EXPERIMENTAL PROCEDURE

3.0 EXPERIMENTAL PROCEDURE

Diabetes has become one of the devastating disorders afflicting the health of many people in recent times and has accounted for a high proportion of health problems worldwide. It is now recognized as one of the leading causes of death in the developing countries. Oral hypoglycemic agents, especially the sulphonylureas and biguanides have been commonly employed in the management of type 2 diabetes. But none are ideal in the treatment due to the toxic side effects and some times diminution in responses after prolonged use. Therefore, there is a great need for a search for an acceptable, cheap and safe blood sugar lowering oral hypoglycemic agent that would be effective in the management of diabetes and devoid of serious side effects of the currently used oral hypoglycemic agents. Herbs and marine sources have been considered the best option. The use of herbs and natural product drugs from various plant sources is now of great interest in the management of diabetes mellitus. Several herbs have been reported in folk medicine to have been successfully employed in the management of diabetes and have shown effectiveness in non-insulin dependent diabetes (Steve *et al.*, 2008).

Helicteres isora L. *(Sterculiaceae)* is a medicinal plant, widely distributed from Southern China to India, South East Asia and Australia. The fruit and the bark of the plant are used for treating various diseases in particular diabetes. Timbers of this plant are used as antihelmintic agents against tapeworm and the fruits are used as colic, anticonvulsant and abdominalgia (Tezuka *et al.*, 1999). The scope of the present study includes phytochemical analysis and pharmacological evaluation of the fruit and the bark of the plant. The methodology adopted is as follows:

PHASE I

3.1 CHARACTERISATION OF THE SELECTED MEDICINAL PLANT TO BE USED AS A HYPOGLYCEMIC AGENT

3.1.1 Collection, identification and processing of the *Helicteres isora*

3.1.2 Characterisation of the medicinal plant *Helicteres isora* for selected biochemical parameters

3.1.3 Estimation of antioxidants in *Helicteres isora*

3.1.4 Free radical scavenging activity of the extracts of *Helicteres isora*

3.1.5 Ames test to assess the antimutagenic / anticarcinogenic effect of the plant extracts of *Helicteres isora*

3.1.6 Toxicological evaluation of the selected medicinal plant

PHASE II

3.2 DETERMINATION OF THE ANTIDIABETIC EFFECT OF THE FRUIT AND THE BARK OF *Helicteres isora* IN RATS

3.2.1 Assessment of blood glucose, liver glycogen, plasma insulin and serum fructosamine

3.2.2 Determination of the activities of selected key enzymes of carbohydrate metabolism

3.2.3 Assessment of lipid profile in rats

3.2.4 Assessment of oxidative stress in streptozotocin-induced diabetic rats

3.2.5 Total protein and A/G ratio of serum

3.2.6 Histopathological studies of rat liver, pancreas and kidney

3.2.7 Statistical analysis

PHASE III

3.3 IDENTIFICATION OF THE ACTIVE COMPONENTS IN THE FRUIT AND THE BARK OF *Helicteres isora*

3.3.1 Preliminary screening for phytochemicals

3.3.2 Identification of the phytochemicals in the plant samples by thin layer chromatography

3.3.3 Analysis of the active components by High Performance Liquid Chromatography

PHASE I

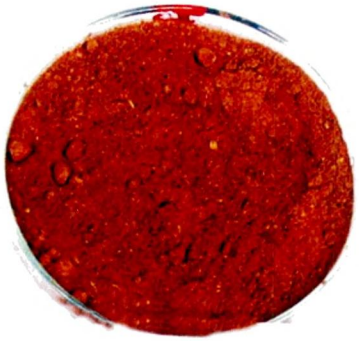
3.1 CHARACTERISATION OF THE SELECTED MEDICINAL PLANT TO BE USED AS A HYPOGLYCEMIC AGENT

3.1.1 COLLECTION, IDENTIFICATION AND PROCESSING OF THE *Helicteres isora*

The fruit and the bark of *Helicteres isora* were collected from the hills near Sultanpatri, Kerala. They were duly authenticated by Mr.R.Chandrasekar, Taxonomist, Botanical Survey of India, Coimbatore. The fruit and the bark were shade dried and powdered (Plate 1). The powder was weighed, packed in airtight containers and stored at 4°C until use.

PLATE 1

Helicteres isora



Fruit powder

Bark powder

3.1.2 CHARACTERISATION OF THE MEDICINAL PLANT *Helicteres isora* FOR SELECTED BIOCHEMICAL PARAMETERS

Estimation of carbohydrate (Hedge and Hofreiter, 1962)

A sample of 100 mg was hydrolysed by keeping it in a boiling water bath for three hours with 5.0 ml of 2.5N HCl and cooled it to room temperature. Neutralised it with solid sodium carbonate. Made up the volume to 100 ml and centrifuged. An aliquot of the supernatant was taken for analysis. A set of standards was obtained by taking 0.2-1.0 mg of glucose in 1.0 ml distilled water. To all the tubes 4.0 ml of anthrone reagent was added and heated for 8 minutes in a boiling water bath. The green colour developed was read at 630 nm.

Estimation of protein (Lowry *et al.*, 1951)

A sample of 500 mg was extracted with 5-10 ml of 0.1M potassium phosphate buffer (pH 7.4). Centrifuged and an aliquot was pipetted out and made upto 1.0 ml with 0.1N NaOH. Standard bovine serum albumin solution (40-200 µg) was also pipetted out and made upto 1.0 ml with 0.1N NaOH. Added 5.0 ml of alkaline copper to all the tubes and allowed to stand for 10 min. Folin's reagent (0.5 ml) was added to each tube and mixed well. The tubes were allowed to stand for 30 minutes at room temperature. The blue colour developed was measured at 660 nm.

Estimation of fibre (Raghuramulu *et al.*, 1983)

Extracted 2.0 g of ground material with ether or petroleum ether to remove fat. After extraction with ether, boiled 2.0 g of dried material with 200 ml of sulphuric acid (1.25 per cent) for 30 min with bumping chips. Filtered through muslin cloth and washed with boiling water until washings were no longer acidic. Boiled with 200 ml of sodium hydroxide solution (1.25 per cent) for 30 min, filtered through muslin cloth again and washed

with 25 ml of boiling 1.25 per cent sulphuric acid, three 50 ml portions of water and 25 ml alcohol. Removed the residue and transferred to a silica crucible (preweighed W_1), dried the residue for 2 hours at 130±2°C. Cooled the crucible in a desiccator and weighed (W_2). Incinerated the residue for 30 min at 600±15°C. Cooled in a desiccator and reweighed (W_3). The percentage of crude fibre in the sample was then calculated.

Preparation of ash solution (Raghuramulu *et al.*, 1983)

The silica crucible was heated upto 600°C and cooled. A sample of 2.0 g was weighed and taken in the crucible. The crucible was placed in an incubator at 100-110°C for 2-3 hours and cooled in a desiccator. The crucible was then placed in a clay pipe triangle and heated over a low flame till all the minerals completely charred. The charred mineral was then heated in a muffle furnace for 6 hours at 600°C. The crucible was then cooled in a desiccator and weighed. The ash thus obtained was used for the estimation of minerals calcium, phosphorus and iron.

Estimation of calcium (Clark and Collip, 1925)

Ash solution of 2.0 ml was taken in a centrifuge tube, added 2.0 ml of 4 per cent ammonium oxalate. Mixed well and allowed it to stand overnight and centrifuged. The precipitate was washed repeatedly with 2 per cent ammonia solution. Centrifuged and discarded the supernatant. This process was repeated till the supernatant gave no precipitate with calcium chloride. Added 2.0 ml of 2N sulphuric acid mixed and warmed in almost boiling water. Removed and titrated with 0.01N potassium permanganate keeping the mixture at 70-75°C to a faint pink colour which persisted for about a minute. A blank was done by titrating 2.0 ml of 2N sulphuric acid to the

same end point. The differences between the two titrations gave the volume of 0.01N potassium permanganate required to titrate the calcium oxalate.

1.0 ml of 0.01N $KMnO_4 \equiv 0.2$ mg of calcium.

Estimation of phosphorus (Oser, 1971)

Working standard solution corresponding to 4 -20 µg was taken and made up the volume to 4.3 ml with water and added 0.5 ml molybdate I solution (2.5 per cent ammonium molybdate in 5N H_2SO_4) and 0.2 ml of amino naphthol sulphonic acid (0.02 g ANSA + 0.12 g sodium bisulphite + 0.12 g sodium sulphite dissolved in 10 ml water). The ash solution (0.1 ml) was taken and treated similarly with molybdate II (2.5 per cent ammonium molybdate in 3N H_2SO_4) and 0.2 ml ANSA. The blue colour developed was read after 20 minutes at 660 nm.

Estimation of iron (Raghuramulu *et al.*, 1983)

Into a series of test tubes added working standard iron solution corresponding to 2-10 µg, 0.25 and 0.5 ml of ash solution. The volume in each tube was made upto 3.85 ml with distilled water. Then added 0.2 ml of saturated potassium persulphate solution, 0.15 ml of concentrated sulphuric acid and 0.3 ml of 3N potassium thiocyanate. The colour developed was read against a reagent blank at 540 nm within 10 minutes.

Estimation of sodium and potassium (Jackson, 1973)

Five gram of the sample was digested with 25 ml of triple acid mix (3:2:1 Conc. HNO_3: $HClO_4$: Conc. H_2SO_4) and the digest was diluted to 100 ml in a volumetric flask. Samples were diluted and used for the assay. The sodium and potassium content was estimated using flame photometer. For this, standard sodium solutions (0-100 ppm) were first injected and the readings were recorded. A standard curve was constructed. Sample extract was then fed and the reading was noted. The ppm of the extract was then

deduced with the use of standard curve. In the same way potassium was also estimated. The ppm was converted to mg/100 g sample.

Estimation of chromium (Krishna and Ranjan, 1991)

Five gram of the sample was digested with 25 ml of triple acid mix (3:2:1 Conc. HNO_3: $HClO_4$: Conc. H_2SO_4) and left aside for 3-4 hours in a fume cupboard. Then heated for 4 hours, cooled, washed 3-4 times with deionised water and made upto 50 ml in a volumetric flask. The standard chromium solution and the sample extract were first aspirated through an air-C_2H_2 flame into atomic absorption spectroscopy and readings were recorded at 358 nm.

3.1.3 ESTIMATION OF ANTIOXIDANTS IN *Helicteres isora*

Antioxidant substances block the action of free radicals which have been implicated in the pathogenesis of many diseases including atherosclerosis, ischemic heart disease, cancer, Alzheimer's disease, Parkinson's disease and in the aging process (Akinmoladun *et al.*, 2007).

