| METHODS & MATERIALS |
4. MATERIALS AND METHODS

4.1 Pharmacognostical analysis

4.1.1 Identification and Collection of the Plant

Whole plant material of *E. littorale* was collected from Gujarat (India) in August – September (2000) at the end of flowering season. The collection was made by uprooting and thereby collecting root also. The plant was identified by comparing it morphologically and microscopically with description given in different standard texts and floras (Kirtikar and Basu 1935). The plant was identified and authenticated by Prof. O. P. Saxena, Head, Botany Department, Gujarat University, Ahmedabad, India and a voucher specimen was deposited. The plant material was cleaned and dried in shade and stored at 25 °C.

4.1.2 Macroscopic Observations

The drug was subjected to macroscopic studies which comprised of study of organoleptic characters of the drugs viz., color, odour, appearance, taste, smell, texture, fracture, etc.

4.1.3 Microscopic Studies

4.1.3.1 Stem/ Root/ Leaf

For microscopical examination of drug, transverse sections of the drug were taken; the sections were treated with phloroglucinol and a drop of concentrated hydrochloric acid to stain lignified material. Lignified elements were colored pink.

4.1.4 Evaluation of Physical Parameters

4.1.4.1 Moisture content

Five grams of accurately weighed drug powder was heated at 105 °C in an oven to a constant weight. Weight loss after drying gave the moisture content of the material.

4.1.4.2 Determination of foreign matter

100-500 g of the drug sample to be examined was weighed accurately, and spread out in a thin layer. Foreign matter was detected by inspection with the unaided
eye or by the use of lens (6 X). The foreign matter was separated and weighed and percentage foreign matter was calculated.

4.1.5 Determination of Ash Values

4.1.5.1 Determination of total ash

Accurately weighed 2 g of the powdered drug was taken in a tared silica dish and it was incinerated at a temperature not exceeding 450 °C until free from carbon. The sample was cooled and weighed. If a carbon free ash cannot be obtained in this way, the charred mass was exhausted with hot water. The residue was collected on an ashless filter paper and the residue and the filter paper were incinerated the filtrate was evaporated to dryness, and ignited at a temperature not exceeding 450°C. The percentage of ash was calculated with reference to the air dried drug.

4.1.5.2 Determination of acid-insoluble ash

The ash obtained as described in the section 4.1.5.1 was boiled for 5 min. with 25 ml of dilute hydrochloric acid. The insoluble matter was collected in a Gooch crucible or on an ashless filter paper and washed with hot water and ignited to constant weight. The percentage of acid-insoluble ash was calculated with reference to the air dried drug.

4.1.5.3 Determination of water-soluble ash

The ash obtained as described in the section 4.1.5.1 was boiled for 5 min with 25 ml of water and insoluble matter was collected in a Gooch crucible, or on an ashless filter paper, washed with hot water and ignited for 15 min. at a temperature not exceeding 450°C. Weight of the insoluble matter was subtracted from the weight of the ash. The difference in weight represents the water-soluble ash. The percentage of water-soluble ash was calculated with reference to the air dried drug.

4.1.6 Determination of Extractive Values

4.1.6.1 Determination of ethanol-soluble extractive

Five grams of the coarsely powdered drug was macerated with 100 ml of ethanol (95 %) in a closed flask for twenty four hours. The flasks were shaken intermittently during six hours and allowed to stand for eighteen hours. The extract was filtered rapidly, taking precautions against loss of solvent. 25 ml of the filtrate was evaporated to dryness in a tared flat bottomed shallow dish, and dried at 100°C, to
a constant weight. The percentage of ethanol-soluble extractive was calculated with reference to the air dried drug.

4.1.6.2 Determination of water-soluble extractive

Water-soluble extractive was obtained by following the same procedure as described for ethanolic-soluble extractive using chloroform water (0.25 % chloroform in water) instead of ethanol.

4.2 Phytochemical analysis

4.2.1 Extraction and Fractionation

Six kilogram of shade-dried herb of *E. littorale* was powdered and boiled with 24 lit water for 8 h. The aqueous extract was concentrated under reduced pressure. Aqueous extract was further fractionated using solvents of varying polarity *viz.*, petroleum ether [60-80°C], toluene, chloroform, ethyl acetate and *n*-butanol, the extract remained after the fractionation with *n*-butanol was the residual extract. The extracts were concentrated under reduced pressure and air dried to remove the solvent completely.
4.2.2. Preliminary Phytochemical Screening

All the extracts prepared as mentioned above, were screened qualitatively for the major groups of chemical constituents using standard reagents. Small quantities of all the extracts were dissolved in ethanol and were subjected to preliminary phytochemical analysis for the detection of the individual components using specific reagents.
Materials and Methods

Alkaloids

A few ml (2-3 ml) of ethanolic extract was evaporated in a watchglass. One ml of dilute hydrochloric acid and a few drops of Mayer’s reagent was added to the residue. White precipitate indicated the presence of alkaloids.

A drop of ethanolic extract was spotted on a small piece of precoated TLC plate. The plate was sprayed with modified Dragendorff’s reagent. Orange coloration of the spot indicated the presence of alkaloids.

Steroids and Terpenoids

To one ml of ethanolic extract of drug, one ml of chloroform and 2 to 3 ml of acetic anhydride was added. To the above mixture, 1 to 2 drops of concentrated Sulphuric acid was added. Dark green coloration of the solution indicated the presence of steroids and dark pink or red coloration of the solution indicated the presence of terpenoids.

Anthraquinones

One ml of ethanolic extract of drug was heated in water containing 10 % ferric chloride solution and 1 ml of concentrated hydrochloric acid. The aqueous layer was separated and shaken with diethyl ether. The ether extract was further extracted with strong ammonia. Pink or deep red coloration of aqueous layer indicated the presence of Anthraquinones.

Flavonoids

To a 2-3 ml of ethanolic extract, a piece of magnesium ribbon and 1 ml of concentrated hydrochloric acid was added. Pink red or red coloration of the solution indicated the presence of flavonoids in the drug.

Phenols

A drop of ethanolic extract was spotted on a filter paper and a drop of phosphomolybdic acid reagent was added on it. The spot was then exposed to ammonia vapor. Blue coloration of the spot indicated the presence of phenols.
Materials and Methods

Tannins

To a 2-3 ml of ethanolic extract, 10 % ethanolic ferric chloride solution was added. Dark blue or greenish grey coloration of the solution indicated the presence of tannins in the drug.

4.2.3 HPTLC Finger Printing

TLC fingerprint profiles of aqueous extract and its pet ether, toluene, chloroform, ethyl acetate and n-butanol fractions were established using HPTLC. Suitably diluted stock solution of aqueous extract and its pet ether, toluene, chloroform, ethyl acetate and n-butanol extract were spotted on pre-coated silica gel 60 F254 TLC plates (E. Merck) using CAMAG Linomat IV Automatic Sample Spotter and the plate were developed in the following solvent systems:

1. For aqueous extract : Ethyl acetate : methanol : water (7.7: 2.0: 0.5)
2. For pet ether fraction : Toluene : ethyl acetate (8: 2)
3. For toluene fraction : Toluene : chloroform : methanol (6: 4:1.5)
4. For chloroform fraction : Chloroform : ethyl acetate : methanol (7.7: 1.5: 0.3)
5. For ethyl acetate fraction : Chloroform : methanol (9.5: 1)
6. For n-butanol fraction : Ethyl acetate : methanol : water (7.7: 1.5: 0.5)

respectively.

