CHAPTER 4
DISCUSSION

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Page No.

(Page no. : 179 – 218)

Weed management in agriculture fields still remains a great challenge. Demand for higher crop yield with sustainable agriculture practices has become one of the prime goal for agriculturists. Weeds being competitors to the crops have to be removed essentially from the crop fields. In the country like India much of the labour and money is involved in weed control and there is a need for introduction of natural means of weed control. Among many others trials, phenomenon of allelopathy has been utilized for natural weed control lately. Allelochemicals are secondary metabolites and are found in abundance in the medicinal plants. The present study was aimed to evaluate the allelopathic potential of some wild or cultivated medicinal plants so that their potential as a natural herbicide can be explored. The phyto-toxicity of selected plant was tested in a step wise progression from *in vivo to in vitro* conditions. Extensive studies were carried out to know the nature of effective compound's probable sites, their targets and their dissipation.

Objectives and steps of the present study were: 1) Screening and identifying medicinal plants with allelopathic potential through field observations and through bioassay, 1a) Segregating the plants for further analysis, based on the potential part (Leaves / stem / root), 1b) Isolation, Screening and confirmation of pharmacologically active fractions of the potent plant part extract (leaves, stem and root) for their allelopathic potential, through bioassay, 2) Conducting the rhizospheric soil analysis of the plant species for studying dissipation of allelochemicals, in the case where the roots are potential part and 3) Studying some indicative cytological and biochemical parameters as a response of the receiver plants. Accomplishment of the research objectives and pertaining findings are individually discussed under separate headings with the relevant supporting literature. The final conclusions are drawn comprehending the entire study.

Secondary metabolites are considered as the agents that are employed in the plants communication (Maffei 2010). Allelopathy, an ecological phenomenon, also termed as chemical interaction, is mediated by the virtue of plant secondary metabolites (Duke 2015). Medicinal plants being already known source of secondary metabolites having associated medicinal significance, turn out to be a natural fascination for the studies intending allelopathic research (Patel and Pandya, 2013). Some of the medicinal plants are known to have even additional biological activities, as the plants own defence mechanism against plant pathogens, herbivores and other plants (Duke 2002). This inspires discovery of agronomic products that are derived from natural sources, such as fungicide, insecticide, nematicide, herbicide etc. However, considering the herbicides or related alternatives, discoveries based on the natural-product are not much successful (Dayan 2012) and there are only a few examples that are derived from natural products. An allelopathic plant/ medicinal plant or its product may perhaps provide a natural weed management alternative or direct herbicidal discovery (Vyvyan 2002). Establishing allelopathic potential of the medicinal plants may increase their existing petite scale of cultivation with an added benefit of natural weed mitigation. Number of the studies have reported allelopathic potential of the medicinal plants against different test plants (Fujii et al. 1990, 1991, 2003; Xuan et al. 2004; Upadhyay et al. 2011). Isolated researches concerning plant allelopathy are conducted as individual analysis of : the aqueous or organic extracts against receptive species (Hong et al. 2003; Iqbal et al. 2004; Ma et al. 2011), use of plant part mulch or residue (Ohno et al. 2000; Jefferson and Pennacchio 2003; Allaie 2006; Batish et al. 2009), *in vitro* allelopathic bioassays (Cruz-Ortega 2002), analysing allelopathy of putative compound (Perez de Luque et al. 2000; Kato-Noguchi and Ino 2005; Rial et al. 2014), mechanism of action (Sampietro et al 2006; Ding et al. 2007; Troc et al.

2011; Owens et al. 2013), analysing plant rhizosphere (Weidenhamer 2005; Loi et al 2008; Li et al. 2011). However, holistic or integrated research involving evaluation of the allelopathic potential of selected medicinal plants on the selected test plant, as in the present study has not been undertaken widely. Hence, with the selected objectives in the present study, to better understand and conceptualize, from the deliberate pool of large number of medicinal plants only the most allelopathic plant were selected and were analysed for: their allelopathic efficacy in agar/ soil medium, for their allelopathic phytoconstituents, probable mechanism of allelopathic action and rhizosphere dynamics.

Medicinal plants were selected with the purpose of gaining dual benefits, 1) they can serve as natural weed control and 2) their cultivation along with crop will supplement to their demand.

4.1. Preliminary allelopathic analysis: Medicinal plants exhibited varied extent of inhibitory effects on the selected test plant i.e. radish. The test plant was highly responsive to the various aqueous extract treatments. The effect of aqueous extract treatments on radish was also evaluated through the membrane damage and MDA content in the radish seedlings treated with the medicinal plant extract.

***Acalypha indica* L.** (Ar): The plant has been reported to have antibacterial (Govindarajan et al. 2008, Mohan et al. 2012), antifungal (Sakthi and Geetha 2011) and insecticidal (Govindarajan et al. 2008) properties. The whole plant (*Acalypha indica* L.) product was found to reduce fresh weight in a noxious aquatic weed i.e. water hyacinth, however the plant was observed to have only moderate inhibitory effects (Kathiresan et al. 2006). Similar effect of the plant was observed in present study, wherein the aqueous extracts of whole plant (leaf, stem and root) were

suppressive only to the radish seed germination while the leaf extract alone was inhibitory to the radicle growth and additionally was found to reduce the seedling fresh weight. The retardation of radish radicle growth may be due to membrane damage induced by Ar extracts however no corresponding increase in the MDA content was observed.

***Adhatoda vasica* (L.) Nees** (Av): The plant has been reported to possess both antifungal and antibacterial properties (Pa and Mathew 2012; Singh and Sharma 2013). Mitra and Prasad (2010) have reported the aqueous leaf extracts of the plant to produce stimulatory effect on the biomass of turnip root. However in the present study, aqueous extracts of Av even though being rich source of pharmacologically active metabolites had no substantial inhibitory effect on the growth parameters of radish where in only leaf extract could bring trivial inhibition of germination and plumule length.

***Aerva lanata* (Linn.) Juss. ex Schult** (Al): The plant possess antibacterial activity (Anita and Retna 2013; Elangovan et al. 2015). In the present study the aqueous leaf and stem extracts from Al decreased the seedling fresh weight in radish only to some extent and correspondingly increase in the MDA content was observed for the same indicating increased stress offered by the extracts treatment.

***Andrographis paniculata* (Burm.f.) Nees** (Ap): The plant has been reported to possess antibacterial (Kumar et al. 2010; Mishra et al. 2013) and antifungal activities (Nidiry et al. 2015). In a study, Upadhyay et al. (2011) have suggested *Andrographis paniculata* (Burm.f.) Nees having potential use in an integrated weed management system. Nagaraja and Deshmukh (2009) found the residue from *Andrographis paniculata* (Burm.f.) Nees to inhibit the growth in *Pathenium hysterophorus* L. by

decreasing the total- sugars, lipids, chlorophyll, polyphenol, aminoacid and protein contents. Alagesaboopathi (2011) has analysed the aqueous extracts of leaf, stem and root of *Andrographis paniculata* (Burm.f.) Nees and has found the leaf extracts to be inhibitory to the germination and growth of *Sesamum indicum* L. However in the present study the leaf aqueous extracts of Ap were observed to inhibit the seed germination, plumule length and seedling fresh weight but only to some extent, when compared to the respective control. Increased membrane damage and MDA level were observed to contribute the reduction in growth of radish seedlings treated by Ap.

***Asparagus racemosus* Willd** (Ar): It is a plant that possesses auto-toxicity and also has been reported to have associated replant problems (Yeasmin et al. a-2013, b-2013). Ar toxicity has been attributed to the organic acids that have been detected in the plant. Considering its auto-toxic potential the plant has also been suggested to have practical application in integrated weed management system (Upadhyay et al. 2011). In the present study the plant root extracts were relatively more toxic and were most inhibitory followed by leaves and stem, to the studied parameters of radish. The observed reduction in growth of radish seedling was accompanied by increased MDA content.

