Chapter II

Materials and Methods
Materials & Methods

Materials

Chemicals

Carbon tetrachloride was purchased from MERK India Ltd., Curcumin and α-Tocopherol (Vitamin E) purchased from Sigma chemicals Co. St Louis. Silymarin suspension purchased from Micro labs, Bangalore. All other chemicals used were of technical grade.

Animal Ethical Clearance

Local Institutional Animal Ethical Committee of our University, obtained ethical clearance for conducting experiments on animals from committee for the purpose of control and supervision of experiments on Animals (CPCSEA) (REGD.No.470/01/a/CPCSEA, DT.24th Aug 2001).

Procurement of Animals and maintenance

Adult male albino rats wistar strain (Rattus norvegicus) weighing 190±30 obtained from Sri Raghavendra Animal Supplier, Bangalore, K.A. They were kept in cages under standard laboratory conditions (25±2°C, 12 hrs dark/light) and were fed with commercial rat feed supplied by Sai Durga Feeds and Foods, Bangalore and water adlibitum. They were allowed to laboratory conditions for ten days after arrival before use.

Methodology

Selection of Dose

The dose of CCl₄ (1ml/Kg Wt) was obtained from previous work of Mc Lean E.K. et al., 1991 and Chanchal et al., 2006. The dose of curcumin 100mg/ kg wt. based on previous work of Sreepriya and Bali, 2006 with minor modifications. The dose of Vitamin E based on previous work of Karima M. Moawad, 2007 with minor modifications.
Experimental Design

Animals were randomly assigned into 7 groups of 6 each.

Group 1. Vehicle control (PBS given daily p.o and liquid paraffin 1ml/kg body w.t twice weekly) for 8 weeks

Group 2. Curcumin control (100mg/Kg w.t p.o., daily) for 8 weeks

Group 3. Curcumin (100mg/Kg w.t p.o., daily) + Vitamin E (40mg/kg body w.t Weekly thrice given p.o.) for 8 weeks.

Group 4. CCl4 Control. (1ml/kg w.t twice weekly) for 8 weeks

Group 5. Curcumin (100mg/Kg w.t given daily) for 8 weeks.

Group 6. CCl4 + Curcumin (100mg/Kg w.t given daily, Vitamin E 40mg/kg w.t, Weekly thrice given p.o.) for 8 weeks

Group 7. CCl4 + Silymarin (50mg/Kg w.t p.o., daily) for 8 weeks.

CCl4 diluted with liquid paraffin (1:1) before administration. Food was withdrawn 12hr before CCl4 administration. Curcumin was suspended in Phosphate Buffer Saline (PBS). Olive oil as a vehicle for vitamin E (1ml/kg, p.o).

Sacrifice of animals and organ collection

The rats were sacrificed at the end of 8 weeks and 24 h after the last dose of CCl4. Rats were sacrificed by cervical dislocation and immediately liver, kidney, brain and thigh muscle were removed and washed thoroughly with ice-cold 0.9% sodium chloride solution (saline). Each organ of every animal was suspended in 0.15 M potassium chloride in polypropylene containers, sealed with parafilm, labelled carefully and stored at -20°C until assays were carried out.

Histopathology

The histological sections of the liver and kidney of rats were taken by adopting the procedure as described by humason (1972). The tissues were isolated and gently rinsed with physiological saline solution (0.9% NaCl) to remove mucus and other debris adhering them. They were fixed in Bouin’s fluid (75 ml saturated aqueous picric acid, 25 ml 40% formaldehyde and glacial acetic acid) for 24 hours.
The fixative was removed by washing through running tap water for overnight. Then the tissues were processed for dehydration. Ethyl alcohol was used as the dehydrating agent, as it is the most suitable and economical besides its hardening effect. The alcoholic transfer schedules were so arranged as to utilize both dehydration and hardening effect. The tissues were passed through successive series containing 30%, 50%, 70%, 80%, 90%, 95% and absolute alcohols. Then the tissues were cleaned in methyl benzoate and embedded in paraffin wax. Sections of 5μ thickness were cut using “SIPCON” rotatory microtome. The sections were stained with Harris hematoxylin (Harris, 1900) and counter stained with eosin, dissolved in 95% alcohol. After dehydration and cleaning, the sections were mounted in Canada balsam. Photomicrographs of the section preparations were taken using Magnus photomicrographing equipment.