The plates were dried at room temperature and scanned using CAMAG TLC scanner 3 at UV 254 and 366 nm and R_f values, absorption spectra of the resolved bands were recorded. Further, the plates were derivatised by spraying with anisaldehyde sulphuric acid reagent followed by heating at 110 °C for 5 min, and the R_f and colours of the bands resolved were recorded.

4.2.4 Extraction and Isolation of Swertiamarin

25 g of powdered plant material was extracted exhaustively with petroleum ether (60-80 °C) and methanol successively by cold maceration. The extract was filtered and the solvent removed under vacuum. The methanolic extract obtained was treated with cold diethyl ether to obtain a precipitate (8.66 g). A fraction of the precipitate (3g) was chromatographed on silica gel (60 g) eluting with petroleum ether (60-80) containing 0-18 % ethylacetate, followed by ethylacetate and then with ethylacetate containing increasing amounts of methanol (0-12 %). Fractions of 25 ml
were collected. Different fractions were monitored by TLC for swertiamarin using the solvent system of ethylacetate: MeOH: H₂O (7.7 : 1.5: 0.5). Fractions of ethyl acetate: methanol eluants containing swertiamarin were pooled and concentrated to dryness (yield 0.53 g) and swertiamarin was confirmed by co-chromatography with standard swertiamarin. It was purified by dissolving the residue in methanol and adding a mixture of chloroform and diethylether.

4.2.4.1. Characterization and purity check by HPTLC, UV, IR, LCMS, and NMR.

Thin-Layer Chromatography.

For TLC experiments, precoated plates of silica gel 60F₂₅₄ (E. Merck) were used and spotting was done on CAMAG LINOMAT IV Automatic TLC spotter. For purity assessment of the isolated compound, and for recording UV spectrum of the compound, the plates were scanned on CAMAG TLC Scanner 3.

Method adopted for testing the purity of the swertiamarin.

The purity of the compound isolated was checked by following:
1. By recording melting point (M.P.) using Tosniwal melting point apparatus.
2. By carrying out TLC in different solvent systems and co-chromatography along with standard swertiamarin.
3. By recording chromatogram and UV absorption spectrum of compound separated on TLC at start, middle and end position of the band.

Characterization by UV, IR, LCMS and NMR

The sample along with the standard of swertiamarin was spotted and developed in a solvent system containing ethyl acetate: methanol: water (7.7: 1.5: 0.5). The UV absorption spectra were recorded on a CAMAG TLC Scanner. UV absorption spectrum of the isolated sample in methanol was recorded on UV/VIS spectrophotometer (ELICO). IR spectra was recorded on BUCK SCIENTIFIC IR Spectrophotometer (model 500). Atmospheric pressure ionisation with ion spray mass spectra of molecular ions were obtained on a PE SCIEX API 165 MS with Waters LCMS. NMR spectra was recorded in CDCl₃ and DMSO using BRUKER DRX FTNMR spectrometer.
4.2.5 Development of Sensitive HPTLC Method for the Estimation of Swertiamarin from *Enicostemma littorale* Blume, and Formulations Containing *E. littorale*

4.2.5.1 Preparation of standard solutions

A stock solution of swertiamarin (800 µg/ml) was prepared by dissolving an accurately weighed 8 mg of swertiamarin standard in 10 ml of methanol in a volumetric flask. Standard solutions of 32 µg/ml, 48 µg/ml, 64 µg/ml, 80 µg/ml, 96 µg/ml, 112 µg/ml were prepared by transferring aliquots (0.4 - 1.4 ml) of stock solution to a 10 ml of volumetric flask adjusting the volume to 10 ml with methanol.

4.2.5.2 Preparation of sample solutions

An accurately weighed 0.1 g of powder of the whole herb of *E. littorale* was extracted with methanol (4 x 25 ml) under reflux (10 min. each time) in a water bath. The extracts were filtered concentrated to 50 ml volumetric flasks, from that 1.25 ml of the solution was diluted to 5 ml in volumetric flask. The extracts were filtered and concentrated to 50 ml volumetric flasks.

4.2.5.3 Calibration curve for swertiamarin

10 µl of each of the standard solutions (320 ng, 480 ng, 640 ng, 800 ng, 960 ng, 1120 ng per respective spot) were applied (band width: 5 mm, distance between the bands: 5 mm) on a precoated silica gel 60 F254 TLC plate (E. Merck, Cat. No. 1.05554.0001) (0.2 mm thick), 10 mm from the bottom edge, using a CAMAG Linomat IV Automatic Sample Spotter. The plate was developed in a solvent system (9.7 ml) of ethyl acetate-methanol-water (7.7: 1.5: 0.5, v/v) in a CAMAG glass twin–through chamber (10 x 10 cm) previously saturated with the solvent for 30 min (temperature 25±2°C, relative humidity 40%). The development distance was 8 cm. After removing the plate from the chamber, it was dried in air and scanned and quantified at 240 nm using a CAMAG TLC Scanner 3 and Cats 4 software. Data of peak area were recorded. A calibration curve was obtained by plotting peak area vs concentration of swertiamarin applied.
4.2.5.4 Validation

The method was validated for the precision, repeatability and accuracy. Precision of the instrument was checked by repeated scanning of the same spot of *E. littorale* (concentration 320 ng) seven times and the coefficient of variation (CV) was calculated. The repeatability of the method was tested by analysing 320 ng / spot of standard solution of swertiamarin after application on TLC plate (n = 5) and % CV was calculated. Variability of the method was studied by analyzing aliquots of different concentrations of standard solutions of swertiamarin (320 ng, and 640 ng) on same day (intra-day precision) and on different days (inter-day precision) and relative standard deviation (RSD) was calculated.

Accuracy of the method was tested by performing the recovery studies at two levels by addition of 50 % and 100 % of swertiamarin to one of the sample powders. To 0.1 g of powder (containing 7.7 mg of swertiamarin), known amounts of standard swertiamarin were added (7.7 and 3.85 mg) and extracted and estimated as described in section 2.6. The percentage recovery as well as the average percentage recovery were calculated.

4.2.5.5 Estimation of swertiamarin from *E. littorale*, and formulations containing *E. littorale*

A 5, and 10 µl of sample solutions of *E. littorale*, and formulations respectively were applied in triplicate on precoated silica gel 60 F\textsubscript{254} HPTLC plate (E. Merck) with the CAMAG Linomat IV Automatic Sample Spotter. The plate was developed and scanned as described in section 4.2.5.1 The peak areas were recorded. The amount of swertiamarin present in the samples was calculated using calibration curve for swertiamarin.

