Chapter 3
MATERIALS AND METHODS

3.1 Preparation of Plant Material and Extraction

Leaf of *Bambusa tulda* were collected from the forests of Kokrajhar District, BTAD, Assam, India during winter season, 2013. After authentication by a plant taxonomist and a voucher specimen (Voucher No. DBT/BU/ Bamboo/007) was deposited at Bodoland University, Kokrajhar, BTAD, Assam, India.

Shade dried *Bambusa tulda* leaves was coarsely powdered (100g) using mechanical grinder and were extracted in a Soxhlet apparatus using 80% aqueous methanol (*v/v*), in the ratio of 1:15 *m/v* (plant material: solvent) at boiling temperature for 6 hours (Singh et al., 2012). The extract was vacuum-dried with the help of rotary vacuum evaporator (Middha et al., 2011) and stored at 4^{o}C until required. Before use, the extract was dissolved in double-distilled water (DDW) in desired concentrations .

3.2 Determination of Plant Extract Yield

Based on dry weight, the yields of extracts were calculated using following equation:

$$Y = \frac{W_1}{W_2} X\ 100$$

where, Y= Yield (g/100 g of dry plant material) (W_1 = the weight of the extract after the solvent evaporation and W_2 = the weight of the dry plant material.

3.3 Gas Chromatography- Mass Spectroscopy Analysis

3.3.1 Derivatization procedure

Two procedures were followed. Firstly, a small amount of concentrated methanol extract was taken in a separating funnel, and shaken by adding water and ethyl acetate in the ratio of 1:4. The supernatant was collected and concentrated in a rotary evaporator to about 1.5 mL. 100µL N, O-Bis(trimethylsilyl)trifluoroacetamide and trimethyl chlorosilane (BSTFA + TMCS) and 20µL pyridine was added and heated at 60°C for the next 30 minutes.

For the layers which were separated from the extract, a small amount of extract was taken and in this acetonitrile was added. In this filtrate 50µL BSTFA+TMCS solution was added. This solution was heated at 60° C in water bath for 30 minutes. The cooled solution was filtered using 0.45µ membrane filter to a vial.

3.3.2 GC-MS Analysis

GC-MS analysis was carried out on a Perkin Elmer Turbo Mass Spectrophotometer (Norwalk, CTO6859,USA) which includes a Perkin Elmer Auto sampler XLGC. Perkin Elmer Elite - 5 capillary column measuring 30cm × 0.25mm with a film thickness of 0.25mm composed of 95% dimethyl polysiloxane column was used. The carrier gas used was Helium at a flow rate of 0.5mL/min. 1µL sample injection volume was utilized. The inlet temperature was maintained at 250°C. The oven temperature was programmed initially at 110°C for 4 min, then an increased to 240°C for a 90 min run time. 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 peak areas and data processing were carried out by Turbo-Mass-OCPTVS-Demo SPL software.

3.3.2.1 Standard phenolics used

Phenolic acid standards namely ferulic acid, 2,4-dihydroxybenzoic acid, o-coumaric acid, p-coumaric acid, p-hydroxybenzoic acid, salicylic acid, vanillic acid were acquired from Sigma Chemical Co., USA. The standard solutions in the range of 1mg per mL were prepared in 80% ethanol. The organic solvents used for the analysis

were of chromatographic/MS grade and all the other reagents were of analytical grade. Water purified in the Milli-Q (Millipore) system was used to prepare the mobile phases. All mobile phases were filtered through membranes with a pore size of 0.45 μm.

3.3.2.2 Calibration Curve

The calibration curve for total and individual phenolic acids was made by using different concentrations. Total phenols were expressed in gallic acid equivalents. Individual phenolic acids and flavonoid were identified and quantified by Multiple Reaction Monitoring(MRM) method in GC-MS-MS knowing their parent mass m/z and most abundant fragmented daughters.

3.4 *In-vitro* analysis

3.4.1 Preliminary Phytochemical Screening

The presence or absence of the phytochemical constituents of powdered plant material was analyzed using the following standard methods for carbohydrates, reducing sugars, tannins, saponnins, flavonoids, steroids, alkaloids, anthraquinones, and glycosides (Evans, 1996):

Carbohydrates: [(500 mg leaves of BT was boiled in 30 mL DDW and filtered); 1 mL filtrate + 1 mL of Molisch's reagent +1 mL conc. H_2SO_4. The presence of carbohydrate is inferred by a reddish ring].

