Chapter -3

Materials and methods

Synopsis

Details of test animals, cell lines, chemicals and instruments used in the present study is given this chapter. Preparation of the hot water extract of saprocarps of P.rimosus, quantitative estimation of carbohydrate by Phenol sulphuric acid and Anthrone reagents, protein estimation by Bradford method are fully described. Statistical method used for the analysis of experimental data is also described.

3.1. Materials

3.1.1. Test animals

Swiss albino mice and Wistar rats used in the experiments were purchased from Small Animal Breeding Centre, Kerela Agricultural University, Mannuthy, Thrissur, India and housed under controlled conditions with free access to standard rodent chow and water ad libitum. Animal experiments were carried out according to the guidelines of Committee for the Prevention of Control and Supervision of Experiments on Animals (CPCSEA) and approval of Institutional Animal Ethic Committee.

3.1.2. Cell lines

Ehrlich’s ascites carcinoma (EAC) and Dalton’s lymphoma ascites (DLA) cell lines were obtained from Cancer Institute, Adayar, Chennai. The cells were maintained by intraperitoneal (i.p.) passages in Swiss albino mice. HCT 116 cells (human colon cancer cell line) were obtained from the National Centre for Cell Science, Pune, India. The cells were grown in monolayer culture in Dulbecco’s Modified Eagle’s Medium (Life Technologies, USA) containing 10% fetal bovine serum (Sigma, USA) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) in a humidified atmosphere of 5% CO₂ at 37 °C.

3.1.3. Chemicals

Phenol, chloroform (CHCl₃), formaldehyde, hydrogen peroxide (H₂O₂),
Sodium nitroprusside and sodium nitrite were purchased from Merck India Ltd., Mumbai. Ascorbic acid, 1,1-diphenyl 2-picryl hydrazyl (DPPH), ferric chloride, methanol, myoglobin, sodium acetate, Trolox (6-hydroxi-2,5,7,8-tetra methyl chroman-2-carboxylic acid), 2,4,6-tripyridyl-s-triazine (TPTZ), 2-thiobarbituric acid, triphenyl phosphene (TPP), trifluoro acetic acid (TFA), carrageenan, 3-(4-5 dimethylthiazol-2-yl) 2-5 diphenyl tetrazolium bromide (MTT), blue dextran, dextran T500, dextran (Mw:60,000), Sephadex G100 were obtained from Sigma Chemical Co., USA. Sodium azide (NaN₃), reduced glutathione (GSH), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), nitroblue tetrazolium (NBT), riboflavin, glucose-6-phosphate, sulphanilamide, nicotinamide adenine dinucleotide phosphate (disodium) (NADP), reduced NADP (NADPH) and Diethyl aminoethyl (DEAE) cellulose were purchased from Sisco Research Laboratories, Mumbai. Dialysis membrane -150 was purchased from Hi-Media and Cisplatin from Dabur India Ltd, New Delhi. Dulbecco’s Modified Eagle Medium (DMEM) obtained from Gibco™, and 10 % Fetal Bovine Serum (FBS) from Life Technologies, Grand Island, USA. All other chemicals and reagents used were analytical reagent grade.

### 3.1.4. Instruments used

The following instruments were used for the study:

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Manufacturer/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold Lab</td>
<td>LKB, Bromma</td>
</tr>
<tr>
<td>Cooling centrifuge</td>
<td>Remi, Chennai</td>
</tr>
<tr>
<td>Deep freezer (-70 and -20°C)</td>
<td>Remi and Tropicana</td>
</tr>
<tr>
<td>Electronic balance</td>
<td>Contech instrument company, Mumbai</td>
</tr>
<tr>
<td>Lyophilyser</td>
<td>Labconco, Missouri</td>
</tr>
<tr>
<td>pH meter</td>
<td>Elico IL 120</td>
</tr>
<tr>
<td>Research Microscope</td>
<td>Meiji, Japan and Labex, India</td>
</tr>
<tr>
<td>Spectrophotometer</td>
<td>Elico SL 159 and Elico SL 177</td>
</tr>
<tr>
<td>Tissue homogenizer</td>
<td>Remi</td>
</tr>
<tr>
<td>ELISA reader</td>
<td>Bio-Tek, Winooski, VT</td>
</tr>
<tr>
<td>Fluorescence microscope</td>
<td>TE-Eclipse 300</td>
</tr>
<tr>
<td>FT - IR Spectroscope</td>
<td>Magna- IR 550</td>
</tr>
</tbody>
</table>
3.2. Methods

3.2.1. Preparation of the hot water extract

Saprocarps of *P. rimosus* growing on the jackfruit tree trunks were collected from the outskirts of Thrissur, Kerala, India. The voucher specimen was deposited in the Herbarium of Centre for Advanced Studies in Botany, University of Madras, Chennai, India (HERB. MUBL 3171). The saprocarps were cut into small pieces, dried at 40-50°C for 48 hrs and powdered. Powdered material was defatted with petroleum ether using a Soxhlet apparatus for 8-10 hrs. The defatted material was extracted with hot water at 95°C for 8-9 hrs, repeatedly thrice. After each extraction, the soluble polymers were separated from residues by filtration and extracts were combined, concentrated under reduced pressure.

