ALLELOPATHIC POTENTIAL OF *THUNBERGIA LAURINFOLIA* LINDL. LEAF EXTRACT AND ITS MODE OF ACTION ON WEED CONTROL

NGUYEN HUY THINH

A THESIS SUBMITTED IN PARTIAL FULFILMENT

OF THE REQUIREMENT FOR THE DEGREE OF

MASTER OF SCIENCE IN AGRICULTURE

FACULTY OF AGRICULTURAL TECHNOLOGY

KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG

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หัวข้อวิทยานิพนธ์	ศักยภาพทางอัลลีโลพาธีของสารสกัดใบรางจืด (*Thunbergia laurifolia* Lindl.) และกลไกการออกฤทธิ์ควบคุมวัชพืช
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อาจารย์ที่ปรึกษาวิทยานิพนธ์	รองศาสตราจารย์ ดร. จำรูญ เล้าสินวัฒนา

บทคัดย่อ

วิทยานิพนธ์นี้ศึกษาเพื่อหาการออกฤทธิ์ทางอัลลีโลพาธีของสารสกัดใบรางจืดโดยใช้ตัวทำละลายต่างๆ และกลไกการออกฤทธิ์ต่อการงอกและการเจริญเติบโตของเมล็ดหญ้าข้าวนก (*Echinochloa crus-galli* (L.) Beauv.) และผักโขม (*Amaranthus gracilis* Deaf.) การศึกษาผลของเฮกเซน เอธิลอะซีเตต และเอทานอลเป็นตัวทำละลายในการสกัดสารสกัดหยาบ และประสิทธิภาพการยับยั้ง ผลจากการทดลองพบว่า เอทานอลสามารถสกัดสารสกัดหยาบจากใบรางจืด ได้ปริมาณสารสกัดหยาบสูงสุด โดยปริมาณสารสกัดหยาบจากใบรางจืดที่สกัดด้วยเอทานอลได้ 4.6 กรัมต่อ 100 กรัมใบแห้ง และสารสกัดหยาบที่สกัดด้วยเฮกเซนได้ปริมาณสารสกัดหยาบน้อยที่สุด คือ 0.63 กรัมต่อ 100 กรัมใบแห้ง ซึ่งสารสกัดหยาบที่สกัดด้วยเฮกเซนมีผลยับยั้งการงอกและการเจริญเติบโตของเมล็ดหญ้าข้าวนก และผักโขมได้ดีที่สุด อย่างไรก็ตามการใช้เอทานอลเป็นตัวทำละลายในการสกัดให้ความปลอดภัยต่อสิ่งแวดล้อม และสุขภาพของมนุษย์มากกว่า และมีผลยับยั้งได้ดี การศึกษาประสิทธิภาพของการใช้สารสกัดเอทานอลต่อน้ำในอัตราส่วนที่แตกต่างกัน (0, 25, 50, 75 และ 100%) ที่มีผลต่อปริมาณสารสกัดหยาบที่สกัดได้ ปริมาณสารฟีโนลิคทั้งหมด และฟลาโวนอยด์ทั้งหมด โดยปริมาณสารสกัดหยาบจะเพิ่มขึ้นตามสัดส่วนของน้ำที่เพิ่มขึ้น ที่อัตราส่วน 100% เอทานอลได้ปริมาณฟีโนลิค และแทนนินทั้งหมด คือ 63.03 มิลลิกรัมสมมูลย์ของกรดแกลลิคต่อสารสกัดหยาบ 1 กรัม และ 7.03 มิลลิกรัมสมมูลย์ของกรดแทนนิกต่อสารสกัดหยาบ 1 กรัม ตามลำดับ และอัตราส่วน 75% เอทานอลได้ปริมาณฟีโนลิค และแทนนินทั้งหมดสูงสุด คือ 97.8 มิลลิกรัมสมมูลย์ของกรดแกลลิคต่อสารสกัดหยาบ 1 กรัม และ 11.81 มิลลิกรัมสมมูลย์ของกรดแทนนิกต่อสารสกัดหยาบ 1 กรัม ตามลำดับ ขณะ 100% เอทานอล มีปริมาณสารฟลาโวนอยด์

ทั้งหมดมากที่สุด คือ 4.2 มิลลิกรัมสมมูลย์เคอร์ซิตินต่อสารสกัดหยาบ 1 กรัม สารสกัดจากใบรางจืดที่ได้จากเอทานอลต่อน้ำที่สัดส่วนที่แตกต่างกัน พบว่า ให้ผล ยับยั้งการงอกและการเจริญเติบโตของเมล็ดหญ้าข้าวนกและผักโขมได้ดี โดยสารสกัด 100% เอทานอลมีผลยับยั้งได้ดีที่สุด เมื่อศึกษาประสิทธิภาพของสารสกัด 100%เอทานอลต่อกลไกการดูดน้ำของเมล็ดและกิจกรรมของเอนไซม์อัลฟา-อะไมเลส ของหญ้าข้าวนก และผักโขม ผลการทดลองพบว่า การดูดน้ำของเมล็ดทั้งสองชนิดเพิ่มขึ้นตามระยะเวลาการแช่สารที่นานขึ้น ในขณะที่ความเข้มข้นทั้งหมดไม่มีความแตกต่างกันในการดูดน้ำ อย่างไรก็ตาม การเพิ่มความเข้มข้นของสารสกัดส่งผลต่อการลดลงของกิจกรรมเอนไซม์อัลฟา-อะไมเลสในเมล็ดของหญ้าข้าวนก และผักโขม ซึ่งสารสกัดจากใบรางจืดสามารถยับยั้งเมล็ดหญ้าข้าวนก และผักโขม โดยอาจไปยับยั้งกิจกรรมเอนไซม์อัลฟา-อะไมเลสในเมล็ดเหล่านี้

Thesis	Allelopathic potential of *Thunbergia laurifolia* Lindl. leaf extract and its mode of action on weed control
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Program	Agriculture
Year	2019
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ABSTRACT

The aim of this study was to investigate the allelopathic activity of *Thunbergia laurifolia* Lindl. leaf extract using various solvents and its mode of action on seed germination and seedling growth of *Echinochloa crus-galli* (L.) Beauv. and *Amaranthus gracilis* Deaf. The effect of hexane, ethyl acetate and ethanol solvents using as sequential extraction solvents on crude extract yield and inhibitory effects were investigated. The results showed that the highest crude extract yield was obtained from ethanol solvent (4.6 g/100 g DW) and the lowest yield was obtained from hexane solvent (0.63 g/100 g DW). The hexane extract had the greatest inhibitory effect on germination and seedling growth of both *E. crus-galli* and *A. gracilis* seeds. However, using ethanol as an extraction solvent is safer for the environment and human health and still has a strong inhibitory effect. The effect of various ratio ethanol (0, 25, 50, 75 and 100%) in water on crude extract yield, total phenolic content, total flavonoid content and total tannin content was determined. The crude extraction yield increased with increase of water ratios and reached the highest crude extraction yield at ratio of 25% ethanol in water. The highest total phenolic and tannin contents were obtained from the 75% ethanol (97.8 mg GAE/g CE and 11.81 mg TAE/ g CE, respectively), while the 100% ethanol extract contained highest flavonoid content (4.2 mg QE/ g CE). Total phenolic and tannin contents of the 100% ethanol extract were 63.03 GAE/g CE and 7.30 mg TAE/ g CE, respectively. All of extracts from *T. laurifolia* showed great inhibitory effect on seed germination and seedling growth of *E. crus-galli* and *A. gracilis* seeds. At the 100% ethanol extract expressed the strongest

inhibitory effect. The effect of the 100% ethanol extract on seed imbibition and α-amylase activity of *E. crus-galli* and *A. gracilis* were studied to find out its mechanism. The results showed that imbibition of both bioassay seeds increased by prolonging time, whereas no significant differences in imbibition among all concentrations. However, increasing the concentration of the extract led to a significant increase of the inhibition on α-amylase activity of *E. crus-galli* and *A. gracilis* seeds. The extracts from *T. laurifolia* inhibited *A.gracilis* and *E. crus-galli* seeds may by inhibited the induction of α-amylase in these seeds.

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Finally, is for my family for their power of love and taking care of my motivation through these years.

Nguyen Huy Thinh

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CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problems

Weeds compete with crop plants to cause a huge loss in their productivity. Yield losses of crops due to weeds are higher than yield losses compared to any other agricultural pests (Jabran *et al.* 2015). Weeds are the cause by 34% of major crop yield loss, and the potential crop yield loss without control was estimated by 43%, on a global scale (Oerke. 2006). Several reports indicated that crop yield loss caused by weeds such as in rice by 10-100%, wheat by 10-60%, and maize by 25-93% (Sharma and Thakur. 1998; Rao, A. N. *et al.* 2014; Jabran and Chauhan. 2015; Yaduraju *et al.* 2015). Weeds can cause rice yield losses of up to 50% and these losses were measured after manual weeding (Chauhan. 2012). However, manual weeding can be performed only when weeds have reached enough size to be pulled out easily by hand. By that time, yield losses have already occurred. In economic terms, weeds caused crop loss amounting to more than 100 billion US dollars over the world and costs have to be paid for herbicides up to US$25 billion (Agrow. 2003). Furthermore, using synthetic herbicides, the most prevalent method for controlling weed nowadays, often leave negative consequences for the environment and human health. Synthetic herbicides can cause a negative effect on human health, from skin rashes to death. A 2010 Organic Consumers Association article stated that a specialized type of cancer known as acute lymphoblastic leukemia is assumed to be directly linked to pesticide exposure in a normal, non-agriculture setting (Sharpley. 2010). Therefore, need to find alternative means to overcome those problems.

The previous reports showed that the ability of allelopathy to control weeds. Allelopathy is a biological phenomenon by which a plant produces biochemicals that influence to inhibit or to stimulate growth of other plants. Allelopathic plants express their allelopathic activity by releasing allelochemical compounds. Products from allelopathic compounds may help to reduce the use of synthetic compounds on weed control. Importantly, it's neither harm the environment nor increase weed management costs and safer for human health with the use of synthetic compounds. Further, it can be applied in combination with other methods to achieve integrated weed management (Jabran *et al.* 2015). Numerous plants are showed to possess allelopathic potential, such as *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). The application of allelopathic plants for control weed can be implemented by growing allelopathic plants in close proximity to weeds (Tesio and Ferrero. 2010) or by using allelochemicals as natural herbicide. Although most plants have allelopathic effective for weed control, the need for finding reliable allelopathic crops to control weeds is importance.

Thunbergia laurifolia Lindl. also known as laurel clock vine or blue trumpet vine is a popular ornamental plant commonly found in tropical gardens (Boonyarikpunchai *et al.* 2014). *T. laurifolia* is a choice to cure fever, mild poisons, and hangover and has been used as medicine for centuries in Thai culture. Some studies indicate that in the *T. laurifolia* contain a high amount of antioxidants (Chan *et al.* 2012; Junsi and Siripongvutikorn. 2016) and have great allelopathy activity (Somkiat. 2012). However, the use of *T. laurifolia* as a natural herbicide is no report. The purpose of this research was to assess the allelopathic potential of *T. laurifolia* on germination and seedling growth of *Echinochloa crus-galli* (L.) Beauv, *Amaranthus gracilis* Desf.

