สำนักหอสมุดกลาง พระจอมเกล้าลาดกระบัง

Project title: Study on DNA degradation of seedling growth of *Amaranthus spp.* by natural herbicide from *Tagetes erecta* L.

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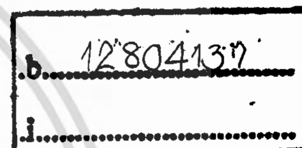

ABSTRACT

Marigold leaf (*Tagetes erecta* L.) was extracted with 75% ethanol in water and evaporated ethanol to obtain crude ethanol extract. The crude ethanol fraction was diluted with distilled water, resulting in aqueous solution which was hydrolized by acidified to pH 3 with 6 N HCl. The filtrate was extracted with ethyl acetate three times. The ethyl acetate solutions were combined, dried over anhydrous $MgSO_4$, and then evaporated to obtain the ethyl acetate soluble hydrolyzed fraction (Hy fraction). The Hy fraction was mixed with adjuvant at the ratio of 30: 70 to give of 30% active ingredient (a.i.) in soluble concentrate formulation (SC). In order to investigate the effects of SC formulation concentrations from *T. erecta* that is lower than concentrations that completely inhibited seed germination and seedling growth of *Amaranthus tricolor* L. (2500 ppm), *Amaranthus gracilis* Desf. and *Amaranthus spinosus* L. (1000 ppm) and obtain samples (leaf and stem) for DNA extraction, a dose of concentration application from 100 to 1000 ppm of *T. erecta* leaf extract in SC formulation was bioassayed on germination, seedling growth on 3 species of amaranth. The results showed that at lower concentration of *T. erecta* leaf extract (less than 1000 ppm) still contains inhibition on germination and seedling growth. Thus, next experiment was

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studied to obtain these samples and extract its DNA genomic to understand molecular mechanisms of allelopathic potential on DNA degradation of *Amaranthus* spp. The results indicated that DNA extracted from 3 *Amaranthus* species treated with leaf extract in SC formulation from *T. erecta* by concentration of 100 - 1000 ppm according to each species showed different genomic DNA degradation. Genomic DNA extracted from *A. spinosus* showed the most sensitively degraded, followed by *A. gracilis* and *A. tricolor*. Hence the use of *T. erecta* leaf extract as a potential natural herbicide for weed control might be possible.

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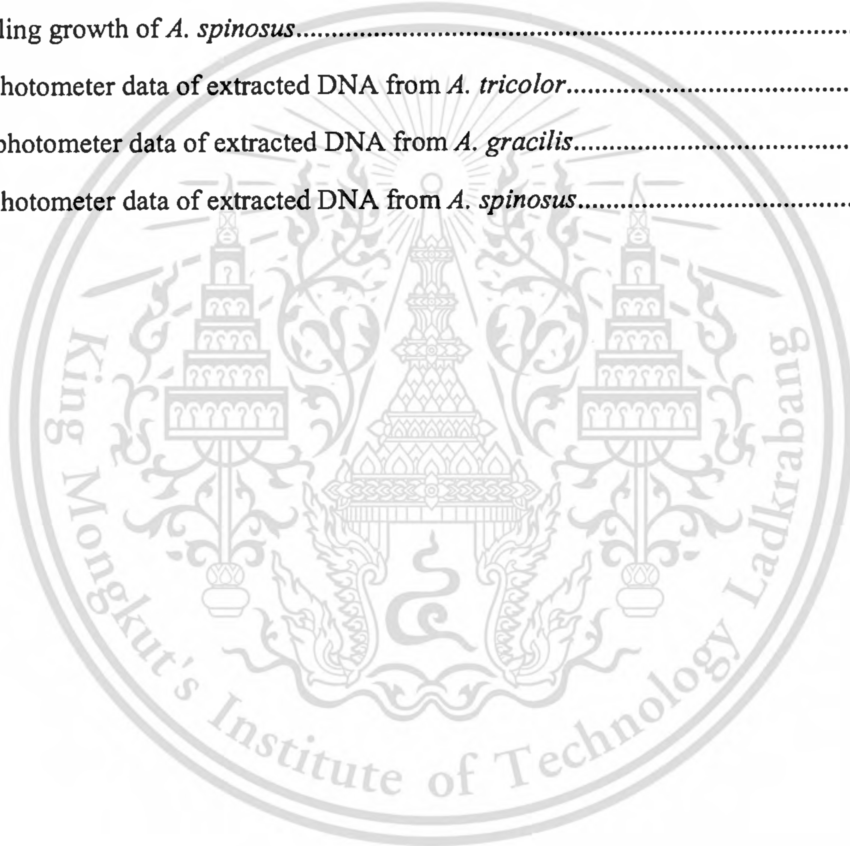

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CHAPTER 1

INTRODUCTION

1.1 The problem and its background

DNA degradation is a process by which DNA breaks down into smaller fragments. The process of degradation can reduce the height of some alleles, making them too low to be distinguished from background noise in the data. In severely degraded DNA samples, no results will be obtained. Two or more biological samples that make up a mixture may show different levels of degradation, which can complicate the interpretation of these samples (NFSTC Science, 2007).

Marigold (*Tagetes erecta* L.) is in Tagetes genus belonging to the family Asteraceae; it is demonstrated with the high allelopathic potential. Almost studies showed that allelopathic activity may inhibit the seeds germination by inhibiting the induction of α-amylase activity (Kato-Noguchi H and Macias FA (2005), Meksawat S and Pornprom T (2010), Laosinwattana C et al.). Crude marigold leaf extract in soluble concentrate formulation (SC) was studied as a natural herbicide because of its high allelochemical. In some cases, herbicides may affect the main crop adversely by interfering with its essential biochemical processes such as respiration, photosynthesis, protein metabolism and hydrolytic enzyme activity (Tim R. Murphy, 1999). However, its molecular mechanism remains unclearly during seed germination, especially in DNA degradation.

