



Methodology

3. METHODOLOGY

The present study centered around evaluating the antioxidant effects evoked by *Triticum aestivum* leaves on oxidant induced events *in vitro*. The layout of the study and the experimental details adopted are presented in this chapter.

The study was conducted in four different phases. In the first phase, the fresh aqueous extracts of the *Triticum aestivum* leaves on different stages of growth were probed for their antioxidant content. In the second phase, the antioxidant response evoked by the *Triticum aestivum* leaf extracts were analyzed against a battery of *in vitro* experimental systems.

The third phase consisted of validating the use of *in vitro* models by following the effects of the *Triticum aestivum* leaf extract in live animals. The fourth phase comprised of the identification of the major active component present in the leaf extracts responsible for its antioxidant effect. The details of the four phases are given below.

CHEMICALS

All the chemicals used in the study were of analytical grade.

PHASE I

The seeds of *Triticum aestivum* were procured from a local market in Coimbatore and were grown within the University campus as pot cultures. The plant was taken at three different stages of growth namely 4, 8 and 12 days after sowing (DAS). The time point beyond 12 DAS could not be included because as the age of the plant increased, the fibre content of the leaf also increased resulting in decreased palatability.

The leaves of the plantlets were collected fresh for each analysis. The leaves were thoroughly washed in running tap water in order to remove any dirt particles adhered and blotted gently between the folds of tissue paper to remove any water droplets. The leaves of the seedlings were analyzed for the enzymic and non-enzymic antioxidants.

ENZYMIC ANTIOXIDANTS

The enzymic antioxidants analyzed were catalase, peroxidase, superoxide dismutase, polyphenol oxidase, glutathione S-transferase and glutathione reductase.

ASSAY OF CATALASE

Catalase activity was estimated by the method proposed by Luck (1974).

PRINCIPLE

The UV light absorption of hydrogen peroxide can be easily measured between 230 and 250nm. On decomposition by catalase, the absorption of H₂O₂ decreases with time and is proportional to the activity of the enzyme. The enzyme activity can be arrived at from this decrease.

REAGENTS

1. Phosphate buffer (0.067M, pH 7.0)
2. H₂O₂ – phosphate buffer (2 mM)

PROCEDURE

PREPARATION OF ENZYME EXTRACT

Triticum aestivum leaves (0.5g) at three stages of growth (4, 8 and 12 DAS) selected were homogenized in 2.5ml of phosphate buffer at 4°C. The debris

present in the homogenate was removed by centrifugation at 2000g for 10 minutes. The supernatant obtained was used for the analysis.

ASSAY

Pipetted out 3.0ml of H₂O₂-phosphate buffer into a quartz cuvette. The enzyme extract (40μl) was rapidly added and mixed thoroughly. The time (in seconds) required for a decrease in absorbance by 0.05 units was recorded. The enzyme solution containing H₂O₂-free phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units.

ASSAY OF PEROXIDASE

Peroxidase activity was estimated by the method of Reddy *et al.* (1995).

PRINCIPLE

In the presence of the hydrogen donor pyrogallol, peroxidase converts H₂O₂ to water and oxygen. During the process, pyrogallol is oxidized to a coloured product called purpurogalli, which is followed spectrophotometrically at 430nm. The formation of the product is proportional to the activity of the enzyme and can be used as a measure of the same.

REAGENTS

1. Pyrogallol (0.05M in 0.1M phosphate buffer, pH 6.5)
2. H₂O₂ (1% in 0.1M phosphate buffer, pH 6.5)

PROCEDURE

PREPARATION OF ENZYME EXTRACT

Triticum aestivum leaves (0.5g) on 4th, 8th and 12th days after sowing were macerated with 2.5ml of 0.1M phosphate buffer (pH 6.5) in a homogenizer. The homogenate was then centrifuged at 3000g for 15 minutes. The supernatant was used as the enzyme source.

ASSAY

Pyrogallol solution (3.0ml) and enzyme extract (0.1ml) were pipetted out into a cuvette. The spectrophotometer was adjusted to read zero at 430nm. One per cent of H₂O₂ (0.5ml) was added to the test cuvette. The change in absorbance for every 30 seconds upto 3 minutes was recorded. One unit of peroxidase activity is defined as the change in absorbance/minute at 430nm.

ASSAY OF SUPEROXIDE DISMUTASE (SOD)

SOD was assayed by the method proposed by Kakkar *et al.* (1984).

PRINCIPLE

The assay of SOD is based on the inhibition of formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The reaction was initiated by the addition of NADH and incubated for 90 seconds. After incubation, the reaction was stopped by the addition of glacial acetic acid. The chromogen formed was extracted into an organic solvent like butanol and estimated at 560nm as a measure of SOD activity.

REAGENTS

1. Sodium pyrophosphate buffer (0.025M, pH 8.3)
2. Phenazine methosulphate (PMS) (186 µmol)

3. Nitroblue tetrazolium (NBT) (300 μ mol)
4. NADH (780 μ mol)
5. Glacial acetic acid
6. n-butanol

PROCEDURE

PREPARATION OF ENZYME EXTRACT

Triticum aestivum leaves (0.5g) were ground with 3.0ml of sodium pyrophosphate buffer, centrifuged at 2000g for 10 minutes and the supernatant was used for the assay.

ASSAY

The assay mixture contained 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of NBT, 0.2ml of enzyme preparation and water in a total volume of 2.8ml. The reaction was started by the addition of 0.2ml of NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1.0ml of glacial acetic acid. The reaction mixture was shaken with 4.0ml of n-butanol. The mixture was allowed to stand for 10 minutes and then centrifuged at 2000g for 5 minutes. The intensity of the chromogen in the butanol layer was measured at 560nm against butanol as blank and the system devoid of enzyme served as control.

One unit of enzyme activity is defined as the amount of enzyme causing a 50% inhibition of NBT reduction/minute.

ASSAY OF POLYPHENOL OXIDASES (PPO)

The polyphenol oxidases (PPO) comprise of catechol oxidase and laccase. The activity of these enzymes was simultaneously assayed spectrophotometrically by the method proposed by Esterbauer *et al.* (1977).

PRINCIPLE

Phenol oxidases are copper proteins which catalyse the aerobic oxidation of phenolic substrates to quinones which are autooxidized to dark brown pigments generally known as melanins, which can be estimated spectrophotometrically at 495nm.

REAGENTS

1. Tris-HCl (50mM, pH 7.2)
2. Sorbitol (0.4M)
3. NaCl (10mM)
4. Phosphate buffer (0.1M, pH 6.5)
5. Catechol (0.01M)

PROCEDURE

PREPARATION OF ENZYME EXTRACT

Triticum aestivum leaves (0.5g) on the selected periods of growth were homogenized in a medium containing Tris-HCl, sorbitol and NaCl and the volume was made upto 2.0ml. The homogenate was then centrifuged at 2000g for 10 minutes at 4°C and the supernatant was used for the assay.

Phosphate buffer (2.5ml), 0.3ml of catechol solution was pipetted into a cuvette and the spectrophotometer was set at 495nm. The enzyme extract (0.2ml) was added to the same cuvette and the change in absorbance was recorded for every 30 seconds upto 5 minutes. One unit of either catechol oxidase or laccase is defined as the amount of enzyme that transforms one μ mole of dihydrophenol to one μ mole of quinone/minute. The activity of PPO can be calculated using the formula,

$$\text{Enzyme unit} = k \times (\Delta A/\text{min})$$

where k for catechol oxidase =0.272, k for laccase = 0.242

ASSAY OF GLUTATHIONE S-TRANSFERASE (GST)

The glutathione S-transferase activity was estimated by the method proposed by Habig *et al.* (1974).

PRINCIPLE

Glutathione S-transferase conjugates with GSH and CDNB, the extent of conjugation causes a proportionate change in the absorption at 340nm.

REAGENTS

1. 1-chloro,2,4-dinitrobenzene (CDNB) (1mM in ethanol)
2. Reduced glutathione (1mM)
3. Phosphate buffer (0.1M, pH 6.5)

PROCEDURE

PREPARATION OF ENZYME EXTRACT

Triticum aestivum leaves (0.5g) at different stages of growth were homogenized in 5ml of phosphate buffer. The homogenate was centrifuged at 5000g for 10 minutes and the supernatant was used for the assay.

ASSAY

The assay mixture contained 0.1ml of GSH, 0.1ml of CDNB and phosphate buffer in a total volume of 2.9ml. The enzyme extract (0.1ml) was added to the assay mixture in order to start the reaction. The readings were recorded against distilled water blank for a minimum of 3 minutes. The complete assay mixture without the enzyme served as the control. The enzyme activity was determined by monitoring the change in absorbance at 340nm in a spectrophotometer. One unit of GST activity is defined as the nmoles of CDNB conjugated per minute.

ASSAY OF GLUTATHIONE REDUCTASE (GR)

Glutathione reductase activity was followed as per the method of David and Richard (1983).

PRINCIPLE

The conversion of oxidized glutathione to reduced glutathione is catalyzed by glutathione reductase employing NADPH as a substrate. The amount of NADPH utilized is a direct measure of enzyme activity.

