3. Materials and Methods

3.1. Experimental Animals

The present study was carried out in male Wistar albino rats weighing 170-200 gm. The animals were obtained from Sun Pharma Advance Research Centre (SPARC), Tandalja. Animals were housed in groups of 2 rats per cage under well maintained condition of temperature and humidity. A 12h/12h light-dark cycle was maintained throughout the period of experiments. Animals were given free access to conventional laboratory chow diet and tap water ad libitum. The protocols for the experiments were approved by the IAEC (Institutional Animal Ethics Committee) as per the guidance of the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), (Protocol Numbers: PIPH 26/13 and PIPH 35/13).

3.2. Collection and authentication of plant material

Aerial parts and bark of the plant *Anogeissus acuminata*, family, Combretaceae, were collected from wastelands of Khedbrahma, Gujarat in the month of March. The herbarium of the collected sample was submitted for authentication at NISCAIR, Delhi. The sample was identified as *Anogeissus acuminata* and was preserved with reference no. NISCAIR/RHMD/consult/2013/2290/70.

3.3. Preparation of extracts

Leaves and bark of plant were dried in the shade and were separately made into a coarse powder. Methanolic extracts of leaves and bark were prepared using a Soxhlet extractor. 50 gm of either powder was extracted in a Soxhlet extractor with methanol (200 ml × 3) as a
Materials and Methods

solvent. The filtered extract was dried in vacuum drier and stored at 4°C. The yield for the leaves and bark was 16% and 14.9% w/w respectively in terms of dried starting material.

3.4. Gas chromatographic evaluation of the extracts for residual solvent

Both leaf and bark methanolic extracts of AA were subjected to gas chromatography analysis for checking residual methanol content using GC-FID with Headspace Auto sampler, having Agilent RTx-130 column. Instrument parameters set according to USP -467. Leaf and bark extracts showed the presence of 271.55 ppm and 1526.39 ppm methanol residues respectively, which were far below the maximum allowable limit of 3000 ppm for methanol. No other solvents were detected in the extract.

3.5. Induction and Characterization of DM models

Induction of T1DM

Type 1 diabetes mellitus was induced in rats by injection of STZ (Himedia Labs, Mumbai) in citrate buffer (pH: 4.5) at a dose of 50 mg/kg i.p. 10% glucose solution was provided to rats instead of drinking water for one day following STZ injection to prevent the hypoglycemia due to sudden release of insulin from pancreatic beta cells by STZ. After 48 hours of STZ injection blood glucose levels (BGL) were determined using a glucometer (One Touch Glucometer, Johnson and Johnson Ltd). Animals with BGL > 250 mg/dl were considered diabetic.

Induction of T2DM

Type 2 DM was induced by Fructose + STZ model (Wilson and Islam, 2012). The animals were given 10% fructose solution in place of drinking water for 3 weeks, after 3 weeks 40 mg/kg, i.p. STZ was injected in 6 hour fasted rats. 48 hours after the STZ injection, blood glucose levels were measured. Animals with blood glucose levels above 250 mg/dl were considered diabetic.
Intravenous Insulin Tolerance Test for detecting insulin sensitivity

On the 15th day of induction of diabetes, Insulin (0.1 IU/kg) was administered by i.v injection and blood samples were collected at 3, 10, 15, 20 minutes for the measurement of plasma glucose. The value is presented as a percentage reduction in plasma glucose level. A graph was plotted for a percentage reduction in plasma glucose level Vs time. Slope ($K_{ITT}$) of the lines was determined. $K_{ITT} > 2.0 \%$/min represented normal insulin sensitivity while that less than $1.5 \%$/min indicated reduced insulin sensitivity or insulin resistance (Patarrão, Wayne Lautt and Paula Macedo, 2014).

3.6. Experimental design

The study was divided in four phases as depicted in figure 3.1. Phase 1 consisted of evaluation of antihyperglycaemic action of extracts in two models of DM (T1DM and T2DM). Phase 2 consisted of evaluation of extracts in complications of DM (Neuropathy, Nephropathy and Cardiomyopathy). Phase 3 was conducted to study the possible mechanisms of action of drug responsible for its protective effect on diabetic complications. Phytochemical evaluation of AA was carried out in Phase 4 of the study, where various standard techniques and tests were used to shed light on the chemical constituents of the plant.
Figure 3.1: Schematic presentation of Experimental design and parameters evaluated
Phase I Study: Evaluation of Antidiabetic action

3.7. Evaluation of antihyperglycemic effect in Type 1 diabetes mellitus

3.7.1. Grouping of animals

Animals with BGL > 250 mg/dl were divided into six groups with 6 animals in each. Moreover, one group of six normal animals (untreated with STZ) was kept as group I or normal control. Treatment was continued for 8 weeks as per following plan for various groups.

