CHAPTER 2

MATERIALS AND METHODS

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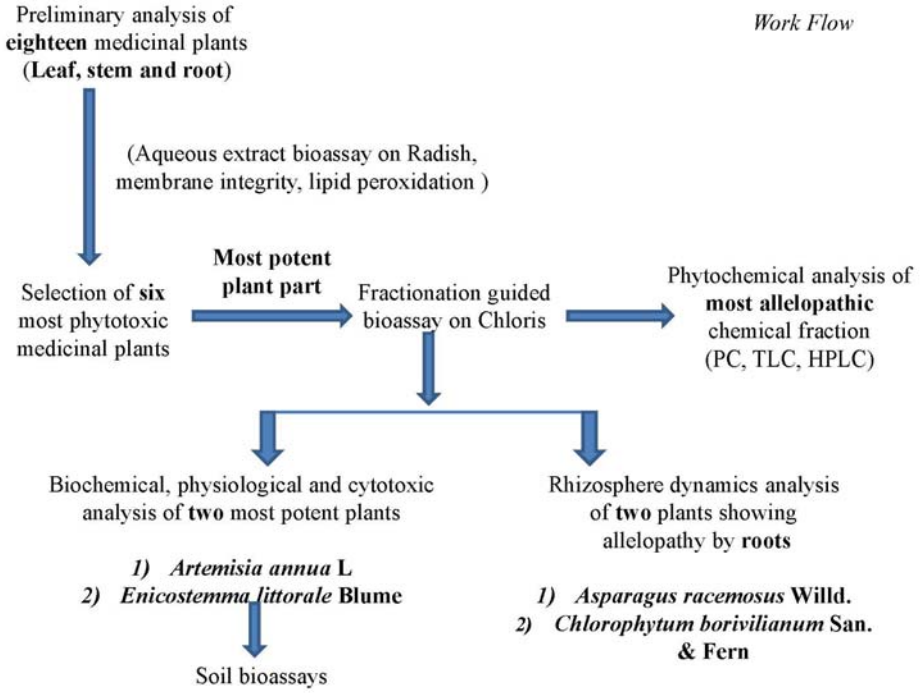
2.1. MedicinalPlant selection and collection: Considering high medicinal importance and utility,eighteen medicinal plants were selected for the preliminary screening. Collection of material for the selected medicinal plants was done from different sources. Most plant material collection was done frompremises of The M.S.University of Baroda (Baroda, Gujarat-India), including university campus, botanical garden, arboretum while some plants were collected from areas aroundJambugodha, Panchmahal, Gujarat.Plant material for,*Acalyphaindica* L., *Adhatodavasica* (L.) Nees,*Aerva lanata* (Linn.) Juss. ex Schult, *Andrographispaniculata*(Burm.f.) Nees, *Asparagus racemosus*Willd.,*Artemisia annua*L. *Boerhaaviadiffusa*L., *Catharanthusroseus*(L.) G. Don, *Chlorophytumborivilianum*San. &Fern.,*Coleus forskohlii*Briq., *Curculigoorchioides*Gaertn., *Euphorbia hirta*L., *Synedrella nodiflora* (L.)Gaertn.,*Solanumnigrum*L. and *Tinosporacordifolia*(Willd.) Miers ex Hook. F. &Thoms, were collected from university premises. Plants like *Dioscoreaalata*L., *Enicostemmalittorale*(Blume) and *Urgineaindica*(Roxb.)Kunth. were collected legitimately from places around theJambugodha region. Plants were collected in their vegetative state and were brought to laboratory. Leaves, stem and roots of each plant were separated, cleaned and washed separately using distilled water.For preliminary analysis, only the freshly collected plant material was used. Considering the results of preliminary bioassay, for the further analysis only the effective part of allelopathic plant was selected, dried (air or oven), powdered and stored for future use.

2.2. Collection of test plants: The allelopathicpotential of the selected plant was analysed against two test plants i.e. *Raphanussativus*L.and*Chlorisbarbata* Sw.

2.2.1.*RaphanussativusL.*: Commercially seeds sold under brand name 912 Queen (produced by Nobel seed private limited, New Delhi)were used for the bioassay, due to high and uniform germination.

2.2.2.*Chlorisbarbata* Sw.: Grass inflorescences were collected from the wild population found to grow in TheM.S.University campus. Fruit coat was gently removed separating the grain or seed. Seeds for bioassay were carefully chosen for uniformity in terms of maturity. All the further analysis for studying allelopathic potential of the medicinal plants were performed only on *Chloris*, considering it as monocot weed and test plant.

The study was primarily initiated with eighteen medicinal plants and through the sequential experiments the study was narrowed to only highly and most potent plants. Work flow of the same is given in the following image.



2.3. Preliminaryallelopathic analysis: In preliminary screening all the eighteen medicinal plants were analysed for their allelopathicpotential using aqueous extract/ filter paper bioassay.Screening was done using filter paper bioassay. Radish *(Raphanussativus*L.) was selected as a test plant for preliminary screening.For preliminary screening, aqueous extracts of leaf, stem and root of each medicinal plant were used.Aqueous extract of each plant part i.e. leaf, stem and root for each medicinal plant were prepared separately at three different concentrations (0.5 %, 1%, 2 %). Fresh plant part werefinely chopped, weighed and soaked in distilled water for 24 hours (in cold and dark), followed by gently macerating the soaked plant material using mortar and pastel in the same aqueous medium and keeping the same in refrigerator for next 24 hours for further extraction. This aqueous preparation was filtered using whatmannno 1. filter paper andthe filtrate was used as aqueous extract or stored in refrigerator for further use.

Table 2.1: Coding used for different aqueous extract treatments for all the medicinal plants

	Plant part	Treatment (% aqueous extract)	Treatment code
Control	-	Water	**1**
Medicinal plant	Leaf	0.5 %	**2**
		1.0 %	**3**
		2.0 %	**4**
	Stem	0.5 %	**5**
		1.0 %	**6**
		2.0 %	**7**
	Root	0.5 %	**8**
		1.0 %	**9**
		2.0 %	**10**

2.3.1. Aqueous extract/ Filter paper bioassay: These bioassay'swere conducted in randomized complete block design. Setup was prepared as per method by Ben-

Hammounda et al. (1995). Petri-plates (Inner diameter- 125 mm)were lined withdouble filter paper and the filter paper was wetted with 10 ml of either the distilledwater or the aqueous extracts (leaf or stem or root).Three replicates were kept for each treatment that is control and medicinal plant aqueous extracts.Treatments for all the medicinal plants were coded as given in Table 2.1. Prepared petri-plates were kept for 24 hours in dark and then were kept in laboratory conditions for incubation. The winter and summer temperatures in laboratory (unconditioned) ranged from 35 ± 5°C and 20 ± 5° C. Before keeping the seeds for bioassay, the uniform sized seeds were surface sterilized with 0.1 % aqueousSodium Hypochloride solution, washed with distilled water atleast for five times and were immediately employed for bioassay use. Ten radish seeds, uniform in size were kept in each prepared petri-plate. Observations were made after one day upon germination. Readings were recorded and measured for germination, seedling parameters like radicle and plumule length, seedling biomass- fresh and dry weight, electrolyte leakage and lipid peroxidation.

2.3.1.1. Seedling health parameters analysed: Readings were recorded or measured for seed germination and seedling parameters like radicle length (mm) and plumule length (mm)and seedling biomass (mg) - fresh and dry weight.

