. Incubation was carried out for 1 hour at 37°C. The reaction was terminated by the addition of 4.0 ml of glycine-NaOH buffer. The reaction mixture was centrifuged and the absorbance of the released p-nitrophenol in the supernatant was measured at 410 nm using UV spectrophotometer.

The enzyme activity was expressed as μmoles of p-nitrophenol liberated /h/mg of protein.

2.8 IN VIVO STUDIES

(PART II: APAP-INDUCED NEPHROTOXICITY)

2.8.1 Experimental induction of nephrotoxicity

The rats were treated with single dose of acetaminophen (APAP 750 mg/kg body weight) for 14 days.
2.8.1.1 Experimental protocol (Adeneye et al., 2008)

**Group 1:** Normal rats were fed orally with normal saline 5 ml/kg body weight daily for 14 days.

**Group 2:** Rats treated with acetaminophen (APAP) of 750 mg/kg body weight for 14 days orally were kept as toxin control.

**Group 3:** APAP induced rats were administrated ethanolic leaf extract of CS (250 mg/kg body wt.) orally by using intragastric tubes for 14 days.

**Group 4:** APAP induced rats were administrated ethanolic leaf extract of, CS (500 mg/kg body wt.) orally by using intragastric tubes for 14 days.

**Group 5:** Animals were treated with ethanolic extract of CS 500 mg/kg only.

**Group 6:** APAP induced rats +250 mg CT extract /kg.

**Group 7:** APAP induced rats +500 mg CT extract /kg.

The same kind of experimental groups were designed for ethanolic extract of CT.
2.8.2  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. The portion 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), granulocytes (Gran) and total leucocytes count (TLC). These parameters were analyzed using automatic hematological system (Sysmex Hematology - Coagulation system, Model MO-1000 I, Trans Asia, Japan).

2.8.3  Biochemical analysis

The second portion of blood was centrifuged for 10 min at 5000 rpm. The clear sera obtained were stored at −20°C for subsequent measurement of urea (UR), creatinine (CR) and uric acid (UA) levels.

2.8.3.1  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.

**2.8.3.2 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.

2.8.3.3 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.
2.8.4 Preparation of renal homogenate

The kidneys were removed and dissected free from the surrounding fat and connective tissue. Each kidney was longitudinally sectioned, and renal cortex was separated and kept at −8°C. Subsequently, renal cortex was homogenized in cold potassium phosphate buffer (0.05 M, pH 7.4). The renal cortical homogenates were centrifuged at 5000 rpm for 10 min at 4°C. The resulting supernatant was used for the determination of antioxidant enzyme levels.

2.8.5 Estimation of lipid peroxide and antioxidants

2.8.5.1 Estimation of TBARS

TBARS content was measured according to the earlier method reported (Nichans and Samuelson 1998) as described in section 2.7.4.1.

2.8.5.2 Estimation of Glutathione (GSH)

The level of total reduced glutathione in the liver tissue was measured by the method of Moron et al. (1979).
**Reagents**

1. Phosphate buffer: 0.2 M, pH 8.0
2. DTNB: 0.6 mM in 0.2 M phosphate buffer, pH 8.0
3. TCA: 10%

**Procedure**

1.5 ml of liver homogenate was precipitated with 1 ml TCA. The contents were mixed well for complete precipitation of proteins and centrifuged. To 0.5 ml of clear supernatant, 2.0 ml DTNB was added and the total volume was made upto 3 ml with phosphate buffer. The absorbance was read at 412 nm against a blank containing TCA instead of sample. A series of standards treated in a similar way were also run to determine the glutathione content.

The amount of glutathione was expressed as nmoles/g wet tissue.

**2.8.5.3 Estimation of Superoxide dismutase (SOD)**

Renal Superoxide dismutase (SOD) activity was estimated by the method of Kakkar et al. (1984) as described in section 2.7.4.2.

**2.8.5.4 Estimation of catalase (CAT)**

Catalase was estimated by the method of Sinha (1972) as described in the section 2.7.4.3.

**2.8.5.5 Estimation of glutathione peroxidase (GPx)**
Renal glutathione peroxidase (GPx) activity was estimated by the method of Rotruck et al. (1973) as described in section 2.7.4.4.

2.9 HISTOPATHOLOGICAL STUDIES OF APAP-INDUCED LIVER AND KIDNEY

2.9.1 Histopathology of liver

The liver was removed and stored immediately in 10% formalin initially for 48 hours; thereafter the materials were transferred to 70% alcohol and stored. After fixation, the tissue was subsequently put in paraffin. Thin (5 µm) sections were drawn using a microtome and then stained with hematoxylin and eosin and mounted in neutral di-styrene-dibutyl propylene (DPX) medium and examined using photomicroscopy (Strate et al., 2005).

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 ceresin 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.

2.9.2 **Histopathology of Kidney**

Pieces of kidney from each group were fixed immediately in 10% neutral formalin for a period of at least 24 h, dehydrated in graded (50-100%) alcohol, embedded in paraffin, cut into 4-5 μm thick sections and stained with hematoxylin-eosin. The sections were evaluated for the pathological symptoms of nephrotoxicity such as necrosis, fatty infiltration, fibrosis, lymphocyte infiltration, etc. as described in section 2.9.1.

2.10 **STATISTICAL ANALYSIS OF DATA**

The obtained results were analyzed for statistical significance using one way ANOVA followed by Dennett’s t-test using Graphpad Prism statistical software for comparison with control group and acetaminophen treated group. P < 0.05 was considered as significant.