3. MATERIALS AND METHODS

3.1 Pharmacognostical and Phytochemical analysis

3.1.1 Identification and collection of samples

Fresh fruits of *Solanum nigrum* were collected from the region of Anand, Gujarat. The plant was authenticated by comparison with voucher specimen No. VSM502 and ARM 2174 at the Prof. G.L.Shah Herbarium of S.P.University, Vallabh Vidyanagar, Anand, Gujarat, India.

Heart wood of *Acacia catechu* was collected from the region of Navsari, Gujarat. The plant was authenticated by comparison with voucher specimen No. YADAV 1049 at the Prof. G.L.Shah Herbarium of S.P.University, Vallabh Vidyanagar, Anand, Gujarat, India. Further differentiation from *Uncaria gambier* was done by performing gambier fluorescin test.

3.1.2 Physico-chemical parameters

**Determination of ash values (The Ayurvedic Pharmacopoeia of India, 1986):**

Ash values of powder of fruits of *Solanum nigrum* and heart wood of *Acacia catechu* were determined by the following methods:

(a) **Total ash:**

2g of accurately weighed powder was incinerated in a crucible at a temperature 500-600° C in a muffle furnace till carbon free ash was obtained. It was then cooled, weighed and percentage of ash was calculated with reference to the air-dried drug.

(b) **Acid insoluble ash:**

The ash obtained above was boiled for 5min with 25 ml of 70 g/L hydrochloric acid and filtered using an ash less filter paper to collect insoluble matter. The ash obtained was washed with hot water and filter paper was burnt to a constant weight in a muffle furnace. The percentage of acid-insoluble ash was calculated with reference to the air-dried powered drug (60#).
(c) **Water soluble ash:**
Total ash was boiled for 5 min with 25 ml of water and insoluble matter collected on an ash-less filter paper washed with hot water and ignited for 15 min at a temperature not exceeded 450° in a muffle furnace. Difference in weight of ash and weight of water insoluble matter gave the weight of water-soluble ash. The percentage of water-soluble ash was calculated with reference to the air-dried powered drug (60#).

**Determination of extractive values (The Ayurvedic Pharmacopoeia of India, 1986):**
Extractive values of powders of fruits of *Solanum nigrum* and heart wood of *Acacia catechu* were determined by the following methods:

(a) **Alcohol soluble extractive:**
4g of the air-dried powdered material (60#) were macerated with 100 ml of alcohol in a closed flask for 24 h, shaking frequently at an interval of 6 h. It was then allowed to stand for 18 h and filtered rapidly to prevent any loss during evaporation. 25 ml of the filtrate was evaporated to dryness in a porcelain dish and dried at 105° to a constant weight. The percentage of alcohol soluble extractive was calculated with reference to the air-dried drug.

(b) **Water soluble extractive:**
4g of the air-dried powdered material were soaked in 100ml of water in a closed flask for 1 h with frequently shaking. It was then boiled gently for 1 h on water bath; cooled and weighed and readjusted the weight. 25 ml of the filtrate was evaporated to dryness in a porcelain dish and dried at 105° to a constant weight. The percentage of water-soluble extractive was calculated with reference to the air-dried powered drug (60#).

3.1.3 **Phytochemical analysis:**
Powders of *Solanum nigrum* fruit and *Acacia catechu* heart wood was subjected to the following tests separately for the presence of various phytoconstituents visually, alkaloids, flavonoids, saponins, carbohydrates, sterols and terpenoids, anthraquinone glycosides, coumarins, carotenoids, tannins and phenolic compounds.
Test for alkaloids (Sim, 1969):
1 g of powder was extracted with 20 ml alcohol by refluxing for 15 min, filtered and
the filtrate was evaporated to dryness. The residue was dissolved in 15 ml of H$_2$SO$_4$
(2N) and filtered. After making it alkaline, the filtrate was extracted with chloroform.
The residue left after evaporation was tested for the presence of alkaloids with
Dragendorff’s reagent. Development of orange colored precipitate indicated the
presence of alkaloid(s).

Test for flavonoids:
(a) Shinoda test (Geissman, 1955):
1g of powder was extracted with 10 ml of ethanol (95%v/v) for 15 min on a boiling
water bath and filtered. To the filtrate was added a small piece of magnesium ribbon
and 3 to 4 drops of concentrated sulphuric acid. Red coloration of the solution
indicated the presence of flavonoid(s).

(b) Fluroscence test (Luckner, 1966):
1 g powder was extracted with 15 ml methanol for 2 min on a boiling water bath,
filtered while hot and evaporated to dryness. To the residue, 0.3 ml boric acid solution
(3% w/v) and 1 ml oxalic acid solution (10% w/v) were added. The mixture was
evaporated to dryness and the residue was dissolved in 10 ml ether. Greenish
fluorescence under U.V. light of the ethereal layer indicated the presence of
flavonoid(s).

