Materials and Methods
MATERIALS AND METHODS

STUDY AREA

The present study focuses on the wild edible tubers, corms and rhizomes consumed by the Kanikkars and Palliyars tribes settled in the South eastern slopes of Western Ghats, Tamil Nadu (Area Map). The Kanikkars are settled in the forest area of Kanyakumari district and Agastiyamalai Biosphere Reserve, Tirunelveli district. The Palliyars are settled in the reserve forest area of Grizzled Giant Squirrel Wildlife Sanctuary (Virudhunagar and part of Madurai district).

The area of investigation lies between 77° E and 78° E longitude and 8.4° N and 10° N latitude. The altitude varies from 100 m to 2210 m (MSL). It receives rainfall both South-west and the North-east monsoons. The varied climatic and topographic conditions prevailing in the study area present remarkable diversity of both the flora and fauna.

Variations in the altitude and rainfall always have a bearing on the vegetation in general. The study area consists of tropical evergreen forests, semi evergreen forests, dry teak forests, southern mixed deciduous forests and dry grassland.

In the study area, the Kanikkars and Palliyars live in several isolated pockets or in small hamlets. Their habitations are known by the following names.

<table>
<thead>
<tr>
<th>Kanikkars</th>
<th>Palliyars</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Thachamalai</td>
<td>1. Sivagiri</td>
</tr>
<tr>
<td>2. Thottamalai</td>
<td>2. Saduragiri hills</td>
</tr>
<tr>
<td>3. Keeriparai</td>
<td>3. Thallaianai</td>
</tr>
<tr>
<td>4. Pechiparai</td>
<td>4. Athikoil</td>
</tr>
<tr>
<td>5. Periamymlar</td>
<td>5. Ayyanarkoil</td>
</tr>
<tr>
<td>7. Injikuzhi</td>
<td></td>
</tr>
<tr>
<td>8. Agastiyamalai</td>
<td></td>
</tr>
</tbody>
</table>

20
Plate I

1. A view of Thacamalai

2. A view of Agastiyamalai
The Kanikkar Tribe

The Kanikkar belong to the Southern tribal Zone. The Kanikkar are also known as Kanikaran or Kani. The Southern tribals are historically more ancient tribes. There are as many as 36 types of scheduled tribes in Tamil Nadu. In the serialized list notified by the Government of Tamil Nadu, the Kanikkar are placed at 7th position. They live in low altitude regions of Western Ghats and live in large numbers. Kanikkar means “hereditary proprietor of land” thus recognizing their ancient rights over the forest lands.

The Kanikkar are generally very short in stature and meager in appearance, from their active habits and scanty food. Some have markedly Negroid features. They are simple and straightforward tribals. They are traditionally a nomadic community (Plate.II). They speak in their own dialect, Kanikkar Bhasha or Malampashi, which is close to the Dravidian language Malayalam (Singh, 1994). They were once Lords of the forest and practised migratory cultivation because of the forest may not set fire to or trees felled at the unrestricted pleasure of individuals.

Most of the Kanikkar tribals have a general knowledge of medicinal plants that are used for first aid remedies, to treat cough, cold, fever, headache, poisonous bites and some other simple ailments. Kanis still supplement their food by gathering roots and tubers from the nearby forest areas. They eat tubers like Manihot esculenta and Dioscorea oppositifolia etc. They are extremely hard working and can survive without the help of modern facilities. They are socio-economically backward and most of them are very poor. They are also engaged in seasonal collection of honey, bee wax and some minor forest produce. They cultivate edible plants like tapioca, banana, millets and cash crops such as pepper, areca nut and cashew nut.
Plate II

1. A tribalman (Mootukkani) at Thachamalai
2. Pichandi kani (Nattu Vaidhiyar) near his house
3. A tribal family
4. A palliyar woman in her house
5. A tribal man holding *Maranta arundinacea*
6. A tribal man digging *Dioscorea tomentosa* tubers.
The Palliyar Tribe

The Palliyars belong to the Southern Tribal Zone. The Southern tribals are historically more ancient tribes. There are as many as 36 types of Scheduled tribes in Tamil Nadu. In the serialized list notified by the Government of Tamil Nadu, the Palliyars are placed at 32nd position. They live in the low altitude regions of Western Ghats and live in large numbers in the study area (Virudhunagar district and part of Madurai district). Of the total population of the Scheduled tribes in Tamil Nadu (5,74,194), the Palliyar tribe accounts for 1890, which ranks 20th among the tribal population (Census of India, 1991). Palliyars can be grouped into three categories based on their lifestyle, namely: (i) Nomadic Palliyars, the hunter-gatherers who live in rock shelters wandering in tracts of forest in search of food and non-timber forest produce (ii) Semi nomadic Palliyars, also the hunter-gatherers, who build huts and do not practise agriculture, go out to collect food and non-timber forest produce and return to their dwellings and (iii) Settled Palliyars, who have land holdings, practising agriculture and living mainly in Kerala (Sankarasivaraman, 2000).

The Palliyars of the study area at present could be called semi nomadic type. Their ancestors were nomadic. They live as individual families. Like other primitive tribes, the Palliyars are short, dark complexioned, curly haired with thick protruding lips and blunt nose with wide nostrils (Plate. II). Palliyars do not have any established mode of dress. They are scantily dressed but freely wear whatever clothes are available to them. They are non-vegetarians; however they abstain from beef as rigidly as the most orthodox Hindus (Dahmen, 1908). Several species of Dioscorea provide the basis of Palliyars staple food. Besides there are a wide variety of greens, stems, tubers, unripe fruits and ripe fruits, which serve as alternative for food. Palliyars also feed on wild animals and birds (like rabbit, rat, deer, hen etc.).
The Palliyars live in small parties as isolated groups. Generally a hamlet has about
20 huts. Their small huts are unique with the walls made up of mud or with wiry interwoven
stems of Lantana camera. Each hut is thatched with the fronds of Cymbopogon citratus or C.
polyneuros or with the leaves of Phoenix pusilla or Cocos nucifera. They sleep on mats
woven with the leaves of the above said taxa.

The Palliyars as a tribe do not possess much cohesiveness. Each settlement has its
headman whose authority is never challenged and he is solely responsible for settling
disputes among the tribals. They are illiterate but in recent years, they have started sending
their children to the nearby schools.

Collection

Wild edible tubers, corms and rhizomes were collected from the South eastern slopes
of Western Ghats, Tamil Nadu (Vide area map). Field visits were made frequently (2007 to
2009). The data regarding the wild edibles mentioned above were collected by interviewing
the Kanikkar / Palliyar tribal and their village leaders. Old experienced tribal were also
interviewed. Specimens of tubers, corms and rhizomes were collected and brought to the
laboratory for botanical identification and nutritive analysis. Voucher specimens were
collected and deposited in the Ethnopharmacology unit, Research Department of Botany,
V.O.Chidambaram College, Thoothukudi-8.

The specimens were identified using the following floras.

i) Flora of the Presidency of Madras, Vol.III. J.S.Gamble, and C.E.C.Fischer,
   (1915-1936).
The wild edible tubers, corms and rhizomes were photographed and the photographs are affixed in appropriate places in the section “results”.

Chemical Analysis

Chemicals

The chemicals used during the experiment were of BDH (AR) and Sigma Chemical Company, St.Louis, U.S.A. Casein and Poly Vinyl Pyrollidone (PVP) were purchased from SISCO Research Laboratories Pvt. Ltd. Mumbai. Porcine pancreatic α-amylase was purchased from E Merck, Germany.

Preparation of Samples

Wild edible samples washed free of dirt were chopped and about 50 g of each were weighed, dried and powered in a Willy mill (Scientific Equipment Works, New Delhi, India) 60 mesh size. The powdered samples were stored in screw cap bottles at room temperature for further analysis.

Proximate analysis

Determination of Moisture content (AOAC, 1975)

The samples were weighed, chopped and incubated in a hot air oven at 80°C for 24 hours. Then the samples were cooled in a desiccator and weighed again. The loss in the weight of the sample was calculated as the moisture content and the average value of triplicate determinations are expressed on percentage basis.

