INTRODUCTION

Plants are considered to be medicinal if they possess pharmacological activities of possible therapeutic use. These activities are often known as a result of millennia of trial and error, but they have to be carefully investigated if we wish to develop new drugs that meet the criteria of modern treatment. Compared with the experience of most modern drugs, the human use and approval of most herbal remedies is awesome.

The requirement by the medical and scientific establishment for research to prove that herbs are effective is not found among the population at large. It is apparent that most ordinary people are content to rely on their impressions of the world to get by it. Judging by the substantial markets for herbal products in the developed world, let alone the vast use in traditional cultures, a great many people have already found herbal medicines useful. The relationship between man and plants has been very close throughout the development of human cultures. It can be recalled that herbal therapy aims to support vital functions of human body.

The role of compounds such as flavonoids and others with activities such as antioxidation and free radical scavenging is largely unknown but could be important in the prevention of chronic inflammatory diseases and cancer (Kinghorn, 1993).
The need to document plant usage and to attempt to confirm their efficacy remains urgent and must be undertaken with same rigour, as would any other scientific study.

Considering the above facts, the present research work has been carried out to evaluate the hepatoprotective action of the combined ethanolic extract of leaves of *Eclipta alba* and the seed of *Piper longum* (BHE)

In Ayurveda the plant *Eclipta alba* is considered a *rasayana* for longevity and rejuvenation. Recent studies have shown that it has a profound antihapatotoxic activity.

*Piper longum* Linn, an important medicinal plant belonging to the family piperaceae has been used in traditional medicine by people in Asia and Pacific islands especially in Indian medicine.

**Plant Collection and Authentication**

The leaves of *Eclipta alba* and seeds of *Piper longum* were collected from the IMPCOPS (Indian Medical practioners co-operative society), Thiruvanmiyur Chennai, India and were authenticated by Dr. P.T. Kalaichelvan, Professor, Advanced Studies in Botany, University of Madras, Chennai, India. The voucher specimen is available in the herbarium file of the Indian Medical practitioners co-operative society, Thiruvanmiyur, Chennai, India.
EXTRACTION

Preparation of *Eclipta alba* extract (EAE)

The leaves of *Eclipta alba* (1 kg) were shade-dried and pulverized to a coarse powder. The powder was passed through 40-mesh sieve and exhaustively extracted with ethyl acetate in soxhlet apparatus at 60°C. The residue left after ethanol extraction was dried and extracted successively with chloroform and ethanol. The extracts were evaporated under reduced pressure using rota flash evaporator till all the solvent had been removed and extract was stored in refrigerator for further use. All these extracts were subjected to HPTLC finger printing analysis. The ethanolic extract alone was used for phytochemical and pharmacological studies. The ethanolic extract was administered to the animals by dissolving each time with 2% v/v aqueous Tween 80.

Preparation of *Piper longum* extracts (PLE)

The seeds of *Piper longum* (1 kg) were shade-dried and pulverized to a coarse powder. The powder was passed through 40-mesh sieve and exhaustively extracted with 90% (v/v) ethanol in soxhlet apparatus at 60°C. The residue left after ethanol extraction was dried and extracted successively with ethyl acetate and chloroform (90%/v/v). The extracts were evaporated under reduced pressure using rota flash evaporator till all the solvent had been removed and extract was stored in refrigerator for further use. All these extracts were subjected to HPTLC finger printing analysis. The ethanolic extract alone was used for phytochemical and pharmacological studies. The
ethanolic extract was administered to the animals by dissolving each time with 2% v/v aqueous Tween 80.

**Preparation of Biherbal extract (BHE)**

The leaves of *Eclipta alba* (1Kg) and seeds of *Piper longum* (1Kg) were shade-dried and pulverized to a coarse powder. Equal quantities of the powder were passed through 40-mesh sieve and exhaustively extracted with 90% (v/v) ethanol in soxhlet apparatus at 60°C. The extract was evaporated under reduced pressure using rota flash evaporator till all the solvent had been removed, the extract was lyophilised and stored in refrigerator for phytochemical and pharmacological studies. The lyophilised material was administered to the animals by dissolving each time with 2% v/v aqueous Tween 80.

**Drugs and Chemicals**

Sodium dodecyl sulphate (SDS), Ethylene diamine tetra acetic acid (EDTA), Trichloro acetic acid (TCA), Thiobarbituric acid (TBA), Sodium nitroprusside, Sulphanilamide, Phosphoric acid, Naphtyl ethylene diamine dihydrochloride were obtained from Sd Fine Chemicals Ltd.). 1,1-Diphenyl, 2-picryl hydrazyl DPPH), Butylated hydroxytoluene (BHT), Bovine Serum Albumin (BSA), Folin-Ciocalteu reagent, 5,5-dithio bis(2-nitrobenzoic acid) (DTNB), 2,2’-bipyridyl were obtained from Sigma Chemicals Company St. Louis, USA. All other chemicals and reagent used were of analytical grade.
Preliminary Phytochemical Screening

The ethanolic extracts of the EAE, PLE, and BHE were subjected to preliminary phytochemical screening for identification of its active constituents by the method of Kokate et al. (1997).

Test for Alkaloids

A small portion of the solvent free extracts were stirred separately with a few drops of dil. Hydrochloric acid and filtered. The filtrate may be carefully tested with various alkaloidal reagents.

(a) Mayer’s reagent - Cream precipitate
(b) Dragendorff reagent - Orange brown precipitate
(c) Hager’s reagent - Yellow precipitate
(d) Wagner’s reagent - Reddish brown precipitate

Test for Carbohydrates

(a) Molisch’s Test

The extracts were treated with 2-3 drops of 1% alcoholic alpha naphthol and 2 ml of conc. Sulphuric acid was added along the sides of the test tube carefully. Formation of violet color ring at the junction of two liquids indicates the presence of carbohydrates.
(b) **Fehling’s Test**

The extracts were treated with Fehling’s solution A and B and heated. Appearance of reddish brown color precipitate indicates the presence of reducing sugars.

(c) **Benedict’s Test**

The extracts were treated with Benedict’s reagent and heated. Appearance of reddish orange colour precipitate indicates the presence of reducing sugars.

**Test for Proteins**

(a) **Biuret Test**

The extracts were treated with copper sulphate solution, followed by the addition of sodium hydroxide solution appearance of violet colour indicates the presence of proteins.

(b) **Millon’s Test**

When the extracts were treated with Millon’s reagent, appearance of pink colour indicates the presence of proteins

**Test for Phytosteroids**

(a) **Libermann Buchard Test**
When the extracts were treated with con. Sulphuric acid, few drops of glacial acetic acid, followed by the acetic anhydride, there is a formation of violet ring between the two layers, and the appearance of green colour in the aqueous upper layer indicates the presence of steroids.

**Test for Phenols**

(a) The different extracts were treated with neutral ferric chloride solution. The appearance of violet color indicates the presence of phenols.

(b) The different extracts were treated with 10% sodium chloride solution. The appearance of cream color indicates the presence of phenols.

**Test for Tannins**

(a) When the extracts were treated with 10% lead acetate solution, appearance of white precipitate indicates the presence of tannins

(b) When the extracts were treated with aqueous bromine solution, appearance of white precipitate indicates the presence of tannins
Test for Flavonoids

(a) 5ml of the each extract solution was hydrolyzed with 10% v/v sulphuric acid and cooled. Then it was extracted with diethyl ether and divided into three portions in three separate test tubes. 1ml of diluted sodium carbonate, 1ml of 0.1N sodium hydroxide and 1ml of strong ammonia solution were added to the first, second and third test tubes respectively. In each test tube, development of yellow color demonstrated the presence of flavonoids.

(b) Shinoda’s Test The extracts were dissolved in alcohol, to that one piece of magnesium followed by conc. Hydrochloric acid were added drop wise and heated. Appearance of magenta color shows the presence of flavonoids.

