DEVELOPMENT OF NATURAL HERBICIDE FROM *TAGETES ERECTA* L. AND ITS MODE OF ACTION

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หัวข้อวิทยานิพนธ์	การพัฒนาสารธรรมชาติกำจัดวัชพืชจากดาวเรือง และกลไกการออกฤทธิ์ของสาร
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บทคัดย่อ

การศึกษาครั้งนี้เพื่อศึกษาศักยภาพทางอัลลีโลพาทีของสารสกัดน้ำจากส่วนใบ ลำต้น ดอก และรากของดาวเรือง (*Tagetes erecta* L.) ที่มีผลต่อการงอกและการเจริญเติบโตของหญ้าข้าวนก (*Echinochloa crus-galli* (L.) Beauv.) และถั่วผี (*Phaseolus lathyroides* L.) ผลการทดลองพบว่า สารสกัดน้ำจากส่วนใบสามารถยับยั้งการงอกและการเจริญเติบโตของพืชทดสอบทั้งสองชนิดได้ดีกว่าสารสกัดจากดอก ลำต้น และราก ตามลำดับ การศึกษาประสิทธิภาพของสารสกัดหยาบเอทานอลต่อน้ำ ในอัตราส่วนที่แตกต่างกัน (100, 75, 50, 25 และ 0 เปอร์เซ็นต์) ปริมาณของสารสกัดหยาบที่ได้ขึ้นอยู่กับตัวทำละลาย อัตราส่วนเอทานอลต่อน้ำที่ 75:25 สามารถสกัดให้สารสกัดหยาบมากที่สุด เมื่อทำการแยกกลุ่มสารออกฤทธิ์จากใบดาวเรืองโดยวิธี acid-base solvent partitioning ได้สารสกัดหยาบ 4 กลุ่ม คือ hydrolyzed (HY), aqueous (AQ), neutral (NE) and acidic (AE) พบว่าสารสกัดหยาบ ในกลุ่ม HY และ AE มีประสิทธิภาพดีที่สุด โดยสามารถยับยั้งการงอก ความยาวต้นและความยาวรากของพืชทดสอบได้โดยสมบูรณ์

จากนั้นทดสอบหาปริมาณฟีนอลิคทั้งหมดและปริมาณฟลาโวนอยด์ทั้งหมด สารสกัดหยาบในกลุ่ม HY และ AE ยับยั้งการงอกและการเจริญเติบโตมากที่สุด และพบว่าสารสกัดหยาบในกลุ่ม HY และ AE มีปริมาณฟีนอลิคและฟลาโวนอยด์สูงกว่าสารสกัดหยาบใน NE, OR และ AQ ทำการแปรรูปสารในกลุ่ม HY ให้อยู่ในรูปสารธรรมชาติกำจัดวัชพืชรูปแบบสารละลายเข้มข้น (soluble concentrate; SCT) พบว่า สารธรรมชาติกำจัดวัชพืชจากดาวเรืองมีประสิทธิภาพในการยับยั้งการงอกและการเจริญเติบโตของหญ้าข้าวนกและถั่วผี การศึกษาผลของการดูดน้ำ และการยับยั้งกิจกรรมเอนไซม์อัลฟาอะไมเลส ในเมล็ดหญ้าข้าวนก และผักโขม (*Amaranthus viridis* L.) พบว่าเปอร์เซ็นต์การดูดน้ำและกิจกรรมเอนไซม์อัลฟาอะไมเลสจะเพิ่มขึ้นเมื่อระยะเวลาการแช่สารเพิ่มขึ้น และจะลดลงในขณะที่ระดับความเข้มข้นของผลิตภัณฑ์จากดาวเรืองเพิ่มสูงขึ้น การทดสอบสารธรรมชาติกำจัดวัชพืชจากดาวเรืองต่อการแบ่งเซลล์ในปลายรากหอมหัวใหญ่ พบว่า มีค่าดัชนี

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การแบ่งเซลล์ลดลง ในขณะที่เซลล์ที่เข้าสู่ระยะเมทาเฟส แอนาเฟส และเทโลเฟสลดลง เกิดลักษณะการขดตัวของโครมาตินในระยะโพรเฟสผิดปกติ เนื่องจากความผิดปกติของสายสปินเดิล การขดตัวกันแน่นของโครโมโซมในระยะเมทาเฟสและแอนาเฟส โครโมโซมไม่จัดเรียงตัวบริเวณกลางเซลล์ในระยะเมทาเฟส จากการทดสอบสารธรรมชาติกำจัดวัชพืชจากดาวเรืองที่อัตรา 10, 20, 40 และ 80 กิโลกรัม(สารออกฤทธิ์)ต่อเฮกตาร์โดยการฉีดพ่นสารผ่านทางใบของหญ้าข้าวนกและถั่วผีหลังได้รับเป็นเวลา 1, 3, 5 และ 7 วัน ทำการตรวจวัดดัชนีความเสถียรภาพของเยื่อหุ้มเมมเบรน ปริมาณรงควัตถุที่เกี่ยวข้องกับการสังเคราะห์แสง (คลอโรฟิลล์ เอ คลอโรฟิลล์ บี และแคโรทีนอยด์) การเกิดปฏิกิริยาลิปิดเปอร์ออกซิเดชันโดยการวิเคราะห์หาปริมาณมาลอนไดอัลดีไฮด์ (malondialdehyde) พบว่าผลิตภัณฑ์จากดาวเรืองที่อัตราสูงสุดและที่ 7 วันหลังได้รับสารใบของหญ้าข้าวนกและถั่วผี มีดัชนีความเสถียรภาพของเยื่อหุ้มเมมเบรน และปริมาณรงควัตถุที่เกี่ยวข้องกับการสังเคราะห์แสงลดต่ำลง และมีปริมาณมาลอนไดอัลดีไฮด์เพิ่มขึ้น

การวิเคราะห์หาองค์ประกอบทางเคมีของน้ำมันหอมระเหยจากใบดาวเรือง โดยใช้วิธี Gas Chromatograph-Mass Spectrometry (GC-MS) มีสารประกอบที่สำคัญได้แก่ piperitone 57, neophytadiene 68, palmitic acid 67, caryophyllene 58 และ 9,12,15-octadecatrien-1-ol 89 นำน้ำมันหอมระเหยจากใบดาวเรืองมาแปรรูปเป็นสารละลายน้ำมัน (emulsifier concentrate) มีประสิทธิภาพในการยับยั้งการงอกของหญ้าข้าวนก และส่งผลให้กิจกรรมเอนไซม์อัลฟาอะไมเลสลดลง

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Thesis	Development of natural herbicide from *Tagetes erecta* L. and its mode of action
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ABSTRACT

The aim of this study was to investigate the allelopathic effects of stem, leaf, flower and root aqueous extracts of *Tagetes erecta* L. on seed germination and seedling growth of *Echinochloa crus-galli* (L.) Beauv. and *Phaseolus lathyroides* L. It was found that leaf extracts had the greatest inhibitory effect. To optimize extraction from *T. erecta* leaves, five different solvent mixtures of ethanol and water (100%, 75%, 50%, 25%, and 0%) were tested. The quantity of crude extracted materials was found to depend on solvent proportion, and recovery increased with water concentrations. A 75:25 (v/v) mixture of ethanol and water was the most effective solvent and gave the greatest quantity of crude extract. Hydroethanolic crude extract (OR) from *T. erecta* leaves was partitioned into four fractions for further evaluation: hydrolyzed (HY), aqueous (AQ), neutral (NE), and acidic (AE). The HY and AE fractions showed the greatest activity, with complete inhibition of germination, shoot length, and root length of *E. crus-galli*. and *P. lathyroides*. The total phenolic and flavonoid contents of each fraction were also determined, with HY and AE having higher concentrations of both phenolics and flavonoids than other fractions.

Due to its strong inhibitory effects and high yield, the HY fraction was selected as having the greatest potential for development as a natural herbicide. This fraction was formulated into a soluble concentrate product (SCT) and its inhibition potential and possible modes of action were investigated. The formulation was confirmed to have a strong inhibitory effect on seed germination and seedling growth of *E. crus-galli* and *P. lathyroides*. Its effects on seed imbibition and α-amylase activity of *E. crus-galli* and *Amaranthus viridis* L. were also determined; imbibition and α-amylase activities of both bioassay seeds were found to be increased by prolonging time but

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decreased by higher concentrations of SCT. Cytogenetic bioassays of SCT on root-tip cells of *Allium cepa* L. clearly indicated it inhibits mitosis and thus cell division, as the proportions of cells in metaphase, anaphase, and telophase were all decreased. Mitotic abnormalities were also observed, including spindle disturbance at prophase and c-metaphase and chromosome stickiness in metaphase and anaphase. Additionally, SCT was foliar sprayed on greenhouse-grown *E. crus-galli* and *P. lathyroides* at rates of 10, 20, 40 and 80 kg (a.i.) ha^{-1} and physiological changes evaluated at 1, 3, 5, and 7 days after treatment. In particular, leaf membrane stability index, photosynthetic pigment content (chlorophyll a, chlorophyll b and carotenoids) , and malondialdehyde (MDA) content as a measure of lipid peroxidation were determined. Results showed that at seven days after treatment, the highest concentration of SCT resulted in decreased membrane stability index, the lowest photosynthetic pigment contents, and increased MDA content for both *E. crus-galli* and *P. lathyroides*.

The major constituents of the essential oil obtained from *T. erecta* leaves were characterized by gas chromatography-mass spectrometry (GC-MS) and included piperitone 57, neophytadiene 68, palmitic acid 67, caryophyllene 58, and 9,12,15-octadecatrien-1-ol 89. The emulsifier concentrate formulation of *T. erecta* essential oil (EC-EOs) was also found to have a strong inhibitory effect on the germination and α-amylase activity of *E. crus-galli* seeds. Taken together, these results indicate that selective extraction from natural resources by appropriate solvents is important for obtaining fractions with high seed germination inhibition activities.

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Pattharin Wichittrakarn

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CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problems

The success of modern agricultural practices is due to the discovery and adoption of chemicals for weed and pest control (Dayan et al., 2009). Recently, synthetic herbicides have been increasingly used to promote productivity, and their overuse has caused environmental pollution and damage to human health. Moreover, the potential impact of pesticides on the environment has become much pressing and stringent pesticide registration procedures have been enacted, such as the Food Quality Protection Act in the United States. Developing alternative weed management tools for sustainable crop production has become a subject of interest. Recently, allelopathy has been introduced as a viable option for alternative weed management in sustainable agriculture (Fujii, 2001; Singh et al., 2003; Hong et al., 2003). Allelopathy can play a beneficial role in various sustainable weed management methods such as cover cropping (Isik et al., 2009; Campiglia et al., 2010), soil surface mulching (Aladesanwa and Adigun, 2008), and soil incorporation (Chon et al., 2005; Kobayashi et al., 2008). However, common methods use bulk plant residues such as mulch, for there are many limitations such as cost-prohibitive heavy fieldwork.

Several natural products or mixtures of natural products have been commercialized as crop protection products for use in organic agriculture, mainly extracts and essential oils of plant origin (Hüter, 2010). For example, Matran® contains up to 50% clove oil and Burnout II® consists of a mixture of 12% clove oil with acetic acid (Dayan et al., 2009). Other natural products produced from allelopathic plant residues have shown potential as natural herbicides, such as corn gluten (Dayan et al., 2009) and organic herbicides from *Aglaia odorata* Lour. (Laosinwattana et al., 2009). Such natural products have been used as active ingredients in weed control, but the market is relatively small; many natural products have demonstrated insufficient biological activity, low persistence under field conditions, or when supplied in industrial quantities cannot be assured to have consistent quality (Hüter, 2010). These characteristics are confirmed by previous reports (Inderjit et al., 2002; Teerarak et al., 2010), and indicate significant room for improvement in natural herbicides.

Products formulated from crude extracts that function on the joint action of allelochemical mixtures from strong allelopathic plants might provide effective and successful natural herbicides.

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Most countries, including the United States of America, European Union member countries, and Thailand, allow patenting of natural pesticides in mixture forms such as essential oil, crude extract, or partially separated gross chemical groups derived from crude extracts. Such a natural herbicide has been successfully developed from *Aglaia odorata* Lour. and dissiminated via national and international conferences and international publication. (Laosinwattana et al., 2009; 2010; 2012). This natural product produced from *Aglaia odorata* Lour. dry leaf and its crude extract in pellet, wettable powder, and water soluble concentrate formulations were patented in Thailand by our group. The output of this research is useful to academic researchers, farmers, and agricultural companies, and also feeds into future academic research on allelopathy and natural herbicides. We continued this work by testing the allelopathic potential of the marigold plant *Tagetes erecta* L.; our results indicated that it had high potential and would be a good candidate for natural herbicide development as we had done with *Aglaia odorata* Lour.

1.2 Objectives of the study

1.2.1 Determine activities of partially separated solvent fractions from *Tagetes erecta* L., isolate and purify main active compounds

1.2.2 Develop natural herbicide from *T. erecta* L.

1.2.3 Determine potential of product as a natural herbicide

1.2.4 Study product effects on physiology and the cellular mechanisms of its compounds

1.2.5 Study the chemical constituents of essential oil from *T. erecta* leaves.

1.3 Scope of the study

1.3.1 Investigate the allelopathic activity of partially separated solvent fractions; isolate and purify main active compounds from *T. erecta* leaves.

1.3.2 Investigate natural herbicide potential and its mode of action.

1.3.3 Isolate the essential oil from *T. erecta* leaves by hydrodistillation.

1.4 Expected benefits and application

1.4.1 Successful separation of gross allelochemical groups and purification of main active compounds from *T. erecta*

1.4.2 Evaluation of the weed control potential of the formulated product.

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1.4.3 Understand the physiological and cellular mechanisms by which natural herbicides inhibit weeds.

1.4.4 Identification of the active compounds in the essential oil obtained from *T. erecta* leaves.

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CHAPTER 2

LITERATURE REVIEW

2.1 Weeds and weed management

Weeds, also known as invasive plants, are defined in agriculture as any plant growing in the wrong place and where it is not wanted. Weeds have been documented as serious plant pests since ancient times (Zimdahl, 2013), and weed management is an important aspect of crop production. Weeds can effectively compete with crop species, lower crop yields, increase labor requirements, and ultimately increase food costs for the consumer; thus, they can have a significant impact on agricultural production systems and lead to more harm than benefit (Craft, 1975; Klingman and Ashton, 1975). Weeds not only interfere in commercial agricultural production but also cause problems for general public in many other ways, for example in regard to health (infestation of small seeds and spores) and maintaining home landscaping, recreational areas, and other non-crop areas. Weeds are highly competitive and are adaptable under adverse conditions if not controlled. Understanding the interactions of weeds with their environment will lead to more effective weed prevention, management, and control (Ghersa et al., 1994; Zimdahl, 2004). Weed management is most successful when it involves an integrated approach using a variety of methods. (Anderson, 1996; Monaco et al., 2002; Zimdahl, 2013). However, combined techniques have limited effectiveness in completely open environments that encourage the arrival of new weeds; these new species may not be controllable with present techniques and therefore may be more difficult to manage (Zimdahl, 2013). This situation requires the development of additional methods for weed control. Currently, common methods used to manage weeds include prevention and cultural, mechanical, biological, and chemical means.

2.1.1 Prevention

Prevention is the most effective method of dealing with weeds. Preventive weed control involves all measures taken to forestall the introduction and spread of unwanted plants, and is usually easier than controlling them after establishment. Preventative practices include cleaning tillage and harvesting equipment of weed seeds and vegetative structures; planting certified, weed-free crop seed; and controlling weeds in barnyards, around structures, and along fencerows and roadways.

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2.1.2 Cultural

Cultural and crop management techniques promote a healthy crop that is best able to compete with weeds. Crop competition can be an inexpensive and effective aid to weed management if used to its fullest advantage. Examples of cultural practices include: crop rotation, crop selection, crop varieties, planting date, plant population and spacing, fertility and irrigation, using a no-tillage system, and cover crops or living mulch.

2.1.3 Mechanical

Mechanical or physical techniques either destroy weeds or make the environment less favorable for seed germination and weed survival. These techniques include hand-pulling, hoeing, mowing, plowing, applying sound and electricity, flooding, salting water, draining, chaining, mulching, tillage, disking, cultivating, digging, and applying artificially high temperatures: hot water, burning, solarization. Mulching (straw, wood chips, gravel, plastic, etc.) can also be considered a mechanical control method since it uses a physical barrier to block light and impede weed growth.

2.1.4 Biological

Biological weed control involves the use of other living organisms such as insects, diseases, or livestock for the management of certain weeds. In theory, biological control is well-suited for an integrated weed management program. There have been a few success stories of weed species being managed with insect or disease biocontrol agents, and herbivores such as sheep and goats can provide successful control of some common pasture weeds. Examples of plant pathogens include: *Alternaria alternata* ITCC4896 for controlling *Parthenium hysterophorus* (Saxena and Kumar, 2010), *Alternaria pellucida* for controlling *Sagitaria trifolia* (Motlagh and Javadzadeh, 2010).

2.1.5 Chemical

Herbicides can be defined as crop-protecting chemicals used to kill weedy plants or interrupt normal plant growth. Since the beginning of agriculture, hand weeding, mechanical weeding, and herbicide applications have been the most relied-upon weed control methods (Griepentrog and Dedousis, 2010; Bergin, 2011; Rueda-Ayala et al., 2011; Chauvel et al., 2012). These methods have served to keep weed infestations low and improve crop productivity throughout the world. Herbicides in particular provide a convenient, economical, and effective way to help manage weeds. However, herbicide use also carries risks that include environmental, ecological, and human health effects. It is important to understand both the benefits and

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disadvantages associated with chemical weed control in order to select an appropriate method. There are many kinds of herbicides from which to choose, and many factors determine when, where, and how a particular herbicide can be used most effectively. Understanding these factors enables using herbicides to their maximum advantage.

Currently, herbicides are the primary method for managing weeds in industrialized countries and are becoming more widely used in developing countries. Potential problems associated with herbicides use are (1) injury to non-target vegetation, (2) crop injury, (3) residues in soil and water, i.e. reduction of soil and water quality, (4) toxicity to other non-target organisms, (5) concerns for human health and safety, and (6) herbicide-resistant weed populations (Li et al., 2003; Cox, 2006; Meksawat and Pornprom, 2010; Pot et al., 2011).

2.2 Herbicide

Herbicides are chemicals used to kill or otherwise manage certain species of plants considered to be pests. Herbicides can be grouped by activity, timing of application, mechanism of action, or type of vegetation controlled (Gilbert, 2014). For herbicides to be effective, they must persist long enough to kill the weeds for which they were intended. Extended persistence, however, may result in injury to nontarget plants and other organisms (Holt, 2001). Most herbicides are transformed into relatively less toxic forms by biochemical processes, but if an herbicide is not degraded, it may remain in the plant or end up in the soil as a contaminant. Thus, while the use of synthetic herbicides for controlling weeds is effective and convenient, their side effects can also have strong negative impacts on humans, animals, and the environment. For example, glyphosate has been documented to reduce populations of *Chordodes nobilii* (Gordiida, Nematomorpha), a poorly-known group of worm-like animals similar to nematodes. (Achiorno et al., 2008).

2.3 Herbicide Formulations

The active ingredient in a herbicide is the chemical that controls the target weed. Typically, most herbicides are formulated with appropriate solvents or surfactants to make the product easier to apply and more convenient to handle. Adjuvants (surfactants, emulsifiers, wetting agents, etc.) and various diluents may also increase the effectiveness of a pure herbicide. Thus, every herbicide product is a combination of active ingredients and inert ingredients, and the same herbicide can be available in more than one formulation. Formulations vary according to the solubility of the active

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ingredient in water, oil, and organic solvents and the manner of application (e.g. dispersed in a carrier such as water or applied directly as a dry formulation). The type of formulation also determines toxicity to plants, uniformity of plant coverage, and stability in storage. May and Manning (2007) have divided types of herbicide formulations as follows:

2.3.1 Dry formulations

2.3.1.1 Wettable powder (WP or W)

Wettable powders are finely ground solids, typically mineral clays, to which an active ingredient is sorbed. They provide an effective way to apply via water spray an active ingredient that is not otherwise readily soluble in water. These dry preparations look like dust, contain a high percentage of the active ingredient (usually 50 percent or more), and are mixed with water for application.

2.3.1.2 Granule (G or GR)

Granule formulations are similar to dust formulations, except granular particles are larger and heavier. The coarse particles are made from materials such as clay, corncobs, or walnut shell. The active ingredient either coats the outside of the granules or is absorbed into them. The amount of active ingredient is relatively low, usually ranging from 1 to 15 percent by weight.

2.3.1.3 Pellet (P)

Pellets are similar to granules, but their manufacture is different. The active ingredient is combined with inert materials to form a thick liquid mixture (slurry). This slurry is then extruded under pressure through a die and cut to produce a particle that is relatively uniform in size and shape but much larger than a granule. Pellets are similar to granules in that they are ready to use, are applied in the dry form, and contain a small amount of active ingredient (usually 10 to 20 percent by weight) combined with inert carrier. Pelleted formulations may be applied by hand or mechanically, and are used for soil treatment. While drift is not a problem with this formulation, pellets should not be applied to frozen soil, on steep slopes, or in close proximity to root systems of nontarget plants. Pellets provide a high degree of applicator safety.

2.3.1.4 Soluble powder (SP)

Soluble powder is a dry formulation that contains a high percentage (usually above 50 percent) of the active ingredient. Soluble powders look like wettable powders but form a true solution when added to water. Few herbicides are available as soluble powders because active ingredients are typically not soluble in water. Agitation in the spray tank

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helpsdissolution; after dissolving, no more agitation is usually needed. Soluble powders are nonabrasive to equipment, and inhalation hazard is a characteristic of this formulation.

2.3.1.5 Water-dispersible granule or dry flowable (WDG or DF)

In recent years, wettable powders (WP) have been improved through a technology that enabled producers to clump particles together into aggregates that would readily disperse in water. These formulations are called water dispersible granules (WDG or DG) or dry flowables (DF), depending on the manufacturer. There is essentially no difference between the two formulations. Another, more recently introduced formulation is the extruded paste (XP). Though formulated with a different process, XP is used in the same manner as the DF and WDG formulations.

2.3.1.6 Dusts (DU)

Also like wettable powders, dusts are very fine particles of clay (or talc or chalk) to which an active ingredient has been added. Unlike WPs, dusts are applied dry. They are formulated for ready-to-use home garden products like rotenone (Derris Vegetable Dust) and are popular for urban pest control of termites and ants – the insects track the dust back to the nest and spread it throughout, giving very good control.

2.3.1.7 Tablets (TA)

Tablets are similar to granules in that they consist of an active ingredient with a dry inert substrate. The most common agricultural tablets are phosphine tablets, used for grain fumigation and rabbit control. On exposure to air, the phosphine tablets absorb moisture and break down to give off phosphine gas, a very toxic fumigant at extremely low concentrations.

2.3.2 Wet formulation

2.3.2.1 Emulsifiable concentrates (EC or E)

Emulsifiable concentrates (EC) are products that contain emulsifiers and so form stable oil/water mixtures. Emulsifiers are able to wrap around oil-soluble chemicals and suspend them in a water-based (aqueous) solution. Milk is an example of an emulsion, with small droplets of oil (fat) suspended in aqueous liquid. When the ingredients of an emulsifiable concentrate herbicide are added to water, the mixture becomes 'milky'. Emulsions require some degree of agitation to prevent separation.

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2.3.2.2 Suspension concentrates (SC)

Suspension concentrates are essentially a pre-mix of water dispersible granules. Fine granules (1-3 microns) are suspended in water, to be further diluted in the spray tank. This is another very popular formulation, used for most types of pesticide.

2.3.2.3 Soluble concentrates (SL)

Soluble concentrates are true solutions where the concentrate is fully dissolved when mixed with water, i.e. they cannot be mechanically separated. These do not require agitation.

2.3.2.4 Ultra low volume (ULV)

ULV solutions consist of the active ingredient in a small amount of organic solvent, and are exclusively used for insecticides. They are not diluted and are specifically designed for aerial application, although it is common now to add a mineral oil and/or water to reduce the risk of drift.

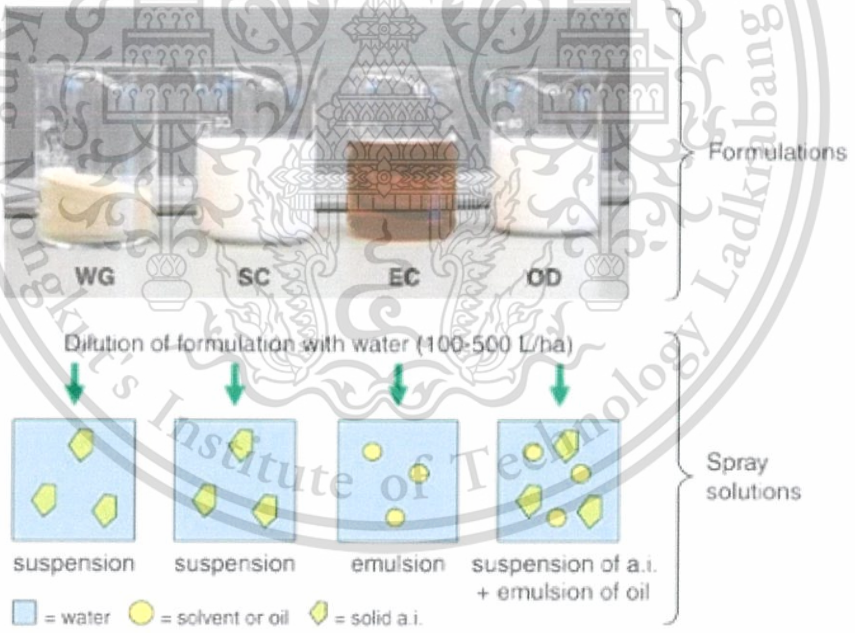

Figure 2.1 Summary of important formulation types. WG: Water Dispersible Granule, SC: Suspension Concentrate, EC: Emulsifiable Concentrate, OD: Oil Dispersion (Source: May and Manning, 2007).