REAGENTS

1. Phosphate buffer (0.12M, pH 7.2)
2. EDTA (15mM)
3. Sodium azide (10mM)
4. Oxidized glutathione (6.3mM)
5. NADPH (9.6mM)

PROCEDURE

PREPARATION OF ENZYME EXTRACT

Triticum aestivum leaves (0.5g) on 4, 8 and 12 days of its growth were extracted by homogenization into 5 ml of phosphate buffer. The homogenate was spun at 5000g for 10 minutes and the supernatant was used for the assay.

ASSAY

The assay system contained 1ml of phosphate buffer, 0.1ml of EDTA, 0.1ml of sodium azide, 0.1ml of oxidized glutathione and 0.1ml of the enzyme source and water in a final volume of 2ml. The assay mixture was incubated for 3 minutes. 0.1ml of NADPH was added after incubation. The absorbance at 340nm was recorded at an interval of 15 seconds for 3 minutes. The complete assay

mixture that contained water instead of oxidized glutathione served as control. One unit of glutathione reductase is defined as the μ moles of NADPH oxidized/minute.

NON-ENZYMIC ANTIOXIDANTS

The non-enzymic antioxidants analyzed in the *Triticum aestivum* leaves were ascorbic acid, α -tocopherol, reduced glutathione, total phenols, flavonoids, total carotenoids, lycopene and chlorophyll.

ESTIMATION OF ASCORBIC ACID

The levels of ascorbic acid in the *Triticum aestivum* leaves were quantified spectrophotometrically by the method described by Roe and Keuther (1943).

PRINCIPLE

Ascorbate is converted to dehydro ascorbate by the treatment with activated charcoal. Dehydro ascorbate reacts with 2,4-dinitrophenyl hydrazine to form osazone, which dissolves in sulphuric acid to give an orange coloured solution, whose absorbance can be measured spectrophotometrically at 540nm.

REAGENTS

1. TCA (4%)
2. Sulphuric acid (9N)
3. 2, 4-dinitrophenyl hydrazine (2%) in 9N H₂SO₄
4. Thiourea (10%)
5. Sulphuric acid (80%)
6. Working standard (100 μ g/ml of ascorbic acid in 4% TCA)

PROCEDURE

EXTRACTION OF ASCORBIC ACID

Triticum aestivum leaves (1g) were homogenized in 4% TCA and the volume was made upto 10ml. The debris in the homogenate were removed by centrifugation. The supernatant obtained was treated with a pinch of activated charcoal, shaken well and allowed to stand for 5 minutes. The charcoal residue was removed by centrifugation. Aliquots (0.5ml) of the supernatants were used for the estimation.

ESTIMATION

Aliquots (ranging between 0.2-1.0ml) of the working standard ascorbate solution and the aliquots of the charcoal treated vitamin extracts (wheat grass homogenate) were made upto 2.0ml with 4% TCA. DNPH reagent (0.5ml) was added to each tube, followed by 2 drops of thiourea solution and incubated at 37°C for 3 hours. The osazone crystals formed were dissolved in 2.5ml of 85% H₂SO₄, drop by drop while mixing so as to avoid local heating. The tubes were cooled in ice and the absorbance was read at 540nm. The concentration of ascorbate in the samples were calculated from the standard curve constructed on an electronic calculator set to the linear regression mode and expressed as mg ascorbate/g leaf.

ESTIMATION OF TOCOPHEROL

Tocopherol levels were estimated in the leaves of *Triticum aestivum* using the Emmerie-Engel reaction, as reported by Rosenberg (1992).

PRINCIPLE

The Emmerie-Engel reaction is based on the reduction of ferric to ferrous ions by tocopherol, which then forms a red colour with 2,2'-dipyridyl. Tocopherols and carotenes are first extracted with xylene and the extinction read at 460nm to

measure carotenes. A correlation is made for this after adding ferric chloride and read at 520nm.

REAGENTS

1. Absolute ethanol
2. Xylene
3. 2,2'-dipyridyl (1.2g/L in n-propanol)
4. Ferric chloride (1.2g/L in ethanol)
5. Standard solution (D, L- α -tocopherol, 10mg/L in absolute ethanol)
6. Sulphuric acid (0.1N)

PROCEDURE

PREPARATION OF PLANT EXTRACT

Triticum aestivum leaves (2.5g) were homogenized in a small volume of 0.1N sulphuric acid and the volume was made upto 50ml with 0.1N sulphuric acid. The contents of the flask were allowed to stand overnight. The homogenate was shaken vigorously on the next day and filtered through Whatmann No. 1 filter paper. Aliquots of the filtrate were used for the estimation of tocopherol.

ESTIMATION

Into three stoppered centrifuge tubes, 1.5ml of plant extract, standard and water respectively were pipetted out. Ethanol (1.5ml) and xylene (1.5ml) were added to all the tubes, mixed and centrifuged. Xylene layer (1.0ml) was transferred into another stoppered tube and 2,2'-dipyridyl reagent (1.0ml) was added to each tube and mixed. The reaction mixture (1.5ml) was pipetted into a spectrophotometer cuvette and the extinction was read at 460nm. Ferric chloride solution (0.33ml) was added and after exactly 15 minutes, the absorbance of the

red colour was read against blank at 520nm. The concentration of tocopherol in the samples were calculated using the formula,

$$\text{Tocopherol } (\mu\text{g}) = \frac{\text{Reading at 520nm} - \text{Reading at 450nm}}{\text{Reading of standard at 520nm}} \times 0.29 \times 15$$

The results are expressed as μg tocopherol/g leaf.

ESTIMATION OF REDUCED GLUTATHIONE

The method proposed by Moron *et al.* (1979) was used for the estimation of reduced glutathione.

PRINCIPLE

Reduced glutathione is measured by its reaction, with DTNB (5,5'-dithiobisnitro benzoic acid) to give a yellow coloured product that absorbs maximally at 412nm.

REAGENTS

1. TCA (5%)
2. Phosphate buffer (0.2M, pH 8.0)
3. DTNB (0.6mM in 0.2M phosphate buffer)
4. Standard GSH (10 nmoles/ml in 5% TCA)

PROCEDURE

PREPARATION OF PLANT EXTRACT

Triticum aestivum leaves (0.5g) were homogenized with 2.5ml of 5% TCA. The protein precipitated was centrifuged at 1000g for 10 minutes. The supernatant (0.1ml) was used for the estimation of GSH.

ESTIMATION

The supernatant (0.1ml) was made upto 1.0ml with phosphate buffer. Freshly prepared DTNB solution (2.0ml) was added and the intensity of the yellow colour formed was read at 412nm in a spectrophotometer after 10 minutes. A standard curve of GSH was prepared using concentrations ranging from 2-10 nmoles. The values were expressed as mg GSH/g leaf.

ESTIMATION OF TOTAL PHENOLS

The levels of total phenols were determined using the method of Mallick and Singh (1980).

PRINCIPLE

Phenols react with phospho molybdic acid in Folin-Ciocalteu reagent in alkaline medium and produce a blue-coloured complex that absorbs maximally at 650nm.

REAGENTS

1. Ethanol (80%)
2. Folin-Ciocalteu reagent (1N)
3. Sodium carbonate (20%)
4. Stock standard solution (catechol 1mg/ml in water)
5. Working standard solution (1 in 10 dilution)

PROCEDURE

Triticum aestivum leaves (0.5g) were homogenized in 10x volumes of 80% ethanol. The homogenate was centrifuged at 10,000g for 20 minutes. The residue was re-extracted with 80% ethanol. The supernatants were pooled and evaporated to dryness. The residue was then dissolved in a known volume of distilled water

and 0.5ml of Folin-Ciocalteu reagent was added to it. Sodium carbonate (20%, 2.0ml) was added after 3 minutes and the tubes were placed in a boiling water bath for exactly 1 minute and cooled. The absorbance was measured at 650nm in a spectrophotometer against a reagent blank. Standard catechol solution (0.2-1.0ml) corresponding to 2.0-10.0 μ g concentrations were added with Folin-Ciocalteu reagent and sodium carbonate.

A standard curve was constructed using an electronic calculator on the linear regression mode, the concentrations of phenols in the samples were quantified. The values were expressed as mg phenols/g leaf.

ESTIMATION OF FLAVONOIDS

Flavonoid content in the samples was measured by the method of Cameron *et al.* (1943).

PRINCIPLE

Flavonoids react with vanillin to produce a coloured product which can be measured spectrophotometrically.

REAGENTS

1. Vanillin reagent (1% in 70% H₂SO₄)
2. Catechin standard (110 μ g/ml)
3. Methanol
4. Hexane

PROCEDURE

PREPARATION OF PLANT EXTRACT

Triticum aestivum leaves (0.5g) were extracted with methanol : water (2:1) and methanol : water (1:1). The two extracts were then combined and evaporated

to 1/3rd of the original volume. The resultant aqueous extract was cleared of low polarity contaminants by extracting with hexane. The solvent-extracted aqueous layer containing the bulk of the flavonoids was then concentrated.