Tests for Gums and Mucilage

(a) About 10ml of various extracts were added separately to 25ml of absolute alcohol with constant stirring and filtered. The precipitate was dried in air and examined for its swelling properties.
Test for Saponins

(a) Foam Test

1ml of the different extracts were diluted with distilled water and shaken well in the test tube. The formation of foam in the upper part of the test tube indicates the presence of saponins.

Test for Glycosides

A pinch of the extracts were dissolved in the glacial acetic acid and a few drops of ferric chloride solution was added, followed by the addition of con. Sulphuric acid, formation of red ring at the junction of two liquids indicates the presence of glycosides.

Tests for fixed oils and fats

(a) Small quantity of the various extracts were separately pressed between two filter papers. Appearance of oil stain on the paper indicates the presence of fixed oil.

(b) Few drops of 0.5N alcoholic potassium hydroxide were added to small quantity of various extracts along with a drop of phenolphthalein. The mixture was heated on a water bath for 1-2 hours. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.
Test for Terpenes

When the extracts were treated with tin and thionyl chloride, appearance of pink colour indicates the presence of terpenes.

Test for Lignin

When the extracts were treated with alcoholic solution of phloroglucinol and con. Hydrochloric acid appearance of red colour shows presence of lignin.

High Performance Thin Layer Liquid Chromatography (HPTLC) fingerprinting

HPTLC fingerprinting was performed on CAMAG TLC scanner – 3 instrument, equipped with Linomat IV applicator and CATS 3.2 software. Precoated aluminium silica gel 60 F<sub>254</sub> (E. Merck) plates, layer thickness of 0.2 mm were used. Fingerprints were obtained by development in CAMAG twin chamber and were scanned at 254 nm.

ESTIMATION OF MACRONUTRIENTS

Estimation of Total soluble sugars

The estimation of total soluble sugars present in the leaves of Eclipta alba and seeds of Piper longum were estimated by using anthrone method.
**Extraction of sugars**

For extracting the sugars 1gm of finely powdered sample was suspended in 40 ml of distilled water and heated in the boiling water bath for 30 min. It was centrifuged for 20 min at 3000rpm. The supernatant was collected and the pellet was suspended in 20 ml of water. The extraction steps were repeated 6 –8 times till the supernatant was free of sugars.

**Reagents**

1. Anthrone reagent: Dissolved 2 gms of anthrone in 1 litre of con. Sulphuric acid. The reagent was prepared freshly.

2. Stock standard glucose solution: 100mgs of glucose was dissolved in 100ml of distilled water.

3. Working standard glucose solution: Stock solution was diluted 1 in 10 times.

**Procedure**

From the supernatant 1ml of the solution was taken. To that 4 ml of anthrone reagent was added. Placed the tubes in the boiling water bath for 10 min. Aliquots of standard glucose was also treated in the same way. A blank was set up with 1ml of water. The test tubes were taken out, cooled and the absorbance of the solution was measured at 625 nm using the colorimeter. From the standard graph the amount of carbohydrate present in the sample
was calculated. The sugar contents of the plant were expressed as mgs/100gms of powder.

**Estimation of Total Proteins**

The estimation of total proteins present in the leaves of *Eclipta alba* and seeds of *Piper longum* were estimated by using Lowry *et al.* (1951) method.

**Extraction of the protein sample**

**Reagents**

1. Ethanol - (80%)
2. Ethanol : Ether mixture (3: 1)
3. Trichloro acetic acid (TCA) -5%

**Procedure**

The fresh leaves of the *Eclipta alba* and seeds of *piper longum* were extracted in hot 80% ethanol by macerating in a motor and pestle. The homogenate was transferred in a centrifuge tube and centrifuged at 2000rpm for 20 min. The supernatant was discarded. The pellet was suspended in a suitable volume of 5% TCA in an ice bath for 15 min. It was centrifuged and the supernatant was discarded. This process was repeated for twice. The pellet was reextracted once with hot absolute ethanol and twice with ethanol: ether mixture, every time discarding the supernatants after centrifugation. This pellet contains the proteins and nucleic acids.
Estimation of the protein sample (Lowry’s method)

Reagents

1. Alkaline copper reagent: 50ml of reagent A (2% Sodium carbonate in 0.1N Sodium hydroxide) was mixed with 1.0ml of reagent B (0.5% copper sulphate in 1% sodium potassium tartarate).

2. Folin’s phenol reagent: This was prepared according to the method of Folin and Ciocalteu’s. The following were mixed together and refluxed for 10hrs.

   Sodium tungstate - 100mg
   Sodium molybdate - 25g
   Water - 700ml
   Phosphoric acid 85% - 15ml
   Con HCl - 100ml

   After refluxing, 150g of Lithium sulphate and 50ml of water were added along with a few drops of bromine. The mixture was boiled for 15min to remove excess of bromine. The contents were cooled, diluted to 1 litre and filtered. This reagent was diluted 1:2 with water before use.

3. Standard protein solution: 10mg of bovine serum albumin dissolved in 100ml of distilled water.
Procedure

Aliquots of the extract were made up to a final volume of 1.0ml with water. A set of standards and blank containing only water were also set up. 5.0ml of alkaline copper reagent was added to all the test tubes, mixed and allowed to stand at room temperature for 10mts. Then 0.5ml of Folin’s phenol reagent was added and shaken well. The blue color developed was read at 640nm after 20mts, in the photoelectric colorimeter. The protein contents of the tissues were expressed as mgs/100gms of powder.

Estimation of Total lipid

The estimation of Total lipids present in the leaves of *Eclipta alba* and seeds of *Piper longum* were estimated by using chloroform methanol mixture by the method of Folsch *et al.* (1957).

Reagents

1. Anhydrous sodium sulphate
2. chloroform : Methanol mixture (2:1)
3. sodium chloride - 1%

Procedure

To 1gm of each of the leaves *Eclipta alba* and seeds of *Piper longum*, 5gms of anhydrous sodium sulphate was added and macerated in the motor and pestle. A small amount of acid washed sand was added as an abrasive since the seed material is tough. To this 20ml of chloroform : methanol
mixture was added and transferred to a tight glass stoppered iodometric flask. The contents of the flask were shaken in a mechanical shaker and filtered through the sintered glass funnel. The extraction was repeated twice and the filtrates were pooled. The solvent present in the residue was removed under vacuum. Since the residue left after drying contain crude lipids it was extracted once again with 10ml of chloroform: methanol mixture containing 1ml of 1% sodium chloride. The pooled sample was transferred to a separating funnel which were shaken thoroughly and allowed to stand for 5 min. The lipids were recovered from the lower layer. The lower layer was drained out and the process was repeated with chloroform: methanol mixture again 3-4 times to extract any residual lipid from it. The lipid containing fractions were collected in a pre weighed beaker. The solvent was evaporated by keeping it in a warm water bath at 50° C. The weight of the beaker with the extract was recorded, and the amount of the crude lipids in the sample was determined by subtracting the weight of the empty beaker. The lipid contents of the tissues were expressed as gms/100gms of fresh tissue

**Estimation of Minerals**

**Preparation of sample for mineral analysis by ashing method**

**Reagents**

1. Desiccator containing fused calcium chloride at the bottom
2. Dilute HCl: Prepared diluting 1ml of HCl with 4ml of water
**Procedure**

About 5 – 10gms of oven-dried samples were taken in a silica crucible and heated first in the bunsen burner on a low flame till it gets charred. The silica crucible was transferred to a muffle furnace and heated at dull red heat (500-550°C) till it was completely converted into white ash. The ash was kept in the desiccator containing fused calcium chloride at the bottom, till it gets cooled down. The ash was moistened with small amount of distilled water and 5ml of dilute HCl was added to it. The solution was evaporated to dryness on a boiling water bath and this process was repeated twice. To the extract 4ml of dil .HCl was added and warmed in the boiling water bath. The extract was filtered through whatmann filter paper and made up to 100ml in the volumetric flask. It was transferred to preacid washed bottles and stored for mineral analysis.