Blood collection, Plasma and Serum separation

The animals were sacrificed 24 hr after last treatment. Blood was collected from the heart (cardiac puncture) of each animal in a eppendorf tubes without anticoagulant allowed to clot for 30 min at room temperature serum was separated by centrifugation. Serum was used for the estimation of Glutamate Pyruvate Transaminase (SGPT), Glutamate Oxaloacetate Transaminase (SGOT) and Alkaline Phosphatase (ALP). The blood collected with anticoagulant containing EDTA (1 mg/ml) was used for estimation of Lipid profile, total protein, albumin, globulins, urea, creatinine.

Determination of Glutamate Pyruvate Transaminase (SGPT)

Serum GPT assayed by using SGPT kit obtained from Erba diagnostics Mannheim, Baddi, H.P.

Reagents: Reagent-1 contained L-Alanine (500 mmol/L), NADH (Yeast) (0.18 m.mol/L), LDH(1820 IU/L), 2-Oxaloacetate (12 mmol/L), Tris buffer pH:7.5±0.1 at 25 °C (80 mmol/L). This was reconstituted with distilled water before experiment.

Procedure: To 0.1 ml of unhemolysed serum, working reagent(1) 1.0 ml was added and mixed well, change in the absorbance was recorded at 1 min intervals at 340 nm
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for 3 min and the activity was calculated using $\Delta A/\text{min} \times \text{Factor (1768)}$. Values expressed as IU/L.

**Determination of Glutamate Oxaloacetate Transaminase (SGOT)**

Serum GPT assayed by using SGPT kit obtained from Erba diagnostics Mannheim, Baddi, H.P.

**Reagents:** Reagent-1 contained 2-Oxoglutarate (12 mmol/L), L-Asparate (200 mmol/L), MDH (545 U/L), LDH (909 U/L), NADH (Yeast)(0.18 mmol/L), Tris buffer pH:7.8±0.1 at 25 °C (80 mmol/L), EDTA (5.0 mmol). This was reconstituted with distilled water before experiment.

**Procedure:** To 0.1 ml of unhemolysed serum, working reagent(1) 1.0 ml was added and mixed well, change in the absorbance was recorded at 1 min intervals at 340 nm for 3 min and the activity was calculated using $\Delta A/\text{min} \times \text{Factor (1768)}$. Values expressed as IU/L.

**Determination of Alkaline Phosphatase (ALP)**

Serum ALP assayed by using ALP kit obtained from Aspen laboratories, Baddi, H.P.

**Reagents:** Alkaline Phosphatase Reagent: p-Nitrophenyl phosphate 17 mM, Magnesium ions 4 mM, Buffer (pH: 10.2±0.2), this was reconstituted with distilled water before experiment.

**Procedure:** To 0.025 ml of unhemolysed serum, Alkaline phosphate reagent 1.0 ml was added and mixed well, change in the absorbance was recorded at 1 min intervals at 405 nm for 3 min and the activity was calculated using $\Delta A/\text{min} \times \text{Factor (1768)}$. Values expressed as IU/L.

**Determination of Total Cholesterol**

Plasma total cholesterol estimated by using kit obtained from M/s Excel Diagnostics Pvt.Ltd, Hyderabad.

**Reagents:**

1. Cholesterol Reagent
2. Cholesterol standard
3. HDL PPT Reagent
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**Procedure:** Pipette in a clean dry test tube labelled as Blank (B), Standard (S), and Test (T). The following were pipette out. Mixed well and the optical density (OD) was read at 500 nm against blank after 5 min incubation (37 °C). The final colour is stable for at least 1 hour.

