CHAPTER 2
MATERIALS AND METHODS
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2.1 MATERIALS

2.1.1 Porcine Pancreas Tissue

Porcine pancreas was collected immediately after slaughtering from meat technology unit, Kerala Agricultural University, Mannuthy. It was packed in ice and brought to laboratory.

2.1.2 Plant materials

Rhizome of *Alpinia galanga* and *Kaempferia galanga* were purchased from Amala Ayurvedic Hospital store. *Rosa damascena* flower was collected locally and identified authentically by Dr. Valsalakumari, Department of Pomology, Horticultural College, Vellanikkara, Mannuthy, Kerala.

2.1.3 Chemicals

1. Guanidium Hydrochloride - Loba chemicals Bombay
2. 6-Aminohexanoic Acid - Loba chemicals Bombay
3. Benzamidine Hydrochloride - Loba chemicals Bombay
4. N-ethylmaleimide - Loba chemicals Bombay
5. DEAE cellulose (DE52) - Sigma chemicals Co. USA
6. Carbazole - Gift from Kerala University
7. Sepharose 6 B - Sigma chemicals Co. USA
8. Agarose - Sigma chemicals Co. USA
9. Silicagel (60-120 mesh) - Sisco Research Laboratories
10. Nitroblue Tetrazolium - Sigma chemical Co. USA
11. Riboflavin - Loba chemicals Bombay
<table>
<thead>
<tr>
<th></th>
<th>Chemical</th>
<th>Supplier</th>
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<tr>
<td>12.</td>
<td>Deoxyribose</td>
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<td>13.</td>
<td>Thiobarbiturate</td>
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<td>14.</td>
<td>Tris. HCl</td>
<td>Sisco Research Laboratories</td>
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<td>15.</td>
<td>Brain thromboplastin reagent</td>
<td>Loba chemicals Bombay</td>
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<td>16.</td>
<td>ADP</td>
<td>Sigma chemical Co. USA</td>
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<tr>
<td>17.</td>
<td>Heparan sulphate</td>
<td>Sigma chemical Co. USA</td>
</tr>
<tr>
<td>18.</td>
<td>Heparin</td>
<td>Amala hospital pharmacy</td>
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<tr>
<td>19.</td>
<td>Carrageenan</td>
<td>Sigma chemical Co. USA</td>
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<tr>
<td>20.</td>
<td>Toludine blue</td>
<td>Loba chemicals Bombay</td>
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All other chemicals were analytical reagent grade

2.1.4 Instruments

<table>
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<tr>
<td>1.</td>
<td>Cooling centrifuge</td>
<td>Remi</td>
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<td>2.</td>
<td>Lyophilizer</td>
<td>Labconco USA</td>
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<td>3.</td>
<td>Cold Lab</td>
<td>LKB Bromma</td>
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<tr>
<td>4.</td>
<td>Microscope</td>
<td>Fugi</td>
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<td></td>
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<td>Nikon</td>
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<td>5.</td>
<td>Electrophoresis system and power supply</td>
<td>Hoefer 500, USA</td>
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<td></td>
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<td>Biotech Madras</td>
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<td>6.</td>
<td>Spectrophotometer</td>
<td>Spectronic 1000 Bausch and Lomb</td>
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<td></td>
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<td>Elico</td>
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<tr>
<td>7.</td>
<td>Aggregometer</td>
<td>Chronolog Corp. USA</td>
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2.1.5. Animals

<table>
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<tr>
<td>1.</td>
<td>Swiss Albino mice</td>
<td>National Institute of Nutrition, Hyderabad</td>
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<tr>
<td>2.</td>
<td>Swiss Albino rabbits</td>
<td>Small animal breeding station, Veterinary College, Mannuthy, Thrissur, Kerala</td>
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</tbody>
</table>
2.1.6 Maintenance of Animals

The animals used for experiments were housed in well ventilated cages, with normal laboratory diet and water *ad libitum*.

2.1.7 Test Compounds

- 70% ethanol extract of *Alpinia galanga* rhizome.

- 70% ethanol extract of *Kaempferia galanga* rhizome.