1.2 Goal and objective of the study

1.2.1. To determine the suitable solvent systems for extraction of crude extract from *Thunbergia laurifolia* Lindl.

1.2.2. To determine total flavonoid, phenolic and tannin content of extracts from *Thunbergia laurifolia* Lindl.

1.2.3. To evaluate inhibition potential of extracts from *Thunbergia laurifolia* Lindl. on seed germination, seedling growth and its mode of action.

1.3 Places

Laboratory of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

CHAPTER 2

LITERATURE REVIEW

2.1 Weeds: A background

Since thousands of years ago, agriculture had to face a variety of harmful factors, such as pest animals, plant pathogen, weeds, etc. Weeds are one of the most serious problems responsible for not only quantity and quality losses in agriculture but also ecosystem and environmental protection (Bajwa *et al.* 2018). In the total annual loss in agricultural production from pests, weeds had the highest loss potential, account 45%, 30% of insects, 20% of diseases and 5% of other pests (Rao, V. S. 2000). There have been numerous definitions of weeds, depending on different factors such as where they grow. Weed usually is defined as an unwanted plant growing where it is not desired and interferes with human activity. One of the harms of weeds is competition. Weeds compete with crops for many physical factors such as nutrients, light, water, and space, that interfere with growth of plants. Weeds increase the cost of crop protection because they can be host to insect pests and diseases. Weeds interfere with crop caused of reducing quality of crops and animals. Weeds growing in ditches could block water irrigation systems on paddy fields. Some poisonous weeds can be grown in crop community and can be toxic to human health (Zimdahl. 2007). The above reasons lead to significant damage by weeds in agriculture. In period of 2007 to 2013, with no weed management method, corn and soybean yield lost by weeds probably was up to 52%, tantamount a decrease in value of approximately 28 and 16 billion US dollars annually, respectively, in Canada and United States (Soltani *et al.* 2016, 2017).

Weed control has been developed to prevent losses due to weeds in fields. Mechanical methods (i.e. hand-pulling, hand-hoeing, tillage, etc.) have a long history and significantly contribute to weed control. Since the starting of agriculture, mechanical methods have been the most applied weed control methods. However, these methods require a large amount of labors so it is difficult to practice on a large scale. In model agricultural systems, farmers often use synthetic herbicides as the main method. They are effective but rather costly and results in various toxicological effects on the environment and living organisms including human. A dependence on herbicides also leads to rapid herbicide resistance evolution in weeds. The combination of different weed control methods has proven to be effective in decrease chances of herbicide resistance

evolution in weeds. (Gniazdowska and Bogatek. 2005; Norsworthy *et al.* 2012). The above problems make it imperative to develop diversity in the current weed control methods.

2.2 Allelopathy

2.2.1 Brief about allelopathy

Allelopathy is a biological phenomenon by which a plant produces biochemicals that influence on the germination, growth, survival and reproduction of other plants. These biochemicals are known as allelochemicals and can have beneficial or detrimental effects on the target plants. Allelochemicals are a subset of secondary metabolites (Stamp. 2003), which are not required for metabolism (i.e. growth, development and reproduction) of the organism. Allelochemicals with negative allelopathic effects are an important part of plant (Stamp. 2003). Any parts of the plants can contain allelochemicals. They can be found in leaves, flowers, roots, fruits, or stems of the plants and also can be found in the surrounding soil. Target plants can be affected by these toxins in many different ways. The toxic chemicals can inhibit shoot, root growth of target plants, they can inhibit nutrient uptake and they also can attack a naturally occurring symbiotic relationship thereby destroying nutrient sources of the plants. Not all plants have allelopathic tendencies. several plants exhibit these allelopathic tendencies, may actually be displaying aggressive competition of a non-chemical form. Most of the controversy about allelopathy is in trying to distinguish the type of competition being displayed. In general, if it is a chemical nature, then the plant is considered allelopathic. Allelochemicals can be classified into 10 categories (Li *et al.* 2010) according to their different structures and properties. Allelopathy offers potential for biological weed control through the production and release of allelepathic chemical. Although this compound causing germination and growth seedling inhibitions, it is not harmful on human and environment. Allelopathic compounds can help to reduce the use of synthetic herbicides, so cause less environmental pollution and safer agricultural products (Singh *et al.* 2003). Allelopathy can play an important role in the various system of sustainable weed control. Many studies showed that allelopathic activity may inhibit seed germination by inhibit activity of α-amylase activity and gibberellins synthesis. Seed imbibitions and activity of α-amylase are consistently linked with the seed germination process (Kato-Noguchi and Macías. 2005; Meksawat and Pornprom. 2010).

2.2.2 Allelopathy for weed control

Exploration of the allelopathic potential of some species allows the introduction of alternative techniques for weed control such as extracts from allelopathic plants can be applied as inhibit germination of seed. The best known examples of natural bioherbicides are phytotoxic water extracts from herbage of sorghum (*Sorghum bicolor* (L.) Moench.) and sunflower (*Helianthus annuus* L.) which can be effectively used in plant protection without yield losses. The highest efficacy of such extract applications has been verified in rice on reduction of barnyard grass biomass by 40%, without significant changes in weed density and accompanied yield increase by 18%. This system of application has reduced biomass of the two most commonly occurring weeds, lambsquarters (*Chenopodium album* L.) and toothed dock (*Rumex dentatus* L.), by 70 and 97%, respectively. In a study about phytotoxic potential of powder and methanol extract of *Tinospora crispa* (L.) leaves as pre-emergence and post-emergence applications on the growth of transplanted rice (*Oryza sativa* L.) and associated weeds were evaluated under glasshouse and field conditions to determine its herbicidal activity as soil additive material in rice fields. In plots amended with 1, 2 and 4ton ha^{-1} leaf powder, weed dry weight was reduced by nearly 80, 97 and 99%, total weed seedling density was inhibited by 73, 94 and 99%, respectively, compared to untreated plots (Aslani *et al.* 2016). There was a significant promotion on grain yield, straw dry weight and number of seed per panicle of rice, when treated with leaf powders and chemical herbicide compared with negative control. High allelopathic potential conditioned by glucosinolates and isothiocyanates is present in *Brassica* sp. (Petersen *et al.* 2001). Isothiocyanates have been strong suppressants of germination of spiny sowthistle (*Sonchus asper* L. Hill), scentless mayweed (*Matricaria inodora* L.), smooth pigweed (*Amaranthus hybridus* L.), barnyard grass (*Echinochloa crus-galli*), blackgrass (*Alopecurus myosuroides* Huds.) and wheat (*Triticum aestivum*) (Petersen *et al.* 2001). Black mustard (*Brassica nigra* L.) extract of different plant parts such as leaf, stem, flower and root have inhibited germination and radicle length of wild oat. Inhibitory effects on germination increased with increasing concentration of extract solution of the fresh plant parts (Turk and Tawaha. 2003).

2.2.3 Mode of action of allelopathy

For over 2,000 years, allelopathy has been reported in with involve to plant interference. Until 1969, the allelopathic concept was firstly applied to plant ecology elucidating the mechanism of plant interference (Muller. 1969). The most widely bioassay of allelopathy of plant on the based seed germination (Reigosa *et al.* 2006). Biological observations include germination percentage, measurement of shoot and root length. There are increasing evidences that changes in plant growth and development may be due to the significant influence of allelochemicals on cell ultrastrcture, ion and water uptake, phytohormone, metabolism, enzyme functions, photosynthesis, respiration, protein synthesis as well as biology and physiology (Gniazdowska and Bogatek. 2005; Reigosa *et al.* 2006; Li *et al.* 2010). This part reviews the allelochemical mode of action at different levels of plant organization.

2.2.3.1 Seed germination

The inhibition of seed germination and growth seedling by allelopathic phenomenon from many species such as Chinese rice flower (*Aglaia odorata* Lour.), common jasmine (*Jasminum officinale* f. var. *grandiflorum* (L.) Kob.), arabian jasmine (*Jasminum sambac* Ait.), sesame plant (Sesamum indicum) were reported and discussed (Laosinwattana *et al.* 2009; Poonpaiboonpipat *et al.* 2011; Teerarak *et al.* 2013; Hussain *et al.* 2017). α-amylase has great influence on seed germination. It regarding starch break down, which necessary for supplying substrates to respiratory metabolism during germination process. Lemongrass (*Cymbopogon citratus*) essential oil decreased α-amylase activity with the increase of inhibition of germination in seeds of barnyard grass (*Echinochloa crus-galli*). At concentration of 8 μL Petridish^{-1}, the germination rate was 9.25% and the inhibition of α-activity in seeds was 57.45% compared with control (Poonpaiboonpipat *et al.* 2013). Similarly, eucalyptus (*Eucalyptus globulus* Labill) leaf leachates also decreased germination rate and α-activity in seeds of fingermilet (*Eleusine coracana* Gaertn cv. AKP-2) (Padhy *et al.* 2000). Furthermore, 6-Methoxy-2-benzoxazolinone (MBOA) greatly inhibited on germination of lettuce (*Lactuca sativa*) seeds. The concentration required for 50% inhibition was 0.15 mmol/L. The concentration required for 50% inhibition of the activity was 0.12 mmol/L, and this value was almost the same as the concentration required for 50% inhibition of seed germination (Kato-Noguchi, Hisashi and Macías. 2005). These results suggest that the decrease of α-amylase activity in seeds lead to inhibition of germination.

The glyoxylate cycle is an anabolic pathway occurring in plants. The glyoxylate cycle plays an important role in the mobilization of triacylglycerides during germination of fat-storing

seeds. Isocitrate lyase, key enzymes involved in the glyoxylate cycle, increase their activity caused by maximum lipid metabolism in storage tissue of germinating seeds. The previous report suggest that isocitrate lyase appear to be one of the most sensitive enzymes in reaction to alllopathy and its decreased activity could be lead to inhibition of seed germination (Gniazdowska and Bogatek. 2005).

2.2.3.2 Seed imbibition

Water is one of the most important conditions of life and the changes in water conditions pose significant effects on plants. Allelochemicals appear to reduce the absorption of water, nutrients and other ions of plants. In the tests with tobacco (*Nicotiana tabacum*), tannic acid was found that it can reduced water uptake and caused stomatal closure (Einhellig. 1971). Lyu and Blum (1990) reported that net uptake of P, K, and water by seedlings was reduced 57, 75, and 29%, respectively, when the whole root system was exposed to ferulic acid. The extract of black mustard (*Brassica nigra*), at concentration of 16g kg^{-1}, was showed that it greatly inhibited water uptake for lentil *(Lens culinaris)* seeds compared to treatment treated with water (Munir and Tawaha. 2002). The similar study was reported by Turk and Tawaha (2003) that increasing the concentration of aqueous leaf extracts from *B. nigra* significantly inhibited the water uptake by germination of wild oat (*Avena fatua* L.) seeds. The greatest inhibition in water uptake occurred at the 20g kg^{-1} extract concentration for seeds soaked for 4 h. Tawaha and Turk (2003) also indicated that the extracts from *B. nigra* inhibited averaging 55% on water uptake of wild barley (*Hordeum spontaneum)* when compared with the water control, at concentration of 20g per 100ml. These results suggestd that allelopathicity of *B. nigra* could be mediated in part through a regulation of water uptake and inhibition of seeds. In the assays for investigate the effects of ginger (*Zingiber officinale* Rosc.) extracts on seed germination and seedling growth of soybean (*G. max*) and chive (*Allium schoenoprasum* L.), the percent of water uptake was 73.5% for soybean and 29.2% for chive which decreased by 21.6% and 28.6%, respectively, compared with the control (Han *et al.* 2008).

