Chapter 4

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 PREPARATION OF PLANT EXTRACTS

Liverwort samples were first carefully examined for the separation of specimen of interest and cleaned cautiously to remove contaminants such as soil and other plant specimens. Samples were then air dried and crushed into powder. Powdered sample of each plant was extracted successively by soxhletion using solvents having different polarity (from non polar to polar). 10g sample was extracted successively with heptane, diethyl ether, ethyl acetate, acetone, butanol and methanol. Solvents were completely evaporated and the crude extract obtained was reconstituted in methanol and stored for further use.

4.2 ANTIOXIDANT ASSAYS

4.2.1 Determination of $DPPH^{\bullet}$ scavenging activity

$DPPH^{\bullet}$ scavenging potential of plants was determined by following the method of Sidduraju *et al*. (2002). Plant extract (200 µl) was added to 2 ml 0.2mM $DPPH^{\bullet}$ solution and the change in color of $DPPH^{\bullet}$ from violet to light yellow was measured spectrophotometrically at 517 nm against reagent blank. The inhibition percentage was calculated by using following equation:

Inhibition percent (%) = $[(A_{control} - A_{sample}) / A_{control} \times 100]$

Where, $A_{control}$ is the absorbance of control and A_{sample} is the absorbance of sample. IC_{50} value was used to express the antioxidant activity. IC_{50} value is defined as concentration of antioxidant required for decreasing the free radical concentration by 50% (Gaikwad *et al*., 2011).

4.2.2 Determination of $ABTS^{+}$ scavenging activity

Method described by Li *et al*. (2009), was followed for estimating $ABTS^{+}$ scavenging activity. $ABTS^{+}$ stock was prepared by dissolving $ABTS^{+}$ to 7 mM concentration. $ABTS^{+}$ stock solution was mixed with 24mM potassium persulfate to prepare $ABTS^{+}$ cation. The solution was kept in dark for 12- 16 h at room temperature before use. Before using solution was diluted with distilled water to give an absorbance of 0.700 (±0.02) at 734 nm. 2ml $ABTS^{+}$ solution was then added to 20 µl plant extract and incubated for 10 minutes.

Absorbance was measured at 734 nm. Inhibition percentage was calculated by using similar chemical equation as mentioned above.

4.2.3 Determination of superoxide scavenging activity

Superoxide scavenging activity was determined by the method described by Fu *et al*. (2010), 1 ml extract and 1ml nitroblue tetrazolium chloride (312µM in phosphate buffer, pH 7.4) were mixed, to this 1ml nicotinamide adenine dinucleotide (936µM in phosphate buffer, pH 7.4) was added after 5 minutes. The mixture was centrifuged to remove precipitate. 10 µl phenazine methosulphate was added to the mixture and was incubated for 30 minutes with exposure to fluorescent light. Absorbance was measured at 560 nm. Formula used for calculating inhibition percentage:

Inhibition percent (%) = $[(A_{control} - A_{sample}) / A_{control} \times 100]$

4.2.4 Determination of nitric oxide scavenging activity

Method of Marcocci *et al*. (1994), was used for estimating nitric oxide scavenging activity. Nitric oxide was generated from sodium nitroprusside. 2 ml 20mM sodium nitroprusside was mixed with 0.5 ml phosphate buffer and 0.5 ml phosphate buffer. The mixture was incubated for 150 minutes at 25ºC. 3 ml Griess reagent was added after the completion of incubation time and again allowed to stand for 30 minutes at room temperature. Absorbance was measured spectrophotometrically at 540 nm. Formula used for calculating inhibition percentage:

Inhibition percent (%) = $[(A_{control} - A_{sample}) / A_{control} \times 100]$

4.2.5 Determination of metal chelating activity

Ferric ion chelating activity was determined using the method described by Dinis *et al* (1994). Plant extract (400 µl), 40 µl 2 mM $FeCl_2$ and 80 µl 5mM Ferrozine was mixed and allowed to equilibrate for 10 minutes. Metal chelating activity was measured by decrease in the absorbance of iron (II)-ferrozine complex at 562 nm. Formula used for calculating inhibition percentage:

Inhibition percent (%) = $[(A_{control} - A_{sample}) / A_{control} \times 100]$

4.2.6 Determination of ferric ion reducing activity

Previously described method of Gulcin (2009), was used for estimating iron reducing ability. At first 1 ml extract and 2.5 ml phosphate buffer (0.2M) was mixed. To this mixture 2.5 ml 1% potassium ferricyanide was added and incubated for 20 minutes at 50ºC. After completion of incubation period, 2.5 ml 10% trichloroacetic acid was added. Mixture was then cooled and centrifuged for 10 minutes. 2.5 ml supernatant was collected; to this 2.5 ml distilled water and 250 µl 0.1 % $FeCl_3$ was added. Iron reducing potential was determined by plotting absorbance value against different concentrations. Absorbance was measured at 700 nm.

