CHAPTER-III

MATERIALS AND METHODS
A. MATERIALS:

In the present investigation some of the representatives of dimorphic pteridophytes have been explored periodically, in the vegetative and reproductive fronds from Sawantwadi, Malvan, Castlerock, Anmode and its environs. The identification and classification of these pteridophytes was done with the help of “The ferns of Bombay” written by E. Blatter and d’Almeda and pteridophytic flora of South India written by Manickam (1992) and ‘The ferns of Southern India’ by Beddome (1873). Identification was also done by personal communication with S.S.Bir, C.R. Fraser-Jenkins and Manickam V.S. Voucher specimens are deposited in the Department of Botany, Shivaji University, Kolhapur.

The leaf tissue were washed first with tap water and then rinsed with distilled water, blotted to dry and then used for analysis.

For the present study, the parameters like chlorophylls, carotenoids, TAN, carbohydrates, proline, polyphenols, nitrogen, protein, amino acids, enzymes like nitrate reductase and nitrite reductase, inorganic constituents Viz; Ca$^{2+}$, Mg$^{2+}$, Na$^+$, k$^+$, Fe$^{3+}$, Mn$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Cl$^-$ were studied separately from the vegetative as well as reproductive fronds.

The fresh plant material was used for chlorophylls, carotenoids, TAN and enzymes like nitrate and nitrite reductase while the remaining material was kept in the oven for drying. This dried material was used for analysis of other parameters.
The Phytochemistry was studied in the vegetative as well as in the reproductive fronds. During survey, dimorphism was observed in the following pteridophytes which can be categorized on the basis of their habitats and life forms.

A) Terrestrial-
2. *Bolbotis presliana* (Fee) Ching.
3. *Bolbitis virens* Schott.
4. *Osmunda hugeliana* Presl.
5. *Acrostichum aureum* L.
6. *Ophioglossum vulgatum* L.

B) Twiner -
7. *Lygodium flexuosum* (L.) Sw.

C) Aquatic-
8. *Ceratopteris thalictroides* Brong.
10. *Marsilea quadrifolia* L.
11. *Azolla pinnata* L.

D) Epiphyte-
12. *Drynaria quercifolia* (L.) J. Sm.
B. METHODS :

1. Organic constituents:
   a. Photosynthetic pigments: (Chlorophylls & Carotenoids)

   The chlorophylls from the leaves were estimated by following the method of Arnon, (1949) and carotenoids by following the method of Robbelen, (1965). For this vegetative (before spore formation) fronds were washed first with tap water and then with distilled water and then were blotted to dry and cut into small pieces. This material was homogenised in 80% acetone and filtered through Whatman No.1 filter paper under suction by using Buchner’s funnel. The residue was washed thoroughly 2-3 times with 80% acetone, collecting all the washings in the same container final volume was made to 100 ml with 80% acetone. The preparation of plant extract for chlorophylls was done at 0 to 4°C in dark.

   The absorbance was taken at 663 nm and 645 nm for chlorophyll ‘a’ and chlorophyll ‘b’ and total chlorophylls were calculated by the formula suggested by Arnon, (1949).

   Chlorophyll a = 12.7 x A 663 - 2.69 x A 645 = X
   Chlorophyll b = 22.9 x A 645 – 4.68 x A 663 = Y
   Total chlorophyll (a + b) = 8.02 x A 663 + 20.2 x A 645 = Z
   Chlorophyll a/ b = X/ Y/ Z x Volume of extract x 100
   or = ....................................................
   total (mg / 100 gm ) 1000 x wt. of plant material (gm)

   For carotenoids the absorbance was taken at 452 nm and carotenoids were calculated by Robbelen,(1965).
Carotenes = 4.75 x A452 – (chl. a + b) x 0.226. The values are expressed as mg 100⁻¹g fresh tissue.

**b. Titrable acid number- (TAN):**

The TAN (Titrable Acid Number) of fresh plant material was determined by the method of Thomas and Beevers, (1949). The fresh tissue was washed and rinsed with distilled water and blotted to dry. It was accurately weighed (5gm). The plant material was taken in 150 ml beaker and 50 ml distilled water was added to it and boiled for half an hour. After boiling it was filtered through muslin cloth and final volume of the extract was recorded. Then 10 ml of this filtrate was titrated against standardised N/ 40 NaOH (dissolve 1 gm of NaOH in distilled water and adjust the final volume to 1000 ml) using phenolphthaleine as an indicator till permanent pink colour is obtained and then the readings were recorded.

