CHAPTER 3

MATERIALS AND METHODS

3. Materials and methods

3.1. Plant material

Common dietary herbs and spices served as the plant materials which were surveyed, collected, identified, enlisted, selected and processed for biological evaluation in the following steps.

3.1.1. Survey and collection

Market survey of various herbs and spices was carried out in different districts/towns of West Bengal (mainly in the northern region) in between January 2011 and January 2012, and total 40 samples (15 herbs plus 25 spices) were purchased from different retail shops and market stalls (Table 3).

Table 3: GIS locations of different sampling sites for different herb and spice samples

Survey area	Sampling site	GIS location
Cooch Behar	Nutun Bazar	26° 18' 57.384" N and 89° 26' 38.167" E
Siliguri	City Centre	26° 43' 28.930" N and 88° 23' 42.342" E
	Shivmandir Bazar	26º42′29.63″ N and 88º21′40.52″ E
	Garden of Medicinal Plants, NBU	26° 42' 26.561" N and 88° 21' 14.363" E
Jalpaiguri	Din Bazar	26° 31' 44.592" N and 88° 43' 22.160" E
	Station Bazar	26° 31' 6.536" N and 88° 43' 29.518" E
Balurghat	Thana Bazar	25° 13' 21.662" N and 88° 46' 43.476" E
	Mini Market	25° 14' 58.359" N and 88° 51' 48.068" E
Malda	Chittaranjan Market	25° 0' 0.212" N and 88° 8' 41.444" E
	Bichitra Market	25° 0' 12.525" N and 88° 8' 12.351" E
Raiganj	Mohanbati Bazar	25° 37' 16.544" N and 88° 7' 41.133" E
	Debinagar Bazar	25° 35' 41.882" N and 88° 7' 32.208" E

Some of the dry spice samples were also purchased from Masala Centre, Broadway, Ernakulam, Cochin (9° 58' 39.986" N and 76° 16' 40.280" E). For authentication, samples were processed into botanical voucher collections. From retailers and

commoners, data on vernacular names, culinary and medicinal uses were collected to get an idea on the importance and the diversity of different dietary herbs and spices in human life.

3.1.2. Selection of plant material

Among the plants served so far, following were selected from the panel to study their phytochemical profiles and biological activities to establish or validate their traditional medicinal uses as these were commonly and extensively used herbs and spices (Table 4 and 5, Figure 3).

Table 4: Plant species selected for the present investigation

Herb samples selected for study	Plant parts tested	Spice samples selected for study	Plant parts tested
Mentha piperita	Whole leaf	*Illicium verum*	Whole fruit
Trigonella foenum-graecum	Whole leaf	*Myristica fragrans*	Aril
Coriandrum sativum	Whole leaf	*Ceiba pentandra*	Floral bud
Murraya koenigii	Whole leaf	*Capsicum annuum*	Whole fruit
Glinus oppositifolius	Aerial part	*Parmelia perlata*	Whole thallus
Foeniculum vulgare	Aerial part	*Dregea volubilis*	Stem bark

Table 5: Herb species along with the accession no. of the voucher specimens

Herb specimen	Accession number
Mentha piperita L.	09863
Trigonella foenum-graecum L.	09864
Coriandrum sativum L.	09865
Murraya koenigii (L.) Spreng.	09866
Glinus oppositifolius (L.) Aug. DC.	09867
Foeniculum vulgare Mill.	09868

3.2. Processing of plant material

A total of 12 plant samples of herbs and spices were selected. Immediately after collection, fresh samples were brought to the laboratory and washed thoroughly with tap water and then distilled water to remove the dirt and extraneous matter, blotted with filter paper, and then shade-dried on laboratory benches in plastic trays for two days and then air-dried in a ventilated oven for 24-48 h at 45 °C. The dry spices were washed only with distilled water, blotted with tissue paper and further dried in a hot air oven for 24 h at 45 °C, wherever to need (commercially available sealed and ready to use products were not required to wash) and used for the preparation of fine powder.

3.2.1. Preparation of fine powder

The dried plant samples were ground into fine powder using a household electric grinder (Bajaj GX8, India) and sieved through a fine mesh. The powdered samples were stored in air-tight screw-capped glass bottles in the dark at -20 °C until extraction.

3.2.2. Preparation of lyophilised extract

Each of the finely powdered samples of herbs and spices was processed for both aqueous and solvent extraction using standard procedures as described below:

3.2.2.1. Aqueous extraction process

Hot water extraction process as described by Aliakbarlu and Tajik (2012) was followed but with some modifications. Ten g of freshly washed and finely chopped leaf samples or powdered plant samples was extracted for 30 min under darkness (flask covered with aluminium foil) by refluxing with HPLC grade water (1:10, w/v) at 100 °C in a temperature controlled water bath shaker (REMI Equipment Pvt. Ltd., India) with gentle agitation. After cooling, sample was filtered through Whatman filter paper (Grade 1, Ø 90 mm) and the solid residues obtained were further treated with same procedure twice. The filtrate fractions from every extraction process were pooled and concentrated under reduced pressure at 40 ± 1°C in a rotary evaporator (RV 10 digital, IKA®, Germany) equipped with chiller (MC3i, IKA®, Germany), followed by lyophilisation in a vacuum freeze-dryer (EYELA FDU-506, Tokyo, Japan) to obtain the lyophilized hot water extracts (LHEs). The lyophilised extracts were weighed and re-dissolved in same fluid to prepare stock solutions of desired concentrations and subsequently stored in air tight vials at -20°C until use for analyses.

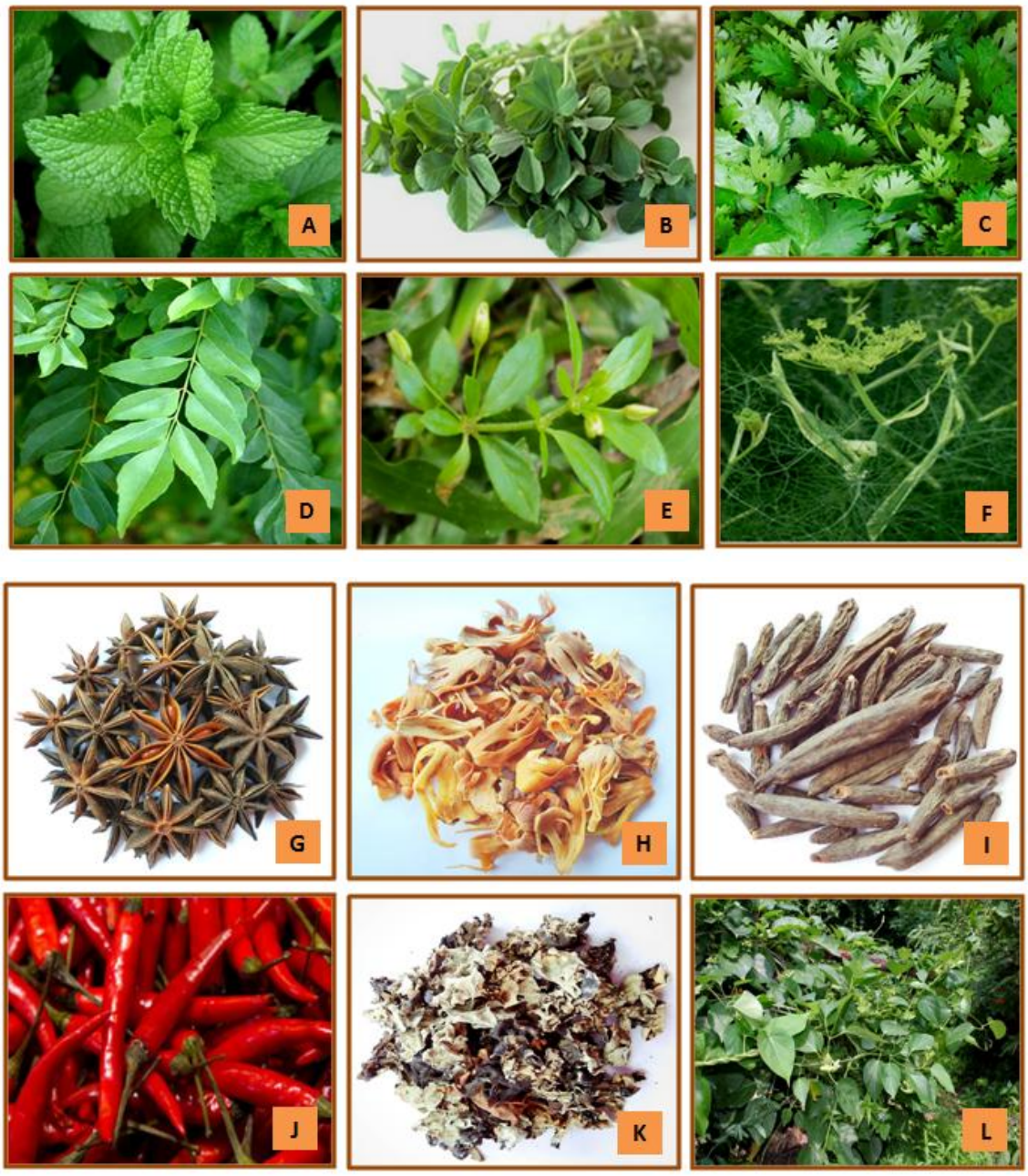

Figure 3. Herbs and spices selected for the bioassay. A: *Mentha piperita*, B: *Trigonella foenum-graecum*, C: *Coriandrum sativum*, D: *Murraya koenigii*, E: *Glinus oppositifolius*, F: *Foeniculum vulgare*, G: *Illicium verum*, H: *Myristica fragrans*, I: *Ceiba pentandra*, J: *Capsicum annuum*, K: *Parmelia perlata* and L: *Dregea volubilis*.