There is also much data on the effect of allelochemicals on membrane bound enzymes such as proton-pumping ATPase localized in plasma membrane (H+-ATPase). H+-ATPase of the plant plasma membrane generates the proton motive force across the plasma membrane that is necessary to activate most of the ion and metabolite transport. H+-ATPase inhibition results in reduction in mineral and water uptake by roots and as a consequence leads to strong effect on essential plant functions such as photosynthesis, respiration or protein synthesis leading finally to reduction of plant growth. Hejl and Koster (2004a, 2004b) reported that juglone, a quinone exuded by the roots

of Juglans *spp.*, inhibited root H+-ATPase activity and leading to decreased water uptake in hydroponically grown corn and soybean seedlings. They also investigated that sorgoleone quinine, the compound extracted from sorghum (*Sorghum bicolor*), decreased nutrient solution use by soybean seedlings and decreased H+-ATPase activity in corn root microsomal membranes.

2.2.3.3 Photosynthesis

Photosynthesis is the basic physicochemical process for plant growth to convert light energy into chemical energy that can later be released to fuel the plants' activities. It is greatly influenced by environmental factors such as light, temperature, water condition, CO_2 and microbes. Disturbances of photosynthesis are one of the most commonly observable phenomenon for physiological effects of many allelochemicals.

Chlorophyll is a green photosynthetic pigment found in plants and plays a major role in the photosynthesis. Any changes in chlorophyll content might results in change in photosynthesis. Allelochemicals affect the photosynthesis by destroying the chlorophyll. The results of (Patterson. 1981) showed that 10-30 µmol/L of caffeic acid, coumaric acid, ferulic acid, cinnamic acid and vanillic acid significantly inhibit the growth of soybean (*Glycine max*) in the strong reducing of photosynthetic products and chlorophyll content of *G. max*. Ervin and Wetzel (2000) reported that the aqueous extract from aboveground of *Juncus effusus* tissues inhibited the growth seedling of *Eleocharis obtusa* due to decreasing on the chlorophyll content. Some allelochemicals such belong to benzoquinones, napthoquinones and triketones such as juglone and sorgoleone inihibited key enzyme for plastoquinon synthesis (Meazza *et al.* 2002). The inhibition of this enzyme disrupted the bopsynthsis of carotenoids and lead to foliar bleaching. Juglone also caused to decrease chlorophyll content in duckweed (*Lemna minor).* Ferulic and p-coumaric acids were reported that reduced the amount of chlorophyll a, b and total chorophyll in soybean on a leaf weight basis. Subsequent reports show benzoic, syringic, protocatechuic, transcinnamic, and caffeic acids reduced the concentration of chlorophyll in leaves of soybean and cowpea, with the major effect on chlorophyll a (Macías *et al.* 2003).

Some studies have shown that allelochemicals or phytochemicals from higher plants exhibited inhibition to ATP synthesis leading to decreased supply of ATP for all energy demanding process (Reigosa *et al.* 2006). In cells, if concentration of cinnamic or benzoic acid reach a high enough, these compounds can inhibit the ATP-generating pathway of chloroplasts. On other hand, flavonoids were reported inhibited hydrolysis of ATP catalyzed by mitochondrial Mg2+ -ATPase.

The major effect of flavonoids seems to be on the ATP-generating pathway, but higher concentrations can inhibit electron transport (Moreland and Novitzky. 1987).

Hence, allelopathic activity can't be explained by only a single mode of action. The majority of effects such as inhibition of germination and seedling growth, decreased water and ion uptake and reduced photosynthetic are caused by a variety of more specific interactions between allelochemicals and molecular systems (Gniazdowska and Bogatek. 2005). Therfore, mode of action of allelopathic activity needs to be studied simultaneously in many aspects.

2.3 *Thunbergia laurifolia* Lindl.

2.3.1 Botany and distribution

Thunbergia laurifolia Lindl. also known as laurel clockvine or blue trumpet vine, and rang jeud in Thailand (Chan and Lim. 2006). It is native to India and the Indomalaya ecozone (Chan *et al.* 2013). *T. laurifolia* is a climbing plant belonging to the Acanthaceae family. The plant grows up to 15 m in height with smooth opposed leaves along the stem. Leaves of *T. laurifolia* are dark green, opposite broadly elliptic to narrowly ovate, 8-10 cm long, 4-5 cm wide, margins entire, usually with scalloped lobes towards the base (Junsi and Siripongvutikorn. 2016). Flowers of the plant is hermaphrodite flower, it is trumpet-shaped with a short broad tube, white outside and yellowish inside. The flowers are not scented and borne on pendulous inflorescences. The corolla is pale blue in color with 5–7 petals, one larger than the others and the flower is up to 8 cm long and 6-8 cm across. The plant flowers bloom almost continuously all year with flowers opening early in the morning and aborting in the evening of the same day. The seed pod is cone-shaped, 1 cm long, with around base (Chan *et al.* 2011). The plant develops a very tuberous root system propagation of *T. laurifolia* is from stem cuttings or shoots from the tuberous roots. *T. laurifolia* is widely cultivated by the color of the flowers is habit of a lot of people.

2.3.2 Chemical composition and some bioactive compounds

In Malaysia, juice from crushed leaves of *T. laurifolia* has been taken for menorrhagia, placed into the ear for deafness, and applied for poulticing cuts and boils. In Thailand, *T. laurifolia* leaves have been used as an antipyretic and an antidote (Junsi and Siripongvutikorn. 2016). It also have been produced and exported as a tea (Chan and Lim. 2006). The tea has been claimed to be able to detoxify the harmful effects of drugs, alcohol and cigarettes. Kanchanapoom *et al.* (2002) has been isolated 8-epi-grandifloric acid and 3'-O-β-glucopyranosyl-stilbericoside along with seven known compounds from *T. laurifolia*. There are several studies showed that *T. laurifolia* contains 5

bioactive phytochemicals for antioxidant, antimicrobial and anticancer activities (Chan and Lim. 2006; Junsi and Siripongvutikorn. 2016). Antioxidant effects of *T. laurifolia* were found that aqueous extract of *T. laurifolia* leaves had high total phenolic content (TPC) and free radical scavenging (DPPH) activity. (Chan *et al.* 2013) reported that the developing leaves had the highest TPC of 513 mg GAE/100 g, mature leaves had the lowest value of 298 mg GAE/100 g and young leaves had the value of 407 mg GAE/100 g. Water, ethanol, and petroleum ether extracts of dried leaf powder of *T. laurifolia* were evaluated for TPC, free radical scavenging, and ferric educing power. Based on TPC, it was found that water extraction (2430 mg GAE/100 g) was the most efficient compared to ethanol (565 mg GAE/100 g) and acetone (142 mg GAE/100 g) extraction. The water extract also yielded the highest free radical scavenging with EC 50 value of 0.13 mg GAE/ml, whereas ethanol and acetone extracts had EC 50 values of 0.26 and 0.61 mg GAE/ml, respectively. The water extract also showed the highest ferric educing power (0.93 mmol/g), compared to extracts of ethanol (0.18 mmol/g) and acetone (0.04 mmol/g). Furthermore, *T. laurifolia* was demonstrated its high allelopathic activity (Somkiat. 2012).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

(1) Ethanol 95% (Merck)

(2) Ethanol 99% (Merck)

(3) Ethyl acetate (Merck)

(4) Hexane (Merck)

(5) Glacial acetic acid (Merck)

(6) Calcium chloride (Sigma - Aldrich)

(7) Sodium hydroxide (Ajax Finechem)

(8) Sodium acetate hydrated (Ajax Finechem)

(9) 3,5-dinitrosalicylic acid (Sigma - Aldrich)

(10) Starch (Sigma - Aldrich)

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

(12) Trichloroacetic acid (Merck),

(13) Iron (II) sulphate (Ajax Finechem)

(14) Folin- Ciocalteu's reagent (Merck)

(15) Sodium carbonate (Ajax Finechem)

(16) Potassium acetate (Quality Reagent Chemical)

(17) Aluminum chloride (Fluka)

(18) Quercetin (Sigma - Aldrich)

3.1.2 Equipments

(1) Centrifuge machine (Universal 320R, Hettich Lab Technology)

(2) Growth chamber (Climace)

(3) Hot-air oven (Memmert)

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

(5) pH meter (Consort)

(6) Rotary evaporator (Rotavapor R-215, BÜCHI)

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

(8) Vortex Mixer (KMC-1300V, Korea)

3.2 Methods

3.2.1 Plant collection and preparation

Barnyard grass (*Echinochloa crus-galli* (L.) Beauv.), slender amaranth (*Amaranthus gracilis* Desf.) seeds and laurel clockvine (*Thunbergia laurifolia* Lindl.) leaves were collected by hand from the experimental field at the King Mongkut's Institute of Technology Ladkrabang and paddy fields in the Ladkrabang district, Bangkok, Thailand. The mature and healthy leaves of *T. laurifolia* were harvested, cleaned from soil immediately by running tap water, then dried-up in a hot-air oven at 45°C for 3 days and ground to small pieces using an electrical blender.

Mature 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. Mature seeds of *A. gracilis* were lightly shaken in collection bags to release seeds. Seeds with damaged coats were eliminated. These species were selected for the experiments because these species are serious problem weeds in paddy rice field and upland crop field.

3.2.2 Effect of sequential solvent extraction on the crude extract yields

One hundred gram of *T. laurifolia* dried leaves were soaked in each 900 ml of hexane at room temperature for 3 days. After 3 days, the solutions were filtered through 2 layers of cheesecloth and re-filtered through Whatman no. 93 filter paper. Following filtration, the solutions were dried by evaporation of the solvent using a rotary evaporator (Buchi R215, Switzerland) under a partial vacuum at 45°C until constant crude extract weight was reached. After that, the residue was re-extracted second time with ethyl acetate and third time as the same condition of the first extraction procedure (Figure 3.1). Extraction yield was compared between the different solvents.