Determination of Crude protein content

Digestion

The nitrogen content of the dried, powdered sample was determined by micro-kjeldahl method (Humphries, 1956). To 100 mg of the dried powdered sample taken in a micro-kjeidahl flask, 2 ml of digestion mixture (5 % (w/v) salicylic acid in Con. H2SO4) was
added and mixed well. After 20 min. 0.3 g of sodium thiosulphate was added and heated gently until fumes disappeared. The contents of the flask cooled and 60mg of catalyst (a mixture of 1 g CuSO₄, 8g K₂SO₄ and 1 g selenium dioxide) followed by 1ml of Con. H₂SO₄ were added. The contents were digested until they turned apple green in colour. The flask was cooled and the contents were made up to a known volume.

**Distillation**

10 ml of the digested solution from the volumetric flask was transferred to Paranas micro-kjeldahl distillation flask. To this, 10 ml of 40% (w/v) NaOH and 2ml of distilled water were added and heated using Bunsen burner. The ammonia from the sample was steam- distilled for 5 min. into a receiver flask, which contained 5 ml of 2% (w/v) boric acid solution and indicator (83.3 mg of bromocresol green + 16.6 mg of methyl red dissolved in 10 ml of 95% ethanol).

The ammonia in the receiving flask produced by the breakdown of organic nitrogen-containing compounds in the sample was titrated against N/50 sulphuric acid. A blank was run simultaneously using all the reagents and the value of the blank was deducted from the value of the sample before calculation. One ml of N/50 H₂SO₄ equaled 0.00028 g of N, which forms the basis for calculation of N content in the sample.

The crude protein content, which is here after called simply the protein content, was calculated by the equation.

\[ \text{Protein content (\%)} = \% \text{ nitrogen} \times 6.25. \]

**Crude Lipid (AOAC, 2005)**

Dried powdered sample (2g) was extracted with ether in a Soxhlet apparatus for 16 hrs. The ether was evaporated and the residue was weighed. The average value of triplicate experiments was expressed as percentage of ether extract or total crude lipid content on dry weight basis.
Determination of Crude fibre content (AOAC, 2005)

The left-out residue after extraction with ether was successively digested with 0.225N H$_2$SO$_4$ solution and 0.313N NaOH solution. After digestion, the sample was washed with boiling water followed by absolute ethanol in a Gooch crucible. The content of the crucible was dried to constant weight, cooled, weighed and ignited in an electric muffle furnace for 30 min. at 600 °C. The contents were reweighed after cooling. The loss in weight was expressed as percentage of crude fibre on dry weight basis.

Ash Analysis (AOAC, 2005)

Two grams of dried powdered sample was placed in a pre-weighed crucible and ignited at 600°C for 2 hrs. The contents of the crucible were cooled in a desiccator and weighed. The difference in weight of the crucible gives the ash content. The ash content was expressed as percentage on dry weight basis.

Determination of Nitrogen Free Extractives (NFE) Or Total Crude Carbohydrate content determination (Muller and Tobin, 1980).

The carbohydrate content was obtained by the difference method: that is, by subtracting the sum of the protein, fat, ash and fibre from the total dry matter.

\[
\% \text{ NFE} = 100 - (\text{CP}\% + \text{EE}\% + \text{Ash} \%)
\]

Where CP = Crude Protein; EE = Ether Extract; CF = Crude Fibre

Calorific value determination

The calorific values of the investigated samples were determined in kJ by multiplying the percentage of crude protein, crude lipid and NFE by the factors 16.7, 37.7 and 16.7 respectively.
Mineral Analysis.

Sample Digestion

Five hundred mg of dried powdered sample was mixed with 10 ml of Con. HNO₃, 4 ml of 60% perchloric acid and 1 ml Con. H₂SO₄ and the contents were kept undistributed overnight.

After that it was heated on a hot plate containing Con. H₂SO₄ in a beaker until the brown fumes ceased coming out and then allowed to cool. After cooling it was filtered through Whatman No.42 filter paper. After filtration the filtrate was made up to 100 ml with distilled water.

Estimation of Sodium and Potassium

Sodium and Potassium were estimated by using Flame Photometer (Model Elico). The sodium and potassium contents were calculated by referring to the calibration curves of sodium and potassium respectively and expressed as mg 100g⁻¹ of powdered samples.

Estimation of Calcium and Magnesium (Jackson, 1973)

Calcium

Five ml of triple acid digested extract was taken in a china dish. To this 10 ml of 10% (w/v) NaOH and 0.1 g mg of murexide indicator powder [40 g of potassium sulphate or potassium chloride was added and ground with 10g ammonium chloride and 0.2 g of murexide (ammonium purpurate)]. The solution was then titred against 0.02 N versenate (19 g of EDTA was dissolved in 5 litres of distilled water) and standardized against 0.2 N Na₂CO₃ solutions and adjusted until the colour changed from red to violet.

Calcium and Magnesium

Five ml of triple acid digested extract was taken in a china dish. To this 10 ml of ammonium chloride-ammonium hydroxide buffer (pH 10) and a few drops of Erichrome
black T indicator (0.1 g of Eriochrome black T dissolved in 25 ml of methanol containing 1 g of hydroxylamine Hydrochloride) were added and titrated against 0.02N versenate solution until the colour changed from red to blue.

Calculation

Percentage of Calcium in the sample = Titre value of Calcium X 100 / 5 X 100/0.5X0.0004

Percentage of magnesium = Titre value of calcium + Magnesium – titre value of calcium

Or

Titre value of calcium = magnesium X 0.96.

Calcium and magnesium contents were expressed as mg 100g⁻¹ of sample.

Estimation of Phosphorus (Dickman and Bray, 1940)

One ml of of triple acid digested extract was pipetted into 100ml volumetric flask. To this 50 ml of distilled water was added, followed by 5 ml ammonium molybdate-sulphuric acid reagent (Solution A: 25g of ammonium molybdate dissolved in 100ml of distilled water. Solution B: 280 ml of Con. H₂SO₄ diluted to 800ml. Solution A was added slowly with constant stirring to solution B and the volume was made upto 1000 ml with distilled water). Blue colour developed after adding 6 drops of 2.5 % (w/v) stannous chloride solution. The total volume was made upto 100ml. The intensity of the blue colour, was measured at 650 nm in a Spectrophotometer. The phosphorus content present in the sample was calculated by referring to a standard graph of phosphorus using potassium dihydrogen phosphate (KH₂PO₄) as standard and expressed as mg 100g⁻¹ of powdered samples.
Estimation of Micronutrients by Atomic Absorption Spectrophotometer (Issac and Johnson, 1975)

By feeding the sample on an Atomic Absorption Spectrophotometer ECIL (Electronic Corporation of India Ltd.) the following elements were estimated with appropriate wavelengths.

<table>
<thead>
<tr>
<th>Name of the Mineral</th>
<th>Wavelength used for estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron</td>
<td>248.3 nm</td>
</tr>
<tr>
<td>Copper</td>
<td>324.5 nm</td>
</tr>
<tr>
<td>Zinc</td>
<td>213.9 nm</td>
</tr>
<tr>
<td>Manganese</td>
<td>279.4 nm</td>
</tr>
</tbody>
</table>

The mineral contents were expressed as mg 100g⁻¹ powdered sample.

Starch (Moorthy and Padmaja, 2002)

Extraction and Estimation

To 1 g of the dried powdered sample, 20 ml of 80% ethanol was added and left overnight to extract the sugars.

The extracted sugars were separated from the residue by filtration with Whatman No.1 filter paper. The filtrates were collected separately for sugar estimation. The residue was transferred back into the conical flask using 20 ml of 2N HCl.

The starch in the residue was then hydrolyzed, cooled, and the volume was increased to 100 ml using distilled water. This supernatant was then directly used for titration.

Four replicas of sample were taken and 2 aliquots were taken from each replica for analysis.
Titrimetric assay

10 ml 1% Potassium ferricyanide was pipetted and 5 ml NaOH (2.5 N w/v) was added and mixed. The contents were boiled and 3 drops of dilute methylene blue was added. The solution immediately turned blue green. The starch hydrolysate was taken in a 2 ml blow pipette and added drop by drop. The end point is the change of colour to golden yellow. The titre value is noted.

Calculation

Each lot of Potassium ferricyanide was calibrated using Std. Glc. Solution and the relation.

