## CHAPTER 4 - MATERIALS AND METHODS

Table 5: Materials and Major Equipments used

<table>
<thead>
<tr>
<th>S. No</th>
<th>Materials and Equipments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Bruker NMR Spectrophotometer</td>
</tr>
<tr>
<td>2.</td>
<td>Buchhi Rotavapor apparatus</td>
</tr>
<tr>
<td>3.</td>
<td>Cholesterol [Loba chemie]</td>
</tr>
<tr>
<td>4.</td>
<td>Cholic acid [Loba chemie]</td>
</tr>
<tr>
<td>5.</td>
<td>Dessicator</td>
</tr>
<tr>
<td>6.</td>
<td>HDL – cholesterol kit [Beacon Diagnostics]</td>
</tr>
<tr>
<td>7.</td>
<td>Hot air oven</td>
</tr>
<tr>
<td>8.</td>
<td>Lard oil [Sigma Chem.]</td>
</tr>
<tr>
<td>9.</td>
<td>Rat Leptin ELISA kit [Crystal Chem. Inc]</td>
</tr>
<tr>
<td>10.</td>
<td>Serum glucose kit [Beacon Diagnostics]</td>
</tr>
<tr>
<td>11.</td>
<td>SGPT and SGOT kits [Erba Mannheim Chem.]</td>
</tr>
<tr>
<td>12.</td>
<td>Shimadzu Mass spectrophotometer</td>
</tr>
<tr>
<td>13.</td>
<td>Shimadzu UV-Visible spectrophotometer</td>
</tr>
<tr>
<td>14.</td>
<td>Sibutramine [Symed Laboratories]</td>
</tr>
<tr>
<td>15.</td>
<td>Star 21 plus Semi-Auto analyzer</td>
</tr>
<tr>
<td>16.</td>
<td>Thermonicolet FTIR 200 spectrophotometer</td>
</tr>
<tr>
<td>17.</td>
<td>Tissue Homogenizer</td>
</tr>
<tr>
<td>18.</td>
<td>Total – cholesterol kit [Beacon Diagnostics]</td>
</tr>
<tr>
<td>19.</td>
<td>Triglycerides kit [Beacon Diagnostics]</td>
</tr>
</tbody>
</table>
4.1. COLLECTION AND STANDARDIZATION OF PLANT MATERIALS:  

The rhizomes of *Alpinia galanga* (AG) and roots of *Argyreia speciosa* (AS) were collected in the month of August 2007 from the fields of Belgaum district, Karnataka. The plant was identified and authenticated by Dr. S. R. Yadav, Prof. and Head, Department of Botany, Shivaji University, Kolhapur, Maharashtra.

The rhizomes and roots were cut into small pieces, cleaned and shade dried, and were subjected to size reduction to get coarse powder and then passed through sieve no.40 to get uniform powder. Then uniform powder was subjected to standardization with different parameters as below.

4.1.1. Physical tests – Nature, colour, odour and taste of the powder.

4.1.2. Determination of Extractive values:

Extractive values help to determine the amount of soluble constituents of a plant material in used solvents. The extraction of any crude drug with a particular solvent yields a solution containing different phytoconstituents. The composition of these phytoconstituents in that particular solvent depends upon the nature of drug and solvent used.

**Determination of alcohol-soluble extractive value:**

The 5 g of the air dried coarsely powdered drug was macerated with 100 ml of 70% ethanol in a closed flask for 24 h, shaking frequently during 6 h and allowed to stand for 18 h. The solution was filtered rapidly taking precautions against loss of ethanol. 25 ml of the filtrate was evaporated to dryness in a tarred flat bottom shallow dish, dried at 105 ± 1°C and weighed. The % of alcohol-soluble extractive was calculated with reference to the air dried drug.
Determination of water soluble extractive value:

The above procedure was used for water soluble extractive value; instead of ethanol, chloroform water IP was used.

Determination of chloroform soluble extractive value:

The above procedure was used for chloroform soluble extractive value; instead of ethanol, chloroform was used.

Determination of petroleum ether soluble extractive value:

The above procedure was used for petroleum ether soluble extractive value; instead of ethanol, pet. ether (40-60°C) was used.

4.1.3. Loss on Drying:

An accurately weighed quantity of the shade-dried coarsely powdered plant material was taken in a tarred glass bottle and the initial weight was taken. The crude drug was heated at 105 ± 1°C in an oven for 5 h, cooled and then weighed. This procedure was repeated till a constant weight was obtained. The moisture content of the sample was calculated using following formula:

\[
\text{Loss on drying (\%)} = \frac{\text{Loss in weight}}{\text{Weight of crude drug}} \times 100
\]

4.1.4. Determination of Total Ash:

The total ash method is designed to measure the total amount of material remaining after ignition. This includes both physiological and non-physiological ash which is the residue of the extraneous matter adhering to the plant surface.

About 2 g of air dried powdered drug was weighed accurately in a tarred silica crucible and incinerated at a temperature not exceeding 450 ± 10°C until free from carbon, cooled and weighed. The % total ash value was calculated using the formula:

\[
\text{Total ash value of sample \%} = \frac{\text{Weight of ash}}{\text{Weight of drug}} \times 100
\]
4.1.5. Acid Insoluble Ash:

Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth. Ash obtained was boiled with 25 ml of 2M hydrochloric acid for 5 min, collected the insoluble matter on an ash less filter paper, washed with hot water, ignited to a temperature not more than 450 ± 10°C, cooled in a dessicator and weighed. The % of acid-insoluble ash was calculated by using the following formula:

\[
\text{Acid insoluble ash value of the sample} \% = \frac{\text{Weight of the residue}}{\text{Weight of air dried drug}} \times 100
\]

4.1.6. Water Soluble Ash:

Water soluble ash is the difference in weight between the total ash and the residue after treatment of the total ash with water. Boil the ash for 5 min with 25 ml of water, the insoluble matter was collected on an ash less filter paper, washed with hot water and ignited for 15 min at a temperature not more than 450 ± 10°C. Weight of the insoluble matter was subtracted from the water-soluble ash to get the weight of water soluble ash. % of water-soluble ash was calculated using the formula:

\[
\text{Water soluble ash value of the sample} \% = \frac{\text{Water soluble ash}}{\text{Weight of air dried drug}} \times 100
\]

The results of standardization of plant materials are tabulated in table no. 7.

4.2. EXTRACTION

The powder of plant materials was charged in to the thimble of a Soxhlet apparatus and extracted separately using petroleum ether (40-60°C) for 18 h, 70% ethanol with 32 h and chloroform for 18 h. Appearance of colourless solvent in the
siphon tube was the indication of exhaustive extraction and based on that the further extraction was terminated. For aqueous extract, the powder was macerated with cold water for 7 days with occasional shaking.

After the effective extraction, solvents were concentrated at room temperature in reduced pressure using a rotary evaporator (Bucchi Rota evaporator) and the extract obtained with each solvent was weighed. The percentage yield was calculated and noted in table no. 8. The perfectly dried extract was then stored in an air tight container till used. The extract was divided into two parts. One part was used for phytochemical analysis and other part was used for pharmacological investigations. The extracts were prepared as suspension by mixing with 1% tween 80 as suspending agent before oral administration to the animals,

4.3. PRELIMINARY PHYTOCHEMICAL TESTS OF EXTRACTS:

All the extracts were subjected for systematic qualitative chemical tests to identify the presence of various phytoconstituents and the results are tabulated in table no. 9.

1. Tests for Carbohydrates:

Molisch's test (General test): To 2-3 ml test solution, added few drops of α-naphthol solution in alcohol, shaken and added concentrated H₂SO₄ from sides of the test tube and observed for violet ring at the junction of two liquids.