Standardization of NaOH was done by N/ 40 oxalic acid (dissolve 1.75 gm of oxalic acid in distilled water, adjust the final volume to 1000 ml) using phenolphthalene as an indicator. From the above readings TAN was calculated by using formula,

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\text{TAN} = \frac{\text{Vol.of oxalic acid taken for titration ml.}}{\text{Titration reading ml.}} \times \frac{\text{Total Vol. of extract ml.}}{\text{Wt.of plant material gm.}} \times \frac{\text{Plant extract reading (ml)}}{\text{Vol.of extract taken for titration ml.}} \times 100
\]
TAN represents the number of ml of decinormal NaOH required to neutralize the acid contents from 100gms of fresh tissue.

c. Carbohydrates: (Reducing sugars, total sugars, starch & carbohydrates)

The sugars were estimated by following the method of Nelson, (1944). The soluble carbohydrates were extracted from 0.5 g oven dried powder with 80% neutral alcohol. The extract was filtered through Buchners funnel using Whatman No. 1 filter paper. The filtrate thus obtained was condensed on water bath to about 5 ml to this 2 g lead acetate and potassium oxalate (1:1) were added for decolorization, 40 ml of distilled water was added and aliquote was filtered. The volume of filtrate was measured and it served as an extract for determination of reducing sugars. A 20 ml aliquote of this extract was hydrolysed with 4 ml conc. HCl by autoclaving at 15 lbs atm. pressure for half an hour. The content was cooled, neutralized with unhydrous sodium carbonate and filtered. The volume of the filtrate was measured and this filtrate was used for the estimation of total sugars.

For estimation of starch, the insoluble residue along with the filter paper obtaines at the beginning after filtering the alcoholic extract was transferred to a 100 ml conical flask with 50 ml distilled water and 5
19 ml conc. HCl and hydrolyzed at 15 lbs atm. Pressure for half an hour. These conical flasks were cooled to room temperature, neutralized by addition of unhydrous sodium carbonate and filtered. The volume of the filtrate was measured as this contains reducing sugars (glucose) formed as a result of hydrolysis of starch. These sugars represent the starch content in the residue.

The sugars from all the three filtrates were estimated by determining the reducing power by employing arsenomolybdate reagent introduced by Nelson (1944) for the colorimetric determination of the cuprous oxide formed in the oxidation of sugars by alkine copper tartarate reagent. For this 0.4 ml aliquots and 0.1 ml aliquot for starch were taken in test tube along with different concentrations of standard glucose solution (0.1 mg/ml) in other test tubes. To this requisite quantity of distilled water was added to make final volume 1 ml. In case of blank instead of filtrate or standard glucose distilled water was added to begin with the reaction 1 ml of Somogyis alkaline copper tartarate solution (4 gm Cuso₄5H₂O, 24 gm anhydrous Na₂CO₃, 16 gm Na- k tartarate and 180 gm anhydrous Na₂SO₄ dissolved in 1000 ml distilled water) was added to each test tube. All the reaction mixtures were then subjected to boiling water bath for about 10 minutes. After cooling to room temperature 1 ml of
arsenomolybdate reagent (25 gm ammonium molybdate in 450 ml distilled water, to which 21 ml concentrated H₂SO₄) were added. To this 3 gm sodium arsenate, Na₂HSO₄ 7H₂O dissolved in 25 ml distilled water. All ingredients were mixed well and the solution was placed in an incubator at 37° c for 48 hours before use was added to each reaction mixture. The contents of each test tube were then diluted with distilled water to a volume of 10 ml. After 10 minutes the absorbance of each reaction mixture was read at 560 nm on spectrophotometer. From the standard curve of glucose, the sugar percentage was determined.

The carbohydrates were estimated by following the method of Hedge and Hofeiter (1962). For this oven dried plant material was taken into a boiling tube. The content was hydrolyzed in a boiling water bath for three hours by adding 5 ml of 2.5N Hcl and cooled to room temperature. Then it was neutralized with solid sodium carbonate until the effervescence ceased. The volume was made to 100 ml and centrifuged. The supernatant was collected and took 0.5 and 1 ml aliquots for analysis. Standard was prepared by taking 0, 0.2, 0.4, 0.6, 0.8 & 1 ml of the working standard ‘0’ served as blank. The volume was made to 1ml in all the tubes including the sample tubes by adding distilled water. Then 4ml of anthrone reagent was added. The samples
were heated in a boiling water bath for eight minutes, cooled and the absorbance was read at 630 nm. Standard curve was prepared by plotting concentration of the standard versus absorbance. From this standard curve the amount of carbohydrate was calculated.