ESTIMATION

An aliquot of the extract was pipetted out and evaporated to dryness. Vanillin reagent (4ml) was added and heated for 15 minutes. The standard was also treated in the same manner. The absorbance was measured at 340nm. The values were expressed as mg flavonoids / g leaf.

ESTIMATION OF TOTAL CAROTENOIDS AND LYCOPENE

Total carotenoids and lycopene were estimated by the method of Zakaria *et al.* (1979).

PRINCIPLE

The total carotenoids and lycopene in the samples were extracted in petroleum ether. The total carotenoids were estimated spectrophotometrically at 450nm. After measuring the total carotenoids at 450nm, lycopene was estimated with the same extract at 503nm. At 503nm, lycopene has a maximum absorbance, while carotenes have only negligible absorbance.

REAGENTS

1. Petroleum ether (40-60°C)
2. Anhydrous sodium sulphate
3. Calcium carbonate
4. Ethanolic potassium hydroxide (12%)

PROCEDURE

All the steps subsequent to the saponification were carried out in the dark to avoid photolysis of carotenoids. *Triticum aestivum* leaves (5.0g) were homogenized and saponified with 2.5ml of 12% ethanolic potassium hydroxide in a water bath at 60°C for 30 minutes. The saponified extract was then transferred into a separating funnel and 10-15ml of petroleum ether was added and mixed. The lower aqueous phase was transferred to another separating funnel and the upper petroleum ether containing the carotenoid pigment was collected. The extraction was repeated until the aqueous phase was colourless. A small quantity of anhydrous sodium sulphate was added to the petroleum ether extract to remove excess moisture, if any. The absorbance of the yellow colour was read at 450nm and 503nm using petroleum ether as a blank. The amount of total carotenoids and lycopene was calculated using the formula,

$$\text{Amount of total carotenoids} = \frac{A_{450} \times \text{Volume of sample} \times 100 \times 4}{\text{Weight of sample}}$$

$$\text{Amount of lycopene} = \frac{3.12 \times A_{503} \times \text{Volume of sample} \times \text{dilution} \times 100}{\text{Weight of sample}}$$

The total carotenoids and lycopene were expressed as mg/g leaf.

ESTIMATION OF CHLOROPHYLL

Chlorophyll was estimated by the method proposed by Witham *et al.* (1971).

PRINCIPLE

The estimation of chlorophyll is based on the absorption coefficient at 663 and 645nm.

REAGENT

80% acetone (pre-chilled).

PROCEDURE

Triticum aestivum leaves (1g) were extracted with 20ml of 80% acetone. The supernatant was transferred to a volumetric flask after centrifugation at 5000g for 5 minutes. The extraction was repeated until the residue was colourless. The supernatant was made upto 100ml with 80% acetone. The absorbance of the green coloured solution was read at 645 and 663nm against 80% acetone blank. The total chlorophyll in the leaf was calculated using the formula,

$$\text{Total chlorophyll} = 202(A_{645}) + 8.02(A_{663}) \times \frac{V}{1000 \times W}$$

where V was the final volume of the extract and W was the fresh weight of the leaves taken for extraction. The values are expressed as mg chlorophyll/g leaf.

The outcome of the first phase (presented in the next chapter) revealed that *Triticum aestivum* leaves are rich sources of the antioxidants studied, with the maximum effect shown by the 4th day plant followed by 8th and 12th day saplings. An attempt was made to test the antioxidant activity on 2nd day plant. The seeds were just sprouting and were very small for the analysis. Hence the analysis were carried out on 4, 8 and 12 days after sowing.

PHASE II

In Phase II, the efficiency of the leaf extracts of *Triticum aestivum* on the time periods selected were studied under *in vivo* simulated *in vitro* conditions to understand the nature of the molecular events taking place. The antioxidant potential of the *Triticum aestivum* leaves were studied against a battery of radicals and oxidants.

In the initial stage of phase II, the leaves were serially extracted into cold solvents of increasing polarity using Soxhlet apparatus. The solvents used were petroleum ether, benzene, ethyl acetate, methanol and water. The solvents were evaporated to dryness and the yields of the extracts were calculated. The residues were redissolved in a known volume of the same solvent in which they were extracted. The crude aqueous extract and different solvent extracts were tested for their SO^{\bullet} and NO scavenging effects as explained below.

RADICAL SCAVENGING EFFECTS OF *Triticum aestivum* LEAVES

The radical scavenging potential of the *Triticum aestivum* leaf extracts against a battery of oxidative radicals was evaluated using superoxide radicals, nitric oxide radicals, DPPH stable free radicals, H_2O_2 generated *in vitro* and hydroxyl radicals in cell-free systems. The experimental details of the same are documented as below.

SUPEROXIDE SCAVENGING ACTIVITY

The efficiency of the leaf extracts of *Triticum aestivum* in inhibiting the *in vitro* generation of SO^{\bullet} was studied by the method of Winterbourn *et al.* (1975).

PRINCIPLE

The extent of superoxide generation was studied on the basis of inhibition in the production of nitroblue tetrazolium formazon of the superoxide ion by the plant extracts and is measured spectrophotometrically at 560nm.

REAGENTS

1. EDTA (0.1M containing 1.5mg of NaCN)
2. Nitroblue tetrazolium (NBT-1.5mM)
3. Riboflavin (0.12mM)

4. Phosphate buffer (0.067M, pH 7.6)
5. Dimethyl sulfoxide (DMSO)

PROCEDURE

The assay mixture (3.0ml) contained 0.02ml of the plant extracts (20mg concentration) with 0.2ml of EDTA, 0.1ml of NBT, 0.05ml of riboflavin and 2.64ml of phosphate buffer. The control tubes were also setup wherein DMSO was added instead of plant extracts. All the tubes were vortexed and the initial optical density was measured at 560nm. The tubes were illuminated uniformly by using a fluorescent lamp for 30 minutes. The optical density was again measured at 560nm. The difference in optical density before and after illumination is the quantum of superoxide production and the per cent inhibition by the plant extract was calculated by comparing with the optical density of the control.

NITRIC OXIDE SCAVENGING ACTIVITY

The extent of inhibition of nitric oxide radical generation *in vitro* was quantified by the method of Green *et al.* (1982).

PRINCIPLE

An aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with oxygen to produce nitrite ions, which is measured spectrophotometrically at 546nm.

REAGENTS

1. Sodium nitroprusside (100mM)
2. Phosphate buffered saline (pH 7.2)
3. Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylethylene diamine dihydrochloride)

PROCEDURE

The reaction mixture (3.0ml) containing 2.0ml of sodium nitroprusside, 0.5ml of phosphate buffered saline and 0.5ml of different plant extracts (20mg), was incubated at 25°C for 30 minutes. After incubation, 0.5ml of Griess reagent was added and allowed to stand for 30 minutes. The control tubes were prepared without the leaf extracts. The absorbance of the pink coloured chromogen was read at 546nm against a reagent blank.

DPPH RADICAL SCAVENGING ACTIVITY

The antioxidant effects of the leaf extracts were studied using their ability to scavenge the DPPH (1,1-diphenyl-2-picryl hydrazyl), a stable free radical, was tested in a rapid dot plot screening assay and quantified using a spectrophotometric assay.

PRINCIPLE

DPPH when acted upon by an antioxidant, is converted into diphenyl-picryl hydrazine. The degree of discolouration from purple to yellow was quantified spectrophotometrically at 518nm, which is a measure of the scavenging effect of antioxidant extracts.

DOT-PLOT RAPID SCREENING ASSAY

The rapid screening assay was performed by the method of Soler-Rivas *et al.* (2000).

REAGENTS

1. TLC plates (silica gel 60 F₂₅₄, Merck)
2. DPPH (0.4mM) in methanol.

PROCEDURE

Aliquots of different solvent extracts (3µl) of *Triticum aestivum* leaf extracts of 4, 8 and 12 DAS were spotted on TLC plate and allowed to air dry. The TLC plate with the dry spots was placed upside down in a solution of DPPH in methanol for 10 seconds. The spots exhibiting radical scavenging antioxidant activity were visible as yellow spots against a purple background.

DPPH SPECTROPHOTOMETRIC ASSAY

The fresh juice of the 4th, 8th and 12th day leaf extracts of *Triticum aestivum* showed better superoxide and nitric oxide radical scavenging activity among the other different solvent extracts tested. Hence the crude aqueous homogenate of the leaves was used in subsequent studies. As a first step, the DPPH scavenging effect of the extracts was quantified using the method proposed by Mensor *et al.* (2001).

REAGENTS

1. DPPH in methanol (0.3mM)
2. Methanol

PROCEDURE

Triticum aestivum leaf extracts, (20 µl corresponding to 4mg) were added with 0.5ml of a methanolic solution of DPPH and 0.48ml of methanol. The mixture was then allowed to stand at room temperature for 30 minutes. Methanol served as a blank and DPPH in methanol without the leaf extracts served as positive control. After incubation, the discolouration of the purple colour was read at 518nm in a spectrophotometer. The per cent radical scavenging activity was calculated as follows,

$$\text{Scavenging activity (\%)} = 100 - \frac{A_{518}[\text{sample}] - A_{518}[\text{blank}]}{A_{518}[\text{blank}]}$$

HYDROGEN PEROXIDE SCAVENGING ASSAY

The ability of the *Triticum aestivum* leaf extracts to scavenge H₂O₂ was determined spectrophotometrically according to the method of Ruch *et al.* (1989).

