1. Collection of Bryophytes

*Plagiochasma intermedium* (Teen-daravaja) Ldbg. et G., *Asterella wallichiana* (Telephone Tower, in south) Lehm. et L., *Targionia hypophylla* (Dhanya-Kothar) L. *sensu stricto* and *Cyathodium cavernarum* (Tabak uddyan) Kunze and a hornwort, *Anthoceros subtilis* (Dhanya-Kothar) St. with rhizoids were collected from the Panhala region of Kolhapur district (Maharashtra) in the month of August-September. Plants irrespective of growth stage (gametophyte or sporophyte) were collected. The care was taken to harvest the pure and uniform patch of particular species of bryophyte to be studied.

2. Physiological and Biochemical studies

A. **Estimation of inorganic constituents:-**
   
a. **Preparation of Acid digest:**

   For acid digestion, method of Toth *et al.* (1948) has been followed for the analysis of inorganic constituents. Plant material was carefully washed in water and blotted to surface drying and subjected to drying at 50°C for 10 days till the dried plant material had constant weight. The oven dried plant material was powdered. Five hundred mg of oven dried powder was transferred to 150 ml capacity beaker to which 20 ml concentrated HNO₃ were added. The beakers were covered with watch glass and kept till the primary reactions subsided. Then these beakers were heated slowly to dissolve solid particles. After cooling to room temperature, 10ml of perchloric acid (60%) were added to it and mixed thoroughly. Then these beakers were heated strongly until a clear and colorless solution (about 2-3 ml) was obtained. It was then cooled and transferred quantitatively to 100 ml capacity volumetric flask, diluted to 100 ml with distilled water and kept overnight. Next day the extract was filtered through dry Whatman No.44 (Ash less) filter paper. Filtrates so obtained were used for estimation of different inorganic constituents.

b. **Estimation of mineral elements:**

   The levels of i) potassium, ii) calcium, iii) magnesium, iv) sodium, v) iron, vi) manganese, vii) zinc, viii) copper, ix) cobalt x)
cadmium, xi) lead and xii) nickel were determined using Atomic Absorption Spectrophotometer (Perkin Elmer 3030). Whenever, needed, appropriate dilution of plant extract was made with distilled water.

xiii) Sulphur:

The sulphur was estimated according to the method of Blanchar et al. (1965) with slight modifications. 10ml of acid digested sample was taken in Nessler’s test tube and to this 1ml of stabilizing reagent [mixture of 95% ethanol and glycerol in 8:2 ratio (v/v)] and 0.5g of BaCl₂ were added and the volume was made to 50ml with distilled water and mixed well. The absorbance was measured at 430 nm against the blank prepared similarly without the sample. The amount of sulphur was calculated by using standard curve of sulphur solution [standard K₂SO₄ solution (2-14ppm sulphur)] and expressed as mg 100 g⁻¹ dry weight.

xiv) Chlorides:

Extraction of Chloride was done according to the method described by Imamul Huq and Larher (1983) with slight modifications, and estimation was performed according to the method of Chapman and Pratt (1961). Five hundred mg oven dried plant material were extracted in distilled water and kept in water bath at 45 °C for 1 hour. After cooling, the extract was filtered through a layer of muslin cloth. The filtrate was collected in 50 ml volumetric flask and volume was made with distilled water. From this, 10 ml extract was taken for titration against standardized AgNO₃. Five drops of 1% potassium chromate solution were added and titrated against standardized 0.05 N Silver nitrate (8.5 g AgNO₃ dissolved in 1 liter distilled water) up to appearance of permanent reddish brown colour.

For standardization 10 ml of 0.1 N sodium chloride (standard) were taken in an Erlenmeyer flask and 50 ml of distilled water and few drops of 1% potassium chromate solution were added. This was titrated against 0.05 N Silver nitrate solution. The chlorides (mg 100 g⁻¹


1 dry weight) were calculated with the help of equation 1 ml 0.05 N AgNO₃ = 1.77 mg Chloride.

**XV) Total Nitrogen:**

Total nitrogen content was determined following the method given by Hawk *et al.* (1948). Oven dried powdered plant material (0.5g) was taken in Kjeldahl’s flask with a pinch of microsalt (200 g K₂SO₄ + 5 g CuSO₄, dehydrated) and to it 5ml H₂SO₄ (1:1) were added. Few glass beads were also added to avoid bumping and the material was digested on low flame. After complete digestion, a faint yellow solution was obtained which was cooled to a room temperature, transferred to a volumetric flask and diluted to 100 ml with distilled water. Then it was filtered through Whatman No. 1 filter paper and used for the estimation of nitrogen.