Total germination (G_T): $\frac{N_T \times 100}{N}$

(Where, $N_{T \text{ is the proportion}}$ of the seeds germinated for the last time measurement
N is number of seeds used in bioassay)(Chiapusioet al. 1997)

Speed of germination ***(S)*** was calculated according to formula given by Einhelling (1982),

$$(N_1 \times 1) + (N_2 - N_1)\frac{1}{2} + (N_3 - N_2)\frac{1}{3} + (N_n - N_{n-1})\frac{1}{n}$$

[Where $N_1, N_2, N_3 \ldots N_{n-1}, N_n$ are proportion of germinated seeds obtained on (1) first, (2) second, (3) third, (n-1) and (n) are day of incubation]

Inhibition (% of Control) = $\left(\frac{\text{Extract Treatment value}}{\text{Control value}}\right) \times 100$

2.3.1.2. Membrane integrity: From the bioassay experiment set up (section 2.3.1), after 48 hours of plant extract treatment,seedlings were collected,washed with distilled water to ward off any adhered surface ions. Then they were immersed in fresh lot of distilled water (1g seedling/10 ml water) for 24 hours, allowed for ionic leakage if any and the ionic conductivity of the bathing medium was measured for 24 hours. Initial conductivity of the distilled water was recorded.Readings for conductivity were taken using, handheld conductivity meter (Model-CD 611, Milwaukee). Finally seedlings were boiled in the same medium and readings for total electrolyte conductivity were taken. The readings obtained were multiplied with the instrument factor (100 for CD 611, Milwaukee) to get readings in ppm. The results were expressed as percentage of the total conductivity.

Percentage Conductivity:$\frac{(C_{24} \times 100)}{C_T}$

[$C_{24:}$ Conductivity after 24 hours, C_T: Total conductivity after boiling]

2.3.1.3. Lipid peroxidation: For analysing lipid peroxidationowing to plant aqueous extracts,bioassayswere performed similar to section 2.3.1.Lipid peroxidation was measured in terms of malondialdehyde content (MDA) as per the method of Heath and Packer (1968). MDA level was used as an index of lipid peroxidation. After 48 hours of incubation, fresh seedlings were washed after removal of cotyledonary leaves. The weight (gm) of axis was recorded. The seedling axisso obtained was macerated in 2.5ml of 1% TCA (Trichloro acetic acid) and centrifuged at 6000 g for 20 min. 0.5 ml of the supernatant was added to 2 ml of freshly prepared 0.5 % TBA (thiobarbituric acid) prepared in 20 % of aqueous TCA. The mixture was incubated at 95ºC for 30 min followed by quick cooling over ice, and then centrifuged at 6000 g

for 20 min. The absorbance of the supernatant was determined at 532 nm and corrected for non-specific absorbance at 600 nm. Absorbance was measured using UV Visible spectrophotometer (Model U 2900-Hitachi, Tokyo, Japan).MDA amount was determined using extinction coefficient of 155mM/cm^{-1} and expressed as µmole g^{-1}fresh weight. Results are presented as percentage of control.

$$\text{MDA (µ moles/ g}^{-1}\text{ FW)} = \frac{(\text{A } 532 - \text{A660}) \times \text{V} \times 10^3 \times \text{W}}{155}$$

(where, A is absorbance, V stands volume of the plant extract, 10^3 is conversion of mM to µM, W is the fresh weight of tissue in grams)

2.4. Evaluation of medicinal plant part toxicity by fractionation guided Bioassay

Considering the results of preliminary screening data, only six medicinal plants showing high allelopathic potential against radish were selected for further use.Also only most potent inhibitory plant part for a medicinal plant was selected for further use. Table-2.2 shows the selected (allelopathic) medicinal plant and its respective part showing inhibitory activity. Respective plant parts were collected as mentioned in section 2.1., were shade dried and finely powdered using electric grinder. The powdered plant part material was subjected to bio-fractionation following Harborne's (1984) protocol and the fractions so obtained were used for conducting allelopathic bioassay. *Chlorisbarbata* Sw. was used as the test plant for these bioassays.

Table 2.2: Allelopathic medicinal plant and its selected potent plant part

Sr. No.	Medicinal plant	Potent part
1	*Asparagus racemosus*Willd.	Root
2	*Chlorophytumborivilianum*San. &Fern.,	Root
3	*Tinosporacordifolia*(Willd.)Miersex Hook. F. &Thoms	Stem
4	*Solanumnigrum*L.	Leaves
5	*Enicostemmalittorale*(Blume)	Leaves
6	*Artemisia annua*L.	Leaves

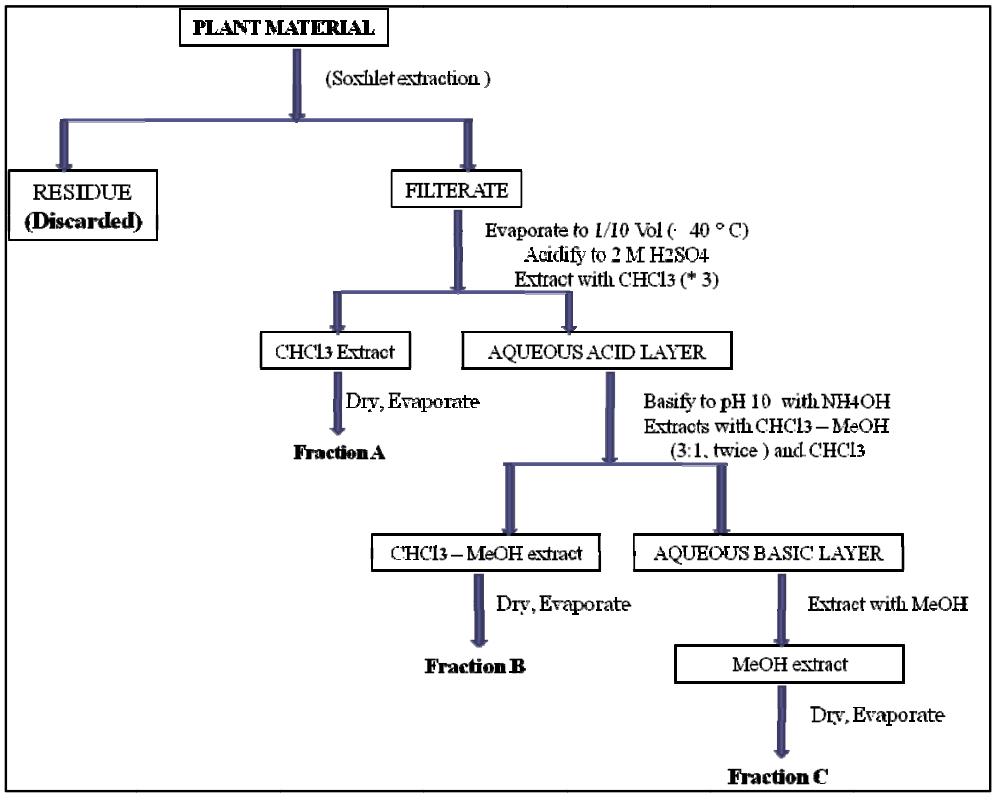
2.4.1. Chemical fractionationof medicinal plant part: Dried powder of medicinal plant part was extracted using soxhlet in the ratio 1g powder: 10ml of 70% aqueous methanol. After extraction the methanolic extract was collected and subjected to fractionation as depicted in Figure 2.1**(**Harborne 1984).Upon fractionation three fractions were obtained i.e. A) Chloroform, B) Chloroform-methanol and C) Methanol(termed as per the respective solvents used). The three fractions so obtained for each of the six medicinal plants were analysed for allelopathicpotential using bioassays.

