PHYTOCHEMICAL STUDY OF *MELIA AZEDARACH* AND

*Piper Longum*

Ayurveda, the ancient healing system of India, flourished in the Vedic era. According to historical facts, the classical texts of Ayurveda, Charaka Samhita and Sushruta Samhita were written around 1000 B.C. The Ayurvedic Materia Medica includes 600 medicinal plants along with therapeutics. Herbs like turmeric, fenugreek, ginger, garlic and holy basil are an integral part of Ayurvedic formulations. These formulations incorporate either a single herb or more than one herb (i.e., polyherbal formulations). Before the availability of synthetic drugs, humans were completely dependent on medicinal herbs for prevention and treatment of diseases. The use of the medicinal herbs for curing disease has been documented in the history of all civilizations. The drugs were used in crude forms like expressed juice, powder, decoction or infusion.

Ancient healers who developed formulations based on medicinal herbs were probably not aware of the chemical composition of these herbs. Nevertheless, the advancement they made despite the non-availability of scientific procedures is astonishing. Medicinal plants are a significant source of synthetic and herbal drugs in India and China and have been on the forefront when we talk about history of herbal drugs. The traditional systems of medicines: Ayurveda, Siddha, Unani, Western Herbal Medicine, Traditional Chinese Medicine and Homeopathy have roots in medicinal herbs.
Scientific validation of herbal drugs always has been questioned, but with recent advances and publications of clinical trials, the researchers and the public are viewing herbal products with more respect. In the commercial market, medicinal herbs are used as raw drugs, extracts or tinctures. Isolated active constituents are used for applied research.

Keeping in view the vast treasure of medicinal herbs, one can expect phytochemicals to play a significant role, as modern science has limited options for diseases like diabetes mellitus, rheumatoid arthritis, alzheimer’s disease liver diseases and parkinson’s disease. Work on the identification and isolation of phytochemicals is an ongoing process and herbal medicine is expected to play critical role in the future health care system.

The purpose of this study was to evaluate experimental proof for the hepatoprotective efficacy of the ethanolic extracts of *M. azedarach* and *P. longum* and the combined biherbal formulation made up of equal concentrations of *M. azedarach* and *P. longum* In this part of the study the processing and phytochemical screening of the plant extracts were done. They were also tested for free radical scavenging activities by using standard models *in vitro*.

**Plant collection and Authentication**

The leaves of *Melia azedarach* and seeds of *Piper longum* were collected from the IMPCOPS (Indian Medical practitioners 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 Melia azedarach extract (MAE)**

The leaves of *Melia azedarach* (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 ethyl acetate 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 ethyl acetate in soxhlet apparatus at 60°C. The residue left after ethyl acetate 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 Biherbal extract (BHE)**

The leaves of *Melia azedarach* (1Kg) and seeds of *Piper longum* (1 kg) were shade-dried and pulverized to a coarse powder. Equal quantities of the powder were passed through 40-mesh sieve and exhaustively extracted with 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 MAE, 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 was treated with 2-3 drops of 1% alcoholic alpha napthol and 2 ml of conc. Sulphuric acid was added along the sides of the test tube carefully. Formation of violet colour 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 colour 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 Bucharad 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 colour indicates the presence of phenols.

(b) The different extracts were treated with 10% sodium chloride solution. The appearance of cream colour 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 colour 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 was added drop
wise and heated. Appearance of magenta colour 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**

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

**Test for Lignin**

(a) 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) Finger Printing**

HPTLC finger printing was performed on CAMAG TLC scanner – 3 instrument, equipped with Linomat IV applicator and CATS 3. 2 software. Precoated aluminium silica gel 60 F$_{254}$ (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 *Melia azedarach* 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.

Estimation 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 and placed 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 mg/100gms of powder.

Estimation of Total Proteins

The estimation of total proteins present in the leaves of Melia azedarach and seeds of Piper longum were estimated by using Lowry’s 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 Melia azedarach 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**

**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
   - Conc. 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 15 min, 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: 10 mg of bovine serum albumin dissolved in 100 ml of distilled water.

**Procedure**

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

**Estimation of Total lipid**

The estimation of Total lipids present in the leaves of *Melia azedarach* and seeds of *Piper longum* were estimated by using chloroform methanol mixture by the method of Folch *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 *Melia azedarach* 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 if 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 mg/100gms of powder.
Estimation of Minerals

Preparation of sample for mineral analysis

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 what man filter paper and made up to 100ml in the volumetric flask. It was transferred to preacid washed bottles and stored for mineral analysis.

Mineral estimation 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 *Melia azedarach* 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% Acryl amide 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 buffers
   (a) for separating gel - 0.375 M pH 8.8
   (b) for stalking gel - 0.125 M pH 6.8

**Preparation of separating gel (6ml)**

1. Tris–HCl Buffer pH 8.8 - 1.5ml
2. Acrylamide - 2.0 ml
3. Sodium dodecyl sulphate 10% - 0.06 ml
4. Ammonium per sulphate (APS) 10% - 0.02 ml
5. TEMED - 0.01 ml
6. 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 - 0.25ml
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 onto 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 - 100 mg dissolved in 100 ml of distilled water

Procedure

One ml of crude extracts of MAE, 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 ºC for 30 min and measured spectrophotometrically at 517nm. Standard antioxidant 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**

Superoxide 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 - 100 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 superoxide radical was generated in 3 ml of sodium phosphate buffer containing 1ml of NBT solution, 1 ml of NADH solution, and different concentrations of the MAE, PLE, and BHE (100 to...
1000µg) 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 superoxide 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 - 10 mg/100 ml 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 1 ml 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. 1 ml 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:

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

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

**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 - 10 mg/100 ml water.

Procedure

Various concentrations of the three different extracts were mixed with sodium nitroprusside in PBS and a final volume of 3 ml was incubated at 25 °C for 150 min. After incubation, 0.5 ml 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.

Nitric oxide scavenging activity (%) = \(\frac{A_0 - A_1}{A_0} \times 100\)

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 MAE, 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)  -  10 mg/100 ml 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 H$_2$O$_2$ was determined by Ruch et al. (1989).