In a set of Nessler’s tubes, 2 ml of plant extract and different concentrations of standard ammonium sulfate solution (0.236g of oven dried ammonium sulfate dissolved in distilled water and few drops of conc. H₂SO₄ were added. The volume was made 1000 ml with distilled water. This solution contains 0.05mg of nitrogen per ml) were taken. To each of these tubes one drop of 8 % KHSO₄ was added and volume was made 35 ml with distilled water. To this 15 ml of freshly prepared Nessler’s reagent was added (Reagent A: 7g KI + 10 g HgI₂ in 40 ml distilled water, Reagent B: 10 g NaOH in 50 ml distilled water. A and B are to be mixed in proportion of 4:5 at the time of estimation). The reaction between sample and the reagent gives the product NH₄Hg₂I₃ which has orange brown colour. The intensity of this colour was measured after 15 minutes at 520 nm on a double beam spectrophotometer (Shimdtzu UV 190).

**xvi) Phosphorus**

The phosphorus was estimated according to the method of Sekine *et al.* (1965). Here phosphorus reacts with molybdate vanadate reagent to give yellow colour complex. By estimating colorimetrically the intensity of the colour developed and by comparing it with the
colour intensity of the known standards, phosphorus content was estimated.

Two ml of acid digest was taken in the test tubes and to this equal amount of 2 N HNO₃ followed by one ml of freshly prepared molybdate vanadate reagent (A - 25 g Ammonium molybdate in 500 ml of distilled water. B - 1.25 g Ammonium vanadate in 500 ml 1 N HNO₃, A and B were mixed equally at the time of using) were added. Then final volume in each test tube was adjusted to 10 ml with distilled water. The ingredients were mixed well and allowed to react for 20 minutes. After 20 minutes colour intensity was measured at 420 nm using a reaction blank containing no phosphorus. Calibration curve of standard phosphorus was prepared from standard phosphorus solution (0.110 g KH₂PO₄ per litre = 0.025 mg P⁵⁺ ml⁻¹) taking different concentrations, other steps being essentially similar to the one described above. Amount of phosphorus in the plant material was expressed in mg 100 g⁻¹ on dry weight with the help of standard curve.

xvi) Silicates:

The insoluble residue of acid digest on ash-less filter paper was used for the quantification of silicates in bryophytes. Ash-less filter paper along with insoluble residues were kept separately in pre weighted crucibles with lids and ignited in the muffle furnace for 5h at about 200°C. Ignition was done until only white powder remained in the respective crucible.

B. Estimation organic components:
a. Photosynthetic Pigments and products:
i) Chlorophylls:

Chlorophylls were estimated following the method of Arnon (1949). Randomly sampled fresh material was brought to laboratory, washed with distilled water and blotted to dry. Chlorophylls were extracted in 80% chilled acetone. From 0.5g of fresh plant material homogenized in cold mortar with pestle in dark. A pinch of MgCO₃ was added to neutralize the acids released during extraction. The extract was filtered through Whatman No.1 filter paper using Buchner’s funnel
under suction. Final volume of the filtrate was made to 100ml with 80% acetone. The filtrate was transferred into a conical flask wrapped with black paper to prevent photo-oxidation of the pigments. Absorbance was read at 663 nm and 645 nm on a double beam spectrophotometer (Shimdtzu-190) using 80% acetone as a blank.

Chlorophylls (mg100⁻¹g fresh weight) were calculated using the following formulae -
Chlorophyll ‘a’ = 12.7 x A₆₆₃ - 2.69 x A₆₄₅ -------- X
Chlorophyll ‘b’ = 22.9 x A₆₄₅ - 4.68 x A₆₆₃ -------- Y
Total chlorophylls (a+b) = (8.02 x A₆₆₃) + (20.20 x A₆₄₅) -------- Z

Chl. a / Chl. b / total Chls. (mg 100 g⁻¹ fresh weight) = X/ Y/ Z x vol. of extract x 100
1000 x weight of plant material (g)

ii) Carotenoids

Carotenoids were extracted from the weighed amount of material as per the procedure described for chlorophylls earlier. Carotenoids were estimated following the method described by Kirk and Allen (1965). The absorbance was recorded at 480 nm on a double beam spectrophotometer (Shimdtzu UV 190). The total carotenoids were calculated using the following formula –

\[ \text{Total carotenoids} = \frac{A_{480} \times \text{vol. of extract} \times 10 \times 100}{2500 \times \text{weight of plant material (g)}} \]