2.4.1.1. Allelopathic analysis of Harborne's fraction's A, B and C against *Chloris*: Modified sandwich method (Fujii et al. 2003) was used for the bioassay**.** Bioassay's studies were conducted using aqueous agar (0.5 %) as a growth medium. The agar based bioassays were carried out in the glass petri-plates (inner diameter - 200 mm). The three Harborne's fraction A, B and C were completely evaporated (to remove and prevent respective solvent toxicity) in water bath, dissolved ultimately in the ethanol and these fractions solubilised with ethanol were used for imparting allelopathic treatments. Three concentrations 0.25, 0.5 and 1 % of each of the three fractions A, B and C were used, constituting nine treatments per medicinal plant. A control was always kept where in pure solvent or plant fractions were not added. In addition to control and the fraction treatments, the solvent used to dissolve extracts was also incorporated as a treatment in order to nullify the inhibitory effect of the pure solvent (eventually ethanol) itself, if any. Required concentrations of the treatments were added to aqueous agar each separately, after the agar was completely melted so as to avoid metabolites degradation. Treatments in case of all the sixmedicinal plants were coded as given in Table 2.3. All the treatments were repeated thrice. For the bioassay, after cooling and solidification of agar, 20 *Chloris*

seeds were uniformly placed in the petri-plates. The setup was kept in dark for an hour and was kept for incubation under the laboratory conditions. Germination in *Chloris* was found to commence after a day of incubation. To determine the speed of germination (S), readings for number of the seeds germinated were recorded at the interval of 24 hour for four days. Seedlings in the treatments were found to undergo decay after fourth day, so readings for the radicle length (mm) were recorded on the fourth day.

Figure 2.1: Chemical fractionation of the methanolic plant extract

(Harborne 1984)



Growth parameters analysed: Speed of germinations and total germination were calculated as per the given formula (2.3.1.1.). Radicle length (cm) was considered and measured as the growth parameter.

Table 2.3: Coding used for Harborne's fraction treatment

	Harbornes's fraction	Treatment	Treatment code
Control	Aqueous agar	-	**1**
Alcohol	Solvent ethanol	1.0 %	**2**
Medicinal plant	A	0.25 %	**3**
		0.50 %	**4**
		1.0 %	**5**
	B	0.25 %	**6**
		0.50 %	**7**
		1.0 %	**8**
	C	0.25 %	**9**
		0.50 %	**10**
		1.0 %	**11**

2.4.1.2 Phytochemical analysis of the allelopathic fraction

For all the six medicinal plants, out of the three analysed fractions the most allelopathicHarborne's fraction was subjected to phytochemical analysis.The allelopathic fraction was analysed for chemical nature of its secondary metabolites to be whether it isphenolic, alkaloidand/ or terpenoid. In case where found allelopathic, the chloroform extract was used for analysis of terpenoids and phenolics, while the chloroform:methanol extract and the methanolic extract were evaluated for alkaloids. The analysis was done using planar chromatographic technique like paper chromatograph (PC) and thin layer chromatography (TLC) and advance techniques such ashigh performance chromatography (HPLC). The phytochemical analysis was performed solely for qualitative purpose

I. Planar chromatography: For paper chromatography,whatmannno 1. filter paperwas used as a stationary phase support. For TLC the plates were prepared using silica gel G (Fisher Scientific, India) and only activated plates were used for the TLC analysis.

Phenolics: Phenolics were analysed using 2 D chromatography (Daniel 1991).The fractions were dissolved with ethanol and spotted at the corner of square cut whatmannno 1. filter paper and was allowed to dry. The chromatogram was developed in organic layer of solvent system Benzene: Acetic acid: Water (6:7:3). After the run was over, the chromatograms were removed from developing solvent system and allowed to dry completely. Then the side of chromatogram flipped with the side having vertically separated metabolite,was kept dipped in the second solvent system (Sodium formate: Formic acid: Water- 10:2:200) and the run was allowed to progress in the second direction. The chromatogram was removed from solvent system after the run was over and was kept for air drying.

Spray reagent:

Reagent 1) p-Nitra aniline: 0.7 g p-Nitra aniline was dissolved in 9 ml HCLand volume was made to 100 ml using distil water. 4 ml of this solution was taken, this was added to 5 ml of cooled 1 % aqueous $NaNO_2$ solution (c) and volume was made to 100 ml using distilled water.

*Reagent 2)Sulfanilic acid:*0.7 g Sulfanilic acid was dissolved in 9 ml HCLand volume was made to 100 ml using distilled water. 4 ml of this was taken to this 5 ml of cooled 1 % aqueous $NaNO_2$ solution (c) was added and volume was made 100 ml using distil water.

All the above stocks were kept in Refrigerator at 4ºC and always used in chilled condition to favour the diazo test.The chromatograms for anextract were always kept in duplicate,for each of the two diazospray reagents.These chromatogramsweredipped in spray reagent and immediately dipped in 10 % Na_2CO_3. The chromatograms sprayed with both the reagent separately were observed for developed colour. A single phenol compound, takes different colour with both the spray reagents. Phenolic

compoundswere identified confirming their colour with both the spray reagents(Daniel 1991) respectively. All the solvents and reagent used were of analytical reagent grade (Fisher Scientific, India).

Terpenoids: Harborne's fractions were analyzed for presence of terpenoid's using thin layer chromatography (Daniel 1990). Fraction was spotted in form of bands or spots solubilising each in ethanol. Hexane: Acetone (ratio 4:1) was used for developing the spotted TLC plates. For colour development 50% H_2SO_4 was used as a spray reagent. TLC plates were heated using hot plate, once sprayed with the spray reagent. TLC plates were observed for the developed colour. All the solvents used for TLC analysis were of analytical reagent grade (Fisher Scientific, India).

Alkaloids: The alkaloids were analysed as per method given by Daniel (1990), using TLC plates. Fraction was solubilised in ethanol for spotting onplates. Developing solvent system used wereChloroform: Methanol: Water (50:45:5) and Methanol: 2M Ammonium nitrate (3:2). Developed TLC plates were sprayed withDragendorffs reagent and thereafter plates were observed for colour development.

Dragendorff's reagent:

Solution a-0.85 g basic bismuth sub-nitrite was dissolved in 10 ml glacial acetic acid. 40 ml of warm water was added to it and filtered.

Solution b- 8 g of potassium iodide was dissolved in 20 ml water.

Solution 'a' and 'b' were mixed in 1:1 ratio when required.

II. HPLC analysis: Considering the results of planer chromatography and respective allelopathicstudies, the potential Harborne's fraction from the six medicinal plants were analysed using HPLC, for the one or more metabolites like PhenolicsTerpenoid, or Alkaloid.For all the HPLC analysis, LC20AT Shimadzu HPLC unit equipped with binary pump and a photo diode array detector was employed.GraceSmart RP-18

column (25mm long, 4.6 mm internal diameter and 5µ pore size) was used for metabolite separation. Solvents of HPLC grade (LobaChemiePvt. Ltd. India and Sisco Research Laboratories Pvt. Ltd. India) were used. All the solvents and mobile phases were filtered through Ultipor® N®66 Nylon 6, 6 membrane (pore size- 0.2µm, 47 mm diameter) Pall India Pvt. Ltd and degassed using sonicator. All the HPLC samples were filtered through Nylon membrane (pore size - 0.45 µm, diameter- 13 mm, Pall India Pvt. Ltd.)

Phenolics: Reverse phase chromatography was performed for the analysis of phenolic compound in the Harborne's fraction A. Method given by Gursoy(2012) was followed for the same with a slight modification. The mobile phase comprised of, solvent (A) as 0.25 % Acetic acid (pH 3) and solvent (B) as Acetonitrile. The phenolics metabolites were separated using a gradient flow, for a run time of 32 minutes. The solvent gradient used was as follows: 0-10 % B in 5 minutes, 10 - 20 % B in 10 minutes, 20 - 80 % B in 13 minutes, 80 -20 % B in 2 minutes, 20 – 10 % B in 2 minutes. A flow rate used was 0.5 ml/min and sample injection volume used was 20 µL.The sample was solubilised in mobile phase(A: B), filtered and then used for HPLC analysis.Spectra were extracted at wavelength of 290 nm and spectrum max for 200 nm to 400nm was also acquired.