4.2.5.6 Estimation of swertiamarin from ethyl acetate and *n*-butanol fractions of aqueous extract of *E. littorale*.

10 µl of sample solutions of ethyl acetate and *n*-butanol fraction were applied in triplicate on precoated silica gel 60 F\textsubscript{254} HPTLC plate (E. Merck) with the CAMAG Linomat IV Automatic Sample Spotter. The plate was developed and scanned as described in section 4.2.5.3 The peak areas were recorded. The amount of swertiamarin present in the samples was calculated using calibration curve for swertiamarin.
4.3 Pharmacological evaluation

4.3.1 Study of Anti-diabetic Activity of *E. littorale*

4.3.1.1 Induction of IDDM

Male Sprague Dawley rats weighing 200-250 g were used for the study. The animals were housed in a group of 3 rats per cage under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12h/12h light-dark cycle. Animals had free access to conventional laboratory diet and tap water *ad libitum*. Diabetes was induced with streptozotocin (STZ) (Sigma Ltd., USA) 40mg/kg dissolved in 0.9% NaCl, administered as a single intravenous (i.v.) tail-vein injection under light ether anesthesia. Control animals were injected with an equivalent volume of 0.9% NaCl. Animals were checked for the extent of glucosuria 48h after the injection of STZ using *Diastix* (Bayer Diagnostics, India). Animals showing glucosuria (>2%) were considered as diabetic. Control rats were randomly divided into two groups, namely control and control treated with test drug. Similarly, diabetic rats were divided in two groups, namely IDDM control and IDDM treated with test drug.

4.3.1.2 Induction of NIDDM

Sprague Dawely rats from an inbred colony were bred under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12h/12h light-dark cycle. Conventional laboratory diet and tap water were provided *ad libitum*. Two-day-old male SD neonates were injected intraperitoneally (i.p.) with 90 mg/kg STZ (Sigma Ltd., USA) in 0.9% sodium chloride solution. Control neonates received equivalent amount of isotonic saline alone. The neonates were left with their own mothers and weaned at four weeks of age. Twelve weeks after the injection of STZ, animals were checked for fasting glucose levels. The animals showing fasting glucose levels >140 mg/dl were considered as diabetic. Control rats were randomly divided into two groups, namely control and control treated with test drug. Similarly, diabetic rats were divided in two groups, namely NIDDM control and NIDDM treated with test drug.

4.3.1.3 Treatment protocol for diabetic rats

The experimental animals were divided into four groups, six animals in each group (1) control, (2) control treated, (3) diabetic control, (4) diabetic treated with *E.*
Materials and Methods

_littorale_ extracts. Treatment was given daily for three weeks in set A and set B and two weeks in set C.

The fourth group was subdivided into groups as follows:

- **Group A**
  - Diabetic treated with aqueous extract (0.5g/kg, p.o.)
  - Diabetic treated with aqueous extract (1g/kg, p.o.)
  - Diabetic treated with aqueous extract (2g/kg, p.o.)

- **Group B**
  - Diabetic treated with toluene fraction (0.5g/kg, p.o.)
  - Diabetic treated with chloroform fraction (0.5g/kg, p.o.)
  - Diabetic treated with ethyl acetate fraction (0.1, 0.5g/kg, p.o.)
  - Diabetic treated with _n_-butanol fraction (0.1, 0.5g/kg, p.o.)
  - Diabetic treated with residual fraction (0.5g/kg, p.o.)

- **Group C**
  - Diabetic treated with aqueous extract (2g/kg, p.o.)
  - Diabetic treated with swertiamarin (50mg/kg, i.p.) (Bhattacharya1976)

The studies for different sets were carried at different times. Each time separate control groups were used. During the study standard food and water were provided _ad libitum_. Changes in body weight, food intake and water intake were recorded.

**4.3.1.4 Blood sample collection and analysis**

Blood samples were collected in clean dry centrifuge tubes at the end of three weeks of treatment in group A and B and two weeks of treatment in group C, after 8h fast by nicking the tip of tail under light ether anesthesia and were allowed to clot for 30min at room temperature. Serum was separated by centrifugation at 3000rpm for 25min and stored at −20°C until the analysis was carried out. Serum samples were analyzed for glucose, cholesterol, triglyceride, creatinine, urea, serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) for group A, B and C while HDL-C was also estimated in group C using diagnostic kits (Bayer Diagnostics, India) colorimetrically using UV-Visible spectrophotometer (Shimadzu UV-1601, Japan). Serum insulin was estimated by radioimmunoassay technique using kits obtained from Board of Radiation and Isotope Technology, Mumbai in a five well gamma counter (Riastar, Packard, USA). VLDL and LDL was calculated as per Friedeavald’s equation.
4.3.1.5 Oral glucose tolerance test (OGTT)

At the end of three weeks of treatment, oral glucose tolerance test was performed after an overnight fast (Olefsky 1981). The animals were orally administered with 1.5 g/kg of glucose and blood samples were collected from the tail vein under light ether anesthesia before i.e. 0 min and 15, 30, 60 and 120 min after oral glucose administration. Samples were allowed to clot for 30min at room temperature. Serum was separated by centrifugation at 3000 rpm for 25 min and analyzed for glucose and insulin as explained earlier. Plotting the glucose or insulin concentration versus time gives a curve showing rise and fall in glucose and insulin levels with time after an oral glucose load. Comparison of such curves gives only a vague idea about alterations in insulin-mediated glucose disposal and insulin release in response to oral glucose load. Therefore, results were expressed as integrated area under the curve (AUC) for glucose and insulin which was calculated by after trapezoid rule \[ \text{AUC} = (C_1 + C_2)/2 \times (t_2 - t_1) \] and changes in glucose and insulin concentrations over 120 min during OGTT were expressed as AUCg (mg/dl.120min) and AUCi (µU/ml.120min) respectively.

4.3.1.6 Insulin tolerance test

Insulin tolerance test (Alford et al., 1971) is used to access peripheral insulin resistance. This test measures insulin sensitivity using \( K_{\text{ITT}} \) as an index of insulin-mediated glucose metabolism. The animals were fasted overnight and 0.2U/100g bodyweight of purified porcine insulin (Actrapid, Novo Nordisk Pharma India Ltd.) was injected by slow intravenous injection through tail vein. Neutral insulin injection was diluted with 0.9% saline to get the final concentration of 0.2U/0.1ml. Blood samples were collected from the tail vein before i.e. 0 min and 5, 10, 20 and 30 min after insulin administration under light ether anesthesia. Serum was separated as described earlier and analyzed for glucose. The KITT, an index of insulin-mediated glucose metabolism, was determined from the slope of a linear portion of the
regression line of natural log of glucose versus time (Alford et al., 1971) and calculated using the formula given by Lundbaek (1962):

$$K_{ITT} = \frac{0.693}{t_{1/2}} \times 100$$

where $t_{1/2}$ represents the half life of plasma glucose decay, obtained by plotting plasma glucose concentration versus time on semilogarithmic graph paper.
4.3.2 Study of Anti-atherosclerotic Activity of *E. littorale*

4.3.2.1 Hyperlipidemic model

Sprague Dawely rats weighing 200-225 g were used for the study. The animals were housed in a group of 3 rats per cage under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12h/12h light-dark cycle. To induce hyperlipidemia in rats cholesterol rich diet (2% cholesterol, 1% sodium cholate and 2% coconut oil) was administered for seven days. The experimental animals were divided into four groups, six animals in each group (1) control (2) cholesterol fed (3) cholesterol fed with aqueous extract of *E. littorale* extracts (2 g/kg p.o.) (4) cholesterol fed with atorvastatin (1.5 mg/kg p.o.) Treatment was given daily for one week orally. Blood was collected and serum samples were analyzed for cholesterol, triglyceride and HDL-C was estimated using diagnostic kits (Bayer Diagnostics, India) colorimetrically using UV-Visible spectrophotometer (Shimadzu UV-1601, Japan). VLDL and LDL was calculated as mention in section 4.3.1.4 while atherogenic index was calculated as per following formula. After blood collection, animals were sacrificed and liver was taken to estimate lipid peroxidation and antioxidant parameters.