Reagents

1. Phosphate buffered saline pH 7.4  -  0.1 M
2. H$_2$O$_2$,  -  2 mM
3. Vitamin E  -  10 mg/100 ml PBS
**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 separate 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 - 100 µg/ml

**Procedure**

Here different concentrations of the three extracts (20 µl to 100µl) was mixed with 200µl of 20mM phosphate buffer pH 6.5 and100µl 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 Fe$^{2+}$)

Metal chelating property for ferrous ion (Fe$^{2+}$) was estimated according to the method of Tripathi et al. (2001).

Reagents

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

Procedure

Different concentrations of the MAE, 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 - 10 mg/100 ml

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 5 ml 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

A aliquots of sample containing 100mg of test drugs 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 - 50mM  
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 - 6.0 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. Vit E under same assay conditions 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**
   - 0.15mM sodium chloride - 3.0ml
   - 0.1 M Tris-HCl pH 7.5 - 1.0ml
   - 1.5mM Magnesium chloride - 0.15ml
   Made up to 100ml with water.

2. **Cell Lysis buffer**
   - 320mM sucrose - 109.6g
   - 1% Triton X-100 - 20ml f 50% Triton X-100
   - 5mM MgCl$_2$ - 5ml of 1M MgCl$_2$
   - 10mM Tris-HCl pH 7.5 - 10ml of 1M Tris-HCl
   - Distilled water - to a final volume of 1 liter

3. **EDTA-NaCl (100ml)**
   - 25mM EDTA PH 8.0 - 5ml of 0.5M EDTA
   - 75mM NaCl - 1.5ml 0.5M NaCl
   - Distilled water - 93.5ml
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

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

**Procedure**

1. About 0.2 gm of liver tissue was homogenised in 2ml 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 8ml of EDTA-NaCl and added 0.8 ml of 10% cell lysis buffer. It was mixed using the vortex mixture.

7. To this mixture 50µl 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 5000 rpm 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).

**STATISTICAL ANALYSIS**

Values reported are the mean ± S.E.M for ‘n’ extracts in each group. The statistical analysis was carried out using analysis of variance (ANOVA) followed by Dunnet’s ‘t’ test. *p* values < 0.05 was considered as significant.
RESULTS

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

In the present investigation the HPTLC chromatographic pattern of the ethyl acetate extract of *Melia azedarach* showed 9 peaks at Rf values 0.21, 0.29, 0.34, 0.42, 0.57, 0.64, 0.71, 0.85 and 0.92 where as the Chloroform extract of *Melia azedarach* showed 11 peaks at Rf values 0.15, 0.23, 0.29, 0.34, 0.40, 0.43, 0.49, 0.70, 0.73, 0.77 and 0.88. The same plant which was extracted in the ethanol showed 11 peaks at Rf values 0.04, 0.10, 0.36, 0.42, 0.47, 0.56, 0.73 and 0.83 at 254nm which were shown in the Figures I - III.

Figures IV - 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.
The SDS-PAGE electrophoresis results of aqueous extract of Melia azedarach showed the presence of protein bands with molecular weight of ranging 6.50 - 97.4 kDa were shown in Figure VII. The Piper longum seeds showed only two protein bands in the molecular weight of 30 and 60 kDa.

Table II depicts the values of the macronutrients like carbohydrates proteins and lipids present in the leaves of Melia azedarach and seeds of the Piper longum. The carbohydrate content present in the seeds of the piper longum is 13.56mg / 100gms, where as the leaves of Melia azedarach contains 23.78mg / 100 gms. The protein content was also found to be high in the leaves of Melia azedarach when compared with the seeds of Piper longum. The same was applicable to the lipid content also, in which the leaves of Melia azedarach contained 27.94mg/ 100 gms and Piper longum contained 3.78mg/ 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 Melia azedarach leaves was found to be in the following order. Ca, > K, > Na, > Mg, > Zn, > Si, > Fe, > Al, > Va, >Mb, >Cu, >Ni, > Hg, > Pb, > Ba>, Mn, >Cr, >Co > and Se. From the values it was clear that the leaves of Melia azedarach 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, > K, > Na, > Mg, > Zn, > Fe, > Al, > Va, > Si, > Cu, > Mb, > Ba, > Hg, > Ni, > Cr, > Mn, > Co, > Pb > and Se .Here also the Piper longum seeds contain highest content of calcium and lowest content of selenium.
Figure VIII shows the DPPH radical scavenging activity of MAE, PLE, BHE and of the positive control ascorbic acid. These extracts demonstrated a concentration dependent scavenging activity by quenching DPPH radicals, out of which the BHE has got a maximum DPPH scavenging activity when comparable to its individual preparations MAE and PLE. The hydrogen donating activity was measured by using DPPH test, showed that the BHE contained 296mg ascorbic acid equivalents/g extract of activity, with EC$_{50}$ value of 148µg/ml. The EC$_{50}$ value of the standard ascorbic acid was found to be 168µg/ml.

Table IV depicts Super oxide scavenging activity of different plant extracts like MAE, PLE, BHE and the positive control ascorbic acid. 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 scavenging superoxide radicals with EC$_{50}$ value of 155µg/ml against the positive control ascorbic acid which showed the EC$_{50}$ value 172µg/ml under the same assay conditions.

The Hydroxyl radical scavenging activity of MAE, BHE, PLE and standard mannitol were shown in the Table V. The plant extracts reduced the hydroxyl radical induced deoxyribose cleavage in a concentration dependent manner, and the hydroxyl radical scavenging activity was more for the BHE when compared with MAE and PLE. BHE reduced hydroxyl radical induced deoxyribose cleavage in a concentration dependent manner with EC$_{50}$ value of 315µg/ml. With this assay, the BHE was found to contain 765mg mannitol equivalent/g extract in nonsite-specific model of hydroxyl radical scavenging.
Figure IX shows the Nitric oxide radical scavenging activity of MAE, BHE, PLE and the positive control rutin. Incubation of solution of sodium nitroprusside in phosphate buffered saline at 25.8 °C for 150 min resulted in the generation of nitric oxide. The BHE effectively reduced the generation of nitric oxide radicals, when compared to its individual preparations MAE and PLE. The scavenging of Nitric oxide by BHE was concentration dependent and the EC$_{50}$ value was found to be 421.65 µg/ml. The EC$_{50}$ value of the standard rutin was found to be 516 µg/ml. This showed marked nitric oxide scavenging activity of the extract.