**Group I**: Normal Control (NC): Blank acacia suspension, 1ml, orally

**Group II**: Diabetic Control (DC): Blank acacia suspension, 1ml, orally

**Group III**: Standard treatment (Std): Human NPH Insulin (4 IU/kg/day), Subcutaneously (Pinheiro et al)

**Group IV**: Test 1 (LE100): Leaf extract suspended in water using acacia as suspending agent, 100 mg/kg bw, orally

**Group V**: Test 2 (LE300): Leaf extract suspended in water using acacia as suspending agent, 300 mg/kg bw, orally

**Group VI**: Test 3 (BE100): Bark extract suspended in water using acacia as suspending agent, 100 mg/kg bw, orally

**Group VII**: Test 4 (BE300): Bark extract suspended in water using acacia as suspending agent, 300 mg/kg bw, orally

3.7.2. Determination of Body Weight, Food Intake and Water Intake

Body weight of animal was determined gravimetrically using calibrated scale. The difference of food placed in the cage and food found at the end of 24 hours gave the food consumed by animal during 24 hours. Similarly, water consumed by animal was estimated by difference of water placed and left at the end of 24 hours.
3.7.3. Determination of Plasma Glucose Levels

Blood was drawn from lightly anaesthetized animals from the retro orbital plexus in gray top tubes at 0, 1, 2, 4 and 8 weeks after starting the treatment. The tubes were inverted 4-5 times to mix the contents. Plasma glucose level was determined by glucose oxidase method using Selectra ELITECH clinical systems fully automated biochemistry analyser.

3.8. Evaluation of antihyperglycemic effect in Type 2 diabetes mellitus

3.8.1. Grouping of animals

Animals were divided into 6 groups with 6 animals in each. Animals untreated with fructose and STZ were kept in normal control group. Treatment was given for 12 weeks in following manner.

**Group I**: Normal Control (NC): Blank acacia suspension, 1ml, orally

**Group II**: Diabetic Control (DC): Blank acacia suspension, 1ml, orally

**Group III**: Standard treatment (Std): Glibenclamide (5 mg/kg b.w.), Orally

**Group IV**: Test 1 (LE100): Leaf extract suspended in water using acacia as suspending agent, 100 mg/kg bw, orally

**Group V**: Test 2 (LE300): Leaf extract suspended in water using acacia as suspending agent, 300 mg/kg bw, orally

**Group VI**: Test 3 (BE100): Bark extract suspended in water using acacia as suspending agent, 100 mg/kg bw, orally

**Group VII**: Test 4 (BE300): Bark extract suspended in water using acacia as suspending agent, 300 mg/kg bw, orally
3.8.2. Determination of Body Weight, Food Intake and Water Intake

Body weight, food intake and water intake of animals were determined at 0, 2, 4, 8 and 12 weeks post induction of DM.

3.8.3. Determination of Plasma Glucose Levels

Blood was drawn from lightly anaesthetized animals from the retro orbital plexus in gray top tubes at 0, 2, 4, 8 and 11 weeks after starting the treatment. The tubes were inverted 4-5 times to mix the contents. Plasma glucose level was determined by glucose oxidase method using Selectra ELITECH clinical systems fully automated biochemistry analyser.

3.8.4. Determination of Glycated Hb levels

At 11 weeks blood was collected from lightly anaesthetized animals in purple top tube containing EDTA. HbA1C was determined by Immunoturbidimetry method using Selectra ELITECH biochemistry analyser.
3.9. Evaluation in diabetic neuropathy

Following methods were used to evaluate the effect of standard and test treatments on diabetic neuropathy during 8 weeks of treatment.

3.9.1. Assessment of thermal nociceptive response

Effect of AA and standard treatment on thermal nociceptive response was determined at the end of 8 weeks of treatment. Nociceptive threshold to radiant heat was quantified using the hot plate paw withdrawal test as previously described (Bianchi et al., 2004). Briefly, a 40 cm high Plexiglas cylinder was kept over the hot plate and the temperature was maintained at 60 ± 0.2°C. Paw withdrawal latency was defined as the time between placing the rat on the hot plate and the time of withdrawal, or licking of hind paw, or discomfort manifested by the animal. (Cut off time: 60 s) Animals were tested twice, with a 30 min interval between tests.