PRINCIPLE

H₂O₂ scavenging activity was measured in terms of a decrease in the absorbance at 230nm spectrophotometrically.

REAGENTS

1. H₂O₂ (4mM)
2. Phosphate buffer (pH 7.4)

PROCEDURE

A solution of H₂O₂ was prepared in phosphate buffer. H₂O₂ concentration was determined spectrophotometrically from its absorption at 230 nm. Plant extracts (10mg, 0.01ml) was added to H₂O₂ and incubated for 10 minutes. The absorbance at 230nm was determined against a blank containing phosphate buffer without H₂O₂. The percentage scavenging of H₂O₂ by plant extract and standard compounds was calculated using the formula,

$$\% \text{ scavenging of H}_2\text{O}_2 = \frac{A(\text{control}) - A(\text{sample})}{A(\text{control})} \times 100$$

HYDROXYL RADICAL SCAVENGING ACTIVITY

The hydroxyl radical scavenging activity was measured by the method proposed by Elizabeth and Rao (1990).

PRINCIPLE

Hydroxyl radicals generated from a Fe^{2+} /ascorbate/EDTA/ H_2O_2 system degrades deoxyribose, producing TBARS. The ability of the plant extracts to inhibit TBARS formation was assayed.

REAGENTS

1. Deoxyribose (2.8mM)
2. FeCl_3 (0.1mM)
3. EDTA (0.1mM)
4. H_2O_2 (1mM)
5. Ascorbate (0.1mM)
6. KH_2PO_4 -KOH buffer (20mM, pH 7.4)
7. Thiobarbituric acid (1%)

PROCEDURE

The reaction mixture (1.0ml) contained 0.1ml of deoxyribose, 0.1ml of FeCl_3 , 0.1ml of EDTA, 0.1ml of H_2O_2 , 0.1ml of ascorbate, 0.1ml of KH_2PO_4 -KOH buffer and 20 μl *Triticum aestivum* leaf extracts (10mg). The reaction mixture was then incubated for 1 hour at 37°C. At the end of the incubation period, 1.0ml of TBA was added and heated in a boiling water bath for 20 minutes. The pink colour produced was measured at 535nm in a spectrophotometer. The per cent TBARS produced for positive control (H_2O_2) was fixed as 100% and the relative per cent TBARS was calculated for the extract treated groups.

EFFECT OF *Triticum aestivum* LEAF EXTRACTS ON MEMBRANE LIPIDS

Oxidation and the production of free radicals are an integral part of human metabolism. The lipid components of biological membranes are especially vulnerable to oxidation and may undergo a chain peroxidation process. The extent of oxidative damage to membrane lipids can be measured by assessing lipid peroxidation.

The three different membrane preparations used in the present study were RBC ghosts which comprises plasma membrane lipids, goat liver homogenate comprising a mixture of both plasma membrane and intracellular membrane lipids and goat liver slices which comprises intact cells. This was done in order to study the effect of *Triticum aestivum* leaf extracts on the damage inflicted to the lipid preparations.

The extent of inhibition of lipid peroxidation in RBC ghosts was determined by the method proposed by Dodge *et al.* (1963) and in goat liver homogenate and goat liver slices by Okhawa *et al.* (1979).

ESTIMATION OF THE EXTENT OF LIPID PEROXIDATION PRINCIPLE

Oxidizing agents (ferrous ions and ascorbate, or H₂O₂) impose a stress on membrane lipids which can be quantified as the extent of thiobarbituric acid reactive substances (TBARS) formed.

EVALUATION OF LPO IN GOAT RBC GHOSTS REAGENTS

1. Isotonic KCl (1.15%)
2. Hypotonic KCl (0.3%)

3. Tris-buffered saline (TBS) (10mM Tris, 0.15M NaCl, pH 7.4)
4. FeSO₄ (10μM)
5. Ascorbic acid (0.06mM)
6. Ethanol (70%)
7. Thiobarbituric acid (TBA) (1%)
8. Acetone

PROCEDURE

PREPARATION OF RBC MEMBRANE GHOSTS

RBC ghosts were prepared by the method of Dodge *et al.* (1963). About 50ml of fresh goat blood was collected in a sterile container. The blood was defibrinated immediately using acid-washed stones. The defibrinated blood was transferred to a fresh sterile container and diluted 1:1 with sterile isotonic KCl. Pelleted the RBCs by centrifugation at 3000g for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed thrice with isotonic KCl.

The washed pellet was suspended in hypotonic KCl (0.5%) and incubated at 37°C for one hour for complete lysis to occur. Complete lysis was indicated by the hemoglobin being released into the supernatant. The lysate was centrifuged at 5000g for 15 minutes at 4°C. The pellet was washed repeatedly with hypotonic KCl until all the hemoglobin washed off and a pale pink pellet containing the erythrocyte ghosts were obtained.

The ghost preparation was suspended in 1.5ml of TBS and used as a source of membrane lipids. The assay was carried out according to the method of Okhawa *et al.* (1979) as given below.

PROCEDURE

The reaction mixture (0.5ml) contained 0.1ml each of RBC ghosts, *Triticum aestivum* leaf extracts, FeSO₄, ascorbate and TBS. A blank containing no lipid source but only FeSO₄, no plant extract, ascorbate and TBS to a final volume of 0.5ml was prepared.

An assay medium corresponding to 100% oxidation was prepared by adding all the other constituents except the plant extracts. The experimental medium corresponding to auto-oxidation contained only the RBC ghosts. The tubes were incubated at 37°C for 1 hour. After incubation 0.5ml of 70% ethanol was added to all the tubes to arrest the reaction. TBA (1.0ml) was added to all the tubes and boiled in a water bath for 20 minutes. After cooling to room temperature, the tubes were centrifuged and the supernatants were added with 0.5ml of acetone. The intensity of the pink colour produced was measured at 535nm in a spectrophotometer.

EVALUATION OF LPO IN GOAT LIVER HOMOGENATE

Fresh goat liver was collected from a local slaughter house and washed free of blood using Tris-HCl buffer (40mM, pH 7.0). A 20% homogenate was then prepared in the same buffer, using Teflon homogenizer. The supernatant was centrifuged in cold to remove the debris and was used as a membrane source of the LPO assay (Okhawa *et al.*, 1979), the same procedure adopted for the evaluation of LPO in RBC ghosts was followed.

EVALUATION OF LPO IN GOAT LIVER SLICES

Fresh goat liver was collected from a slaughter house, plunged into cold sterile PBS and maintained at 4°C till the assay. Very thin (1mm) slices of the goat liver were cut using a sterile scalpel. The determination of lipid peroxidation

(Okhawa *et al.*, 1979) in goat liver slices was carried out as for the liver homogenate, in which ferrous sulphate (10 μ M) alone was added as a prooxidant during the incubation.

EFFECT OF *Triticum aestivum* LEAF EXTRACTS ON DNA DAMAGE INDUCED BY THE OXIDANT

Oxidative damage to DNA is the seemingly inevitable consequence of cellular metabolism. Despite protective mechanisms, cellular levels of damage may increase under conditions of oxidative stress, arising from the exposure to a variety of physical or chemical insults (Cooke *et al.*, 2006). Deoxyribonucleic acid (DNA) is a particularly important target for oxidation, as the damage may lead to heritable alterations.

The effect of *Triticum aestivum* leaf extracts were evaluated for their DNA-protective effects against the damage induced in different sources of DNA by the standard oxidant H₂O₂. The oxidant damage to DNA was followed *in vitro* using purified, commercially available preparations of DNA and in intact cells. The pure DNA used were of different evolutionarily hierarchical levels. They were λ DNA (linear, phage DNA), pUC18 plasmid DNA (circular, bacterial DNA) and herring sperm (genomic, haploid DNA from higher eukaryote). The extent of DNA damage was also followed in Hep2 (laryngeal carcinoma) and KB (oral carcinoma) cell lines.

EVALUATION OF THE EXTENT OF DNA DAMAGE IN λ DNA AND pUC18 DNA

The extent of damage induced in λ DNA and pUC18 DNA was followed by the variation in the migration pattern in agarose gel (Chang *et al.*, 2002).