3.2.2.2. Solvent extraction process

The solvent extracts of plant materials were prepared by using methanol as solvent according to a method described previously by Cheesbrough (2000) with minor modifications. Under darkness and normal room temperature, a 10-15 g of fine powder was extracted thrice with HPLC grade methanol (1:10, w/v) by stirring at 150 r min^{-1} for 3 x 24 h intermittently with fresh solvent each time and filtered through Whatman filter paper (Grade 1, Ø 90 mm). The filtrate fractions from every single extraction process were pooled and concentrated*in vacuo*at 40 ± 1°C in a rotary evaporator (RV 10 digital, IKA®, Germany) equipped with chiller (MC3i, IKA®, Germany), followed by lyophilization in a vacuum freeze-dryer (EYELA FDU-506, Tokyo, Japan) to obtain the lyophilized methanolic extracts (LMEs). The lyophilized extracts were then weighed using a microbalance (Sartorius, Germany) and were dissolved in the same solvent (HPLC grade methanol) to make stock solutions of desired concentrations and preserved at -20°C until further use.

3.2.2.3. Determination of extractive value

The extractive value or yield is a measure of the solvent's efficiency to extract specific active compounds from the original plant material to be extracted and it is defined as the amount of extract recovered in mass compared with the initial amount of plant material used. It was presented in percentage (%) and was determined using the formula: Extractive value or % yield = (W_{CE} /W_{DP}) X 100; where W_{CE} = weight of lyophilised crude extract, W_{DP} = weight of dried powdered plant material used. The experiment was performed in triplicate (*n* = 3).

3.2.2.4. Determination of total moisture content

Moisture content of fresh and dried samples was determined using a laboratory oven kept at 105 ± 3°C for 24 h. The moisture content (%) was calculated on a weight basis from the difference between the wet and dry weight divided by the wet weight (AOAC, 1975), using the following formula: Total moisture content (%) = [(Initial weight of the sample before drying - Final weight of the sample after drying) / Initial weight of the sample before drying] x 100. One g of sample in pre-weighed Petri dish was placed in an oven for 24 h, cooled and reweighed. The dry weight of the sample was determined by repeated consistent weighing. The experiment was performed in triplicate (*n* = 3).

3.3. Analysis of nutritional components

3.3.1. Carbohydrate content

Total and reducing sugar was extracted by the method described by Harborne (1998) with minor changes. About 0.2 g of dried powdered or fesh sample was crushed properly in 10 mL of 95% ethanol. The alcoholic fraction was evaporated by keeping the beaker on a boiling water bath. The aqueous fraction was dissolved in 5 mL of distilled water and then centrifuged at 5000 rpm for 10 min. The supernatant was collected and used for further estimation after making the final volume to 5 mL by addition of distilled water.

3.3.1.1. Total soluble sugar content

Total sugar estimation was done according to the method explained by Plummer (1978). In brief, 1 mL of test solution was added with 4 mL of Anthrone's reagent (0.2 % Anthrone in concentrated H_2SO_4). The reaction mixture was mixed thoroughly and allowed to incubate in boiling water for 10 min (precaution were taken to prevent the water loss). The reaction mixture was then cooled under running tap water and the absorbance was measured in a colorimeter at 620 nm against a suitable blank solution. Total soluble sugar content (TSC) was calculated from a D-glucose calibration curve and results were expressed as mg of glucose equivalents (GLE) g^{-1} of tissue weight. The experiment was performed in triplicate (n = 3).

3.3.1.2. Reducing sugar content

The estimation of reducing sugar was done following the Somogyi-Nelson's method as described by Plummer (1978). One mL of the test solution was mixed with 1 mL of alkaline copper tartarate solution (4 g of $CuSO_4.5H_2O$, 24 g of Na_2CO_3 anhydrous, 16 g of Na^+-K^+ tartarate, 180 g of Na_2SO_4 anhydrous in 1000 mL of distilled water) and heated over a boiling water bath for 20 min. After cooling the reaction mixture under running tap water, 1 mL of commercially available Nelson's arseno-molybdate reagent and 2 mL of distilled water was added sequentially and mixed thoroughly. The optical density values were recorded at the wavelength of 515 nm in a colorimeter against a suitable blank solution. The reducing sugar content (RSC) was calculated from a D-glucose calibration curve and results were expressed as mg of glucose equivalents (GLE) g^{-1} of tissue weight. The experiment was performed in triplicate (n = 3).

3.3.2. Total soluble protein content

Totalsoluble protein was extracted using the standard protocol given by Chakraborty *et al.* (1995) and quantification was done according to Lowry *et al.* (1951) using BSA as standard. One g of fresh or dried powdered sample was crushed in phosphate buffer (0.05M, pH 7.2) solution using mortar and pestle. The extracted material was then centrifuged in a cold centrifuge at 10,000 r min^{-1} for 15 min. The supernatant was collected and made up to 5 mL volume and stored at 4 ºC. For protein estimation, the sample was diluted 10 times or 100 times. To 1 mL of diluted solution 5 mL of alkaline reagent was added, mixed thoroughly and incubated at room temperature for 15 min. Then 0.5 mL of Folin-Ciocalteau solution was added, mixed thoroughly and incubated at room temperature for 20 min. Absorbance was measured in a colorimeter at 690 nm. Total soluble protein (TSP) content was calculated as BSA equivalents (BSAE) from a calibration curve of BSA and expressed as mg of BSAE g^{-1} of tissue weight. The experiment was performed in triplicates (n = 3).

3.3.3. Total lipid content

The total lipid extraction and estimation was performed following the method described by Bligh and Dyer (1959) with minor changes. Briefly, 1 g of dried powdered or fresh sample was macerated with 10 mL of distilled H_2O for 5min, followed by the addition of 30 mL of chloroform-methanol (2:1, v/v). The resultant mixture was mixed thoroughly in a beaker and kept overnight at room temperature. To this, 20 mL of chloroform and 20 mL of distilled H_2O was added and centrifuged at 1000 rpm for 10 min. Three distinct layers were formed after centrifugation, out of which the lowermost layer of chloroform containing lipid was collected in a beaker. The chloroform phase was then kept in an oven for one hour at 50 ºC for complete evaporation of chloroform. Weight of the beaker was taken and the lipid content was expressed as mg g^{-1}tissue weight.

3.3.4. Ascorbic acid content

Ascorbic acid (Vitamin C) was determined according to the method of Klein and Perry (1982). A fine powder of sample (200 mg) was extracted with *m*-phosphoric acid (1%, 10.0 mL) for 45 min at room temperature and filtered through Whatman filter paper (Grade 4, Ø 90 mm). The filtrate (1.0 mL) was mixed with 9.0 mL of50 mM 2, 6-dichloroindophenol (DCIP) and the absorbance was measured within 30 min at 515 nm

against a blank. Content of ascorbic acid was calculated on the basis of the calibration curve of standard L-ascorbic acid and the results were expressed as mg of ascorbic acid equivqlents g^{-1} of dry weight sample. The assay was performed in triplicate (n = 3).

3.3.5. α-Tocopherol content

α-Tocopherol (Vitamin E) in plant sample was extracted and estimated following the method of Jayaraman (1996) with minor modifications. The dried sample (0.5 g) was taken with 6 mL of hexane and shaken vigorously. The mixture was then filtered using whatman filter paper. The filtrate (2 mL) was taken in the test tube and 2 mL of absolute ethanol was added to it and mixed thoroughly. Further, 0.2 mL of 2, 2'-Bipyridyl solution (0.5 % in ethanol) and 0.2 mL of ferric chloride solution (0.2 % in ethanol) was added, shaken properly and incubated in dark for 15 min. After incubation, 4 mL of distilled water was added to the red colored solution and mixed well. Two distinct layers were formed which was then separated by the separating funnel. The red coloured aqueous layer was collected which was observed to be stable for 30 min. The absorbance was measured against the appropriate blank at wavelength 520 nm in a spectrophotometer. The α-tocopherol content was quantified using a standard curve of α-tocopherol and expressed as mg Tocopherol equivalent (TE) g^{-1}dry weight sample. The experiment was performed in triplicate (n = 3).

3.4. Analysis of major phytochemical constituents

3.4.1. Qualitative analysis

A number of chemical screening tests are in use to detect different biologically active chemical constituents present in different plant extracts and therefore help to characterize them phytochemically. Preliminary phytochemical analyses of the crude plant extracts were performed following standard test methods (Harborne, 1973; Trease & Evans, 1989; Sofowora, 1993; Kokate, 1994; Kokate *et al.*, 1995; Raman, 2006).

