MATERIAL AND METHODS

MATERIAL

The nutraceutical analysis were conducted on three wild edible tuberous plants such as *Brachystelma edulis*, *Ceropegia bulbosa* and *C.hirsuta.*

Collection of Plant Material:

The *Ceropegia hirsuta* and *C.bulbosa* were collected in vegetative, flowering and fruiting stages during frequent visits to the places of collection in the month of June–Octomber. Simultaneously *Brachystelma edulis* was collected during rainy season (June-Sept.) in vegetative and summer (May-June) and in flowering and fruiting from various places of Satara and Kolhapur district: Kas, Thoseghar, Kartikswami ghat, Wai, Pateshwar, Katyani, Ramling, Nesari, Tarewadi and Bahubali by randomly in the wild at scattered places. The local floristic keys were used for determining the species. Approximately, 3 kg material of selected species was collected. The collected material was placed in a polythene bag to prevent loss of moisture during transportation to the laboratory.

Sample Preparation:

The plants were washed thoroughly until no extraneous material remained. They were blotted till the excess moisture absorbed, air dried and weighted to obtain fresh weight. The sample used for mineral analysis was washed using double deionised water. Then the plant material cut into small pieces and placed in paper envelop and dried in the oven at 40 ^{0}C until constant weight was obtained. After complete drying the sample was ground to a fine powder by using an electric grinder. The sample was packed into airtight sample bottles and used for the nutrient analysis. All analyses were conducted in duplicate by using analytical grade reagents.

METHODS

I. Ethnobotanical studies:

Rural people of Satara and Kolhapur district are using various wild edible tuberous plants as a food and medicine. These people mostly relied on the wild plants resources for healthcare. Frequent visit were made to meet different rural people of Satara and Kolhapur district. Information on the traditional uses of plants and plant products useful in day to day life for healthcare was noted down. Remote villages of Satara; namely Shindewadi, Wai, Pateshwar, Ambheri and Nesari, Tarewadi, Katyani, Ramling from Kolhapur were surveyed during August 2008 to June 2010. Ethnobotanical information was collected by interviewing local medicine men, chief old men, experienced informant, old women and cowboys who prescribed their own herbal medicine and data were properly stored. The plant part used, local name, period of collection, place of collection, traditional preparations, doses and mode of administration of plants were recorded.

II. Proximate Nutrient Analysis:

Dry matter and Moisture:

The dry matter of the sample represents the amount of material left after the complete removal of moisture from it. The moisture of the sample was lost by volatilization caused by heat. The amount of material left after the removal of the moisture was the dry matter. Dry matter and moisture of the material were determined by following the method by AOAC (1990). Dishes were washed with detergents and then taken dried at 105 ^{0}C in oven for overnight. Then dishes were removed from oven and then kept in dessicator for cooling and weights. Two gm sample was taken