Where, 2500 = average extinction.

iii) Carbohydrate (Soluble sugars, Reducing sugars and Starch)

The sugars were estimated following the method described by Nelson (1944). Five hundred mg oven dried powder of tissue was extracted with 80% alcohol. The extract was filtered through Buchner’s funnel using Whatman No. 1 filter paper. The filtrate was condensed to
5 ml on water bath and to this 2 g each of lead acetate and potassium oxalate (1:1) were added for decolourization, 40 ml distilled water was added and aliquot was filtered through Buchner's funnel. The volume of filtrate (a) was measured and it served as an extract for determination of reducing sugars. For the estimation of starch, the insoluble residue along with the filter paper obtained at the beginning after filtering the alcoholic extract was transferred to a 100ml conical flask. To this 50ml distilled water and 5ml concentrated HCl were added and the contents were hydrolysed at 15 lbs pressure for half an hour. These conical flasks were cooled to room temperature, and the contents were neutralized by addition of anhydrous sodium carbonate and filtered through Buchner's funnel. The volume of filtrate (b) was measured and this contains reducing sugars (glucose) formed as a result of hydrolysis of starch. The amount of glucose so formed is equivalent to the starch content in the residue.

For estimation of reducing sugars and starch respectively 0.4 ml (a) and 0.1 ml (b) filtrates were taken in a set of other test tubes. Different concentrations of glucose (0.1 mg ml⁻¹) were taken in other test tubes. In each test tube requisite amount of distilled water was added to make final volume 1 ml. In case of blank 1 ml distilled water was taken instead of filtrate or standard glucose. To this 1 ml Somogyi’s alkaline copper tartarate reagent (4g CuSO₄, 5H₂O, 24 g anhydrous Na₂CO₃, 16 g Na-K-tartarate and 180g anhydrous Na₂SO₄ dissolved in 1 liter distilled water) was added and then the tubes were kept in boiling water bath for 10 minutes. After cooling to room temperature, 1 ml Nelson’s Arsenomolybdate reagent (25g Ammonium molybdate dissolved in 450 ml distilled water, 3g sodium arsenate dissolved in 25 ml distilled water, 21 ml concentrated HCl. These ingredients were mixed well and digested for 48 hours at 37°C) was carefully added. The reaction mixtures were further diluted to 10 ml with distilled water. The absorbance readings were recorded on a double beam spectrophotometer (Schimadzu-190) at 660 nm. The amount of reducing sugars was estimated with the help of calibration curve of standard glucose (0.1 mg ml⁻¹) and the values were expressed as g 100g⁻¹ dry tissue.
The extract of reducing sugars was used for the further estimation of soluble sugars. The soluble sugars were estimated following the method of Dey (1990) (Phenol-sulphuric acid) with slight modification. For the estimation, in 0.2 ml plant extract in a test tube 1 ml 0.5% phenol (was carefully added) and mixed thoroughly. Five ml of analytical grade sulphuric acid were added very carefully to the above test tube. This was mixed thoroughly by vertical agitation with a glass rod with a broadened end. The contents were cooled in air and the absorbance was read at 485 nm. The amount of soluble sugars was estimated with the help of standard glucose (0.1 mg ml⁻¹). The values were expressed in g 100 g⁻¹ dry tissue.

C. Secondary metabolites

a. Free amino acids and amid content:

i) Amino acid contents (Quantitative analysis):

Free amino acid contents of bryophytes were estimated following the method by Moore and Stein (1948). Hundred milligram of dry plant material was extracted in 10 ml 80% ethanol. The extract was filtered through Whatman No.1 filter paper using Buchner’s funnel. The residue was washed twice with 5 ml 80% ethanol. The filtrate and washings were collected together and used for assay.

To 0.1 ml of above preparation, 1 ml ninhydrin reagent (0.02g stannous chloride dissolved in 50 ml 0.02M citrate buffer, pH 5 was added to 2 g ninhydrin dissolved in 50 ml methoxyethanol and the mixture was filtered) were added and mixture was boiled for 20 minutes in water bath. After cooling 5 ml diluent (prepared by mixing equal amounts of n-propanol and distilled water) was added and shaken vigorously. After 20 min, the intensity of purple colour developed was read at 570 nm on UV-VIS spectrophotometer. Blank was prepared using 80% ethanol. Standard curve was obtained by taking different concentrations of standard amino acid (0.1 mg ml⁻¹). The free amino acid concentration was expressed as mg 100 g⁻¹ dry weight.