Enzymic antioxidants in *Helicteres isora*

The enzymic antioxidants such as catalase, superoxide dismutase, glutathione peroxidase and glutathione-S-transferase were assessed in the fruit and the bark of *Helicteres isora.*

Determination of catalase activity (Luck, 1974)

Catalase is an enzyme, which is present in most cells and catalyzes the decomposition of H_2O_2 to water and oxygen and thereby protects the cells from oxidative damage of H_2O_2 and $OH^{\bullet}$. Homogenized the sample in a blender with M/15 phosphate buffer (pH 7.0) at 4°C and centrifuged. Stirred the sediment with cold M/15 phosphate buffer (pH 7.0), allowed to stand in the cold with occasional shaking and then repeated the extraction once

or twice. The combined supernatants were used for the assay. Read against a control cuvette containing the enzyme solution as in the experimental cuvette, but containing H_2O_2 free M/15 phosphate buffer (pH 7.0). Pipetted into the experimental cuvette 3.0 ml of H_2O_2-phosphate buffer (0.16 ml of H_2O_2 made upto 100 ml with M/15 phosphate buffer, pH 7.0). Mixed in 0.01- 0.04 ml sample and noted the time, Δt, required for a decrease in absorbance from 0.45 to 0.40. Calculated the concentration of H_2O_2 using the extinction coefficient 0.036 per μmole per ml.

Determination of superoxide dismutase activity (Misra and Fridovich, 1972)

Superoxide anion is known to inactivate enzymes and initiate the damaging chain reactions of lipid peroxidation. Cellular defence mechanisms against superoxides include a series of linked enzyme reactions which remove the toxic radicals and repair the radical induced damage. The first of these enzymes is superoxide dismutase that converts superoxide anion to hydrogen peroxide (Evans and Halliwell, 2001).

The incubation medium contained in a final volume of 3.0 ml, 50 mM potassium phosphate buffer (pH 7.8), 45 μM methionine, 5.3 μM riboflavin, 84 μM NBT and 20 μM KCN. The tubes were placed in an aluminium foil-lined box maintained at 25°C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophotometrically at 600 nm after exposure to light for 10 minutes. The maximum reduction was evaluated in the absence of the enzyme. One unit of enzyme activity was defined as the amount of enzyme causing 50 per cent inhibition of the reduction of NBT.

Determination of glutathione peroxidase activity (Reddy *et al.*, 1985)

Peroxidases catalyze the oxidation of a variety of electron donors with the help of H_2O_2 and thus scavenge the endogenous H_2O_2. Pipetted out 3.0 ml of pyrogallol solution (0.05M pyrogallol in 0.05M phosphate buffer, pH 6.5) and 0.05 to 0.1 ml of enzyme extract in cuvettes. Added 0.5 ml of 1 per cent H_2O_2 in the test cuvette. Recorded the change in absorbance every 30 seconds upto 3 minutes at 430 nm.

Determination of glutathione-S-transferase (Beutler, 1984)

Glutathione-S-transferase catalyses the reaction of 1-chloro-2, 4 dinitro - benzene (CDNB) with the -SH group of glutathione.

CDNB + GSH $\longrightarrow$ CDNB-S-glutathione

The activity of the enzyme was measured by following the increase in absorbance at 340 nm.

Ground about 5.0 g of the sample in a medium and made upto 20 ml with the medium containing 50 mM Tris HCl (pH 7.2, 0.4 M sorbitol and 10 mM NaCl). Centrifuged the homogenate at 2000 g for 10 min and used the supernatant for the assay. K_2HPO_4 buffer (0.5 ml, 0.5 M, pH 6.5) was taken in a test tube and 0.1 ml of CDNB (25 mM) was added. Added 8.8 ml of distilled water. Incubated the tubes at 37°C for 10 min. Then 0.5 ml of 20 mM glutathione was added to the reaction mixture. Added 0.2 ml of enzyme extract to the reaction mixture. Run a blank like test without the addition of enzyme. Measured the absorbance at 340 nm.

Glutathione-S-transferase activity in the extract is expressed as μmoles of CDNB-GSH conjugate / min / mg protein.

Non-enzymic antioxidants

The antioxidants belonging to the second line of defence include glutathione (GSH), vitamin C, vitamin E, carotenoids, flavonoids, tannins and glutathione. Glutathione is the most abundant non-protein synthesized in the liver and act as a substrate for glutathione peroxidase enzyme. This serves as a scavenger of different free radicals. Similarly vitamin C and vitamin E is important antioxidant scavengers, which cannot be synthesized by most mammals including human are therefore required from external source (Irshad and Chaudhuri, 2002).

Estimation of vitamin E (Rosenberg, 1992)

Vitamin E, the fat soluble vitamin functions as a scavenger of free radicals to prevent their peroxidative effects on unsaturated lipids of membrane. It was estimated by Emmerie-Engel method, as described by Rosenberg (1992). The samples were weighed (2.5 g) and added 50 ml of 0.1N sulphuric acid slowly without shaking. Stoppered and allowed to stand overnight. The next day, the contents of the flask were shaken vigorously and filtered through Whatman No.1 filter paper, discarding the initial 10-15 ml of the filtrate. Aliquots of the filtrate were used for the estimation. Into three stoppered centrifuge tubes (test, standard and blank) pipetted out 1.5 ml of extract, 1.5 ml of the standard (D, L-α-tocopherol 10 mg/L in absolute alcohol, 91 mg of α-tocopherol is equivalent to 100 mg of tocopherol acetate) and 1.5 ml of water respectively. To the test and blank added 1.5 ml of ethanol and to the standard, added 1.5 ml of water. Added 1.5 ml of xylene to all the tubes, stoppered, mixed well and centrifuged. Transferred 1.0 ml of xylene layer into another stoppered tube. Added 1.0 ml of 2, 2'-dipyridyl reagent (1.2 g/L n-propanol) to each tube, stoppered and

mixed. Pipetted out 1.5 ml of the mixture into spectrophotometer cuvettes and read the extinction of test and standard against the blank at 460 nm. Then, in turn, beginning with the blank, added 0.33 ml of ferric chloride solution. Mixed well and after exactly 15 minutes read the test and standard against the blank at 520 nm.

Estimation of vitamin C (Roe and Kuether, 1953)

Vitamin C is an electron donor, a reductant and a free radical scavenger. One gram of the sample was homogenized in 10 ml of 4 per cent TCA. Centrifuged at 2000 rpm for 10 minutes. The supernatants obtained were treated with a pinch of activated charcoal. Shaken well and kept for 10 minutes. Centrifuged and 0.5 and 1.0 ml aliquots of this supernatant were taken for the assay. The assay volumes were made upto 2.0 ml with 4 per cent TCA. The working standard solution containing 20-100 µg of ascorbate were pipetted out, the volumes of which were also made upto 2.0 ml with 4 per cent TCA. Added 0.5 ml of 2 per cent DNPH (2,4-dinitrophenyl hydrazine in 9 N H_2SO_4) reagent to all the tubes, followed by 2 drops of 10 per cent thiourea solution. Incubated at 37°C for 3 hrs. The osazones formed were dissolved in 2.5 ml of 85 per cent sulphuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone, DNPH reagent and thiourea were added after the addition of sulphuric acid. After incubation for 30 minutes at room temperature, the absorbance was read spectrophotometrically at 540 nm.

Estimation of flavonoids (Cameron *et al.*, 1943)

A portion of the sample was weighed out and the extraction was carried out in two steps, first, with methanol: H_2O (9:1) and second, with methanol: H_2O (1:1). At each step, sufficient solvent was added to make liquid slurry and the mixture was left for 6-12 hrs. Filtration to separate the

extract from the sample was carried out rapidly by using a glass wool or cotton wool plug in the neck of a filter funnel. The two extracts were then combined and evaporated to about 1/3 the original volume or until most of the methanol had been removed. The resultant aqueous extract was cleared off the contaminants such as fats, terpenes, chlorophylls and xanthophylls by extraction (in a separatory funnel) with hexane or chloroform. This was repeated several times and the extracts combined. The solvent-extracted aqueous layer containing the bulk of the flavonoids was then concentrated. An aliquot of the extract was pipetted into a test tube and evaporated to dryness. Then added 4.0 ml of vanillin reagent (1 per cent vanillin in 70 per cent H_2SO_4) and heated for 15 minutes in a boiling water bath. The standard (catechin 20-100 μg) was also treated in the same manner. Then the absorbance was measured at 360 nm.

Estimation of tannins (Schanderl, 1970)

Tannins are important polyphenolic compounds that act as antioxidants. Weighed 0.5 g of the sample in a 250 ml conical flask and added 75 ml water. Heated the flask gently and boiled for 30 min. Centrifuged at 2000 rpm for 20 min and collected the supernatant in 100 ml volumetric flask and made up the volume. Transferred 1.0 ml of the sample extract to a 100 ml volumetric flask containing 75 ml alcohol. Added 5 .0 ml of commercially available Folin-Denis reagent, 10 ml of 35 per cent sodium carbonate and diluted to 100 ml with water. Mixed well and read the absorbance at 700 nm after 30 minutes. The entire procedure was followed with a blank (water) and standards (20-100 μg tannic acid).

Estimation of carotenoids (Zakaria *et al.*, 1979; Raghuramulu *et al.*, 1983)

Carotenoids are lipophilic compounds that can inhibit free radical reactions. They also contribute towards immuno enhancement by limiting oxidative membrane damage and altering membrane structure and integrity. Weighed 5-10 g (w) of the sample and saponified for 30 minutes in a shaking water bath at 37°C with 12 per cent alcoholic KOH. Transferred the saponified extract into a separatory funnel (packed with glass wool and $CaCO_3$) containing 10 to 15 ml of petroleum ether and mixed gently to extract the carotenoid pigments into the petroleum ether layer. Transferred the lower aqueous phase to another separating funnel and the petroleum ether extract containing the carotenoid pigments to an amber-coloured bottle. Repeated the extraction of the aqueous phase similarly with petroleum ether, until it was colourless. Discarded the aqueous phase. To the pooled petroleum ether extracts, added a small quantity of anhydrous Na_2SO_4 to remove turbidity. Noted the final volume of the petroleum ether extract and diluted by a known dilution factor (v). The absorbance of the extract at 450 nm (p) was noted in a spectrophotometer using petroleum ether as a blank.

Estimation of reduced glutathione (Moron *et al.*, 1979)

One gram of the sample was homogenized in 5 per cent TCA to give a 20 per cent homogenate. The precipitated protein was centrifuged at 1000 rpm for 10 minutes. The homogenate was cooled on ice and 0.1 ml of the supernatant was taken for the estimation. The volume of the aliquot was made upto 1.0 ml with 0.2M sodium phosphate buffer (pH 8.0). Two ml of freshly prepared DTNB solution (0.6 mM in 0.2 M phosphate buffer, pH 8.0) was added to the tubes and the intensity of the yellow colour formed

was read at 412 nm in a spectrophotometer after 10 minutes. The standards (2-10 nmoles GSH in 1.0 ml of 5 per cent TCA) were also treated in a similar manner.