This study was carried out to evaluate the effects on allelopathic potential of marigold leaf extract in different concentrations; to understand physiological and molecular interactions mechanisms of the allelopathic effects of crude leaf extract from marigold on seed germination during *Amaranthus* spp. germination. Finally, this information on marigold extracted application could be applied for customer safety as one of the potential natural herbicides.

1.2 The purpose of the research project

1. To evaluate the effects of allelopathic potential of different concentrations from marigold leaf extract *(T. erecta)* on seed germination and seed growth through bioassays of *Amaranthus spp.* (*Amaranthus spinosus* L., *Amaranthus tricolor* L. and *Amaranthus gracillis* Desf.)

2. To further clarify the molecular mechanism of crude extract from marigold on germination of *Amaranthus spp.* though effects of herbicide from *T. erecta.* Therefore it can be an application for safe use of herbicides in other crop.

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1.3 The scope of the research project

1. Determine extract concentration of *T. erecta* effects inhibition on seed germination and seedling growth of *A. spinosus*, *A. tricolor* and *A. gracilis*.

2. Determine the effects of marigold extract on the DNA degradation in plant cells.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction of marigold

Mexican marigold (*Tagetes erecta* L.) is in *Tagetes* genus belonging to the family *Asteraceae;* comprises about 56 species distributed around the world. It was almost studied as an antibiotic and antioxidant plant. Owino (1992) studied effects of marigold leaf extract and captafol on fungal parasitism of root knot nematode egg-kenyan isolates. Olabiyi and Oyedunmade (2007) studied on marigold as interplant with cowpea for the control of nematode pests (*Meloidogyne spp*., *Pratylenchus spp*., and *Helicotylenchus* spp*.)*. Besides Dasgupta et al. (2012) studied on the antibacterial effect of marigold leaf extract at room temperature against 10 gram positive and 6 gram negative. The maximum antibacterial effect of Mexican Marigold leaf extract among micro- organism was obtained for *Acinetobacter* and *Propioni* bacterium acne. The results suggest that species of Mexican marigold can be useful in developing drugs for diseases like dermatitis, acne, skin races and also can be developed as antiseptic. Moreover antioxidant activity of marigold essential oil was researched by Martha Perez Gutierrez et al. (2006). The essential oil from flowers of marigold was evaluated for antioxidant activity in vitro using diphenyl-1-picrylhydrazyl (DPPH), thiocyanate, β-carotene bleaching, free radical scavenging activity and oxidation of deoxyribose assay. The GC-MS analysis of the oil has resulted in the identification of 18 components; β-caryophyllene, limonene, methyleugenol, (E)-ocimene, piperetone, piperitenone and terpinolene were the main components. It is demonstrated with the high allelopathic activity in the bioassay.

2.2 DNA degradation in plant cell review

DNA degradation is a process by which DNA breaks down into smaller fragments. As DNA molecules randomly break down into smaller fragments, the STR regions of the DNA molecule can be fractured. If the STRs do not stay intact, amplification of these regions will not be successful. Degradation is more likely to occur at a large STR locus before occurring in a smaller STR locus. Generally, degradation can be easily identified because the peak heights exhibit a downward slope across the electropherogram. The process of degradation can reduce the height of some alleles, making them too low to be distinguished from background noise in the data. In severely degraded DNA samples, no results will

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be obtained. Two or more biological samples that make up a mixture may show different levels of degradation, which can complicate the interpretation of these samples (NFSTC Science, 2007).

Transgenic procedure is a reason of DNA degradation in plant cell that inhibit the germination. MariaTeresa et al. (2003) studied on degradation and transformability of DNA from transgenic leaves. This result indicated that while most of the DNA will be degraded inside plant cells, sufficient DNA persists to be released into the soil. The fate of transplastomic (chloroplast genome contains the transgene) tobacco plant DNA in planta was studied when the plant leaves were subjected to decay conditions simulating those encountered naturally, including grinding, incubation with cellulase or enzymes produced by *Erwinia chrysanthemi,* and attack by the plant pathogen *Ralstonia solanacearum.* Direct visualization of DNA on agarose gels, gene extraction yield (the number of amplifiable aadA sequences in extracted plant DNA), and the frequency that recipient bacteria can be transformed by plant DNA were used to evaluate the quality and quantity of plant DNA and the transgene. These measurements were used to monitor the physical and biological degradation of DNA inside decaying plant tissues.

Although various nucleases are known to function in nuclear DNA degradation in animal apoptosis, it is unclear what hydrolase is involved in nuclear degradation in plants. During Programmed Cell Death of Tracheary Elements Tracheary elements (TEs) have a unique cell death program in which the rapid collapse of the vacuole triggers the beginning of nuclear degradation. Jun Ito and Hiroo Fukuda (2002) determined ZEN1 is a key enzyme in the degradation of nuclear DNA. In this study, they demonstrated that an S1-type nuclease, Zinnia endonuclease 1 (ZEN1), functions directly in nuclear DNA degradation during programmed cell death (PCD) of TEs. In-gel DNase assay demonstrated the presence of a 24-kD $Ca^{2+/}Mg^{2+}$-dependent nuclease and a 40-kD $Zn2^{+}$-dependent nuclease as well as ZEN1 in 60-h-cultured cells that included differentiating TEs. Such cell extracts possessed the ability to degrade the nuclear DNA isolated from *Zinnia elegans* cells in the presence of Zn^{2+}, and its activity was suppressed by an anti-ZEN1 antibody, indicating that ZEN1 is a central DNase responsible for nuclear DNA degradation. The introduction of the antisense ZEN1 gene into Zinnia cells cultured for 40 h specifically suppressed the degradation of nuclear DNA in TEs undergoing PCD but did not affect vacuole collapse. Therefore based on these results, a common mechanism between animal and plant PCD is discussed.