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.

Table VII shows the hydrogen peroxide scavenging activity of MAE, BHE, PLE and standard Vitamin E. In the present investigation the BHE effectively scavenged the H$_2$O$_2$ radicals by 74.00±0.56% and 79.03±0.67% at the concentration of 100 and 200 micrograms where as the vitamin E inhibited only 67.89±0.89 % and 74.96±0.56% at the same concentration. The hydrogen peroxide scavenging potential of MAE was 53.56% and 56.90% at the concentration of 100 and 200µg /ml where as PLE exhibited 65.00% and 67.00 % respectively.
A significant result were obtained from BHE on the Fe$^{2+}$ and Fe$^{3+}$ metal chelating activities as 53.08% and 55.19% respectively at 100µg concentration. 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 the control. EDTA exhibited 78.64% chelation for Fe$^{2+}$ and 85.42% for Fe$^{3+}$ respectively at 100 µg concentration. 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 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. Lower absorbance indicates a higher level of antioxidant activity. Table IX shows the changes in the absorbance under the influence of different concentrations of the extract (100-1000µgm/ml) at 37°C compared to BHT a positive control. 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 compared with those of individual preparation MAE and PLE.

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, metal-ion dependent reactions, and autoxidation of lipids and sugars.
Major molecular mechanisms, leading to structural changes in proteins are free-radical mediated protein oxidation characterized by carbonyl formation (PCO). The protein oxidation was determined in terms of inhibition protein carbonyl formation (PCO). As shown in Table X BHE exhibited inhibitory effects of PCO formation by 28.46%, 41.34%, 55.24%, 64.93%, 70.93%, and 78.94% at the extract concentration of 100, 200, 400, 600, 800, and 1000 µgm/ml respectively. At the same time the individual preparation MAE and PLE exhibited lower inhibitory effects of PCO formation at the same concentration.

The antioxidant activity of BHE is probably due to its phenolic contents. It is well known that phenolic compounds are constituents of many plants, and they have attracted a great deal of public and scientific interest because of their health promoting effects as antioxidants. 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 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 79.4 mg gallic acid and 52.4 mg catechin. 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 hepatic tissue 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) such as singlet oxygen (\(\cdot O_2\)), hydrogen peroxide (\(H_2O_2\)) and hydroxyl (\(\cdot OH\)) radical are often generated as byproducts of biological reactions or from exogenous factors. The involvement of these species in the pathogenesis of a large number of diseases including rheumatoid arthritis, atherosclerosis, aging, nephrites, reperfusion injury, asthma, diabetes mellitus, and carcinogenesis are well documented (Stadtman and Oliver, 1991; Freig et al., 1994; Dandona et al., 1996). In situations of increased free radical generation the reinforcement of endogenous antioxidants via intake of dietary antioxidants may be of particular importance in attenuating the cumulative effects of oxidatively damaged molecules. Antioxidants such as Vitamins C and E are essential for the protection against ROS. However, the majority of the antioxidant activity of a fruit or vegetable may be from compounds such as phenolic acids and flavonoids, rather than from Vitamin C, E and \(\beta\)-carotene as stated by (Hanasaki et al., 1994) According to (Guo et al., 1997), intake of controlled diets rich in fruits and vegetables increased significantly the antioxidant capacity of plasma. As per the findings by (Cao et al., 1998), flavonoids, a group of polyphenolic compounds, found widely in foods and beverages are responsible for antioxidant capacity.

Polyphenolics is a highly inclusive term that covers many different subgroups of phenolic acids and flavonoids. According to (Kahkonen et al., 1999), polyphenols are very important antioxidants, because of their high redox potentials, which allow them to act as reducing agents, hydrogen
donors and singlet oxygen quenchers. In addition, their metal chelating potentials were also reported by Rice-Evans et al. (1995). 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); Black et al. (1995) reported that the administration of an antioxidant source comprising of multiple components could offer protection against cancer and combat oxidative stress-induced physiological malfunctions. Although initial research on antioxidants was mostly on isolated pure compounds, recent focus is more on natural formulations. It has been found according to Hagerman et al. (1998) that compounds in their natural formulations are more active than their isolated preparations. Hence the phytochemical screening of these plant extracts was carried out.

The phytochemical screening of chemical constituents of the test drugs showed that the leaves of M. azedarach and seeds of P. longum were rich in alkaloids, flavonoids, tannins, steroids, terpenoids glycosides and lignins. It is generally accepted that a synergistic relationship amongst phytochemicals has been adduced to be responsible for the overall beneficial effect derivable from plants. It was stated by Fallah et al. (2005) that medicinal values of plants and vegetables are indicated by their phytochemicals. According to Hill (1952) most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic
compounds which are found in our plant to a considerable amount. They were known to show medicinal activity as well as exhibiting physiological activity.

Micronutrients consist of vitamins and minerals required by the body in small quantities for the normal function of cellular metabolic processes. They usually 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 disease conditions. Electrolyte disturbances may lead to severe and even life-threatening metabolic abnormalities such as liver diseases, one associated with abnormal serum sodium concentrations, with hyponatremia as the most common alteration. Sodium together with potassium assists in the maintenance of the body's electrolyte and water balance. In addition, potassium and sodium play an important role, in transmission of nerve impulses, muscle contraction, and the transport of substances across membranes. As mentioned by Damodara Reddy (2007) supplementation of plant extracts to CCl$_4$ rats significantly maintained acid-base balance probably by increasing the absorption of electrolytes and minerals from intestine and inhibited electrolytes elimination through urine. The same effect can be expected in our study also because the plant extracts under present investigation contains these minerals.

The presence of selenium in minute quantities in our plant might be responsible for its hepatoprotective activity. The main function of selenium is to act as a cofactor in the enzyme selenium-glutathione peroxidase which is considered as an *in vivo* antioxidant enzyme in protecting the cells from free radicals. The antioxidant properties of selenoproteins help to prevent cellular
damage from free radicals, regulate thyroid function and play a role in immune system as reported by Levander, 1997; Mckenzie et al., 1998. According to Haas (2001), selenium and vitamin E work together synergistically in that they carry out antioxidant and immuno stimulating function better together than individually.

