3.0 EXPERIMENTAL PROCEDURE

The present research focused on “Antioxidative, antitumor and immunomodulatory efficacy of protein fraction of *Cynodon dactylon* and *Terminalia catappa* leaves on experimentally implanted ELA cells in *Swiss albino mice*” was conducted in four different phases. The first two phases constituted the *in vitro* studies and the last two phases constituted the *in vivo* studies.

**IN VITRO STUDIES**

**PHASE I**

3.1 ASSESSMENT OF THE PROTEIN CONTENT AND SEPERATION OF AMMONIUM SULPHATE PROTEIN FRACTIONS OF *C. dactylon* AND *T. catappa*

Fresh leaves of *C. dactylon* (Plate I) and *T. catappa* (Plate II) were collected in an area free of pesticides and other contaminants from Tiruchengode, Namakkal District, Tamilnadu. The collected leaves were washed thoroughly in tap water and blotted dry using filter paper and used for the protein preparation.

The 20 per cent extracts of fresh leaves of *C. dactylon* and *T. catappa* were prepared in Phosphate Buffered Saline (PBS) of pH 7.2 and centrifuged at 5000 rpm for 10 minutes. The supernatant obtained was used for the experiments.

3.1.1 Preparation of protein fractions of *C. dactylon* and *T. catappa* leaves

Supernatant obtained was subjected to ammonium sulphate fractionation using 10-100 per cent saturation of ammonium sulphate, as shown in Appendix I (Jayaraman, 1981). The precipitates thus obtained were dissolved in a known amount of PBS of pH 7.2. Dialysis is commonly used for removing salts from the proteins since presence of salts in proteins interferes in many ways. So, the above separated protein precipitate were redissolved in 3ml of PBS and desalting of protein fractions were carried out by dialysis using 0.01M PBS by the method of Jayaraman, (1981) as in Appendix II.
PLATE I

*Cynodon dactylon*

<table>
<thead>
<tr>
<th>Common name</th>
<th>Cough grass, Dhoub grass, Arugampul</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class</td>
<td>Monocotyledons</td>
</tr>
<tr>
<td>Subclass</td>
<td>Glumaceae</td>
</tr>
<tr>
<td>Order</td>
<td>Poales</td>
</tr>
<tr>
<td>Family</td>
<td>Poaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Cynodon</td>
</tr>
<tr>
<td>Species</td>
<td>dactylon</td>
</tr>
</tbody>
</table>
PLATE II

*Terminalia catappa*

**Common name**  - Tropical almond, badam
**Class**  - Dicotyledons
**Subclass**  - Rosidae
**Order**  - Myrtales
**Family**  - Combretaceae
**Genus**  - *Terminalia*
**Species**  - *catappa*
3.1.2 Assessment of the protein content of the protein fractions of *C. dactylon* and *T. catappa*

Proteins are involved in almost all cellular processes and fulfill many functions. Protein content of 10-100 per cent saturation of ammonium sulphate precipitates in PBS was determined by the method of Shakir *et al.* (1994) as shown in Appendix III. The percentage saturation of ammonium sulphate which showed maximum protein contents of *C. dactylon* and *T. catappa* were selected for the following *in vitro* and *in vivo* studies.

3.1.3 Purification and separation of ammonium sulphate protein fractions of *C. dactylon* and *T. catappa* by PAGE/SDS PAGE

There are tens to thousands of proteins in the cell, differing in abundance over six orders of magnitude. For the purification of ammonium sulphate protein fractions, *C. dactylon* and *T. catappa* were subjected to PAGE and SDS PAGE as per the method of Laemmli, (1970) as indicated in Appendix IV and V. The major bands of 10-100 per cent saturation of ammonium sulphate precipitate of the *C. dactylon* and *T. catappa* were eluted by the method of Stone *et al.* (1989) as shown in Appendix VI.

**PHASE II**

3.2 ASSESSMENT OF *IN VITRO* ANTIOXIDATIVE AND ANTITUMORIGENIC EFFECT OF *C. dactylonPF* AND *T. catappaPF*

The antioxidative activity was evaluated by DPPH, NO and H$_2$O$_2$ radical scavenging assays. The antitumorigenic activity of *C. dactylonPF* and *T. catappaPF* was assessed by Trypan blue exclusion method using intraperitoneally propagated ELA tumor cells.