\[
\text{Atherogenic index} = \frac{\text{Total serum triglycerides}}{\text{Total serum HDL-C}}
\]
Materials and Methods

4.3.2.2 In vivo assay for evaluation of antioxidant activity of E. littorale

a. Estimation of Free radicals

The Liver was homogenized in ice-cold tris-hydrochloride buffer (pH 7.2). Homogenate was centrifuged at 800g for 10 min followed by centrifugation of the supernatant at 12,000g for 15 min. The supernatant obtained was used for the following estimations.

b. Measurement of lipid peroxidation

Lipid peroxidation was estimated as per the method described by Ohkawa et al (1979). Supernatant (1.0 ml) was mixed with 0.2 ml of 4% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid in 0.27 M hydrochloric acid (pH 3.5) and 1.5 ml of 0.5% thiobarbituric acid (pH 7.4). The mixture was heated in a waterbath at 85 °C for 1 hour, cooled and centrifuged at 1200 g for 10 min. The absorbance was read at 532 nm. The amount of malinoaldehyde (MDA) (thiobarbituric acid reactive material) was calculated using molar extinction coefficient 1.56 \times 10^5 M^{-1} \cdot Cm^{-1} and reported as nmoles of MDA/mg protein.

c. Superoxide dismustase (SOD))

SOD was estimated as per the method described by Misra et al (1972). Supernatant (0.1 ml) of sample was mixed with 0.1 ml EDTA \((1 \times 10^{-4} \text{ M})\), 0.5 ml of carbonate buffer (pH 9.7) and 1 ml of Epinephrine \((3 \times 10^{-3} \text{ M})\). The optical density of formed adrenochrome was read at 480 nm for 3 min at an interval of 30 sec.

d. Catalase

Decomposition of H\(_2\)O\(_2\) in presence of catalase was estimated by Aeibi et al., 1974. A 50uM supernatent was added to buffered substrate (50 mM phosphate buffer, pH 7 containing 30 mM H\(_2\)O\(_2\)) to make total volume 3 ml. The decrease in the absorbance was read at 240 nm for 2.5 min at an interval of 15 sec. Results were expressed as mean absorbance of catalase activity.
e. GSH level (Reduced Glutathione)

Reduced GSH levels in tissue homogenates were estimated as per the method described by Beutler et al (1963). The supernatant (2 ml) was mixed with 10% chilled trichloroacetic acid. The mixture was kept in ice bath for 30 min and centrifuged at 1000g for 10 min at 4 °C. Supernatant (0.5 ml) was mixed with 2.0 ml 0.3 M disodium hydrogen phosphate and 0.25 ml 5, 5'-dithiobis-2-nitrobenzoic acid (40 mg/100ml in 1% sodium citrate) was added just before measuring the absorbance at 412 nm. Standard curve for GSH was prepared using glutathione. Results were expressed as µmole of GSH/g tissue.

4.3.3 In Vitro Assay for Evaluation of Antioxidant Activity of Aqueous Extract of E. littorale

Assay for antiradical activity with DPPH

Antiradical activity was measured by a decrease in absorbance at 516 nm of a methanolic solution of colored DPPH brought about by sample (Vani et al., 1997). A stock solution of DPPH (1.3 mg/ml in methanol) was prepared such that 75 µl of it in 3 ml methanol gave initial absorbance of 0.9. Decrease in the absorbance in the presence sample at different concentration was noted after 15 min. EC₅₀ was calculated from % inhibition. Ascorbic acid was used as a standard.
4.3.4 Hepatoprotective Activity of *E. littorale*

4.3.4.1 Induction of liver injury

Swiss albino mice weighing 20-25 g were used for the study. The animals were housed in a group of 6 mice per cage under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12h/12h light-dark cycle. Animals had free access to conventional laboratory diet and tap water *ad libitum*. Liver injury was induced with CCL₄ 0.8 ml/kg *i.p.* (30% solution in liquid paraffin) given for 7 days. Animals were checked for the extent of hepatic damage 7 days after the injection of CCL₄. The experimental animals were divided into four groups, six animals in each group: (1) control (2) CCl₄ control (3) CCL₄ with silymarin (4) CCL₄ treated with aqueous extract of *E. littorale* extracts. Treatment was given daily for seven days orally.

The fourth group was subdivided into three groups as follows:

1. CCL₄ treated with aqueous extract of *E. littorale* (0.25g/kg)
2. CCL₄ treated with aqueous extract (0.5 g/kg)
3. CCL₄ treated with aqueous extract (1 g/kg)

Blood was collected from all the groups and serum samples were analyzed for serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) alkaline phosphatase (ALP) total bilirubin, direct bilirubin, total protein (TP) and albumin (Alb) using diagnostic kits (Bayer Diagnostics, India) colorimetrically using UV-Visible spectrophotometer (Shimadzu UV-1601, Japan).

4.3.4.2 Pentobarbitone-induced sleeping time

In a group of mice, the sleeping time was measured using sodium pentobarbitone (35 mg/kg, i.p.). The sleeping time was calculated as the interval lapping between the loss and recovery of the righting reflux.
4.3.5 Details of Biochemical Parameters Used

Glucose (GOD/POD method)

Principle
Glucose is oxidized by the enzyme glucose oxidase (GOD) into gluconic acid and hydrogen peroxide. Hydrogen peroxide in the presence of enzyme peroxidase (POD) oxidizes the chromogen 4-aminoantipyrine/phenolic compound to a red coloured compound. The intensity of the colour produced is proportional to glucose concentration in the sample and is measured at 505nm. This final colour is stable for two hours.

\[
\text{GOD} \quad \text{Glucose + O}_2 \quad \rightarrow \quad \text{gluconic acid + H}_2\text{O}_2
\]

\[
\text{POD} \quad \text{H}_2\text{O}_2 + \text{Phenolic} + 4\text{-amino} \quad \rightarrow \quad \text{Red compound + 2H}_2\text{O}
\]

Preparation of working solution
One buffer/enzyme/chromogen tablet was gently dissolved in 20 ml of distilled water in a clean beaker, with continuous stirring.

Procedure
One ml of the working solution was added to test tube containing 10 μl of serum sample. Similarly standard and blank were prepared by using 10 μl of glucose standard (provided in the kit) and distilled water respectively. They were then mixed and incubated at room temperature for 30 min (end point reaction). Absorbance of test and standard was measured against blank at 505nm using UV-Visible spectrophotometer (UV-1601 Shimadzu, Japan).

The concentration of glucose in test samples was calculated using following formula.

\[
\text{Serum Glucose (mg/dl)} = \frac{\text{O.D. of test}}{\text{O.D. of std}} \times 100
\]
**Materials and Methods**

**Insulin (Radioimmunoassay method)**

*Principle*

The assay is based upon the competition between unlabeled insulin in the standard samples and radioiodinated ($^{125}$I) insulin for the limited binding sites on a specific antibody. At the end of incubation, the antibody bound and free insulin are separated by the second antibody polyethylene glycol (PEG) separation method. Insulin concentration of samples is quantitated by measuring the radioactivity associated with the bound fraction of sample and standards. The amount of radiolabeled insulin bound to the antibody is inversely proportional to the amount of insulin in the serum. A standard curve with the known amounts of test substances can thus be constructed and the amount of insulin in the unknown samples can be calculated.

