Chapter 3

Materials and methods

3. Materials and methods

3.1. Plant materials

3.1.1. Collection

Some of the ferns locally available in the Darjeeling district of North Bengal regions, West Bengal, with or without the ethnobotanical importance were collected for the present study. The district of Darjeeling, West Bengal is located in The Mahabharat range or lower Himalaya at the elevation of 6700ft and extends between 27.05°N and 88.263°E with the total geographical area of 10.5 km^2 (4.08 sq mi). Nestled among the rolling mountains with glistening Mt. Kanchenjunga towering over the azure sky, Darjeeling fondly called as "Queen of Hills" has delightful flora and fauna. Sometimes, even referred as "Plantlover's paradise", is the house of around 300 varieties of ferns. Ferns were collected from different areas of Darjeeling town (Hooker road, Singamari, and Lebong) from various sub-division of Mungpoo (Labdah, Reshep and Rambi) and from Siliguri shibmandir area (Medical more, Satyan Bose Road and NBU campus) (Figure 1A-C, 2; Table 1).

3.1.2. Identification

The healthy and mature fronds of the selected ferns were collected in polythene bags and brought to the laboratory for further processing and identification. Plants were identified by Professor AP Das of Department of Botany and the voucher specimens have been deposited in the North Bengal University Herbarium, Department of Botany, University of North Bengal, India (Figure 3, Table 2).

3.2. Preparation of plant extract

3.2.1. Preparation of dried plant powder

The collected fronds were initially washed thoroughly (3-4 times) with tap water and then with double distilled water and dried using blotting paper. The washed and cleaned plant materials were covered with thin cloth and allowed to dry under the shade for about 7 days. The air and shade dried plant materials were ground to obtain fine powder using mixer grinder (Jaipan, Super Deluxe, India) and was stored in plastic bottles at 4°C till further use (Figure 4).

3.2.2. Preparation of Hot water extract (HWE)

The hot water extracts were prepared following the method of Coban and Konuklugil (2005) with slight modification. The finely ground powdered samples were mixed with boiling distilled water (dH_2O) in the ratio of 1:10 and kept in hot water bath for 15min.The mixtures were then kept

overnight and filtered using Whatman No.1 filter paper which were concentrated with rotary evaporator (IKA RV10) and lyophilized using Eyela Freeze Dryer FDU-506. The lyophilized extracts were then stored at -20°C till further analysis.

Figure 1A: Collection sites of neighbouring areas of Darjeeling town A. Lebong B: Hooker Road C: Singamari

3.2.3. Preparation of Methanolic extract (ME)

The method described by Okwori *et al.* (2006) with slight modification was used for the preparation of methanolic extraction. In brief, methanol and powdered samples were mixed in the ratio of 1:10 (sample:solvent) and shaken vigorously for 5min and then kept for 72h at room temperature while stirring for 30min at an interval of 24h. The mixture was then filtered and the supernatant obtained were concentrated at 40°C in rotary evaporator, lyophilized and stored at -20°C until further use.

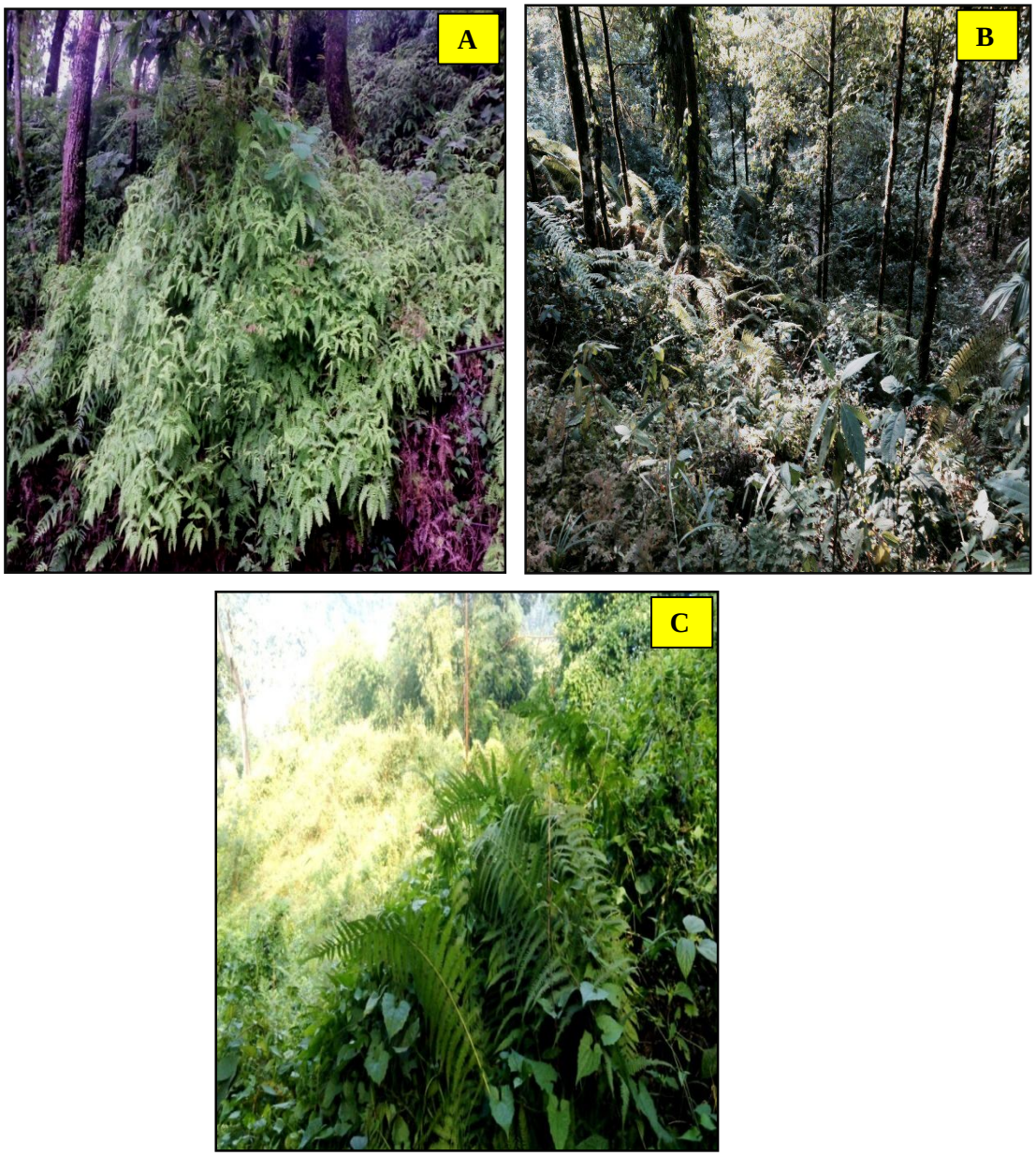

Figure 1B: Collection sites of Mungpoo subdivisions A. Reshep B: Labdah C: Rambi

3.2.4. Preparation of Ethanolic extract (EE)

The ethanolic extract of the samples were prepared following the slightly modified method described by Okwori *et al.* (2006). Briefly, 10g of the powdered samples were mixed with