Besides regulation of gene expression by a biologist first requires accurate identification of the target allelochemicals, to determine enzymes and the genes encoding them.

A lot of effort has been done to explore the nature of allelopathic interactions. Some allelochemicals such as: phenoxy, benzoic acid, picolinic acid readily absorbed by foliage, less so by roots, extensively translocated, interfere with DNA, RNA and protein synthesis, results in uncontrolled

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cell division and elongation (Murphy et al., 1999) suggested that the stronger ability of PI312777 to suppress target weeds could be attributed to the stronger activation of the genes that function in de novo synthesis of allelochemicals. Rice accession PI312777 exhibited high allelopathic potential to suppress the growth of accompanying weeds, especially when the culture solution had low N content. Additionally, subtractive hybridization suppression was used to construct a forward cDNA library of PI312777 to investigate gene expression profiles under low N treatment. A total of 35 positive clones from the cDNA library were sequenced and annotated. According to the function category, 24 genes were classified into five groups related to primary metabolism, phenolic allelochemical synthesis, plant growth/cell cycle regulation, stress response/signal transduction, and protein synthesis/degradation.

Allelopathy is a quantitative trait. A genetic analysis of quantitative trait loci (QTL) is a promising approach to identify genes underlying this trait. Only a few crops are under genetic screening for its allelopathic properties including: rice, wheat, barley and oat. The first QTL map associated with allelopathic properties was developed in rice. A segregating population derived from a cross of two cultivars varying with allelopathic potential against barnyardgrass. The map contained 140 DNA markers with four main-effects QTL located on chromosome. Proteomic studies on allelopathy of rice against barnyardgrass confirmed the crucial role of three enzymes: phenylalanine ammonia-lyse (PAL), thioredoxin and 3-hydroxy-3-methilglutaril coenzyme. A reductase 3 (HMGR) is highly involved in phenols biosynthesis. Such a genetic approach may allow the location of the gene in the genome and better understanding of its function in plant allelopathy and create the chance of applying marker assisted selection (MAS) (Jensen et al., 2002; Belz, 2007).

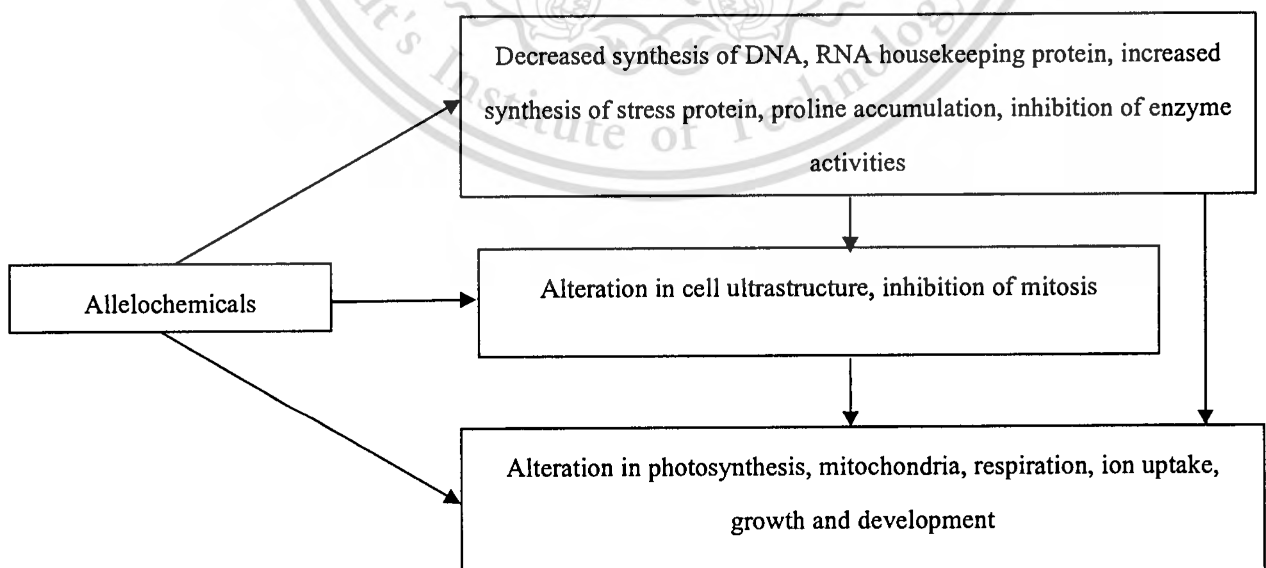

Figure 2.1 Multi site actions of allelochemicals (Gniazdowska and Bogatek, 2005)

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CHAPTER 3

RESEARCH METHODOLOGY

3.1 Chemicals and instruments

3.1.1 Reagent solution preparation for DNA extraction

1. Tris HCl 1 M (PH: 8.0)

Dissolve 60.57 g of Tris (hydroxyl methyl) aminomethane or Tris base MW and bring to 400 ml by distilled water. Then add 21 ml concentrated HCl dilute to a final volume of 500 ml, PH = 8 and autoclave.