Tests for saponins (Fishcer, 1952; List and Horhammer, 1967):
(a) Froth test:
0.1g of powder was vigorously shaken with 5 ml of distilled water in a test tube for 30
sec and was left undisturbed for 20 min. Persistent froth indicated the presence of
saponin(s).
(b) Haemolytic Zone:
0.5 ml of blood was mixed with gelatin solution (3 g gelatin powder dissolved in 100 ml of 0.85% NaCl solution) at 60 °C and taken on a glass slide. 0.1 g of powder was placed on it. Haemolysis of red blood cells indicates the presence of saponin(s).

Test for carbohydrates (Trease and Evans, 2002):

(a) Molisch’s test:
1 g powder was extracted with 10 ml ethanol for 15 min on a boiling water bath and filtered. Appearance of purple color on addition of α-naphthol and concentrated H₂SO₄ to the filtrate indicates the presence of carbohydrates.

(b) Fehling test:
1 ml Fehling A and 1 ml Fehling B solutions were mixed and boiled for 1 min. 2 ml of test solution was added. It was heated on boiling water bath for 5-10 mins. Appearance of first yellow and then brick red precipitate indicated the presence of carbohydrate(s).

Tests for sterols and triterpenoids:

(a) Liberman Buchardt test (Griffin et al., 1968):
1 g powdered drug was moistened with 1.0 ml of acetic anhydride and 2 drops of sulphuric acid on a clean tile. The powder was mixed well and the color gained by the powder was observed. Purple to violet color indicated the presence of sterol(s) or triterpenoid(s).

(b) Salkowski reaction (Robinson, 1964):
To the 2 ml of extract, 2 ml chloroform and 2 ml concentrated H₂SO₄ were added and shake well. Presence of sterol(s) and triterpenoid(s) indicated if chloroform layer appeared red and acid layer showed greenish yellow fluorescence.
Tests for tannins (Simmes et al., 1959):
Aqueous extract of the leaves was prepared by refluxing 10g of leaves powder with 50 ml of water for about 1h on water bath and was used for the following tests;

(a) Test with gelatin (Freudenberg and Weinger, 1962):
To 2-3 ml of aqueous extract, add 1 %w/w gelatin solution containing NaCl. Heavy white precipitate indicated the presence of tannin(s).

(b) Reaction with lead acetate (Robinson, 1964):
To the aqueous extract of drug, add 2 ml of 10 %w/w solution of lead acetate. Precipitate indicated the presence of tannin(s).

Tests for phenolic compounds:

(a) Test with FeCl₃ (Clerk et al., 1947):
Methanolic extract of powdered seeds was taken. To this, a drop of freshly prepared FeCl₃ solution was added. Brownish green color indicated the presence of phenolic compound(s).

(b) Test with Folin ciocalteu reagent (Harborne, 1984):
To a drop of methanolic extract of seeds, a drop of Folin ciocalteu reagent was added. Appearance of bluish green color indicated the presence of phenolic compound(s).

Tests for Coumarins (Trease and Evans, 2002):

(a) With Ammonia:
Take a drop of ammonia on a filter paper, a drop of aqueous extract of seeds was added. Fluorescence indicated the presence of coumarin(s).

(b) With Hydroxylamine hydrochloride:
The ethereal extract was treated with one drop of saturated alcoholic hydroxylamine hydrochloride and a drop of alcoholic KOH. The mixture was heated, cooled and acidified with 0.5 N hydrochloric acid and a drop of 1% w/v FeCl₃ was added to it. Violet color indicated the presence of coumarin(s).
Tests for anthraquinone glycoside (Mukherjee, 2002):

(a) Borntrager’s test:
Powder of the drug was taken and extracted with ether. To the filtered ethereal extract, ammonia was added. The aqueous layer was not show pink red or violet color after shaking indicated the presence of anthraquinone glycoside(s).

(b) Modified borntrager’s test:
To the aqueous solution of drug, ferric chloride and dilute HCl were added. The mixture was heated, cooled and filtered. Filtrate was shaken with ether. The ethereal extract was shaken with dilute ammonia. Rose pink to cherry red color of the aqueous layer indicated the presence of anthraquinone glycoside(s).