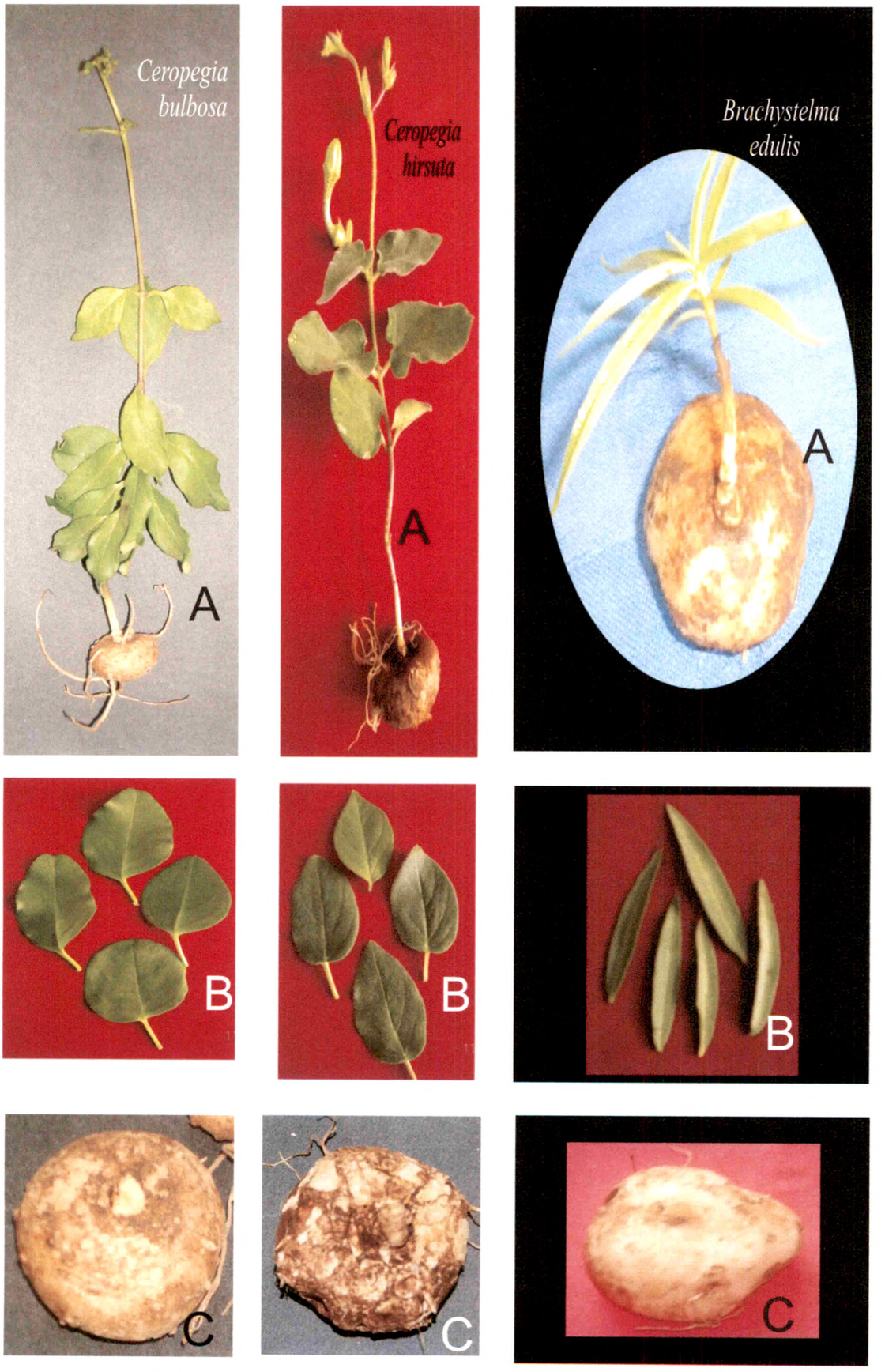

Plate 4 Comparative lifeforms of three wild edible tuberous plants: A. Habit; B. Leaves; C. Tuber

in dishes and placed in oven at 105 ^{0}C overnight. The dry matter and moisture were calculated by using the following formulae:-

$$\text{Dry matter (\%)} = \frac{\text{(Weight of dish + Weight of dried sample)- Weight of dish}}{\text{Weight of sample before drying}} \times 100$$

$$\text{Moisture content (\%)} = \frac{\text{(Weight of fresh sample - Weight of dry sample)}}{\text{Weight of fresh sample}} \times 100$$

Ash:

Ash value was determined by following the method of AOAC (1990). For this crucible were kept muffle furnace at 600 ^{0}C for 1h. Then they were transfer crucible from furnace to a desiccator and cooled to room temperature and weighed as quickly as possible to prevent moisture absorption. Two gram material was taken in tared silica crucible and placed in a muffle furnace at 600 ^{0}C for 6h. Then crucible was transferred to a desiccator and cooled to room temperature, crucible was transferred as quickly as possible to avoid moisture absorption. The percentage of ash was calculated by using the following formula:-

$$\text{Ash (\%)} = \frac{\text{Weight of Ash}}{\text{Weight of sample}} \times 100$$

Crude fibre:

Crude fibre content was determined by following the method of Sadasivam and Manikam (1992). Two gm of dried sample was boiled with 200ml H_2SO_4 for 30 minutes with bumping chips. Then it was filtered through muslins cloth and washed

with boiling water until washings were free of acid. Then the residue was boiled with 200 ml of NAOH for 30 minutes. Again it was filtered through musline cloth and washed with 25 ml of boiling H_2SO_4 three 50 ml portion of water and 25 ml of alcohol. Then residue was removed and transfered to pre-weighed ashing dish (W_1 g). The residue was analysed for 2h. at 130^0C, cooled in desiccator and weighed (W_2 g). It was ignite 30 minutes at 600^0C. After cooling in desiccator it was again reweighed (W_3 g). The percentage of crude fibre was calculated by using following formula:-

$$\text{Crude fibre content (\%)} = \frac{\text{Loss in weight on ignition } (W_2 - W_1) - (W3 - W_1)}{\text{Weight of sample}} \times 100$$

Where, W_1= Preweighted ashing dish,

W_2= Ashing dish with dry residue,

W3= Ashing dish with ash.