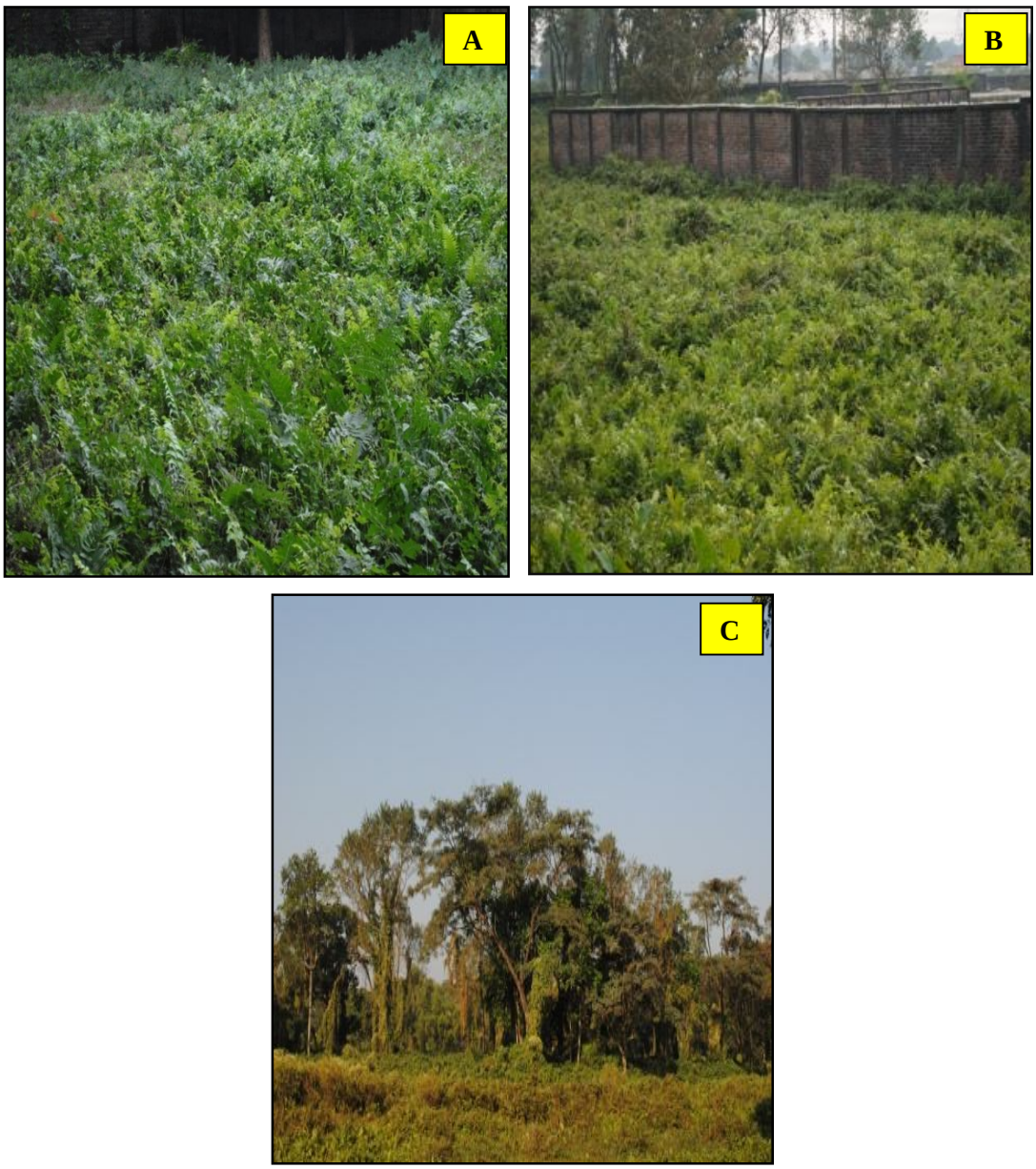

Figure 1C: Collection sites of Siliguri (Shibmandir neighbouring areas) A: Medical More B: Satyan Bose Road C: NBU campus

100 mL of ethanol and kept at room temperature for 72h with constant stirring for 30min after every 24h interval. The mixture was then filtered and processed as described above till further use

