CHAPTER II
MATERIAL AND METHODS

A. Material

1. Procurement of leaf litter and seedlings.

Senescent leaves of *Chromolaena odorata* L. were obtained from the coastal area of Sawantwadi taluka, Maharashtra. The leaf litter was preserved in polythene bags and stored in dry conditions. The fresh matured, insect and disease free leaves of *Chromolaena odorata* were gathered from same place at the time of experimental work.

Seedlings of *Sonneratia alba* Sm., *Acanthus ilicifolius* L., *Derris trifoliata* Lour., *Salvadora persica* L., *Crotalaria verrucosa* L., *Crotalaria retusa* L. and *Ipomoea pes-caprae* (L.) R. Br. were collected from Aronda, Sawantwadi taluka and potted in pots.

2. Preparation of leaf leachate and leaf extract.

The senescent leaf litter was weighted, thoroughly cleaned with tap water and blotted to dry then 200g leaf material was placed to soak in 1 litre of distilled water for 24 hours at room temperature. After 24 hours leachate was filtered through Whatman No.1 filter paper. The leaf leachate (filtrate) was stored in refrigerator until used for further studies.

The fresh leaves were washed thoroughly with tap water, chopped into 1 cm long pieces and were grated with mechanical grater. The 200g ground leaf matter was weighted and soaked in 1 litre of distilled water for 24 hours and filtered through muslin cloth followed by then Whatman filter paper No 1. The aqueous extract was stored in a refrigerator until used for further studies.

Leaf leachate and leaf extract treatments have given to seedling pots by immersing pots into tray containing leaf leachate and leaf extract. When the surface soil of pot is wet then pots removed from the tray and kept in polyhouse. These treatments were continued for three weeks. Then the leaves
of each plant species are harvested, dried, powdered and used for further analysis.

B. Method

1. Photosynthetic Pigments

a. Chlorophylls

By following the method of Arnon the Chlorophylls were estimated (1949). Randomly sampled fresh leaves from controlled and treated plants were gathered and brought to laboratory, cleaned with distilled water and blotted to dry. Five hundred milligram of plant material was homogenized in 80% chilled acetone containing 4ml ammonia per litre and the pigments were extracted. A pinch of MgCO₃ was added to neutralize the acids released during extraction. It is through Whatman No. 1 filter paper the extract was filtered using Buchner’s funnel under suction. The final volume of the filtrate was determined 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 for chlorophyll a and 645 nm for chlorophyll b, on a double beam spectrophotometer (Shimadzu) using 80% acetone as a blank. Chlorophylls (mg100⁻¹ g 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 = (8.02 x A₆₆₃) + (20.2 x A₆₄₅) = .........Z

\[
\text{Chlorophyll a or Chlorophyll b or Total Chlorophylls} = \frac{X/Y/Z \times \text{volume of extract} \times 100}{1000 \times \text{weight of plant material in gram}}
\]

b. Carotenoids:

Carotenoids were estimated by recording the absorbance of 80 % acetone extract of leaf material at 480 nm (Kirk and Allen, 1965). The
carotenoids were estimated from the same extract used for chlorophyll estimation, by recording the absorbance at 480 nm on UV-VIS double beam spectrophotometer, using 80% acetone as a blank. The carotenoids were calculated by with the formula given below and expressed in mg 100⁻¹ g fresh material

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

2. Carbohydrates

According to the method by Nelson (1944) Carbohydrates were projected. The 0.5 g oven dried plant material was then homogenized in mortar with pestle which then extracted with 80% alcohol. It was filtered with Whatman No.1 filter paper by using Bucher’s funnel. Then with 80% alcohol the residue was washed repeatedly on filter paper. Then the filtrated and the washed material were mixed together. This filtrate was then used in order to observe estimation of soluble sugars while the residue was saved for the further estimation of starch.

a Reducing sugars:

To decolourised the extract the filtrate was reduced on the water bath to about 2-3 ml. the lead acetate and potassium oxalate were added to it. With the help of glass rod it was mixed together with the addition of some water. Then the it is filtered and washed for 2-3 times with distilled water. While doing this the filtrate and washings are collected. With distilled water then the final volume of filtrate was arranged to 50ml. This filtrate was used for estimation of reducing sugars (A).

b Total sugars:

From the above filtrate, 20 ml were taken into the conical flask and hydrolyzed with 2-3 ml conc. HCL in an autoclave at 15 lbs pressure for half
an hour. The contents were cooled, neutralized with Na$_2$CO$_3$ and filtered. This filtrate was used for the estimation of total (reducing + non-reducing) sugars (B). The volume of the filtrate was noted down.

c Starch:

With 50 ml of distilled water and 3-5 ml of conc. HCL the residue on the filter paper which was saved for estimation of starch was transferred to it. This was hydrolyzed, neutralized and filtered as stated above. This filtrate contains reducing sugars that is produced as a result of hydrolysis of starch. The sugars so available were estimated to determine the starch present in the tissue (C). The volume of the filtrate was also noted down.

The requisite quantity, (2 ml each of A and B and 0.1 ml of filtrate C) were separately taken in 10 ml marked test tubes. 1 ml of alkaline copper tartarate reagent- (4 g CuSO$_4$·5H$_2$O, 24 g unhydrous Na$_2$CO$_3$, 16 g Na-K- tartarate and 180 g unhydrous Na$_2$SO$_4$ 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 min and then cooled to room temperature. 1 ml of arsenomolybdate reagent (25 g ammonium molybdate in 450 ml distilled water and to this were added 21 ml of conc. H$_2$SO$_4$. This was mixed with solution containing 3 g sodium arsenate which was dissolved in 25 ml distilled water. At the temp. of 37% the mixture of the solutions was placed in an incubator for 48 hours and then added to each test tube and shaken vigorously. The volume of the reaction mixture in each test tube was made 10 ml with distilled water. In the same way a blank was prepared but without sugar solution. After 10 minutes, on double beam spectrophotometer the absorbance was read at 560 nm (Shimadzu, UV-VIS 190). The sugar content was calculated by preparing a standard curve of glucose (0.1 mg.ml$^{-1}$).
3 Enzymatic antioxidants

a. Enzyme Catalase (EC 1.11.1.6)

By following the method of Luck (Sadasivam and Manikam (1992)) a Catalase activity was assayed. Controlled and treated plant’s leaves were collected freshly and brought to the laboratory. Further washed with distilled water, blotted to dry and cut into small pieces. Five hundred milligrams of leaf material was homogenized in 10 ml ice cold (1/15 M) phosphate buffer (pH-6.8) and filtered through 4 layered muslin cloth. At 10000 rpm for 20 minutes the crude enzyme extract was centrifuged and then was used as source of enzyme. The reaction mixture contained 3 ml of 0.05 % H₂O₂ in phosphate buffer (pH-7) and 0.1 ml enzyme extract. It was mixed well and change in optical density after addition of enzyme was recorded at 240 nm. The soluble proteins in the enzyme extract were determined according to the method of Lowry et al., (1951) which has been described earlier. The enzyme activity is expressed as ΔOD min⁻¹ mg⁻¹ protein.

b. Enzyme Peroxidase (EC 1.11.1.7)

Peroxidase activity was studied according to the method of Horiguchi, (1988). Five hundred milligrams leaf material was homogenized in 10 ml ice-cold (1/15 M) phosphate buffer (pH-6.8) and filtered through 4 layers of muslin cloth. The crude enzyme extract 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, 0.5 ml, enzyme extract, 2 ml distilled water, 0.5 ml, 0.08 % H₂O₂ which was incubated at 30 °C. Zero minute reaction was immediately terminated by adding 1 ml, 1 N H₂SO₄ while the reaction was carried out in another test tube for 15 minutes and it was then terminated by addition of H₂SO₄. The difference in the absorbance of 0 and 15 min. reaction mixtures was recorded by taking absorbance at 470 nm. The soluble proteins in the enzyme extract were determined according to the method of Lowry et al., (1951) which has been
described earlier. The enzyme activity was expressed as $\Delta \text{O.D. h}^{-1} \text{mg}^{-1}$ protein.