Terpenoid: The Harborne'sfraction A wasalso analysed for terpenoids. Method given by Kalyani and Laddha (2009) was followed for the same with a slight method modification. The analysis was performed using isocratic flow. The mobile phase used was methanol: water (95:5) for a run time of 30 minutes and flow rate was 1 ml/ minute. Samples were solubilised in the mobile phase (A: B), filtered and then used for HPLC analysis. The spectra were extracted at 210 nm and spectrum max plot was acquired at 200 nm to 400 nm.

Alkaloid:Harborne's fraction B was analysed for Alkaloids using HPLC analysis.The method given by Sheludko et al (1999) was followed with a slight modification. The mobile phase consisted of Acetonitrile (mobile phase B): 2.5 mMhexanesulfonic acid buffer (pH 2.5) (mobile phase A). The analysis was performed using gradient as follow: 0.01 minute – 15 % B, 5 min – 20 % B, 40 min- 40 % B, 20 min- 80 % B, 10 min - 15 % B. Flow rate was 1 ml / minute and run time was 80 minute. Samples were solubilised in the mobile phase (A: B), filtered and then used for HPLC analysis. Data were extracted at 254 nm, 260 nm, 210 nm and spectrum max plot for 200 nm - 400 nm.

2.5. Analysis of biochemical, physiological and cytotoxiceffects:

Considering the results of allelopathic analysis, conducted using fractionation bioassay, out of the six plants, only two medicinal plants i.e. *Enicostemmalittorale* (Blume) and *Artemisia annua*L were selected for analysis of biochemical, physiological and cytological effects. Only the most effective Harborne's fraction from each of the two medicinal plants,were selected for studying the possible mechanism of action. Table2.4, shows treatment coding used for all the following experiments. All the studies intending the biochemical and physiological analysis were performed on *Chlorisbarbata* Sw. except the cytotoxicity analysis which was performed on *Allium cepa* L.

Tables 2.4: Coding used for biochemical and physiological analysis in *Chloris*

	Harborne's fraction	Treatment	Treatment code
Control	Aqueous agar	-	**1**
Alcohol	Solvent ethanol	1.0 %	**2**
Enicostemmalittorale (Blume)	**B**	0.125 %	**3**
		0.25 %	**4**
		0.50 %	**5**
*Artemisia annua*L.	**A**	0.125 %	**6**
		0.25 %	**7**
		0.50 %	**8**

2.5.1. Lipid peroxidation: Lipid peroxidation was measured as mentioned in the section (2.3.1.3.) following the method by Heath and Packer (1968). The seedling axis (1 gm) was used for extraction. Malondialdehyde content was determined using an extinction coefficient (e) of 155 $mM^{-1}cm^{-1}$ and expressed as µM/ $gm^{-1}FW$. Absorbance was measured using UV Visible spectrophotometer (Model U 2900-Hitachi, Tokyo, Japan).

MDA (µM/ g^{-1} FW)$=\frac{(A\,532-A660)\times V\times 1000\times W}{155}$,

(where, A is absorbance, V stands volume of the plant extract, 10^3 is conversion factor for converting mM to µM, W is the fresh weight of tissue in grams)

2.5.2. Antioxidant enzymes assays: Activities for antioxidant enzymes such as Super oxide dismutase, Catalases and Peroxidase were analyzed.For each of the enzyme essay, amount of protein in the enzyme extract was also estimated.

2.5.2.1. Super oxide dismutase: Activity of Superoxide dismutase (SOD) was estimated by recording the decrease in absorbance of the sample as described by Dhindsa et al (1981). Fresh 500 mg seedling was homogenized in 2 ml of 0.1 N of phosphate buffer (pH 7.5). The extract was centrifuged at 10000 g for 20 min at 4ºC and supernatant was used as enzyme source. Three ml of reaction mixture containing 0.1 ml of1.5 M Na_2CO_3, 0.2 ml of 200mM methionine, 0.1 M of 3mM EDTA, 0.1 ml of 2.25 mM NBT, 1.5 ml of 100 mM potassium phosphate buffer (pH 7.5), 1ml of distilled water and 0.05 ml of enzyme samples. The tube without enzyme extract was taken as control. Reaction was started by adding 0.1 ml 60 µM riboflavin and placing the tubes below a light source of two 15 W fluorescent lamps for 15 min. The reaction was stopped byswitching of the light and covering the tubes with black cloth. Absorbance was recorded at 560 nm using (Model U 2900-Hitachi, Tokyo, Japan).One unit of SOD activity was defined as the quantity of SOD required to

obtain a 50% inhibition of the reduction of NBT. The activity was expressed as Unit of SOD.

- % Inhibition= $\frac{\text{Control OD}-\text{Treatment OD}}{\text{Control OD}} \times 100$ = X %
- 50 % Inhibition= 1 enzyme unit, than X % inhibition $= \frac{X}{50} =$ Y unit of enzyme activity

2.5.2.2. Catalase: The Catalase activity was determined according to the method given by Luck (1974). 250 mg of seedlings were homogenized in 3ml of 0.1M phosphate buffer (pH 7). The extract was centrifuged at 10000 g for 20 min at 4º C and supernatant was taken as the enzyme source. The three ml of assay mixture contained0.5 ml of 0.2 M phosphate buffer (pH 7), 0.3 ml of H_2O_2 and 0.1 ml of enzyme and 2.1 ml of distilled water. The reaction was started by adding enzyme extract, optical density was measured at 240 nm and change in optical density was recorded at 0 min and 3 min using UV Vis spectrophotometer(Model U 2900-Hitachi, Tokyo, Japan). Extinction coefficient for H_2O_2 is ε = 39$mM^{-1}cm^{-1}$, Amount of Catalase was expressed as enzyme Unit /mg$Protein^{-1}$.One unit of Catalase is defined as the amount of enzyme able to decompose one μM H_2O_2 in 1minute at 25℃.

Specific enzyme activity:$\frac{\Delta \text{ Absorbance} \times \text{Cuvette Volume} \times 10^3}{\varepsilon \times \Delta \text{ Time} \times \text{Sample volume} \times P}$

= X μMH_2O_2/min/mg of protein

[Where, Δ Absorbance = Absorbance of sample – Absorbance of blank

10^3= Is the conversion factor of Ɛ

Ɛ = Molar extinction coefficient of H_2O_2 (36.0mM^{-1} cm^{-1})

Δ Time = Timeinterval in which absorbance was measured (minute)

P = concentration of protein in the extract (mg/ml)]

2.5.2.3. Peroxidase: Peroxidase activity was assayed as described by Putter (1974). 250 mg of seedling was homogenized in 5 ml of 0.1 M phosphate buffer (pH 7). The

enzyme extract were centrifugedat 10000 g for 20 min at 4ºC and supernatant was taken as enzyme source. The 3 mlof assay mixture contained 1.5 ml of 0.1 M phosphate buffer (pH 7.0), 0.5 ml freshly prepared 10mM Guaicol, 0.1 ml enzyme extract, 0.1 ml of 12.3 mM $H_2O_2$and volume was made 3 ml using water.Absorbance was read at 436 nm at 0 minute and then increase in the absorbance was noted at the interval of 0.5 minute on UV-Visible spectrophotometer (Model U 2900-Hitachi, Tokyo, Japan).Extinction coefficient of Guaicolis 27 mM^{-1} cm^{-1}.Amount of Peroxidase was expressed as enzyme Unit /mg Protein $^{-1}$.