4.3 ANTI-DIABETIC ACTIVITY

4.3.1 Determination of α-glucosidase inhibitory activity

The α-glucosidase enzyme inhibitory activity was determined by the method of Kim *et al*. (2005). For the assay, 2.5 ml 0.2mM phosphate buffer, 0.1 α-glucosidase enzyme and 0.1 ml 3 mM reduced glutathione was mixed and incubated for 15 minutes at 37ºC. Later 0.5 ml sample and 0.25 ml 3 mM ρ-NPG was added and incubated for 15 minutes. 0.1 M Na_2CO_3 was added after 15 minutes to stop the reaction. Absorbance measured at 450 nm. Formula used for calculating inhibition percentage:

Inhibition percentage (%) = $[1-(A_s - A_b) / A_c] \times 100$

Where, A_s = absorbance of sample, A_b = absorbance of blank, A_c = absorbance of control

4.3.2 Determination of α-amylase inhibitory activity

α-amylase inhibitory activity was estimated by the method of Kim *et al*. (2005) mixture of 0.1 ml extract, 0.1 ml α-amylase enzyme and 0.3 ml 0.02 M sodium phosphate buffer was incubated for 10 minutes. 500 µl starch solution was added after 10 minutes. 1 ml dinitrosalicylic acid added at the end to terminate the reaction. The reaction mixture was heated at 100ºC for 15 minutes. Absorbance measured at 540 nm. Formula used for calculating inhibition percentage:

Inhibition percentage (%) = $(A_{Control} - A_{Sample}) / A_{Control} \times 100$

4.4 ANTI- CANCER ACTIVITY

4.4.1 MTT assay for Mammalian Cell Viability

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by mitochondrial succinate dehydrogenase of living cell to a blue formazan product. The effect of liverworts *P. cordatum*, *A. wallichiana*, *P. nepalensis*, *L. cruciata* and *M. paleacea* on 'Kidney cancer cell line (ACHN) was measured by following the MTT assay method described by Denizot and Lang (1986) with few modifications. The cancerous cell line ACHN was seeded in 96 well plates at a density of 5×10^3 cells/well in 100 µl culture medium and incubated for 24 hrs at 37ºC in a humified incubator with 5% CO_2. After incubation and attachment of cells on well, the cells were washed with 1X PBS (Phosphate-buffered saline) twice and treated with different aliquots of studied liverwort extract and kept for 24 hrs. The media was replaced with MTT solution prepared in PBS (10 µl of 5mg/ml/well) after 24 hrs of treatment and again incubated for 3 hours at 37ºC. 50 µl isopropanol was added to solubilize the formazan crystals. The plates were then gently shaken for 1 min and the absorbance was measures at 620 nm by microtiter plate reader (BMG LABTECH SPECTRO star^{Nano} Germany).

4.5 PHYTOCHEMICAL ANALYSIS

4.5.1 Quantitative phytochemical test

4.5.1a Total phenol estimation

Phenol content estimated following the method of Kadam *et al*. (2013) using Folin-Ciocaolteu reagent. For this purpose 1 ml ethanol, 1 ml extract, 0.5 ml 50 % Folin Ciocalteu reagent and 5 ml distilled water were mixed. 1 ml 5% sodium carbonate added after 5 minutes and the mixture was incubated for 1 hour. Absorbance was measured at 725 nm. Gallic acid was used as standard.

4.5.1b Total flavonoid estimation

Method described by Atanassova *et al*. (2011) was followed for the estimation of flavonoid. 0.5 ml extract, 0.3 ml 5% sodium nitrite and 4 ml distilled water was mixed and allowed to stand for 5 minutes. After 5 minutes 0.3 ml 10% aluminium chloride was added and the mixture was left for 6 minutes. After completion of incubation time 2 ml 1 M sodium

hydroxide and 2.4 ml distilled water were added subsequently. Absorbance was measured at 510 nm. Quercetin was used as standard.

4.5.1c Total orthodihydric phenol estimation

Orthodihydric phenol content was estimated by following the method of Mahadevan and Sridhar (1982). Arnow's reagent was prepared by dissolving 10g sodium nitrite and 10g sodium molybdate in 100 ml water and stored in brown bottle. 0.5 ml Arnow's reagent was added to 0.5 ml plant extract. To this mixture 5 ml water and 1 ml of 1 (N) NaOH was added. Absorbance was measured at 515 nm. Catechol was used as standard.

4.5.1d Total Tannin estimation

Tannin content was estimated by following the method described by Thimmaiah (1999). Reaction was initiated by adding 5 ml water and 1 ml sodium carbonate to 0.1 ml plant extract. After 30 minutes of incubation, absorbance was measured at 700 nm. Tannic acid was used as standard.