The mineral analysis of the test drugs showed the presence of zinc. Zinc is widely recognized as an essential micronutrient with a catalytic role in over 100 specific metabolic enzymes in human metabolism. It is one of the most ubiquitous of all trace elements involved in human metabolism and 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.

Calcium salts provide rigidity to the skeleton and calcium ion plays a role in many if not most, 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 signalling pathways (FAO/WHO, 1998). Phosphorous is also important in bone formation and many essential metabolic activities in the body such as phosphorylation reactions. As reported by Al-Ghamdi et al. (1994), Mg plays an important role in the metabolism of calcium. 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 vitamin 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.

The presence of different proteins in the leaves of *M. azedarach* and seeds of *P. longum* also have some hepatoprotective activity against the free radical damage on liver. 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). So in future any one of the protein present in the plants can be targeted for the development of vaccines against liver diseases.

The plants present in the BHE are being traditionally used as an herbal remedy for liver diseases in many parts of south India. To clarify the mechanism of action of this medicinal plant, particularly with respect to its anti-hepatotoxic effects, the antioxidative and free radical scavenging capabilities of the extract were evaluated.

In the DPPH test, the antioxidants reduce the DPPH radical to a yellow-colored compound, diphenyl picrylhydrazine, and the extent of the reaction will depend on the hydrogen donating ability of the antioxidants (Bondent *et al.*, 1997). The BHE demonstrated a concentration dependent scavenging activity by quenching DPPH radicals. It has been documented by
(Blois, 1958) that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol and gallic acid), reduce and decolourise 1,1-diphenyl-2-picrylhydrazine by their hydrogen donating capabilities. Several unsaturated amides, aristolactams, lignans, long and short chain esters, terpenes, steroids and alkaloids have previously been reported by Parmar et al. (1997) from *P. longum* might be responsible for DPPH scavenging activity of the BHE.

It has been suggested that superoxide anions are the most common free radicals *in vivo* and are generated in a variety of biological systems and the concentration of superoxide anions increases under conditions of oxidative stress. From NBT assay it is clear that the BHE scavenged superoxide anions and was found to contain 230mg ascorbic acid equivalents/g extract. As stated by Chang et al. (1994) ascorbic acid is a critical antioxidant that acts as a free radical scavenger and may regenerate other antioxidants, including vitamin E. So the presence of ascorbic acid might account for the superoxide scavenging activity of the BHE.

Rollet-Labelle et al. (1998) in his work stated that hydroxyl radicals are known to be the most reactive of all the reduced forms of dioxygen and are thought to initiate cell damage *in vivo*, capable of modifying almost every molecule in the living cells. This radical has the capacity to cause strand damages in DNA leading to carcinogenesis, mutagenesis, and cytotoxicity. Moreover, according to Aruoma (1998) hydroxyl radicals are capable of quickly initiating the lipid peroxidation process as by abstracting hydrogen atoms from unsaturated fatty acids. BHE neutralized hydroxyl radical
induced deoxyribose cleavage in a concentration dependent manner. These results clearly demonstrated the capacity of BHE to quench hydroxyl radicals and also to chelate the iron metal ions. Wu et al. (2004) reported the presence of nine amides namely N-isobutyl-eicosa-2,4-dienamide, N-isobutyl-eicosa-2,4,14-trienamide, N-isobutyl-ocatadeca-2,4,12-trienamide, guineensine, pipernonaline, pellitorine, piperine, piperanine, and piperlonguminine from *Piper longum* and Wagner and Bladt (1996) reported the presence of flavonoids, alkaloids, lignans, and triterpenes in the leaf extracts of *Melia azedarach* were the possible phytoconstituents responsible for the hydroxyl radicals scavenging activity of the BHE.

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

Hsu et al. (2006) in his findings stated that the reducing capacity of compound may serve as a significant indicator of its potential antioxidant
activity. The reducing potential of BHE increased in a dose-dependent manner. The compounds piperlongumine, piperine, apigenin, dimethyl ether and β-sitosterol were isolated by Stohr et al. (2001) showed the presence of phenolic structures that might be responsible for the reducing capacity of the plant extracts.

Hydrogen peroxide is formed by two-electron reduction of O₃ which is not a free radical, but an oxidizing agent. In the presence of O₃ and transition metal ions, the H₂O₂ can generate OH radical via Fenton reaction. Mallakckakron et al. (2004) reported, that in addition, 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 (So the removal of H₂O₂ is important for the antioxidant defence mechanism. In the present investigation the BHE effectively scavenged the H₂O₂ radicals. According to Brash and Harve (2002), inhibition of free radical induced damage by supplementation of antioxidants has become an attractive therapeutic strategy for reducing the risk of diseases. From the plant Melia azedarach limonoids like pyroangolensolide, fraxinellone and its 30-hydroxyfraxinellone, 9a- and 9b-hydroxyfraxinellone, were also isolated by Ascher et al. (1995). Limonoids are classed as tetrnortriterpenes wide variety of therapeutic effects such as antiviral, antifungal, antibacterial, antineoplastic and antimalaria which exerts their biological activity through furonolacton structure (Nakatani 1998). This could also be accounted for the antioxidant activity of the BHE.
A significant result were obtained from BHE on the metal chelating activities of Fe$^{2+}$ Fe$^{3+}$. 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. The presence of phenolic compounds in the BHE acted as antioxidant by chelating metal ions, preventing radical formation, and improving the antioxidant endogenous system. Similar results were reported by Tanaka et al. (1988). The antioxidant activity of BHE is probably due to its phenolic contents. It is well known that phenolic compounds are constituents of many plants, and they have attracted a great deal of public and scientific interest because of their health promoting effects as antioxidants. Piperlonguminine Piperine Asarinin, Guineensine Retrofractamide are some of the phenolic compounds reported in Piper longum, which is one of the ingredient of the BHE were responsible for the metal chelating activity.

According to Pietta (2000), flavonoids are a class of secondary plant phenolics with powerful antioxidant properties (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 was stated by Harborne (1996). The quantitative estimation of BHE revealed considerable amount of the flavonoid and phenolic compound which could be accounted for the inhibition of the activation of procarcinogens or by binding carcinogens to
macromolecules in addition to their free radicals scavenging activities as stated by Krishnaswamy (1996).