3.2.1 Assessment of *in vitro* antioxidative potential of *C. dactylonPF* and *T. catappaPF*

The antioxidative potential of *C. dactylonPF* and *T. catappaPF* with maximum protein content was chosen and the different concentrations of the selected protein fractions ranging from 10-100 µg were taken for the assessment of scavenging of DPPH radical, Nitric oxide radical and Hydrogen peroxide.
3.2.1.1 DPPH radical scavenging assay

DPPH radical scavenging activity was assessed by the method of Mensor et al. (2001) as given in Appendix VII.

3.2.1.2 Nitric oxide scavenging activity

Nitric oxide generation was analyzed by the method of Green et al. (1982) as given in Appendix VIII.

3.2.1.3 Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging assay was carried out by the method of Ruch et al. (1989) as given in Appendix IX.

3.2.2 Assessment of \textit{in vitro} antitumorigenic effect of \textit{C. dactylonPF} and \textit{T. catappaPF} against ELA tumor cells

3.2.2.1 Tumor cell lines maintenance

Seven to eight weeks old Swiss albino female mice weighing about 25-30g were brought from small animal breeding station, Thrissur, Kerala. Ehrlich’s Lymphoma Ascites (ELA) tumor cell lines were procured from Amala Cancer Research Centre, Thrissur, Kerala (Plate III).

The mice were acclimatized for two weeks and cells were propagated by intraperitoneal transplantation of $1 \times 10^6$ cells in 100 $\mu$l of PBS. After 10-15 days, the cells were drawn from the intraperitoneal cavity and used for the \textit{in vitro} studies.

3.2.2.2 \textit{In vitro} cytotoxic studies

\textit{In vitro} cytotoxic studies were carried out to find out the 50 per cent effective concentration (EC$_{50}$) of ammonium sulphate protein fraction of \textit{C. dactylon} and \textit{T. catappa} by trypan blue exclusion method of Salomi and Panikkar, (1989) as shown in Appendix X. The fraction which showed minimum concentration of protein as EC$_{50}$ was selected for the \textit{in vivo} studies.

Dialyzed 60 per cent (\textit{C. dactylon}) and 70 per cent (\textit{T. catappa}) ammonium sulphate fractions were subjected to PAGE and the bands were eluted. These eluted protein fractions of \textit{C. dactylon} and \textit{T. catappa} were referred as \textit{C. dactylonPF} and \textit{T. catappaPF} respectively.
IN VIVO STUDIES

PHASE III

3.3 ASSESSMENT OF ANTIOXIDATIVE AND ANTITUMORIGENIC EFFECT OF C. dactylonPF AND T. catappaPF IN ELA TUMOR INDUCED SWISS ALBINO MICE

In vivo studies were carried out by the intraperitoneal administration of 52 µg (EC₅₀) of C. dactylonPF and 40 µg (EC₅₀) of T. catappaPF to examine their antioxidative and antitumorigenic effect.

3.3.1 Maintenance of experimental animals

In bred Swiss albino mice weighing on an average 20-25 g procured from Small Animal Breeding Station, Medical College, Perundurai were used to evaluate the antioxidant role against standard antioxidant silymarin and antitumorigenic effect in ELA tumor induced Swiss albino mice (Plate III). These animals were maintained for two weeks under environmentally controlled conditions with free access to standard food (Lipton, India) and water ad libitum, prior to the experiments.

PLATE III

SWISS ALBINO MICE

Normal mice          ELA Transplanted mice

All animal experiments were carried out according to the guidelines prescribed by Animal Welfare Board and with the approval of Animal Ethic Committee (Register no: 623/02/b/CPCSEA).
3.3.2 Grouping of Animals

The mice were divided into eight groups with 6 mice in each for each treatment period.

The groupings are as follows:

1. PBS control
2. Paraffin oil control
3. Silymarin
4. *C. dactylon* PF
5. *T. catappa* PF
6. *C. dactylon* PF + ELA
7. *T. catappa* PF + ELA
8. ELA

**Treatment Schedule**

**Group 1** received (i.p) 0.1 ml of PBS every day and served as a vehicle control for the experimental groups 4 to 8.

**Group 2** received (i.p) 0.1 ml of paraffin oil, which constituted the vehicle control for the standard antioxidant silymarin group.