**Estimation of Minerals by Atomic Absorption**

The minerals present in the dried powdered plants were analysed quantitatively using atomic absorption spectrophotometer. (AAS400-HGA 900-Perkin Elmer) and expressed as ppm of plant powder.

**SDS-PAGE ANALYSIS OF PROTEINS**

A SDS-PAGE analysis of proteins was performed with the aqueous extracts of the seeds of *Piper longum* and with the leaves of *Eclipta alba* and the molecular weight of the different proteins present in the plants were identified by comparing with the marker proteins.
Reagents

Stock solutions

1. Acrylamide stock solution: 30% Acrylamide and 0.8% N, N’, methylene bis acrylamide was dissolved in 50ml of distilled water.
2. Sodium dodecyl sulphate (SDS)- 10%
3. Ammonium per sulphate (APS)- 10%
4. Tetra ethyl methylene ethylene diamine (TEMED)
5. Tris – HCl buffer: Two buffets
   (a) for separating gel- 0.375 M pH 8.8
   (b) for stalking gel -0.125 M pH 6.8
   1. Preparation of separating gel (6ml)
   2. Tris – HCl buffer pH 8.8 - 1.5ml
   3. Acrylamide - 2.0 ml
   4. Sodium dodecyl sulphate 10% - 0.06 ml
   5. Ammonium per sulphate (APS) 10% - 0.02 ml
   6. TEMED - 0.01 ml
   7. Water - 2.41 ml

Preparation of stalking gel (3ml)

1. Tris – HCl buffer pH 6.8 - 0.75ml
2. Acrylamide - 0.5 ml
3. Sodium dodecyl sulphate 10% - 0.03 ml
4. Ammonium per sulphate (APS) 10% - 0.015 ml
5. TEMED - 0.005 ml
6. Water - 1.7 ml

Preparation of sample buffer

1. 0.025M Tris PH 6.8 - 0.25
2. 2 mercaptoethanol - 0.1ml
3. SDS - 0.04ml
4. Glycerol - 0.2ml
5. Bromophenol Blue - 0.001%
6. Water - to make up to 1ml

Preparation of tank buffer PH 8.3

1. Tris 0.025M - 1.5g
2. Glycine 0.192M - 7.2g
3. SDS 0.1% - 0.5g
4. Water - 500ml

Protein sample preparation

The protein isolated from the plants as above mentioned procedure was taken and mixed with equal volume of sample buffer in sterilized microfuge tubes and boiled for 3 min in a boiling water bath. It was cooled at room temperature and then used for loading.
Protein Staining Solutions

0.1 gms of coomassie brilliant blue (CBB R 250) was weighed, dissolved completely in 40 ml of methanol, 10 ml of acetic acid was added and made-up to 50 ml using water.

Procedure

1. The polyacrylamide slab was prepared between the two glass plates, a notched upper plate and a lower plate that were separated by the spacers. The sides of the gel plates were sealed using 5% agar solution. The plates were checked for leaks before pouring the separating gel solution.

2. The separating gel was poured in between the plates without any air bubble. 100µl of water was laid over the separating gel to get a uniform layer. The separating gel was allowed to get solidify. After the gel gets solidifies the water layer was removed.

3. The stalking gel was added on the top of the separating gel. The comb was introduced on the top without air bubble, and allowed to get solidify.

4. After solidification of the stalking gel the comb was removed, the spacers kept in between the plates were also removed.

5. The plates with the gel were clamped in the electrophoretic apparatus. The top electrolyte compartment was filled with the
running buffer and checked for leaks. If it was perfect the bottom compartment was also filled with the same.

6. About 25µl of the processed protein sample was applied on the wells by using the micropipette.

7. Known molecular weight protein markers were also loaded onto the wells.

8. After loading the samples and the markers on to the wells the power pack was “switched on” and a voltage of 15 mA was applied which gives good resolution and avoid heating effects.

9. Once the tracking dye reaches the bottom of the gel the current was “switched off” and the plates were removed and the gel present in between the plates were taken out for staining purposes.

10. The gel was stained for an hour in the freshly prepared staining solution and destained in the destaining solutions till the bands of required intensity was obtained.

11. The proteins present in the sample were studied using the marker proteins.

**Molecular weight determination**

SDS-PAGE is frequently used to determine the molecular weight of a protein. Since the protein migration is generally proportional to the mass of the protein, a standard curve is generated with proteins of known molecular
weight and the molecular weight of unknown protein can be calculated by extrapolating the graph.

**IN- VITRO ANTIOXIDANT STUDIES**

1,1-Diphenyl-2 picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity was assessed according to the method of Shimada *et al.* (1992).

**Reagents**

1. Acetate buffer, pH 5.5 - 0.1 M
2. DPPH in ethanol - 0.5 mM
3. L- Ascorbic acid - 10mg/100ml water

**Procedure**

One ml of crude extracts of EAE, PLE, and BHE at variable concentrations ranging from 100 to 1000 µg in ethanol were mixed in 1 ml of freshly prepared DPPH solution and 2 ml of 0.1 M acetate buffer at pH 5.5. The resulting solutions were then incubated at 37 °C for 30 min and measured spectrophotometrically at 517nm. Standard antioxidants like L-Ascorbic acid was used as positive control under the same assay conditions. Negative control was without any inhibitor or extract. Lower absorbance at 517 nm represents higher DPPH scavenging activity. DPPH radical scavenging activity of the extracts was calculated accordingly from the decrease in absorbance at 517 nm in comparison with the negative control.
**Super oxide scavenging activity**

Super oxide anion scavenging activity was measured based on the described method by Robak and Gryglewski (1988).

**Reagents**

1. Sodium phosphate buffer, pH 7.4 - 100 mM
2. Nitro blue tetrazolium (NBT) - 150 mM
3. NADH - 468 mM
4. Phenazine methosulphate (PMS) - 60 mM
5. L-Ascorbic acid - 10 mg/100 ml water

**Procedure**

Superoxide radicals were generated in nicotinamide adenine dinucleotide, phenazine methosulphate (PMS–NADH) system by the oxidation of NADH and assayed by the reduction of nitro blue tetrazolium (NBT). In this experiment, the super oxide radical was generated in 3 ml of sodium phosphate buffer containing 1 ml of NBT solution, 1 ml of NADH solution, and different concentrations of the EAE, PLE, and BHE (100 to 1000 µg) in water. The reaction was started by adding 1 ml of PMS solution to the mixture. The reaction mixture was incubated at 25 ºC for 5 min and the absorbance was measured against the corresponding blank solution. L-Ascorbic acid was used as the positive control. The decrease of NBT reduction measured by the absorbance of the reaction mixture correlates with
the super oxide radical scavenging activity of the BHE. The superoxide radical scavenging activity was calculated using the following formula:

\[
\text{Superoxide radical scavenging activity (\%)} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100,
\]

where \( A_0 \) is the absorbance of the control and \( A_1 \) is the absorbance of plant extract or the standard sample.

**Hydroxyl radical scavenging activity**

The hydroxyl radical scavenging activity was determined according to the method of Halliwell *et al.* (1987).

