Chapter 3
Materials and Methods

"Experience without theory is blind, but theory without experience is mere intellectual play."

-Immanuel Kant

3.1. The study area

Various regions of northern part of the Bengal province of India, especially Malda, North Dinajpur and Darjeeling districts were covered in the present study.

Malda district comprises total geographical area of 3455.66 sq km with Latitude and Longitude of 24°40’20" N to 25°32’08" N and 88°28’10" E to 87°45’50" E respectively with an average elevation of 17 metres. The region is surrounded by Bangladesh and South Dinajpur in the east, Santal Parganas of Jharkhand state in the west, Uttar Dinajpur in the north and Murshidabad in the south (Sengupta, 1969). The district is also characterized by its diversified wetland and forest vegetation. Plenty of rain throughout the year promote the growth of natural flora. The Adh soi wetland (beel), located at Harischandrapur-II block, is one of the largest among the wetlands of the state comprising rich vegetation due to its macrophytic diversity (Chowdhury and Das, 2011). Adina and Bhalluka forests are two other important forest areas of the district.

North Dinajpur District (latitudes of 25°11' N and 26°49' N and longitudes of 87°49' E and 90°00' E) is in the gangetic belt of West Bengal, occupying an area of 3142 sq km. Bangladesh is in the East, Bihar on the West, Darjeeling and Jalpaiguri Districts are on the North and Malda District is on the South of this district. The territory is also covered by several green forests (Sengupta, 1985).

Darjeeling (Latitude 27.03° N, Longitude 88.18° E), is the most beautiful hill station of West Bengal in India with a total area of 3,149 sq. km. The district is situated at foothills of the Himalayas and famous for its spectacular tea gardens. Tea also the major cash crop and main backbone of local economy along with tourism. The district extends from the tropical tarai plains, at about 300 feet above sea level, to the Sandakphu-Phalut ridge at 12,000 feet. Darjeeling is surrounded by the borders of Sikkim to the North, Bhutan to the East and Nepal to the West. Besides, the district owns a worth resource of diversified

natural vegetation. In essence, the whole town is ablaze with a wide range of flowers such as, rhododendrons, magnolias, gladioli, tiger lilies, hydrangeas, sweet peas, corn flowers, roses and dahlias unite to make Darjeeling bloom.

3.2. Exploitation of local ethno-medicinal plants

Practice of medicinal plants is widespread among the tribal people of Malda and North Dinajpur districts. Moreover, it is deeply rooted in their socioeconomic culture. Considering immense cultural and ethnolinguistic assortment of the ethnic people of these territories, several field interviews were designed to collect the ethnic knowledge and the plant species employed in traditional remedy with special emphasis on the members of Mimosaceae. A survey was conducted during March 2012 to May 2013 in the above mentioned districts. Several interviewing procedures, including group discussion, semi-structured questionnaire, direct interview, open-ended conversations etc. were followed to obtain the information from the native traditional healers (commonly known as Kavirajs, Baidyas or Ojhas) as well as from elderly knowledgeable persons regarding the use of different medicinal Mimosoids curing several ailments. The purpose of this survey was explained to the healers in details, and prior informed consent was taken as per ethical guidelines of the International Society of Ethnobiology (Posey, 1990). Every plant was pointed out by the informants and their local names, used plant parts, formulation and dosages were also recorded for further purposes.

3.3. *In-vitro* Antioxidant profiling

3.3.1. Selection of Plant material

A total of 4 plant species under Mimosaceae were selected for the assessment of their antioxidative aptitude based on the local knowledge. The name of plant species are as follows: *Acacia catechu, A. nilotica*, *Mimosa pudica* and *M. invisa*. The species were collected from above mentioned three districts and authenticated by the Taxonomists of Department of Botany, University of North Bengal, India and voucher specimens were (Table 3.1) deposited at the herbaria of the same Department and also in the Department of Botany, Raiganj University, India. The fresh leaves of each plant were collected during the month of August-September 2013.

3.3.2. Ethical statement

3.3.2.1. Plan collection and conservation

The plant samples collected from the different parts of Malda, North Dinajpur and Darjeeling districts were not within a National Park /Reserve Forest /Govt. protected area and the study did not involve any endangered or protected species.

Table 3.1. Selected plant species selected for antioxidant activities.

Name of Plant	Abbreviation used	Voucher no.
Acacia catechu (L.f.) Willd	ACL	NBU/UD/1039
Acacia nilotica (L.) Delile	ANL	RUC/MLD/433
Mimosa pudica L.	MPD	NBU/MLD/308
Mimosa invisa Mart. ex Colla	MIN	NBU/MLD/311

3.3.2.2. Animal maintenance and approval

All the experiments using animals were reviewed and approved by the Animal Ethical Committee of the University of North Bengal (840/ac/04/CPCSEA, Committee for the Purpose of Control and Supervision on Experiments on Animals) and performed in accordance with the legislation for the protection of animals used for scientific purposes.

3.3.3. Chemicals and reagents

Chemicals and reagents in the present study for varied experiments were of analytical grade and purchased either from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), or Merck (Mumbai, India), or Sigma-Aldrich (USA), unless mentioned otherwise. Milli-Q ultrapure water (grade >1.83) was used for all the experiments.

3.3.4. Preparation of plant extracts

The disease-free leaves of each plant (15 g each) were washed twice thoroughly in double distilled water and shade-dried separately for 21-27 days and then pulverized into powder by electric grinder (Lords Hummer 1100). Extensive extraction was performed in Soxhlet apparatus for 9-11h using suitable solvent (ethanol) with dried plant material (plant:solvent, 1:10 v/v). The extracts were then concentrated under reduced vacuum pressure at 40ºC in a rotary vacuum evaporator (Buchi Rotavapor R-3, Switzerland). The concentrated extracts were further lyophilized separately using Eyela Freeze Dryer (FDU-506, USA) and weighed subsequently. The yields (13%) of lyophilized crude extracts were calculated following the formula:

$$y = \frac{w_2 - w_1}{w_0} \times 100$$

where, W_2 is the weight of lyophilized extract and container; W_1= weight of the container only; W_0= weight of the initial dried plant sample. Finally, the lyophilized extracts were stored in sterile container and placed in -20ºC until further use. The extracts were freshly dissolved in Milli-Q water prior to experiments.

3.3.5. Various *in-vitro* antioxidant assays

For each assay in the present study, comparative analyses were drawn with scavenging potentialities of the different plant extracts with that of the standards, corresponding to the assays. Besides, the concentration of each extract was selected based on the physiological standards to

reflect their dose-dependent inhibitory effects.

3.3.5.1. DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The free radical scavenging activity through DPPH assay was performed as per Chew *et al.* (2009) with a brief modification. Different concentrations of plant extracts (0-100µg/ml) were prepared and mixed properly with freshly prepared DPPH solution (1mM; diluted in 95% methanol) and kept in dark. Optical density (OD) was measured after 30 minutes of reaction at 517nm using UV-Vis Spectrophotometer (Thermo UV1, Thermo Electron Corporation, England, UK). Ascorbic acid was taken as standard. The percent radical scavenging activity was calculated using following Equation I:

$$DPPH = \frac{A_0 - A_1}{A_0} \times 100$$

Where, DPPH= %age scavenging of DPPH; A_0= absorbance of the control and A_1= absorbance in the presence of samples and standard.

3.3.5.2. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging assay of each plant extract was carried out on the basis of Fenton reaction (Kunchandy and Rao, 1990) with a few changes. Briefly, reaction mixture was prepared containing 2-deoxy-2-ribose (2.8mM), monopotassium phosphate-potassium hydroxide buffer (KH_2PO_4-KOH; 20mM; pH 7.4), $FeCl_3$ (100µM), ethylenediaminetetraacetic acid (EDTA; 100µM), hydrogen peroxide (H_2O_2; 1.0mM), ascorbic acid (100µM) and various concentrations of extracts (0–200µg/ml) up to a final volume of 1ml and the reaction mixture was left for 1h incubation at 37°C. Following incubation, the incubated mixture (0.5ml) was taken into another tube and mixed with 1ml of TCA (2.8%) and 1 ml of aqueous thiobarbituric acid (TBA; 1%). The final mixture was incubated at 90°C for 15 min then cooled down to room temperature and the absorbance was measured at 532nm against a blank solution. Mannitol was used as positive control. Percentage of inhibition was evaluated following Equation- I.

3.3.5.3. Superoxide radical scavenging assay

The assay was performed by the reduction of nitro-blue tetrazolium (NBT) as described by Fontana *et al.* (2001) with brief modifications. Usually, the nonenzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/ NADH) system generates superoxide radicals, which reduce NBT to a purple formazan. A reaction mixture (1ml) was prepared containing phosphate buffer (20mM, pH 7.4), NBT (50µM), PMS (15µM), NADH (73µM), and various concentrations (0-100µg/ml) of plant extracts and mixed properly subsequent to incubation for 5 min at ambient temperature. The absorbance at 562nm was measured against an appropriate blank

to determine the quantity of formazan generated. Quercetin was used as standard.

3.3.5.4. Singlet oxygen scavenging assay

The production of singlet oxygen ($_1O^2$) was determined by monitoring the bleaching of N, N-dimethyl-4-nitrosoaniline (RNO) using the method of Pedraza-Chaverri *et al.* (2004) with minor modifications. The reaction mixture contained 45mM phosphate buffer (pH 7.1), 50mM NaOCl, 50mM H_2O_2, 50mM L-histidine, 10µM RNO and various concentrations (0-200µg/ml) of plant extracts to make final volume of 2ml. The mixture was then incubated for 40 min at 30°C and decrease in the absorbance of RNO was measured at 440nm. Lipoic acid was used as a reference compound. Singlet oxygen scavenging activity was calculated using the Equation I.

3.3.5.5. Nitric oxide radical scavenging assay

Nitric oxide radical quenching activity was performed following the GriessI-llosvoy reaction (Garratt, 2012) with minor modifications. In brief, phosphate buffered saline (pH 7.4), sodium nitroprusside (SNP; 10mM) and different concentrations of plant extracts (0-100µg/ml) were mixed to make final volume of 3 ml. Following incubation for 150 minutes at 25°C, 1ml of sulfanilamide (0.33%; diluted in 20% of glacial acetic acid) was added to 0.5ml of the pre-incubated reaction mixture and left for 5 min of incubation. Then, 1ml of N-(1 -Naphthyl)ethylenediamine dihydrochloride (NED; 0.1%) was added and incubated for 30min at 25°C to develop the color. The absorbance was measured spectrophotometrically at 540 nm against blank sample. Curcumin was used as standard. The percentage inhibition was calculated using Equation I.

3.3.5.6. Peroxynitrite scavenging assay

Peroxynitrite ($ONOO^-$) was prepared using the method of Beckman *et al.* (1994). An acidic solution (0.6M HCl) was prepared mixing with 5ml of H_2O_2 (0.7M) and 5ml of KNO_2 (0.6M) on an ice bath for 1 min. Subsequently, 5ml of ice-cold NaOH (1.2M) was added to the mixture. Then, excess H_2O_2 was removed by the treatment with granular MnO_2 pre-washed with NaOH (1.2M) and the reaction mixture was left overnight at -20°C. Finally, peroxynitrite solution was collected from the top of the frozen mixture and the concentration was measured spectrophotometrically at 302 nm (ε = 1670 M-1 cm-1).