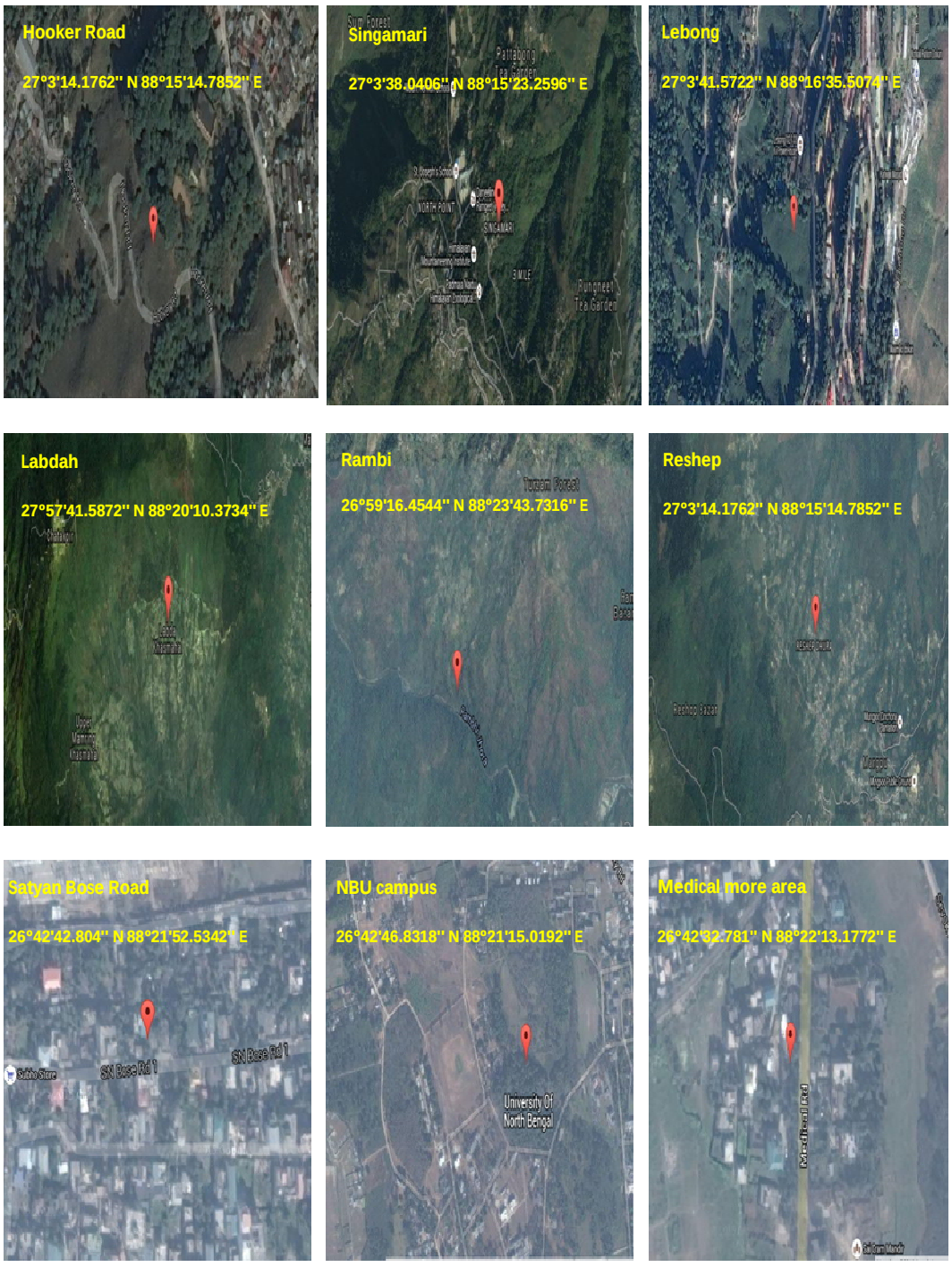

Figure 2: GIS location map of the collection sites

Table 1: Table showing the GIS locations of the sampling sites

Area	Collection sites	GIS location of sampling
Darjeeling town	Hooker Road	27º 3' 14.1762" N 88º 15' 14.7852" E
	Singamari	27º 3' 38.0406" N 88º 15' 23.2596" E
	Lebong	27º 3' 41.5722" N 88º 16' 35.5074" E
Mungpoo (Sub division)	Labdah	26º 57' 41.5872" N 88º 20' 10.3734" E
	Reshep	26º 58' 39.1440" N 88º 21' 45.543" E
	Rambi	26º 59'16.4544" N 88º 23'43.7316" E
Siliguri (shibmandir area)	Medical more area	26º42'32.781" N 88º22'13.1772" E
	Satyan Bose Road	26º42'42.804" N 88º21'52.5342" E
	NBU campus	26º 42 46.8318" N 88º 21' 15.0192" E

3.3. Phytochemical screening

3.3.1. Test for phenol

The powdered plant samples were mixed with 10mL double distilled water and stirred in magnetic stirrer for 10 min. The mixture was then filtered using Whatman filter paper No.1. To the filtrate (1mL), equal volume (1mL) of 1% $FeCl_3$ was added and observed for the appearance of blue or green color indicating the presence of phenols (Martinez and Valencia, 2003).

3.3.2. Test for flavonoid

The method described by Evans (2002) was followed to detect the presence of flavonoid in the samples. Briefly, 2g of the sample was mixed thoroughly with 10mL of acetone which was evaporated by keeping the flask in a hot water bath for 5 min. Further, the sample was extracted using 10mL of warm double distilled water. The solution was thoroughly mixed, filtered while hot and allowed to cool at room temperature. To the filtrate (5mL), equal volume of 20% NaOH was added and change in appearance/color of the solution to yellow indicated the presence of flavonoid**.**

Figure 3: Plant samples collected for the study. A: *Nephrolepis cordifolia* (L.) C. Persl. B: *Cyclosorus dentatus* (Forsk.) C: *Dicranapteris linearis* (N. Burm.) Underw D: *Phymatosorus cuspidatus* (D.Don) Pic. Serm. E: *Pteris biaurita* L. F: *Pteris vittata* L. G: *Drynaria quercifolia* (L.) J. Smith H: *Microsorum punctatum* (L) Copel.

Table 2: Identification of the plant samples along with the accession no. of the deposited samples

Name	Accession number
Drynaria quercifolia (L.) J. Smith	09871
Pteris vittata L.	09872
Phymatosorus cuspidatus (D.Don) Pic. Serm.	09873
Nephrolepis cordifolia (L.) C. Persl	09874
Pteris biaurita L.	09883
Cyclosorus dentatus (Forsk.) Ching.	09885
Dicranapteris linearis (N. Burm.) Underw.	09886
Microsorum punctatum (L.) Copel.	09887

3.3.3. Test for tannin

The crude plant powder (200mg) was mixed with 10mL of double distilled water and allowed to stand for 10 min before filtration. Then, 1mL of 5% $FeCl_3$ was added to 2mL of the filtrate. The formation of yellow brown precipitate indicates the presence of tannin (Jigna and Sumitra, 2007).

3.3.4. Test for alkaloid

The powdered sample (200mg) was mixed vigorously with 10mL of methanol for 1h at room temperature. The mixture was then filtered and to 2mL of methanolic filtrate, 2mL of 1% HCl was added. The solution was kept in boiling water bath for 5min. Then, Mayer's/Wagner's reagent (6-7 drops) was added to the filtrate. Formation of creamish/brown/red/orange precipitate indicates the presence of alkaloid **(**Trease and Evans, 1989)

3.3.5. Test for cardiac glycosides

The method described by Trease and Evans (1989) with minor modification was followed for the qualitative screening of cardiac glycosides in the samples. The methanolic filtrate (2mL) was mixed with 1mL glacial acetic acid, to which further 3-4 drops of 5% $FeCl_3$ was added. Then, 1mL of concentrated H_2SO_4 was added carefully to the solution. Development of brown ring at the interface indicates the presence of cardiac glycosides. A violet color may also appear below the brown ring.