Crude fat:

The Crude fat content was determined by following the method of Sadasivam and Manikam (1992). Two gram dried sample was taken in a thimble (prepared from Whatman No.41 filter paper) and kept it in the soxhlet apparatus. A dry pre-weighted solvent flask ('a' g) was connected beneath the apparatus and to it added the required volume of solvent (petroleum ether) and then connected to the condensor. Then the heating was adjusted to give a condensation rate of 2-3 drops and extracted for 16 h. After thimble was removed and ether was retained from the apparatus. The excess of ether was evaporate from the solvent flask on a hot water bath and dried the flask. Then it was cooled in desiccator and weighed ('b' g). Crude fats were calculated by using the following formula:-

$$\text{Crude fat content (\%)} = \frac{(b - a)}{\text{Weight of sample}} \times 100$$

Crude Protein:

The sample was subjected to micro Kjeldahl method to obtain its nitrogen content. Oven dried 0.5 g powder of each plant material was taken in Kjeldahl's flask and to it added10 ml 1:1 H_2SO_4. A pinch of microsalt (200g K_2SO_4 +5 g dehydrated $CuSO_4$) and a few glass beads were added to accelerate the digestion and to avoid bumping of solution in flask, respectively. Digestion was carried out till a clear solution was obtained. After cooling to room temperature, it was transferred quantitatively to 100 ml volumetric flask and the volume was made with distilled water and then stored overnight at room temperature. Next day, it was filtered through Whatman No.1 filter paper and from the filtrate; nitrogen was estimated following the method by Hawk *et al.* (1948). For this, the assay mixture contained, 1ml plant extract, a drop of 8% $KHSO_4$ and 15 ml Nessler's reagent (Reagent A-7 g KI + 10 g HgI_2 in 40 ml distilled water, Reagent B- 10 g NaOH in 50 ml distilled water. Reagents A and B were mixed in the proportion of 4:5, diluted to 50 ml with distilled water. In place of any extract, distilled water along with other assay mixture served as blank. The absorbance was recorded at 520 nm on a double beam spectrophotometer (Shimadzu UV 190). A standard curve of Ammonium sulphate (0.05 mg $N.ml^{-1}$) was prepared and the nitrogen content was calculated. The total nitrogen content of the sample was multiplied by factor 6.25 to calculate the crude protein content.

Carbohydrates:

i. Reducing Sugar, ii. Total Sugar and iii. Starch.

Carbohydrates were estimated according to the method described by Nelson (1944). Five hundred mg of oven dried plant material was homogenized in morter and pestle and extracted with 80% alcohol. It was filtered through Buchner's funnel using Whatman No.1 filter paper. The filtrate was condensed to 5 ml on the water bath and added 2g Lead acetate and Potassium oxalate (1:1) for decolourization, 40 ml distilled water added and aliquot was filtered through Buckner'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 100 ml conical flask. To this 50 ml distilled water and 5 ml conc. HCl were added and hydrolyzed at 15 lbs pressure for 30 minutes. The contents were cooled to room temp. The contents were neutralize by addition of anhydrous sodium carbonate and filtered through Buckner'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, 0.4ml (a) and 0.1 ml (b) filtrates were taken in a set of other test tubes respectively. Different concentrations of glucose (0.1 $mg.ml^{-1}$) were taken in a set of 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 alkaline copper tartarate reagent (4 g $CuSO_4.5H_2O$, 24 g unhydrous Na_2CO_3, 16 g Na-K-tartarate and 180 g unhydrous Na_2SO_4 were dissolved in distilled water and volume

was made to 1000 ml) was added to each test tube. All the test tubes containing the reaction mixtures were subjected to boiling water bath for about 10 minutes and then cooled to room temparature. One ml of Nelsons arsenomolybdate reagent (25 g ammonium molybdate dissolved in 450 ml distilled water, 3 g sodium arsenate dissolved in 25 ml distilled water, 21 ml of conc. HCl These ingredients were mixed well and digested for 48 hours at 37 ^{0}C) was carefully added and reaction mixture diluted to 10 ml by distilled water. The absorbance was recorded on a double beam spectrophotometer (Shimadzu, UV-VIS 190) at 660 nm. The amount of reducing sugar was estimated with help of calibration curve of standard glucose (0.1mg.ml^{-1}) and values were expressed as g.100g^{-1}dry tissue.