Amides were estimated following the same procedure described for free amino acids except that Asparagine was used in place of
Leucine as standard. The std. curve was prepared with different concentrations of Asparagine (0.1mg.ml-1). The values of optical densities for free amino acids were used to calculate amide content. The relationship used was, 1 ml std. Asparagine = 0.1 mg amides.

ii) **Amino acid contents (qualitative analysis):**

Thin layer chromatography technique was employed for the qualitative analysis of free amino acids in bryophytes.

**Preparation of plant extract:**

One gram of fresh thallus of liverworts and a hornwort were homogenized in 50 ml of 80% ethyl alcohol and filtered through Buchner’s funnel using Whatman No. 1 filter paper. The filtrate was condensed to 3 – 4 ml on water bath and some quantity of water was added in each sample. Then the extracts (samples) were quantitatively transferred to centrifuge tube and centrifuged at 5000 rpm for 10 minutes. Supernatants served as extract for the further analysis.

**Preparation of TLC plates:**

Thin layer plates of silica gel-G were used for qualitative (chromatographic) analysis of secondary metabolites (i.e. for separation of amino acids, flavonoids, terpenoids and lipid compounds). Thin plates of uniform thickness were prepared using silica gel-G (300 mesh, containing CaSO4 as binder). The plates were activated in an oven at 100°C for 1h and stored in dry pace and used.

The thallus extracts (35λ) of each bryophyte were loaded on TLC plate with the help of micropipette for the separation of amino acids. The solvent employed for amino acid separation was N–butanol, Acetic acid and Distilled water in the proportion of 8:2:2. When the TLC plate was sufficiently developed it was removed from the glass jar, air dried and sprayed with Ninhydrin reagent (0.5 % solution of Ninhydrin in 100 ml 80% acetone). The amino acids present in the extract were identified with the help of standard Rf values. The Rf values of developed spots were calculated and the amino acids were identified
and confirmed by co-chromatography of authentic amino acids samples following their colour reactions and Rf values.

iii) Free Proline

Proline was estimated from the oven dried plant material, following the method described by Bates et al. (1973). 0.2 g plant material was homogenized in 10 ml, 3% sulfosalicylic acid and the extract was filtered through Whatman No. 1 filter paper. For assay, 2 ml of the filtrate was mixed with 2 ml of glacial acetic acid and 2 ml of acid Ninhydrin reagent (mixture of 1.25 g Ninhydrin, 30 ml glacial acetic acid and 20 ml 6 M orthophosphoric acid, heated for few minutes so that Ninhydrin was completely dissolved and kept in freeze at 0°C). The contents were boiled for 1 h on boiling water bath and then cooled rapidly in ice bath. 4 ml toluene was added to each test tube and vigorously shaken for few seconds. The absorbance of the toluene chromophore was recorded at 520 nm against toluene as a blank. Standard curve of proline (0.1mg.ml⁻¹) was prepared by taking different concentrations of L-proline. From this standard curve, the proline content was calculated.

b. Total Polyphenols

i) Total Polyphenols (Quantitative analysis):-

The method of Folin and Denis (1915) was employed for determination of the total polyphenol content of bryophyte tissue (from four liverworts and a hornwort). Fresh thallia (0.5 g) were homogenized in 80 % acetone and filtered through Buchner’s funnel. The residue was washed several times with 80 % acetone and the final volume was made 50 ml with 80% acetone. Two ml of plant extract along with a series of standard tannic acid (0.1 mg/ml) were taken in separate Nessler’s tubes and to each tube 10 ml of 20% Na₂CO₃ and 2 ml of Folin Denis reagent (100 g of sodium tungstate mixed with 20 g Phosphomolybdic acid in about 800 ml distilled water to this 200 ml 25% Phosphoric acid was added and the mixture was refluxed for 2-3 hours to room temperature and volume was made 1000 ml with distilled water) were added. The final volume of reaction mixture was
made 50 ml with distilled water. After 20 minutes absorbance was read at 660 nm with reagent blank. Total polyphenols were calculated with the help of std. curve of tannic acid and expressed as mg 100 g⁻¹ fresh weight.

**ii) Total polyphenol (Qualitative estimation):-**

Polyphenols from bryophytes were analyzed qualitatively following the technique of two dimensional paper chromatography. Whatman paper No. I of A4 size was used for the chromatographic separation.

**Preparation of extract : -**

Extract preparation was the same described for qualitative analysis of amino acids.

