Chapter-3
Material and Method

3.1 Study Area

Kokrajhar district occupies the north western part of Assam, India. Located in 89°46′ to 90°38′E longitudes and 26°19′to 26°54′N latitudes, total geographical area of this district is 3,169 sq. km. with population of 8, 86,999 according to 2011 census projection. It is surrounded by Bhutan on the North, the Sokosh River, and Jalpaiguri district of West Bengal on the west and reverend tract of Brahmaputra valley (Goalpara and Dhuburi districts) on the south and on the east side Chirang, and Bongaigaon districts. As such this has separate geographical entity so far its location is concerned. The flora and fauna looks interesting, because of its geographical occupancies. The major part of tract is surrounded by the forest which can be divided in two terrains of Bhabar and the Terai (Nath and Mwchahary 2012).

Topography: The land surface of Kokrajhar district undulating with a general slope from north to south and it may be divided into two distinct physiological zones-(a) North foothill zone of the Himalaya and (b) The plain or the built up zone. The zone has isolated hillocks, depressions, perennial rivers, tributaries and minor streams and abandoned canals (Phukan 1990).

Soil: the soil of the Kokrajhar district is alluvial in origin. The soil varies from sandy loam to silty clay loam, silty clay and clay. It consists of sand and clay of varying proportion ranging from poor sand stiff clay towards south tract. In the Northern part i.e. in the sub Himalayan area the soil is mostly light gravel; which originates from old mountain valley alluvial soil. The soil is silt in organic matter and acidic in nature (Bhowmick et al. 2015).

Climate: The district is situated in sub -tropical climate with moderately hot summer and cold winter with high humidity. Dust storms are also not uncommon during February to April of the year because of proximity to the Brahmaputra River. Generally December to February is the coldest month of the year. During hot summer day's temperature goes up to 32°C and

in winter comparatively cooler temperature goes below 11°C. Monsoon is the month of June to September with heavy rainfall, record average ranging up to 747mm (Kour and Sharma 2016).

3.2 Plant Material Processing

3.2.1 Collection: The complete plant materials of climbing stem, leaves, flower and inflorescence, root and fruit of *Hodgsonia heteroclita* were procure from the forest of Chakrachila wild life sanctuary, Kokrajhar, Bodoland Territorial Council (BTC), Assam, India by the month of September to November, 2014.

3.2.2 Authentication: The collected plant materials were pressed dried, processed, tagged, recorded as the specimen at the departmental herbarium. The specimen was authenticated as a *Hodgsonia heteroclita* (Roxb.) Hook. f. & Thomson by a complete proved identification in the BSI Shillong. After authentication the specimen has been deposited at Bodoland University with a voucher specimen no. DBT/BU/Herb/2014/005

3.2.3 Processing of *Hodgsonia* Fruit Pulp (HFP): The well washed fruit with cleaned water was allowed to air dry and surface sterilised by the 70% ethanol. The fruit was cut longitudinally with a sterilised sharp knife. After eliminating the six large seeds, the fruit peel was peeled off followed by pulp was sliced in the thin sections. The pulp sections were allowed to dry under constant temperature of 40°C in hot air oven. After complete drying the fruit pulp was grinded to make powder with mechanical grinder. Then the plant material was kept in an air tight container for further process.

3.2.4 Extraction: The 300g of dried powdered pulp was wrapped by the Whatman filter paper (No. 1) and placed in the thimble of the soxhlet apparatus. The extraction was done using 70% methanol solvent in the ratio of 1:15 mg/mL (plant material: solvent) as per Middha et al. (2013). The solvent cycling of soxhlet apparatus was allowed to process for more than eight cycles setting the apparatus heat at 70°C, 80°C, and 90°C, respectively. The plant material extract was collected in the round bottom flask and was allowed to evaporate at 50°C under pressure (12 torr) to a constant weight. After

drying the whole solvent using rota evaporator the left out product was further lyophilised using lyophilizer (Telstar Lyoquest Freeze Dryer). The extract (HFP) was stored at 4°C for further future testing .

3.2.5 Determination of Plant Extract Yield: The yield of evaporated HFP extracts based on dry weight basis was calculated using the following equation:

$$Y = W1 \times 100 / W2$$

[Where, Y= Yield in g/100g of dry plant material, W1 and W2 were the weight of the extract after the solvent evaporation and the weight of the dry plant material, respectively].