2. EDTA 0.5 M (Ethylenediaminetetraacetic acid)

Dissolve 93.06 g of EDTA and bring to 300 ml sterile water. Then add 100 g of NaOH and adjust PH to 8 (NaOH 5 N). Dilute to a final volume of 100 ml by distilled water and autoclave

3. NaOH 5 N (Sodium hydroxyl)

Dissolve 20 g NaOH in 80 ml H_2O. Then bring to 100 ml distilled water and autoclave

4. HCl 1 N (Hydroxyl chloride)

Dissolve 8.62 ml concentrated HCl by 91.38 ml distilled water

5. NaCl 5 M (Sodium chloride)

Dissolve 29.25 g of NaCl by 80 ml H_2O. Then bring to 100 ml by distilled water and autoclave

6. TE buffer (Tris EDTA buffer)

Dissolve 5 ml of Tris HCl 1 M (PH 8.0); 1 ml EDTA 0.5 M and bring to 500 by distilled water and autoclave

7. Ethidilium bromide 10 mg/ml

Dissolve 1 g of ethidilium bromide and bring to 100 ml sterile water

8. TAE 50X stock solution (Tris acetate EDTA)

Dissolve 60.5 g of Tris base by distilled water. Then add 14.3 ml of acetic acid and 25 ml EDTA 0.5 M. Adjust the pH to 8. Dilute to a final volume of 250 ml by distilled water and autoclave

9. CTAB buffer (Cetyltrimethyl ammonium bromide buffer)

Dissolve 11.688 g of NaCl 2 M. Add 5 ml of EDTA 25 mM and 10 ml of Tris HCl 100 mM PH 8.0. Then bring to 100 ml by sterile water and autoclave. Add 2 g of CTAB and then add 2 β-mercaptoethanol with propotion: 1 ml CTAB solution: 10 μl 2 β – mercaptoethanol

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3.1.2 Other chemicals

Ethanol 99%, ethanol 70%, isopropanol, ethyl acetate, Phenol: Chloroform: isoamylalcohol = 25: 24: 1

3.1.3 Apparatus

1. Petri dish (9 and 15 cm in diameter)
2. Eppendorf 1.5 ml and kinds of tip
3. Beaker
4. Cylinder
5. Test tube
6. Curvet
7. Spatula
8. Micropipette
9. Stirring rod
10. Germination paper
11. Filter paper Whatman no. 1
12. Parafilm
13. Sterile pestle and mortar

3.1.4 Instruments

1. Hot air oven machine
2. Magnetic stirrer
3. Rotary evaporator
4. Growth chamber
5. Autoclave machine
6. pH meter
7. Vortex machine
8. Centrifuged machine
9. Water bath
10. Gel DocTM 2000
11. UV transilluminator

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3.2 Test plant preparation

The healthy seeds of *A. spinosus, A. tricolor* and *A. gracilis* were manually collected from experimental field at King Mongkut's Institute of Technology Ladkrabang and farmer fields in the Ladkrabang district, Bangkok, Thailand. Their seeds were removed from panicle by lightly shaking in collection bags to release seeds. Seeds of these species were examined and seeds with damaged seed coats were discarded.

3.3 Crude marigold leaf extracts preparation

Marigold plant was grown at the experimental field at King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. The mature and healthy leaves of *T. erecta* were collected at 50 days after planting, then cleaned from soil immediately with running tap water, dried-up in a hot-air oven at 45°C for 5 days and ground into powder (100 mesh) in an electrical blender. According to method of Laosinwattana (2010), the crude extracts were prepared from *T. erecta* leaf powder by extraction with 75% ethanol in water for 48 hours at room temperature and repeatedly extracted 3 times, followed by filtration through three layers of cheese cloth to remove debris.

After filtration using Whatman No. I filter paper, the filtrates was combined and evaporated in the rotary evaporator at 45°C, leaving a sticky residue (crude ethanol fraction). This residue was then diluted with 500 mL of distilled water and stirred vigorously on a magnetic stirrer at 45°C for 20 min, resulting in an aqueous solution which was acidified to pH 3 by 6N HCl. The filtrate was extracted with ethyl acetate three times. The ethyl acetate solutions was combined, dried over $MgSO_4$ and then evaporated to obtain the ethyl acetate soluble hydrolyzed fraction (Hy fraction) and the remains of the aqueous phase was discarded (Figure 3.1). The Hy fraction was mixed with adjuvant at the ratio of 30 : 70 to give of 30% active ingredient (a.i.) in soluble concentrate formulation (SC). The inhibitory activities from each fraction were prepared by dissolved crude of each fraction to contain different concentrations.