The extract of reducing sugar was used for the further estimation of soluble sugar. The total 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 5% phenol (was carefully added) and mixed thoroughly. Five ml of analytical grade sulphuric acid were added very carefully to the above test tubes. 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 total sugar was estimated with the help of std. glucose (0.1mg.ml^{-1}) and values were expressed as g 100 g^{-1}dry tissue.

Energy:

The Atwater system was used to determine the energy values. This system uses factor to estimate available energy from protein, fat, carbohydrates and alcohol component of food item. Energy was calculated by using the general Atwater factor of 4 kilocalorie (kcal) per 'g' protein, 9 kcal per 'g' fat and 4 kcal per 'g' carbohydrate.

These conversion factors were multiplied by 4.186 in order to obtain energy values in kilojoules (kJ) (WHO, 1985).

Energy (kcal) = (4 kcal/g **x** g protein) + (4 kcal/g **x** g carbohydrate) + (9 kcal/g **x** g fat).

III. Mineral Analysis:

Total nitrogen:

Total nitrogen was estimated according to the method of Hawk *et al.* (1948). Oven dried powered of plant material 0.5 gms of each part was taken in Kjeldahl's flask with pinch of microsalt (200g K_2SO_4 +5 g dehydrated $CuSO_4$) and to it 5 ml H_2SO_4 (1:1) were carefully added. A few glass beads were added to accelerate the digestion and to avoid bumping of solution in flask, respectively. Digestion was carried out till a clear solution was obtained. After cooling to room temperature, it was transferred quantitatively to 100 ml volumetric flask and the volume was made with distilled water and then stored overnight at room temperature. Next day, it was filtered through Whatman No.1 filter paper and used for the estimation of nitrogen. For this, the assay mixture contained, 1ml plant extract, a drop of 8% $KHSO_4$ and 15 ml Nessler's reagent (Reagent A-7 g KI + 10 g HgI_2 in 40 ml distilled water, Reagent B- 10 g NaOH in 50 ml distilled water. Reagent A and B was mixed in the proportion of 4:5), diluted to 50 ml with distilled water. In place of any extract, distilled water along with other assay mixture served as blank. The absorbance was recorded at 520 nm on a double beam spectrophotometer (Shimadzu UV 190). A standard curve of Ammonium sulphate (0.05 mg $N.ml^{-1}$) was prepared and the nitrogen content was calculated.

Preparation of acid digests:

The acid digestion method of Toth *et al.* (1948) has been followed for the analysis of inorganic constituents. Tubers and leaves were washed with water. Blotted to dry and then kept in oven at 60^0C till a constant weight was obtained. The oven dried plant material was randomly mixed and powdered. Five hundred mg oven dried powdered was transferred to 150 ml clean borosil beaker and to that 10 ml concentrated HNO_3 were added. It was covered with watch glass and kept for an hour till the primary reactions subsided. Then, it was then heated on hot plate till all the material was completely dissolved. It was allowed to cool to room temperature and then 10 ml of Perchloric acid (60%) were added to it and mixed thoroughly. Then, it was then heated strongly on the hot plate until the solution became colourless and reduced to about 2-3 ml. While heating, the solution was not allowed to dry. After cooling, it was 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 Whatman No. 44 (Ashless) filter paper. The filtrate was stored properly and used for analysis of inorganic constituents.

The level of Calcium, Magnesium, Sodium, Iron, Manganese, Zinc, and Copper were estimated by using Atomic Absorption Spectrophotometer. In case needed, appropriate dilution of plant extract was made with distilled water. Sodium and Potassium were estimated flame photometrically following the standard method of flame photometer (Model-Elico, ch-22A). For standardization, various concentrations of sodium and Potassium were prepared by ranging from 10 to 80 ppm by diluting stock solution of NaCl (100 ppm).

The remaining inorganic elements viz. Calcium, Potassium, Magnesium, Iron, Manganese, Zinc, Copper and Cobalt were estimated by using Atomic absorption spectrophotometer (Perkin-Elmer, 3030 A).