The peroxynitrite scavenging aptitude was measured by Evans Blue bleaching assay. The assay was carried out as per the method of Bailly *et al.* (2000) with a slight modification. Briefly, a reaction mixture was prepared containing phosphate buffer (50mM; pH 7.4), DTPA (0.1mM), NaCl (90mM), KCl (5mM), 12.5µM of evans blue, various doses of plant extracts (0–200µg/ml) and 1mM of peroxynitrite in a

final volume of 1ml. After incubation at 25°C for 30 min the absorbance was measured at 611 nm. The scavenging percentage of ONOO- was calculated by comparing the results of the test and blank samples. Gallic acid was used as the reference compound.

3.3.5.7. Hypochlorous acid scavenging assay

In-vitrocally, hypochlorous acid (HOCl) was prepared freshly by mixing 10% (v/v) solution of NaOCl and 0.6M of H_2SO_4 by adjusting the pH (6.2) and the concentration was determined by measuring the absorbance at 235nm using the molar extinction coefficient of $100M^{-1}$ cm^{-1} as per Aruoma and Halliwell (1987) with few changes. In brief, a reaction mixture was prepared containing 50mM phosphate buffer (pH 6.8), catalase (7.2µM), HOCl (8.4mM) and plant extracts of different concentrations (0–100µg/ml) into a final volume of 1ml. Then, mixture was incubated for 20 min at 25°C and absorbance was measured against an appropriate blank. The quenching activity was accessed by measuring the decrease in absorbance of catalase at 404nm. Ascorbic acid was used as standard.

3.3.5.8. Iron chelation assay

Ferrous ion chelating activity was carried out as per the method of Haro-Vicente *et al.* (2006) with slight changes. Each plant extract (0–200µg/ml) was mixed properly with ferrous sulfate solutions (12.5µM) in HEPES buffer (20mM; pH 7.2) followed by the addition of ferrozine (75µM) to initiate reaction. Reaction mixture was shaken vigorously and incubated for 20 min at room temperature. Absorbance was measured at 562nm. EDTA was used as positive control.

3.3.5.9. Hydrogen peroxide scavenging assay

The assay was determined by the method of Long *et al.* (1999) with minor modifications. A mixture was prepared with H_2O_2 (50mM) and various concentrations of plant samples (0-2000µg/ml) and left for 30 min of incubation at room temperature followed by the addition of 90µl H_2O_2, 10µl of Methanol (HPLC grade) and 0.9ml of FOX reagent (prepared by mixing 9 volumes of 4.4mM BHT in HPLC grade methanol with 1 volume of 1mM xylenol orange and 2.56mM ammonium ferrous sulfate in 0.25M H_2SO_4). Entire mixture was then vortexed and left for incubation for 30 min. Absorbance was measured at 560nm. Sodiun pyruvate was used as positive control.

3.3.5.10. Lipid peroxidation inhibition assay

Lipid peroxidation assay was followed by the method of Kizil *et al.* (2008) with a few modifications. Swiss albino mice brain homogenate was prepared by centrifuging (20±2 ×g) with phosphate buffer (50mM;

pH 7.4) and potassium chloride (KCl; 120mM) at 3000 rpm for 10 min. Different concentrations of extracts (0-25µg/ml) were mixed with the homogenate (100µl) followed by addition of ferrous sulfate ($FeSO_4$; 0.1mM) and ascorbic acid (0.1mM) and incubated for 1h at 37°C. After incubation, TCA (500µl; 28%) and TBA (380µl; 2%) were added in the reaction mixture and then heated at 95°C in water bath for 30 min. After that, mixtures were cooled down to room temperature and centrifuged at 8000 rpm for 2 min. Absorbance of the supernatant was measured at 532 nm. Trolox was used as positive control.

3.3.5.11. Total antioxidant activity

Standardized modified method of Prieto *et al.* (1999) was followed to study the total antioxidant activity (TAA) of each plant extract. Various concentrations of extracts (0-150 µg/ml) were mixed with 1 mL of reaction mixture containing 0.6 M of sulfuric acid (H_2SO_4), sodium phosphate (28 mM) and ammonium molybdate (1%). Then the mixture was kept on a water bath at 95°C for 1 h and subsequent cooling to room temperature. The absorbance was read at 695 nm against a suitable blank. Ascorbic acid was used as a standard. The antioxidant activity was measured by the capacity of the yam extract to reduce molybdenum (VI) to molybdenum (V) using equation II:

$$TAA = \frac{OD_o - OD_1}{OD_0} \times 100$$

where,, TAA= % of TAA; OD_0 was the absorbance of the blank and OD_1 was the absorbance in the presence of the samples and standard (ascorbic acid).

3.3.5.12. Quantification of total phenolic content

Total phenolic content (TPC) was determined using Folin-Ciocalteu reagent (Singleton and Rossi, 1965) with slight changes. In brief, each extract (100µl) was mixed with 0.75ml of Folin-Ciocalteu reagent (previously diluted 1000-fold with distilled water) and left for 5 min at room temperature followed by the addition of sodium carbonate (Na_2CO_3; 0.06%) to the mixture. Following incubation of 90 min at room temperature, the absorbance was measured at 725nm. Phenolic content was measured against a gallic acid standard curve ($R^2 = 0.9708$).

3.3.5.13. Quantification of total flavonoid content

The total flavonoid content was measured using aluminum chloride ($AlCl_3$) method (Zhishen *et al.*, 1999) with few modifications. In brief, each extract (100µl) was added to 0.3ml of distilled water followed by addition of $NaNO_2$ (5%; 0.03ml). Following 5 min of incubation at room temperature, $AlCl_3$ (10%; 0.03ml) was added and left for 5min. Reaction mixture was then treated with 0.2ml of sodium hydroxide (NaOH; 1mM) and diluted to 1ml with water. Absorbance was

measured at 510nm. The flavonoid content was determined from a quercetin standard curve ($R^2 = 0.9891$).

3.4. PCA and HCA of different antioxidant traits

To analyze the relationship between the different antioxidant traits and the quantified phytochemicals, principal component analysis (PCA) based on the correlation matrix were drawn for selected samples. Two factors were extracted under varimax method. The data obtained from the antioxidant profiling were analyzed by multivariate statistical approach, employing hierarchical cluster analysis (HCA). The method employed was 'proximity' matrix using between group linkages. The differences between the measured variables were calculated by square Euclidean distances. Transform values of variables (average zero and S.D. 1) called Z scores was carried out as a pre-treatment of the data. Horizontal dendrogram with all clusters icicle chart was carried out to elucidate the similarity or nearness of the various measured variables.

3.5. Assessment of cytotoxicity of plant extracts

3.5.1. *In-vitro* haemolytic assay

The haemolytic consequence of each extract was evaluated according to the standardized method of Malagoli (2007) with few modifications. In brief, human blood was collected in citrated tubes and erythrocytes were then washed (150 ×g for 5 min) three times with 20mM Tris–HCl containing 144mM NaCl (pH 7.4). Then, the erythrocyte suspension (100µL) was prepared with the same solution and plated into each well of the 96-well plate. Sodium chloride solution (0.85%) containing calcium chloride (10mM) was added (100µL) to each well. First lane of the plate served as the negative control without plant extract. Various concentrations (0-200µg/ml) of extracts were added into the different wells beginning from the second lane. In another set of lanes, 100µL of Triton X-100 (0-200µg/ml) in 0.85% saline were plated and used as standard. Thereafter, the plate was incubated for 30 min at 37°C. Following incubation, the suspension was centrifuged (604 ×g for 5 min) and the supernatant was taken to a fresh 96-well plate and measured the absorbance of liberated haemoglobin at 540nm. Percentage of haemolytic activity was calculated using the following equation:

$$Haemolysis = \frac{H_0 - H_1}{H_0} \times 100$$

where, haemollysis= %age of haemolysis; H_0 was the absorbance of the blank and H_1 was the absorbance in the presence of the samples and standard (Triton X-100).

3.5.2. Erythrocyte membrane stabilizing activity (EMSA)

Standardized method of Navarro *et al.* (1993) was followed to estimate the

erythrocyte membrane stabilizing activity of juice. Briefly, a reaction mixture of 1ml was prepared containing 50mM of phosphate buffer (0.2mL; pH 7.2), 0.4ml of distilled water, 0.1ml of RBC suspension (10%; diluted in PBS), EDTA (40µl; 12mM), 60µl of nitro blue tetrazolium (NBT; 1%), 40µl of riboflavin and varying concentrations of extracts (0-200µg/ml) and mixed properly followed by bright light exposure for 30s and then incubated for 30 min at 50ºC. After incubation, the reaction mixture was centrifuged at 1000 rpm for 10 min and absorbance of the supernatant was measured at 562nm. Quercetin was taken as reference standard. The EMSA was measured using the equation:

$$protection\ (\%age) = \frac{A_s}{A_b} \times 100$$

where, A_s and A_b are the absorbance value of sample and blank, respectively.

3.5.3. MTT cell cytotoxicity assay

The assay was performed to evaluate the cytotoxic properties of each extract on murine splenocytes and macrophages. The splenocyte and macrophage cells were collected from Swiss albino mice (Dey and Chaudhuri, 2016). The mouse was sacrificed under mild ether anesthesia and the spleen was aseptically removed from the body and washed thrice (1000 rpm) with RPMI-1640 and splenocytes suspension was prepared and resuspended in 0.16M NH_4Cl (in 0.17M Tris; pH 7.2) to remove any trace of erythrocytes. The reaction was stopped after 5 min, using chilled RPMI-1640 and the cells were washed as previously mentioned. The peritoneal exudate macrophages were collected by washing the mouse peritoneal region with RPMI-1640. A cell suspension (2×10^6 cells/ml) was prepared with penicillin (50U/ml), streptomycin (50U/ml), nystatin (50U/ml) and fetal bovine serum (FBS, 10%) in RPMI-1640 medium. Hundred µl of cell suspension was added with 100µl of different concentrations (0-200µg/ml) of plant extracts (dissolved in RPMI-1640) to the wells of 96-well plate. Plates were then covered and incubated under 5% CO_2 and humidified atmosphere of 90% air at 37°C temperature for 48h. The assay was performed with EZcount™ MTT Cell Assay Kit (HiMedia) according to the manufacturer's instructions.

3.6. Neurotherapeutic effects of plant extract

3.6.1. Preparation of plant extracts and chemicals

In this study, methanol was selected as extracted solvent for *M. pudica* and *M. invisa* while *A. catechu* was extracted in ethanol and the chemicals were of analytical grade procured either from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), or Merck (Mumbai, India), or Sigma-Aldrich (USA), unless mentioned otherwise.

3.6.2. Screening of acetylcholinesterase

(AChE) inhibitory activity (*in-vitro*)

3.6.2.1. Selection of Plant material

The plant ethanolic leaf extracts of *Acacia catechu* (ACL), *Mimosa pudica* (MPD), and *M. invisa* (MIN) were chosen in this study depending on their ethnopharmacological significance.