3.3 *In vitro* Studies

3.3.1 Preliminary Phytochemical Screening: The presence or absence of the phytochemical constituents of powdered plant material was analyzed using the following standard methods. *Hodgsonia* fruit pulp (dry, 500 mg) was boiled in 30mL DDW and filtered, as per Goyal et al. (2011).

Carbohydrates: 1mL filtrate + 1mL of Molisch's reagent + 1mL conc. H_2SO_4. The presence of carbohydrate is inferred by a reddish ring.

Reducing sugars: 1mL of the above filtrate + 2mL of Fehling's solution were boiled for 5min. A brick red precipitate indicates the presence of reducing sugar.

Tannins: 2mL of the filtrate + 1mL $FeCl_3$. A blue-black or greenish-black precipitate confirms tannins.

Saponnins: Frothing test: 0.5mL filtrate + 5mL DDW, shaken for 30sec, persistent frothing indicates saponnins.

Flavonoids: Shinoda's Test: 200mg plant material was extracted with 5mL ethanol and filtered; 1mL filtrate + magnesium ribbon + conc. HCl were added. A pink or red color indicates the presence of flavonoids.

Steroids: Liebermann–Burchard's test: 200mg of the dry plant material in 10mL chloroform, filtered. 2mL filtrate + 2mL acetic anhydride + 1mL conc. H_2SO_4 are added. A blue-green ring shows the presence of steroids.

Alkaloids: Plant material (dry, 200mg) was boiled in 20mL of 1% H_2SO_4 in 50% ethanol and filtered. Filtrate + 5drops of conc. NH_4OH + 20mL chloroform were added and the two layers were separated. The

chloroform layer was extracted with 20mL dilute H_2SO_4. On addition of extract + 5 drops of Mayer's reagent, a creamy/ brownish red/ orange-red precipitate indicates the presence of alkaloids. (Mayer's reagent; freshly prepared mercuric chloride (1.36 g) and potassium iodide (5g) in water (100 mL).

Anthraquinones: Borntrager's test: 100 mg of powdered plant material was added to 5mL of chloroform and filtered. 2mL filtrate + 2mL 10% NH_4OH were added. A bright pink color confirms the presence of anthraquinones.

Glycosides: Keller–Kiliani test: 2mL filtrate + 1mL glacial acetic acid + 1mL $FeCl_3$ + 1mL conc. H_2SO_4 were added. A green-blue color indicates the presence of glycosides (Mikail 2010).

3.3.2 Determination of the Total Phenolic Content: Total phenolic content was determined as per Singleton and Rossi (1965) using the Folin-Ciocalteu (FC) reagent with slight modification (Goyal et al. 2010) using the gallic acid standard. The 0.5mL of fruit pulp extract was mixed with 0.5mL of 1:1 FC reagent diluted with distilled water and incubated for 5min at RT, then 1mL of 2% Na_2CO_3 solution was added. After incubation at RT for 10min, the absorbance was measured at 730nm. All tests were performed in triplicates. The total phenolic content was expressed as micro gram of gallic acid equivalents (GAE) per 100 micro gram extract.

3.3.3 Determination of the Total Flavonoid Content: The total flavonoid content was determined as per Zhishen et al. (1999) with minor modifications (Usha et al. 2014) with the aluminum chloride ($AlCl_3$) method using quercetin standard. The 0.25mL of plant extract was added to 1.25mL DDW followed by 75µL of 5% $NaNO_2$. After 5min. $AlCl_3$ (0.15mL, 10%) was added at room temperature (RT). Further 6min. incubation at RT, the reaction mixture was treated with 0.5mL of 1mM NaOH. The reaction mixture was diluted with 275µL of DDW. Further incubation for 20minutes at RT the absorbance of reaction was measured at 510nm. All tests were repeated trice. Finally the flavonoid content was calculated from a quercetin standard curve.

3.3.4 Determination of the Total Flavonol Content: The total flavonols content was estimated

according to Kumaran and Karunakarn (2007) using quercetin standard. 2mL of fruit extract was mixed with 2mL $AlCl_3$ (2%) followed by 3mL of sodium acetate. After 2.5h the absorbance was measured at 440nm at 20°C. All the tests were carried out in triplicates and the total flavonoid content was calculated using the different concentrations of quercetin standard curve.