10 mg of Glc. = 10ml of Potassium ferricyanide.

The starch content was calculated by the formula.

\[
\text{Starch (g 100g-1DM) } = \frac{\text{Starch content}}{\text{DM%}} \times 10^2 \times 100^b \times 0.9^c \times 100
\]

\[= T \times 2^d \times 1000\]

a - Titre obtained for ferricyanide reagent against Glc. Std.

b - Total volume of starch hydrolysate.

c - Morris factor for converting sugar to starch.

d - Weight of the sample (g)

T – Titre value for starch hydrolysate.

Vitamins

Ascorbic acid and niacin content were extracted and estimated as per the method given by Sadasivam and Manickam (1996). For the extraction of ascorbic acid, 3g air dried powdered sample was ground with 25ml of 4% oxalic acid filtered. Bromine water was added drop by drop to 10ml of the filterate until it turned orange-yellow to remove the enolic
hydrogen atoms. The excess of bromine was expelled by blowing in air. This filterate was made up to 25ml with 4% oxalic acid and used for ascorbic acid estimation. Two millilitres of the extract was made up to 3ml with distilled H2O in a test tube. One millilitre of 2% 2, 4-dinitrophenyl hydrazine reagent and a few drops of thiourea were added. The contents of the test tube were mixed thoroughly. After 3hr incubation at 37°C, 7ml of 80% H2SO4 was added to dissolve the osazone crystals and the absorbance was measured at 540nm against a reagent blank.

For the extraction of niacin, 5g air-dried powdered sample was steamed with 30ml concentrated H2SO4 for 30min. After cooling, this suspension was made up to 50ml with distilled H2O and filtered. Five millilitres of 60% basic lead acetate was added to 25ml of the filtrate. The pH was adjusted to 9.5 and centrifuged to collect the supernatant. Two millilitres of concentrated H2SO4 was added to the supernatant. The mixture was allowed to stand for 1hr and centrifuged. The 5ml of 40% ZnSO4 was added to the supernatant. The pH was adjusted to 8.4 and centrifuged again. Then the pH of the collected supernatant was adjusted to 7 and used as the niacin extract. For estimation, 1ml extract was made up to 6ml with distilled water in a test tube, 3ml cyanogens bromide was added and shaken well, followed by addition of 1ml of 4% aniline. The yellow colour that developed after 5min was measured at 420nm against a reagent blank. The ascorbic acid and niacin contents present in the sample were calculated by referring to a standard graph and expressed as milligrams per 100grams of powdered samples.

**Amino acid analysis**

The total protein was extracted by a method of Basha *et al.* (1976). The extracted proteins were purified by precipitation with cold 20% trichloroacetic acid (TCA). A protein sample of 30mg was hydrolysed by 6N HCL (5ml) in an evacuated sealed tube, which was kept in an hot air oven maintained at 110°C for 24 hr. The sealed tube was broken and the
acid removed completely by repeated flash evaporation after the addition of de-ionized water. Dilution was effected by means of citrate buffer pH 2.2 to such an extent that the solution contained 0.5 mg protein ml⁻¹. The solution was passed through a Millipore filter (0.45μM) and derivitized with O-phthalaldehyde by using an automated pre-column (OPA). Amino acids were analysed by a reverse-phase HPLC (method L 7400, HITACHI, Japan) fitted with a Denali C₁₈ 5 micron column (4.6X 150mm). The flow rate was 1 ml min⁻¹ with fluorescence detector. The cystine content of protein sample was obtained separately by the Liddell and Saville (1959) method. For the determination of tryptophan content of proteins, aliquots containing known amounts of proteins were dispersed into glass ampoules together 1 ml 5M NaOH. The ampoules were flame sealed and incubated at 110°C for the 18 hr. The tryptophan contents of the alkaline hydrolysates were determined colorimetrically using the method of Spies and Chambers, (1949) as modified by Rama Rao _et al._ (1974). The contents of the different amino acids were expressed as g 100g⁻¹ proteins and were compared with FAO / WHO (1991) reference pattern. The essential amino acid score was calculated as follows:

\[
\text{Essential amino acid score} = \frac{\text{grams amino acid in 100g of total protein}}{\text{grams of essential amino acid in 100g of FAO/WHO (1991) reference pattern}} \times 100
\]

**Lipid extraction and Fatty acid analysis**

The total lipid was extracted from the samples according to the method of Folch _et al._ (1957) using chloroform and methanol mixture in ratio of 2: 1 (v/v). Methyl esters were prepared from the total lipids by the method of Metcaife _et al._ (1966). Fatty acid analysis was performed by gas chromatography (ASHMACO, Japan; Model No : ABD20A) using an instrument equipped with a flame ionization detector and a glass column (2m x 3mm) packed with 1% diethylene glycol succinate on chromosorb W. The temperature conditions for GC
were injector 200°C and detector 210°C. The temperature of the oven was programmed from 180°C and the carrier gas was nitrogen at a flow rate of 30ml/min. Peaks were identified by comparison with authentic standards, quantified by peak area integration and expressed as weight percentage of total methyl esters; the relative weight percentage of each fatty acid was determined from integrated peak areas.

**In vitro protein digestibility determination (Padmaja, 2001)**

100mg of the dried powdered sample was weighed and 10 ml distilled water was added. The sample was allowed to hydrate for 1 hour and equilibrated to pH 8.0 at 37°C. Panzynorm-N (Enzyme tablet) was dissolved in 5 ml of 0.02 M sodium phosphate buffer solution (pH 6.9) 1 ml of enzyme solution was added and incubated at 37°C for 1 hr. The reaction was stopped by heating to 100°C. Control was run without the sample. The amino acids released were estimated by Lowry et al method (1951).

**Calculation**

The process was standardized using a Bovine Serum Albumin standard and the factor was calculated to be 1 mg.

**In vitro** protein digestibility was calculated as

\[
\text{Protein liberated in test sample as a result of enzyme digestion} = T \times F \text{ mg}
\]

\[
T = \text{Test OD - Control OD}
\]

\[
F = \text{Factor (1 mg)}
\]

**In vitro starch digestibility determination (Padmaja, 2001)**

100mg of powdered sample was weighed and 10ml of the buffer (0.02 M Sodium phosphate buffer) was added. The volume was then made upto 20 ml using the buffer after the samples were homogenized over a boiling water bath.
**Colorimetric assay**

To nullify the effect of free reducing sugars, controls were set up 0.2 ml of the samples was pipetted out and the volume was made to 1.0 ml using distilled water. To the remaining sample 0.5 ml of pancreatic amylase was added and incubated at room temperature for 30 seconds. The flasks were placed in a boiling water bath immediately to inactivate the enzyme. On cooling 0.2 ml aliquots from each sample were pipetted out to obtain the test values. The reducing sugars formed by the action of α – amylase on the starch were estimated by Nelson – Somogyi’s method and the absorbance was read at 520 nm.

The in vitro starch digestibility was expressed as mg reducing group formed /hr / g starch taken.

**Calculation**

\[
\text{In vitro digestibility units} = T \times 407.33 \text{ units}
\]

Where 100 mg of the sample was used

\[
T = \text{Test OD value} - \text{Control OD VALUE}
\]

**Antinutritional components**

**Extraction and Estimation of Total free phenolics**

**Extraction (Maxon and Rooney, 1972)**

Five hundred milligram of air dried powdered sample was taken in a 100 ml flask, to which 50 ml of 1% (v/v) HCl in methanol was added. The samples were shaken on a reciprocating shaker for 24 hrs.at room temperature. The contents were centrifuged at 10,000 x g for 5 minutes. The supernatant was collected separately and used for further analysis.

**Estimation of Total free phenolics (Sadasivam and Manickam, 1996)**

One ml of aliquot of the above extract was pipetted into different test tubes to which 1ml of Folin-ciocalteu’s reagent followed by 2 ml of 20% (w/v) Na2CO3 solution were added and the tubes were shaken and placed in a boiling water bath for exactly 1 min.
The test tubes were cooled under running tap water. The resulting blue solution was diluted to 25 ml with distilled water and the absorbance was measured at 650 nm with the help of a Spectrophotometer. If precipitation has occurred, it was removed by centrifugation at 5000 x g for 10 min. before measuring the absorbance. The amount of phenolics present in the sample was determined from a standard curve prepared with catechol. A blank containing all the reagents except plant extract was used to adjust the absorbance to zero. Average value of triplicate estimation was expressed as g 100g⁻¹ of powdered sample on dry weight basis.

