3. MATERIAL AND METHODS

3.1. Ethnobotanical survey and selection of plant materials

3.1.1. Survey of wetland plants

Locally available wetland plants in the districts of North Bengal region of West Bengal were collected. The plants were selected mostly on the basis of their importance in this area. Useful wetland plants were collected through survey based field observations. Several field trips to different wetlands of North Bengal were conducted to collect the plant samples from December 2012 to January 2015. Traditional medical practitioners of this region like Kaviraj, Jahakri, Janguru and locales with knowledge related to the use of wetland plants were interviewed to collect information regarding the importance of these plants. This study was also based on information gathered from the seller and consumers of the plants in market as well as from the people living in the vicinity of these wetland studied. The sellers and consumers were interviewed and discussed with questionnaires for collecting the information on the uses of these plants and the daily need, market value, seasonal variation of availability in markets. The collected data is based on first hand information and after that various literature were also discussed. The collected plants were dried and herbarium specimens were prepared which later on identified with the help of floras, herbaria as well as in consultation with Taxonomy experts. Final authentication was done at Taxonomy & Environmental Biology Laboratory, Department of Botany, University of North Bengal. Herbarium sheets of selected plants were submitted to the North Bengal University Herbarium, Department of Botany, University of North Bengal, India and accession numbers were obtained.

3.1.2. Selection of plant material for experimentation

Out of the surveyed plants with medicinal and other beneficial properties, 15 plants were finally selected for studying the potentiality of these plants as reported.

3.2. Preparation of plant extract

3.2.1. Preparation of dried plant powder

The collected wetland plants were primarily washed thoroughly (4-5 times) with normal tap water and then with distilled water and dried using blotting paper. The cleaned plant materials were covered with thin blotting paper and allowed to dry under the shade for about 10 to 21 days. Special care was taken to stop spoilage due to fungal degradation of plant material. The dried plant materials were ground to

obtain fine powder using mixer grinder (Jaipan, Super Deluxe, India) and was stored in glass bottles at 4°C till further use.

3.2.2. Preparation of methanol (MEOH) extract

With slight modification, the method described by Okwori *et al.* (2006) was used for the preparation of methanol extract. Powdered samples and methanol were mixed in the ratio of 1:10 (sample:solvent) and vigorously shaken for 20 min and then kept for 72h at room temperature while stirring for 40 min at an interval of 24h. The mixture was then filtered and the supernatant were concentrated in rotary evaporator, at 40°C, lyophilized and stored at -20°C until further use.

3.3. Phytochemical screening

3.3.1. Test for phenol

Presence of phenol in the methanolic extract was determined by following the method of Martinez and Valencia (2003). Briefly, 1 mL extract was mixed equal volume of 1% $FeCl_3$ and observed for the formation of blue or green colour which indicated the presence of phenols.

3.3.2. Test for flavonoid

The presence of flavonoid in the samples was done following the method of Evans (2002). Briefly, 2g of the sample was mixed with 10 mL of acetone and the acetone was evaporated by keeping the flask in a hot water bath for 5 min. After that the sample was extracted using 10 mL of warm double distilled water. The solution was mixed, filtered while hot and allowed to cool at room temperature. 5mL filtrate was then mixed with equal volume of 20% NaOH and observerd for the change in color of the solution to yellow indicating the presence of flavonoid**.**

3.3.3. Test for tannin

Presence of tannin in powered plant samples were determined by following the method of Jigna and Sumitra (2007) where 200 mg crude plant powder was mixed with 10 mL of double distilled water and incubated at room temperature for 10 min followed by filtration. 1mL of 5% $FeCl_3$ was added to 2 mL of the filtrate, the formation of yellow brown precipitate indicated the presence of tannin.

3.3.4. Test for alkaloid

200 mg of powdered sample was mixed with 10 mL methanol for 1h at room temperature and after filtration 2 mL of 1% HCL was added to equal volume of the filtrate. Then few drops of Mayer's / Wagner's reagent were added to the filtrate.

Apperance of creamish / brown / red / orange precipitate indicated the presence of alkaloid **(**Trease and Evans, 1989).

3.3.5. Test for cardiac glycosides

The method described by Trease and Evans (1989) was followed for the qualitative screening of cardiac glycosides. 2 mL methanolic filtrate was mixed with 1 mL glacial acetic acid, added 3-4 drops of 5% $FeCl_3$ and then 1 mL of concentrated H_2SO_4 was added carefully. Appearance of brown ring at the interface indicated the presence of cardiac glycosides.

3.3.6. Test for carbohydrates

Qualitative analysis of carbohydrates was done following the protocol of Evans (2002). Plant sample (0.5 g) was boiled in 30 mL of double distilled water and filtered. 2mL of Molish's reagent (5% α-naphthol in absolute ethanol) was then added to 2 mL of aqueous extract and shaken vigorously followed by addition of concentrated H_2SO_4. Presence of carbohydrate was confirmed by the formation of reddish-ring at the junction of two liquids.

3.3.7. Test for reducing sugars

Qualitative analysis of reducing sugars was done following the protocol of Evans (2002). Aqueous extract of the sample was prepared by boiling 500 mg sample in 30 mL of double distilled water followed by filtration. 1 mL of the filtrate was then mixed with 2 mL of Fehling's solution and boiled for 5 min. Appearnce of brick red precipitation at the bottom of test tube indicated the presence of reducing sugars.

3.3.8. Test for protein

About 1 g of the plant sample was mixed thoroughly with 10 mL of double distilled water following the method of Pullaiah (2006). Then 2 mL of the filtrate was mixed with 1 mL of 40% NaOH and 1-2 drops of $CuSO_4$. Change in the color of solution to violet indicated the presence of proteins.