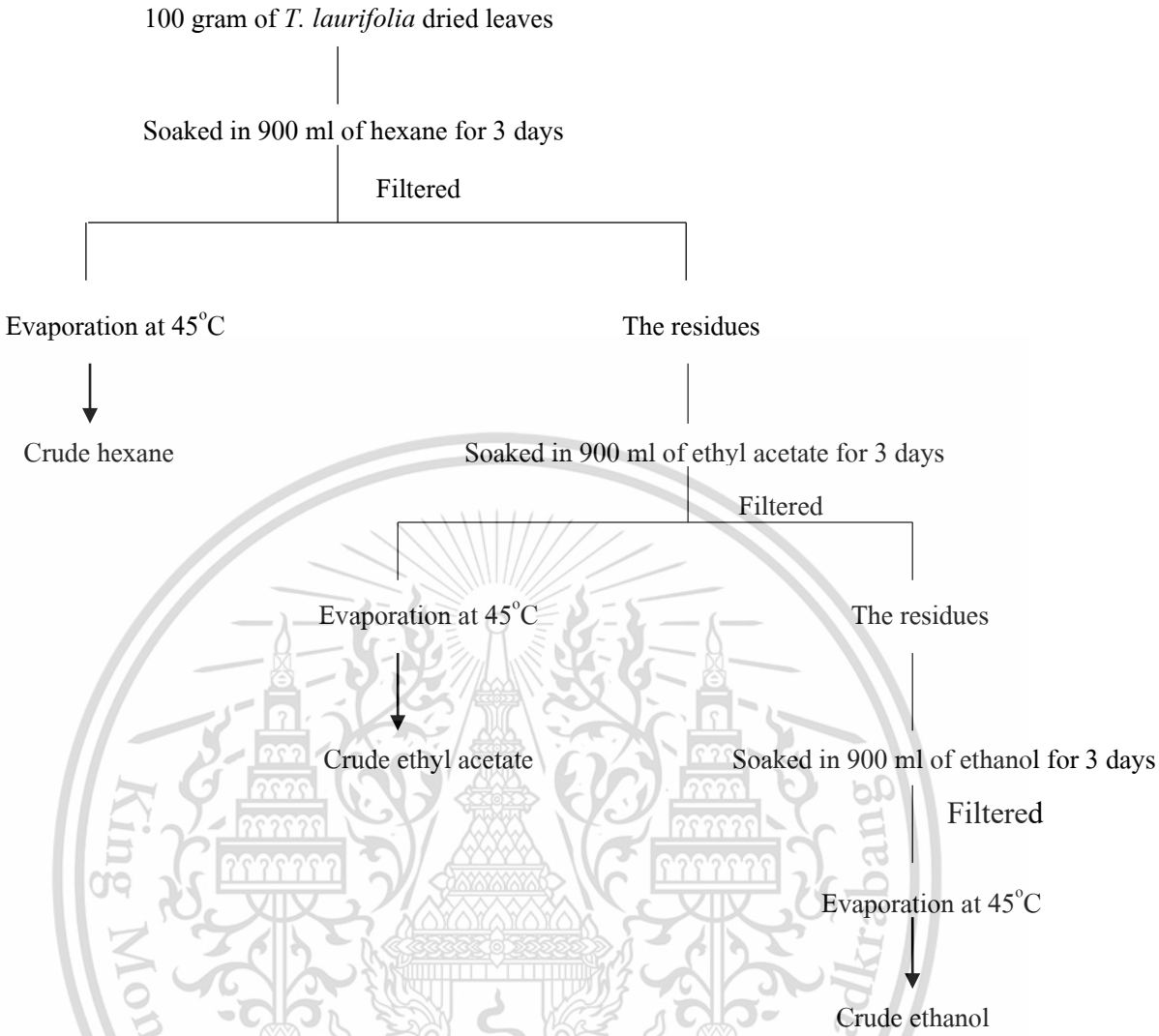

Figure 3.1 Sequential solvent extraction from dried leaves of *T. laurifolia*

3.2.3 Bioassay effect of sequential solvent extraction from *T. laurifolia* on seed germination and seedling growth of *A. gracilis* and *E. crus-galli*

For this study, the crude extracts (hexane, ethyl acetate and ethanol) were prepared by dissolving 2 g of the extract in 100 ml solvent (hexane, ethyl acetate and ethanol, respectively), to have a stock solution of 2% concentration. The extracts were prepared to obtain serial concentration of 125, 250, 500, 1,000, 5,000, 10,000, 15,000 and 20,000 ppm from stock extracts.

The crude extracts of *T. laurifolia* were tested for their effect on germination of seed of *A. gracilis* and *E. crus-galli.* Five ml of concentration (125, 250, 500 and 1,000 ppm) was added to each petri dishes (9 cm diameter) containing germination paper, and then 20 seeds of *A. gracilis* were placed on the germination paper. Five ml of concentration (5,000, 10,000, 15,000 and 20,000

ppm) was added to each petri dishes, and then 20 seeds of *E. crus-galli* were placed. The petri dish that has only distilled water was used as control. The treatments were replicated four times in a completely randomized design (CRD). The germination was deemed to have occurred only after the radicle had protruded beyond the seed coat by at least 2 mm at seven days after treatment. All petri dishes were covered and placed at room temperature. After seven days, germination percentage, shoot and root length were recorded in all treatments. Inhibition percentage relative to control was calculated as:

Inhibition (% of control) = 100 - [(sample extracts/control) x 100]

3.2.4 Effect of different ethanol ratios on the crude extraction yields

The method described by Laosinwattana and Teerarak (2014) was applied in this experiment. Twenty gram of *T. laurifolia* dried leaves was soaked in each 180 ml of different solvent system at room temperature (with solvent is distilled water keep at 4^{o}C) for 3 days. The extraction solvent ratios of ethanol in distilled water (0, 25, 50, 75 and 100% (v/v) ethanol) were used in this study. After 3 days, the solutions were filtered through 2 layers of cheesecloth and re-filtered through Whatman no. 93 filter paper. Following the filtration, the solutions were dried up by a rotary evaporator (Buchi R215, Switzerland), under a partial vacuum at 45^{o}C until constant crude extract weight was reached. After that each residue was re-extracted 2 times more with the same extraction solvent as the same condition of the first extraction procedure, and then crude extract of the extraction first time, second time and third time were pooled (Figure 3.2). Extraction yield was compared between the different solvent systems.

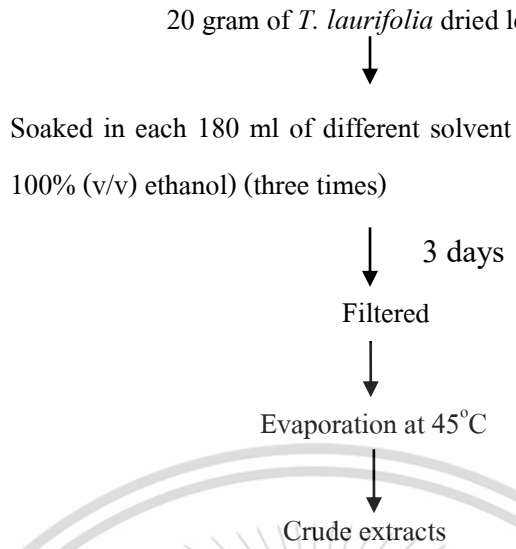

Figure 3.2 Flow chart for extraction and diffirent solvent systems of active compounds from *T. laurifolia* dried leaves

3.2.5 Determination of total polyphenol content

3.2.5.1. Determination of total phenolic content

The Folin-Ciocalteu method described by (Chumyam *et al.* 2013) was used to investigate the total phenol content of the extracts. 1 ml of the extracts (at concentration 0.25, 0.5 and 1 mg/ml) was added to 4.5 ml distilled water, 0.5 ml of 2N Folin-Ciocalteu reagent. The reaction mixtures were vortexed for 5 second and added 4 ml of sodium carbonate (7.5%). Afterwards, the mixtures were vortexed for 15 second and incubated at room temperature for 60 minutes in the dark. Next, the mixtures were centrifuged at 6000 rpm for 5 minutes at 25°C. The absorbance was read at 765 nm with a spectrophotometer. The experiment was replicated three times in a completely randomized design (CRD). The total phenolic content was calculated on the basis of the calibration curve of gallic acid standard. Results were showed as mg gallic acid equivalent per gram crude extract (mg GAE/g CE).

3.2.5.2. Determination of total flavonoid content

The aluminum chloride colorimetric method according to Patel *et al.* (2010) was used to quantify the total flavonoid content of the extracts. 0.5 ml of the extracts (at concentration 1.0, 2.0 and 4.0 mg/ml) were diluted with 1.5 ml methanol. Subsequently, the extracts were added to 0.1 ml of aluminum chloride (10%), 0.1 ml of 1M potassium acetate and 2.8 ml distilled water. Then, the mixtures were kept for 30 minutes at room temperature. The maximum absorbance of the

mixture was measured at 415 nm using a spectrophotometer. The experiment was replicated three times in a completely randomized design (CRD). The total flavonoid content was showed as milligram quercetin equivalent per gram crude extract (mg QE/g CE).

3.2.5.3. Determination of total tannin content

The total tannin content was determined using Folin-Ciocalteu method described by (Tamilselvi *et al.* 2012). 0.1 ml of the extracts (at concentration 2.5, 5.0, 10 mg/ml) were mixed 7.5 ml of distilled water. Then 0.5 ml of Folin-Ciocalteu reagent, 1 ml of 35% sodium carbonate solution and 0.9 ml of distilled water were added into mixtures. The mixtures were shaken well, kept for 30 minutes at room temperature. Finally, absorbance was measured at 725 nm. Blanks were prepared with distilled water instead of the extracts. The experiment was replicated three times in a completely randomized design (CRD). The total tannin content was showed as mg tannic acid per gram crude extract (mg TAE/g CE).

3.2.6 Bioassay effect of different ethanol ratio extracts from *T. laurifolia* on germination and seedling growth of *A. gracilis* and *E. crus-galli*

For this study, the crude extracts (0, 25, 50, 75, 100% ethanol) were prepared by dissolving 1 g of the extract in 100 ml distilled water (100% ethanol crude extract dissolving in ethanol), to have a stock solution of 2% concentration. The extracts were prepared to obtain serial concentration of 625, 1,250, 2,500, 5,000, 10,000, 15,000 and 20,000 ppm from stock extracts.

The crude extracts of *T. laurifolia* were tested for their effect on seed germination of *A. gracilis.* 5 ml of each concentration (625, 1,250, 2,500, 5,000 and 10,000 ppm) were added to each petri dish (9 cm diameter) containing germination paper, and then 20 seeds of testing weeds were placed on the germination paper. Five ml of concentration (2,500, 5,000, 10,000 and 20,000 ppm) was added to each petri dishes, and then 20 seeds of *E. crus-galli* were placed. The petri dishes that have only distilled water was used as control treatments. All of treatments were replicated four times in a completely randomized design (CRD). The germination was deemed to have occurred only after the radicle had protruded beyond the seed coat by at least 2 mm at seven days after treated. All petri dishes were covered and placed at room temperature. After seven days treated, germination percentage, shoot, and root length were recorded in all of treatments. Inhibition percentage over control was calculated as:

Inhibition (% of control) = 100 - [(sample extracts/control) x 100]

3.2.7 Seed imbibition and α-amylase activity bioassay

3.2.7.1. Effect on seed imbibition

To investigate the imbibition of seeds, a method according to Turk and Tawaha (2003) was used. Four replicates of 100 healthy seeds of *A. gracilis* and 30 seeds of *E. crus-galli* were weighed and recorded as starting seed weight (W1). These seeds were separately germinated in crude extracts of *T. laurifolia* and distilled water as control (according to above treatment). After imbibition period, seed weights were recorded as final seed weight (W2) for each treatment and exposure time. Seed imbibition percentage of the seeds was calculated from following the equation:

Water uptake (%) = [(W2-W1)/W1] x 100, while: W1 is the weight of the seeds before imbibition period, W2 is the weight of the seeds after imbibition period.