3.2.2. Carbohydrate estimation by Phenol sulphuric reagent

Quantitative examination of carbohydrate content was carried out according to Dubois’s et al. method (1956) using glucose as the standard.

**Principle**

In hot acidic medium carbohydrate is dehydrated to hydroxymethyl furfural. This forms a colored complex with phenol and has absorption maximum at 490 nm.

**Procedure**

The stock standard solution was prepared by dissolving 100mg of glucose in 100 ml of distilled water. 10ml of this stock solution was made up to 100ml with distilled water to prepare the working standard. 0.1, 0.2, 0.4, 0.6, 0.8 and 1ml of standard solution was taken in six test tubes. 0.1 and 0.2ml of the test solutions having unknown concentrations were added to the test tubes, marked as T1 and T2. All the solutions were made up to 1ml with distilled water. The blank containing 1ml of distilled water was also taken. To all tubes, 1ml of 5% phenolic solution was added. Then 5ml of concentrated sulphuric acid was added to all tubes without disturbing the solution and shaken well. After 10min, the
contents of the tube were mixed well and placed in a water bath. Optical density was measured at 490nm. A calibration curve was constructed by plotting optical density reading on Y-axis against standard glucose mg/tube. The amount of total carbohydrate present in the test solutions were calculated using the standard graph.

3.2.3. Carbohydrate estimation by Anthrone reagent

Anthrone method (Yemm and Wills, 1954) was utilized to monitor the presence of carbohydrate in the test solutions.

Principle

Carbohydrate reacts with concentrated sulphuric acid to give derivative of furfural which on reaction with anthrone forms a bluish green colored complex and has absorption maximum at 630nm.

Procedure

Anthrone reagent is prepared by dissolving 0.2g anthrone in 100ml of chilled concentrated sulphuric acid. To all the test tubes 4ml of anthrone reagent was added. Then without disturbing the reagent in the test tubes, 0.5ml of the test solution having unknown concentration of carbohydrate was added. All the solutions were made up to 1ml using distilled water. The blank containing 1ml of distilled water was also taken. Then the contents of the tubes were mixed well and heated in a boiling water bath for 8min. It was then immediately cooled and optical density was measured at 630nm.

3.2.4. Protein estimation by Bradford method

Quantitative examination of protein content was carried out using Protein Estimation Kit (GENEI Cat # KT33) by Bradford Macro Method (Bradford, 1976). Bradford’s assay is a rapid and accurate method for estimation of protein concentration. Moreover, when compared with the Lowry’s method it is subject to less interference by common reagents and nonprotein components of biological sample.

Principle

The assay relies on the binding of the dye Coomassie blue G250 to protein. The quantity of protein can be estimated by determining the amount of dye in
the blue ionic form. This is usually achieved by measuring the absorbance of the solution at 595nm.

**Procedure**

Standard protein is prepared by reconstituting one vial containing 5mg Bovine serum albumin (BSA) with 1ml distilled water to get 5mg/ml. Just before use 0.2ml of 5mg/ml BSA solution was diluted with 0.8ml of distilled water to get 1mg/ml standard protein solution. 10, 20, 40, 60 and 80μl of standard BSA solution were taken in five test tubes. 40 and 60μl of the test solutions having unknown concentrations were added to the test tubes, marked as T1 and T2. All the solutions were made up to 200μl with distilled water. The blank containing 200μl of distilled water was also taken. To all the tubes 2ml of Bradford’s reagent was added, mixed well and kept for 10min. Optical density was measured at 595nm. A calibration curve was constructed by plotting optical density reading on Y-axis against standard protein μg/tube. The amount of total protein present in the test solutions were calculated from the standard graph.

**3.2.5. Statistical analysis**

The data were analysed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. The formula for Tukey's test is:

\[ q_s = \frac{Y_a - Y_b}{SE} \]

where \( Y_a \) is the larger of the two means being compared, \( Y_b \) is the smaller of the two means being compared, and SE is the standard error of the data. This \( q_s \) value can then be compared to a \( q \) value from the studentized range distribution.

\[ q = \frac{\text{Range}}{S} \]

Where **range** is the maximum minus the minimum of first sample and **S** is standard deviation of the second sample. The \( q_{\text{critical}} \) value is then obtained on the basis of \( q \), degrees of freedom of first and second sample from the standard statistical table. If the \( q_s \) value is *larger* than the \( q_{\text{critical}} \) value obtained from the distribution, the two means are said to be significantly different. A p value <0.05 was considered as significant.