***Artemisia annua* L.** (Aa): It is also well documented to have allelopathic potential against large number of test plants (Duke et al. 1987; Lydon et al. 1997; Morvillo et al. 2011; Moussavi-Nik et al. 2011; Abate et al. 2011; Jessing et al. 2014). Moreover in the present study, leaf extracts of Aa were strongly inhibitory to all the measured parameters of radish and the inhibitory effect was observed to be concentration dependant. As a result of allelopathic stress offered by Aa leaf extracts, increase in the content of MDA was also observed in radish seedlings

***Boerhaavia diffusa* L. (Bd)**: Earlier studies have reported the plant to have antibacterial (Baskaran et al. 2011) and antifungal properties (Agrawal et al. 2013). In the present study all the extracts from the plant were suppressive to the seed germination while leaf extracts reduced the seedling biomass. Leaf, stem and root extracts caused the membrane damage and the former two increased MDA content in the radish seedling.

***Catharanthus roseus* L (G) Don.** (Cr): The plant is reported to possess insecticidal (Ramya et al. 2008) and antibacterial (Ibrahim et al. 2011) properties. Although, the plant possesses number of medicinally important secondary metabolites, the plant has no remarkable phytotoxic potential. Plant leaf extracts, only to some extent inhibited the radicle length and seedling biomass in radish when compared to the same in the control. Increase in MDA content and membrane damage was observed in the radish seedlings as a result of Cr treatment.

***Chlorophytum borivilianum* San. and Fern. (Cb):** Chakraborthy and Aeri (2009) have reported the antimicrobial activity of the plant. In the present study the aqueous root extracts of Cb were found to considerably suppress all the analysed parameters in radish except the membrane integrity and MDA content.

***Coleus forskohlii* Briq.** (Cf): The plant has been reported to possess antifungal (Nilani et al. 2006; Nidiry et al. 2015) and antibacterial (Saklani et al. 2011) properties. In the present study root extracts of Cf were found to inhibit only the radicle growth followed by leaf and stem extracts producing little effect on radicle.

***Curculigo orchioides* Gaertn** (Co): The plant possess antifungal (Raaman et al. 2009) and antibacterial (Raaman et al. 2009; Nagesh and Shanthamma 2009) properties. In

the present study the aqueous leaf and stem extracts from Co affected the radicle length and the seedling fresh weight.

***Dioscorea alata* L.** (Da): Leaf and stem aqueous extracts from the plant inhibited the radicle length and the inhibitory effect seen in case of tuber was concentration dependant.

***Enicostemma littorale* (Blume)** (El): Earlier studies have reported the plant to possess antifungal (Gopal et al. 2011) properties. The leaf extracts of El in the present study were the most inhibitory of all, affecting germination and growth parameters of radish. Germination, radicle, plumule and fresh weight were severely affected by the plant leaf extracts in a concentration dependant manner and the radish seedling also showed corresponding increase in MDA content and loss of membrane integrity.

***Euphorbia hirta* L.** (Eh): Aqueous extracts of leaf, stem and root were suppressive to the radish seed germination, accompanied by increased MDA content caused by leaf extracts and membrane damage caused by root extracts. Earlier studies have reported leaf extracts from Eh to be moderately allelopathic to the test plants (Hong et al. 2003; Saswade and Dhumal 2010) and also reported is the antibacterial properties (Suresh et al. 2008) of the plant.

***Solanum nigrum* L.** (So.n): In an earlier study made by Verma and Rao (2006), they found that the aqueous extracts from the plant, can inhibit the growth of soybean (*Glycine max* (L.) Merill). The plant is also reported to have anti microbial property (Modilal et al. 2015) and antifungal properties (Muto et al. 2006). In the present study also the aqueous leaf extracts of So.n were the most affective of all the plant part extracts and were found to significantly inhibit the seed germination and growth

parameters of radish. Aqueous leaf extracts of So.n increased the MDA content and damaged the seedling membrane.

***Synedrella nodiflora* (L.)** (Sy.n): Earlier works have reported insecticidal (Rathi et al. 2006), larvicidal (Ghayal et al. 2010) and allelopathic potential (Ray et al. 2013; Ghayal et al. a-2013, b-2014) of the plant. However in the present study, aqueous extracts from *Synedrella nodiflora* (L.) Gaertn. had no considerable inhibitory effect on radish growth parameters, other than a modest inhibition observed in case of the seed germination.

***Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms** (Tc): The plant has been reported to have antibacterial (Nagaprashanthi et al. 2012) and antifungal properties (Singh et al 2010; Mathur 2011; Nagaprashanthi et al. 2012). Aqueous extracts of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms (Singh et al. 2009; Abdul Raoof and Siddiqui 2012) in earlier studies, have exhibited allelopathic effect on some weed species. In the present study also aqueous leaf, stem and root extracts of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms were inhibitory retarding the growth of radish and inducing the membrane damage in radish seedling.

***Urginea indica* (Roxb.) Kunth**. (Ui): The plant is reported to possess different biocide properties including antifungal activity (Deepak et al. 2003; Shenoy et al. 2006; Temikotan et al. 2013), antibacterial activity (Temikotan et al. 2013; Manigandan et al.) and insecticidal (Shiva kameshwari et al. 2012) activity.

In the present study, the plants aqueous extracts were analysed for their allelopathic activity and the aqueous extracts from leaf, stem and root were found to inhibit/ reduce the seed germination, seedling growth (radicle and plumule length) and seedling fresh weight. Seed germination (Chiapusio et al. 1997) and growth bioassays

are primary tools through which allelopathic potential or phytotoxicity of a plant on target plant can be assessed. Primarily, the onset of germination is marked by imbibition of water and subsequent emergence of radicle accompanied by a sporadic outburst of number of physiological processes such as respiration. Allelochemicals are supposed to inhibit germination by disrupting the mitochondria and affecting glycolysis, Kreb cycle, electron transport and hindering the mobilization of reserved food available of catabolism (Bogatek et al. 2005). The allelochemicals upon absorption by the radicle may inhibit the antioxidant enzyme and increases the free radicle content there by increasing the membrane lipid peroxidation and subsequently damaging the whole membrane system in the test plant (Cheng and Cheng (2015). Cheng and Cheng (2015) have also suggested that the allelochemicals can have effect on plant growth regulators which may be in terms of an imbalance in the content of various phytohormones that ultimately brings about inhibition of seed germination and seedling growth. In the present study as the aqueous medicinal plant extracts which are likely to possess various polar to semi polar metabolites exhibited the inhibitory potential, it can be inferred that the metabolites may be inhibiting the germination, seedling growth or seedling biomass by one or the other mechanism as discussed above.

Considering the test plant response, in terms of germination and growth parameters to the dose/concentration of treatments supplied in form of aqueous extracts, the effect was concentration dependant in some aqueous extract treatments that is, a gradual increase in concentration resulted into increase in the inhibitory effect offered. However, rather unusual to that it was also observed that in certain case, at low concentration the response was stimulatory and as the concentration increases the response becomes inhibitory. Such dose/ paired responses (stimulation

and inhibition) phenomenon is termed as hormesis (Belz 2007). This type of response has been observed in many growth regulatory chemicals and even herbicides (An 2005). Occurrences of such phenomenon in allelopathy have also been reported, where by hormesis responses has been observed and studied (Belz 2007).

Apart from vital medicinal uses, all the studied plants have some or the other biocide use and have shown effect against microbial pathogens, insects or other plants. Thus if utilized appropriately they can be employed for multiple purposes such as fungicide, insecticide, herbicide etc. Results of the preliminary analysis revealed the possible allelopathic potential of the selected medicinal plants and their respective part (leaf, stem or root). It was observed that the aqueous extracts from leaf, stem and root of a medicinal plant can differentially offer inhibition to the seed germination and seedling growth and this is true as for a medicinal plant the metabolite distribution may vary across the different plant parts. The phytotoxic ability of all the medicinal plants in case if any, was found to be related to the plant parts assayed, selected concentrations and parameters analysed in the test species.