3.2.7.2. Bioassay of α-amylase activity

The method according to Bernfeld (1955) and Sadasivam (1996) was used to investigate activity of α-amylase of the seeds. After measuring imbibition, the seeds (100 seeds of *A. gracilis*. for one determination) were homogenized with 4 ml ice-cold solution of 0.1M $CalCl_2$ and then centrifuged at 10,000 rpm for 20 minutes at 4°C. Supernatant was used as enzyme extract. The α-amylase activity was then assayed by measuring rate of generation of reducing sugar from soluble starch. 1 ml of supernatant was mixed with 1 ml of 1% soluble starch in acetate buffer solution at pH 5.5. After that, the assay medium was incubated for 15 minutes at 37°C. Next, 1 ml of DNS reagent (40 mM 3.5 dinitrosalicylic acid, 0.4 N NaOH and 1M K-Na tartrate) was added, and immediately heated in a boiling water bath for 5 minutes. The mixture was cooled under running tap water. The intensity of color was measured as absorption at 560 nm by a spectrophotometer. The experiment was replicated four times in a completely randomized design (CRD). A standard graph was prepared using maltose, and the amount of α-amylase present in sample was calculated from standard curve and expressed as μmol maltose/min/g (fresh weight).

3.2.8 Statistical analysis

The experimental design was carried out as a completely randomized design (CRD) with three replications and was repeated three times using one-way analysis of variance (ANOVA). Whenever ANOVA indicated significant effects ($p<0.05$), a pairwise comparison of means by Tukey's studentized range test is carried.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Effect of sequential solvent extraction on crude extraction yield and allelopathic activity from *T. laurifoia* leaves

4.1.1 Extraction yield

Solvent is an important factor for obtaining extracts. It has a great impact on extraction yield and allelopathic activity. Effect of sequential solvent extraction (hexane, ethyl acetate, and ethanol) on crude yields was exhibited as gram per 100 g of dry weight (g/100 g DW) presented in Figure 4.1. The results showed that various extraction solvents have a great influence on the extraction yields. The extraction yield of *T. laurifolia* was found highest when extracted by ethanol solvent (4.16 g/100 g DW). Ethyl acetate solvents for extraction yield only 1.24 g/100 g DW and hexane had the lowest yield of 0.63 g/100 g DW, respectively.

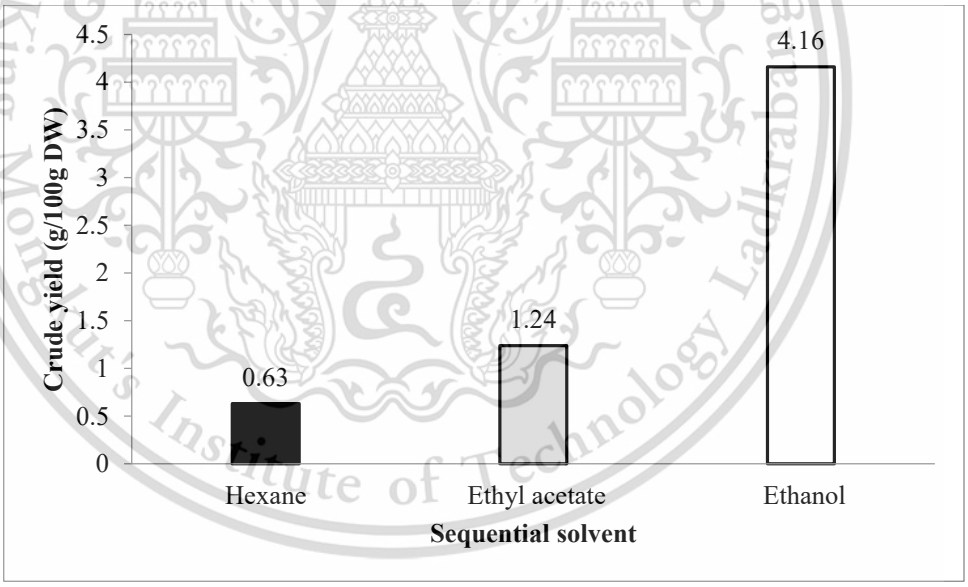

Figure 4.1 The effect of sequential solvent extraction on crude extract yields from *T. laurifolia* dried leaves

4.1.2 Effect of sequential solvent extract from *T. laurifolia* on seed germination and seedling growth of *A. gracilis* and *E. crus-galli*

The inhibitory effect of extracts obtained by hexane, ethyl acetate and ethanol solvent on seed germination and seedling growth of *A. gracilis* is shown in Figure 4.2. The results indicated that different extract significantly affected on seed germination and seedling growth. The inhibitory effect also increased with the increase of concentration of the extracts. Figure 4.2 exhibit that the hexane and ethyl acetate extract completely inhibited germination, shoot length and root length of *A. gracilis*, at concentration of 500 ppm. Besides, the ethanol extract at concentration of 1000 ppm only inhibited the germination of *A. gracilis* seeds by 89.04%. The ethyl acetate was the highest inhibitory effect compared with the hexane and ethanol extracts, the extract inhibited germination, shoot length and root length of *A.gracilis* seeds by 95.89, 51.56 and 79.06%, respectively, at concentration of 250 ppm.

The inhibitory effect of hexane, ethyl acetate and ethanol extract on seed germination and seedling growth of *E. crus-galli* is expressed in Figure 4.3. The results found that seed germination and seedling growth of the seeds treated by different extracts were differed. The inhibitory effect also increased with the increase of concentration of the testing extracts. At concentration of 20,000 ppm, the hexane, ethyl acetate and ethanol inhibited seed germination by 87.01, 15.58 and 12.99%, respectively. At concentration of 10,000 ppm, the hexane extract completely inhibited shoot and root length of *E. crus-galli*, following by the ethyl acetate extract (inhibited shoot and root length by 32.4 and 59.43%) and ethanol extract (inhibited shoot and root length by 20.72 and 55.66%).

This result indicated that extracts from *T. laurifolia* inhibited root length more than shoot length on both of *A.gracilis* and *E. crus-galli*. It could be explained by the sensitivity of root growth. Many previous studies agree with our results. Meksawat and Pornprom (2010) who suggested that root length has a high sensitivity to allelochemicals. Jelassi *et al.* (2016) who also reported that extracts from three Tunisian species of Acacia (*Acacia cyclops, Acacia mollissima* and *Acacia cyanophylla*) inhibited on root length stronger than shoot length. Sitthinoi *et al.* (2017) studied allelopathic effects of jungle rice extract on seed germination and seedling growth of rice and reported that root growth was the most sensitive variable affected by the jungle rice extracts in the current study.

Wu *et al.* (2000) reported that short-chain fatty acids (aliphatic acids) have been claimed as a third category of compounds implicated in wheat allelopathy. The production of short-chain

fatty acids as a result of anaerobic fermentation of the insoluble polysaccharides which represent the major constituents of wheat straw, can also adversely affect crop development in soils of low redox potential. Suzuki *et al.* (1996) suggested that (5Z,8Z,11Z,14Z,17Z)-eicosapentaenoic acid on the basis displays growth-inhibitory activity and spore-settlement suppressive activity. Fatty acids often are extracted by using hexane as a solvent because of the polarity of hexane. In this study, the hexane extract showed the strongest inhibitory effect, it could be explained the fatty acids are one of the main allelochemicals in *T. laurifolia* leaves.

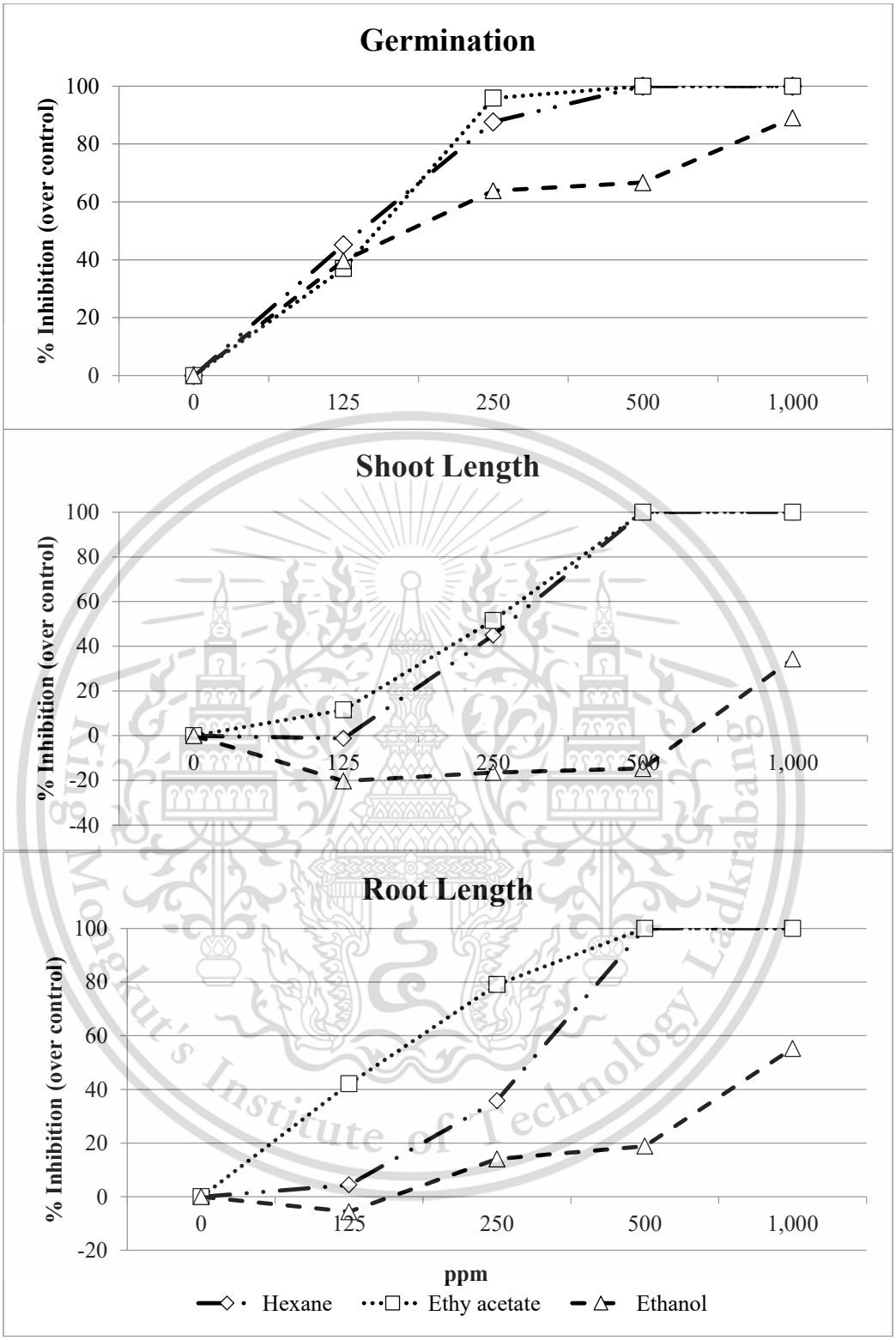

Figure 4.2 Inhibitory effect of sequential solvent extraction from *T. laurifolia* dried leaves concentrations of the extracts (125, 250, 500, and 1,000 ppm) on seed germination and seedling growth of *A. gracilis*