**Estimation of Tannins (Burns, 1971)**

From suitable aliquots of the above extract tannin content was quantified by the Vanillin – HCL method of Burns (1971) using phloroglucinol as a standard at 500 nm with a Spectrophotometer. The average values of triplicate estimates of all samples were expressed as g 100g⁻¹ powdered samples on dry weight basis.

**Extraction and Estimation of Hydrogen cyanide, (Jackson, 1967)**

**Extraction**

Three gms of air-dried powdered sample was mixed thoroughly with 62.5ml of distilled water and 3 to 4 drops of chloroform in a distillation flask. The above suspension was steam distilled. The delivery end of the condenser was kept below the surface of 5 ml of 2% KOH solution in a beaker. Approximately 30 ml of distillate was collected and it was used as the source material.

**Estimation**

Five ml of aliquots of the above extract was pipetted into different test tubes and 5 ml of alkaline picrate solution was added to each test tube. The contents of the test tubes were mixed and digested in boiling water bath for 5 min. and the absorbance was measured at 520 nm against a reagent blank. The Hydrogen cyanide content present in the sample was
calculated by referring to a standard graph of potassium cyanide as standard and expressed as mg 100g⁻¹ of powdered samples.

**Total oxalate estimation (AOAC, 1984)**

One gm dried powdered sample was extracted twice with 0.25 N HCl in a water bath (60°C) for 1 hour each. The centrifuge was collected in a conical flask. This extract was precipitated by adding tungstophosphoric acid kept overnight and centrifuged. Then it was neutralized with 1: 1 dil. Ammonium solution. It was precipitated overnight by adding 5ml of acetate buffer with CaCl₂ (pH 4.5). The precipitate was centrifuged and washed twice with wash liquid (Dilute acetic acid + Calcium oxalate, decanted and filtered).

The precipitate was then dissolved in 15 ml 2 N H₂SO₄ and titrated against 0.01 N KMnO₄ solutions at 60°C.

**Calculation**

\[
\text{Total oxalate (\% \ (dry weight))} = \frac{0.063 \times \text{vol. of } N \ \text{KMnO}_4}{\text{Weight of sample (gm)}}
\]

**Trypsin inhibitor assay (Sasikiran and Padmaja, 2003)**

Caseinolytic assay was used for the study of trypsin inhibitor. Three types of assay systems were used. The control system consisted of casein (2%, 2.0ml), trypsin inhibitor extract (0.3 ml) and 1.7 ml sodium phosphate buffer (0.01 M, pH 8.0) trypsin (0.2 ml from a 1mg / 10ml stock). In the inhibited system, trypsin (0.2 ml) and the inhibitor extract (0.3ml) were pre-incubated for 30 min. for maximum inhibitor response. To this, casein (2.0 ml) was added followed by 1.5ml buffer. The system was incubated for 15min. at 30°C and the reaction was stopped by adding 4.0 ml of 10% TCA. The TCA system was kept for one hour to complete protein precipitation and the precipitate was centrifuged off at 1000 x g for 10 min. One ml of the supernatant was used for the quantification of TCA soluble peptide fragments by the method of Lowry et al., (1951).
Calculation of trypsin inhibitor activity

A calibration curve was prepared using Bovine Serum Albumin (BSA) as standard. One unit of trypsin activity is defined as one mg peptide fragments released per minute at 30°C under the assay conditions.

Trypsin inhibitor unit (TIU) is defined as the number of trypsin units inhibited and is expressed on a dry weight basis. The effect of free amino acids in the inhibitor was nullified by subtracting the readings of control system from the inhibited system readings.

Assay of amylase inhibitor (Rekha and Padmaja, 2002)

The amylase inhibitor activity was studied using 0.5 % soluble starch as substrate. Porcine pancreatic α – amylase (Emerck, Germany) was used as the enzymatic source uniformly throughout the study. Three assay systems were used to quantify the amylase inhibitor activity. The control system consisted of starch (0.5ml) and 0.02 M sodium phosphate buffer pH 6.9 (2.35 ml). In the uninhibited system, 0.5 ml starch and 2.0 ml buffer were allowed to react with α-amylase (0.25 ml from a solution containing 100 μg porcine pancreatic α – amylase / ml). After incubation for 10 min. the reaction was stopped by adding 1.0 ml of 0.1N HCl. In order to elicit maximum inhibitor response, the inhibitor extract (0.5 ml) was preincubated with the buffer (1.5 ml) and α – amylase (0.25 ml) for 30 min. at room temperature (30 ± 2°C). To this inhibited system, 0.5 ml starch was added to initiate enzyme action. After incubation for 10 min., the reaction was terminated by adding 1.0 ml 1N HCl.

The residual starch in the uninhibited and inhibited system as well as the starch in the control system was quantified by the method of Mohammed and Sharma (1986) using iodine reagent (0.5 ml of an iodine solution containing 0.2 g iodine crystals and 2g potassium iodide in 100 ml distilled water). The volume was increased to 20 ml with distilled water and the absorbance of the blue colour was measured at 620 nm in an ATI – Unicam UV – Vis Spectrophotometer.
Calculation of amylase inhibitor activity

A calibration curve was prepared using soluble starch (500 – 3000 µg), phosphate buffer and iodine reagent. One α - amylase unit is defined as one µg starch hydrolyzed per minute at 30°C under the assay conditions.

One α-amylase inhibitor unit (AIU) is defined as the amount of inhibitor that reduces the α-amylase activity by one unit.

The amount of starch hydrolyzed in the uninhibited system was computed by subtracting the respective readings from the control system readings.

Three replications were maintained for each sample and duplicate analysis was performed for each replicate.

PHARMACOLOGICAL STUDIES

Preparation of plant extract for pharmacological studies

Freshly collected tubers of *Dioscorea esculenta*, *Nymphaea pubescens* and corms of *Xanthosoma sagittifolium*, *Amorphophallus paeoniifolius var. campanulatus* were dried in shade, and then coarsely powdered separately in a Willey mill. The coarse powder (100g) was extracted with ethanol, each ml in a Soxhlet apparatus for 24 hours. The extracts were filtered through Whatman No. 41 filter paper. The ethanol extracts were concentrated in a rotary evaporator. The concentrated ethanol extracts were used for pharmacological studies.

Acute toxicity study

Acute oral toxicity study was performed as per OECD – 423 guidelines (acute toxic class method), albino rats (n = 6) of either sex selected by random sampling were used for acute toxicity study (OECD, 2002). The animals were kept fasting for overnight and provided only with water, after which the extracts were administered orally at 5 mg/kg body weight by gastric incubations and observed for 14 days. If mortality was observed in two out of three animals, then the dose administered was assigned as toxic dose. If mortality was
observed in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for higher doses such as 50, 100 and 1000 mg/kg body weight.

ANTIFERTILITY ACTIVITY

Experimental Animal

Mature adult male Wister strain albino rats weighing about 180-200 gm body weight were selected for this work. They were maintained in a well-ventilated animal house with constant 12 hours of darkness and 12 hours of light schedule. Clean water and standard pellet diet (Hindustan Lever Ltd., India) were available to them ‘ad libitum’.

Experimental Design

The animals were divided into four groups, each consisting of 5 animals.

Group I : Rats received given normal saline daily for 7 days consequently orally (by using an intragastric catheter tube (IGC). (Control)
Group II: Rats received ethanol extract of Dioscorea esculenta tuber 100mg/kg body weight, daily for 7 days, orally by IGC for seven consecutive days (Low)
Group III: Rats received ethanol extract of Dioscorea esculenta tuber 300mg/kg body weight, daily for 7 days, orally by IGC for seven consecutive days (Moderate)
Group IV: Rats received ethanol extract of Dioscorea esculenta tuber 600mg/kg body weight, daily for 7 days, orally by IGC for seven consecutive days (High)

Suitable controls were maintained for each duration of treatment. However, as there was no obvious difference on any parameter among control groups, a common control was employed in the present study.