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

Superoxide dismutase activity was determined following the method described by Giannopolitis and Ries (1977). Enzyme was extracted by homogenizing 0.5 g fresh leaves in 10 ml, 150 mM cold potassium phosphate buffer (pH 7.8) containing 1% PVP (to protect the 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 rpm 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 (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. The total volume of the assay mixture was made 3 ml. Immediately, the absorbance of the assay mixture was measured at 560 nm on UV-VIS double beam spectrophotometer (Shimadzu-190, Japan). Then the assay mixtures were exposed to full sunlight for 30 min and again the changed absorbance was read at 560 nm. The enzyme activity is expressed as $\Delta \text{O.D. h}^{-1} \text{mg}^{-1}$ of protein.

d. **Polyphenol oxidase (E.C. 1.10.3.2):**

The activity of an oxidative enzyme, polyphenol oxidase, was studied spectrophotometrically by using the extraction and assay procedure suggested by Mahadevan and Shridhar (1982).

0.5 g thoroughly washed leaf material was cut into small pieces and extracted in 10 ml cold 0.1 M phosphate buffer pH 6.1, in prechilled mortar with pestle. The homogenate was filtered through 4 layered muslin cloth and centrifuged for 10 min at 10,000 rpm using refrigerated centrifuge (Remi-Model c-24). The supernatant was used as an enzyme source. In order to score the activity of polyphenol oxidase, the oxidation of catechol was measured.
from a reaction mixture containing 3.0 ml phosphate buffer (0.1 M, pH-6.1), 0.4 ml enzyme and 2.0 ml catechol (0.02 M) at 495 nm on UV-VIS double beam spectrophotometer (Shimadzu- 190). The change in optical density was recorded for 1 min. The activity of enzyme is expressed as Δ O.D. min⁻¹mg⁻¹ protein.

4 Antioxidant Assays

a. DPPH free radical scavenging activity

On the basis of free radical scavenging effect of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity, the antioxidant activities of plant extracts and the standard were assessed with the help of modified method (Braca et al., 2002). In methanol the diluted working solutions of the test extracts were prepared. As a standards an Ascorbic acid (10mg/ml) was used. In methanol, 0.002% DPPH was prepared and 3 ml of this solution was mixed with 1 ml of sample solution and standard solution separately. Nearly for 30 minutes these solution mixtures were kept in dark and by using Spectrophotometer optical density was measured at 517 nm. As a blank Methanol (1 ml) with DPPH solution (0.002%, 3 ml) was used. By using following formula, the optical density was recorded and % inhibition was calculated:

\[
\text{Percent inhibition of DPPH activity} = \frac{\text{absorbance(continue)} - \text{absorbance(sample)}}{\text{absorbance(continue)}} \times 100
\]

b. Ferric-reducing / antioxidant power (FRAP)

To calculate the total antioxidant power of leaf extracts the ferric reducing/antioxidant power (FRAP) assay was used. In the FRAP assay, reductants (antioxidants) in the sample reduce Fe3+ / tripyridyltriazine complex, present in stoichiometric excess, to the blue colored ferrous form, with an increase in absorbance at 593 nm. The method described by Beenzie
and Strain (1996) is used to perform antioxidant activity assays. The results are expressed as ascorbic acid equivalent antioxidant capacity.