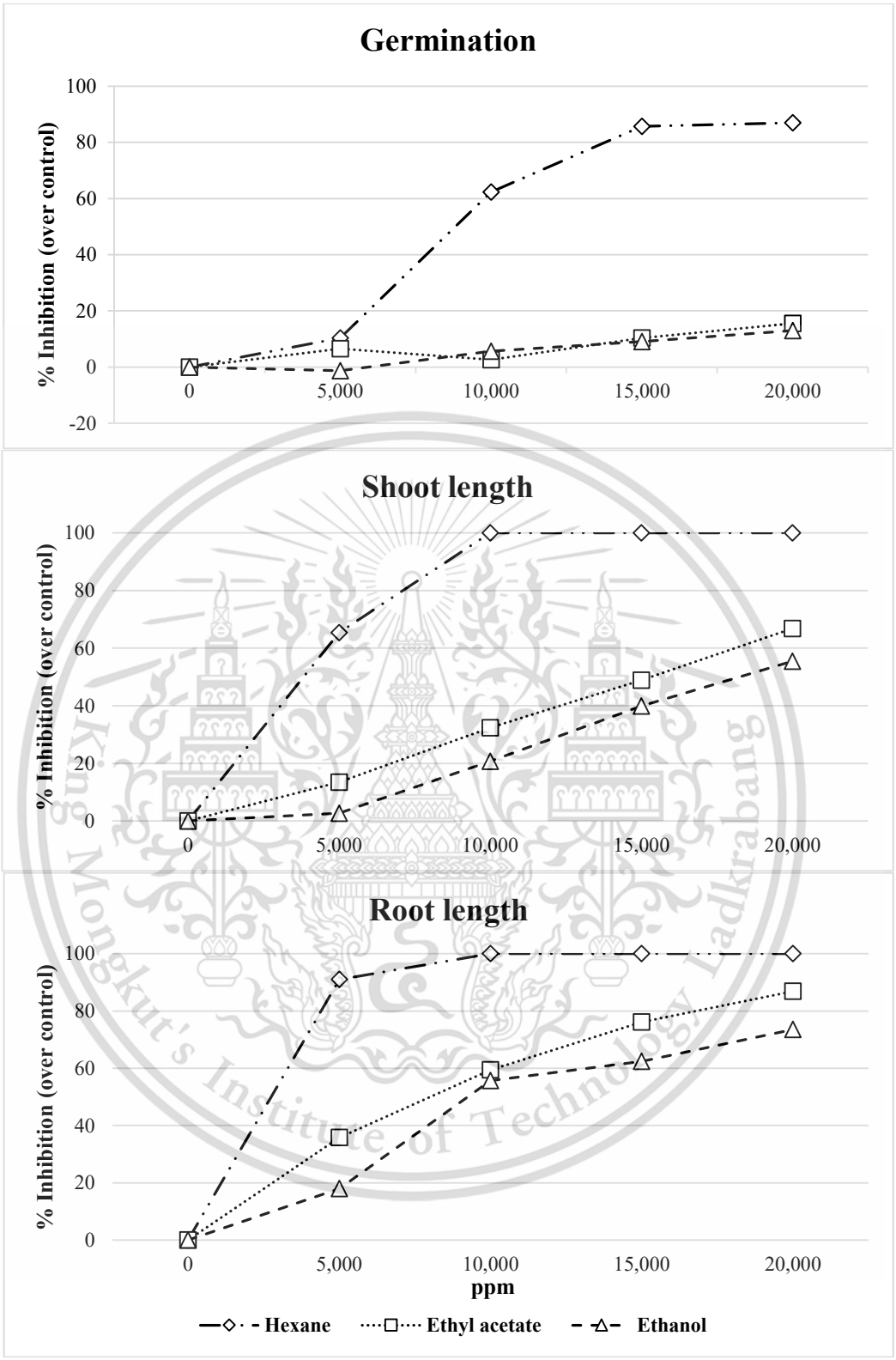

Figure 4.3 Inhibitory effect of sequential solvent extraction from *T. laurifolia* dried leaves concentrations of the extracts (5,000, 10,000, 15,000, and 20,000 ppm) on seed germination and seedling growth of *E. crus-galli*

4.2 Effect of different ethanol ratio solvents on the crude extraction yields, total polyphenolic content and inhibitory effect of the extracts

4.2.1 Extraction yield

Solvent selection is one of the most important factors for obtaining extracts with an amount of crude yields and strong bioactivities. In this study, the extraction solvents significantly affected on crude yield of *T. laurifolia* leaf extracts (Figure 4.4). The results showed that the extraction yields of various solvents decreased in the following order: 25% ethanol > 50% ethanol > 75% ethanol > 0% ethanol > 100% ethanol. That could be seen that the extraction yield of 25% ethanol was the highest (4.31 g/20 g DW) and the extraction yield of 100% ethanol was lowest (1.62 g/20 g DW). The results exhibited that the major chemicals in *T. laurifolia* leaves are mostly high in polarity. This finding was in an agreement with the study of Wichittrakarn *et al*. (2011), who reported that the greatest recovery was achieved by using ethanol at 25%. The results could be explained by several factors such as composition of each particular plant, differences in the solubility of extractive from *T. laurifolia* and their polarity. Water has high polarity so it could dissolve and remove high polar compounds from the leaves, while ethanol (non-polar solvent) can only dissolve non-polar compounds. The mixture of ethanol in solvents could dissolve both of polar and non-polar compounds, it may explain why this solvent could give more crude extract yield than only water or ethanol solvents.

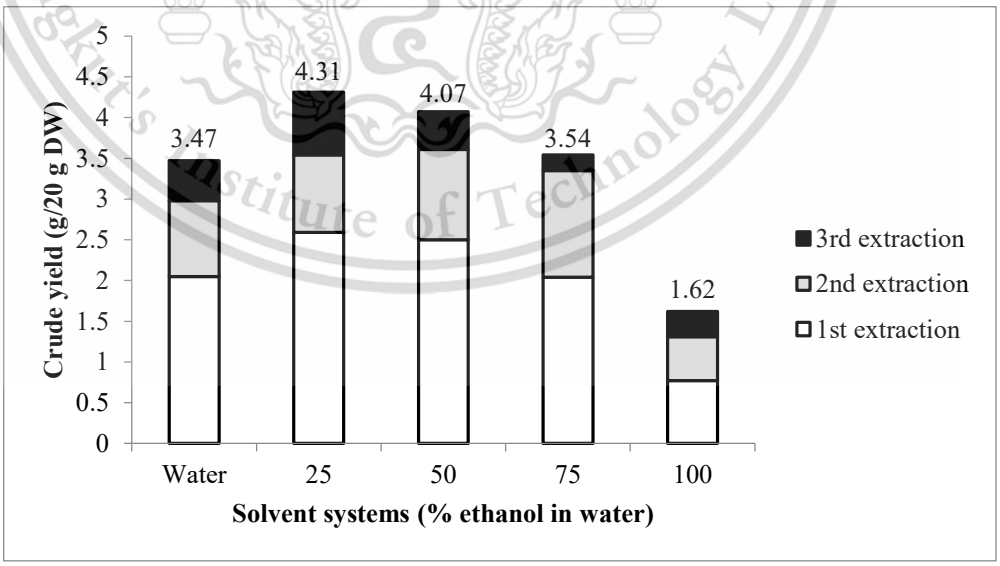

Figure 4.4 The effect of different ethanol ratios on crude extract yields from *T. laurifolia* dried leaves

4.2.2 Determination of total polyphenolic content

4.2.2.1 Total phenolic content (TPC)

Table 4.1 shown the TPC of the extracts measured using Folin-Ciocalteu method. Results of these assays demonstrated variability in total phenolic content ($P<0.05$). The TPC values of the extract range from 48.47 mg GAE/g CE for water extract to 97.80 mg GAE/g CE for 75% ethanol extract and they decreased in the following order: 75% ethanol > 50% ethanol > 25% ethanol > 100% ethanol > water. The results were agreement with the report of Sun *et al.* (2015), who suggested that 75% ethanol in water solvent could be the best extraction solvent for phenolic propolis. Junsi *et al.* (2017) also reported that TPC in crude, dry extract of *T. laurifolia* leaves was 106 mg GAE/g crude, dry extract. However, in the case of Oonsivilai (2006), who suggested that aqueous extract of *T. laurifolia* leaves had higher TPC compared with ethanol and acetone extract, these differences in phenolic content might be due to variable factors such as planting location, extraction preparation and stage of leaf development.

4.2.2.2 Total flavonoid content (TFC)

Total flavonoid contents of different extracts are given in Table 4.1. The TFC can be grouped into three levels. The highest level with the highest value belongs to the 100% ethanol extract (4.20 mg QE/g CE). The second level (2.26-2.39 mg QE/g CE) includes the 50% ethanol extract (2.26 mg QE/g CE) and the 75% ethanol extract (2.39 mg QE/g CE). The final level with the lowest TFC (1.33-1.58 mg QE/g CE) includes the water extract (1.33 mg QE/g CE) and the 25% ethanol extract (1.58 mg QE/g CE). This finding was in an agreement with the study of Rojsanga *et al.* (2012) who reported the presence of flavonoid compounds in *T. laurifolia.*

4.2.2.3 Total tannin content

The recovery of tannin content was shown in Table 4.1. The tannin content ranged from 7.30 mg TAE/g CE to 11.81 mg TAE/g CE. The highest tannin content belongs the 50% ethanol extract (11.42 mg TAE/g CE) but no different ($P<0.05$) was observed between 75% ethanol extract (11.81 mg TAE/g CE). Followed by the 25% ethanol extract (10.66 mg TAE/g CE) and the lowest tannin content are the water extract (7.69 mg TAE/g CE) and the 100% ethanol extract (7.30 mg TAE/g CE). No results have been published on the tannin composition of *T. laurifolia.*

Table 4.1 Content of total phenolic, flavonoid and tannin in *T. laurifolia* extracts

Solvents (% ethanol)	Phenolic (mg GAE/g CE)	Flavonoid (mg QE/g CE)	Tannin (mg TAE/g CE)
Water	48.47 ± 0.67 [e]	1.33 ± 0.03 [c]	7.69 ± 0.70 [c]
25	71.66 ± 1.33 [c]	1.58 ± 0.03 [c]	10.66 ± 0.39 [b]
50	88.23 ± 2.00 [b]	2.26 ± 0.11 [b]	11.42 ± 0.33 [a]
75	97.80 ± 1.11 [a]	2.39 ± 0.07 [b]	11.81 ± 0.38 [a]
100	63.03 ± 1.27 [d]	4.20 ± 0.34 [a]	7.30 ± 0.25 [c]

[1]/ [a-e] Means sharing different letters in same column are significantly different (P<0.05)

[2]/ All values were expressed as mean ± standard deviation

4.2.3 Bioassay effect of different ethanol ratio extracts from *T. laurifolia* on seed germination and seedling growth of *A. gracilis* and *E. crus-galli*

The effect of crude extracts from *T. laurifolia* dried leaves on germination and seedling growth of *A. gracilis* are shown in Figure 4.5. The results revealed that the extracts obtained from different ethanol ratio significantly effect on seed germination and seedling growth of *A. gracilis*. The inhibitory effect also increased with the increase of concentration of the extracts. The extracts of 75 and 100% ethanol in water completely inhibited seed germination, shoot length and root length, at concentration of 2,500 ppm. Moreover, the extracts from all ratios completely inhibited seed germination, at concentration of 10,000 ppm. The 100% ethanol extract expressed the strongest inhibitory effect on seed germination of *A. gracilis* followed by the 75% ethanol, 50% ethanol, 25% ethanol and the water extracts (inhibited seed germination by 100, 84.29, 78.57, 42.86 and 35.71%, respectively), at concentration of 1,250 ppm.