Estimation of thiamine (Gopalan *et al.*, 1993)

Weighed 2.5 g of sample in a 250 ml conical flask. Added 100 ml of 0.1N H_2SO_4 slowly. The flask was stoppered and allowed to stand overnight. Then it was shaken vigorously and filtered through Whatman No. 1 filter paper. Pipetted out 10 ml of the extract in duplicate into 100 ml separatory funnels. Pipetted out 10 ml of working standard (6 replicates) into separate separatory funnels. Three ml of 15 per cent NaOH was added into each separatory funnel immediately followed by 4 drops of 1 per cent potassium ferricyanide solution. It was shaken gently for 30 seconds. Then added 15 ml of isobutanol rapidly from a quick delivery burette. Stoppered immediately and shaken vigorously for 60 seconds and the layers were allowed to separate. The bottom layer was drained off carefully and added one spatulaful of sodium sulphate. The clear extract from the top layer was collected using a Pasteur pipette in to a clean dry test tube. A set of sample blank was prepared by pipetting out 10 ml of the extract except for the addition of ferricyanide. A blank was prepared for the standard separately. The primary (366 nm) and secondary filters (485 nm) were selected and adjusting the standard blank to 0 reading (a) and standard to 100 (a^1), set the fluorimeter. Then the sample blank and sample readings were recorded.

Estimation of riboflavin (Gopalan *et al.*, 1993)

Weighed 2.0 g of sample in a 250 ml conical flask. Added 75 ml of 0.1N H_2SO_4 and mixed. The flask was immersed in boiling water for 30 min and shaken frequently. It was cooled to room temperature. Then added 5.0 ml of 2.5M sodium acetate solution mixed and allowed to stand for

1 hour. This was then transferred to a 100 ml volumetric flask and made up with water. Filtered through Whatman No. 2 filter paper. The first 10-15 ml was discarded.

The fluorimeter was set and the fluorescence of the solutions was determined. To the sample cuvette, 20 mg sodium hydrosulphite was added, stirred and blank fluorescence was noted at 440-500 nm.

3.1.4 FREE RADICAL SCAVENGING ACTIVITY OF THE EXTRACTS OF *Helicteres isora*

Extract preparation

The fruit and the bark powders of *Helicteres isora* were macerated with methanol for 7 days, filtered, concentrated under reduced pressure at 40° C and evaporated to dryness under vacuum. The resulting brown coloured gummy residue was used for the study. From 100 g each of fruit and the bark, 45.0 and 54.2 g of residues were obtained respectively.

Extent of inhibition of *in vitro* lipid peroxidation (Okhawa *et al.*, 1979)

An *in vitro* model of goat liver homogenate was used for induction of lipid peroxidation, mediated by 10 μM $FeSO_4$ ($FeSO_4$ was prepared fresh in TBS) pro-oxidant. Application of the relevant plant tissue extract in the medium was tried with an objective of assessing the extent of inhibition of *in vitro* lipid peroxidation by the measurement of thio barbituric acid reactive substances (TBARS) in the experimental mixtures.

A 5 per cent liver homogenate was prepared in TBS cold (10 mM Tris, 0.15M NaCl, pH 7.4) and 50 μl of it was used in the assay. Fifty μl of the methanol extracts were used in the assay. Ferrous sulphate ($FeSO_4$ in TBS) at a final concentration of 10 μmoles was added in the assay medium to induce oxidation. The final volume in the test tubes was made upto

500 µl with cold TBS. Controls were prepared for each sample, containing the respective plant extract (50 µl), liver homogenate (50 µl) and TBS to make up the final volume to 500 µl. Pro-oxidant was not added to the control tubes. A blank containing no plant extract, no liver homogenate, but only $FeSO_4$ and TBS to make a final volume of 500 µl, was also prepared. An assay medium corresponding to 100 per cent oxidation was prepared by adding all the other constituents except the relevant extracts, and the volume made upto 500 µl with cold TBS. The experimental medium corresponding to auto oxidation contained only the liver homogenate and TBS to make up the final volume to 500 µl. All the tubes were incubated at 37°C for 1 hour.

Following the incubation period, 500 µl of 70 per cent alcohol was added to all the tubes to stop the reaction. One ml of 1 per cent TBA was added to all the tubes, followed by boiling in a hot water bath for 20 minutes. After cooling to room temperature, the tubes were centrifuged. To the clear supernatants collected *in toto* added 500 µl of acetone and measured the TBARS at 535 nm in a spectrophotometer.

Extent of inhibition of superoxide production (Winterbourn *et al.*, 1975)

The extent of superoxide generation was studied on the basis of inhibition in the production of nitroblue tetrazolium formazon of the superoxide ion by the test sample measured colorimetrically at 560 nm.

The assay tubes contained 0.02 ml of the sample (corresponding to 20 mg/ml of the methanolic extract), 0.2 ml EDTA (0.1M containing 1.5 mg NaCN / 100 ml), 0.1 ml nitroblue tetrazolium (NBT, 1.5 mM), 0.05 ml riboflavin (0.12 mM) and 2.55 ml 0.067M phosphate buffer (pH 7.8). Control tubes were set up without the extracts. The initial optical densities of the solutions were recorded at 560 nm and the tubes were illuminated

uniformly with a fluorescent lamp for 30 minutes. A_{560} was measured again and difference in OD taken as the quantum of superoxide production. The percentage inhibition by the samples was calculated by comparing with the OD of the control tubes.

Extent of inhibition of nitric oxide generation (Green *et al.*, 1982)

Aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with oxygen to produce nitrite ions. The reaction mixture containing 0.3 ml 100 mM sodium nitroprusside, 2.68 ml PBS (0.88 per cent NaCl, 0.02 per cent KCl, 0.02 per cent KH_2PO_4 and 0.115 per cent Na_2HPO_4, pH 7.2), and 20 µl of the extracts (20 mg extract) was incubated at 25°C for 15 minutes. Control tubes (100 per cent generation) were prepared without the extracts. After incubation, 0.5 ml of the reaction mixture was removed and 0.5 ml of Griess reagent (1 per cent sulphanilamide, 2 per cent H_3PO_4 and 0.1 per cent naphthalenediamine dihydrochloride) was added to it. The absorbance of the chromophore formed, indicative of the quantum of NO generated, was read at 546 nm. The percentage inhibition of nitric oxide generation was measured by comparing the absorbance values of control and those of test samples.

DPPH free radical scavenging activity (Mensor *et al.*, 2001)

Free radical scavenging activity was determined by 2, 2′-diphenyl-1- picryl - hydrazyl (DPPH) method by spectrophotometry and compared with the ascorbic acid.

A methanolic solution of DPPH (100 mM, 2.95 ml) was added to different concentration of test samples (10-50 mg/ml) and allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH

in methanol without the extracts served as the positive control. The methanolic solution of ascorbic acid was taken as standard. After 30 minutes, the absorbance was measured at 518 nm. The per cent reduction in the absorbance was calculated from the initial and final absorbance at each level. Concentration of extracts required for 50 per cent reduction was calculated from the calibration curve of the concentration of the extracts Vs per cent reduction in absorbance.

Hydroxyl radical scavenging activity (Elizabeth and Rao, 1990)

Deoxy ribose assay was used to detect hydroxyl radical scavenging activity and iron binding ability of the test sample. Hydroxyl radicals are generated in a reaction mixture containing ascorbate, H_2O_2 and iron–III-EDTA at pH 7.4 and measured by their ability to degrade the sugar deoxyribose. The extent of inhibition is dependent on the concentration of the scavenger and its rate constant for reaction with hydroxyl radicals.

The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3.6H_2O$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbate (0.1 mM), KH_2PO_4 – KOH buffer (20 mM, pH 7.4) was taken in a set of clean test tubes and final volume was made upto 1.0 ml. To this 0.5 ml each of 1 per cent trichloroacetic acid, 1 per cent thiobarbituric acid and 500 µl of various concentrations (10 to 50 mg/ml) of the extracts were added. Concentration of extracts required for 50 per cent reduction in absorbance was calculated from the calibration curve of concentration of the extracts Vs percent reduction in absorbance. Copper sulphate solution of 0.1 mM was used as reference standard. ($CuSO_4.5H_2O$)

3.1.5 Ames TEST TO ASSESS THE ANTIMUTAGENIC / ANTI-CARCINOGENIC EFFECT OF THE PLANT EXTRACTS OF *Helicteres isora*

The mutant bacterial strains of *Salmonella typhimurium* TA 98, TA 100 and TA 102, were obtained from Prof. B.N. Ames, Biochemistry Department, University of California, Berkeley, California, U.S.A.

Preparation of organic extract

In order to check whether the components of the powder of *Helicteres isora* fruit and bark show mutagenic/non-mutagenic effect, an organic extract was prepared from 10 g each of dry powder of the fruit and the bark. It was carefully transferred into a flask and extracted using petroleum ether (40° - 60°C). The extraction was carried out in the dark at 4°C for 72 hours with occasional shaking of the contents. After 72 hours the contents were filtered through cotton gauze and allowed to evaporate at room temperature in the dark. The extracts were transferred using minimum amount of petroleum ether into pre-weighed containers. The ether was then evaporated and the container was weighed again to obtain the weight of the extract.

The extract was dissolved in sterile dimethyl sulfoxide (DMSO) to give the required concentration and used for mutagenicity assay. An arbitrary concentration of 100 µg in 10 µl DMSO / plate of the extract were used.

The co-mutagenicity/antimutagenicity of the sample extracts was tested as follows.

The standard mutagens namely sodium azide for the bacterial strain TA 98 and daunomycin for the strain TA 100 and TA 102 were plated along with the sample extract and the test carried out.

The concentrations of standard mutagens used were as follows

Sodium azide – 1.0 mg / ml

Daunomycin – 1.0 mg / ml

Ames bacterial mutagenicity assay (Maron and Ames, 1983)

A set of histidine requiring strains is used for mutagenicity testing. These strains are incapable of growth in the absence of histidine in the growth medium. When a mutagen is added to the culture, the strain is mutated back, thereby losing the histidine dependence for its growth. The number of revertant colonies resulting after the action of the mutagen depends on the potency of the compound.

The type of mutation in the histidine operon in the strains is different, thereby enabling the identification of frame shift mutagens (those which are mutagenic towards TA 98), base-pair substituting mutagens (those which are mutagenic towards TA 100) and oxidative mutagens (those which are mutagenic towards TA 102).