3.9.2. Assessment of chemical nociception in formalin test

Diabetic neuropathy results in marked chemical induced hyperalgesia which worsens between 4 to 8 weeks. Response to chemical stimulus was assessed by formalin test at 6 weeks after starting the treatment by formalin test. Briefly, the animal was acclimatized to the observation box before any testing began. Then, 50 μl of 0.2% formalin was injected subcutaneously into the plantar surface of one hind paw. The animal was observed for next 60 min and number of hind limb flinches was counted for each animal. (Dubuisson and Dennis, 1977).

3.9.3. Assessment of autonomic function by charcoal meal test

At 8 weeks of treatment, overnight fasted animals of different groups were orally administered 2 ml charcoal meal (10% charcoal in 5% gum acacia) and 25 min later the rats were killed by cervical dislocation. The abdomen was opened and the intestine was removed from pyloric junction to caecal end. The farthest distance travelled by the charcoal meal through the small intestine and total length of the small intestine were measured. Gastrointestinal transit was expressed as the percentage of the distance travelled by the charcoal meal relative to the total
length of small intestine and expressed as percentage of intestinal transit using following formula (Janssen and Jageneau, 1957):

\[
% \text{Transit} = \frac{\text{Distance travelled by charcoal meal} \times 100}{\text{Total length of small intestine}}
\]

### 3.9.4. Determination of Nerve Conduction Velocity

At the end of 8 weeks of treatment, the animals were sacrificed by cervical dislocation under anaesthesia. Sciatic nerve was isolated from the animal and mounted in the nerve chamber (BSL MP36 assembly, Biopac Systems, Inc., Goelta, CA). The nerve was stimulated at knee end and the time to travel the ankle end was recorded (frequency 20 Hz, duration 0.1 ms, amplitude 1.5 V, sampling 20 k/s). The ratio of Distance between two ends and the time was calculated as nerve conduction velocity (Zangiabadi et al., 2011).

### 3.10. Evaluation in Diabetic Nephropathy

Following methods were used to evaluate the effect of standard and test treatments on diabetic nephropathy during 8 weeks of treatment.

**3.10.1. Collection of urine**

At the end of 8 weeks of treatment animals were placed in metabolic cages for 24 hours and the volume of urine collected was measured. The urine collected in container was centrifuged to remove solid particles if any. The volume of supernatant fluid was recorded and considered as 24 hr urinary volume.

**3.10.2. Determination of urinary protein**

Urinary protein content was measured by sulphosalicylic acid method. Standard plot was prepared by taking absorbance (at 500 nm) of reaction mixtures of albumin at different concentrations as mentioned in table 3.1. From test tube number 1-5, 1.25 ml was transferred in test tubes labelled a-e respectively. To each of these test tubes a-e, 3.75ml of 3% sulphosalicylic acid solution was added. Absorbance was recorded after 5 min at 500 nm. A
graph of concentration Vs Absorbance was plotted. $R^2$ value and formula for line was determined using Microsoft Excel, 2010.

**Table 3.1. Preparation of standard curve for sulphosalicylic acid**

<table>
<thead>
<tr>
<th>Test tube no.</th>
<th>Volume of albumin 10mg/ml solution added (ml)</th>
<th>0.9% NaCl (ml)</th>
<th>Final Protein Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
<td>9.95</td>
<td>0.05</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>9.9</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>9.8</td>
<td>0.2</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>9.5</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>9.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

For test samples, 1.25 ml of filtered urine was mixed with 3.75ml of 3% sulphosalicylic acid solution in the test tube. Blank solution was prepared by mixing 3.75 ml of 0.9% NaCl solution instead of sulphosalicylic acid. Absorbance was measured after 5 min in a spectrophotometer at 500 nm. Concentration of protein in urine as sample was calculated using formula derived from standard curve (Kaminskas and Mazeikiene, 2012).

### 3.10.3. Serum Creatinine and BUN Levels

Serum creatinine and blood urea nitrogen levels in overnight fasted animals were determined using Selectra ELITECH clinical systems fully automated biochemistry analyser (Matsumoto and Hatano, 1989). Creatinine was determined using Jaffé’s reagent and measuring the absorbance of coloured complex formed. BUN determination was based on Kinetic Enzymatic method.