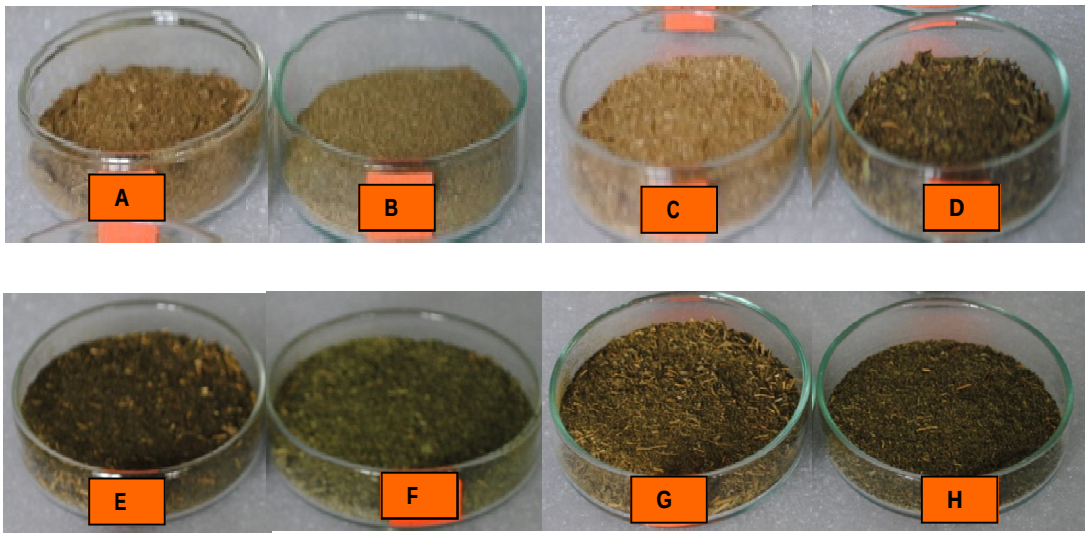

Figure 4: Dried and powdered frond samples of the study plants. A: *Cyclosorus dentatus* B: *Microsorum punctatum* C: *Nephrolepis cordifolia* D: *Phymatosorus cuspidatus* E: *Pteris biaurita,* F: *Drynaria quercifolia* G: *Dicranopteris linearis* H: *Pteris vittata*.

3.3.6. Test for carbohydrates

The plant samples (500mg) were boiled in 30mL of double distilled water and filtered. Then, 2mL of aqueous extract was mixed with 2mL of Molish's reagent (5%-α-naphthol in absolute ethanol) and shaken vigorously. To it, 2mL of concentrated H_2SO_4 was added carefully along the wall of the test tube. The presence of carbohydrate was inferred by the formation of reddish-ring at the junction of two liquids (Evans, 2002).

3.3.7. Test for reducing sugars

The sample (500mg) was boiled in 30mL of double distilled water and filtered to obtain an aqueous extract/filtrate. The aqueous filtrate (1mL) was then mixed with 2mL of Fehling's solution (A: 7%CuSO4 in dH_2O containing 2 drops of dil.H_2SO_4, B: 12% KOH and 35% Sodium potassium tartarate in dH_2O. Mix A and B in equal amount) and boiled for 5mins. Formation of a brick red precipitate indicates the presence of reducing sugars (Evans, 2002).

3.3.8. Test for protein

The method described by Pullaiah (2006) was followed for the detection of protein in the plant samples. About 1g of the plant sample was mixed thoroughly with 10mL of double distilled

water by a magnetic stirrer for 10h and filtered. 2mL of the filtrate was then mixed with 1mL of 40% NaOH in a test tube. Then, 1-2 drops of $CuSO_4$ was gently added to the solution. Change in the color of solution to violet indicates the presence of peptide linkages in a solution which in turn is an indication of the presence of proteins.

3.3.9. Test for saponin

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

3.3.10. Test for terpenoid

The methanolic filtrate (2mL) was mixed with 5mL of chloroform and 2mL of acetic anhydride. Then, to the mixture 1mL of concentrated H_2SO_4 was added carefully along the wall of the test tube. The formation of reddish brown ring at the interface indicates the presence of terpenoid (Harborne, 1973).

3.3.11. Test for steroid

The methanolic filtrate (5mL) was treated with 0.5mL of anhydrous CH_3COOH and cooled on an ice bath for 15mins. Then, 0.5mL of chloroform and 1mL of concentrated H_2SO_4 was added to the cold solution. Presence of the steroid may be inferred by the formation of reddish-brown ring at the junction of two liquid phase (Gokhale and Kokate, 2008).

3.3.12. Test for anthraquinone

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

3.4. Quantification of biochemical components

3.4.1. Extraction and estimation of total phenols

3.4.1.1. Extraction of total phenols

The method given by Mahadevan and Sridhar (1982) with minor modification was employed for the extraction of phenol. Briefly, 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 then filtered using Whatman No.1 filter paper. The residue was re-extracted with 80% alcohol making the final volume up to 10mL. The entire procedure was carried out in dark chamber.

3.4.1.2. Estimation of total phenols

Estimation of total phenol was done following the method described Bray and Thorpe (1954). 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. The reaction mixture was cooled under running tap water and further diluted with distilled water to make the final volume up to 25mL. The optical density value was taken at 650nm in a colorimeter (Digital Colorimeter 112, Systronics) against a proper blank solution. The concentration of total phenols was expressed as mg Ferulic acid (FAE) equivalents/ g dry weight sample (dw), using the standard curve of ferulic acid.

3.4.2. Quantification of protein content

3.4.2.1. Extraction

Extraction of protein from the dried powdered material was done using the method of Chakraborty *et al.* (1995). The powdered sample (1g) was homogenized in a pre-chilled motor and pestle with 5mL of 50mM 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. The supernatant was collected and used for further estimation.

3.4.2.2. Estimation

Estimation of protein content in the extract was done according to the method of Lowry *et al.* (1951). In brief, 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. Then, 0.5mL of 1N Folin Ciocalteau's phenol reagent was added to the mixture which was further incubated for 20 min. The absorbance was recorded at 690 nm in spectrophotometer against a proper blank. The total protein was quantified 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 of flavonoid

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

3.4.3.2. Quantification of flavonoid

The flavonoid content was quantified using the method of Sultana *et al*. (2009). Briefly, the extract (1mL) was mixed with 4mL of distilled water and 300µL of 5% $NaNO_2$ and allowed to incubate for 5min at room temperature. After the 5min of incubation 300µL of 10% $AlCl_3.6H_2O$ was added. Further, at 6^{th} min 2mL of NaOH, followed by 2.4mL of distilled water was added and mixed well. Absorbance of the reaction mixture (pink colour) 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 Catechin (+) equivalents (CE)/ g dry weight sample (dw) using the standard curve of Catechin (+). The entire procedure was performed in a dark condition.

3.4.4. Extraction and estimation of tannin

3.4.4.1. Extraction

The method given by Sadasivam and Manickam (1992) with slight changes was followed for the extraction of tannin from the samples. Briefly, 1g of powdered sample was dissolved in 50mL of methanol and mixed occasionally by swirling and kept in a dark chamber for 20-28h. The mixture was centrifuged at 5000rpm for 5min and the supernatant was collected for further estimation.