3.3.9. Test for saponin

0.5 mL aqueous filtrate prepared as above was mixed with 5 mL of double distilled water and shaken vigorously for about 30 seconds following the protocol of Trease and Evans (1989). The presence of saponins was indicated by the formation and persistent frothing.

3.3.10. Test for terpenoid

For the test of terpenoid 2 mL of plant extract was mixed with 5 mL of chloroform and 2 mL of acetic anhydride. Then, 1mL of concentrated H_2SO_4 was

added carefully along the wall of the test tube, and the formation of reddish brown ring at the interface indicated the presence of terpenoid (Harborne, 1973).

3.3.11. Test for steroid

The methanolic filtrate (5 mL) was treated with 0.5 mL of anhydrous CH_3COOH followed by cooling on an ice bath for 15 mins. To the cold solution 0.5 mL of chloroform and 1 mL of concentrated H_2SO_4 was added. Presence of steroid was confirmed by the formation of reddish-brown ring at the junction (Gokhale and Kokate, 2008).

3.3.12. Test for anthraquinone

0.5g of powdered sample was added to 5 mL of chloroform, shaken for 5 min and filtered using Whatman No.1 filter paper. 3 mL filtrate was mixed with equal volume of 10% ammonia solution and shaken properly. Development of red / pink /violet color in the aqueous layer after shaking indicated the presence of free anthraquinone (Evans, 2002).

3.3.13. Test for amino acid

Few drops of ninhydrin reagent was added to 0.5 mL methanolic plant extract and then heated in a water bath. The presence of amino acid was indicated by the formation of purple colour (Kumar *et al*. 2009).

3.3.14. Test for resins

0.5 mL of extract was evaporated and dissolved in 2 mL of petroleum ether. After that, 2mL of 2% cupper acetate solution was then added, vigorously mixed and allowed to separate. Green colour of the solution indicated the presence of resin (Trease and Evans, 1983).

3.3.15. Test for glycosides

Methanolic extract (0.5 mL) was added to 2 mL of 50% hydrochloric acid and placed on a water bath for 2 hrs. 1 mL pyridine, few drops of 1% sodium nitroprusside solution and 5% sodium hydroxide solution were then added to the hydrolysed mixture. Appearance of pink to red colour indicated the presence of glycosides (Kumar *et al*. 2009).

3.3.16. Test for triterpenoids

0.5 mL of methanolic plant extract was evaporated and the residue was dissolved in 1mL chloroform. 1mL acetic anhydride was then added and after cooling, conc. H_2SO_4 was added. Appearance of violet colour confirmed the presence of triterpenoids (Kumar *et al*. 2009).

3.4. Quantification of biochemical components

3.4.1. Extraction and estimation of total phenols

3.4.1.1. Extraction

With minor modification the method given by Mahadevan and Sridhar (1982) was employed for the extraction of phenol. 1g of the powdered material was immersed in a beaker containing 10mL of boiling absolute alcohol and kept in dark for 10 mins. The sample was allowed to cool at room temperature and then crushed in motor pestle using 80% alcohol and filtered using Whatman No.1 filter paper. The residue was re-extracted with 80% alcohol making the final volume up to 10mL. The total procedure was carried out in diffuse light.

3.4.1.2. Estimation

The extract (1mL) was mixed with 1mL of 1N Folin ciocalteu's phenol reagent followed by 2mL of 20% Na_2CO_3 solution and was kept in boiling water bath for 1min. Under running tap water the reaction mixture was cooled and after that diluted with distilled water to make the final volume up to 25mL. The absorbance was taken at 650nm in a colorimeter and the concentration of total phenols was expressed as mg gallic acid (GAE) equivalents/ g dry weight sample (dw), using the standard curve of gallic acid (Bray and Thorpe, 1954).

3.4.2. Quantification of total soluble protein

3.4.2.1. Extraction

From dried powdered material the extraction of protein was done using the method of Chakraborty *et al.* (1995). 1g powdered sample was homogenized in a refrigerated motor and pestle with 5mL of 50 mM sodium phosphate buffer (pH-7.2) and polyvinyl-pyrrolidone under ice cold condition. The mixture was then centrifuged at 10,000 rpm at -4^0C for 15min. For further estimation the supernatant was collected and used.

3.4.2.2. Estimation

According to the method of Lowry *et al.* (1951) estimation of protein content in the extract was done. In short, 1mL of the extract was mixed with 5mL of freshly prepared alkaline reagent (2% Na_2CO_3 in 0.1N NaOH, to which 1mL each of 1% $CuSO_4$ and 2% Na^+ - K^+ tartarate was added just before use) and incubated for 15min. After that, 0.5mL of 1N Folin Ciocalteau's phenol reagent was added to the mixture which was further incubated for 20 min at room temoerature. The absorbance was measured at 690 nm in spectrophotometer against a proper blank. Quantification of

total protein was done by using a standard curve of Bovine serum albumin and expressed as mg Bovine serum albumin equivalent (BSAE)/ g dry weight sample (dw).

3.4.3. Extraction and quantification of flavonoid

3.4.3.1. Extraction

The extraction procedure of flavonoid, described by Mahadevan and Sridhar (1982) was followed with minor modification. 1g dried powdered material was immersed in boiling methanol (10mL) for 5-10 min and then filtered. The final volume was made upto 10 mL.

3.4.3.2. Quantification of flavonoid

The flavonoid content was estimated using the method of Sultana *et al*. (2009). The extract (1 mL) was mixed with 4mL of distilled water and 300µL of 5% $NaNO_2$ and allowed to incubate for 5min at room temperature. After 5min of incubation 300 µL of 10% $AlCl_3.6H_2O$ was added. Next, at 6^{th} min 2mL of NaOH, and 2.4 mL of distilled water was added and mixed properly. Absorbance of the reaction mixture was recorded at 510 nm in UV-VIS spectrophotometer (Model 118 systronics) against a proper blank solution. The total flavonoid content was expressed as mg quercetin equivalents (QE)/ g dry weight sample (dw) using the standard curve of quercetin.