### 3.10.4. Kidney Weight to Body Weight Ratio

At the end of 8 weeks of treatment, rats were sacrificed by cervical dislocation under anaesthesia. Body weight of the animals was determined just before sacrifice. Kidneys were removed, washed with physiological saline, cleared of fatty tissue and weighed. Kidney weight /100 gm of body weight were calculated for each animal as per following formula:
Kidney weight /100 gm of body weight = \( \frac{\text{Kidneyweight(gm)} \times 100}{\text{Bodyweight (gm)}} \)

### 3.11. Evaluation in Diabetic Cardiomyopathy

Parameters for cardiomyopathy were evaluated in animals with T2DM over 12 weeks of treatment period.

#### 3.11.1. Determination of lipid levels

Blood was collected in yellow top (Serum separator tubes) for determination of lipid levels at 2 and 12 weeks after starting treatment. All the samples were stored below 4°C before sending to the pathology lab. Endpoint enzymatic PAP method was used for determination of Total Cholesterol, Triglyceride, LDL and HDL levels in serum samples.

#### 3.11.2 Determination of LDH and CK-MB levels

Serum LDH and CK-MB levels were determined at 12 weeks post treatment. Kinetic UV SFBC and Immunoinhibition Kinetic UV methods were used respectively for determination of LDH and CK-MB levels using autoanalyzer.

#### 3.11.3 Measurement of Haemodynamic parameters

**Measurement of mean blood pressure**

The animals were anaesthetized by Ketamine (100 mg/kg, i.p.) + Xylazine (7 mg/kg, i.m.). The body temperature was maintained at 37 ± 1 °C during the experiment. Carotid artery behind the trachea was exposed and cannulated for the measurement of mean blood pressure using a transducer attached to Student physiography (Parasuraman and Raveendran, 2012).

**Determination of heart rate and force of contraction**

After determination of mean blood pressure animals were sacrificed with cervical dislocation and thoracic cavity of the animals were rapidly opened. Heart was isolated and mounted as per the Langendorff heart technique. Chenoweth-Keolle buffer (119.8 mmol/L NaCl, 5.6 mmol/L KC1, 2.88 mmol/L CaCl2, 4.5mmol/L MgCl2, 3.8 mmol/L NaHCO3 and 5mmol/L
glucose) was used for perfusion of heart (Goyal et al., 2011). It was continuously bubbled with air. The responses were recorded using force transducer and strain gauge coupler attached to the student’s physiograph. The assembly was calibrated using 500 mg weight attached to the transducer in place of heart. This calibration factor was used for calculation of force of contraction (mg) of hearts.

**Determination of Cardiac Hypertrophy Index and Left Ventricular Hypertrophy Index**

At the end of the experiment, the hearts were blotted with filter paper to remove excess of water. Extraneous tissues from heart were removed and weight of the heart was noted. Index of hypertrophy was calculated as ratio of heart weight to body weight (HW/BW) using following formula (Goyal and Patel, 2011).

\[
\text{Cardiac Hypertrophy Index} = \frac{\text{Heart weight (mg)}}{\text{body weight (gm)}}
\]

Left ventricle of the heart was isolated and weighed. Left Ventricular Hypertrophy Index was calculated using following formula:

\[
\text{Left Ventricular Hypertrophy Index} = \frac{\text{Left ventricular weight (mg)}}{\text{body weight (gm)}}
\]
3.12. In vivo anti-oxidant activity in type 1 and type 2 diabetic rats

3.12.1. Determination of Malondialdehyde (MDA) level

Method described by Ohkawa et al (1979) was used for estimation of lipid peroxidation product MDA. Supernatant (0.2 ml) was mixed with 0.2 ml of 8% w/v sodium dodecyl sulfate, 1.5 ml of 20% acetic acid in 0.27 M hydrochloric acid, 1.5 ml freshly prepared solution of thiobarbituric acid (TBA) (1% w/v) and 0.6 ml of distilled water. The mixture was heated in a water bath at 95°C for 45 minute, cooled and 2 ml of the mixture was mixed with 2 ml of 10% (TCA) trichloro acetic acid. The resulting mixture was centrifuged at 1000 rpm for 5 min. The intensity of pink color developed was read against blank at 532 nm. The amount of (MDA) (thiobarbituric acid reactive material) was calculated using molar extinction coefficient $1.56 \times 10^5 M^{-1} \text{cm}^{-1}$ and reported as nmoles of MDA/ml.