c. Reducing Power Assay

According to the method of Oyaizu (1986) the reducing power was determined. With 1 ml phosphate buffer (0.2M, pH 6.6) and 1 ml 1% K3Fe(CN)6 an aliquot of 0.5 ml plant extract (0.2, 0.4, 0.6, 0.8, 1 mg/ml) was mixed. The mixture was shaken and then for 20 min the mixture was incubated at 50°C. In order to stop the reaction 1 ml TCA (10%) was added to the mixture after incubation. The mixture then centrifuged at 3000rpm for 10 min. 1.5 ml supernatant, 1.5 ml D.W. and 0.1 ml FeCl3 (0.1%) solution were mixed and incubated for 10 min and absorbance was read at 700nm on Spectrophotometer. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations. The extract concentration providing 0.5 of absorbance (EC50) was calculated from the graph of absorbance at 700 nm against extract concentration. As a standard an Ascorbic acid was used. Higher absorbance indicates higher reducing power.

d. TAC by Phosphomolybdenum method

It is by using method described by Prieto et al, the total antioxidant capacity of methanol extracts of leaves was measured (1999). 5 ml reagent solution (0.6 M sulfuric acid, 28 mm sodium phosphate and 4 mM ammonium molybdate) was mixed with an aliquot of 0.5 ml of sample solution (0.2, 0.4, 0.6, 0.8, 1 mg/ml). The at the temperature of 95°C tubes were closed with cap and incubated in a boiling water bath for 90 min. The absorbance of the aqueous solution was measured at 695nm on the contrast to blank after the samples had cooled to room temperature, on Spectronic 20 visible spectrophotometer. A typical blank solution contained 5 ml of reagent solution. The appropriate volume of the same solvent (methanol) used for the sample.
The solvent then under the same conditions were incubated. For samples antioxidant capacity is expressed as equivalents of ascorbic acid.

**e. Metal chelating ability assay**

By using the method explained by Decer and Welc (1990) the ferrous ion-chelating ability was determined with minor modifications. The extract stock solution of 1.6ml each (0.2, 0.4, 0.6, 0.8 and 1 mg/ml) was combined with 2.16 ml of distilled water and 80 µl of 2 mM FeCl$_2$ in a test tube. By adding 160 µl of 5 mM ferrozine the reaction was started. At room temperature the mixed solutions were kept for 10 min. On Spectrophotometer at 562nm, after incubation, the absorbance was measured. As a control, instead of distilled water (1.6 ml) sample solution was used. As a blank ferrozine solution is used instead of distilled water (160 µl). It was used for error correction because of unequal colour of the sample solutions. As reference standard L-ascorbic acid was used. In triplicate all measurements were performed. The ferrous ion-chelating ability was calculated as follows:

$$\%\text{ scavenging activity} = \frac{(Ac - AE) \times 100}{Ac}$$

Where,

- $Ac$ is the Absorbance of Control reaction.
- $AE$ is the Absorbance of plant extract.
- $As$ is the Absorbance of standard.

**f. Total polyphenols**

Total polyphenols in the leaves were calculated by the procedure of Folin and Dennis (1915). 500mg fresh leaves were homogenized in 30 ml 80% acetone and then by using Buchner funnel it was filtered. With 80% acetone the residue was washed again and again. The final volume was made 50ml with 80% acetone. Clean Nesseler’s tubes were taken. They were labelled as Std.-1,
2, 3, 4 and extract. Different concentrations of polyphenol standard (Std. tannic acid, 0.1mg per ml.) solutions and corresponding plant extracts were accurately transferred in the above labelled test tubes. Then in each Nesseler’s tube 2 ml Folin-Dennis reagent (100g sodium tungstate was mixed with 20g phosphomolybdic acid in about 800ml distilled water to this 200ml 25% phosphoric acid was added and the mixture was refluxed for 3 hours at room temperature and volume was made 1000ml with distilled water.) and 10 ml 20% fresh Na₂CO₃ were added. The final volume of the reaction mixture was made 50ml with distilled water and the reaction mixtures were thoroughly mixed by frequent shaking of the tubes. The reaction mixture was kept for 20 minutes and after 20 minutes absorbance of blue coloured complex was read at 660 nm using reagent blank. Total polyphenols were calculated with the help of standard curve of tannic acid and expressed as mg 100g⁻¹ fresh weight.