For Reducing Sugars:

a) Fehling's test: 1 ml Fehling’s A and 1 ml Fehling’s B solutions was mixed and boiled for one minute. Added equal volume of test solution. Heated in boiling water bath for 5-10 min was observed for a yellow, then brick red precipitate.

b) Benedict's test: Equal volume of Benedict's reagent and test solution in test tube were mixed. Heated in boiling water bath for 5 min. Solution may appear green, yellow or red
depending on amount of reducing sugar present in test solution.

2. Tests for Proteins:

a) Biuret test (General test): To 3 ml test solution added 4% NaOH and few drops of 1% CUSO₄ solution observed for violet or pink colour.

b) Million’s test (for proteins): Mixed 3 ml test solution with 5 ml Million’s reagent, white precipitate obtained. Precipitate warmed turns brick red or precipitate dissolves giving red colour was observed.

c) Xanthoprotein test (For protein containing tyrosine or tryptophan): Mixed 3 ml test solution with 1 ml concentrated H₂SO₄ and observed for white precipitate.

3. Tests for sterols: The test solution was dissolved in chloroform, filtered and the filtrate was tested for sterols.

a). Salkowski test: Few drops of concentrated sulphuric acid was added to the chloroform solution, shaken and allowed to stand, appearance of red colour in lower layer indicates the presence of sterols.

b). Liebermann - Burckhardt test: To the chloroform solution, few drops of acetic anhydride were added and mixed well. 1 ml of concentrated sulphuric acid was added from the sides of the test tube, appearance of green fluorescence in chloroform layer indicates the presence of sterols.

4. Tests for flavonoids:

The test solution was prepared by dissolving extracts in ethanol and the following test were carried-out.

a). Shinoda test: To the test solution of extract, a few fragments of magnesium ribbon and concentrated hydrochloric acid was added. Appearance of magenta colour after few min indicates the presence of flavonoids.

b). Ferric chloride test: Few drops of neutral ferric chloride solution were added to little
quantity of test solution. Formation of violet colour indicates the presence of phenolic nucleus.

\textit{c). Zinc-Hydrochloric acid reduction test:} The test solution was treated with a pinch of zinc dust and few drops of concentrated hydrochloric acid. Formation of magenta colour after few min indicates the presence of flavonoids.

\textbf{5. Tests for Alkaloids:} 0.5 g extract was dissolved in 10 ml of dilute hydrochloric acid and filtered. The filtrate was used to test the presence of alkaloids

a) \textit{Dragendorff’s test:} To 2-3 ml filtrate added few drops Dragendorff’s reagent observed for orange brown precipitate.

b) \textit{Mayer's test:} 2-3 ml filtrate with few drops Mayer's reagent observed for precipitate.

c) \textit{Hager’s test:} 2-3 ml filtrate with Hager’s reagent observed for yellow precipitate.

d) \textit{Wagner's test:} 2-3 ml filtrate with few drops of Wagner's reagent observed for reddish brown precipitate.

\textbf{6. Tests for Tannins and Phenolic Compounds:} To 2-3 ml test solution, added few drops of following solution and observed for the corresponding colour formation.

a) 5\% Ferric chloride solution: Deep blue-black colour.

b) 10\% Lead acetate solution: - White precipitate.

c) Gelatin solution: - White precipitate.

d) Bromine water: Discoloration of bromine water.

e) Acetic acid solution: Red colour solution.

\textbf{7. Tests for Glycosides:}

\textit{General test for Glycosides:}

Part A: To 2-3 ml of extract added dil H\textsubscript{2}SO\textsubscript{4} and heat on a water bath for 1-2 min. Neutralize with 10\% NaOH, check with litmus paper and to resulting solution add Fehling’s A and B. Increased red precipitate in this case shows glycosides are present.
Part B: To 2-3 ml of extract added water and heat. According to NaOH added for neutralization added equal quantity of water. To the resulting solution added Fehling’s A and B. Increased red precipitate in this case shows absence of glycosides.

Compare Part A and B.

Tests for Cardiac Glycosides:

a) Baljet's test: A test solution observed for yellow to orange colour with sodium picrate.

b) Legal's test: To aqueous or alcoholic test solution, added 1 ml pyridine and 1 ml sodium nitroprusside and observed for pink to red colour.

c) Test for deoxysugars (Keller Killani test): To 2 ml extract added glacial acetic acid, one drop of 5% FeCl₃ and concentrated H₂SO₄ observed for reddish brown colour at junction of the two liquid and upper layers bluish green.

Tests for Saponin Glycosides:

a) Foam test: The drug extract or dry powder was shaken vigorously with water. Persistent foam was observed.

b) Haemolytic test: Added test solution to one drop of blood placed on glass slide. Haemolytic zone whether appeared was observed.

Test for anthroquinones glycosides:

a) Borntrager's test: Boil powder drug with 5ml of 10% Sulphuric acid for 5 min. Filter while hot, cool the filtrate shake gently with equal volume of benzene. Benzene layer was separated, and then treated half of its volume with solution of ammonia (10%). Allowed to separate, ammonical layer acquires rose pink colour due to the presence of anthroquinones.

4.4. EXPERIMENTAL ANIMALS:

Albino wistar rats weighing 150 – 200 g of either sex were obtained from central animal house, KLEU College of Pharmacy, Belgaum. They were housed in clean
polypropylene cages under standard laboratory conditions at 22 ± 2°C with light (12 h): dark (12 h) cycle. The animals were provided with normal pellet chow and water *ad libitum* for a period of 1 week for acclimatization. In all experimental sets, 6 rats were used for each group. All animal studies were performed in accordance to guideline of CPCSEA and Institutional Animal Ethical Committee (IAEC) of J. N. Medical College, KLE University, Belgaum, Karnataka. (CPCSEA registration number: 627/02/a/CPCSEA).

4.5. **ACUTE ORAL TOXICITY:**

The acute oral toxicity was carried out as per the guidelines set by Organization for Economic Co-operation and Development (OECD), revised draft guidelines 423, received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.\(^{171}\)

It involved a stepwise procedure, each step using 03 animals. The study was started at 300 mg/kg body weight and proceeded further at lower dose (50 and 5 mg/kg) or higher dose (2000 mg/kg) depending on the presence or absence of compound related mortality of the animals, to determine the LD\(_{50}\) value. Absence or presence of compound related mortality of the animals dosed at the step will determine the next step of;

- No further testing is required
- Dosing of three additional animals with the same dose
- Dosing of 3 animals at the next higher or the next lower dose level

**Description of the method:**

1) *Selection of animal species:* Healthy young albino rats of either sex weighing 150-200 g were used for acute toxicity study to determine LD\(_{50}\) of all the extracts.
2) **Housing and feeding condition:** The temperature in the experimental room was around 25°C. Lighting was natural sequence being 12 h dark and 12 h light. The conventional laboratory diet was fed with adequate supply of drinking water.

3) **Preparation of Animals:** The animals were randomly selected, marked to permit individual identification and kept in polypropylene cages for one week prior to dosing to allow acclimatization of them to laboratory conditions.

4) **Preparation of doses:** All the extracts were prepared as a suspension by triturating with water and 1% Tween 80.

5) **Administration of doses:** The test substances are administered in a single dose by gauge using a stomach tube. Prior to dosing, animals were kept for 12 h of fasting. Then animals were weighed and test substance was administered. After the dose was administered food was withheld for further 3-4 h.

6) **Number of animals and dose levels:** In each step three animals were used in each group. Study was begun at 300mg/kg body weight and continued up to 2000 mg/kg body weight. The procedure of dose selection and finalizing LD$_{50}$ cut off values is as below.

**Observations:**

Animals were observed individually after dosing at least once during the first 30 min, periodically during the first 24 h, with special attention given during the first 4 h, and daily thereafter, for a total of 14 days.

**Results:** No toxic symptoms or mortality was observed till the end of the study. All the extracts were found to be safe and devoid of any drug related mortality, when tested even at the maximum dose of 2000 mg/kg body weight in rats. The LD$_{50}$ value for all the extracts was considered to be 2000 mg/kg body weight and 1/4$^{th}$ of LD$_{50}$ value i.e. 500
mg/kg body weight was considered as experimental dose for the further pharmacological screening of the extracts in laboratory animals.