The values are expressed as $g \, 100g^{-1} \, g$ dry tissue.

d. **Free proline:**

Free proline content in leaflet in vegetative frond as well as reproductive frond has been determined following the method of Bates *et al.*, (1973). For this 0.5 gm oven dried plant material was homogenized in 10 ml suphosalicylic acid (3%) and the extract was filtered through whatman No 1 filter paper. For assay known quantity of filtrate was mixed with 2 ml of acid Ninhydrin reagent (Mixture of 1.25 gm Ninhydrin, 30 ml glacial acetic acid and 20 ml 6m Orthophosphoric acid, heated for few minutes till ninhydrin was completely dissolved and kept in freeze at $0^\circ$C). The contents were boiled for 1 hour on boiling water bath and then cooled rapidly in freeze ice bath. 4 ml of toluene was added to each test tube and vigorously shaken for few seconds. The absorbance of toluene chromophere was recorded at 520 nm against toluene as a blank. Standard curve of proline ($0.1mg \, ml^{-1}$) was prepared taking different concentrations of proline. From this standard curve the proline content of plant material was calculated. The values are expressed as $g \, 100^{-1} \, g$ dry tissue.
e. Polyphenols:

Polyphenols were estimated using the oven dried powdered material by Folin-Denis method (1915). Polyphenols from dried plant material were extracted in 80% acetone (30 ml). The extract was filtered through whatman No. 1 filter paper using Buchner’s funnel under suction. Polyphenols were extracted repeatedly from the residue. The volume of the filtrate was made to 50 ml. This filtrate was used for the estimation of polyphenols. 2 ml of this filtrate was taken in 50 ml marked Nesseler’s tube. In other such tubes different concentrations (0.5, 1.0, 2.0 and 4.0 ml) of standard polyphenol solution (tannic acid, 0.1 mg/ml) were taken, 10 ml of 2% Na$_2$CO$_3$ were then added to each test tube to make the medium alkaline, 2 ml of Folin denis reagent (100 gm of sodium tungstate and 20 gm of phosphomolybdate acid, dissolved in 200 ml of distilled water were mixed with 25% phosphoric acid. It was then refluxed for two and half hour, cooled to room temperature and diluted to 1 litre with distilled water ) were then added to each tube and finally the volume was made to 50 ml with distilled water. A blank was prepared without polyphenol solution. The ingredients were allowed to mix thoroughly. After 10 minutes the optical density of each mixture was read at 660 nm on spectrophotometer. The values are expressed as g 100$^{-1}$ g dry tissue.
f. Nitrogen metabolism:

i. Total nitrogen and Crude proteins:

Total nitrogen was estimated from leaflet in vegetative as well as reproductive stage by following the method of Hawk et al., (1948). 0.5 gm oven dried plant material was digested in a kjeldhals flask with sulphuric acid (1:1) and a pinch of microsalt (mixture of anhydrous copper sulphate and potassium sulphate in 1: 40 proportion) till the colourless liquid is obtained at the bottom of the flask. It was then cooled to room temperature and transferred quantitatively to volumetric flask and the volume was made to 100 ml with distilled water. It was kept overnight and it was filtered through the filter paper. The filtrate was used for the estimation of nitrogen. 2 ml of this filtrate was taken in Nesseler’s tube (35 and 50 ml marked) in other such tubes different concentrations (0.5, 1.0, 2.0 and 4.0 ml) of standard ammonium sulphate (0.05 mg nitrogen/ml) were taken. One tube is kept as blank without ammonium sulphate. To these test tubes a drop of 8 % potassium bisulphate and 1ml of H₂SO₄ (1:1) were added. The volume of Nesseler’s reagent was then added to each tube Nesseler’s reagent is a mixture of A ( 7gm KI and 10 gm HgI₂ dissolved in 40 ml distilled water) and B (10 gm NaoH, dissolved in 50 ml distilled water ) in the proportion of 4:5. The colour density of the orange brown product produced by the reaction between
NH$_3$ liberated from the sample and the reagent was measured at 520 nm on spectrophotometer. The amount of nitrogen in the sample was calculated from the standard curve of ammonium sulphate.

Crude Protein content was calculated by multiplying the total nitrogen with the factor 5.7. The values of nitrogen and protein are expressed as g 100$^{-1}$ g dry tissue.