*Procedure*

1. All reagents were brought to room temperature before use and reconstituted as described in the leaflet supplied along with the kit.
2. Tubes were arranged and labeled as total, blank, standards, insulin controls and unknowns.
3. 0.3 ml of assay buffer was added to zero standard tube while 0.2 ml of assay buffer was added to insulin standard tubes and 0.4 ml of assay buffer was added to the blank tube. Assay buffer was not added to the total count tubes.
4. 100 μl of insulin standards (7.5 to 200 μU/ml) and unknown samples (serum samples) were added to the appropriate tubes.
5. 100 μl insulin free serum was added to blank, zero standard and insulin standard tubes.
6. 100 μl of anti-insulin serum was added to all the tubes except total and blank tubes.
7. The contents of the tubes were mixed gently and refrigerated at 2°C to 4°C overnight.
8. 100 μl of $^{125}$I-insulin reagent was added to all the tubes.
9. Contents of the tubes were mixed gently and incubated at room temperature for 3h.
10. 100 μl second antibody was added to all tubes except total count tubes.
11. One ml of precipitating reagent (PEG) was added to all tubes except total count tubes.
12. All tubes were vortexed and incubate at room temperature for 20 min.
13. Later the tubes except the total count tubes were centrifuged at 4500 rpm for 20 min.
14. After centrifugation the tubes were decanted and radioactivity in the precipitate was measured using gamma counter (Riastar, Packard, USA)

Calculations
1. Background counts were subtracted from all the counts to get actual counts.
2. All the duplicates were averaged.
3. Blank count was subtracted from all the other counts to give corrected average counts.
4. 
   \[
   \% \text{ B/Bo} = \frac{\text{Corrected counts of sample/ standard}}{\text{Corrected count of zero standard}} \times 100
   \]
5. The standard curve of % B/Bo on the logit was plotted against μl/ml of insulin on the logarithmic scale using logit-log graph paper.
6. The concentration of insulin in sample was read from the standard curve by extrapolation.

Cholesterol

Principle

Cholesterol Ester + O₂ \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids}.

Cholesterol + O₂ \xrightarrow{\text{Cholesterol oxidase}} \text{Dehydroacetone phosphate} + \text{H₂O₂}

2H₂O₂ + Phenol + 4-aminoantipyrine \xrightarrow{\text{peroxidase}} \text{Red quinone} + 4H₂O

The intensity of the red complex (red quinone) formed during the reaction is directly proportional to the cholesterol concentration in the sample and is measured at 500nm.
Procedure

Reagents are reconstituted as described in the leaflet supplied along with the kit. 10 μl of serum samples, distilled water serving as control and standard triglyceride (200 mg/dl) serving as standard were mixed well with 1.0 ml reconstituted reagent i.e. enzyme/chromogen mixture. They were incubated at 37°C for min and absorbance was read against blank at 500nm.

Calculations

\[
\text{Serum cholesterol (mg/dl)} = \frac{\text{O.D. of test}}{\text{O.D. of std}} \times 200
\]

Triglyceride

Principle

Triglycerides are enzymatically hydrolysed to glycerol according to the following reactions

\[
\text{Triglycerides} + \text{H}_2\text{O} \xrightarrow{\text{lipoprotein lipase}} \text{Glycerol} + \text{free fatty acids}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{Glycerol-3-Phosphate} + \text{ADP}
\]

\[
\text{Glycerol-3-Phosphate} + \text{O}_2 \xrightarrow{\text{GPO}} \text{Dehydroacetone phosphate} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{4-aminoantipyrine} + \text{ADPS} \xrightarrow{\text{peroxidase}} \text{Red quinone} + 4\text{H}_2\text{O}
\]

GPO = Glycerol-3-Phosphate Oxidase
ADPS = N-Ethyl-N-Sulphopropyl-n-anisidine

The intensity of the red complex (red quinone) complex formed during the reaction is directly proportional to the triglyceride concentration in the sample and is measured at 546nm. The final colour is stable for at least 30 min.
Materials and Methods

Procedure

Reagents are reconstituted as described in the leaflet supplied along with the kit. 10 µl of serum samples, distilled water serving as control and standard triglyceride (200 mg/dl) serving as standard were mixed well with 1.0 ml reconstituted reagent 1 i.e. enzyme/chromogen mixture. They were incubated at 37°C for min and absorbance was read against blank at 546nm.

Calculations

Serum triglyceride (mg/dl) = \( \frac{O.D. \text{ of test}}{O.D. \text{ of std}} \times 200 \)

HDL-Cholesterol

Principle

Chylomicrons, VLDL, and LDL fractions in serum or plasma are separated from HDL by precipitating with phosphotungstic acid and magnesium chloride. After centrifugation, the cholesterol in HDL fraction, which remains in the supernatant is assayed with enzymatic cholesterol method, using cholesterol esterase, cholesterol oxidase, peroxidase and the chromogen 4-amino antipyrine/phenol.

Procedure

Reagents are reconstituted as described in the leaflet supplied along with the kit. 0.2 ml of serum sample was mixed well with 0.2 ml of precipitating reagent (Reagent 2) and centrifuged at 3500-4000 for 10 min. Supernatant 20 µl and 1 ml of reconstituted reagent 1 was added. In case on blank 1 ml reconstituted reagent 1 was taken. Absorbance of test samples was measured against reagent blank at 500nm.

Calculations

Serum HDL-C (mg/dl) = \( \frac{O.D. \text{ of test}}{O.D. \text{ of std}} \times 50 \)

Creatinine

Principle

Creatinine in a protein free solution reacts with alkaline picrate and produces a red coloured complex, which is measured colorimetrically at 520nm.
Materials and Methods

Procedure
Deproteinization of test sample

0.5 ml of serum sample was mixed well with 0.5 ml distilled water and 3 ml picric acid (Reagent 1). It was kept in boiling water bath exactly for one minute and cooled immediately under running tap water and centrifuged.

2.0 ml of the supernatant from the above step is mixed with 1.0 ml sodium hydroxide solution (Reagent 2). 0.5 ml of distilled water and working creatinine standard mixed with 1.5 ml picric acid and 0.5 ml sodium hydroxide solution served as blank and standard respectively. All the tubes were allowed to stand at room temperature after thorough mixing for 20 min. The absorbance of blank, standard and samples were measured immediately against distilled water at 520nm.

Calculations
Serum creatinine concentration was calculated using following formula

\[
\text{Serum Creatinine (mg/dl)} = \frac{\text{O.D. test - O.D. blank}}{\text{O.D. std - O.D. blank}}
\]

Urea
Principle

The test is based on the Berthelot’s reaction. Urease splits urea into ammonia and carbon dioxide. The ammonia reacts with phenol in the presence of hypochlorite to form an indophenol which with alkali gives a blue coloured compound. The intensity of the colour is proportional to the concentration of urea in the sample and is measured at 546 nm. The colour of the reaction is stable for 8h.