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<tr>
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<th>B</th>
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<tr>
<td>Enzyme reagent</td>
<td>1 ml</td>
<td>1 ml</td>
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<tr>
<td>Deionised water</td>
<td>0.01 ml</td>
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<tr>
<td>Standard</td>
<td>--</td>
<td>0.01 ml</td>
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</tr>
<tr>
<td>Plasma</td>
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<td>0.01 ml</td>
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Total cholesterol (in mg%) was calculated using following formulae:

\[
\text{OD of the test/OD of standard} \times 200 \quad (\text{Std.Conc.})
\]

**Determination of Triglycerides**

Plasma triglycerides estimated by using kit obtained from M/s Excel Diagnostics Pvt.Ltd, Hyderabad.

**Reagents:**

1. Enzyme Reagent
2. Standard (200 mg %)

**Procedure:** Pipetted in a clean dry test tube labelled as Blank (B), Standard (S), and Test (T). The following were pipette out. Mixed well and incubated for 10 min at 37 °C. The optical density (OD) was read at 546 nm against blank.

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<tbody>
<tr>
<td>Enzyme reagent</td>
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<td>1 ml</td>
<td>1 ml</td>
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<tr>
<td>Deionized water</td>
<td>0.01 ml</td>
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<tr>
<td>Standard</td>
<td>--</td>
<td>0.01 ml</td>
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<tr>
<td>Plasma</td>
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<td>0.01 ml</td>
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Triglycerides were calculated using following formulae:

\[
\text{Triglycerides Conc.in mg \%} = \frac{\text{OD of the test}}{\text{OD of standard}} \times 200 \text{ (Std.Conc.)}
\]

**Determination of Total Proteins**

Plasma total protein estimated by using kit obtained from Span Diagnostics Ltd, Surath.

**Reagents:**

1. Biuret Reagent
2. Protein Standard (6.5 g/dL)

**Procedure:** Pipetted a clean dry test tube labelled as Blank (B), Standard (S), and Test (T). The following were pipette out. Mixed well and incubated for 5 min at 37 °C. The optical density (OD) of standard and test against blank was read at 578 nm.

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<td>Reagent-1</td>
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<td>1 ml</td>
<td>1 ml</td>
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<tr>
<td>Deionized water</td>
<td>0.01 ml</td>
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<td>—</td>
</tr>
<tr>
<td>Standard (Reagent-2)</td>
<td>—</td>
<td>0.01 ml</td>
<td>—</td>
</tr>
<tr>
<td>Plasma</td>
<td>—</td>
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<td>0.01 ml</td>
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Total proteins was calculated using following formulae:

\[
\text{Total protein conc.in g/dL} = \frac{\text{OD of the test}}{\text{OD of standard}} \times 6.5
\]

Globulins = Total proteins - Albumin

Conversion factor g/L = Total protein conc.in g/dL x 10

**Determination of Albumin**

Plasma albumin estimated by using kit obtained from Span Diagnostics Ltd, Surath.

**Reagents:**

1. Albumin Reagent
2. Albumin Standard (4 g/dL)
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Procedure: Pipetted a clean dry test tube labelled as Blank (B), Standard (S), and Test (T). The following were pipette out. Mixed well and incubated for 1 min at room temperature. The optical density (OD) of standard and test against blank was read at 630 nm.

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<tr>
<td>Albumin Reagent</td>
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<tr>
<td>Deionized water</td>
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</tr>
<tr>
<td>Standard (Reagent-2)</td>
<td>--</td>
<td>0.01 ml</td>
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</tr>
<tr>
<td>Plasma</td>
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<td>0.01 ml</td>
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Albumins were calculated using following formulae:

Albumin conc.in g/dL = OD of the test/OD of standard x 4
Conversion factor g/L = Total protein conc.in g/dL x 10

Determination of Urea

Plasma urea estimated by using kit obtained from M/s Excel Diagnostics Pvt.Ltd, Hyderabad.

Reagents:

1. Urease Reagent
2. Enzyme concentrate
3. Colour reagent
4. Urea standard (40 mg%)

The entire enzyme concentrate (2) was transferred into urease reagent (1).