3.6.2.2. AChE inhibitory assay

Acetylcholinesterase inhibiting activity of selected plant extracts was carried out based on Ellman *et al.* (1961) method with concise modification. Briefly, a reaction mixture was prepared containing sodium phosphate buffer (0.1mM), 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB; 0.1mM), various concentrations of plant extracts (0-200µg/ml) and acetylcholinesterase (2U/ ml) in a 96 well micro plate and incubated for 15 min at 25ºC. Following incubation, acetylthiocholin iodide (0.05mM) was added as substrate in the reaction mixture and the enzyme activity was measured immediately after 3 min in a Bio-Rad iMark™ microplate absorbance reader at 412nm. Eserine was used as positive control. Percentage of inhibition was calculated in terms of percentage by dividing the difference of sample absorbance from control with control absorbance × 100.

3.6.3. Memory-Enhancing Behavioral Study (*in-vivo*)

3.6.3.1. Selection of Plant material

Mimosa pudica (MPD) and *M. invisa* (MIN) leaf extract were chosen for performing memory impairment paradigm based on their ethnic use.

3.6.3.2. Animal maintenance

Swiss albino mice (9-10 weeks old; 30-35 gm) of both sexes were first acclimatized for a period of 7 days in the Animal House of the Department of Zoology, University of North Bengal prior to experiment. Mice were maintained at an ambient temperature of 25±2°C, humidity of 55–60%, with12h dark/light cycle and fed pellet diet and water *ad libitum*. The animal trials were evaluated and permitted by the ethical committee (840/ac/04/CPCSEA) of University of North Bengal and performed in accordance with the legislation for the protection of animals used for scientific purposes.

3.6.3.3. Acute toxicity study

Acute oral toxicity of selected plant extracts have been performed as per OECD guidelines (OECDiLibrary, 2002). No symptoms of toxicity and death were recorded at maximum dose of 1000 mg/kg body weight (BW) of respective plant extract. Therefore, it was inferred to be safe. Thus, 100 mg/kg BW was selected as the starting lower dose and a higher dose of 200 mg/kg BW were also employed for further testing.

3.6.3.4. Experimental design and drug administration

A total of seven groups of mice, six in

each group received the following treatment schedule:

Group I: normal control, received normal saline for 7 days; Group II: Scopolamine-treated mice (0.5mg/kg; dissolved in Mili-Q water), received normal saline for 7 days; Group III: Scopolamine-treated mice, received standard drug, piracetum at 200mg/kg BW per day (dissolved in Mili-Q water) for 7 days; Group IV: Scopolamine-treated mice, received low dose MPD extract (in Mili-Q water) at 100 mg/kg BW per day for 7 days; Group V: Scopolamine-treated mice, received high dose MPD extract (in Mili-Q water) at 200 mg/kg BW per day for 7 days; Group VI: Scopolamine-treated mice, received MIN dose plant extract (in Mili-Q water) at 100 mg/kg BW per day for 7 days; Group VII: Scopolamine-treated mice, received high dose MIN extract (in Mili-Q water) at 200 mg/kg BW per day for 7 days.

On 8th day, plant extracts/standard drug/ normal saline was injected after 90 min. of scopolamine treatment followed by behavioral tests performed after 45 min of injection. The retention of learned task was examined after 24h of last injection (9th day). All the trial and examining sessions were carried out at night, preferably between 19:00 and 23:00h due to restless movement of the mice during night.

3.6.3.5. Step-through Passive Avoidance Task

The task was carried out as per previously described methods of Reddy (1997), using a wooden two-compartment passive avoidance apparatus (dark and light chamber, partitioned by a wall with a openable door in the middle part), with slight alterations of the time intervals. After compliance of the trial, on day 7th, the animal groups were kept in the experimental room. One hour later, each mouse was placed in the light chamber for the attainment trial and was left to familiarize to the apparatus. After 100 s, the middle door was opened, and the mouse was allowed to enter the dark compartment. The latency time with which the animal crossed into the dark compartment was recorded. Animals that fail to enter the dark compartment within 100s were removed from the experiments. Once the mouse entered with all its four paws to the dark compartment, the middle door was closed, and the animal was transferred into its home cage. The same trial was again repeated after 30min of first test, but the door was opened after 5s. Once the animal reached to the dark compartment, the door was closed and a mild foot shock (25V, AC, 5s) was immediately delivered to the grid floor of the dark room. Afterward, the mouse was immediately transferred to its home cage. In this test, the initial latency (IL) period of entrance into the dark chamber was noted within 120s (selected as maximum time). A retention test was performed to determine short term memory of mice after

24h of last training (i.e. 8th day). Briefly, each animal was placed in the light compartment for 20s and the middle door was opened. The step through latency (STL) was measured for entering into the dark compartment and the test session ended when the animal reached the dark chamber or waited in the light compartment for 300s. During these sessions, no electric shock was applied.

3.6.3.6. Preparation of brain tissue samples

After behavioral evaluations, the animals were anesthetized and sacrificed. The brain of the sacrificed mice of each group were removed quickly from the skull and washed consciously in ice cold normal saline followed by homogenization in 1X KH_2PO_4 saline (10%, w/v, pH 8), to which BHT (0.004%, w/v) was added to prevent autoxidation of the samples. Subsequently, the homogenate was centrifuged at 10,000 ×g for 30 minutes at 4ºC and an aliquot of supernatant was separated for further biochemical studies.

3.6.3.7. Determination of AChE activity in the brain tissue

The brain AChE activity was measured as per Ellman's method (1961) using DNTB (5, 5-dithiobis (2-nitrobenzoate). The absorbance was measured at 412 nm.

3.6.3.8. Determination of SOD, CAT and GSH activity in the brain tissue

SOD activity of tissue was measured following the method of Kakkar *et al.* (1984). Briefly, a reaction mixture was prepared containing PMS (phenazine methosulphate; 200µl), sodium pyrophosphate buffer (pH 7; 2.4ml), homogenate supernatant (600µl) and NADH (400µl) by mixing properly. Glacial acetic acid (1ml) was added after 60s. of incubation to terminate the reaction. The chromogen color intensity was measured at 560nm. Results were expressed in units/mg protein. Further, CAT activity of tissue was determined as per the standardized method of Kar and Mishra (1976) and the absorbance of the reaction solution was appraised at 240nm. whereas reduced glutathione (GSH) was estimated by Ellman's method (1961) with the modified method of Jollow *et al.* (1974), using DTNB as a substrate and the yellow colour intensity was read immediately at 412nm.

3.6.3.9. Statistical analysis

For reproducibility, all data were prepared as the mean ± SD of six measurements. Statistical analysis were performed by one-way analysis of variance (ANOVA) with Dunnett's test using KyPlot version 5.0 (32 bit) for windows. $P < 0.05$ was considered as significant.

3.7. Anti-diabetic Activity of plant extract

3.7.1. Selection of Plant material

The plant, ethanolic extract of *A. nilotica* leaf (ANL) was selected in this study

based on its ethnopharmacological relevance.

3.7.2. α-amylase inhibitory activity of plant extract

The study was performed as per the standard method of Hansawasdi *et al.* (2000) with slight modifications. In brief, 1% of starch azure was suspended in Tris–HCl (0.5M; pH 6.9) containing 0.01M of $CaCl_2$ and boiled on water bath for 5min. Then the tubes were cooled down to room temperature and different concentrations (0 –200mg/ml) of plant extract and amylase enzyme (2U/ml) in Tris–HCl buffer was added to it, followed by 5 min of incubation at 37°C. Reaction was stopped by addition of 250ml of 1M HCl followed by centrifuge at 3,000 rpm for 10 min. The supernatant was collected and absorbance was read at 595nm. The percentage of inhibition was measured from the following formula:

$$PI(\alpha - amylase) = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0 was the absorbance of the blank and A_1 was the absorbance in the presence of the varying concentrations of extract; PI= Percentage of inhibition.

3.7.3. Acute oral toxicity study

To investigate the acute toxicity of ANL, we followed OECD (test 423: Acute oral toxicity – Acute toxic class method; 2002) guidelines (OECDiLibrary, 2002). All the animals were kept on fasting overnight before treatment. ANL extract at the dose range of 100mg–2000mg/kg body weight (BW) were administered orally to six different groups of mice comprised of six animals in each group. The groups were kept under critical surveillance to observe about any kind of toxicological or mortality symptoms, if any, at 1, 2, 4, 8, 24, 48 and 72h of interval.

3.7.4. Induction of diabetes in experimental animals

Mice were made diabetic by a single intra-peritoneal injection (0.2ml) of freshly prepared alloxan monohydrate in saline (154mM NaCl) with a dose of 150mg/kg BW. On the 3rd day of alloxan injection, blood glucose level of each animal was recorded and the mice with blood glucose level of >200mg/dl were considered as diabetic and chosen for further study.

3.7.5. Experimental design and drug administration

A total of five groups of mice, six in each group received the following treatment schedule.

Group I: normal control (non-diabetic mice), received normal saline; Group II: type 1 diabetic control (alloxan-treated mice), received normal saline; Group III: diabetic mice (alloxan-treated mice), received standard drug, glibenclamide at 5 mg/kg BW per day; Group IV: low dose extract administrated diabetic mice, received ANL extract (in Mili-Q water) at 50mg/kg BW per day; Group V: high dose extract administrated diabetic mice,

received ANL extract (in Mili-Q water) at 200mg/kg BW per day. These treatments were continued for 20 consecutive days.

3.7.6. Blood glucose level estimation

Fasting blood glucose levels (12h after feed delivery) of mice were measured from the 1st day of extract treatment. Blood glucose estimation was done by single touch glucometer (Bayer, contour TS meter) on every 10-day interval from the tail vein.

3.7.7. Collection of blood, serum and tissue sample

On the 21st day of experiment, all the mice were sacrificed at fasting condition under mild ether anesthesia and blood was collected by puncturing the heart. Blood sample (50µl) was used to estimate glycated hemoglobin (HbA1c) level by ion -exchange HPLC using Bio-Rad D-10™ Dual HbA1c program 220-0201 following the manufactures instructions. The serum was separated by centrifugation at 1000 rpm for 10 min and kept at -20°C until further use. Liver, kidney and skeletal muscle (collected from thigh muscle) were separated from body parts followed by gentle washing in PBS (phosphate buffer saline) and stored at -20°C.

3.7.8. Study of different serum biochemical parameters

Serum insulin level was measured by the ELISA method using Accu-Bind Universal ELISA kit (Monobind Inc., USA) according to manufactures instructions. Besides, different enzymatic assays like acid phosphatase (ACP), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were carried out for all the experimental groups using commercially available kits (Crest Biosystems, Goa, India).

3.7.9. Measurement of protein and lipid metabolic disorders

3.7.12.1. Estimation of serum urea, uric acid, creatinine, cholesterol and triglyceride

Levels of serum urea, uric acid, creatinine, cholesterol and triglyceride were measured spectrophotometrically by the addition of enzymes present in the reagent kits of Crest Biosystems (Goa, India) following manufactures instruction and the values were expressed in mg/dL in all cases.

3.7.10. Estimation of glycogen level

Hepatic glycogen level was estimated according to the standard method of Carroll *et al.* (1956) with a few alterations using anthrone reagent. The intensity of the green to dark-green coloration of the reaction mixture was estimated at 630nm. Glycogen content of tissue was determined from the standard curve prepared with glucose solution.

3.7.11. Estimation of lipid peroxidation

Commercially available TBARS assay kit (Cayman Chemical, USA) was used to

measure the malondialdehyde (MDA) content in serum of lipid peroxidation in all experimental groups.