For two dimensional paper chromatography the solvent systems used were -

**Direction I**- n-Butanol:Acetic acid:Water (40:10:50 v/v, upper layer)

**Direction II**- 2 % Acetic acid

The phenolic compounds separated on chromatogram were spotted (35λ) with micropipette, identified and confirmed by co-chromatography of authentic samples following their colour reactions and Rf values. The position of individual phenolic compound on chromatogram was determined by marking its fluorescent area under UV light as well as under UV light in presence of ammonia.

**C. Flavonoids**

**i) Flavonoids (Quantitative):-**

Total flavanoid content was determined by the method of Luximon-Ramma *et al.* (2002). 0.5g plant material was homogenized in 10 ml, 80 % acetone in a mortar using pestle. The homogenate was filtered through Buchner's funnel using Whatman No. 1 filter paper and final volume of filtrate was made 25 ml with 80 % acetone. 1.5 ml of the extract was taken into a test tube and 1.5 % 1.5 ml Methanolic aluminum chloride (2 g aluminium chloride dissolved in 100 ml pure methanol) was carefully added. Blank was prepared as above but with
distilled water in place of standard rutin. Absorbance was measured at 368nm (367.5 nm) on a UV double beam spectrophotometer (Shimadzu). Amount of total flavonoids was calculated from the standard curve of rutin obtained using different concentrations of standard rutin (0.03 mg/ml) and values are expressed as mg of rutin equivalent g⁻¹ of fresh weight.

**ii) Flavonoids (Qualitative):**

Qualitative analysis of flavonoids from bryophyte thalli was done by employing one dimensional thin layer chromatography.

**Preparation of plant extract:**

5g fresh bryophyte thalli were taken and exhaustively extracted with 90% methanol. Extract was filtered through Buchner’s funnel using Whatman No. 1 filter paper. The filtrate was condensed to 5 – 7 ml on water bath, transferred to centrifuge tube and centrifuged at 5000 rpm for 10 minutes. Supernatant served as extract for the further analysis.

The extract (50μl) of each sample was loaded on TLC plate of uniform thickness for separation of flavonoids. Solvent system comprises Ethyl acetate:Fumaric acid:Acetic acid:Water (100:11:11:26 v/v)(Wagner and Bladt, 2003) was employed. After the TLC was sufficiently developed, the plate was removed from glass jar and air dried. It was sprayed with benzidine spray reagent [0.5g Benzidine, 20ml glacial acetic acid and 80ml ethanol (Bolliger et al.,1965)]. After spraying the TLC plate it was air dried. The plate was heated at 100°C for 15 min and Rf values were measured.

d. **Terpenoid compounds**

i) **Terpenoid compounds (Quantitative estimation):**

Estimation of terpenoids was done following the method of Banthorp (1991). 5g dry thalli of each liverwort and a hornwort were taken and exhaustively extracted with methanol and water mixture (4:1). Each extract was filtered through Buchner funnel with Whatman no.1 filter paper. Residue was discarded and filtrate was reduced to 1/10 at less than 40°C on waterbath. Concentrated extract was then
extracted with chloroform water mixture (1:1). Chloroform and aqueous layer were separated and evaporated till dry residue was obtained. Total non-polar terpenoids i.e. chloroform residue and total polar terpenoids i.e. aqueous residue were calculated by using following formulae,

\[
\text{Weight of Chloroform residue} = \frac{\text{Total non-polar terpenoids}}{\text{Amount of sample taken}} \times 100
\]

\[
\text{Weight of Aqueous residue} = \frac{\text{Total polar terpenoids}}{\text{Amount of sample taken}} \times 100
\]

ii) **Terpenoid compounds (Qualitative analysis) :-**

Qualitative analysis of terpenoids from bryophyte thalli was done by employing one dimensional thin layer chromatography technique. TLC plate prepared for this technique was similar to that described for amino acid separation. While extract used for loading TLC plates was prepared by redissolving separately the chloroform and aqueous residues of quantitative estimation of terpenoids in 5-7ml 90% methanol. 50\(\mu\)l sample of extracts of various bryophytes was loaded on TLC plate and developed in a solvent containing Tolune:Chloroform:Ethanol (40:10:10 v/v) (Wagner and Bladt, 2003). After development, i.e. when the solvent had reached the top, the plate was dried in air and sprayed with the developing freshly prepared anisaldehyde-sulphuric acid reagent [0.5ml of anisaldehyde to 10ml glacial acetic acid and 85ml methanol. 5ml concentrated sulphuric acid was added to that mixture (Bolliger et al., 1965)]. The plate was heated to 100\(^\circ\)C for 10 min and \(R_f\) values of spots were measured.

e. **Total lipids**

i) **Total lipids (Quantitative estimation):-**

Method employed for quantitative analysis of total lipid content was that described by Chiu et al. (1985). 5g dry plant thalli of various bryophytes were extracted with 25ml chloroform:methanol (2:1) with soxhlet extractor. The extract was evaporated under reduced pressure.
The residue was redissolved in chloroform and filtered through Whatman I filter paper. The filtrate was evaporated and the remaining residue was defined as the total lipid of the plants.

ii) **Total lipids (Qualitative estimation):**

Qualitative analysis of lipids was carried out using the technique of one dimensional thin layer chromatography described by Mangold and Malins (1960).