3.1.4 Determination of total phenolic content
Total phenolic content of SNE extract and ACE extract were determined by the Folin–Ciocalteu procedure by using gallic acid as a standard phenolic compound (Slinkard and Singleton, 1977). 1 ml of extract solution (1000μg /ml) in a volumetric flask diluted with distilled water (46 ml). Folin–Ciocalteu reagent (1 ml) was added and the contents of the flask were mixed thoroughy. After 3 min, 3 ml of Na₂CO₃ (2%) was added, then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in a spectrophotometer (Shimadzu Pharma Spec UV – 1700, Japan). The amount of total phenolic compounds in the SNE extracts was determined in micrograms of gallic acid equivalent, using the equation obtained from the standard gallic acid graph:
Absorbance = 0.0048 × Total phenols [Gallic acid equivalents (μg)] – 0.0932

3.1.5 Extraction
Extracts were prepared by soxhlet extraction of the crushed fresh fruits of Solanum nigrum and powdered heart wood of Acacia catechu with ethanol, and were evaporated to dryness. The extracts were stored in desiccators for use in subsequent experiments.
3.2 Pharmacological studies

3.2.1 Fixation of Doses:
Healthy rats of either sex fasted overnight, but allowed access to water ad libitum were randomly divided into five groups (n = 5, either sex). The first group (control group) received water. Groups 2–5 were orally treated with the ethanolic extract of *Solanum nigrum* at the doses of 0.5, 1.0, 1.5, 2.0 and 2.5 g/kg, respectively. Animals were observed for hazardous symptoms and mortality for a period of 3 days after treatment. The results indicated that acute treatment of *Solanum nigrum* extract by oral route at dose of 2.5 g/kg did not produce any sign of toxicity or death in rats during 14 days of observation. Therefore, the LD$_{50}$ could not be estimated, and it is possibly more than 2.5 g/kg.

Healthy rats of either sex fasted overnight, but allowed access to water ad libitum were randomly divided into five groups (n = 5, either sex). The first group (control group) received water. Groups 2–5 were orally treated with the ethanolic extract of *Acacia catechu* at the doses of 0.5, 1.0, 2.0, 4.0 and 5.0 g/kg, respectively. Animals were observed for hazardous symptoms and mortality for a period of 3 days after treatment. The results indicated that acute treatment of *Acacia catechu* extract by oral route at dose of 5.0 g/kg did not produce any sign of toxicity or death in rats during 14 days of observation. Therefore, the LD$_{50}$ could not be estimated, and it is possibly more than 5.0 g/kg.

3.2.2 Induction of type 1 diabetes (IDDM)
Male albino wistar rats of 20-24 weeks age, weighing 250-300 gm were used for the study. The animals were housed in group of 6 rats per cage under well controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12h/12h light-dark cycle. Animals have free access to conventional laboratory diet and tap water ad libitum. Diabetes was induced with streptozotocin (STZ) (Sisco research lab Limited, India) 50 mg/kg dissolved in 0.9% NaCl, administered as a single intraperitoneal injection. The destruction of pancreatic β-cells by both drugs is associated with a huge release of insulin which makes animals more susceptible to severe hypoglycemia that may be lethal. Thus, following treatment with either STZ, animals are fed with glucose solution (5%) for 24 h. Control animals were injected with an equal volume
of 0.9% NaCl. Animals were checked for the extent of glucosuria 48 hours after the injection of STZ using diastix (Bayer diagnostics, India). Animals having glucosuria (> 2%) were considered as diabetic. Control animals injected with NaCl were used as normal control. Diabetic rats were divided into two groups, namely diabetic control and diabetic treated with test drugs.

Study of effect of plant extracts on type 1 diabetic rats involved 2 sets of experiments. The groups of animals in two different set were as follows:

Set 1: Study of effect of ethanolic extract of *Solanum nigrum* fruits (SNE) on STZ induced type 1 diabetic rats:
- GP I Non diabetic control
- GP II Normal control treated with SNE (250 mg/kg)
- GP III Diabetic control
- GP IV Diabetic treated with SNE (100 mg/kg)
- GP V Diabetic treated with SNE (250 mg/kg)
- GP VI Diabetic treated with Insulin (5 IU/kg, i.p.).

Set 2: Study of effect of ethanolic extract of heart wood of *Acacia catechu* (ACE) on STZ- induced type 1 diabetic rats:
- GP I Non diabetic control
- GP II Normal control treated with ACE (500mg/kg)
- GP III Diabetic control
- GP IV Diabetic treated with ACE (250 mg/kg)
- GP V Diabetic treated with ACE (500 mg/kg)
- GP VI Diabetic treated with Insulin (5 IU/kg, i.p.).

All the studies were carried out for a period of 21 days. At the end of which blood samples were collected under fasting conditions, the serum was separated and was subjected to biochemical estimation. During the study, food intake, water intake and body weights of animals were recorded in all the groups.