g. Ascorbic Acid Content (Vitamin C)

Ascorbic Acid content in leaves was estimated following the titrimetric method of Aberg (1958). The leaves were washed and cleaned with distilled water and immediately blotted to dry. They were cut in to small pieces with the help of razor. 3g of leaf material was transferred to mortar and crushed thoroughly with pestle in 12 ml 0.4% oxalic acid. The oxalic acid was added at the rate of 4 ml/g tissue. The extract was filtered through 2 layers of cheese cloth. Then it was centrifuged at 1000g for 20 minutes. The volume of supernatant was made to 15ml (1g tissue in 5ml oxalic acid) with oxalic acid reagent (0.4% w/v). Ascorbic acid in extract was estimated by visual titration method based on reduction of 2, 6, dichlorophenol indophenol dye.

Standard ascorbic acid solution was prepared by adding 50mg of ascorbic acid to 50ml of 0.4 percent oxalic acid solution in 250ml volumetric flask and finally volume was made to 250ml with oxalic acid. One ml of this solution contains 0.2 mg of ascorbic acid.
Indophenol reagent was prepared as follows. 150ml of glass distilled water was added to 200ml volumetric flask. Then 50mg of sodium 2, 6, dichlorophenol indophenol was added to it. The flask was gently heated in a hot water bath to dissolve the dye. Then 42mg of NaHCO$_3$ were added. The flask was allowed to cool. After cooling the volume of solution was made to 200ml with glass distilled water and the reagent was stored in dark glass bottles at 2°C. For standardizations of indophenol reagents 5ml ascorbic acid solution was taken in white porcelain dish and then it was titrated against the indophenol dye kept in burette until the solution became pink. (The pink colour persisted for at least 15 seconds).

After standardization of indophenol reagent 5ml of plant extract (prepared as described earlier) were titrated against standardized indophenol reagent as above.

The ascorbic acid content of the extract was calculated by using formula-

$$\frac{I \times S \times D}{A \times 100/W} = \text{mg of ascorbic acid} \times 100 \text{g}^{-1} \text{ fresh tissue.}$$

Where,

- $I = \text{ml of indophenol reagent used in the titration.}$
- $S = \text{mg of ascorbic acid reacting with 1ml of the reagent.}$
- $D = \text{Volume of the extract in ml.}$
- $A = \text{the aliquot titrated in ml.}$
- $W = \text{the weight of the sample in grams.}$

5 Osmolytes

a. Free proline

Free proline was calculated from leaves according to method of Bates et al., (1973). 500mg fresh plant material was homogenized in 10 ml of 3% sulfosalicylic acid. After complete homogenization it was then filtered by using Buchner’s funnel by using Whatman No. 1 filter paper. Then filter paper was washed repeatedly with small amount of 3% sulfosalicylic acid. The collected filtrate volume was made to 20 ml with 3% sulfosalicylic acid. 0.5 ml extract
was taken in clean dry test tube and 2 ml of acid ninhydrin reagent was added to it. (Acid ninhydrin was prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml of 6M phosphoric acid with agitation cooled and stored at 4°C). Then 2 ml of glacial acetic acid were added to the reaction mixture. The reaction was allowed to continue for about 1 hour by keeping test tubes in boiling water bath at about 100°C. At the same time reaction mixtures for standard proline curve were prepared by taking different concentrations of proline (0.1ml, 0.2ml, 0.3ml, 0.4ml, 0.8ml). Standard proline solution contains 0.1 mg proline ml⁻¹ of sulfosalicylic acid. After 1 hour the reactions in all test tubes were terminated by keeping them in ice bath. Then 4ml of toluene was added to each test tube with vigorous shaking for 15 to 20 seconds. The reaction mixtures were brought to room temperature. Toluene chromophore layer was pipetted out with the help of vacuum pipette. The absorbance of toluene chromophore layer was read at 520 nm using toluene blank. The values are expressed as mg 100g⁻¹ fresh tissue.

b. Glycinebetaine:

Glycinebetaine content was determined from fresh leaves of controlled and treated plants by following the method by Ishitani et al. (1993). 0.1/0.2 g oven dried powdered plant material was incubated in 20 ml of 1 N H₂SO₄ for 18 h at 25°C. The suspension was centrifuged at 2000 x g for 10 min and supernatant was collected which served as extract.