3.4.1.1. Test for reducing sugars

Fehling's test: To 1 mL of crude extract, 3 mL of distilled water was added. Then few drops of Fehling's A and Fehling's B reagents were mixed and gently boiled for few min. Formation of orange-red or brick-red precipitation at the bottom of the test tube confirmed the presence of reducing sugars.

Benedict's test: One mL of crude extract when mixed with few drops of Benedict's reagent and boiled for few min, a reddish-brown precipitation was formed indicating the positive result for reducing sugars.

3.4.1.2. Test for phenols

Ferric chloride test: One mL of crude extract was treated with 1 mL of neutral ferric chloride solution. Formation of a deep bluish-green solution gave an indication of the presence of phenols.

3.4.1.3. Test for flavonoids

Alkaline reagent test: To 1 mL of crude extract, 1 mL of dilute NaOH solution was added. Appearance of a yellow fluorescent color which turned colorless on addition of few drops of dilute HCl depicted the presence of flavonoids.

Shinoda test: To 1 mL of crude extract, 1-2 magnesium turnings were added followed by the addition of 1 or 2 drops of concentrated HCI and then boiled it for few min. Appearance of reddish pink or magenta-red or dirty brown or orange color indicated the positive result.

3.4.1.4. Test for resins

To 1 mL of crude extract, few drops of acetic anhydride solution and 1 mL of concentrated H_2SO_4 were added. Appearance of color ranging from orange to yellow suggested the presence of resins.

3.4.1.5. Test for free amino acids

Ninhydrin test: One mL of crude extract when boiled with few drops of 1% Ninhydrin (5.0 mg of ninhydrin in 100 mL of actone), would result in the formation of blue or violet colour indicating the presence of free amino acids.

3.4.1.6. Test for tannins

Lead acetate test: To 1 mL of crude extract, few drops of 10 % lead acetate solution was added to it. Formation of a bulky white precipitation confirmed the presence of tannins.

3.4.1.7. Test for alkaloids

Mayer's Test: To 1 mL of extract, a drop or two of Mayer's reagent were added along the side of the test tube. A white or creamy precipitation indicated the test as positive.

Wagner's test: To 1 mL of extracts, 2-3 drops of Wagner's reagent were added along the side of the test tube. A reddish-brown precipitation confirmed the presence of alkaloids.

3.4.1.8. Test for glycosides

NaOH test: To 1 mL of sample extract, 1 mL of distilled water and 1 mL of aqueous solution of NaOH were added. Formation of yellow color confirmed the presence of glycosides.

3.4.1.9. Test for steroids

One mL extract extract was taken in a test tube and 4 mL of acetic anhydride was added to it and shaken carefully. Then 2-3 drops of concentrated H_2SO_4 was added slowly. Brown ring at the junction of mixture suggested the presence of steroids. Test tube was kept in ice as it's an exothermic reaction.

3.4.1.10. Test for saponins

Frothing test: One mL of sample extract was added to 2 mL of distilled water. The mixture was shaken vigorously for few min. Formation of a stable persistent froth was indicated the positive result for saponins.

3.4.1.11. Test for cardiac glycosides

Keller-Kiliani test: To 1 mL of sample extract, 1 mL each of glacial acetic acid, ferric chloride ($FeCl_3$) solution and 1 mL of concentrated H_2SO_4 were added and mixed carefully. Formation of blue or green precipitation confirmed the presence of cardiac glycosides.

3.4.1.12. Test for phlobatannins

HCl test: To 1 mL of crude extract, few drops of 1 % HCl were added to it and heated for few min. Formation of red precipitation confirmed the presence of phlobatannins.

3.4.1.13. Test for cardenolides

Benzene test: One mL of crude extract was treated with 2 mL of benzene. Formation of a turbid brown color was indicated the presence of cardenolides.

3.4.2. Quantitativeanalysis

3.4.2.1. Total polyphenol content

The total polyphenol content was assayed with Folin-Ciocalteu reagent (FCR) using gallic acid as the standard (Taga *et al*., 1984). A 0.1 mL of extract was added to 2.0 mL of 2% aqueous solution of sodium carbonate. After 2 min, 0.1 mL of 50% FCR was added and the mixture was shaken thoroughly and left to stand for 30 min in the dark at RT. Absorbance was read spectrophotometrically at 750 nm. TPC was calculated as gallic acid equivalents (GAE) from a calibration curve of gallic acid standard solutions and expressed as mg of GAE g^{-1} of lyophilized extract. The experiment was performed in triplicate (n = 3).

3.4.2.2. Total flavonoid content

Total flavonoids were estimated using method described by Ordon-ez *et al*. (2006). To 0.5 mL of extract, 1.5 mL of methanol, 0.1 mL of 10 % aluminium trichloride hexahydrate, 0.1 mL of 1.0 M potassium acetate solution and 2.8 mL of ddH_2O water was added. After one and half hour of incubation at RT, the absorbance was measured spectrophotometrically at 420 nm against a blank. TFC was calculated as catechin equivalents (CAE) from a calibration curve of (+)-catechin standard solutions and expressed mg of CAE g^{-1} of lyophilized extract. The experiment was performed in triplicate (n = 3).

3.4.2.3. Total flavonol content

Total flavonols in the spice extracts were estimated using the method of Kumaran and Karunakaran (2007). To 2.0 mL of sample or standard, 2.0 mL of 2% aluminum trichloride (in methanol) solution and 3.0 mL of aqueous solution of sodium acetate (50 g L^{-1}) were added. The absorption at 440 nm was read spectrophotometrically after 2.5 h at 20 °C. Total flavonols (TFO) content was calculated as quercetin equivalents (QRE) and expressed as mg of QRE g^{-1} of lyophilized extract. The experiment was performed in triplicate (n = 3).

3.4.2.4. Total chlorophyll content

Chlorophylls were extracted from the samples following the method of Harborne (1998) with minor changes. About 1 g of the dried powder was crushed with 80% acetone in a mortar pestle and filtered through Whatman No.1 filter paper in a dark chamber. The residue was re-extracted with 80% acetone until it became completely

colorless attaining final volume up to 10 mL. Estimation for total chlorophyll, chlorophyll a and b was done using the method of Harborne (1998). The filtrate obtained (either crude or diluted) was taken directly in the test tube and the OD was measured at 645nm and 663 nm respectively in a UV-Vis spectrophotometer against a blank of 80% acetone. The content was calculated using the formula given by Arnon (1949): Total chlorophyll = (20.2 A_{645} + 8.02 A_{663}) mg g^{-1} fresh tissue weight. The experiment was performed in triplicate (n = 3).

3.4.2.5. Total carotenoid content

Carotenoids were extracted and estimated according to the method given by Lichtenthaler (1987). 1 g of the powdered material was soaked and crushed with 100% methanol in dark chamber and filtered using Whatman filter paper (Grade1). The total volume was made up to 10 mL with methanol and used for further analysis. Absorbances of the sample were observed spectrophotometrically at $\lambda_{663,}$ λ_{645}andλ_{480} against a blank in UV-Vis spectrophotometer 118 Systronics and the total carotenoid content (TCR) was calculated by using the formula: TCR = A_{480} – (0.114 A_{663} – 0.638 A_{645}) µg g^{-1}fresh tissue weight. The experiment was performed in triplicate (n = 3).

3.5. *In vitro* evaluation of antioxidant activity of herb and spice extracts

3.5.1. DPPH• free radical scavenging activity

The DPPH• free radical scavenging activity was monitored using the method of Yen & Duh (1994), with slight changes. Briefly, different aliquots (0.1 mL) of plant extract were added to 2.9 mL of freshly prepared solution of DPPH• (6 x 10^{-5} M in methanol). The mixtures were vortexed thoroughly for 15 s, and left in the dark at RT for 30 min. After incubation, the decrease in absorbance was recorded spectrophotometrically at 517 nm for all samples. Methanol was used to zero the spectrophotometer. Absorbance of the radical without antioxidant (control) was measured daily. Ascorbic acid was used as reference. Free radical scavenging activity ($FRSA_{DPPH•}$)expressed as percentage inhibition (% I) of the DPPH• radical was calculated according to the formula given by Viuda-Martos *et al*. (2010): DPPH• radical scavenging activity (% I) = [(A_c – A_s)/ A_c] x 100, where A_c refers to the absorbance (λ_{517}) of control (t=0 min) and A_s is the absorbance (λ_{517})of sample plus DPPH• (t = 30 min). The experiment was performed in triplicate (n = 3). Further, IC_{50} values for each extract and ascorbic acid was also calculated.