Procedure: Pipetted a clean dry test tube labelled as Blank (B), Standard (S), and Test (T). The following were pipette out. Mixed well and incubated for 5 min at 37° C/10 min RT. The absorbance of standard and test against blank was read at 570 nm.

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Urea

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<th>B</th>
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<tr>
<td>Urease (working)</td>
<td>1 ml</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Deionized water</td>
<td>0.01 ml</td>
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</tr>
<tr>
<td>Urea Standard</td>
<td>--</td>
<td>0.01 ml</td>
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<tr>
<td>Plasma</td>
<td>--</td>
<td>--</td>
<td>0.01 ml</td>
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Incubate for 5 min. at 37° C/10 min at room temperature (RT)

Colour reagent | 1 ml | 1 ml | 1 ml

Plasma urea was calculated using following formulae:

Urea conc. in mg % = OD of the test/OD of standard x 40 (Std. conc.)

Blood urea nitrogen in mg % = Plasma urea x 0.467

Determination of Creatinine

Plasma creatinine estimated by using kit obtained from M/s Excel Diagnostics Pvt. Ltd, Hyderabad.

Reagents:

1. Picric acid reagent
2. Alkaline buffer reagent
3. Standard Creatinine (2 mg %)
4. Acid reagent

Mix equal volumes of Picric acid reagent (1) and Alkaline buffer reagent (2).

Procedure: Pipetted in a clean dry test tube labelled as Standard (S) and Test (T). The following were pipette out. Mixed well allow it to stand at RT for 5 min. Read the absorbance for AS₁ and AT₁ against distilled water at 520 nm.

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<tr>
<td>Working reagent</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Creatinine standard</td>
<td>0.1 ml</td>
<td>--</td>
</tr>
<tr>
<td>Plasma</td>
<td>--</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

Mix well allow it to stand at RT for 5 min. Read absorbance for AS₀ and AT₀ against distilled water at 520 nm.

| Acid reagent | 0.05 ml | 0.05 |
Plasma creatinine was calculated using following formulae.

Plasma creatinine conc. in mg % = $\frac{\Delta AT}{\Delta AS} \times 2$ (Std. conc.)

$\Delta AS = AS_0 - AS_1$

$\Delta AT = AT_0 - AT_1$

**Studies on Carbohydrate metabolism: Glycogen**

**Preparation of tissue extracts for glycogen estimation**

Liver extracts were made following the procedure outlined by Carrol (1956). The excised tissues were blotted between Whatman filter paper and weighed. Ten percent tissue homogenates were prepared in 5% TCA, centrifuged at 6000 X g for 15 min and the supernatant was used for glycogen estimation.

**Glycogen (Liver)**

**Principle:** Glycogen released from protein free supernatant of TCA homogenized tissues were precipitated with alcohol. The precipitated glycogen was then hydrolysed under acidic condition and the liberated glucose was estimated by anthrone reagent method as adapted by Carrol *et al.*, 1956.

**Reagents:**

1. 5% TCA
2. 95% ethanol
3. Anthrone reagent: 0.4 gms of anthrone in 200ml of H$_2$SO$_4$
4. Standard Glucose solution: 20-100µg/ml

**Procedure:** To 1.0 ml of protein free supernatant 5.0 ml of ethanol was added and allowed to stand overnight at 4°C. After precipitation was completed, centrifuged at 6000×g for 15 min, the supernatant decanted and the residue was dissolved in 1.0 ml of distilled water. Five ml of anthrone reagent was added by constant mixing and incubated in boiling water bath for 15 min with a marble on top to prevent loss of water by evaporation, cooled and the blue-green colour developed was read at 620 nm against reagent blank. A system devoid of glycogen was chosen as blank and series of standard glucose (20-100 µg) were treated in a similar manner. Values are expressed as mg glucose/ gm tissue weight.
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Protein

Principle: Tyrosine and truptophan present in the proteins reacts with Folin-Ciocalteau reagent in the presence of alkaline copper to give coloured complex with a maximum absorbance at 750 nm (Lowry et al., 1951).