**Reagents**

1. Phosphate buffer pH 7.4 - 20 mM
2. Deoxyribose - 8 mM
3. Ferric chloride - 0.1 mM
4. Ascorbic Acid - 0.1mM
5. EDTA - 0.1 mM
6. \( \text{H}_2\text{O}_2 \) - 1 mM
7. Thiobarbituric acid - 1%
8. Trichloroacetic acid - 2%
9. Standard Mannitol - 10mg/100ml water
**Procedure**

Briefly for the non site-specific hydroxyl radical system, the reaction mixture containing 0.1 ml deoxyribose, 0.1 ml FeCl₃, 0.1 ml ascorbic acid, 0.1 ml EDTA, and 0.1 ml H₂O₂ were mixed with or without various concentrations of the three extracts in 1ml of final volume made with KH₂PO₄–KOH buffer pH 7.4 and was incubated in a water bath at 37 ºC for 1 hr. The extent of deoxyribose degradation was measured by thiobarbituric acid (TBA) method. 1ml of TBA and 1 ml trichloroacetic acid were added to the mixture and heated at 100 ºC for 20 min. After cooling to room temperature the absorbance was measured at 532 nm. Mannitol, a classical hydroxyl radical scavenger was used as positive control. The hydroxyl radical scavenging activity was calculated using the following formula:

\[
\text{Hydroxyl radical scavenging activity (\%)} = \left(\frac{A₀ - A₁}{A₀}\right) \times 100,
\]

where \(A₀\) is the absorbance of the control and \(A₁\) is the absorbance of plant extract or the standard sample.

**Nitric oxide scavenging activity**

Nitric oxide generated from sodium nitro prusside was measured by the Griess reagent by the method of Marcocci *et al.* (1994).

**Reagents**

1. Sodium nitroprusside - 5 mM
2. Phosphate buffered saline
3. Griess reagent: 1% sulpha-nilamide, 2% o-phosphoric acid, and 0.1% naphthylethylenediamine dihydrochloride were mixed together.

4. Rutin -10mg/100 ml

**Procedure**

Various concentrations of the three different extracts were mixed with sodiumnitroprusside in PBS and a final volume of 3 ml was incubated at 25 °C for 150min. After incubation, 0.5ml of sample was removed and diluted with 0.5 ml of Griess reagent. The absorbance of the chromophore formed was read at 546 nm. The inhibition of nitric oxide generation was estimated by comparing the absorbance value of control. Rutin was used as positive control under the same assay conditions.

\[
\text{Nitric oxide scavenging activity (\%) = } \left[ \left( A_0 - A_1 / A_0 \right) \times 100 \right],
\]

where \(A_0\) is the absorbance of the control and \(A_1\) is the absorbance of plant extract or the standard sample.

**Reducing power assay**

The reducing power of the prepared EAE, PLE and BHE was determined according to method of Oyaizu (1986).

**Reagents**

1. Phosphate buffer pH 6.6 - 0.2 M
2. Potassium ferricyanide solution - 1%
3. TCA - 10%
4. Ferric chloride solution - 0.1%
5. Butylated hydroxyl toluene (BHT)-10mg/100ml water

Briefly, various concentrations of the extracts and the standard compound (BHT) in 1 ml of distilled water were mixed with 2.5 ml of phosphate buffer and 2.5 ml of a potassium ferricyanide solution. The mixture was incubated in a water bath at 50 °C for 20 min. Then 2.5 ml of a 10% TCA solution was added and the mixture was then centrifuged at 3000g for 10 min. 2.5 ml aliquot of the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of a ferric chloride solution, the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture correlates with greater reducing power.

Hydrogen peroxide scavenging activity

The ability of samples to quench H₂O₂ was determined by Ruch et al. (1989).

Reagents

1. Phosphate buffered saline pH 7.4 - 0.1 M
2. H₂O₂ - 2 mM
3. Vitamin E - 10mg/100ml
Procedure

The samples were dissolved in 3.4 ml of phosphate buffered saline (PBS) and mixed with 0.6 ml of 2 mM solution of H$_2$O$_2$. Absorbance of H$_2$O$_2$ at 230 nm was determined 10 min later in a spectrophotometer. For each concentration, a separated blank sample was used for background subtraction. Vitamin E was used as the standard conditions. The inhibition of H$_2$O$_2$ production was calculated as follows: Hydrogen peroxide radical scavenging activity (%) = \[(A_0 - A_1/A_0) \times 100\],

where $A_0$ is the absorbance of the control, and $A_1$ is the absorbance of BHE or the standard sample.

Test for Ferric ion reducing capacity (Fe$^{3+}$ to Fe$^{2+}$)

The Ferric ion reducing capacity was determined according to the method of Wang et al. (2003) with minor modifications.

Reagents

1. Phosphate buffer, pH 6.5 - 20mM
2. Ferric chloride - 2mM
3. Potassium thiocyanate - 4mM
4. EDTA - 100µg/ml
**Procedure**

Here different concentrations of the three extracts (20 µl to 100µl) was mixed with 200µl of 20mM phosphate buffer pH 6.5 and 100µl of ferric chloride (2mM). The mixture was incubated for 30 min. At the end of the incubation 1ml of potassium thiocyanate (4mM) was added and absorbance of ferric-thiocynate complex (reddish brown complex) was measured at 460nm using spectrophotometer. The results were compared with standard EDTA which were treated similarly.

**Test for ferrous ion chelating activity (binds Fe²⁺)**

Metal chelating property for ferrous ion (Fe²⁺) was estimated according to the method of Tripathi et al. (2001).

**Reagents**

1. Ferrous sulphate - 10µg.
2. 2,2’–bipyridyl - 1mM
5. EDTA - 100µg/ml

**Procedure**

Different concentrations of the EAE, PLE, and BHE (10 µl to 100µl) were mixed with a fixed concentration of ferrous sulphate (10µg). The mixture was incubated for 30 min. At the end of the incubation, 2ml of 2,2’–bipyridyl (1mM) was added and absorbance of ferrous–bipyridyl complex
(pink colored complex) was measured at 525nm. The results were compared with EDTA which were treated similarly.

**Total antioxidant activity**

The total antioxidant activity of the extracts were measured by use of a linoleic acid system by the method of Mitsuda *et al.* (1996).

**Reagents**

1. Linoleic acid - 0.2804 g
2. Tween - 20
3. phosphate buffer, pH 7.0 - 0.2 M
4. Ethanol
5. Ammonium thiocyanate - 30%
6. Ferrous chloride. - 20 mM in 3.5% HCl
7. Butylated hydroxytoluene - 10mg/100ml

**Procedure**

The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid in Tween 20 and 50 ml of phosphate buffer (pH 7.0). The mixture was then homogenized. A 0.5 ml of different concentration of the extract and standard sample (in ethanol) was mixed with 2.5 ml of linoleic acid emulsion and 2 ml phosphate buffer. The reaction mixture was incubated at 37°C in the dark to accelerate the peroxidation process. The levels of peroxidation were determined according to the thiocyanate method by sequentially adding 5ml
of 75% ethanol, 0.1 ml of ammonium thiocyanate, 0.1 ml sample solution and 0.1 ml ferrous chloride. Butylated hydroxytoluene (BHT) was used as positive control which was treated similarly. After mixing for 3 min, the peroxide values were determined by reading the absorbance at 500 nm.

**Determination of Total Phenolic Content**

Total phenolic content in the lyophilized extract was determined with the Folin–Ciocalteu’s reagent (FCR) according to a published method of Slinkard and Singleton (1977).

**Reagents**

1. Folin–Ciocalteu’s reagent: Prepared as previously mentioned.
2. Sodium carbonate solution -7.5%,
3. Gallic acid - 100mg dissolved in 100ml of water.

**Procedure**

100mg of the sample was dissolved in 0.5ml of water, mixed with 2.5 ml Folin–Ciocalteu’s reagent (diluted 1:10, v/v) followed by 2 ml of sodium carbonate solution. The absorbance was then measured at 765 nm after incubation at 30°C for 90 min. Results were expressed as gallic acid equivalents (mg gallic acid/g dried extract).
**Determination of Total Flavonoid Content**

The total flavonoid content of the extracts were determined by a colorimetric method as described in the literature of Zhishen *et al.* (1999).