3.12.2. Determination of catalase level

Decomposition of $H_2O_2$ in presence of catalase was estimated by Aeibiand Bergmeyer(1974). A 50µl supernatant was added to buffered substrate (50 mM phosphate buffer, pH 7 containing 10 mM $H_2O_2$) to make total volume 3 ml. The decrease in the absorbance was read at 240 nm for 2.5 min at an interval of 15 sec. The activity was calculated using the following formula:

$$\text{Volume activity (U/ml)} = \frac{(\Delta A_{50} - \Delta A_{0}) \times \text{total volume (ml)} \times df}{0.0436 \times \text{enzyme containing solution (ml)}}$$

3.12.3. Determination of GSH (Reduced Glutathione) level

Reduced GSH levels were estimated as per the method described by Beutler et al (1963). The supernatant (2 ml) was mixed with 10% chilled (TCA) trichloroacetic acid. The mixture was kept in ice bath for 30 min and centrifuged at 1000g for 10 min at 4°C. 0.5 ml supernatant was mixed with 2.0 ml 0.3 M disodium hydrogen phosphate and 0.25 ml 5, 5’-dithiobis-2-nitrobenzoic acid (40 mg/100ml in 1% sodium citrate) was added just before measuring the absorbance at 412 nm. Standard curve for GSH was prepared using glutathione. Results were expressed as µg of GSH/ml.
3.13. **In vitro anti-oxidant activity**

The antioxidant activity of AA extracts was determined by different in vitro methods such as DPPH free radical scavenging assay, Reducing power assay, TBA assay. The different extracts were dissolved in methanol. All the assays were carried out in triplicate, and average values were considered.

3.13.1. **DPPH assay**

DPPH scavenging activity of the plant extracts was carried out according to the method of (Gyamfi, Yonamine and Aniya, 1999). Methanolic solution of plant extracts (50 µl) at different concentrations (10 µg/ml-10 mg/ml) was mixed with 450 µl of trisHCl buffer (50 mM, pH 7.4). One milliliter 0.1 mM DPPH in methanol was added to the above mixture. The mixture was shaken vigorously and incubated for 30 min at room temperature. Absorbance of the resulting solution was measured at 517 nm UV Visible Spectrophotometer. Methanolic solution of leaf or bark of AA was used as blank and DPPH methanolic solution served as control. The Vitamin C was used as a standard antioxidant in this method. Percentage of DPPH scavenging activity was determined as follows:

\[
\% \text{ inhibition} = \left( \frac{A_{\text{Control}} - A_{\text{test}}}{A_{\text{Control}}} \right) \times 100
\]

Where \( A_{\text{Control}} \) = absorbance of control, \( A_{\text{test}} \) = absorbance of test

Decreased absorbance of the reaction mixture indicates stronger DPPH radical scavenging activity.

3.13.2. **Reducing Power assay**

One milliliter of methanolic solution of plant extracts (final concentration 1 to 50 µg/ml) was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide [K3Fe(CN)6] (10 g/l). After 20 min incubation at 50°C, 2.5 ml of trichloroacetic acid (100 g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 20 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml FeCl₃ (1 g/l). Absorbance of the resultant solution was measured at 700 nm in UV-Visible Spectrophotometer. The assay was carried out in triplicate. 2.5 ml solution of ascorbic acid (concentration 1 to 50 µg/ml) and phosphate buffer were used as standard and control.
solutions, respectively. Methanolic solution of plant extracts was used as blank. Increased absorbance of the reaction mixture indicates stronger reducing power. Percentage reducing power for each solution was calculated by considering highest absorbance of vitamin C as 100% (Oyaizu, 1986).

3.13.3. TBA method

The method of Ottolenghi was used. Two ml of 20% trichloroacetic acid and 2 ml of 0.67% 2-thiobarbituric acid was added to 1 ml of 10μg/ml sample solution. The mixture was placed in a boiling water bath. The solution after cooling was centrifuged at 3000 rpm for 20 min. Absorbance of supernatant was measured at 552 nm. Water instead of sample was used as blank. Vitamin C was used as standard. Antioxidant activity was based on the absorbance observed at 14th day (Alam, Bristi and Rafiquzzaman, 2013).

