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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation
ALLELOPATHY OF FIVE LAMIACEAE MEDICINAL PLANT SPECIES
(5 種のシソ科薬用植物のアレロパシー)

学位論文要約：
Dissertation Summary

Weed management is an important and challenging task in sustainable agriculture as weeds cause substantial loss in crop yields and quality (Davies and Welsch 2002). On an average approximately 9.7% of total crop yields are lost every year by the effects of 1800 kind of weeds worldwide (Li et al. 2003). Since the introduction of first commercial herbicide 2, 4–D in 1940s the farmers of different agricultural countries use thousand tonnes of herbicide per year in order to control weeds. Farmers intention to rely on synthetic herbicides for weed control, perhaps due to their easy accessibility and more rapid out return. Over reliance on synthetic herbicides may develop herbicide-resistant weed biotypes and also creates severe environmental hazards. Therefore, for the sake of pollution free earth ecosystem and sustainability in crop production, conventional agriculture has to be improved by reducing the use of synthetic herbicides.

The word 'allelopathy' derived from two Greek words *allelon*, 'of each other' and *pathos* 'to suffer'; hence, it means the adverse effect of one plant species upon another. Hans Molisch, an Austrian plant physiologist, in 1937 first coined the term 'allelopathy'. On the basis of Molisch's concept Rice (1984) defined allelopathy 'as any direct or indirect harmful effect by one plant (including microorganisms) on another through production of chemical compounds/substances that escape into the environment'. Allelopathic substances are released into the surrounding environment through volatilization from the leaves (Oleszek 1987), leaching from the above ground parts by precipitation (Overland 1966), decomposition of leaf litter or sloughed root tissues (Hedge and Miller 1990), microbial transformation from the decayed plant organs (Chick and Kielbaso 1998), through root exudates (Tang and Young 1982), from pollen of some crop plants (Cruz-Ortega et al. 1988), or other processes (Figure 1). These substances upon release, may suppress the germination, growth and establishment of neighboring native plants, even the secreting plant itself either directly by affecting their physiological properties (Weir et al. 2004; Yu et al. 2003), or indirectly by modifying the rhizosphere soil properties through influencing the microbial biomass carbon and microbial community (Xingjun et al. 2005; Zhou et al. 2013). Because of the growth suppressing potential of allelopathic plants, they are suggested as a viable option for alternatives of weed management under sustainable agriculture (Fujii 2001; Macías et al. 2007). Substantial number of reports has been documented in the literature about the successful use of allelopathic plants, their extracts/residues, or the allelopathic substances into the crop fields as a substitute of synthetic herbicides to control weeds. Emphasis is given on allelopathic medicinal plants because of their possibility to contain more bioactive compounds than other plant species.

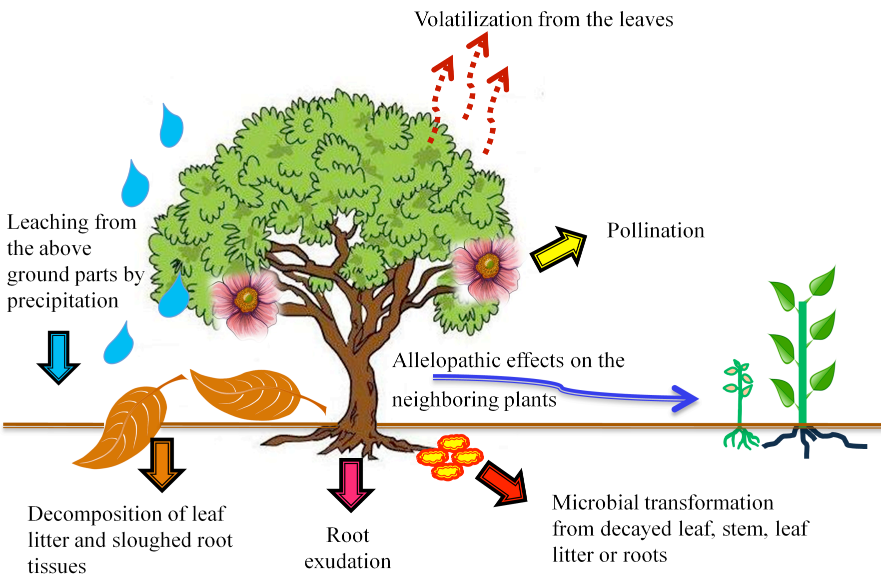

Figure 1. Possible routes of entry of allelopathic substances to the surrounding environment from the allelopathic plants (modified from Chick and Kielbaso 1998)