3.4.4. Extraction and quantification of Amino acid

Free amino acids were estimated with standardized protocol with slight modifications (Moore and Stein 1948). 0.5 g leaf tissue was extracted in ethanol and filtered using Whatman No. 1 filter paper. To 1 ml of the filtrate, 1 ml of ninhydrin reagent was added and boiled in a water bath for 20 min. The absorbance was read at 570 nm and the free amino acid content was estimated using a standard curve of L-proline.

3.4.5. Extraction and estimation of carbohydrates

3.4.5.1. Extraction of total and reducing sugar

Total and reducing sugar was extracted following the method of Harborne (1973). 1 g of tissue was extracted in 10 ml of 95% ethanol and the alcoholic fraction was evaporated on a boiling water bath. The residue was reextracted with ethanol and the process was repeated 3 times. Then the residue was dissolved in dH_2O and the

final volume was made up to 5 ml which was then centrifuged at 5000 rpm for 10 min. The supernatant was collected and used for estimation.

3.4.5.2. Estimation of total soluble sugar

Estimation of total sugar was done by Anthrone reagent following the method of Plummer (1978). To 1 ml of test solution, 4 ml of Anthrone reagent (0.2% Anthrone in conc. H_2SO_4) was added. The reaction mixture was mixed thoroughly and was incubated in boiling water bath for 10 mins. Then the reaction mixture was cooled under running tap water and absorbance was measured in a colorimeter at a 50 wavelength of 620 nm and sugar content was quantified using a standard curve of D-glucose.

3.4.5.3. Estimation of reducing sugar

Reducing sugar was estimated by Nelson-Somogyi method as described by Plummer (1978). 1 ml of the test solution was mixed with 1 ml of alkaline copper tartarate solution (2 g $CuSO_4$, 12 g Na_2CO_3 anhydrous, 8 g Na-K tartarate, 90 g Na_2SO_4 anhydrous in 500 ml of dH_2O) and heated over a boiling water bath for 20 mins. The reaction mixture was then cooled under running tap water and 1 ml Nelson's Arsenomolybdate reagent was added along with 2 ml of dH_2O and mixed vigorously. A blue colour was developed, the absorbance of which was the measured in a colorimeter at 515 nm and reducing sugar content was quantified using a standard curve of D-glucose.

3.4.6. Extraction and estimation of chlorophylls

3.4.6.1. Extraction of chlorophylls

From the samples chlorophyll was extracted following the method of Harborne (1998) with minor changes. 1g of the dried powder was crushed with 80% acetone in a mortar pestle and filtered through Whatman No.1 filter paper in a dark room. The residue was re-extracted with 80% acetone until it became completely colorless attaining final volume upto 10mL.

3.4.6.2. Estimation of chlorophylls

Total chlorophyll, chlorophyll a and b were estimated using the method of Harborne (1998). The crude or diluted filtrate was taken directly in the cuvettes and the absorbance was measured at 663nm and 645nm respectively in a UV-VIS spectrophotometer against a blank of 80% acetone. Using the formula given by Arnon (1949), the chlorophyll content was calculated.

Total chlorophyll = $(20.2\ A_{645} + 8.02\ A_{663})$ mg g^{-1} dry weight

Chlorophyll a = $(12.7\ A_{663} - A_{645})$ mg g^{-1} dry weight

Chlorophyll b = $(22.9\ A_{645} - 4.68\ A_{663})$ mg g^{-1} dry weight

3.4.7 Determination of carotenoid content

3.4.7.1. Extraction

Following the method of Lichtenthaler (1987), the carotenoid was extracted from the samples. About 1g of the powdered material was soaked and crushed with 100% methanol in dark condition and filtered using Whatman filter paper No.1. The final volume was made upto 10mL with methanol and used for further experiment.

3.4.7.2. Estimation

Carotenoid content was estimated by the procedure of Lichtenthaler (1987). The desired amount of crude or diluted filtrate was taken directly into the cuvate and the absorbance was taken at 480nm, 645nm and 663nm wavelength against a blank in UV-VIS spectrophotometer 118 systronics. The amount of carotenoid was calculated using the standard formula as follows:

$$A_{480} - (0.114 \times A_{663}) - 0.638\ (A_{645})\ \mu g\ g^{-1} \text{ dry weight}$$

3.4.8. Quantification of Ascorbic Acid (Vit C)

3.4.8.1. Extraction

Mukherjee and Choudhuri (1983) described method for the extraction of Ascorbic acid from the powdered samples. The samples were crushed in pre-chilled motor and pestle on ice using 6% trichloroacetic acid (10mL) and filtered at chilled condition (0°C). The final volume of the filtrate was made upto 10mL by adding trichloroacetic acid and used for further estimation of ascorbic acid.

3.4.8.2. Estimation

For this estimation, 4mL of the extract, 2mL of 2% Dinitrophenylhydrazine (in acidic medium) and 1 drop of 10% Thiourea (70% ethanol) was added one after another and mixed thoroughly. The reaction mixture was then kept in boiling water for 15min and cooled at chilled condition in ice. To the reaction mixture, 5mL of 80% (v/v) sulphuric acid (H_2SO_4) was added at 0°C. The absorbance was taken at 530nm against a blank in UV-VIS spectrophotometer. The concentration was determined from the standard curve of ascorbic acid and expressed as mg ascorbic acid equivalent (AAE)/g dry weight sample (Mukherjee and Choudhuri 1983).