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2.4 Allelopathy

The word allelopathy is derived from two separate Greek root words: *allelon* which means "mutual or of each other", and *pathos* which means "to suffer or feel" (Rizvi and Rizvi, 1992; Willis, 2007), and refers to the chemical inhibition of one plant species by another. In natural ecosystems, allelopathy is a biological phenomenon that maintains balance among the various plant communities. Some plants release chemicals referred to allelochemicals that often affect the growth, development, survival, and reproduction of neighboring plants (Rice,1984; Toshiki and Asaduzzaman, 2012). The impact can be beneficial (positive allelopathy) or detrimental (negative alleolopathy) and can affect many aspects of plant ecology including occurrence, growth, plant succession, the structure of plant communities, dominance, diversity, and plant productivity. Chemicals that impose allelopathic influences are called allelochemicals or allelochemics. Allelochemicals are mostly plant secondary metabolites and may be found in flowers, stem, leaves, or roots (Weston, 1996; Ahn and Chung, 2000; Batish et al., 2007; Duke et al., 2007). In order to have any effect on the target plant the allelochemicals have to be released from the donor plant (Rice, 1984). Allelochemicals may be released by any of the following processes: root exudation, leaching from dead or live plant tissues, volatilization from aboveground plant parts, and plant residue decomposition (Scrivanti, 2010).

2.4.1 Runoff and leachate from leaves and stem of plants. For example, the leaf leachates of three multiple-use trees, neem (*Azadirachta indica*), sandal (*Santalum album*), and poovarasu (*Thespesia populnea*) which have allelopathic potential against paddy rice (*Oryza sativa* L.) var. ponmani (Neelamegam and Dhanusha, 2013).

2.4.2 Volatile phytotoxic compounds from the green parts of a plant. For example, *Ginkgo biloba* (Kato-Noguchi et al., 2013) and *Drimys brasiliensis* Miers (Anese et al., 2015).

2.4.3 Phytotoxic compounds from decomposing plant material. Examples include *Catharanthus roseus*, *Coreopsis tinctoria*, *Cosmos sulphureus*, *Gomphrena haageana* and *Impatiens balsamina*, when used as mulching material. Apart from shading and keeping the soil moist, rye mulch also inhibits germination of weed seeds in the soil (Hanim et al., 2014).

2.4.4 Phytotoxic compounds released from the plant roots. Rice is an example, where living rice plants are able to selectively suppress weed growth (Navarez and Olofsdotter, 1996; Olofsdotter et al., 1997; Ahmed et al., 2014).

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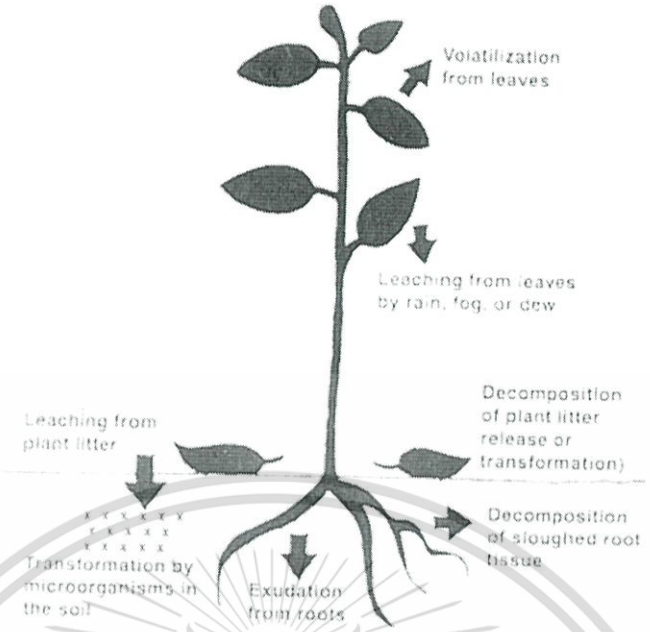

Figure 2.2 Methods of allelopathic chemicals exudation from plants.
(source: https://edis.ifas.ufl.edu/hs186)

Allelopathic chemicals are products of secondary metabolism or are non- nutritional primary metabolites (Weir et al., 2004; Iqbal and Fry, 2012). These compounds belong to numerous chemical groups including: triketones, terpenes, benzoquinones, coumarins, flavonoids, terpenoids, strigolactones, phenolic acids, tannins lignin, fatty acids, and nonprotein amino acids. A wide range of these biochemicals are synthesized through the shikimate pathway (Hussain and Reigosa, 2011) or, in the case of essential oils, from the soprenoid pathway. Allelochemicals can be classified into ten categories (Li et al., 2010) according to their different structures and properties:

1) Water-soluble organic acids, straight-chain alcohols, aliphatic aldehydes, and ketones;

2) Simple lactones;

3) Long-chain fatty acids and polyacetylenes;

4) Quinines (benzoquinone, anthraquinone and complex quinines);

5) Phenolics;

6) Cinnamic acid and its derivatives;

7) Coumarins;

8) Flavonoids;

9) Tannins; and

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10) Steroids and terpenoids (sesquiterpene lactones, diterpenes and triterpenoids).

Allelochemicals can potentially be used as growth regulators, herbicides, insecticides, and antimicrobial crop protection products. Allelochemicals act to affect the development and growth of organisms (Rice, 1984) by influencing processes such as:

1) Cell division, cell elongation, and ultrastructure of cell;
2) Hormone-induce growth;
3) Membrane permeability;
4) Mineral uptake;
5) Easily available phosphorus and potassium in soils;
6) Stomata opening and photosynthesis;
7) Respiration;
8) Protein synthesis and changes in lipid and organic acid metabolism;
9) Possible inhibition of porphyrin synthase;
10) Inhibition and stimulation of specific enzymes
11) Corking and clogging of xylem elements;
12) Stem conductance of water and internal water relations; and
13) Miscellaneous mechanisms.

Nowadays, allelopathy has become a significant subject of research involving sustainable agriculture, like biological weed and pest control (Sodaeizadeh and Hosseini, 2012).

2.5 Allelopathic effect on weed control

In a prior study, aqueous extracts from the roots, leaves, inner stem bark, and flowers of *Tecomella undulata* were studied for their allelopathic effects on seed germination and early seedling growth of four important monocotyledonous and dicotyledonous ornamental plants. The flower and root extracts of *T. undulata* inhibited germination of tall fescue (*Festuca arundinacea* Schreb.) and perennial ryegrass (*Lolium perenne* L.) seeds, but leaf and inner stem bark extracts had no significant effects on either of these turf grasses (Karami et al., 2017). Mubeen et al. (2011) also reported the effects of aqueous extracts from weeds on the germination and seedling growth of rice (*Oryza sativa* L.). Monem et al. (2012) reported effects for barley shoot aqueous extract on germination, seedling growth, cell membrance permeability, and the malondialdehyde content of

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corn (*Zea mays* L.) weeds. These and other studies point to the considerable potential of allelopathy as an agricultural management tool.

Einhellig (1995) suggested that nearly all allelopathic activities are due to the presence of several compounds in a mixture. The concentration of each compound in that mixture might be significantly less than would be needed to cause growth inhibition using the pure chemicals. This hypothesis was confirmed by study of dichloromethane extracts of annual wormwood leaves, which contain artemisinin; the impure extract inhibited seed germination and seedling growth of redroot pigweed more strongly than did similar amounts of artemisinin alone (Inderjit et al., 2002). Furthermore, aqueous extracts without artemisinin had similar activity to that of artemisinin alone. This illustrates the significance of joint action of allelochemicals in mixtures, and suggests that products formulated from crude extracts and including joint action of mixed allelochemicals have promising potential as natural herbicides.

To successfully produce effective natural herbicide products requires not only the allelochemical mixtures from strong allelopathic plants, but also obtaining the maximum yield of bioactive compounds from plant materials. Extracted yield is dependent on the solvent and the method of extraction used (Goli et al., 2004), and investigation is needed to determine which solvents give efficient extraction of high-quality bioactive compounds. Water and aqueous mixtures of ethanol, methanol, and acetone are commonly used in plant extraction (Sun and Ho, 2005), and alcoholic solvents have been commonly employed to extract bioactive compounds from plant materials, particularly as mixtures of alcohol and water (Spigno et al., 2007). Aqueous ethanol has been reported as superior to methanol and acetone for extracting flavonoids from tea (Wang and Helliwell, 2001). However, water was reported to be a better solvent for extracting tea catechins than either 80% methanol or 70% ethanol (Khokhar and Magnusdotti, 2002). In addition to the solvent, which plant parts used in extraction are a critical consideration. Raoof and Siddiqui (2012) found that aqueous extract of *Tinospora cordifolia* leaves exhibited allelopathic effects while stem extracts had the least effect on weeds. Similarly, aqueous extracts prepared from the roots and mature leaves aqueous extracts of *Sapindus saponaria* had inhibitory effects on the germination of diaspores and seedling growth of lettuce and onion. Thus, there is a great deal of opportunity for optimizing extraction protocols in order to obtain the greatest allelopathic potential.

Over many years, various types of allelochemicals have been isolated and characterized from hundreds of plants. Some of the allelochemicals exploited as commercial herbicides are

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cineole (shell), benzoxazinones (BASF), quinolic acid (BASF), and leptpspermones (Zeneca) (Kohli et al., 1998). Similarly, the natural fatty acid pelargonic acid and maize gluten (a byproduct of the maize-milling process) are used in organic farming and have limited use in the USA (Duke et al., 2000). Other allelopathic plants with products that show potential as natural herbicides include *Aglaia odorata* Lour. (Laosinwattana et al., 2009), *Jasminum officinale* f. var. *grandiflorum* (L.) Kob. (Teerarak et al., 2013), *Jasminum sambac* Ait. (Poonpaiboonpipat et al., 2011), *Anisomeles indica* (Batish et al., 2007), and the essential oils from *Artemisia scoparia* and *Cymbopogon citratus* (Kaur et al., 2010; Poonpaiboonpipat et al., 2013). Additional examples include hairy vetch (*Vicia vilosa* L.), which is a promising cover crop for weed control in fields, grasslands, and orchards in Japan (Fujii, 2001); buckwheat (*Fagopyrum esculentum* Moench) pellets, which significantly reduce paddy weeds when applied at 2 ton ha^{-1}; kava root (*Piper methysticum* L), which provides weed control in a paddy when applied at 1 ton ha^{-1} six and ten days after the soil was saturated with water (Xuan et al., 2003); and dried Saururaceae (*Houttuynia cordata* Thunb.) powder, which significantly reduces *Echinochloa* and *Monochoria* paddy weeds when applied at 150 g m^{-2} and also increases the grain yield of rice (Lin et al., 2006). However, most allelochemicals having potential herbicidal activity are not commercially used because they are extremely expensive to manufacture. The development of natural herbicides that are both potent and cost-effective is thus important for enhancing sustainability in agriculture, reducing and replacing the use of synthetic pesticides for pest management, and overall making agricultural production safer for humans, animals, and the environment (Khanh et al., 2007; Singh et al., 2003; Sodaeizadeh et al., 2010).

2.6 Essential oils

Essential oils are volatile, highly concentrated substances extracted from flowers, leaves, stems, roots, seeds, bark, resin or fruit rinds. They contain the aromatic compounds that give plants their wonderful scents, a characteristic aroma and odour determined by the specific mixture of a variety of aromatic phenols, oxides, ethers, alcohols, esters, aldehydes, and ketones (Langenheim, 1994; Batish et al., 2008). Plant volatile oils play important roles in many aspects of human behavior and industries such as pharmaceuticals, nutrition, and perfumes (Baldwin, 2010).

The secondary metabolites and chemical compounds present in extracts or essential oils of some plants have been shown to have allelopathic effects on seed germination and seedling growth (Romagni et al., 2000; De Feo et al., 2002; Abrahim et al., 2000; Kordali et al., 2007; Paudel and

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Gupta, 2008; Ramezani et al., 2008; Brito et al., 2010; Ismail et al., 2012; Rassaeifar et al., 2013). In one very recent study, Synowiec et al. (2017) reported the phytotoxic potential of 12 essential oils (EOs) collected from plants growing in natural or cultivated stands in a temperate climate on germination of *Amaranthus retroflexus*, *Avena fatua*, *Bromus secalinus* and *Centaurea cyanus*. The tested oils were extracted from *Achillea millefolium*, *Acorus calamus*, *Carum carvi*, *Chamomilla recutita*, *Foeniculum vulgare*, *Lavandula angustifolia*, *Melissa officinalis*, *Mentha* × *piperita*, *Salvia officinalis*, *Solidago canadensis*, *Tanacetum vulgare* and *Thymus vulgaris*. They found four Eos to be the most phytotoxic group, namely *Carum carvi*, *Thymus vulgaris*, *Mentha* × *piperita* and *Salvia officinalis*. These EOs were composed mainly of oxygenated monoterpenes at proportions of 64.1–93.3%. The least phytotoxic EO was from *Solidago canadensis* and composed mainly of mono- and sesquiterpene hydrocarbons (92.3%). In addition, principal component analysis indicated that the phytotoxic effects of EOs also depended on the sensitivity of the subject species, with crops being more tolerant than weeds to the majority of EOs. Small-seeded species, namely *A. retroflexus* and *C. cyanus*, were the most sensitive, while the kernels of *Z. mays* and the seeds of *A. fatua* were the most tolerant. Another study demonstrated the phytotoxic effect of *Eucalyptus astringens* essential oil on seed germination, root and shoot length, chlorophyll content, membrane integrity, and malondialdehyde (MDA) and proline contents of *Triticum durum*, *Vicia faba*, *Phaseolus vulgaris*, *Sinapis arvensis*, *Erica vesicaria*, and *Scorpiurus muricatus* (Grichi et al., 2015). The results showed *E. astringens* essential oil to have strong phytotoxicity activity against weeds and to possesses weed-suppressing abilities. Allelopathic activity has also been reported for the essential oil from basil (*Ocimum basilicum*) against seed germination and the initial growth of lettuce, tomato, and lemon balm seedlings (Yoshimura et al., 2011). Hence, these EOs could be effective bases for developing bioherbicides, and may be valuable alternatives for integrated weed control technology under organic farming systems (Tworkoski, 2002; Batish et al., 2008; Dayan et al., 2009; Kordali et al., 2015; Isman, 2000).

2.7 Marigold

The *Tagetes* genus, belonging to the family Asteracaea and commonly known as marigolds (Figure. 2.3), comprises about 56 species distributed around the world. Its members are common ornamental or biennial plants having pinnate green leaves and white, golden, yellow, orange, or almost red floral heads that are typically 0.1 to 4-6 cm in diameter, generally with both ray florets and disc florets (Edward, 1999; BaliČeviĆ et al., 2014). The species *Tagetes erecta* (Rhama and

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Madhavan, 2011; Coelho et al., 2017). *T. minuta* (Tereschuk et al., 1997; Garcia et al., 2012), and *T. patula* (Munhoz et al., 2014) are most common worldwide; other species referred to are often region-specific, such as *T. lucida* (Capunzo et al., 2003) or *T. mendocina* (Lima et al., 2009). It is grown as an ornamental crop for loose flowers and as a landscape plant due to its variable height (1-3 feet) and colour shades of flowers, often planted to form a solid mass of color in beds.

Every part of the marigold contains many types of chemical compounds and essential oils. It can be used to inhibit the growth of microorganisms, as a pesticide, and also as a major source of antioxidants (Tereschuk et al., 1997; De las Heras et al., 1998; Abad et al., 1999). The pharmacological activities of marigolds result from to their containing several classes of secondary metabolites such as flavonoids, sterols, carotenoids, tannins, saponins, triterpene alcohols, polysaccharides, a bitter principle, mucilage, and resin (Jacobs et al., 1994 and Piccagliy et al., 1998). Marigold has also been reported to contain 5-(3-buten-1-ynyl) 2,2-bithienyl, and alpha terthienyl (Morallo-Rejesus and Decena, 1982). Alpha terthienyl in a synthetic form has been reported to be the active component in marigold (Kanagy and Kaya, 1986). Furthermore, marigold roots have been reported to contain flavonoids (Olabiyi, 2006), including di-hydro flavonoid, flavones, and flavonones lacking a free 5-OH (Bamiduro, 2001). The roots have also been reported to contain amines, amides, phenols, and ketones (Olabiyi, 2004). Marigold flower petals are an excellent and important source of carotenoids, particularly the yellow carotenoids such as μ- and β-carotenes and the xanthophylls, lutein and zeaxanthin (Hojnik et al., 2008). In a yolk colour assay, Hasin et al. (2006) reported highly significant differences scores between marigold and other (orange skin and control diet) dietary groups resulted from the birds on the 4% marigold diet consuming more xanthophylls. The essential oil derived from marigold capitula and leaves, tagetes oil, is composed of a substance in terpenes, almost entirely (Z)- and (E)-ocimenones, along with limonene, caryophyllene, piperitone, and piperitenone (Vasudevan et al., 1997). This oil also has the features of a pesticide. Dharmagadda et al. (2005) found that tagetes oil from *T. patula* was effective in eliminating larval mosquitoes of the species *Aedes aegypti*, *Anopheles stephensi*, and *Celex quinnquefaciatus*. Natural plant products are at the present research focus because of their ability to produce environmentally less harmful but efficacious chemical substances (Schmutterer, 1990). It is envisaged that natural plant products would replace or minimize the use of highly toxic and persistent synthetic chemicals (Jackai et al., 1992).

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Figure 2.3 Botanical characteristics of *Tagetes erecta* L.

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CHAPTER 3

RESEARCH METHODOLOGY

3.1 Chemicals and Instruments

3.1.1 Chemicals

3.1.1.1 Ethanol 95% (Merck)

3.1.1.2 Ethanol 99% (Merck)

3.1.1.3 Ethylacetate (Merck)

3.1.1.4 Acetone (Merck)

3.1.1.5 Methanol (J.T. Baker)

3.1.1.6 Ammonium hydroxide (Merck)

3.1.1.7 Hydrochloric acid 37% (ACI-Labscan)

3.1.1.8 Glacial acetic acid (Merck)

3.1.1.9 N,N-dimethylformamide (Fluka)

3.1.1.10 Nonylphenol ethoxylate (NP40) (Sigma - Aldrich)

3.1.1.11 Tween80 ((Sigma - Aldrich)

3.1.1.12 Magnesium sulfate anhydrous (Ajax Finechem)

3.1.1.13 Sodium bicarbonate (Ajax Finechem)

3.1.1.14 Calcium chloride (Sigma - Aldrich)

3.1.1.15 Sodium hydroxide (Ajax Finechem)

3.1.1.16 Sodium acetate hydrated (Ajax Finechem)

3.1.1.17 3,5-dinitrosalicylic acid (Sigma - Aldrich)

3.1.1.18 Starch (Sigma - Aldrich)

3.1.1.19 Potassium sodium (+) - tartrate (Ajax Finechem)

3.1.1.20 D (+) - Maltose monohydrate (Sigma - Aldrich)

3.1.1.21 L-Ascorbic acid (Sigma - Aldrich)

3.1.1.22 Trichloroacetic acid (Merck)

3.1.1.23 2-Thiobarbituric acid (Sigma - Aldrich)

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3.1.1.24 2,6-di-tert-butyl-4-methylphenol (BHT) (Sigma - Aldrich)

3.1.1.25 Cellulose (Fluka), Pectinase (Fluka)

3.1.1.26 Iron (II) sulphate (Ajax Finechem)

3.1.1.27 Talcum Powder (chemipan)

3.1.1.28 Folin- Ciocalteu's reagent (Merck)

3.1.1.29 Sodium carbonate (Ajax Finechem)

3.1.1.30 Potassium acetate (Quality Reagent Chemical)

3.1.1.31 Aluminum chloride (Fluka)

3.1.1.32 Quercetin (Sigma - Aldrich)

3.1.1.33 Gallic acid monohydrate (Sigma - Aldrich)

3.1.2 Apparatus

3.1.2.1 Erlenmeyer flask

3.1.2.2 Beaker

3.1.2.3 Graduated cylinder.

3.1.2.4 Filter paper Whatman No. 93

3.1.2.5 Petri-dish 9 cm.

3.1.2.6 Micropipette

3.1.2.7 Stirring rod

3.1.2.8 Test tube

3.1.2.9 Glass column

3.1.2.10 Glass funnel

3.1.2.11 Thermometer

3.1.2.12 Germination paper

3.1.2.13 Parafilm

3.1.2.14 Mortar jar

3.1.3 Scientific Instruments

3.1.3.1 Hot-air oven (Memmert)

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3.1.3.2 Magnetic stirrer (Fisher scientific)

3.1.3.3 Rotary evaporator (Rotavapor R-215, BÜCHI)

3.1.3.4 Analytical balance (Denver Instrument Company TC-254)

3.1.3.5 Clevenger-type apparatus

3.1.3.6 Growth chamber (Climace)

3.1.3.7 UV-VIS Spectrophotometer (Spectronic™ GENESYS 20 spectrophotometer, Thermo Fisher Scientific)

3.1.3.8 pH meter (Consort) and Accumet electrode (Fisher scientific)

3.1.3.9 Centrifuge (Universal 320R, Hettich Lab Technology)

3.1.3.10 Hotplate and magnetic stirrer (ST0707V2, Favorit, PLT Scientific)

3.1.3.11 Gas Chromatography - mass spectrometer (GC: Agilent Technologies Model 6890N, MS: Agilent Technologies Model 7683)

3.2 Research methodology

3.2.1 Plant materials and plant bioassay

3.2.1.1 Plant materials

Mature, healthy leaves of *Tagetes erecta* L. were collected 45 days after planting in an experimental field at King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. Soil residues were washed off under running tap water and the leaves were dried to constant weight in a fan oven at 45 °C for 3 days. They were then ground to a powder (100 mesh) in an electrical blender.

3.2.1.2 Plant bioassay

Seeds of barnyardgrass (*Echinochloa crus-galli* L. Beauv.), wild pea (*Phaseolus lathyroides* L.), and slender amaranth (*Amaranthus viridis* L.) were collected from paddy fields and upland fields in the Ladkrabang district, Thailand. These species were selected due to being serious problem weeds in both paddy rice fields and upland crop fields. Healthy seeds of *E. crus-galli* were placed in the shade at room temperature for 3 months and then incubated at 60 °C in a hot-air oven for 48 hours to break dormancy. Hard seed coats of *P. lathyroides* were

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scrubbed with No. 0 sandpaper to break their dormancy. Healthy seeds of *A. viridis* were released from panicles by lightly shaking in collection bags. Seeds with damaged coats were be discarded. Commercial radish (*Raphanus sativas* L.) seed was also used in this experiment. Healthy and equal-sized onion (*Allium cepa* L.) bulbs were used for cytogenetic experiments.

3.2.2 Experiment 1. Inhibitory potential of aqueous extract and optimal extraction solvent

3.2.2.1 Aqueous extracts bioassay

Aqueous extracts were prepared from leaf, stem, flower and root materials of *T. erecta* by dissolving 10 grams of each powered material in 100 mL of distilled water at 8 °C for 72 h, followed by filtration through three layers of cheesecloth to remove any debris. The supernatant was then filtered through Whatman No.1 filter paper (Whatman Inc. Clifton, NI, USA), adjusted to a concentration of 100 mg mL^{-1}, and stored in a refrigerator at 4 °C until bioassays were performed. Dilutions of each *T. erecta* extract were prepared in distilled water to produce final concentrations of 12.5, 25, 50 and 100 mg mL^{-1}. Bioassays were performed in 9 cm diameter Petri dishes lined with filter paper, in which twenty healthy seeds of *E. crus-galli* and *P. lathyroides* were placed in each Petri dish along with 5 mL of diluted extract. Four replicates were maintained per treatment in a completely randomized manner, and dishes were incubated in a growth chamber with a temperature of 25–32 °C, a 12-12 hours dark-light photoperiod, a light intensity (cool white 840) of 100 µmol m^{-2} s^{-1}, and a relative humidity of around 80%. Treatment with distilled water was used as the control. Germination was deemed to have occurred only after the radicle had protruded beyond the seed coat by at least the dimension of the seed. Seedling growth was measured as the root and shoot lengths at seven days after treatment.

3.2.2.2 Effect of solvent extraction on the crude extracts yield and bioassay

Ten grams of 100 mesh *T. erecta* leaf powder were extracted (ratio 20 g:200 mL) at room temperature for 48 hours in different solvent systems consisting of either absolute ethanol mixed with distilled water (75%, 50%, 25% and 0%) or pure distilled water was carried out based on the method of Poonpaiboonpipat et al. (2011). After 48 hours, the brown

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supernatants were filtered through three layers of cheesecloth, then re-filtered through Whatman No. 1 filter paper. Following filtration, the supernatants were dried using a rotary evaporator (BUCHI Rotavapor R255, BUCHI, Lausanne, Switzerland) under partial vacuum at 45 °C until a constant crude extract weight was obtained. Subsequently, each residue was re-extracted 2 times with the same extraction solvent as used in the first extraction procedure, and the three crude extracts were combined. Stock solutions of each crude extract generated using ethanol: water mixtures were prepared by dissolving the sticky crude extract with acetone in a mortar jar and adding wettable powder (bentonite: anionic surfactant; 95:5 (w/v)) at a 3:7 ratio of crude fraction:wettable powder. The mixture was slowly pulverized until completely dried, with acetone added three times, and stored in the dark at low temperature until used. Each crude extracts was then dissolved in distilled water to produce four solutions with concentrations ranging from 1,250 to 10,000 ppm. Seed germination and seedling growth bioassays were performed as described above. Four replicates and complete randomization were used in all experiments.