3.5.2. Hydrogen peroxide scavenging activity

The hydrogen peroxide (H_2O_2) scavenging assay was carried out following the procedure of Ruch *et al*. (1989). The principle of this method is that there is a decrease in absorbance upon oxidation of H_2O_2. A solution of 43.0 mM H_2O_2 was prepared in 0.1M phosphate buffer (*p*H 7.4). 1.0 mL extract in 3.4 mL phosphate buffer (0.1 M, *p*H 7.4) was added to 0.6 mL of 43.0 mM H_2O_2 solution and the absorbance of the reaction mixture was recorded at 230 nm after 10 min incubation at room temperature. A blank solution contained the sodium phosphate buffer (0.1 M, *p*H 7.4) without H_2O_2. The percentage of H_2O_2 scavenging by the extracts and standard were calculated using the following equation: H_2O_2 scavenging (HPS) activity (%) = $[(A_c - A_s) / A_c] \times 100$, where A_c was the absorbance (λ_{230}) of the control (without test sample) and A_s was the absorbance (λ_{230}) in presence of test sample. The experiment was performed in triplicate ($n = 3$).

3.5.3. Superoxide anion scavenging activity

The superoxide anion radicals ($O_2^{\bullet -}$) generated in a non-enzymatic PMS-NADH-O_2 system by oxidation of NADH and were detected by the reaction with NBT (Wang *et al*., 2002) based on the method described by Nishikimi *et al.* (1972). Reaction mixture contained 1.0 mL sample, 1.0 mL of NBT solution (312 µM prepared in phosphate buffer, *p*H 7.4) and 1.0 mL of NADH solution (936 µM prepared in phosphate buffer, *p*H 7.4). Finally, the reaction was accelerated by adding 0.1 mL PMS solution (120 µM prepared in phosphate buffer, *p*H 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min and absorbance at 560 nm was measured against a suitable blank. L-ascorbic acid was used as a positive control. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anions in the reaction mixture indicating the increased scavenging activity. Percentage of superoxide anion radicals scavenged was measured using the formula: Superoxide anion scavenging (SAS) activity (% I) = $[(A_c - A_s)/A_c] \times 100$, where A_c was the absorbance (λ_{560}) of the control, and A_s was the absorbance (λ_{560}) of the plant extract or L-ascorbic acid. The experiment was performed in triplicate ($n = 3$). Further, IC_{50} values for each extract and ascorbic acid was also calculated.

3.5.4. Nitric oxide (NO) scavenging activity

Nitric oxide scavenging activity of the extracts was determined using the protocol described by Packer and Ong (1998), and Jagetia *et al.* (2004). The reaction mixture containing 2 mL of sodium nitroprusside (10 mM), 0.5 mL of phosphate buffer saline (*p*H:7.4,0.1M) and 0.5 mL of the extract/standard (100-500 µg mL^{-1}) was incubated for 2.5 h at 25^{o}C. From the incubated solution, 0.5 mL solution was taken in the test tube and mixed with 1 mL of sulphanilic acid (0.33% in 20% glacial acetic acid). The mixture was allowed to stand for 5 min after which 1 mL of naphthylethylene diamine dihydrochloride (NED) (0.1% in distilled water) was added, mixed thoroughly and incubated for another 30 min at 25°C. The absorbance of the solution (pink chromophore) was read out at 540 nm against appropriate blank. Control was prepared taking all the reagents except the plant extract. L-ascorbic acid was taken as the reference standard. The nitric oxide scavenging percentage was calculated according to the formula: Nitric oxide (NO) scavenging activity (% I) = $[(A_c - A_s)/A_c] \times 100$, where A_c was the absorbance (λ_{540}) of the control, and A_s was the absorbance (λ_{540}) of the sample extract or L-ascorbic acid. The experiment was performed in triplicate ($n = 3$). Further, IC_{50} values for each extract and ascorbic acid was also calculated.

3.6. Evaluation of antibacterial activity of methanolic extracts of herbs and spices

The effect of methanolic extracts of herbs and spices on different bacterial strains was determined by standard Disc-agar diffusion (DAD) assay method. For quantitative determination of antibacterial activity, the minimum inhibitory dose (MID), *i.e.* minimum amount of extract per discrequired to inhibit the growth of the target bacterial strains was indicated by the presence of a zone of growth inhibition around the paper disc.

3.6.1. Preparation of media

Microbiological media, Nutrient agar, Mueller Hinton agar, Tryptone Soya broth used for the study were procured from Hi-Media, Mumbai, India. For antimicrobial assay, media were prepared according to the manufacturer's instructions. Media were suspended in distilled water and were dissolved completely by heating in microwave oven. The media were autoclaved for 15 min at 15 lbs pressure (121ºC) and then used in the experiments.

3.6.2. Preparation of filter-sterilized methanolic extract

The lyophilized crude extracts were reconstituted in the minimum volumes of the extracting solvent *i.e.*, methanol for desired concentrations. Prior to use, the reconstituted extracts were filter sterilized using disposable Millipore filter (pore size 0.22 µm) in the laminar air flowand further tested their sterility by streaking the extracts on sterile nutrient agar before being incubated for 24 h at 37 °C. A sterile extract was indicated by the absence of visible microbial growth in agar plate after incubation.

3.6.3. Target bacterial strains

Both Gram-positive and Gram-negative bacterial strains were used in the present study. The Gram-positive bacterial strains tested were *Bacillus cereus* MTCC 10655and *Baccilus pumilus* MTCC 1684 and Gram-negative bacterial strains tested were *Serratia marcescens* soilisolate (NCBI GenBank accession number JN 020963.1)and *Pseudomonas aeruginosa* MTCC 2453. The organisms were selected for antibacterial assay as these are the common pathogens either to animals or cause food spoilage. The pure culturess of these microbial strains were either obtained from the Microbial Culture Collection of Immuno-Phytopathology Laboratory, Department of Botany of University of North Bengal or from the Microbial Type Culture Collection and Gene Bank, Chandigarh, India.

3.6.4. Growth conditions and maintenance

The stock cultures of bacterial strains were maintained on slants of Nutrient Agar (NA), stored at 4 °C, and routinely sub-cultured once in a month to prevent morphological and metabolic transformations. Working bacterial cultures were prepared on Mueller Hinton Agar (MHA) slants on the day before experiment and were incubated for 24 h at 37 °C.

3.6.5. Preparation of bacterial inoculum

Active cultures were generated by inoculating a loopful of cells from a 24 h-old pure culture of the respective bacterial strains into 15 mL of Tryptone Soya Broth (TSB). The broth suspension was then incubated for 6-8 h at 37 °C on an orbital shaker (150 r min^{-1}). Bacterial cell suspension in physiological saline (0.85 % NaCl)was prepared from the flask broth culture in such a way that yielded a cell concentration of about 10^8 cfu mL^{-1}. The bacterial suspension so obtained was used as the final inoculum for the

susceptibility assay. The suspension of the inoculum was tested within 10-15 min or stored temporarily at 4 ^{o}C until use. Besides, dilutions of the inoculum in saline solution were plated on solid medium (NA) to verify the absence of contamination and to check the validity of the inoculum.

3.6.6. Preparation of paper disc

Paper discs (Ø 5.5 mm) were prepared from Whatman filter paper (Grade 1) with the help of punching machine and were sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 min. After sterilization, the moist discs were dried on hot air oven for 5-10 min at 50 ºC. The methanolic plant extracts of different concentrations (doses) ranging from 4-10 mg disc^{-1} were prepared by flooding the discs with adequate volumes of respective extracts from the stock solutions and evaporating the solvent from the discs completely in the laminar air flow. The commercially available discs of standard antibiotics were used as positive control and solvent disc so prepared as the negative control.

3.6.7. Disc-agar diffusion assay

The antibacterial activity of the methanolic extracts was carried out following standard Disc-agar diffusion method(Bauer *et al*., 1966). About 20 mL of MHA medium (45 ºC) was poured in the sterilized Petri dishes (Ø 70 mm) and allowed to stand about 15 min for solidify in the laminar air flow. A 0.1 mL of standardized inoculum suspension was pipetted out and swabbed to inoculate the entire surface of a solidified MHA plate using a sterile non-toxic cotton swab. About 5-10 min were allowed to dry and then previously prepared discs with specific concentration of plant extracts were aseptically placed onto the seeded medium with sterile forceps and gently pressed down to ensure complete contact of the discs with the agar surface. Each Petri plate contained four radially placed discs along with a disc of solvent control in the middle. All Petri plates were sealed with a strip of parafilm and incubated for 18-24 h at 37 ^{o}C. All the tests were performed in duplicate. Antibacterial activity was determined by measuring the diameter of the zones of inhibition, a clear area devoid of visible bacterial growth produced by the respective extracts. The diameter of the zone of inhibition around each disc was recorded in millimetre (mm).

3.6.8. Determination of minimum inhibitory dose

The minimum inhibitory dose (MID) is regarded as the lowest amount of an antimicrobial agent that produces a visible zone of growth inhibition. The MID of a

plant extract to a microbial strain gives a quantitative estimation in antimicrobial assay. In the present study, MID method was applied to the extracts that proved their high efficacy against the test bacterial strains. The MID of a bacterial strain was determined by impregnating paper discs with plant extracts at a concentration ranging from 10-0.5 mg $disc^{-1}$ (10, 9.5, 9.0, 8.5, 8.0, 7.5, 7.0, 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5, 1.25, 1.0, 0.5 mg $disc^{-1}$).The discs loaded with plant extracts were then placed aseptically into MHA plates inoculated with the target bacterial strains for accessing growth inhibition (Kariba *et al*., 2001; Mwitari *et al*., 2013).