REAGENTS

1. λ DNA (2 μ g)
2. pUC18 DNA (2 μ g)
3. Tris buffer (50mM, pH 7.4)
4. H₂O₂ (30%)
5. FeCl₃ (500 μ M)
6. Agarose (1%) in 1x TAE buffer
7. EtBr (10mg/ml)
8. Gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, glycerol)
9. 50x TAE buffer (Tris base 24.2g, EDTA 18.612g, glacial acetic acid 5.71ml and the volume was made upto 100ml, pH 8.0)

PROCEDURE

The reaction mixture (30 μ l) contained 5 μ l of Tris buffer or pUC18 DNA/ λ DNA (2 μ g) and 5 μ l of tris buffer or plant extract. H₂O₂ (10 μ l) and 5 μ l of FeCl₃ were added to the test samples and then incubated at 37°C for 15 minutes for pUC18 DNA and 30 minutes for λ DNA. The assay mixture was mixed with 6 μ l of the gel loading dye and loaded onto 1% agarose gel with 5 μ g/ml EtBr. In a submarine gel electrophoretic apparatus, electrophoresis was carried out at 100V for 15 minutes. The DNA was visualized under the UV transilluminator and photographed using an Alpha Digidoc digital gel documentation system.

EVALUATION OF THE EXTENT OF DAMAGE IN HERRING SPERM DNA

The DNA damage caused by H₂O₂ to DNA was measured spectrophotometrically by the method of Aeschlach *et al.* (1994).

PRINCIPLE

Oxidizing agents (H_2O_2 , Ferrous ions, magnesium chloride) impose an oxidative stress on DNA which can be measured as the extent of TBARS generated.

REAGENTS

1. Herring sperm DNA (0.5mg/ml in 500 mM tris buffer).
2. H_2O_2 (30%)
3. $MgCl_2$ (5mM)
4. $FeCl_3$ (50 μ M)
5. EDTA (0.1M)
6. TBA (1%)
7. HCl (25% v/v)

PROCEDURE

The reaction mixture (0.5ml) contained 0.05ml of herring sperm DNA, 0.167ml of H_2O_2 , 0.05ml of $MgCl_2$, 0.05ml of $FeCl_3$ and leaf extracts (10mg) or buffer to the same volume. The mixtures were incubated at 37°C for 1 hour. The reaction was terminated by the addition of 0.05ml of 0.1M EDTA. The colour was developed by adding 0.5ml of thiobarbituric acid and 0.5ml HCl, followed by incubation at 37°C for 15 minutes. After centrifugation, the extent of DNA damage was measured by the increase in absorbance at 532nm.

EVALUATION OF THE EXTENT OF DNA DAMAGE IN INTACT CELLS

The extent of DNA damage was followed in intact cells by alkaline single cell gel electrophoresis assay (comet assay) (Singh *et al.*, 1988). The intact cells used were cultured human carcinoma cell lines namely Hep2 (laryngeal carcinoma cells) and KB (oral carcinoma cells).

The Hep2 and KB cell lines were procured from the National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). The cells were grown confluent and were harvested by trypsinization (0.25%). The harvested cells were collected in DMEM containing 10% FBS and were held on ice till the analysis. Prior to the assay, the cells were spun down at 2000g for 5 minutes at 4°C in a microfuge and resuspended in Hank's Balanced Salt Solution (HBSS).

PRINCIPLE

The alkaline (pH > 13) single cell gel (SCG) electrophoresis assay, commonly known as comet assay, combines the simplicity of biochemical techniques for detecting DNA single strand breaks, alkaline labile sites and the single cell approach typical of cytogenetic assays. This assay is one of the most sensitive assays to detect DNA damage.

REAGENTS

1. H₂O₂ (30%)
2. Low melting point agarose (LMPA – 0.5%)
3. Normal melting point agarose (NMPA – 1%)
4. Lysis solution (1M tris, pH 8.0)
5. Alkaline electrophoresis buffer (10N NaOH, 0.2M EDTA, pH >13)
6. Neutralizing solution (1M tris, pH 7.5)
7. EtBr (5µg/ml)
8. EDTA (0.5M)
9. NaCl (2.5M)
10. DMSO (10%)
11. Triton X-100 (1%)

PROCEDURE

Hep2 and KB cells were treated with H₂O₂ in the presence and the absence of *Triticum aestivum* leaf extracts (20mg) and incubated at 37°C for 1 hour. At the end of the incubation period, an aliquot (150µl) of the cell suspension was mixed with equal volumes of 0.5% molten LMPA and maintained at 37°C. This suspension (75µl) was layered carefully onto 1% NMPA coated glass slides and spread evenly using a coverslip (22x40mm). The slides were then immediately placed on slide trays held on ice in order to solidify the agarose. The coverslip was removed carefully and the cell spreaded was overlaid with a layer of LMPA without cells. After solidification, the ‘mini gels’ were placed in cold lysing solution taken in coplin jars and incubated overnight at 4°C. The lysed cells were denatured in the alkaline electrophoresis buffer for 20 minutes and electrophoresed in the same buffer at 25V to 20 minutes. The slides were neutralized in Tris buffer for 10 minutes (pH 7.5) and stained in ethidium bromide solution. The slides were washed to remove excess ethidium bromide and air dried. The slides were then scored for the presence of comet ‘tails’ under oil immersion in a Nikon fluorescent microscope. Totally 100 cells per slide were scored and the frequency of DNA damage as the number of comet bearing cells were noted.

ASSESSMENT OF CELL VIABILITY

The cytotoxicity of *Triticum aestivum* leaf extracts were determined by performing MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay in Hep2 and KB cells by the method of Igarashi and Miyazawa (2001).

PRINCIPLE

Living cells convert MTT into its formozan derivative. The number of surviving cells can be determined by the amount of MTT formozan produced, which is measured in an ELISA reader after solubilization with a suitable solvent.

REAGENTS

1. Phosphate buffered saline (PBS) (pH 7.2)

Sodium chloride	8.8g
Potassium chloride	0.2g
Disodium hydrogen phosphate	7.15g
KH ₂ PO ₄	0.2g in 1000ml water
2. MTT (3mg/ml in PBS)
3. 2-propanol in 0.04N HCl
4. H₂O₂ (200µM)

PROCEDURE

An aliquot of the incubated sample (350µl containing 10⁶ Hep2 and KB cells, 87.5µl of H₂O₂, with or without 20µl (20mg) of plant extract) was mixed well with 50µl of MTT. The mixture was then incubated at 37°C for 3 hours. After incubation 200µl of PBS was added to all the samples. The liquid was then carefully aspirated after centrifugation at 10,000g for 3 minutes. The cells were then resuspended in 200µl of acid-propanol and left overnight in the dark. The absorbance was read at 650nm in an ELISA plate reader. The optical density of the control cells were fixed to be 100%, the per cent viability of the cells in the other treatment groups was calculated.

EVALUATION OF THE EFFECTS OF *Triticum aestivum* LEAF EXTRACTS ON THE ANTIOXIDANT STATUS OF THE CELLS *in vitro*

The first part of the phase II analysis revealed the 4th, 8th and 12th day aqueous extracts of *Triticum aestivum* leaves possessed good antioxidant effects against the oxidative assaults. The DNA damage was also reverted in intact live cells. In the next step, the antioxidant response evoked by the *Triticum aestivum* leaf extracts were studied in the cells of the liver in order to assess its activity in

the cellular environment and to validate its use as an alternative to the current *in vivo* tests, as a means to replace animals in experiments.

ANTIOXIDANT STATUS *in vitro*

The *in vitro* model used in the study as an alternative to live animals was goat liver slices. Precision cut liver slices were taken as the *in vitro* alternative in the place of live animals. Recently, much effort has been devoted to the development of *in vitro* systems and precision-cut liver slices are a widely used tool for *in vitro* metabolic and toxicological studies (Martignoni *et al.*, 2004).

Fresh goat liver was obtained from a local slaughter house and transported to the laboratory on ice. The liver was then washed with isotonic KCl and processed for the assays. Liver was the organ of choice because it is the metabolic organ and is responsible for the metabolic clearance of many xenobiotics (Tingle and Helsby, 2006). Very thin slices (1mm thick) were cut from the liver using a sterile scalpel. The slices were taken in sterile Hank's balanced salt solution (HBSS) at a proportion of 0.25g in 1ml. HBSS simulated the peritoneal fluid in live animals. H₂O₂ was used as the oxidant to induce oxidative stress in the liver slices and was used at a final concentration of 500µM. The plant extract (20µl corresponding to 20mg) was used to study the antioxidant effects on the cells.

The following groups were setup for every assay

1. Untreated (negative control)
2. H₂O₂ treated (positive control)
3. Group treated with 4th day extract of *Triticum aestivum* leaves
4. Group treated with H₂O₂ and 4th day extract of *Triticum aestivum* leaves
5. Group treated with 8th day extract of *Triticum aestivum* leaves
6. Group treated with H₂O₂ and 8th day extract of *Triticum aestivum* leaves
7. Group treated with 12th day extract of *Triticum aestivum* leaves
8. Group treated with H₂O₂ and 12th day extract of *Triticum aestivum* leaves

After addition of the respective agents, the tissue slices were incubated at 37°C for 1 hour with mild shaking. After the incubation period, the tissue was homogenized in a Teflon homogenizer with HBSS. The estimation of various parameters indicative of antioxidant potential were analyzed in the homogenate.