4.5.1e Total steroid estimation

Total steroid content was estimated according to the method described by Rai *et al* (2013). At first 1 ml extract and 4 ml chloroform was mixed and shaken vigorously. From this mixture, 1 ml chloroform layer was taken and evaporated. The extract remaining on the test tube after evaporation of chloroform was then dissolved in 2 ml Liebermann Burchard's Reagent which is prepared by adding 0.5 ml H_2SO_4 in 10 ml acetic anhydride. Absorbance was measured at 640 nm. Solasodine was used as standard.

4.5.2 Qualitative phytochemical analysis

4.5.2a Test for amino acid

0.5 ml methanolic plant extract was reacted with few drops of ninhydrin reagent (30 mg ninhydrin dissolved in 10 ml n-butanol and mixed with 0.3 ml 98% acetic acid). The mixture was heated in a water bath. Purple colour of the mixture indicated the occurrence of amino acid (Kumar *et al*., 2009).

4.5.2b Test for anthraquinones

1 ml plant extract was evaporated and dissolved in 2 ml chloroform. To this, 2 ml ammonia wad added. Mixture turning into red or orange colour confirms the presence of anthraquinones (Kumar *et al*., 2009).

4.5.2c Test for phytosterol

0.5 ml methanolic plant extract was evaporated and reconstituted in 2 ml chloroform. 2 ml concentrated H_2SO_4 was added. Formation of red colour ring indicated the presence of steroid (Kumar *et al*., 2009)

4.5.2d Test for tannin

0.5 ml methanolic plants extract and 1% lead acetate was mixed. Appearance of brownish to yellowish precipitation indicated the presence of tannin (Kumar *et al*., 2009).

4.5.2e Test for triterpenoids

0.5 ml extract was evaporated and reconstituted in 2 ml chloroform. 1 ml acetic anhydride was added to this mixture and chilled. After cooling 2 ml H_2SO_4 was added. Turning of the mixture into reddish violet colour confirms the presence of triterpenoids (Kumar *et al*., 2009)

4.5.2f Test for resins

From 0.5 ml methanolic extract, the entire methanol was evaporated in a water bath and the remaining plant extract was dissolved in 1 ml petroleum ether. 2 ml 2% cupper acetate was added, shaken vigorously and allowed to separate. Appearance of green colour on the lower layer confirms the presence of resins (Trease and Evans, 1983).

4.5.2g Test for cardiac glycosides

Methanolic extract (0.5 ml) was evaporated and the remaining plant extract was dissolved in 1 ml glacial acetic acid. To this mixture, 1 drop of 10% ferric chloride was added. After the addition of ferric chloride, 1 ml concentrated H_2SO_4 added by the side of the test tube. Appearance of brown ring at interface and green colour of upper layer confirms the presence of cardiac glycoside (Ngbede *et al*., 2008).

4.5.2h Test for alkaloids

Reaction was initiated by mixing 0.5 ml methanolic extract and 0.2 ml 36.5% HCl. To this mixture 0.2 ml Dragendroff's reagent was added. Formation of orange or red precipitation indicated the presence of alkaloids (Kumar *et al*., 2009).

4.5.2i Test for glycosides

For the detection of glycosides 0.5 ml methanolic extract was mixed with 2 ml of 50% HCl and hydrolyzed for 2 hours on a water bath. After 2 hours 1 ml pyridine, few drops of 1% sodium nitropruside and 5% sodium hydroxide were added. Appearance of red to pink colour confirms the presence of glycosides (Kumar *et al*., 2009)

4.5.2j Test for reducing sugar

Reaction initiated by mixing 0.5 ml extract and 2 ml (1:1) mixture of Fehling's solution A and Fehling's solution B. The mixture was then heated in water bath for 5 minutes. Formation of brick red precipitate confirms the presence of reducing sugar (Ibrahim, 2009).

4.5.2k Test for flavonoids

Few drops of 10% ferric chloride were added to 1 ml methanolic extract. Turning of the reacting mixture to blue or green colour confirms the presence of flavonoids (Ibrahim, 2009).

4.5.3 Phytochemical screening by Thin Layer Chromatography (TLC) analysis

To screen the presence of secondary metabolites, TLC analysis was performed following the standard method of Wagner and Bladt, 1996. Silica gel 60 F_{254} pre-coated plates (Merck, Darmstadt Germany) were used in this method.

4.5.3a Test for anthraglycosides

Sample preparation: 1 g powdered sample was extracted with 5 ml methanol by heating on a water bath for 10 minutes. 20 µl filtrate was for the analysis.

Separation and identification: Running solvent used for the separation of anthraglycosides was ethylacetate: methanol: water (100: 13.5: 10). For detection, plate was sprayed with 10%

ethanolic potassium hydroxide and evaluated under UV- 365 nm. Red band confirms the presence of anthraquinones.