**Preparation of plant extract:**

The thick and sticky residues of various bryophytes extracted in chloroform were evaporated and redissolved in 5-7ml methanol and used for the chromatographic analysis of neutral and phospholipids.

TLC plates were loaded with 100μl of samples by micropipette. The spots were air dried and then the plates were run in a chromatographic chamber. The two different solvent systems were used for separation of phospholipids and neutral lipids. In order to get good separation two different solvent systems were used for separation of phospholipids (*Bolliger et al, 1965*) which were-

**Neutral lipids:** Pet ether : Diethyl ether: Acetic acid(90:10:1v/v).

**Phospholipids:**

**Solvent system I:** Chloroform : Methanol : Acetic acid : Distilled water (85:15:10:04 v/v)

**Solvent system II:** Chloroform : Methanol : Distilled water (65:25:04 v/v).

The plates were run to reach the solvent upto 15cm from the origin. After development plates were removed and dried in air at room temperature. Spots on chromatograms were detected by using iodine vapours. Developed plates were placed in a jar containing vapours of iodine. Within 5-10min brown spots started appearing against yellow background on the plates. Rf values of these spots were measured.
D. Antioxidative enzymes

a. Catalase (EC 1.11.1.6)

Catalase activity was assayed by following the method of Luck (1974) as described by Sadashivam and Manikam (1992). The fresh thalli of liverworts and hornworts were washed, blotted to dry. Five hundred milligram of plant material was homogenized in 10 ml ice cold (1/15 M) phosphate buffer (pH-6.8) and filtered through 4 layered muslin cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes and supernatant was used as source of enzyme. The reaction mixture contained 3 ml of 0.05 M H₂O₂ in 100 ml phosphate buffer (pH-7) and 0.5 ml enzyme extract for Asterella and Targonia while 2 ml of 0.05 M H₂O₂ in 100 ml phosphate buffer (pH-7) and 1ml enzyme extract for Plagiochasma, Cyathodium and Anthoceros sp. were mixed well and change in OD was recorded at 240 nm. The soluble proteins in the enzyme extract were determined according to the method of Lowry et al. (1951). The enzyme activity is expressed as unit h⁻¹ mg⁻¹ protein.

b. Peroxidase (EC 1.11.1.7)

To study peroxidase activity the method of Kondo and Monita (1951) was followed. Five hundred miligram fresh plant tissues (thalli) were homogenized in 15 ml ice-cold (1/15 M) phosphate buffer (pH-6.8) and filtered through 4 layers of muslin cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes and supernatant was used as source of enzyme. The reaction mixture contained 5 ml of 1/15 M Acetate buffer (pH-5), 0.5 ml of 0.1% guaiacol, 2 ml enzyme extract , 2 ml distilled water and 0.5 ml 0.08% H₂O₂. The reaction mixture was incubated at 30°C. After 15 minutes of incubation, 1 ml 1 N H₂SO₄ was added to stop the reaction, followed by measurement of absorbance at 470 nm. The soluble proteins in the enzyme extract were determined according to the method of Lowry et al. (1951).The enzyme activity was expressed as unit h⁻¹ mg⁻¹ protein.

c. Polyphenol oxidase (EC 1.10.3.2)

Activity of enzyme polyphenol oxidase from thalli of bryophytes was studied according to the method of Mahadevan and Sridhar
(1982). Five hundred milligram of plant material was homogenized in 15 ml cold 0.1 M phosphate buffer (pH-6.1). The resultant homogenate was filtered through 4 layers of muslin cloth. The filtrate was centrifuged at 10,000 rpm on cooling centrifuge for 20 minutes. The supernatant served as enzyme source. The assay mixture contained 4 ml 0.1 M phosphate buffer (pH-6.1), 1 ml 0.01 M catechol prepared in 0.1 M phosphate buffer (pH-6.1) and 1 ml enzyme and it was mixed well. The increase in OD at 30 seconds interval up to 180 seconds at 495 nm was recorded. The soluble proteins in the enzyme extract were determined according to the method of Lowry et al. (1951). The enzyme activity is expressed as ΔOD min⁻¹ mg⁻¹ protein.