DETERMINATION OF ENZYMIC ANTIOXIDANTS

The enzymic antioxidants analyzed in the tissue homogenate were superoxide dismutase, catalase, peroxidase, glutathione reductase and glutathione S-transferase. The procedures adopted for the determination of the activities of enzymic antioxidants were the same as those for the leaf analysis (Phase I). An aliquot of the liver homogenate was used instead of plant extract.

DETERMINATION OF NON-ENZYMIC ANTIOXIDANTS

The non-enzymic antioxidants determined in the liver homogenate were ascorbic acid, vitamin E, reduced glutathione and vitamin A.

Vitamin C, vitamin E and reduced glutathione were assayed by the methods as elaborated in phase I of this chapter. Instead of the plant extract, the liver homogenate prepared after incubation was used for the estimations.

ESTIMATION OF VITAMIN A

Vitamin A was estimated by the method proposed by Bayfield and Cole (1980).

PRINCIPLE

The colour produced by vitamin A, its acetate and palmitate with TCA is proportional to its concentration, which property is used for its spectrophotometric estimation.

REAGENTS

1. Saponification mixture (2N KOH in 90% ethanol)
2. Petroleum ether (40-60°C)
3. Anhydrous sodium sulphate
4. Chloroform
5. Vitamin A palmitate
6. TCA reagent (60% TCA in chloroform)

PROCEDURE

To 1.0ml of the liver homogenate, 1.0ml of saponification mixture was added. The mixture was refluxed for 20 minutes at 60°C in the dark. It was then cooled and 20ml of water was added. Vitamin A was extracted twice with 10ml portions of petroleum ether (40-60°C). The extracts were pooled, washed thoroughly with water and the layers were separated using a separating funnel, when the petroleum ether fraction was clear, a pinch of sodium sulphate (anhydrous) was added to remove the excess moisture. The volume of the extract was noted and 1.0ml of it was evaporated to dryness at 60°C. The dried residue was dissolved in 1.0ml of chloroform. Aliquots of the standard (vitamin A palmitate) were pipetted out into a series of clean, dry test tubes in the concentration range of 0-7.5µg. The volume was made upto 1.0ml with chloroform in all the tubes. TCA reagent (2.0ml) was rapidly added and mixed well. The absorbance of blue colour formed was recorded immediately at 620nm in a spectrophotometer. Vitamin A levels were expressed as µg/g tissue.

PHASE III

Phase III was planned to evaluate the protective effects of the aqueous extract of the leaves of *Triticum aestivum* against ethanol-CCl₄ induced oxidative

stress and also for confirming the results obtained in the *in vitro* studies under *in vivo* conditions. The layout of the study is explained below.

EXPERIMENTAL ANIMALS

Male Wistar rats weighing between 150-190g and about 8 weeks old were purchased from the small animal breeding centre in Trissur, Kerala. All rats were allowed to acclimatize for two weeks before experimentation. Throughout the experimental period, the rats were provided with standard pellet diet and water *ad libitum*. The animal studies were followed according to the Institute animal ethics committee regulations approved by CPCSEA (623/02/b/CPCSEA).

TREATMENT GROUPS

The animals were randomly divided into 11 groups of 6 each. The experimental period was 21 days. All the treated rats received 10% ethanol in drinking water throughout the experimental period to induce cytochrome P450 2E1, the isoenzyme that metabolizes CCl₄ to its oxidant form. CCl₄ was administered as a single subcutaneous injection on day 21 alone (2ml/kg body weight) diluted 1:1 in paraffin oil.

- Group I - Untreated control
- Group II - Ethanol alone treated group
- Group III - Ethanol + CCl₄
- Group IV - Ethanol + Aqueous extract of 4th day leaves of *Triticum aestivum*
- Group V - Ethanol + CCl₄ + Aqueous extract of 4th day leaves of *Triticum aestivum*
- Group VI - Ethanol + Aqueous extract of 8th day leaves of *Triticum aestivum*
- Group VII - Ethanol + CCl₄ + Aqueous extract of 8th day leaves of *Triticum aestivum*
- Group VIII - Ethanol + Aqueous extract of 12th day leaves of *Triticum aestivum*
- Group IX - Ethanol + CCl₄ + Aqueous extract of 12th day leaves of *Triticum aestivum*
- Group X - Ethanol + Silymarin
- Group XI - Ethanol + CCl₄ + Silymarin

The aqueous extracts of *Triticum aestivum* leaves were prepared by grinding the leaves with distilled water using mortar and pestle to obtain a final concentration of 1.25%. The extract was clarified by centrifugation and administered at a dose of 500mg/kg body weight/day and were prepared fresh each day, while silymarin was administered at a dose 25mg/kg body weight/day. Both the plant extracts and silymarin were administered by gastric intubation (gavage) using an intubation tube, for 21 days. On day 21, CCl₄ injection was given subcutaneously and the animals were sacrificed by cervical dislocation on day 22. The response elicited by the *Triticum aestivum* leaf extracts were compared with a standard antioxidant silymarin.

The animals were dissected quickly and the blood was collected by cardiac puncture. The liver and kidney tissues were quickly excised and plunged into sterile, ice-cold saline for removal of blood. The washed organs were blotted dry on sterile filter paper and stored in 0.1M tris-HCl buffer (pH 7.4) at -85°C until analysis. A small portion of the organs were cut and fixed in 10% formalin for processing for histopathological observation. The blood collected were centrifuged, the serum was separated and the samples were stored at -85°C, till analysis.

Liver marker enzymes and lipid profile were analysed in the serum and the antioxidant status in the liver and kidney.

ASSAY OF SERUM MARKER ENZYMES

The markers for hepatic damage namely aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), γ -glutamyl transferase (γ -GT) and bilirubin were estimated in serum. All the hepatic markers were analysed using kits from Span Diagnostics Limited, Sachin, India.

ASSAY OF AST AND ALT (Bergmeyer *et al.*, 1978)

PRINCIPLE

AST and ALT catalyse the transfer of amino group from L-aspartate and L-alanine respectively to α -ketoglutarate to yield oxaloacetate/pyruvate along with L-glutamate. Oxaloacetate/pyruvate can oxidize NADH to NAD^+ in the presence of malate dehydrogenase/lactate dehydrogenase. The conversion of $\text{NADH} + \text{H}^+$ to NAD^+ causes a decrease in the absorbance at 340nm, the rate of which is proportional to AST/ALT activity.

REAGENTS

1. Reagent I Buffer

Tris	80mmol/L (pH 7.8)
L-Aspartate /L-alanine	240 mmol/L
LDH	≥ 600 U/L

2. Reagent II Substrate

2-oxoglutarate	12mmol/L
NADH	0.18mmol/L

PROCEDURE

The reagent kit contained solution A (aspartate/buffer/enzyme) for AST and (alanine/buffer/enzyme) for ALT and solution B (α -ketoglutarate/coenzyme). The working reagent was prepared by mixing 4ml of reagent 1 with 1ml of reagent B.

To 0.1ml of serum, 1.0ml of working reagent was added, mixed well, incubated for 60 seconds and the absorbance was read at 340nm. Noted the change in absorbance for 2 minutes. The enzyme activities were expressed as IU/L.

ASSAY OF ALP (Schlebusch *et al.*, 1974)

PRINCIPLE

At alkaline pH, ALP catalyzes the hydrolysis of p-nitrophenyl phosphate to yellow coloured p-nitrophenolate and phosphate. The change in absorbance can be measured at 415nm and is proportional to the enzyme activity.

REAGENT

1. Buffer
2. pNPP

PROCEDURE

The reagent kit contained buffer and p-nitrophenyl phosphate (pNPP) substrate. The working reagent was prepared by mixing one vial of pNPP substrate to 5.0ml of buffer.

The working reagent (1.0ml) was added to 20 μ l of serum and incubated for 1 minute. The increase in absorbance was measured at 415nm. The ALP activity was expressed as IU/L.

ASSAY OF γ -GT (Persijn and Van der Slik, 1978)

PRINCIPLE

γ -Glutamyl transferase catalyzes the transfer of amino group between L- γ -Glutamyl-3-carboxy-4-nitroanilide and glycylglycine to form L- γ -glutamylglycylglycine and 5-amino-2-nitrobenzoate. The rate of formation of 5-amino-2-nitrobenzoate is measured as an increase in absorbance at 450nm, which is proportional to γ -GT activity.

REAGENTS

1. Buffer
2. Substrates pNPP tablets

PROCEDURE

The kit contained buffer reagent and substrate tablets. The working reagent was prepared by dissolving 1 substrate tablet in 2.2ml of buffer reagent.

The working reagent (1.0ml) was incubated at 37°C for 1 minute and then 0.1ml of serum was added, mixed well. The absorbance was read after 1, 2 and 3 minutes. The mean absorbance change/minute was calculated and the enzyme activity was expressed as IU/L.

ESTIMATION OF BILIRUBIN

Bilirubin was estimated by the method of Jendrassik and Grof (1938).

PRINCIPLE

Both conjugated and unconjugated bilirubin react with diazotized sulfanilic acid in the presence of the accelerator, dimethyl sulfoxide to produce red-purple azobilirubin. The change in absorbance due to the formation of azobilirubin is recorded at 546nm and is directly proportional to the concentration of bilirubin (total and direct).