Lamiaceae, a large dicotyledonous family belongs to the Angiosperm order Tubiflorae (Rendle 1959). The family also designated as Labiatae or mint family, and comprising at least 3500 species in about 180 genera (Lovett and Weerakoon 1983). There are about 175 species of 45 genera of that family are considered as weeds in different parts of the world (Holm et al. 1979). The plants of that family mostly attracted the attention of many researchers in pharmacological interest because of their toxic potential and medicinal properties. Although the first allelopathic report of Lamiaceae plant species is published about four hundreds years ago (Culpeper 1633), only few investigations have been done to date to explore their allelopathic potential. Moreover, most of the allelopathic research of this family concentrated on one or two genera particularly *Salvia*, and on their preliminary phytotoxic bioassay studies. A vast majority of the allelopathic activities of Lamiaceae plants species particularly their allelopathic substances are remain unknown. Therefore, current research has been undertaken to explore the allelopathic potential of five Lamiaceae medicinal plant species (Figure 2): *Leucas aspera* (Willd.) Link., *Hyptis suaveolens* (L.) Poit., *Mentha sylvestris* L., *Leonurus sibiricus* L. and *Ocimum tenuiflorum* L., and further isolation and characterization of active allelopathic substances from the plant species that possess strong allelopathic properties.

Figure 2. Five Lamiaceae medicinal plant species used in this research

The whole parts (leaves, stems and roots) of these five Lamiaceae medicinal plant species were collected from Bangladesh, and extracted separately with 70% (v/v) aqueous methanol. An aliquot of the extract of each plant materials was then evaporated to dryness at 40 °C and dissolved in methanol to prepare four different extract concentrations of 3, 10, 30 and 100 mg dry weight [DW] equivalent extract mL^{-1} for each plant species. Eight test plant species; cress (*Lepidum sativum* L.), lettuce (*Lactuca sativa* L.), alfalfa (*Medicago sativa* L.), rapeseed (*Brassica napus L.*), timothy (*Phleum pratense* L.), crabgrass (*Digitaria sanguinalis* L. Scop.), barnyard grass (*Echinochloa crus-galli* L.) and Italian ryegrass (*Lolium multiflorum* Lam.) were used in the present research to determine the biological activity of five Lamiaceae medicinal plants. Among these eight test species, the first four are dicotyledonous and the rest are monocotyledonous. Cress, lettuce, alfalfa, rapeseed and timothy were chosen due to their known seedling growth, whereas crabgrass, barnyard grass and Italian ryegrass were chosen because they are most common weeds in the crop fields and distributed throughout the world.

As germination bioassay is the most widely used method to examine the allelopathic activity (Putnam and Tang 1986; Rice 1984), the allelopathic potential of *L. aspera*, *H. suaveolens*, *M. sylvestris* and *L. sibiricus* were determined by the total germination percent (GP) of cress and barnyard grass. Whereas, that of *O. tenuiflorum* plant extract was evaluated by GP, germination index (GI), germination energy (GE), speed of emergence (SoE), seedling vigour index (SVI), coefficient of the rate of germination (CRG), time required for 50% germination (T_{50}) and mean germination time (MGT) of cress, lettuce, alfalfa, timothy, barnyard grass and Italian ryegrass.

L. aspera, *H. suaveolens*, *M. sylvestris* and *L. sibiricus* plant extracts inhibited and/or delayed the germination of both cress and barnyard grass at 100 mg DW equivalent extract mL^{-1} except barnyard grass by *L. sibiricus* plant extracts. Furthermore, *L. aspera* and *M. sylvestris* plant extracts completely inhibited the germination of cress at the same concentration. The inhibition of the plant extracts was more prominent on cress than barnyard grass. At concentrations greater than 30 mg DW equivalent extract mL^{-1} *O. tenuiflorum* reduced significantly the GP, GI, GE, SoE, SVI and CRG of all test species except barnyard grass and GP of lettuce. In contrast, T_{50} and MGT were increased at the same or higher than this concentration. The increasing trend of T_{50} and MGT, and the decreasing trend of GP, GI, GE, SoE, SVI and CRG indicated a significant inhibition or delay of germination of the test species by *O. tenuiflorum* plant extracts, and vice-versa.