3.14. Determination of Insulin level and Calculation of HOMA-IR and HOMA-β

Insulin levels were determined by radioimmunoassay method at 11 weeks after starting the treatment. The fasting blood sample for determination of insulin was collected in red top tube containing clot activator. All the samples were stored below 4°C before sending to the pathology lab. HOMA-IR and HOMA-β scores were calculated using fasting serum insulin and fasting plasma glucose levels at 11 weeks of treatment according to the following formula (Matthews et al., 1985) using conversion factors:

Insulin (1U/l = 7.174 pmol/l)

Blood glucose (1 mmol/l = 18 mg/dl).

\[ \text{HOMA-IR} = \frac{[\text{Insulin (U/l)} \times \text{Blood glucose (mmol/l)}]}{22.5} \]

\[ \text{HOMA-β} = \frac{[20 \times \text{Insulin (U/l)}]}{[\text{Blood glucose (mmol/l)} - 3.5]} \]

3.15. PTP1B inhibitory activity *in vitro*

PTP1B inhibition colorimetric assay kit (ab139465) was purchased from Abchem (Cambridge, UK). Phosphate curve was prepared by using phosphate solution in
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concentration range of 0.25 – 3.0 nM. Each experiment was performed in triplicate. Leaf and bark extracts were tested in final concentration range of 1- 400 μg/ml. Suramin in final concentration of 1-100 μg/ml was used as standard. Solutions of test and standard were prepared in phosphate buffer provided with kit at concentrations 15 times that of expected final concentrations. To each well of a 96 well plate, 10μl of test or standard, 85μl of assay buffer, 5 μl of (0.5 ng/μl) PTP 1B enzyme and 50 μl of 75 μM PTP1B substrate were added and incubated at 30 °C. After 30 min reaction was terminated by addition of 25 μl of provided red assay reagent and allowed to stand for 20 min before reading the absorbance at 620 nm on ELISA plate reader (Na et al., 2009). The curve-fitting program Prism 5 (GraphPad Software, San Diego, CA) was used to calculate IC50 values.
3.16 Preliminary phytochemical screening

Leaf and bark AA extracts were subjected to following preliminary phytochemical tests for the identification of chemical constituents like, carbohydrates, glycosides, alkaloids, amino acids, flavonoids, fixed oil, tannins, gum and mucilage, phytosterolsetc using standard methodology (Khandelwal, 2007).

3.17 HPTLC fingerprinting of AA extracts

Optimization of mobile phase was done using various mobile phases to get optimum separation of bands (Wagner and Bladt, 1996). However, toluene: ethyl acetate: formic acid (4.5:3.0:0.2 \( \text{v/v/v} \)) showed best result. This mobile phase was further used for HPTLC fingerprinting analysis. Chromatography was performed on Merck Silica gel 60F254 TLC precoated aluminum plates. 20 \( \mu \text{l} \) of freshly prepared samples were applied on the plate as a band of 10mm width with the help of LINOMAT V \(^\text{®} \) Automatic Sample Spotter at the distance of 10 mm from the edge of the plate. The plate was developed to a distance 80mm in a CAMAG \(^\text{®} \) twin trough chamber (10x 10 cm) previously equilibrated with mobile phase for 20 minutes. After development, densitometric evaluation of plate was performed at 254nm in absorption mode using TLC scanner 3 linked to WinCats Software (CAMAG \(^\text{®} \)).

3.18 Quantification of tannins in extracts

25 ml extract was added to 25 ml indigo sulphonic acid in conical flask. This mixture was titrated against 0.1 M potassium permanganate solution until golden yellow colour was obtained (Horwitz, Chichilo and Reynolds, 1975). Tannin content was calculated using factor - each ml of 0.1 M potassium permanganate solution is equivalent to 0.004157g of tannin compound calculated as tannic acid.

3.19 Quantification of flavonoids in extracts

Flavonoids were quantified using aluminum chloride method. In this method Quercetin was used as standard. Calibration curve of quercetin (6.25, 12.5, 25, 50, 100 \( \mu \text{g/ml} \)) was prepared.
1ml of standard or extract solution was taken into 10ml volumetric flask, containing 4ml of distilled water. 0.3ml of 5%NaNO$_2$ was added to the flask. After 5min, 0.3ml 10%AlCl$_3$ was added to the mixture. 2 ml of 1M NaOH was added and volume was made up to 10 ml with distilled water. The absorbance of the resulting solution was noted at 510 nm using UV-Visible spectrophotometer (Zhishen, Mengcheng and Jianming, 1999).
3.20 Statistical Analysis

Data were expressed as mean ± SEM. Statistical analysis was done by one-way or two way analysis of variance (ANOVA) followed by appropriate post hoc analysis, using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA. P values <0.05 were considered as significant.