In the present study, the medicinal plants were primarily screened using aqueous extracts bioassay as the one of the motive behind the present piece of work was its possible practical application. Thus considering the 'in field' applications of allelopathic medicinal plant in the weed mitigation program, it would be more appropriate if the utility of a plant is both economic and conducive while it is serving the purpose of weed mitigation as in case of an agricultural field. As manifested by the results of preliminary analysis many of the plant exhibited phytotoxicity by their aqueous extracts. Water is by enlarge considered as the universal, inexpensive and non hazardous solvent. Thus by critically standardizing the dose, method of application, its suitability to the crop plants, the aqueous extracts of allelopathic

medicinal plants may be employed for weed mitigation either singly or in combination with other weed eradicating tools.

4.2. Evaluation of medicinal plant part toxicity by fractionation guided bioassay

Based on the results of preliminary analysis six medicinal plants were selected for further studies. The selection of these medicinal plants was done on the basis of two criteria, 1) phytotoxicity and 2) medicinal significance. While accounting the phytotoxicity, a medicinal plant was observed for its overall extent of inhibition to the studied parameters of radish and number of parameters being inhibited by the same. It was observed that for all the six medicinal plants, the plant parts that confer medicinal importance and utility to the plant, were also found to possess allelopathic activity. Appraising the results and taking into account the above mentioned criteria, the six medicinal plants and respective plant part, that were selected for the further study were *Artemisia annua* L. leaf, *Asparagus racemosus* Willd. root, *Chlorophytum borivilianum* San. & Fern. root, *Enicostemma littorale* (Blume) leaf, *Solanum nigrum* L. leaf and *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms stem. The selected allelopathic plants were further subjected to Harborne's fractionation. All the three fractions (at three concentrations) so obtained from each of the six medicinal plants part were individually analysed for their toxicity against a common monocot weed that is *Chloris barbata* Sw. using agar based bioassays. From the results of fractionation guided bioassay, conducted for each of the six medicinal plants, the chemical fraction contributing towards the inhibitory behaviour of the plant were ultimately exposed. Thus one and the most inhibitory fraction for each medicinal allelopathic plant was analysed for its phyto-constituents using classic and advance chromatographic methods. Fractions obtained following the Harborne's fractionation protocol possess metabolites of a particular chemical nature and accordingly

considering the same, fraction A may possess Terpenoid or Phenolic where as fraction B and C may possess different Alkaloids. Thus for the plants for which the fraction A was inhibitory, that fraction from the plant was analysed for both terpenoids and phenolics and for the plants where the fraction B or C was inhibitory, that fraction from the plant was analysed for alkaloids. The possible compound identification was attempted comparing the results from the available literature for a given medicinal plant.

1) *Artemisia annua* L. (Aa)**:** Terpenoids are a group of metabolites that dominate the plant and owe the plant its diverse medicinal uses. Bhakuni et al. (2001) have reviewed large number of secondary metabolites that are found in *Artemisia annua* L. including Monoterpens, Sesquiterpenes, Triterpenes, Coumarins, and Flavonoids. They have also reviewed associated biological activity exhibited by the Aa metabolites. Han et al. (2008) have identified some 40 phenolics and Skowyra et al. (2014) have shown presence of caffeic acid, rutin and apigenin as principle phenolic found in Aa.

It was observed in the present study that the Harborne's fraction A in *Artemisia annua* L. was the most inhibitory of the three fractions analysed and it was found to inhibit germination, seedling growth (radicle and plumule length) and reduce seedling biomass in Chloris. The inhibitory effect imparted by fraction A was observed to be concentration dependent and increase in concentration was observed to hinder the Chloris growth correspondingly. Thus Harborne's fraction A was analysed for its possible phytotoxic metabolites. Simple planar chromatographic analysis such as PC and TLC helped to identify the chemical nature of fraction A. Fraction A was found to possess phenolic and terpenoid type of compounds. PC and HPLC analysis of fraction A conducted to evaluate phenolic compounds resulted in to separation of eleven

phenolic compounds and atleast three of which were simple phenols. The phenolic compounds found in fraction A were, Protocatechuic acid, Vanillic acid and trans-Ferulic acid. The TLC and HPLC analysis of fraction A performed for terpenoids revealed presence of eight metabolites that were terpenoid in nature with atleast one of the metabolite being artemisinin. In earlier studies, the allelopathic activity of Aa, has been analysed on different test plants, where in different components from Aa, such as extracts, mulch and isolated compounds found in Aa have been studied for their phytotoxicity (Duke et al. 1987; Lydon et al. 1997; Morvillo et al. 2011; Moussavi-Nik et al. 2011). Different terpenoidal metabolites from the plant have been suspected and studied to have allelopathic ability. So far the phytotoxicity of Aa has been attributed to the different terpenoid compounds such as artemisinin and its analogues that are found in the plant (Lydon et al. 1997; Dayan et al. 1999; Hussain and Reigosa 2014). However the occurrence of phenolic compounds in addition to terpenoids in the fraction A which was also the allelopathic fraction from the plant, observed in the present study, suggests that the allelopathic compounds from Aa may be either phenolic or terpenoid in nature and that phenolics singly or together with terpenoid may be contributing to the phytotoxicity of Aa. Number of allelopathic studies conducted earlier, have proved that the pure phenolic compounds as well as when present in plant extracts, have various deleterious effect on the plants growth. The phenolics compound detected in the Fraction A, such as Protocatechuic acid, Vanillic acid and trans-Ferulic acid are also been reported to have detrimental effects on other plants. Thus the fraction A containing phenolic and terpenoid metabolites, is a potential chemical fraction from *Artemisia annua* L. which is responsible for the inhibitory activity of the plant on different test plants.

2) *Chlorophytum borivilianum* San. & Fern. (Cb)**:** The plant is reported to possess number of metabolites, principle metabolites which are of therapeutic value are steroidal saponins (Acharya et al. 2008, Joshi et al. 2013) and alkaloids (Singh et al. 2012). The plant owes high medicinal utility in number of formulations and nutraceutics. In the present study fraction A was found to be the most allelopathic chemical fraction of the three fractions analysed from Cb. It was found to inhibit the germination and growth parmaters of Chloris. Hence fraction A was analysed for its phytoconstituents such as phenolics and terpenoids. In the planar chromatographic analysis, one simple phenol was detected in fraction B which may be Caffeic acid or Cis- Sinapic acid. However the HPLC analysis could detect presence of ten phenolic metabolites. TLC analysis of fraction A performed for terpenoids resulted in to separation of four compounds where as the HPLC analysis of the same resulted in to separation of seven metabolites. Thus the fraction A from Cb roots which is also an allelopathic fraction, showed presence of phenolics and terpenoid compounds, which are also medicinally significant metabolites from the plant.

3) *Solanum nigrum* L. (So.n)**:** Leaves are rich source of different pharmocologicaly important alkaloids (Mohy-Ud-Din et al. 2010). Subjecting the methanolic leaf extracts to fractionation following the Harborne's fractionation, alkaloids were extracted in the fraction B and C. In the present study all the three fractions from So.n were analysed for their phytotoxic ability and fraction A was found to be highly toxic. The fraction A was found to affect the Chloris growth, retarding its germination and seedling health. Phytochemical analysis of fraction A revealed presence of phenolics and terpenoid compounds. PC analysis of fraction A could detect two simple phenols which may be Vanillic acid and Syringic acid. HPLC analysis of the fraction A for phenolics compound could separate ten compounds. The separated compounds

require further analysis for confirmation of their identity. TLC and HPLC analysis of fraction A for terpenoids could detect presence of four and twelve terpenoid metabolites respectively. Thus, as the pharmacologically important compounds in the plant So.n are alkaloidal metabolites whereas the pehnolic and terpenoid metabolites from the plant may be further explored and employed for their allelopathic use.

4) *Enicostemma littorale* (Blume) (El)**:** Principle secondary metabolites reported in the plants are flavonoids, alkaloid, saponins (Leelaprakash and Dass 2012) and secoiridoid glycoside such as swertiamarin (Vishwakarma et al. 2004). In the present study, the three fractions were analysed for their inhibitory potential against various growth parameters of Chloris. Fraction B was found to be highly phytotoxic to the weed i.e. Chloris. Chloris seed germination and seedling growth were severely affected by fraction B treatment. Phytochemical analysis was performed for fraction B using TLC and HPLC. TLC analysis revealed presence of single compound and HPLC analysis reveals presence of fourteen compounds that are alkaloid in nature. Thus El exhibits allelopathy owing to its fraction B which is chemically alkaloid in nature.