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

Tannins: [2 mL filtrate + 1 mL $FeCl_3$, blue-black or greenish-black precipitate confirms tannins].

Saponnins: [frothing test: 0.5 mL filtrate + 5 mL DDW, shaken for 30 sec, persistence frothing indicates saponnins].

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

Steroids: [Liebermann - Burchard's Test: (200 mg plant material in 10 mL chloroform, filtered); 2 mL filtrate + 2 mL acetic anhydride + 1 mL of conc. H_2SO_4. A blue – green ring shows the presence of steroids].

Alkaloids: [200 mg plant material was boiled in 20 mL of 1% H_2SO_4 in 50% ethanol and filtered; filtrate + 5 drops conc. NH_4OH + 20 mL chloroform and the two layers separated. Chloroform

layer was extracted with 20 mL dilute H_2SO_4. Extract + 5 drops of Mayer's reagents, a creamy/brownish-red/ orange-red precipitate indicates the presence of alkaloids].

Anthraquinones: [Borntrager's test: 100 mg of powdered plant in 5 mL of chloroform, filtered. 2 mL filtrate + 2 mL 10% NH_4OH. A bright pink color confirms the presence of anthraquinones].

Glycosides: [Keller- Kiliani test: 2mL filtrate + 1 mL glacial acetic acid + 1 mL $FeCl_3$ + 1 mL conc. H_2SO_4, green-blue color indicates the presence of gycosides].

3.4.2 Determination of Total Phenolic Content

Modified Folin-Ciocalteu (FC) method was used to determine the total phenolic content (Singleton et al., 1965). 10-100 mg/mL concentrations of standard gallic acid solution were prepared in methanol. 0.5mL of FC reagent (1:1 dilution with double distilled water) was added to 0.5ml of different concentrations of *Bambusa tulda* leaf extract (0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL) and incubated at room temperature for 5 minutes. 1mL of 20% Na_2CO_3 was added to this and incubated for 10 minutes at room temperature (RT). The absorbance was measured at 730nm. The test was performed in triplicates. Absorbance of various gallic acid concentrations was used to prepare the standard curve and the total phenolic content of the different fractions of *Bambusa tulda* extract was determined from this standard curve. It was expressed as mg gallic acid equivalent (GAE)/g of extract.

3.4.3 Determination of Total Flavonoids

The total flavonoid content (TFC) was determined according to Zhishen et al. (1999) with minor modifications using quercetin equivalent (mg QE/g) as a standard.0.25mL of different concentrations of *Bambusa tulda* leaf extract (0.1, 0.2, 0.3, 0.4 and 0.5 mg/ mL) was taken. 1.25mL of millipore water and 0.075ml of 5% $NaNO_2$ was added to it. It was incubated for 5 minutes at room temperature. 0.15mL of 10% $AlCl_3$ was added to the reaction mixture and was incubated for 6 minutes at room temperature. 0.5mL of 1mM NaOH and 0.275mL of millipore water was added and the absorbance was measured at 510nm after incubation for 20 minutes at room temperature. The test was performed in triplicates and the standard curve was prepared using different concentrations

of quercetin solution (10-80µg/mL in methanol) as the standard. The quercetin (QE) standard curve was used to determine the total amount of flavonoid and it was expressed in µg of quercetin equivalents (QE)/g of extract.

3.4.4 Determination of Total Proanthocyanidins

The proanthocyanidin content was determined using the procedure previously reported by Sun et al. (1998). 0.5mL of different concentrations of the leaf extract (0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL) were taken and mixed with 3ml of 4% vanillin-methanol solution and 1.5mL of 36% hydrochloric acid. The reaction mixture was incubated for 15 minutes

$$S = \frac{A_0 - A_1}{A_0} \text{X} (100)$$

at room temperature and the absorbance was read at 500nm. The test was performed in triplicates and Catechin solutions at concentrations 5-25µg/mL in methanol were used to prepare the standard curve. Total proanthocyanidins contents was calculated using the standard catechin curve and were expressed in terms of µg catechin equivalence (CE)/g of extract.