Even though, germination bioassay is widely used method, early seedling growth is reported to be most sensitive parameter to test the allelopathic activity (Gong et al. 2001; Wardle et al. 1991). Therefore, present research mainly focuses on the growth inhibitory potential of those plants extracts against cress, lettuce, alfalfa, rapeseed, timothy, crabgrass, barnyard grass and Italian ryegrass at four different extract concentrations as mentioned earlier.

The aqueous methanol extracts of *L. aspera* and *H. suaveolens* significantly inhibited the hypocotyl/coleoptile and root growth of all test plant species at or greater than 10 mg DW equivalent extract mL^{-1} except Italian ryegrass and alfalfa. On the other hand, *M. sylvestris, L. sibiricus* and *O. tenuiflorum* extracts significantly inhibited the hypocotyl/coleoptile and root growth of all test species at 100 mg DW equivalent extract mL^{-1}. The concentrations lower than the thresholds for inhibition have tendency to stimulate the hypocotyl/coleoptile and root growth. The inhibitory effects of all extracts were concentration and test plant species dependent. The total average inhibitions on the hypocotyl/coleoptile growth of all test plant species by *L. aspera, H. suaveolens, M. sylvestris, L. sibiricus* and *O. tenuiflorum* were 46, 39, 15, 20 and 17%, respectively (Figure 3). However, that of the root growth was 67, 53, 42, 32 and 30%, respectively (Figure 3). The average concentrations required for 50% hypocotyl/coleoptile growth inhibition (I_{50}) of the test species by *L. aspera*, *H. suaveolens*, *M. sylvestris*, *L. sibiricus* and *O. tenuiflorum* were 22, 33, 58, 91 and 70 mg DW equivalent extract mL^{-1}, respectively (Figure 4). Whereas that for the root growth of the test species was 8, 13, 27, 46 and 55 mg DW equivalent extract mL^{-1}, respectively (Figure 4). The lowest the I_{50} values, the highest the sensitivity of the test species to that plant extracts. The root growth of all test plants was more sensitive to five Lamiaceae plant extracts than the hypocotyl/coleoptile growth. The inhibitory potentials of five Lamiaceae medicinal plant species were in the order of *L. aspera* > *H. suaveolens* > *M. sylvestris* > *L. sibiricus* > *O. tenuiflorum*.

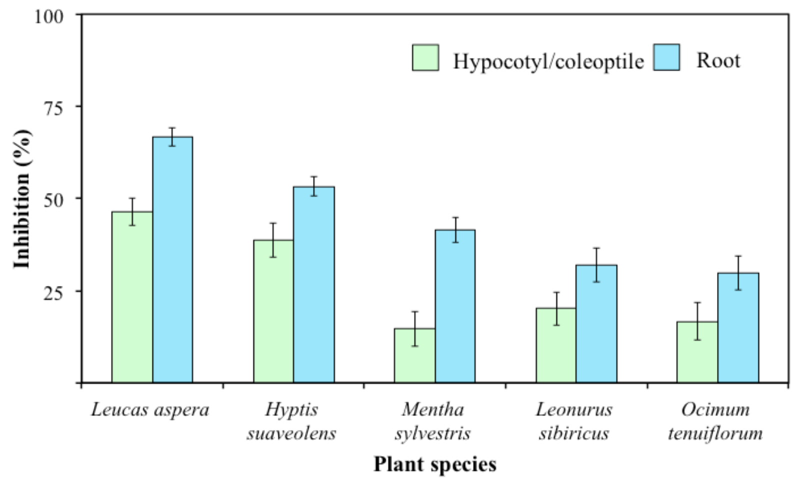

Figure 3. Overall inhibition percent of hypocotyl/coleoptile and root growth of eight test plant species by five Lamiaceae medicinal plant species