5) *Asparagus racemosus* Willd. (Ar)**:** Medicinal importance of the plant is owing to its steroidal saponins (Hayes et al. 2008) and sapogenins (Negi et al. 2011). In the present study all the three fractions resulting from the Harborne's fractionation of plant root extracts, were analysed for their phytotoxicity on Chloris. Out of the three, fraction B was observed to exhibit highest inhibitory potential on Chloris. Thus fraction B was analysed for its phytoconstituents. The TLC analysis confirmed the alkaloid nature of fraction and could detect a single alkaloid metabolite in the fraction. The HPLC analysis of same could separate eleven compounds. Moreover in earlier studies, the plant is reported to exhibit auto-toxicity and the allelochemicals

responsible for the autotoxicity of Ar were certain organic acid such as oxalic, succinic and tartaric (Yeasmin et al. a-2013, b-2013). However the results of present study reveal the allelopathic metabolites from Ar to be alkaloid in nature.

6) *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms (Tc)**:** The plants possess high medicinal utility owing to its rich secondary metabolite content. It is reported to possess alkaloids, terpenoids (Sankhala et al. 2012; Kakkar et al. 2013). Highest inhibitory effect was exhibited by the fraction B and all the parameters were found to be affected by the fraction B treatment. In the present study, it was observed that out of the three fractions analysed, fraction B was found to be most inhibitory to the growth parameters of Chloris. TLC and HPLC analysis of fraction B resulted in to separation of two and eleven alkaloid metabolites respectively. Thus the allelopathic effect exhibited by the plant Tc is due to alkaloids that the plant possesses.

Diverse allelopathic activities have been attributed to different group of secondary metabolites detected in the present study, wherein the metabolites have been found to have different mechanism of action in the test plants, ultimately resulting in to deleterious effect on its growth. Large number of the phenolic compounds has been studied to have various physiological effects on the test plant. Some phenolics like ferulic acid and cinnamic acid can be inhibitory to the protein synthesis (He et al. 2001). Both, Ni (2000) and Zeng et al. (2001) have found that all the phenolics could reduce nucleic acid (DNA and RNA) integrity. Phenolic compounds such as salicylic acid and ferulic acid could significantly affect mineral absorption by plants (Harper and Balke 1981) and both the acids inhibited potassium ion (K+) absorption in excised root tissue of *Avena sativa* L. cv. Goodfield however salicylic acid was most inhibitory. Reigosa and Pazos-Malvido (2007) have observed

vanillic acid and sinapinic to delay seed germination in *Arabidopsis thaliana* L. and the vanillic acid in addition to ferulic acid, protocatechuic, syringic, were able to affect even the radicle length in *Arabidopsis thaliana* L. Phenolics such as caffeic acid and its derivatives were found to inhibit the growth of a coexisting species and thereby were observed to regulate the plant community composition (Scognamiglio et al. 2012). Various terpenoids and alkaloids have been known to behave as allelochemicals. The tropane alkaloids such as scopolamine and hyoscyamine, is a group of alkaloids found in the members of Solanaceae, were extremely toxic to sunflower (*Helianthus annuus* L.) seedling growth, as well as that of several cereal. In case of terpenoids some of the popular metabolites that manifest allelopathy are parthenin (Kanchan and Jayachandra 1980) and artemisinin and its analogues (Dayan et al. 1999). Birkett et al. (2001) reported that the saponins, a type of terpenoids have plant membranes disrupting capability and thus can be used as potential herbicides.

Thus detection of the above discussed metabolites, in the allelopathic plant fractions suggest that the effectiveness of the plant fraction must be due to presence of one or the other metabolites such as phenolics or terpenoid or alkaloid. In the medicinal plants such as Aa, Cb and So.n, where the fraction A was inhibitory, the plant must be offering growth suppression by the allelochemicals that are phenolic or terpenoid in nature. As mentioned earlier, Aa, Cb and So.n exhibiting allelopathy by fraction A, are already known to possess different terpenoid metabolites, however presence of the same was also confirmed in the present study. In plants such as Ar, El and Tc, where the fraction B was the allelopathic fraction for a given medicinal plant, the allelochemicals in the plants are alkaloid in nature. Also, Ar, El and Tc are already known to possess alkaloids and the same was also confirmed by in the present study. However in each of the medicinal plants, the identity of allelochemical needs to be

confirmed by more advanced and precise techniques such as LCMS, MSMS, NMR and X-Ray crystallography. Also the effectiveness of the plant extracted metabolite needs to be studied individually, in order to confer physiological effect to the metabolite.

Also the medicinal plant fraction treatments at different applied dose could bring stimulatory or inhibitory responses in Chloris. Apart from the effect that was directly dependant on concentration, certain treatments at lower doses could bring stimulation of the studied parameter in Chloris, however the subsequent higher doses resulted in to inhibition of the same. This must be due to existences of the phenomenon of hormesis, between treatment concentration and the test plants response. Although not for the present study, the stimulatory effects of such inhibitory chemicals/extracts are gaining tremendous attention in the field of agriculture and others. Recently efforts are underway to utilize the low doses for achieving higher crop yields while achieving its prime benefit as that of weed mitigation observed in case of herbicides (Belz et al. 2011).

Considering the magnitude of inhibitory effects of all the six plants two of the most allelopathic medicinal plants i.e. *Artemisia annua* L. and *Enicostemma littorale* (Blume) were further evaluated for possible mechanism of action (analysed by biochemical, physiological and cytotoxic studies) by which they are able to bring growth retardation in the test plant. As these two plants were most phytotoxic of all the plants evaluated using agar based bioassay, phytotoxicity of these plants were also studied by conducting soil bioassay. Two of the medicinal plants exhibiting allelopathy by roots i.e. *Asparagus racemosus* Willd. and *Chlorophytum borivilianum* San. and Fern were selected for their rhizosphere analysis.

4.3. Analysis of biochemical, physiological and cytotoxic effects: According to Bartoli et al. (2013), the plants respond to different environmental stress through a fine balance between the hormonal growth regulators and the redox signalling system, where in the information from the environment and the plant's metabolism and physiology are been integrated. When the allelochemicals are toxic to the receptive plant they also induce stress (Cruz-Ortega et al. 2007) in the plant (Chloris in the present study) and there by generate corresponding responses in the plant. Bogatek and Gniazdowska (2007) have reported sunflower allelochemicals to have induced disturbances in hormonal balance between ABA and ethylene in germinating mustard seeds. Hence knowing that the particular fraction and corresponding metabolites from a plant are phytotoxic, it becomes crucial to know as to what is the possible mechanism that is responsible for the obstructed growth in the receptor plant. The fraction A from *Artemisia annua* L. leaf and fraction B from *Enicostemma littorale* (Blume) leaf were highly toxic to the test plant and the treatments resulted in to reduced growth in Chloris. In order to have better understanding towards their mode of action, the respective plant fractions were analysed for their possible mechanism of action on Chloris, where in the seedlings treated with the fractions were evaluated for MDA contents, Antioxidant enzymes, Protein and RNA content, Chlorophyll content. The Cytotoxicity of fractions were analysed on Onion root cells.

4.3.1. Lipid peroxidation: Weir (2004) suggested that one of the effects of allelochemicals on target plant may be uncontrolled production and accumulation of ROS accompanied by activation of cellular antioxidant system. In a study by Oracz et al. (2007), sunflower extract was found to induce an oxidative burst that was expressed as an increased H_2O_2 concentration in germinating mustard seeds, enhancement in MDA level and increased electrolyte leakage (Bogatek et al. 2006)

due to associated membrane damage. They correlated this with loss of mustard seed viability in response to sunflower allelochemicals, which must be a resultant effect owing to increased lipid peroxidation. In the present study the El fraction B was found to alleviate the MDA level in Chloris seedlings in a concentration dependant manner, whereas Aa fraction A had no effect on the same. Thus increase in MDA content must be a factor that contributes to reduced seed germination and radicle growth in Chloris exposed to El fraction B treatment.