Specific enzyme activity: $\frac{\Delta \text{ Absorbance} \times \text{Cuvette Volume} \times 10^3}{\varepsilon \times \Delta \text{ Time} \times \text{Sample volume} \times P}$

[Where, Δ Absorbance = Absorbance of sample – Absorbance of blank

10^3= conversion factor of ε (only if the value is in moles)

ε = Extinction coefficient of Guaicol(27mM^{-1} cm^{-1})

Δ Time = Time interval in which absorbance was measured (minute)

P =concentration of protein in the extract (mg/ml)]

2.5.2.4. Estimation of protein content in enzyme extracts: Protein content was estimated following Bradford (1976) assay. It was required for calculating activity of all the three enzymes.

Preparation of CBB G 250 (Coomassie Brilliant Blue) dye: CBB was dissolved in 5 ml of 95 % ethanol. 10 ml of 85% Ortho-Phosphoris acid was added to it and was diluted to 100 ml using distil water.

Estimation of protein in enzyme samples:100 µl of enzyme extract was taken and 900 µl of distilled water was added to it. Finally 4 ml of CBB reagent was added and absorbance was recorded at 595 nm using the UV-Vis spectrophotometer (Model U 2900-Hitachi, Tokyo, Japan). The absorbance for each enzyme extract sample was

plotted on the protein standard graph (constructed using Albumin), the concentration values were recorded and used for calculating enzyme activity respectively

2.5.3. Proteinanalysis: Protein from the seedlings was isolated as per method described by Isaacson et al. (2006). SDS-PAGE was performed as per method given by Laemmli (1970).

2.5.3.1. Isolation of protein: *Chloris*seedlings for protein isolation were pooled from following setup,

- ✓ Control (aqueous agar)
- ✓ Solvent alcohol
- ✓ *Enicostemmalittorale*(Blume) (Harborne's fraction B applied at 0.125, 0.25, 0.5 % concentration)
- ✓ *Artemisia annua* L. (Harborne's fraction A applied at 0.125, 0.25, 0.5 % concentration)

The seedlings were macerated using liquid nitrogen. To this 10% TCA acetone solution was added and itwas kept overnight. This was decanted and the filtrate was centrifuged at 8500g for 30 minutes at 4° C. This was decanted and to the residueacetone was added, which was again centrifuged at 8500g and 4° C for 10 minutes. This was repeated twice. Acetone was aspirated and to the residue 500 µl of sample buffer (prepared as in section IV.)was added. This was boiled in a water bath for 5 minutes.

2.5.3.2. Preparation of solutions:

Preparation of running gel (10% Acrylamide): The gel was prepared by adding chemicals in the following sequence,

- 1.9 ml of 1.5M Tris HCL(pH 8.8, adjusted using Conc. HCL)
- 38 µl 20% SDS
- 2.5 ml Acrylamide stock (6 g Acrylamide+0.16g Bis-acrylamide +20 ml Water

- 38 µl APS (freshly prepared)
- 5µ TEMED
- 3.075 ml Distil water

This was poured immediately in to prior sealed (used 1 % aqueous agar and cello tapes for sealing) glass slabs (Checked for leakage using water)well spaced using spacers and kept aside for gel polymerization and setting.

Preparation of stacking gel (4% Acrylamide): The gel was prepared by adding chemicals in the following sequence,

- 6.25 µl 0.6M Tris (pH 6.8 adjusted using HCL)
- 13 µl20% SDS
- 3.35 µl Stock Acrylamide (6g Acrylamide + 0.16 g Bis-acrylamide+20 ml water)
- 13 µl Freshly prepared APS
- 2.5 µl TEMED
- 1.5 ml Water

Immediately the stacking gel was poured in between the properly placed comb (to make protein loading wells) on the polymerized running gel. Was kept aside and allowed to polymerize.

Preparation of running buffer: Buffer was prepared by adding chemicals in the following sequence,

- 3.2 g Tris base
- 14.8 g Glycine
- 1 g SDS
- Dissolved in one litre of water

Preparation of protein sample buffer (5x 20 ml): Buffer was prepared by adding chemicals in the following sequence,

- 2g SDS was taken in a flask
- 14.5 µl Beta Mercaptoethanol
- 4 ml Glycerol
- 0.24 g Tris HCL added to 10 ml Distil water (pH 6.8)

Preparation of protein stain: Was prepared by adding chemicals in the following sequence,

- 0.1 g CBB R 250
- Dissolved in 50 ml Methanol
- 7 ml Glacial Acetic Acid was added
- Volume was made to 100 ml using water

Preparation of gel destaining solution: Was prepared by adding chemicals in the following sequence,

- 10 Glacial acetic acid
- 40 ml Methanol
- 50 ml water

Preparation of tracking dye:0.5 % Bromo phenol blue dissolved in distilledwater

2.5.3.3. SDS PAGE

- Total 15 µl volume of protein sample [13 µlof protein extract (prepared in section 2.5.3.2.) + 2 µlof tracking dye] was loaded in each well.
- After loading the protein sample the PAGE gel was kept vertically in the electrophoresis unit having the running buffer.
- After connecting the electrodes the voltage of 50 volt was applied and the proteins were allowed to separate on the gel.

- The run was monitored using tracking dye.

Staining and destaining: After the run was over, the gel was kept in stain for overnight. This was destained using the destaining solution.

The destained gel was washed using distil water and was observed for protein bands using gel doc (GelDocIt^2 Imager, Ultra Violet Products Ltd, UK).

2.5.4. Analysis of RNA: *Chloris* seedlings for RNA isolation were pooled from following setup,

- ✓ Control (aqueous agar)
- ✓ Solvent alcohol
- ✓ *Enicostemmalittorale*(Blume) (Harborne's fraction B applied at 0.125, 0.25, 0.5 % concentration)
- ✓ *Artemisia annua* L. (Harborne's fraction A applied at 0.125, 0.25, 0.5 % concentration)

2.5.4.1.RNA extraction(Trizol method)

- 100 mg of tissue was homogenizedin 1000 µlTrizol (notto be vortex), directly centrifuge at 12000 X g for 10 minutes at 4° C
- Homogenate was transferredto a fresh tube and was incubated for 5 minutes at room temperature (20 – 30° C)
- 200 µl of chloroform was added, tube was vigorously shaken by hand for 10-15 minutes and was incubated at room temperature for 2-3 minutes.
- The sample was centrifuge at 12000 X g for 15 minutes at 4° C(RNA remains in upper aqueous phase)
- Aqueous phase was transferred to a fresh tube and 500 µl of Isopropyl alcohol was added to it
- Sample was incubated at room temperature for 10 minutes, centrifuged at 12000X g for 10 minutes at 4° C.

- The supernatant was removed and RNA pelletwas washed (often invisible and forms a gel like pellet on side and bottom of the tube) with 1000 µl of cold 75% ethanol
- Centrifuged at 7500 X g for 5 minutes at 4° C.
- Ethanol was aspirated and the pellet was air dried at room temperature for 5 – 10 minutes and suspended in 50 µl of DEPC water

These samples were analysed for RNA using gel electrophoresis and measuring absorbance at wavelength of 260 nm and 280 nm.