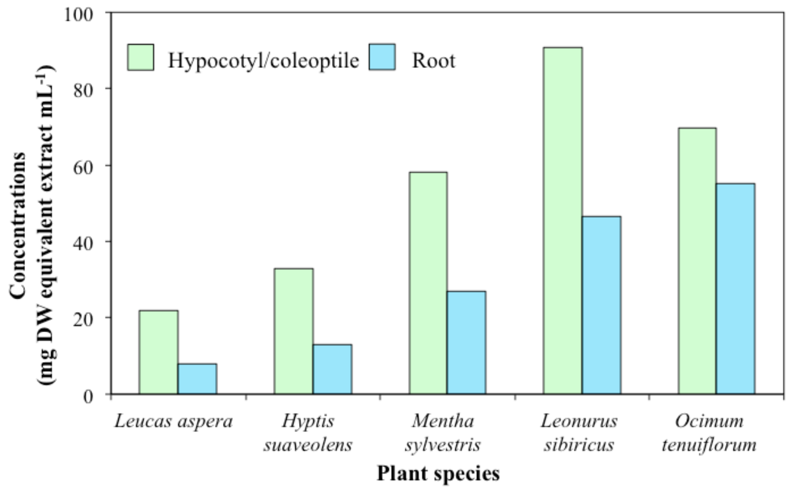

Figure 4. Average concentration required for 50% growth inhibition (I_{50}) of the eight test plant species by five Lamiaceae medicinal plant species. The values were determined by a logistic regression analysis after bioassays.

In the present research, seedling growth was observed as more sensitive to the five Lamiaceae medicinal plants extract than seed germination. The higher sensitivity of early seedling growth to allelopathic plant extracts than germination could be due to: (i) the presence of seed coat which act as a barrier in between the embryo and its surrounding environment (Araùjo and Monteiro 2005); (ii) the selective permeability of seed coat (Wierzbicka and Obidzińska 1998) which may protect the inhibitory activity of allelopathic extract/substances if they can not pass through seed coat, and (iii) the parameter that was used to measure germination (the protrusion of the root through the seed coat which does not necessarily mean growth by cell division) etc. (Salvatore et al. 2008).

The growth inhibition of the test plant species, in presence of allelopathic extracts/substances could be for the reason of lower cell division, elongation and expansion rate which are growth pre-requisites (Cruz-Ortega et al. 1988; Einhellig 1996). The chemical agents that inhibit cell division can act in two ways: (i) by affecting the synthesis or the structure of DNA-RNA, and (ii) by inhibiting the energy production necessary for the process of mitosis (Kilhman 1966). Both processes are important for cell division, and interferences with them generally cause inhibition of the whole process. Moreover, allelopathic substances inhibit the respiration (Inderjit and Keating 1999), ion absorption process (Qasem and Hill 1989), enzyme activity (Sato et al. 1982), plant endogenous hormones and protein synthesis (Jacob and Sarada 2012), alteration of the phytochrome control of germination (Leather and Einhellig 1988) and thus, results in arrested plant growth (dos Santosh et al. 2004). Allelopathic substances may produce more than one effect of the above on the cellular processes that are responsible for reduced plant growth. However, the details of the biochemical mechanism through which allelopathic substances exert a toxic effect on the growth of plants are still not well known (Zhou and Yu 2006).