4.6. CAFETERIA AND ATHEROGENIC DIETS:

The cafeteria diet (CD) consisted of 3 diets (condensed milk 48 g + bread 48 g), (chocolate 18 g + biscuits 36 g + dried coconut 36 g) and (cheese 48 g + boiled potatoes 60 g). The three diets were given to each group of 6 rats on day 1, 2 and 3 respectively and then repeated in same succession for six weeks in addition to normal pellet chow. The atherogenic diet (AD) consisted of normal pellet chow mixed with cholesterol (1%), cholic acid (0.5%) and lard oil (5%). It was provided to each group of 6 rats everyday for six weeks. The diets were prepared by mixing the powdered pellet diet with high fat diet ingredients to obtain a soft mass.

Table 6: Composition and Calorie value of Cafeteria diet.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Calorie value (kcal/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condensed milk</td>
<td>335</td>
</tr>
<tr>
<td>Bread</td>
<td>230</td>
</tr>
<tr>
<td>Chocolate</td>
<td>550</td>
</tr>
<tr>
<td>Biscuit</td>
<td>360</td>
</tr>
<tr>
<td>Dried coconut</td>
<td>660</td>
</tr>
<tr>
<td>Cheese</td>
<td>320</td>
</tr>
<tr>
<td>Boiled potato</td>
<td>80</td>
</tr>
</tbody>
</table>

4.7. DETERMINATION OF ANTI-OBESITY ACTIVITY:

The anti-obesity activity of various extracts of AG rhizomes and AS roots was studied by using two different models of high fat diet induced obesity in experimental rats i.e. Cafeteria diet (CD) induced obesity and Atherogenic diet (AD) induced obesity.
Materials and Methods

4.7.1. Cafeteria Diet Induced Obesity in Rats:

Albino rats of either sex were randomly selected and were divided into following 11 groups of 6 animals each and given respective treatment for 6 weeks.

Group I  -  Normal control (normal pellet chow + water *ad libitum*).
Group II -  Cafeteria diet control (CD + Normal diet +1 ml tween 80 solution).
Group III -  CD + Normal diet + Sibutramine (2 mg/kg/day i.p.)
Group IV -  CD + Pet. ether extract of AG rhizomes (500 mg/kg/day p.o.)
Group V  -  CD + Chloroform extract of AG rhizomes (500 mg/kg/day p.o.)
Group VI -  CD + Ethanol extract of AG rhizomes (500 mg/kg/day p.o.)
Group VII -  CD + Aqueous extract of AG rhizomes (500 mg/kg/day p.o.)
Group VIII -  CD + Pet. ether extract of AS roots (500 mg/kg/day p.o.)
Group IX -  CD + Chloroform extract of AS roots (500 mg/kg/day p.o.)
Group X  -  CD + Ethanol extract of AS roots (500 mg/kg/day p.o.)
Group XI -  CD + Aqueous extract of AS roots (500 mg/kg/day p.o.)

4.7.2. Atherogenic Diet Induced Obesity in Rats:

Albino rats of either sex were randomly selected and were divided into following 11 groups of 6 animals each and given respective treatment for 6 weeks.

Group I  -  Normal control (normal pellet chow + water *ad libitum*).
Group II -  AD control (AD + Normal diet + 1 ml of tween 80 solution).
Group III -  AD + Normal diet + Sibutramine (2 mg/kg/day i.p.)
Group IV -  AD + Pet. ether extract of AG rhizomes (500 mg/kg/day p.o.)
Group V  -  AD + Chloroform extract of AG rhizomes (500 mg/kg/day p.o.)
Group VI -  AD + Ethanol extract of AG rhizomes (500 mg/kg/day p.o.)
Group VII -  AD + Aqueous extract of AG rhizomes (500 mg/kg/day p.o.)
Materials and Methods

Group VIII - AD + Pet. ether extract of AS roots (500 mg/kg/day p.o.)
Group IX  - AD + Chloroform extract of AS roots (500 mg/kg/day p.o.)
Group X   - AD + Ethanol extract of AS roots (500 mg/kg/day p.o.)
Group XI  - AD + Aqueous extract of AS roots (500 mg/kg/day p.o.)

4.7.3. Common parameters studied:

Measurement of Body weight

The individual animals were weighed at the start of the experiment and then every week thereafter. The % change in body weight produced by various extracts with respect to normal diet and high fat diet control rats was calculated.

Measurement of Food intake

Food intake of each group of animals was determined initially and then every week thereafter by measuring the difference between the preweighed chows and weight of the food that remained after 24 h.

Blood Biochemical Analysis

On day 42, blood was collected by retro-orbital puncture in ether-anaesthetized rats and subjected to centrifugation to obtain serum. The serum levels of glucose, total-cholesterol, HDL-cholesterol and triglycerides (TGs), aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT) were estimated using the biochemical kits. The serum VLDL–cholesterol and LDL–cholesterol levels were calculated using Friedwald’s formula:\textsuperscript{174}

\[
\text{LDL – cholesterol} = \text{Total cholesterol} – (\text{HDL – cholesterol} + \text{VLDL - cholesterol}).
\]

Where VLDL – cholesterol = Triglycerides/5
The atherogenic index of plasma (AIP) was calculated by using: AIP = log (TGs / HDL). The concentration of leptin in the serum was also measured using rat leptin ELISA kit.

**Determination of Liver weight and Parametrial adipose tissue weight**

Animals were then killed with an overdose of diethyl ether. The livers and parametrial adipose tissues were quickly removed and weighed. The liver tissues were rinsed with ice cold distilled water and immediately stored at −20°C until biochemical analysis was performed.

**Estimation of liver triglyceride content**

The liver triglycerides content was determined directly by using modified method of Van Handel and Zilversmit.175

**Estimation of liver lipid peroxidation and antioxidant enzymes**

The liver tissue was homogenized in 10 volume of 0.1 M phosphate buffer (pH 7.4) containing 5 mM EDTA and 0.15 M NaCl, and centrifuged at 9000 rpm for 30 min at 4°C. The supernatant was collected and used for the assay of lipid peroxidation and antioxidant enzyme activities viz., superoxide dismutase, catalase and glutathione peroxidase.

**Histopathological studies of liver tissue**176:

The isolated liver was cut into small pieces, preserved and fixed in 10% formalin for two days. Then the liver piece was rinsed with running water for about 5 to 10 min to remove the formalin and was followed by dehydration with isopropyl alcohol of increasing strength (70%, 80% and 90%) for 1 h each. Then finally dehydration was done using absolute alcohol with about three changes for 1 h each. Dehydration was performed to remove all traces of water. Further alcohol was removed by using
chloroform and chloroform removed by paraffin infiltration. The clearing was done by using chloroform with two changes for 15 to 20 min each. After paraffin infiltration the liver pieces were subjected to automatic tissue processing unit.

*Embedding in paraffin vacuum:*

Hard paraffin was melted and the hot paraffin was poured into L-shaped blocks. The liver pieces were then dropped into the molten paraffin quickly and allowed to cool.

*Sectioning:*

The blocks were cut using microtome to get sections of thickness of 5μ. The sections were taken on a micro slide on which egg albumin i.e., sticking substance was applied. The sections were allowed to remain in an oven at 60 ± 1°C for 1 h. Paraffin melts and egg albumin denatures, thereby fixing tissue to slide.

*Staining:*

Haematoxylin, a basic stain which stains all the acidic cell components blue i.e.: DNA in the nucleus. Eosin is an acid stain, hence it stains all the cell constituents pink which are basic in nature i.e., cytoplasm. Haematoxylin was allowed to act for 1 min followed by washing under tap water for 2 min, dehydration with alcohol of increasing strength each for 2 min. Then the counter stain Eosin made to act for 1 min followed by dehydration as above. The stained sections were observed microscopically for the histological changes.