Overnight grown cultures of TA 98, TA 100 and TA 102 of *Salmonella typhimurium* in nutrient broth were used for the mutagenicity test. Mutagenicity of the extract was tested using the method detailed by Maron and Ames (1983). One hundred μg of the extract dissolved in 0.01 ml of DMSO was added along with 0.1 ml of the overnight grown culture of the bacterial strain to 2.0 ml molten top agar held at 45°C. The contents were mixed gently and thoroughly, poured over the basal agar and spread evenly. After the top agar had solidified, the plates were incubated inverted at 37°C for 48 hours. At the end of 48 hours, the number of histidine revertant colonies was counted.

After testing the mutagenicity of the extract alone, their (co - or antimutagenic) effect on a standard oxidizing mutagen was carried out in the same way. For this purpose, 0.1 ml of the overnight grown culture was plated with 0.01 ml of DMSO containing 100 μg of the extract and 10 μl of sodium azide (1.0 mg/ml) for TA 98 and 6 μl of daunomycin (1.0 mg/ml) for the strains TA 100 and TA 102.

Each assay included 5 sets of plates as follows:

The overnight grown cultures were serially diluted and known amounts of these cultures were plated on nutrient agar plate to determine the number of viable bacteria in the inoculum. The plates were incubated at 37°C overnight and the number of colonies was counted. The spontaneous revertants in the inoculum were determined as above; with exception that 0.1 ml of the culture alone was added to the top agar before pouring onto the plate. The number of revertants induced by the extract was determined by plating 0.1 ml of the culture and 0.01 ml of appropriate concentration (100 μg) of the test compound on minimal glucose agar plates supplemented with trace amounts of histidine. The number of revertants induced by the standard mutagen was determined by plating 0.1 ml of the culture and 10 ml of standard mutagen containing 250 μg of phenyl hydrazine. The effect of the extract on the mutagenicity of the standard mutagen was assayed by plating the microbes with the standard mutagen in the presence of the extracts in the same doses as above.

All the groups (SR, SM, Extracts - fruit, bark) were set up in four replicas of four plates per group. The plates were incubated inverted at 37°C in the dark for 48 hrs (24 hours for group one). At the end of the incubation period, the number of colonies in each plate was counted and

recorded. After calculating the number of viable bacteria in the inoculum (from group one plates), the number of induced revertants was finally converted per 2×10^8 bacterial cells and was referred to as revertants per plate.

3.1.6 TOXICOLOGICAL EVALUATION OF THE SELECTED MEDICINAL PLANT

Evaluation of the toxicity and the adverse drug reaction of the herbal preparation has been a neglected area, as herbs are considered natural products and therefore safe. This lack of information makes it difficult to compare the benefit risks profile of herbal medicines. Further, the comparison of traditional medicines with modern drugs with comparative efficacy has not been conducted for most of the drugs.

Even if no adverse drug reaction is reported, the long term toxicity, mutagenicity and genotoxicity studies need to be conducted, as they are not evident clinically. Toxicity studies for herbal products should be conducted as per the regulatory requirements of the country to which they are marketed (Seth and Sharma, 2004).

In order to study any possible toxic effect or changes in the normal behaviour of experimental animals, the toxicity of the extracts was studied by checking the symptoms, the posture, the mortality, hematological parameters, biochemical parameters and blood glucose levels.

Induction of diabetes mellitus in experimental animals

Streptozotocin (STZ) is a mixture of α and β-stereoisomers, which occurs as pale yellow crystals or platelets. It is originally derived from the soil microorganism, *Streptomyces achromogens*. It has been investigated for use in diabetes, since it has specific toxic action on pancreatic β-cells.

However, the compound has been shown to artificially induce diabetes in rats (Tripathy, 1994).

Diabetes mellitus was induced in selected rats after 18 hour fasting by a single intraperitoneal injection of streptozotocin (Upjohn company, Kalamazoo, MI, USA) at a dose of 70 mg / kg body weight dissolved in 10 mM citrate buffer (pH 4.5) (Siddique *et al.*,1987). After 15 days, diabetes was confirmed by the presence of high blood glucose level. Control rats were given a vehicle injection at the same time when the diabetic condition was induced in experimental animals.

Treatment protocol for the animal study

Healthy male white Sprague Dowley rats of approximately same age, weighing 170-210 g were procured from small animals breeding station, Thrissur. These were maintained as per the principles and guidelines of ethical committee for animal care of Avinashilingam University in accordance with the Indian National law on animal care and use (Reg No: 623/02/b/CPCSEA). The rats were fed with normal laboratory diet and water *ad libitum* and acclimatized for a week under laboratory conditions. The rats were divided into eleven groups of 10 each to determine the effective dose and toxicity of the fruit and the bark extracts of *Helicteres isora.*

Earlier studies in the laboratory reveals that the alcoholic extracts of *Helicteres isora.* showed better antidiabetic effect than that of the aqueous extracts. Hence the present study was carried out with the alcoholic extract of the *Helicteres isora.* The alcoholic extract of the fruit and the bark of *Helicteres isora* were prepared by continuous hot percolation of 10 g of sample with 500 ml of alcohol (95 per cent) in Soxhlet apparatus. After completion of extraction it was filtered and the solvent was removed by distillation under reduced pressure. A brown coloured gummy residue was obtained. It was then dissolved in 1 per cent carboxymethyl cellulose and used for the study. Rats were given oral dose of extracts after 15 days of induction of diabetes. The treatment protocol for animals study is given in Table 1.

TABLE 1

TREATMENT PROTOCOL FOR ANIMAL STUDY

Group	Designation	Treatment
1	UC	Untreated control (1.0 ml of distilled water)
2	VC	Vehicle control (1.0 ml of 1 per cent carboxy methyl cellulose - CMC)
3	HF1	1.0 ml alcoholic extract of *Helicteres isora* fruit (150 mg/kg b.w/day)
4	HF2	1.0 ml alcoholic extract of *Helicteres isora* fruit (200 mg/kg b.w/day)
5	HB1	1.0 ml alcoholic extract of *Helicteres isora* bark (150 mg/kg b.w/day)
6	HB2	1.0 ml alcoholic extract of *Helicteres isora* bark (200 mg/kg b.w/day)
7	DC	Diabetic control (Streptozotocin induced - 1.0 ml of distilled water)
8	DHF1	Diabetic (1.0 ml alcoholic extract of *Helicteres isora* fruit - 150 mg/kg b.w/day)
9	DHF2	Diabetic (1.0 ml alcoholic extract of *Helicteres isora* fruit - 200 mg/kg b.w/day)
10	DHB1	Diabetic (1.0 ml alcoholic extract of *Helicteres isora* bark - 150 mg/kg b.w/day)
11	DHB2	Diabetic (1.0 ml alcoholic extract of *Helicteres isora* bark - 200 mg/kg b.w/day)

Administration of all the compounds and the vehicles were done using an intragastric tube for 45 days. Base line body weight, fluid intake and food consumption pattern were established and monitored during the following 45 days of oral treatment.

HEMATOLOGICAL STUDIES

The animals were sacrificed by cervical dislocation and blood was collected immediately by cardiac puncture and different hemotological parameters namely RBC count, total and differential counts of WBC, platelet count and hemoglobin were estimated in both the experimental as well as control rats.

Estimation of hemoglobin (Drabkin and Austin, 1932)

The reaction mixture in a volume of 5.02 ml contained 5.0 ml of Drabkin's reagent and 0.02 ml of blood. Varying concentrations of cyanmethemoglobin standards (16 g / dl) were also treated similarly. The reaction mixture was kept at room temperature for 5 minutes to ensure the completion of the reaction and read at 540 nm against a reagent blank.

Enumeration of Red Blood Corpuscles (Sanderson and Phillips, 1981)

Blood was drawn exactly upto the 0.5 ml mark in the RBC pipette and the diluting fluid (Hayem's fluid – 5.0 g of sodium sulphate, 1 g of sodium chloride, 0.5 g of mercuric chloride dissolved in 200 ml of distilled water) was drawn immediately upto the mark and the blood mixed thoroughly with the diluting fluid. It was left for 2-3 min for proper mixing. The Neubauer counting chamber was placed along with its cover glass in position. The capillary stem of the pipette was emptied which contains only the diluting fluid. This was done by discarding the first 3-5 drops.

Charging of the counting chambers

One drop of diluted blood was released into the groove of the Neubauer counting chamber. The chamber square is divided into 25 smaller squares by means of triple lines: counted the number of cells in 5 smaller squares (4 corner smaller square and central) including those touching the upper and left line in each square and deleting those touching the lower and right lines. Erythrocytes were counted, after allowing the cells to settle for 2 min, in the 5 squares of the counting area of 1 mm square. The average number of cells found in 5 groups of 16 squares is multiplied by 10,000 to give the number of cells in millions / mm^3 of blood.

Enumeration of White Blood Corpuscles (Sanderson and Phillips, 1981)

WBC dilution fluid or Truk's fluid (glacial acetic acid 5.0 ml + Gentian violet 1 per cent + water 95 ml) was used as the diluent, which can destroy RBCs. The method of counting is similar to RBC counting except that the count is made in 4 corner squares of the Neubauer counting chamber. The average number of cells in 4 squares is multiplied by a factor of 2500 to give the count / mm^3 of blood.

Determination of Differential Leucocyte Count (Sanderson and Phillips, 1981)

The blood film was placed in a level position and the dry blood film was covered with Leishman's stain, which should be evenly distributed over the entire slide. At the end of one minute, the quantity of buffer solution or distilled water was doubled carefully and mixed with the stain by means of the clean pipette. The film was allowed to stain for 7 to 8 minutes and the excess stain was removed by washing with distilled water for 2 minutes. The film was dried in air and then examined microscopically.

Platelet Count (Sanderson and Phillips, 1981)

Venous blood collected with EDTA was used for platelet count. Blood (0.05 ml) was diluted with 0.95 ml of Dacies fluid and mixed well using a narrow bore Pasteur pipette, the counting chamber was filled with the diluted blood. The cells were allowed to settle to the bottom of the chamber for 15 minutes to prevent from drying and the chamber was placed in a petridish, which contained a piece of wet filter paper. Using the 40 X objective with reduced condenser aperture the platelets were counted in 1/5 sq.mm^{-5} of the small squares of the large center square. From this the number of platelets in cu.mm of blood was calculated.

BIOCHEMICAL PARAMETERS

Estimation of blood urea (Netelson, 1957)

To 0.2 ml of blood, 1.8 ml of 10 per cent TCA was added, mixed well and after 10 min centrifuged. The supernatant (0.5 ml) was taken and made up the volume to 3.0 ml with water and then added 2.0 ml of reagent I [Mixed 0.25 ml of Reagent A (50 mg of ferric chloride, 0.2 ml of water, 1.0 ml of O-phosphoric acid and 2.5 ml of water) + 500 ml of Reagent B (50 ml of conc. sulphuric acid and 450 ml of water)] followed by 2.0 ml of fresh reagent II [Mixed 33.5 ml of reagent C (1.0 g of diacetyl monoxime in 50 ml water) + 33.5 ml of Reagent D (250 mg of thiosemicarbazide in 50 ml of water)] and diluted to 500 ml]. Mixed well, stoppered with marbles and heated vigorously in a boiling water bath for 20 min. Blank and standards (10-50 µg) were treated similarly. Removed the tubes, cooled and read against the blank.