4.3.2. Antioxidant enzymes assays: Antioxidant enzymes play a vital role in detoxification of ROS and the mediated stress offered by allelochemicals (Cruz-Ortega et al 2007). The levels of different stress related enzymes such as SOD, CAT and POD, in a test plant increases or decrease, in response to various stress experienced by the plant. Hence in response to the allelopathic stress imparted by the fraction from the two plants, the levels of different antioxidant enzymes in Chloris seedlings were analysed. SOD activity showed distinct response to the chemical fractions from both the phytotoxic medicinal plant (El and Aa). The effect of fractions from both the plants on SOD activity was observed to be concentration dependant that is increase in concentration of fractions resulted in to increase in SOD activities. This suggests that phenolics and terpenoids from Aa and alkaloids present in El induces allelopathic stress in the test plant where by the SOD enzyme activities in the Chloris seedlings are affected. Catalase activity in the Chloris seedlings treated with the fraction A from Aa were higher as compared to the seedlings in control and the increase in Catalase activity was concentration dependant for Aa treatments. This suggest that the Catalase content in the test plant increases as a response to the stress offered by phenolic and terpenoid present in the plant. Fraction B from El and its respective alkaloids had no inhibitory effect on Catalase activity in Chloris. Both the

plant fractions with their respective metabolites had no effect on the POD activities in Chloris. Also out of the three enzymes evaluated for response to various allelochemicals, SOD was more responsive in comparison to CAT and POD. Also as antioxidant enzymes in the test plant respond to the ROS, the molecules employed in plant-plant signalling, the study on effect of these allelopathic plant metabolites on the receptive plant can be extended to the more natural environment with a profound understanding.

4.3.3. Protein analysis: Protein synthesis pathway offers one of the primary metabolite target sites to the allelochemicals. Alteration in the protein contents depicts the plant health. Requirement of certain proteins may alleviate under some condition while it may go down at the other. Earlier studies by Romero-Romero et al. (2002) have suggested that the environmental stresses such as biotic and abiotic ones induce the synthesis of some new proteins in the plants and for analysing the stress associated changes in the protein profile they have utilized 2D PAGE, wherein they have compared the protein expression in the allelopathic analysis. Hence similar comparison was attempted in the present study.

A unique pattern of protein expression was observed in fraction treated Chloris seedling. Amount of Chloris tissues taken for isolation of proteins was kept same for all the pooled protein samples. In case of Aa treated seedlings, number of protein bands appeared to be high then its respective control and alcohol treatment, indicating requirement and synthesis of the same in the treated seedlings. Also, number of the protein bands resolved, were higher in the lowest concentration treatment followed by an increase in the second lowest and a sudden decrease in the highest concentration treatment. It was observed that the percentage inhibition of germination in them was 3.0, 4.7 and 65.7 % and corresponding percent inhibition of

radicle and plumule growth was 86.5, 91.2, 95.6 % and 86.5, 91.2, 95.6 respectively. This shows that due to comparatively lesser inhibition of germination, in the lowest and second lowest concentrations the seedling growth was relatively high and this may contribute to the observed high protein content in them as compared to the highest treatment where already the seed germination was low and so was the seedling growth and associated protein expression. The proportion of inhibition offered to germination and to the seedling growth are important determinants of protein content/ expression of test plant.

In case of El treatment treated seedling also the protein band number was higher than that in the control and alcohol treated seedlings, again suggesting the higher requirement of the same in the stressed seedlings. The protein band resolved for the El treatments applied at three rates was: higher number of bands in lowest concentration, least number of bands in second lowest concentration and high number in highest concentration. Percentage inhibition of germination observed for the same was 37.2, 86.4 and 87.5 respectively. Percentage inhibition experienced by the radicle and plumule were, 60.0, 70.0, 80.0 % and 25.9, 74.9, and 84.0 % respectively. This indicates that due to relatively higher germination in the treatment applied at lowest rates the seedling growth was high and so was the protein content, lesser germination with relatively lesser seedling growth, contributed to low protein content in the second lowest and highest treatments.

Thus it was observe that the protein profile and content is remarkably affected in response to Aa and El treatments. It was inferred from the studied association between the protein content and the germination/seedling growth ratio, that the physiological stage where by the allelopathic treatment is effective on the seedlings is also responsible for the observed difference in protein content. The allelochemicals found

in Aa fraction A were phenolics and terpenoid and the once found in El fraction B were alkaloids, where in the treatment with these allelochemicals are reported to inhibit cell DNA and RNA and ultimately affect protein synthesis (Cheng and Cheng 2015). In contrast to these number of researches fail to explain that when the effect of such allelochemical can reduce the DNA, RNA and protein synthesis, how are certain enzymes such as antioxidant (CAT, SOD, POD etc) which are also protein in nature are alleviated owing to the allelopathic treatments. Hence studies concerning synthesis of the type of proteins being stimulated or inhibited in response to the allelopathic treatments, needs to be addressed.

4.3.4. Analysis of RNA: RNA is directly or indirectly institutionalized in the condition of Plant stress. Thus RNA analysis allows dissecting the physiological and metabolic intricacies in a plant thriving in a particular set of conditions. Horvath et al. (2006) have compared the RNA isolated from corn grown in the presence/ absence of *Abutilon theophrasti* Medik. under field conditions. Similar kind of studies may help understanding the underlying mechanism for the reduced growth in the test plant. Thus as an additional measure of allelopathic stress RNA from the treated (solvent alcohol and Harborne's fraction) and the non treated Chloris seedlings (control) through a very preliminary analysis were compared in terms of concentration of RNA. The method followed in present studies enabled separation of total RNA. The RNA isolation was analysed for its purity by analysing the bands separated in the developed gel, absorbance ratio of the RNA samples at 260 nm and 280 nm of wavelength. Pure RNA has an approximate A260/A280 ratio of 1.8 - 2.2. Considering the intensity of upper 28 S RNA band and lower 18 S RNA band, in a good RNA preparation the 28 S RNA band is two times more intense than the 18 S RNA band and also there is less or almost no shearing of bands so the bands are distinct. Samples of RNA which were

found highly impure as in the case of treatment with alcohol, the El fraction B (0.125, 0.25 %) and Aa FA (0.25, 0.5 %) are not discussed. In terms of relative intensities of 28 S and 18 S RNA, most samples had minor contamination.

In case of Chloris seedlings pooled from the control, considering A260/280 ratio the RNA isolation was pure, however the band intensities were not in expected trend. RNA isolated from alcohol treatment was impure in terms of the A 260/280 ratio and the band intensities were also not in trend. In case of the El, the Chloris seedlings treated at the highest concentration of fraction B (0.5 %) was having a good quality RNA with the A260/280 ratio of 1.82. For the Aa, a good quality RNA was obtained in case of Chloris seedlings treated with lowest concentration of fraction of fraction A (0.125 %) where in the A260/280 ratio was 1.92.

Comparing the concentration of RNA in the control and treated seedlings, the RNA content of seedlings treated with El (8440 µg/ml) and Aa (5760 µg/ml) was high as compared to the control seedlings (3520 µg/ml). Higher amount of RNA in the treated seedlings suggest that as a response to the allelopathic treatments imposed by El and Aa to the Chloris seedlings, the seedlings must have produce higher RNA. However, the result was contradictory to the earlier reports where in the allelochemicals offer inhibitory effect on the transcription and translation of DNA (Cheng and Cheng 2015).

4.3.5. Analysis of Chlorophyll content: Effect imparted in form of any treatment on the seedling, can be directly correlated to the observed alteration in its growth. Chloris seedlings in the present study were highly responsive to the treatments applied in form of chemical fraction from the plants. One of the responses observed was reduction in seedling growth. Thus it was hypothesised that this growth reduction must be a result of effect of treatment on the seedling chlorophyll content. Thus

Chlorophyll content was estimated in the treated and non treated Chloris seedlings as a representative of seedling health. Chlorophyll concentration was relatively low in the plant fraction treated seedlings in trend with the pattern of observed growth. The effect of treatments, were visible as bleaching induced in the treated seedlings. Treatment with El fraction B had significantly reduced Chlorophyll content in the Chloris seedlings, where in the seedlings treated with highest concentration had highest impact on chlorophyll concentration. The effect was more on Chlorophyll b which was contributing to the reduction observed in total chlorophyll content.