3.7.12. Determination of peroxidase (Px) and catalase (CAT) activity

The method of Sadasivam and Manickam (2008) was followed to estimate the peroxidase activity in liver, kidney and skeletal muscle tissues of experimental groups through the oxidation of guiacol and the absorbance was recorded at 436nm. The catalase activity of above said organs of each group were estimated according to a standard method of Luck (1963).

3.7.13. Statistical analysis

Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). Group-difference were measured using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. Data of OGTT was analyzed using two-way ANOVA. $P<0.05$ was considered significant. All data are reported as the mean ±SD of six measurements

3.8. Chemical Characterizations of selected plant extracts

Characterizations of plant extracts provide major opportunities for new drug discovery as well as for pharmaceutical industry. In essence, natural products or phyto-chemicals are constantly being screened due to their safe and less adverse effects. Several modern techniques of isolation are being included in characterization of bioactive molecules from plant extracts. In the present study, Fourier transform infrared (FTIR) spectra, Gas Chromatography-Mass Spectroscopy (GC-MS) and Nuclear Magnetic Resonance (NMR) spectra were employed and summarized below.

3.8.1. FTIR analysis

Fourier Transform Infrared (FTIR) has been established to be a helpful tool for the characterization and detection of compounds or functional groups (i.e. chemical bonds) present in an unknown mixture of plant extracts (Eberhardt *et al.*, 2007). Briefly, previously prepared selected extract (10µg) was taken in calcium fluoride (CaF_2) vessel and placed in a sample cup of a diffuse reflectance accessory. Thereafter, IR spectrum was obtained using Shimadzu 8300 FTIR spectrophotometer at ambient temperature (25ºC). The background correction was done by taking IR spectrum of de-ionized water as the reference in identical condition. The sample was scanned from 400 to 4000 cm^{-1} for 16 times to increase the signal to noise ratio. The functional groups were identified from the IR peaks (Silverstein *et al.*, 2006).

3.8.2. GC-MS analysis

In the present study, GC-MS analysis was done following two different procedures which are summarized below.

Procedure-I:

Sample preparation and analysis

Here, GC-MS analysis was performed following the method of Kind *et al.* (2009) after slight alteration. In brief, the leaf extract (*A. catechu*) was derivatized with 10µl of methoxyamine hydrochloride (20mg/ml in Pyridine) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS), 2µl FAME (Fatty Acid Methyl Esters) markers [a mixture of internal Retention Index (RI) markers was prepared using fatty acid methyl esters of C8, C10, C12, C14, C16, C18, C20, C22, C24 and C26 linear chain length, dissolved in chloroform (HPLC) at a concentration of 0.8mg/ml (C8-C16) and 0.4mg/ml (C18-C30)] was added.

HP-5MS capillary column (Agilent J & W; GC Columns (USA) with length of 30m plus Duraguard 10m, diameter 0.25mm narrowbore, (film 0.25µm) was used. The analysis was performed under the following oven temperature programme: Injection in sandwich mode with fast plunger speed without viscosity delay or dwell time, oven ramp 60ºC (1 minute hold) to 325ºC at 10ºC/minute, 10 minute hold before cool-down, 37.5 minute run time. The injection temperature was set at 250ºC; the MS transfer line at 290ºC and the ion source at 230ºC. Prior to analysis the method was calibrated with the FAME standards available with the Fiehn GC/MS Metabolomics library (2008) (Agilent ChemStation, Agilent Technologies Inc., Wilmington, USA). Helium was used as the carrier gas at a constant flow rate of 0.723ml/min (carrier linear velocity 31.141 cm/sec). Samples (1µl) were injected via the split mode (Split ratio 1:5) onto the GC column. Automated mass spectral deconvolution and identification system (AMDIS) was used to deconvolute GC-MS results and to identify chromatographic peaks. Identification of the metabolites was carried out by comparing the fragmentation patterns of the mass spectra, retention times and retention indices with entries of those in Agilent Fiehn Metabolomics library using Agilent retention time locking (RTL) method. Response ratio was calculated as peak area ratios of metabolite and ribitol as internal standard. Relative response ratio (RRR) is normalized response ratio per gram crude extract.

Procedure-II:

Sample preparation and Silylation derivation for GC-MS

The plant (*A. catechu*, *Mimosa pudica* and *M. invisa*) extracts (50mg) were separately mixed with 2 ml n-hexane with occasional shaking at laboratory temperature (20-22° C). After 24 h of incubation, 20µl of BSTFA and TMCS (99:1 v/v) mixture was added to the previously prepared solution and left for 60 min of incubation at 20-22° C with occasional shaking. Then the mixture was centrifuged at 15,000 rpm for 20 min at 25°C. The supernatants were

collected and passed through the anhydrous Na_2SO_4 and activated charcoal (2:1; w/w) in a 10 cm mini-column $1/10^{th}$ packed with non-absorbent cotton to remove moisture and color (Dey and Chaudhuri, 2015). The eluted liquid was re-centrifuged at 15,000 rpm for 20 min and passed through Whatman filter paper no. 1 (11µm). The resultant clear solutions were used for analysis.

Analysis

Thermo Scientific Trace 1300 gas chromatography coupled with Thermo Scientific ISQ QD single quadrupole mass spectrophotometer was used to analyze the samples. The GC was equipped with TG-5MS column (30 m × 0.25 mm × 0.25 µm). The inlet temperature was maintained at 250°C and initial temperature was set at 60°C (solvent delay 5 min) with a hold of 4 min, followed by a ramp of 5°C to 290° C with a hold of 10 min (60 min program). Each sample (1µl) was injected in a splitless mode (split flow 50ml/min) with splitless time of 0.80 min, using a Thermo Scientific AI-1310 auto-sampler. Helium (99.99%) was used as the carrier gas at a constant flow rate of 1ml/min, passed through hydrocarbon and dehydrating columns. MS transfer line temperature was set at 290°C with an Ion source temperature of 230°C (electron ionization). Individual sample was analyzed at electron energy 70 eV (vacuum pressure- 2.21e-0.5 Torr). The mass analyzer range was set to 50-650 atomic mass unit (amu). Automated mass spectral deconvolution and identification system (AMDIS; version 2.70) was used to analyze GC-MS results and to identify chromatographic peaks (Dey and Chaudhuri, 2015). The major and essential compounds were identified by mass fragmentation patterns of the reference of parent compound using MS interpreter Version 2.0 and by matching with the reference database of National Institute Standard and Technology (NIST) with a MS library version 2011.

3.8.3. NMR analysis

The ^{1}H and ^{13}C NMR-spectra of plant extracts (*Acacia catechu*, *A. nilotica*, *Mimosa pudica* and *M. invisa*) were recorded with a 300 MHz Bruker FT-NMR (Avance AV-300) spectrometer. Deuterated chloroform and dimethylsulfoxide were used as solvents. All experiments were done at room temperature (24°C). Calibrations of spectra were done on the basis of solvent residual peak and the chemical shifts were reported in δ values (Silverstein *et al*., 2006).

3.9. *In-silico* drugability prediction and Pharmacokinetic study

Following successful characterization of phytochemicals from different extracts, *in-silico* docking techniques towards the discovery of potent drug was undertaken to establish whether the phytochemicals have any role in the management of different imperative diseases including neurodegeneration and diabetes which are

explained below.

3.9.1. Drugability prediction and ROS

High resolution structures of FAS Ligand (FasL; PDB ID- 4MSV) (Liu *et al.*, 2016), NADPH oxidase (PDB ID- 1OEY) (Wilson *et al.*, 2003) and Toll like receptors (TLR; PDB ID- 5AWA) (Beesu *et al.*, 2015) of human were retrieved from RSCB Protein Data Bank (PDB). These protein structures were generated through X-ray diffraction method and all of them have resolution ranging from 2.0-2.5Å. This range of resolution is quite considerable since they are assumed of having correct folding pattern, less errors in surface loops and fewer number of sidechains in wrong rotamer (https://en.wikipedia.org/wiki/Resolution_(electron_density).

3.9.2. Drugability prediction and neurodegeneration

During this study, human dopamine receptor D3 protein (PDB ID- 3PBL) was considered (Chien *et al.*, 2010) as the target protein for performing computational docking investigations.

3.9.2.1. Pharmacokinetic study

Generally, 'Lipinski's rule of five' is considered as "rule of thumb" while predicting a chemical or compound as novel drug (http://www.rsc.org/chemistryworld/Issues/2008/June/BreakingTheRules.asp). This rule is purely based on the physiochemical properties of the ligand molecule. Therefore, I predicted physiochemical properties using number of hydrogen donor and acceptor, molecular weight as well as partition coefficient (logP or lipophicity) via ALOGPS (http://www.vcclab.org/lab/alogps/) and Chemicalize (http://www.chemicalize.org/). I further screened the target drug molecules through BBB predictor (http://www.cbligand.org/BBB/index.php) using SVM and AdaBoost algorithms with four different fingerprints, namely, MACCS, Openbabel (FP2), Molprint 2D and PubChem to verify whether the target compounds can indeed cross the blood brain barrier (BBB). Besides, another tool, BioZyne (http://pgp.biozyne.com/) was employed to study the P-glycoprotein (P-gp) substrate property of considered drug target.

3.9.3. Drugability prediction and diabetes

Nrf2 transcription factor (PDB ID: 5FNQ), responsible for defense mechanism, was employed in the present study as a target protein using the identified phytocompounds including catechol, β-sitosterol, α-tochopherol and γ- tocopherol.

3.9.4. Docking study

The PDB protein structures were prepared for docking after deletion of water atoms followed by addition of polar hydrogen atoms. A supplementary file was created with information regarding the dimensions for the purpose of docking. Gasteiger

charges were further added to both proteins and ligands on the basis of electronegativity equilibration. The torsions of all ligands (i.e. selected phytocompounds) were allowed to rotate freely to obtain better outcome. Molecular docking was performed by Autodock Vina and the docked complexes were visualized by means of PyMol protein structure visualization package through which the relative distance between the atoms of ligands and proteins were also generated [in the unit of Angstrom (Å)]. The chemical structures for the identified phytochemicals were downloaded in .sdf format from NCBI-PUBCHEM (http:// www.ncbi.nlm.nih.gothrv/pccompound) followed by converted into .pdb format through SMILES server (https:// cactus.nci.nih.gov/translate/).

3.10. Study of Molecular diversity of Mimosoids

3.10.1. Study area

Different areas of Malda, Uttar Dianpur and Darjeeling districts were chosen for germplasm collection.

3.10.2. Collection of plant material

Selected places (Chanchal, Ratua, Samsi, Raiganj, Kaliyaganj, Sibmandir and NBU campus) of above mentioned 3 districts (Table 3.2) of West Bengal in India were visited for the collection of the germplasm. A total of eleven months were taken to complete the field survey depending upon the season. Nine different plant materials (Table 3.2) under the subfamily Mimosoideae (Mimosaceae) were collected and authenticated by the plant taxonomist of Department of Botany, University of North Bengal. The voucher specimens were deposited at the Botany Department Herbarium, University of North Bengal. The habit, habitat, locality, collection time and morphology of each plant specimen were recorded in field data sheet (Fig. 3.1).