The assay mixture contained 0.5 ml plant extract, 0.5 ml 1N H₂SO₄ and 1 ml KI I₂ reagent (15.7 g Iodine, 20.0 g Potassium iodide dissolved in 100 ml D. W.). The contents were mixed and cooled to 0°C for 2 h in an ice bath with constant stirring of the reaction mixture. The contents were centrifuged at 10,000 x g, supernatant was decanted and 10 ml of ethylene dichloride was added to dissolve the precipitate and absorbance was measured at 365 nm on UV-VIS double beam spectrophotometer. Glycinebetaine concentration was
calculated from calibration curve with 0.1 mg.ml$^{-1}$ betaine (Lancaster). The values are expressed as μmoles. g$^{-1}$ dry tissue.

c. Reduced Glutathione contents (Sulphydryl groups)

The content of Sulphydryl groups was determined following the method of Ellman (1959). The plant leaves were washed with water and blotted to dryness. Then they were cut into small segments. 500mg material was accurately weighed and crushed in 10ml phosphate buffer (pH 7). Extract was filtered through Buchner’s funnel using Whatman No.1 filter paper. The reaction mixtures were prepared for 0 minutes and 5 minutes. 0 minutes reaction mixture contained 2.3 ml of phosphate buffer (pH 7), 0.2ml of 1 N NaOH and 0.5 ml of extract/ to this reaction mixture 0.02ml of Ellman’s reagent {5-5’-dithiobis (2-nitrobenzoic acid) (DTNB)} was added. (It was prepared by dissolving 39.6mg of {5-5’-dithiobis (2 nitrobenzoic acid)} in 10ml phosphate buffer pH=7). The absorbance was immediately recorded (0 minutes) at 470 nm against phosphate buffer blank and 5 minutes reaction mixture was prepared in similar manner and the absorbance was recorded 5 minutes after the addition of Ellman’s reagent at 412nm.

\[
\frac{A}{\epsilon} = \frac{C_o}{X}D
\]

Where,

$C_o =$ Original concentration.

$A =$Absorbance at 412 nm. (Difference between 5 min. and 0 min. reading)

$\epsilon =$ Extinction coefficient = 13600/m/cm.

$D =$ Dilution factor.

\[
D = \frac{\text{Vol at 5 min. (after addition of DTNB)}}{\text{Vol at 0 min. (without addition of DTNB)}}
\]

The results are expressed on fresh weight basis.
d. Free amino acids

The free amino acid content of the plant material was estimated following the method by Moore and Stein (1954). Five hundred milligrams of leaf material was extracted in 5 ml hot 80% ethanol. The extract was filtered through Whatman No. 1 filter paper using Buchner’s funnel. The residue was washed twice with 5 ml of hot 80% ethanol. The filtrate and washings were collected together and used for assay. 0.2 ml of above preparation, 1 ml ninhydrin solution (0.02g stannous chloride dissolved in 50 ml 0.02M citrate buffer, pH 5 was added to 2 g ninhydrin dissolved in 50 ml 2 methoxyethanol and the mixture was filtered) was added and mixture was boiled for 20 minutes in water bath. After cooling, 5 ml diluent (prepared by mixing equal amount of n-propanol and distilled water) were added and shaken vigorously. After 20 minutes, the intensity of purple colour developed was read at 570 nm on UV-VIS spectrophotometer. Blank was prepared using 80% ethanol instead of plant extract. Standard curve was obtained by taking different concentrations of standard Leucine (10 mg 100ml⁻¹). The free amino acid concentrations were calculated by using the relationship, 1ml standard leucine solution = 0.1 mg amino acid ml⁻¹.