3.5. Determination of anti-oxidative activities of extracts

3.5.1. DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity

The DPPH (2,2-diphenylpicrylhydrazyl) assay measures the free radical scavenging capacity of the extracts under investigation (Blois 1958). Different concentrations of plant extracts (0.1 mL) were put in the test tube and 2.9 mL of a methanol solution of DPPH (0.1 mM) was added. The mixture was kept in the dark at room temperature for 30 min and absorbance was measured at 517 nm against a blank. The same procedure was used for the vitamin C (1 mg/mL) used as standard. The following equation was used to determine the percentage of the radical scavenging activity of each extract.

$$\text{Scavenging effect (\%)} = 100 \times (A_o - A_s)/A_o$$

Where, A_o is the absorbance of the blank and A_s the absorbance of the sample.

3.5.2 Scavenging effect of the $ABTS^+$ radical

The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay was based on method of Re *et.al.*(1999) with slight modifications. ABTS radical cation ($ABTS^+$) was produced by the reaction of a 7 mM ABTS solution with potassium persulphate (2.45 mM). The $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.05 at 734 nm. The mixture was stored in the dark at room temperature for 12 h before use. After addition of 25 µL of extract sample or vitamin C used as standard to 2 mL of diluted $ABTS^+$ solution, absorbance was measured at 734 nm after exactly 6 min. The decrease in absorption was used for calculating scavenging effect values. The following equation was used to determine the percentage of the radical scavenging activity of each extract.

$$\text{Scavenging effect (\%)} = 100 \times (A_o - A_s)/A_o$$

3.5.3. Nitric oxide (NO) scavenging activity

Nitric oxide scavenging activity was determined according to the Griess Illosvoy reaction (Garratt 1964). The reaction mixture contained 2 mL of sodium nitroprusside (10 mM) in 0.5 mL phosphate buffer (0.5 M; pH 7.4). Various concentrations of the extracts (0.5 mL) were added in a final volume of 3 mL. After incubation for 60 min at 37°C, Griess reagent [N-(1-Naphthyl) ethylenediamine (0.1%) and sulphanilic acid (1%) in H_3PO_4 (5%)] was added. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with N-(1-Naphthyl) ethylenediamine was measured spectrophotometrically

at 540 nm. Ascorbic acid was used as a positive control. The scavenging ability (%) of the nitric oxide was calculated using the formula:

$$\text{Scavenging effect (\%)} = 100 \times (A_o - A_s)/A_o$$

Where A_o is the absorbance of the blank and A_s the absorbance of the sample.

3.5.4. Superoxide anion radical scavenging activity

Determination of superoxide anion radicals scavenging activity was based on the method described by Nishikimi *et al.* (1972) with minor changes. The superoxide anions were generated in a non-enzymatic system Phenazine methosulfate-Nicotinamide adenine dinucleotide (reduced form) (PMS-NADH), through the reaction of PMS, NADH and oxygen which was detected by the reaction with 2,2'-di-p- nitrophenyl -5,5'- diphenyl –(3,3'- dimethoxy-4,4'-diphenyline) di-tetrazolium chloride (nitro blue tetrazolium-NBT). The reaction mixture contained 1mL of sample/reference standard at differrrent concentration (10-50µg/mL), 1mL of NBT (312µM prepared in potassium phosphate buffer pH 7.4,0.2M) and 1mL of NADH (936 µM in phosphate buffer pH 7.4). Finally, the reaction was accelerated by adding 200µL of PMS solution (120 µM) to the mixture. After 5min incubation at 25°C the absorbance was taken at 560nm against proper blank sample containing phosphate buffer. The control was prepared with all the reagents except the plant extract. As a reference standard L-ascorbic acid was used. Superoxide anion radical scavenging percentage was measured using the equation as follows:

$$\text{\% Superoxide anion scavenged} = (A_0 - A_1)/A_0 \times 100$$

Where, A_0 was the absorbance of the control and A_1 was the absorbance of the extract/standard. IC_{50} values of all extracts and ascorbic acid were also calculated.

3.5.5. Total antioxidant activity by ferric reducing antioxidant power assay (FRAP)

The FRAP was determined using the method of Benzie & Strain (1996) with slight modifications. The fresh FRAP reagent consisted of 500 mL of acetate buffer (300 mM; pH 3.6), 50 mL of 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) (10 mM), and 50 mL of $FeCl_3 \cdot 6H_2O$ (50 mM). The colorimetric measurement was performed at 593 nm and the measurement was monitored up to 12 min on 75 µL of each extract and 2 mL of FRAP reagent. The vitamin C was used to draw a standard curve and the butylated hydroxy toluene (BHT) was used for the comparison. The results were expressed as mg equivalent vitamin C/g of dried extract (mg eq VitC/g DE).

3.5.6. Metal chelating activity

With slight modification the method of Dinis *et al,* (1994) was followed to estimate the ability of the extract to chelate ferrous ions. The extract was added to a solution of 2 mM $FeCl_2$ (0.05 mL and the reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). The mixture was then shaken vigorously and left to stand at room temperature for 10 min. Spectrophotometrically the absorbance of the solution was measured at 562 nm. The percentage inhibition of ferrozine-Fe2+ complex formation was calculated as Chelating rate (%) = (A0 - A1) / A0 × 100, where *A*0 was the absorbance of the control, and *A*1 of the mixture containing the extract or the absorbance of a standard solution.