All the treatment was given between 8.00 and 11.00 hour in the morning. After 24 hours of last treatment, the final body weight was recorded and the animals were sacrificed by decapitation. Blood was collected, sera separated by centrifugation at 3000 x g for 10 minutes and stored at -20°C until used for various biochemical assays. Then testis,
epididymis, vas deferens, seminal vesicle and ventral prostate were dissected out, trimmed off extraneous tissues and weighed accurately on a torsion balance. The organs weights were expressed in terms of mg/100 gm body weight.

Assessment of Sperm motility

Immediately after animals sacrifice, cauda epididymis was punctured using hypodermic needle; sperm suspension was collected and transferred into a droplet of physiological saline. The motility of the spermatozoa was observed under microscope at 100 x magnification. The distance traversed by the sperm was determined using an occulometer and expressed as micro (\(\mu m\)) meter traversed per minute.

Sperm count determination

Collection of epididymal fluid

Epididymal fluid (for sperm count) was collected from caput and cauda segments, separately, minced in 2 ml Sorenson’s buffer (pH 7.2) and passed through nylon mesh of 75 \(\mu\) size. The separated fluid was taken for sperm count.

Sperm count was carried out by using Neubauer haemocytometer as described by Zaneveld and Polakoski (1977).

For the standard sperm analysis, a 20 fold dilution was made by mixing the epididymal fluid with Sorenson’s buffer. 0.1 ml epididymal fluid was added to 1.9 ml of Sorenson’s buffer. The preparation was thoroughly mixed and one drop was added to both side of a standard blood cell haemocytometer (Neubauer). The numbers of spermatozoa in the appropriate square of the haemocytometer were counted under the microscope at x 100. Both sides of the haemocytometer were counted and an average was taken.

Calculation

The sperm concentration refers to the number of spermatozoa per ml epididymal fluid. The haemocytometer is a grid containing 5 major squares called E1, E2, E3, E4 and
the central small square is E5. While counting all the spermatozoa within the designated squares and those that cross the lines at the top and right hand sides were included.

Major Square E is 1 mm long, 1 mm wide and the thickness of the fluid between the cover slip and the haemocytometer is 0.1 mm. The total volume represented by major square is thus 0.1 mm$^3$ or $10^{-4}$ ml. The multiplication factor of corner square E is therefore $10^{-4}$ or 10,000. When all the spermatozoa in the major square E were counted, the number was multiplied with the multiplication factor, 10,000 to get the number of spermatozoa per ml of the solution applied to the haemocytometer. When this was multiplied with a dilution factor (the amount of sperm dilution normally was 20 times), the concentration of spermatozoa in the original sample was obtained. When the spermatozoa in small square E1, E2, E3, E4 and E5 were counted, the multiplication factor was 5 times greater than when the entire E square was counted and was thus 50,000. When all the major squares were counted as in the case of very low sperm concentration, the multiplication factor was 5 times smaller i.e. 2000. The sperm concentration is expressed in counts x $10^6$/ml. The basic formula that is applied to obtain the sperm concentrations is:

$$\text{Sperm concentration} = \text{Number of spermatozoa} \times \text{multiplication factor} \times \text{dilution factor}$$

**Sperm motility, viability and counts**

The rats were anaesthetized with 25% urethane at a dose of 0.6 ml/100 g intraperitoneally. The caudal epididymis was then dissected. An incision (about 1 mm) was made in the caudal epididymis and drops of sperm fluid were squeezed onto the microscope slide and 2 drops of normal saline were added to mobilize the sperm cells. Epididymal sperm motility was then assessed by calculating motile spermatozoa per unit area and was expressed in percentage.
Epididymal sperm counts were also done by homogenizing the epididymis in 5 ml of normal saline. Counting was then done using the counting chamber in the haemocytometer. The sperm viability was also determined using Eosin/Nigrosin stain.

**Fertility test**

Fertility test of individual rat was done before the experiment and after 7 days of treatment. Each male rat was cohabitated with proestrous females in 1:2 ratio. Vaginal smear was examined every morning positive mating and number of litres delivered were recorded.

**Hormonal analysis**

Blood removed from the animals by intracardiac method. Blood was centrifuged at 2000 rpm (Revolution per minute) to separate the serum for the measurement of FSH, LH, and Testosterone and Estrogen. The quantitative determination of hormones was done by using Enzyme Immunoassay Method (EIA). The EIA kit was obtained from Immunometrics (London, UK) and contained a testosterone EIA enzyme label testosterone EIA substrate reagent and EIA quality control sample. An enzyme-based immunoassay (EIA) system was used to measure all the hormone in the serum samples collected. A quality control was carried out at the beginning and the end of the assay to ascertain the acceptability with respect to bias and within batch variation. The EIA kit used had a sensitivity level of approximately 0.3-0.1 respective of all reproductive hormones. The intra- and inter- assay variations were 10.02 %, 10.12 %, 9.7% and 10.4 for Testosterone, FSH, LH, and Estrogen respectively.

**ANTIDIABETIC ACTIVITY**

**Experimental induction of diabetes in rats**

Three month old male Wistar albino rats weighing 180-240g were obtained from the animal house of the laboratory of Agricultural University, Trissur, and Kerala. All animals were kept in an environment controlled room with a 12h light/12h dark cycle.
animals had free access to water and standard rat diet. The rats were injected alloxan monohydrate dissolved in sterile normal saline at a dose of 150 mg/kg body weight, intraperitoneally. Since alloxan is capable of producing fatal hypoglycaemia as a result of massive pancreatic insulin release, rats were treated with 20 % glucose solution intraperitoneally after 6h. The rats were then kept for the next 24h on 5 % glucose solution bottles in their cages to prevent hypoglycemia (Dhandapani et al., 2002). After a fortnight rats with moderate diabetes having glycosuria (indicated by Benedict’s test for Urine) and hyperglycemia with blood glucose range of 200 – 260 mg / 100 ml were used for the experiment.

**Experimental design**

In the present investigation non-diabetic control rats and diabetic induced rats were used. Diabetics were induced in rats 2 weeks before starting the treatment. The rats were divided into nine groups as follows after the induction of diabetics. Each groups consisting of 5 numbers of rats.

**Group I** : Rats given normal saline daily for 14 days consequently orally (by using an intragastric catheter tube (IGC). (Normal control)

**Group II** : Diabetic rats given normal saline daily for 14 days consequently orally by using IGC.(Diabetic control).

**Group III** : Diabetic rats given ethanol extract of *Xanthosoma sagittifolium* corm at the dose of 200 mg / kg body weight daily orally for 14 days consequently (IGC)

**Group IV** : Diabetic rats given ethanol extract of *Nymphaea pubescens* tuber, at the dose of 200mg / kg body weight daily orally for 14 days consequently (IGC).

**Group V** : Diabetic rats given ethanol extract of *Xanthosoma sagittifolium* corm, at the dose of 500 mg / kg body weight daily orally for 14 days consequently (IGC).

**Group VI** : Diabetic rats given ethanol extract of *Nymphaea pubescens* tuber, at the dose of 500 mg / kg body weight daily orally for 14 days consequently (IGC).
Group VII: Diabetic rats given glibenclamide at the dose of 600 μg / kg body weight daily orally for 14 days consequently (IGC).

Group VIII : Diabetic rats given ethanol extract of Xanthosoma sagittifolium corm (200 mg / kg body weight) and Nymphaea pubescens tuber (200 mg / kg body weight) daily orally for 14 days consequently (IGC).

Group IX : Diabetic rats given ethanol extract of Xanthosoma sagittifolium corm (500 mg / kg body weight) and Nymphaea pubescens tuber (500 mg / kg body weight) daily orally for 14 days consequently (IGC).

All the plant drug treatment was given between 9.30 to 10.00 hour in the morning. All rats were sacrificed on the morning of the respective experimental day, by decapitation. Blood was collected, sera separated by centrifugation at 3000 x g for 10 min. and stored at - 20° C until used for enzyme and biochemical assays.

Estimation of Insulin (Anderson et al., 1993)

The UBI MAGIWEL™ INSULIN QUANTITATIVE is solid phase enzyme-linked immunosorbtant (ELISA).