3.7. Evaluation of anti-quorum sensing activity of different herbs and spices

The anti-QS activity or QS-inhibitory activity of herb and spice extracts was evaluated using the *Chromobacterium violaceum* and *Pseudomonas aeruginosa* biosensor systems. *Chromobacterium violaceum* MTCC 2656, a typical pigmented indicator strain produces a short acyl-homoserine lactone (acyl-HSL) signaling molecule which regulates production of dark purple pigment, violacein under QS-control. Thus, inhibition of short acyl-HSL in *C. violaceum* MTCC 2656is indicated by inhibition of violacein production. Disc-agar diffusion (DAD) assay was performed to detect inhibition of violacein production by plant extracts, wherein a colorless, opaque, but viable halo due to loss of pigmentation around the discs against a purple lawn. The plant extracts which inhibited violacein production were further tested to explore their effects on QS-mediated virulence factors or QS-modulatory activities such as pigment production, motility pattern and biofilm-forming ability of *Ps. aeruginosa* MTCC 2453.

3.7.1. Preparation of media

Microbiological media such as Nutrient Agar, Nutrient Broth, Luria Bertani Broth and Luria Bertani Agar used for the anti-QS study were procured from Hi-Media, Mumbai, India. Media were prepared according to the manufacturer's instructions. Briefly, media were suspended in distilled water and were dissolved completely by heating in microwave oven. The media were autoclaved for 15 min at 15 lbs pressure (121ºC) and then used in anti-QS assay.

3.7.2. Preparation of filter-sterilized methanolic extract

The powdered samples were subjected to methanolic extraction following the modified method of Cheesbrough (2000) as described earlier. Extracts were weighted and reconstituted in HPLC grade methanol for making desired concentrations and stored at

-20°C until use. The reconstituted extracts were filter sterilized using disposable Millipore filter (Ø 0.22 µm) in the laminar air flowand further tested their sterility by streaking the extracts on sterilized agar medium before overnight incubation at 37 °C. A sterile extract was indicated by the absence of visible microbial growth on agar medium.

3.7.3. Growth conditions and maintenance

The pure cultures of *Chromobacterium violaceum* MTCC 2656 and *Pseudomonas aeruginosa* MTCC 2453 were maintained on slants of Nutrient Agar (NA) and stored at 4 °C, and routinely sub-cultured once in a month. Working bacterial cultures were prepared on Luria Bertani Agar (LB agar) slants from frozen stocks and incubated at 37 °C for 24 h before the day of experiment.

3.7.4. Preparation of standard inoculum

Active cultures were generated by inoculating a loopful of cells from a 24 h-old pure culture of *C. violaceum* 2656 and *Ps. aeruginosa*2453 into 15 mL of Luria Bertani broth (LB broth).The broth suspensions were then incubated for 6-8 h at 37 °C on an orbital shaker at 150 r min^{-1}. Bacterial cell suspensions in sterile physiological saline (0.85 % NaCl)were prepared from the flask broth cultures in such a way that yielded a cell concentration of aproximately 10^8 cfu mL^{-1}. The bacterial suspensions so obtained were used as the standard inoculum for the anti-QS activity test. The suspension of the inoculum was tested within 10-15 min or stored temporarily at 4 ^oC until use. The validity of inoculum was checked by pour plating.

3.7.5. Preparation of paper disc

Paper discs (Ø 5.5 mm) were prepared from Whatman filter paper (Grade 1) with the help of punching machine and were sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 min. After sterilization, the moistened discs were dried on hot air oven for 3-5 min at 50 ºC, whenever necessary. The methanolic plant extracts of different concentrations (doses) ranging from 3-10 mg $disc^{-1}$ were prepared by flooding the discs with adequate volumes of respective extracts from the stock, followed by the complete evaporation of the solvent part from the discs in the laminar air flow.

3.7.6. Preliminary screening test for anti-QS activity against *C. violaceum* 2656

Preliminary screening test for anti-QS potency was evaluated by placing the ethanol-washed whole or fragment of herb or spice sample directly onto a prepared *C.violaceum* plate. Briefly, LB agar plates (Ø 70 mm, 20 mL) were prepared and swabbed with 0.1 mL of standard suspension ofthe indicator strain *C. violaceum* 2656. Then surface sterilized plant sample (dipped in ethanol for 15 min and then dried in laminar air flow) was placed in agar plate and anti-QS activity was detected by the presence of an area of pigment inhibition. Control plate (without *C. violaceum* 2656) was also set for individual plant sample to check the surface sterilization. The test was performed in duplicate set.

3.7.7. Bioassay for anti-QS activity against *C.violaceum* 2656

3.7.7.1. Qualitative bioassay

3.7.7.1.1. Paper disc-agar diffusion assay

The integral paper disc-agar diffusion method proposed by Adonizio *et al.* (2006) was employed to detect anti-quorum sensing activity qualitatively. A 0.1 mL of standard inoculum suspension of *C. violaceum* 2656 was swabbed over the surface of LB agar using sterilized cotton swabs. After few minutes, previously prepared discs with specific concentration of plant extracts were aseptically placed onto the seeded medium with sterile forceps and gently pressed down to ensure complete contact of the discs with the agar surface. Each Petri plate contained a disc of solvent control (methanol) also. Petri plates were sealed with a strip of parafilm and placed in incubator for 18-24 h at 37 ^{o}C. The tests were performed in duplicate. After O/N incubation Growth and/or pigment inhibition around the disk was observed. A positive quorum-sensing inhibitory result was indicated by de-pigmentation of the indicator strain in the vicinity of the test extract. Digital photographs were taken of all the plates using a Nikon camera.

3.7.7.2. Quantitative bioassay

3.7.7.2.1. Violacein inhibition assay

Inhibition of violacein production in the presence of methanolic extracts was quantified using previously described protocol with some modifications (Choo *et al.*, 2006).Briefly, 15 mL LB broth containing different concentrations of plant extract (100-1000 μg mL^{-1}, 250 μL) was inoculated with 100 μL of *C. violaceum* 2656 and

incubated at 37 °C for 24 h under shaking at 150 r min^{-1}. After O/N incubation, bacterial cell culture were lysed by 10% SDS (1:1, v/v) and incubating them at room temperature for 5 min. Then 1 mL of cell lysed culture was centrifuged at 13,000 r min^{-1} for 10 min at 28 °C to precipitate the insoluble violacein. The culture supernatant was discarded and the cell pellet was re-suspended evenly in equal volume of water saturated n-butanol (n-butanol:distilled water, 5:1, v/v) and centrifuged again (13,000 r min^{-1}, 10 min, 28 °C) to remove the cell debris. The supernatant (upper n-butanol phase) containing violacein was quantified spectrophotometrically at 585 nm. The percentage of violacein inhibition was calculated by following the formula: % violacein inhibition = $(C_{OD585} - T_{OD585} / C_{OD585}) \times 100$, where C_{OD585} and T_{OD585}were optical density of control and test extract at 585 nm respectively (Packiavathy *et al*., 2012). The cell viability in the culture medium was tested by standard plate count method for validation. The experiment was performed in triplicate ($n = 3$).

3.7.8. Determination of effects on *PA* 2453 QS-mediated virulence factors

Effect of plant extracts on widely studied QS-mediated virulence factors such as pyocyanin synthesis, swarming motility and biofilm-forming ability was tested in *Ps. aeruginosa* MTCC 2453.

3.7.8.1. Pyocyanin inhibition assay

The quantitative estimation of pyocyanin is based fundamentally on the spectrophotometric absorbance of extracted pyocyanin at 520 nm in acidic medium. Pyocyanin was extracted and estimated from *Ps. aeruginosa* 2453culture supernatant as previously described method (Essar *et al*., 1990; Kong *et al*., 2005) with slight modifications. Briefly, 250 µL of extracts (100-1000 µg mL^{-1}) were mixed thoroughly with 15 mL of freshly prepared LB broth. Afterwards, the broth was inoculated with 100 µL of 24 h-old culture of *Ps. aeruginosa* 2453 (OD_{600}=0.1) and thus incubated for 48 h at 37 °C. Broth culture without plant extract was set as the control. After 48 h of cultivation, culture of *Ps. aeruginosa* 2453was centrifuged at 10,800 *g* for 5 min at 28 °C. The resulting supernatant was mixed vigorously with chloroform in a ratio of 5:3 (v/v) and centrifuged again at 4600 *g* for 10 min at 28 °C. The lower chloroform phase was transferred to another centrifuge tube, mixed with 1 mL of 0.2 M HCl by shaking and then centrifuged for another 10 min at 4600 *g*. The upper HCl phase (pink to deep red coloration indicating the presence of pyocyanin) was taken to read at OD_{520} in an UV-Vis spectrophotometer against a blank of 0.2 M HCl. The OD_{520} reading was

normalized by dividing the final OD_{600} value of the culture. The experiment was performed in triplicate set (n = 3).