3.5.7. Hydroxyl radical scavenging activity

The scavenging activity of the extracts on hydroxyl radical was measured according to a method of Yu. *et al*. (2004) with slight modifications. To 1.5 mL of each diluted extract, 60 µL of $FeCl_3$ (1 mM), 90 µL of 1,10-phenanthroline (1 mM), 2.4 mL of phosphate buffer (0.2 M; pH 7.8) and 150 µL of H_2O_2 (0.17 M) were added respectively. The mixture was then homogenized using a vortex and incubated at room temperature for 5 min. The absorbance was read at 560 nm against the blank. The percentage of the radical scavenging activity of each extract was calculated from the equation below:

$$\text{Scavenging effect (\%)} = 100 \times (A_o - A_s)/A_o$$

where A_o is the absorbance of the blank and A_s the absorbance of the sample.

3.6. Sample preparation for HPLC analysis of phenolics

Extraction of total phenolics from the dried samples for HPLC analysis was done following the method described by Pari & Latha (2005) with minor changes. Two gram of powdered sample was soaked overnight in10mL of absolute methanol in dark. After 12h of soaking, the suspension was filtered and the filtrate was completely evaporated using a rotary evaporator at 40°C and lyophilized. The lyophilized extract was re-dissolved in 1mL of HPLC grade methanol and filtered through Millipore membrane filter (0.45µm).

3.7. Fingerprint analysis of total phenolics

HPLC fingerprint analysis of total phenolics was done following the protocol of Pari *et al*. (2007). The analysis was done using High Performance Liquid Chromatograph (Shimadzu) equipped with LC 10ATVP pumps, UV-Vis detector and C18 column. The HPLC program used a flow rate of 1 ml/min, binary gradient elution of

HPLC grade acetonitrile–water–acetic acid (5:93:2, v/v/v) [solvent A] and acetonitrile–water–acetic acid (40:58:2, v/v/v) [solvent B], starting with solvent B from 0 to 100% over a period of 50 min were applied. An injection volume of 20 µl of the filtrate was used and the separation of phenolics was monitored at 278 nm.

3.8. Testing of *in vitro* hypoglycemic activities

3.8.1. *In vitro* α-amylase inhibition activity

In vitro α-amylase inhibition activity of the plant extracts was tested following the method of Bernfield (1955) with minor changes. Plant extracts and positive control acarbose (500µL) of different concentrations was allowed to react with 500µL of freshly prepared α-amylase (3.246mg in 100mL of cold dH_2O) for 20min at room temperature. Then, 1% starch (1g in 0.1M, pH 4.7 acetate buffer) was added to the solution and incubated further for 15min at room temperature. In order to stop the reaction, 1mL DNSA solution (prepared in 1% NaOH) was added to the reaction mixture and kept in boiling H_2O for 5min. The mixture was cooled under running tap water. The final volume was made upto 4mL by adding 1.8mL of dH_2O and absorbance was recorded at 540nm against appropriate blank solution. Control solution was prepared with all the reagents except the plant samples. Percentage of α-amylase inhibition activityof the extracts was calculated according to the formula:

$$\% \alpha \text{ - amylase inhibition} = (A0 - A1)/A0 \times 100$$

Where, A_0 was the absorbance of the control and A_1 was the absorbance of the extract/standard. IC_{50} values for all the extracts and acarbose were also calculated.

3.9. Testing of antimicrobial activities

3.9.1. Sample preparation

To determine the antimicrobial activity the extracts were prepared using the method described by Okwori *et al.* (2006) and Coban and Konuklugil (2005). The solvent used here was 50% methanol and sterile distilled water (sdH_2O) in the ratio of 1:10 (w/v). After, extraction the solvents was evaporated using a rotary evaporator under reduced pressure and further lyophilized for complete solvent removal. The extracts were stored in sterilized glass vials at -20°C until further analysis. To avoid any contamination and alterations of chemical constituents, the extracts were used within 2-3days of preparation (Singh *et al*., 2012).

3.9.2. Preparation of media

3.9.2.1. Potato Dextrose Agar (PDA)

For maintaining fungal culture potato dextrose agar was prepared using fresh potato decoction (400g/L). Then the decoction was filtered through muslin cloth and final volume was adjusted to 1L. To this, dextrose (20g/L) and agar (20g/L) was added and heated until the uniform mixture was obtained. The media was autoclaved at 15lbs for 15min at 121ºC. For the media preparation distilled water was used.

3.9.2.2. Nutrient Broth (NB) and nutrient agar (NA)

Bacterial cultures were grown and maintained both in nutrient broth and agar media. Both the media were prepared taking 13g of available media (Himedia) in 1L of distilled water and warmed the media until dissolved completely. The NA media contains peptone (5g/L), NaCl (5g/L), Beef extract (1.5g/L), Agar (15g/L)Yeast extract (1.5g/L) and pH 7.2±0.2 (at 25ºC) whereas in NB all the ingredients are same as of NA except agar. The nutrient media were then sterilized at 15lbs for 15min at 121ºC.

3.9.3. Antibacterial activities

3.9.3.1. Disc diffusion method

Agar disc-diffusion method of Murray (1995) was followed to evaluate the antibacterial activities of the crude plant extracts. The bacterial strains were maintained and tested on nutrient agar (NA). The media was sterilized at 15lbs (121°C) for 15min prior to pouring it into the sterilized petridishes. A final inoculum of 100µL suspension containing 10^{8}CFU/mL of each bacterium was mixed with the sterilized nutrient agar media and allowed to solidify in the laminar air flow. Crude extracts were sterilized in disposable Milliporefilter (0.22µm) prior to use. After 15min, the plates were impregnated with sterile Whatman No.1 filter paper dics (6mm) containing desired concentrations viz. 500 mg/mL, 250 mg/mL and 100mg/mL of methanolic extract (ME) of the plants. The solvents were completely evaporated from the disc in the laminar air flow before aseptically placing it on the agar surface. Negative control (sterile hot water), solvent control (50% MeOH) and positive control plates were also prepared. Positive control discs (7mm) of A= Azithromycin (15 mcg/disc), S=Streptomycin (10 mcg/disc) were used in the study. The plates were then incubated at 37°C for 24 h after which the diameter of inhibition zones was noted. All the assays were performed in triplicates.

