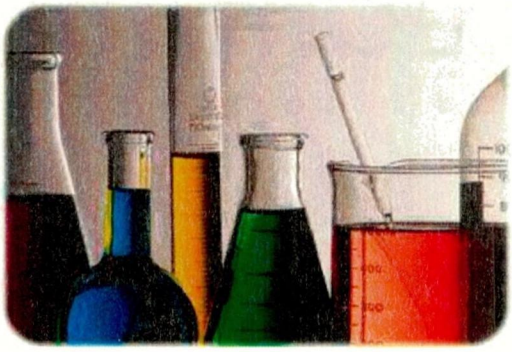

EXPERIMENTAL PROCEDURE

3.0 EXPERIMENTAL PROCEDURE

The present study has been undertaken "**To evaluate the Antioxidant potential, Hepatoprotective and Thrombolytic effect of *Alternanthera sessilis*" (L). R.Br.ex DC** under the following headings:

3.1 COLLECTION OF PLANT SAMPLE

3.2 PREPARATION OF THE PLANT EXTRACT

3.3 EXPERIMENTAL DESIGN

3.4 SELECTION AND GROUPING OF ANIMALS

3.5 PREPARATION OF THE SAMPLE FOR BIOCHEMICAL ANALYSIS

3.6 DETERMINATION OF BIOCHEMICAL PARAMETERS

3.7 HISTOPATHOLOGICAL EXAMINATION

3.8 BIOSAFETY SCREENING OF THE PLANT SAMPLE

3.9 ASSESSMENT OF THROMBOLYTIC ACTIVITY

4.0 STATISTICAL ANALYSIS

3.1 COLLECTION OF PLANT SAMPLE

The leaves of *Alternanthera sessilis* used in the study were procured from Salem district of Tamil Nadu. The plant was identified by the Botanical survey of India, TamilNadu Agricultural University, Coimbatore.

3.2 PREPARATION OF THE PLANT EXTRACT

i. Fresh leaves of the plant were washed with water inorder to remove dirt and soil particles adhered and blotted between the folds of tissue paper to remove any water droplets. 5 g of the plant leaves was homogenized with 50ml of water and the extract was filtered using Whatmann No.1 filter paper. Similarly alcoholic and petroleum ether extract were prepared by using 50 ml of ethanol and petroleum ether respectively. All the three extracts were used for the phytochemical analysis.

ii. The plant leaves were dried under shade at room temperature, powdered and stored in an airtight container. The powder (20g) was extracted with ethanol (200ml) in

a Soxhlet extraction. The extract was concentrated to dryness under reduced pressure and controlled temperature (40–50°C) and used for the determination of antioxidant, hepatoprotective, cytotoxic and thrombolytic effect of the selected plant.

PLATE I

***Alternanthera sessilis* - Medicinal plant**

PLATE II

***Alternanthera sessilis* - Leaf powder**

3.3 EXPERIMENTAL DESIGN

The study was carried out in four phases.

- Phase I involves the qualitative screening of phytochemical constituents and determination of antioxidant potential of the plant extract.
- In phase II, hepatoprotective effect of the plant was evaluated using experimental rats.
- Biosafety screening of the plant extract was carried out in phase III using Brine Shrimp Lethality Bioassay.
- Phase IV involves the assessment of thrombolytic activity of the plant using human blood.

3.3.1 PHASE I

SCREENING OF PHYTOCHEMICAL CONSTITUENTS

Phytochemical screening was performed using standard procedures. The procedures used for detection of alkaloids, flavonoids, saponins, phenols, glycosides (Raaman, 2006), anthraquinones (Ayoola *et al.*, 2008), tannins, carbohydrates (Iyengar, 1995), steroids and terpenoids (Siddiqui and Ali, 1997) are given in Appendix I.

DETERMINATION OF ANTIOXIDANT POTENTIAL

The sample was analyzed for total antioxidants, total phenols and total flavonoids.

TOTAL ANTIOXIDANT CAPACITY (TAC)

The total antioxidant capacity was determined by the Phosphomolybdenum method of Prieto *et al.*, (1999) as described in Appendix II.

DETERMINATION OF TOTAL PHENOLS

Total phenols were measured by the method of Malick and Singh, (1980) as depicted in Appendix III.

DETERMINATION OF TOTAL FLAVONOIDS

Colorimetric method Zhinsen *et al.*, (1999) was used for flavonoid determination in the ethanolic extract of the plant. Details are furnished in Appendix IV.

3.3.2 PHASE II

CCl_4 induced hepatotoxicity in rats was used as a model to determine the hepatoprotective effect of the leaf extract of *Alternanthera sessilis*. The dried ethanolic extract was suspended in DMSO and administered to experimental animals.

3.4 SELECTION AND GROUPING OF ANIMALS

Male Wistar strain albino rats weighing between (100-200g) were obtained from animal breeding station, Thrissur, Kerala. The animals were placed at random and allocated to treatment groups in plastic cages with paddy husk as bedding. They were allowed free access to water *ad libitum* and fed with standard commercial pelleted feed throughout the experimental period. All procedures described were reviewed and approved by the Institutional Animal Ethical Committee.