Principle

UBI MAGIWEL™ Insulin is a solid phase enzyme-linked immunosorbtant assay (ELISA). The wells are coated with monoclonal antibody with higher activity for insulin. When the samples, and controls are incubated in the wells with enzyme conjugate, which is another antibodies linked to horse radish peroxidase to form a sandwich complex bound to the well. Unbound conjugate are then washed off with wash buffer. The amount of bounded peroxidase is proportional to the concentration of the insulin present in the sample. Upon addition of the substrate and chromogen, the intensity of the colour developed is proportional to the concentration of insulin in the samples.
Assay procedure

- Secured the designed number of coated wells in the holder. Marked data sheet with sample identification.
- Dispensed 25 µl of serum sample, control and reference into the assigned wells.
- Dispensed 100 µl of enzyme conjugate into each well and mixed for 5 secs.
- Incubated for 30 min. at 25° C.
- Removed incubation mixture and rinsed the wells five times with washing buffer.
- Dispensed 100 µl of solution A and then 100 µl of solution B in to each well.
- Incubated for 15 min. at room temperature.
- Stop reaction by adding 50 µl of 1N sulphuric acid or 2N HCl to each well and read O.D at 450 nm with a micro well reader.

Estimation of Glucose (Sasaki et al., 1972)

Principle

Ortho toludine reacts with glucose in hot acetic acid solution to produce blue colour, which is measured at 630nm.

Reagents

1. Ortho toludine boric acid reagent: This reagent consists of 2.5g of thiourea and 2.4g of boric acid in 100ml of a mixture of water, acetic acid and ortho toludine (distilled) in the ratio of 10:75:15.

2. Standard glucose: 100mg of glucose in 0.1% benzoic acid. 10ml of the above solution was diluted to 100ml to give 100µg of glucose per ml.

Procedure

To 0.2 ml of serum was added 0.8ml of 10% TCA. Mixed well and centrifuged. 0.5 ml of the supernatant was taken. To this 2.0 ml of ortho toludine reagent was added and heated in a boiling water bath for 15min. along with standard solution containing 20-100 µg
of glucose. The blue colour developed was read at 640nm. The result was expressed as mg/dl in serum

**Estimation of Urea (Varley, 1976)**

**Principle**

Di-acetyl monoxime in the presence of acid, hydrolysis to produce the unstable compound diacetyl. This reacts with urea to produce a yellow diazone derivative. The colour of this product becomes pink by addition of thiosemicarbazide which is measured colorimetrically at 520nm.

**Reagents**

1. TCA, 10%
2. Stock Diacetylmonoxime, 25g/L
3. Stock Thiosemicarbazide 2.5g/L
4. Acid ferric chloride solution: Added 1.0ml sulphuric acid to 100ml of ferric chloride solution containing 50 g/L in water.
5. Acid reagent: Added 10ml of ortho phosphoric acid, 80 ml sulphuric acid and 10ml acid ferric chloride solution to 1 litre of water and mixed.
6. Colour reagent: To 300 ml acid reagent added 200ml water, 10ml stock diacetylmonoxime and 2.5 ml thiosemicarbazide.
7. Stock urea standard: 5,10,15,20,30,40, and 50 mmol/L (30, 60, 90, 120, 180, 240 and 300 mg/100 ml).

**Procedure**

To 0.2 ml of serum were added 1.0 ml water and 1.0 ml of 10%TCA. Mixed well and centrifuged. 0.2 ml of the supernatant was taken and added 3.0 ml of colour reagent. At the same time took 0.2ml of water bath for 20 min. Cooled to room temperature and read the colour developed at 520 nm within 15 min. The result was expressed as mg/dl in serum.
**Estimation of Creatinine (Owen et al., 1954)**

**Principle**

Creatinine forms a coloured complex with picrate in alkaline medium. The rate of formation of the complex is measured at 540nm.

**Reagents**

1. Picric acid: 8.02g/L
2. Sodium hydroxide: 12.8g/L
3. Standard creatinine: Dissolved 100 mg of creatinine in 100ml with distilled water.
4. Working standard: Diluted 2.0 ml of stock solution was diluted to 100 ml with distilled water. This contains 20μg of creatinine / ml.
5. Reagent mixture: Mixed one part by volume of diluted NaOH with one part by volume of picric acid at least 30 minutes before the assay.

**Procedure**

0.2ml of the serum and 2.0ml of the reagent mixture were pipetted into a cuvette. Simultaneously, a blank was set up with the reagent mixture and distilled water. Mixed well and the change in absorbance was measured after 30 sec., which was taken as A₁ and exactly after 2 min. the absorbance was read as A₂ at 490nm. Sets of standards were also treated in the same manner. A₁-A₂ gives the change in absorbance, which was the measure of the creatinine present in the sample. The result was expressed as mg/dl in serum. The values are expressed as mg of creatinine/dl.

**Estimation of Glycosylated Haemoglobin (HbA₁C)**

At the end of the experimental period, animals were killed and blood samples (5ml) were collected in heparinized tubes by cardiac puncture. Plasma was separated and cells were washed twice (0.154 M saline) and stored at -20° C until HbA₁C concentration
were determined by the method of Karunanayake and Chandrasekharan, (1986). The normal values of HbA1C in Wistar rats comparable age and weight were established by determining HbA1C concentration in 10 untreated Wistar rats.

Estimation of Protein (Lowry et al., 1951)

Principle

The Blue colour developed by the reduction of the phosphomolybdic phosphotungstic components in the Folin-ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured at 660nm.

Reagents

1. 1.2% Sodium carbonate in 0.1N NaOH (Reagent A)
2. 0.5% Copper sulphate in 1% potassium sodium tartarate (Reagent B)
3. Alkaline copper reagent: Mixed 50ml of A and 1.0ml of B prior to use
4. Folin-ciocalteau reagent: Mixed 1 part of reagent with 2 part of water.
5. Stock standard: Weighed 50mg of Bovine Serum Albumin and made up to 50ml in a standard flask with saline.
6. Working standard: Diluted 10ml of the stock to 50ml with distilled water. 1.0ml of this solution contains 200μg of protein.

Procedure

0.2ml to 1.0ml working standard solution was pipetted into test tubes. 0.1ml of the sample was taken. The volume in all the tubes was made upto 1.0ml with distilled water. Added 5.0 ml of alkaline copper reagent to each tube. Mixed well and allowed to stand for 10min. Then added 0.5ml of folin-ciocalteau reagent. Mixed well and incubated at room temperature for 30 min. A reagent blank was also prepared. After 30 minutes, the blue colour developed was read at 660nm. The result was expressed as g/dl in serum.
Serum albumin is determined by quantitative colorimetric method by using bromocresol green reagent. The test was performed by adding 10μl of serum to 1ml of albumin reagent. This test did not require any pretreatment. This reagent forms a coloured complex specifically with albumin. The intensity of the colour measured at 620nm, is directly proportional to the albumin concentration in the serum. The total protein minus the albumin gives the globulin.

**Estimation of Serum Glutamate Pyruvate Transaminase (SGPT or ALT) (Reitman and Frankel, 1957)**

**Principle**

The enzyme catalyses the following reaction:

\[
\text{L-Alanine} + \alpha\text{-oxoglutarate} \rightarrow \text{Pyruvate} + \text{L-glutamate}
\]

The oxaloacetate is measured by the reaction with 2, 4- dinitrophenylhydrazine giving a brown coloured hydrazone after the addition of sodium hydroxide. The colour developed is read at 520 nm.

**Reagents**

1. Phosphate buffer: 0.1M, pH 7.5
2. Substrate: Dissolved 146 mg of α- ketoglutarate and 17.8 g of L-alanine in 1N NaOH with constant stirring. Adjusted the pH to 7.4 and made up to 1000 ml with phosphate buffer.
3. Standard pyruvate, 2 mM: Dissolved 22 mg of sodium pyruvate in 100ml of phosphate buffer, 0.2 ml of standard contained 0.4 μM of sodium pyruvate.
4. Dinitrophenyl hydrazine reagent, 1 mmol/L: 200mg/L in 1 mol/L HCl.
5. 0.4 N NaOH: Dissolved 16 g of NaOH in 1000 ml water
Procedure

0.2 ml of sample and 1.0 ml of the buffer substrate were incubated for 30 min. at 37°C. To the control tubes, enzyme was added after arresting the reaction with 1.0ml of DNPH and the tubes were kept at room temperature for 20 min. Then 10ml of 0.4 N Na OH was added. A set of standard pyruvate was also treated in a similar manner. The colour developed was read at 520 nm. The enzyme activities were expressed as units/L in serum and units/protein in tissues.