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. According to Jiang et al. (2007) flavanoids generally occur as $O$-glycosides in which one or more of the hydroxyl groups are bound to sugars. As stated by Robards and Antolovvich (1997) this glycosylation renders flavonoids more water soluble, making them store readily in the cell vacuole where they are commonly found. 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 aging and several pathological events as stated by Stadtman and Levin (2000). 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 by Jenner (1991) including liver diseases, Alzheimer's disease, Parkinson's disease, Hodgkin's disease and Bloom's syndrome. According to Imlay and Linn (1988) interaction of ROS with DNA can induce a multiplicity of products of varying structures and with differing biological impacts. Studies by Marnett (2002) stated the antioxidant cell defence system intercepts ROS and normally inhibits cellular and nuclear
damage. When the amount of ROS produced overwhelms these endogenous defences, an increase in oxidative DNA injury occurs. The decrease in the Fenton's reaction-mediated degradation of DNA by the presence of BHE suggest that these extracts have compounds which may combat against free radical-mediated degradation to the deoxyribose sugar moiety of DNA.
REFERENCES


Bondent V, Brand-Williams W, Bereset C. Kinetic and mechanism of antioxidant activity using the DPPH free radical methods. Lebensmittel Wissenschaft and Technologie1997; 30:609


Haas EM. Selenium. 2001. Health world on-line


Tripathi YB, Singh AV, Dubey GP. Antioxidant property of the bulb of Scilla indica Science 2001;80:1267.


Table 1: Preliminary Phytochemical Screening of the test drugs

<table>
<thead>
<tr>
<th>S.No</th>
<th>Phytochemicals</th>
<th>MAE</th>
<th>PLE</th>
<th>Bi herbal extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloid</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Carbohydrate</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Protein</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Phytosterol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Tannins</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Phenolic compounds</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Gums and Mucilage</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Saponins</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Oils and fats</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Terpenes</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>Lignin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) Indicates the presence of the chemical
(-) Indicates the absence of the chemical
Figure I: The HPTLC finger print analysis of the Ethyl acetate extract of *M. azedarach*
Figure II: The HPTLC fingerprint analysis of the Chloroform extract of *M. Azedarach*
Figure III: The HPTLC finger print analysis of the Ethanolic extract of *M. Azedarach*
Figure IV: The HPTLC finger print analysis of the Ethyl acetate extract of *P. longum*
Figure V: The HPTLC finger print analysis of the Chloroform extract of *P. longum*
Figure VI: The HPTLC fingerprint analysis of the ethanolic extract of *P. longum*
Figure VII: SDS-PAGE electrophoresis results of aqueous extract of *Melia azedarach*

Lane 1 - shows the marker protein bands with their molecular weight ranging 6.50 – 97.4 kDa.

Lane 2 - shows the Melia azedarach leaf protein bands with their molecular weight of ranging 6.50 – 97.4 kDa.

Protein used as markers with their molecular weight expressed in kDa: Phosphorylase b-97.4; BSA-66; Ovalbumin-43; Carbonic Anhydrase-29; Soyabean; Trypsin Inhibitor-20.1; Lysozyme-14.3; Aprotinin-6.5.
### Table II: Concentration of Macronutrients

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Macronutrients</th>
<th>Leaves of <em>Melia azedarach</em> (Expressed in mg/100gms)</th>
<th>Seeds of <em>Piper longum</em> (Expressed in mg/100gms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Total sugars</td>
<td>23.78</td>
<td>13.56</td>
</tr>
<tr>
<td>2.</td>
<td>Total protein</td>
<td>36.23</td>
<td>7.80</td>
</tr>
<tr>
<td>3.</td>
<td>Total lipid</td>
<td>27.94</td>
<td>3.78</td>
</tr>
</tbody>
</table>

### Table III: Concentration of Minerals present in the plants

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Macronutrients</th>
<th>Leaves of <em>Melia azedarach</em> (Expressed in PPM)</th>
<th>Seeds of <em>Piper longum</em> (Expressed in PPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Aluminum</td>
<td>3.286</td>
<td>2.294</td>
</tr>
<tr>
<td>2.</td>
<td>Barium</td>
<td>0.299</td>
<td>0.420</td>
</tr>
<tr>
<td>3.</td>
<td>Calcium</td>
<td>280</td>
<td>340</td>
</tr>
<tr>
<td>4.</td>
<td>Copper</td>
<td>0.746</td>
<td>0.574</td>
</tr>
<tr>
<td>5.</td>
<td>Chromium</td>
<td>0.186</td>
<td>0.149</td>
</tr>
<tr>
<td>6.</td>
<td>Cobalt</td>
<td>0.107</td>
<td>0.081</td>
</tr>
<tr>
<td>7.</td>
<td>Iron</td>
<td>2.198</td>
<td>2.633</td>
</tr>
<tr>
<td>8.</td>
<td>Lead</td>
<td>0.311</td>
<td>0.025</td>
</tr>
<tr>
<td>9.</td>
<td>Magnesium</td>
<td>36.171</td>
<td>26.115</td>
</tr>
<tr>
<td>10.</td>
<td>Molybdenum</td>
<td>0.810</td>
<td>0.745</td>
</tr>
<tr>
<td>11.</td>
<td>Mercury</td>
<td>0.371</td>
<td>0.259</td>
</tr>
<tr>
<td>12.</td>
<td>Manganese</td>
<td>0.136</td>
<td>0.132</td>
</tr>
<tr>
<td>13.</td>
<td>Nickel</td>
<td>0.429</td>
<td>0.181</td>
</tr>
<tr>
<td>14.</td>
<td>Potassium</td>
<td>136.5</td>
<td>117</td>
</tr>
<tr>
<td>15.</td>
<td>Sodium</td>
<td>92</td>
<td>138</td>
</tr>
<tr>
<td>16.</td>
<td>Silicon</td>
<td>2.343</td>
<td>1.399</td>
</tr>
<tr>
<td>17.</td>
<td>Selenium</td>
<td>0.0117</td>
<td>0.0094</td>
</tr>
<tr>
<td>18.</td>
<td>Vanadium</td>
<td>1.2945</td>
<td>2.107</td>
</tr>
<tr>
<td>19.</td>
<td>Zinc</td>
<td>4.944</td>
<td>4.130</td>
</tr>
</tbody>
</table>
Figure VIII: Effect of different plant extracts on DPPH radical scavenging activity

DPPH radical scavenging activity of MAE, PLE, BHE and ascorbic acid. Each value represents the mean ± SD (n = 3).