The animals were divided into 5 main groups each group containing 6 animals.

Group I	Control
Group II	CCl_4
Group III	CCl_4 +Silymarin
Group IV	CCl_4 +Plant extract (200mg/kg)
Group V	CCl_4 +Plant extract (400mg/kg)

Normal healthy rats in Group I served as control. The Group II served as the induction control which received CCl_4 (2ml/kg, b.wt, i.p. dose 1:9 dilution with olive oil on 7^{th} and 14^{th} day).Group III received standard drug silymarin (1ml/kg b.wt,) i.p dose for 21 days and CCl_4 induction on 7^{th} and 14^{th} day. Group IV and V, received ethanolic extract of leaves 200mg/kg and 400mg/kg respectively for 21 days and CCl_4 induction on 7^{th} and 14^{th} day. After the experimental periods of 21 days animals were fasted overnight and anaesthetized using chloroform. Blood was collected by heart puncture.

3.5 PREPARATION OF THE SAMPLE FOR BIOCHEMICAL ANALYSIS

Preparation of serum from blood

The blood samples after coagulation were centrifuged at 2500 rpm for 10-15 minutes and the sera were used for the estimation of total protein, albumin, bilirubin and TBARS. Total cholesterol, HDL, LDL, Triglycerides, AST and ALT were also estimated in the same sample.

Preparation of liver homogenate

The liver tissues from the animals were surgically removed, blotted and weighed. 1.0g was crushed in mortar and pestle and then homogenized in phosphate buffered saline. The liver homogenate was centrifuged at 2000g for 20 minutes in a refrigerated centrifuge. The supernatant was separated and immediately used for biochemical parameters AST, ALT, Total cholesterol, HDL, LDL and Triglycerides. The liver tissue was homogenised with 1% TCA and centrifuged. The supernatant was used for the assay of TBARS. A portion of liver sample from each group was fixed in 10% formaldehyde and analyzed for histopathological examination.

3.6 DETERMINATION OF BIOCHEMICAL PARAMETERS

3.6.1 ESTIMATION OF TOTAL PROTEIN

Total protein was estimated by the method of Lowry *et al.*, (1951) as outlined in Appendix-V

3.6.2 ESTIMATION OF ALBUMIN

Albumin was assayed by the method of Rasanayagam *et al.,* (1986) as described in Appendix-VI

3.6.3 ESTIMATION OF BILIRUBIN

Bilirubin was determined by the method of Dangerfield and Finlayson (1953) and it is explained in Appendix-VII

3.6.4 ESTIMATION OF TOTAL CHOLESTEROL

The method Allain, (1974) was used to determine the total cholesterol and the procedure is stated in Appendix-VIII.

3.6.5 ESTIMATION OF HIGH DENSITY LIPOPROTEIN (HDL)

HDL was estimated by the method of Burstein *et al.*, (1970) as described in Appendix-IX

3.6.6 ESTIMATION OF LOW DENSITY LIPOPROTEIN (LDL)

LDL was determined by the method of Rifai and Warnick, (1994) and detailed procedure is given in Appendix-X

3.6.7 ESTIMATION OF TRIGLYCERIDES

Triglycerides were analysed by the method of Trinder, (1969) as explained in Appendix-XI

3.6.8 ESTIMATION OF ASPARTATE TRANSAMINASE (AST)

AST was determined by the method Bradley *et al.*, (1972) and the detailed procedure is depicted in Appendix-XII

3.6.9 ESTIMATION OF ALANINE TRANSAMINASE (ALT)

The method of ALT was measured by Bradley *et al.*, (1972 the procedure is explained in Appendix-XIII

3.6.10 ESTIMATION OF THIOBARBITURIC ACID AND REACTIVE SUBSTANCES (TBARS)

Thiobarbituric acid was measured according to the method of Nadigar *et al.*, (1986) as given in Appendix-XIV

3.7 HISTOPATHOLOGICAL EXAMINATION

A portion of liver sample from each group was fixed in 10% formaldehyde and stained with hematoxylin and eosin for histopathological observation Culling, (1979) as given in Appendix- XV

3.3.3 PHASE III

3.8 BIOSAFETY SCREENING OF THE PLANT SAMPLE

Brine shrimp (*Artemia salina*) lethality bioassay was carried out to investigate the cytotoxicity of ethanolic extract of *Alternanthera* sessilis leaf powder. The procedure is presented in Appendix- XVI as described by Zakaria *et al.*, (2007).

3.3.4 PHASE IV

3.9 ASSESSMENT OF THROMBOLYTIC ACTIVITY

The thrombolytic effect of the ethanolic extract of *Alternanthera sessilis* leaf powder was evaluated using human blood according to the *in vitro* clot lysis method of Prasad *et al.*, (2007) as described in Appendix-XVII.

STATISTICAL ANALYSIS

The data are expressed as mean± standard deviation. Statistical significance of the data was assessed by analysis of variance (ONE-WAY ANOVA).