**Reagents**

1. Sodium nitrite - 15%
2. Aluminium chloride - 10%
3. Sodium hydroxide - 4%
4. Catechin - 100mg dissolved in 100ml of water.

**Procedure**

An aliquots of sample containing 100mg of test drugs (0.5 ml) was mixed with 2 ml of distilled water and subsequently with 0.15 ml of sodium nitrite solution was added. After 6 min, 0.15 ml of aluminium chloride solution was added and allowed to stand for 6 min, then 2 ml of NaOH solution (4%) was added to the mixture. Immediately, water was added to bring the final volume to 5 ml and the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was then determined at 510 nm versus prepared water blank. Results were expressed as catechin equivalents (mg catechin/g dried extract).

**Assay of Protein Oxidation**

The effects of the plant extracts on protein oxidation were carried out according to the slightly modified method of Wang *et al.* (2006).
Reagents

1. Potassium phosphate buffer, pH 7.4 - 20 mM
2. Bovine serum albumin (BSA) - 4 mg/ml
3. Ferric chloride - 50 mM
4. Hydrogen peroxide - 1 mM
5. Ascorbic acid. - 100 mM
6. 2,4-dinitrophenylhydrazine (DNPH) - 10 mM in 2 M HCl
7. TCA - 10%,
8. Ethanol
9. Ethyl acetate
10. Guanidine Hydrochloride, pH 2.3 - 6 M

Procedure

Bovine serum albumin (BSA) was oxidized by a Fenton-type reaction. The reaction mixture (1.2 ml), containing sample extract (100–1000 µg/ml), potassium phosphate buffer, BSA, Ferric chloride hydrogen peroxide and ascorbic acid was incubated for 30 min at 37°C. For determination of protein carbonyl content in the samples, 1 ml of 2,4-dinitrophenylhydrazine (DNPH) was added to the reaction mixture. Samples were incubated for 30 min at room temperature. Then, 1 ml of ice cold TCA was added to the mixture and centrifuged at 3000g for 10 min. The protein pellet was washed three times with 2 ml of ethanol/ethyl acetate (1:1 v/v) and dissolved in 1 ml of guanidine hydrochloride. The absorbance of the sample was read at 370 nm. The data were expressed in terms of percentage inhibition, calculated from a control
measurement of the reaction mixture without the test sample. Vitamin E under same assay condition was used as standard.

**In vitro assay of DNA fragmentation study**

The DNA was subjected to oxidation by the Fenton’s reactants and subjected to fragmentation. The protection offered by the plant extract against the DNA fragmentation was studied according to the method of Sultan et al. (1995), with minor modifications.

**Isolation of DNA from Hepatocytes**

**Reagents**

1. **Isotonic buffer**
   - (a) 0.15mM sodium chloride - 3.0ml
   - (b) 0.1 M Tris–HCl pH 7.5 - 1.0ml
   - (c) 1.5mM Magnesium chloride - 0.15ml
   
   Made up to 100ml with water.

2. **Cell lysis buffer**
   - (a) 320mM sucrose - 109.6g
   - (b) 1% Triton X-100 - 20ml of 50% Triton X-100
   - (c) 5mM MgCl₂ - 5ml of 1M MgCl₂
   - (d) 10mM Tris–HCl pH 7.5 - 10ml of 1M Tris–HCl
   - (e) Distilled water - to a final volume of 1 liter

3. **EDTA-NaCl (100ml)**
   - (a) 25mM EDTA PH 8.0 - 5ml of 0.5M EDTA
   - (b) 75mM NaCl - 1.5ml 0.5M NaCl
4. Proteinase K (10mg/ml)
   (a) Proteinase K - 10 mg
   (b) Distilled water - 1ml

   Stored in -20°C

5. 5M sodium acetate (100ml)

   About 68.5 g of sodium acetate was dissolved in 70 ml of water, pH was adjusted to 5.2 by adding acetic acid. Volume was made up to 100ml with distilled water.

6. Phenol- chloroform – Isoamyl alcohol (50 ml)
   (a) phenol - 25 ml
   (b) chloroform - 24ml
   (c) isoamyl alcohol - 1ml

7. Tris EDTA Buffer
   (a) 10 mM Tris HCl pH 8.0 - 1ml from 1M Tris HCl pH 8.0
   (b) 1mM EDTA pH 8.0 - 0.5ml from 0.5 ml EDTA pH 8.0
   (c) Distilled water - 98.5ml

8. 20 mM potassium phosphate buffer, pH 7.4

9. 150mM sodium chloride

10. Fenton reaction mixture
    (a) 100mM ascorbate
    (b) 100mM FeCl₂
    (c) 1mM H₂O₂
(d) 104 mM EDTA

The above contents were mixed in 20 mM potassium phosphate buffer, pH 7.4

10. Tris acetate EDTA buffer pH 8
   (a) 40 mM Tris
   (b) 20 mM sodium acetate,
   (c) 2 mM EDTA,
   (d) 18 mM NaCl, pH 8

Procedure

1. About 0.2 gm of liver tissue was homogenised in 2 ml of isotonic buffer.

2. The animal cells were collected by centrifugation at 2000 rpm for 10 min at 4°C.

3. The cell pellet was resuspended in cold cell lysis buffer.

4. The cells were homogenised in a glass homogeniser with a loose fitting pestle.

5. The above treated homogenate was centrifuged at 4000 rpm for 20 min at 4°C to pellet the nuclei.

6. Resuspended the pellet in 8 ml of EDTA-NaCl and added 0.8 ml of 10% cell lysis buffer. It was mixed using the vortex mixture.

7. To this mixture 50 µl of the proteinase K was added and incubated at 37°C for 3-5 hrs.
8. Then 0.5ml of sodium acetate buffer and 8 ml of Phenol-chloroform – Isoamyl alcohol were added. The contents were mixed by inverting the tube for one hr.

9. The above treated solution was centrifuged at 12000 rpm for 10 min at 4°C.

10. The upper aqueous layer was collected. Equal volume of Phenol-chloroform – Isoamyl alcohol was added. It was mixed gently by inverting the tube for 1 min.

11. Once again the solution was centrifuged at 12000 rpm for 10 min at 4°C. The upper aqueous layer was collected.

12. About 2 volumes of 100% ethanol was added to the above collected solution to precipitate the DNA.

13. Centrifuged at 5000rpm for 5 min at 4°C. The DNA pellet was washed in 70 % ethanol.

14. The precipitated DNA was dissolved in 20µl of TE buffer.

**Assay of DNA fragmentation**

1. 1 mg of the extracted DNA was mixed well with in 20 mM potassium phosphate buffer, pH 7.4. To this 1 ml of 150mM sodium chloride was added and left at 4°C for 24 hrs for complete solubilisation
2. 100 µg of the above DNA was treated with 1ml of potassium phosphate buffer, the reaction mixture containing FeCl₃ (100 µM), EDTA (104 µM), H₂O₂ (1 mM).

3. To the above mixture two concentrations of BHE (20 and 40 µg) were added. A standard antioxidant BHT was also treated in the same way. A negative control without the fenton reactants were also used in the assay. A positive control without the extract or standard was also taken.

4. All these reaction mixtures were incubated for 37°C for 30 min, and placed on the ice bath for 10 min to stop the reaction.

5. These were then mixed with the loading buffer (0.5% bromophenol blue and 50% glycerol)

6. Agarose gel electrophoresis was carried out by mixing 1% agarose with ethidium bromide (1 µg/ml)

7. About 10 µl of the treated samples were placed in the wells in the following order. Control, positive control, BHE treated sample (20 and 40 µg), standard BHT.