Estimation of Phosphorus:

Phosphorus was estimated from the same acid digest by following the method described by Sekine *et al.* (1965). Two mls of acid digest were pipetted out in a test tube, to which 2 ml of 2 N HNO_3 were added followed by 1 ml of Molybdate-Vanadate reagent (Reagent A: 1.25 g ammonium vanadate dissolved in 1 N HNO_3 and volume was made to 500 ml with 1 N HNO_3. Reagent B: 25 g ammonium molybdate dissolved in distilled water and volume was made to 500 ml. Then reagent A and B were mixed in equal volumes). The volume was made to 10 ml with distilled water. The ingredients were mixed well and allowed to react for 20 minutes. After 20 minutes, yellow colour intensity was measured at 420 nm by using a reaction mixture blank containing no phosphorus. The colour developed by standards of known concentration of phosphorus in KH_2PO_4 solution (0.110 g KH_2PO_4 per litre = 0.025 mg $P.ml^{-1}$) with Molybdate-Vanadate reagent was used for plotting the standard curve. With the help of standard curve, the concentration of phosphorus in the plant material was expressed in mg 100 g^{-1}on dry weight basis.

IV. Antioxidants Analysis:

Total Polyphenols:

The method of Folin and Denis (1915) was employed for determination of the total polyphenols content in plant material. Fresh plant material (0.5 g) was homogenized in 30 ml 80% acetone and filtered through Buckner funnel. The residue was washed several times with 80% acetone and the final volume was made 50 ml

with 80% acetone. One ml of plant extract along with a series of standard tannic acid (0.1 mg ml^{-1}) were taken in a set of Nesselers tubes and to each test tube 10 ml 20% Na_2CO_3 and 2 ml of Folin-Denis reagent (100 g sodium- tungstate mixed with 20 g phosphomolybdic acid was dissolved in about 800 ml distilled water, to this 200 ml 25% phosphoric acid was added and the mixture was refluxed for 2 to 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 standard curve of tannic acid and expressed as mg $100g^{-1}$ fresh tissue.

Ascorbic Acid:

A titrimetric method described by Sadasivam and Manikam (1992) was followed to determine the tubers and leaves ascorbic acid content. The extract was prepared from plant material in 4% oxalic acid to reduce the pH and to stabilize its content by preparing catalytic oxidation. After centrifugation clear supernatant was used to estimate the amount of Ascorbic acid.

Ascorbic acid was oxidized to dehydroascorbic acid by reducing 2, 6 dichlorophenolindophenol (blue dye) to a pink coloured solution. Oxalic acid was used as a titrant. The capacity of plant extract to reduce the dye is directly proportional to ascorbic acid content. Standard Ascorbic acid (100 µg ml^{-1}) was titrated against the dye till the pink colour appeared.

The amount of the dye consumed was equivalent to the amount Ascorbic acid taken for titration and was calculated as follows,

$$\text{Ascorbic acid } (mg.100\ g^{-1}) = \frac{0.5}{\text{Burrete reading (Std.)}} \times \frac{\text{Burrete reading (extract)}}{5} \times \frac{\text{Volume} \times 100}{\text{Weight of sample (g)}}$$

Peroxidase:

To study the enzyme peroxidase activity the method of Maehly (1954) was followed. Five hundred milligram of tubers and leaves of each plant were homogenized in 15 ml ice-cold (0.1M) phosphate buffer (pH 7) and filtered through 4 layered of musline cloth. The filtrate was centrifuged 10,000 rpm for 20 minutes and supernatant was used as source of enzyme. The reaction mixture contained 2 ml 0.1M phosphate buffer (pH 7), one ml 20 mM guaiacol and one ml enzyme extract. The reaction was initiated by the addition of 0.05 ml H_2O_2 (1 mM) changes in optical density due to oxidation of guaiacol was recorded after 30 minutes 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 min^{-1} mg^{-1} Protein.

Catalase:

Catalase activity was assayed by following the method of Luck (1974) as described by Sadasivam and Manikam (1992). Fresh tubers and leaves were collected, washed and blotted to dry and cut into small segment. Five hundred milligram of plant material was homogenized in 10 ml (1/15M) phosphate buffer (pH 6.8) and

filtered through four layered muslin cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes at 4 ^{0}C and supernatant was used as an enzyme source.