3.3.5 Assessment of Antioxidant Activity:

3.3.5.1 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity: The free radical scavenging capacity of the extracts was determined using DPPH as per Hasan et al. (2009). The DPPH solution (0.006% w/v) was freshly prepared in 95% methanol. The extract of the HFP was mixed with 95% methanol to prepare the stock solution (1 mg/mL). Freshly prepared DPPH solution was taken in test tubes and extracts were added followed by serial dilutions (20–200µg) to every test tubes to fill the final volume 2mL, incubated at 37°C for 30min in the dark and discoloration was measured at 517nm. The tests were performed in triplicate. Ascorbic acid was dissolved in DDW to make 1.0mg/mL stock solution with the same concentration as a reference standard. The 95% methanol of same volume without extract was used as a control sample. The scavenging percent of the DPPH free radical was calculated using the equation: S = (Ao – A1) / Ao x100. [Where, S = (%) scavenging effect of DPPH, Ao is the absorbance of the control and A1 is the absorbance in presence of the sample]. The decrease in absorption induced by the test compound was compared with the positive controls. The EC_{50} value was calculated using the dose inhibition curve.

3.3.5.2 Reducing Power Assay: This method is based on the principle of increase in the absorbance of the reaction mixtures. Increase in the absorbance indicates an increase in the antioxidant activity. In this method, antioxidant compound forms a colored complex with potassium ferricyanide, trichloro acetic acid and ferric chloride. Increase in absorbance of the reaction mixture indicates the reducing power of the samples. The reducing power of the HFP extract was determined as per Oyaizu (1986) with minor changes. Different

concentrations of the extract (0.2–0.12mg/mL) in 1ml of DDW were prepared and mixed with the phosphate buffer (2.5mL, 0.2M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5mL, 1%). The mixture was incubated at 50°C for 20min. A portion (2.5mL) of TCA (10%) was added to the mixture and centrifuged at 3,000rpm for 10min. The upper layer of the solution (2.5mL) was mixed with DDW (2.5mL) and $FeCl_3$ (0.5mL, 0.1%) and the absorbance was recorded at 700nm. Butyl Hydroxyl Toluene (BHT) and phosphate buffer (pH 6.6) were used as a reference standard and blank, respectively. Here the yellow coloured solutions were transformed to the pale green.

Absorbance of the final reaction mixture of two parallel experiments were recorded and expressed as mean ± standard deviation.

3.3.6 GC-MS analysis

3.3.6.1 Derivatization: Concentrated crude methanol extract was taken in a separating funnel and shaken by adding water and ethyl acetate in the ratio of 1:4. The upper layer was concentrated in a rotary evaporator to about 1.5mL, added 100µL of N, O-Bis (trimethylsilyl) trifluoro-acetamide (BSTFA) and trimethyl chlorosilane (TMCS) and 20µL pyridine and then heated at 60°C for 30min. From the layers which are separated from the crude extracts, a small amount of the extract was taken and totally evaporated out. To the acetonitrile was added and filtrate was added to 50µL BSTFA+TMCS solution. The solution was heated at 60°C at water bath for 30min. The cooled solution was filtered using 0.45µ membrane filter to a vial.

3.3.6.2 GC-MS analysis of HFP: Gas Chromatography and Mass Spectroscopy analysis was carried on Perkin Elmer Autosystem XLGC with Autosampler (SpectraLab Scientific Incorporation). The column used was Perkin-Elmer Elite-5 capillary column measuring 30m × 0.25mm (Length x diameter) with a film thickness of 0.25mm composed of 95% Dimethyl polysiloxane. Helium was used as the carrier gas at a flow rate of 0.5 mL min^{-1}. 1µL sample volume was utilized for injection. The inlet temperature was maintained as 250°C. The oven temperature was

programmed initially at 110°C for 4min, then an increase to 240°C and then programmed to increase to 280°C at a rate of 20°C ending with a 5min. Total run time was 90min. The MS transfer line was maintained at a temperature of 200°C. The source temperature was maintained at 180°C. GC-MS was analyzed using electron impact ionization at 70eV and data was evaluated using total ion count (TIC) for compound identification and quantification. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS library. Measurement of retention time (RT), height occupied, concen- tration peak area % and data processing were carried out by Turbo-Mass-OCPTVS -Demo SPL software (Thomas et al. 2013).