3.2.3 Experiment 2. Inhibitory potential, total phenolic content, and flavonoid content of various extract fractions

3.2.3.1 Effect of solvent extraction on crude extract yields and bioassay results

Crude extract was prepared according to the method of Laosinwattana et al. (2007); Teerarak et al. (2010) (Figure 1). Briefly, *T. erecta* L. leaf powder was extracted three times for three days at room temperature with 75% ethanol in distilled water, filtered through three layers of cheesecloth to remove debris, and filtered once more through filter paper (Whatman No.1). The three filtrates were combined and evaporated in a rotary evaporator at 45°C, leaving a sticky residue (original crude fraction; OR fraction). This residue was then diluted with 500 mL of distilled water with vigorous stirring on a magnetic stirrer at 45 °C for 20 min, resulting in an aqueous solution that was then acidified to pH 3 using 6 N HCl. The filtrate was extracted with ethyl acetate (500 mL) three times. After adjusting the pH to neutral, the aqueous phase was dried by reducing pressure at 45 °C, and an aqueous residue obtained (AQ fraction). The ethyl acetate solutions were combined and treated with anhydrous magnesium sulfate, concentrated to about 500 mL, and then

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extracted three times with saturated aqueous $NaHCO_3$ (500 mL). The resulting ethyl acetate phase was dried with anhydrous magnesium sulfate and concentrated by reducing pressure, yielding an ethyl acetate-soluble neutral fraction (NE fraction). Finally, the combined sodium bicarbonate phase was evaporated to about 1 L, adjusted to pH 7 using 6 N HCl, and then extracted three times with ethyl acetate (500 mL). These ethyl acetate solutions were combined, dried over $MgSO_4$, and then evaporated to obtain the ethyl acetate-soluble acidic fraction (AE fraction). The remaining aqueous phase was discarded. Each dried crude fraction was weighed and stored at 4 °C in a dark container to minimize light and temperature degradation.

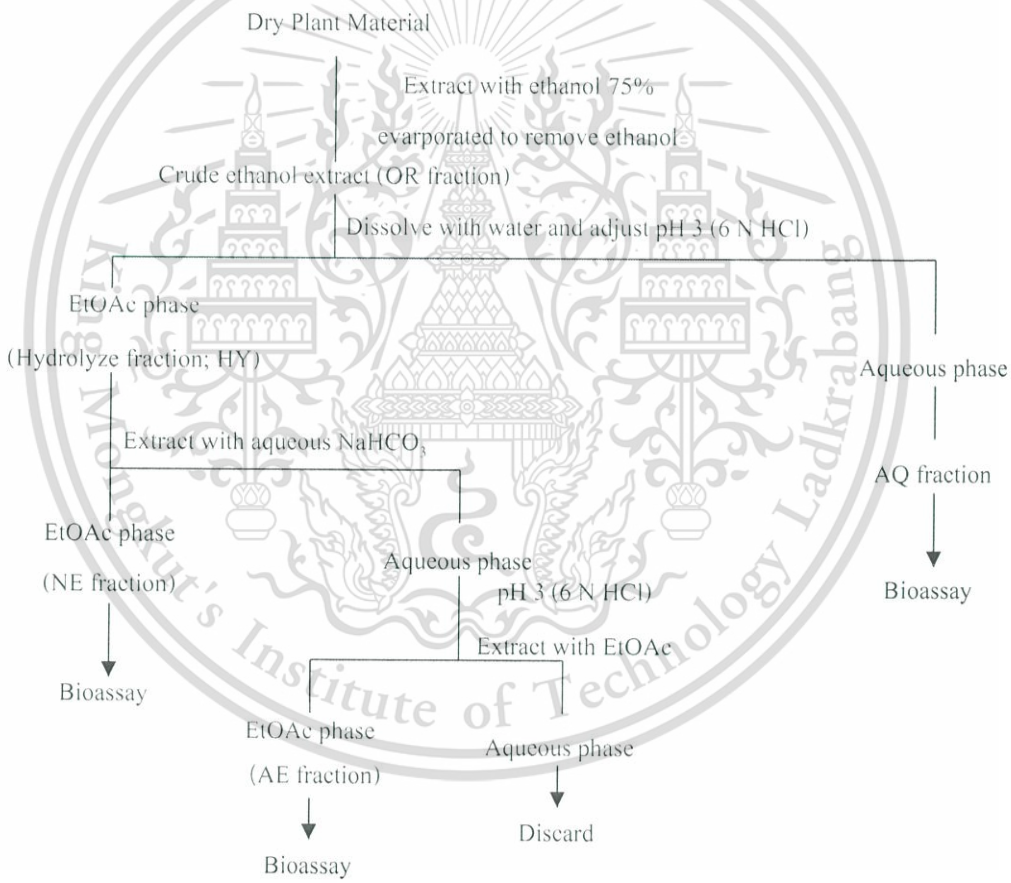

Figure 3.1 Flow chart for acid-base solvent partitioning from crude extract.

(Source: Laosinwattana et al., 2007)

3.2.3.2 Solvent fraction bioassays

Each of the five fractions (OR, AQ, HY, NE and AE) was dissolved as appropriate in distilled water, ethanol, ethyl acetate to the following final concentrations: 0, 2,000,

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4,000, 8,000, and 16,000 ppm. Five mL of each dilution was placed in a Petri dish (9 cm diameter) containing a double layer of germination paper and evaporated to dryness at room temperature, after which distilled water (5 mL) was added to each. Dishes containing only distilled water served as controls. Twenty healthy seeds of *E. crus-galli* and *P. latyroides* were placed in each dish with uniform spacing, and the dishes placed in a growth chamber (Climacell 707, Munich, Germany) with a temperature of 25-32 °C, a 12 h-12 h dark-light photoperiod, a light intensity (cool white 840) of 100 mmol $m^{-2}s^{-1}$, and relative humidity of around 80%. Germination was deemed to have occurred only after the radicle had protruded beyond the seed coat by at least the dimension of the seed. Seedling growth was measured as the root and shoot lengths at seven days after treatment. A completely randomized manner with four replicates was used in all experiments.

3.2.3.3 Determination of total phenolics and flavonoids

Total phenolic contents of the crude fractions (OR, AQ, HY, NE, and AE) were determined using the Folin-Ciocalteu method as slightly modified by Škerget et al. (2005) and Sripakdee et al. (2015). A 1 mL aliquot of extract was mixed with 4.5 mL of distilled water and 0.5 mL of Folin-Ciocalteu's phenol reagent (2 N), placed in a test tube, and shaken well. After 5 min, 4 mL of aqueous sodium carbonate (7.5%) was added to the mixture. The sample was incubated for 1 h at room temperature (32-36 °C) in the dark. Its absorbance was then measured at 765 nm using a spectrophotometer (Universal 320R, Hettich Zentrifugen, Germany). The total phenolic content was calculated from a standard calibration curve obtained using gallic acid. Results are expressed as mg gallic acid equivalent per gram dry weight of crude extract (mg GAE/g of crude extract). All procedures were repeated three times. The total flavonoid content of each crude extract (OR, AQ, HY, NE and AE) was determined using the aluminum chloride method ($AlCl_3$) (Kalita et al., 2013; Kiranmai et al., 2011). An aliquot of 0.5 mL of sample extract was added to 1.5 mL of methanol, 0.1 mL of aluminum chloride (10% w/v), 0.1 mL of potassium acetate (1M), and 2.8 mL of distilled water and mixed well. The samples were incubated for 30 min at room temperature (32-36 °C). The absorbance was measured at 415 nm using a spectrophotometer. The total flavonoid content was calculated on the basis of a calibration curve obtained from a quercetin standard. Results are expressed as mg quercetin equivalent per gram dry

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weight (mg QE/g of crude extract) of crude extract. The result of each assay was determined as the average of three replicates.

3.2.4 Experiment 3. Natural herbicide formulation and evaluation of its potential

3.2.4.1 Preparation of natural herbicide

The hydrolyzed fraction obtained from the hydroethanolic crude extract was formulated into a soluble concentrate (SCT) by mixing the sticky crude hydrolyzed fraction with adjuvant in the ratio of 30:67:3 (w:v:v) crude hydrolyzed fraction: N,N-dimethylformamide: nonylphenol ethoxylate (NP40), resulting in a mixture containing 30% active ingredient. The SCT was diluted with distilled water to obtain four concentrations: 2,000, 4,000, 6,000 and 8,000 ppm. For each treatment, 5 mL of diluted SCT was added to two sheets of germination paper in a 9 cm Petri dish along with twenty seeds of *E. crus-galli* and *P. latyroides*; these dishes were incubated as described above. Germination and seedling growth were determined after 7 days. Four replicates and complete randomization were used in all experiments.

3.2.4.2 Bioassay of seed imbibition and α-amylase activity

To determine seed imbibition and alpha amylase activity, another set of Petri dishes was prepared as described above. Thirty *E. crus-galli* seeds and one hundred *A. viridis* seeds were weighed (W_1) before being placed in the Petri dishes. The controls received 5 mL of distilled water, while treatments received an equal volume of prepared extract. Seeds were harvested 6, 12, 24, 36 and 48 h after of exposure to the treatment. The final seed weight (W_2) was recorded for each concentration and exposure time. Seed imbibition was calculated as:

$$\text{Seed imbibition (\%)} = [(W_2 - W_1) / W_1] \times 100$$

The activity of α- amylase (EC 3. 2. 1. 1) was assayed using the dinitrosalicylic acid (DNS) method, measuring the reducing sugars liberated from soluble starch, based on Teerarak et al. (2012) with slight modification. After measuring seed imbibition, seeds (30 and 100 per determination) were ground to a fine paste in 4 mL of iced-cold aqueous 0.1 M $CaCl_2$. The homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatant was used as the enzyme source. The α-amylase was assayed by measuring the reducing sugars liberated

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from soluble starch. The reaction medium (3 mL) contained 1 mL of enzyme with 1 mL of 1% starch soluble substrate in acetate buffer solution (pH 5.5). The assay medium was incubated for 15 min at 37 °C, and then the reaction terminated by addition of 1 mL of dinitrosalicylic acid reagent (40 mM 3,5-dinitrosalicylic acid, 0.4 N NaOH and 1 M K-Na tartrate) and immediate heating in a boiling water bath for 5 min. The mixture was then cooled under running tap water. Color intensity was measured as the absorption at 560 nm in a spectronic GENESYS 20 spectrophotometer (Thermo Electron Corporation, USA). A standard graph was prepared using maltose, and α-amylase activity was calculated from the standard graph and expressed as μmol maltose min^{-1} g^{-1} (FW). A completely randomized manner with four replicates was used in all experiments.

3.2.4.3 Cytogenetic bioassay

A cytogenetic bioassay was carried out based on the method of Teerarak et al. (2010), with adaptation to our conditions. Onion (*Allium cepa*) root tip cells were used as the study material. Onion bulbs were cut near their basal ends and placed in containers with the basal ends dipping in tap water. The cytogenetic bioassay was performed when the newly-emerging roots reached 1.50-2.00 cm in length (FiskesjÖ, 1985). Samples were treated with SCT at 15.625, 31.25, 62.5 and 125 μM for 18 h, or with distilled water for controls. At the end of the exposure period, root tips were cut and fixed in a freshly-prepared mixture of absolute ethanol and glacial acetic acid (3:1 v:v). To remove the fixative, fixed root tips were washed in distilled water three times and then macerated at 37 °C for 60 min in an enzyme mixture containing 8% cellulase (Fluka) and 6% pectinase (Fluka) in buffer containing 0.01 mM Na citrate and 0.01 mM citrate. To determine the mitotic index, mitotic phases, and the presence of chromosomal aberrations, the root tips were squashed in a drop of absolute ethanol and acetic acid and then stained with 2% Giemsa solution (Merck Co., Ltd.) for 10 min, rinsed with distilled water, and dried. Four replicates were used per treatment and scoring was determined as the average of three roots from each replicate. The mitotic index was calculated as the ratio of dividing cells and total cells, analyzing at least 4,000 cells per treatment. The frequencies of each mitotic phase and of chromosomal abnormalities

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were calculated as percentages of the dividing cells counted in mitosis. Chromosomal abnormalities are presented in a number of micrographs.

3.2.5 Experiment 4. Herbicidal potential of natural herbicide from *T. erecta*

3.2.5.1 Bioassay demonstrating efficacy of SCT by soil application

Determination of the effects of SCT on germination and seedling growth of *E. crus-galli* was performed in a pot experiment. The soil used in this experiment was sandy loam with a pH of 6.5, prepared by mixing soil with compost manure (2:1 ratio) and poured into plastic pots (15 cm diameter). Twenty seeds of *E. crus-galli* were placed in separated pots at 0.5 cm below the soil surface. The SCT was applied to the soil surface at quantities of 25, 50, and 100 kg a.i. ha^{-1}, and a non-treated pot served as a control. A solution of 2.5% of mixture from N,N-dimethylformamide and nonylphenol ethoxylate was used as the negative control. Seed germination was counted at 7 days after treatment, and plant height was determined at 7, 14, 21, and 28 days after treatment. The upper and lower ground parts of surviving seedlings were measured separately at 28 days after treatment. A completely randomized manner with four replicates was used in all experiments.

3.2.5.2 Bioassay demonstrating efficacy of SCT by foliar application

The effects of foliar-applied SCT on the growth and physiological mechanisms of *E. crus-galli* and *P. lathyroides* were studied in a greenhouse setting. Plastic pots of 15 cm diameter and 15 cm depth were filled with loam soil (soil: sand: manure 3:1:1, w/w). Fifteen seeds of *E. crus-galli* and *P. lathyroides* were sown at a depth of 1 cm in each pot. Two weeks after sowing, emergent plants were thinned to ten equal-sized healthy plants per pot. Various rates of SCT (10, 20, 40, and 80 kg a.i. ha^{-1}) were foliar-applied to growing weeds at 28 days after sowing using a hand pressure sprayer at a rate of 1,000 L ha^{-1}. The control was sprayed with distilled water. Four replicates per treatment were maintained in a completely randomized design. Cell membrane integrity, photosynthetic pigment content (chlorophyll a, chlorophyll b and carotenoids), and leaf malondialdehyde (MDA) content were evaluated at 1, 3, 5, and 7 days after treatment.

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3.2.5.3 Membrane integrity

Membrane integrity was estimated through the leakage of elelctrolytes, following the method of Lutts et al. (1996) and Ghobadi et al. (2011) with minor modifications. Ten freshly-cut leaf discs (0.6 cm diameter) were floated abaxial side up on 5 mL of distilled water in a test tube. The solution was incubated in a 40 °C water bath for 30 min before measuring the initial electrical conductivity (EC_1). Samples were then boiled at 100 °C for 15 min to release all electrolytes, cooled down to 25 °C, and their final electrical conductivities (EC_2) have measured. Electrolyte leakage (EL) was calculated according to the following formula (Lutts et al., 1996):

electrolyte leakage (%) = $(EC_1 / EC_2) \times 100$

3.2.5.4 Photosynthetic pigment contents

Chlorophyll and carotenoid contents were determined according to the method of Poonpaiboonpipat et. al. (2013). The assay was conducted using thirteen freshly-cut leaf 13 discs (0.6 cm diameter) extracted with 5 ml of aqueous 80% (v/v) acetone in a mortar jar and pestle. After 3 h incubation in the dark at room temperature, the suspension was filtered through Whatman No. 1 filter paper. Pigment contents in the supernatant were determined spectrophotometrically at 663, 647 and 470 nm for chlorophyll a, b and carotenoids using a Spectronic GENESYS 20 spectrophotometer (Thermo Electron Corporation, USA). Photosynthetic pigment contents were calculated using Lichtenthaler's equation (Lichtenthaler, 1987) and expressed as mg cm^{-2}.

chlorophyll a (mg/L) = $12.25 \times A663 - 2.79 \times A647$

chlorophyll b (mg/L) = $21.50 \times A647 - 5.10 \times A663$

carotenoids (mg/L) = $(1000 \times A470 - 1.82 \times Chl\ a - 85.02 \times Chl\ b)/198$

3.2.5.5 Lipid peroxidation (malondialdehyde [MDA] content)

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) content in the leaves using the thiobarbituric acid method described by Dong et al. (2014). Approximately 0.5 g fresh leaves was homogenized with 5 ml of 0.1% trichloroacetic acid (TCA) in an ice bath using a mortar jar and pestle. The homogenate was centrifuged at 10000 xg for 20 min. A 1 ml aliquot of supernatant was added to a test tube containing 4 ml of 0.5% thiobarbituric

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acid (TBA) prepared in 20% TCA and 400 µL 4% butylated hydroxytoluene (BHT) in ethanol. This mixture was heated in a boiling water bath at 95 °C for 30 min. After boiling, the reaction was quickly cooled in an ice bath for 10 min and then centrifuged at 6000 xg for 15 min. The absorbance of the supernatant was determined at 532 and 600 nm, and the concentration of MDA content was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}. The results were expressed as nmol g^{-1} FW.

$$\text{MDA (nmol } g^{-1} \text{ FW)} = (A532 - A600 / 155000) \times 10^6$$

3.2.6 Experiment 5. Phytotoxic effects of essential oil from *T. erecta* leaves

3.2.6.1 Plant materials and extraction of essential oils

Fresh leaves of *Tagetes erecta* were collected 45 days after planting in an experimental field at King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. Essential oil (EO) was produced from fresh, mature, healthy leaves by the boiling hydrodistillation method, using a 4 h period and a Clevenger-type apparatus. EOs was recovered from directly above the distillate without adding any solvent and then dried over anhydrous sodium sulfate until the water was completely removed. The EOs produced were kept at 4 °C in amber sealed sterile glass vials until use.

3.2.6.2 Identification of essential oil constituents by gas chromatography-mass spectrometry (GC-MS)

The compositions of *T. erecta* leaf essential oils were determined by gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890N gas chromatograph coupled to an Agilent 5973 mass detector and employing a HP-5 (Hewlett-Packard, Palo Alto, CA, USA) fused-silica capillary column (30 m x 250 µm ID, 0.25 µm film thickness). An electron ionization system; with ionization energy of 70 eV was used for this analysis. The carrier gas was helium, run at a flow rate of 1 ml min^{-1} with linear velocity of 29.6 cm s^{-1}. Typically, a 1 µl sample was injected with a split ratio of 1:100. The injection and detector temperatures were maintained at 250 °C and 290 °C, respectively. The oven temperature was set to increase from 50 °C to 165 °C at 4 °C min^{-1}, hold constant for 5 min, and then continue increasing to 290 °C at 10 °C min^{-1} before remaining isothermal for 10 min. Identifications of EO components were assigned by comparison

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of the Koviats retention indices (KIs) determined with reference to a homologous series of n-alkanes. Percentage composition was based on GC peak areas and retention times that were calculated with a Shimadzu CR6A data processor. GC operating parameters were identical with those of the GC analysis.

3.2.6.3 Preparations of emulsifier concentrate formulation

Emulsifier concentrate formulations of *T. erecta* essential oils (EC-EOs) were prepared using Tween 80 (30% v/v), DMF (20% v/v), and *T. erecta* EOs (50% v/v). The mixture was stirred using a homogenizer for 10 min. The obtained emulsifier concentrate formulations were evaluated for their soluble in water.

3.2.6.4 Allelopathic activity of essential oil

Allelopathic activity was determined by a seed germination and seedling growth bioassay. Mature seeds of *E. crus-galli* were collected from paddy fields at Ladkrabang, Bangkok, Thailand. After collection, the seeds were kept in packets at room temperature for 3 months, and then incubated in a hot-air oven at 45 °C for 48 h to break their dormancy. A preliminary test was carried out in order to discard unwanted and underdeveloped seeds. In order to insure the seeds were capable of germination, a primary evaluation was carried out, obtaining a germination percentage of 90%. To test the allelopathic activity of EC-EOs on *E. crus-galli* seeds, 5 ml of essential oil at different concentrations (250, 500, 1000, and 2000 $\mu L\ L^{-1}$) were added to Petri dishes lined with two sheets of Germitest paper (JProlab®). Then 20 seeds of *E. crus-galli* were placed in each Petri dish. A solution of Tween 80 mixed with DMF in distilled water served as the control. To prevent evaporation, the plates were sealed with parafilm and incubated in a growth chamber (Climacell 707, Munich, Germany) at 25-32 °C, 12 h/12 h dark/light photoperiod, light intensity of 100 $\mu mol\ m^{-2}\ s^{-1}$, and a relative humidity of ~80%. The rate of germination and length measurements of both root and shoot were measured at 7 days after treatment. Four replicates were performed for each treatment in a completely randomized design.

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3.2.6.5 Bioassay of seed imbibition and α-amylase activity

Seed imbibition in *E. crus- galli* seeds germinated in different concentrations of EC- EOs (250-2000 μL L^{-1}) and control was measured according to a previously described method (Turk and Tawaha, 2003). Imbibition was evaluated in 30 *E. crus-galli* seeds at 24, 36, and 48 h after exposure to treatments. Four replicate seed weights were recorded as the original seed weight (W_1), and then the final seed weight (W_2) taken for each concentration and exposure time. The seed imbibition percentage was calculated by the following equation:

$$\text{Seed imbibition (\%)} = [(W_2 - W_1) / W_1] \times 100$$

The activity of α- amylase (EC 3. 2. 1. 1) was assayed by the dinitrosalicylic acid (DNS) method, modified from Teerarak et al. (2012). After measuring water uptake, *E. crus-galli* seeds were ground to a fine powder in 4 ml of ice-cold 0.1 M $CaCl_2$. The homogenate was centrifuged at 12,000 rpm for 20 min, and the resulting supernatant used as the enzyme source. Enzyme activity was determined by measuring the reducing sugars liberated from soluble starch. One ml of enzyme solution was incubated for 15 min at 37 °C with 1 ml of 1% soluble starch in acetate buffer solution at pH 5.5 as substrate. The reaction was terminated by the addition of 1 ml DNS reagent (40 mM 3,5 dinitrosalicylic acid, 0.4 N NaOH, and 1 M K-Na tartrate) and immediately heated in a boiling water bath for 5 min before being cooled under running tap water. Color intensity was measured as absorption at 560 nm in a Spectronic GENESYS 20 spectrophotometer (Thermo Electron Corporation, USA). The blank solution used was identical with the test solution, except for including no enzyme. A standard graph was prepared using maltose, and α-amylase activity was calculated and expressed as μmol maltose min^{-1} g^{-1} (FW).

3.2.8 Statistical analysis

All experimental data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's Studentized Range Test to determine significant differences among mean values at the p=0.05 level.

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CHAPTER 4

RESULTS

4.1 Experiment 1. Inhibitory potential of aqueous extract and optimal solvent extract

4.1.1 Bioassay of aqueous extracts

Inhibition effects of stem, leaf, flower, and root aqueous extracts of *T. erecta* were assayed at concentrations of 12.5, 25, 50, and 100 mg mL^{-1} on seed germination and seedling growth of *E. crus-galli* and *P. lathyroides*, compared with the effects of distilled water control. Results indicated that degree of inhibitory was significantly different depending on concentration and plant parts extracted. The degree of inhibition increased with increasing concentrations. With respect to plant parts, leaf aqueous extract had the greatest inhibitory effect on germination of both *E. crus-galli* and *P. lathyroides*, with stronger effects on *P. lathyroides*. After leaf extract, flower extract showed the greatest inhibitory effect on *E. crus-galli*, followed by stem and root extracts, respectively (Figure 4.1, Figure 4.3); conversely, root extract had the second-strongest effect on *P. lathyroides*, followed by flower and stem extracts, respectively (Figure 4.2, Figure 4.4). For seedling growth (shoot and root length), results showed that all concentrations of aqueous extract, from all plant parts, acted to promote shoot and root growth of surviving *E. crus-galli* seedlings, except for the highest concentration of 100 mg mL^{-1}. In contrast, *P. lathyroides* shoot length was significantly inhibited by all extracts tested, while root length was inhibited by leaf extracts but other extracts (stem, flower, and root) either stimulated root growth of *P. lathyroides* or had no significant effect. In all cases tested, *P. lathyroides* was more sensitive to extracts than *E. crus-galli*.