6. GC-MS analysis of Fatty acids

The phyto-components of the fresh leaf extract and leaf litter leachate extracts of the leaves of *C. odorata* were qualitatively analyzed in detail as per the standard methods (Kokate, 2000, Harbone, 1999 and Tiwari et al., 2011). Preparation of extract

The fresh leaves of Insect-free, disease-free plants of *C. odorata* and Fallen matured senescent leaves were collected from the old plants growing were collected from the coastal area from Sawantwadi, Maharashtra, where it was growing abundantly. They were washed thoroughly with distilled water and air-dried at room temperature for 96 h. Both, fresh as well as fallen matured leaves chopped into 1-cm long pieces, and were grated with
mechanical grater. The ground plant was soaked in 1 L of water for 24 hr. The extracts were then filtered with muslin cloth followed by Whatman filter paper No. 1.

**GC-MS analysis**

With Hewlett-Packard (HP) 890 the samples were analyzed. To a HP 5973 N mass spectrometer the gas chromatograph fitted with a Gerstel MPS2 auto sampler and coupled. The carrier gas was helium (BOC gases, Ultra High Purity), flow rate 1.2 ml min$^{-1}$. At 50°C the oven temperature started and held at this temperature for 1 min, which then increased to 220°C at 10° min$^{-1}$ for 10 min. The injector was held at 200°C and the transfer line at 250°C. The mass spectra were recorded for quantification of the compounds in the Selective Ion Monitoring (SIM) mode using NIST library.

**7. Nitrogen fractions**

**a. Total Nitrogen**

Total nitrogen content from treated and controlled plant leaves were estimated following the method given by Hawk *et al.*, (1948). Oven dried powdered plant material (0.5 g) was taken in Kjeldahl’s flask with a pinch of micro salt($200$ g K$_2$SO$_4$ + $5$ g CuSO$_4$ dehydrated) and to it $5$ ml H$_2$SO$_4$ (1:1) were added. Few glass beads were added to prevent bumping and the plant material was digested on low flame for first few minutes and then on strong blue flame. After complete digestion, 1-2 ml colorless digest was obtained which was then cooled to room temperature, and transferred quantitatively to 100ml capacity 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 (0.236 g of oven dried ammonium sulfate dissolved in distilled water and few drops of conc. H$_2$SO$_4$ 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% KHSO$_4$ was added and volume was made 35 ml with distilled water. To each tube 15 ml of fresh Nessler’s reagent were added (Reagent A: 7 g KI + 10 g HgI$_2$ in 40 ml distilled water, Reagent B: 10 g NaOH in 50 ml water. A and B were mixed in proportion of 4:5 at the time of estimation).

The reaction between NH$_4$ in the sample and the reagent gives the product NH$_4$Hg$_2$I$_3$ which has orange brown colour. The intensity of this colour was measured after 15 minutes at 520 nm on a Shimadzu, UV-190 double beam spectrophotometer.

**b. Nitrate Reductase (NR; EC.1.6.6.1):**

*In vivo* activity of this enzyme was determined following the method of Jaworsky (1971). Fresh leaf material was cut into small pieces of about 0.5 cm$^2$ and incubated in 10 ml incubation medium containing 1ml, 1M KNO$_3$, 2ml 5% n-propanol, 5ml 0.2 M phosphate buffer, pH 7.5 and 2ml, 0.5% Triton-X-100, for 1 h in dark. After incubation, 1ml of the reaction medium was taken out for determination of nitrite and was mixed with 1ml each of 1% sulfanilamide in 1M HCl and 0.02% NEEDA. The absorbance was read at 540 nm on double beam spectrophotometer using reagent blank. The standard curve was prepared with 0.03mM KNO$_2$ (0.0026 mg NO$_2$ ml$^{-1}$) against a mixture of 1ml incubation medium, 1 ml sulfanilamide and 1ml NEEDA as a blank.