REAGENTS

1. Total bilirubin
2. Sodium nitrite
3. Total bilirubin standard
4. Total bilirubin blank

PROCEDURE

The working reagent was prepared by mixing 10 ml of total bilirubin reagent and 50 μ l of sodium nitrite, mixed well and let stand at room temperature for 15 minutes before use.

Serum (50 μ l) was added to 1.0ml of working bilirubin reagent and mixed well. A standard was setup by adding 50 μ l of total bilirubin standard to 1.0 ml of working bilirubin reagent. A sample blank was setup by adding 50 μ l of serum to 1.0ml of total bilirubin blank and all samples were incubated at 37°C for 5 minutes. The absorbance was read at 546nm. The total bilirubin was expressed as mg/dl.

DETERMINATION OF SERUM LIPIDS

The levels of cholesterol, triglycerides, free fatty acids and phospholipids were estimated in serum and analyzed using commercially available diagnostic kits purchased from Ark Diagnostics Pvt. Ltd., Mumbai.

ESTIMATION OF CHOLESTEROL (Allain *et al.*, 1974)

PRINCIPLE

Cholesterol esters are hydrolyzed by cholesterol esterase to free cholesterol and fatty acids. The free cholesterol produced and the pre-existing ones were oxidized by cholesterol oxidase to 3-cholestenone and H₂O₂. H₂O₂ in the presence of peroxidase, oxidizes the chromogen (4-amino antipyrine and phenol) to quinoneimine dye and the absorbance of quinoneimine measured at 505nm, is proportional to cholesterol concentration.

REAGENTS

1. Cholesterol mono reagent

Goods buffer	50mmol/L (pH 6.70)
Cholesterol oxidase	≥ 50U/L
Cholesterol esterase	≥ 100U/L
Peroxidase	≥ 3KU/L
4-aminoantipyrine	0.4mmol/L
2. Cholesterol standard 200mg/dl

PROCEDURE

The reagent kit contained cholesterol mono reagent (buffer/enzyme/chromogen) and cholesterol standard.

To 1.0ml of the cholesterol mono reagent, 0.01ml each of serum and standard were added, mixed well and incubated at 37°C for 10 minutes. The colour developed was read at 505nm against a reagent blank. The serum cholesterol was expressed as mg/dl.

ESTIMATED OF TRIGLYCERIDES (Bucolo and David, 1973)

PRINCIPLE

Triglycerides are quantified after enzymatic hydrolysis with lipases. Peroxidase catalyses the conversion of H₂O₂, 4-amino-antipyrine and N-methyl-N-sulphopropyl-N-anisidine (ESPAS) to a purple coloured quinonemine complex which can be measured at 546nm.

REAGENTS

1. Triglyceride mono reagent
2. Triglyceride standard 200mg/dl

PROCEDURE

To 0.01ml of triglyceride mono reagent taken in three tubes named blank, standard and test, 0.01ml of standard and serum were added in the respective tubes and incubated at 37°C for 10 minutes and the absorbance of the test and the standard were read against blank at 546nm. The values were expressed as mg/dl.

ESTIMATION OF FREE FATTY ACIDS

Non-esterified free fatty acids were estimated by the method of Falholt *et al.* (1973).

PRINCIPLE

The lipid is shaken well with high density copper reagent (pH 8.1) in the presence of phosphate buffer. The copper soap remaining in the upper organic layer is quantified spectrophotometrically with diphenyl carbazide at 550nm.

REAGENTS

1. Phosphate buffer (33moles/L, pH 6.4)
2. Extracting solvent (chloroform:hexane:methanol in the ratio 5:5:1)
3. Copper reagent (500nmoles/L, CuSO₄, triethanolamine, sodium hydroxide, sodium chloride, pH 8.1)
4. Standard palmitic acid (2mM/L)
5. Diphenyl carbazide

PROCEDURE

To 1.0ml of phosphate buffer, 6.0ml of extracting solvent, 2.5 ml of copper reagent and 0.1ml of serum was added. The tubes were shaken vigorously for 90 seconds and kept for 15 minutes. The tubes were centrifuged and 3.0ml of the upper layer was transferred to another tube containing 0.5ml of diphenyl carbazide

and mixed. Standard palmitic acid was treated with copper reagent and diphenyl carbazide. The absorbance was read at 550nm against phosphate buffer as blank. The serum free fatty acids were expressed as mg/dl.

ESTIMATION OF PHOSPHOLIPIDS

The levels of serum phospholipids were estimated by the method of Zilversmit and Davis (1950).

PRINCIPLE

Phospholipids are digested with sulphuric acid and the inorganic phosphorus formed reacts with ammonium molybdate and amino naphthol sulphonic acid (ANSA) to form a blue colour, which is measured at 680nm.

REAGENTS

1. Sulphuric acid (5N)
2. Concentrated nitric acid (HNO₃)
3. Ammonium molybdate (2.5%)
4. ANSA (0.4%)
5. Standard phosphate (KH₂PO₄ - 8µg phosphorus/ml)

PROCEDURE

To 0.1ml of 5N sulphuric acid, 0.1ml of serum was added and digested in a digestion rack till light brown. Concentrated nitric acid (2-3 drops) was added and the digestion was continued till it became colourless. To that 1.0ml of water was added and heated in a boiling water bath for 5 minutes. After cooling to room temperature 1ml of ammonium molybdate and 0.1ml of ANSA were added. The volume was then made upto 10.0ml with distilled water. The standard phosphate

was treated in a similar manner. The absorbance was measured at 680nm within 10 minutes. The serum phospholipids were expressed as mg/dl.

ANTIOXIDANT STATUS IN LIVER AND KIDNEY

The enzymic and non-enzymic antioxidants were determined both in the liver and kidney of all experimental rats. A known weight of the tissue was minced and homogenized in 0.1M Tris-HCl (pH 7.5). The volume was adjusted to obtain a 20% homogenate, which was subjected to the antioxidant analysis.

The enzymic antioxidants analyzed were SOD, CAT, Px, GR, GST and G6PD. The procedures adopted for the assay of CAT, Px, GR and GST were exactly the same as those followed for the tissue slices, which are explained in detail in the methodology of earlier phases.

ASSAY OF SUPEROXIDE DISMUTASE (SOD)

SOD was assayed by the method proposed by Misra and Fridovich (1972).

PRINCIPLE

Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating system and catalyses the inhibition of NBT reduction, the extent of which can be assayed spectrophotometrically at 600nm.

REAGENTS

1. Potassium phosphate buffer (50mM, pH 7.8)
2. Methionine (45 μ M)
3. Riboflavin (5.3mM)
4. Nitroblue tetrazolium (NBT) (84 μ M)
5. Potassium cyanide (20 μ M)

PROCEDURE

The assay mixture contained 3.0ml of tissue homogenate, 300 μ l potassium phosphate buffer, 300 μ l methionine, 300 μ l riboflavin, 300 μ l NBT, 300 μ l potassium cyanide and 1.25ml water. Reduced NBT was measured spectrophotometrically at 600nm after exposure to light for 10 minutes. The maximum reduction was evaluated in the absence of the enzyme. One unit of SOD activity was defined as the amount of enzyme giving a 50% inhibition of the reduction of NBT.

ASSAY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD)

G6PD was assayed by the method of Ballinsky and Bernstein (1963).

PRINCIPLE

The assay is based on the enzymatic transfer of electrons from glucose-6-phosphate to NADP where it is reduced to NADPH and the increase in absorbance can be measured at 340nm.

REAGENTS

1. Tris HCl buffer (0.1M, pH 8.2)
2. NADP (0.2mM)
3. Magnesium chloride (0.1M)
4. Glucose-6-phosphate (6 mM)

PROCEDURE

Tris-HCl buffer (0.4ml), 0.2ml of NADP, 0.2ml of magnesium chloride, 1.0ml of water and 0.2ml of tissue homogenate were taken in a cuvette. The reaction was started by the addition of 0.2ml of glucose-6-phosphate and the increase in absorbance was measured at 340nm. One unit of the enzyme was

expressed as the amount of enzyme that brought about a change in absorbance of 0.01/minute.

The non-enzymic antioxidants analyzed were vitamin C, vitamin E, vitamin A, GSH and protein thiols. The experimental details of vitamin C, vitamin E and vitamin A and GSH are exactly the same as explained in phase II methodology for liver tissues.

ESTIMATION OF PROTEIN THIOLS

The levels of protein thiols were estimated by the method proposed by Sedlack and Lindsey (1968).

PRINCIPLE

The sulphhydryl groups in tissues can be determined using Ellman's reagent. DTNB is reduced by SH group to form 1mole of 2-nitro-5-mercapto benzoic acid per mole to give a yellow coloured chromogen, the intensity of which can be measured at 420nm.