d. **Superoxide dismutase (SOD; E.C. 1.15.1.1):**

Superoxide dismutase was determined following the method described by Giannopolitis and Ries (1977), with slight modifications. Enzyme was extracted by homogenizing 0.5 g fresh plant material (bryophyte thalli) in 10 ml, 150mM cold potassium phosphate buffer (pH-7.8) containing 1% PVP, to protect enzyme from the action of polyphenols. Then it was filtered through 4-layered muslin cloth and the filtrate so obtained was centrifuged at 10,000 x g for 20 min at 0-4°C. The supernatant was used as an enzyme source. An enzyme assay mixture contained 2 ml potassium phosphate buffer pH-7.8, 0.2 ml methionine (13mM), 0.1 ml Nitroblue tetrazolium (75μM), 0.5 ml EDTA (0.1mM), 1 ml enzyme and 0.1 ml riboflavin (2mM) was added lastly and immediately the absorbance was measured at 560 nm on UV-VIS double beam spectrophotometer (Shimadzu-190). Then the assay mixture was exposed to full sunlight for 30 min and again the absorbance was read at 560 nm. The enzyme activity is expressed as Δ O.D. h⁻¹ mg⁻¹ of protein

e. **Enzyme Phenylalanine Ammonia Lyase (EC 4.1.1.5)**

The activity of phenylalanine Ammonia Lyase (PAL) was determined according to slightly modified method of Bopp and Murach (1980). Five hundred milligram plant material (bryophyte thalli) were homogenized in 10 ml ice-cold 0.2 M sodium borate buffer (pH-8.8)
and filtered through 4 layered muslin cloth. Filtrate was centrifuged at 10,000 rpm for 20 minutes. Supernatant served as enzyme source. Assay mixture containing 2.8 ml 0.2 M sodium borate buffer (pH-8.8), 0.2 ml enzyme, 0.2 ml 0.1 M L-phenylalanine, was incubated at 36°C. Absorbance of the reaction mixture was read at 0 min, 10 min, 20 min and 30 min at 290 nm. The soluble proteins in the enzyme extract were determined according to the method of Lowry et al. (1951). The enzyme activity is expressed as ΔOD min⁻¹ mg⁻¹ protein.

f. Soluble Proteins

The soluble proteins in the enzyme extract were determined according to the method of Lowry et al. (1951). In test tube, 0.1 ml enzyme extract was taken and diluted to 1 ml with distilled water. To this 5 ml of ‘Reagent – C’ solution (50 ml of ‘A’ containing 2% sodium carbonate in 0.1 N aqueous NaOH was mixed with 1 ml of ‘B’ containing 0.5% copper sulphate in 1%, Na-K tartarate), was added, mixed well and allowed to stand for 15 minutes at room temperature. After 15 minutes 0.5 ml Folin Ciocalteau phenol reagent was added with immediate mixing. This was allowed to stand for 30 minutes in dark and intensity of developed blue colour was measured at 660 nm on UV-VIS double beam spectrophotometer (Shimduz-190). Protein content was calculated by comparing this absorbance with standard curve of different concentrations of Bovine serum albumin (0.1 mg ml⁻¹) prepared in a similar manner. The values were expressed as mg g⁻¹ fresh tissue.

3. Allelopathic effect of bryophytes on wheat and jowar:

Bryophytes (four liverworts and a hornwort) growing at Panhala region (Kolhapur district) were collected in sporophytic conditions and brought to the laboratory during months of August-September. Samples were thoroughly washed first with tap water then with distilled water and blotted to dry. 5g fresh whole plant body i.e. thallus of each bryophyte was crushed in 100 ml distilled water
separately. The extracts were filtered through muslin cloth; and supposed as ‘stock’. At the time of experiment different concentrations (dilutions) like 3:1(1.25%), 1:1(2.50%), 1:3 (3.75%) were prepared from stocks separately. The treatments were of four concentrations [3:1(1.25%), 1:1(2.5%), 1:3(3.75%), and Direct (5%)] of the bryophytes. Twenty seeds of jowar and wheat were placed in petri dish (9cm) lined with paper towel (germination paper). Seeds and germination paper were moistened with 6ml of each concentration of the extracts. For control, 6ml distilled water was used. The experiments were done at room temperature in dark. The effect of these extracts was observed in three ways. In one set the seeds were kept for germination directly in different dilutions in respective extracts (6ml) and in the second set the seeds were soaked (6 h) in distilled water and then were kept in different dilutions of respective extract (6 ml) for germination. In the third set the seeds were soaked (6 h) in the respective extracts and then were kept in distilled water (6 ml) for germination. Jowar (M-35-1), Wheat (NIAW-34) seeds used for the study were procured from Agriculture College, Kolhapur.