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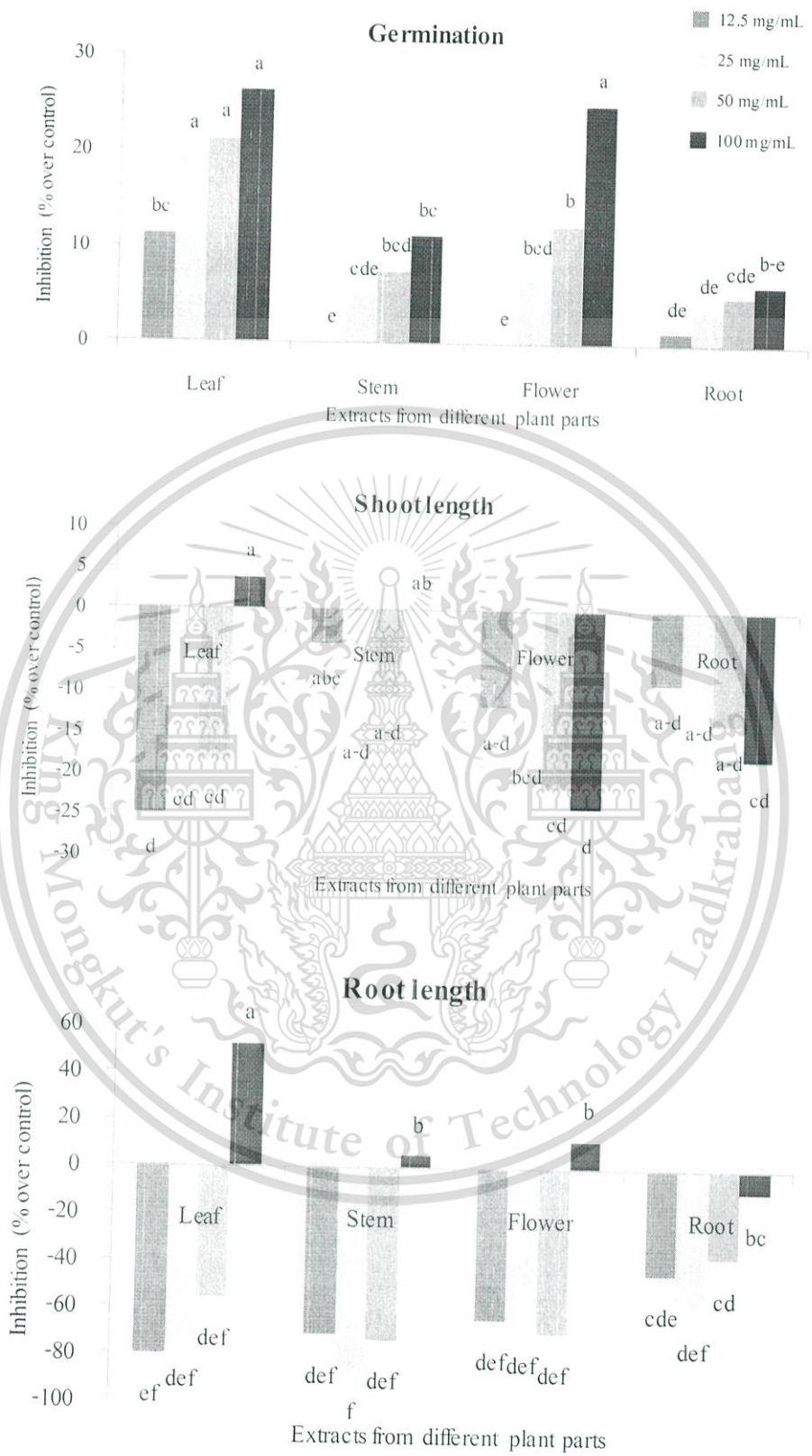

Figure 4.1 Effects of aqueous extracts of from different *T. erecta* plant parts on germination and seedling growth of *E. crus-galli* seeds at seven days after treatment. Means followed by the same letter(s) are not significantly different by Tukey's range test (p=0.05).

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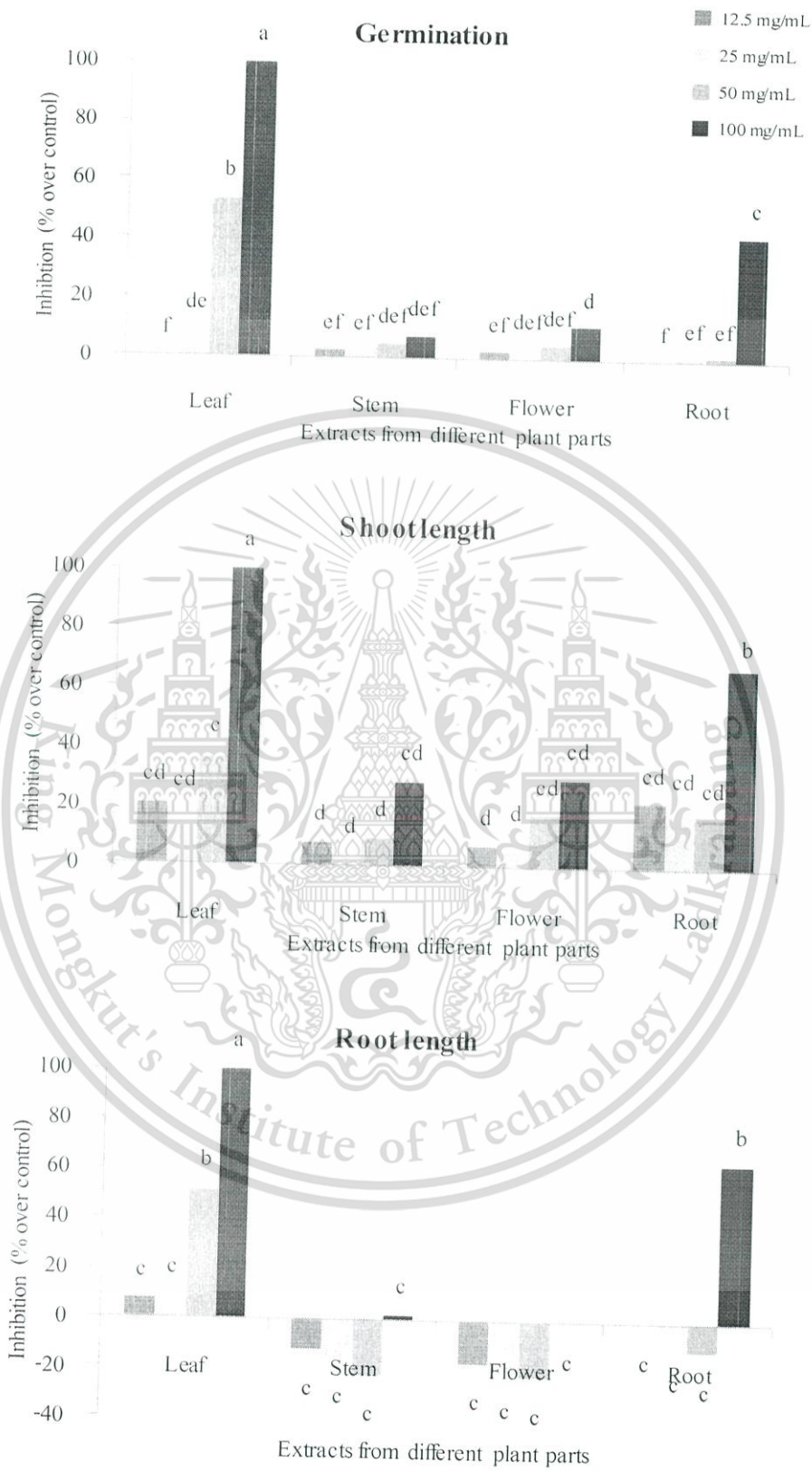

Figure 4.2 Effects of aqueous extracts of from different *T. erecta* plant parts on germination and seedling growth of *P. lathyroides* seeds at seven days after treatment. Means followed by the same letter(s) are not significantly different by Tukey's range test (p=0.05).

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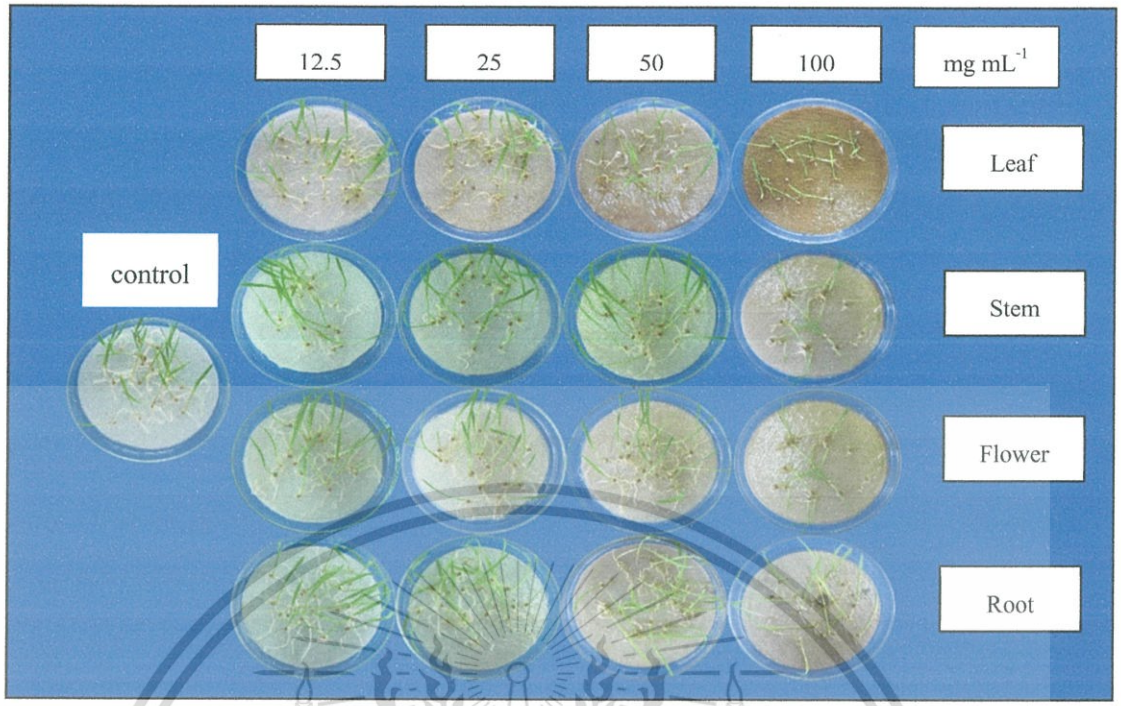

Figure 4.3 Inhibition effects of leaf, stem, flower, and root aqueous extracts of *T. erecta* on germination, shoot length, and root length of *E. crus-galli* seeds at seven days after treatment.

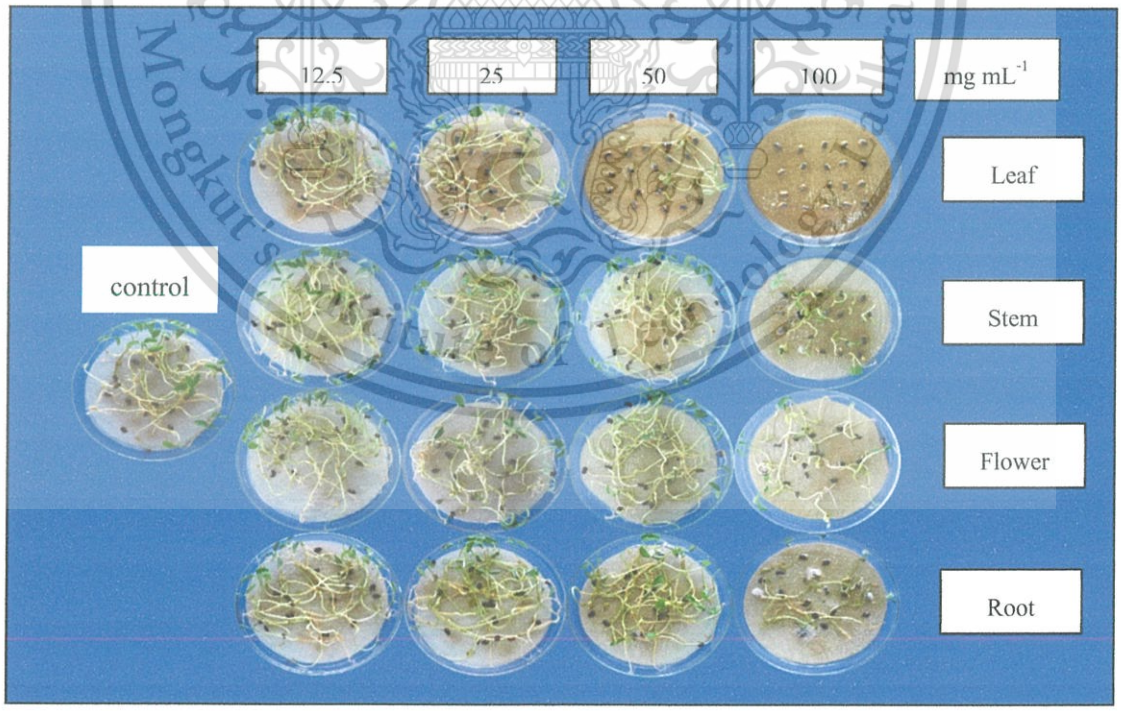

Figure 4.4 Inhibition effects of leaf, stem, flower, and root aqueous extracts of *T. erecta* on germination, shoot length, and root length of *P. lathyroides* seeds at seven days after treatment.

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4.1.2 Effect of solvent extraction method on crude extracts yield

Figure 4.5 shows the effects of different solvent systems and repeat extractions on the yield of the crude extract. For all solvent systems, total extraction yield was found to increase with additional extractions. *T. erecta* dried leaves powder was extracted with a mixture of ethanol in water with various ethanol percentages from 0-100% for 24 hours, three times of extraction was the optimal number. There was a markedly significant difference in the recoveries of total crude extracts from *T. erecta* dried leaves. Extraction yield increased with decreasing ethanol concentration, with the greatest recovery achieved by using 25% ethanol.

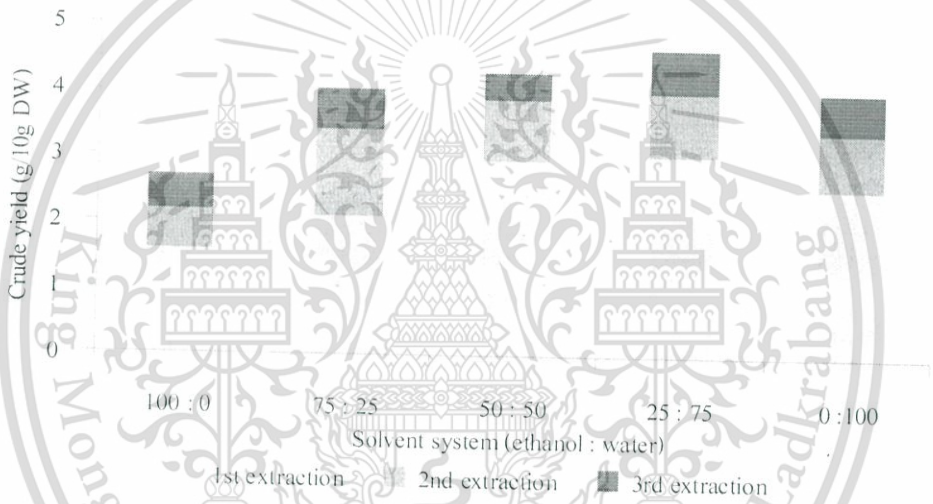

Figure 4.5 The effect of different ethanol percentage in water and extraction number on crude extraction yield from from *T. erecta*. dried leaves (1st extraction, 2nd extraction, 3rd extraction).

4.1.3 Bioassay of solvent extraction

Figure 4.6 and Figure 4.7 show that extracting the dried leaves of *T. erecta* with different solvent systems resulted in different effects on *E. crus-galli* and *P. lathyroides* seed germination and seedling growth in various ways. The degree of inhibitory was different depending on sources of crude extract and concentrations being tested. At 10,000 ppm concentration of crude extract obtained from 100%, 75%, 50%, 25%, and 0% ethanol in water, *E. crus-galli* seed germination was reduced by 3.75, 6.25, 2.5, 2.5 and 0% over control (distilled water) (Figure 4.8), and *P. lathyroides* seed germination was reduced by 36.25, 12, 5, 5, and 5% over control (Figure 4.9), respectively.

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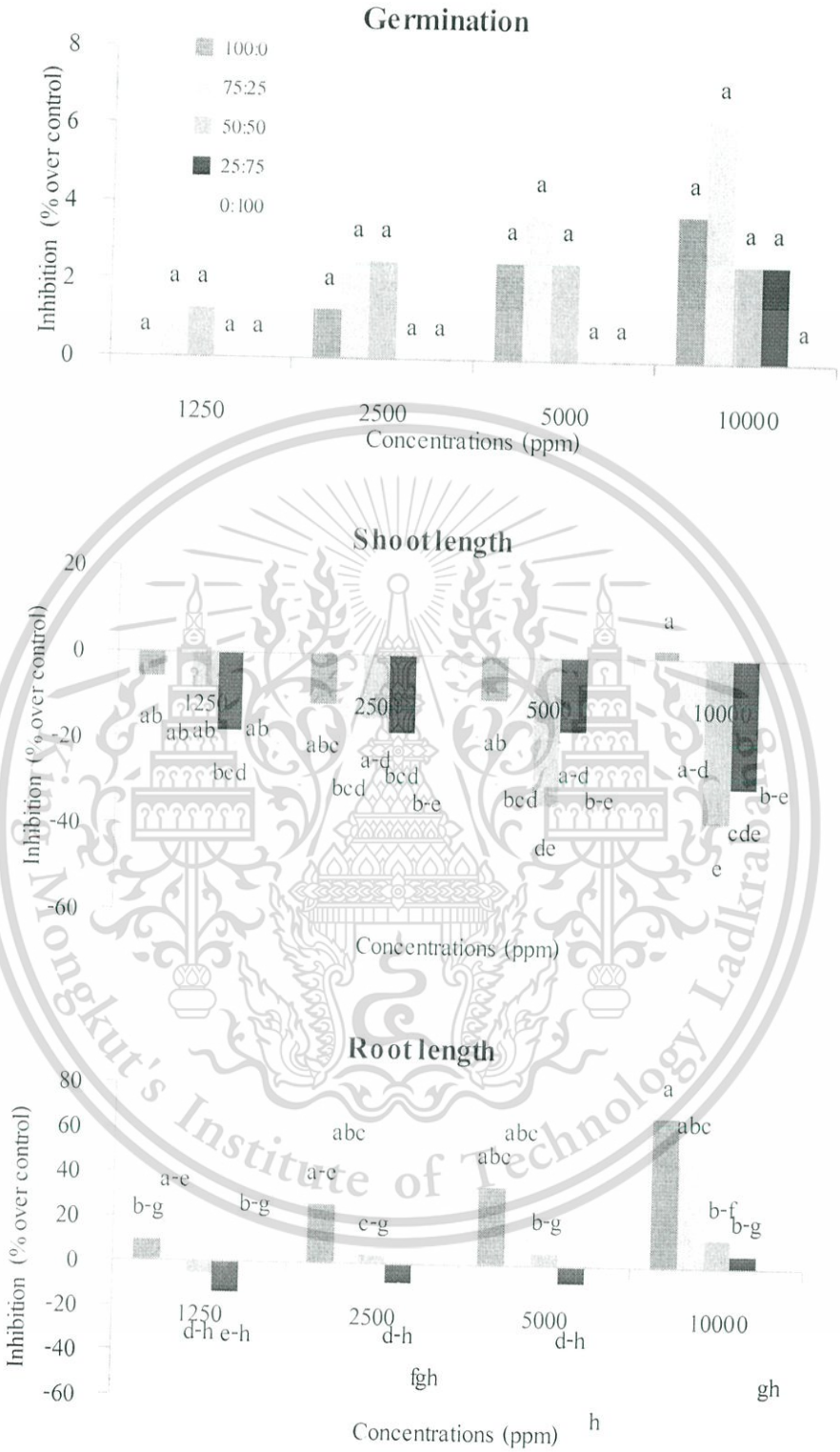

Figure 4.6 Effects of different solvent systems on inhibitory potential of *T. erecta* leaf crude extract against germination and seedling growth of *E. crus-galli* seeds at seven days after treatment. Means followed by the same letter(s) are not significantly different by Tukey's range test (p=0.05).

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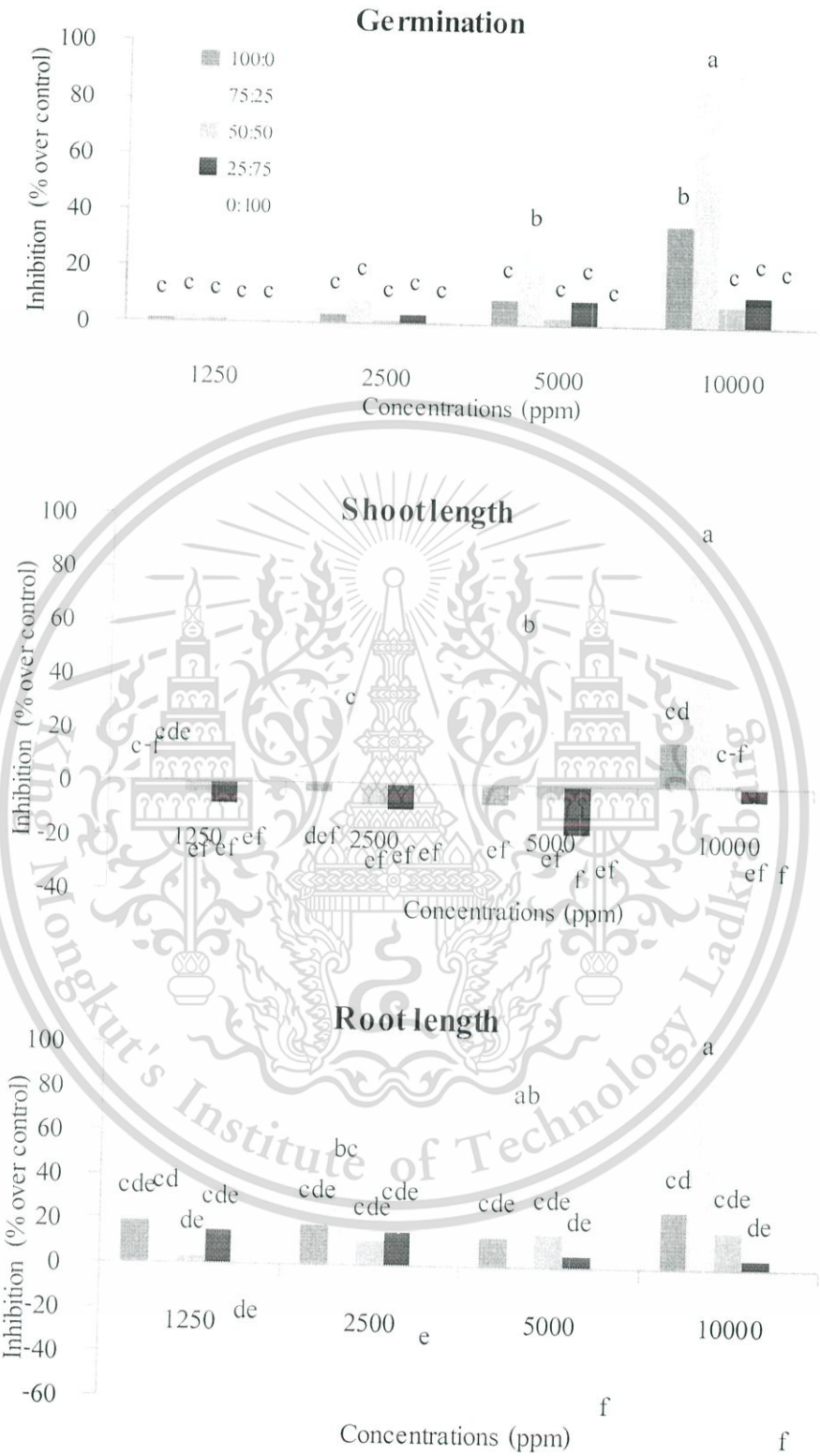

Figure 4.7 Effects of different solvent systems on inhibitory potential of *T. erecta* leaf crude extract against germination and seedling growth of *P. lathyroides* seeds at seven days after treatment. Means followed by the same letter(s) are not significantly different by Tukey's range test (p=0.05).

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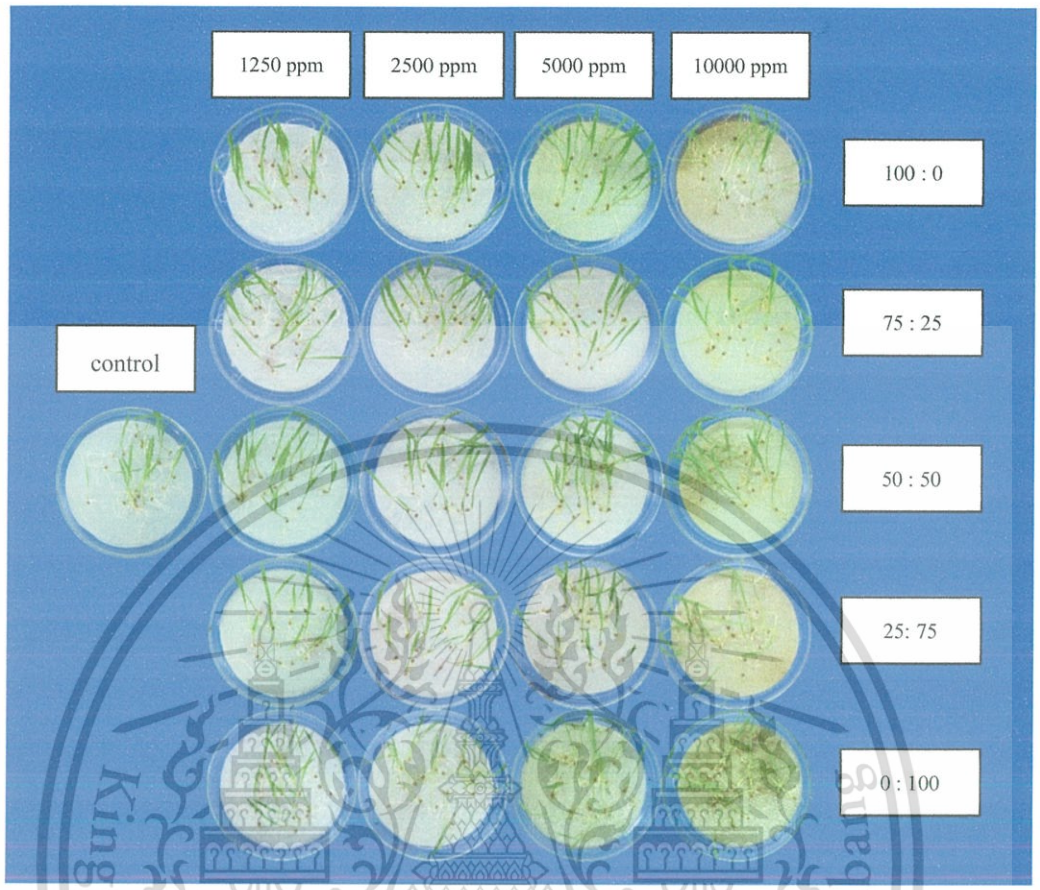

Figure 4.8 Effects of different solvent systems on inhibitory potential of *T. erecta* leaf crude extract against germination, shoot length, and root length of *E. crus-galli* seeds at seven days after treatment.