**Group 3** received (i.p) 25mg standard antioxidant silymarin in 100 μl of paraffin oil / kg body weight (Fraschini *et al.*, 2002).

**Group 4** received (i.p) 52μg (EC50) of *C. dactylon* PF in 100μl of PBS.

**Group 5** received (i.p) 40μg (EC50) of *T. catappa* PF in 100μl of PBS.

**Group 6** received 1x10^6 ELA tumor cells and 52μg of *C. dactylon* PF (i.p) on the same day and *C. dactylon* PF administration was continued for 60 days (*C. dactylon* PF + ELA).

**Group 7** received 1x10^6 ELA tumor cells and 40μg of *T. catappa* PF (i.p) on the same day and *T. catappa* PF administration was continued for 60 days (*T. catappa* PF + ELA).

**Group 8** received 1x10^6 ELA tumor cells (i.p) that served as ELA control.

The study was continued for the period of 15 days, 30 days, 45 days and 60 days. At the end of the study the mice were sacrificed after an overnight fasting. The blood of the animals was collected by heart puncture and the serum separated was used for the estimation of liver marker enzymes. The organs such as liver, lung, kidney, heart, spleen and brain were dissected,
blotted of blood and washed with PBS of pH 7.2. A part of the liver homogenate was prepared using PBS and used for the determination of enzymic and nonenzymic antioxidants. A part of the liver and the other organs such as lung, kidney, heart, spleen and brain homogenates were prepared using Tris HCl for the assessment of the rate of lipid peroxidation.

3.3.3 Assessment of the activities of liver marker enzymes in serum

Liver plays a major role in detoxification and excretion of many exogenous and endogenous compounds. To assess the normal functioning of the liver treated with *C. dactylon* PF and *T. catappa* PF, in the presence and absence of ELA tumor cells, selected enzyme activities were determined in the serum.

3.3.3.1 Glutamic Oxaloacetic Transaminase (GOT, EC. 2.6.1.1)

Glutamic Oxaloacetic Transaminase was assayed by the method of Reitman and Frankel, (1957) as given in Appendix XI.

3.3.3.2 Glutamic Pyruvic Transaminase (GPT, EC. 2.6.1.2)

Glutamic Pyruvic Transaminase was assayed by the method of Reitman and Frankel, (1957) as given in Appendix XII.

3.3.3.3 Alkaline phosphatase (ALP, EC. 3.1.3.1)

Alkaline Phosphatase was assayed by the method of King, (1965) as in Appendix XIII.

3.3.4 Assessment of the activities of enzymic antioxidants in the liver

Enzymic antioxidants such as Catalase (CAT), Superoxide dismutase (SOD) and Glutathione Peroxidase (GPx) were assessed in the liver of Swiss albino mice.

3.3.4.1 Estimation of Catalase (CAT, EC.1.11.1.6)

Catalase activity in the liver homogenate was assessed by the method of Luck, (1974) as shown in Appendix XIV.

3.3.4.2 Estimation of Superoxide dismutase (SOD. EC.1.15.1.1)

The activity of Superoxide dismutase was estimated by the method of Misra and Fridovich, (1972) as shown in Appendix XV.

3.3.4.3 Estimation of Glutathione Peroxidase (GPx. EC.1.11.1.9)

The activity of Glutathione Peroxidase was determined by the method of Rotruck *et al.* (1973) as shown in Appendix XVI.
3.3.5 Assessment of non-enzymic antioxidants in the liver

The levels of the non-enzymic antioxidants such as Vitamin A, E and Reduced glutathione were also assessed in the liver of Swiss albino mice.

3.3.5.1 Estimation of Vitamin A

Vitamin A was estimated by the method of Bayfield and Cole, (1980) as in Appendix XVII.

3.3.5.2 Estimation of Vitamin E

Vitamin E content was determined by the method of Rosenberg, (1992) as shown in Appendix XVIII.

3.3.5.3 Estimation of Reduced glutathione (GSH)

The reduced glutathione was determined by the method of Moron et al. (1979) as in Appendix XIX.