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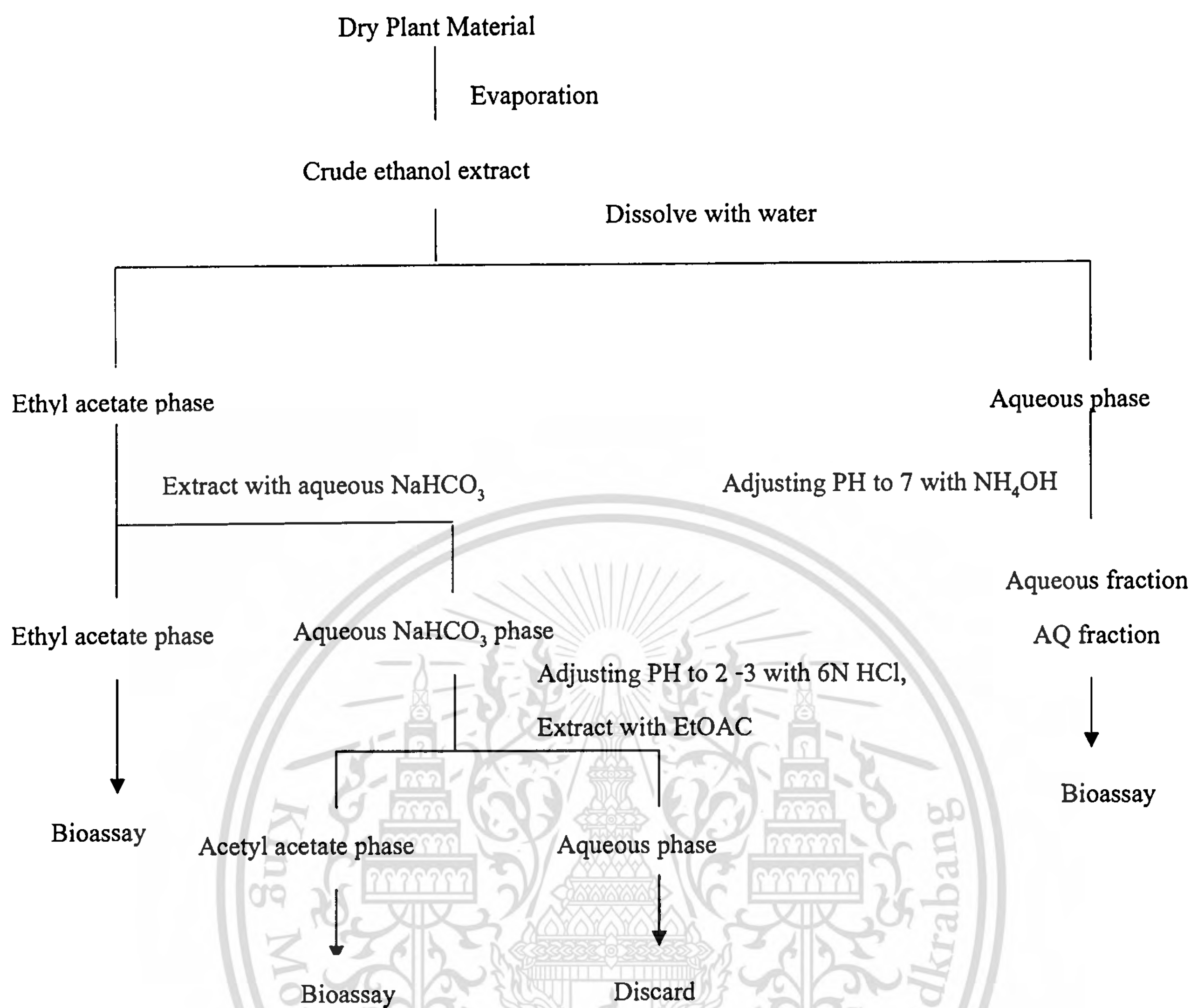

Figure 3.1 Flow chart for extraction and partially separation of active compounds from marigold dried leaves

3.4 Methodology

Experiment 1. Effect of different concentrations of SC formulation from *T. erecta* on seed germination of *A. spinosus* and *A. tricolor* and *A. gracilis* for DNA extraction

The SC formulation from *T. erecta* was diluted with distilled water to100 ppm to 1000 ppm according to each species. Five ml of each concentration was added to each petri dish (9 cm in diameter) containing 2 layers of germination paper and then 20 healthy seeds of test plant were placed as per treatment. The control was only received distilled water. Each treatment had 4 replications in a completely randomized design (CRD):

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For *A. tricolor,* the SC formulation from *T. erecta* was diluted with distilled water to 250, 500, 750 and 1000 ppm

Control: Distilled water

Treatment 2: 250 ppm

Treatment 3: 500 ppm

Treatment 4: 750 ppm

Treatment 5: 1000 ppm

For *A. gracilis* and *A. spinosus*, the SC from *T. erecta* was diluted with distilled water to 100, 200, 300and 400 ppm.

Control: Distilled water

Treatment 2: 100 ppm

Treatment 3: 200 ppm

Treatment 4: 300 ppm

Treatment 5: 400 ppm

Then all petri dishes were placed in a growth chamber with condition (cool white 840 Climacell 707, Munich, Germany) at 25 - 32°C, 12h dark/ light photoperiod light intensity of 100 µmol $m^{-2}s^{-1}$, and relative humidity of 80%. After 7 days, germination percentage (%) (SG), shoot length (SL) and root length (RL) were observed and recorded in all treatments.

The inhibition percentage relative to control was calculated from following equation:

G, SL or RL (% of control) = 100 – [(sample extracts/control) x 100] (5)

Experiment 2. Effect of different concentrations of SC formulation from *T. erecta* on DNA degradation in plant cells

The SC formulation from *T. erecta* was diluted with distilled water to 250, 500, 750, 1000 ppm for *A. tricolor* while 100, 200, 300 and 400 ppm for *A. spinosus* and *A. gracilis*. Ten ml of each concentration was added to each petri dish (15 cm in diameter) containing 2 layers of germination paper and then 100 healthy seeds of *Amaranthus* spp. were placed as per treatment. The control was only received distilled water. Each treatment had 3 replications in a completely randomized design (CRD):

All petri dishes were sealed with parafilm and placed in a growth chamber.

After 12 days, Amaranths leaves and stems were collected and thencovered by alumium foil and kept in -80°C refrigerator for 3 days before DNA extraction.

Method of extraction of genomic DNA from plant tissue (CTAB method):

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A hundred mg leaf and stem of *Amanranthus* spp. for each determination was ground in liquid nitrogen with prechilled mortar and pestle. A 500 µl hot CTAB solution (60°C) was immediately added to the homogenized solution. The flour sample was then transferred into 1.5-mL DNase-free microcentrifuge tube (eppendorf). After mixing the content with vigorous vortex mixing, flour sample was incubated in water bath at 60°C for 15 minutes. Samples were then centrifuged immediately at 12,000 rpm for 10 min at 4°C. The upper aqueous phase was carefully transferred to a new 1.5-mL eppendorf. A 700 µL phenol: chloroform: isoamyl alcohol mixture (25:24:1) was added. The samples was mixed well by inversion and placed on ice for 10 min and then centrifuged at 12,000 × g for 10 min at 4°C. The upper aqueous phase will be transferred to a new 1.5-mL eppendorf. To the precipitated DNA, 500 µL isopropanol will be added. The sample were placed in -80°C refrigerator for 1 hour and centrifuged to obtain DNA for 12,000 rpm for 15 min at 4°C. Then the supernatant was discarded, and the DNA pellets were washed carefully with 400 µL 70% ethanol at RT and dissolved by 30 µl TE buffer and kept in 4°C refrigerator.