The EC$_{50}$ value of the BHE was found to be 148µg/ml.

The EC$_{50}$ value of the standard ascorbic acid was found to be 168µg/ml.
Table IV: Superoxide scavenging activity of different plant extracts

<table>
<thead>
<tr>
<th>Concentration in µgms / ml</th>
<th>Inhibitory activity of BHE (%)</th>
<th>Inhibitory activity of MAE (%)</th>
<th>Inhibitory activity of PLE (%)</th>
<th>Inhibitory activity of Ascorbic acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>15.67±2.76a*</td>
<td>12.97±2.80</td>
<td>13.67±1.76</td>
<td>17.82±1.82b*</td>
</tr>
<tr>
<td>200</td>
<td>32.86±1.72a*</td>
<td>27.84±1.60</td>
<td>28.84±2.18</td>
<td>34.76±2.84b*</td>
</tr>
<tr>
<td>400</td>
<td>56.25±1.32a*</td>
<td>50.50±2.1</td>
<td>51.89±1.48</td>
<td>58.64±2.23b*</td>
</tr>
<tr>
<td>600</td>
<td>69.49±2.72a*</td>
<td>63.80±2.4</td>
<td>62.81±1.94</td>
<td>72.35±2.34b*</td>
</tr>
<tr>
<td>800</td>
<td>77.86±1.35a*</td>
<td>74.78±1.98</td>
<td>72.54±1.80</td>
<td>84.45±1.36b</td>
</tr>
<tr>
<td>1000</td>
<td>82.52±2.37a*</td>
<td>79.74±1.50</td>
<td>80.88±1.44</td>
<td>91.34±1.86b*</td>
</tr>
</tbody>
</table>

Superoxide radical scavenging activity of MAE, PLE, BHE and ascorbic acid. Each value represents the mean ± SD (n = 3).

Comparison between

a - BHE vs. MAE, PLE
b - BHE vs. Ascorbic acid

*p<0.05, * *p<0.01

The EC_{50} value of the BHE was found to be 155µg/ml

The EC_{50} value of the standard ascorbic acid was found to be 172µg/ml
Table V: Hydroxyl radical scavenging activity of the different extracts

<table>
<thead>
<tr>
<th>Concentration in µgms / ml</th>
<th>Inhibitory activity of MAE (%)</th>
<th>Inhibitory activity of PLE (%)</th>
<th>Inhibitory activity of BHE (%)</th>
<th>Inhibitory activity of Mannitol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>21.41±0.98</td>
<td>22.84±1.8</td>
<td>24.40±1.72a*</td>
<td>54.20±2.30b**</td>
</tr>
<tr>
<td>400</td>
<td>47.84±0.18</td>
<td>48.46±1.2</td>
<td>49.86±1.45a*</td>
<td>65.60±2.41b**</td>
</tr>
<tr>
<td>600</td>
<td>56.41±1.80</td>
<td>60.51±2.0</td>
<td>59.90±1.75a*</td>
<td>72.40±3.82b**</td>
</tr>
<tr>
<td>800</td>
<td>64.71±1.64</td>
<td>62.84±1.1</td>
<td>68.7±2.80a*</td>
<td>82.80±1.72b**</td>
</tr>
<tr>
<td>1000</td>
<td>76.84±1.85</td>
<td>78.64±1.6</td>
<td>79.82±1.50a*</td>
<td>91.4±2.84b**</td>
</tr>
</tbody>
</table>

Hydroxyl radical scavenging activity of MAE, BHE, PLE and standard Vitamin E Each value represents the mean ± SD (n = 3).

Comparison between
a - BHE vs. MAE, PLE
b - BHE vs. Mannitol

*p<0.05, **p<0.01

The EC<sub>50</sub> value of the BHE was found to be 315µg/ml

The EC<sub>50</sub> value of the standard ascorbic acid was found to be 420µg/ml
Figure IX: Nitric oxide scavenging activity of different plant extracts

Nitric oxide radical scavenging activity of MAE, BHE, PLE and the positive control Rutin. Each value represents the mean ± SD (n = 3).

Comparison between

a - BHE vs. MAE, PLE

b - BHE vs. Rutin

*p<0.05, **p<0.01

The EC$_{50}$ value of the BHE was found to be 421µg/ml

The EC$_{50}$ value of the standard Vitamin E was found to be 516 µg/ml
Table VI: Reducing power of the different plant extracts

<table>
<thead>
<tr>
<th>Concentration in µgms / ml</th>
<th>Absorbance of BHE in (O.D)</th>
<th>Absorbance of MAE in (O.D)</th>
<th>Absorbance of PLE in (O.D)</th>
<th>Absorbance of BHT in (O.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.27±0.04a*</td>
<td>0.18±0.03</td>
<td>0.23±0.02</td>
<td>0.30±0.03b*</td>
</tr>
<tr>
<td>200</td>
<td>0.38±0.04a*</td>
<td>0.28±0.03</td>
<td>0.33±0.01</td>
<td>0.38±0.03b NS</td>
</tr>
<tr>
<td>400</td>
<td>0.42±0.02a*</td>
<td>0.39±0.05</td>
<td>0.46±0.03</td>
<td>0.49±0.02b*</td>
</tr>
<tr>
<td>600</td>
<td>0.56±0.03a*</td>
<td>0.46±0.04</td>
<td>0.52±0.45</td>
<td>0.62±0.20b*</td>
</tr>
<tr>
<td>800</td>
<td>0.62±0.06a*</td>
<td>0.57±0.03</td>
<td>0.64±0.03</td>
<td>0.75±0.05b*</td>
</tr>
<tr>
<td>1000</td>
<td>0.78±0.04a*</td>
<td>0.68±0.24</td>
<td>0.62±0.34</td>
<td>0.90±0.34b**</td>
</tr>
</tbody>
</table>

Reducing capacity of MAE, BHE, PLE and standard BHT. Each value represents the mean ± SD (n = 3).