- *Rosa damascena* flower juice and its partially purified fractions

- Proteoglycans isolated from Porcine Pancreas

2.1.8 Preparation of test materials

2.1.8a Isolation and characterisation of proteoglycans

Proteoglycans were isolated according to the method of J. Mangnus *et al.* (1992) (200). In brief, tissue was macerated (1:2w/v) with 4M Guanidium hydrochloride containing various proteolytic inhibitors such as 50 mM sodium acetate, 10 mM aminohexanoic acid, 10 mM Benzamidine hydrochloride, 10 mM EDTA, 5 mM N-ethylmaleimide and 0.5 mM Poly methane sulphonyl fluorde (PMSF), pH 5.0. The homogenate was gently mixed for 24 h and then centrifuged at 10000 rpm for 30 minute at 4°C. The supernatant was collected.

Ice cold 100% (w/v) trichloroacetic acid was added to the above supernatant to get a final concentration of 10% (v/v). The precipitate was centrifuged off and the supernatant was neutralized to pH 7.
Nuclease Digestion

The supernatant was then dialysed exhaustively against 6M Urea buffer containing 50 mM sodium acetate, 10 mM amino hexanoic acid, 10 mM Benzamidine hydrochloride, 10 mM EDTA, 5 mM N-ethylmalaimide and 0.3 M NaCl pH 8.0. There after benzone nuclease (50 units / gm tissue) was added and the mixture was incubated for 2 h at 37°C and the materials were subjected to ion exchange chromatography.

Ion exchange chromatography

DE 52 was packed on to a column 1x10 cm. The column was then washed several times with 6M urea buffer containing 0.15 M NaCl. Batches of dialysate was loaded on to the column and washed with the same buffer. Elution was carried out using urea buffer containing increasing concentrations of Sodium chloride (0.3 M - 1 M).

3 ml fractions were collected and the absorbance of each fraction at 280 nm (protein) was read and each fraction after dialysis under went carbazole reaction (201) and the carbazole positive materials (530 nm) were pooled.

The pooled carbazole positive materials were then dialysed against Urea buffer and the proteoglycans were precipitated out by adding 3 volume 95% ethanol containing 1% potassium acetate (KAC).

Gel filtration of proteoglycans

The precipitated carbazole positive materials were then dissolved in D.W; and passed through a column of sepharose 6B (1x108cm). Elution was carried out
with 6M urea buffer containing 5M NaCl. 3ml fractions were collected and absorbance at 280 nm and 530 nm were monitored. Carbazole positive materials containing fractions were pooled. Proteoglycans were precipitated from the pooled fraction after dialysis by the addition of 3 volume. 95% ethanol containing 1% KAC.

Alkali – Borohydrate digestion

Aliquots of precipitated PGs were individually treated with 0.05 M NaOH with 1.0 M NaBH₄ for 48 hrs at 40°C and there after neutralized with HCl and subsequently gel filtered on a sepharose 6 B column as described earlier. Elution profiles based on hexuronic acid determination were compared to those of untreated samples (202).

HNO₂ digestion

Proteoglycan samples were digested with HNO₂ (1:2 w/v) for 1 hr. at room temperature. The digest were then neutralized and electrophoresed.

Electrophoresis of HNO₂ digest

Electrophoresis was carried out in 0.9% Agarose gel with 0.6 M phosphate buffer for 1 hr. Gels were stained with toludine blue (203).

2.1.8 b Plant extracts

Dried powder of A. galanga and K. galanga rhizome (1:10w/v) were separately extracted twice with 70% ethanol by stirring overnight. Filtrates of two extractions were pooled, evaporated to dryness over a water bath. The residues were then dissolved in distilled water and made up to a known volume.
Petals of *Rosa damascena* (25 gm) were squashed and the juice was collected after filtration through a cheese cloth. The yield was 0.5 ml / gm tissue. Juice was then diluted 1-10 (v/v) with distilled water and used for preliminary studies.

The original juice was lyophilized and the Lyophilized powder (2 gm) was loaded on to a column of silica gel (1 x 50 cm). Elution was carried out using solvants of increasing polarity. The different solvant fractions eluted were collected, evaporated to dryness and used for further studies.

2.1.9 Antioxidant studies

The antioxidant potentials of the partially purified plant materials were checked by three different assay system given below.