4.5.3b Test for bitter principle

Sample preparation: 1g powdered sample was extracted by heating on a water bath for 10 minutes with 5 ml methanol. 20 µl filtrate was used for the analysis.

Separation and identification: Running solvent used for separation was ethylacetate: methanol: water (100: 13.5: 10). For detection, plate was sprayed with 10 ml 1% ethanolic vanillin solution followed immediately by 10 ml 10% ethanolic sulphuric acid. Plate was then heated at 110ºC for 10 minutes. Plate was evaluated visually. Appearance of Red/ yellow/ brown or blue-green coloured band confirms the presence of bitter principle.

4.5.3c Test for flavonoids

Sample preparation: 1 g powdered sample was extracted with 5 ml methanol by heating on a water bath for 10 minutes. 20 µl filtrate was used for the analysis.

Separation and identification: Running solvent used for flavonoid separation was ethyl acetate: formic acid: glacial acetic acid: water (100: 11: 11 diphenylboric acid-β-ethylamino ester: 26). For detection, plate was sprayed with 10 ml 1% methanolic followed by 8 ml 5% etahnolic polyethylene glycol-4000. Plate was evaluated in UV-365 nm. Yellow/ green/orange coloured band indicates the presence of flavonoids.

4.5.3d Test for arbutin

Sample preparation: 1g powdered sample was extracted with 5 ml methanol by heating on a water bath for 10 minutes. 20 µl filtrate was used for the analysis.

Separation and identification: Solvent combination ethylacetate: methanol: water in the proportion of 100: 13.5: 10 was used as a running solvent. For detection, plate was sprayed with 10g ferric chloride and 0.5g potassium hexacyanoferrate in 100 ml water. Plate was evaluated visually. Blue coloured band indicated the presence of arbutin.

4.5.3e Test for alkaloids

Sample preparation: Powdered sample was moistened with 1 ml 10% ammonia solution. 5 ml methanol was added to the sample and extracted for 10 minutes in a water bath. 20 µl of the filtrate was used for analysis.

Separation and detection: Running solvent used for separation of alkaloids was ethylacetate: methanol: water (100: 13.5: 10). For detection, plate was sprayed with 1 ml Dragendroff reagent mixed with 2 ml glacial acetic acid and 10 ml water. Plate was evaluated visually. Orange- brown band confirms the presence of alkaloids.

4.5.3f Test for cardiac glycosides

Sample preparation: 1 g powdered sample was mixed with 5 ml 50% methanol and 10 ml 10% lead (II) acetate solution and in a water bath for 10 minutes. The filtrate was then cooled to room temperature and extracted with 10 ml dichloromethane for two times. The extract was then evaporated and the residual extract was dissolved in dichloromethane- methanol (1:1) solution. 100 µl of the filtrate was used for the analysis.

Separation and detection: Running solvent used for separation of cardiac glycoside was ethylacetate: methanol: water (100: 13.5: 10). For detection, plate was sprayed with 15-20 ml 20% solution of antimony-II-chloride in chloroform and heated for 5-6 minutes at 110ºC. Plate was evaluated visually. Appearance of blue coloured band indicates the presence of cardiac glycosides.

4.5.3g Test for coumarins

Sample preparation: Sample was prepared by heating 1g powdered sample with 10 ml dichloromethane for 15 minutes under reflux. Filtrate was evaporated and the residue was dissolved in 0.5 ml toluene. 20-40 µl filtrates was used for analysis.

Separation and detection: Running solvent used for separation of coumarins was toluene: ethyl acetate (93:7). For detection, plate was sprayed with 10 ml 10% ethanolic potassium hydroxide and evaluated in UV 365 nm. Light blue or brown coloured band confirms the presence of coumarins.

4.5.3h Test for phenol

Sample preparation: Sample was prepared in the same way as for coumarins.

Separation and detection: Solvent combination Ethyl acetate: methanol: water in the ratio 100: 13.5: 10 were used as running solvent for separation of phenols. For detection, plate was sprayed with 8 ml Fast blue reagent prepared by dissolving 0.5g fast blue salt B in 100 ml water. Evaluation of plate was done visually. Formation of reddish brown band confirms the presence of phenols.

4.6 SEASONAL CHANGES IN ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT IN LIVERWORTS

4.6.1 Sample preparation

Liverwort *Marchantia paleacea* was collected during two seasons: one during unfavorable growth condition *i.e.* when there is scarcity of water during the month of October to March and the other during favorable environmental condition *i.e*. when water is present in sufficient amount in the environment. Collected samples were then air dried and crushed into powder. 10g powdered sample was then extracted successively with heptane, diethyl ether, ethyl acetate, acetone, butanol and methanol. Solvents were completely evaporated and the crude extract obtained was reconstituted in methanol and stored for further use.