**c. Nitrate content**

By using the procedure explained by Cataldo *et al.* (1975) the nitrate contents were treated and controlled plant leaves were determined. In 10 ml of deionized water, 100mg of dry bark powder was suspended in 50mL test tube. At 45°C for one hour the suspension was incubated. At 5000g after incubation in order to sediment the residue the sample was centrifuged for 15 minutes. Only the supernatant was taken for nitrate estimation. In 50ml test tubes, 0.2 ml of the extract mixed thoroughly with 0.8 ml of 5% (w/v) salicylic acid
(prepared in concentrated H$_2$SO$_4$), 2 N NaOH was slowly added more than 12 after 20 minutes at room temperature, to raise the pH 19 ml. On double beam spectrophotometer absorbance was determined at 410nm by cooling the solution to room temperature. With the help of a standard curve which was retrieved at different concentration of KNO$_3$ the amount of nitrate (mg NO$_3^-$ g$^{-1}$ dry tissue) was measured.

8. Inorganic Mineral Constituents

a. Preparation of acid digest

Acid digestion method of Toth et al., (1948) has been followed for the analysis of inorganic constituents. Leaves were collected and washed with water blotted to dry and then kept in oven at 60$^\circ$C till a constant weight was obtained. The oven dried plant material was randomly mixed and powdered. Five hundred milligrams of oven dried powder of leaves was transferred to 150 ml capacity beaker to which 20 ml concentrated HNO$_3$ were added. The beaker was covered with watch glass and kept till the primary reactions subside. 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 the beaker was heated strongly until a clear and colourless 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 Whatman No.44 (ashless) filter paper. Filtrate so obtained was used for estimation of different inorganic constituents.

b. Estimation of Potassium, Calcium, Magnesium, Iron, Zinc, Copper and Manganese

c. Estimation of sulphur

The sulphur content was estimated according to the method of Blanchar et al., (1965). Ten ml of acid digested sample was taken in Nesseler’s tube and
to this 1ml of stabilizing reagent [95% ethanol and glycerol mixed in 8:2 (v/v)] and to this 0.5g of BaCl$_2$ and the volume was made to 50ml with distilled water. The ingredients were mixed well and allowed to react for 30 minutes. Colour intensity was measured at 430 nm using a reaction blank without sulphur.

Calibration curve of standard sulphur was prepared from standard sulphur solution [standard K$_2$SO$_4$ solution (14ppm) in water or 7.60g K$_2$SO$_4$ dissolved in 100 ml distilled water]. By taking different concentrations (0.01, 0.02, 0.04 and 0.08 ml) of standard sulphur, other steps being essentially similar to one described above. With the help of standard curve, amount of sulphur in plant material was calculated and expressed as mg 100$^{-1}$mg dry tissue.

Various inorganic constituents like Na$^+$, K$^+$, Mg$^{2+}$, Fe$^{3+}$, Mn$^{2+}$, Ca$^{2+}$, Cu$^{2+}$, Zn$^{2+}$ were estimated from the young, mature and senescent leaves. Oven dried plant material was powdered and 0.5 g of sample was acid digested following the standard method of Toth et al. (1948).

In a 150ml clean borosil beaker, plant material samples were taken. HNO$_3$ was then added to 10 ml concentrated were added. It was covered with watch glass and kept for an hour till the primary reactions subsided. 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. 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 it was filtered through Whatman No. 44 filter paper. The filtrate was stored properly and used for inorganic constituents analysis.

Sodium and potassium were estimated flamephotometrically using standard procedure on flame photometer (Model-Elico, ch-22A). For
standardization, various concentrations of sodium were prepared 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 and copper were estimated using Atomic absorption spectrophotometer (Perkin-Elmer, 3030 A).