3.4 *In vivo* Studies

3.4.1 Experiment Approval: A permission of animal model experimentation and study protocol was approved and cleared by Internal Ethical Approval Committee (IEAC-1368/ac/10/CPCSEA) of Maharani Lakshmi Ammanni College for Women, Maleswarm, Bengaluru.

3.4.2 Experimental Animals Homogenization: Four months old male albino rats (n=48, 140-160g) of wistar strain (*Rattus norvegicus*) and Swiss albino mice (n=60, 25-30g) of both sexes were acclimatized under standard laboratory conditions of 12 hours light and dark cycles, temperature of 25±2°C and relative humidity of 68±1% for 15 days for homogenization. The animals were given standard rat pellet (Lipton, India Ltd. Bangalore, India) and added tap water ad libitum (Dash et al. 2007).

3.4.3 Acute toxicity (LD_{50} dose detection): Swiss albino mice (25-30g) of both sexes were segregated in to 6 groups of 10 animals. All animals were overnight fasted providing only water. Aqueous extract of *Hodgsonia* fruit pulp (HFP) at different dose ranges (200, 400, 600, 800, 1000 and 1200 mg/mL/Kg bw) were administered once to each animal of every groups. The mice were observed for 24 hours and the mortality rates were recorded and the median lethal dose LD_{50} was determined using the Karber method modified by Aliyu and Nwude (1982). The toxicity of LD_{50} was calculated using the formula: $LD_{50} = LDy- {}^{I-r}\sum (Dd \times Md)/n$.

[Where: LDy= Highest dose, n=number of animals per group (n=10), Dd=Dose difference, Md= Mean dead]

3.4.4 Induction of Diabetes: Diabetes mellitus was induced to rats as per Middha et al. (2013) by a single intra-peritoneal injection of freshly prepared alloxan monohydrate in citrate buffer (150mg/mL 0.1M, pH 4.0) in a volume of one mL/kg following the overnight fasting excluding the normal control group. After two days of alloxan injection the diabetic animals were confirmed by blood samples collected from the tail vein method. The fasting blood glucose (FBS) was measured by electronic glucometer Accu-Chek (Roche, USA)

3.4.5 Experimental Design: Based on the body weight the experimental wistar rats were divided into six groups of eight animal each with a normal control group in each case as per et al. (2013). The **group-1; NL** (Normal) that served as normal control where the animals were not treated with alloxan and even not fed with drug extract, providing only the functionary food chow chow pellet (Lipton India) with tap water *ad libitum*. The **group-2; DC** (Diabetic Control) served as diabetic control. In this group the rats were induced alloxan *i.p* and animals were not treated with drug extract but provided only with water and rat pellets. The **group-3; LH** (Low *Hodgsonia*), the rats were alloxan induced and the animals were orally feeding with low dose (20mg/ Kg.bw/mL/day) of drug extract suspended in 0.1% di-methyl sulphoxide (DMSO) providing rat pellets and tap water. The **group-4; HH** (High *Hodgsonia*), here the animals were alloxan induced and feeding with high dose (40mg/Kg.bw/ mL/day) of drug extract suspended in 0.1% DMSO providing rat pellets and water. The **group-5; DG** (Diabetic glibenclamide), here the alloxan induced diabetic animals were orally treated with a standard drug glibenclamide at a dose of 0.06mg/ Kg.bw/day but given pellet with normal water and food . The **group-6; DI** (Diabetic Insulin), here the alloxan induced diabetic animals were treat with insulin at a dose of 2U/Kg. bw/ day by i.p. route injection having provided pellet with normal water. All the experimental cases were properly tagged and recorded for the whole assessments. After a period of one week the induced animals were used for experimental purposes.

3.4.6 Drug Extract Feeding: Based on the LD_{50} toxicity the low and high dose (LH and HH) of drug extracts were considered. The drug extract was fed using 16 gauge ball point needle. From the third day (72 hours) of alloxan induction the drug treatment were carried out every day morning for a period of six weeks as per Middha et al. (2013). After half an hour of drug treatment only the usual food and water were provided. The body weights (BW) of animals were determined every day morning.