3.4.4.2. Estimation

For estimation, 1mL of the supernatant was taken in a test-tube to which 5mL of vanillin hydrochloride reagent (8% hydrochloric acid in methanol and 4% vanillin in methanol mixed in equal volumes, just before use) was added quickly and mixed thoroughly. The reaction mixture was allowed to incubate for 20min at room temperature in dark condition. The absorbance was measured in spectrophotometer (UV-VIS spectrophotometer 118 Systronics) at 500nm against a blank solution prepared with vanillin hydrochloride reagent alone. The tannin content was expressed as mg Catechin (+) equivalents (CE)/ g dry weight sample (dw) using the standard

curve of Catechin (+) prepared using the concentration of 20-100µg/mL (Sadasivam and Manickam, 1992).

3.4.5. Extraction and estimation of carbohydrates

3.4.5.1. Extraction of total soluble and reducing sugar

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

3.4.5.2. Estimation of total soluble sugar

Total sugar estimation was done according to the method explained by Plummer (1978). In brief, 1mL of test solution was added with 4mL of Anthrone's reagent (0.2% Anthrone in conc. H_2SO_4). The reaction mixture was mixed thoroughly and allowed to incubate in boiling water for 10min (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 620nm against a suitable blank solution. The total sugar content was calculated from a glucose standard curve and expressed as mg glucose equivalent (GE)/g dry weight sample.

3.4.5.3. Estimation of reducing sugar

The estimation of reducing sugar was done following the Somogyi-Nelson's method as described by Plummer (1978). 1mL of the test solution was mixed with 1mL of alkaline copper tartarate solution (4g-$CuSO_4.5H_2O$, 24g- Na_2CO_3 anhydrous, 16g- Na^+-K^+ tartarate, 180g- Na_2SO_4 anhydrous-in 1000ml of distilled water) and heated over a boiling water bath for 20min (taking necessary precautions). After cooling the reaction mixture under running tap water, 1mL of commercially available Nelson's arseno molybdate reagent and 2mL of distilled water was added sequentially and mixed thoroughly. The optical density values were recorded at the wavelength of 515nm in a colorimeter and the reducing sugar content was calculated from the calibration curve of glucose and expressed as mg glucose equivalent (GE)/g dry weight sample.

3.4.6. Extraction and estimation of chlorophylls (total, chl a and chl b)

3.4.6.1. Extraction

Chlorophyll was extracted from the samples following the method of Harborne (1998) with minor changes. About 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 chamber. The residue was re-extracted with 80% acetone until it became completely colorless attaining final volume upto 10mL.

3.4.6.2. Estimation

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 663nm and 645nm 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} 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. Extraction and estimation of total lipid

The total lipid extraction and estimation was performed following the standard method described by Bligh and Dyer (1959) with minor changes. Briefly, 1g of dried powdered sample was macerated with 10mL of dH_2O for 5min, followed by the addition of 30mL of chloroform-methanol (2:1 v/v). The resultant mixture was mixed thoroughly in a beaker and kept overnight at room temperature. To this, 20mL of chloroform and 20mL of dH_2O was added and centrifuged at 1000rpm for 10min. Three distinct layers were formed after centrifugation, out of which the lowermost layer of chloroform containing lipid was collected in a beaker. The mixture was then kept in an oven for one hour at 50ºC for complete evaporation of chloroform. Weight of the beaker was taken and the lipid content was expressed as mg/g dry weight sample (dw).

3.4.8. Quantification of carotenoid content

3.4.8.1. Extraction

The carotenoid was extracted from the samples following the method of Litchtenthaler (1987). 1g of the powdered material was soaked and crushed with 100% methanol in dark chamber and filtered using Whatman filter paper No.1. The total volume was made upto 10mL with methanol and used for further analysis.

3.4.8.2. Estimation

Estimation of carotenoid content was done by the procedure of Litchtenthaler (1987). The desired amount of filtrate was taken directly into the test tube either in the diluted or undiluted form and the absorbance was noted at 480nm, 645nm and 663nm wavelength against a blank in UV-VIS spectrophotometer 118 systronics. The contents were calculated using the standard formula as follows:

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

3.4.9. Quantification of vitamin C (ascorbate) content

3.4.9.1. Extraction

The method described by Mukherjee and Choudhuri (1983) was followed for the extraction of vitamin C/ascorbate from the powdered samples. The samples were homogenised in pre-chilled motor and pestle on ice using 6% trichloroacetic acid (10mL) and filtered at chilled condition ($0^{\circ}C$). The final volume of the filtrate was made upto 10mL by adding trichloroacetic acid and used for further estimation.

3.4.9.2. Estimation

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

3.4.10. Estimation and extraction Vitamin E (α-tocopherol) content

3.4.10.1. Extraction of vitamin E

Vitamin E (α-Tocopherol) was extracted by the method of Jayaraman (1996) with slight changes. The dried sample (0.5g) was taken with 6 mL of hexane and shaken vigorously.The mixture was then filtered using whatman filter paper.

3.4.10.2. Estimation of vitamin E

Vitamin E (α-Tocopherol) content in the powdered sample was estimated following the method of Jayaraman (1996) with minor modifications. The filtrate (2mL) was taken in the test tube and 2mL of absolute ethanol was added to it and mixed thoroughly. Further, 0.2mL of 2,2'- Bipyridyl solution (0.5% in ethanol) and 0.2mL of ferric chloride solution (0.2% in ethanol) was added, shaken properly and incubated in dark for 15min. After incubation, 4mL 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 30min. The absorbance was measured against the appropriate blank at wavelength 520nm in a spectrophotometer. The α-tocopherol content was quantified using a standard curve of α-tocopherol and expressed as mg tocopherol equivalent (TE)/g dry weight sample (dw).

3.5. Determination of anti-oxidative activities of extracts

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

The DPPH radical scavenging activity of the plant extracts and positive standard was determined using the method described by Lim & Quah (2007). Briefly, different concentration of EE, ME and HWE and positive standard (1mL) was mixed with 1mL of DPPH methanolic solution (100µM) and incubated in dark chamber for 30 min at room temperature. The absorbance was recorded at 517nm against blank solution (100% methanol). The control was prepared taking all the reagents except the plant extracts. L-ascorbic acid was used as the positive standard. The scavenging activity of the extracts based on percentage inhibition was calculated according to the formula:

$$\% \text{ DPPH inhibition} = \frac{(A_0 - A_1)}{A_0} \text{ X } 100$$

Where, A_0 was the absorbance of the control and A_1 was the absorbance of the extract/standard. Results were also calculated as IC_{50} values, which is the concentration of the extract or ascorbic acid required to scavenge 50% of DPPH radicals.