3.3.6 Assessment of lipid peroxidation in the selected organs

Lipid peroxidation plays a major role in mediating oxidative damage in biological systems. Malondialdehyde, a major end product and index of lipid peroxidation, cross-links DNA and protein and nucleotides on the same and opposite strands thereby promoting carcinogenesis. Therefore, it is found to be increased in tumor conditions (Bartsch, 1996). The rate of lipid peroxidation was assessed in the liver, lung, kidney, spleen, heart and brain homogenate of different groups of mice using the method of Bishayee and Balasubramaniam, (1971). The detailed procedure is given in Appendix XX.

3.3.7 Assessment of antitumor effect of *C. dactylon*PF and *T. catappa*PF by percentage of mortality rate in in vivo cytotoxic studies

*In vivo* cytotoxic studies were carried out using the EC50 of *C. dactylon*PF and *T. catappa*PF to follow the antitumor activity in terms of Increase in Life Span (ILS) of Swiss albino mice transplanted with ELA tumor cells. Three groups of (6 mice/group) Swiss albino mice were used for the *in vivo* cytotoxic studies. To the control group 1x10^6 ELA tumor cells were administered intraperitoneally on the 1st day of the experiment for the development of tumor. At the end of 24 hours, to the experimental groups the protein fractions of selected medicinal plants were administered intraperitoneally. This was repeated for 60 days. The mortality of the animals dying off tumor was noted and the percentage of increase in life span was calculated as described by Geran et al. (1972).
Percentage increase in life span = \( \frac{T - C}{C} \times 100 \)

Where, \( C \) = Average life span of control mice  
\( T \) = Average life span of treated mice  
The value more than twenty five per cent is taken as significant.

### 3.3.8 Assessment of histological status of liver

The sample of the liver from mice administered with protein fractions and their controls were fixed in 10 per cent formalin and then embedded in paraffin. Microtome sections of 6 µm thicknesses were prepared from each portion of liver and stained with haemotoxylin-eosin for pathological observation using the method of Culling, (1974) as shown in Appendix XXI.

### PHASE IV

#### 3.4 ASSSESSMENT OF IMMUNOMODULATORY ACTIVITY OF C. dactylonPF AND T. catappaPF IN SRBC/ELA TUMOR INDUCED SWISS ALBINO MICE

In the fourth phase, the selected EC\(_{50}\) protein fractions were evaluated for the immunomodulatory role in the SRBC/ELA tumor induced and Carbon ink induced Swiss albino mice.

**Preparation of SRBC:**

Sheep blood was collected from a local slaughter houses in sterilized container in the presence of Alseiver’s solution. SRBCs were obtained by centrifugation and the cells were washed three times in 0.9 per cent saline and adjusted to concentration of \( 0.5 \times 10^9 \) cells per ml for immunization and challenge.

**3.4.1 Grouping of animals**

The mice were divided into twelve groups with 6 mice in each for each treatment periods. The hematological parameters such as total leukocyte count, neutrophil adhesion and circulating antibody titre were carried out in the serum of the mice on 7\(^{th}\) day and 14\(^{th}\) day. All the twelve groups of mice received \( 0.5 \times 10^9 \) Sheep Red Blood Cells (SRBCs) in 100µl of PBS on the 1\(^{st}\), 8\(^{th}\) and 15\(^{th}\) day and indicated as SRBC induced mice.
Group 1 SRBC induced mice received pyrogallol (50mg/g body weight) in 100µl of PBS from 2nd to 14th day (i.p).

Group 2 SRBC induced mice received 100µl of PBS from 2nd to 14th day (i.p).

Group 3 SRBC induced mice received C. dactylon PF 52µg in 100µl PBS from 2nd to 14th day (i.p).

Group 4 SRBC induced mice received T. catappa PF 40µg in 100µl PBS from 2nd to 14th day (i.p).

Group 5 SRBC induced mice received C. dactylon PF 52µg in 100µl PBS and 100µl of pyrogallol (50mg/g body weight, Joharapurkar et al., 2004) from 2nd to 14th day (i.p).

Group 6 SRBC induced mice received T. catappa PF 40µg in 100µl PBS and 100µl of pyrogallol (50mg/g body weight) from 2nd to 14th day (i.p).

Group 7 SRBC induced mice received 1x10^6 ELA tumor cells on the 1st day (i.p).