**4.8. PROCEDURE FOR SERUM AND LIVER BIOCHEMICAL ESTIMATIONS**

**4.8.1. Estimation of Serum Glucose Levels:**

The glucose levels were estimated by using GOD – POD endpoint colorimetric method by Trinder.
This method is simple, single stepped, rapid, reliable and there is precision. Trinder’s method (1969) utilizes two enzymes glucose oxidase (GOD) and peroxidase (POD) along with the chromogen L-amino antipyrine and phenol. This method is intended for in vitro quantitative determination of glucose in serum / plasma or cerebrospinal fluid. There was no interference due to the substances like creatinine, fructose, galactose, reduced glutathione, ascorbic acid and xylose.

**Principle:**

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide so formed reacts under catalysis of peroxidase with phenol and 4-amino antipyrine (4AAP) to form a red coloured quinoneimine compound which is measured at 505nm and the intensity is directly proportional to glucose concentration.

\[
\text{Glucose + O}_2 + \text{H}_2\text{O} \xrightarrow{\text{GOD}} \text{Gluconic Acid + H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{AAP} + \text{Phenol} \xrightarrow{\text{POD}} \text{Red Quinoneimine Complex + H}_2\text{O}
\]

**Kit contents:**

- Reagent –1 Glucose reagent -- 5 vials
- Reagent –2 Glucose standard (100mg/dl) -- 5 ml

**Working reagent components:**

1. Glucose oxidase - > 15 KU/L
2. Peroxidase - > 3 KU/L
3. 4-Aminoantipyrine - 0.3 mM/L
4. Phenol - 5 mM/L
5. Phosphate buffer - 200 mM/L
Preparation of working reagent:

Allowed the vial to attain room temperature. Dissolved the contents of each vial using Glucose diluent with special lipid clearing agent. Make up the final volume to 500 ml and transferred to a clean dry amber colored bottle.

Procedure:

<table>
<thead>
<tr>
<th>Pipette into tubes marked</th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
<td>--</td>
<td>--</td>
<td>0.01 ml (10 μl)</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>0.01 ml (10 μl)</td>
<td>--</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.01 ml (10 μl)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Working Reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

Mixed and incubated for 10 min at 37 ± 1°C. The absorbance of test and standard at 500 nm was recorded against reagent blank.

Calculation:

\[
\text{Glucose (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 100
\]

4.8.2. Estimation of Serum Cholesterol Level\textsuperscript{178}:

Method: Estimation of serum cholesterol by the end point enzymatic method.

Principle: Cholesterol estimation is an enzymatic method using cholesterol esterase, cholesterol oxidase and peroxidase. Cholesterol esterase hydrolyses cholesterol esters into free cholesterol and fatty acids. Cholesterol oxidase converts cholesterol to cholest-4-en-3- one and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide oxidatively couples with 4-aminoantipyrine and phenol to produce quinoneimine dye, which has absorbance maximum at 510 nm. The intensity of red colour is proportional to the amount of cholesterol.
Materials and Methods

Cholesterol esterase
Cholesterol - ester + H₂O → Cholesterol + Fatty acid.

Cholesterol oxidase
Cholesterol + O₂ → H₂O₂ + Cholesterol-4-en-3-one.

Peroxidase
2H₂O₂ + 4 Amino-antipyrine + Phenol → Quinoneimine + 4H₂O.

Reagents: Sodium cholate 0.5mM/L, phenol 28 mM/L, Cholesterol esterase >0.2 U/ml, cholesterol oxidase >0.1 U/ml, peroxidase >0.8 U/ml, 4-aminoantipyline 0.5 mM/L, pH 7.0 Cholesterol standard: - Cholesterol 200 mg/dl.

Procedure: Three test tubes labelled as test, standard and blank were taken. The following solutions were pipetted into the respective tubes.

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol STD</td>
<td>--</td>
<td>10 μl</td>
<td>--</td>
</tr>
<tr>
<td>Sample</td>
<td>10 μl</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Working Reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>--</td>
<td>--</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

The above solutions were mixed well and incubated at 37 ± 1°C for 5 min. The absorbance of standard and test was measured against the blank at 500 nm.

Calculations:

\[
\text{Cholesterol (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 200
\]
4.8.3. Estimation of Serum Triglycerides:

Method: Estimation of serum triglycerides by glycerol phosphate oxidase method.

Principle: Lipoprotein lipase hydrolyses triacylglycerol to glycerol and free fatty acids. The glycerol formed with ATP in the presence of glycerol kinase forms glycerol-3-phosphate, which oxidized by glycerol phosphate oxidase to form hydrogen peroxide. The hydrogen peroxide further reacts with phenolic compound and 4-amino antipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of triglyceride present in the sample.

\[
\begin{align*}
\text{Lipoprotein lipase} & : \text{Triglyceride} \rightarrow \text{glycerol + free fatty acids} \\
\text{Glycerol kinase} & : \text{Glycerol + ATP} \rightarrow \text{Glycerol – 3-phosphate + ADP} \\
\text{Glycerol phosphate oxidase} & : \text{Glycerol-3-Phosphate} \rightarrow \text{Dihydroxyacetone Phosphate + H}_2\text{O}_2 \\
\text{Peroxidase} & : \text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine + Phenol} \rightarrow \text{Red Quinoneimine + H}_2\text{O}.
\end{align*}
\]

Procedure:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides reagent</td>
<td>1000 μl</td>
<td>1000 μl</td>
<td>1000 μl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>--</td>
<td>--</td>
<td>10 μl</td>
</tr>
<tr>
<td>Triglyceride Std</td>
<td>--</td>
<td>10 μl</td>
<td>--</td>
</tr>
<tr>
<td>Serum</td>
<td>10 μl</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
The above solutions were mixed well and incubated at 37 ± 1°C for 5 min. The absorbance of standard and test was measured against the blank at 540 nm.

**Calculations:**

\[
\text{Triglycerides (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Std.}} \times 200
\]

**4.8.4. Estimation of Serum HDL-Cholesterol Level**

**Method:** The estimation of serum HDL-C was carried out by phosphotungstate precipitation method.

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

**Reagent – 1 (Enzymes / chromogens)**

- Cholesterol esterase > 200 U/L
- Cholesterol oxidase > 250 U/L
- Peroxidase > 0.5 mM /L

**Reagent 1A (Buffer)**

- Pipes buffer; PH 6.90 50 mM /L
- Phenol 24 mM/L
- Sodium cholate 0.5 mM/L

**Reagent 2 (Precipitating reagent)**

- Phosphotungstic acid 2.4 mM/L
- Magnesium chloride 39 mM/L
- Standard (HDL-cholesterol) 50 mg/dl
Materials and Methods

**Reagent constitution:** The reagents were allowed to attain room temperature. The contents of Reagent 1 were dissolved into Reagent IA. They were mixed by gentle swirling till completely dissolved.

**Procedure:**

Precipitation: The following were dispensed into the centrifuge tube.

Serum: 0.20 ml

Precipitating reagent: 0.20 ml

The above were mixed well and centrifuged at 3500-4000 rpm for 10 min. The clear supernatant was separated.

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reconstituted reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>--</td>
<td>--</td>
<td>0.02 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>0.02 ml</td>
<td>--</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.02 ml</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

The above solutions were mixed and incubated for 5 min at $37 \pm 1^\circ C$. The absorbance of the test and standard was measured against blank at 500 nm.