4.6.2 Antioxidant assays

$DPPH^{\cdot}$, $ABTS^{+}$, super oxide, nitric oxide scavenging, metal chelating and reducing power assays were performed following the methods of Sidduraju *et al*. (2002) Li *et al*. (2009), Fu *et al*.(2010), Marcocci *et al*. (1994), Dinis *et al*.(1994) and Gulcin (2009) described earlier in the methodology section for antioxidant assays.

4.6.3 Quantitative phytochemical estimation

Phenol, flavonoid and ortho-dihydric phenol content was estimated following the methods of Kadam *et al*. (2013) Atanassova *et al*. (2011) Mahadevan and Sridhar (1986) described earlier in this methodology section for antioxidant assays.

4.7 BIOASSAY GUIDED PURIFICATION

4.7.1 Extraction of plant sample

1 kg plant sample (*Marchantia paleacea)* was cleaned initially under tap water, dried and ground into fine powder. It was then finely crushed into powder and extracted successively by soxlation method with hexane, toluene, diethyl ether, ethyl acetate,

chloroform, acetone, acetonitryl, ethanol, methanol and water according to the increasing polarity of solvents. The fraction showing the best antioxidant, anti-diabetic activities and flavonoid content was subjected to column chromatography separation method for isolation of active constituents.

4.7.2 Bioassay guided screening of the fractions

$DPPH^{\bullet}$ scavenging assay, anti-diabetic assay and flavonoid content estimation was done following the method of Sidduraju *et al*. (2002), Kim *et al*. (2005) and Atanassova *et al*. (2011) as described earlier.

4.7.3 Column chromatography

Powdered and dried plant sample (1Kg) was sequentially extracted with hexane, toluene, diethyl ether, ethyl acetate, chloroform, acetone, acetonitryl, ethanol, methanol and water. Each fraction was analyzed for antioxidant, antidiabetic activity and flavonoid content. The diethyl ether showed the highest antioxidant, anti-diabetic activities as well as flavonoid content. The diethyl ether fraction was then chromatographed on silica gel (75g) column. 10g extract was subjected to column chromatography for separating the extracts into its successive fractions. Silica gel 200-400 M was utilized as the stationary phase and different solvent combinations with increasing polarity were utilized as the mobile phase for the analysis. For the preparation of column, at first a small piece of cotton was stocked at the bottom of the glass column with the help of glass rod to prevent the straining out of the silica gel. Thin layer of sea sand was deposited above the cotton wool for the uniform deposition of silica. The slurry of 75g silica gel and 150 ml benzene was poured carefully into the column. While pouring the slurry, the tap of the column was kept open for free flow of solvent. The solvent was drained freely until a clear solvent carrying no silica gel or cotton wool was obtained. The tap was closed and allowed to stand for 24 hours for stabilization. After 24 hours, the solvent on top of silica gel was drained to the meniscus of silica gel. The sample was prepared by absorbing 10g extract on 20g silica gel by applying a gentle heat. Low heat was given until the sample completely gets attached to the silica gel firmly and dry powder was obtained. Powder was gently layered on the top of silica gel packed in the column. Benzene was then poured gently into the column. Tap of the column was opened and the eluent was allowed to flow at the rate of 30 drops per minute. Solvent systems of increasing polarity were used for the elution of the extract. Solvent systems used were: Benzene: diethyl ether 100: 0, 75: 25, 50: 50, 25: 75; 0: 100; diethyl ether: chloroform 75: 25, 50: 50, 25: 75, 0:

100; chloroform: ethyl acetate 75: 25, 50: 50, 25: 75; 0: 100; ethyl acetate: methanol 75: 25, 50: 50, 25: 75; 0: 100; methanol: water 75: 25, 50: 50, 25: 75; 0: 100. 500 ml of each solvent combination was added gradually by the side of the mouth of the column. This was done carefully as the distortion of the silica gel layer results in non-uniform draining. The eluted sample was collected in the test tube in aliquots of 10 ml. A total of 208 sub fractions were collected and combined on the basis of their antioxidant activity and TLC profiles. Antioxidant property was screened through DPPH radical scavenging activity. Sub fractions with good DPPH radical scavenging activity were subjected to analytical thin layer chromatography.