Estimation of creatinine (Owen *et al.*, 1954)

To 0.2 ml of serum added 3.0 ml of water, 1.0 ml of 10 per cent sodium tungstate and 2.0 ml of 2/3 N sulphuric acid. Kept for 10 min and centrifuged. To 3.0 ml of supernatant added 1.0 ml of 0.04 M picric acid solution and 1.0 ml of 0.75 N sodium hydroxide and allowed to stand for 20 min. Blank and standards (10-50 µg) were treated similarly. The colour developed was read at 500 nm.

Determination of total bilirubin (Malloy and Evelyn, 1937)

To 0.2 ml of serum added 5.4 ml of water. Pipetted out 2.8 ml of this into a second tube for blank. To the test added 0.7 ml of diazo reagent [0.3 ml of solution A (0.5 per cent of sodium nitrite) + 10 ml of solution B (1 g of sulphanilic acid in 15 ml of conc. HCl and made upto one litre with water)] and to the blank 0.7 ml of 1 per cent sulphanilic acid solution. Mixed, allowed to stand for 5 minutes and added 3.5 ml of methanol to each tube. To the standard bilirubin (20-100 µg/ml of chloroform) added 3.5 ml of methanol, 0.7 ml of diazo reagent and 1.8 ml of water, read at 540 nm.

Estimation of serum aspartate transaminase (AST) and alanine transaminase (ALT) (Reitman and Frankel, 1957)

Added 2.0 ml of serum to 1.0 ml of buffer substrate [100 mM phosphate buffer and 2 mM α-oxoglutarate with 100 mM L-aspartate included for AST or 200 mM DL-alanine for ALT. Dissolved 15 g K_2HPO_4, 2.0 g KH_2PO_4 and 300 mg α-oxo- glutaric acid in 700-800 ml of water and (a) for AST added 15.7 g L-aspartate, monosodium salt or (b) for ALT added 17.8 g DL-alanine. In both cases the pH was adjusted to 7.4 with sodium hydroxide, mixed and incubated for 60 min for AST or 30 min for ALT at 37°C in a water bath. Then added 1.0 ml of DNPH (1mM in 1M HCl), allowed to stand at room temperature for 20 min. Added 10 ml of

400 mM sodium hydroxide solution, mixed and after 5 min, read at 500-550 nm. For the blank put up 0.2 ml of serum, 1.0 ml of buffer substrate and 1.0 ml of DNPH, mixed and completed as for test. A set of pyruvate standards (10-100 μg) was run similarly and plotted the absorbance against concentration.

Estimation of alkaline phosphatase (ALP) (Raghuramulu *et al.*, 1983)

Pipetted out 4.0 ml of buffer substrate [50 ml of 100 mM disodium phenyl phosphate + 50 ml of 100 mM sodium carbonate-bicarbonate buffer] in a test tube and placed in a water bath at 37°C for a few minutes, added 0.2 ml of serum, mixed, stoppered and left in the water bath for 15 minutes exactly. Removed, added 1.8 ml of diluted (1:3 dilution) phenol reagent of Folin-Ciocalteau. Set up control containing 4.0 ml buffer substrate and 0.2 ml of serum to which added 1.8 ml of diluted phenol reagent. Mixed and centrifuged. Took 4.0 ml of supernatant from each and added 2.0 ml of sodium carbonate (150g of Na_2CO_3/L). To the standard (2-10 μg/ml) added 2.0 ml of sodium carbonate and varying concentrations containing phenol reagent. Placed the tubes in a water bath at 37°C for 15 minutes and read at 700 nm against a blank. The King-Armstrong unit corresponds to the liberation of 1.0 mg of phenol by 100 ml of serum under the assay condition.

Determination of blood glucose (Hjelm and de Verdier, 1963)

Blood glucose was estimated every 5 days in untreated control as well as in experimental animals for 45 days. Added 0.1 ml of blood to 1.0 ml of 0.5M NaOH. Then added 0.1 ml of 10 per cent zinc sulphate, mixed well and centrifuged. To 0.2 ml of the supernatant added 4.0 ml of the enzyme-dye reagent (125 mg glucose oxidase, 5.0 mg peroxidase and 0.5 ml of 1 per cent o-dianizidine in 95 per cent ethanol per 100 ml of phosphate

solution (pH 7.0). Standards (40-200 μg glucose in 3 per cent benzoic acid) were also treated in the same way as the test. For the blank 0.2 ml of distilled water and 4.0 ml of enzyme-dye reagent were taken. All the tubes were placed in the water bath at 37°C for 45 minutes and read at 430 nm.

PHASE II

3.2 DETERMINATION OF THE ANTIDIABETIC EFFECT OF THE FRUIT AND THE BARK OF *Helicteres isora* IN THE EXPERIMENTAL RATS

For further studies a single dose of both the extracts of the fruit and the bark of *Helicteres isora* (200 mg/kg b.w) and a combination of both HF (100 mg/kg b.w) and HB (100 mg/kg b.w) were taken and compared with that of the standard allopathic drugs glibenclamide and metformin. Grouping of the experimental animals is shown in Table 2

TABLE 2
GROUPING OF EXPERIMENTAL ANIMALS

Group	Designation	Treatment
1	UC	Untreated control (1.0 ml of distilled water daily)
2	HF	1.0 ml alcoholic extract of *Helicteres isora* fruit (200 mg/kg b.w/day)
3	HB	1.0 ml alcoholic extract of *Helicteres isora* bark (200 mg/kg b.w/day)
4	HF+HB	1.0 ml alcoholic extract of *Helicteres isora* in combination HF (100 mg) + HB (100 mg/kg b.w/day)
5	DC	Diabetic control: Streptozotocin induced rats - 1.0 ml of distilled water daily
6	DHF	Diabetic - 1.0 ml alcoholic extract of HF (200 mg/kg b.w/day)
7	DHB	Diabetic - 1.0 ml alcoholic extract of HB (200 mg/kg b.w/day)
8	DHF+DHB	Diabetic - 1.0 ml alcoholic extract of *Helicteres isora* in combination HF (100 mg) + HB (100 mg/kg b.w/day)
9	DG	Diabetic - 1.0 ml glibenclamide (100 mg/kg b.w/day) in distilled water
10	DM	Diabetic – 1.0 ml metformin (100 mg/kg b.w/day) in distilled water

3.2.1 ASSESSMENT OF BLOOD GLUCOSE, LIVER GLYCOGEN, PLASMA INSULIN AND SERUM FRUCTOSAMINE

Estimation of blood glucose

Blood glucose was estimated by glucose oxidase method as described in section 3.1.6

Estimation of glycogen (Good *et al.*, 1933)

The liver was taken out rapidly from the animal and the excess blood removed by blotting between folds of filter paper and weighed quickly to the nearest 0.1 g. Minced the liver and a portion of it was immediately put into a weighed stoppered test tube containing 30 per cent KOH and weighed again. The difference between this weight and the original weight of the tube plus the KOH solution gives the weight of the liver sample used. It was digested in a boiling water bath for 1½ hour. Cooled in ice-cold water. Two volumes of 95 per cent ethanol were then added and the mixture was heated just to boiling. Spurting was avoided. It was left to stand overnight in the cold, then homogenized and centrifuged; the precipitate was dissolved in 5-10 ml warm water. The glycogen was reprecipitated with 2 volumes of 95 per cent ethanol. It was centrifuged and washed several times with 60 per cent ethanol. Added 2.0 ml of 2N H_2SO_4 per gram of liver and hydrolysed in a boiling water bath for 3-4 hours. The solution was neutralised with NaOH using phenol red as indicator, then made to a known volume and filtered. Glucose was determined in an aliquot. The factor 0.93 was used to convert glucose to glycogen.

Quantitative determination of plasma insulin

A substudy was conducted by taking two samples and the plasma insulin is analysed. The plasma insulin was assayed by the modified method of Hales and Randle (1963) by Boehringer Mannheim kit (Boehringer analyser ES 300). Plasma (0.1 ml) was injected into the plastic tubes coated with monoclonal anti-insulin antibodies. Phosphate buffer (40 mM, pH 7.0) and anti-insulin POD conjugate were added to form anti-insulin antibody-POD conjugate. Substrate [phosphate/citrate 10 mM, pH 4.4/H_2O_2 (sodium-perborate) 3.2 mM] and chromogen [di-ammonium 2, 2-azino-bis (3-ethyl benzothiazoline-6-sulphonate)] solutions were then added to form indicator reaction. A set of standards (insulin in bovine serum matrix) was also treated in a similar manner. The absorbance was read at 420 nm. The values were expressed as μU / ml of plasma.

Estimation of serum fructosamine (Armbruster, 1987)

The term fructosamine refers to glycosylated albumin and other proteins. Hemoglobin is not the only protein, which reacts with glucose to form a carbohydrate protein derivative. Serum albumin and a wide variety of other proteins in blood and tissues also undergo this reaction (Watanabe *et al.*, 2004).

The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 100 μM nitro blue tetrazolium (NBT) and 0.1 ml serum in a final volume of 3.0 ml. Incubated at 25°C for 30 minutes. The standards (fructose 40–200 μg) were also treated as above and read the absorbance at 530 nm.

3.2.2 DETERMINATION OF THE ACTIVITIES OF SELECTED KEY ENZYMES OF CARBOHYDRATE METABOLISM

Glycolysis and gluconeogenesis are the two prime complementary events balancing the glucose load in our body. During diabetes the activity of the glycolytic enzyme is lowered leading to reduced disposal of glucose as glucose-6-phosphate. On the contrary the activity of gluconeogenic enzymes are enhanced during diabetes.

Liver glucokinase (Brandstrup *et al.*, 1957)

Glucokinase is a key glycolytic enzyme. It phosphorylates glucose to glucose-6-phosphate with the help of ATP. Glucokinase has low K_m value like 50 mM for glucose and high V_{max}. Glucokinse is allosterically inhibited by glucose-6-phosphate. Increased glycolysis and β-oxidation in diabetes enhances the concentration of ATP and raises the ATP/ADP ratio. These inhibit phosphofructokinase, leading to the accumulation of fructose-6-phosphate and glucose-6-phosphate, which in turn inhibit the glucokinase, stopping further glycolysis.