2.5.4.2. Gel electrophoresis:

Preparation of TBE buffer (10 X)

- 10.8 g Tris base
- 5.5 g Boric acid
- 2 ml 0.5 M EDTA (pH 8, autoclaved)
- Volume was made 100 m using RO water

Preparation of Gel: The gel was prepared by adding chemicals in the following sequence,

- 1% Agarose gel was prepared using the TBE buffer
- This was allowed to boil and then Ethidium bromide (solution of 10 mg/ 1 ml distil water) was added after cooling
- This was poured in sealed glass slabs, after inserting the comb, the gel was allowed to polymerize

Preparation of loading dye:

- 0.25%Bromophenol blue
- 10 % Glycerol
- 10 mMTris- HCL (pH 7.5)

- 50 mM EDTA (pH 8.0)

The 13 µl volume ofRNA sample (10 µl RNA sample + 3 µl of loading dye) was loaded per well. TBE buffer was used as a running buffer for gel electrophoresis. The Gel run was performed using horizontal electrophoretic unit, electrodes were connected and was supplied with voltage of 50 volts. After the run, the gel was observed for RNA (appearing fluorescent red coloured in UV light) using Gel doc (GelDocIt^2 Imager, Ultra Violet Products Ltd, UK).

Purity check for RNA: The RNA was analysed using spectrophotometer for its quality. Absorbance of RNA samples diluted in ratio 1:100 (RNA sample: DEPC water) was recorded at both, 260 nm and 280 nm using UV-Visible spectrophotometer (Model U 2900-Hitachi, Tokyo, Japan).

Absorbance ratio: The ratio of OD values (260 nm /280 nm) was compared and RNA purity was analysed.

Concentration of RNAin the samples was determined as follow:

$A\ 260\ \times$ dilution factor $\times\ 40\ =$ µg RNA/mL

1 unit of A at 260 nm = 40 µg RNA/mL, where 40 is the extinction coefficient

2.5.5. Analysis of Chlorophyll content: *Chloris*leaf blades were used for chlorophyll extraction. Chlorophyll extraction and estimation was performed following the Arnon (1949) method. 100 mg of leaf tissue was taken in a glass test-tube. To this 10 ml of DMSO (Dimethyl sulfoxide) was added. This was kept in boiling water bath (95 ℃) for 30 minutes. Absorbance was recorded on a UV- Visible spectrophotometer (Model U 2900-Hitachi, Tokyo, Japan)at 645 nm and 663 nmalong with reference blank. Chlorophyll a, Chlorophyll b andtotal Chlorophyll were calculated placing the OD values in the below given formulas:

$$\textbf{Chlorophyll a (g l}^{-1}\textbf{)} = 0.0127(\mathrm{A663}) - 0.00269(\mathrm{A645})$$

$$\textbf{Chlorophyll b (g l}^{-1}\textbf{)} = 0.0229(A645) - 0.00468\ (A663)$$

$$\textbf{Total Chlorophyll (g l}^{-1}\textbf{)} = 0.0202(A645\ ) + 0.00802\ (0.00802\ )$$

2.5.6. Cytotoxicity analysis: Cytotoxic effect was analysed on the onion root-tip cells, following squash technique by Armbruster et al. (1991). Rooting in onion bulbs was obtained by placing the peeled bulbs in wet sand with a perennial water supply maintained for four days. After rooting, a set up (Figure 2.2) was prepared by placing the bulbs in sand filled plastic pots.Three pots were prepared, **1**) supplied with only water as control, **2**) supplied with 1% aqueous ethanol and **3**) supplied with 1 % aqueous Harborne's fraction A (*Artemisia annua* L.) or 1 % Harborne's fraction B (*Enicostemmalittorale*Blume). These treatment pots already having hole at the bottom were placed in plastic containers slightly bigger than the pots. After placing onion bulbs with roots buried in the sand, treatment solutions were separately poured into the sand as one treatment per pot. Containers were filled with respective treatment solutions. This set up was kept for a day. Five roots were pooled from each of three pots and microscopic slides of roots were prepared separately for each of the three treatments. In total 25 readings were taken, 5 areas from each of the five slides for a single treatment. Microscopic preparations were observed for total number of cells, total number of cells undergoing mitosis and cells in different phases of mitotic cell division.

Figure 2.2: Set up depicting allelopathic treatment imparted to onion bulb

2.6. *Artemisiaannua*L., artemisinin and the plant metabolite absorbance by *Chloris*:

2.6.1. Agar based bioassays for *Artemisia annua*L. related treatments:

2.6.1.1. Preparation of ethanolic extract from *Artemisia annua*L. leaf and its bioassay on *Chloris*:

The method forAametabolites extraction as given by Lapkin et al. (2009) was followed. 10 g of dried leaf powder was extracted for 10 minutes using 100 ml of chloroform at room temperature with gentle and intermittent shaking. This was filtered through whatmannno1. filter paper and the filtrate was collected. Solvent from filtrate was vacuum evaporated and the residue was dissolved in 10 ml ethanol to make stock of concentration worth 1000 mg/ ml of ethanolic leaf extract. Final concentrations for the bioassays were prepared using the same stock. All the treatments (seven in total) were coded numerically as given in Table 2.5.

Table 2.5: Coding used ethanolic leaf treatments against *Chloris*

	Treatment	Treatment code

Control	Water	**1**
Alcohol	Solvent ethanol	**2**
Ethanolic Leaf extracts (mg/ml)	1.25	**3**
	2.5	**4**
	5.0	**5**
	7.5	**6**
	10.0	**7**

2.6.1.2.Preparationof standard artemisinin stock and its bioassay on *Chloris*:

Artemisinin stock (0.1 Molar) was prepared by dissolving standard artemisinin in ethanol. Same stock was used to make the artemisinin treatment concentrations, coded serially as given in the Table 2.6.

Table 2.6: Coding used for artemisinin treatments against *Chloris*

	Treatment	Treatment code
Control	Water	**1**
Alcohol	Solvent ethanol	**2**
Artemisinin (μM)	11	**3**
	22	**4**
	33	**5**
	44	**6**

For both the above bioassay, aqueous agar (0.5 %) was used as a growth medium. Required concentrations of the treatments were added each separately after completely melting the agar to avoid metabolites degradation. The bioassays were carried out in the glass petriplates (inner diameter - 20 cm). In both the bioassay, after cooling and solidification of agar, 20 seeds of *Chloris* were uniformly placed in the petriplates. The setup was kept in dark for an hour and was incubated under the laboratory conditions. Total germination (as given in section 2.4.1.1) and radical length (cm) were recorded on the fourth day after germination.

2.6.2. Qualitative analysis:

2.6.2.1. Spectrophotometric evaluation of standard artemisinin:to evaluate purity of standard artemisinin, it was analysed spectrophotometricaly with and without derivation. Preparation and derivatization of artemisinin was performed following the method by Qian et al. (2005). Artemisinin stock was prepared by dissolving artemisinin in ethanol (1mg/ml) and five aliquots equivalent viz 0.0, 0.25, 0.5, 1.0, and 2.5 ml were pipetted into five 50mL flasks respectively using the stock. The solutions were then diluted to 5.0mL with ethanol and mixed with 20mL of 0.2% (w/v)NaOH solution respectively. These mixtures were then warmed in a water bath at 45ºC for 30min to obtain Q292. After being cooled to room temperature with water, these mixtures were neutralized and adjusted to mark with 0.08M acetic acid to obtain the resulting compound Q260. The standard artemisinin, the derivatizedArtemisinin samples and ethanolic leaf extract (prepared as in section 2.6.1.1.) were analyzedspectrophotometricaly. Absorbance were recorded at 216, 292 and 260 nm using Spectrophotometer (Model U 2900-Hitachi, Tokyo, Japan).

2.6.2.2. HPLC analysis of artemisinin, ethanolic leaf extract and Harborne's fraction A: The analysis of standard artemisinin (dissolved in ethanol), ethanolic leaf extract (prepared as given in section 2.6.1.1.) and Harborne's fraction A (as prepared in section 2.4.1.) was performed as per method given by Qian et al. (2005) with a slight modification. The mobile phase was 45/10/45 (by volume) Methanol/Acetonitrile/0.9mM Na_2HPO_4 - 3.6mM NaH_2PO_4 buffer (pH 7.76), with a flow rate of 0.5mL/min. The samples were analyzedIsocratically. Volume of 20 µl was injected in to the injection loop. Mobile phase was filtered and degassed before use. For all the other samples runtime was 40 minutes. Spectra were observed for210, 216 nm, 292 nm, 260 nm and spectrum max for 200 nm to 400 nm was also obtained.Harborne's fraction A was analysed as given in section 2.4.1.2.