4.7.4 Analytical thin layer chromatography (TLC) and merging of fractions

The content of the selected sub fractions were spotted on TLC plates pre-coated with silica gel 60 F_{254}; Merck, Darmstadt, Germany. TLC plate was cut into small strips (2cm × 8cm) and 20 µl of concentrated sample (sub fraction of interest) was loaded on the plate with micro pipette above 0.5 cm from the edge. The sample spot was dried by applying hot air. The plate was then gently lowered into a chromatographic jar that contained running solvent system (ethyl acetate: methanol: water:: 100: 13.5: 10). The jar was closed with a lid. The solvent was allowed to run on the plate. The plate was removed and heated in an oven for few minutes to remove the solvents. Dried plate was then viewed under UV lamp at 365nm. On the basis of TLC profiles, sub fractions were merged into eleven main fractions as follows: F1A, F1B, F1C, F1D, F1E, F1F, F1G, F1H, F1I, F1J, F1K. These main fractions were used for antioxidant and anti-diabetic assays. $ABTS^{+}$ and $DPPH^{\cdot}$ scavenging assays were done to evaluate the free radical scavenging activity of the merged fractions. Earlier described method was followed for screening $ABTS^{+}$ and $DPPH^{\cdot}$ scavenging activity. Anti-diabetic activity were screened by measuring their potential to inhibit the activity of α- glucosidase enzyme. Earlier described method was followed. Fraction F1F showed best antioxidant and antidiabetic activity and was subjected to additional fractionation. F1F fraction was chromatographed again on a silica gel column. Column was prepared in the similar method as described earlier. The solvent combinations used for elution process were petroleum ether: benzene 100: 0, 70:30, 50:50, 30:70, 0:100; benzene: diethyl ether 100:0, 70:30, 50:50, 30:70, 0:100; diethyl ether: chloroform 100: 0, 70:30, 50:50, 30:70, 0:100; chloroform: ethyl acetate 100: 0, 70:30, 50:50, 30:70, 0:100; ethyl acetate: methanol 100: 0, 70:30, 50:50, 30:70, 0:100. The eluents were collected at the rate of 30 drops per minute as aliquots of 5 ml. A total of 546 sub fractions were collected and screened for antioxidant anti-diabetic

activities (DPPH˙ scavenging assay) and phytochemical content (flavonoid content). Sub fractions showing good antioxidant activity and high flavonoid content were combined on the basis of their TLC (ethyl acetate: methanol: water:: 10: 1.3: 1) profiles into two main fractions and were subjected to Gas chromatography-mass spectrometry for the identification of active compounds present in the fraction.

4.7.5 Gas Chromatography – Mass Spectrometry analysis

For the analysis, Perkin-Elmer GC Clarus 500 system with Gas chromatograph interfaced to a Mass Spectrometer and AOC-20i auto sampler was utilized. For separations Elite-5MS (5% diphenyl/ 95% dimethyl poly siloxane) and capillary tube (30 × 0.25 µm ID × 0.25 µm film thickness) was used. Carrier gas helium (99.9%) was used at a constant flow rate of 1 ml for 1 minute with a 10.0 mL/ min split flow rate. Mass spectrometer was operated at 70ev ionization energy and a scan interval of 0.5 s scanning from 45 to 450 m/z. Temperature of the column was maintained at 250ºC and temperature 200ºC was maintained for ion source. Column was programmed at temperature 110ºC for 2 minutes with an increase of 10ºC/minute to 200ºC, 5ºC/ minute to 280º C and finally held for 9 minutes at 280º C. The total runtime was 36 minutes. 2 µl samples were loaded in a mode injector. TurboMass Gold^{TM} PerkinElmer was used as the mass detector and Turbo-Mass ver-5.2 was used as software for mass spectral analysis and chromatogram. NIST Library database was used for comparing MS fragmentation patterns and retention time of the compound. Compounds relative percentage was estimated by comparing average peak to total area.

4.7.6 Network pharmacology

4.7.6a Data collection for network analysis

Active phytochemicals present in *M. paleacea* was identified by performing Gas Chromatography –Mass Spectrometry analysis of bio-active fractions obtained from column chromatography. Chemical information like Canonical SMILES, PubChemID etc were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov). Human proteins targeted by the active phytochemicals present in the liverwort were predicted from Binding Data Base (BindingDB) [https://www.bindingdb.org]. To access the high confidence human protein targets, phytochemical-protein interactions having similarity search value ≥ 0.75 was screened. BindingDB is an open access database having binding affinities of approximately 20,000 protein-ligand complexes. It works on the basic principle that similar compounds most likely bind identical proteins. BindingDB aims to support drug discovery through

literature awareness and validation of computational chemistry, development of structure activity relations and molecular modeling approaches. Gene ID of the human protein targets were obtained from UniprotKB (http://www.uniport.org).

Target associated Diseases of all target proteins was determined from Therapeutic Target Database (TTD) [http://bidd.nus.edu.sg]. The diseases were classified according to ICD 10 codes (https://www.icd10data.com). TTD provides information of known, earlier explored therapeutic drugs, pathway information, targeted disease, nucleic acid targets and corresponding drugs directed at targets.

4.7.6b Target-Target interactions

First-degree interaction of the target proteins was identified using STRING (https://string-db.org). STRING (Search Tool for the Retrieval of Interacting Genes/ Proteins) is a biological database of known and predicted protein- protein interactions.