### 3.2.2.1 Blood Sample collection and analysis

At the end of the treatment period, animals were kept for overnight fasting and blood samples were collected in to centrifuge tubes. The blood samples were allowed to clot
for 30 min at room temperature. Serum were separated by centrifugation at 3000 rpm for 25 min and stored at -20 °C until the analysis was carried out. Serum samples were analyzed for glucose, triglycerides, cholesterol, creatinine, urea, Serum glutamate oxaloacetate transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT) and HDL using diagnostic kits (Span Diagnostics Ltd, India) colorimetrially using UV-Visible spectrophotometer (Shimadzu UV-1601, Japan). VLDL, LDL and atherogenic index were calculated as per Friedewald’s equation. VLDL = Total Serum Triglycerides/5; LDL = Total Serum Cholesterol – (Total Serum Triglycerides/5) – Total Serum HDL-C; Atherogenic index = LDL + VLDL/ HDL Serum insulin was estimated by radio immunoassay method using kits obtained from Linco Inc. in a five well gamma counter.

All the animals were sacrificed by an overdose of diethyl ether and liver was excised immediately. Rat livers were excised and immediately frozen in dry ice and stored at -20°C. Frozen tissue from each rat was homogenized in ice cold 0.1 M Tris- HCL buffer (pH 7.4). The homogenate was centrifuged and supernatant was used for the estimation of proteins, lipid peroxidation, Superoxide dismutase, catalase and reduced glutathione.

3.2.2.2 Oral Glucose Tolerance Test (OGTT)

At the end of 21 days of treatment, oral glucose tolerance test were performed after an overnight fasting (Olefsky, 1981). The animals were orally administered with 1.5 g/kg of glucose and blood samples were collected from the retro orbital plexus under light ether anesthesia before i.e. 0 min and 30, 60 and 120 min after oral glucose administration. Samples were allowed to clot for 30 min at room temperature. Serum were separated by centrifugation at 3000 rpm for 25 min and analyzed for glucose as explained earlier. Plotting the glucose concentration versus time gives a curve showing rise and fall in glucose with time after an oral glucose load. Comparison versus time gives a curve showing rise and fall in glucose with time after an oral glucose load. Comparison of such curves gives only a vague idea about alterations in insulin-mediated glucose disposal. Therefore, results were expressed an integrated area under the curve (AUC) for glucose.
This was calculated by trapezoid rule \[ AUC = (C_1 + C_2)/2 \times (t_2-t_1) \] and changes in glucose concentrations over 120 min during OGTT were expressed as \( AUC_{\text{glucose}} \) (mg/dl.120 min).

### 3.2.3 Induction of type 2 diabetes (NIDDM)

Sprague Dawley (SD) rats of either sex from in bred colony were bred under well controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5 %) and 12h/12h light dark cycle. NIDDM was induced in overnight fasted rats weighing 250–280 g by a single intraperitoneal injection of 60 mg/kg streptozotocin (Sisco research lab, Mumbai), 15 min after the i.p. administration of 120 mg/kg of nicotinamide (Sisco research lab, Mumbai). Streptozotocin (STZ) was dissolved in citrate buffer (pH 4.5) and nicotinamide was dissolved in normal saline. Hyperglycemia was confirmed by the elevated glucose levels determined at 72 h and then on day 7 after injection by One Touch Glucometer (Life Scan, Inc., Johnson & Johnson Company, USA). The threshold value of fasting plasma glucose to diagnose diabetes was taken as >140 mg/dl. Only rats found with permanent NIDDM were used for the antidiabetic study.

Study of effect of plant extracts on type 2 diabetic rats’ involved 2 sets of experiments. The groups of animals in two different set were as follows:

Set 1: Effect of ethanolic extract of *Solanum nigrum* fruits (SNE) on normal rats and on STZ - Nicotinamide induced type 2 diabetic rats:

- GP I Non diabetic control
- GP II Diabetic control
- GP III Diabetic treated with SNE (100 mg/kg),
- GP IV Diabetic treated with SNE (250 mg/kg)
- GP V Diabetic treated with Glipizide (10 mg/kg, p.o.).
Set 2: Study of effect of ethanolic extract of heart wood of *Acacia catechu* (ACE) on STZ-Nicotinamide induced type 2 diabetic rats:

**GP I** Non diabetic control  
**GP II** Diabetic control  
**GP III** Diabetic treated with ACE (250 mg/kg)  
**GP IV** Diabetic treated with ACE (500 mg/kg)  
**GP V** Diabetic treated with Glipizide (10 mg/kg, p.o.).

All the studies were carried out for a period of 21 days. At the end of which, blood samples were collected under fasting conditions, the serum was separated and were subjected to biochemical estimation. During the study, food intake, water intake and body weights of animals were recorded in all the groups. Blood collections, biochemical analysis, OGTT were carried out as explained earlier in type 1 diabetes studies.