2.6.3. HPLC analysis for metabolite absorption: This analysis was preformed for artemisinin absorption, where in *Chloris*seedling from the artemisinin treatment bioassay's (section 2.6.1.2.) were analysed for the metabolite absorbed. Seedlings for this analysis were pooled from the control, the Ethanol treated and the artemisinin (concentration at rate of 33 μM) treated seedlings. Seedlings were washed, dried and subjected to extraction with the same method as used for ethanol leaf extract preparation (2.6.1.1.). The samples were filtered and sonicated before use. HPLC analysis was performed for the seedling extracts, following the HPLC method as given in section 2.6.2.2.

2.7. Bioassay using soil as medium:Normal field soil was used for the bioassay studies so as to mimic agricultural field conditions. Plastic pots filled with one kilogram equivalent of soil were used for the allelopathic bioassay. Pots were prepared by watering two days prior to setting up the allelopathic experiments. In total 33 pots were prepared for eleven treatments and the set up was kept in wire house at botanical garden (The M.S.University of Baroda) as shown in the Figure 2.3.

The soil from all treatments was analyzed for its pH and water holding capacity to study if these are not the factors contributing to the reduced growth in *Chloris*. The readings for seed germination were recorded for four days.

Analysis of pH: Soil pH was determined using Eco Testr pH 1 (Eutech Instrument Singapore, Accuracy: ± 0.01 pH). Soil and deionized water was taken for each sample in the ratio 3 g: 5 ml. Water was added to weighed soil, was stirred for one minute and soil was allowed to stand for five minute. pH of the flooded water in each sample was determined using pH meter.

Water holding capacity: Finite volume (X= 20 ml) of water was supplied to the equal amount of soil pooled from all the experimental pots. Filtering water was

collected (Y). The volume of water retained by the soil was considered as thewater holding capacity (WHC) of soil and was measured as follow: WHC= $(X - Y)$ ml, difference in water of volume was used to infer water holding capacity of soil.

2.7.1.Soil bioassay for *Artemisia annua* L.: For this bioassay, dried leaf mulch, Harbornes fraction A and pure artemisinin were used at three different rates with triplicates for each. Leaf mulch was applied on dry weight basis while the other two treatments were applied in the form of aqueous solutions. Leaf mulch was applied at the rates, 0.5 %, 1% and 2% (W/W). Harborne's fraction A was applied at the rates, 0.5 %, 1% and 2 % (V/V). Artemisinin (stock prepared in ethanol) was applied at the concentrations, 16.5 µM, 33 µM and 66 µM (W/V). Two sets of pots were kept without any treatment where in, in one ofthe set only water was added while in another 2 % ethanol was added. Treatments were coded as given in Table 2.7. Fraction A and artemisinin (prepared in ethanol) were poured in water and supplied as a treatment, where as leaf mulch was directly added to soil and subsequently water was supplied. Equal amount of water (100 ml) was added to all the pots. Moisture was maintained by keeping the pots in water-filled trays. Twenty seeds were uniformly placed in each pot.

Table 2.7: Coding used for the soil bioassay for*Artemisia annua* L. treatments

		Treatment	Treatment code
Control	Water	-	**1**
Alcohol	Solvent ethanol	2.0 %	**2**
Aa related treatment	Leaf mulch	0.50 %	**3**
		1.0 %	**4**
		2.0 %	**5**
	Fraction A	0.50 %	**6**
		1.0 %	**7**
		2.0 %	**8**
	Artemisinin	0.50 %	**9**
		1.0 %	**10**
		2.0 %	**11**

Figure 2.3: Experimental setup for soil bioassay

2.7.2.Soil bioassay for *Enicostemmalittorale*Blume: For this bioassay, Harborne's fraction A, Harborne's fraction B and dried leaf mulch were used at three rates with triplicates of each. Leaf mulch was applied on dry weight basis while the two fraction treatments were applied in the form of aqueous solutions. Leaf mulch was applied at the rates, 0.5 %, 1% and 2% (W/W). Both the Harborne's fraction i.e. A and B were applied at the rates, 0.5 %, 1% and 2 % (V/V). Two sets of pots were kept without any treatment, in one of the set only water was added while in another 2 % ethanol was added. Treatments were coded as given in Table 2.8. Fraction A and B were poured in water and supplied as a treatment, where as leaf mulch was directly added to soil and subsequently water was supplied. Equal amount of water (100 ml) was added to all the pots. Moisture was maintained by keeping the pots in water-filled trays. Twenty seeds were uniformly placed in each pot. Readings for seed germination were recorded till fourth day.

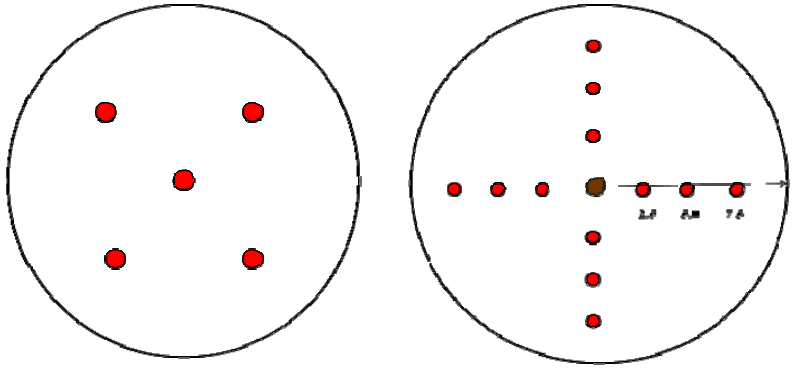
Table 2.8: Coding used for the soil bioassay for*Enicostemmalittorale*Blume treatments

		Treatment	Treatment code

Control	Water	-	**1**
Alcohol	Solvent ethanol	2.0 %	**2**
El related Treatment	Fraction A	0.50 %	**3**
		1.0 %	**4**
		2.0 %	**5**
	Fraction B	0.50 %	**6**
		1.0 %	**7**
		2.0 %	**8**
	Leaf mulch	0.50 %	**9**
		1.0 %	**10**
		2.0 %	**11**

2.8. Rhizosphere analysis: The experiment was established in wire house placed at univeristy botanical garden and conducted in the month of June (Monsoon). Twomedicinal plants, *Asparagus racemosus*Willd.(Ar) and *Chlorophytumborivilianum*San. and Fern. (Cb) were selected for the rhizosphere analysis. Before setting up experiment the earthen pot (internal diameter 36 cm and height 20 cm) were filled with 15 kg garden soil, assured to be free of plant debris and watered regularly for ten days. Pots were watered to force germinate any seeds present in the soil. The weeds that grew in pots as a result of watering were manually removed. Young plant saplings for both the medicinal plants were collected from the university arboretum. Saplings of both the plants were planted after weed removal and watered regularly for one month for the growth and establishment of the plants (Figure 2.5).

Figure 2.4: The schematic diagram showing placement of PDMS probes in soil



a) Pot without allelopathic plant

b) Pot with allelopathic plant (probes inserted at different distances from the plant axis)

Figure 2.5: Pots without PDMS probes for rhizosphere analysis

Figure 2.6: Pots with inserted PDMS probes for rhizosphere analysis

Note: red tapes depicts location of PDMS probes

2.8.1. Preparation of soil probes: PDMS probes were prepared as describe by Weidenhamer et al. (2009). Silastic® tubing (0.64 mm ID×1.19 mm OD, Fisher Scientific catalog no. 11-189-15B) was cut into five cm length pieces. The soil probes (Figure 2.7 Source: Weidenhamer et al. 2009) were prepared by soaking the tubes in Hexane and followed by insertion of 22-gauge steel wire into the swollen tubes.