3.5.2. Hydrogen peroxide (H_2O_2) scavenging activity

The assay was carried out according to the standard method of Ruch *et al*. (1989) with slight modifications. In brief, 5mL each of the EE, ME and HWE (50-500 µg/ml) was mixed with 1mL of H_2O_2 (2mM prepared in phosphate buffer 0.1M, pH 7.4) and incubated for 10 min at room temperature in dark condition. The absorbance was read at 230nm against a blank solution

containing phosphate buffer without hydrogen peroxide. Control was prepared with H_2O_2 and phosphate buffer. L- ascorbic acid was used as a positive control. The percentage of hydrogen peroxide scavenged was calculated using the following formula:

$$\% \text{ Scavenged } (H_2O_2) = \frac{(A_0 - A_1)}{A_0} \text{ X } 100$$

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

3.5.3. Nitric oxide (NO) scavenging activity

Nitric oxide scavenging capacities of the extracts was determined using the standard protocol described by Jagetia *et al.* (2004) and Packer *et al.* (1998). The reaction mixture containing 2mL of sodium nitroprusside (10mM), 0.5mL of phosphate buffer saline (pH:7.4,0.1M) and 0.5mL of the extracts/positive control (200-1000µg/mL) was incubated at 25°C for 2h 30min. Then, from the incubated solution 0.5mL solution was taken in the test tube and mixed with 1mL of sulphanilic acid (0.33% in 20% glacial acetic acid). The mixture was allowed to stand for 5min after which 1mL of naphthylethylene diamine dihydrochloride (NED) (0.1% in distilled water) was added, mixed thoroughly and incubated for another 30min at 25°C. The absorbance of the solution (pink chromophore) was read at 540nm against appropriate blank solution. Control was prepared taking all the reagents except the plant sample. L-ascorbic acid was taken as the reference standard. The nitric oxide scavenging percentage was calculated according to the formula:

$$\% \text{ Nitric oxide (NO)scavenged} = \frac{(A_0 - A_1)}{A_0} \text{ X } 100$$

Where, A_0 was the absorbance of the control and A_1 was the absorbance of the extract/standard. Further, IC_{50} values for each extract and ascorbic acid was also calculated.

3.5.4. Superoxide anion radical scavenging activity

Measurement of superoxide anion radicals scavenging activity was based on the method described by Nishikimi *et al.* (1972) with minor modificaition. 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 various 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 incubation for 5min at 25°C the absorbance was measured at 560nm against appropriate blank sample containing phosphate buffer. Control was prepared with all the reagents except the plant extract. L-ascorbic acid was used as a reference standard. Percentage of superoxide anion radical scavenged was measured using the equation as follows:

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

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. Ferric reducing antioxidant power assay (FRAP)

The ferric reducing power of ethanolic, methanolic and hot water extract was assayed using the method described by Oyaizu (1986) with slight changes. The reducing power was based on Fe^{3+} to Fe^{2+} transformation in the presence of the extracts which can be examined by measuring the formation of Perl's Prussian blue at 700nm. One mL of various concentrations (20-100µg/ml) of extract/standard was mixed with 2.5mL (0.2M, pH 6.6) of phosphate buffer and 2.5 mL of 1% potassium ferricyanide. The reaction mixture was incubated at 50°C for 20 min. The solution was allowed to cool at room temperature after which 2.5mL of 10% tri-carboxylic acid (TCA) was added and centrifuged at 3000rpm for 10 min. 1mL of upper layer of the centrifuged solution was taken and mixed with 1mL of double distilled water and 0.5mL of 0.1% ferric chloride and was allowed to incubate for 10min at room temperature. Absorbance was recorded against appropriate blank solution containing phosphate buffer at 700nm. L-ascorbic acid was taken as a positive control. Higher absorbance of the reaction mixture indicates stronger reducing power. Results were also expressed as mg gallic acid equivalent (GAE)/g dry weight using standard graph of gallic acid.

3.6. Principal Component Analysis (PCA) of antioxidant parameters

PCA was performed to understand the relationships or influence of various factors (phytochemical parameters studied) towards the measured antioxidative parameters. Factors taken for the study was flavonoid, phenol, tannin, lipid, total sugar, reducing sugar, protein vitamin E, vitamin C, total chlorophyll (total chl), chlorophyll a (chl a), chlorophyll b (chlb) and carotenoid which were consecutively labeled as "a,b,c,d,e,f,g,h,i,j,k,l and m". Likewise,

antioxidant activities were labeled as “n” for DPPH scavenging activity, “o” for hydrogen peroxide scavenging activity, “p” for superoxide scavenging activity, “q” for nitric oxide scavenging activity and “r” for ferric reducing antioxidant power.

A factor rotation method used was Varimax with Kaiser Normalization. The analysis was performed using software IBM SPSS statistic version 21.

3.7. HPLC analysis of phenolics

3.7.1. Sample preparation

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).

3.7.2. Analysis of total phenolics

HPLC fingerprint analysis of total phenolics present in the sample was done following the method described by Pari *et al.* (2007). The analysis was done using High Performance Liquid Chromatograph (Shimadzu) equipped with HPLC pumps (model LC 10ATVP), UV-Vis detector (modesl SPD-10AVP) and C18 column. The flow rate of 1mL/min, injection volume of 20 μL and 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. The separation of compounds was monitored at 280 nm. The identification and quantification of the phenolic compounds were done using the standards such as caffeic acid, caffeine, catechin, catechol, chlorogenic acid, cinnamic acid, 3,4-dihydroxybenzoic acid, ferulic acid, gallic acid, phloroglucinol, pyrogallol, resorcinol, salicylic acid and vanillic acid.

3.8. Testing of antimicrobial activities

3.8.1. Sample preparation

The extracts used for antimicrobial activity was prepared using the method described by Okwori *et al.* (2006) and Coban and Konuklugil (2005) as described earlier. Here, the solvent used was 50% methanol and 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. In order to avoid any contamination and alterations of chemical constituents, the extracts were used within 2-3days of preparation (Singh *et al*., 2012).

3.8.2. Preparation of media

3.8.2.1. Potato Dextrose Agar (PDA)

Potato dextrose agar for maintaining fungal culture was prepared using fresh potato decoction (400g/L). 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 then autoclaved at 15lbs for 15min at 121ºC. Distilled water was used for the media preparation.

3.8.2.2. 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 composition was as follows peptone (5g/L), NaCl (5g/L), Beef extract (1.5g/L), Agar ( )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. Nutrient media were then sterilized at 15lbs for 15min at 121ºC.

3.8.3. Antibacterial activities

3.8.3.1. Disc diffusion method

The antibacterial activities of the crude plant extracts were evaluated following the agar disc-diffusion method of Murray (1995). 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 petriplates. A final inoculum of 100µL suspension containing 10^8CFU/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 Millipore filter (0.22 µm pores) 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 the hot water extract (HWE) and methanolic extract (ME) of the plants. 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 Chloramphenicol (C_{25}) (25mcg), Kanamycin (K)

(30mcg) and Ampicillin (A_{25}) (25mcg) 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.