Reagents:
1. Alkaline Solution (A): 2% Na₂CO₃ in 0.1 M NaOH
2. Alkaline Solution (B): 0.5% Copper sulphate in 1% sodium potassium tartrate solution
3. Alkaline Solution (C): Mixed 50 ml of (A) and 1.0 ml of (B), prepared just before use.
4. 2 N Folin-Ciocalteau reagent: Diluted to 1:1 and used
5. Bovine Serum Albumin standard: 0.2 mg/ml.

Procedure: Aliquot of test sample was made up to 1.0 ml with distilled water and 5.0 ml of alkaline solution (C) was added, mixed thoroughly and allowed to stand at room temperature for 10 min. 0.5 ml of Folin-Ciocalteau reagent was added rapidly with immediate mixing and the intensity of the colour developed were read at 750 nm after 30 min. A series of standards (40-200 µg) were also treated in a similar manner along with a blank. Values are calculated from the standard graph.

Studies on Antioxidant and Oxidative stress

Preparation of tissue extracts for glutathione and lipid peroxidation

Immediately after separation of liver, 10% tissue homogenate was prepared in 0.15 M potassium chloride using homogenizer at 0° C. The whole homogenate was used for estimation of glutathione and lipid peroxidation.

Lipid peroxidation

Principle: Lipid peroxidation was estimated as evidence by the formation of thiobarbutic acid reactive a substance like malondialdehyde (MDA) according method of Utely et al., 1967.

Reagents:
1. 10% TCA
2. 0.67% TBA
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Procedure: To 1.0 ml of the liver homogenate, 2.0 ml of TCA and 4.0 ml of TBA were added, heated in water bath for 30 minutes. After cooling and centrifugation, the absorbance of the supernatant was read at 535 nm. A reagent blank was prepared using water instead of tissue homogenate. The extent of lipid peroxidation was expressed as nmol MDA formed/mg protein, using a molar extinction co-efficient of MDA as 1.56 X 10^5 M^-1 cm^-1.

Reduced Glutathione

Principle: Total reduced glutathione content was measured following the method of Ellman’s (1959). This method was based on the development of a yellow colour, when 5,5'-dithio-2-nitro benzoic acid (DTNB) reacts with the compounds containing sulphhydryl groups with maximum absorbance at 412 nm.

Reagents:
1. Phosphate buffer 0.2 M, pH: 8.0
2. 5% TCA
3. Ellman’s reagent: 19.8 mg DTNB in 100 ml of 0.1% sodium citrate (prep.fresh)
4. GSH standard: 20 µg/ml

Procedure: 0.5 ml of liver tissue homogenate was deproteinized with 3.5 ml of 5% TCA and centrifuged. To 0.5 ml of the supernatant, 3.0 ml phosphate buffer and 0.5 ml of Ellman’s reagent were added and the yellow colour developed was read at 412 nm. A series of standards (4-20 µg) were treated in a similar manner along with blank. Values expressed as µg GSH/mg protein.

Assay of Antioxidants

Sample preparation

Ten percent of the tissue homogenate (liver/kidney) in 0.15 M potassium chloride was prepared at 0°C and centrifuged in cold (0-4°C) at 12,000 rpm for 45 min, in Remi (SL-) cooling centrifuge. The supernatant thus obtained into eppendorf tubes, labelled and stored at -20°C and all the antioxidant enzymes were assayed at the earliest.
Glutathione peroxidise

**Principle:** A known amount of the enzyme preparation was allowed to react with H$_2$O$_2$ in the presence of GSH for a specified time period according to the method of Rotstruck (1973) and remaining GSH was measured by Ellman's method (1959) as described for GSH estimation.