**Calculations:**

\[
\text{HDL – Cholesterol (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Std.}} \times 100 \times 1.1
\]

**4.8.5. Estimation of SGPT levels**

**Principle:**

SGPT catalyses the transfer of amino group from L-Alanine to 2-oxo glutarate with the formation of pyruvate and L-glutamate. The pyruvate so formed is allowed to
react with NADH to produce L-lactate. The rate of this reaction is monitored by an indicator reaction coupled with LDH in the presence of NADH (nicotinamide adenine dinucleotide). The oxidation of NADH in this reaction is measured as a decrease in the absorbance of NADH at 340 nm, which is proportional to SGPT activity.

\[
\text{L- alanine} + 2\text{- oxoglutarate} \xrightarrow{\text{ALT}} \text{pyruvate} + \text{L glutamate}
\]

\[
\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{L- Lactate} + \text{NAD}
\]

ALT: Alanine amino transferase  
LDH: Lactate dehydrogenase  

**Procedure:**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Quantity (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000</td>
</tr>
<tr>
<td>Sample</td>
<td>100</td>
</tr>
</tbody>
</table>

The above solutions were mixed well and measured the absorbance at 340 nm.

4.8.6. Estimation of SGOT Levels\textsuperscript{181}:

**Principle:**

SGOT catalyses the transfer of amino group from L- Aspartate to 2-oxo glutarate with the formation of oxaloacetate and L-glutamate. The rate of reaction is monitored by an indicator reaction coupled with malate dehydrogenase (MDH) in which the oxaloacetate formed is converted to malate ion in the presence of NADH (nicotinamide adenine dinucleotide). The oxidation of NADH in this reaction is measured as a decrease in the absorbance of NADH at 340 nm, which is proportional to SGOT activity.
Materials and Methods

L- Aspartate + 2- oxoglutarate $\xrightarrow{\text{AST}}$ oxaloacetate + L glutamate

Pyruvate + NADH $\xrightarrow{\text{MDH}}$ L- Malate + NAD

Sample + NADH $\xrightarrow{\text{LDH}}$ L- Lactate + NAD

Where:-

AST: Aspartate amino transferase

MDH: Malate dehydrogenase

LDH: Lactate dehydrogenase

Procedure:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Quantity (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000</td>
</tr>
<tr>
<td>Sample</td>
<td>100</td>
</tr>
</tbody>
</table>

The above solutions were mixed well and measured the absorbance at 340 nm.

4.8.7. Estimation of Serum Leptin Levels:

The serum level of leptin was estimated by using leptin ELISA kit. The antibody-coated micro plate was affixed to the frame. Washed each well two times with wash buffer (Each well washed with 300 µl of wash buffer. Aspirated the wells completely to remove all excess solution). 45 µl of sample diluent, 50 µl of guinea pig anti-mouse leptin serum and 5 µl of the sample (or working rat leptin standard) per well was dispensed. Incubated the micro plate overnight (16 – 20 h) at 4 ± 0.5°C. Washed each well for 5 times with wash buffer. Dispensed 100 µl of anti-guinea pig IgG enzyme conjugate per well. Incubated the micro plate for 3 h at 4 ± 0.5°C. Washed each well seven times with wash buffer. Dispensed 100 µl of enzyme substrate solution per well
Materials and Methods

and incubated the micro plate for 30 min at room temperature while avoiding exposure to light. The enzyme reaction was stopped by adding 100 μl of enzyme reaction stop solution per well. Measure A450 and subtract A630 values within 30 min. The leptin concentrations were calculated using the standard curve.

4.8.8. Estimation of Liver Triglyceride Content:

The estimation of liver triglycerides consists of multiple steps-

- Homogenization of liver tissue with phosphate buffer
- Adsorption of phospholipids onto Zeolite.
- Extraction of triglycerides into chloroform
- Oxidation of TGs to fatty acids and glycerol
- Oxidation of glycerol with NaIO₄ to form formic acid and formaldehyde
- Formation of coloured complex of formaldehyde and chromotropic acid
- Measure the absorbance at 570 nm.

Procedure

A portion of liver tissue was homogenized with 9 volumes of phosphate buffer. One ml of homogenate solution was immediately transferred to 25 ml glass stoppered graduated cylinder containing about 4 g of activated Zeolite moistened with 2 ml of chloroform. 18 ml of chloroform was gradually added with intermittent shaking for 10 min (The tissue TGs are now diluted 1:200). The solution was filtered, andpipetted 0.125 to 1 ml of filtrate (containing about 0.05 mg of TGs) in to each of three glass stoppered tubes. 1 ml of standard corn oil solution (0.05 mg/ml) was added to each tube. The chloroform from all the tubes was evaporated by placing in a water bath maintained at 80°C.
Materials and Methods

To two out of three each standard solution and test sample, added 0.5 ml of alcoholic KOH (saponified sample); to the third standard and test sample, 0.5 ml of 95% alcohol was added (unsaponified sample). The tubes were maintained at 60 -70°C for 20 min and then 0.5 ml of 0.2 N H₂SO₄ was added. Alcohol was removed by placing the tubes in a boiling water bath for about 15 min. The tubes were cooled and 0.1 ml of periodate solution was added. 0.1 ml of sodium arsenite solution was added after 10 min. 5 ml of chromotropic acid reagent was added after several min. Mixed well, heated for 30 min in the absence of excessive light. After cooling, measured the absorbance at 570 nm.

The triglycerides content in mg/g tissue was calculated as \[ \text{mg/g tissue} = \frac{200}{A} \times R \times 0.05 \]

\[ = 10 \times \frac{R}{A} \]

Where \( A \) = volume of chloroform extract taken and

\[ R = \frac{(\text{Abs. of test saponified} - \text{Abs. of test unsaponified})}{(\text{Abs. of std. saponified} - \text{Abs. of std. unsaponified})}. \]

4.8.9. Estimation of Lipid peroxidation\textsuperscript{182}:

**Principle:** Oxidative stress is associated with peroxidation of cellular lipids, which is determined by measurement of thiobarbituric acid reacting substance (TBARS). The concentration of lipid peroxide products may reflect the degree of oxidative stress. The increased level of TBARS results in increased levels of oxygen free radicals, which attacks the polyunsaturated fatty acids in cell membranes and cause lipid peroxidation. Malondialdehyde is the end product of lipid peroxidation by reactive oxygen species. The malondialdehyde (MDA) content, a measure of lipid peroxidation was determined using TBARS assay.
Materials and Methods

Reagents used:

1. 0.1M Tris HCl buffer (pH 7.4)
2. 10% W/V Trichloroacetic acid (TCA).
3. 0.67% W/V Thiobarbituric acid (TBA).

Procedure:

0.5 ml of liver homogenate was taken and to it was added 0.5 ml of Tris hydrogen chloride buffer and incubated at 37 ± 1°C for 2 h, and then 1ml of ice cold trichloroacetic acid was added, centrifuged for 10 min. From the above, 1ml of supernatant was taken and added to 1ml of thiobarbituric acid and the tubes were kept in boiling water bath for 10 min. The tubes were removed and brought up to room temperature and 1ml of distilled water was added. Absorbance was measured at 532 nm against blank solution prepared without tissue homogenate.

4.8.10. Estimation of Superoxide Dismutase (SOD):

Principle: SOD is a metalloprotein and is the first enzyme involved in the antioxidant defense against ROS by lowering the steady state level oxygen. SOD scavenges the superoxide ions produced as cellular byproducts. SOD is a major defense for aerobic cells combating the toxic effect of superoxide radicals. The SOD activity was determined by the method of Beauchamp and Fridovich (1971)\(^{183}\). It is based on the ability of the enzyme to inhibit the reduction of Nitroblue tetrazolium (NBT) by superoxide radical which is generated by the reaction of photoreduced riboflavin with oxygen.

Reagents used: M/15 Phosphate buffer (pH 7.8), 0.1 M EDTA containing 1.5 mg of sodium cyanide per 100 ml, 0.12 nM Riboflavin solution, and 1.5 mM NBT solution.
**Procedure:**

Control: 0.1 ml of NBT, 0.2 ml EDTA/NaCN, 0.05 ml riboflavin and phosphate buffer to give total volume of 3 ml.

Test: 0.1 ml of NBT, 0.2 ml EDTA/NaCN, 0.1 ml liver homogenate, 0.05 ml riboflavin and phosphate buffer to give total volume of 3 ml.