3.8.4. Antifungal activities

3.8.4.1. 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 1000rpm to separate the debris from the spores and was washed with sterile distilled water (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:

$$\text{Percentage (\%) of spore germination} = \frac{\text{Number of spores germinated}}{\text{Total number of spores counted}} \text{ X } 100$$

3.8.4.2. Radial growth bioassay

The antifungal activity of the plant extracts were evaluated using the food poisoning technique of Kumar *et al*. (2008). The plant extracts were re-dissolved in respective solvents to make the stock solution of 2000 mg/mL. Prior to use, the extracts were sterilized in disposable Millipore filter (0.22 µm pores) and mixed with sterile potato dextrose agar medium (PDA) to obtain the final concentration of 500 mg/mL of each plant extract and then poured in sterile petridishes. Control plates were mixed with sdH_2O (negative control), 50% methanol (solvent control) and griseofulvin (1mg/mL). The agar block (discs) of about 7mm diameter of phytopathogenic fungi were cut from the periphery of 7 days old cultures and inoculated aseptically to the centre of the poured petridishes of treatment and control sets. The plates were then incubated at 25 ± 2°C

for 3 days and the zone of inhibition were noted. All the assays were performed in triplicates. Fungal colony diameter of treatments and control sets were measured and percentage of mycelia inhibition was calculated using the following formula:

$$\text{Percentage of mycelial inhibition} = \frac{(C - T)}{C} \text{ X } 100$$

Where, C and T are the growth diameter (mm) in control and treatment respectively.

3.9. Testing of hypoglycemic activities

3.9.1. Extraction

The dried plant powder used for analyzing hypoglycemic activities was extracted following the method of Coban and Konuklugil (2005) with slight modification. For extraction, the sample was dissolved in normal drinking H_2O in the ratio of 1: 10 (w/v). After the extraction, the solvent was evaporated, lyophilized and stored in glass vials at -20°C until further use. Prior, to the use, both the extracts were re-dissolved in normal drinking H_2O to make the stock solution of 2000 mg/mL.

3.9.2. *In vitro* α-amylase inhibition activity

The *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 various 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 activity of the extracts was calculated according to the formula:

$$\% \, \alpha - amylase \; inhibition = \frac{(A_0 - A_1)}{A_0} \; X \; 100$$

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.

3.9.3. *In vivo* test

3.9.3.1. 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 (Cicer seeds) and water *ad libitum* (Figure 5).

3.9.3.2. Acute toxicity study

The extracts, *Nephrolepis cordifolia* aqueous extract (NCAE) and *Cyclosorus dentatus* aqueous extract (CDAE) 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).

3.9.3.2.1. Permission

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

3.9.3.3. 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).

Streptozotocin freshly prepared in citrate buffer (0.1M, pH 4.5) was administered intraperitoneally (i.p) at a single dose of 65 mg/kg bw and in a volume of 1mL/kg. Development of diabetes was confirmed by measuring the blood sugar level after 48h of administration. 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 (Figure 5). Rats with blood glucose levels above 200mg/dL were considered to be diabetic and used for further experimentations.

3.9.3.4. Treatment of diabetic animals

For the treatment of diabetic animals, an experiment was conducted for 15days 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 *Nephrolepis cordifolia* (NCAE) (250mg/kg b.w.) once daily.

Group-V: Diabetic rats orally administered with aqueous extract of *Nephrolepis cordifolia* (NCAE) (500mg/kg b.w) once daily.

Group-VI: Diabetic rats orally administered aqueous extract of *Cyclosorus dentatus* (CDAE) (250mg/kg b.w) once daily.

Group-VII: Diabetic rats orally administered with aqueous extract of *Cyclosorus dentatus* (CDAE) (500mg/kg b.w) once daily.

All the extracts fed were prepared freshly, just before the time of treatment (Figure 5).

Figure 5: Illustration of maintenance and handling of rats. A. Rats housed in the polypropylene cage. B: Blood being withdrawn from the tail vein of rats. C: STZ injected intraperitonially (i.p.). D. Feeding the rats with standard drug(metformin)/plant extracts.

3.9.3.5. Analytical procedure

3.9.3.5.1. Measurement of body weight

The total body weight of all the experimental rats were recorded on day1, day5, day 10 and day 15 of the treatment.

3.9.3.5.2. Estimation of blood sugar level

Blood sugar level was also recorded on day1, day5, day 10 and day 15 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.

3.9.3.5.3. Collection of serum

Blood was collected in a centrifuge sterile glass tube without anticoagulant 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. The serum was then collected without disturbing the residue and used for studying further biochemical parameters.

3.9.3.5.4. Study of serum biochemical parameters

3.9.3.5.4.1. Lipid profile analysis

- Estimation of Total Cholesterol in the serum

  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). 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 (pH 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 (15-30ºC). Then, 2mL of the working reagent was mixed well with 40µL of test sample (unhaemolyzed serum of the experimental rats) and allowed to incubate for 37ºC for 10min. Blank and standard was prepared by taking distilled water and available cholesterol standard (200mg/dL) instead of the serum. The reaction mixture was aspirated

and the absorbance was recorded at 505nm. The cholesterol content was calculated using the following formula (provided in the kit):

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

- Estimation of Triglycerides in the serum

  The content of triglycerides 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 (pH 7.0±0.1 at 20ºC) 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 2mL of the working reagent was then mixed with 20µL of the serum/standard (200mg/dL) and incubated for 10min at 37 ºC). The absorbance was recorded at 505nm. The triglycerides content was calculated using the following formula (provided in the kit):

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

- Estimation of HDL-cholesterol in the serum

  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 Cholesterol esterase (pancreatic), cholesterol oxidase (microbial), peroxidase (horseradish), sodium phenolate, 4-aminoantipyrine, phosphate buffer (pH 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,

500µL of sample was mixed thoroughly with 1000µL of precipitating reagent and allowed to stand for 10min at room temperature (15-30ºC). The reaction mixture was centrifuged at 4000rpm (1800 x g) for 10min to obtain the clear supernatent. The clear supernatant (100 µL) was then mixed well with 2000µL of cholesterol working reagent and incubated for 10min at 37ºC. The absorbance for each test samples and standard was read at 505nm or 505/670nm against appropriate reagent blank. The supplied HDL cholesterol standard was 25mg/dL. The HDL cholesterol content was calculated using the following formula (provided in the kit):

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

3.9.3.5.4.2. Liver function test

- Analysis 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, 1000µL 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 (15-30ºC). 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 (given in 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 ALT at 37 ºC (IU/L) = (ΔA/min) x Factor (1768)

- Analysis 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 the AST 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 (15-30ºC). The working reagent was prepared mixing the content of one reagents bottle with 20mL of Aqua-4 (double deionized, 0.2 micron, membrane filtered, particle free water). For estimation, 1000µL of the working reagent was mixed thoroughly with 100 µL of the test sample (unhaemolysed serum) and aspirated. The absorbance was immediately recorded at 340nm at an interval of 60 seconds.