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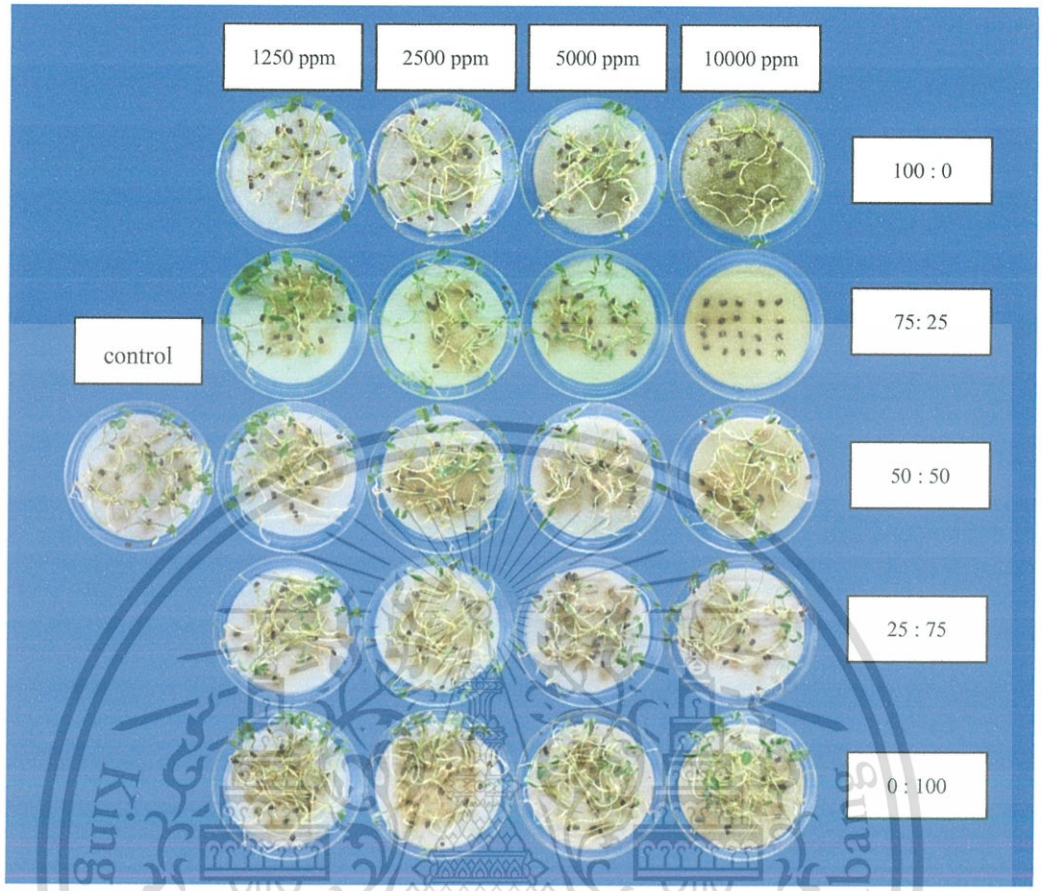

Figure 4.9 Effects of different solvent systems on inhibitory potential of *T. erecta* leaf crude extract against germination, shoot length, and root length of *P. lathyroides* seeds at seven days after treatment.

4.2 Experiment 2. Inhibitory potential, total phenolic content, and flavonoid content of various crude extract fractions

4.2.1 Crude extract yields after solvent partitioning

Table 4.1 shows the recovery yields of each crude fraction after solvent separation. The greatest yield was achieved in the aqueous fraction (AQ; 79.26%), followed by the neutral (NE; 10.25%) acidic (AE; 6.22%), and hydrolyzed (HY; 3.06%) fractions respectively. The total weight across the AQ, NE, and AE fractions was 98.79%; the reduction in weight is mainly because the regarding fraction was not corrected.

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Table 4.1 Recovered yields of each crude fraction after partial solvent partition from *T. erecta* crude extract.

Fraction	% crude extract
Crude ethanol (OR)	100.00
Aqueous (AQ)	79.26
Hydrolyzed (HY)	3.06
Neutral (NE)	10.25
Acidic (AE)	6.22

Crude extract obtained from *T. erecta* using 75% ethanol in water (OR) was separated into four fractions: aqueous (AQ), hydrolyzed (HY), neutral compound (NE), and acidic compound (AE). Bioassays were performed for all fractions to evaluate inhibitory activity on *E. crus-galli* seed germination and seedling growth at concentrations of 2,000 4,000, 8,000 and 16,000 ppm. The results showed that inhibitory activity differed significantly depending on both concentration and fraction (Figure 4.10). At 16,000 ppm, HY, NE, and AE fractions completely inhibited germination, shoot length, and root length; less effect was observed for OR and AQ fractions. Inhibition percentages for seed germination of HY, AE, NE, OR and AQ fractions at 8,000 ppm were 96.25%, 95.00%, 57.50%, 6.25%, and 2.50% over control, respectively. Regarding early seedling growth, the OR and AQ fractions promoted shoot growth at all concentrations, excepting the highest concentration of OR (16,000 ppm), which inhibited shoot growth by 19%, compared to the control. Conversely, shoot length was significantly inhibited at all concentrations of the HY, NE, and AE fractions. At a concentration of 8,000 ppm, shoot lengths for the HY, NE, and AE fractions were 55.51%, 29.69%, and 67.59% over control, respectively. Root length results were similar to the findings for shoot length (Figure 4.12).

With regard to *P. lathyroides* (Figure 4.11), the OR fraction at a concentration of 16,000 ppm showed strong inhibitory activity, giving 88.75%, 98.63%, and 98.73% inhibition of *P. lathyroides* germination, shoot length, and root length, respectively. After solvent partitioning, relative inhibition increased compared to the OR fraction. The HY and AE fractions showed the greatest activity with complete inhibition of germination, shoot length, and root length of *P. lathyroides* seedlings at a concentration of 16,000 ppm. At 8,000 ppm, seed germination inhibition percentages for the AE, HY, OR, AQ, and NE fractions were 93.75%, 63.75%, 31.25%, 7.50%, and 6.25% over control, respectively. The NE and AQ fractions showed weaker inhibitory effects

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on *P. lathyroides* germination, shoot length, and root length when compared with the OR fraction (Figure 4.13). These results indicate that most of the phytotoxic compounds produced by *T. erecta* could be present in the HY and AE fractions.

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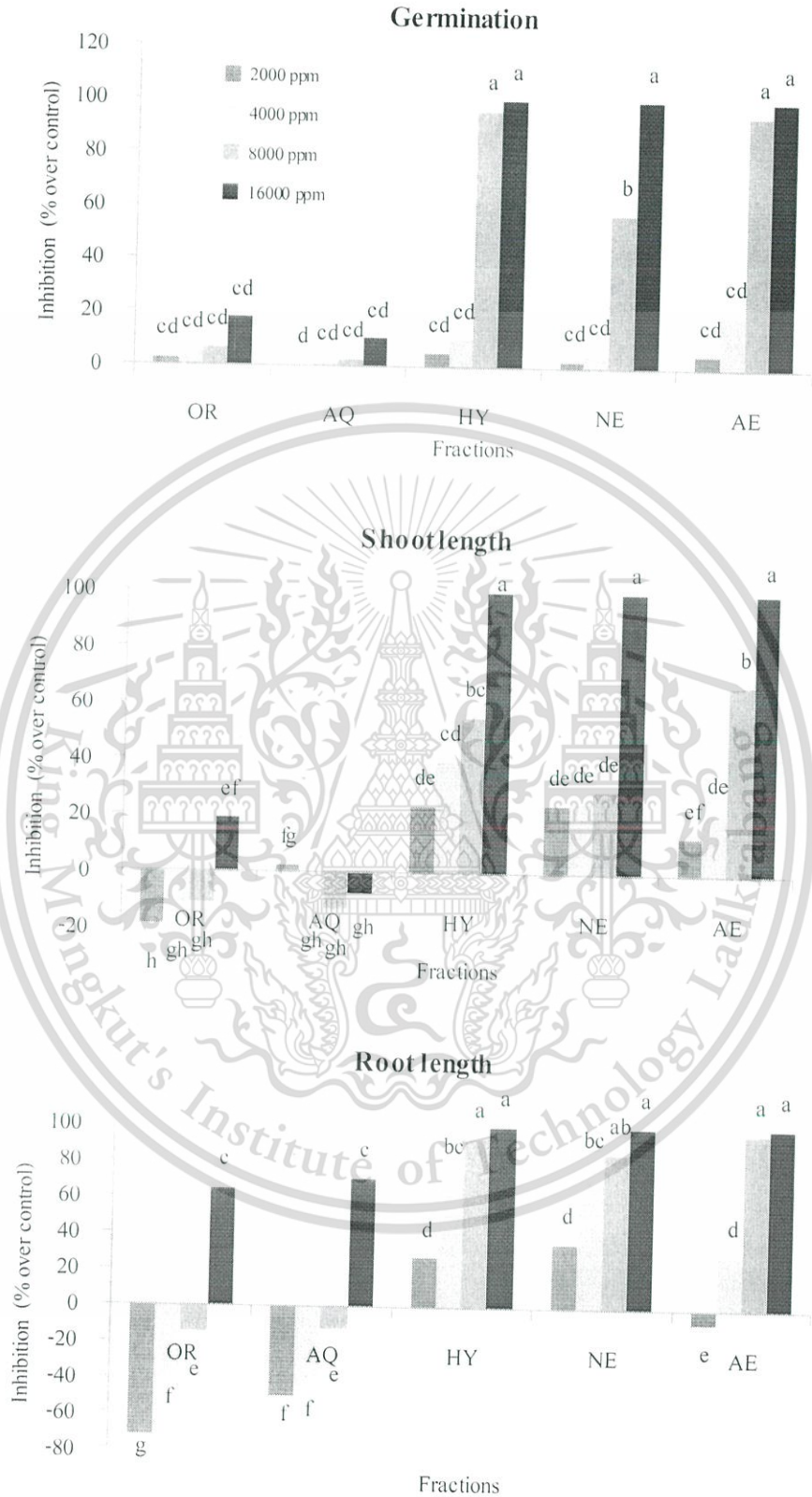

Figure 4.10 Effects of acid-base solvent partitioned *T. erecta* leaf extracts on germination and seedling growth of *E. crus-galli* at seven days after treatment. Means followed by the same letter(s) are not significantly different by Tukey's range test (p=0.05).

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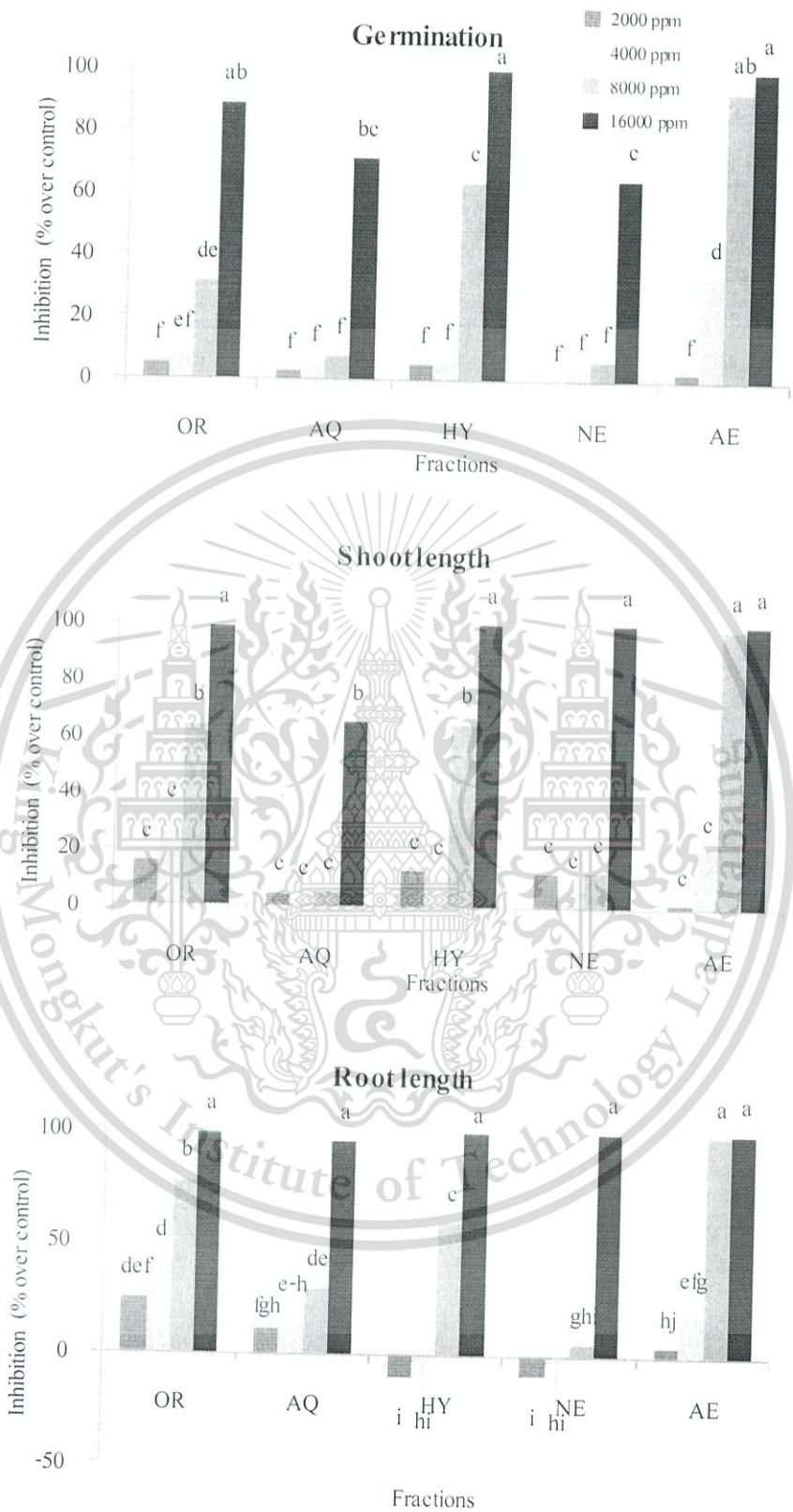

Figure 4.11 Effects of acid-base solvent partitioned *T. erecta* leaf extracts on germination and seedling growth of *P. lathyroides* at seven days after treatment. Means followed by the same letter(s) are not significantly different by Tukey's range test (p=0.05).

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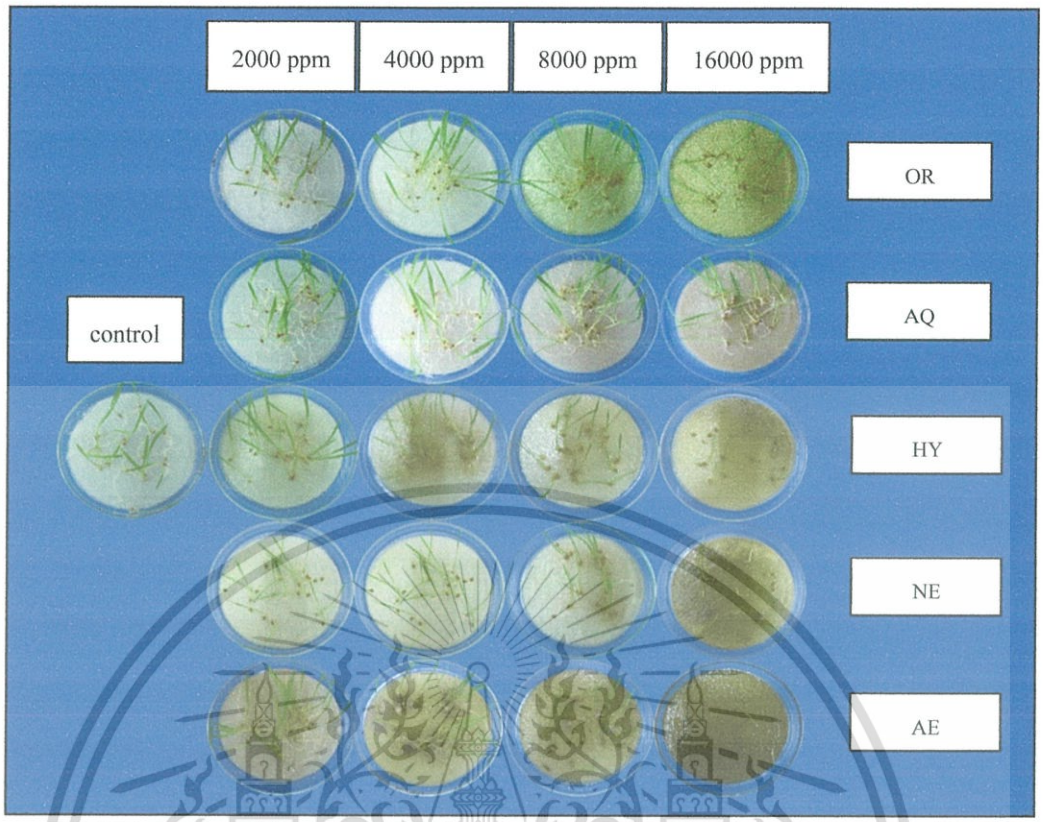

Figure 4. 12 Inhibitory effects of acid- base solvent partitioned *T. erecta* leaf extracts on germination, shoot length, and root length of *E. crus-galli* seeds at seven days after treatment.

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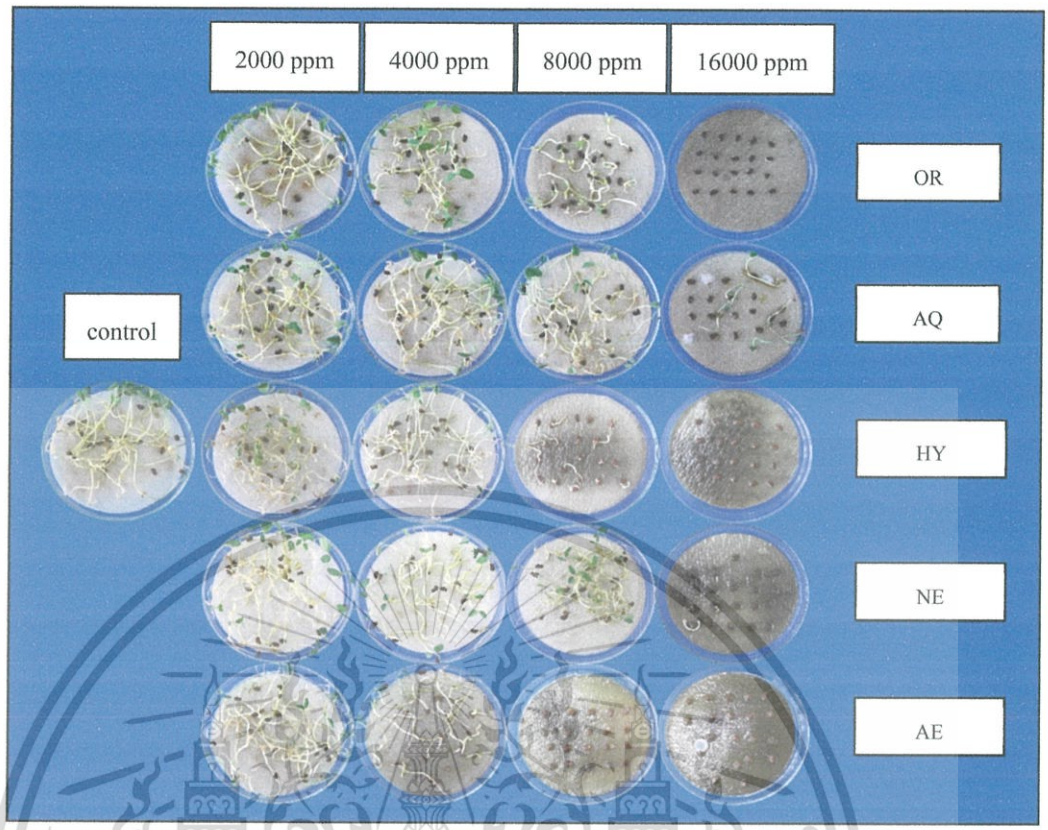

Figure 4. 13 Inhibitory effects of acid- base solvent partitioned *T. erecta* leaf extracts on germination, shoot length, and root length of *P. lathyroides* seeds at seven days after treatment.

4.2.2 Determination of total phenolics and flavonoids

Standard curves was used for the determination of total phenolics and flavonoids using different concentrations of gallic acid and quercetin. Results for the various fractions are presented in Table 4.2.

Total phenolic contents of the fractions ranged from 13.65 to 217.71 mg gallic acid equivalent of crude (mg GAE/g of crude). Among the five fractions, the AE (217.71±14.98) and HY (150. 72±2. 59) fractions had higher amounts of total phenolic compounds than OR (68.08±0.31), AQ (22.06±1.73) and NE (13.65±0.36). Similarly, concentrations of flavonoids were significantly higher in HY (5.11±0.19 mg of quercetin equivalent/g of crude [mg QE/g of crude]) and AE (2.52±0.15) fractions, while in the OR, AQ and NE fractions these were 2.04±0.09, 0.29±0.02 and 0.58±0.05 mg QE/g of crude, respectively. The greater concentrations of phenolic compounds could explain the higher inhibition responses observed for HY and AE. These differences between the various fractions are expected because each contains different types of

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phenolic compounds. There was a very good relationship between total phenolic content and the seed germination results for the original extract and the four fractions used in this study (R = 0.76). The relationship between flavonoid content and seed germination was also reasonably good (R = 0.67) (Table 4.3).

Table 4.2 Total phenolic and flavonoid contents of different crude fractions of dried-leaf extract of *T. erecta*. OR, hydroethanolic crude extract; the four fractions: HY, hydrolyzed; AQ, aqueous; NE, neutral; and AE, acidic; GAE, gallic acid equivalent; QE, quercetin equivalent. See text for further details.

Fraction	Phenolic acid content mg GAE/1g of crude extract	Flavonoid content mg QE/1g of crude extract
OR	68.08 ± 0.31	2.04 ± 0.09
AQ	22.06 ± 1.73	0.29 ± 0.02
HY	150.72 ± 2.59	5.11 ± 0.19
NE	13.65 ± 0.36	0.58 ± 0.05
AE	217.71 ± 14.98	2.52 ± 0.15

Table 4.3 Correlation coefficients (r) of total phenolic and flavonoid contents in *T. erecta* extracts and inhibition bioassay results.

	Total phenolic content	Total flavonoid content	Germination	Shoot growth	Root growth
Total phenolic content	-	0.7050	0.7551	0.7895	0.5688
Total flavonoid content	0.7050	-	0.6674	0.6067	0.4777

4.3 Experiment 3. Studies on physiological effects of compounds and formulated product

4.3.1 Mode of action of natural herbicide from *T. erecta*

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The HY fraction obtained from the hydroethanolic crude extract was formulated into a soluble concentrate (SCT), prepared as described above. Test concentrations (2,000 to 8,000 ppm) ranged in pH from 5.32 to 6.41 and in EC values from 2.23 to 3.43 mS cm^{-1} (data not shown). These results indicated that pH and EC values of all test concentrations could be considered as non-influential factors in bioassays. It is clear from the bioassay results (Figure 4.14) that SCT strongly inhibited seed germination and seedling growth of *E. crus-galli* compared to controls. At seven days after treatment, germination of seeds treated with SCT at concentration of 2,000, 4,000, 6,000, and 8,000 ppm was inhibited by 43.75%, 66.25%, 92.5%, and 100%, respectively. In general, inhibition of root growth was greater than of shoot growth. At 6,000 ppm SCT, root and shoot length declined by 96.96% and 76.19%, respectively. Bioassay results for the seed germination and seedling growth of *P. Lathyroides* are shown in Figure 4.14. At seven days after treatment, 8,000 ppm SCT completely inhibited germination and seedling growth. At 6,000 ppm SCT, inhibition of seed germination, shoot length, and root length was 55.00%, 40.60%, and 62.32% over control, respectively.