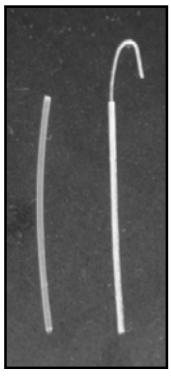


Figure 2.7:

PDMS probes

2.8.2. Design of experiment: Three sets were kept: one as control, second set with planted Aa and third with planted Cb, each set had three plants. Pots were kept for ten days (for the plants to establish and grow) and then the PDMS probes were inserted subsquently at the interval of ten days. Thus readings were taken on 10^{th} day, 20^{th} day and 30^{th} day of plantation. For control, five PDMS probes (approximately one each at end of arbitraryly taken two intersecting diagonals and one at the point of intersaction) were inserted in to soil, as shown in Figure 2.4 a. In pots with medicinal plant, PDMS probes were inserted in to soil as shown in Figure 2.4 b. Four PDMS probes, one each at the radius of 2.5 cm from the plant axis were placed in four opposite directions. Another set of probes similary, was placed at a radius of 5.0 cm from the plant axis. For first two reading taken at an interval of ten days, probes were kept at only two radius that is 2.5 cm and 5.0 cm, however for the third reading in addition to the PDMS placed at the two radius another set of PDMS (one each at the radius in four opposite direction) were placed at a radius of 7.5 cm . This pattern of PDMS placement, was followed forboth the plants i.e. Aaand Cb.

Pots were watered one day before placing the soil probes. The inserted PDMS probes were kept as such (Figure 2.6) and were removed from soil after 24 hours of insertion. For all the plants, four probes (and pooled soil) kept at a particular distance from plant axis for a single plant were combined and then extracted to get one extract per distance. Rhizosphere (both PDMS and Soil) sampling was done as given in Table 2.9. Probes were gently washed with water to remove adhered dirt, PDMS tube than was teared from probe and kept in an eppedorf tube having 1 ml of 90 % aqueous methanol. These were refered to as methanol extracts. The soil at the placment of PDMS was also collected separately similar to collection of PDMS probes.The probes

and soil after sampling were kept at 0ºC till extraction. Three such readings were taken at the interval of ten days.

Table2.9: Rhizosphere analysis sampling

Sampling at interval of 10 days →		Reading 1			Reading 2			Reading 3		
Distance of probe → from plant axis (cm)		**2.5**	**5**	**7.5**	**2.5**	**5**	**7.5**	**2.5**	**5**	**7.5**
Distance code		**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Soil Without Plant (SWP)		SWP1			SWP2			SWP3		
Ar	**Plant 1**	Ar111*	Ar112	-	Ar211	Ar212	-	Ar311	Ar312	Ar313
	Plant 2	Ar121	Ar122	-	Ar221	Ar222	-	Ar321	Ar222	Ar223
	Plant 3	Ar131	Ar132	-	Ar231	Ar232	-	Ar331	Ar332	Ar333
Cb	**Plant 1**	Cb111	Cb112	-	Cb211	Cb212	-	Cb311	Cb312	Cb313
	Plant 2	Cb121	Cb122	-	Cb221	Cb222	-	Cb321	Cb322	Cb323
	Plant 3	Cb131	Cb132	-	Cb231	Cb232	-	Cb331	Cb332	Cb333
Note: * Numerals in sequence (e.g. Ar111)starting from left, first numeral- Reading number, second numeral- plant number, third numeral – distance of probe from plant axis										

2.8.3. Preparation of extracts for HPLC analysis:

2.8.3.1. Extraction of PDMS probes: The refrigerated eppendorf tubes containing methanol with PDMS probes were vortexed and the PDMS probe were removed from the methanol.

2.8.3.2. Extraction of rhizosphere soil: Soil samples were collected in a manner similar to the probes and from the respective probes insertion sites. Approximately 20 g of soil lump was collected. Collected soil was separately air dried. Soil lumps were broken and sieved to remove plant debries. 20 g of the soil for each sample was extracted in 10 ml 90 % aqueous methanol, for 24 hours. This was vortexed, decanted in centrifuged tube and centrifuged at 10000 g for 10 min.

The probe extracts were combined with respective soil extracts and was filtered using whatmann no 1. filter paper. The solvent from all the extracts was evapourated and remains were re-solubilized in 90 % 1 ml of aqueous methanol (HPLC grade). These final extracts (total of 45) were analyed for rhizosphere metabolites analysis using HPLC.

2.8.3.3.Root washing: The plant roots were washed using water. This washing was collected, air dried and extracted with 90 % aqueous methanol. This was filtered and used for HPLC analysis. Thus there were two samples of plant root washing, one for each of the two medicinal plant i.e Ar and Cb.

2.8.3.4. Root extracts: 1 g dried root powder was extracted in 1 ml of (90 % aqueous methanol) for 24 hours and filtered. This was used for HPLC analysis.

2.8.4. HPLC analysis: For all the HPLC analysis, GraceSmart RP-18 column (25mm long, 4.6 mm internal diameter and 5µ pore size) and LC20AT Shimadzu HPLC unit equipped with binary pump and a Photo diode array detectorwere employed. Solvent for HPLC analysis were of HPLC grade (LobaChemiePvt. Ltd. India and Sisco Research Laboratories Pvt. Ltd. India). All the solvents and mobile phase were filtered through Ultipor® N®66 Nylon 6, 6 membrane (pore size- 0.2µm, 47 mm diameter) Pall India Pvt. Ltd and degassed using sonicator. All samples were filtered through Nylon membrane (pore size - 0.45 µm, diameter- 13 mm, Pall India Pvt. Ltd.)

2.8.4.1.*Asparagus racemosus*Willd. (Ar): The samples from Control (Without plant), combined*(*PDMS and soil) samples from Ar rhizosphere, Ar root washing and Ar root extracts were analysed using HPLC (Kumeta et al. 2013) and chromatogram were compared. The extract were analysed using a gradient of 0.3 % Acetic Acetonitril (Solvent B): 0.3% aqueous Acetic acid (Solvent A) (3:7). The run time was 20 minutes, injection volume was 20 µl, flow rate was adjusted to 0.4 ml/ minute and the spectra were observed for absorbance at 254 nm and spectrum max for 200 nm to 400 nm.

2.8.4.2*Chlorophytumborivilianum*San. & Fern. (Cb): The samples from Control (Without plant), Cb rhizosphere (PDMS and soil combined), Cb root washing and Cb root extracts ,were analysed using HPLC (Joshi et al.2013)and chromatogram were

compared. The extract were analysed using a gradient of Acetonitril (Solvent B): Water (Solvent A) (3:7). Chromatography condition include,run time of 30 minutes, 20 µl sample were injected, flow rate adjusted to 0.2 ml/ minute. The spectra were extracted at the wavelength of 210 nm and spectrum max polt for 200 nm to 400 nm. The HPLC chromatograms from the rhizosphere (PDMS+ Soil extract) of each plant were compared to the HPLC chromatograms from respective plant root and root washing.

2.9. Data analysis: Statistical analysis was performed using SPSS Statistics 20.0 (IBM, USA). In order to find significance of differences between control and treatment means, results were subjected to one way ANOVA followed by separation of means using Tukeys post hoc test ($P < 0.05$). For selected experiments regression analysis was performed to find out any type of relation between the considered variables, if any.