Riboflavin was added last and subsequent exposure to bright light was avoided until after mixing. Then the tubes were placed in bright illumination for 15 min. Absorbance was measured at 560 nm. Enzyme activities were calculated from the inhibition of reduction using a standard curve constructed by varying amounts of standard enzyme. One unit of enzyme activity is defined as amount of enzyme giving 50% inhibition of the reduction of NBT and expressed as units/mg protein.

**4.8.11. Estimation of Catalase:**

**Principle:** Catalase is a heme protein, localized in the microperoxisomes. It reduces hydrogen peroxide produced by dismutation reaction and prevents generation of hydroxyl radicals thereby protecting the cellular constituents from oxidative damage in peroxisome. It catalyses the decomposition of hydrogen peroxide (H$_2$O$_2$) to water and oxygen and thus protecting the cell from oxidative damage by H$_2$O$_2$. The decomposition of H$_2$O$_2$ catalyzed by Catalase can be followed by spectroscopy, due to the absorbance of H$_2$O$_2$ in this region. At 240 nm the molecular extinction coefficient for H$_2$O$_2$ is 43.6/min.

**Preparation of reagents:**

1. 60 mM Phosphate buffer (pH=7.0): Dissolved 0.35 g of KH$_2$PO$_4$ in distilled water and make up the volume to 25 ml with distilled water (Solution A). Dissolved 1.45 g of Na$_2$HPO$_4$ in distilled water and make up the volume to 100 ml with distilled water (Solution B). Mixed 16 ml of solution A and 84 ml of solution B and adjust pH to 7.0.
2. 10 mM H$_2$O$_2$ in phosphate buffer: Adjusted the absorbance of H$_2$O$_2$ in phosphate buffer (pH=7.0) between 0.4-0.5 at 240 nm.

**Procedure:**

Test: 3 ml of H$_2$O$_2$ phosphate buffer was used mixed with 0.05 ml of liver homogenate.

Blank: It was prepared by mixing 0.05 ml of liver homogenate with phosphate buffer.

Absorbance was measured for both blank and test at 240 nm for 2 min with 60 sec interval.

**Calculation:**

($\Delta$A / min) 43.6 = U/mg Protein absorbance values of control and those of test compound.

### 4.8.12. Estimation of Glutathione peroxidase:

Glutathione peroxidase catalyzes the reduction of hydroperoxides including H$_2$O$_2$ by reduced glutathione and functions to protect the cell from oxidative damage. Glutathione peroxidase activity was determined according to the method of Lawrence and Burk. This method measures glutathione peroxidase activity indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione produced upon reduction of hydroperoxide by glutathione peroxidase is recycled to its reduced state by glutathione reductase and NADPH.

**Procedure**

Liver homogenate solution (100 µl) was mixed with 800 µl of 100 mM/L potassium phosphate buffer (pH 7.4), containing 1 mM/L EDTA, 1 mM/L NaN$_3$, 0.2 mM/L NADPH, 1 U/ml glutathione reductase (GRd) and 1 mM/L reduced glutathione (GSH). After 5 min, 2.5 mM/L H$_2$O$_2$ (100 µl) was added to start reaction. The absorbance change at 340 nm in 3 min was recorded. The activity was calculated using
value of E340 = 6220/M per cm and result was expressed in units of nmol NADPH/min per mg protein.

4.9. DETERMINATION OF IN VITRO ANTIOXIDANT ACTIVITY:

The ability of the ethanol extracts of AG and AS to scavenge DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical, nitric oxide radical and hydroxyl radical was determined at different concentrations.

4.9.1. DPPH• scavenging assay:

Mechanism:

DPPH is a molecule containing a stable free radical. In the presence of an antioxidant which can donate an electron to DPPH, the purple color which is typical to free DPPH radical decays and changes to yellow coloured compound. The change in absorbency at 517 nm is determined spectrophotometrically. Free radical scavenging potentials of the extracts were tested against a methanolic solution of DPPH. Antioxidants react with DPPH (purple) and convert it to 1-1-diphenyl -2-picryl hydrazine (yellow). The degree of discoloration indicates the scavenging potential of the antioxidant extract. The change in the absorbance produced at 517 nm has been used as a measure of antioxidant activity.

Procedure:

The ethanol extracts of AG rhizomes and AS roots (2.0 ml) at various concentrations (10, 20, 40, 60, 80 and 100 µg/ml) were added to 1.0 ml of 0.5 mM DPPH solution in methanol. After 30 min of incubation at room temperature, the absorbance was measured at 517 nm. Ascorbic acid was used as standard free radical scavenger. The % scavenging of DPPH radical was calculated by comparing the absorbance between the test (extract) and diluent control reaction mixture. The antioxidant activity of each sample is expressed in terms of IC$_{50}$ (µg/ml concentration
Materials and Methods

required to scavenge DPPH radical by 50%) calculated from log % activity vs log concentration curves.

\[
\text{% scavenging} = 100 \times \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{blank}}}
\]

where

\( A_{\text{blank}} \) = Absorbance of blank.

\( A_{\text{sample}} \) = Absorbance of sample.

4.9.2. Nitric oxide scavenging assay:

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction\(^{187}\).

**Procedure:**

1.0 ml of Sodium nitroprusside (10mM) in distilled water was mixed with different concentrations (10, 20, 40, 60, 80 and 100 µg/ml) of the ethanol extracts of AG rhizomes and AS roots and incubated at 25°C for 150 min. A control experiment without test compound but with the equivalent amount of the vehicle was also conducted. Following the incubation period, 0.5 ml of Griess reagent (1 % sulfanilamide, 2 % \( \text{H}_3\text{PO}_4 \) and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance was read at 546 nm and the % scavenging of nitric oxide was calculated by comparing the absorbance between the test and diluent control reaction mixture and IC\(_{50}\) values were calculated. Quercetin was served as positive control.

4.9.3. Hydroxyl radical scavenging assay\(^{188}\):

**Principle:**

Scavenging capacity of components for hydroxyl radicals was evaluated by measuring the degradation of 2-deoxyribose with OH radicals, generated in a Fenton reaction. The degradation products are the 2-thiobarbituric acid (TBA) –reactive
Materials and Methods

substances, which would be determined spectrophotometrically at 532nm. In this activity the Fenton reaction describes the oxidation of H$_2$O$_2$ by Fe$^{++}$ to •OH and Fe$^{+++}$. In the model employed in this experiment, the production of •OH induced oxidation of the 2-Deoxy-D-ribose, which in turn reacted with the thiobarbituric acid in presence of trichloroacetic acid to produce a TBA reactive chromophore that was detectable at 532 nm.

Procedure:

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Mol. Wt</th>
<th>Std. Conc.</th>
<th>Qty. Added</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disodium-EDTA</td>
<td>372</td>
<td>1 mm</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Ferrous sulfate</td>
<td>278</td>
<td>1 mm</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>34</td>
<td>10 mm</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>2-Deoxy-d-ribose</td>
<td>134</td>
<td>10 mm</td>
<td>0.36 ml</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>pH-7.4</td>
<td>0.33 mm</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

Added to various conc.(10-100 μg) of ethanol extracts under study and incubated at 37°C for 1 h and then added

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>0.5 % w/w</th>
<th>1 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiobarbituric acid</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trichloroacetic acid</td>
<td>--</td>
<td>10 % w/w</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

All the solutions and reagents were dissolved in phosphate buffer and used freshly. All the sample solutions were prepared in phosphate buffer (pH-7.4). In the test tubes, different concentrations of sample i.e. 10-100 μg/ml prepared in phosphate buffer were taken and mixed with 0.1 ml H$_2$O$_2$, 0.1 ml FeSO$_4$ and 0.36 ml 2-deoxy-D-ribose and filled up with 0.5 ml phosphate buffer of pH 7.4, and kept the test tubes for incubation at 37°C for 1 h. After incubation for 1 h, 1.0 ml of TBA solution and 1.0 ml of TCA solution were added and the tubes were heated at 100°C for 20 min. After cooling the absorbance was read against a blank (containing a buffer solution instead of
sample) at 532 nm. The absorbance was used for the calculation of the percentage inhibition of deoxyribose degradation by the sample by using the following formula.

\[
\% \text{inhibition} = 100 \times \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}
\]

A blank = Absorbance of blank.