The AST activity was determined using the following formula (given in 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)

3.9.3.5.4.3. Kidney function test

- 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 available in the manufacturer's instructions of commercially available kit (Erba diagnostics Manheim GmbH, Mallaustr, Manheim/Germany). The urea working reagent (consisting the mixture of α-

ketoglutarate, NADH, urease, glutamate dehydrogenase (GLDH), ADP, Tris buffer (pH 7.9±0.1) was used to determine the concentration of serum in the 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 (15-30ºC). The working reagent was then prepared by gently mixing the content of one reagent bottle with 20mL of Aqua-4. For estimation, 2000µL of the working reagent was mixed well with 20µL of the standard/test samples and aspirated. The absorbance was immediately recorded at 340nm at an interval of 60 seconds against the appropriate blank. The rate of decrease in absorbance is directly proportional to urea concentration in the sample.

The concentration of urea was calculated using the following formula (provided in the kit):

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

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

- Estimation of creatinine level

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

  The creatinine concentration was calculated as follows:

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

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

3.10. Characterization of active compounds

Since the methanolic extracts of *Nephrolepis cordifolia* and *Cyclosorus dentatus* 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 column chromatography and Gas Chromatography mass spectrometry (GC-MS).

3.10.1. Column chromatography

Column chromatography is a type of adsorption chromatography wherein the separation of bioactive components of the plant depends on the differential adsorption of the substance by the adsorbent. For successful separation, the component must possess varying degree of affinity towards adsorbent and even varying reversible interaction. Low affinity compounds are eluted first in column chromatography.

The method described by Tomer *et al*. (2009) with minor modification was followed for column chromatography. The methanolic extract was separated by column chromatography using column of an appropriate size (60cm length and 4cm diameter). Initially the column was washed thoroughly with water and rinsed with suitable solvent and then dried completely. Non-absorbant cotton was placed at the bottom of the column. Initially the column was filled with n-hexane to about 20cm height. Then, the column was packed with the solution of silica gel with hexane using the wet slurry method. The slurry of silica gel was prepared by adding 280g of silica gel (60-120 mesh) into 350mL of n-hexane. Simultaneously, the column was tapped continuously to ensure proper loading of the gel and to prevent the occurrence of air bubbles in the column. Finally, the column was loaded upto the $3/4^{th}$ of the column size. The loaded column was rinsed with hexane before loading the methanolic extract of *Nephrolepis cordifolia* and *Cyclosorus dentatus*. The extracts were mixed with silica gel until the free flowing powder was obtained. The powder was then slowly poured into the column containing hexane. Simultaneously, the poured powder was stirred slightly with the glass rod to avoid clogging without disturbing the loaded silica gel. The solvent was released by slowly opening the knob. The elution was done using hexane, ethyl acetate and methanol in different ratios like Hexane (100%- broad fraction 1), Hexane: Ethyl acetate (50:50- broad fraction 2), Ethyl acetate (100%- broad fraction 3), Ethyl acetate: Methanol (50:50 – broad fraction 4) and methanol (100%- broad fraction 5).

Various parameters such as the weight of the individual eluted fraction, colour and the biological activities such as antioxidant activity mainly DPPH and α-amylase activity of was tested.

3.10.2. Gas Chromatography mass spectrometry (GC-MS)

The collected fractions of both the plant samples were further analysed through GC-MS to identify the various bioactive constituents. For analysis, samples were sent to Sophisticated Analytical Instrument Facility, 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 110ºC and maintained as such for 2min, after which the oven temperature was

increased to 280ºC at the rate of 5ºC /min and maintained further at this temperature for 9 min. The helium flow rate was maintained at 1mL/min and the injection port temperature was kept at 250ºC. 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.

3.11. *In silico* molecular docking studies

3.11.1. Preparation of protein and ligand structure

The X-ray crystallographic structure of diabetic molecular targets like peroxisome proliferators activated receptor gamma (PPAR-γ) (PDB ID: 3DZY), 11-β-hydrosteroid dehydrogenase type 1 (PDB ID: 2BEL) (Berman *et al*., 2000), glucokinase (PDB ID: 1V4S) (Kamata *et al.,* 2004) and fructose 1,6-bisphosphatase (PDB ID: 2JJK) (Hebeisen *et al.*, 2008) were retrieved from the Protein Data Bank (PDB) (http:://www.pdb.org.) database.

The ligand structures or the structures of natural compounds were downloaded from Pubchem database (http://www.pubchem.ncbi.nlm.nih.gov).

Ligand and protein preparation was done using Molecular Operating Environment (MOE) 2009.10 (Chemical Computing Group Inc., 2005) and Pymol 1.0 was used for visualization (DeLano, 2009). All water molecules associated with the crystallographic structure was removed and non polar hydrogen was merged. Further, Gasteiger charges were assigned to the protein and ligand structures. The pdb files were then converted to pdbqt files using Autodock tools. Energy optimization and minimization was done using the MMFF94 force field implemented in MOE.

3.11.2. Docking studies

Docking calculations were conducted with AutoDock Vina (Trott & Olson 2010). The ligand is docked automatically in the AutoDock program with the user-specified dihedral flexibility within a rigid binding site of a protein. Several runs are performed by the program in each docking experiment wherein each run provides one predicted binding mode. During docking, all the torsions were allowed to rotate. The auxiliary program AutoGrid generated the grid maps. Each grid was centered at the crystallographic coordinates of the crystallographic compound. The Lamarckian genetic algorithm was applied for the search using default parameters. The number of docking runs was 100. After docking, the best 100 solutions were clustered into groups. The

clusters were ranked by the lowest energy representative of each cluster. In order to describe the ligand-binding pocket interactions, the top ranked binding mode found by AutoDock vina in complex with the catalytic binding site of target enzymes was subject to full energy minimization using the MMFF94 force field implemented in MOE until the gradient 0.05 was reached. Protocol described by Hernández-Campos *et al.* (2010) was followed to refine the docking poses.

3.11. Statistical analysis

All the data was subjected to various statistical analyses and expressed as mean ± standard deviation (SD). Analysis like one way and two way analysis of variance followed by Fisher's Least significance difference test (LSD), Student's t-test, one way ANOVA with Dunnett's test and Principle component analysis (PCA) was employed for the statistical analysis of data. Softwares like KyPlot (version 2 beta15) and IBM SPSS statistic version 21 was used for the analysis.