8. The electrophoresis was conducted in TAE buffer, pH 8 at 60 V for 6hr

9. DNA was visualized under a UV transilluminator (Bio-Rad, Sydney, Australia).
RESULTS

Table I shows the preliminary phytochemical analysis of the various extracts. In the present investigation preliminary phytochemical screening of the EAE, PLE and BHE shows the presence of constituents like alkaloid, carbohydrates, phytosterol, tannins, phenol, flavonoids, glycosides, terpenes, saponins and lignin. In all these extracts proteins, gums and mucilage were found to be absent.

Figure I to III shows the present chromatographic pattern of ethyl acetate, chloroform and ethanolic extract of *Eclipta alba*. Ethyl Acetate extract showed 14 peaks at Rf values 0.07, 0.14, 0.18, 0.30, 0.41, 0.49, 0.58, 0.63, 0.72, 0.80 and 0.95. Chloroform extract of *Eclipta alba* showed 11 peaks at Rf values 0.07, 0.14, 0.18, 0.30, 0.41, 0.49, 0.58, 0.63, 0.72, 0.80 and 0.95. The ethanolic extract of *Eclipta alba* showed 12 peaks at Rf values 0.12, 0.19, 0.31, 0.43, 0.51, 0.60, 0.65, 0.73, 0.81, 0.86 and 0.90 at 254nm.

Figure IV to VI shows the HPTLC finger printing of the different extracts of *Piper longum*. The ethyl acetate extract of *Piper longum* showed 11 peaks at Rf values 0.03, 0.08, 0.13, 0.23, 0.30, 0.40, 0.51, 0.61, 0.76, 0.88 and 0.94 where as the Chloroform extract showed 10 peaks at Rf values 0.09, 0.16, 0.24, 0.32, 0.53, 0.63, 0.77, 0.86 and 0.94. The ethanolic extract of the same plant showed 12 peaks at Rf values 0.03, 0.06, 0.11, 0.19, 0.27, 0.37, 0.47, 0.59, 0.69, 0.80, 0.86 and 0.92 at 254nm.

Figure VII shows the SDS-PAGE electrophoresis results of aqueous extract of *Eclipta alba*. The presence of protein bands with
molecular weight of ranging 7.50 – 123 kDa were seen. The *Piper longum* seeds showed only two protein bands in the molecular weight of 30 kDa and 60 kDa.

The values of the macronutrients like carbohydrates, proteins and lipids present in the leaves of *Eclipta alba* and seeds of the *Piper longum* were shown in the Table II. The carbohydrate content present in the seeds of the *Piper longum* was 15.56mgs / 100gms, where as the leaves of *Eclipta alba* contains 27.78mgs/ 100 gms. The protein content was also found to be high in case of leaves of *Eclipta alba* when compared with the seeds of *Piper longum*. The same was applicable to the lipid content also, in which the leaves of *Eclipta alba* contained 24.94mgs/ 100 gms and *Piper longum* contained 3.89mgs/ 100gms respectively.

Table III shows the concentration of different minerals like Ca, K, Na, Mg, Zn, Si, Fe, Al, Va, Mb, Cu, Ni, Hg, Pb, Ba, Mn, Cr, Co, and Se expressed in ppm. The concentration of these minerals in *Eclipta alba* leaves was found to be in the following order, Ca > Na > K > Mg > Al > Si > Zn > Fe > Va>Cu >Ni >Ba>Mg > Cr > Mb> Co>Hg >Pb > and Se. From the values it was clear that the leaves of *Eclipta alba* has got a high calcium content and has a low content of selenium. At the same time the concentration of these minerals in the seeds of *Piper longum* was found to be in the following order, Ca > Na > K> Mg > Zn > Fe > Al > Va > Si > Mb > Cu > Ba > Hg> Ni > Cr > Mn > Co > Pb > Se showing that the Piper longum seeds also contained highest content of calcium and lowest content of selenium.
The hydrogen donating activity was measured by using DPPH test. The DPPH radical scavenging activity of EAE, PLE, BHE and the positive control ascorbic acid were shown in the Figure VIII. These extracts quenched the DPPH radicals in a concentration dependent manner. The BHE has got a maximum DPPH scavenging activity when compared with its individual preparations, EAE and PLE. The BHE contained 252mg ascorbic acid equivalents/g extract of activity, with EC\textsubscript{50} value 139µg/ml. The EC\textsubscript{50} value of the standard ascorbic acid was found to be 188µg/ml.

Table IV depicts Superoxide scavenging activity of different plant extracts. The extracts like EAE, PLE, BHE and the positive control ascorbic acid demonstrated a concentration-dependent scavenging activity of superoxide radicals. The inhibitory activity was minimum in low concentration of plant extract and increases with increase in the concentration. The BHE demonstrated a maximum scavenging activity by neutralizing superoxide radicals with EC\textsubscript{50} value of 165µg/ml. The EC\textsubscript{50} value of the standard ascorbic acid was found to be 182µg/ml.

The plant extracts reduced the hydroxyl radical induced deoxyribose cleavage in a concentration dependent manner. The hydroxyl radical scavenging activity of EAE, BHE, PLE and standard mannitol were shown in the Table V. The hydroxyl radical scavenging activity was more for the BHE when comparable with EAE and PLE. The EC\textsubscript{50} value of BHE was found to be 288µg/ml. With this assay, the BHE was found to contain 696 mg mannitol equivalent/g extract in non site-specific model of hydroxyl radical
scavenging. The EC$_{50}$ value of the standard mannitol was found to be 460µg/ml.

Incubation of solution of sodium nitroprusside in phosphate buffered saline at 25.8 ºC for 150 min resulted in generation of nitric oxide. Figure IX shows the Nitric oxide radical scavenging activity of EAE, BHE, PLE and the positive control Rutin. The BHE effectively reduced the generation of nitric oxide radicals, when compared to its individual preparations EAE and PLE. The scavenging of Nitric oxide by BHE was concentration dependent and the EC$_{50}$ value was found to be 431µg /ml. The BHE was found to contain 415.22 mg/gm equivalent of Rutin. The EC$_{50}$ value of the standard Rutin was found to be 616 µg/ml.

Table VI depicts the reducing capability of BHE (measured at 700 nm) relative to BHT, a well-known antioxidant. The reducing potential of the extracts increased in a dose-dependent manner. The absorbance values of the extract at different concentrations were found to be less than that of the reference compound.

In the present investigation the BHE effectively scavenged the H$_2$O$_2$ radicals by 78.00 % and 82.03% in the concentration of 100 and 200 micrograms where as the vitamin E inhibited only 65.89±0.89 % and 75.96±0.56% at the same concentration which was shown in the Table VII. The scavenging potential of EAE was 55.56% and 58.90% at the concentration of 100 and 200 micrograms per ml where as PLE exhibited 63.00% and 64.00 % respectively.
Table VIII shows the chelating ability of the BHE for metal transition ions (Fe$^{2+}$, Fe$^{3+}$) that increases in a dose dependent manner when compared with control. The metal chelating activities of BHE on Fe$^{2+}$, Fe$^{3+}$ was 56.08% and 55.19% respectively at 100µg concentration respectively. EDTA exhibited 78.64% chelation for Fe$^{2+}$ and 85.42% for Fe$^{3+}$ respectively. The phenolic compound may contribute directly to antioxidative action. Based on these results, it might be concluded that BHE is an electron donor capable of neutralizing free radicals.

Table IX shows the changes in the absorbance under the influence of different concentrations of the extract (100-1000µg/ml) at 37 °C compared to BHT as a positive control. The total antioxidant activities of the plant extract were measured using ferric thiocyanate test, which determines the amount of peroxide produced at the initial stage of lipid peroxidation. According to this the extent of inhibition of lipid oxidation is moderate at low (100 µg/ml) doses of BHE. However, at higher concentrations (800 and 1000 µg/ml), BHE suppressed lipid oxidation to a considerable extent when comparable with its individual preparation EAE and PLE. Lower absorbance indicates a higher level of antioxidant activity.