Comparison between

a - BHE vs. MAE, PLE

b - BHE vs. BHT

*p<0.05,  * *p<0.01
Table VII: Hydrogen peroxide scavenging activity of different plant extracts

<table>
<thead>
<tr>
<th>Concentration in µg/ml</th>
<th>Inhibitory activity of MAE (%)</th>
<th>Inhibitory activity of PLE (%)</th>
<th>Inhibitory activity of BHE (%)</th>
<th>Inhibitory activity of Vitamin E (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.99±0.40</td>
<td>10.34±0.69</td>
<td>12.78±0.34a*</td>
<td>15.90±0.67b**</td>
</tr>
<tr>
<td>100µg /ml</td>
<td>53.56±0.56</td>
<td>65.00±0.87</td>
<td>74.00±0.56a**</td>
<td>67.89±0.89b*</td>
</tr>
<tr>
<td>200µg /ml</td>
<td>56.90±0.56</td>
<td>67.00±0.67</td>
<td>79.03±0.67a**</td>
<td>74.96±0.56b*</td>
</tr>
</tbody>
</table>

Hydrogen peroxide scavenging activity of MAE, BHE, PLE and standard Vitamin E. Each value represents the mean ± SD (n = 3).

Comparison between

a - BHE vs. MAE, PLE

b - BHE vs. Vitamin E

*p<0.05, * *p<0.01
Table VIII: Iron chelating activity of Bi herbal Extract (BHE)

<table>
<thead>
<tr>
<th>Concentration in µg/ml</th>
<th>O.D at 525nm</th>
<th>Chelation of Fe²⁺ (%)</th>
<th>O.D at 460nm</th>
<th>Chelation of Fe³⁺ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.308</td>
<td>0</td>
<td>1.021</td>
<td>0</td>
</tr>
<tr>
<td>BHE (20µg/ml)</td>
<td>0.246</td>
<td>20.26±0.194</td>
<td>0.884</td>
<td>13.44±0.093</td>
</tr>
<tr>
<td>BHE (40µg/ml)</td>
<td>0.208</td>
<td>32.69±0.308</td>
<td>0.793</td>
<td>22.33±0.171</td>
</tr>
<tr>
<td>BHE (60µg/ml)</td>
<td>0.200</td>
<td>35.02±0.259</td>
<td>0.716</td>
<td>29.94±0.006</td>
</tr>
<tr>
<td>BHE (80µg/ml)</td>
<td>0.195</td>
<td>47.25±0.177</td>
<td>0.697</td>
<td>36.01±0.006</td>
</tr>
<tr>
<td>BHE (100µg/ml)</td>
<td>0.163</td>
<td>53.08±0.433</td>
<td>0.654</td>
<td>55.19±0.006</td>
</tr>
<tr>
<td>EDTA (100µg/ml)</td>
<td>0.067</td>
<td>78.64±0.204</td>
<td>0.149</td>
<td>85.42±0.006</td>
</tr>
</tbody>
</table>

Metal chelating activity of BHE and EDTA.
Each value represents the mean ± SD (n = 3)
Table IX: The average total antioxidant activity of different plant extract

<table>
<thead>
<tr>
<th>Concentration in µgms / ml</th>
<th>BHE (Absorbance at 500 nm)</th>
<th>MAE (Absorbance at 500 nm)</th>
<th>PLE (Absorbance at 500 nm)</th>
<th>BHT (Absorbance at 500 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.98±0.04a*</td>
<td>0.88±0.05</td>
<td>0.84±0.03</td>
<td>0.92±0.07b*</td>
</tr>
<tr>
<td>200</td>
<td>0.88±0.02a*</td>
<td>0.92±0.06</td>
<td>0.85±0.45</td>
<td>0.84±0.02b*</td>
</tr>
<tr>
<td>400</td>
<td>0.82±0.04a*</td>
<td>0.89±0.05</td>
<td>0.76±0.05</td>
<td>0.79±0.04b*</td>
</tr>
<tr>
<td>600</td>
<td>0.64±0.02a*</td>
<td>0.74±0.03</td>
<td>0.77±0.56</td>
<td>0.64±0.01b*</td>
</tr>
<tr>
<td>800</td>
<td>0.54±0.02a*</td>
<td>0.62±0.04</td>
<td>0.65±0.45</td>
<td>0.42±0.02b*</td>
</tr>
<tr>
<td>1000</td>
<td>0.35±0.02a*</td>
<td>0.46±0.05</td>
<td>0.54±0.45</td>
<td>0.22±0.04b*</td>
</tr>
</tbody>
</table>

The average total antioxidant activity of MAE, BHE, PLE and standard BHT.

Values are represented by mean ±SEM. (n=3)

Comparison between

a - BHE vs. MAE, PLE

b - BHE vs. BHT

*p<0.05, * *p<0.01
Table X: The Percentage inhibition of protein carbonyl formation by different plant extracts

<table>
<thead>
<tr>
<th>Concentration in µgms / ml</th>
<th>Inhibitory activity of BHE (%)</th>
<th>Inhibitory activity of MAE (%)</th>
<th>Inhibitory activity of PLE (%)</th>
<th>Inhibitory activity of Vitamin E (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>28.46±1.38a*</td>
<td>26.89±0.04</td>
<td>24.56±0.7</td>
<td>32.15±1.07b*</td>
</tr>
<tr>
<td>200</td>
<td>41.34±1.23a*</td>
<td>36.98±0.03</td>
<td>35.98±0.02</td>
<td>51.68±1.24b*</td>
</tr>
<tr>
<td>400</td>
<td>55.24±1.17a*</td>
<td>43.89±0.03</td>
<td>46.02±0.45</td>
<td>63.22±1.04b*</td>
</tr>
<tr>
<td>600</td>
<td>64.93±1.25a*</td>
<td>56.67±0.02</td>
<td>54.23±0.4</td>
<td>72.18±1.05b*</td>
</tr>
<tr>
<td>800</td>
<td>70.93±2.23a*</td>
<td>68.34±0.03</td>
<td>62.78±0.23</td>
<td>80.26±1.1b*</td>
</tr>
<tr>
<td>1000</td>
<td>78.94±2.22a*</td>
<td>72.98±0.05</td>
<td>72.06±0.45</td>
<td>91.06±1.05b**</td>
</tr>
</tbody>
</table>