Germination, root length and shoot length of seedlings were recorded after every 24 h intervals upto 120h. Emergence of radical was considered as criterion for seed germination. Average root and shoot lengths for each treatment were calculated. The data were statistically analyzed.

4. **Antimicrobial Activity of bryophyts:**

A. **Plant material and Extraction Procedure:**

Five different bryophytes viz. *Plagiochasma intermdium*, *Asterella, wallichiana*, *Targonia hypophylla*, *Cyathodium cavernarum* and *Anthoceros subtilis* with rhizoids were collected from the Panhala region of Kolhapur district of Maharashtra in the months of August and September. The care was taken to harvest the pure and uniform patch of particular species of bryophyte to be tested.

Fresh samples of said bryophytes were extensively washed with tap water and distilled water and surface dried on blotting paper. Any
dirt and senescent parts of plants were removed during the washing process.

5g air dried plant material along with rhizoids was powdered in mortar with paste and soxhlet extracted in 150-160ml of two different solvents i.e. dichloromethane and methanol at 40°C. Methanol extract was evaporated to 30ml under vacuum pressure at 40°C. While dichloromethane extract was dried and redissolved in 30ml of acetone. While 15 g fresh thalli of the bryophytes were soaked for 8 days in ethanol, crushed and filtered through Buchner’s funnel and directly used for antimicrobial activity.

B. Test microorganisms

Two fungal strains were procured from NCIM, NCL, Pune. In vitro antifungal activity was tested against 4 different fungal species such as *Aspergillus flavus* (NCIM-524), *A. niger* (NCIM-512) and *Fusarium oxysporium* (NCIM-1008). Fungal species of *Microfomina phaseolina*, was collected from department of Botany Shivaji University, Kolhapur. Antibacterial activity was tested against plant pathogenic bacteria *Erwinia carotovora*, collected from Microbiology department of Rajaram College, Kolhapur. The standard cultures were stored in slants at 4°C. Bacterial cell suspensions were prepared separately in nutrient broth by transferring a loop full of 24 h grown culture from standard culture slant.

C. Preparation of Media:-

**Nutrient Broth :-**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g / L</th>
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</thead>
<tbody>
<tr>
<td>Beef Extract</td>
<td>10</td>
</tr>
<tr>
<td>Peptone</td>
<td>10</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5</td>
</tr>
</tbody>
</table>

The ingredients were dissolved in 1000 ml of distilled water and the pH was adjusted to 7. 4 before sterilization. Nutrient Agar and PDA (Hi-media) were used for antimicrobial and antifungal activity respectively.
D. **Determination of Antifungal Activity:-**

The methanol, dichloromethane and ethanol extracts of bryophytes were tested for their antifungal activity employing poisoned food technique (Dubey, 2001). Sterile PDA media was prepared and poured in the pre-sterilized petriplates. Different concentrations viz. 0.2, 0.3, 0.4 ml of different extracts of different bryophytes were mixed in the respective plates in the liquid medium and the media was allowed to solidify. The plates were left open aseptically to facilitate moisture content and organic solvent to evaporate. Each plate is inoculated with an agar disc (about 3mm diameter) containing young mycelium growth respective fungal pathogen and incubated at room temperature. 50µgml⁻¹ bavistin which served as +ve control and methanol and/or acetone and/ ethanol (Hi-Media) used as –ve control. Plates are incubated at 35°C for 72h. Diameter of the colony was measured and inhibition was calculated.

E. **Determination of antibacterial Activity:-**

*In vitro* antibacterial assay for the methanol, dichloromethane and ethanol extracts of bryophytes was done by agar-plate diffusion method (Perez et al., 1990). Sterile nutrient agar plates were prepared, 48 hrs cultured suspensions were made and inoculated on sterile agar medium in the respective culture plates. The 10mm sterile cork borer was used for making wells. The extracts, 0.2 and 0.4ml were added in assay well carefully. 50µgml⁻¹ tetracycline (Hi-Media) which served as +ve control and methanol and/or acetone and/ ethanol (Hi-Media) used as –ve control were added in the wells. All the plates were kept at low temperature for 1-1 ½ h for sample diffusion and incubated at 35°C for 48 h. After incubation, the zone of inhibition was measured.