A sample = Absorbance of sample.

BHT was used as a positive control. From the obtained % inhibition values, the IC\textsubscript{50} values were calculated.

**4.10. DETERMINATION OF TOTAL PHENOLIC CONTENT:**

The total phenolic content of the ethanol extracts of AG rhizomes and AS roots was determined by using Folin-Ciocalteu (FC) reagent according to the method of Singleton and Rossi\textsuperscript{189}. Under alkaline conditions, phenolic compounds will be oxidized by the Folin-Ciocalteu reagent containing a mixture of phosphotungstic acid and phosphomolybdic acid. The reagent becomes partly reduced producing the molybdentungstic blue complex.

**Procedure:**

The ethanol extracts (200 µl) of AG rhizomes was mixed with 1.0 ml of FC reagent and 0.8 ml 7.5% sodium bicarbonate solution. The tube was incubated at room temperature and allowed to stand for 30 min. After incubation the absorbance was measured at 765 nm and used to calculate the phenolic contents using gallic acid as a standard. The total phenolic content was then expressed as mg gallic acid equivalent (GAE)/g sample. All experiments were carried out in duplicate.

The same procedure was followed for the ethanol extract of AS roots.
4.11. DETERMINATION OF TOTAL FLAVONOID CONTENT\textsuperscript{190}:

The total flavonoid content was determined with aluminium chloride (AlCl\textsubscript{3}) according to a known method using quercetin as a standard. The ethanol extract of AG rhizomes (0.1 ml) was added to 0.3 ml distilled water followed by NaNO\textsubscript{2} (0.03 ml, 5\%). After 5 min at 25°C, AlCl\textsubscript{3} (0.03 ml, 10\%) was added. After 5 min, the reaction mixture was treated with 0.2 ml 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm. All tests were performed in duplicate. The flavonoid content was calculated from Quercetin standard curve. The same procedure was followed for the ethanol extract of AS roots.

4.12. ISOLATION AND CHARACTERIZATION OF PHYTOCONSTITUENTS

The ethanol extracts of \textit{Alpinia galanga} rhizomes and \textit{Argyreia speciosa} roots have shown promising and highly significant anti-obesity and antioxidant effects. These extracts were subjected for isolation and characterization of the active phytoconstituents responsible for the activity by using thin layer chromatography (TLC), column chromatography, and spectral analysis. A wealth of information indicated numerous bioactive components from nature are potentially useful in obesity treatments particularly phenolic compounds including flavonoids and phytosterols\textsuperscript{191}. Hence, the phytochemical investigation was directed towards the isolation of phenolic compounds and sterols.

4.12.1. Isolation and Characterization of \textit{Alpinia galanga} extract

The ethanol extract of AG rhizomes was further fractionated with Petroleum ether (40 - 60°C), chloroform, ethyl acetate and water to isolate the bioactive flavonoids. The obtained fractions were screened for \textit{in vitro} antioxidant activity by using DPPH radical scavenging assay.
The results indicated that, ethyl acetate fraction is having better free radical scavenging activity as compared to remaining fractions. Hence, the ethyl acetate fraction was further subjected to TLC studies, which showed two spots of Rf values 0.44 and 0.66 indicating the presence of two flavonoids. For the separation and purification of these flavonoids, the ethyl acetate fraction was subjected for column chromatography.

**Colum Chromatography:**

Adsorbent: Silica gel for column chromatography

Activation: 110 ± 1°C for 1 h.

Length of the column: 45 cm.

Length of adsorbent packed: 31 cm

Diameter of the column: Outer -3 cm, Inner –2.8 cm

Rate of elution: 15-18 drops per minute

Volume of elute collected: 10 ml

Type of elution: Gradient elution

**Column Packing:**

175 g of silica gel was activated in hot air oven at 110 ± 1°C for 1 h. The slurry made in the chloroform and loaded in the column with gentle tapping to ensure the

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>% DPPH radical scavenging activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol extract</td>
<td>60 µg/ml</td>
<td>70.03</td>
</tr>
<tr>
<td>Pet. Ether fraction</td>
<td></td>
<td>72.56</td>
</tr>
<tr>
<td>Chloroform fraction</td>
<td></td>
<td>74.02</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td></td>
<td>78.64</td>
</tr>
<tr>
<td>Aqueous fraction</td>
<td></td>
<td>62.56</td>
</tr>
<tr>
<td>Ascorbic acid (Standard)</td>
<td></td>
<td>87.73</td>
</tr>
</tbody>
</table>
uniform packing . The small quantity of solvent was allowed to remain at the top of the column in order to avoid the drying or cracking of the column.

**Separation of Flavonoids by Column Chromatography:**

**Preparation of sample:**

10 g of ethyl acetate fraction was dissolved in 10 ml of chloroform and mixed with silica gel (60-120 mesh size) and dried in vacuum oven at 45 ± 1°C. The adsorbed material obtained was loaded on top of the column.

**Gradient Elution:**

The column was then eluted successively with hexane, hexane: chloroform (1:1), chloroform, chloroform: methanol (1:1), and finally with methanol. Fractions were collected in 25 ml portions and subjected to TLC. Chloroform: methanol, 9:1 as developing solvent and 5% Vanillin solution as detecting agent in order to combine the fractions with the same compounds.

<table>
<thead>
<tr>
<th>Solvent ratio</th>
<th>Fraction No.</th>
<th>TLC result</th>
<th>Eluent colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>1 to 10</td>
<td>No spots</td>
<td>Colourless</td>
</tr>
<tr>
<td>Hexane: Chloroform:1:1</td>
<td>11 to 20</td>
<td>No spots</td>
<td>Colourless</td>
</tr>
<tr>
<td>Chloroform</td>
<td>21 to 30</td>
<td>2-3 bands with tailing</td>
<td>Straw</td>
</tr>
<tr>
<td>Chloroform: Methanol:1:1</td>
<td>31 to 40</td>
<td>1 spot</td>
<td>Yellowish brown</td>
</tr>
<tr>
<td>Methanol</td>
<td>41 to 50</td>
<td>1 spot</td>
<td>Brown</td>
</tr>
</tbody>
</table>

The fractions 31 to 40 obtained with chloroform: methanol (1:1) showed single spot when exposed to vanillin reagent on TLC plate. Therefore, they were combined and solvent was evaporated to dryness at room temperature. The obtained residue of light yellow powder was labeled as F1 and subjected for further characterization.
Similarly, the fractions 41 to 50 obtained with Methanol showed single spot when exposed to vanillin reagent on TLC plate. Therefore, they were combined and kept for evaporation to dryness at room temperature. The obtained residue of dark yellow powder was labeled as F2 and subjected for further characterization.

The isolated compounds F1 and F2 were further characterized by qualitative chemical tests and spectral studies. The spectral studies include UV, FT-IR, $^1$H-NMR, $^{13}$C-NMR and Mass spectroscopy. The UV spectrum was recorded in methanol using Shimadzu UV-Visible Spectrophotometer. The IR spectrum was obtained by using KBr disc with Thermonicolet FTIR 200 spectrophotometer. The $^1$H-NMR Spectrum was recorded in MeOD using Bruker 400. The $^{13}$C-NMR Spectrum was recorded in MeOD using Bruker 100. The mass spectrum was recorded by using Shimadzu mass spectrophotometer.