3.7.8.2. Anti-swarming assay

A modified method after Rashid and Kornberg (2000) was adopted for anti-swarming assay. Briefly, 24 h-old single colony of *Ps. aeruginosa*2453 was point inoculated by using the sharp end of a sterilized toothpick at the center of the Petri plate containing LB agar (0.5% agar, supplemented with filter sterilized 1.0 % D-glucose) with or without varied concentrations of test extract (250 µL of extracts, 250-1000 µg mL^{-1}). After 24-48 h of incubation in upright position at 37 ℃, the extent of motility was determined by measuring the diameter of the bacterial colony and the mean value of the swarmer's diameter was assigned.Anti-swarming assay was performed in triplicate set (n = 3).

3.7.8.3. Protease inhibitory assay

To determine the efficacy of the plant extracts for inhibiting extracellular protease production, skim milk agar assay was followed. Briefly, 24 h-old single colony of *Ps. aeruginosa* 2453 was point inoculated by using the sharp end of a sterile toothpick at the center of the Petri plate containing 20 mL of skim milk agar with or without plant extract of different concentrations (250 µL, 200-500 µg mL^{-1}). After 24-48 h of incubation at 37 ℃ in upright position, the zone of proteolysis was determined by measuring the halo area around the colonies formed due to casein hydrolysis. Also diameter of the colony was measured and relative proteolytic activity was determined by the formula: diameter of the zone of proteolysis/diameter of the colony. The experiment was performed in triplicate set (n = 3).

3.7.8.4. Biofilm inhibition assay

Biofilm inihibition assay was performed following the method of Chong *et al.* (2011) with some modifications. An overnight broth culture of *Ps. aeruginosa* 2453 was adjusted to an OD_{600} of 0.4 and 0.1 mL of culture further grown in 15 mL of fresh LB medium supplemented with filter-sterilized 0.5% (w/v) D-glucose and 250 µL of test extract of different concentrations ranging from 100-1000 µg mL^{-1}. Cells grown in the absence of plant extract served as negative control. To facilitate biofilm formation, *Ps. aeruginosa* 2453 cells were incubated statically for 24 h at 37 ºC in sterile tissue culture tubes. The planktonic bacterial cells were discarded by washing with sterile distilled

water, and the tubes were air dried for 15 min and latter on stained with 1 mL of 0.1 % crystal violet (v/v) for 30 min. The stained biofilms were washed several times with sterile distilled water to rinse off the excess crystal violet, followed by the addition of 4 mL of ethanol (95%, v/v). The resulting solution (2 mL) was transferred to an ethanol-washed cuvette, and the absorbance was read spectrophotometrically at 590 nm against an ethanol blank. The experiment was performed in triplicate (n = 3).

3.8. Evaluation of *in vivo* antidiabetic activity of methanolic extracts of herb and spice

3.8.1. Selection of plant material

Two plant species selected for the screening of antidiabetic activities were *Glinus oppositifolius* (*Go*) and *Illicium verum* (*Iv*). Such plants were selected on the basis of the following reasons:

- traditionally used as antidiabetics in folk medicine;
- written records in Ayurvedic formulations;
- scientific validation of anecdotal evidence, and
- searching for new lead compounds for drug discovery, etc.

3.8.2. Reconstitution of lyophilized methanolic extract into aqueous extract

For evaluating the hypoglycaemic or antidiabetic activity powdered plant samples were subjected to solvent extraction process following the method of Cheesbrough (2000) as described earlier. Prior to experimentation, solvent free crude methanolic extracts were weighted and reconstituted in sterile distilled water to make stock solutions of desired concentrations ranging from 2000 mg mL^{-1} to 250 mg mL^{-1} and stored at 4 °C during the experiments.

3.8.3. *In vivo* test for antidiabetic activity

3.8.3.1. Animal and housing condition

Healthy Swiss albino adult male rats (*Rattus norvegicus*, 150-200 g) were procured from the Ghosh Enterprise, Kolkata-55, West Bengal. They were acclimatized for a week to the experimental room (Animal House, Department of Botany) at normal atmospheric temperature of 25±2 ^{0}C, humidity (40-60%) and normal 12 h light/12 h dark photoperiod (Niyonzima and Vlietinck, 1993). At the end of adaptation period, the animals were divided randomly into groups and housed in well aerated

polypropylene cages and were fed with standard food pellets (Hindustan Lever, Kolkata, and India.) alternating with some soaked cereals (*Cicer* seeds) and water *ad libitum*.

3.8.3.2. Animal ethical clearance

Institutional Animal Ethical Committee (NBU) obtained ethical clearance for conducting experiments on animals from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). All procedures employed were reviewed and approved by the Animal's ethical Committee of University. The present work was carried out with a prior permission from the Animal's ethical Committee of University.

3.8.3.3. Acute TOX study

The extracts were studied for acute toxicity prior to the antidiabetic experimentation on animals according to OECD (Organization for Economic Cooperation and Development) guidelines (test 423: Acute oral toxicity- Acute toxic class method; 2002) (OECDiLibrary, 2002). Briefly, the rats were dosed once with 2000 mg kg^{-1} BW and monitored for 14 days for general clinical or toxicological signs and symptoms as well as mortality (Turner, 1965; Sundarranjan *et al.* 2011). Initially, the animals were observed continuously for 2 h under the following profiles a) Behavioural profile: alertness, restlessness, irritability, and fearfulness; b) Neurological profile: spontaneous activity-reactivity, touch response, pain response and gait; c) Autonomic profile: defecation and urination. After a period of 24 and 72 h they were observed for any lethality or death.

3.8.3.4. Induction of experimental diabetes

Experimental diabetes was induced by a single dose intraperitoneal (*i.p*) injection (1 mL kg^{-1} BW) of freshly prepared Streptozotocin (65 mg kg^{-1} BW) in citrate buffer (0.1M, pH 4.5) maintained at 37 ^{o}C, to rats fasted for 14 h (Siddique *et al.* 1987). Control rats received a similar volume of citrate buffer alone. STZ treated animals were allowed to drink 5% of D-Glucose for 2-4 h to prevent the potentially fatal hypoglycaemia shock as a result of partial destruction of pancreas. After 48 hours of streptozotocinization, fasting blood glucose levels of the animals were estimated using a glucometer (Accu-Chek, Active Blood Glucose meter, Roche Diagnostics India Pvt. Ltd.) through glucose oxidase-peroxidase method. Development of diabetes was

confirmed by elevated level of blood glucose, loss of body weight, polyuria, and glycosuria. Only rats that had fasting blood glucose levels above 250 mg dL^{-1} and glycosuria were considered as diabetic and were included in the present investigation.

3.8.3.5. Experimental design

In the present study, an experiment with 48 rats (6 normal plus 42 STZ-diabetic surviving rats) was conducted for three weeks (Table 6). The rats were distributed into eight groups each having six in number (n=6) as in the following manner:

Table 6: Experimental design followed in antidiabetic investigation

Group	Label	Treatment	Dose	Route	Schedule
I	NCR	CBS	1 mL kg^{-1} BW	*i.p*	Once in 3 Wk
II	DCR	STZ	65 mg kg^{-1} BW	*i.p*	Once in 3 Wk
III	DST	MET	10 mg kg^{-1} BW	*p.o*	OD for 3 Wk
IV	D*Iv*$_{250}$	*Iv*ME	250 mg kg^{-1} BW	*p.o*	OD for 3 Wk
V	D*Iv*$_{500}$	*Iv*ME	500 mg kg^{-1} BW	*p.o*	OD for 3 Wk
VI	D*Go*$_{250}$	*Go*ME	250 mg kg^{-1} BW	*p.o*	OD for 3 Wk
VII	D*Go*$_{500}$	*Go*ME	500 mg kg^{-1} BW	*p.o*	OD for 3 Wk

Note: NCR-Normal control rats, DCR-Diabetic control rats, DST-Diabetic rats with standard drug Metformin treatment, D*Iv*$_{250}$-Diabetic rats with *Iv*$_{250}$ treatment, D*Iv*$_{500}$-Diabetic rats with *Iv*$_{500}$ treatment, D*Go*$_{250}$-Diabetic rats with *Go*$_{250}$ treatment, D*Go*$_{500}$-Diabetic rats with *Go*$_{500}$ treatment, CBS-Citrate buffer solution, STZ-Streptozotocin, MET-Metformin, IvME-*Illicium verum*methanolic extract, GoME-*Glinus oppositifolius*methanolic extract, BW-Body weight, OD-Once a day, Wk-Week, *i.p*-intraperitoneal and *p.o*-per os/per orem.

The extracts were administered orally to the animals once daily for three weeks. For each group the dosage was adjusted every week according to the change in body weight to maintain similar dose per kg body weight of rat over the entire period of study. Blood glucose level (post-treatment) of rats was monitored on week 1, week 2, and week 3 as described earlier (pre-treatment).