All the animals were sacrificed by an overdose of diethyl ether and liver was excised immediately. Rat livers were excised and immediately frozen in dry ice and stored at -20°C. Frozen tissue from each rat was homogenized in ice cold 0.1 M Tris-HCL buffer (pH 7.4). The homogenate was centrifuged and supernatant was used for the estimation of proteins, lipid peroxidation, Superoxide dismutase, catalase and reduced glutathione.

### 3.2.4 Experimental induction of hyperlipidemia

Albino rats of wistar strain at the age of 20-24 weeks, weighing 250-300 gm were used in this 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. Hyperlipidemia was induced by supplementing normal drinking water with 10 % fructose in drinking water. Control rats received only normal drinking water without supplementation of fructose in it. Normal rats received only vehicle where as hyperlipidemic treated rats received drug treatment for 3 weeks in addition to fructose supplementation.
Study of effect of plant extracts on fructose induced hyperlipidemia involved 2 sets of experiments. The groups of animals in two different set were as follows:

Set 1: Effect of ethanolic extract of *Solanum nigrum* fruits (SNE) on fructose induced hyperlipidemic rats.
- GP I Normal control
- GP II Hyperlipidemic control
- GP III Hyperlipidemic treated with SNE (100 mg/kg, p.o.)
- GP IV Hyperlipidemic treated with SNE (250 mg/kg, p.o.)

Set 2: Study of effect of ethanolic extract of heart wood of *Acacia catechu* (ACE) on fructose induced hyperlipidemic rats.
- GP I Normal control
- GP II Hyperlipidemic control
- GP III Hyperlipidemic treated with ACE (250 mg/kg, p.o.)
- GP IV Hyperlipidemic treated with ACE (500 mg/kg, p.o.)

All the studies were carried out for a period of 21 days at the end of which blood samples were collected under fasting conditions, the serum was separated and were subjected to biochemical estimation. During the study, food intake, water intake and body weights of animals were recorded in all the groups. In these studies, biochemical parameters such as serum glucose, insulin, cholesterol, triglycerides, HDL-cholesterol, LDL- cholesterol, VLDL- cholesterol were estimated. Blood collection, biochemical analysis was carried out as explained in type I diabetes studies.

### 3.2.5 Estimation of Biochemical parameters

(A) 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 colored
compound. The intensity of the color produced is proportional to glucose concentration in the sample and is measured at 505 nm.

\[
\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2 \\
H_2O_2 + \text{Phenolic compound} + 4\text{-amino antipyrin} \xrightarrow{\text{POD}} \text{Red Compound} + 2H_2O_2
\]

**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 10μl of serum sample. Similarly standard and blank were prepared by using 10μl of glucose standard (provided in the kit) and distilled water respectively. They were then mixed and incubated and room temperature for 30 min (end point reaction). Absorbance of test and standard was measured against blank at 505 nm using UV-Visible spectrophotometer (UV-1700 Shimadzu, Japan).

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

\[
\text{Serum Glucose (mg/dL)} = \frac{\text{O.D. of Test}}{\text{O.D. of Std}} \times 100
\]

**B) Insulin (Radio immunoassay method)**

**Principle**

The assay is based upon the competition between unlabeled insulin in the standard samples and radioiodinated (I\(^{125}\)) 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. Measuring the radioactivity associated with the bound fraction of sample and standards quantitates insulin concentration of samples. 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 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 tubes except total and blank tubes.
7. The contents of the tubes were mixed gently and refrigerated at 2-4 °C overnight.
8. 100 μl of I^{125}-insulin reagent was added to all the tubes.
9. Contents of tubes were mixed gently and incubated at room temp. for 3 hr.
10. 100-μl second antibody was added to all tubes except total count tubes.
11. 1 ml of precipitating reagent (PEG) was added to all tubes except total count tubes.
12. All tubes were vortexed and incubated 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.

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. \[ \%B/Bo = \frac{\text{Corrected count of sample/standard test}}{\text{Corrected count of zero standard}} \times 100 \]
5. The standard curve of %B/Bo on the logit was plotted against μl/ml of insulin on the logarithmic scale using log-log graph paper.
6. The concentration of insulin in sample was read from the standard curve by extrapolation.

(C) Cholesterol

**Principle**

\[
\begin{align*}
\text{Cholesterol Ester} & + O_2 \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids} \\
\text{Cholesterol} & + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Dehydroacetone phosphate} + H_2O_2 \\
2H_2O_2 + \text{Phenol} + 4\text{-aminoantipyrine} & \xrightarrow{\text{Peroxidase}} \text{Red quinone} + 4H_2O_2
\end{align*}
\]

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 505 nm.

**Procedure**

Reagents were 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 ºC for min and absorbance was read against blank at 505 nm.