### 4.12.2. Isolation and Characterization of *Argyreia speciosa* extract

The ethanol extract of AS roots was further fractionated with Petroleum ether (40-60°C), Chloroform, Ethyl acetate and water to isolate the bioactive components. The obtained fractions were screened for *in vitro* antioxidant activity by using DPPH radical scavenging assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>% DPPH radical scavenging activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol extract</td>
<td></td>
<td>67.53</td>
</tr>
<tr>
<td>Pet. Ether fraction</td>
<td>60 µg/ml</td>
<td>75.86</td>
</tr>
<tr>
<td>Chloroform fraction</td>
<td></td>
<td>54.08</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td></td>
<td>52.26</td>
</tr>
<tr>
<td>Aqueous fraction</td>
<td></td>
<td>80.66</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td></td>
<td>87.73</td>
</tr>
</tbody>
</table>
The results indicated that, aqueous and pet. ether fractions produce marked free radical scavenging activity as compared to remaining fractions. Hence, these fractions were subjected to TLC studies. In which, the aqueous fraction showed multiple pink colour bands turning to tan-brown colour by using chloroform: ethanol (5:5) developing solvent when exposed to iodine vapors and pet. ether fraction showed multiple bands with pet. ether: acetone (9:1) as developing solvent when exposed to antimony trichloride solution. For the separation and purification of these components, the fractions were subjected for column chromatography.

**Column chromatography of aqueous fraction of ethanol extract of AS roots:**

**Preparation of sample:**

10 g of aqueous fraction was dissolved in 30 ml of chloroform and mixed with silica gel (60-120 mesh size) and dried in vacuum oven at 45 ± 1°C. The adsorbed material obtained was loaded on top of the column.

**Gradient Elution:**

The column was then eluted successively with chloroform and chloroform: methanol (9:1). Fractions were collected in 25 ml portions and subjected to TLC using chloroform: ethanol (5: 5) as developing solvent and iodine vapors as detecting agent and the fractions showing similar spots were combined together.

The fractions 38 to 68 obtained with chloroform: methanol (9:1) showed single pink coloured band turning to tan-brown colour with chloroform: ethanol (5:5) developing solvent when exposed to iodine vapors. So they were combined and kept for evaporation to dryness at room temperature. The obtained residue of light brown coloured powder was named as compound 1 and was further characterized by qualitative chemical tests and spectral studies.
**Materials and Methods**

<table>
<thead>
<tr>
<th>Solvent ratio</th>
<th>Fraction No.</th>
<th>TLC result</th>
<th>Eluted sample colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>1 to 10</td>
<td>No spots</td>
<td>Colorless</td>
</tr>
<tr>
<td>Chloroform: Methanol:9:1</td>
<td>11 to 12</td>
<td>No spots</td>
<td>Colorless</td>
</tr>
<tr>
<td>Chloroform: Methanol:9:1</td>
<td>13 to 16</td>
<td>3 fluorescent bands</td>
<td>Straw</td>
</tr>
<tr>
<td>Chloroform: Methanol:9:1</td>
<td>17 to 22</td>
<td>Multiple</td>
<td>Straw</td>
</tr>
<tr>
<td>Chloroform: Methanol:9:1</td>
<td>23 to 30</td>
<td>2 bands with tailing pattern</td>
<td>Straw</td>
</tr>
<tr>
<td>Chloroform: Methanol:9:1</td>
<td>31 to 33</td>
<td>3 bands with tailing pattern</td>
<td>Green</td>
</tr>
<tr>
<td>Chloroform: Methanol:9:1</td>
<td>34 to 37</td>
<td>2 bands with tailing pattern</td>
<td>Straw</td>
</tr>
<tr>
<td>Chloroform: Methanol:9:1</td>
<td>38 to 68</td>
<td>1 band</td>
<td>Light brown</td>
</tr>
</tbody>
</table>

**Column Chromatography of Pet. Ether fraction of ethanol extract of AS roots:**

**Preparation of sample:**

10 g of pet. ether fraction was dissolved in 20 ml of chloroform and mixed with silica gel (60-120 mesh size) and dried in vacuum oven at 45 ± 1°C. The adsorbed material obtained was loaded on top of the column.

**Gradient Elution:**

The column was then eluted successively with chloroform and chloroform: methanol solvent combination in the ratio of 95:5, 90:10 and 80:20. Fractions were collected in 25 ml portions and subjected to TLC using pet. ether: acetone (9:1) as developing solvent and antimony trichloride solution as local visualizing and the fractions showing similar spots were combined together.

The fractions 26 to 34 obtained with chloroform: methanol (90:10) showed single spot when exposed to antimony trichloride solution. So they were combined and kept for evaporation to dryness at room temperature. The obtained residue of buff coloured powder was labeled as compound 2 and was further characterized by qualitative chemical tests and spectral studies.
**Materials and Methods**

<table>
<thead>
<tr>
<th>Solvent ratio</th>
<th>Fraction No.</th>
<th>TLC result</th>
<th>Eluent colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>1 to 08</td>
<td>No spots</td>
<td>Colorless</td>
</tr>
<tr>
<td>Chloroform: methanol:95:5</td>
<td>09 to 25</td>
<td>No spots</td>
<td>Colorless</td>
</tr>
<tr>
<td>Chloroform: methanol:90:10</td>
<td>26 to 34</td>
<td>1 band</td>
<td>Greenish yellow</td>
</tr>
<tr>
<td>Chloroform: methanol:80:20</td>
<td>35 to 44</td>
<td>No spot</td>
<td>colourless</td>
</tr>
</tbody>
</table>

**4.13. MEASUREMENT OF PANCREATIC LIPASE INHIBITORY EFFECT**

The two compounds each isolated from ethanol extracts from AG rhizomes and AS roots were subjected to study their influence on pancreatic lipase enzyme activity in an attempt to find out the mechanism of beneficial effects produced by ethanol extracts in cafeteria diet and atherogenic diet induced obesity in rats.

**Procedure**

Lipase activity was determined by measuring the amount of release of fatty acid from Triolein. A suspension of triolein (80 mg), phosphatidylcholine (10 mg) and taurocholic acid (5 mg) in 9 ml of 0.1 M N-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) buffer (pH 7.0) containing 0.1 M NaCl was sonicated for 5 min. This sonicated substrate suspension (0.1 ml) was incubated with 0.05 ml (final concentration 5 units per tube) pancreatic lipase and 0.1 ml (10 mg/ml) of various isolated compounds from *Alpinia galanga* rhizomes and *Argyreia nervosa* roots for 30 min at 37°C in a final volume of 0.25 ml and the released fatty acid was determined by titrating the solution against 0.02M NaOH (Standardized by 0.01M oxalic acid) using phenolphthalein as an indicator. % inhibition of lipase activity was calculated using the formula-

\[
\text{Lipase inhibition} = \frac{A - B}{A} \times 100
\]

Where A is control lipase activity and B is lipase activity in presence of extract.
4.14. MEASUREMENT OF PLASMA TRIACYLGLYCEROL CONTENT:

To test the possibility of inhibition of pancreatic lipase enzyme in experimental animals, the effect of isolated compounds on plasma triacylglycerol concentration after oral administration of a lipid emulsion containing corn oil was also determined\textsuperscript{198}.

Lipid emulsions were prepared with 6 ml of corn oil, 80 mg cholic acid, 2 g of cholesteryoleate and 6 ml of saline in the absence or presence of isolated compounds of AG rhizomes and AS roots (final concentration of 50 mg/kg body weight). Male wistar rats were fasted overnight and divided into 4 groups of 6 animals each. All the group of animals administered orally 3 ml of lipid emulsion. Blood samples were collected at 0, 1, 2, 3 and 4 h after administration of lipid emulsion with or without test drug using a heparinized capillary tube and centrifuged at 5000 rpm for 5 min to obtain plasma. The plasma triacylglycerol content was determined by following the procedure of estimation of serum triglycerides\textsuperscript{179}.

4.15. STATISTICAL ANALYSIS:

The data obtained were analyzed using the Graph Pad Prism 5 software package. The significance of the differences between the means of control and test studies was established by one-way Analysis of Variance (ANOVA) followed by Dunnett’s test for multiple groups comparison. A value of p<0.05 was considered statistically significant.