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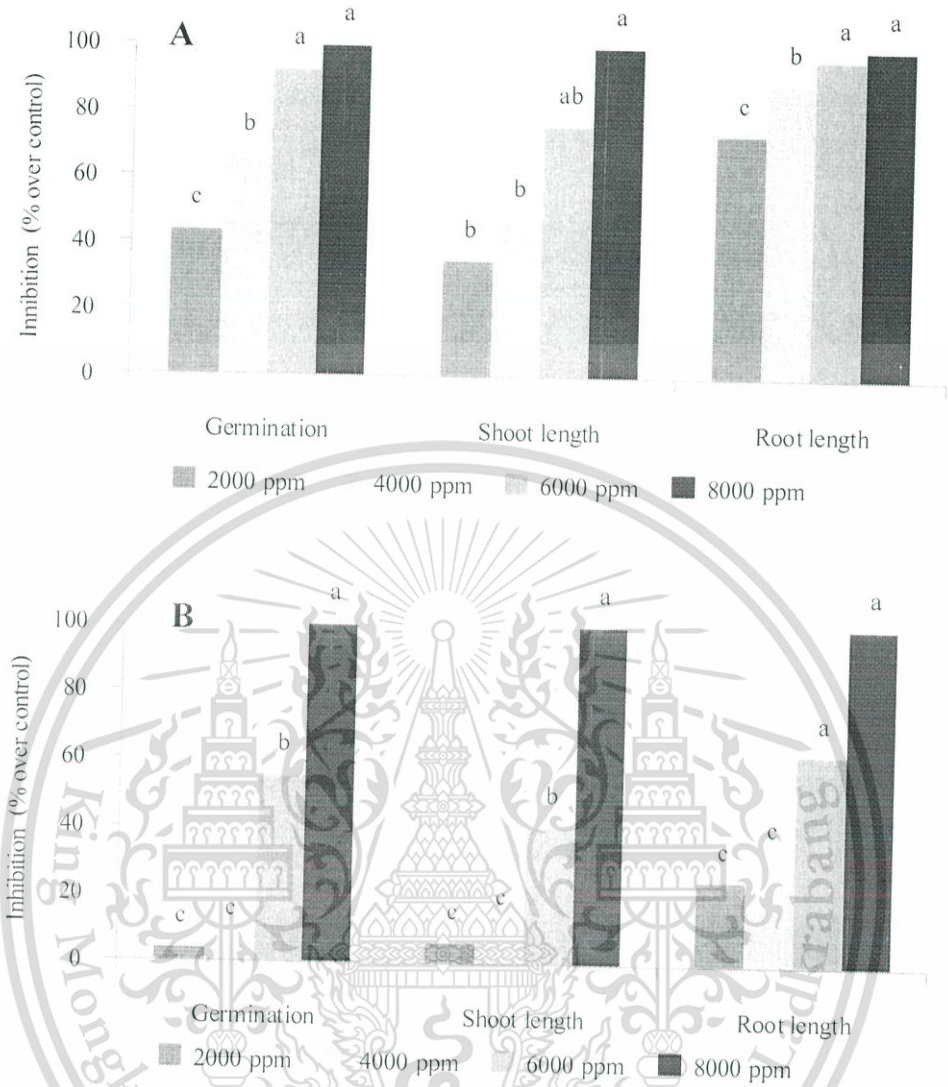

Figure 4.14 Inhibitory effect of natural herbicide soluble concentrate formulation (SCT) produced from *T. erecta* on the germination and seedling growth of *E. crus-galli* (A) and *P. lathyroides* (B) at seven days after treatment. Means followed by the same letter(s) are not significantly different by Tukey's range test (p=0.05).

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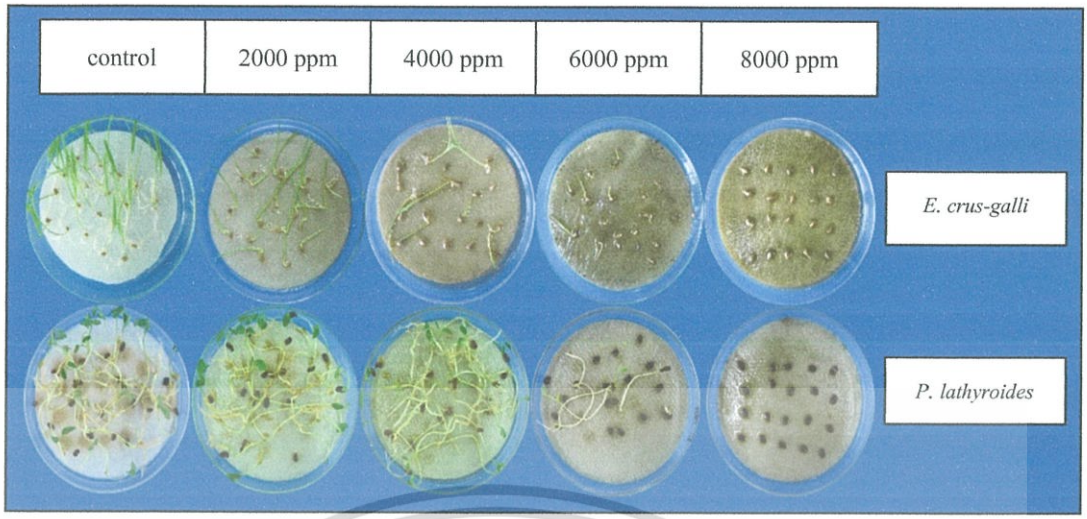

Figure 4.15 Inhibitory effects of natural herbicide soluble concentrate formulation (SCT) produced from *T. erecta* on the germination, shoot length, and root length of *E. crus-galli* and *P. lathyroides* seeds at seven days after treatment.

4.3.2 Effect on seed imbibition and α-amylase activity

The results of seed imbibition and α-amylase activity assays on *E. crus-galli* after soaking for different periods (24, 36, and 48 h) in various concentrations of SCT are shown in Figure 4.15. Seed imbibition and α-amylase activity increased with soaking time but decreased with increasing SCT concentrations. The percentage seed imbibition with soaking in water (control) for 24, 36, or 48 h was 23.08%, 27.09%, and 45.11%, respectively. Meanwhile, α-amylase activities for the same periods were 2.78, 3.28, and 14.37 μmol maltose min^{-1} g^{-1} (FW), respectively. There was significant reduction in seed imbibition after 48 h with any SCT concentration; imbibition values for 2,000, 4,000, 6,000 and 8,000 ppm were 38.49%, 37.02%, 29.14%, and 28.25%, respectively. Treatment with SCT inhibited α-amylase activities after 48 h at all concentrations (2,000 to 8,000 ppm). Increasing the concentrations of SCT increased α-amylase inhibition, and to a greater degree than for imbibition.

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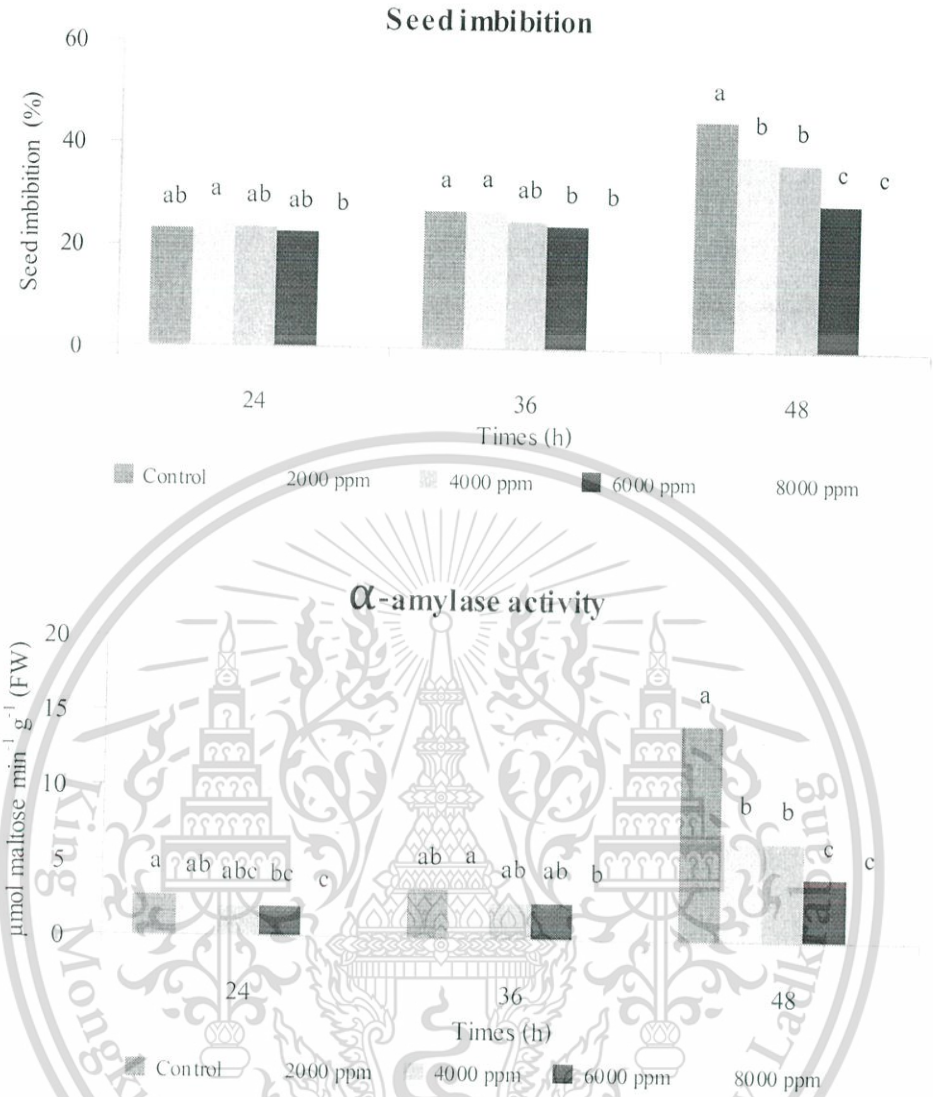

Figure 4.16 Effects of natural herbicide soluble concentrate formulation (SCT) produced from *T. erecta* in on imbibition and α-amylase activity in *E. crus-galli* seeds after different soaking times. Means followed by the same letter(s) are not significantly different by Tukey's range test (p=0.05).

Seed imbibition and α- amylase activity results for *Amaranthus gracilis* after different soaking periods (6, 12, and 24 h) in SCT concentrations of 500, 750, 1,000, and 1,250 ppm are shown in Figure 4.16. Seed imbibition percentages for the control at 6, 12, and 24 h were 22.86%, 26.37%, and 31.46%, respectively. The α-amylase activity values for the control at 6, 12 and 24 h were 11.52, 23.50, and 31.55 µmol maltose min^{-1} g^{-1} (FW). After soaking time at 24 h, α-amylase activityvalues for SCT concentrations of 500, 750, 1,000, and 1,250 ppm of were 24.44,

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22.41, 21.03, and 20.71 μmol maltose min^{-1} g^{-1} (FW), respectively. Seed imbibition and α-amylase activity was increased by prolonging the soaking period and with decreasing concentrations of SCT.

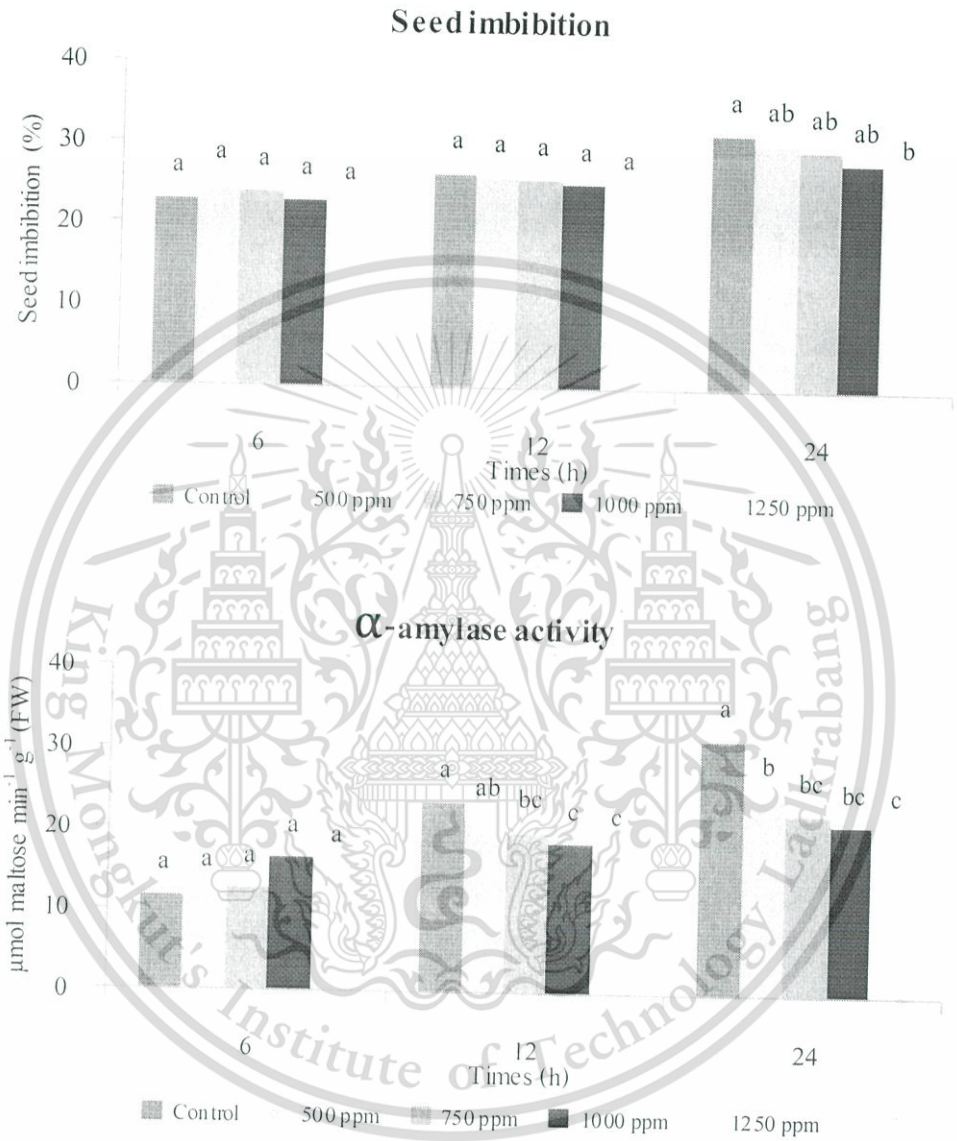

Figure 4.17 Effects of natural herbicide soluble concentrate formulation (SCT) produced from *T. erecta* on imbibition and α-amylase activity in *A. gracilis* seeds after different soaking times. Means followed by the same letter(s) are not significantly different by Tukey's range test (p=0.05).

4.3.3 Cytotoxicity

Differences in mitotic indices of treated onion root tips are presented in Table 4.4. The results clearly indicate that mitotic index decreases with exposure time to SCT, with all SCT

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concentrations causing total inhibition of mitotic activity. Treatment with SCT also changed the proportions of mitotic phases, depending on concentration. Phase proportions in the controls were 78.59% (prophase), 17.17% (metaphase), 3.48% (anaphase), and 1.25% (telophase). Treatment with SCT increased the proportion in prophase and decreased those in metaphase, anaphase, and telophase. There were significant differences between control and treated groups in the mitotic phase index.

Treatments also induced a wide range of mitotic abnormalities (see Table 4.5 and Figure 4.17). The predominant abnormalities were spindle disturbance in late prophase (Figure 4.17f), c-mitosis (Figure 4.17g), and sticky metaphase (Figure 4.17h). In addition, low frequencies of sticky anaphase (Figure 4.17i), delayed anaphase (Figure 4.17j), and diagonal at telophase (Figure 4.17k) were recorded. The percentage of total abnormalities observed did not depend on SCT concentration; all abnormality frequencies were higher with SCT than in the controls, although not all such differences were significant. The percentage of total abnormalities after SCT treatment increased by a range of 2.27% to 2.91%.

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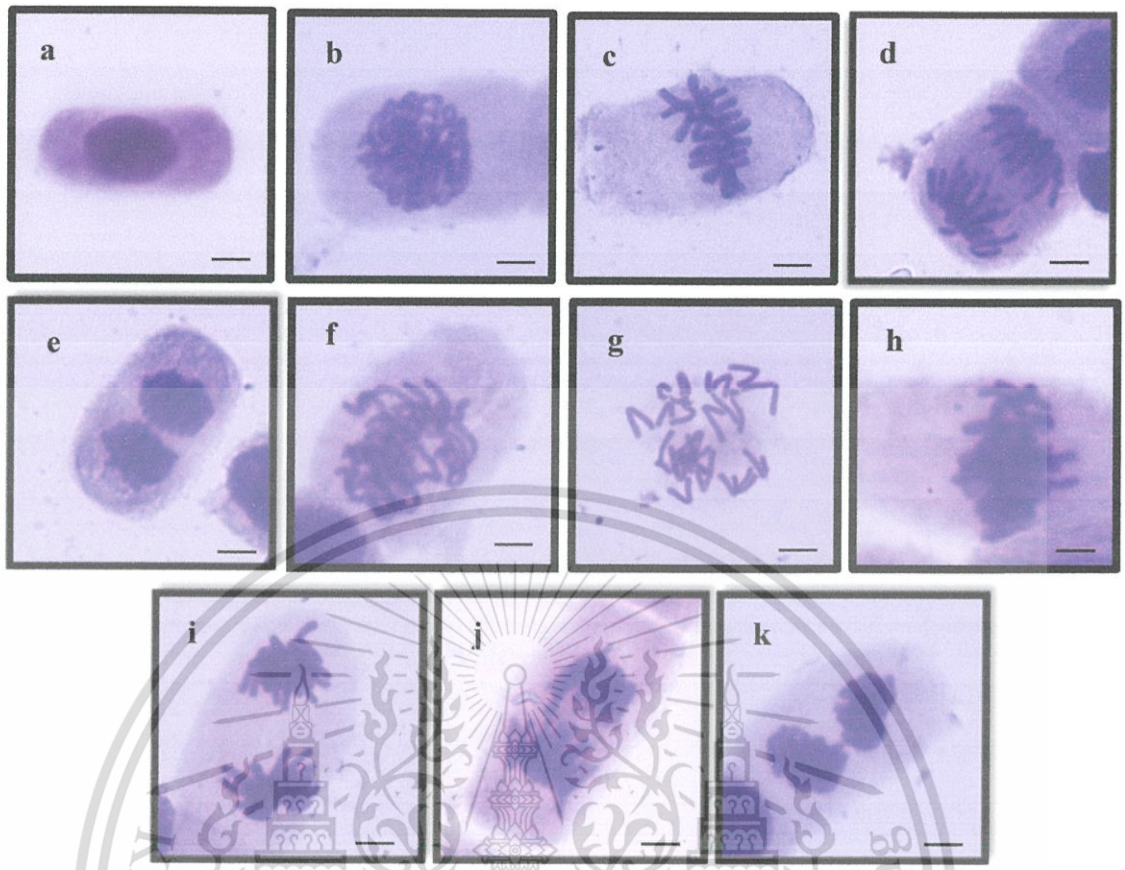

Figure 4.18 Meristematic cells of *Allium cepa* L. exposed to different concentrations of soluble concentrate formulation produced from *T. erecta*. (a) normal interphase; (b) normal prophase; (c) normal metaphase; (d) normal anaphase; (e) normal telophase; (f) spindle distribution at prophase; (g) c-metaphase; (h) sticky metaphase; (i) sticky anaphase; (j) anaphase bridge; (k) diagonal at anaphase. Bars represent 10 μm.

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Table 4.4 Mitotic index and mitotic phase index at 18 h in the meristematic cells of *Allium cepa* L. roots treated with various concentrations of the soluble concentrate formulation extracted from *T. erecta*.

Concentration (ppm)	Cells examined (n)	Cells in mitosis (mean ± S.E.)	Mitotic index (mean ± S.E.)	Mitotic phase index			
				Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)
control	4479	100.25 ± 2.87 a	8.95 ± 0.39 a	78.59 d	17.17 a	3.48 a	1.25 a
12.5	4543	98.75 ± 5.56 ab	8.69 ± 0.46 ab	87.63 c	9.91 b	1.23 b	1.47 a
25	4621	99.50 ± 1.29 ab	8.61 ± 0.19 ab	89.45 bc	8.29 bc	1.00 b	1.26 a
50	4692	98.50 ± 2.38 ab	8.40 ± 0.15 ab	90.62 bc	7.35 bc	0.76 b	1.27 a
100	4612	92.50 ± 3.79 bc	8.03 ± 0.49 bc	93.00 ab	5.41 cd	0.53 b	1.06 a
200	4628	86.75 ± 2.22 c	7.50 ± 0.18 c	96.54 a	2.58 d	0.29 b	0.59 a

In each column, means having the same letter are not significantly different (p<0.05) based on Tukey's Studentized Range Test.

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Table 4.5 Chromosome aberrations at 18 h in the meristematic cells of *Allium cepa* L. roots treated with various concentrations of the soluble concentrate formulation extracted from *T. erecta*.

Concentration (ppm)	Cells examined (n)	Spindle disturbance at late prophase (%)	Sticky metaphase (%)	c-metaphase (%)	Diagonal at anaphase (%)	Sticky anaphase (%)	Delayed anaphase (%)	Total abnormalities (%)
control	4,479	0.00	0.00	0.00	0.00	0.00	0.00	0.00 ± 0.00 b
12.5	4,543	1.39	0.73	0.26	0.11	0.13	0.09	2.71 ± 0.37 a
25	4,621	1.66	0.69	0.17	0.09	0.11	0.11	2.83 ± 0.51 a
50	4,692	1.88	0.62	0.15	0.06	0.11	0.11	2.91 ± 0.39 a
100	4,612	1.95	0.48	0.11	0.04	0.09	0.06	2.74 ± 0.29 a
200	4,628	1.84	0.28	0.06	0.02	0.04	0.02	2.27 ± 0.20 a

In each column, means having the same letter are not significantly different ($p<0.05$) based on Tukey's Studentized Range Test.

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4.4 Experiment 4. Herbicidal potential of natural herbicide from *T. erecta*

4.4.1 Soil application bioassay

The effectiveness of soil-applied soluble concentrate formulation (SCT) of natural herbicide produced from *T. erecta* was tested by bioassay. Emergence and seedling growth (plant height and dry weight) of *E. crus-galli* was found to be significantly suppressed by soil surface application of SCT when compared with the control (Figure 4.18). However, the lowest dose (25 kg a.i. ha^{-1}) of SCT did not reduce emergence and growth of *E. crus-galli*; instead, plant growth and dry weight was slightly increased at this amount. With increasing doses, emergence of *E. crus-galli* as well as the plant height and dry biomass all declined. At the highest dose of 100 kg a.i. ha^{-1}, seed germination was 82.50% inhibited, while plant growth and dry weight were reduced by 65.00% and 87.50%, respectively.

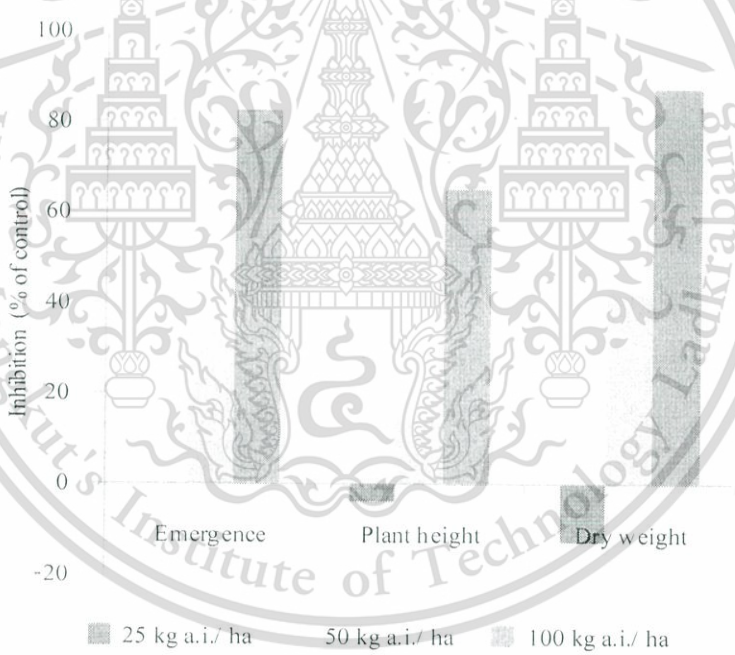

Figure 4.19 Effects of soil-applied natural herbicide in soluble concentrate formulation (SCT) produced from *T. erecta* on emergence, plant height, and dry weight of *E. crus-galli* (L.) Beauv.

4.4.2 Effects of foliar spray on membrane stability

Electrolyte leakage is an indicator of cell membrane stability, and was determined for *E. crus-galli* and *P. lathyroides* leaves at 1, 3, 5, and 7 days after treatment. Marked differences

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were observed depending on the concentration of SCT and timepoint after treatment. At seven days after treatment, foliar spraying at 10, 20, 40, and 80 kg a.i. ha^{-1} resulted in *E. crus-galli* leaf membrane stability indexes of 88.31%, 88.01%, 87.72%, and 86.64%, respectively, while *P. lathyroides* leaves had membrane stability indexes of 88.31%, 88.01%, 87.72%, and 86.64%, respectively (Table 4.6 and Table 4.7).

Table 4.6 Membrane stability indexes for leaf discs of *E. crus-galli* after treatment with SCT for 1, 3, 5, and 7 days.

SCT rate	Membrane stability index (%)			
(kg a.i. ha^{-1})	1 DAT	3 DAT	5 DAT	7 DAT
Control	94.57 a	95.54 a	95.74 a	96.18 a
10	93.03 a	92.88 b	92.17 ab	88.31 a
20	92.10 a	91.91 bc	90.98 ab	88.01 a
40	91.85 a	90.77 c	89.85 ab	87.72 a
80	88.12 b	87.54 d	87.45 b	86.64 a

In each column, means having the same letter are not significantly different according to Tukey's Studentized Range Test at the $p<0.05$ level. DAT, days after treatment.