The prominent root growth inhibition over hypocotyls/coleoptiles could be due to: (i) the more intensive contact in between the roots and plant extracts and subsequently with allelopathic substances (Tefera 2002), (ii) roots; which are the first organ to confront with allelopathic substances from the environment (Turk and Tawaha 2002), (iii) the reduced rate of cell division in presence of allelopathic substances, which might inhibit gibberellin and/or indoleacetic acid function (Tomaszewski and Thimann 1966), (iv) the lower mitotic division in root apex in presence of allelopathic substances (Levizou et al. 2002), or (v) the hypocotyls/coleoptiles growth of seedling that is largely depends on cell expansion (which is relatively insensitive to the allelopathic substances), whereas root growth requires not only cell expansion but also cell proliferation (which is sensitive to the allelopathic substances), and thus exerts higher root growth inhibition than the hypocotyls/coleoptiles (Nishida et al. 2005). Because of the higher sensitivity of roots, they are considered as the main parameters to assess allelopathic effects of any plant extracts or compounds on target species (Inderjit and Duke 2003). Based on the above discussion it may conclude that the plant growth inhibitory activities of all five Lamiaceae medicinal plants on the germination and seedling growth of the test plant species could be due to their allelopathic properties. Therefore, they may contain allelopathic substances to inhibit the germination and growth of other species. As *L. aspera* and *H. suaveolens* have higher allelopathic potential than others, these two plant materials were used for further isolation and identification of allelopathic substances. The extracts of *L. aspera* or *H. suaveolens* were then divided into two equal parts, and each part was adjusted to pH 7.0 with 1M phosphate buffer and partitioned three times against an equal volume of ethyl acetate to yield aqueous and ethyl acetate fractions. As the inhibitory activity of ethyl acetate fractions of *L. aspera* or *H. suaveolens* was greater than that of the aqueous fraction, the purification process was further continued with the ethyl acetate fraction. This fractions of *L. aspera* or *H. suaveolens* were then purified by silica gel column, Sephadex LH 20 column and C_{18} cartridges through bioassay guided fractionation using garden cress as a test plant. The final purification was achieved by reversed-phase HPLC.

An equilibrium (or inseparable) 3:2 mixture of two novel labdane type diterpenes Compounds **1** and Compound **2** have been isolated and characterized from the aqueous methanol extract of *L. aspera* through spectroscopic analyses. These two compounds were unable to separate, even though different solvent compositions and a number of columns have been used. This may either due to their co-existence in nature as equilibrium, or their fast inter-conversion reactions. A mixture of these two compounds inhibits the germination of both cress and barnyard grass at concentrations greater than 30 μM, whereas that of seedling growth was at concentrations greater than 3 μM. The I_{50} values of these two compounds mixture for the seedling growth of cress and barnyard grass were ranged from 31–80 μM, which suggests that the mixture of these compounds were responsible for the allelopathic activity of *L. aspera* plant extracts. On the other hand, a growth inhibitory substance was isolated and identified from the *H. suaveolens* plant extracts by high-resolution ESI-MS, ^{1}H-, ^{13}C- NMR, CD and specific rotation. The isolated compound inhibited the hypocotyl/coleoptile growth of cress, lettuce, Italian ryegrass and barnyard grass with different inhibition values.

The results of this research suggest that *L. aspera* and *H. suaveolens* plant extracts and their isolated substances possess strong allelopathic potential against several weed species including most noxious barnyard grass. Therefore, both plants could be introduced in alternative weed management strategies through application of their crude extracts or their residues as green manure/mulch, or their isolated substances as natural herbicides or templates of new herbicides classes. The application of plants extracts/residues may provides dual benefits to the farmers; (i) environment friendly bio-herbicides for weed control, and (ii) organic matter to improve the soil properties. Although the amount, concentration, and environmental conditions are important factors determining the effectiveness of any substances under field settings, our results may be helpful for large-scale production of these compounds or their synthetic analogs to develop natural product based herbicides for weed control.

Allelopathic activity is considered as one of the important mechanism of plant dominance. It has been reported that *L. aspera* and *H. suaveolens* forms dense thickets and suppress the growth of other neighboring species. To some extent, such type of bio-invasion may cause the loss of indigenous biodiversity. The compounds isolated from *L. aspera* and *H. suaveolens* may play an important role in their interactions with other neighboring plants under natural settings. A deeper knowledge of their underlying releasing mechanisms into the environment, and their interaction with neighboring plants should necessary to raise new control mechanisms and to avoid the loss of indigenous biodiversity. Therefore, future investigations should be directed to understand their releasing mechanisms in environment, and their interactions with other neighboring plant species.

The bioassay results after each chromatography of both *L. aspera* and *H. suaveolens* plant extracts showed the presence of some other active fractions. However, the present research only focuses on the most active fraction, and subsequently isolated and identified those allelopathic substances. There have some possibility to identify some other promising allelopathic substances from other active fractions. As allelopathic activity of any plants is the synergistic effects of many bioactive compounds, further investigation with those fractions to purify the allelopathic substances, and their contribution with our isolated substances on the total allelopathic activity of respective plants is crucial.

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