Procedure

Reagents are reconstituted as described in the leaflet supplied along with the kit. 10 μl of serum sample, urea standard (40 mg/dl) and distilled water as blank were mixed with 100 μl of urease solution (Reagent 1). Contents are mixed and incubated at 37°C for 10 min. 1.5 ml of phenol (Reagent 2) and sodium hypochlorite solution (Reagent 3) were added to all test tubes and mixed well. The absorbance was read at
Materials and Methods

546nm UV-Visible Spectrophotometer (UV-1601 Shimadzu, Japan). The final colour developed is stable for at least 30 min.

The concentration of urea was calculated by using following formula

\[
\text{Serum Urea (mg/dl)} = \frac{\text{O.D. of test}}{\text{O.D. of std}} \times 40
\]

**Serum Glutamate Pyruvate Transaminase (SGPT)**

*Principle*

SGPT catalyses transfer of amino group from L-alanine to α-ketoglutarate with formation of pyruvate and glutamate. The pyruvate so formed, is allowed to react with 2,4 DNPH to produce 2,4-dinitrophenylhydrazone derivative which is brown coloured in alkaline medium. The absorbance of this hydrazone derivative is correlated to SGPT activity by plotting a calibration curve using pyruvate standard.

\[
\text{L-alanine} + \text{SGPT} + \alpha\text{-ketoglutarate} \rightarrow \text{Pyruvate} + \text{L-glutamate}
\]

\[
\text{Pyruvate} + \text{2,4-DNPH} \rightarrow \text{2,4-dinitrophenyl hydrazone (Brown coloured)}
\]

*Procedure*

**Calibration Curve**

In five clean test tubes buffered substrate, pyruvate standard distilled water and DNPH colour reagent were added as per mentioned in the leaflet supplied with the kit. Contents were mixed well and incubated at room temperature for 20 min. 5 ml of working sodium hydroxide was added to all the tubes, mixed and allowed to stand at room temperature for 10 min. Absorbance of tubes 2 to 5 was measured against tube 1 as reagent blank at 505nm. A graph of absorbance was plotted against enzyme activity (supplied in leaflet).

**Assay**

0.5ml buffered substrate was incubated at 37°C for 3 min. 0.1 ml serum sample was added to buffered substrate and incubated at 37°C for 60 min. To this DNPH colour reagent was added and allowed to stand at room temperature for 20
min. Finally 5.0 ml working sodium hydroxide solution was added to all the tubes. After mixing well the tubes were allowed to stand at room temperature for 10 min. In case on blank similar procedure was followed except that instead of serum 0.1 ml distilled water was added. Absorbance of test samples was measured against reagent blank at 505 nm and was read on calibration curve to find out enzyme activity.

Serum glutamate oxaloacetate transaminase (SGOT)

Principle

SGOT catalyses transfer of amino group from L-aspartate to $\alpha$-ketoglutarate with formation of oxaloacetate and glutamate. The oxaloacetate so formed, is allowed to react with 2,4 DNPH to produce 2,4-dinitrophenylhydrazone derivative which is brown coloured in alkaline medium. The absorbance of this hydrazone derivative is correlated to SGOT activity by plotting a calibration curve using pyruvate standard.

\[
\begin{align*}
\text{L-aspartate} & \quad + & \quad \text{SGOT} & \quad \rightarrow & \quad \text{Oxaloacetate} \\
\text{\(\alpha\)-ketoglutarate} & \quad \text{pH 7.4} & \quad \rightarrow & \quad \text{L-glutamate} \\
\text{Oxaloacetate} & \quad \text{Alkaline} & \quad \rightarrow & \quad 2,4\text{-dinitrophenyl} \\
\text{2,4-DNPH} & \quad \text{medium} & \quad \rightarrow & \quad \text{hydrazone (Brown coloured)}
\end{align*}
\]

Procedure

Calibration Curve

In five clean test tubes buffered substrate, pyruvate standard distilled water and DNPH colour reagent were added as per mentioned in the leaflet supplied in with the kit. Contents were mixed well and incubated at room temperature for 20 min. 5 ml of working sodium hydroxide was added to all the tubes, mixed and allowed to stand at room temperature for 10 min. Absorbance of tubes 2 to 5 was measured against tube 1 as reagent blank at 505nm. A graph of absorbance was plotted against enzyme activity (supplied in leaflet).

Assay

0.5 ml buffered substrate was incubated at 37°C for 3min. 0.1 ml serum sample was added to buffered substrate and incubated at 37°C for 60 min. To this DNPH colour reagent was added and allowed to stand at room temperature for 20
Materials and Methods

min. Finally 5.0 ml working sodium hydroxide solution was added to all the tubes. After mixing well the tubes were allowed to stand at room temperature for 10 min. In case on blank similar procedure was followed except that instead of serum 0.1 ml distilled water was added. Absorbance of test samples was measured against reagent blank at 505nm and was read on calibration curve to find out enzyme activity.

**Alkaline phosphatase (ALP)**

**Principle**

Alkaline Phosphatase from serum converts Phenyl Phosphate to inorganic phosphate and Phenol at pH 10.0. Phenol so formed reacts in alkaline medium with 4-aminoantipyrine in presence of the oxidising agent potassium ferricyanide and forms an orange-red coloured complex, which can be measured colorimetrically. The color intensity is proportional to the enzyme activity.

The reaction can be represented as:

\[
\text{Alk. Phosphatase} \quad \text{Phenyl Phosphate} \quad \xrightarrow{\text{pH 10.0}} \quad \text{Phenol} + \text{Pi}
\]

\[
\text{Pot. Ferricyanide} \quad \text{Phenol} + 4\text{-Aminoantipyrine} \quad \rightarrow \quad \text{Orange-red colored complex}
\]

**Procedure**

Reagents are reconstituted as described in the leaflet supplied along with the kit. 100 µl of serum samples, distilled water serving as control and standard triglyceride (10 mg %) serving as standard were mixed well with 3.0 ml reconstituted reagent i.e. enzyme/chromogen mixture. They were incubated at 37°C for min and absorbance was read against blank at 510nm.

**Bilirubin**

**Principle**

Direct: (Conjugated) Bilirubin couples with diazotized Sulfanilic acid, forming Azobilirubin, a red-purple colored product in acidic medium.
**Indirect: (Uncoujugated) Bilirubin** is diazotized only in the presence of its dissolving solvent (methanol). Thus the red-purple colored Azobilirubin produced in presence of methanol originates from both direct and indirect fractions and thus represents Total Bilirubin concentration. The difference of total and Direct Bilirubin gives Indirect Bilirubin.

The intensity of red-purple color so developed above is measured colorimetrically and it is proportional to the concentration of the appropriate fraction of Bilirubin.

This reaction can be represented as:

\[
\text{H}^+ \quad \text{Bilirubin + Diazotized Sulfanilic Acid} \quad \overset{\rightarrow}{\text{Azobilirubin}} \quad \text{H}^+ \quad \text{Red-purple color}
\]

**Procedure**

Reagents are reconstituted as described in the leaflet supplied along with the kit. 50 µl of serum samples, distilled water serving as control and standard bilirubin (10 mg %) serving as standard were mixed with distilled water, diazo reagent and methanol. Mix well and measure the optical density at 540 nm against distilled water as blank.

**Total proteins and albumin**

**Principle**

A. **Total Proteins:** Proteins in serum react with copper of Biuret Reagent in alkaline medium to form a blue purple complex with absorption maximum at 550 nm.