The reaction mixture in a total volume of 5.0 ml contained the following viz., 1.0 ml of 5 mM glucose solution, 0.5 ml of 72 mM ATP, 0.1 ml of 50 mM magnesium chloride solution, 0.4 ml of 12.5 mM potassium dihydrogen phosphate, 0.4 ml of 0.1M potassium chloride, 0.4 ml of 0.5 M sodium fluoride and 2.2 ml of 0.01M Tris-HCl buffer (pH 8.0). The mixture was pre-incubated at 37°C for 5 min. The reaction was initiated by the addition of 2.0 ml of tissue homogenate. Removed 1.0 ml of the reaction mixture immediately to the tubes containing 1.0 ml of 10 per cent TCA which was considered as zero time. A second aliquot was removed after 30 minutes incubation at 37°C. The protein precipitate was removed by centrifugation and residual glucose in the supernatant was estimated by the

O-toluidine method. The enzyme activity is expressed as μmoles of glucose phosphorylated / min / mg protein.

Liver glucose-6-phosphate dehydrogenase (Kornberg and Horecker, 1955)

Measured into a test tube 2.0 ml of 0.05 M triethanolamine buffer (pH 7.6), 0.1 ml 0.01 M NADP and 1.0 ml of liver homogenate. Mixed and allowed to stand for 5 min at 25°C. Added 0.05 ml of 0.031 M glucose-6-phosphate and after about 2 min read the extinction at 340 nm for every minute for 5 minutes. Used a blank with tissue homogenate plus buffer but without NADP and glucose-6-phosphate. The glucose-6-phosphate dehydrogenase activity was measured by the initial rate of reduction of $NADP^+$ at 25°C by following the increase in absorption at 340 nm.

Liver fructose-1,6 bisphosphatase (Gancedo and Gancedo, 1971)

Fructose-1,6-bisphosphatase, the key gluconeogenic enzyme hydrolyses, fructose-1,6-bisphosphate to fructose 6- phosphate and inorganic phosphate. This enzyme occurs in the hepatic and renal cytosol. It is strongly and allosterically inhibited by AMP, but is activated by citrate. Insulin represses the enzyme and reduces gluconeogenesis. Decreased insulin concentration, increased glucagon in diabetes enhances gluconeogenesis by inducing the activity of fructose-1,6-bisphosphatase (Bhavapriya *et al.*, 2001).

The assay mixture in a final volume of 2 ml contained 1.2 ml of 0.1M Tris HCl buffer (pH 7.0), 0.1 ml of substrate (0.05M fructose-1,6-bisphosphate), 0.25 ml of 0.1M magnesium chloride, 0.1 ml of 0.1 M potassium chloride solution, 0.25 ml of 0.001M EDTA solution and 0.1 ml of enzyme homogenate. The incubation was carried out at 37°C for 15 min. The reaction was terminated by the addition of 10 per cent TCA.

The suspension was centrifuged and the supernatant was used for phosphorus estimation by the method of Fiske and Subbarow (1925). The supernatant was made upto a known volume. To this 1.0 ml of ammonium molybdate (2.5 g of ammonium molybdate in 100 ml 3N H_2SO_4) was added followed by 0.4 ml of ANSA (0.02 g of ANSA + 0.12 g sodium bisulphite + 0.12 g sodium sulphite dissolved in 10 ml water). The blue colour developed after 20 min was read at 660 nm. Enzyme activity was expressed as µmoles of inorganic phosphorus liberated / min / mg protein.

Liver glucose-6-phosphatase (Koida and Oda, 1959)

Glucose-6-phosphatase occurs in hepatic microsomal membrane, hydrolyses glucose-6-phosphate to release glucose in the endoplasmic reticulum. It is induced by glucagon, adrenaline and glucocorticoid. It enhances gluconeogenesis. Insulin represses the enzyme. The activity of the glucose-6-phosphatase is enhanced during diabetes (Bhavapriya *et al.*, 2001).

The incubation mixture contained 0.7 ml of 0.1M citrate buffer pH 6.4, 0.5 ml of substrate (0.01M glucose-6-phosphate) and 0.3 ml of tissue homogenate. The reaction mixture was incubated at 37°C for 1 hr. Addition of 1.0 ml of 10 per cent TCA to the reaction tubes terminated the reaction of the enzyme. The suspension was centrifuged and the phosphorus content of the supernatant was estimated by the method of Fiske and Subbarow (1925). Enzyme activity was expressed as µmoles of inorganic phosphorus liberated / min / mg protein.

3.2.3 ASSESSMENT OF LIPID PROFILE IN RATS

Uncontrolled diabetes mellitus may result in hyperlipidemia. It leads to the lipid mediated secondary complications including atherosclerosis. The

lipid profile (levels of total cholesterol, triacylglycerols, VLDL, LDL and HDL) was estimated in serum and liver homogenate in the experimental animals.

Extraction of serum lipids (Folch *et al.*, 1957)

One ml of serum was added drop wise to 5.0 ml of methanol in a stoppered tube. Then 5.0 ml of chloroform was added and mixed. This mixture was incubated at 55°C for 15 minutes. At the end of the incubation period, another 5.0 ml of chloroform was added so that the proportion of chloroform to methanol was 2:1 (v/v). After filtration and washing the residue 3 times with chloroform: methanol (2:1) and the combined filtrate was washed with 0.7 per cent KCl solution (20 per cent of the total volume of the extract). The aqueous upper phase was removed and the lower layer was washed four times with 5.0 ml of chloroform: methanol: KCl solution (3:48:47 v/v). The washed lower layer was evaporated to dryness and dissolved in a known volume of chloroform. Aliquots were used for the estimation of lipids.

Extraction of liver lipids (Radin, 1981)

For each gram of tissue added 18 ml extraction solvent [hexane: isopropanol (3:2 v/v)] and homogenized thoroughly. After 30-60 seconds of mixing, centrifuged and transferred the supernatant into a 25 ml graduated flask. The insoluble residue was resuspended in 3.0 ml extraction solvent and centrifuged after 5 minutes. Repeated with another 3.0 ml of solvent and finally made upto 25 ml. Aliquots were used for the estimation of lipids.

Estimation of total cholesterol (Abell *et al.*, 1952)

Pipetted out 0.01 ml of sample and 200 mg/dl cholesterol standard in to different tubes and added 1 ml of enzyme reagent (100 mM Phosphate buffer pH 6.5, 0.25 mM amino phenazone, 5 mM Phenol, 5 kU Peroxidase,

100 U Cholesterol oxidase, 0.05 per cent Sodium azide, 150 U Cholesterol esterase) and 1.0 ml of reagent alone served as reagent blank. The contents were mixed and incubated for 5 minutes at 37°C and read the absorbance at 500 nm.

An aliquot of the lipid extract was pipetted out into a glass stoppered centrifuge tube and was evaporated to dryness. To this, 5.0 ml of ethanolic KOH (6.0 ml of 33 per cent KOH made upto 100 ml with absolute ethanol) was added, stoppered and shook well. It was then warmed in a water bath at 37-41°C for 55 minutes. After cooling to room temperature, 10 ml of petroleum ether (60-80°C) was added and mixed. To this was then added 5.0 ml of water and was shaken vigorously for 1 minute. It was then centrifuged at a low speed for 5 minutes. Four ml of petroleum ether layer was pipetted out into test tube and evaporated to dryness at 60°C. Standards (0.2-1 mg / ml chloroform) were also treated in the same manner. Six ml of colour reagent (20 ml acetic anhydride + 1.0 ml conc. H_2SO_4 + 10 ml glacial acetic acid) was added to each tube and kept at 25°C after thorough shaking. After 30-35 minutes, the absorbance was measured at 620 nm against a reagent blank.

Estimation of High Density Lipoproteins (HDL), Very Low Density Lipoproteins (VLDL) and Low Density Lipoproteins (LDL) (Lopes- Virella, 1977)

Chylomicrons, VLDL and LDL are precipitated by adding phosphotungstic acid and magnesium ions to the sample. Centrifugation leaves only the HDL in the supernatant and the cholesterol content is determined.

Pipetted out 0.2 ml of sample and 0.5 ml of HDL cholesterol precipitant reagent in a test tube. Mixed and allowed to stand for 10 minutes

at room temperature, then centrifuged at 4000 rpm. After centrifugation, the clear supernatant was separated within two hours. To 0.1 ml of supernatant, 1.0 ml of cholesterol reagent was added. Mixed and then incubated at 37°C for 15 minutes and measured the absorbance at 500 nm. A reagent blank with 0.1 ml of distilled water and 1.0 ml of reagent solution was taken and treated similarly.

Concentration of HDL cholesterol (c) =219.2 x A_{sample} mg / dl

Total cholesterol was estimated in the whole serum by the method of Abell *et al.* (1952). The procedure described by Warnick and Albers was used for the separation of HDL and LDL + VLDL. LDL + VLDL were precipitated from the serum by treating with heparin and manganese chloride (final concentration of heparin 0.144 per cent and $MnCl_2$ 0.091 M). After keeping at room temperature for 10 minutes, it was centrifuged for 30 minutes at 4000 rpm at 4°C. The supernatant, which contained HDL, was analysed for cholesterol to obtain HDL -cholesterol.

$$\text{VLDL (mg/dl)} = \frac{\text{Triglycerides}}{5}$$

$$\text{LDL (mg/dl)} = \text{Total cholesterol} - \frac{\text{Triglycerides}}{5} - \text{HDL-cholesterol}$$

Estimation of triglycerides (Van Handel and Zilversmit, 1957)

Two gram of florisil was taken in glass-stoppered tube and 3.0 ml of chloroform was added. An aliquot of the extract was layered on the top of the florisil and mixed. It was then stoppered and shaken intermittently for 10 minutes. It was then made upto 10 ml with chloroform and filtered. One ml of the filtrate was pipetted out into three tubes. The solvent was evaporated at 60-70°C and 0.5 ml of ethanolic KOH (0.4 per cent) was then added to two out of three tubes (saponified sample) and 0.5 ml of ethanol

was added to the third tube (unsaponified sample). The tubes were closed and kept at 60-70°C for 15 minutes. To each tube, 0.5 ml of 0.2N H_2SO_4 was added and the tubes were then placed in a boiling water bath for 15 minutes to remove alcohol. They were then cooled to room temperature. Added 0.1 ml of sodium metaperiodate (0.05M) to each tube and kept for 10 minutes. Then 0.1 ml of sodium arsenate (0.5M) solution was added and kept for 10 minutes. A yellow colour of iodine appeared and vanished within a few minutes. Five ml of chromotropic acid reagent (2.0 g of chromotropic acid in 1000 ml of 70 per cent H_2SO_4) was added to each tube and mixed. The tubes were then closed and heated in a boiling water bath for 30 minutes. They were then cooled and the absorbance was read at 500 nm.