3.4.5 Determination of total flavonol content

The protocol developed by Kumaran and Karunakaran (2007) was used to estimate the total flavonols using quercetin as a standard. Briefly the leaf extract (2mL) was mixed with 2mL $AlCl_3$ (2%) followed by 3mL sodium acetate. The absorbance at 440 nm was read after 2.5 h at 20˚C. All determinations were carried out in triplicates. The total flavonols was calculated using a standard curve obtained from different concentration of quercetin.

3.4.6 Assessment of Antioxidant Activity

3.4.6.1 Free Radical scavenging assay-

2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) method

The antioxidant activity of *B. tulda* leaf extracts and standard were assessed on the basis of the radical scavenging effect of the stable DPPH free radical as per the modified protocol by Goyal et al. (2010). DPPH solution (0.06% w/v) was prepared in 95% methanol. The stock solution of *Bambusa tulda* leaf extract (1mg/mL) was prepared using 95% methanol. 1mL of freshly prepared DPPH solution was pipetted out into separate test tubes and

different concentrations of the extract (100-500µg/mL) were added. The final volume was made upto 2mL with millipore water and the test tubes were incubated for 30 minutes at RT, in the dark. The change in colour was

$$S = \frac{A_0 - A_1}{A_0} \text{ x } (100\ )$$

measured by reading the absorbance 517nm. The experiment was performed in triplicates. The reference standard used was ascorbic acid and DPPH without any extract was used as the control. The percentage of scavenging of the DPPH free radical was measured using the following equation:

where, S= % scavenging activity; A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample.

The absorbance of test compounds was compared with positive control to determine the actual decrease in absorption induced by the sample. IC_{50}, that is the concentration of the extract which reduces 50% of free radical DPPH, was calculated using dose inhibition curve.

3.4.6.2 Ferric reducing power assay (FRP)

The reducing power of the extracts was determined according to the method of Oyaizu(1986). Different concentrations of BME (0.2–2.0 mg/mL) in 1 mL of DDW was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of TCA (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with DDW (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. Ascorbic acid was used as a reference standard. Phosphate buffer (pH 6.6) was used as blank solution.

3.4.6.3 Hydrogen peroxide scavenging activity

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Ruch et al (1989). An aliquot of H_2O_2 (2mM) and sample at various concentrations (0.2–2.0 mg/mL) were mixed (1:0.6 v/v) and incubated for 10 min at room temperature. After incubation, absorbance was read at 230 nm and was determined against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging activity of

hydrogen peroxide was calculated using the following equation.

where, S= % scavenging activity; A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample.

3.5 *In vivo* studies

3.5.1 Experimental design

3.5.1.1 Acclimatization

Six months old male albino *wistar* rats (Weight 200-250g) were housed in a group of four and were acclimatized for 15 days. The sheltered rats were kept under standard laboratory conditions of light-dark cycles of 24 hours, temperature of 26°C. The animals were given standard rat pellet

(Lipton India Ltd., Bangalore) and tap water *ad libitum.*

3.5.1.2 Acute toxicity test

Swiss albino mice (25-30g) of either sex were divided into 10 groups of 10 each. Animals were fasted overnight but water was made available prior to experiment. Aqueous extract of *B. tulda* leaf at different dose levels (0.2, 0.4, 0.8, 1.0, 2.0, 4.0g/kg BW/mL) was administered once to experimental groups. The mice were observed for 24 hours, mortality was recorded and median lethal dose LD_{50} (median lethal dose) was determined using the arithmetic method of Karber modified by Aliyu and Nwude (Middha et al., 2011). The acute toxicity (LD_{50}) was calculated using the formula:

$LD_{50} = LDy - {}^{I\text{-}r}\Sigma$ (Dd X Md)/n

where: LDy = highest dose and n = number of animals per group (n= 10), Dd = Dose difference, Md = Mean dead

3.5.1.3 Induction of Diabetes

Hyperglycemia (diabetes) was induced in rats by a single intra-peritoneal (*ip*) injection of freshly prepared alloxan (130 mg/kg body weight) in 50mM Phosphate buffer (pH 7.2) per mL per kg body weight. Three days after alloxan treatment rats with a blood glucose range (FBS) of 220–260 mg/ dL were deemed as diabetic and accounted for the study.