3.8.3.6. Analytical procedure

3.9.3.6.1. Periodical measurement of body weight

Individual body weight (g) of all the experimental rats was recorded initially on week 0 and thereafter on week 1, week 2 and week 3 after the treatment.

3.8.3.6.2. Periodical estimation of blood glucose level

Fasting blood glucose level (mg dL^{-1}) was also recorded on week 0, week 1, week 2 and week 3 of the treatment. Blood was collected from the tail tip and the glucose levels were determined using glucometer-strip (Accu-Chek, Active Glucose meter) by glucose oxidase-peroxidase method.

3.8.3.6.3. Animal sacrifice

Three weeks after treatment, animals were fasted overnight and anaesthetized by dropping each in a transparent plastic jar saturated with chloroform vapour. Blood sample was collected through cardiac puncture and divided into plain and EDTA-containing centrifuge tubes. Humane procedure was adopted throughout the experiment.

3.8.3.6.4. Collection of blood and preparation of serum

Blood was collected in sterile Eppendorf tubes without anticoagulant and allowed to stand for 20-30 min at 4°C. Then, it was centrifuged at 1500 *g* for 10 min at 4°C. The serum got separated from the blood as an upper transparent liquid over the clotted blood. The serum was then collected in a separate sterile Eppendorf tubes without disturbing the residue and was store at –20 °C for studying different biochemical parameters.

3.8.3.6.5. Analysis of biological parameters

Sera were analyzed for the activities of alkaline phosphatise (ALP), alanine amino transferase (SGPT/ALT), aspartate amino transferase (SGOT/AST), total cholesterol, HDL-cholesterol, triglycerides, and for the bilirubin, creatinin and urea concentration.

3.8.3.6.5.1. Analysis of serum lipid profile

3.8.3.6.5.1.1. Estimation of total cholesterol

The total cholesterol level in the serum samples collected from all the groups of experimental rats were analysed using the manufacturer's instructions of

commercially available kit (Erba diagnostics Manheim GmbH, Mallaustr, Manheim/Germany). The estimation of cholesterol was performed by Dynamic extended stability with lipid clearing agent (CHOD-PAP method or modified Roeschlau's method) (Allian *et al.,* 1974; Roeschlau *et al.,* 1974).

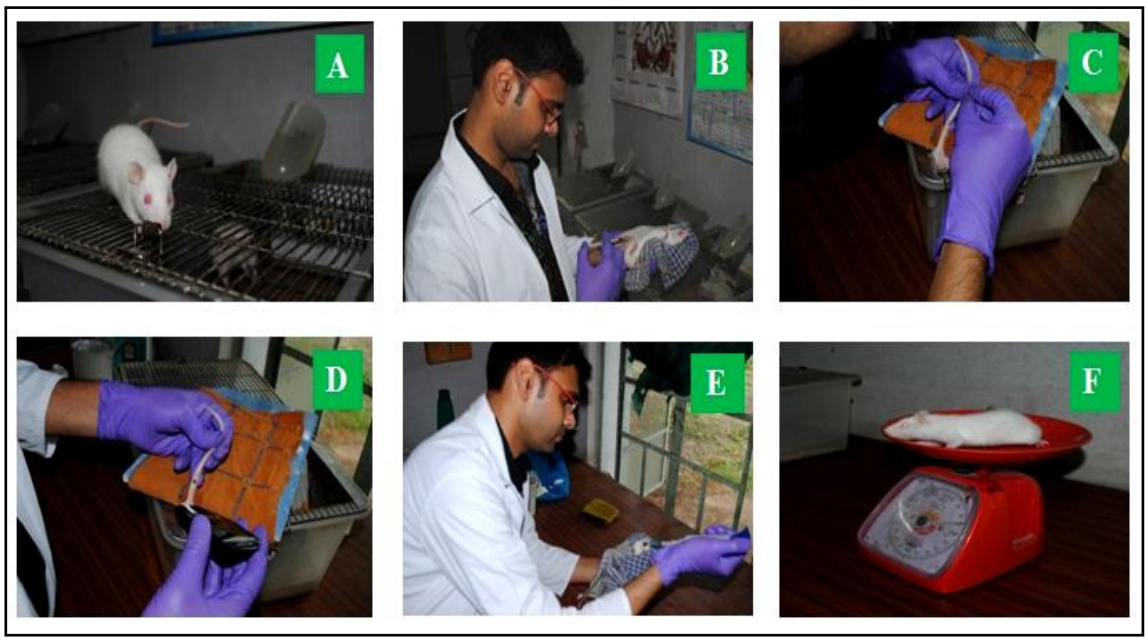

Figure 4: Illustration of maintenance and handling of rats for antidiabetic test. A. Rats housed in the polypropylene cage, B: STZ injected intraperitonially (i.p.) C: Blood being withdrawn from the tail vein of rats with the help of sterile needle, D: Estimation of blood glucose with glucometer, E: Feeding the rats with standard drug (Metformin)/plant extracts, F: Measurement of body weight.

Prior to experimentation working reagent was prepared by mixing available Cholesterol reagent (*i.e.*, the mixture of Cholesterol esterase (pancreatic), cholesterol oxidase (microbial), peroxidase (horseradish), sodium phenolate, 4-aminoantipyrine, phosphate buffer (*p*H 6.5±0.1) and lipid clearing agent) with appropriate amount of Aqua 4 (double deionized, 0.2 micron, membrane filtered, particle free water). Prior to use, all available reagent were first allowed to attain the room temperature (15-30 ºC). Then, 2 mL of the working reagent was mixed well with 40 µL of test sample (unhaemolyzed serum of the experimental rats) and allowed to incubate for 37 ºC for 10 min. Blank and standard was prepared by taking distilled water and available cholesterol standard (200 mg dL^{-1}) instead of the serum. The reaction mixture was aspirated and the absorbance was recorded at 505 nm. The cholesterol content was calculated using the formula as provided in the kit:

$$\text{Cholesterol mg dL}^{-1} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \text{ x Conc. of standard (mg dL}^{-1})$$

3.8.3.6.5.1.2. Estimation of triglycerides

Triglyceride content in the serum samples were analysed using the manufacturer's instructions of commercially available kit (Erba diagnostics Manheim GmbH, Mallaustr, Manheim/Germany). Triglycerides were estimated by Dynamic extended stability with lipid clearing agent (Glycerol Phosphate Oxidase-Trinder method) (Product Data sheet; McGowan *et al.*, 1983; Fossati and Prencipe, 1982; Trinder 1969). Briefly, supplied Triglycerides Des reagent (consisting of ATP, Mg^{2+}, 4-aminoantipyrine, 3-5 DHBS, Peroxidase, Glycerol Kinase, Glycerol Phosphate Oxidase (GPO), Lipoprotein Lipase, Buffer (*p*H 7.0±0.1 at 20 ºC) was mixed with Aqua 4 (double deionized, 0.2 micron, membrane filtered, particle free water) by gently swirling the mixture and was allowed to stand for 10min at room temperature. Following, reagent reconstitution 2 mL of the working reagent was then mixed with 20 µL of the serum/standard (200 mg dL^{-1}) and incubated for 10 min at 37 ºC). The absorbance was recorded at 505 nm. The triglycerides content was calculated using the following formula:

$$\text{Triglycerides (mg dL}^{-1}) = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \text{ x Conc. of standard (mg dL}^{-1})$$

3.8.3.6.5.1.3. Estimation of HDL-cholesterol

The estimation of HDL-cholesterol was done following the phosphotungstic acid method as described by Burstein *et al.* 1970 in manufacturer's instructions of commercially available kit (Erba diagnostics Manheim GmbH, Mallaustr, Manheim/Germany).The cholesterol working reagent (consisting the mixture of pancreatic Cholesterol esterase, microbial cholesterol oxidase, horseradish peroxidase, sodium phenolate, 4-aminoantipyrine, phosphate buffer (*p*H 6.5±0.1) and lipid clearing agent) was used to determine the concentration of HDL cholesterol in the sample. Prior, to the use of sample the HDL was precipitated from the sample using precipitating reagent (consisting of Phosphotungstic acid and magnesium chloride) was used to determine the concentration of HDL cholesterol in the sample. Briefly, 500 µL of sample was mixed thoroughly with 1000 µL of precipitating reagent and allowed to

stand for 10 min at room temperature (15-30 ºC). The reaction mixture was centrifuged at 4000 r min^{-1} (1800 *g*) for 10 min to obtain a clear supernatant. The clear supernatant (100 µL) was then mixed well with 2000 µL of cholesterol working reagent and incubated for 10 min at 37 ºC. The absorbance for each test samples and standard was read at 505 nm or 505/670 nm against appropriate reagent blank. The supplied HDL cholesterol standard was 25 mg dL^{-1}. The HDL cholesterol content in the serum sample was calculated using the following formula:

$$\text{HDL cholesterol (mg dL}^{-1}) = \frac{\text{Abs. of test}}{\text{Abs. of standard}} \text{ x Conc. of standard (mg dL}^{-1}) \text{ x dilution factor}$$