Method of quality assessment of extracted DNA:

Spectrophotometer was used to determine the purity of the extracted DNA. Nucleic acids absorbed light at 260nm and the amount of light absorbed was used to calculate the purity and amount of DNA. By using a spectrophotometer that emits a light at 260nm that passed through the sample, the concentration of DNA in the sample was determined. The more light was absorbed the more nucleic acid is present in the sample. Interference by contaminants was calculated by using a ratio. Since proteins absorbed at 280nm, the ratio of absorbance A of a sample at 260nm and the absorbance A at 280nm was used to estimate the purity of the DNA sample.

Two µl of the DNA sample was mixed with 498 µl pure HPLC-grade water to make a 1/ 250 dilution with a total volume of 500 µl in a 1.5 ml eppendorf tube. In order to concentrate the fluid in the bottom of the tube, the tube was mixed well and centrifuged for a few seconds. Sixty µl of the dilution was pipetted in to the bottom of a plastic disposable cuvette. Sixty µl pure water were placed a cuvette containing the blank in position in the spectrophotometer. The clear sides were placed in line with the direction of the light ray. The photometer and programme were switched on to measure double stranded DNA. The blank was measured by pushing the button marked "Blank" and made sure the result reads 0. The cuvette containing the diluted sample was placed in position in the photometer and pressed the "Sample" button to measure. The instrument showed its readings for absorbance at 230, 260, 280 and 320 nm as well as the ratio of the absorbance A260/ A280 nm and A260/ A230 nm. It also showed the concentration of DNA in the sample in ng/µl.

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The DNA degradation was accessed by electrophoresis in highly percentage of agarose gel (approximately 2% w/v) containing 5 mg/ml ethidium bromide. Then, the degradation of plant genomic DNA was determined by Gel Doc™ 2000 and UV transilluminator.

3.5 Data analysis/ Statistical analysis

Each treatment consists of four replications in completely randomized design (CRD). Analysis of variance was calculated for all data and comparisons between treatments will be made at probability level $p \leq 0.05$ using Tukey's test.

3.6 Duration and location of research work

My thesis was carried out during 6 months (10/ 2014 – 3/ 2015) in laboratory and green house, Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand.

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 Effect of different concentrations of SC formulation from *T. erecta* on seed germination of *A. tricolor, A. gracilis* and *A. spinosus* for DNA extraction

4.1.1 Effect of different concentrations of SC formulation from *T. erecta* on seed germination of *A. tricolor* for DNA extraction

In order to investigate the effects of SC formulation from *T. erecta* under the concentration that completely inhibited seed germination and seedling growth of *A. tricolor* (2500 ppm) and obtain samples (leaf and stem) for DNA extraction, a dose of concentration application from 250 to 1000 ppm of *T. erecta* leaf extract in SC formulation was treated with *A. tricolor*. Data showed the differences in the percentage of seed inhibition (% of control). At dose of 250 to 1000 ppm *T. erecta* leaf extract in SC formulation, the inhibition (% of control) of seed germination and shoot length was less than 10% and not differ significantly by Tukey's Studentized Range Test ($p=0.05$) whereas the inhibition of root length was 12.37 to 53.67% on *A. tricolor* depending on the concentration application.

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Table 4.1 Effect of different concentrations of SC formulation from *T. erecta* on seed germination and seedling growth of *A. tricolor.*

Concentration (ppm)	Seed inhibition (% of control)		
	Seed germination	Shoot length	Rootlength
250	7.5a[1]	7.26a	12.37c
500	7.5a	6.85a	15.93bc
750	10a	17.34a	38.76ab
1000	7.5a	20.16a	53.63a