Table 4.7 Membrane stability indexes for leaf discs of *P. lathyroides* after treatment with SCT for 1, 3, 5, and 7 days.

SCT rate	Membrane stability index (%)			
(kg a.i. ha^{-1})	1 DAT	3 DAT	5 DAT	7 DAT
Control	82.79 b	85.20 a	87.34 a	89.22 a
10	87.18 a	85.43 a	84.65 ab	83.86 b
20	86.98 a	85.19 a	84.29 ab	83.23 b
40	86.15 a	84.63 a	83.05 b	81.47 b
80	85.76 a	83.42 a	81.79 b	79.38 b

In each column, means having the same letter are not significantly different according to Tukey's Studentized Range Test at the $p<0.05$ level. DAT, days after treatment.

4.4.3 Effects of foliar spray on photosynthetic pigment contents

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Photosynthetic pigment content is an indicator of photosynthetic activity, and was evaluated for *E. crus-galli* and *P. lathyroides* leaves at 1, 3, 5, and 7 days after treatment. The photosynthetic pigments measured were chlorophyll a, chlorophyll b and carotenoids content. All photosynthetic pigments of treated *E. crus-galli* and *P. lathyroides* leaves were found to be significantly different depending on SCT concentrations and tested species (Figure 4.20 and Figure 4.21). For treated *E. crus-galli* leaves, with the increasing SCT concentrations, all photosynthetic pigment contents showed no significant difference from the control throughout the experiment. For *P. lathyroides* leaves, chlorophyll a was significantly decreased with an increasing SCT concentrations from 3 to 7 days after treatment meanwhile chlorophyll b and carotenoid contents showed no significant difference from the control except on 7 days after treatment. At the highest concentration (80 kg a.i. ha^{-1})and longest evaluation time (seven days), *E. crus-galli* leaves had chlorophyll a, b, and carotenoid contents of 20.06, 5.39 and 3.31 μg cm^{-2}, respectively, compared to control values of 23.37, 8.22, and 5.56 μg cm^{-2}, respectively. At the same treatment and timepoint, *P. lathyroides* leaves had chlorophyll a, b, and carotenoid contents of 10.91, 4.57, and 2.89 μg cm^{-2}, respectively, compared to control values of 19.10, 7.28, and 4.76 μg cm^{-2}, respectively.

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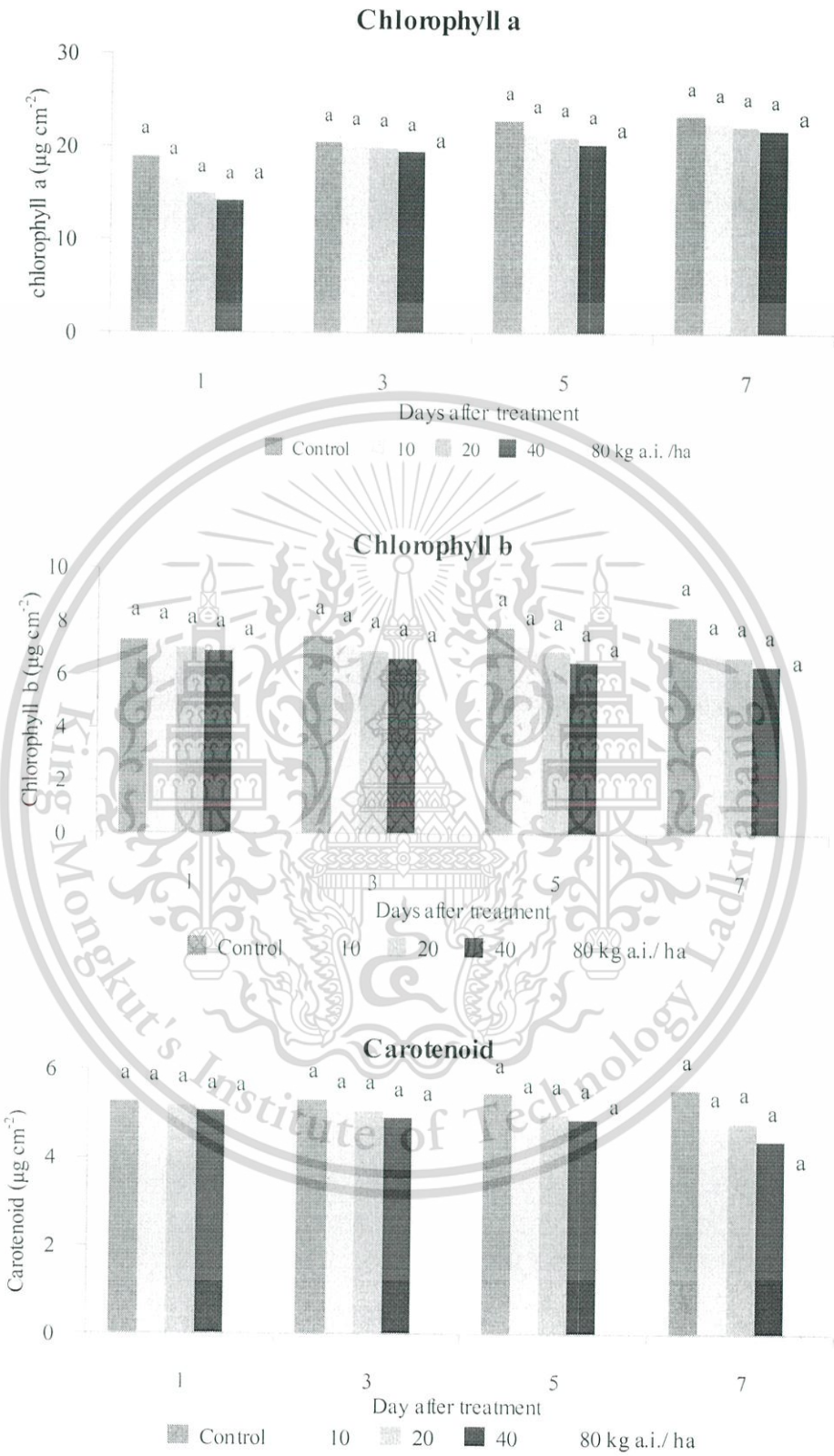

Figure 4.20 Effects of foliar applied SCT on chlorophyll a, b, and carotenoid contents (µg cm^{-2}) of *E. crus-galli* leaves at 1, 3, 5, and 7 days after treatment. Means followed by the same letter(s) are not significantly different by Tukey's range test (p=0.05).

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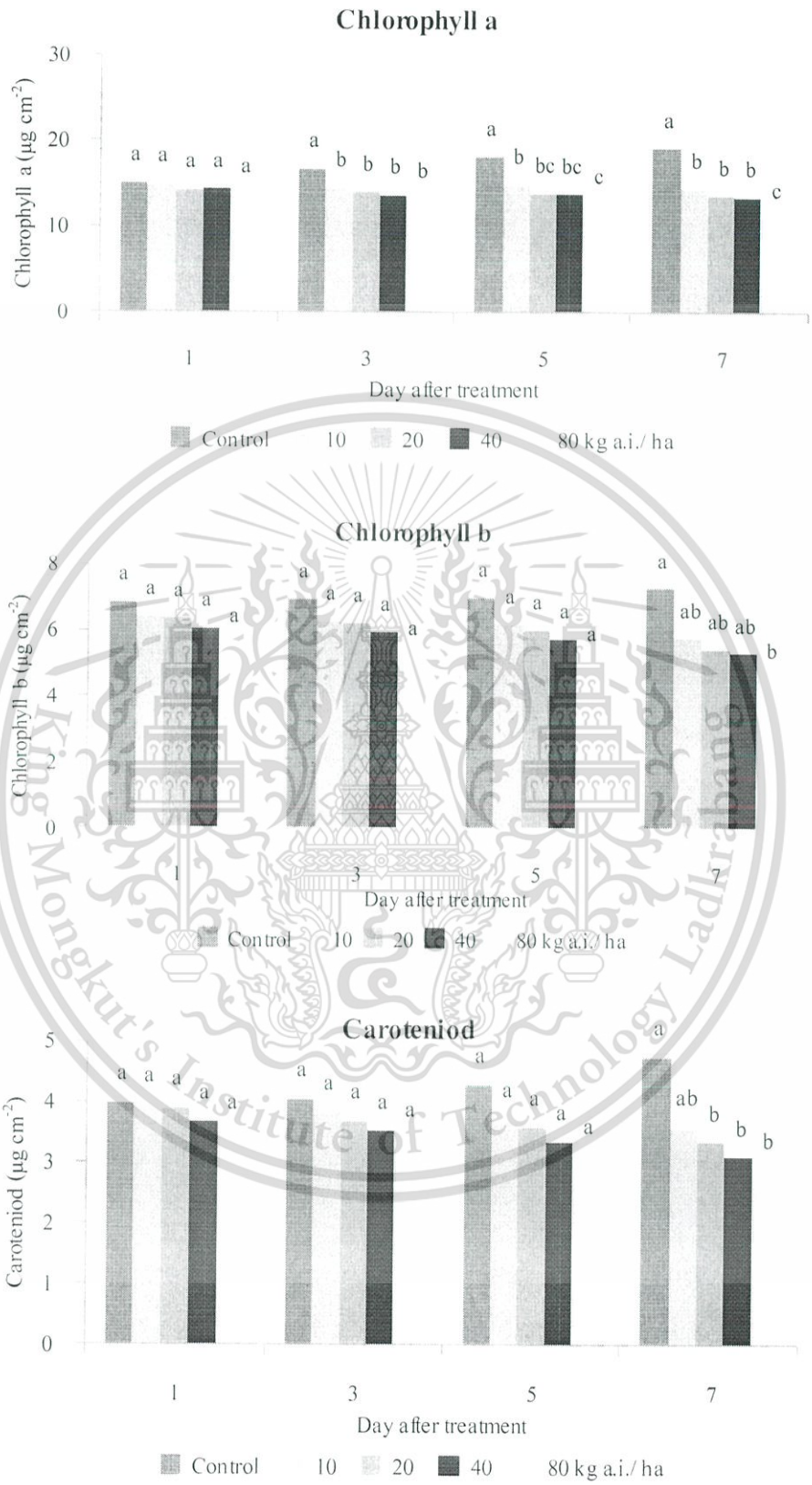

Figure 4.21 Effects of foliar applied SCT on chlorophyll a, b, and carotenoid contents (µg cm^{-2}) of *P. lathyroides* leaves at 1, 3, 5, and 7 days after treatment. Means followed by the same letter(s) are not significantly different by Tukey's range test (p=0.05).

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4.4.4 Effects of foliar spray on malondialdehyde content

The MDA content in leaves of E. *crus-galli* and *P. lathyroides* was measured using the TBARS assay. As shown in Figure 4.19, the amount of MDA differed significantly depending on applied rates and length of time after treatment. For *E. crus-galli* leaves, differences were significant seven days after treatment, with MDA contents of 10.12, 10.46, 11.04, and 12.53 nmol g^{-1} (FW) at 10, 20, 40 and 80 kg a.i. ha^{-1}, respectively, compared to a control value of 9.23 nmol g^{-1} (FW). For *P. lathyroides* leaves, MDA contents seven days after treatment were 24.84, 25.14, 26.61, and 28.80 nmol g^{-1} (FW) at 10, 20, 40, and 80 kg a.i. ha^{-1}, respectively, while the control had a value of 23.14 nmol g^{-1} (FW). Accumulation of MDA in both in *E. crus-galli* and *P. lathyroides* leaves was significantly higher than in control with increased treatment concentrations.

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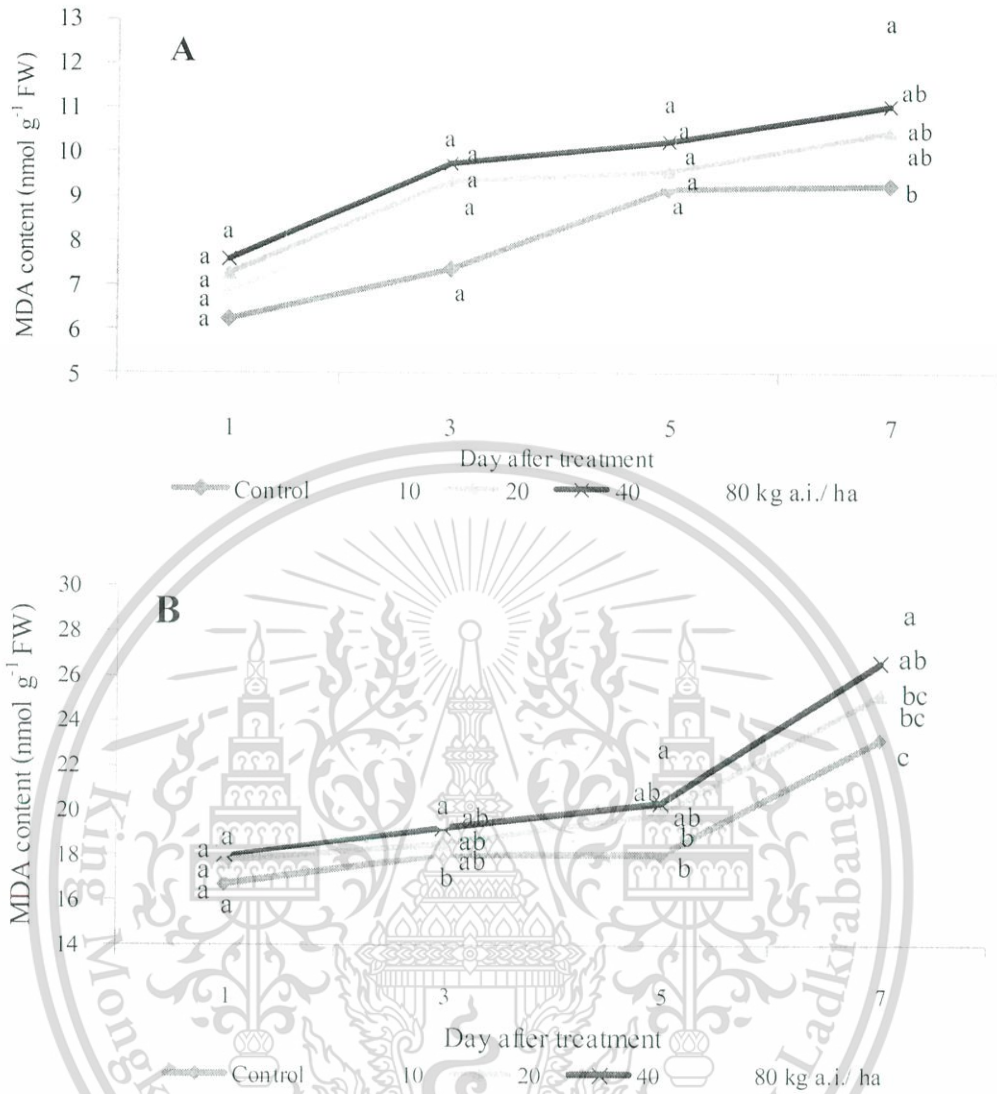

Figure 4.22 MDA content of *E. crus-galli* and *P. lathyroides* leaves at 1, 3, 5 and 7 days after foliar treatment with SCT. Means followed by the same letter(s) are not significantly different by Tukey's range test (p=0.05).

4.5 Experiment 5. Phytotoxic effects of essential oil from *T. erecta* leaves

4.5.1 Identification of chemical compositions of essential oil from *T. erecta* leaves

Essential oil was obtained by boiling hydrodistillation of *T. erecta* leaves, which gave yields of 0.25% on a fresh weight basis. Results obtained by GC–MS analysis of the essential oil of *T. erecta* are presented in Table 4.8. Twenty-one compounds were identified, constituting 91.12% of the total oil. The predominant components in the oil were piperitone **57** (17.12%),

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neophytadiene **68** (16.18%), palmitic acid **67** (11.62%), caryophyllene **58** (11.10 %), and 9,12,15-octadecatrien-1-ol **89** (11.10%).

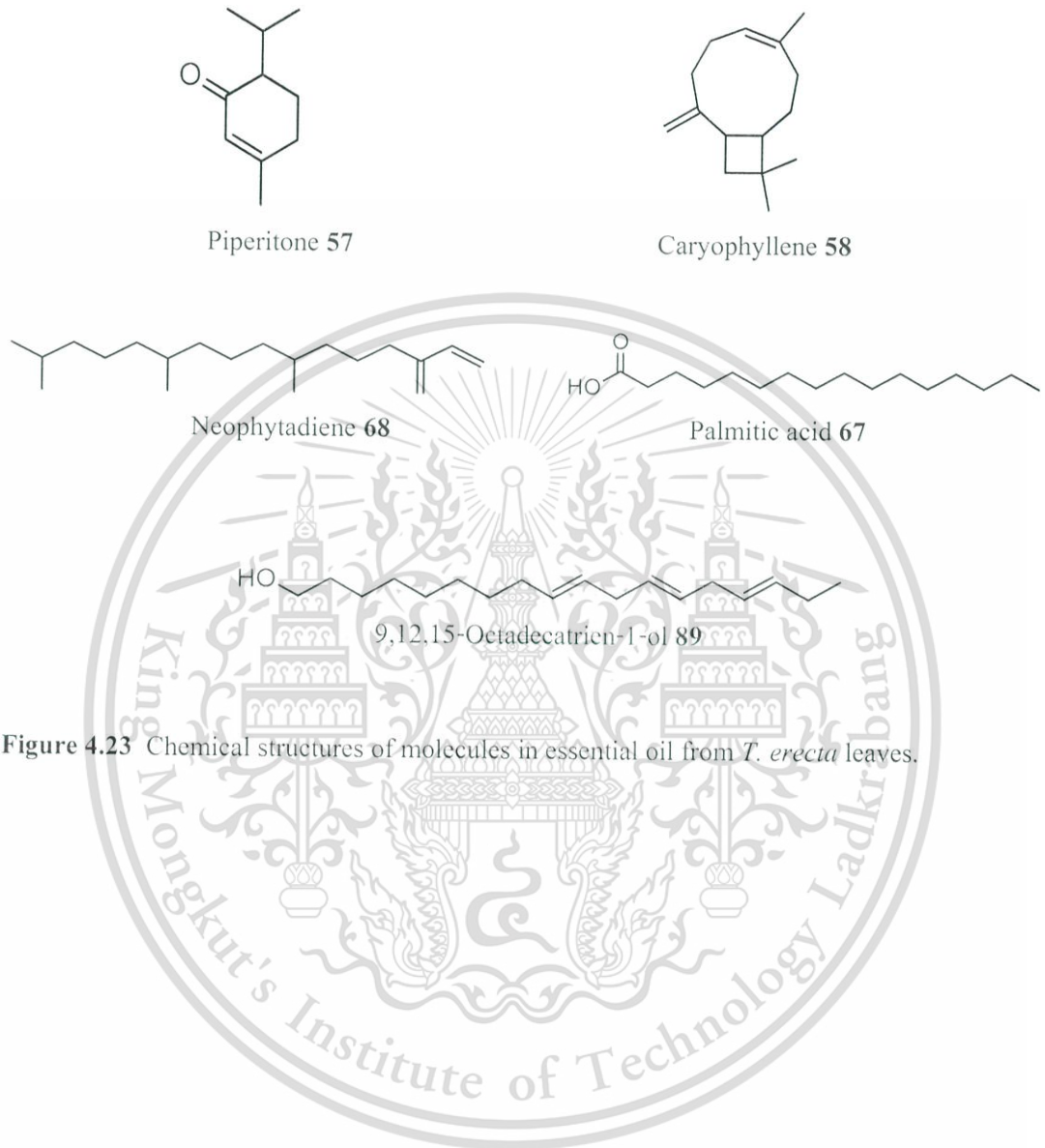

Figure 4.23 Chemical structures of molecules in essential oil from *T. erecta* leaves.

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Table 4.8 Twenty-one compounds identified in essential oil from *T.erecta* leaves.

Compounds	Peak area (%)	RT	Formula	MW	Fragment ion (m/z)
Limonene **55**	1.28	6.86	$C_{10}H_{16}$	136	136 $[M]^+$, 121, 107, 93, 79, 68, 65, 53, 39
Ocimene	0.59	7.11	$C_{10}H_{16}$	136	136 $[M]^+$, 121, 105, 93, 79, 67, 53, 41
Moslene	0.19	7.32	$C_{10}H_{16}$	136	136 $[M]^+$, 121, 105, 93, 77
Terpinolene **56**	4.96	7.81	$C_{10}H_{16}$	136	136 $[M]^+$, 121, 105, 93, 79, 67, 53, 43, 39
Linalool **27**	1.07	7.92	$C_{10}H_{18}O$	154	154 $[M]^+$, 136, 121, 93, 80, 71, 67, 55, 43, 39
Borneol	0.22	9.01	$C_{10}H_{18}O$	154	154 $[M]^+$, 139, 121, 110, 95, 67, 55, 41
4-Terpineol	0.27	9.16	$C_{10}H_{18}O$	154	154 $[M]^+$, 136, 111, 93, 86, 83, 77, 71, 67, 55, 43, 39
α-Terpineol	0.52	9.35	$C_{10}H_{18}O$	154	154 $[M]^+$, 136, 121, 93, 81, 67, 59, 43
Piperitone **57**	17.12	10.38	$C_{10}H_{16}O$	152	152 $[M]^+$, 137, 110, 95, 82, 67, 54, 39
3-Carene	0.38	10.75	$C_{10}H_{16}$	136	136 $[M]^+$, 121, 93, 81, 77, 53
Dihydro-edulan I	0.33	10.89	$C_{13}H_{22}O$	194	194 $[M]^+$, 179, 107, 95, 91, 69, 55
Piperitenone **72**	2.46	11.52	$C_{10}H_{14}O$	150	150 $[M]^+$, 135, 121, 107, 91, 79, 67, 53, 39
Caryophyllene **58**	11.10	12.63	$C_{15}H_{24}$	204	204 $[M]^+$, 189, 161, 147, 133, 120, 105, 93, 79, 69, 55, 41
α-Bergamotene	0.41	13.39	$C_{15}H_4$	204	204 $[M]^+$, 161, 119, 107, 93, 79, 69, 55, 41
Spathulenol **65**	0.96	14.58	$C_{15}H_{24}O$	220	220 $[M]^+$, 205, 187, 177, 159, 147, 131, 119, 105, 91, 79, 69, 55, 43

Table 4.8 Twenty-one compounds identified in essential oil from *T.erecta* leaves (Cont.).

Compounds	Peak area (%)	RT	Formula	MW	Fragment ion (m/z)
Caryophyllene oxide	1.73	14.66	$C_{15}H_{24}O$	220	220 $[M]^+$, 205, 177, 161, 121, 109, 93, 79, 69, 55, 43
Neophytadiene **68**	16.18	17.48	$C_{20}H_{38}$	278	278 $[M]^+$, 137, 123, 109, 95, 82, 68, 57, 43
Palmitic acid **67**	11.62	19.69	$C_{16}H_{32}O_2$	256	256 $[M]^+$, 213, 185, 171, 157, 129, 115, 97, 83, 73, 60, 43
Phytol **70**	6.56	22.99	$C_{20}H_{40}O$	296	296 $[M]^+$, 278, 196, 123, 111, 95, 81, 71, 57, 43
Linoleic acid **69**	2.07	23.52	$C_{18}H_{32}O_2$	280	280 $[M]^+$, 109, 95, 81, 67, 55, 41
9,12,15-Octadecatrien-1-ol **89**	11.10	23.66	$C_{18}H_{30}O$	264	264 $[M]^+$, 149,135,121, 108,95, 79,67, 55, 41

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4.5.2 Effects of essential oil on seed germination and seedling growth

In the dose-response bioassay, the emulsifier concentrate formulation of *T. erecta* essential oil (EC-EOs) delayed and reduced seed germination rates of *E. crus-galli* (Figure 4.24). Inhibition percentages on germination at seven day after treatments were 23.68%, 40.79%, 51.32%, and 100% for concentrations of 250, 500, 1,000, and 2,000 µl L^{-1}, respectively. Growth of the germinated seeds was determined by measuring shoot and root lengths on day seven, and a similar pattern was seen in seedling growth as for germination. At the lowest concentrations of 250 µl L^{-1}, there were significantly effects on shoot and root lengths; seedling growth decreased further with increasing concentrations and was completely inhibited at 2,000 µl L^{-1}. Thus, the phytotoxic effects of essential oil from *T. erecta* on *E. crus-galli* are clearly evident.

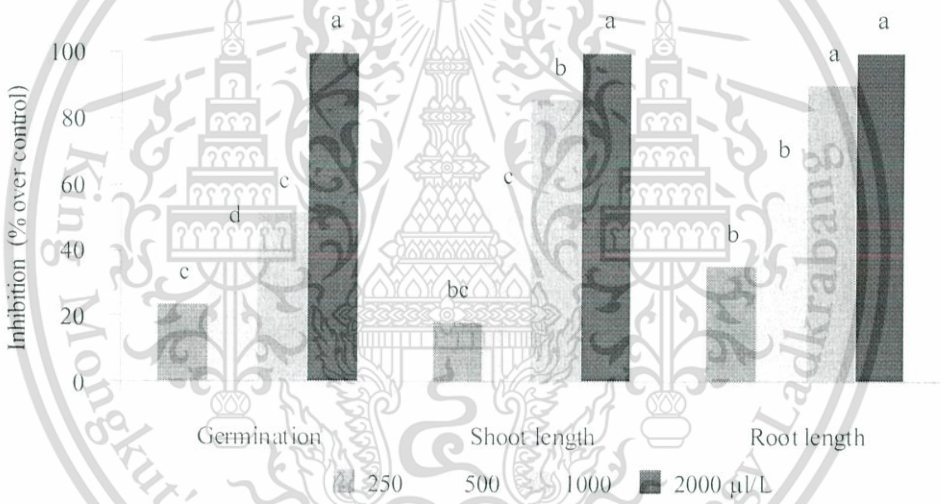

Figure 4.24 Effects of the emulsifier concentrate formulation of *T. erecta* essential oil on germination and seedling growth of *E. crus-galli* at seven days after treatment. Means followed by the same letter(s) are not significantly different by Tukey's range test (p=0.05).