3.9.4. Antifungal activities

3.9.4.1. Spore gerimation bioassay

The spore germination bioassay against the tested fungal spores was performed following the method of Trivedi and Sinha (1976). Spore suspension was prepared by filtering the fungal culture from the broth through muslin cloth. The suspension was centrifuged for 5min at 1000 rpm to separate the debris from the spores and was washed with sdH_2O for about 3-4 times. The spores were collected and suspended in 1mL of sdH_2O till further use. The methanolic and hot water extracts of concentration 500 mg/mL (100µL) were placed at the two ends of each clean, grease free slide and allowed to dry inside a laminar air flow. After drying, about 10 µL of spore suspension of test fungus was placed on top of the dried extract spots. Similarly the slides for positive control (Griseofulvin 1mg/mL), solvent control (50% MeOH) and negative control (sdH_2O) were prepared. All the slides were kept on the glass rods in a petri plates with 5mL of sdH_2O and incubated for 24h. Precautions were taken to avoid the drying of the spores. Following the incubation, the spores (or the spots) were stained with lactophenol cotton blue, fixed and observed under the microscope. The microscopic observation was done from about 5-8 microscopic fields and a total of 500 spores were counted for each case. Further, the percentage of germination was calculated using the formulae:

% of spore germination = Number of spores germinated/Total number of spore counted × 100

3.10. Testing of hypoglycemic activities

3.10.1. Preparation of crude extract for *in vivo* experiments

The dried plant extract used for analyzing hypoglycemic activities was extracted following the method of Coban and Konuklugil (2005) with slight modification. Prior, to the use the extract was mixed with normal drinking H_2O to make the stock solution.

3.10.2. *In vivo* test

3.10.2.1. Animals

Swiss albino male rats (150-200g) were procured from the Ghosh Enterprise, Kol-55. The animals were acclimatized to the experimental room at the temperature of 25 ± 2^0C and 12h light and dark cycles for one week (Niyonzima & Vllietinck, 1993). The animals were then grouped and kept in polypropylene cages with a

maximum of two animals and were fed with standard food pellets (Hindustan Lever, Kolkata, and India.) alternating with some soaked cereals (Black gram seeds) and water.

3.10.2.2. Acute toxicity study

The extracts, *C. retrospiralis* aqueous extract and *H. sibthorpioides* aqueous extract were studied for acute toxicity prior to the 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). The rats were dosed once with 2000mg/kg b.w. and monitored for 14 days for general clinical or toxicological signs and symptoms as well as mortality (Sundarranjan *et al.* 2011).

3.10.2.2.1. Permission

All procedures employed were reviewed and approved by the Animal's ethical Committee of University.

3.10.2.3. Induction of experimental diabetes in test animals

The animals were deprived of food and water for 14h prior to the induction of diabetes to the experimental rats (Siddique *et al.* 1987).

Freshly prepared streptozotocin in citrate buffer (0.1M, pH 4.5) was administered intraperitoneally (i.p) at a single dose of 65 mg/kg body weight and in a volume of 1mL/kg. Diabetes development was confirmed by measuring the blood sugar level after 48h of administration. From the tail tip blood was collected and the glucose levels were determined by glucose meter (Accu-Chek, Active Glucose meter) by glucose oxidase-peroxidase method using strips. Rats with blood glucose levels above 200mg/dL were considered to be diabetic and used for further experimentations.

3.10.2.4. Treatment of diabetic animals

For the treatment of diabetic animals, an experiment was conducted for 25days with 42 rats distributed into six groups (n=6) in the following manner:

Group-I: Normal control (treated with normal water).

Group-II: Streptozotocin –induced diabetic control (treated with normal water).

Group-III: Diabetic rats treated with metformin (10mg/kg b.w).

Group-IV: Diabetic rats orally administered with aqueous extract of *Cryptocoryne retrospiralis* (CR) (200mg/kg b.w.) once daily.

Group-V: Diabetic rats orally administered with aqueous extract of *Cryptocoryne retrospiralis* (CR) (400mg/kg b.w) once daily.

Group-VI: Diabetic rats orally administered aqueous extract of *Hydrocotyle sibthorpioides* (HS)(200mg/kg b.w) once daily.

Group-VII: Diabetic rats orally administered with aqueous extract of *Hydrocotyle sibthorpioides* (HS)(400mg/kg b.w) once daily.

3.10.2.5. Analytical procedure

3.10.2.5.1. Measurement of body weight

The total body weight of all the experimental rats were recorded on day 1, day 5, day 10, day 15, day 20 and day 25 of the treatment.

3.10.2.5.2. Estimation of blood sugar level

Blood sugar level was also recorded on day 0, day5, day 10, day 15,day 20 and day 25 of the treatment. Blood was collected from the tail tip and the glucose levels were determined using glucose meter (Accu-Chek, Active Glucose meter) by glucose oxidase-peroxidase method using strips.

3.10.2.5.3. Collection of serum

In a sterile centrifuge glass tube (without anticoagulant) blood was collected and allowed to stand in room temperature for 20-30min.Then, it was centrifuged at 1500g for 10min at 20°C. The serum gets separated from the blood as an upper transparent liquid over the clotted blood. Then the serum was collected without disturbing the residue and used for studying further biochemical parameters.