3.10.3. Molecular diversity of selected Mimosoids

Genetic diversity of 9 different Mimosoids were studied by using different types of PCR based molecular techniques namely, RAPD, PCR-RFLP and barcoding markers. Isolation of DNA is the foremost approach to carry out these molecular fingerprinting. The method developed by Doyle and Doyle (1987) was used for the isolation of genomic DNA with minor alterations as summarized below.

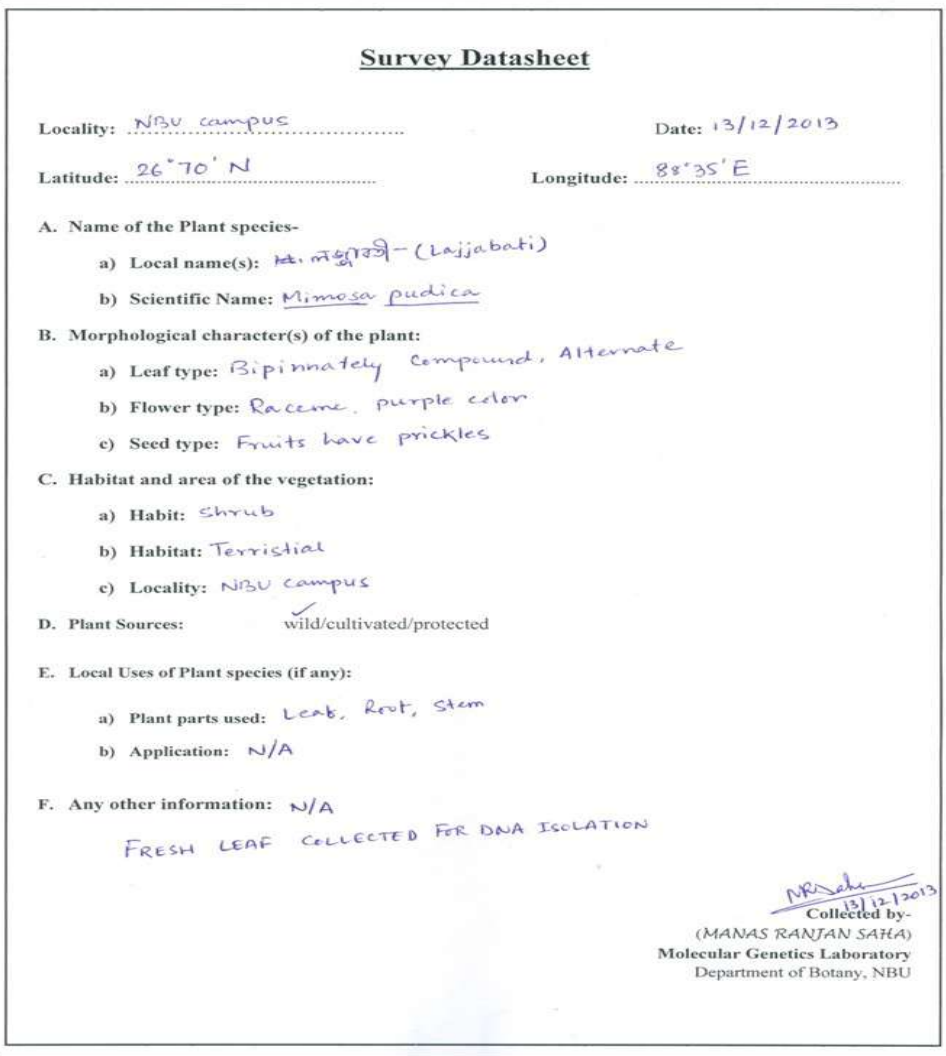
3.10.3.1. Extraction of DNA from leaf

The DNA extraction procedures from the selected plant samples are as follows:

- Fresh tender leaves (approx. 5 gm) were grounded in a mortar and pestle using liquid nitrogen followed by quick transfer of those pulverized leaf materials into Oakridge tube (Tarsons, Cat#541040; 30 ml) containing 15 ml of pre-warmed CTAB extraction buffer (Refer Appendix- B for composition) and mixed properly.

Table 3.2. Collection sites of 9 different plant samples.

Name of the plant species	Sample ID	Voucher No.	(West Bengal) District	Location	Latitude	Longitude
Mimosa pudica L.	M1	NBU/MLD/308	Malda	Chanchal	25°38′ N	88°01′ E
Mimosa invisa Mart. ex Colla	M2	NBU/MLD/311		Ratua	25°19′ N	87°92′ E
Acacia nilotica (L.) Delile	M3	RUC/MLD/433		Samsi	25°27′ N	88°00′ E
Acacia nilotica var. *indica* (Benth.) A.F.Hill.	M4	RUC/UD/454	Uttar Dinajpur	Raiganj	25°61′ N	88°12′ E
Acacia catechu (L.f.) Willd	M5	NBU/UD/1039		Kaliya-ganj	25°63′ N	88°32′ E
Acacia concinna (Willd.) DC.	M6	NBU/UD/756		Raiganj	25°61′ N	88°12′ E
Albizia lebbeck (L.) Willd.	M7	NBU/DJ/902	Darjee-ling	Siliguri	26°72′ N	88°39′ E
Albizia chinensis (Osbeck) Merr.	M8	NBU/DJ/1002		NBU campus	26°70′ N	88°35′ E
Samanea saman (Jacq.) Merr.	M9	NBU/DJ/1022		NBU campus	26°70′ N	88°35′ E



Survey Datasheet

Locality: NBU campus Date: 13/12/2013

Latitude: 26°70' N Longitude: 88°35' E

A. Name of the Plant species-

a) Local name(s): [illegible] – (Lajjabati)

b) Scientific Name: Mimosa pudica

B. Morphological character(s) of the plant:

a) Leaf type: Bipinnately Compound, Alternate

b) Flower type: Raceme, purple color

c) Seed type: Fruits have prickles

C. Habitat and area of the vegetation:

a) Habit: Shrub

b) Habitat: Terristial

c) Locality: NBU campus

D. Plant Sources: ✓ wild/cultivated/protected

E. Local Uses of Plant species (if any):

a) Plant parts used: Leaf, Root, Stem

b) Application: N/A

F. Any other information: N/A

FRESH LEAF COLLECTED FOR DNA ISOLATION

13/12/2013

Collected by-

(MANAS RANJAN SAHA)

Molecular Genetics Laboratory

Department of Botany, NBU

Fig. 3.1. Datasheet used during the collection of germplasm.

- The tube was incubated in a water bath (Rivotek, Cat#50121002) for 1h at 65ºC with occasional mixing by gentle swirling.
- Subsequently, an equal volume of chloroform (Merck India, Cat#822265): isoamyl alcohol (Merck India, Cat#8.18969.1000) (24:1) was added and gently mixed.
- The entire mixture was then centrifuged (REMI, Model No.C-24) for 20 min at about 6,500 rpm (5,000xg) at room temperature (25ºC) and the supernatant was carefully transferred to a fresh tube without disturbing the middle cell debris layer.
- Approximately 0.6 volume of ice cold isopropanol (Merck India, Cat#17813) was added to the supernatant and mixed gently.
- The mixture was then incubated for 2h at -20ºC.
- Following, the mixture was then centrifuged at about 6,500 rpm (5,000xg) for 30 minutes at 4ºC.
- Afterward, the upper aqueous layer was removed and the pellet was washed gently with chilled 70% ethyl alcohol (BDH, Cat#10107) and air dried for 1-2h.
- Then, the pellet was allowed to dissolve in 500µl of 1X TE buffer (pH 7.4) (Refer Appendix- B for composition).
- Next, the buffered DNA solution was extracted with an equal volume of equilibrated phenol (pH 8.0) (Sigma, Cat#P4557-400ML), mixed properly and centrifuged at 13,000 rpm (16,000xg) for 20 minutes.
- Following centrifugation, the upper aqueous layer was then transferred into a fresh tube and extracted with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 10,000 rpm (10,000xg) for 15 minutes at room temperature.
- The upper aqueous layer was again taken to a fresh tube and 0.1 volume of 3M sodium acetate (pH 5.2) (SIGMA, Cat#S-9513) was added followed by the addition of 2 volume of ice cold absolute ethyl alcohol (BDH, Cat#10107) and mixed gently for 10 min. and then centrifuged at 4ºC for 30 min at 13,000 rpm (16,000xg).
- Finally, the pellet thus obtained was washed gently with chilled 70% ethanol, air dried and dissolved in 500µl of 1X TE buffer (pH 7.4).

3.10.3.2. Purification of crude *DNA*

RNA, protein and polysaccharides are the main contaminants found in crude DNA hampering further downstream process. Therefore removal of these impurities is the main rational pathway for purification of crude DNA. Utilization of CTAB helps in elimination of polysaccharides from DNA. Subsequently, phenol: chloroform

and RNase treatment facilitate in the elimination of RNA and most of proteins to a large extent from crude DNA. The following procedure of DNA purification is as follows:

- Freshly prepared RNaseA (50µg/ml) (SIGMA, Cat#R-4875) (Refer Appendix- B for composition) was added into the genomic DNA, dissolved in 500µl of 1X TE buffer (pH 7.4) and incubated at 37ºC for 1h in a Dry water bath (GeNei™, Cat#107173).
- Then, the equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed properly and centrifuged at 10,000 rpm (10,000xg) for 15 min at room temperature.
- The upper aqueous phase was then transferred to a fresh micro centrifuge tube (Tarsons, Cat#500010) and 0.1 volume of 3M sodium acetate (pH 5.2) was then added into the tuve followed by double volume of absolute ethyl alcohol for DNA precipitation. Next, the tube was centrifuged at 13,000 rpm (16,000xg) for 30 min at 4ºC.
- Finally, the DNA pellet was collected and washed gently with chilled 70% ethyl alcohol, air dried and finally dissolved in 100µl of 1X TE buffer (pH 7.4).

3.10.3.3. Quantification of DNA

Proper DNA quantification is required in molecular biology for the amplification of target DNA by polymerase chain reaction and complete digestion of DNA by restriction enzymes. In essence, DNA quantification is carried out by spectrophotometric measurements or by agarose gel analysis. Both the methods were used in the present study.

3.10.3.3.1.Spectrophotometric measurement

- At first, spectrophotometer (Thermo UV1 spectrophotometer, Thermo Electron Corporation, England, UK) was calibrated at 260nm as well as 280nm by taking 600µl 1X TE buffer in a cuvette.
- The purified DNA (6µl diluted in 594µl of 1X TE) was taken in a cuvette, mixed properly and the optical density (OD) was recorded at both 260nm and 280nm. DNA concentration was estimated by using the following formula:

$$DNA = \frac{OD_{260} \times 50 \times DF}{1000}$$

where, DNA=Amount of DNA (ng/µl), DF= Dilution factor.

- Next, the quality of DNA was considered from the OD values recorded at 260nm and 280nm. The DNA showing A_{260}/A_{280} ratio around 1.8 was chosen for further PCR amplification by using RAPD, PCR-

RFLP and barcoding techniques.