**ii. Nitrate reductase:**

Activity of this enzyme in vivo was determined by following the method described by Jaworski, (1971). The leaf tissue was incubated in the medium containing 1 ml 1 M KNO$_3$, 2 ml 5 % n- propanol, 5 ml 0.2 M Phosphate buffer PH 7.5 and 2 ml 0.5 % titron- x- 100 for 1 hour in dark under anaerobic conditions. After 1 hour, 1 ml of reaction mixture was taken out and mixed with 1 ml 1 % sulphanilamide in 1 ml HCl and 1 ml 0.02 % NEEDA ( N-1 Napthylethylene diamine dihydrochloride). The absorbance was read at 540 nm on spectrophotometer (Elico). Standard curve was prepared with 0.03 Mm KNO$_2$ (0.0026mgKNO$_2$/ml incubation medium, 1 ml sulphanilamide and 1 ml NEEDA served as a blank. Enzyme activity is expressed as nm NO$_3$ liberated/hr/gm fresh tissue.
iii. Nitrite reductase:

The activity of enzyme nitrite reductase was determined following the same method described already for nitrate reductase except that KNO$_3$ was replaced by 0.3 Mm KNO$_2$ in the incubation medium and the incubation was done in the light. Changes in KNO$_2$ present in the incubation medium was determined by reading the optical density of the reaction mixture containing 1 ml incubation medium, 1 ml sulphanilamide and 1 ml NEEDA. The difference between the two readings one at zero minute and other after the enzymatic reaction gives an amount of KNO$_2$ utilized by the enzyme (reduced). The values are expressed as NO$_2$ reduced/hr/gm fresh tissue.

g. Total free amino acids:

The amino acids were estimated by following the method of Moore, S and Stein, W.H (1948).

500 mg of the plant sample was grinded in a pestle and mortar with a small quantity of acid washed sand. To this homogenate 5 to 10 ml of 80% ethanol was added. Then it was filtered or centrifuged. Save the filtrate or supernatant. The extraction was repeated twicely with the residue and pooled all the supernatants. The volume was reduced by evaporation and the extract was used for the quantitative estimation of total free amino acids.
Estimation- 0.1 ml of extract was taken and 1 ml of ninhydrin solution was added. Volume was made to 2 ml with distilled water. The tube was heated in a boiling water bath for 20 minutes. Then 5 ml of the diluent was added and mixed the contents. After 15 minutes the intensity of the purple colour was read at 570 nm. The colour is stable for 1 hour. The reagent blank was prepared as above by taking 0.1 ml of 80 % ethanol instead of the extract.

Standard was prepared by dissolving 50 mg of leucine in 50 ml of water in a volumetric flask. 10 ml of this stock standard was taken and diluted to 100 ml in another volumetric flask for working standard solution. A series of volume from 0.1 to 1 ml of this standard solution gives a concentration range 10 g to 100 g. Proceed as that of the sample and read the colour.

Standard curve was drawn using absorbance versus concentration of the total free amino acids in the sample and expressed as percentage equivalent of leucine. The values are expressed as mg g\(^{-1}\) dry tissue.

2. INORGANIC CONSTITUENTS:

a. Preparation of acid digest:

For the estimation of different inorganic constituents (except chlorides) an acid digest was prepared following the method of Toth et al., (1948). For acid digestion, 0.5 gm plant material was taken in 150 ml
beaker. Then 20 ml concentrated HNO₃ was added to it and allowed to stand for 30 minutes till initial reaction subsides. Then the mixture was heated until plant material was dissolved, cooled to room temperature and added to it 10 ml perchloric acid and heated it gradually. The mixture becomes clear. Then volume was reduced to 2-3 ml by heating, then cooled to room temperature and adjusted the volume to 100 ml with distilled water and allowed it to stand overnight. On next day it was filtered through whatman No.1 filter paper. The solution was used for estimation of some elements viz; Sodium, Potassium, Calcium, Magnesium, Iron, Manganese, Copper and zinc. These elements were estimated by using Atomic Absorption Spectrophotometer (Perkin Elmer).

b. Estimation of chlorides:

Chlorides were estimated according to the method described by Imamul Huq with slight modification given by Volhard, (1915). The chlorides were extracted in distilled water at 45°C for 1 hour and hot distilled water was added to prevent drying. After cooling the extract was filtered through the layer of cheese cloth. The filtrate was collected in 50 ml volumetric flask and the volume was made with distilled water. From this 10 ml extract was taken for titration against standardized AgNO₃.
Few drops of acetic acid (20 %) solution (dilute 200 ml concentrated acetic acid with 800 ml distilled water) were added to a filtrate until the pH of the solution was 6 to 7. The five drops of potassium chromate (1%) were added and titrated with standardized 0.05 N silver nitrate (dissolve 8.5 gm AR grade AgNO3 in distilled water. Transfer it to 1 liter volumetric flask and make up the volume with distilled water to 1 liter) until the first permanent reddish brown colour appears.

The values are expressed as g 100\(^{-1}\) g dry tissue.

STANDARDIZATION- 10 ml 0.1 N sodium chloride standard was put into Erlenmeyer flask and 50 ml distilled water was added. This was titrated with prepared silver nitrate solution.

1 ml 0.05N AgNO3 = 1.77 mg chlorides for 1 gm of sample % of chloride = ml 0.05 N AgNO3 X 0.177.