3.10.2.5.4. Study of serum biochemical parameters

3.10.2.5.4.1. Lipid profile analysis

Estimation of Total Cholesterol in the serum

The total cholesterol level in the serum samples of experimental rats was done using commercially available kit following the manufacturer's guidelines (Erba diagnostics, Germany). The cholesterol was estimated by dynamic extended stability with lipid clearing agent (Allian *et al.,* 1974; Roeschlau *et al.,* 1974). Prior to experimentation, the working reagent was prepared by mixing the providedrol reagents (i.e, mixture of cholesterol esterase, cholesterol oxidase, peroxidase, sodium phenolate, 4-aminoantipyrine, phosphate buffer (pH 6.5 ± 0.1) and lipid clearing agent) with appropriate amount of sterilized distilled water. 2 ml of the working reagent was then mixed with 40 µl of test sample and allowed

to incubate for 37 ºC for 10 min. The reaction mixture was aspirated and the absorbance was recorded at 505 nm. The cholesterol content was calculated using the formula provided with the kit:

Cholesterol mg dL^{-1} = Absorbance of test / Absorbance of standard× Conc. of standard(mg dL^{-1})

Estimation of Triglycerides in the serum

The content of triglycerides in the serum samples of experimental rats was done using commercially available kit following the manufacturer's guidelines (Erba diagnostics, Germany). Triglycerides were estimated by dynamic extended stability with lipid clearing agent (McGowan *et al*., 1983; Fossati and Prencipe, 1982; Trinder 1969). Briefly, supplied triglycerides reagent (consisting of ATP, Mg^{2+}, 4- aminoantipyrine, 3-5 DHBS, peroxidase, glycerol kinase, glycerol phosphate oxidase (GPO), lipoprotein lipase, buffer (pH 7.0 ± 0.1 at 20 ºC) was mixed with appropriate amount of sterilized distilled water and allowed to stand for 10 min at room temperature. 2 ml of the working reagent was then mixed with 20 µl of the serum sample and incubated for 10 min at 37 ºC and the absorbance was recorded at 505nm. The triglycerides content was calculated using the formula provided with the kit:

Triglycerides mg dL^{-1} = Absorbance of test / Absorbance of standard× Conc. of standard(mg dL^{-1})

Estimation of HDL-cholesterol and LDL cholesterol in the serum

The estimation of HDL-cholesterol was done following the phosphotungstic acid method using commercially available kit following the manufacturer's guidelines (Erba diagnostics, Germany) as described by Burstein et al. 1970. The cholesterol working reagent (consisting the mixture of cholesterol esterase, cholesterol oxidase, peroxidase, sodium phenolate, 4-aminoantipyrine, phosphate buffer (pH 6.5 ± 0.1) and lipid clearing agent) was used to determine the concentration of HDL cholesterol. Briefly, 500 µl of sample was mixed thoroughly with 1 ml of precipitating reagent and allowed to stand for 10 min at room temperature (15-30ºC). The reaction mixture was centrifuged at 4000 rpm for 10 min to obtain the clear supernatant. The clear supernatant was then mixed well with cholesterol working reagent and incubated for 10 min at 37 ºC. The

absorbance was then read at 505 nm. The HDL and LDL (Freidewald's Formula) cholesterol content was calculated using the formula provided with the kit.

HDL cholesterol (mg dL^{-1}) = Abs.of test/ Abs. of standard × Conc. of standard (mg dL^{-1}) × dilution factor

LDL Cholesterol (mg/dL) = (Total Cholesterol) – (Triglycerides/5) – (HDL Cholesterol)

3.10.2.5.4.2. Liver function test

Analysis of SGPT/ALT (Alanine aminotransferase) level

The ALT level in the serum samples was done using commercially available kit following the manufacturer's guidelines (Erba diagnostics, Germany) according to IFCC method (International Federation of Clinical Chemistry method, 1980). Briefly, 1000 µl of the working reagent (composed of L-Alanine, NADH, lactate dehydrogenase, 2-oxoglutarate and tris buffer) was mixed thoroughly with 100 µL of the test sample and the absorbance was immediately recorded at 340 nm at an interval of 1 min.

The ALT activity was calculated using the formula provided 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 = (ΔA/min) 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 340 nm= 6.22

P = cuvette lightpath = 1cm

Activity of ALT at 37 ºC (IU/L) = (ΔA/min) x Factor (1768)

Analysis of SGOT/ AST (Aspartate aminotransferase) level

The AST level in the serum samples was done using commercially available kit following the manufacturer's guidelines (Erba diagnostics, Germany) according to IFCC method (International Federation of Clinical Chemistry method, 1980). The working reagent (2-oxoglutarate, L-aspartate, malate dehydrogenase, lactate dehydrogenase, NADH, tris buffer and EDTA). For estimation, 1000 µl of the

working reagent was mixed thoroughly with 100 µl of the test sample and aspirated. The absorbance was immediately recorded at 340 nm at an interval of 1 min.

The AST activity was calculated using the formula provided 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 = (ΔA/min) 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) = (ΔA/min) x Factor (1768)

3.10.2.5.4.3. Kidney function test

Estimation of urea level

The estimation of urea level in the serum samples was done using commercially available kit following the manufacturer's guidelines (Erba diagnostics, Germany) according to GLDH-Urease method, as described by Talke and Schubert (1965); Triffany *et al.*(1972). The urea working reagent (mixture of α-ketoglutarate, NADH, urease, glutamate dehydrogenase (GLDH), ADP, Tris buffer (pH 7.9 ± 0.1) was used for estimation of urea level. For estimation, 2000 µl of the working reagent was mixed with 20 µl of the test samples and aspirated and the absorbance was immediately recorded at 340 nm at an interval of 1 min. The rate of decrease in absorbance was directly proportional to urea concentration.