3.10.3.3.2. Gel analysis

- The agarose gel (0.8%, gelling temperature 36°C) (SIGMA, Cat#A9539) was casted in 0.5X TBE buffer [Tris-Borate-EDTA)] (Refer Appendix- B for composition) containing 0.5µg/ml Ethidium bromide (Himedia, Cat#RM813) on gel platform (100×70mm) (Tarsons, Cat#7024).
- Sample DNA (5µl) mixed with 3µl of 6X gel loading dye (Refer Appendix-B for composition) was loaded.
- The lambda DNA/*Eco*RI/*Hin*dIII double digest (1µl) (GeNei™, Cat#106000) was loaded as molecular marker to determine the molecular size of the adjacent genomic DNA.
- The gel was run at 40 Volt (V) for 1hr in a Mini Submarine Gel Electrophoresis Unit (Tarsons, Cat#7030) using Electrophoresis Power Supply Unit (Tarsons, Cat#7090).
- Afterwaerd, the run time was over the gel was visualized under UV light on a UV Transilluminator (GeNei™, Cat#SF850).
- The DNA quality was considered by the presence of a single compact band at the corresponding position to λ DNA/*Eco*RI/*Hin*dIII double digest indicating high molecular weight of the DNA.
- The quantity of the DNA was estimated by comparing the sample DNA with the control by visualizing the band intensity under UV.
- The pure DNA thus obtained was used for various fingerprinting studies.

3.10.3.4. Gel Photography

Gel photographs were taken for using gel documentation system fitted with Cannon SLR camera (EOS350D) and Marumi orange filter (58mm YA2, Marumi, Japan). Besides, the EOS utility software was used for this purpose.

3.10.4. RAPD analysis (Random Amplified Polymorphic DNA)

A total of 45 random 10 mer primers were tested for 9 different species and varieties of Mimosoids (Table 3.3).

3.10.4.1. RAPD-PCR amplification

An autoclaved sterile PCR tube (Tarsons, 0.2ml, Cat#500050) was taken containing 25 µl of PCR reaction mixture for PCR amplification. The mixture was consisted of following solutions:

- PCR master Mix (2X; Promega; Cat# M7122) - 12.5µl
- Primer - 1.25µl (0.25µM)
- Template DNA - 2µl (25ng)
- Pyrogen free water- 9.25µl

One negative control PCR mix tube was prepared without DNA. The PCR reactions

Table 3.3. List of RAPD primers used in the Present Study.

Primer ID	Sequence (5′-3′)
OPA01	CAGGCCCTTC
OPA02	TGCCGAGCTG
OPA03	AGTCAGCCAC
OPA04	AATCGGGCTG
OPA05	ATTTTGCTTG
OPA06	GGTCCCTGAC
OPA07	GAAACGGGTG
OPA08	GTGACGTAGG
OPA09	GGGTAACGCC
OPA10	GTGATCGCAG
OPA11	CAATCGCCGT
OPA12	TCGGCGATAG
OPA13	CAGCACCCAC
OPA16	AGCCAGCGAA
OPA17	GACCGCTTGT
OPA18	AGGTGACCGT
OPA19	CAAACGTCGG
OPA20	GTTGCGATCC
OPB01	GTTTCGCTCC
OPB02	TGATCCCTGG
OPB03	CATCCCCCTG
OPB04	GGACTGGAGT
OPB05	TGCGCCCTTC
OPB06	TGCTCTGCCC
OPB07	GGTGACGCAG
OPB08	GTCCACACGG
OPB11	GTAGACCCGT
OPB12	CCTTGACGCA
OPB13	TTCCCCCGCT
OPF09	CCAAGCTTCC
OPG19	GTCAGGGCAA
OPH04	GGAAGTCGCC
OPN04	GACCGACCCA
OPN05	ACTGAACGCC
OPN13	AGCGTCACTC
OPN19	GTCCGTACTG
MGL01	GCGGCTGGAG
MGL02	GGTGGGGACT
MGL03	GTGACGCCGC
MGL04	GGGCAATGAT
MGL05	CTCGGGTGGG
MGL06	CGTCTGCCCG
MGL07	CTGTCCCTTT
MGL08	GTATTGCCCT
MGL09	TGTACGTGAC

were performed on Applied Biosystems Thermocycler-2720 (Germany). The RAPD-PCR amplification cycle consists of the following specifications:

- Cycle 1 - Denaturation at 94ºC for 4 min, Primer annealing at 37ºC for 1 min, Primer extension at 72ºC for 2 min.
- Cycle 2-44 - Denaturation at 94ºC for 1 min, Primer annealing at 37ºC for 1 min, Primer extension at 72ºC for 2 min.
- Cycle 45 - Denaturation at 94ºC for 1 min, Primer annealing at 37ºC for 1 min., Primer extension at 72ºC for 10 min.

After the compliance of total PCR cycle, the PCR products were separated on 1.8% (W/V) agarose gel containing Ethidium bromide solution (0.5µg/ml) run in 0.5X TBE buffer.

Next, the PCR product (12µl) was mixed with 4µl of 6X Gel loading dye (Refer Appendix- B for composition) and the samples were loaded and electrophoresis was carried out at 50V for 2.5h.

Finally, the gels were visualized with a UV transilluminator (GeNeiTM, Cat#107161) and photographed with Gel Documentation System as mentioned earlier. One DNA ladder (λDNA/EcoRI/HindIII double digest) (GeNeiTM, Cat#106000) and 100bp DNA ladder (Sigma, Cat#D3687) were used as a molecular size marker. All PCR reactions were run at least thrice and only reproducible and clear bands were scored and aligned by diversity data base software (NTSYSpc).

3.10.5. PCR-RFLP analysis

3.10.5.1. Primers used

Tab c-f in trnL-trnF (Taberlet *et al.*, 1991) region of the genome of selected Mimosoids was amplified. The primer sequence Tab c- 5′-CGAAATCGGTAGACGCTACG-3′ and

Tab f - 5′-ATTTGAACTGGTGACACGAG-3′

was used on the basis of the known sequence from the Taberlet region of the other plant species.

3.10.5.2. PCR amplification of trnL-trnF region

The polymerase chain reaction was carried out in a 25µl volume containing the following components:

- PCR master Mix (2X; Promega; Cat# M7122) - 12.5µl
- Primer - 1.25µl Tab c (0.25µM) and 1.25µl Tab f (0.25µM)
- Template DNA - 2µl (25ng)
- Pyrogen free water- 8µl

One negative control PCR mix tube was prepared without DNA. The PCR reactions were performed on Applied Biosystems Thermocycler-2720 (Germany). The RFLP -PCR amplification cycle consists of the following specifications:

- Cycle 1 - Denaturation at 95ºC for 5 min, Primer annealing at 54ºC for 45 sec, Primer extension at 72ºC for 2 min.
- Cycle 2-34 - Denaturation at 95ºC for 45 sec, Primer annealing at 54ºC for 45 sec, Primer extension at 72ºC for 2 min.
- Cycle 35 - Denaturation at 95ºC for 45 sec, Primer annealing at 54ºC for 45 sec, Primer extension at 72ºC for 7 min.

After the compliance of total PCR cycle, the PCR products were separated on 1.8% (W/V) agarose gel containing Ethidium bromide solution (0.5µg/ml) run in 0.5X TBE buffer (pH- 8.0).

Next, the PCR product (12.5µl) was mixed with 3µl of 6X Gel loading dye and the samples were loaded and electrophoresis was carried out at 50 V for 2 h 20 min.

Finally, the gels were visualized with a UV transilluminator (GeNeiTM, Cat#107161) and photographed with Gel Documentation System as mentioned earlier. One DNA ladder (λDNA/*Eco*RI/*Hin*dIII double digest) (GeNei^{TM}, Cat#106000) and 100bp DNA ladder (Sigma, Cat#D3687) were used to determine the band size.

3.10.5.3. PCR product restriction digestion

The PCR products were subjected to restriction digestion with 8 different restriction endonucleases, namely EcoRI (Promega, Cat#R6011), HinfI (Promega, Cat# R6201), HaeIII (Promega, Cat#R6171), HpaI (Promega, Cat# R6311), MboI (Promega, Cat# R6711), AluI (Promega, Cat# R6281), MspI

Table 3.4. List of restriction endonucleases used in the present study.

Restriction Enzyme	Cutting Site	Temperature
*Alu*I	AG↓CT	37 ºC
*Hin*fI	G↓ANTC	37 ºC
*Hpa*I	GTT↓AAC	37 ºC
*Hae*III	GG↓CC	37 ºC
*Msp*I	C↓CGG	37 ºC
*Taq*I	T↓CGA	65 ºC
*Eco*RI	G↓AATTC	37 ºC
MboI	↓GATC	37 ºC

(Promega, Cat# R6401) and TaqI (Promega, Cat# R6151). All enzymes are 4 -base cutters. The list of the enzymes along with their cutting sites and other information's are described in Table 3.4.

The protocol of PCR-RFLP restriction digestion (20µl for each sample) is as follows:

- Restriction Enzyme buffer - 2µl
- Enzymes - 5units (0.5µl)
- PCR product - 5µl
- Pyrogen free water- 12.5µl

[N.B.- In case of enzyme *Taq*I, BSA (0.2µl) was also added separately in the reaction mixture.]

The reaction mixture tube was spinned for a while for proper mixing.

The tube was then incubated at specific temperature for each enzyme (as mentioned in Table 3.4) for a period of 1h in a Dry bath (GeNei^{TM}, Cat#107173).

Following 1h of incubation, the restriction digestion mix product was separated on a 2% (W/V) agarose gel and run in 0.5X TBE buffer (pH-8.0).

Next, the restriction digestion produc (20µl) was mixed with 5µl of 6X Gel loading dye and the samples were loaded and electrophoresis was carried out at 50 V for 2h.

Finally, the gels were visualized with a UV transilluminator (GeNeiTM, Cat#107161) and photographed with Gel Documentation System as mentioned earlier. One DNA ladder (λDNA/*Eco*RI/*Hin*dIII double digest) (GeNei^{TM}, Cat#106000) and 100bp DNA ladder (Sigma, Cat#D3687) were used to determine the band size.

3.10.6. Barcoding analysis

3.10.6.1. Primers used

Two primers namely, matK and TrnL-F were used in the present study. The sequences of the primers are described in Table 3.5.

3.10.6.2. PCR amplification of matK region

The study was undertaken in a 25µl

volume containing the following components:

- PCR master Mix (2X; Promega; Cat# M7122) – 12.5µl
- Primer – 1.25µl each (forward and reverse)
- Template DNA - 2µl (25ng)
- Pyrogen free water- 8µl

The PCR reactions were performed on Applied Biosystems Thermocycler-2720 (Germany). The amplification cycle consists of the following specifications:

- Cycle 1 - Denaturation at 94ºC for 4 min, Primer annealing at 48ºC for 30 sec, Primer extension at 72ºC for 1 min.
- Cycle 2-34 - Denaturation at 94ºC for 1 min, Primer annealing at 48ºC for 30 sec, Primer extension at 72ºC for 1 min.
- Cycle 35 - Denaturation at 94ºC for 1 min, Primer annealing at 48ºC for 30 sec, Primer extension at 72ºC for 7 min.

After the compliance of total PCR cycle, the PCR products were separated on 2% (W/V) agarose gel containing Ethidium bromide solution (0.5µg/ml) run in 0.5X TBE buffer (pH- 8.0).

Next, the PCR product (12µl) was mixed with 4µl of 6X Gel loading dye and the samples were loaded and electrophoresis was carried out at 50V for 2h 20 min.