4.7.6c Network construction and analysis

Pharmacological properties of the phyto-compounds were studied by constructing tripartite network through interactions between phytochemical-target protein-associated diseases using Cytoscape (http://www.cytoscape.org). Cytoscape is a platform that analyzes complex molecular networks and integrates these with gene expression profiles.

4.7.6d Enrichment analysis

The functional enrichments were highlighted in list of isolated centralized proteins using KEGG (Kyoto Encyclopedia of Genes and genomes) pathway (https://www.genome.jp/keg/pathway).

4.7.6e Drug-likeliness prediction:

Pharmacokinetic and toxicity properties of phytochemicals were studied by using pKCSM that utilizes graph based signatures using predictive models of ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) properties for drug development.

4.8 *IN VITRO* PROPAGATION AND COMPARISON OF BIOACTIVITIES OF AXENICALLY AND NATURALLY GROWN LIVERWORT *LUNULARIA CRUCIATA*

4.8.1 Plant material collection and method of *in vitro* culture

Liverwort *Lunularia cruciata* was collected from Darjeeling and the voucher specimen was deposited in the herbarium of Botanical Survey of India, Kolkata. Axenic culture was initiated from the gemmae as sporophytes were not found in proper developmental stage. Gemmae present in the gemma cup were carefully taken out and rinsed carefully with distilled water. Sterilization of the gemmae was done in 1, 2, 3 and 4% solution of sodium hypochlorite for different time intervals like 30 seconds, 1 minute, 2 minutes and 4 minutes. Gammae was then immediately washed with sterilized double distilled water. Different culture mediums like half and full strength of Murashige and Skoog medium, Knop's medium and Gamborg G5 medium were used at first to standardize the suitable media for micropropagation. Media was gelled using 0.8% agar and the pH was maintained at 5.8. All the glasswares and media were sterilized by autoclaving them at 15 lb/sq inch for 15 minutes. In the Laminar Air Flow cabinet, the sterilized gammae was inoculated into the media. The culture was carefully maintained under controlled and aseptic condition under illumination of alternating 4000-5000 lux light and dark period of 14 and 10 hours respectively at 21±2ºC. Germinated gemmae were transferred into another media supplemented with growth regulators like 1-Naphthaleneacetic and 6-Benzylaminopurine.

4.8.2 Screening of phytochemicals and biological activities

4.8.2a Sample preparation

Naturally grown as well as *in vitro* grown *L. cruciata* were dried carefully and ground into fine powder. Powder was then refluxed separately in methanol for 4 hours. The extracts were concentrated and stored for future use.

4.8.2b Antioxidant activities

DPPH radical scavenging assay, ABTS radical scavenging assay and metal chelating assay were performed to evaluate the free radical scavenging activity of *in vitro* and *in vivo* grown plants. Earlier described methods for $DPPH^{\bullet}$, $ABTS^{+}$ and metal chelating assay were followed.

4.8.2c Anti-diabetic activity

α- glucosidase and α- amylase enzyme inhibitory assays were performed to evaluate anti-diabetic potential of *in-vitro* grown and naturally grown plants. Earlier described methods for α- glucosidase and α- amylase inhibitory assays were followed.

4.8.3 Phytochemical analysis through Gas Chromatography – Mass Spectrometry analysis

Phytochemicals present in axenically and naturally grown liverwort *L. cruciata* were studied by Gas Chromatography – Mass Spectrometry analysis. Previously described method for GC-MS was followed.

4.9 VARIATION IN THE ABUNDANCE OF EPIPHYTIC LIVERWORTS IN RELATION TO PHYSICO-CHEMICAL ATTRIBUTES

4.9.1 Study area

Senchal Wildlife Sanctuary, Darjeeling, West Bengal, India was studied for habitat study. It is one of the oldest wildlife sanctuaries of India situated at an elevation of 1500 to 2600 m and covers an area of 38.88 km^2. The mean annual temperature ranges from 8.9ºC to 15.98ºC and the annual precipitation in the area is 29.81.8 mm. Studied sample plot was selected randomly. In this area, *Cryptomeria japonica* is the dominant tree species and it also favours the luxuriant growth of epiphytic liverworts. Thus, the study of abundance of liverworts was conducted on a total of fifty *C. japonica* trees of different age groups. Density, presence or absence of the epiphytic liverworts was recorded up to diameter at breast height (dbh). Five quadrats of 5× 5cm size were placed randomly on each tree for the sampling of bryophytes along the underlying bark.

4.9.2 pH measurement of the bark

Bark has been cleaned carefully by removing all the epiphytic species growing on it. 0.5g bark was cut into small pieces. 20 ml 1M KCl solution was added and shaken vigorously. After 1 hour pH was measured using pH meter.