3.5.1.4 Experimental Groups

The following groups of rats having 8 randomly selected rats in each group were made as follows:

Group 1: Normal Control rats (NL)

Group 2: Diabetic Control rats (DC)

Group 3: Diabetic rats given glibenclamide (600 µg/kg body weight) daily for 45 days (DG)

Group 4: Diabetic rats given insulin (2U /kg body weight) daily for 45 days (DI)

Group 5: Diabetic rats supplemented with low *Bambusa tulda* (orally, 100mg/kg BW) daily for a period of 45 days (LBT)

Group 6: Diabetic rats supplemented with *Bambusa tulda* (orally, 200mg/kg BW) daily for a period of 45 days (HBT)

Rats with blood glucose levels of more than 400 mg/dL were considered as hyperglycemic. After the last treatment, the rats were fasted over night. Blood was collected in tubes containing ethylenediaminetetraacetate (EDTA) as an anticoagulant for glucose estimation.

3.5.2 Tissue Preparation

At the end of the experimental period rats in all groups were anaesthetized with xylazine (0.1mL/ 100g BW) and sacrificed by cervical dislocation. The liver and kidney tissue was isolated (Glowinski et al., 1966) and supernatant was used for further analysis.

3.5.3 Enzyme Assays

3.5.3.1 Glutathione peroxidase (GSH-Px):

GSH-Px activity will be measured at 37 ºC by the method of Flohe (Flohe and Gunzler, 1984). For the estimation, tissue samples were homogenized with 10% TCA and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was used for the estimation of GSH. To 1 mL of supernatant, 6mL of 0.2M of phosphate buffer (pH 8) and 1mL of 0.06mM prepared DTNB (Ellman's reagent) solution was added and the intensity of the yellow color formed was read at 412 nm in a spectrophotometer after 10 minutes. The experiment was done in triplicates. The GSH concentration was calculated from the standard curve. The values are expressed as μmoles of GSH/g tissue.

3.5.3.2 Super-oxide dismutase (SOD)

SOD activity was determined at room temp., according to the method of Misra (Misra and Fredovich, 1972). 100μl of supernatant of 5% tissue extract was mixed with 880μL of carbonate buffer. 20μL of 0.05 M epinephrine in 0.05% acetic acid was added to the mixture and the absorbance was measured for the next four minutes at 480nm. The experiment was done in triplicates. 1 unit of activity is equal to the amount of enzyme that results in 50%

inhibition of epinephrine auto oxidation.

3.5.3.3 Lipid Peroxidation (LPO)

Lipid peroxidation was used to analyze malondialdehyde (MDA) using 1,1,3,3 -tertamethoxypropane (TMP) as the standard (Ohkawa et al., 1979). Lipid peroxidation was expressed as nano moles of MDA per mg protein. 50µL of 8.1% sodiumdodecylsulphate (SDS) was added to 0.1mL of 5% homogenate and incubated for 10 minutes at room temperature. To the reaction mix 375µL of 20% acetic acid (pH3.5) and 375µ of 0.6% thiobarbituric acid (TBA) was added and then boiled in boiling water bath (60°C) till a pink-orange colour is was obtained. The sample was cooled and was followed by addition of 1.25mL of 50:1 (butanol:pyridine) mixture. It was then centrifuged at 1000 rpm for 5 minutes and the upper (pinkish) layer was separated and analyzed at 532nm using an ELISA reader. The experiment was done in triplicates.

3.6 Total Protein Estimation

Protein concentration was estimated by using Lowry et al., (1951) method using BSA as standard.

3.7 Histopathological studies

At the end of the study, the whole pancreas of sacrificed animal's sections were used for the islet cell characteristics studies using a binocular compound microscope. Organ specimens were fixed in the 10% Buffered Formalin. Specimens were placed in plastic casket and processed in automatic tissue processor. The tissues were embedded in melted paraffin wax. Trimmed sections were fitted and cut for 5µ thickness in the rotary microtome. The ribbon sections were placed in floating water bath for 10-15 minutes at 40°C. The sections were passed through the xylene and alcohol grades and stained with haematoxylene stain. After passing through the 1% acid alcohol and 2% potassium acetate the sections were again stained with eosin stain. Slides were mounted in the DPX and labeled. Observed under the pathological binocular microscope.