3.8.3.6.5.2. Analysis of liver function test (LFT)

3.8.3.6.5.2.1. Estimation of SGPT/ALT (Alanine aminotransferase) level

The ALT level in the serum samples were analysed using IFCC method (International Federation of Clinical Chemistry method, 1980) given in the manufacturer's instructions of commercially available kit (Erba diagnostics Manheim GmbH, Mallaustr, Manheim/Germany). Briefly, 1000 µL of the working reagent was mixed thoroughly with 100 µL of the test sample and aspirated. Then, the absorbance was immediately recorded at 340nm at an interval of 1min. The working reagent was prepared by reconstituting the reagents supplied in the kit. The reagent was composed of L-Alanine, NADH (yeast), lactate dehydrogenase, 2-oxoglutarate and tris buffer. Prior, to the use all the reagent bottle and Aqua-4 (double deionized, 0.2 micron, membrane filtered, particle free water) was allowed to attain room temperature (15-30 ºC). About 20 mL of Aqua-4 was added to the reagent containing vial, swirled gently and was used for the estimation. The ALT activity was determined using the following formula as given with the kit. First, the mean absorbance change obtained was converted into International Units (IU) of activity using the general formula provided in the kit as follows:

$IU\ L^{-1} = (\Delta A\ min^{-1})$ x T.V. x 10^3/ S.V. x Absorptivity x P; Where:

T.V. = Total reaction volume in µL

S.V. = Sample volume in µL

Absorptivity = millimolar absorptivity of NADH at 340nm= 6.22

P = cuvette lightpath= 1cm

Activity of ALT at 37 ºC (IU L^{-1}) = ($\Delta A\ min^{-1}$) x Factor (1768)

3.8.3.6.5.2.2. Estimation of SGOT/AST (Aspartate aminotransferase) level

The IFCC method (International Federation of Clinical Chemistry method, 1980) provided in the manufacturer's instructions of commercially available kit (Erba diagnostics Manheim GmbH, Mallaustr, Manheim/Germany) was used to determine theAST level in the serum samples of all the experimental animals. The working reagent supplied had the composition of 2-oxoglutarate, L-aspartate, malate dehydrogenase, lactate dehydrogenase, NADH (yeast), tris buffer and EDTA. Prior to the use, the supplied reagent bottle and Aqua-4 (double deionized, 0.2 micron, membrane filtered, particle free water) was allowed to attain the room temperature (15-30 ºC). The working reagent was prepared mixing the content of one reagents bottle with 20mL of Aqua-4 (double deionized, 0.2 micron, membrane filtered, and particle free water). For estimation, 1000 µL of the working reagent was mixed thoroughly with 100 µL of the test sample (unhaemolysed serum) and aspirated. The absorbance was immediately recorded at 340 nm at an interval of 60 sec. The AST activity was determined using the following formula as given with the kit. First, the mean absorbance change obtained was converted into International Units (IU) of activity using the general formula provided in the kit as follows:

IU L^{-1} = ($\Delta A\ min^{-1}$) x T.V. x 10^3/ S.V. x Absorptivity x P; Where:

T.V. = Total reaction volume in µL;

S.V. = Sample volume in µL

Absorptivity = millimolar absorptivity of NADH at 340nm= 6.22

P = cuvette lightpath= 1cm

Activity of AST at 37 ºC (IU L^{-1}) = ($\Delta A\ min^{-1}$) x Factor (1768)

3.8.3.6.5.3. Analysis of kidney function test (KFT)

3.8.3.6.5.3.1. Estimation of urea level

The estimation of urea level in serum was done following the GLDH-Urease method, as described by Talke and Schubert (1965); Tiffany *et al.* (1972) and provided with the commercially available kit (Erba diagnostics Manheim GmbH, Mallaustr,

Manheim/Germany). The urea working reagent (consisting of a mixture of α-ketoglutarate, NADH, urease, glutamate dehydrogenase (GLDH), ADP, Tris buffer (*p*H 7.9±0.1) was used to determine the concentration of urea in the serum sample. Prior to the use, the supplied reagent bottle and Aqua-4 (double deionized, 0.2 micron, membrane filtered, particle free water) was allowed to attain the room temperature (15-30 ºC). The working reagent was then prepared by gently mixing the content of one reagent bottle with 20 mL of Aqua-4. For estimation, 2000 µL of the working reagent was mixed well with 20 µL of the standard/test serum and aspirated. Immediately, absorbance was recorded at 340nm at an interval of 60 sec against appropriate blank. The rate of decrease in absorbance is directly proportional to urea concentration in the sample.The concentration of urea in the serum sample was estimated using the following formula:

$$\text{Urea (mg dL}^{-1}) = \frac{\Delta\text{A of test}}{\Delta\text{A of standard}} \text{ x Conc. of standard (mg dL}^{-1})$$

Where, ΔA (absorbance change for the standard and test) = A_1-A_2.

3.8.3.6.5.3.2. Estimation of creatinine level

A modified method after Jaffe described by Slot (1965) and Bartel *et al.* (1972) that was provided with the commercial kit (Erba diagnostics Manheim GmbH, Mallaustr, Manheim/Germany) was employed for the estimation of creatinine level in the sera. The working reagent was prepared by mixing equal amount of picric acid and sodium hydroxide reagent. The mixture was then allowed to stand for 15 min. For estimation, 2000 µL of the working reagent was mixed with 200 µL of standard/serum sample and the initial absorbance (A_1) was read at 20 sec after mixing followed by final absorbance (A_2) at 80 sec after mixing at wavelength 505 nm. The absorbance of the orange-yellow colour (Jaffe's reaction) formed is directly proportional to the creatinine concentration.

The creatinine concentration was calculated by the formula:

$$\text{Creatinine (mg dL}^{-1}) = \frac{\Delta\text{A of test}}{\Delta\text{A of standard}} \text{ x Conc. of standard (mg dL}^{-1})$$

Where, ΔA (absorbance change) = A_2 (final) - A_1 (initial).

3.9. Isolation, partial purification and characterization of bioactive compounds

Since the methanolic extracts of *Illicium verum (Iv*ME*)* and *Glinus oppositifolius* (*Go*ME) were found to possess significant pharmacological activity compared to other extracts, an attempt has been made to partially purify and characterize the various chemical compounds present in these extracts through solvent partitioning followed by Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

3.9.1. Preparation of methanolic extract by solid-liquid extraction

Twenty g of the fine powder of samples was extracted with 200 mL of HPLC grade methanol for 24 h under darkness and normal room temperature. The solution was filtered through a Whatman filter paper (Grade 1). This liquid phase of methanolic extract was used in solvent partitioning or liquid-liquid fractionation.

3.9.2. Solvent partitioning by liquid-liquid fractionation

The methanolic extract was transferred to a separating funnel and successively partitioned with n-hexane and ethyl acetate by liquid-liquid fractionation (LLF) based on polarity of the solvents. Briefly, the methanolic extract was poured in a separatingfunnel and n-hexane was added in equal quantity to the methanol phase and gently shaken. This process was repeated three times; hexane phase was separated from methanol phase and collected in a conical flask, and evaporated to dryness under reduced pressure at 40˚C. This fraction was further lyophilized to get solvent free mass fraction. Same procedure was also applied for ethyl acetate fraction. Thus three types of fractions i.e., methanolic fraction (ME), hexane fraction (HE) and ethyl acetate (EA) fraction were obtained.

3.9.3. Gas Chromatography-Mass Spectrometry analysis

In the present study, methanol, hexane and ethyl acetate fractions of *Illicium verum* and *Glinus oppositifolius* was used separately for carrying out the GC-MS analysis for various phytochemical compounds present in the plant samples. GC–MS analysis of the extract was carried out with GCMS-QP-2010 Ultra, Shimadzu, Japan with Thermal Desorption System TD 20. The instrument was equipped with programmable head space auto sampler and auto injector. The capillary column used was DB-1/RTXMS (30 m) with pure helium (99.999%) as a carrier gas, at a constant flow rate of 3 mL min^{-1} and 1 µL injection volume. Column oven temperature and injection temperature were set at 60 °C and 260 °C respectively. Samples were analyzed with the column

held at different temperatures. Pressure was established as 72.3 kPa and the sample was run for 60 min. Temperature and column flow for flame ionization detectors were set as 230 °C and 1.20 mL min^{-1}, correspondingly. MS parameters were as follows: scan range: 40 to 650 m/z with a scan speed of 3333. The relative percentage of the chemical constituents present in the samples was expressed as percentage by peak area normalization.

3.9.4. Identification of chemical compounds

The chemical compounds were identified on the basis of comparison of their retention time and mass spectra and computer matching with WILEY8.0 libraries and National Institute of Standards and Technology (NIST14.0) database provided with computer controlling the GC-MS system. The spectrum of the unknown component was compared with the spectrum of the known compounds stored in the library. The name, molecular weight and structure of the compounds of the test plants were ascertained.

3.10. Statistical analysis

All the data were subjected to various statistical analyses and expressed as mean ± standard deviation (SD). One way and two way analysis of variance (ANOVA) and LSD were employed for the statistical analysis of data using IBM SPSS statistic v. 21software. Level of significance *p*-value of less than 0.05 was considered to be statistically significant.