4.9.3 Total phenol estimation

3g bark was powdered and refluxed with methanol for two hours. The filtered extract was used for phenol estimation. Method of Kadam *et al*. (2013) was followed. One ml

sample, 1 ml 95% ethanol, 5 ml distilled water and 0.5 ml 50% Folin Ciocalteau reagent were mixed, followed by addition of 1 ml 5% Na_2CO_3 after 5 minutes. The absorbance was measured at 725 nm. Standard curve was calibrated using different concentrations of gallic acid.

4.9.4 Total flavonoid estimation

Flavonoids were estimated following the method of Atanassova *et al*. (2011). 0.5 ml extract, 4 ml distilled water and 0.3 ml 5% $NaNO_2$ was mixed. To this, 0.3 ml 10% $AlCl_3$ and 2 ml 1.0 M NaOH was added after 5 minutes and 6 minutes respectively. The whole mixture was diluted by adding 2.4 ml of distilled water. Absorbance was measured at 510 nm. Quercetin was used as standard.

4.9.5 Terpenoid estimation

Method of Theng and Korpenwar (2013) was followed. Two gram bark powder was soaked in 50 ml 95% ethanol for 24 hours. It was then filtered. The filtrate was extracted with petroleum ether (60°C – 80°C). After extraction, petroleum ether fraction was kept and dried. Content of total terpenoids were determined from extractive weight of the petroleum ether fraction.

4.9.6 Total soluble sugar estimation

One hundred milligram (100 mg) bark powder was boiled for 3 hours with 5 ml 2.5N HCl in water bath. Extract was neutralized by adding sodium carbonate and the volume was made up to 100 ml. The extract was then centrifuged and supernatant collected was used for estimation of total soluble sugar following the earlier described method of Thimmaiah (1999). One milliliter (1 ml) aliquote was mixed with 4 ml anthrone reagent and heated for 8 minutes in a water bath. The reactant was cooled rapidly and the absorbance as measured at 630 nm.

4.9.7 Moisture content

Three gram bark was dried at 50°C for 1 day until it lost all the moisture content and its weight was stabilized. Bark was weighed again. Moisture content was measured using following formula

$$Moisture\ \% = \frac{initial\ weight - weight\ after\ drying}{initial\ weight} \times 100$$

4.9.8 Light intensity

Intensity of light falling on a particular area was measured using Lutron lux meter LX-101.

4.9.9 Data analysis

Effects of physical and biochemical parameters on epiphytic distribution pattern were studied by Box plot and Histogram test using XLSTAT 2014 software. Correlation co-efficient matrix was prepared using SPSS (Version 12.00) for determining the relation of liverwort density with environmental and biochemical factors. Regression value was calculated using XLSTAT 2014 software. Relationship between epiphytic liverwort density, environmental factors and chemical attributes of trees were studied by Principle component analysis (PCA) and Heatmap using Multivariate Statistical Package (MSVP 3.1) and R software (version 3.4.0) respectively.

4.10 VARIATION IN THE ABUNDANCE OF LIVERWORTS GROWING ON SOIL IN RELATION TO PHYSICAL FACTOR AND SOIL PROPERTIES

4.10.1 Study area

The study of abundance of liverworts was conducted on a total six different locations. Five quadrats of 10 × 10 cm size were set at an interval of 10 cm along the studied sites. A screen with 25 grids (2 cm x 2cm) was placed on each quadrate. The percentage cover of the bryophytes was calculated on the basis of space and number of grids occupied by bryophytes (Fernando *et al*., 2008).

4.10.2 Soil sampling and determination of physicochemical properties

Soil samples are collected from five different sites and processed for physicochemical analysis viz. moisture content, pH, carbon nitrogen ratio of soil, organic carbon, available form of nitrogen, phosphorous and potash content (Jackson, 1973).

4.10.3 Moisture content

Soil sample was dried at room temperature until it lost all the moisture content and its weight was stabilized. Soil was weighed again. Moisture content was measured using following formula

$$Moisture\ \% = \frac{initial\ weight - weight\ after\ drying}{initial\ weight} \times 100$$

4.10.4 Light intensity

Intensity of light falling on a particular area was measured using Lutron lux meter

4.11 STATISTICAL ANALYSIS

The data were collected in triplicate. IC_{50} values and standard error of estimates was determined using MS Excel 2007 (Microsoft, Redmond, WA, USA). Duncan's Multiple Range Test (DMRT) through DSAASTAT software (version 1.002; DSAASTAT, Peruglia, Italy) was used for comparing means; $p < 0.05$ was considered to be statistically significant for differences in the mean level of components. Correlation co-efficient matrix using SPSS (Version 12.00, SPSS Inc., Chicago, IL, USA) and a principal component analysis (PCA) using Multivariate Statistical Package (MVSP 3.1) was used for drawing relation between pharmacological attributes and the phytochemical constituents of liverworts. Heatmap by Multivariate Statistical Package (MSVP 3.1) and R software (version 3.4.0) used for analyzing the function of different biological solvents on extraction of phytochemicals from studied liverworts.