The protein oxidation was determined in terms of inhibition of protein carbonyl formation (PCO). As shown in Table X, BHE dose-dependently exhibited inhibitory effects of PCO formation. At the same time the individual preparation EAE and PLE exhibited lower inhibitory effects of PCO formation at the same concentration. The oxidative protein damages, provoked by free radicals, have been demonstrated to play a significant role in aging and several pathological events. Radical mediated damages to proteins...
might be initiated by electron leakage. Major molecular mechanisms, leading to structural changes in proteins are free-radical mediated protein oxidation characterized by carbonyl formation (PCO).

The antioxidant activity of BHE is probably due to its phenolic contents. Flavonoids are a class of secondary plant phenolics with powerful antioxidant properties. Therefore, it would be valuable to determine the total phenolic and flavonoid content of the plant extracts. The extracts were investigated regarding their composition by different colorimetric techniques, such as the content of total phenolic compounds by the Folin–Ciocalteu’s assay and flavonoids by AlCl₃ reagent. The total phenolic and flavonoid contents of the plant extracts, were expressed in terms of gallic acid and catechin equivalents. Total phenolic and flavonoid contents of each gram of dried BHE were estimated to be equivalent to 73.4 mg gallic acid and 55.4 mg catechin which was shown in Table XI. The antioxidant activity of BHE is probably due to its phenolic content and the secondary plant phenolics the flavonoids.

The inhibitory action of the BHE on DNA fragmentation of the isolated hepatocytes were shown in the Figure X. In the CCl₄ intoxicated group II animals the extent of DNA damage was detected by the increased mobility of the DNA molecule due to the decrease in the molecular weight which was shown in the lane 2. On the other hand in the BHE at the concentration of 20µg/ml and 40µg/ml protected the DNA from damage which was shown in the lane 3 and 4. This was evidenced by the decreased mobility of the DNA molecule in the agarose gel electrophoresis. The protective effect of BHT the standard antioxidant was shown in lane 5.
DISCUSSION

Reactive oxygen species (ROS) and free transition metal ions cause extensive oxidative damage to cellular biomolecules such as DNA, proteins and lipids. Consequently, they contribute to the pathogenesis of oxidative stress-related diseases (Droge 2002; Hippeli and Elstner, 1999). Although synthetic antioxidants seem to be promising, their toxicity and unwanted side effects rules out their extensive prescription. Hence, there is great interest in the use of naturally occurring antioxidants for treatment or prophylaxis of various oxidative stress-related diseases (Maxwell, 1995). The administration of an antioxidant source comprising of multiple components could offer protection against cancer (Black et al., 1995) and combat oxidative stress-induced physiological malfunctions.

ROS include free radicals such as superoxide •O, hydroxyl radical (•OH) as well as non radical species such as hydrogen peroxide (H$_2$O$_2$) (Cerutti, 1991). In vivo, some of these ROS play a positive role as energy production, phagocytosis, regulation of cell growth and intracellular signaling (Halliwell and Gutteridge, 1999). On the other hand, ROS are also capable of damaging a wide range of essential biomolecules such as proteins, DNA and lipids (Farber, 1994). ROS are not only strongly associated with lipid peroxidation resulting in deterioration of food materials, but also are involved in development of a variety of diseases including aging, carcinogenesis, coronary heart disease, diabetes and neuro degeneration (Harman, 1980; Moskovitz et al., 2002) Cells have several antioxidant defense mechanisms that help to prevent the destructive effects of ROS. These defense
mechanisms include antioxidative enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase and of small molecules such as glutathione, vitamins C and E (Fridovich, 1999). The efficiency of the antioxidant defense system is altered under pathological conditions and, therefore, the ineffective scavenging and/or overproduction of free radicals may play a crucial role in determining tissue damages (Aruoma, 1994).

Substances termed antioxidants can influence the oxidation process through simple or complex mechanisms including prevention of chain initiation, binding of transitional metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging (Shahidi and Wanasundara, 1992). Antioxidants are believed to play an important role in preventing or alleviating chronic diseases by reducing the oxidative damage to cellular components caused by ROS (Ceriello, 2003). There is growing interest in natural phenolic antioxidants, present in medicinal and dietary plants, that might help attenuate oxidative damage (Silva et al., 2005). These natural antioxidants not only protect food lipids from oxidation, but may also provide health benefits associated with preventing damages due to biological degeneration.

High consumption of fruits and vegetables is associated with low risk for these diseases, which is attributed to the antioxidant vitamins and other phytochemicals. Polyphenols are especially important antioxidants, because of their high redox potentials, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Kahkonen et al., 1999). In addition, they have metal chelating potentials (Rice et al., 1995).
The antioxidant activity of the dietary polyphenolics is considered to be much greater than that of the essential vitamins, therefore contributing significantly to the health benefits of fruits and vegetables. The phytochemical screening of these plant extracts were carried out because compounds in their natural formulations are more active than their isolated preparations.

The phytochemical screening showed, that the leaves of *Eclipta alba* and seeds of *P. longum* were rich in alkaloids, flavonoids, tannins, steroids, terpenoids, glycosides and lignins. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds. They were known to show medicinal activity as well as exhibiting physiological activity (Sofowara, 1993). The *in vivo* hepatoprotective activity was mainly due to the presence of these phytochemicals. The medicinal values of plants and vegetables are indicated by their phytochemical and other chemical constituents (Fallah *et al.*, 2005)

Micronutrients consist of vitamins and minerals required by the body in small quantities. They function as essential cofactors in the numerous enzyme catalyzed reactions and their absence can result in impairment of metabolic functions which can lead to serious diseased conditions. Sodium together with potassium assists in the maintenance of the body's electrolyte and water balance. In addition, potassium and sodium plays an important role in nerve conduction, muscle contraction, and the transport of substances across membranes. Supplementation of BHE to CCl₄ rats significantly
maintained acid-base balance by increasing the absorption of electrolytes and minerals from intestine and inhibited electrolytes elimination through urine (Damodara Reddy et al., 2007).

The presence of selenium an important mineral in the BHE is responsible for its antioxidant activity. The main function of selenium is as an antioxidant in the enzyme selenium-glutatline peroxidase. The antioxidant properties of selenoproteins help prevent cellular damage from free radicals, regulate thyroid function and play a role in immune system (Mckenzie et al., 1998; Levander, 1997). Selenium and vitamin E work together synergistically in that they carry out antioxidant and immuno stimulating function better together than individually (Haas, 2001).

Zinc is widely recognized as an essential micronutrient with a catalytic role in over 100 specific metabolic enzymes in human metabolism which plays multiple roles in the perpetuation of genetic materials including transcription of DNA, translation of RNA, and ultimately in cellular division. So supplementation of zinc in the form of plant drug would be helpful in maintaining the normal metabolism of the cells.

Ca salts provide rigidity to the skeleton and calcium ion plays an important role in many metabolic processes. Many neuromuscular and other cellular functions depend on the maintenance of the ionized calcium concentration in the extracellular fluid. Calcium fluxes are important mediators of hormonal effects on target organs through several intracellular signaling pathways (FAO/WHO, 1998) Phosphorous is also important in bone
formation and many essential metabolic activities in the body such as phosphorylation reactions. Mg plays an important role in the metabolism of calcium (Al-Ghamdi et al., 1994). Soft tissue magnesium functions as a cofactor of many enzymes involved in energy metabolism, protein synthesis, RNA and DNA synthesis, and maintenance of the electrical potential of nervous tissue and cell membranes. The presence of these minerals in the plant extract might be responsible for the overall free radical scavenging properties. The important minerals and vitamins found in the plant might also be major contributors to the medicinal value of the plant. Mineral elements may have more roles to play, than presently acknowledged, in the synergy of phytochemicals for the health benefit of man.