Treatment in form of Harborne's fraction A from Aa, also reduced the chlorophyll concentration in the Chloris seedlings and the effect was observed to be concentration dependant. Owing to the plant fraction treatments, the most affected was Chlorophyll b which showed decrease in concentration of about half of that observed in the control. Effect on Chlorophyll b was contributing to the effect observed on the total Chlorophyll. Earlier studies have reported the inhibitory effect of Artemisia metabolites on the Chlorophyll contents (Dayan et al. 1999). Ferulic acid detected in the allelopathic Fraction A from Aa is reported to have effect on biosynthetic and degradative pathways of Chlorophyll (Yang et al. 2004). Bharati et al. (2012) have reported that artemisinin (a metabolite from *Artemisia annua* L.) is employed in the inhibition of photosynthetic electron flow affects photosynthesis and ultimately results in reduction in the plant growth. This supports our results, where in the overall inhibitory effect of Aa fraction on the Chloris growth can be attributed to the corresponding effect of fraction A on Chlorophyll content in Chloris. However in the phyto-chemical analysis of Aa fraction A, it was found to possess phenolic compounds in addition to artemisinin and its terpenoid analogues. Thus the artemisinin may be individually able to affect photosynthesis in the receptive plant as

seen in earlier studies (Dayan et al. 1999; Bharati et al. 2012) where artemisinin alone was used. However in present study it was found that treatment with crude extract and fraction had an additive inhibitory effect. The observed effect of plant fraction must be due to presence of the additional matrix of metabolites in the same. Results of our study also confirm, the inhibitory fraction to possess phenolic compounds in addition to artemisinin and other terpenoids, thus there exist possibilities of phenolic compounds such as ferulic acid found in Aa, to have decreased chlorophyll content in the treated seedlings.

4.3.6. Cytotoxicity analysis: Plant toxins can inhibit plant growth by directly affecting the cell division (Hess, 1987). In the present study toxicity of chemical fractions from the plant was analysed on the onion root cells. The results were compared to infer the results observed in the test plant that is Chloris. Mitotic index was highly reduced in the treatment with fraction B from El as compared to that in the control and was reduced by 12 %. Most of the dividing cells were observed to be severely arrested in Interphase. In case of El fraction B treatment the reduced growth of Chloris radicle must be due to both lower mitotic index as well as delayed cell division. The phytochemical analysis of fraction B could detect fourteen alkaloids. These secondary metabolites are also reported to affect the DNA synthesis by inhibiting the DNA polymerase I enzyme (Cheng and Cheng 2015). However in the present study no effect was observed on interphase where the cell undergoes DNA synthesis and where in the polymerase is employed. Thus the metabolites must be acting upon the subsequent stages of cell division.

Treatment with fraction A from Aa had no effect on the mitotic index. However cells were severely arrested in interphase. This suggest that the compounds present in the Aa fraction i.e. phenolics and terpenoid compounds, retard the rate of cell division

and hence delays the cell division. The effect of delay in cell division must be responsible for the reduced growth in the treatments. Artemisinin and its analogues (Duke et al. 1987; Bagchi et al. 1997; Dayan et al. 1999) have been known to affect mitosis in certain test species. As observed in the present work, the retarded growth in Chloris radicle treated with fraction A from Aa must be due to the delayed cell division and not due to effect on mitotic index.

4.4. *Artemisia annua* L., artemisinin and the plant metabolite absorbance by *Chloris*: Considering all the assays, as *Artemisia annua* L. was the most phytotoxic plant, its inhibitory potential was assessed using additional bioassays. Ethanolic leaf extracts from Aa and Artemisinin a metabolite from Aa were also analysed for their phytotoxicity on Chloris using agar based bioassay.

4.4.1. Agar based bioassays for *Artemisia annua* L. related treatments: Leaf extracts were found to retard the speed of seed germination in Chloris and was found to inhibit the seed germination as well as radicle growth. The inhibitory effect imposed by ethanolic leaf extracts on the germination and radicle length was observed to be concentration dependent, where in increase in extracts concentration resulted in to decrease in both the parameters. Treatment with standard artemisinin was found to inhibit the germination of agar grown Chloris, however radicle length of Chloris was highly reduced owing to the artemisinin treatments.

4.4.2. Soil bioassay for *Artemisia annua* L.: *Artemisia annua* L. exhibited high allelopathic potential in the agar based bioassay's. For analysing consistency of phytotoxic potential exhibited by the Artemisia treatments (Harborne's fraction A, ethanolic leaf extract and artemisinin) in agar based bioassay, certain Artemisia treatments were further analysed by means of soil based bioassay. As the leaf was the

most allelopathic part, the possible allelopathic potential of leaf mulch was compared with that of other Aa treatments. The Harborne's fraction A, plant leaf mulch and standard artemisinin were selected to impart treatments on Chloris in the soil bioassays. The effect of phytotoxicity was evaluated on the speed of germination and total germination of the soil grown Chloris seeds. The inhibitory effect of leaf mulch was observed to be concentration dependent and increase in concentration of leaf mulch decreased the seed germination. It was observed that the Chloris seeds subjected to Fraction A treatment failed to germinate at all the applied rates, thus there was no seed germination. Contrary to the agar grown Chloris, treatment supplied in form of standard Artemisinin to the soil grown Chloris was found to impart stimulatory effects, where in the speed and total germination was observed to be higher than the respective control.

The agar based bioassays were used to the study the effect of treatments on the test plant in a neutral medium such as agar while nullifying the additional effects as the ones expected in soil, while the soil bioassays were conducted to evaluate application value of the work. While comparing the effectiveness of Aa treatments on both soil and agar grown Chloris, it was inferred that the inhibitory effect of Harborne's fraction A was consistent in both agar and soil grown Chloris. The dose response studies for Aa treatments reveal, the ethanolic leaf extract, Harborne's fraction A and leaf mulch (all applied at rate of 1 %) to be potential enough to inhibit Chloris growth. However in case where Chloris radicle was analysed, delayed germination can be a reason for the observed reduction in radicle growth, even after the emergence, radicle could not grow with the pace it was found growing in both the control and ethanol treatment. In the agar based bioassays, Harborne's fraction A was most allelopathic of all the studied Aa treatments. In agar bioassay, at the

concentration of 1 %, Harborne's fraction A could bring 77.3 % of inhibition in germination of Chloris as compared to the same concentration of the ethanolic leaf extract, effective to inhibit the germination by 45.1 % only, which is comparatively almost 30 % less. In the soil based bioassays, even at 0.5 % concentration the seed germination was completely inhibited by Harborne's fraction A. Thus Harborne's fraction A was found to be more effective then both, the ethanolic leaf extract and leaf mulch even at the same concentration. The remarkable effectiveness observed in the allelopathic potential of Harborne's fraction A, must be possibly due to the reason that Harborne's fraction A was obtained after fractionation of the alcoholic leaf extract of Aa, which results into separation of metabolites into different fractions. Fractionation in such a manner results into compartmentation and eventual concentration of allelopathic metabolites in to the fractions according to their chemical affinity. Use of the fractionation guided bioassays (Rimando et al. 2001; Baratelli et al. 2012; Wahyuni et al. 2013; Araniti et al. 2013) for the allelopathic analysis is currently increasing, where in the fractions obtained after fractionation are subjected to toxicity bioassays. Our results support the earlier studies conducted using Aa leaf mulch (Lydon et al. 1997), where in the leaf mulch and extracts were found to inhibit some other test plants. However the higher inhibitory effect of Harborne's fraction A in the soil grown Chloris increases the application value of the present work. The pure artemisinin concentration used in present study was selected based on results by Duke et al. (1987). The results so obtained were contradictory as it was found to be inhibitory only in case of agar grown Chloris where as it was stimulatory to the soil grown Chloris. Germination and radicle growth in Chloris could be inhibited by pure artemisinin but it was less effective as compared to inhibition induced by Harborne's fraction A, Aa leaf mulch and ethanolic extract respectively. This implies that

artemisinin alone is not the only metabolite responsible for allelopathic potential of *Artemisia annua* L.