Figure 4.6 expressed inhibitory effect of *T. laurifolia* extracts on seed germination and seedling growth of *E. crus-galli*. In this study, all of extracts from *T. laurifolia* showed great

allelopathic activity. The 100% ethanol extract exhibited greater on inhibitory effect compared with the other extracts. At concentration of 20,000 ppm, the 100% ethanol extract inhibited seed germination, shoot length and root length by 24.05, 44.49 and 72.36%, respectively.

The variation in inhibition of germination and seedling growth of the extracts in different solvents may be attributed to the different polarity of the solvents. Different chemicals were dissolved in different polar solvents that led to the variability of the extracts of same plant in different ethanol ratio solvents. This result was supported by the study of Wichittrakarn *et al.* (2011) who studied the optimal extraction solvent for extraction of *Tagetes erecta* Linn. and found that 75% ethanol in water demonstrated the highest inhibitory effect. Poonpaiboonpipat *et al.* (2011) also reported that *Jasminum sambac* Ait. crude extract from 50% ethanol gave the highest inhibitory activity compared with other extracts. At concentration of 4,000 ppm, the 50% ethanol extract reduced seed germination of *Sesbania aculeate* by 78% over control. The difference may be due to allelochemicals presented in the plants are different.

Simple phenolic acids have been the most frequently identified allelopathic agents. Phenolic compounds are chemical compounds consisting of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group. Phenolic compounds are generally thought of as containing a range of compound types that include structures such as simple aromatic phenols, hydroxy and substituted benzoic acids and aldehydes, hydroxy and substituted cinnamic acids, coumarins, tannins, and perhaps a few of the flavonoids (Blum 2011). This results exhibited the correlation between flavonoids and allelopathic activity. The 100% ethanol extract not only showed the strongest inhibitory effect but also contained the highest total flavonoid content compared with other extracts.

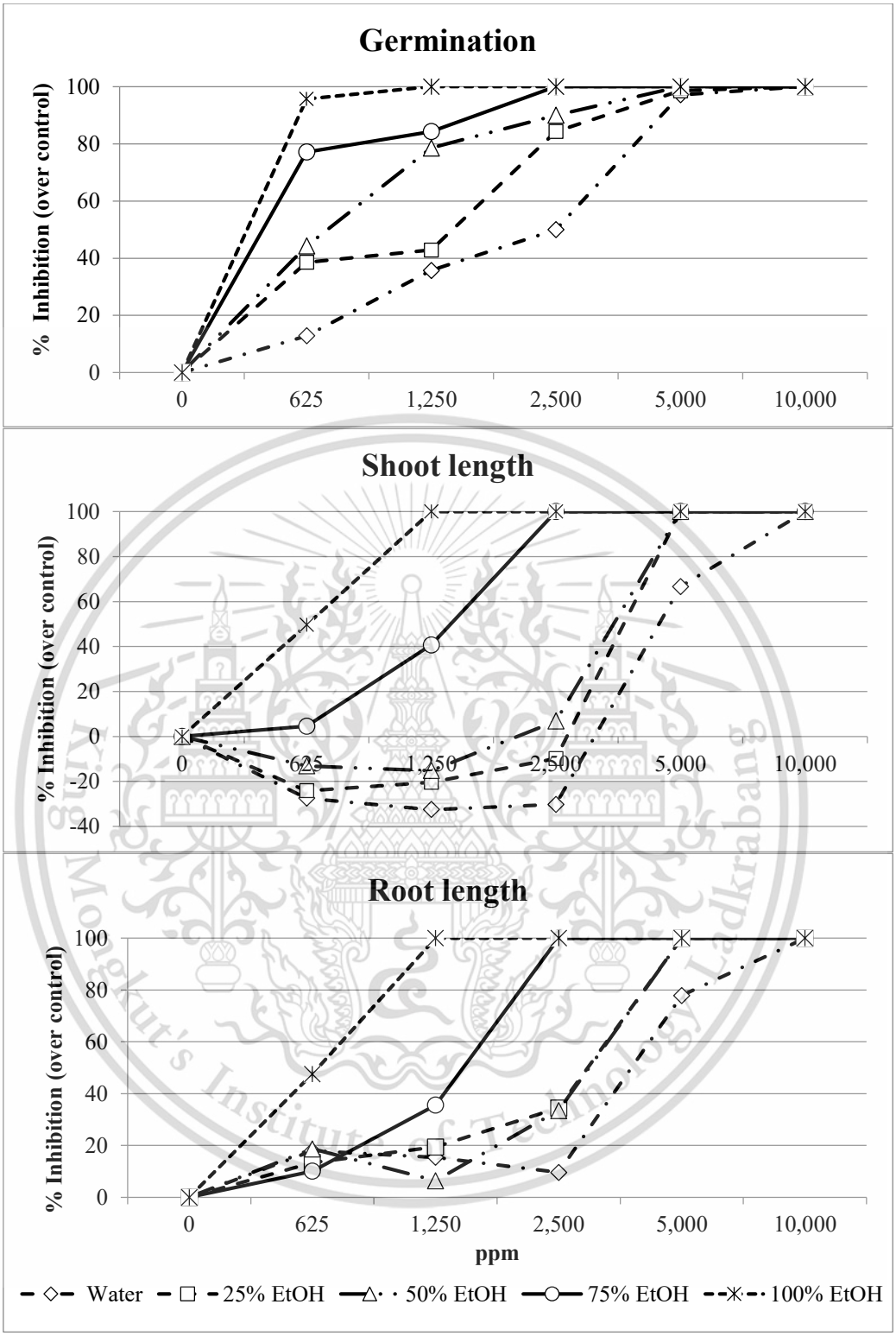

Figure 4.5 Inhibitory effect of crude extracts obtained by different ratios of ethanol in water from *T. laurifolia* dried leaves and concentrations of the extracts (625, 1,250, 2,500, 5,000 and 10,000 ppm) on germination and seedling growth of *A. gracilis*

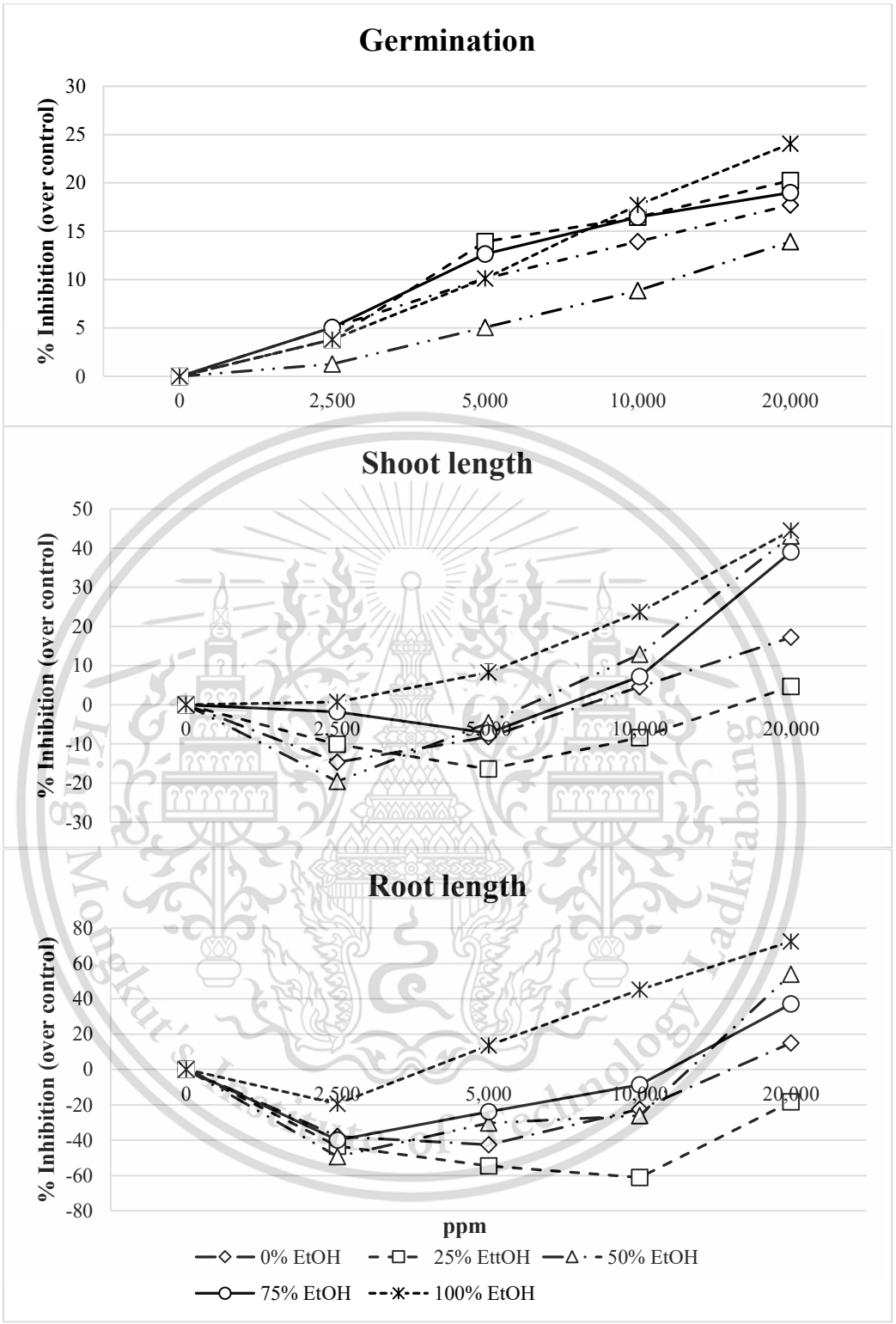

Figure 4.6 Inhibitory effect of crude extracts obtained by different ratios of ethanol in water from *T. laurifolia* dried leaves and concentrations of the extracts (2,500, 5,000, 10,000, and 20,000 ppm) on germination and seedling growth of *E. crus-galli*

4.4 Seed imbibition and α-amylase activity bioassay

Percentage of imbibition and α-amylase experiments were studied to understand the mechanism of inhibition on *A. gracilis* and *E. crus-galli* seed germination. Figure 4.7 and 4.8 exhibited the percentage of imbibition at different imbibition periods and concentration of the 100% ethanol extract. The results exposed that the percentage of imbibition increased by prolonging the imbibition period, at the same concentration. In the control seeds, the percentage of imbibition at different imbibition periods of 12, 18 and 24 hours were 18.52, 23.50 and 28.67%, respectively. Under the same imbibition period, no significant differences in imbibition among all concentrations that were observed. It may explain that the extract from *T. laurifolia* inhibits seed germination and seedling growth of *A. gracilis* not by inhibiting the imbibition of the seeds. However, on *E. crus-galli*, the percentage of imbibition of control and treated seed was significant difference ($P<0.05$). This finding was consistent with the study of Teerarak *et al.* (2012), who reported that the wettable powder formulation of crude extract from *Jasminum officinale* f. var. *grandiflorum* (Linn.) Kob. inhibited the imbibition of *Echinochloa crus-galli* seeds. The difference could be due to allelochemical compounds exist inside plants are different.