**Calculations**

\[
\text{Serum Cholesterol (mg/dL)} = \frac{\text{O.D. of Test}}{\text{O.D. of Std}} \times 200
\]

(D) Triglyceride

**Principle**

Triglycerides are enzymatically hydrolyzed to glycerol according to the following reactions.

\[
\begin{align*}
\text{Triglycerides} & + H_2O_2 \xrightarrow{\text{Lipoprotein lipase}} \text{Glycerol} + \text{Free fatty acids} \\
\text{Glycerol} & + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol-3-phosphate} + \text{ADP} \\
\text{Glycerol-3-phosphate} & + O_2 \xrightarrow{\text{GPO}} \text{Dehydroacetone phosphate} + H_2O_2 \\
2H_2O_2 + 4\text{-aminoantipyrine} + \text{ADPS} & \xrightarrow{\text{Peroxidase}} \text{Red quinone} + 4H_2O_2
\end{align*}
\]
GPO = Glycerol-3 phosphate oxidase
ADPS = N=Ethyl-N-Sulfopropyl-n-anisidine

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

Procedure
Reagents were 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 a min and absorbance was read against blank at 505 nm.

Calculations

\[
\text{Serum Triglyceride (mg/dL)} = \frac{\text{O.D. of Test}}{\text{O.D. of Std}} \times 200
\]

(E) HDL-Cholesterol

Principle
Chylomicrons, VLDL and LDL fractions in serum or plasma are separated from HDL by precipitating with phosphotungustic acid and magnesium chloride after centrifugation, the cholesterol method, using cholesterol esterase, cholesterol oxidase, peroxidase and the chromogen 4-amino antipyrine/phenol.

Procedure
Reagents are reconstituted as described in the procedure given with kit. 0.2 ml of serum sample/standard (50mg/dL) 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 blank, 1 ml of reconstituted reagent 1 was taken and absorbance of test samples was measured against reagent blank at 505 nm. The concentration of HDL-cholesterol in test samples was calculated using following formula. The results were expressed as mg/dL.
Materials and Methods

\[
\text{Serum HDL - C (mg/dL)} = \frac{\text{O.D. of Test}}{\text{O.D. of Std}} \times 50
\]

(F) Serum Glutamate Pyruvate Transaminase (SGPT)
(Reitman & Frankel’s method)

Principle

SGPT catalyses transfer of amino group from L-alanine to \(\alpha\)-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, colored in alkaline medium. The absorbance of this hydrazone derivative is correlated to SGPT activity by plotting a calibration curve using pyruvate standard.

\[
\begin{align*}
\text{L-alanine + } \alpha\text{-ketoglutarate} & \xrightarrow{\text{SGPT}} \text{Pyruvate + L-glutamate} \\
\text{Pyruvate + 2, 4-DNPH} & \xrightarrow{\text{Alkaline medium PH 7.4}} \text{2,4-dinitrophenyl hydrazone} \\
\text{Brown colored}
\end{align*}
\]

Procedure

Calibration Curve

In five test tubes buffered substrate, pyruvate standard, distilled water and DNPH color 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 505 nm and was read from calibration curve to find out enzyme activity.

Assay

0.25 ml buffered substrate was incubated at 37 °C for 5 min. 0.05 ml serum sample was added to buffered substrate, mix well and incubated at 37 °C for 30 min. to this DNPH color reagent was added, mix well and allowed to stand at room temperature for 20 min. finally 2.5 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. Absorbance of test samples was measured against purified water at 505 nm and was read on calibration curve to find out enzyme activity.

**G) Serum Glutamate Oxaloacetate Transaminase (SGOT)**

**(Reitman and Frankel’s Method)**

**Principle**

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

\[
\text{L-aspartate + α-ketoglutarate} \xrightarrow{\text{SGOT}} \text{Oxaloacetate + L-glutamate}
\]

\[
\text{Oxaloacetate + 2, 4-DNPH} \xrightarrow{\text{Alkaline medium}} 2, 4\text{-dinitrophenyl hydrazone}
\]

(Brown colored)

**Procedure**

**Calibration Curve**

In five clear test tubes buffered substrate, pyruvate standard distilled water and DNPH color 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 was measured against purified water at 505nm. A graph of absorbance was plotted against enzyme activity (supplied in leaflet).

**Assay**

0.25 ml buffered substrate was incubated at 37 °C for 5 min. 0.05 ml serum sample was added to buffered substrate, mix well and incubated at 37 °C for 60 min. to this DNPH color reagent was added, mix well 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. Absorbance of test samples was measured against purified water at 505 nm and was read on calibration curve to find out enzyme activity.

(H) 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 indophenols, which with alkali gives a blue colored compound. The intensity of the color is proportional to the concentration of urea in the sample and is measured at 546 nm. The color of the reaction is stable for 8 hrs.