**Reagents:**
1. Phosphate buffer 0.4 M, pH: 7.0 containing 0.4 mM EDTA, 10 mM Sodium Azide
2. 2 mM GSH
3. 10% TCA
4. 0.2 mM H$_2$O$_2$

**Procedure:** To 0.5 ml buffer, 0.2 ml enzyme source, 0.2 ml GSH, 0.1 ml H$_2$O$_2$ were added and incubated at room temperature for 10 min along with a control tube containing all reagents except enzyme source. The reaction was arrested by adding 0.5 ml of 10% TCA, centrifuged at 4000 rpm for 5 min. and GSH content in 0.5 ml of supernatant was estimated. The activity was expressed as µg of GSH consumed/min/mg protein.

Glutathione reductase

**Principle:** Glutathione reductase catalyses the reduction of oxidised glutathione (GSSG) by NADPH to GSH. The activity of the enzyme was measured by following the oxidation of NADPH spectrophotometrically at 340 nm according to the method of Pinto and Bartley (1969).

**Reagents:**
1. Potassium phosphate buffer, 0.25 M, pH:7.4
2. 25 mM EDTA
3. 50 mM GSSG
4. 1 mM NADPH

**Procedure:** The system contained 0.5 ml of buffer, 0.1 ml of EDTA, 0.1 ml of NADPH, 0.96 ml of distilled water and 0.1 ml of enzyme source (150 µg protein). The reaction was initiated by the addition of 0.24 ml GSSG. The change in absorbance was recorded at 1 min intervals at 340 nm for 5 min. The specific activity
is expressed as $\mu$mol of NADPH oxidized/min/mg protein using an extinction coefficient for NADPH of 6.22 cm$^{-1}$ mmol$^{-1}$.

**Glutathione-S-transferase**

**Principle:** Glutathione-S-Transferase activity was measured by monitoring the increase in the absorbance at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate according to the method of Habig et al. (1974).

**Reagents:**
1. 0.14 M Sodium phosphate buffer, pH:6.5
2. 0.01 M CDNB dissolved in 50% ethanol
3. 30 mM GSH

**Procedure:** The assay system contained 1.7 ml of buffer, 0.2 ml GSH and 0.04 ml enzyme source (40 $\mu$g protein). The reaction was initiated by 0.06 ml CDNB. The change in absorbance was recorded at 1 min intervals at 340 nm for 5 min and the activity was calculated using an extinction coefficient of CDNB-GSH conjugate as 9.6 mM$^{-1}$ and expressed as mmole of CDNB-GSH conjugate formed/min/mg protein.

**Catalase**

**Principle:** Catalase catalyses the breakdown of $\text{H}_2\text{O}_2$, and it was measured spectrophotometrically at 240 nm following the method of Beers and Seizer (1952).

**Reagents:**
1. 0.05 M Sodium phosphate buffer, pH:7.0
2. 0.059 $\text{H}_2\text{O}_2$ in buffer.

**Procedure:** The assay system contained 1.9 ml buffer and 1.0 ml $\text{H}_2\text{O}_2$. The reaction was initiated by addition of 0.1 ml enzyme source (45$\mu$g protein). The decrease in absorbance was monitored at 1 min interval for 5 min at 240 nm and activity was expressed as “n” moles of $\text{H}_2\text{O}_2$ decomposed/min/mg protein.

**Superoxide dismutase**

**Principle:** SOD activity was measured based on the ability of the enzyme to inhibit the autoxidation of pyrogallol. A modified procedure described by Marklund and Marklund and Marklund (1974) was adopted as followed by Soon and Tan (2002).
Reagents:
1. 50 mM Phosphate buffer, pH:7.8 containing 1 mM EDTA
2. 10 mM Pyrogallol in 0.01 N HCl.

Procedure: The assay system contained 2.1 ml of buffer, 0.02 ml of enzyme source (35 μg protein) and 0.86 ml of distilled water. The reaction was initiated with 0.02 ml of pyrogallol and change in absorbance was monitored at 420 nm. The percent inhibition was calculated on the basis of comparison with a blank assay system. One unit of SOD was defined as that amount of enzyme required to inhibit the autooxidation of pyrogallol by 50% in standard assay system of 3 ml. The specific activity was expressed as units/min/mg protein.