3.4.7 Collection and Estimation of Blood Glucose: The 44was determined as per Schmidtke et al. (1998) early in the morning in every week for the six weeks from the day of drug treatment. The blood sample was withdrawn using retro orbital method from overnight fasting animals. The blood of overnight fasting rats were collected in clinical tubes containing EDTA as an anticoagulant for the glucose estimation. The blood glucose level was measured using Accu Chek (Roche USA), the one touch electronic glucometer to record.

3.4.8 Sacrifice and Cervical Dislocation: On 42 days of last treatment the animals were sacrificed using the over dose of Ketamine: Xylazine, in the ratio of 1:4. Abdomens of the animals were dissected and the pancreas of the animals were dislocated and isolated. The organs were immediately chilled in ice cold 0.9% sodium chloride as per the method used by Glowinski et al. (1966).

3.4.9 Organ Tissue segregation: Each pancreas were cut into three sections. One of the each section was conserved in -80^{o}C for further studies. Second part was used for enzyme assays (SOD, GPx and MDA) and third part was used for histopathological study using the haematoxylene and eosin stain.

3.4.10 Enzyme Assays: The pancreatic tissues of experimental animals of different groups were prepared for enzyme assays as per Usha et al. (2014). The tissues were collected on ice, weighed and rinsed several times in ice cold tyrodes medium and homogenized in 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA to obtain a 5% homogenate for the malondialdehyde (MDA) tests. The analytical procedures for SOD and GPx were

assessed in the supernatant obtained after centrifugation of the 5% homogenate at 600xg for10 min at 4°C (RV/FM, super spin, Plastocraft, India). The supernatant was collected and used for analytical procedures.

3.4.10.1 Glutathione peroxidase (GPx): EC 1.11.1.9; GPx activity was measured as per Flohe and Gunzler (1984). 100µL of tissue extract was added to the reaction mixture consist of 500µl of phosphate buffer, 100µL of 0.01M reduced glutathione (GSH), 100µL of 1.5mM NADPH and 100µL of GR(0.24U). The reaction mixture was incubated at 37°C for 10 min. Then 50µL of 12mM t-butyl hydro-peroxide was added to 450µl of tissue reaction mixture and measured at 340nm for 180sec in a spectrophotometer. [A molar absorptivity of $6.22x10^3$ M/cm was used to determine enzyme activity. One unit of activity is equal to mM NADPH oxidised per min per mg protein.]

3.4.10.2 Super-oxide dismutase (SOD): EC 1.15.1.1; The SOD activity was determined according to the method of Misra and Fredovich (1972). The tissue extract was added to 0.05M carbonate buffer (pH 10.2) containing 0.1mM EDTA and 30mM epinephrine in 0.05% acetic acid and changes in activity were measured at 480nm for 4min. Activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equivalent to one unit and is expressed in terms of units/mg protein.

3.4.10.3 Lipid Peroxidation (LPO): The LPO assay was used to analyze malondialdehyde (MDA) as per the method of Ohkawa et al. (1979) using 1, 1, 3, 3-tetramethoxy propane as standard. In the homogenated tissue 8.1% SDS (sodium dodecyl sulfate) was added and incubated for 10 min. at room temperature (RT), following by boiling with 20% acetic acid and 0.6% thiobarbituric acid for 60 min. in water bath. On cooling, the butanol : pyridine (15:1 v/v) was added and centrifuged at 600xg for 5min. The absorbance of supernatant the upper colored layer was measured at 532nm and the concentration of MDA was expressed in terms of nM/mg protein. Data were expressed as mean±SEM in comparison between the different groups. It was done using one way ANOVA followed by Turkey's multiple comparison test's ($p<0.05$)

which was considered to be statistically significant.

3.5.11 Histopathology: Pancreatic organ specimens were fixed in the 10% buffered formalin. Specimens were placed in plastic cassettes and processed using automatic tissue processor. The tissues were embedded in melted paraffin wax. The trimmed sections were fit and cut for 3-5µ thickness in the rotary microtome. The ribbon sections were placed in floating water bath for 10-15 minutes at 40°C and passed through the xylene and alcohol grades. Sections were stained with the haematoxylene and eosin stain by rinsing with running water, 1% acid alcohol and 2% potassium acetate. Slide were mounted in the DPX and labelled. The slides were observed under the pathological microscope and snapped the photographs and recorded as per Singh and Gupta (2005).