Finally, the gels were visualized with a UV transilluminator (GeNeiTM, Cat#107161) and photographed with Gel Documentation System as mentioned earlier. One DNA ladder (λDNA/*Eco*RI/*Hin*dIII double digest) (GeNei^{TM}, Cat#106000) and 100 bp DNA ladder (Sigma, Cat#D3687) were used to determine the band size.

3.10.6.3. PCR amplification of TrnL-F region

The amplification was carried out in a 25 µl volume containing the following components:

- PCR master Mix (2X; Promega; Cat# M7122) – 12.5µl
- Primer – 1.25µl each (forward and reverse)
- Template DNA - 2µl (25ng)
- Pyrogen free water- 8µl

Table 3.5. Primers used for amplification of matK and TrnL-F gene segments.

Primer name	Binding	Primer sequence (5′– 3′)
matK F	Forward	CGATCTATTCATTCAATATTTC
matK R	Reverse	TCATGCACACGAAAGTCGAAGT
TabC	Forward	CGAAATCGGTAGACGCTACG
TabF	Reverse	ATTTGAACTGGTGACACGAG

The PCR reactions were performed on Applied Biosystems Thermocycler-2720 (Germany). The amplification cycle consists of the following specifications:

- Cycle 1 - Denaturation at 95ºC for 5 min, Primer annealing at 54ºC for 45 sec, Primer extension at 72ºC for 2 min.
- Cycle 2-34 - Denaturation at 95ºC for 45 sec, Primer annealing at 54ºC for 45 sec, Primer extension at 72ºC for 2 min.
- Cycle 35 - Denaturation at 95ºC for 45 sec, Primer annealing at 54ºC for 45 sec, Primer extension at 72ºC for 7 min.

After the compliance of total PCR cycle, the PCR products were separated on 2% (W/V) agarose gel containing Ethidium bromide solution (0.5µg/ml) run in 0.5X TBE buffer (pH- 8.0).

Next, the PCR product (12µl) was mixed with 4µl of 6X Gel loading dye and the samples were loaded and electrophoresis was carried out at 50V for 2h 20 min.

Finally, the gels were visualized with a UV transilluminator (GeNeiTM, Cat#107161) and photographed with Gel Documentation System as mentioned earlier. One DNA ladder (λDNA/*Eco*RI/*Hin*dIII double digest) (GeNei^{TM}, Cat#106000) and 100bp DNA ladder (Sigma, Cat#D3687) were used to determine the band size.

3.10.7. DNA fingerprinting data analysis

In the present study, each polymorphic band was considered as binary character and was scored as 1 (presence) or 0 (absence) for each sample and accumulated in a data matrix. Further, a similarity matrix was calculated on the basis of band-sharing from the binary data using Dice coefficient (Nei and Li, 1979) while a dendrogram of similarities was generated using the group average agglomerative clustering tool. The analysis was done using the software package NTSYSpc (version 2.0) (Rohlf, 1998a). In addition, correspondence analysis (2D and 3D plot) of right vectors from the binary data was performed to exhibit a graphical representation among the varieties using NTSYSpc (version 2.0).

3.10.8. Sequencing of amplified products

A total of 4 species namely, *Mimosa pudica*, *M. invisa*, *Acacia concinna* and *Albizia chinensis* were subjected for DNA sequencing purposes using mat-K and TrnL-F gene. Amongst these species, *M. invisa* and *A. chinensis* were selected for the sequencing of both of the mat-K and taberlet regions of chloroplast genome of these species. In case of *M. pudica*, only taberlet region was subjected to DNA sequencing while only mat-K region of *A. concinna* was selected for sequencing purposes. The DNA sequencing was done by means of outsourcing to a private company, Chromous Biotech Pvt. Ltd. [#842, II, Floor, Shankar Bhawan, A Block, Shankar Nagar, Bangalore-5600092

(www.chromous.com)].

3.10.9. Submission of sequence

The raw sequences of all four above mentioned species of Mimosoides were rearranged with the help of Sequin Application Version 12.30 Standard Release [Nov 13, 2012] for Database submission to GenBank along with all necessary information viz., definition of the sequence (i.e. the specific region of the genome), source of the sequence (chloroplast DNA in this case; name of the plant species along with its taxonomic position, date and place of collection, tissue type etc.).

3.10.10. Sequence analysis and construction of phylogenetic tree

The six sequences of matK and TrnL-F region of selected species obtained in the present study were compared with other existed global sequences [downloaded from NCBI (http://www.ncbi.nlm.nih.gov)] in GeneBank under the subfamilies Mimosoideae, Papilionoideae and Caesalpinioideae (Table 3.6).

The DNA sequences were aligned by using ClustalW2 (www.ebi.ac.uk/Tools/clustalw2) and T-Coffee (www.ebi.ac.uk/Tools/t-coffee) software. The phylogenetic tree was constructed by Neighbour Joining (NJ) method using Molecular Evolutionary Genetics Analysis (MEGA 4.0). The tree was analyzed by both NJ and UPGMA (Unweighed Pair Group Mean Average) methods. In addition, parsimony analysis, various clades, transition/transversion (ns/nv) ratio and variability in different regions were also determined by MEGA4.0 in accordance with the Kimura 2-Parameter (K2P) model. The tree was generated employing 16 (matK) and 15 (TrnL-F) sequences from Mimosoideae, Papilionoideae and Caesalpinioideae as well as 1 outgroup taxa from Cannabaceae (Table 3.6).

3.11. Medicinal and molecular assortment of Microsymbionts

3.11.1. Medical aspect

It was further intended to investigate the probable medicinal value of micro-symbionts in Mimosoids. Therefore, antioxidant activity between root and root nodule comprizing *Rhizobium* were subjected in this study. Three well-studied species namely, *M. pudica*, *M. invisa* and *A. nilotica* have chosen as host plant to collect root and root nodule. Initially, a few *in-vitro* antioxidant assays were performed among root and root nodule separately to evaluate their potentiality.

3.11.1.1. Preparation of extracts

The root and root nodule extract were prepared separately as mentioned in section 3.3.4.

3.11.1.2. In-vitro antioxidative tests of root and root nodule

The free radical scavenging activity of roots and root nodules through DPPH

assay was performed as per Saha *et al*. (2016). The Optical density (OD) was measured at 517 nm and the positive control was ascorbic acid. Further, hydroxyl radical scavenging assay of each extracts were carried out on the basis of Fenton reaction (Kunchandy and Rao, 1990) and mannitol was used as positive control. The nitric oxide radical quenching activity was performed following the GriessI-llosvoy reaction (Garratt, 2012) with minor modifications of Saha *et al*.

Table 3.6. Taxa, specimens and GenBank accession numbers for sequences used in the present study.

Taxa	Family	Sub-family	matK accession number	TrnL-F accession number
Mimosa invisa	Fabaceae	Mimosoideae	LM643807*	LM643811*
Mimosa pudica	Fabaceae	Mimosoideae	JQ587776	LM643810*
Acacia nilotica	Fabaceae	Mimosoideae	FJ711574	AF522973
Acacia catechu (Syn: *Senegalia catechu*)	Fabaceae	Mimosoideae	KF531964	DQ371870
Acacia concinna	Fabaceae	Mimosoideae	LM643808*	---
Samanea saman	Fabaceae	Mimosoideae	JQ587830	AF522965
Albizia lebbeck	Fabaceae	Mimosoideae	GU134994	EU440023
Albizia chinensis	Fabaceae	Mimosoideae	LM643809*	LM643812*
Abrus precatorius	Fabaceae	Papilionoideae	GU135022	EF543423
Crotalaria juncea	Fabaceae	Papilionoideae	JQ041057	---
Indigofera circinella	Fabaceae	Papilionoideae	KR735049	KR738539
Senna tora	Fabaceae	Caesalpinioideae	KJ638441	KP338336
Glycine max	Fabaceae	Papilionoideae	KF022416	EU717321
Medicago sativa	Fabaceae	Papilionoideae	HQ593363	GQ488614
Pisum sativum	Fabaceae	Papilionoideae	----	AY839473
Phaseolus vulgaris	Fabaceae	Papilionoideae	KF022429	EU717342
Senna occidentalis	Fabaceae	Caesalpinioideae	KU551092	---
Cannabis sativa	Cannabaceae		HQ619806	AY958392

*Present Study (submitted to GeneBank)

Table 3.7. Details of the collected *Rhizobium* from different location with ID.

Host plant	Collection Site	Latitude	Longitude	Sample ID
M. pudica	Samsi (Malda)	25°27′ N	88°00′ E	MPD-SAM-**I**
	Sibmandir (Darjeeling)	26°70′ N	88°35′ E	MPD-SIB-**II**
M. invisa	Samsi (Malda)	25°27′ N	88°00′ E	MIN-SAM-**III**
	Sibmandir (Darjeeling)	26°70′ N	88°35′ E	MIN-SIB-**IV**
A. nilotica	Samsi (Malda)	25°27′ N	88°00′ E	ANL-SAM-**V**
	Sibmandir (Darjeeling)	26°70′ N	88°35′ E	ANL-SIB-**VI**

Collection Data Sheet

Sample No- MIN-SAM-III **Date:** 11/10/2015

A. General Information:

1. Collection Site- Samsi (Malda, West Bengal)
2. Date- 11/10/2015
3. Time- 11:15 am
4. Scientific name of the host: Mimosa invisa
5. Local Name: সাদা লজ্জাবতী - (Sada Lajjabati)
6. Nodule Collected: Yes
7. Root Collected: Yes
8. Seeds Collected: No
9. Soil Collected: No
10. Twig Collected: Yes

B. Habits:

1. Tree/Shrub/Herb: Shrub
2. Flowering Time: September - December
3. Seeding Time: November - January
4. Planting Time: N/A

C. Habitat and area of the vegetation:

1. Rain fall season: Moderate
2. Altitude: Above sea level
3. Temperature: 28°C
4. Topography: Plain
5. Vegetation type: Natural forest/ Road side/ Social forest/Others
6. Management: Cutting/Burning/Natural/Habitat preserved

D. Specific collection Site:

1. Soil pH: N/A
2. Colour of the nodule: Reddish white
3. Drainage: Flooded/Poorly drained/well drained

E. Nodules:

1. Grows on: Crown Area/Tap Root/Lateral Root
2. Growth form: Clumped/Scattered

11/10/2015
Collected by-
(MANAS RANJAN SAHA)
Molecular Genetics Laboratory
Department of Botany, NBU

Fig. 3.2. Datasheet used during sample collection of root nodule (*Rhizobium*).

(2016). The absorbance was determined spectrophotometrically at 540 nm against blank sample. Curcumin was used as standard.

3.11.2. Molecular documentation

3.11.2.1. Collection of germplasm

The nodules of young plant species namely, *Mimosa pudica*, *M. invisa* and

Acacia nilotica (host plant) were collected from different regions (Table 3.7) in their respective growing season (August-November). Essential data were recorded during the collection of germplasm (for details see Fig. 3.2).

3.11.3. Isolation of *Rhizobium*

3.11.3.1. Surface sterilization

Prior to isolation experiment, the surface sterilization of root nodules was performed.