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

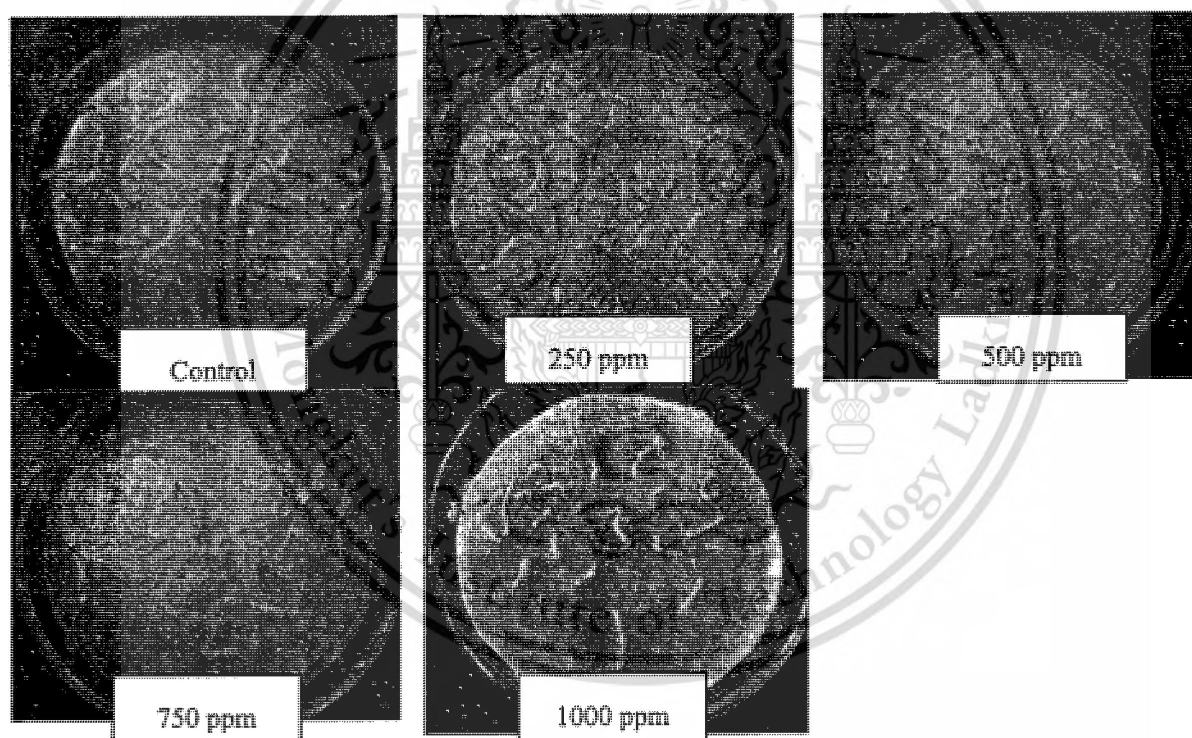

Figure 4.1 Effect of different concentrations of SC formulation from *T. erecta* on seed germination and seedling growth of *A. tricolor*

4.1.2 Effect of different concentrations of SC formulation from *T. erecta* on seed germination of *A. gracilis* for DNA extraction

Table and Figure below showed the inhibition of *T. erecta* leaf extract in SC formulation from 100 to 400 ppm on seed germination and seedling growth of *A. gracilis.* At concentration of 100 ppm,

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germination of seed of *A. gracilis* was inhibited significantly by 30%. Its shoot and root length inhibition were 46.54 and 29.18%, respectively. By increasing the dose of application at 400 ppm, germination of seed of *A. gracilis* was inhibited by 78.75% whereas its shoot and root length inhibition were 92.26 and 97.77%, respectively. This suggests that at concentration less than complete inhibition concentration of *T. erecta* leaf extract (1000 ppm) still contains some inhibitory principles upon inhibited germination and seedling growth. Hence, next experiments were carried out to obtain these samples and extract genomic DNA to understand interaction mechanisms of allelopathic potential about inhibition on DNA degradation.

Table 4.2 Effect of different concentrations of SC formulation from *T. erecta* on seed germination and seedling growth of *A. gracilis*.

Concentration (ppm)	Seed inhibition (% of control)		
	Seed germination	Shoot length	Rootlength
100	30b[1]	46.54c	29.18c
200	40b	49.47bc	32.96c
300	72.5a	63.03c	65.14b
400	78.75a	92.29a	97.77a

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

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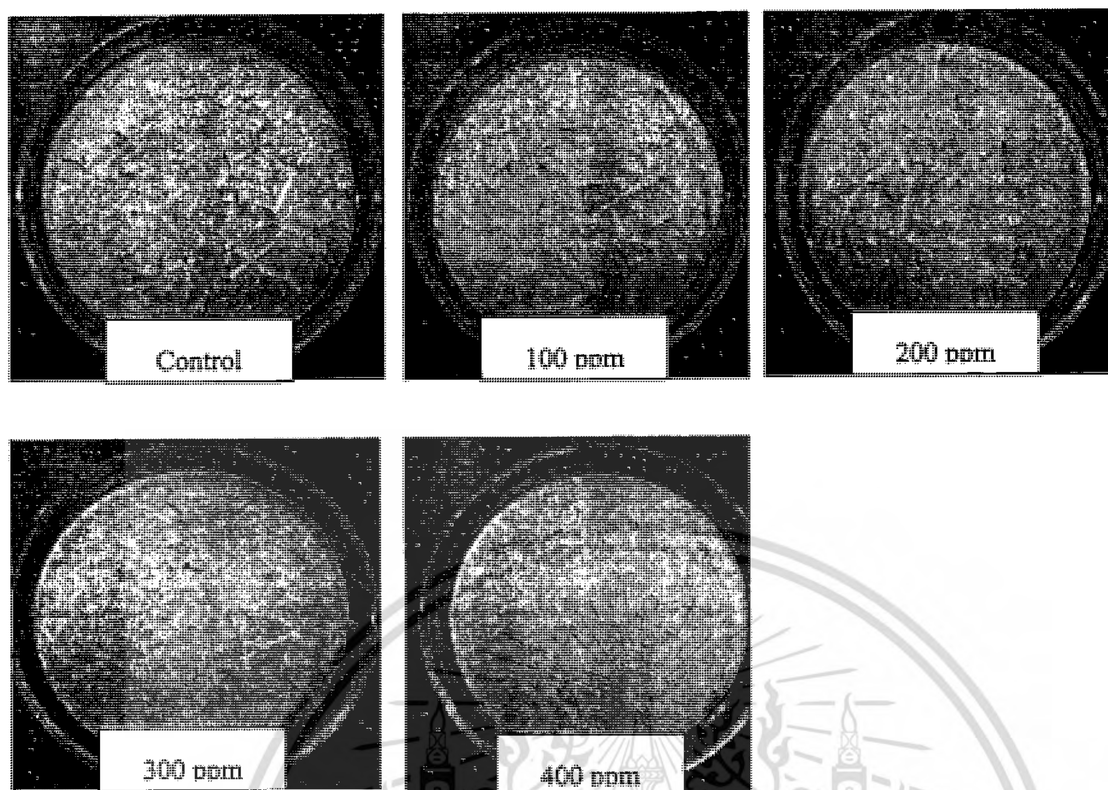

Figure 4.2 Effect of different concentrations of SC formulation from *T. erecta* on seed germination and seedling growth of *A. gracilis*