4.4.3. Qualitative analysis:

Spectroscopic absorption analysis of artemisinin and ethanolic leaf extract was attempted, so as to evaluate artemisinin and its analogues where in the absorbance properties of metabolites have been utilized for their qualitative analysis. Earlier studies have attempted studying allelopathic potential of Artemisinin and alike metabolites from *Artemisia annua* L. (Duke et al. 1987). Absorption maxima of artemisinin (qinghaosu-QHS) lie around 216nm. Upon derivatization, an intermediate Q292 is formed on treatment of artemisinin with sodium hydroxide solution having maximum absorbance at 292nm and Q292 on further conversion with acetic acid it forms another compound Q260, having a maximum absorbance at 260nm (Qian et al. 2005). However in the present study absorption of both derivatised and underivatised artemisinin sample at 292nm and 260 nm, indicates presence of both Q292 and Q260 metabolites. The derivatized artemisinin samples showed consistent increase in the Q260 corresponding to increase in concentration. However even derivatized sample showed some absorbance at other two wavelengths indicating some amount of pure artemisinin remaining underivatized at either NaOH or Acetic acid stage. Ethanolic leaf extracts also showed absorption at all the three wavelengths that are 216, 292 and 260 nm, indicating presence of all three, with maximum absorption at 216 suggesting artemisinin to be present in highest concentration. Obvious amount of artemisinin in leaf extract was less than that in pure artemisinin, however still the ethanolic leaf extract offered more inhibition to germination and radicle growth of Chloris as compared to pure artemisinin alone. This suggests that the metabolite responsible for allelopathic effect of *Artemisia annua* L. can not only be artemisinin but it could be

synergistic and additive effect of two or more compounds. Also both plant extracts and pure artemisinin were found to absorb at Q260 and Q292 indicating possibilities of these derivatives to be allelopathic.

HPLC analysis was performed purely for qualitative purpose. Number of major peaks in the HPLC chromatograms of Harborne's fraction A and ethanolic leaf extract were same. The HPLC analysis also confirms presence of three metabolites absorbing, at two different but nearly close wavelengths, 210 nm and 216 nm. One of the metabolite in both, the Harborne's fraction A and ethanol extract is artemisinin (absorption maxima around 216nm), as the spectral pattern resembles that in the HPLC chromatogram of pure artemisinin.

The presence of artemisinin in Harborne's fraction A and ethanolic leaf extract and inhibitory effects of both along with the artemisinin and its analogues, suggest that artemisinin alone can inhibit the seed germination and seedling growth in neutral medium like agar, but the enhanced inhibitory effect imposed by Aa extracts strongly point out that the growth reduction effect of artemisinin is potentiated when it is coupled with other compounds. It also emphasizes that artemisinin being the potent antimalarial drug source can be preserved for that purpose and other compounds of Artemisia can be used as bioherbicide. The dose response comparison between plant treatment and pure artemisinin also support the view that when artemisinin is working along with other compounds its effect increase.

4.4.4. Metabolite absorbance: As the artemisinin was inhibitory to the Chloris, in metabolite absorption experiment, it was hypothesized that the Chloris seedling manifests if any result in-terms of reduced growth as compared to control, then probably the seedlings should absorb metabolite from the donor plant. For this

purpose the extracts of artemisinin fed Chloris and Chloris in the control were analysed by HPLC. The HPLC chromatograms were compared to study the possible allelopathic compound absorbed by treated Chloris seedlings, if at all it is artemisinin. However with the present method no artemisinin was found in artemisinin treated seedlings. There may be two reasons for the observed result, 1) compound other than artemisinin were absorbed and reduced the seedling growth (as seen in Figure 3.43 b) or after absorption artemisinin is converted into some other related compound. This needs to be studied in detail.

The results from soil bioassay study suggest that allelopathic effect of Artemisia in soil is consistent with that in agar and that the Harborne's fraction A and the leaf mulch must also be standardized before practically being employed in the agricultural field. The estimated allelopathic effect was found to be more with matrix of compounds like Harborne's fraction, leaf mulch and ethanolic leaf extracts as compared to pure artemisinin.

4.5. Soil bioassay for *Enicostemma littorale* Blume: The plant exhibited high allelopathic potential in the agar based bioassay's. To assess the consistency and application value of phytotoxicity exhibited by El, observed in the agar based bioassay, effective treatments from the plants were analysed for their efficacy in soil. The El leaf was the most allelopathic part and thus possible allelopathic potential of the leaf mulch was compared with that of its isolated chemical fraction. The most allelopathic fraction that is fraction B, a relatively much less phytotoxic fraction, that is fraction A and the plant leaf mulch were used for imparting treatment to Chloris seeds. The phytotoxic effect was evaluated on the parameters such as speed of germination and total germination of the soil grown Chloris. All the selected treatments were applied at the three different rates each. Both the selected parameters

were most affected by the plant leaf mulch treatment followed by the fraction B treatment, applied at the same rates. Whereas it was least affected by fraction A treatment. It was observed that fraction B though, was expected to be more concentrated in terms of extracted metabolites it was relatively less allelopathic than the leaf mulch. Although the inhibitory effect of El fraction B was consistent in both agar and soil where in the effect was relatively more pronounced in soil.

4.6. Rhizosphere Analysis: *Asparagus racemosus* Willd. and *Chlorophytum borivilianum* San. and Fern, were the two medicinal plants, where in plant roots were the found to exhibit allelopathic potential. According to Weston et al. (2012), the allelochemicals and other metabolites that are released by plant roots play important roles in rhizosphere signalling, plant own defence and other responses to different abiotic stresses. Considering its significance and application, number of studies have been conducted so far for the rhizosphere analysis pertaining to plant allelopathy (Perez et al. 1991; Yoshitomi and Shann 2001; An et al. 2001; Batish et al. 2006; Cui et al. 2012; Jandova et al. 2014), however the studies intending the analysis of soil grown live plant's rhizosphere, are relatively less. Thus to study the release of metabolites from the plant and to observe temporal and spatial variation in the spread of the them, the present study was conducted. According to Weidenhamer (2005) PDMS material can be used to sorb and measure root exuded allelochemicals in the undisturbed rhizosphere of living plants. In an experiment Weidenhamer et al. (2009) have studied the thiophene dynamics in the root zone of *Tagetes patula* and they found the distribution of the same to be spatially and temporally heterogeneous. Employing the PDMS probes an attempt was made to study the spatial and temporal dynamics of compound released from the allelopathic plant roots. However, the experiment also intended to study whether or not the metabolites that were presumed

to be allelopathic in the bioassays conducted in the present study, are leached by the plant roots or not. Simultaneously the data was also analysed for the flux of number of metabolites found for a reading taken in the given duration of time. The experiment was designed by placing PDMS probes in the soil, at a finite distance from the plant axis. The PDMS probes are known to have affinity towards non polar compounds (Weidenhamer et al. 2009), thus for the polar compounds, soil samples from the respective probe insertion sites were also collected, extracted similar to the probes. Subsequently, soil extracts containing polar metabolites were combined with the respective probe extracts to form a single methanol extract directly used for HPLC analysis. Thus the extracts were expected to possess both polar (soil extracts) and non polar (PDMS extracts) metabolites. The experiments were solely meant for qualitative purpose and no quantitation of metabolites was done. In case of both the plants, the plant root metabolites were observed to travel away from the plant axis in to its rhizosphere.

4.6.1. *Asparagus racemosus* Willd. (Ar): Comparing the ArRE (Ar Root Extract) and ArRW (Ar Root Washing), of the six metabolites (compound 1, 2, 3, 4, 5 and 6) found in root extract, three (compound 4, 5, 6) were also detected in the root washing (Figure 3.48). This suggests that the metabolites are leached to the surface of the roots and this increases the possibilities of their movement to the area around root rhizosphere and subsequently exhibit allelopathy on the neighbouring plants.