Estimation of phospholipids (Zilversmit and Davis, 1950)

An aliquot of the extract was pipetted out into a Kjeldhal flask and evaporated to dryness. To this, 1.0 ml of 5N H_2SO_4 was added and digested in a digestion rack till it became light brown. It was then cooled to room temperature; one or two drops of 2N HNO_3 were added, digested again till it became colourless. The Kjeldhal flask was cooled and 1.0 ml of water was added and heated in a boiling water bath for 5 minutes. One ml of 2.5 per cent ammonium molybdate was then added followed by 0.1 ml of ANSA. The volume was made upto 10 ml with distilled water. The absorbance was measured at 660 nm within 10 minutes.

3.2.4 ASSESSMENT OF OXIDATIVE STRESS IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

During diabetes mellitus persistent hyperglycemia causes an increased production of free radicals via autooxidation of glucose and non-enzymatic protein glycations, which may lead to disruption of cellular functions and

oxidative damage to membranes. Free radicals affect the cell components such as lipid, protein, DNA and carbohydrate, of which lipids are the most sensitive part. The levels of reactive oxygen species are controlled by antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase and non-enzymic scavengers such as reduced glutathione and vitamin C. Thus, the tissue antioxidant status seems to have an important role in the etiology of diabetic complications (Bukan *et al.*, 2003).

Estimation of thiobarbituric acid reactive substances (TBARS) (Nichans and Samuelson, 1968)

One gram of tissue was homogenized with 0.1M phosphate buffer (pH 7.0). Added 0.2 ml of TCA-TBA-HCl reagent (15 per cent w/v TCA and 0.37 per cent w/v 2-thiobarbituric acid in 0.25N HCl) was added to 1.0 ml of tissue homogenate (0.2 ml of tissue homogenate + 0.8 ml of buffer) and mixed thoroughly. The contents were heated in a boiling water bath for 15 minutes. After cooling the flocculent precipitate was removed by centrifugation at 1000 g for 10 minutes. The supernatant was shaken with n-butanol to extract the coloured complex into organic phase. The absorbance of the butanol layer was read at 535 nm against a blank that did not contain the sample. The concentration of MDA (TBARS) can be calculated using extinction co-efficient of 1.56×10^5 M^{-1} cm^{-1}.

Estimation of lipid hydroperoxides (Mair and Hall, 1977)

One ml of the aqueous tissue homogenate was mixed thoroughly with 5.0 ml of chloroform: methanol (2:1) followed by centrifugation at 1000 g for 5 minutes to separate the phases. The lower chloroform layer was evaporated to dryness under a stream of nitrogen at 45°C. One ml of acetic acid: chloroform (3:2) mixture and 0.05 ml of KI (6.0 g KI / 5.0 ml water) were quickly added to the evaporated sample and the test tube was stoppered

and mixed. The tubes were placed in dark at room temperature for exactly 5 minutes followed by the addition of 3.0 ml of cadmium acetate (0.5 per cent). The solution was mixed and centrifuged at 1000 g for 10 minutes. The absorbance of the upper layer was read at 353 nm against a blank containing the complete assay mixture without the tissue homogenate. Standardisation of the reaction was done using cumene hydroperoxide as the peroxide standard. The molar extinction coefficient of cumene hydroperoxide is $1.73 \times 10^4 M^{-1} cm^{-1}$.

ASSESSMENT OF ENZYMIC ANTIOXIDANTS IN RAT LIVER

Catalase (Sinha, 1972)

To 0.9 ml of 0.01M phosphate buffer (pH 7.0), 0.1 ml of tissue homogenate and 0.4 ml of 0.2M H_2O_2 were added after 60 sec. Two ml of dichromate acetic acid mixture (1:3 ratio of potassium dichromate was mixed with glacial acetic acid. From this 1.0 ml was diluted again with 4.0 ml acetic acid) was added. The tubes were kept in boiling water bath for 10 min and the colour developed was read at 620 nm. Standards of H_2O_2 in the range of 2-10 µM were taken and preceded as test with blank containing reagent alone.

The activity is expressed as µM of H_2O_2 consumed / min / mg protein.

Glutathione peroxidase (Rotruck *et al.*, 1984)

Glutathione peroxidase was estimated by the method of To 2.0 ml of 0.4M Tris buffer (pH 7.0), 0.2 ml of 0.4 mM EDTA, 0.1 ml of 10 mM sodium azide and 0.5 ml of tissue homogenate were added to the mixture, 0.2 ml of 2 mM glutathione followed by 0.1 ml of 20 mM hydrogen peroxide were added. The contents were mixed well and incubated at 37°C for 10 min along with a tube containing all the reagents except sample. After 10 min the reaction was arrested by the addition of 0.5 ml of

10 per cent TCA, centrifuged and the supernatant was assayed for glutathione by the method of Moron *et al.* (1979).

The activity is expressed as µg of GSH consumed / min / mg protein.

Superoxide dismutase (Kakkar *et al.*, 1984)

Assay mixture contained 1.2 ml of sodium pyrophosphate (0.025M, pH 8.3), 0.1 ml of 186 µM phenazine methosulfate, 0.3 ml of 300 µM NBT, and 0.2 ml of 780 µM NADH, appropriately diluted enzyme preparation and water in a total volume of 3.0 ml. Reaction was started by the addition of NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1.0 ml glacial acetic acid. Reaction mixture was stirred vigorously and shaken with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged and the butanol layer was collected. The colour intensity of the chromogen in the butanol layer was measured at 560 nm against butanol. A system devoid of the enzyme served as control. One unit of enzyme activity is defined as the enzyme concentration, which gave 50 per cent inhibition of NBT reduction in 1 min under the assay conditions and expressed as specific activity in units/mg protein.

Glutathione - S - transferase (Habig *et al.*, 1974)

The enzyme was assayed by its ability to conjugate GSH and CDNB, the extent of conjugation causing a proportionate change in the absorption at 340 nm.

The enzyme activity was determined by monitoring the change in absorbance at 340 nm in a spectrophotometer. 0.1 ml of both substrates (1 mM, glutathione and 1 mM, 1-choro- 2,4-dinitrobenzene) were taken in 0.1M phosphate buffer (pH 6.5) at room temperature to make a volume of 2.9 ml. The reaction was started by the addition of 0.1 ml of liver

homogenate to this mixture. The readings were recorded against distilled water blank for a minimum of 3 minutes. The complete assay mixture without the enzyme served as the control. Care was taken to ensure that the final concentration of ethanol in the mixture was always less than 4 per cent.

ASSESSMENT OF NON-ENZYMIC ANTIOXIDANTS IN RAT LIVER

Estimation of vitamin C (Omaye *et al.*, 1979)

Liver homogenate (0.5 ml) was mixed thoroughly with 1.5 ml of 6 per cent TCA and centrifuged for 20 min at 3500 g. The supernatant was shaken vigorously with a pinch of acid-washed Norit and filtered. To 0.5 ml of the filtrate, 0.5 ml of DNPH reagent (2.0 g of DNPH was dissolved in 100 ml of 9N sulphuric acid. To this 4.0 g of thiourea was added and mixed) was added and mixed well. The tubes were allowed to stand at room temperature for 3 hr. Removed, placed in ice-cold water and added 2.5 ml of 85 per cent sulphuric acid and allowed to stand for 30 min. A set of standards containing 10-50 µg of ascorbic acid were taken and processed similarly with a blank. The absorbance was read at 530 nm.

Estimation of vitamin E (Baker *et al.*, 1951)

To 0.1 ml of lipid extract from liver, 1.5 ml of ethanol and 2.0 ml of petroleum ether were added, mixed and centrifuged. The supernatant was evaporated to dryness at 80°C, to this was added 0.2 ml of 2,2'- dipyridyl solution (0.2 per cent 2,2'-dipyridyl in ethanol) and 2.0 ml of ferric chloride solution (0.5 per cent ferrric chloride in ethanol). Mixed well and kept in dark for 5 min and added 2.0 ml of butanol. The intense red colour developed was read at 520 nm. Standard tocopherol in the range of 10-100 µg was taken and treated similarly along with a reagent blank containing only the reagent.

Estimation of vitamin A (Bayfield and Cole, 1980)

Vitamin A was estimated by the method of Bayfield and Cole, (1980). Aliquots of standards ranging from (0-7.5 µg) were pipetted into clean dry test tubes and volume of all the tubes was made upto 0.1 ml with chloroform. To this 2.0 ml of TCA was added rapidly and mixed the contents. Samples were also treated similarly and the absorbance was recorded immediately at 620nm.

Estimation of reduced glutathione

The glutathione content was determined by the method of Moron *et al.* (1979) as given in section 3.1.3

3.2.5 TOTAL PROTEIN AND A/G RATIO OF SERUM

Changes in total protein concentration and A/G were estimated in the serum of the rats.

Protein estimation (Lowry *et al.*, 1951)

An aliquot of suitably diluted serum or tissue homogenate (0.1 ml) was made upto 1.0 ml with water and protein was precipitated by adding 1.0 ml of 10 per cent TCA. Centrifuged and the residue was dissolved in 1.0 ml of 0.1 N NaOH. From this an aliquot was pipetted out and made upto 1.0 ml with 0.1 N NaOH. Standard albumin solution was also pipetted out and made upto 1.0 ml with 0.1N NaOH. Added 5.0 ml of alkaline copper reagent to all the tubes and allowed to stand for 10 minutes. To each tube 0.5 ml of Folin's reagent was added and mixed well. The tubes were allowed to stand for 30 minutes at room temperature. Optical density was measured at 670 nm against a reagent blank.

Determination of Albumin to Globulin ratio (A/G ratio) (King and Wootton, 1956)

To 5.8 ml of 28 per cent sodium sulphate in a glass-stoppered tube, 0.2 ml of plasma was added and mixed by inversion. One ml of ether span reagent was added and stoppered the tube gently, inverted 20 times and centrifuged for 10 minutes. The globulin forms a button at the ether-water interface. A pipette was inserted and 3.0 ml of supernatant was withdrawn and added to 3.0 ml of Biuret reagent, after mixing, kept at 37°C for 10 minutes. The solution was cooled and the colour read at 540 nm.

3.2.6 HISTOPATHOLOGICAL STUDIES OF RAT LIVER, PANCREAS AND KIDNEY (Culling, 1979)

The rats were sacrificed by cervical dislocation and an autopsy was carried out to obtain liver, pancreas and kidney of the rats. Tissue samples were taken and preserved in 10 per cent formalin solution for a minimum of one hour. Formalin was removed from the tissue samples with running water. Dehydration of the fixed tissue was done by giving three changes of acetone (each 100 ml). Cleaning of tissue from acetone was followed by three changes of xylene (each 500 ml) in a total duration of three hours. Incubation of processed tissue in melted paraffin was done by two changes for 3-4 hours in an incubator maintained at 58-60°C. Embedding of the tissue in paraffin wax was then done by immersing the tissue in molten paraffin and then cooling it to harden the paraffin. Sections of the paraffin embedded tissue were done using a microtome adjusted to 1-3 μ thickness. The paraffin sections were carefully taken on glass slides. The sections were then cleaned by immersing in xylene. The sections were stained with

hematoxylin and eosin stain and screened to evaluate the morphology and cellular composition.