2.1.9a Superoxide radical production by photo reduction of riboflavin (204)

The assay system contained 0.6 M phosphate buffer pH 7.4, 0.12 M riboflavin (50 μl) 0.1 m EDTA containing 0.0015% sodium cyanide (200 μl), 1.5 mM Nitobluetetrazolium (100 μl) and various concentrations of test materials, in a total volume of 3 ml. The tubes were then illuminated under an incandescent lamp for 15 minutes. Optical density at 530 nm were measured before and after illuminations and the percentage inhibition was calculated using the formula

\[ \frac{\text{Optical density of control tubes} - \text{optical density of treated tube}}{\text{Optical density of control tube}} \times 100 \]

2.1.9b Hydroxyl radical generation (205)

Assay system contained potassium phosphate buffer (20 mM, pH 7.4)
EDTA, Deoxyribose, Ascorbic acid, Ferric chloride and Hydrogen peroxide 0.3 mM each and various concentrations of drug in a final volume of 1 ml. Incubation was carried out at 37°C for 1 hr. and Thiobarbituric acid reacting substances were estimated. Percentage inhibition was calculated from the optical measurements at 560 nm of control and experimental tubes.

2.1.9c Lipid peroxide formation (206)

The reaction system contained mice liver homogenate 25% (200 µl) Tris HCl buffer (0.2M, pH 7), Ascorbic acid (0.3 mM), potassium chloride (1.15 mM), Ferrous Ammonium sulphate (0.3 mM), 100 µl each and various concentrations of drug in a final volume of one ml. The mixture was then incubated at 37°C for 1 hr. Thiobarbituric acid reacting substances were then estimated and percentage inhibition was calculated from the optical measurements at 530 nm of control and experimental tubes.

2.1.10 Lipoprotein lipase releasing activity

Effect of plant extracts and the isolated proteoglycans on lipoprotein lipase releasing activity in vivo were evaluated by using the method of Korn (1959) (207). Heparin was used as reference standard.

For each test compound, healthy male rabbits weighing 1½ kg were used. Heparin (1 mg kg body wt) as well as test compounds (5 mg and 6 mg / kg body wt) were injected through the ear vein of rabbits. Blood was drawn to EDTA tubes after 10-12 minutes of injection. The plasma was separated and used as enzyme source. Human lipemic serum (TG<400 mg / dl) was used as substrate. The assay system contained.
Sorenison's phosphate buffer (pH7.4) - 0.5 ml
Enzyme - 0.2 ml
Substrate - 0.2 ml
D. water - 0.1 ml

Incubation was carried out at 37°C and the aliquots (0.05 ml) were drawn to tubes containing 100 µl N/12 H₂SO₄ at intervals 0, ½, 1 and 1½ hr. The liberated glycerol was then estimated by chromotropic acid method.

2.1.11 Anticoagulant studies

Different PGs as well as plant extracts were tried for their effect on blood coagulation in vivo.

For this PGs 1 mg / kg body wt, plant extracts 5 & 10 mg / kg body wt were individually given to rabbits intravenously through ear vein. Blood was collected 10-12 minute after drug administration to tubes containing 0.1M sodium acetate as anticoagulant.

2.1.11a Plasma recalcification time

Plasma was separated by centrifugation at 1500 rpm for 10 min in a centrifuge. 0.2 ml each plasma was taken in triplicate tube and kept at 37°C. 0.1ml M/100 CaCl₂ was then added to each tubes and started a stop watch immediately. The time for the formation of a firm clot was noted. The recalcification time of untreated, treated and a heparin treated blood were compared.

2.1.11b Activated partial thromboplastin time test

In this particular experiment instead of CaCl₂ activated thromboplastin
reagent commercial thromboplastin was added (0.1 ml) to previously warmed (37°C) plasma and the time for the formation of a firm clot was noted. Clotting time of untreated (normal blood) treated and heparin treated blood samples were compared.

2.1.12 Platelet aggregation studies (208)

Platelet rich plasma was prepared by centrifugation of blood collected from normal aspirin free blood bank donors. For collection of every 8.5 ml blood, 1.5 ml of acid citrate dextrose (ACD) was used as anticoagulant. Blood collected into plastic tubes were spun at 2500 rpm on Hitachi SCR 20 BA centrifuge using RPP-20-2 fixed angle rotor. The platelet count was adjusted to 2500 / μl and 0.45 ml PRP was taken into siliconized glass cuvetts and incubated with 50μl saline or PGA to get the described final concentration. The cuvetts were incubated at 37°C for 5’, without stirring. The aggregation was initiated by adding 10 μl (10 μM) ADP (sigma chemicals C. USA) Aggregation was recorded for 5’ using a chronology humi aggregometer (chronology cropn. USA). The data was processed using AGG hink integrator soft ware run on an IBM compatible computer.