REAGENTS

1. Tris-EDTA (0.2M, pH 8.0)
2. DTNB (0.01M)
3. Methanol
4. Standard glutathione (50mg of GSH in 100ml of 0.2M Tris-EDTA, pH 8.0)

PROCEDURE

0.2ml of the tissue homogenate was mixed with 1.5ml of tris-EDTA and 1.0ml of DTNB. The mixture was made upto 10.0ml with methanol. A reagent blank without the sample and sample blank with DTNB were prepared in the same

manner. The tubes were stoppered and allowed to stand for 15 minutes at room temperature, with occasional shaking. Centrifuged the assay mixture at 3000g for 15 minutes. The absorbance of the yellow colour produced was measured at 420nm. Calibration curves were obtained with reduced glutathione as standard. The levels of protein thiols were expressed as mg/g tissue.

The activities of enzymic antioxidants were expressed as specific activity (units/mg protein). The protein content of the enzyme extracts were estimated using the method proposed by Lowry *et al.* (1951). The experimental details for the same are as follows.

ESTIMATION OF PROTEINS

PRINCIPLE

The blue colour developed by the reduction of the phosphomolybdic phosphotungstic components in the Folin-Ciocalteu reagent by the amino acid residues like tryptophan present in the protein and the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate is measured at 660nm.

REAGENTS

1. Sodium carbonate (2% in 0.1N NaOH)
2. Copper sulphate (0.5% in 1% potassium sodium tartarate)
3. Alkaline copper solution : 50ml of solution was mixed 1 with 1ml of solution 2
4. Folin-Ciocalteu reagent (1N)
5. Stock standard (50mg BSA/ml)
6. Working standard (1 in 5 dilution)
7. Tris buffer (0.1M, pH 7.5)

PROCEDURE

The enzyme preparation from each assay was used as the protein source for estimation.

The working standard (0.2 to 1.0ml) was pipetted out into a series of test tubes and 1.0ml of the sample was used for the estimation. The volume was made upto 1.0ml in all the tubes with distilled water. Water (1.0ml) served as blank. Reagent 3 (5.0 ml) was added in all the tubes, mixed well and allowed to stand at 37°C for 3 minutes. Folin-ciocalteau reagent (0.5ml) was added, mixed well and incubated at 37°C for 3 minutes. The blue colour developed was read at 660nm.

The antioxidant status of the liver tissue was further analyzed and confirmed by studying the end product of oxidative damage namely lipid peroxidation and also the DPPH scavenging efficiency of the tissue extract was followed. These parameters could be analyzed only in the liver, as the kidney tissue was exhausted for the antioxidant analysis.

ESTIMATION OF THE EXTENT OF LPO

Lipid peroxidation is involved in the oxidative damage *in vivo* and pathogenesis of several disorders and diseases induced by reactive oxygen species. Lipid peroxidation may cause damage directly to biological molecules and membranes and may also induce the generation of toxic and signaling molecules (Tang *et al.*, 2002). LPO was analysed as reflected by the levels of thiobarbituric acid reactive substances (TBARS) produced (Okhawa *et al.*, 1979) as explained in phase II (liver slices).

ESTIMATION OF DPPH SCAVENGING ACTIVITY

DPPH scavenging activity was analyzed spectrophotometrically in the liver slices as described by Mensor *et al.* (2001) which was elaborately explained in phase II.

HISTOLOGICAL ARCHITECTURE OF THE HEPATIC AND RENAL TISSUES OF THE RATS

The cellular and tissue architecture of the hepatic and renal tissues were followed in support of the biochemical parameters analyzed. Small pieces of the tissues were fixed in 10% formalin immediately after autopsy. The tissues were then processed as per the procedure proposed by Luna (1968).

PROCESSING OF TISSUES

The tissues were placed in 10% formal saline (10% formalin in 0.9% NaCl) for one hour in order to rectify the shrinkage caused by the higher concentration of formalin. The tissues were then left overnight in running water after securing the mouths of the vessels with cotton gauze. The tissues were then dehydrated with ascending grades of isopropanol by immersing in 80% isopropanol overnight followed by 100% isopropanol for one hour. The dehydrated tissues were then cleared in two changes of xylene, one hour each. Histology grade paraffin wax (melting point 58-60°C) was impregnated into the tissues at 60°C. The wax impregnated tissues were embedded in paraffin blocks using the same grade wax. The paraffin blocks were mounted and cut with rotary microtome at 3 micron thickness. The sections were floated on a tissue floatation bath at 40°C and taken on a glass slide smeared with equal parts of egg albumin and glycerol. The sections were then melted in an incubator at 60°C and allowed to cool after 5 minutes.

TISSUE STAINING

The sections were deparaffinised by immersing in xylene for 10 minutes in a staining jar. The deparaffinised sections were washed in 100% isopropanol and stained in Ehrlich's hematoxylin for 8 minutes. After staining, the sections were washed in tap water and then dipped in acid ethanol (8.3% HCl in 70% ethanol) to remove excess stain. They were placed in running tap water for 10 minutes and were then counterstained with 1% aqueous solution of eosin for 1 minute. The excess stain was washed in tap water and were allowed to dry. Care was taken to ensure complete dehydration of the stained sections by placing them in the incubator at 60°C for 5 minutes. The sections were then cooled and were mounted in DPX mountant. The histological architecture in the liver and kidney tissues were observed under high power objective in a microscope.

PHASE IV

The results of the first three phases showed that the aqueous leaf extracts of *Triticum aestivum* of selected time points of growth were effective in evoking an antioxidant response against oxidative assaults induced. In phase IV, the phytochemical component responsible for eliciting the protection rendered by the leaves of *Triticum aestivum* against oxidative damage was probed. Initially, a preliminary phytochemical screening of the leaf extracts was carried out.

PRELIMINARY PHYTOCHEMICAL SCREENING

Crudely cut leaves were successively extracted into solvents of increasing polarity (petroleum ether, benzene, ethyl acetate, methanol and water) using Soxhlet extraction. The extracts were evaporated to dryness and the yields of the extracts were calculated gravimetrically. The residues were redissolved in their respective solvents and subjected to qualitative analysis for the presence of

alkaloids, phenolics and flavonoids as described by the method of Khandelwal (2002).

DETECTION OF ALKALOIDS

a) Mayer's test

An aliquot of the extract was treated with Mayer's reagent (1.36g of mercuric chloride and 5g of potassium iodide in 100ml of distilled water) and observed for the formation of cream coloured precipitate.

b) Dragendorff's test

An aliquot of the extract was treated with Dragendorff's reagent and observed for the formation of reddish orange coloured precipitate.

c) Wagner's test

An aliquot of the extract was treated with Wagner's reagent (1.27g of iodine and 2g of potassium iodide in 100 ml distilled water) and observed for the formation of reddish brown coloured precipitate.

DETECTION OF PHENOLICS

a) Ferric chloride test

An aliquot of the extract was treated with 5% Ferric chloride reagent and observed for the formation of deep blue-black colour.

b) Lead acetate test

An aliquot of the extract was treated with 10% lead acetate solution and observed for the formation of white precipitate.

DETECTION OF FLAVONOIDS

a) Aqueous sodium hydroxide test

An aliquot of the extract was treated with 1N aqueous sodium hydroxide solution and observed for the formation of yellow orange colouration.

b) H₂SO₄ test

An aliquot of the extract was treated with concentrated sulphuric acid and observed for the formation of orange colour.

c) Schinodo's test

An aliquot of the extract was treated with a piece of magnesium turnings followed by a few drops of concentrated hydrochloric acid, heated slightly and observed for the formation of dark pink colour.

The results of the preliminary qualitative analysis showed the presence of alkaloids, phenols and flavonoids in the leaves of *Triticum aestivum*. Further, the leaves were subjected to extraction using the specific extraction protocols for alkaloids, phenolics and flavonoids (Harborne, 1973, Vitale *et al.*, 1995) as given below.

FT-IR ANALYSIS

Infrared light from a suitable source passes through a scanning Michelson interferometer and Fourier Transformation gives a plot of intensity versus frequency. When a powdered plant sample is placed in the beam, it absorbs particular frequencies, so that their intensities are reduced in the interferogram and the ensuing Fourier transform is the infrared absorption spectrum of the sample.

NMR ANALYSIS

The powdered plant material was also subjected to ¹H-NMR (Bruker, 200 MHz in CdCl₃, internal standard TMS) and ¹³C-NMR (Bruker, 75MHz in CdCl₃, internal standard TMS). The chemical shift values were recorded as δ (delta) value ppm, relative to the TMS.

GC-MS ANALYSIS

The powdered plant material was analysed using a Shimadzu gas chromatography apparatus (Model qp 5000 GC-MS) using a DB-S capillary column (30m) equipped with QP MS detector (EI, 70 ev) with helium as a carrier gas at a flow rate of 1ml/minute. The compounds were identified by computer searching, followed by matching the mass spectral data with those held in the database library.

STATISTICAL ANALYSIS

The biochemical parameters studied were subjected to statistical analysis using SigmaStat statistical package (Version 3.1). Paired 't' test was followed for phase I and one-way ANOVA, followed by post-hoc analysis using Fischer's LSD was adopted to all the other parameters under study to test the level of statistical significance.

The results obtained for the various parameters analyzed in the four major phases and the salient findings made during the study are presented in the next chapter.