**Procedure**
Reagents are reconstituted as described in the leaflet supplied along with the kit. 10 μl of serum sample, urea standard (40 mg/dL) (reagent 3) were added to reconstituted solution (solution I) (reagent 1) and mixed well. 0.25 ml diacetylmonoxime (Reagent 2) were added to all test tubes. Contents were mixed well and kept the tubes in the boiling water exactly for 10 min. cool immediately under running water for 5 minutes, mix by inversion and measured color intensity within 10 min 525 nm UV-Visible Spectrophotometer (UV-1700 Shimadzu, Japan).

The concentration of urea was calculated by using following formula.

\[
\text{Serum Urea (mg/dL)} = \frac{\text{O.D. of Test}}{\text{O.D. of Std}} \times 30
\]

(I) Creatinine

**Principle**
Creatinine in a protein free solution reacts with alkaline picrate and produces a red colored complex, which is measured colorimetrically at 520 nm.
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**Procedure**

*Deproteinization of test sample.*

0.5 ml of serum 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 was 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 (150 mg %) respectively. All the tubes were allowed to stand at room temperature after through mixing for 20 min. the absorbance of blank, standard and samples were measured immediately against distilled water at 520 nm.

**Calculations**

Serum creatinine concentration was calculated using following formula

\[
\text{Serum Creatinine (mg/dL)} = \frac{\text{O.D. of Test} - \text{O.D. of blank}}{\text{O.D. of Std} - \text{O.D. of blank}} \times 3
\]

**(J) In vivo assay for evaluation of antioxidant activity of Solanum nigrum**

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

0.1 ml of supernatant was mixed with 4 ml of alkaline copper sulphate solution. After 10 min 0.4 ml of folin’s ciocolteau reagent (33%) was added and optical density was measured after 10 min at 610 nm against similarly prepared blank using distilled water. The protein content was obtained by calculating with the help of standard curve prepared by using bovine albumin and was expressed in terms of mg/ml of tissue.

(b) **Measurement of lipid peroxidation**

Lipid peroxidation was measured by the method reported by Raja et al. (2007). Acetic acid 1.5 ml (20%; pH 3.5), 1.5 ml of thiobarbituric acid (0.8%) and 0.2 ml of sodium dodecyl sulfate (8.1%) were added to 0.1 ml of supernatant and heated at 100°C for 60 min. The mixture was cooled, 5 ml of n-butanol-pyridine (15:1) mixture and 1 ml of distilled water was added and vortexed vigorously. After centrifugation at 1200 x g for 10 min, the organic layer was separated and absorbance was measured at 532nm.
using spectrophotometer. Malonyldialdehyde (MDA) is an end product of lipid peroxidation which reacts with thiobarbituric acid to form pink chromogen (TBARS). It was calculated using MDA as a standard. It was expressed as nanograms of TBARS/mg of protein.

(c) Superoxide dismutase (SOD)
SOD activity was analyzed by the method described by Raja et al. (2007). Assay mixture contained 0.1 ml of supernatant, 1.2 ml of sodium pyrophosphate buffer (pH 8.3; 0.052 M), 0.1 ml of phenazine methosulphate (186 µm), 0.3 ml of nitro blue tetrazolium, 300 µM, 0.2 ml of NADH (750 µM). Reaction was started by addition of NADH. After incubation at 30 °C for 90 s, the reaction was stopped by addition of 0.1 ml of glacial acetic acid. Reaction mixture was stirred vigorously with 4.0 ml of n-butanol. Colour intensity of the chromogen in butanol was measured spectrophotometrically at 560 nm and concentration of SOD was expressed as U/mg of protein.

(d) Catalase (CAT)
Catalase activity was measured by the method of Beers et al. (1952) and Stern (1937). 1 ml of supernatant was added to cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). Reaction was started by addition of 2.9 ml of freshly prepared 0.036 (%w/w) H₂O₂ and mixing by inversion. Time required for the absorbance to decrease from 0.45 to 0.40 AU at 240nm was recorded. Activity of catalase was calculated using the formula:

\[
\text{Units/ml of supernatant} = \frac{(3.45) (df)/(min)(0.1)}
\]

\[
\text{Units/ mg of protein} = \frac{\text{Units/ml of supernatant}}{\text{mg of protein/gm of tissue}}
\]

(e) Reduced glutathione (GSH)
The GSH was determined by the method of Elman et al. (1959). 0.2 ml of tissue homogenate was mixed with 1.8 ml of EDTA solution. To this 3.0 ml of precipitating reagent (1.67 g of ortho phosphoric acid, 0.2 g of EDTA disodium salt, 30 g sodium chloride in 1 l of distilled water) was added, mixed thoroughly and kept for 5 minutes before centrifugation. To 2 ml of this filtrate, 4 ml of 0.3 M disodium hydrogen
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phosphate solution and 1 ml of 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) reagent were added and read at 412 nm. The concentration was expressed as µg/mg of protein.