2.1.13 Platelet arachidonate pathway (209)

Effect on Diethyl malaimide induced platelet arachidonate pathway by different test materials was studied by the following procedure.

Platelet rich plasma (PRP) was prepared by centrifugation of blood (1500 rpm 10’) collected from normal aspirin free blood bank donors. The platelets were then pelleted by centrifugation at 3000 rpm for 15’. Pellets was suspended in Hepes - Tyroids buffer pH 7.4 and the count was adjusted 25000/μl. Reaction system
contained 1 ml platelet suspension, different concentrations of test material. Incubation was carried out for 10 minutes at room temperature. To this mixture added 10μl diethyl maleimide (10mM). Incubation was carried out at 37°C for 1 hr. The thiobarbaturic acid formed in the reaction system was estimated. Percentage inhibition was calculated from the optical measurements at 530 nm of control (without drug) and experimental tubes by the formula

\[
\text{Percentage inhibition} = \frac{\text{OD of control tube} - \text{OD of treated tube}}{\text{OD of control tube}} \times 100
\]

2.1.14 Antiinflammatory studies

Carrageenan induced pedal oedema formation in mice as described by Langrange et al (210) was adopted for the studies.

For this 18 Swiss albino mice were group into 3 of 6 animals each received carrageenan (3 mg / kg body wt) in 0.1ml saline to the paw of right hind limb and kept as control. The group ii and iii were received 250 and 500 mg / kg body wt respectively of test materials 1 hr. prior to carrageenan infection. The pedal oedema formation was measured using a vernier callipers before carrageenan injection (0 hr) and at 1hr. intervals there after upto 4 hr. The percentage inhibition was then calculated.

2.1.15 Anti-atherogenic study

Animals used - Male albino rabbits weighing 1-1.5 kg
Test compounds and doze - Acetone fraction of Rosa damascena 50 mg / kg wt. proteoglycan fraction A 5 mg / kg body wt.
Group I (Normal) - 6 animals fed normal rabbit chaw
Group II (Control) - 6 animals fed high fat diet alone

Group III (Treated) - H.F.D + Rosa damascena, AF 50 mg/kg body wt.

Group IV - H.F.D + proteoglycan fraction A 5 mg / kg body weight

Duration - 4 months

Drugs were administered intravenously through ear vein. The H.F.D contain the following ingredients. Bengal gram - 30%, sucrose - 25%, milk power - 15%, salt mixture - 0.4%, yeast - 1%, shark liver oil - 2% Hydrogenated ground nut oil - 10%, cholesterol 5% and sodium taurocholate - 1% (211)

Parameters studied - Serum:- Total cholesterol, HDL cholesterol, LDL cholesterol (212), Triglycerides Phospholipids (213).

Serum parameters were checked once in a month. At the end of experimental period the animals were fasted overnight and sacrificed on the next day. The aorta were taken and kept stored at -20°C.

**Aortal staining for fat deposition (214)**

Segments of aorta were kept overnight in formalin (10%), washed several time in distilled water and then stained in 0.5% oil Red O in 60% iso propanol for 1 hr at 4°C. The aorta were cut longitudinally and visualised the deeply stained areas of fat deposition. Photographs were taken immediately.

**2.1.16 Statistical evaluation**

Statistical significance were determined using students ‘t’ test (215), according to the formula. To find out statistical significance between group (x) and group (y). Value of ‘t’ was found out from the equation $$ t = \frac{(\bar{x} - \bar{y})}{\sqrt{\frac{1}{(nx)} + \frac{1}{(ny)}}} $$ where
$\bar{x}$ is the mean value of group (x) $\bar{y}$ is the mean value of the group Y. $ar{y}$ is the mean of sample (y).

$nx$ - no. of sample x

$ny$ - no. of sample y

$S$ was found out from the equation

$$S = \sqrt{\frac{sx^2(nx - 1) + sy^2(ny - 1)}{(nx + ny - 2)}}$$

Where $Sx$ – Standard deviation of sample x

$Sy$ – Standard deviation of sample y

$nx + ny - 2$ is the degree of freedom by working the degree of freedom, statistical significance (p values) are found out from 't' distribution table.