3.2.7 STATISTICAL ANALYSIS

The data given in the tables and figures are the mean of the values from the number of animals specified in the respective tables and figures Statistical significance was determined by One-way Analysis of Variance (ANOVA). 'p' value of 0.05 or less was considered as significant.

PHASE III

3.3 IDENTIFICATION OF THE ACTIVE COMPONENTS IN THE FRUIT AND THE BARK OF *Helicteres isora*

3.3.1 PRELIMINARY SCREENING FOR PHYTOCHEMICALS

The fruit and the bark powder of *Helicteres isora* were analysed for the presence of phytochemicals such as flavonoids, phenols, tannins, alkaloids, saponins and steroids.

Test for alkaloids (Akilandeshwari *et al.*, 2001)

Two gram of potassium iodide and 1.2 g of iodine were dissolved in 5.0 ml of sulphuric acid and the solution was diluted to 100 ml. 10 ml of alcoholic extract of the sample was acidified by adding 1.5 % v/v of HCl and a few drops of Wagner's reagent. Formation of yellow or brown precipitate confirmed the presence of alkaloid.

Test for flavonoids (Shinoda, 1928)

The sample was dissolved in ethanol and magnesium ribbon was added to it and then hydrochloric acid was added along the sides of the test tube. A deep blue colour shows the presence of flavonoids.

Test for phenols (Benze and Schmid, 1954)

Neutral ferric chloride reagent was prepared and added to the plant extract. The appearance of blue colour shows the presence of phenols.

Test for terpenoids (Akilandeshwari *et al.*, 2001)

Dissolved 0.5 g sample in 2.0 ml of chloroform in a dry test tube. Added equal volume of concentrated H_2SO_4. Shook gently. The upper layer of chloroform turns red and then sulphuric acid layer shows a green fluorescence.

Test for tannins (Akilandeshwari *et al.*, 2001)

The test solution of the extract was treated with a few drops of lead acetate solution. The appearance of white precipitate indicates the presence of tannins.

Test for saponins (Akilandeshwari *et al.*, 2001)

Five ml of aqueous extract was taken in a test tube and a drop of sodium bicarbonate was added. The mixture was shaken vigorously and kept for 3 min. A honey comb-like froth formed showing the presence of saponins.

3.3.2 IDENTIFICATION OF THE PHYTOCHEMICALS IN THE PLANT SAMPLES BY THIN LAYER CHROMATOGRAPHY (Riose *et al.*, 1986)

The petroleum ether, benzene, chloroform, acetone and methanol extracts were prepared from fruit, bark and a combination of both. These extracts were analysed by thin layer chromatography (TLC) for the identification of phytochemicals.

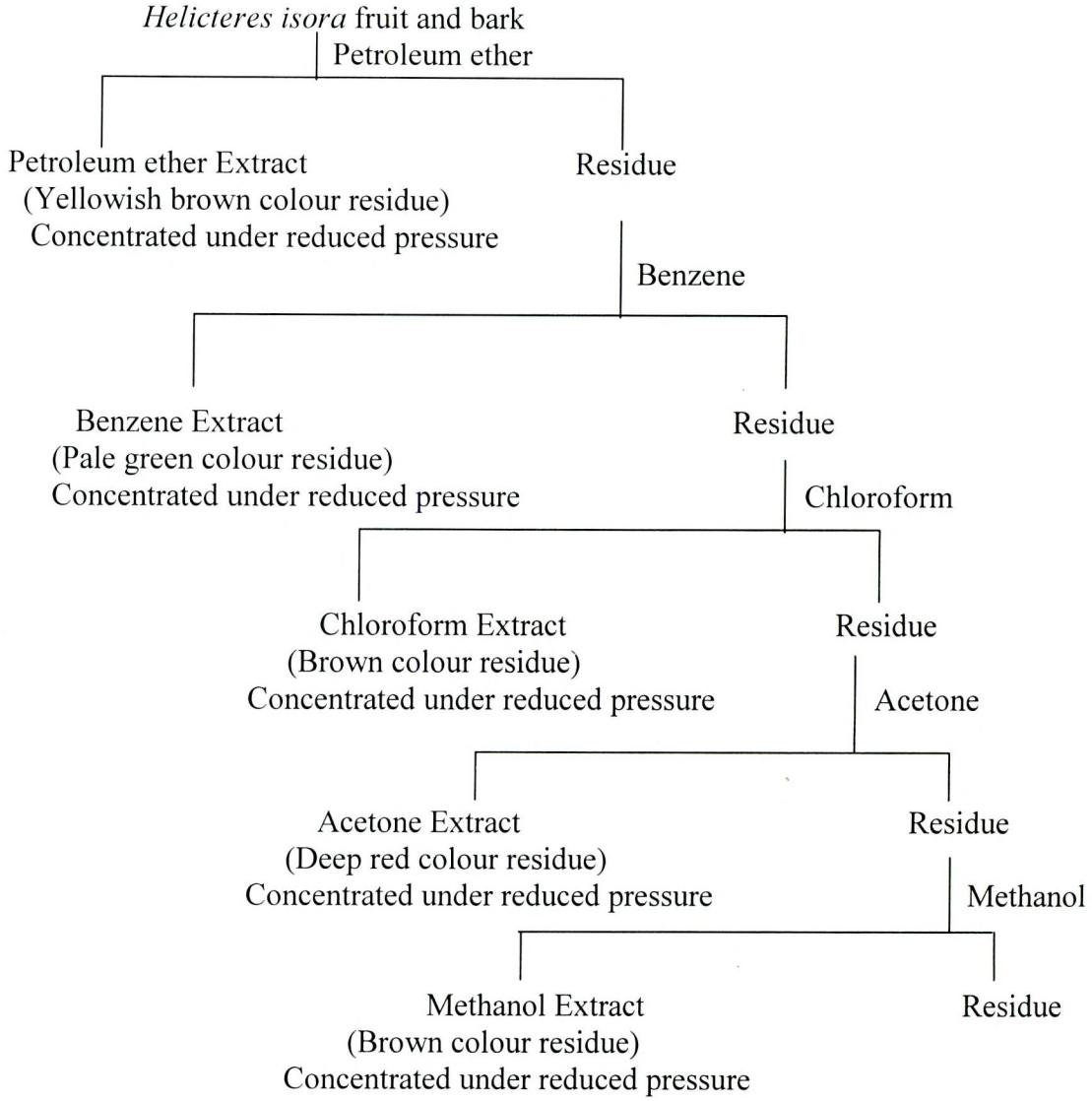
Preparation of the extracts

The extraction was done using a Soxhlet apparatus (Fig 2). About 10 g of fruit, bark and combination of the fruit and the bark of

Helicteres isora was extracted using the solvents petroleum ether, benzene, chloroform, acetone and methanol.

FIGURE 2

FLOW CHART FOR THE PREPARATION OF THE PLANT EXTRACT USING SELECTED ORGANIC SOLVENTS



Thin layer chromatography

Thin layer chromatography technique is easy to perform and require simple apparatus. The mixture of compounds to be separated is placed near one end of the TLC plate and allowed to dry. The plate is then placed with this end dipping in the solvent mixture, taking care that the sample spot is not immersed in the solvent. As the solvent moves towards the other end of the plate, the test mixture separates in to various components. This is known as development. The plate is removed after an optimal development time and dried and the spots are detected using a suitable location reagent. The silica gel acts as an inert support, the interstices of which hold the more polar phase of the solvent mixture which thus acts as the stationary phase, the less polar phase acting as the mobile phase. Separation results from differences in partition equilibrium of the components in the mixture. However, the silica gel interacts with the components and these effects the separation.

Preparation of the plate

The precoated alumina silica gel G60 plates incorporated with the fluorescent dye F254 were obtained from EMerck. The aluminium sheets were activated by drying it in hot air oven at 110°C for 30 minutes.

Application of the extract for separation

The extracts (3.0 µl) were taken in a capillary tube and it was spotted on a TLC plate, 2 cm above its bottom. The solution for application was between 0.1 to 1 per cent strength. The starting points were equally sized as far as possible and had a diameter ranging from 2 to 5 mm.

They were developed in separate solvent systems and the spots were identified. The solvent systems used for the identification of the compounds are as follows

Determination of alkaloids

Cyclohexane: Chloroform: Diethylamine	5:4:1
Spraying reagent	0.03 g paratarmaldehyde is dissolved in 100 ml of 85 per cent phosphoric acid

Determination of terpenes

Benzene: Methanol	5: 4
Spraying reagent	20 g antimony chloride and dissolved in a mixture of 20 ml of acetic acid and 60 ml of chloroform

Determination of flavonoids

Benzene: Methanol: Butane	3:1:1
Spraying reagent	1 per cent aluminium chloride

Determination of phenols

Acetic acid: Chloroform	1:9
Spraying reagent	Spray with dilute Folin-Ciocalteau reagent (1:1 with water) followed by spraying with 20 per cent Na_2CO_3

3.3.3 ANALYSIS OF THE ACTIVE COMPONENTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (Kim *et al.*, 2006)

Preparation of the standard

The standard rosmarinic acid was obtained from Cayman Chemical Co. (Ann Arbor, MI) and scuttelarein from Sigma Chemical Co. (Chromadex, USA). All HPLC analytical grade solvents were obtained from Fischer Scientific (Suwanee, GA). A quantity of 0.1 ml of the standards were drawn from 250 µg / ml of the standards and mixed with equal quantity of methanol and injected into LC- 10 AT HPLC system (Shimadzu, Kyota, Japan) for the elution of standard peaks.

Separation and identification of the compounds

To separate and identify the active compounds in the *Helicteres isora* (fruit and bark) extracts, reverse phase C_{18} high performance liquid chromatography (HPLC) was used. The Pinnacle II C_{18} column (150 x 4.6mm, 5µm; Restek, PA) was connected to the LC- 10 AT HPLC system (Shimadzu, Kyota, Japan) and equilibrated with 0.05 per cent aqueous trifluoroacetic acid (TFA). Fifty microliters of methanolic extracts were injected and eluted with 0.25 per cent triethylamine, 5 per cent tetrahydrofuran, 20 per cent acetonitrile and 75 per cent methanol (HPLC grade) at a flow rate of 1 ml / min. Collected 1.0 ml fractions of the eluant and the absorbance of the eluant was scanned from 200 to 500 nm by a SPD-M10V photodiode array detector (PDA).