B. **Albumin:** Albumin in serum binds with the dye Bromocresol green at pH 3.68 to form a green colored complex the absorbance of which is measured at 600 nm.
Materials and Methods

Procedure

2.5 ml of Biuret reagent was mixed with 50μl serum sample/standard and incubated at 37°C for 5 min. Absorbance of test samples was measured against reagent blank at 550 nm.

The concentration of total protein was calculated by using the following formula:

\[
\text{Con. of Total protein} = \frac{\text{O.D. test}}{\text{O.D. std}} \times \text{Conc. of std protein. (g/100 ml)}
\]

Procedure

3 ml of Buffered Dye reagent mix with 20μl serum sample/standard and incubated at 37°C for 1 min. Absorbance of test samples was measured against reagent blank at 600 nm.

\[
\text{Con. of serum albumin} = \frac{\text{O.D. test}}{\text{O.D. std}} \times \text{Conc. of std albumin. (g/100 ml)}
\]

Estimation of Protein

100 mg of liver tissue was weighed and homogenised in 5 ml of distilled water. 0.2 ml of homogenate was added to 4 ml of solution C (Solution A: 2 g of sodium hydroxide, 10 g of sodium carbonate, 0.1 g of sodium potassium tartarate in 500 ml of distilled water, Solution B: 0.5 g of cuprous sulphate in 100 ml of distilled water, Solution C: 10 ml of solution A and 0.2 ml of Solution B) and 0.6 ml of distilled water was added and allowed to stand for 15 min at 37°C. Folin-phenol reagent 0.4 ml was added and incubated at 30 min absorbance was read at 540 nm.

Amount of protein in 100 mg of tissue was calculated as

\[
\text{Protein (mg/100 mg tissue)} = \frac{X \times \text{Dilution factor (5)} \times 100}{\text{Aliquot volume (2 ml)} \times \text{Tissue wt} \times 100}
\]

Where \( X = 8070351 \times (Y - 74.80) \) and \( Y \) is optical density.
4.3.6 Histopathological study

Histopathological study of kidney and liver was carried out to study the effects of chronic treatment with aqueous extract and its fraction i.e. ethyl acetate and butanol on degenerative changes induced by diabetes and also to assess the nephrotoxic and hepatotoxic potential of these heavy metals under the conditions of the present investigation.

Fixation of the tissues

Dissected kidney and liver were washed with normal saline and then kept in 10% formal saline. The tissues were then kept in Bouin’s fixative for 18 to 24h. Tissues were then washed twice with distilled water and kept in 70% alcohol. A pinch of lithium carbonate was added to remove excessive stain. The tissues were washed and kept in 70% alcohol again. After that tissues were transferred to 90% alcohol and kept in it overnight. Next morning all tissues were transferred into 100% alcohol and kept for 3h. Then tissues were transferred to xylene and kept till they become transparent.

Microtomy

Tissues were fixed in melted paraffin in wooden blocks, so that sectioning can be performed. Several sections of 3 µm thickness were taken from each tissue and sections with uniform shape and size were selected for histology. Selected sections were fixed on the clear glass slide with the help of egg albumin.
Staining

Tissues were stained using Hematoxylin and Eosin (H & E) stain.

```
[Materials and Methods]
Staining
Tissues were stained using Hematoxylin and Eosin (H & E) stain.

Slides ---------►  Xylene ------*400% alcohol
20 min 2-3 dips 90% alcohol
1-2 min

Distilled Water
30% alcohol
50% alcohol
70% alcohol
2-5 min

Heamatoxyllin stain
1% acid
2-4 drops

Ammonia water

Running tap water alcohol 2-4 drops

Acetone
Eosin stain
Running tap water
2-3 min
2 min

Distilled water

Dry
Xylene
DPX mounting

[Diagram]
```
4.3.7 Estimation of Glucose Transporters in Cardiac Membrane

I. Membrane and cytosol preparation

Immediately after the weights of separated hearts were recorded the tissue was frozen in liquid nitrogen (-20°C). Frozen cardiac tissue (approx. 700 mg) was homogenized in a solution containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 mM ethylenediamine tetra acetic acid (EDTA) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) using polytron at 12000 rpm. This homogenate was centrifuged at 1000 g (Beckman, USA, rotor JA-20) at 0°C. Supernatant was subjected again to centrifugation at 10,000 g at 0°C. Supernatant from this was finally subjected to centrifugation at 40,000 g at 0°C. Pellets obtained from this final centrifugation are rich in crude membrane fraction and the supernatant are rich in cytosol. Pellets were resuspended in a solution containing 10 mM histidine and 250 mM Sucrose (pH 7.0). Protein estimation for the suspension was done using Lowry’s method and equal amount of protein (2mg/ml) was loaded by diluting with Laemmli buffer for the gel electrophoresis.

II. Western blotting

Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% resolving gel. Each sample was run in duplicate. Gels were run at 200 V ~ 45 min. Prestained molecular weight standards (Sigma, USA) were run in one corner well, whereas Laemmli buffer was added to another corner well to ensure the evenness of the electrophoresis. Electrophoretic transfer of proteins was done to PVDF membranes (Amersham, USA) using Bio-Rad assembly at 100 V~2 hr. Then the membranes were incubated in (Tris)-buffered saline containing 50 mM Tris and 750 mM NaCl (pH 7.4) with 5% non fat dry milk for 1 hr at room temperature. The membranes were washed with TBS, added with GLUT 1(antirabbit) or GLUT 4 (antigoat) antibody (Santa Cruz Biotechnology Inc, USA) in 1:750 dilution in TBS containing 5% milk. These were incubated overnight at 4°C. Membranes were then washed with TBS containing 0.1%Tween 20 (TBST) the next day. Appropriate second antibody (Santa Cruz Biotechnology Inc, USA) was added in 1: 3000 dilution in TBS with 5% milk and incubated for 1 hr at room temperature, washed again with TBST and then finally the membranes were incubated with anti-
rabbit horseradish-peroxidase labeled antibody for 30 min at room temperature. After washings with TBST the membranes were dipped in chemiluminescence solution (Amersham, USA) for 1 min and then immediately exposed on the Kodak X-ray film for duration of 1-2 min. The relative levels of proteins were quantified by densitometry using Camag 3 Cats software.

III. Western blotting

Stock solutions:

A) 1M Tris-HCl (pH 6.8): 12.1g Tris base dissolve in 60ml double distilled water (DDW), adjust pH to 6.8 with 1N HCl. Make the volume to 100ml with DDW and store at 4°C.

B) 1.5M Tris-HCl (pH 8.8): 18.15g Tris base dissolve in 60ml DDW, adjust the pH to 8.8 with 1N HCl. Make the volume to 100ml with DDW and store at 4°C.

C) Laemmli buffer: 3.2% sodium dodecyl sulfate (SDS), 8% β-mercaptoethanol, 16.25% glycerol, 0.00188% bromophenol blue, 0.1M Tris-HCl, pH 6.8, store at room temperature.

D) 10% SDS: 10g SDS in 80ml DDW, stir gently and bring to 100ml with DDW, store at 4°C.

E) 10% ammonium persulfate (APS): 100mg APS + 1ml DDW (prepare fresh).