Group 8 SRBC induced mice received 1x10^6 ELA tumor cells on the 1st day and pyrogallol (50mg/g body weight) in 100µl of PBS from 2nd to 14th day (i.p).

Group 9 SRBC induced mice received 1x10^6 ELA tumor cells on the 1st day and C. dactylon PF 52µg in 100µl PBS from 2nd to 14th day (i.p).

Group 10 SRBC induced mice received 1x10^6 ELA tumor cells on the 1st day and T. catappa PF 40µg in 100µl PBS from 2nd to 14th day (i.p).

Group 11 SRBC induced mice received 1x10^6 ELA tumor cells on the 1st day, C. dactylon PF 52µg in 100µl PBS and 100µl of pyrogallol (50mg/g body weight) from 2nd to 14th day (i.p).

Group 12 SRBC induced mice received 1x10^6 ELA tumor cells on the 1st day, T. catappa PF 40µg in 100µl PBS and 100µl of pyrogallol (50mg/g body weight) from 2nd to 14th day (i.p).

3.4.2 Assessment of lymphoid organ weight of mice

The weight of the lymphoid organs such as spleen and thymus were analyzed by sacrificing the animal on the 7th day and 14th day.

3.4.3 Assessment of Cell Mediated Immune Response of C. dactylon PF and T. catappa PF

The cell mediated immune response of C. dactylon PF and T. catappa PF was assessed by Total leukocyte count, Neutrophil adhesion and Phagocytic index.
3.4.3.1 Assessment of Total Leukocyte count of *C. dactylon*PF and *T. catappa*PF

Total leukocyte count present in blood sample on the 7th day and 14th day was counted using the haemocytometer. Blood (20 µl) was added to 380 µl of WBC diluting fluid (acetic acid 1.5 ml, 1 per cent crystal violet 1 ml and 97.5 ml distilled water). A drop of sample was put on a naeuber’s chamber and number of leucocytes per cubic millimeter was determined by observing under the microscope (Subramoniam *et al.*, 1996).

3.4.3.2 Assessment of Neutrophil adhesion of *C. dactylon*PF and *T. catappa*PF

On the 7th day and 14th day of the protein treatment, blood samples were collected (before challenge) by puncturing the retro orbital plexus into heparinised vials and analyzed for Total Leukocyte Counts (TC) and Differential Leukocyte Counts (DC) by fixing blood smears and staining with Field stain I and II Leishman’s stain. After initial counts, blood samples were incubated with 80 mg/ml of nylon fibers for 15 minutes at 38°C. The incubated blood samples were again analyzed for TC and DC. The product of TC and per cent neutrophil gives Neutrophil Index (NI) of blood sample (Wilkonson, 1978). Per cent neutrophil adhesion was calculated as shown below:

\[
\text{Percentage Neutrophil adhesion} = \frac{NI_U - NI_T}{NI_U} \times 100
\]

Where,

- \(NI_U\) = Neutrophil index of untreated blood sample
- \(NI_T\) = Neutrophil index of treated blood sample

3.4.3.3 Assessment of Phagocytic index of *C. dactylon*PF and *T. catappa*PF

The mice were divided into three groups with 6 mice in each for each treatment period. The phagocytic index was followed by carbon clearance test using the serum of all three groups of mice on the 7th day.

**Group 1** received 100 µl of PBS (i.p) for 5 days.

**Group 2** received 52 µg of *C. dactylon*PF in 100 µl of PBS (i.p) for 5 days.

**Group 3** received 40 µg of *T. catappa*PF in 100 µl of PBS (i.p) for 5 days.

For all the above groups, Carbon ink (10 µl/g body weight) was
administered on the 7th day and the blood samples were drawn from the retro orbital vein at 0th and 15th minutes.

Group I served as control and was given 100 µl of PBS for 5 days intraperitoneally. Group II and Group III served as test which was administered with the C. dactylonPF and T. catappaPF for 5 days. After 48 hours of administration of the last dose of the proteins mice were injected 0.1ml of Carbon ink via the tail vein. Blood samples were withdrawn at 0 and 15 minutes after injection. 25 µl of blood samples were mixed with 2 ml of 0.1per cent sodium carbonate solution and the absorbance of this solution was determined at 660 nm (Jayathirtha and Mishra, 2004). The Phagocytic index $K$ was calculated using the following equation:

$$K = \frac{(\log_{e} OD_1 - \log_{e} OD_2)}{15}$$

Where, $OD_1$ and $OD_2$ are the optical densities at 0 and 15 minutes respectively.