4.5.3 Effects of essential oil on seed imbibition and α-amylase activity

The results of seed imbibition and α-amylase activity assays on *E. crus-galli* seeds treated with the emulsifier concentrate formulation of *T. erecta* essential oil (EC-EOs) are presented in Figure 4.25. In the control, seed imbibition progressively increased with imbibition time, with values of 20.48%, 25.75%, and 26.56% at about 24, 36, and 48 h, respectively. Only non-significant differences in inhibition values were observed for any essential oil treatment in the range of 250 to

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2,000 µl L^{-1}. Consistent with the control, the percentage of seed imbibition increased for any given treatment concentration with longer imbibition periods.

The effects of essential oil treatment on α-amylase activity in *E. crus-galli* seeds are shown in Figure 4.25. The α-amylase activity of seeds increased with germination, where radicals emerged almost at 48 h after sowing. Treatment with *T. erecta* essential oil initially decreased the induction of α-amylase activity at 24 h after treatment, and this inhibitory effect was greater with increasing oil concentrations. At 48 h, the inhibitions of activities in treated seeds were 2.63, 2.50, 2.29, and 2.09 µmol maltose min^{-1} g^{-1}(FW) for concentrations of 250, 500, 1,000, and 2,000 µl L^{-1}, respectively. In particular, a concentration of 2,000 µl L^{-1} non-significantly decreased α-amylase activity in *E. crus-galli* seeds at 48 h compared to the control and other concentrations.

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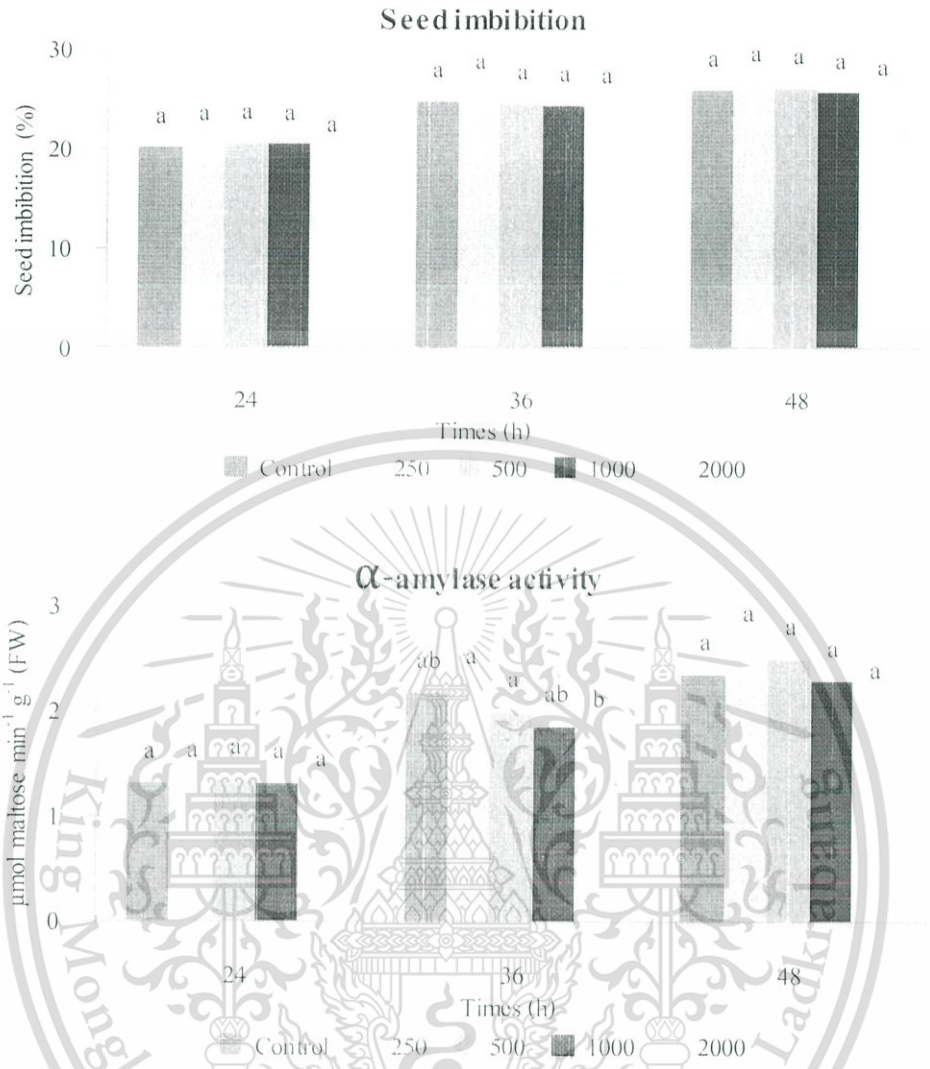

Figure 4.25 Effects of emulsifier concentrate formulation of *T. erecta* essential oil concentration and soaking time on imbibition and α-amylase activity of *E. crus-galli* seeds. Means followed by the same letter(s) are not significantly different by Tukey's range test (p=0.05).

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CHAPTER 5

DISCUSSION

5.1 Experiment 1. Inhibitory potential of aqueous extract and optimal extraction solvent

Inhibition effects of stem, leaf, flower, and root aqueous extract of *T. erecta* were assayed. Leaf extract showed the greatest inhibitory effect on *E. crus-galli* seeds, followed by flower, stem, and root extracts, respectively; for *P. lathyroides* seeds, leaf extract again had the greatest effect, followed by root, flower, and stem extracts, respectively. The effect of leaf extract on *P. lathyroides* seed germination and seedling growth was greater than for *E. crus-galli*. In both bioassay species, the inhibition effect was found to increase with increasing concentrations of aqueous extract (Sisodia and Siddiqui, 2008; 2009). Results were in congruent with findings of Phuwiwat et al., (2012) who reported that leaf aqueous extracts of *Melia azedarach* L. had inhibited seed germination and seedling growth of barnyardgrass and wild pea. Yan et al. (2012) who reported that root stem and leaf aqueous extracts of *Paeonia decomposita* inhibited seed germination and seedling growth of wheat and the degree of inhibition increased with the incremental extracts concentration.

Compounds in these extracts may also have direct effects on various plant growth and metabolism processes (Blum, 2002; Gniazowska and Bagatek, 2005). The biological responses of receiver plants to allelochemicals are known to be affected by concentration, species, cultivar/genotypes within species, and stages of plant growth (Khanh et al., 2005; Yarnia et al., 2009; Sodaeizadeh et al., 2010). Reductions in seed germination by an allelochemical could be due to the reduction or delay of reserve mobilization under allelopathy stress conditions (Gniazowska and Bagatek, 2005). These results are similar to those of Laosinwattana et al. (2009), who reported that leaf and branch aqueous extracts of *Aglaia odorata* had inhibitory effects on barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.) and wild pea (*Phaseolus lathyroides* L.). Lin et al. (2006) reported that the inhibitory effects of Saururaceae (*Houttuynia cordata* Thunb.) varied with the weed species. In contrast to our results, Sisodia and Siddiqui (2010) reported that the

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allelopathic effects of the *Croton bonplandianum* weed on seed germination and seedling growth of both crop plants (*Triticum aestivum* L., *Brassica oleracea* var. *botrytis* L., and *Brassica rapa* L.) and weed plants (*Melilotus alba* Medik., *Vicia sativa* L., and *Medicago hispida* Gaertn). In their study, aqueous extracts from root, stem, and leaf had no effect on the germination of test plants; stem extracts had a stimulatory effect on shoot length, while leaf extracts had an inhibitory effect. Gulzar and Siddiqui (2014) also reported that the allelopathic effects of *Eclipta alba* leaf aqueous extract caused maximum inhibition compared to root and stem extracts.

When optimizing the leaf extraction method, increasing the number of extractions was found to increase total extraction yield for all solvent systems tested. The greatest recovery was achieved using 25% ethanol. Many authors agree that extraction yield is dependent on the solvent used (Goli et al., 2004; Li et al., 2009). The differences in yields obtained using various ethanol-water ratios could be caused by several factors such as the composition of each particular plant, differences in the solubility of extractive compounds, and the polarity of compounds. Extracts produced by different solvent systems also showed different inhibitory influences on seed germination and seedling growth; thus, that the solvent used to produce an extract affects its inhibitory activity. These results indicate that selective extraction by appropriate solvent systems is important for obtaining fractions with high allelopathic potential and high crude extraction yield. This finding is supported by Li et al. (2006); Luthria et al. (2007); Garcia et al. (2010), who reported that different solvent systems have been used for the extraction of secondary metabolites from plant materials because extraction efficacy depends on chemical nature.

5.2 Experiment 2. Inhibitory potential, total phenolic content, and flavonoid content of various fractions separated from original crude extract

To obtain the fraction with highest inhibitory activity, crude hydroethanolic extracts of dried leaves of *T. erecta* were successively fractioned by the acid-base solvent separation method. Specifically, the 75% ethanol in water crude extract (OR) was separated into four fractions: aqueous (AQ), hydrolized (HY), neutral compound (NE), and acidic compound (AE) fractions. Comparative bioassays showed that the highest inhibitory effects were obtained with the HY and AE fractions, followed by NE, OR and AQ, respectively. This finding is supported by

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Poonpaiboonpipat et al. (2011), who reported that the AE fraction produced from *Jasminum sambac* contained the most allelochemical compounds, and by Teerarak et al. (2010), who reported that an allelopathic seco-iridoid glucoside named oleuropine from was identifyied the AE fraction of a related *Jasminum officinale* var. *grandiforum*.

Phenolic and flavonoid compounds are the most common constituents of allelochemicals (Chon et al., 2005; Deba et al., 2007), and as reported by several authors (Araniti et al., 2013; El Hadj Ali et al., 2014), fractions high in phenolics and/or flavonoids show highly inhibitory effects on seed germination and seedling growth of weed seeds. Here, the total phenolic and flavonoid contents of fractions were tested and found to vary significantly. The AE and HY crude fractions had the highest contents for both phenolics (217.71 and 150.72 mg (GAE)/1 g of crude) and flavonoids (2.52 and 5.11 mg (QE)/ 1 g of crude), respectively. In particular, the high phenolic concentrations may explain the higher inhibition effects of the HY and AE fractions. The HY fraction had a much higher crude yield than AE and NE, so was selected for further development as a soluble concentrate (SCT) formulation.

5.3 Experiment 3. Studies on the physiologic action of compounds and formulated product

The mode of action of the *T. erecta* SCT might be via various physical mechanisms (e.g. imbibition) and physiological/biochemical mechanisms (e.g. alpha amylase activity). Roots are more sensitive to phytotoxins than shoots, so are better indicators of allelochemical activity (Omezzine et al., 2011; Ladhari et al., 2013). Inhibitory effect on roots may occur through a variety of mechanisms such as a reduced rate of ion uptake, inhibition of respiration, or inhibition of cell division (Gulzar et al., 2015). The initial stage of germination is imbibition, which is substantially a physical process. After few hours, this is followed by the formation of α-amylase, which is essential for the conversion of starch reserves in the endosperm to soluble sugars that drive respiration (Beck and Ziegler, 1989; Perata et al., 1997). Here, we found that imbibition and α-amylase activity increased with soaking time but decreased with SCT concentration. Decreased α-amylase activity in seeds and seedlings indicates metabolic inhibition by SCT, and there may be a relationship between the allelochemicals found in *T. erecta* that inhibit imbibition and α-amylase activity and the reduced seed germinationobserved in extract bioassays. This finding is supported

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by Teerarak et al. (2010), Phuwiwat et al. (2012); Poonpaiboonpipat et al. (2013) who reported that imbibition and α-amylase activity in *E. crus-galli* was inhibited by allelochemicals extracted from *Jasminum officinale* L. f. var. *grandiflorum* (L.) Kob, *Melia azedarach*, and *Cymbopogon citrates*. Lordan et al. (2013) evaluated the inhibitory effects of 15 seaweed species and found that at 10 mg/ml, extracts of *Ascophyllum nodosum*, *Fucus serratus*, *Fucus vesiculosus*, and *Pelvetia canaliculata* significantly ($p<0.05$) reduced α-amylase activity to <20%.

Exposure of seedlings to SCT also resulted in significant reductions in root length and shoot length. At all concentrations, SCT markedly reduced germination and growth in a concentration-dependent manner (Ladhari et al., 2013; Gulzar et al., 2015). Cytology assays were used to obtain detailed information regarding the effects of SCT on the production of genetic material and cell division. Results showed that the proportion of cells in each mitotic phase (mitotic index) was reduced, indicating that the cytotoxic potential of SCT is mitodepressive and may block DNA and nucleoprotein synthesis (Mercykutly and Stephen, 1980; Schulze and Kirschner, 1986). The frequencies of mitotic phases were also altered by SCT. The accumulation of dividing cells at prophase indicates that SCT may interfere with the sequence of mitotic division, perhaps due to interaction with the spindle, with subsequent arrest of cell division at metaphase (Duan and Wang, 1995; Liu et al., 1996).

Treated roots additionally exhibited various mitotic abnormalities including spindle disturbances at prophase, c-metaphase, sticky metaphase, sticky anaphase, anaphase bridge, and diagonal at anaphase. In particular, all concentrations of SCT tested induced elevated frequencies of sticky chromosomes at metaphase. These abnormalities could be due to SCT effects on splitting of the spindle fibers or partial suppression of spindle formation (Mukherjee et al., 1990). The synthesis and formation of microtubules and the formation of the division-spindle are normally responsible for segregating chromosomes, leading to exclusion from the daughter nuclei (Bond, 1987). Hence, these processes are potential targets for aneugenic effects (Singh, 2002). Another chromosomal abnormality induced by SCT was c-mitosis. The presence of c-mitosis can result from inactivation of mitotic spindles (FiskesjÖ, 1985; FiskesjÖ, 1993), which reinforces the formation of multiple-nucleated cells. Sticky anaphase was also observed in some treatments, and appears as a consequence of improper chromatin organization (El-Ghamery et al., 2003).

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5.4 Experiment 4. Herbicidal potential of natural herbicide from *T. erecta*

Emergence and seedling growth (plant height and dry weight) of *E. crus-galli* was significantly suppressed by soil surface application of SCT when compared with the control (Figure 4.18). However, the lowest dose (25 kg a.i. ha^{-1}) of SCT did not reduce emergence and growth; instead, plant growth and dry weight were slightly increased. With increasing doses, emergence as well as plant height and dry biomass declined. At a dose of 100 kg a.i. ha^{-1}, seed germination was 82.50% inhibited while plant growth and dry weight were reduced by 65.00% and 87.50%, respectively. These results are congruent with Batish et al. (2007), who reported that treatment of bioassay weed species with allelochemicals from allelopathic plants gave a greater negative effect on dry biomass than emergence or plant height. Furthermore, the differential effects of SCT against the two weed species are consistent with several previous reports, which noted that *E. crus-galli* possessed stronger resistance against phytotoxins from other plants than other bioassay seeds (Seal et al., 2004; Khanh et al., 2005; Laosinwattana et al., 2010).

The effects of foliar-applied SCT on leaf membrane integrity were evaluated using electrical conductivity as an indicator of membrane leakage (Table 4.6 and Table 4.7). In *E. crus-galli* and *P. lathyroides* leaves treated with SCT, solute leakage indicated a loss of membrane stability that can negatively affected biochemical functions and cell viability (Jaspers and Kangasjärvi, 2010). This finding is congruent with the results of Kim et al. (2004), who observed a loss of membrane stability in corn leaves after treatment with the herbicide fluridone. Furthermore, essential oil from *Artemisia scoparia* has been reported to cause severe electrolyte leakage from *E. crus-galli* and *Cassia occidentalis*, indicating membrane disruption and loss of integrity (Kaur et al., 2010).

Our work additionally showed that foliar spraying with the *T. erecta* SCT reduced photosynthetic pignment content, namely of chlorophyll a, b, and carotenoids, in both *E. crus-galli* and *P. Lathyroides*; effects were significantly different depending on SCT concentration and tested plant species (Figure 4.20 and Figure 4.21). For treated *E. crus-galli* leaves, chlorophyll a, chlorophyll b and carotenoid contents decreased with increasing SCT concentration but showed no significant difference to the control. Meanwhile, all photosynthetic pigment contents were reduced in *P. lathyroides* leaf at higher concentrations of SCT. Conversely, all photosynthetic pigment contents were reduced in *P. lathyroides* leave when receiving high concentrations of SCT. At seven days after treatment, the highest SCT concentration (80 kg a.i. ha^{-1}) resulted in chlorophyll a, b, and carotenoid values in *E. crus-galli* of 20.06, 5.39, and 3.31 $\mu g\ cm^{-2}$, while values in *P. lathyroides*

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were 10.91, 4.57 and 2.89 μg cm^{-2}, respectively. These results are congruent with Teerarak et al. (2013), who reported that PORGANIC™ foliar spraying reduced photosynthetic pigment contents in the leaves of *E. crus-galli* and *P. lathyroides*. Similar findings were reported by Oyerinde et al., (2009), who showed decreases in chlorophyll-a, chlorophyll-b and total chlorophyll accumulation in young plants after being treated with fresh shoots of *Tithonia diversifolia* that possess allelopathic characteristics. However, whether the reduction in chlorophyll content is due to a decrease in *de novo* biosynthesis of chlorophylls or their enhanced degradation is unknown. The observed decline in carotenoid content may lead to carotenoid deficiency-induced photo-oxidation of chlorophylls (Dankov, et al., 2009), thus potentially causing the decrease in chlorophylls (Teerarak et al., 2013). Moreover, carotenoids are important for photosystem (PS) assembly, the stability of light harvesting complex proteins, and thylakoid membrane stabilization (Niyogi et al., 2001). Another marker of physiological damage is MDA content, which serves as an indicator of the extent of lipid peroxidation and thus provides an indirect reflection of the extent of cell damage (Meloni et al., 2003; Li et al., 2010). The greater the MDA content, the less stable the membranes become. Here, MDA content in leaves of *E. crus-galli* and *P. lathyroides* foliar-treated with SCT were measured using the TBARS assay (Figure 4.22). The MDA content differed significantly depending on applied rate and time after treatment, with significant changes observed in *E. crus-galli* seven days after treatment and in *P. lathyroides* three days after treatment. At higher doses of SCT, MDA accumulation was significantly higher in both *E. crus-galli* and *P. lathyroides* leaves than in controls. MDA continually increased with increasing SCT concentration and extended time. The same result was reported in pea (*Pisum sativum* L., cv. sugar snap boys) after treatment with imazethapyr, an imidazolinone herbicide (Zabalza et al., 2007).

5.5 Experiment 5. Phytotoxic effects of essential oil from *T. erecta* leaves

The results obtained by GC–MS analysis of the essential oil of *T. erecta* are presented in Table 4.8. Twenty-six compounds were identified, constituting 91.12% of the total oil. Piperitone, neophytadiene and palmitic acid were main components of *T. erecta* EOs, representing 17.12%, 16.18%, and 11.62% of the total, respectively. These results are congruent with Armas et al. (2012), who reported that piperitone is the main component in EOs from *T. erecta* (Ogunwande and Olawore, 2006; Kishore et al., 1991). Another study by Marques et al. (2011) reported main compounds of piperitone **57** (45.725%), limonene **55** (9.67%), and piperitenone **72** (5.89%) for *T.*

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erecta essential oil. However, a third study (Tripathi et al., 2012) reported the major components of essential oil from the aerial parts (leaves, flowers, and shoots) of *T. erecta* (Var. Pusa Narangi Genda) as *cis*-ocimene (18.46%), *(E)*-oscimene (8.65%), l-limonene (11.16%), *(E)*-tagetone (10.56%), β-caryophyllene (6.9%), and dl-limonene (4.16%). In another study on essential oils from the related *T. patula*, the major constituents were identified as l-limonene, δ-cadinene, dl-α-cadinol, α-terpinolene, piperitone, ocimene, α-caryophyllene, piperitenone, and tagetone (Krishna et al., 2002). Differences between componants of essential oils could be mainly related to factors such as geographic origin and the season of collection (Rustayian et al., 2006).

As indicated by Figure 4.24, *T. erecta* essential oil affected the seed germination and seedling growth of *E. crus-galli*. The activity of α-amylase plays an important role in degradation of reserve carbohydrates to soluble sugars (Perata et al., 1997), which is necessary for seed germination and subsequent seedling growth until photosynthesis can provide sufficient support (Kato-Noguchi et al., 2010; Beck and Ziegler, 1989). Thus, to understand the mechanisms inhibiting seed germination, the effects of *T. erecta* essential oil on α-amylase activity were analyzed. Results showed a decrease in induction of α-amylase activity with SCT treatment. The results found in this study are inconsistent with those of Poonpaiboonpipat et al. (2013), who reported that high concentration of essential oil from plant significantly inhibited affecting α-amylase activity of *E. crus-galli* seeds. The herbicidal effect of essential oil from *Teucrium polium* has also been attributed to decreased activities of α-amylase (Bigham et al., 2010) and essential oil derived from the wood of *Cedrus libani* has been reported to exhibit α-amylase inhibitory activity (Jumepaeng et al., 2013; Loizzo et al., 2007). Thus, the decreased seed germination rates and seedling growth observed with essential oil treatment of *E. crus-galli* may result from the essential oil's inhibition of α-amylase activity.

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CHAPTER 6

CONCLUSION AND SUGGESTION

6.1 Conclusion

6.1.1 Inhibitory potential of aqueous extract and optimal extraction solvent

This work assayed the allelopathic effects of stem, leaf, flower, and root aqueous extracts of *T. erecta* on seed germination and seedling growth of *E. crus-galli* and *P. lathyroides*. The assays were run using Petri dish tests under laboratory conditions. Aqueous extracts from leaves had the greatest inhibitory effects on bioassay plant, more than root, flower, and stem extract. A The optimal extraction solvent was determined for leaf extracts using a range of ethanol: water solutions (100:0, 25:75, 50:50, 75:25, and 0:100) and evaluating both total yield and effects on seed germination and seedling growth of *E. crus-galli* and *P. lathyroides*. The extracting solvent and number of extractions significantly affected both yield and inhibitory effect, with the greatest inhibitory effect obtained using 75:25 ethanol:water. Regardless of the solvent system used, three successive extractions gave optimal yield. These results indicate that selective extraction from natural resources using appropriate solvents and number of extractions is important for obtaining fractions with high inhibitory activity.

6.1.2 Inhibitory potential, total phenolic content, and flavonoid content of various fractions separated from original crude extract

The 75% ethanol in water crude extract fraction (OR) from *T. erecta* was separated into four fractions: aqueous (AQ), hydrolized (HY), neutral compound (NE), and acidic compound (AE). The HY and AE fractions showed the greatest activity, with complete inhibition of germination, shoot growth, and root growth for both *E. crus-galli.* and *P. lathyroides.* The results further indicate that this inhibitory effect is associated with high phenolic content, as there was a very good relationship between total phenolic content and seed germination results. The HY fraction had a much higher crude yield than AE and NE, so was selected for further development as a soluble concentrate formulation (SCT).

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6.1.3 Mode of action of the herbicidal potential of SCT

A soluble concentrate formulation was prepared from the crude HY fraction of *T. erecta* (SCT) as described above. This formulation had strong inhibitory effects on the seed germination and seedling growth of both *E. crus-galli.* and *P. lathyroides.* Seed imbibition and α-amylase activities were found to increase with increasing soaking times but decreased with higher concentrations of SCT. These results suggest that the inhibitory effects of SCT might be caused by reduction of seed imbibition and α- amylase activities during germination. Additionally, the reduced growth may be due to observed cytotoxic effects on cell division (mitosis). The SCT formulation offers good potential for further natural herbicide development.

6.1.4 Herbicidal potential of SCT as a foliar application

Plants treated with foliar applied SCT had lower chlorophyll a, chlorophyll b, and carotenoid contents compared with untreated plants. These results may be related to loss of membrane integrity that interferes with the photosynthetic process. Furthermore, treated leaves exhibit an increase in MDA content, suggesting lipid peroxidation. Thus, it was concluded that SCT damaged plant tissues, especially in the cell walls and cell membranes of the leaf, leading to the disturbance of lipids and membrane integrity.

6.1.5 Phytotoxic effects of essential oil from *T. erecta* leaves

Essential oil was extracted from *T. erecta* leaves by hydrodistillation and its constitutents analysed by GC- MS. Major constituents were identified as piperitone **57**, neophytadiene **68**, palmitic acid **67**, caryophyllene **58**, and 9,12,15-octadecatrien-1-ol **89**. The emulsifier concentrate formulation of *T. erecta* essential oil (EC-EOs) had shown the greatest inhibition of seed germination and seedling growth in test weeds; therefore, as a potential mode of action, the effect of EC-EOS on α-Amylase activity in *E. crus-galli* seeds was investigated. Results showed that *T. erecta* essential oil decreased α-amylase induction, thus causing inhibition of seed germination and seedling growth.

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6.2 Suggestion

6.2.1 Study the effective storage period of natural herbicide produced from *T. erecta*

6.2.2 Determine suitable environmental conditions for natural herbicide produced from *T. erecta*, such as temperature, light, moisture, etc.

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