F) 30% Acrylamide mix (Filter and store at 4°C in the dark maximum for 30 days). Make up the volume with DDW.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Mol. Wt. (MW)</th>
<th>For 50ml</th>
<th>100ml</th>
<th>200ml</th>
<th>500ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide (g)</td>
<td>71.08</td>
<td>14.6</td>
<td>29.2</td>
<td>58.4</td>
<td>146.0</td>
</tr>
<tr>
<td>Bisacrilamide (g)</td>
<td>154.17</td>
<td>0.4</td>
<td>0.8</td>
<td>1.6</td>
<td>4.0</td>
</tr>
</tbody>
</table>

G) 5 x Tank buffer

<table>
<thead>
<tr>
<th>Reagents</th>
<th>MW</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1L</td>
</tr>
<tr>
<td>Tris (g)</td>
<td>121.1</td>
<td>125mM</td>
<td>15.14</td>
</tr>
<tr>
<td>Glycine (g)</td>
<td>75.07</td>
<td>960mM</td>
<td>72.1</td>
</tr>
<tr>
<td>SDS (G)</td>
<td>288.38</td>
<td>0.5%</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Materials and Methods

H) 5 × TBS (Tris saline buffer) pH 7.4

<table>
<thead>
<tr>
<th>Reagents</th>
<th>MW</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1L</td>
</tr>
<tr>
<td>Tris (g)</td>
<td>121.1</td>
<td>50mM</td>
<td>6.05</td>
</tr>
<tr>
<td>NaCl (g)</td>
<td>58.44</td>
<td>750mM</td>
<td>43.83</td>
</tr>
</tbody>
</table>

SDS-PAGE gel solutions:

a) For preparation of 5% stacking (upper) gels.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>For 5ml gel</td>
</tr>
<tr>
<td>DDW</td>
<td>3.4</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>0.83</td>
</tr>
<tr>
<td>1M Tris (pH 6.8)</td>
<td>0.63</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005</td>
</tr>
</tbody>
</table>

10% APS needs to be prepared freshly. TEMED is added immediately before making the gel.

b) For resolving (lower) gels:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>For 15ml gel</td>
</tr>
<tr>
<td>8%</td>
<td></td>
</tr>
<tr>
<td>DDW</td>
<td>6.9</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>4.0</td>
</tr>
<tr>
<td>1.5M Tris (pH 8.8)</td>
<td>3.8</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.15</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>0.15</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.009</td>
</tr>
<tr>
<td>10%</td>
<td></td>
</tr>
<tr>
<td>DDW</td>
<td>5.9</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Materials and Methods

1. Preparation of samples
   a) Determine protein concentration in the sample and dilute it to 2mg/ml using Laemmli buffer.
   b) Take about 50 μl of sample and add equivalent amount of Laemmli buffer to it in a 1.5ml Eppendorf tube and place it in boiling water bath for 5 min (for protein solubilization).

2. SDS-PAGE gel electrophoresis
   a) Set the gel sandwich: two glass plates, two spacers, and acrylic pressure plate.

Transfer buffer (From SDS-PAGE to PVDF membrane):

<table>
<thead>
<tr>
<th>Reagents</th>
<th>1L</th>
<th>2L</th>
<th>3L</th>
</tr>
</thead>
<tbody>
<tr>
<td>25mM Tris (g)</td>
<td>3.03</td>
<td>6.06</td>
<td>9.08</td>
</tr>
<tr>
<td>192mM Glycine (g)</td>
<td>14.42</td>
<td>28.84</td>
<td>43.26</td>
</tr>
<tr>
<td>Methanol (ml)</td>
<td>200.0</td>
<td>400.0</td>
<td>600.0</td>
</tr>
</tbody>
</table>

4. Blocking buffer:
   5g non-fat powdered milk + 100ml TBS.

5. Washing solutions (TBST):
   TBS buffer + 0.1% Tween 20.

Procedure:

1. Preparation of samples
   a) Determine protein concentration in the sample and dilute it to 2mg/ml using Laemmli buffer.
   b) Take about 50 μl of sample and add equivalent amount of Laemmli buffer to it in a 1.5ml Eppendorf tube and place it in boiling water bath for 5 min (for protein solubilization).

2. SDS-PAGE gel electrophoresis
   a) Set the gel sandwich: two glass plates, two spacers, and acrylic pressure plate.
b) Put the gel sandwich on the casting stand with thin layer of parafilm underneath.

c) Prepare the resolving gel mixture as mentioned earlier depending on the molecular weight of the protein to be identified.

d) Fill the gel sandwich with the resolving gel solution and immediately overlay a thin layer of DDW.

e) After the gel is set, pour out the overlaid DDW and try it gently with filter paper.

f) Place the comb in the gel sandwich and keep it on one side of the sandwich.

g) Prepare stacking gel solution and immediately place in the sandwich carefully from one corner of the sandwich without trapping air bubbles and move the comb immediately to the center of the sandwich.

h) After this gel is set remove the comb and attach the gel sandwich to the inner cooling core.

i) Wash the wells with tank buffer completely and load the samples in the wells using gel loader tips. For the marker, 5 µl is made up to the volume of samples loaded by adding Laemmli buffer. Any empty wells are loaded with Laemmli buffer too.

j) Put the inner cooling core into the tank and add the remainder of tank buffer to the chamber, put on the lid and run the gel at 200V for 45-60 min.

3. Transfer of proteins to polyvinylidifluoride (PVDF) membrane.

a) Prepare the transfer buffer during the period of electrophoresis and place the buffer in cold room.

b) Cut the membrane to the dimensions of the gel and label it with a pencil to identify the gel and the orientation of membrane. Soak the membrane in 100% methanol for 15 seconds and then into transfer buffer for at least 20 min at 4°C.

c) Once the electrophoresis is done, remove the separating gel from the stacking gel cut it in right bottom corner and rinse with transfer buffer in transfer vessel.

d) Soak the pre cut filter papers in transfer buffer and pack the filter paper, gel and membrane according the following order: cathode (-): filter papers: gel: membrane: filter papers: anode(+) inside the cassette. Before closing the cassette scratch the upper fiber pad so as to remove gently any entrapped bubbles inside.
e) Close the cassette and put it into holder.

f) Connect the power system and start the transfer at 1.5 mA for 0.5-1 hr.

4. Immunostaining

a) After the transfer is complete, remove the transfer membrane and cut the lower right corner of membrane to mark orientation of the gel.

b) Rinse the membrane in DDW and then block unoccupied protein binding sites on membrane by incubating the membrane with blocking buffer overnight at 4°C or 1-2 hr at room temperature.

c) Pour off the blocking buffer and incubate the membrane in primary antibody at appropriate dilution in TBST overnight at 4°C on a shaker.

d) Wash the membrane three times for 5min each with TBST.

e) Incubate the membrane with appropriate secondary antibody at appropriate dilution at room temperature for 1hr.

f) Wash the membrane three times for 5min each with TBST.

i) Dip the membrane into ECL solutions (ECL1: ECL2: DDW in 1:1:2 proportion) for 1min. Wrap the membrane in 2 layers of plastic sheet and put it in the cassette to expose protein bands.

j) The bands are visualized by developing the chemilluminigrams on hyperfilm ECL.

k) Determine protein content by an imaging densitometer model CAMAG 3 CATS software.

4.4 Statistical analysis

Results are presented as mean ± SEM. Statistical differences between the means of the various groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey test. Data were considered statistically significant at P value ≤ 0.05.