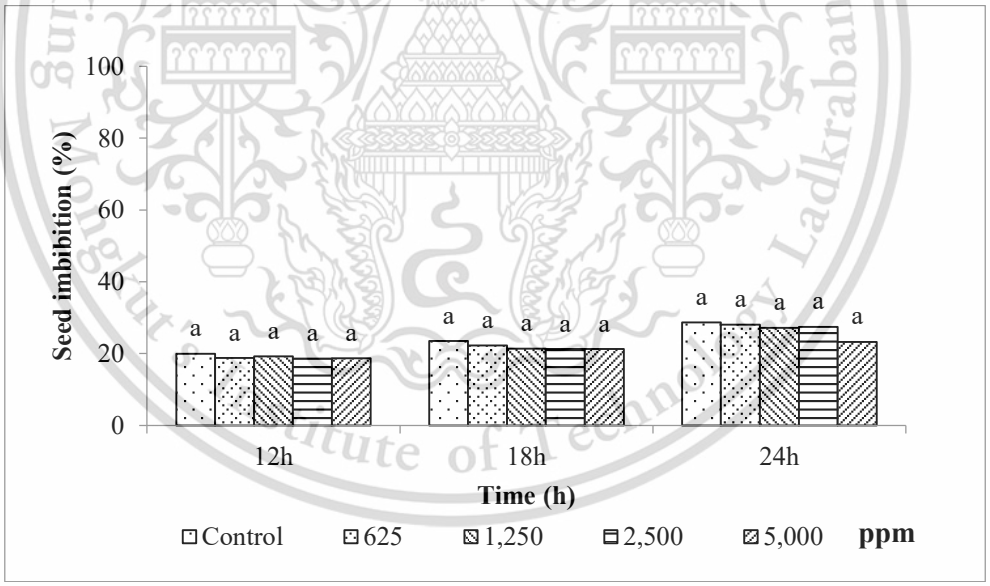

Figure 4.7 Effects of the 100% ethanol extract from *T. laurifolia* on imbibition of *A. gracilis* seeds at different imbibition periods. Different letters are significantly different ($p<0.05$)

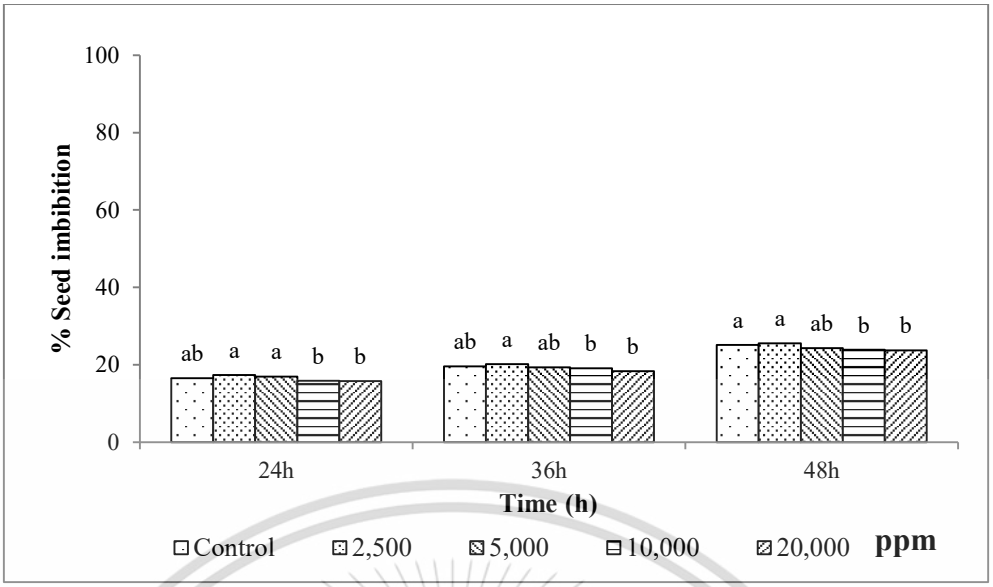

Figure 4.8 Effects of the 100% ethanol extract from *T. laurifolia* on imbibition of *E. crus-galli* seeds at different imbibition periods. Different letters are significantly different ($p<0.05$)

During seed germination, α-amylase plays an important role in the breakdown starch and proteins, which provide the energy for the growth of roots and shoots. The α-amylase activity of *A. gracilis* and *E. crus-galli* seeds was analyzed during they were assayed with the crude extract and the results were shown in Figure 4.9 and 4.10. The results indicated that increasing on the concentration of the extract lead to a significant increase of the inhibition on α-amylase activity of the seeds. Moreover, the α-amylase activity also increased by prolonging the imbibition period, under the same concentration. For the imbibition periods of 12 and 24 hours, the inhibitory effect on α-amylase activity at each concentration was significantly different. At the same imbibition period, the α-amylase activity of *A. gracilis* and *E. crus-galli* seeds were strongly inhibited at the concentration of 5,000 and 20,000 ppm, respectively. The results found in this study were consistent with those of Kato-Noguchi and Macías (2008), who suggested that 6-methoxy-2-benxozazolinone may inhibit the seed germination by inhibiting the induction of α-amylase activity.

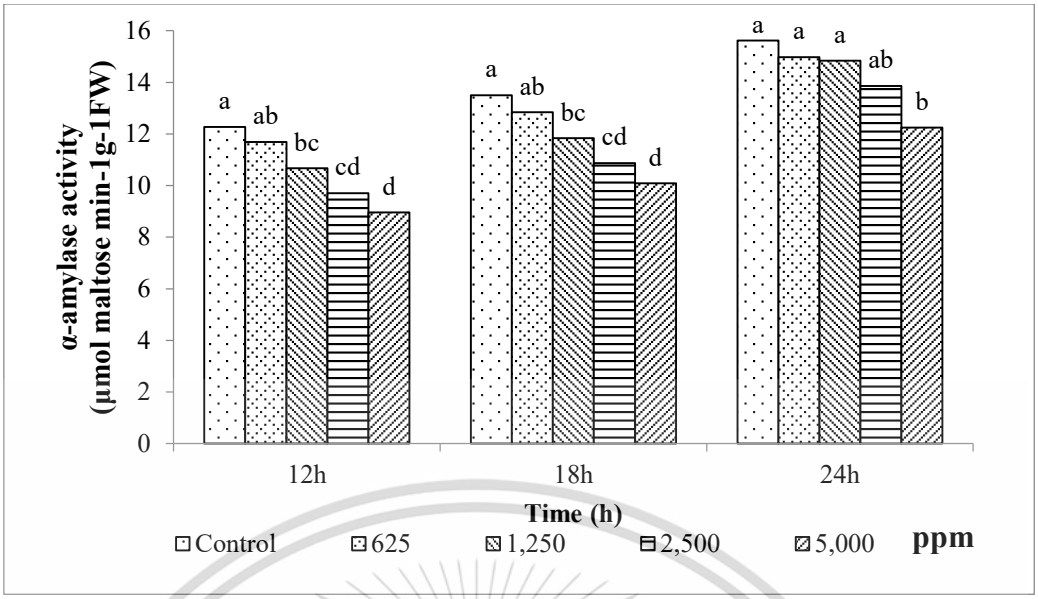

Figure 4.9 Effects of the 100% ethanol extract from *T. laurifolia* on α-amylase activity of *A. gracilis* seeds at different imbibition periods. Different letters are significantly different ($p<0.05$)

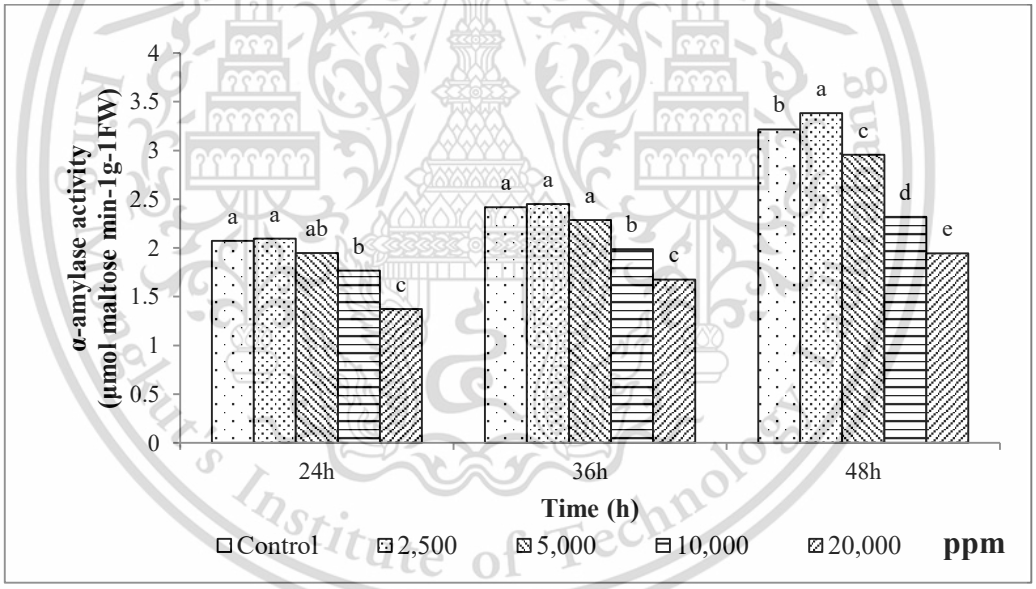

Figure 4.10 Effects of the 100% ethanol extract from *T. laurifolia* on α-amylase activity of *E. curs-galli* seeds at different imbibition periods. Different letters are significantly different ($p<0.05$)

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

5.1.1 Effect of different solvents on extraction yield and allelopathic activity from *T. laurifoia* leaves

The highest crude extraction yield was obtained from ethanol solvent.

The strongest inhibitory effect on seed germination and seedling growth of *E. crus-galli* and *A. gracilis* was the hexane extract.

5.1.2 Effect of different ethanol ratio solvents on the crude extraction yields, total polyphenol content and inhibitory effect of the extracts

The highest crude extraction yield was obtained from 25% ethanol in water solvent.

The highest total phenolic content from *T. laurifolia* leaf was found in the 75% ethanol extract.

The highest total flavonoid content from *T. laurifolia* leaf was found in the 100% ethanol extract.

The highest total tannin content from *T. laurifolia* leaf was found in the 50% ethanol extract but no different ($P<0.05$) was observed between 75% ethanol extract.

5.1.3 Seed imbibition and α-amylase activity bioassay

The percentage of imbibition increased by prolonging the imbibition period, at the same concentration. Under the same imbibition period, no significant differences in imbibition among all concentrations.

α-amylase activities of both bioassay seeds were increased by prolonging time whereas decreased with higher concentration of *T. laurifolia* extract.

5.2 Recommendation

T. lauriflia leaf extracts have a great allelopathic activity that can apply for weed control.

Further studies should be studied the natural herbicide product from *T. lauriflia* leaf, combination of *T. laurifolia* with other plants, and its activity in the field condition.

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