The Percentage inhibition of protein carbonyl formation of MAE, BHE, PLE and Vitamin E

Values are represented by mean ± SEM. (n=3)

Comparison between

a - BHE vs. MAE, PLE
b - BHE vs. Vitamin E

*p<0.05, **p<0.01
Table XI: Total Phenolic and flavonoid content of Biherbal ethanolic extract

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Total phenolic content (mg/g)</th>
<th>Total flavonoid content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAE</td>
<td>56.67±2.67</td>
<td>43.46±2.89</td>
</tr>
<tr>
<td>PLE</td>
<td>63.00±2.37</td>
<td>46.98±2.67</td>
</tr>
<tr>
<td>BHE</td>
<td>79.4±1.64</td>
<td>52.4±1.57</td>
</tr>
</tbody>
</table>

Each value represents the mean ±SEM. (n=3).

Total phenolic content was expressed as mg gallic acid equivalents/g dried extract.

Total flavonoid content was expressed as mg catechin equivalent/g dried extract.
Figure X: *In Vitro* assay of DNA fragmentation study

Lane 1 - Untreated Hepatic DNA

Lane 2 - H$_2$O$_2$ induced DNA damage

Lane 3 - DNA damage protection by BHE at concentration of 20µg/ml

Lane 4 - DNA damage protection by BHE at concentration of 40µg/ml

Lane 5 - DNA damage protection by BHT at concentration of 40µg/ml
HISTOPATHOLOGY AND REPORT WITH PHOTOGRAPHS

Figure I: The histology of Lung from the control and treated groups (the 10x and 40x magnifications)

- Normal
- BHE 25mg/kg treated
- BHE 50 mg/kg treated

Figure II: The histology of Heart from the control and treated groups (the 10x and 40x magnifications)

- Normal
- BHE 25mg/kg treated
- BHE 50 mg/kg treated

Figure III: The histology of Stomach from the control and treated groups (the 10x and 40x magnifications)

- Normal
- BHE 25mg/kg treated
- BHE 50 mg/kg treated
Figure IV: The histology of Spleen from the control and treated groups (the 10x and 40x magnifications)

Normal   BHE 25mg/kg treated   BHE 50 mg/kg treated

Figure V: The histology of Liver from the control and treated groups (the 10x and 40x magnifications)

Normal   BHE 25mg/kg treated   BHE 50 mg/kg treated

Figure VI: The histology of Pancreas from the control and treated groups (the 10x and 40x magnifications)

Normal   BHE 25mg/kg treated   BHE 50 mg/kg treated
Figure VII: The histology of Brain from the control and treated groups (the 10x and 40x magnifications)

Normal  |  BHE 25mg/kg treated  |  BHE 50 mg/kg treated

Figure VIII: The histology of Kidney from the control and treated groups (the 10x and 40x magnifications)

Normal  |  BHE 25mg/kg treated  |  BHE 50 mg/kg treated

Figure IX: The histology of Ovaries from the control and treated groups (the 10x and 40x magnifications)

Normal  |  BHE 25mg/kg treated  |  BHE 50 mg/kg treated

Figure X: The histology of testis from the control and treated groups (the 10x and 40x magnifications)

Normal  |  BHE 25mg/kg treated  |  BHE 50 mg/kg treated
HISTOPATHOLOGY CHANGES

Normal

LUNG: shows normal alveoli
HEART: shows normal cardiac muscle bundles.
STOMACH: shows normal mucosal glands.

BHE 25mg/Kg treated

LUNG: shows congested alveolar wall with mild thickening and mild emphysematous changes 400x)
HEART: shows congestion and mild inflammatory infiltration in between cardiac muscle bundles.
STOMACH: shows near normal mucosal gland with mild exudates

BHE 50mg/Kg treated

LUNG: shows congestion, narrowed alveolar space and thickened alveolar wall.
HEART: shows hypertrophic cardiac muscle bundles.
STOMACH: shows stomach with superficial erosion and congestion.
**Normal**

SPLEEN: shows normal spleen with lymphoid aggregation.
LIVER: shows normal hepatocytes.
PANCREAS: shows pancreas with acini and normal islets

**BHE 25mg/Kg treated**

SPLEEN: shows congestion with lymphoid hyperplasia.
LIVER: shows hepatocytes with focal mild fatty change (400x)
PANCREAS: shows pancreas with acini and normal islets.

**BHE 50mg/Kg treated**

SPLEEN: shows lymphoid hyperplasia
LIVER: shows almost normal hepatocytes and occasional binucleate cells.
PANCREAS: shows atrophic islet cells.
Normal

BRAIN: shows normal brain with nerve fibers and astrocytes  
KIDNEY: shows normal renal tissue with glomeruli and tubules.  
OVARY: shows ovarian stroma with follicles and corpus leuteum.  
TESTIS: shows normal tubules with spermatogenesis.

BHE 25mg/kg treated

BRAIN: shows brain with micro cystic change and astrocytic proliferation (400x).  
KIDNEY: shows renal tissue with focal tubular damage, interstitial inflammatory  
OVARY: shows ovarian stroma with follicles and corpus leuteum  
TESTIS: shows normal tubules with spermatogenesis.

BHE 50mg/kg treated

BRAIN: shows brain with edema. Astrocytes show degenerative changes.  
KIDNEY: shows renal tissue with tubular epithelial damage. RBC with in the tubules.  
OVARY: shows ovarian follicles and corpus leuteum  
TESTIS: shows normal tubules with spermatogenesis
HISTOPATHOLOGY

Liver

Figure XVIII(a)  Figure XVIII(b)

Figure XVIII(c)  Figure XVIII(d)
Kidney

Figure XIX(a)       Figure XIX(b)       Figure XIX(c)       Figure XIX(d)

Heart

Figure XX(a)            Figure XX(b)            Figure XX(c)            Figure XX(d)

Intestine

Figure XXI(a)            Figure XXI(b)            Figure XXI(c)            Figure XXI(d)