In future any one of the protein present in the plants under investigation may be targeted for the production of vaccine against liver diseases, since the leaves of Eclipta alba and seeds of P. longum showed the presence of many proteins. Plants have been actively targeted for the production of medically important proteins, including vaccine antigens and monoclonal antibodies especially against Hepatitis viruses (Mason et al., 1998).

In traditional medicine these two plants are regularly used for the treatment of liver diseases. To clarify the mechanism of action of particularly with respect to its anti-hepatotoxic effects, the antioxidative and free radical scavenging capabilities of the extract were evaluated.
The DPPH radical is a stable organic free radical with absorption of maximum band around 515–528 nm and thus it is a useful reagent for evaluating antioxidant activity of compounds (Sanchez-Moreno, 2002). The BHE demonstrated a concentration dependent scavenging activity by quenching DPPH radicals. It has been documented that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol, gallic acid), reduce and decolorize 1,1-diphenyl-2-picrylhydrazine by their hydrogen donating capabilities (Blois, 1958). The plant is reported to contain the phytoconstituents ecalbatin, alpha-amyrin, ursolic acid, oleanolic acid (Upadhyay et al., 2001) might be responsible for DPPH scavenging activity of the BHE.

Super oxide anions are the most common free radicals in vivo and are generated in a variety of biological systems and the concentration of super oxide anions increases under conditions of oxidative stress (Lee et al., 2002).

In the NBT the BHE demonstrated a concentration-dependent scavenging activity by neutralizing super oxide radicals. Moreover BHE was found to contain 252 mg ascorbic acid equivalents/g extract The earlier photochemical studies indicate the presence of various long-chain esters and amides, alkaloids, lignans, neolignans, amides, terpenes, steroids, chalcones, flavones and flavanones in P. longum extract (Parmar et al., 1999; Stohr et al., 2001) could account for the quenching of super oxide radicals.

BHE neutralized hydroxyl radical induced deoxyribose cleavage in a concentration dependent manner. Hydroxyl radicals are extremely reactive
oxygen species capable of modifying almost every molecule in the living cells and also has the capacity to cause strand damages in DNA leading to carcinogenesis, mutagenesis, and cytotoxicity. These are capable of quickly initiating the lipid peroxidation process as by abstracting hydrogen atoms from unsaturated fatty acids (Aruoma, 1998). These results clearly demonstrated the capacity of BHE to quench hydroxyl radicals and also to chelate the iron metal ions. Several unsaturated amides, aristolactams, lignans, long and short chain esters, terpenes, steroids and alkaloids were already been reported in the plants which were used for the preparation of BHE may be responsible for the hydroxyl radical scavenging activity.

In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Moncada et al., 1991). A potential mechanism of oxidative damage is the nitration of tyrosine residues of proteins, peroxidation of lipids, degradation of DNA and oligonucleosomal fragments (Hemnani and Parihar, 1998). Nitric oxide or reactive nitrogen species, formed during its reaction with oxygen or with super oxide, such as NO₂, N₂O₄, N₃O₄, nitrate and nitrite are very reactive. These compounds alter the structure and function of many cellular components. The BHE was effective in reducing the generation of nitric oxide radicals in our study. Any compound, natural or synthetic, with antioxidant properties might contribute towards the partial or total alleviation of this damage (Lin et al., 1995).

The reducing potential of BHE increased in a dose-dependent manner. The reducing capacity of compound may serve as a significant
indicator of its potential antioxidant activity (Meir et al., 1995). The absorbance values of the extract at different concentrations were found to be less than that of the reference compound. The phenolic compounds may contribute directly to antioxidative action. Based on these results, it might be concluded that BHE is an electron donor capable of neutralizing free radicals. This would have the effect of converting free radicals to more stable products and thus terminating free radical initiated chain reactions. Yahara et al., 1997 reported the presence of phytoconstituents such as triterpene glycoside, saponins like eclalbasaponin, 3β, 2Oβ, 16β -trihydroxytaraxastane; 3 β,2O β,28-trihydroxytaraxastane and sulphated saponins in the leaves of E.alba. These phytochemicals might be account for the reduction reactions exhibited by BHE.

Hydrogen peroxide is formed by two-electron reduction of O₃ which is not a free radical, but an oxidizing agent. In the present investigation the BHE effectively scavenged the H₂O₂ radicals. Inhibition of free radical induced damage by supplementation of antioxidants has become an attractive therapeutic strategy for reducing the risk of diseases (Brash and Harve, 2002). H₂O₂ can easily cross the cell membrane and exerts an injurious effect on tissues through a number of different mechanisms such as, perturbing intracellular Ca²⁺ homeostasis, increasing intracellular ATP, inducing DNA damage, and cell apoptosis (Mallakckakron et al., 2004). The compounds piperlonguminine, piperine, apigenin, dimethyl ether and β sitosterol were reported by Parmar et al., in the P. longum species which was used in the preparation of BHE could be responsible for its H₂O₂ scavenging activity.
A significant result were obtained from BHE on the metal chelating activities of Fe$^{2+}$ and Fe$^{3+}$. The phenolic compound may contribute directly to antioxidative action. Based on these results, it might be concluded that BHE is an electron donor capable of neutralizing free radicals. The extracts of spices and herbs may well act as electron donors and they can react with free radicals to convert them into more stable products and terminate radical chain reactions. Phenolic compounds in plants act as antioxidants by chelating metal ions, preventing radical formation, and improving the antioxidant endogenous system (Tanaka et al., 1998). The antioxidant activity of BHE is probably due to its phenolic contents. Polyphenolics is a highly inclusive term that covers many different subgroups of phenolic acids and flavonoids. More than 5000 polyphenolics, including over 2000 flavonoids have been identified, and the number is still growing (Harborne, 1996). BHE contained considerable amount of the flavonoid and phenolic compounds. The phenolic compounds exhibit considerable free radical scavenging activities, through their reactivity as hydrogen- or electron-donating agents, and metal ion chelating properties (Rice et al., 1996). According to Pietta, 2000 flavonoids are a class of secondary plant phenolics with powerful antioxidant properties. So the presence of considerable amount of these compounds could be accounted for the free radical scavenging activities.

The total antioxidant activity of BHE was measured using ferric thiocyanate test which determines the amount of peroxide produced at the initial stage of lipid peroxidation. Lower absorbance indicates a higher level of antioxidant activity. Flavonoids exert a protective effect against lipid per
oxidation *in vitro* as free radical scavengers and metal-chelating agents (Afanasev *et al.*, 1989). They generally occur as *O*-glycosides in which one or more of the hydroxyl groups are bound to sugars. This glycosylation renders flavonoids more water soluble, making them store readily in the cell vacuole where they are commonly found (Robards and Antolovich, 1997). The above-mentioned effects might be expected for the BHE in reducing the lipid peroxidation.

The oxidative protein damages, provoked by free radicals, have been demonstrated to play a significant role in ageing and several pathological events (Stadtman and Levin, 2000). Major molecular mechanisms, leading to structural changes in proteins are free-radical mediated protein oxidation characterized by carbonyl formation (PCO). The test drug BHE was effective in reducing the PCO formation. This might be due to the cumulative effect of the phytochemicals present in it.

Oxidative DNA damage has been implicated to be involved in various degenerative diseases (Jenner, 1991) including Alzheimer's disease, Parkinson's disease, Hodgkin's disease and Bloom's syndrome (Imlay and Linn, 1988). The decrease in the Fenton's reaction-mediated degradation of DNA by the presence of BHE suggested that these extracts have compounds which may combat against free radical-mediated degradation to the deoxyribose sugar moiety of DNA.
REFERENCES


Ceriello A. New insights on oxidative stress and diabetic complication may lead to a “causal” antioxidant therapy. Diabetes Care 2003;26: 1589-1596.


Sanchez-Moreno C. Methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Science and Technology International 2002;8:121-137.