The concentration of urea was calculated using the formula provided with the kit:

Urea (mg/dL^{-1}) = ΔA of test / ΔA of standard × Conc. of standard (mg/dL^{-1})

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

Estimation of creatinine level

The creatinine level was estimated in the serum samples was done using commercially available kit following the manufacturer's guidelines (Erba diagnostics, Germany) according to the modified Jaffe's method described by Slot (1965) and Bartel *et al.* (1972). The working reagent was prepared by mixing equal amount of

picric acid and sodium hydroxide reagent provided in the kit and allowed to stand for 15 min. For estimation, 2000 µl of the working reagent was mixed with 200 µl of sample and the initial absorbance (A_1) was read at 20 seconds after mixing followed by final absorbance (A_2) 80 seconds after mixing at a wavelength of 505 nm. The absorbance of the orange–yellow colour was directly proportional to the creatinine concentration.

The creatinine concentration was calculated using the formula provided with the kit:

Creatinine (mg/dL^{-1}) = ΔA of test / ΔA of standard × Conc. of standard (mg/dL^{-1})

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

3.11. Cell culture and Cytotoxic effect of plant extract

From the cell line repository at NCCS, Pune, human hepatocarcinoma cell line ($HepG_2$) was purchased and maintained in Ham's F-12 medium, with 10% Foetal Bovine Serum in a humidified CO_2-Incubator at 5% CO_2 level and 37° C. confluent cells were trypsinized with 0.25% trypsin-EDTA and seeded in a 96-well microtiter plate (104 cells per well) and incubated under the same conditions for 24 hours. In a rotary evaporator the methanolic extract of these plant samples were dried and redissolved in DMSO to obtain a concentration of 500 mg/ml. Further these extract was diluted for experiment again (Acharya *et al*. 2017). The percentage of DMSO at all circumstances was not greater than 1%. The cells seeded in the 96-well plate were treated with different concentrations of the extract. After 24 hours of incubating of the plates, cytotoxicity was measured with iMark™ Microplate Absorbance Reader (BIO-RAD, USA) employing WST-I reagent (TaKaRa) according to manufacturer protocol.

3.12. Characterization of active compounds

Since the methanolic extracts of *Cryptocoryne retrospiralis* and *Hydrocotyle sibthorpioides* was found to possess significant better pharmacological activity compared with the other extracts, an attempt has been made to partially characterize the various components present in these extracts through FTIR spectroscopy and Gas Chromatography mass spectrometry (GC-MS).

3.12.1. Fourier Transform Infrared Spectroscopy (FTIR)

The interference pattern obtained from a two beam interferometer as the path difference between the two beams is altered, when Fourier transformed, gives rise to

the spectrum. The transformation of the interferogram into spectrum is carried out mathematically with a dedicated on-line computer. For analysis, samples were sent to Sophisticated Analytical Instrument Facility, Indian Institute of Technology, Madras. The Perkin Elmer Spectrum1 FT-IR instrument consists of globar and mercury vapor lamp as sources, an interferometer chamber comprising of KBr and mylar beam splitters followed by a sample chamber and detector. Entire region of 450-4000 cm-1 is covered by this instrument. The spectrometer works under purged conditions. Solid samples are dispersed in KBr or polyethylene pellets depending on the region of interest. This instrument has a typical resolution of 1.0 cm-1. Signal averaging, signal enhancement, base line correction and other spectral manipulations are possible.

Instrument details

Model:	Spectrum one : FT-IR Spectrometer
Scan Range :	MIR 450-4000 cm-1
Resolution :	1.0 cm-1
sample required	50 mg, solid or liquid.

3.12.2. Gas Chromatography mass spectrometry (GC-MS)

Two plant samples, *viz. Cryptocoryne retrospiralis* and *Hydrocotyle sibthorpioides* were further analysed through GC-MS to identify the various bioactive constituents. For analysis, samples were sent to SAIF, Indian Institute of Technology, Madras. The analysis was performed using a JEOL GCMATE II GC-MS spectrometer. Initially, the instrument was kept at temperature of 110 ºC and maintained as such for 2 min, after which the oven temperature was increased to 280 ºC at the rate of 5 ºC /min and maintained further at this temperature for 9 min. The helium flow rate was maintained at 1 ml/min and the injection port temperature was kept at 250 ºC. The samples were injected in split mode at 10:1. The ionization voltage was 70 eV. Mass spectral scan range was at the rate of 45-450 (m/z). National Institute of Standards and Technology (NIST) Ver.2.1 MS data library was used to identify the compounds present in the plant extracts. The spectrum obtained through GC-MS of each compounds were compared with the NIST data library for the identification.

3.13. *In silico* molecular docking studies

Molecular docking

The coordinates of Peroxisome proliferators activated receptor gamma (3DZY.pdb), one of the diabetic molecular targets, were obtained from the RCSB protein data bank (www.rcsb.org). The biologically active compounds were docked into the active pocket of the enzyme by using docking program Autodock 4.0 (Huey *et al*. 2004, Huey *et al*.2007 and Morris *et al*. 2009).

Initially the structures of the compounds have been generated by ChemSketch software [ACD/ChemSketch, 2009] and the hydrogen atoms were added to the enzyme. The Lamarckian genetic algorithm (LGA) was applied to look out for the best conformers. A grid map with 80x80x80 points and 0.375 Å spacing was used in Autogrid program to evaluate the binding energies between the biologically active compounds and 3DZY. The grid centre was set at the catalytic site of the enzyme and the default settings were used. For each compound ten docking poses were saved and ranked by binging energy. The lowest energy docking pose was selected for analyzing the type of interactions.The binding site was analyzed with molegro molecular viewer software (Thomsen and Christensen 2006).

3.14. Statistical analysis

All the data was subjected to statistical analyses and the results were expressed as mean ± standard deviation (SD). Analyses like one way and two way analysis of variance followed by Duncan's multiple range test (DMRT). IBM SPSS statistic version 21 was used for the analysis.