Estimation of Serum Glutamate Oxalo Transaminase (SGOT or AST) (Reitman and Frankel, 1957)

Principle

The enzyme catalyses the following reaction:

L-Aspartate + α-oxoglutarate → Oxaloacetate + L-glutamate

The oxaloacetate is measured by the reaction with 2,4-dinitrophenylhydrazine giving a brown coloured hydrazone after the addition of sodium hydroxide. The colour developed is read at 520 nm.

Reagent

1. Phosphate buffer 0.1M, pH 7.5
   SolutionA: 0.1M solution of monobasic sodium phosphate (13.9g/l).
   SolutionB: 0.1M solution of dibasic sodium phosphate (6.8 of Na₂PO₄.7H₂O g/L)
   16ml of A and 84 ml of B, diluted to a total of 200ml.

2. Substrate: Dissolved 146 mg of α-Ketoglutarate and 13.3 g of aspartic acid in 1 N NaOH with constant stirring. Adjusted the pH to 7.4 and made up to 1000 ml with phosphate buffer.

3. Standard pyruvate, 2m mol/L: Dissolved 22 mg of sodium pyruvate in 100ml of phosphate buffer. 0.2ml of standard contained 0.4 μM of sodium pyruvate.
4. Dinitrophenylhydrazine reagent, 1 mmol/L: 200 mg in 1 mol/L HCl.
5. 0.4 N NaOH: Dissolved 16 g of NaOH in 1000 ml water.

**Procedure**

0.2 ml of sample and 1.0 ml of the buffer substrate was incubated for 60 min. at 37\(^\circ\) C. To the control tubes, enzyme was added after arresting the reaction with 1.0 ml of DNPH and the tubes were kept at room temperature for 20 min. Then 10 ml of 0.4 N NaOH was added. A set of standard pyruvate was also treated in a similar manner. The colour developed was read at 520 nm. The enzyme activity was expressed as units/L in serum and units/protein in tissue.

**Estimation of Alkaline Phosphatase (ALP) (King and Armstrong, 1934)**

**Principle**

The method used was that of King and Armstrong in which disodium phenyl phosphate is hydrolysed with the liberation of phenol and inorganic phosphate. The liberated phenol is measured at 700 nm with Folin-Ciocalteau reagent.

**Reagent**

1. Sodium carbonate-sodium bicarbonate buffer, 100 m mol/L: Dissolved 6.36 g anhydrous sodium carbonate and 3.36 g sodium bicarbonate in water and made to a litre.
2. Disodium phenyl phosphate, 100 m mol/L: Dissolved 2.18 g in water, heated to boil, cooled and made to a litre. Added 1.0 ml of chloroform and stored in the refrigerator.
3. Buffer substrate: Prepared by mixing equal volume of the above two solutions. This has a pH of 10.
4. Folin-Ciocalteau reagent: Mixed 1.0 ml of reagent with 2.0 ml of water.
5. Sodium carbonate solution, 15%: Dissolved 15 g of anhydrous sodium carbonate in 100 ml of water.
6. Standard phenol solution, 1 g/L: Dissolved 1 g pure crystalline phenol in 100 mmol/L HCl and made to a litre with the acid.

7. Working standard solution: Added 100 ml dilute phenol reagent to 5.0 ml of stock standard and diluted to 500 ml with water. This contained 10 μg phenol/ml.

**Procedure**

4.0 ml of the buffer substrate was pipette into a test tube and incubated at 37°C for 5 min. Added 0.2 ml of serum or tissue homogenate and incubated further for exact 15 min. Removed and immediately added 1.8 ml of diluted phenol reagent. At the same time a control was set up containing 4.0 ml buffer substrate and 0.2 ml sample to which 1.8 ml phenol reagent was added immediately. Mixed well and centrifuged. To 4.0 ml of supernatant added 2.0 ml of sodium carbonate. Take 4.0 ml of working standard solution and for blank taken 3.2 ml water and 0.8 ml of phenol reagent. Then add 2.0 ml of sodium carbonate. Incubated all the tubes at 37°C for 15 min. Read the colour developed at 700 nm. The activity was expressed as units/L in serum and units/protein in tissue.

**Estimation of Total Cholesterol (TC) (Parekh and Jung, 1970)**

**Principle**

Cholesterol reacts with ferric chloride in the presence of concentrated sulphuric acid to give a pink colour. The intensity of colour developed is directly proportional to the amount of cholesterol present and is read at 540 nm in a colorimeter.

**Reagents**

1. Stock ferric chloride: 840 mg of pure dry ferric chloride was weighed and dissolved in 100 ml of glacial acetic acid.

2. Ferric Chloride precipitation reagent: 10 ml of stock ferric chloride reagent was taken in 10 ml of standard flask and made up to the mark with pure glacial acetic acid.
3. Ferric chloride diluting reagent: 8.5 ml of stock ferric chloride is diluted to 100ml with pure glacial acetic acid.

4. Standard cholesterol solution: 100 mg of cholesterol was dissolved in 100 ml of glacial acetic acid.

5. Working standard: 10 ml of stock was dissolved in 0.85 ml of stock ferric chloride reagent and made up to 100 ml with glacial acetic acid. The concentration of working standard is microgram/ml.

Procedure

To 0.1 ml of the serum was added 4.9 ml of ferric chloride precipitating reagent. Centrifuged and to 2.5ml of supernatant added 2.5 ml of ferric chloride diluting agent. Add 4.0ml of concentrated sulphuric acid. A blank was prepared simultaneously by taking 5.0 ml of diluting reagent and 4.0ml of concentrated sulphuric acid. A set of standards (0.5-2.5ml) were taken and made up to 5.0 ml with ferric chloride diluting reagent. Then add 4.0 ml of concentrated sulphuric acid. After 30 min., the intensity of colour developed was read at 540 nm against a reagent blank. The amount of cholesterol in the sample is expressed as mg/dl.

Estimation of Triglycerides (TG) (Rice, 1970)

Principle

The glycerol moiety is oxidized to formaldehyde and the later condensed with ammonia and 2,4-pentanедione (acetyl acetone) to produce 3,5-diacetyl 1,4-dihydrotoludine, which is yellow in colour and has absorption at 450nm.

Reagents

1. Chloroform-methanol mixture (2:1)

2. Activated silicic acid: It was activated by washing silicic acid with 4N or 2N HCl and then with water until the washings become natural. After drying, ether was added. Silicic acid was then dried at 60°C and activated at 100°C over night prior to use.
3. 0.2 N H₂SO₄

4. Saponification reagent: Dissolved 5g of KOH in 60ml water and added 40ml of isopropanol.

5. Sodium-metaperiodate reagent: To 77g of anhydrous ammonium acetate in 700ml water, added 60ml acetic acid and 650mg of sodium metaperiodate. Dissolved and diluted in 1litre with distilled water.

6. Acetyl acetone reagent: Added 0.75ml of acetyl acetone to 20ml of isopropanol and mixed well. Added 80ml of distilled water and mixed.

7. Tripalmitin standard was containing 100µg/ml in chloroform.

**Procedure**

0.1ml of the serum or dried lipid extract was taken. The volume was made upto 4.0ml with isopropanol. Mixed well and added 400mg of silicic acid. Placed them in a mechanical shaker and centrifuged.

To 2.0ml of the supernatant was added 0.6 ml of saponification reagent and incubated at 60-70°C for 15min. After cooling added 1.0ml of sodium metaperiodate and mixed well. Then added 0.5ml of acetyl acetone reagent and mixed again. Incubated the tubes at 50°C for 30min. After cooling read the colour at 405nm. Standard tripalmitin (20-100µg) were taken in tubes and treated similarly. Triglycerides are expressed as mg/100ml in serum.

**Determination of LDL Cholesterol (LDL-C) and very low density Lipoprotein – Cholesterol (VLDL-C)**

LDL cholesterol and VLDL- Cholestrol level in serum was calculated by Friedwald *et al.*, (1972) formula.
Estimation of HDL – Cholesterol (HDL-C) (Warnick et al., 1985)

Principle

Cholesterol reacts with hot solution of ferric per chlorate, ethyl acetate and sulphuric acid (Cholesterol reagent) and gives a lavender coloured complex which is measured at 560 nm.