Assessing the spatial dynamics of Ar metabolites it was observed that their release and movement from the pant must be highly ambiguous in terms of distance travelled from the plant axis. Considering the first reading of rhizosphere analysis, for the three plants of Ar, it was observed that the probes extracted for the distance of 5.0 cm had one or two metabolites less than the ones kept at a distance of 2.5 cm. In the second reading,

two of the three Ar plants, that is Ar22 and Ar23, extracts at both the distance showed same number of metabolites however only for Ar21 it was observed that number of metabolite was more for the extract Ar212 (representing 5.0 cm) and Ar211 (representing 2.5 cm). In the third reading two of the three Ar plants viz. Ar31 and Ar32, the number of metabolites were more for the extracts representing distance of 7.5 cm as compared to that at distance of 2.5 and 5.0 cm, indicating long distance movement of metabolites. However only for Ar33 it was observed that number of metabolite was more at 5.0 cm then at 7.5 cm suggesting that partial movement of metabolites. More number of metabolites at further distances as compared to the nearer once suggests relatively faster and long distance movement of the plant root metabolites and that more number of metabolites could travel long distance. Whereas less number of the metabolites at farther distances as compared to the near once indicates presence of metabolites (in the near distance) that has yet to travel further distance from the plant.

All the three readings (taken at interval of 10, 20 and 30 days) for the three Ar plants, were compared and a great temporal difference in metabolites persistence was observed. For a given plant, data for the considered distances were combined (for example, of six root metabolite, their presence in any of the Ar11, Ar12 or Ar13 was accounted). For Ar plant one (Ar11), for the first reading taken after ten days all the six root metabolites were observed, after twenty days the Ar root metabolites 3, 4, 5 and 6 were observed and after thirty days the Ar root metabolite 1, 3, 4, 5 and 6 were observed. Considering Ar plant two, for first reading taken after ten days metabolite 5 and 6 were detected, after twenty days Ar metabolite 1, 3, 4, 5 and 6 were observed and after thirty days Ar metabolite 2, 3, 4, 5 and 6 were obtained. For Ar plant three, after ten days Ar root metabolite 1, 4 and 6, after twenty days Ar root metabolite 3, 5 and 6 and after thirty days Ar root metabolite 2 and 6 were detected. This suggests the metabolite flux in the Ar rhizosphere is heterogenous.

Considering the Ar rhizosphere results, it was inferred that the release and travel of metabolites from Ar roots in to its rhizosphere is highly unpredictable and must be homogenous in some case while heterogeneous in others.

It was observed that the metabolite 6 from Ar root having retention time of 17.78 minutes was the only root metabolite present in root washing and also in all of the Ar samples which strongly suggest occurrence of the metabolite in Ar rhizosphere at different distance and time.

From the results of Harborne's fractionation bioassay's, fraction B from Ar was the most allelopathic in nature and planar chromatographic analysis of the same gave positive results for the presence of alkaloids. Inferring the same, all the chromatograms were extracted at 254 nm, a wavelength specific to alkaloidal metabolite detection. Hence the various peaks observed in the Ar chromatograms may be of alkaloids. However in the HPLC analysis of the same direct methanolic extracts were used, without subjecting them to any process as is followed in case of alkaloid isolation. Thus the metabolite separated in the HPLC chromatograms may be alkaloid or even some of the Steroidal saponins (Hayes et al. 2008; Kumeta et al. 2013) that are already reported in the root.

Also the plant is reported to have replanting problems and the auto-toxicity exhibited by the plants has been attributed to the organic acid released by roots in the exudates (Yeasmin et al. 2013). In the present study the rhizosphere analysis of *Asparagus racemosus* Willd. suggest that the plant roots releases metabolites, absorbance of which is in UV range and release of this compounds by roots is heterogeneous across both, the space and the time.

Results also indicate possibility of alkaloids present in roots being allelopathic in nature and this enhances the practical value of *Asparagus racemosus* Willd. roots where in the plant saponins can be used for the medicinal purpose and other compounds for herbicidal use.

4.6.2. *Chlorophytum borivilianum* San. and Fern (Cb): Plant root extract showed presence of four metabolites (compound 1, 2, 3, 4) (Figure 3.49). The plant root washing showed presence of five metabolites one of which was coinciding with the root metabolite 2. This indicates exudation of root metabolite to the root surface which increases the possibilities for release of compounds from the Cb roots, followed by their subsequent movement in the rhizosphere and ultimately exhibit allelopathy on the plants growing in vicinity.

Analysing spatial dynamics of the metabolites released from Cb roots it was observed that the movement of metabolites is extremely heterogeneous. For the first reading it was observed that for the Cb plants viz. Cb12 and Cb13, the number of metabolites were more at distance 5.0 cm then that at a distance of 2.5 cm, where as in case of Cb11, reverse was the case, i.e. number of metabolite was more at distance of 2.5 cm then that at distance of 5.0 cm. In the second reading it was observed that for the Cb plants viz. Cb21 and Cb23 number of metabolites at distance 2.5 cm were more than that at distance 5.0 cm from the plant axis, where as in case of Cb22, number of metabolites was less at 2.5 cm as compared to those at 5.0 cm. For the third reading, it was observed that in all the three Cb plants that number of metabolites at the nearer distance were either more or equal as compared to the farther distance. More number of metabolites at nearer distances as compared to the farther once suggests relatively slower movement of the plant root metabolites and that more number of metabolites are yet to move away from the plant axis.

All the three readings (taken at interval of 10, 20 and 30 days) for the Chlorophytum plants, were compared and a great temporal difference in metabolites persistence was observed. For a given plant, data for the considered distances were combined (for example, of six root metabolite, their presence in any of the Cb11, Cb12 or Cb13 was accounted). For the first reading taken after ten days, in first plant, root metabolite 1 and 2 were observed, for the reading taken after twenty days root metabolite 1, 2 and 3 were observed and after thirty days also root metabolite 1, 2 and 3 were observed. For the Cb plant two, for reading taken after ten days root metabolite was observed and for reading taken after two and three days root metabolite 1, 2 and 3 were observed in both. For Cb plant three, after ten days root metabolite 1, 2, 3, 4 were observed, after twenty days root metabolite 1, 2 and 3 were observed and after thirty days root metabolite 1 and 2 were observed.

Considering the Cb rhizosphere results, it was observed that the plant roots do release the metabolites into its rhizosphere however the release and movement of metabolites from the Cb roots into its rhizosphere must be homogenous or heterogeneous.

Cb root metabolite 2 eluting at 10.78 minutes was present in the Cb rhizosphere at almost all the distance and in the reading taken for all time intervals. The metabolites found in the Cb extracts must be terpenoidal in nature, as they were found to absorb at 210 nm. The plant is already reported to have number of steroidal saponins (Deore and Khadabadi 2010; Joshi et al. 2013). However in the present study the results from bioassay shows that Harborne's fraction A from Cb was the most allelopathic in nature and chromatographic analysis of fraction A showed presence of both phenolic and terpenoid compound in the same. Thus the metabolites found in Cb rhizosphere must be many of the reported steroidal saponins or some

phenolics present in the plant. This increases the chance of exploring allelopathic potential of Cb. The results of the present study indicate that *Chlorophytum borivilianum* San. and Fern. roots releases metabolites that move away from root in the CB rhizosphere and are terpenoidal or phenolic in nature.

Advance analysis (such as LCMS, MSMS/ NMR) and quantification of the metabolites for both *Asparagus racemosus* Willd. and *Chlorophytum borivilianum* San. and Fern, may aid better understanding of the metabolites and related processes such as degradation and persistence of metabolites happening in the plants rhizosphere. Weidenhamer et al. (2009) suggest that probes are inexpensive, low cost, easy made, suitable for large-scale sampling and disposable. Also the use of such probes have associated limitation, better experimental designs may work miracle. Such studies if efficiently utilized can be of greater use to address the problems such as exotic plant invasions, applications of allelopathic plants in agriculture field etc. Such probes may be utilized to study the real time concentration of allelochemicals in the soil at given point of time in addition to study the spatial and temporal dynamics of allelochemicals. Development and establishment of proper agricultural modal, this tendency to release the metabolites may be utilized by means of companion cropping or intercropping the *Asparagus racemosus* Willd. with the suitable crop species where by ecofriendly and low cost weed mitigation can be achieved.