- Firstly, the nodulated roots were washed thoroughly with tap water to remove all the soil particles and organic materials.
- Only healthy and undamaged nodules were selected and washed with few drops of detergent (Dextran) followed by washing with distilled water.
- Then, the nodules were treated with 70% ethanol for 30 sec followed by several washes with sterile distilled water.
- The nodules were further treated with 30% sodium hypochlorite (NaOCl) for 4-6 min. Final rinsing of the nodules was done thoroughly with sterile distilled water for 4-5 times to remove the traces of NaOCl.

3.11.3.2. Media for isolation

Yeast extract mannitol (YEM) medium was used for the isolation of *Rhizobium.*

3.11.3.2.1. Isolation of Rhizobium in liquid and solid media

The following isolation procedure was carried out:

- Surface sterilized nodule lobes were crushed on a grease free sterile slide.
- Next, a loopful of the crushed nodule sap was then put into liquid YEM medium containing conical flask.
- The flask was then transferred to an orbital shaker at 200 rpm maintained at an optimum temperature of 28 ± 2ºC for 2-3 days for proper incubation. The media turned to be turbid with the growth of the bacteria.
- A loopful of culture was streaked on YEM-agar plates (1.8%).
- The YEMA plates were incubated at 28±2^{o}C for 2-3 days.
- The single pure colony obtained was then sub cultured and maintained in YEM slants for further use. The strains were sub cultured at regular interval of one month.

The pure strains were isolated from different regions (Table 3.7) were then named with their respective codes.

3.11.3.3. Morphological characterization

Fresh cultures of 24 to 48h were used for morphological characterization.

3.11.3.3.1. Colony morphology

A fresh culture from broth was streaked on the YEM-agar plates and incubated for 24-48h at 28±2ºC. The morphological

characteristics of the colonies were observed based on the diameter, shape, color, transparency, form and production of mucous as per Aneja (1996).

3.11.3.3.2. Gram staining

Gram-staining was further performed as per the standard method of Aneja (1996). The isolates were observed under light microscope to differentiate gram negative from the gram positive bacteria.

3.11.4. Genomic diversity study

The genomic DNA of *Rhizobium* strains obtained from selected plant species from different regions was isolated through following procedures:

3.11.4.1. Isolation of Genomic DNA from pure culture

- The copious amount of exopolysaccharides produced by *Rhizobium* acts as a hindrance in the isolation of genomic DNA. The CTAB/NaCl method was followed to isolate the *Rhizobium* genomic DNA using standard protocol of William and Feil (2012) with minor modifications.
- The bacterial strains were grown in YEM for 24-48h in OSI until the O.D. value reached 0.8 at 600nm.
- Thirty ml of 24-48 hr culture was put into a sterile oakridge tube and kept in the ice bucket for 1h of incubation. Then, the tubes were centrifuged (REMI, Model No.C-24) at 5000xg for 20 min at 4ºC.
- The supernatant was discarded and the pellet obtained was used for further isolation process.
- The pellet was suspended in 5.67ml 1XTE buffer (pH 8.0) (Refer Appendix- B for composition).
- Fifty five µl of lysozyme (conc. 10mg/ ml) was added to lyse the bacterial cell wall. The mixture was incubated for 5min at room temperature.
- To the mixture 300µl of 10% sodium dodecyl sulphate (SDS) was added and mixed properly.
- Afterward, 50µl of proteinase K (20mg/ml) was added and blended well with gentle swirling.
- The mixture was then incubated in water bath (Rivotek, Cat# 50121002) for 1h at 37ºC. The tubes were mixed occasionally by gentle swirling in between the incubation period.
- Following incubation, 1ml of 5M NaCl was added to the solution and mixed well. To that mixture, 0.8ml of hot (65°C) CTAB/NaCl (Refer Appendix- B for composition) was added, mixed well and incubated at 65°C for 10 min in water bath.
- The mixture was then extracted with equal volume of chloroform:isoamylalcohol (24:1) followed by gentle spinning at 5000xg for 10 min at room temperature. A white interface containing the debris

could be visible after centrifugation. The clear upper aqueous layer was transferred to a fresh oakridge tube.

- Further, equal volume of phenol/ chloroform/isoamyl alcohol (25:24:1) was added to the solutions, mixed properly and centrifuged at 5000xg for 20 min. The upper clear aqueous layer was transferred to another fresh oakridge tube.
- Approximately 0.6 vol (8ml) of chilled isopropanol stored at -20°C was added to precipitate the DNA. The tubes were shaken back and forth gently until a white cottony form of DNA was visible.
- Finally, the tubes were stored at -20°C for 2h. After incubation, the tubes were spinned at 5000xg for 30 min at 4°C. The pellet thus obtained was washed with chilled 70% ethyl alcohol, air dried and dissolved in 500µl 1X TE buffer (pH 8.0).

3.11.4.2. Purification of DNA

To eliminate the major contaminants like, RNA and protein the following procedures were followed:

3.11.4.2.1. RNase A treatment

RNase A (10mg/ml) (Refer Appendix- B for composition) was dissolved in 500µl 1X TE buffer (pH 8.0) containing the crude DNA. The mixture was incubated at 37°C for 1h in a Dry water bath (GeNei™ make, Cat#107173).

3.11.4.2.2. Protein removal

The protein impurities were removed using 500µl of phenol/chloroform/ isoamylalcohol (25:24:1) to the crude DNA and kept at 4°C. Micro centrifuge was done at 14000Xg for 2 min at room temperature. The aqueous phase was then transferred to a fresh micro centrifuge tube (Tarsons, Cat# 500010).

3.11.4.3. Ethanol precipitation of DNA

- The volume of the DNA was measured visually and 0.1 volume of 3M Sodium acetate (pH 5.2) was added and mixed well.
- It was followed by the addition of 2 volume of ice cold absolute ethyl alcohol and blended well by gentle swirling.
- The tubes were placed on ice at -20 ºC for 30 min and centrifuged at 14,000xg for 10-15 min at 4ºC.
- The DNA pellet was washed with 1 ml of chilled 70% ethanol, air dried and finally dissolved in 100µl of 1XTE buffer (pH 8.0).

3.11.4.4. Quantification of DNA

Proper DNA quantification is required in molecular biology for the amplification of target DNA by polymerase chain reaction and complete digestion of DNA by restriction enzymes. In essence, DNA quantification is carried out by spectrophotometric measurements or by agarose gel analysis. Both the methods

were used in the present study.

3.11.4.4.1. Spectrophotometric measurement

First, spectrophotometer (Thermo UV1 spectrophotometer, Thermo Electron Corporation, England, UK) was calibrated at 260 nano meter (nm) as well as 280 nm by taking 600µl 1X TE buffer in a cuvette.

The purified DNA (6µl diluted in 594µl of 1XTE) was taken in a cuvette, mixed properly and the optical density (OD) was recorded at both 260nm and 280nm. DNA concentration was estimated by using the following formula:

$$DNA = \frac{OD_{260} \times 50 \times DF}{1000}$$

where, DNA=amount of DNA (ng/µl); DF=dilution factor.

Next, the quality of DNA was considered from the OD values recorded at 260nm and 280nm. The DNA showing A_{260}/A_{280} ratio around 1.8 was chosen for further PCR amplification by using RAPD techniques.

3.11.4.4.2. Gel electrophoresis

- DNase free Agarose (0.8%, gelling temperature 36°C) was used to cast the gel in 0.5X TBE (Tris-Borate-EDTA) buffer (Refer Appendix- B for composition) containing 7µl of Ethidium bromide (10mg/ml) on gel platform (100x70mm) (Tarsons, Cat # 7024).
- Five µl of DNA samples were mixed with 3µl of 6X gel loading dye (refer appendix for composition) and loaded in the wells carefully. Lambda DNA/ *Eco*RI/*Hind*III double digest (2µl) and 100 bp ladder were used as molecular markers to determine the size of genomic DNA.
- The gel was run at 50 volt (V) and 100mAmp for 1.5h in the Midi Submarine Electrophoresis Unit (Tarsons, Cat #7050) connected to the Electrophoresis Power Supply Unit (Tarsons, Cat #7090).
- Finally, the gel was viewed on a UV Transilluminator (GeNei™, Cat #SF850). The molecular sizes of the genomic DNA were detected in the form of bands. The sizes of the bands were estimated with Photo-Capt Version 12.4, (Vilber Lourmat, USA).

3.11.4.5. Gel photography

Please refer section 3.10.3.4.

3.11.5. RAPD analysis

Table 3.8. List of RAPD primers used in the Present Study.

Primer ID	Sequence (5′-3′)
OPA01	CAGGCCCTTC
OPA02	TGCCGAGCTG
OPA03	AGTCAGCCAC
OPA04	AATCGGGCTG
OPA05	ATTTTGCTTG
OPA06	GGTCCCTGAC
OPA07	GAAACGGGTG
OPA08	GTGACGTAGG
OPA09	GGGTAACGCC
OPA10	GTGATCGCAG
OPD 03	GTCGCCGTCA
DAF 9	CCGACGCGGC
OPI 06	AAGGCGGCAG
OPY 04	AAGGCTCGAC
OPQ 01	GGGACGATGG

3.11.5.1. Primers used

A total of 15 random 10 mer primers were tested for 6 different *Rhizobium* species (Table 3.8).

3.11.5.2. RAPD-PCR amplification

An autoclaved sterile PCR tube (Tarsons, 0.2ml, Cat#500050) was taken containing 25µl of PCR reaction mixture for PCR amplification. The mixture was consisted of following solutions:

- PCR master Mix (2X; Promega; Cat# M7122) - 12.5µl
- Primer - 1.25µl (0.25µM)
- Template DNA - 2µl (25ng)
- Pyrogen free water- 9.25µl

The ingredients were mixed evenly in a SpinWin PCR micro centrifuge (Tarsons, Cat# 1000).

The PCR reactions were performed in Applied Biosystems, Thermal Cycler, 2720 PCR machine.

The amplification cycle (modified protocol of Elboutahiri *et al.,* 2009) set at 35 cycles were as follows:

- Cycle 1: Denaturation at 94ºC for 4 min, primer annealing at 36ºC for 1 min, primer extension at 72ºC for 2 min.
- Cycle 2-34: Denaturatin at 94ºC for 1 min, primer annealing at 36ºC for 1 min, primer extension at 72ºC for 2 mins.
- Cycle 35: Denaturatin at 94ºC for 1 min, primer annealing at 36ºC for 1 min, final extension at 72ºC for 7 min.

After the compliance of total PCR cycle, the PCR products were separated on 1.8% (W/V) agarose gel containing Ethidium bromide solution (0.5µg/ml) run in 0.5X TBE buffer.

Next, the PCR product (12µl) was mixed with 4µl of 6X Gel loading dye (Refer Appendix- B for composition) and the samples were loaded and electrophoresis was carried out at 50V for 2.5h.

Finally, the gels were visualized with a UV transilluminator (GeNeiTM, Cat#107161) and photographed with Gel Documentation System as mentioned earlier. One DNA ladder (λDNA/*Eco*RI/*Hin*dIII double digest) (GeNei^{TM}, Cat#106000) and 100bp DNA ladder (Sigma, Cat#D3687) were used as a molecular size marker. All PCR reactions were run at least thrice and only reproducible and clear bands were scored and aligned by diversity data base software (NTSYSpc).

3.11.5.2. DNA fingerprinting data analysis

Please refer section 3.10.7 for more details.