High density lipoproteins (HDL) are obtained in the supernatant after centrifugation. The cholesterol in the HDL fraction is also estimated by this method.

Procedure

(i) HDL – Cholesterol separation

Mixed well, kept at room temperature for 10 min. and then centrifuged at 2000 rpm for 15 min. to obtain a clear supernatant. Proceed to step II.

(ii) HDL – cholesterol estimation

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1: Cholesterol reagent</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Reagent 2 : Working cholesterol Standard, (200 mg%)</td>
<td>-</td>
<td>0.015 ml</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 (μl)</td>
<td>0.12 ml</td>
</tr>
<tr>
<td>Supernatant from step – 1</td>
<td>-</td>
<td>-</td>
<td>(120 μl)</td>
</tr>
</tbody>
</table>

Mixed well and kept the tubes immediately in the boiling water bath exactly for 90 seconds (1 ½ minutes). Cooled them immediately at room temperature, under running tap water. Measured the O.D of standard (S) and Test (T) against Blank (B) on a colorimeter with a yellow green filter or on a spectrophotometer at 560 nm.
Determination of Phospholipids

Lipids were extracted from 2 ml of the serum by following a modification in the procedure of Folch et al., (1957) using a chloroform-methanol mixture in the proportion of 2:1 (v/v) containing 15mg of butylated hydroxyl tobose (BHT). This lipid extract was used for the estimation of the phospholipids by using the method of Takagama et al., (1977).

Estimation of Oxidised Glutathione (GSSG)

An enzymatic method for quantitative determination of amounts of total (reduced and oxidized) glutathione. The method employs Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid or DTNB), which reacts in the GSH to form a spectrophotometrically detectable product at 412 nm. GSSG can be determined by the reduction of GSSG to GSH, which is then determined by the reaction with Ellman's reagent. In brief, the Tietze method (1969) utilizes the change in colour development during the reaction, and the reaction rate is proportional to the GSH and GSSH concentrations.

GSH / GSSG Ratio

The GSH/GSSG ratio is then calculated by dividing the difference between the GSH and GSSG concentration (reduced GSH) by the concentration of GSSG.

\[
\frac{\text{GSH} - 2\text{GSSG}}{\text{GSSG}} = \text{Ratio}
\]

Estimation of Lipid peroxidation (LPO) (Uchiyama and Mihara, 1978)

Principle

Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red colour, absorbing at 535 nm.
Reagents

1. 15% KCl
2. 1% Phosphoric acid
3. n-butanol
4. 0.6% thiobarbituric acid
5. 10 mM ferrous sulphate
6. 0.2 mM ascorbate

Procedure

0.5 ml of aliquot of the serum was mixed with 3.0 ml of 1% phosphoric acid and 1.0 ml of 0.6% thiobarbituric acid. The mixture was heated for 45 min. in a boiling water bath and after addition of 4.0 ml of n-butanol vigorously, vortexed and centrifuged at 2000 rpm for 20 min. the absorbance of the upper organic layer at 535 nm was measured in a spectrophotometer and compared with a standard of freshly prepared 1,1,3,3 tetraethoxy propane at concentration of 5.125, 10.25 and 20.5 nmol ml$^{-1}$ or using an extinction coefficient of the chromophore $1.56 \times 10^{-5}$ M$^{-1}$ cm$^{-1}$ and the results were expressed as n moles of MDA formed / mg protein

Extraction of Glutathione Reductase (GR)

The Glutathione Reductase activities in blood were assayed by the method of Goldbery and Spooner (1983). The Glutathione Reductase activity in blood has been expressed as nM NADPH oxidized to NADP / of Hb / min.

Estimation of Glutathione peroxidase (GPx) and Reduced Glutathione (GSH)

GPx activity was measured by the method described by Rotruck et al., (1984). Briefly, reaction mixture contained 0.2 ml of 0.4 mmol phosphate buffer (pH 7.0), 0.1 ml of 10 mmol sodium azide, 0.2 ml of serum, 0.2 ml of GSH and 0.1 ml of 0.2 mmol H$_2$O$_2$. The contents were incubated at 37°C for 10 min. The reaction was arrested by 0.4 ml of 10%
TCA, and centrifuged. Supernatant was assayed for GSH content by using Ellmans reagent (19.8 mg of 5,5'-dithiobisnitro benzoic acid in 100 ml of 0.1% sodium nitrate). GSH was determined by the method of Ellman (1959). 1.0 ml of supernatant was treated with 0.5 ml of Ellmans reagent and 3.0 ml of phosphate buffer (0.2 mmol, pH 8.0). The absorbance was read at 412 nm. GPx activity was expressed as µg of GSH consumed/min/mg protein and reduced GSH as mg/dl.

**Estimation of Glutathione S-Transferase (GST) assay**

The GST activity was determined spectrophotometrically according to the method Habig et al.,(1974). The total reaction mixture contained 2.79 ml 0.1M phosphate buffer (pH 6.5), 0.15 ml 20mM 1-chloro-2,4-dinitrobenzene (CDNB), 0.03 ml of 0.1M GSH. The reaction mixture was pre incubated at 37°C which after the reaction was started by the addition of 0.03 ml diluted Cytosol and the absorbance was read at 30sec, 1min, 2min, 3min, interval at 340 nm. The reaction mixture without the enzyme was used as blank. The specific activity of GST is expressed as nmol of GSH – CDBN conjugate formed/min/mg protein using an extinction co-efficient of 9.6m M⁻¹ CM⁻¹.

**ANTIINFLAMMATORY ACTIVITY**

**Carrageenan induced hind paw edema**

Albino rats of either sex weighing 150-200 grams were divided into four groups of six animals each. The dosage of the drugs administered to the different groups was as follows.

- Group 1 - Control (normal saline 0.5 ml/kg) standard drug
- Group 11 - Indomethacin (10mg/ kg p.o) was used as reference drug.
- Group111- Animals received ethanol extracts of *Amorphophallus paeoniifolius* var. *campanulatus* corm at the dose of 200mg/kg body weight
Group IV - Animals received ethanol extract of *Amorphophallus paeoniifolius* var. *campanulatus* corm at the dose of 400mg / kg body weight.

After one hour of the administration of the drugs, 0.1 ml of 1% w/v carrageenin solution in normal saline was injected into the subplantar tissues of the left hind paw of the rat and right hind paw was served as the control. The paw volume of the rats were measured in the digital plethysmograph (Ugobasile, Italy), at the end of 60 min., 120 min., 180 min. and 240 min.. The percentage increase in paw edema of the treated groups was compared with that of the control and the inhibitory effect of the drugs were studied. The relative potency of the drugs under investigations was calculated based upon the percentage inhibition of the inflammation.

\[
\text{Percentage inhibition} = \frac{(\text{Control (% increase in paw volume in 3}^{\text{rd}} \text{ hour)}) - (\text{Test (% increase in paw volume in 3}^{\text{rd}} \text{ hour)})}{\text{Control (% increase in paw volume in 3}^{\text{rd}} \text{ hour})} \times 100
\]

**Statistical Analysis**

The data were statistically analysed and expressed as mean ± standard error of mean (S.E.M). The S.E.M were calculated as follows (Ostle, 1966).

\[
\text{S.E.M} = \frac{\Sigma X^2 - (\Sigma X)^2}{n (n-1)}
\]

Where \(X\) = individual observations; \(n\) = number of observations.

Students’ t’ test was used to compare the mean values of two groups = values were calculated by the following formula:

\[
t = \frac{X_1 - X_2}{S^\frac{1}{n_1} + S^\frac{1}{n_2}}
\]
Where $S = \sqrt{\frac{X^2_{1} - \frac{X^2}{n_1} + X^2_{2} - \frac{X^2}{n_2}}{n_1 + n_2}}$

$n_1$ and $n_2$ stand for the number of observations in the two classes being compared.

The number of values of probability was obtained from the degree of freedom by using the standard table given by Fisher and Yates (1963). If the calculated value was more than the table value, it was significant at that probability level.