The reaction mixture contained 3 ml of 10% H_2O_2 [0.16 ml of H_2O_2 (60 %w/v was diluted to 100 ml with phosphate buffer (pH 7.0)] and 0.1ml enzyme extract. It was mixed well and ΔOD was recorded at 240 nm. The enzyme activity was expressed as unit $\Delta OD min^{-1}.mg^{-1}$ protein described by Bergmeyer (1974).

Superoxide dismutase:

Superoxide dismutase was determined by following the method described by Giannopolitis and Ries (1977), with slight modifications. Enzyme was extracted by homogenizing 0.5 g fresh plant material (Tubers and leaves) in 15 ml, 150 mM 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. The filtrate was centrifuged at 10,000 rpm for 20 min at 0 to 4^{0}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 (13 mM), 0.1 ml Nitroblue tetrazolium (75μM), 0.5 ml EDTA (0.1 mM), 0.1 ml enzyme and 0.1 ml riboflavin (2M) was added lastly, and immediately, the absorbance was measured at 560 nm on UV-VIS double beam spectrophotometer (Shimadzu-190, Japan). 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 $\Delta OD\ h^{-1}\ mg^{-1}$ of protein.

Soluble Protein:

The soluble proteins were determined by following the method of Lowry *et al.* (1951). In the assay mixture, 0.5 ml plant extract was diluted to 1ml with distilled water and 5 ml of freshly prepared reagent 'C' (50 ml 2% Na_2CO_3 in 0.1 N aqueous

NaOH mixed with 1ml 0.5% $CuSO_4.5H_2O$ in 1% Na-K- tartarate) was added to it. After 15 min 0.5ml Folin Phenol reagent (100g Sodium tungstate mixed with 25g Sodium molybdate dissolved in 700 ml distilled water, 50 ml 85 % phosphoric acid and 100 ml concentrated HCl together were refluxed gently for 10 hours using water condenser. To this, 150 g Lithium sulphate, 50 ml distilled water and a few drops of bromine water were added. This was boiled for 15 min without water condenser to remove excess bromine. It was cooled and then adjusted to 1N by titrating it against 1 N NaOH) was added. The colour was allowed to develop for 30 minutes and then absorbance was recorded at 660 nm. Albumin (0.1 mg. ml^{-1}) was used for preparation of standard curve of protein. Blank was prepared with distilled water.

Carotenoids:

The carotenoids were estimated following the method of Kirk and Allen, 1965. Fresh 0.5 g plant material was homogenized in 80% chilled acetone in cold mortar and pestle. A pinch of $MgCO_3$ was added to neutralize the acids released during extraction. The extract was filtered through Whatman No.1 filter paper using Buchner funnel under suction. Final volume of the filtrate made to 100 ml with 80% acetone. The filtrate was transferred into a conical flask wrapped with black paper to prevent photo-oxidation of the pigment. The absorbance was recorded at 480 nm on UV- VIS double beam spectrophotometer, using 80% acetone as blank. The carotenoids were calculated by using following formula:-

$$\text{Total Carotenoids } (mg.100^{-1}g \text{ fresh weight}) = \frac{A_{480} \times \text{volume of extract} \times 10 \times 100}{2500 \times \text{weight of plant material (g).}}$$

Where, 2500 = average extinction.

V. Qualitative analysis of free Amino acids:

Paper chromatography technique of (Zweig and Whitakar, 1971) was employed for the qualitative analysis of free amino acids in tubers and leaves.

Preparation of Plant extract:

Two gm tubers and leaves were homogenized in 50 ml 80% ethyl alcohol and filtered through Buckner's funnel by using Whatman paper no.1. The filtrate was condensed to 3 to 4 ml on water bath. Then, the extracts quantitatively transferred to centrifuge tubes were centrifuge at 5000 rpm for 10 minutes. The supernatants were served as extract for the further analysis.

Loading of extract:

The leaves and tubers extracts of each category loaded on Whatman chromatograph paper with help of micropipette for the separation of amino acid. Solvent system employed for Amino acid separation was N-Butanol, Acetic acid and Distilled water in proportional of 12:3:5.When the chromatogram paper was sufficiently developed, the paper was removed from the glass jar, it was air dried and sprayed with Ninhydrine reagent (0.3% solution of Ninhydrin in Butanol containing 3 ml of acetic acid and diluted to 100 ml distilled water). After spraying Ninhydrine reagent, chromatogram paper was air dried and slightly warmed in oven until the Amino acid were visible. The Amino acid presents in the extract were identified with the help of standard Rf values.