In vitro assay for evaluation of antioxidant activity of Solanum nigrum fruit extract

(A) Reducing power ability
The samples prepared for ferric cyanate method was used for this and the other antioxidant assays. The reducing power of plant extract was determined by the method of Oyaizu (1982). The capacity of extract to reduce the ferric-ferricyanide complex to the ferrous-ferricyanide complex of Prussian blue was determined by recording the absorbance at 700 nm after incubation. For this purpose, different concentrations of plant extract (50-1000 µg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. Aliquots (2.5 ml) of Trichloroacetic acid (TCA, 10%) were added to the mixture. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1%). The absorbance was measured at 700 nm by spectrophotometer. Increased absorbance of the reaction mixture indicates increased capability.

(B) DPPH radical scavenging activity (Navarro et al., 1993)
The free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH. 0.1mM solution of DPPH in methanol was prepared and 1.0 ml of this solution was added to 3.0 ml of extract solution in water at different concentrations (50-1000µg/ml). it was incubated at room temperature for 45 minutes and the absorbance was measured at 517 nm against corresponding blank solution. Ascorbic acid was taken as reference. Percentage inhibition of DPPH free radical was calculated based on the control reading, which contain DPPH and distilled water without any extract using the following equation

\[
\text{DPPH Scavenged} \text{ (%) = } (A_{\text{cont}} - A_{\text{test}}) / A_{\text{cont}} \times 100
\]

Where \( A_{\text{cont}} \) is the absorbance of the control reaction and \( A_{\text{test}} \) is the absorbance in the presence of the sample of the extracts.
The antioxidant activity of the extract was expressed as IC\textsubscript{50}. The IC\textsubscript{50} value was defined as the concentration (in μg/ml) of extracts that inhibits the formation of DPPH radicals by 50%.

(C) Nitric oxide scavenging activity assay.
Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract at various concentrations and the mixture incubated at 25°C for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 ml maphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min. The absorbance at 540 nm was measured with a spectrophotometer. The nitric oxide radicals scavenging activity was calculated according to the following equation:

\[
\% \text{ Inhibition} = \left[ \frac{(A_0 - A_1)}{A_0} \times 100 \right]
\]

Where \(A_0\) was the absorbance of the control (blank, without extract) and \(A_1\) was the absorbance in the presence of the extract.

(D) H\textsubscript{2}O\textsubscript{2} scavenging activity
H\textsubscript{2}O\textsubscript{2} scavenging ability of extract was determined according to the method of Ruch et al. (1989). A solution of H\textsubscript{2}O\textsubscript{2} (40mM) was prepared in phosphate buffer (pH 7.4). Extract at the different concentrations (50-1000μg/ml) in 3.4 ml phosphate buffer were added to a H\textsubscript{2}O\textsubscript{2} solution (0.6ml, 40mM). The absorbance value of the reaction mixture was containing the phosphate buffer without H\textsubscript{2}O\textsubscript{2}. The percentage of H\textsubscript{2}O\textsubscript{2} scavenging of extract and standard compounds was calculated as:

\[
\% \text{ Scavenged} = \left[ \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \right] \times 100
\]

Where \(A_{\text{control}}\) is the absorbance of the control, and \(A_{\text{sample}}\) is the absorbance in the presence of the sample of extract.

(E) β-Carotene–linoleate bleaching assay
The antioxidant activity of ethanolic extract of \textit{Solanum nigrum} was assayed based on the β-carotene bleaching method. Ascorbic acid was used as the standard, β-carotene
(0.2 mg in 1 ml chloroform), linoleic acid (0.02 ml) and Tween 20 (0.2 ml) were transferred into a round-bottomed flask. The mixture was then added to 0.2 ml of extract or standard or methanol (as control). Chloroform was removed at room temperature under vacuum at reduced pressure using a rotary evaporator. Following evaporation, 50 ml of distilled water was added to the mixture, and then shaken vigorously to form an emulsion. Two ml aliquots of the emulsion were pipetted into test tubes and immediately placed in a water bath at 50°C. The absorbance was read at 30 min intervals for 2 h at 470 nm.

Antioxidant activity (AA) was expressed as percent of inhibition relative to the control, using the following formula:

$$AA = \frac{[(R_{\text{CONTROL}} - R_{\text{SAMPLE OR STANDARDS}})/R_{\text{CONTROL}}] \times 100}{\text{Where, Rcontrol and Rsample represent the bleaching rates of } \beta\text{-carotene without and with the addition of antioxidant, respectively.}}$$

**Statistical analysis**

Results were expressed as mean ± SEM. Differences among data were determined using one way analysis of variance (ANOVA) followed by Dunnet's multiple comparison tests. p<0.05 were considered to be statistically significant.