3.4.4 Assessment of Humoral Immune Response of C. dactylonPF and T. catappaPF

The humoral immune response of C. dactylonPF and T. catappaPF were assessed by Circulating antibody titre and protein profiles through SDS PAGE, immunodiffusion and immunoelectrophoresis.

3.4.4.1 Assessment of circulating antibody titre of C. dactylonPF and T. catappaPF

Blood samples were collected from individual animals by retro-orbital puncture on day 7 and 14, serum separated and heat inactivated at 56°C. Antibody titre was determined by the haemagglutination method (Puri et al., 1993) using SRBC as the antigen. 25 µl of 0.1per cent SRBC suspension was added to 25 µl of two-fold diluted serum samples in V-shaped microtitration plates. The micro titer plates were incubated at room temperature for two hours and examined visually for agglutination. The reciprocal of the highest dilution of serum showing 50 per cent agglutination has been expressed as HA titre (Mitra et al., 1999).
3.4.4.2 Assessment of the protein profile of *C. dactylon*PF and *T. catappa*PF

The mice were divided into six groups and the blood was collected on the 15th day and the serum was separated.

**Group 1** received (i.p) 0.1 ml of PBS every day and served as a vehicle control for the experimental groups 2 to 6.

**Group 2** received (i.p) 52μg of *C. dactylon*PF in 100μl of PBS.

**Group 3** received (i.p) 40μg of *T. catappa*PF in 100μl of PBS.

**Group 4** received *C. dactylon*PF and 1x10⁶ ELA tumor cells (i.p) on the same day and *C. dactylon*PF administration was continued for 15 days (ELA+C. dactylonPF).

**Group 5** received *T. catappa*PF and 1x10⁶ ELA tumor cells (i.p) on the same day and *T. catappa*PF administration was continued for 15 days (ELA+T. catappaPF).

**Group 6** received 1x10⁶ ELA tumor cells (i.p) that served as ELA control.

**Precipitation and Separation of serum proteins by SDS PAGE**

The proteins in the serum of experimental animals were precipitated by ammonium sulphate fractionation. The precipitate was dissolved in a known amount of PBS of pH 7.2 and dialyzed. The dialyzed protein fraction was subjected to further separation and purification process followed by SDS PAGE as described by the method of Laemmli, (1970) as indicated in Appendix V.

**Double Immunodiffusion and Immunoelectrophoresis**

In the preliminary slide test, the *C. dactylon*PF and *T. catappa*PF showed agglutination with the antiserum of mice administered with *C. dactylon*PF and *T. catappa*PF. So, the double immunodiffusion in agar plates was carried out by the method of Ouchterlony, (1948) as mentioned in Appendix XXII.

Immunoelectrophoresis indicates the production of specific antibodies due to immunomodulatory activity of protein and hence immunoelectrophoresis was carried out using the method of Weir, (1986) as given in Appendix XXIII.
Statistical Analysis

The data presented here are the means ± SD of 6 mice in each group. The biochemical results of *in vivo* studies using mice for 15 days treatment period alone including ELA were subjected to one-way ANOVA and the results of 15 days to 60 days treatment periods excluding ELA treated group were analysed using two way ANOVA using SigmaStat statistical package to test the level of statistical significance at $P<0.05$. The results of phase IV were subjected to one-way and two way ANOVA.
FIGURE 6: EXPERIMENTAL DESIGN OF THE STUDY

Phase I

PBS extract of leaves → Protein precipitation → 10-100% Ammonium Sulphate → Protein estimation → PAGE and SDS PAGE → Elution of major Protein Bands

Phase II

In vitro antioxidative activity

Cytotoxic studies for the assessment of EC \(_{50}\)

Phase III

In vivo antitumorigenic and antioxidative activity

- Liver marker enzymes
- Enzymic and nonenzymic antioxidants
- Lipid peroxidation
- Percentage mortality rate
- Histological status

Immunomodulatory activity

- Lymphoid organ weight
- Cell mediated immune response
- Humoral immune response

Phase IV