4.1.3 Effect of different concentrations of SC formulation from *T. erecta* on seed germination of *A. spinosus* for DNA extraction

Table and Figure 4.3 showed that *A. spinosus* seeds were treated *T. erecta* leaf extract in SC formulation from 100 - 400 ppm inhibited seed germination and seedling growth according to the increasing of *T. erecta* concentration. At concentration of 100 ppm, germination of seed of *A. spinosus* was inhibited significantly by 20% while its shoot and root length inhibition were 42.29 and 26.73%, respectively. By increasing the dose of application at 400 ppm, seed germination of *A. spinosus* was inhibited by 78.75% whereas its shoot and root length inhibition were 84.31 and 98.11%, respectively. This suggests that at lower concentration of *T. erecta* leaf extract (less than 1000 ppm) still contains inhibition on germination and seedling growth. Thus, next experiment was studied to obtain these samples and extract its DNA genomic to understand molecular mechanisms of allelopathic potential on DNA degradation of *Amaranthus* spp.

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Table 4.3 Effect of different concentrations of SC formulation from *T. erecta* on seed germination and seedling growth of *A. spinosus.*

Concentration (ppm)	Seed inhibition (% of control)		
	Seed germination	Shoot length	Rootlength
100	20c[1]	42.29c	26.73c
200	35c	47.61bc	31.74c
300	53.75b	59.57b	65.37b
400	78.75a	84.31a	98.11a

[1]Means followed by the same letter in a column do not differ significantly by Tukey's Studentized Range Test (p=0.05)

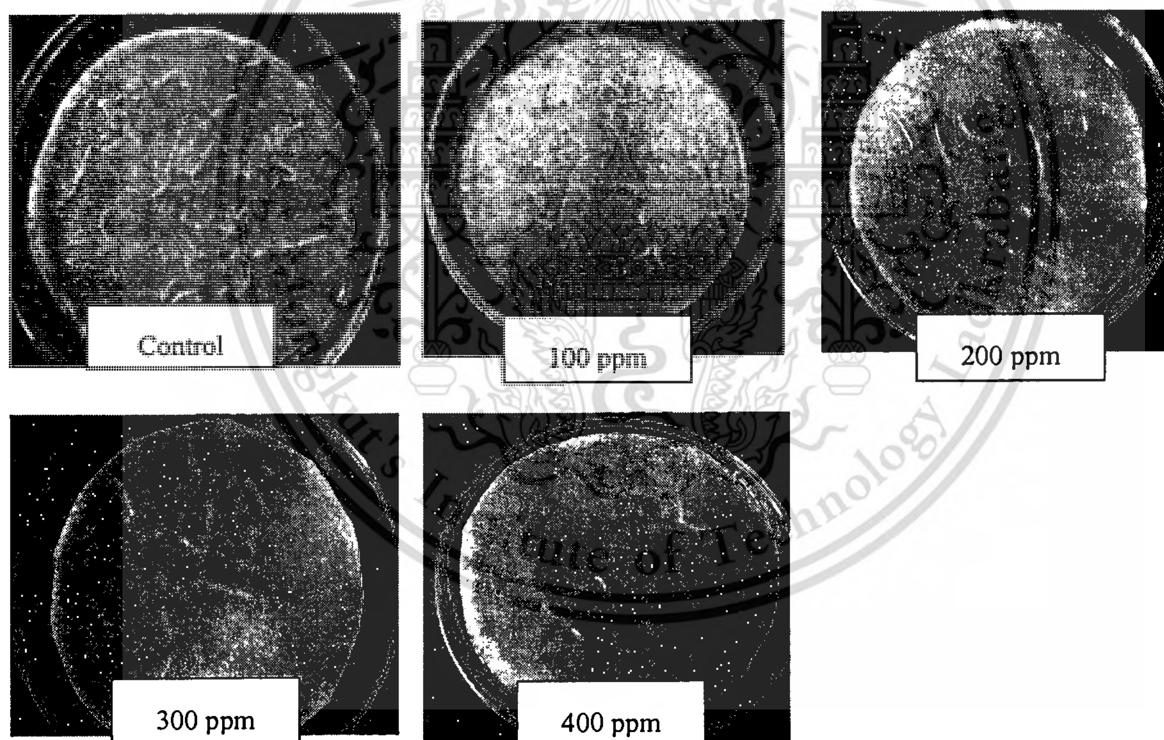

Figure 4.3 Effect of different concentrations of SC formulation from *T. erecta* on seed germination and seedling growth of *A. spinosus*

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4.2 Effects of different concentrations of SC formulation from *T. erecta* on DNA degradation in plant cells

4.2.1 Spectrophotometer results

Samples were analyzed with a spectrophotometer that measured absorbance at 230 nm, 260 nm, 280 nm and 320 nm, as well as calculating the ratios A260/ A280 and A260/ A230 and the concentration of DNA in ng/ µl of the samples. A260/ A280 ratios were used to estimate the sample purities obtained with each of the three species of amaranth that treated with natural herbicide from *T. erecta.* In general, the ratio A260/ A280 were summarized in Tables 4.4 to 4.6 with the displays all the spectrophotometer results of all the samples between 1.6 and 2.0. Therefore, these samples can be used for electrophoresis to determine DNA degradation by natural herbicide from *T. erecta.*

Table 4.4 Spectrophotometer data of extracted DNA from *A. tricolor*

	Sample (ppm)	Concentration (ng/ µl)	A260/ A280	A260 /A230	A230	A260	A280	A340
Rep1	Control	3989.3	**1.83**	1.95	0.1625	0.3195	0.175	0.025
	250	1982.25	**1.69**	1.515	0.1045	0.1585	0.0935	0.02495
	500	5948.4	**1.64**	1.215	0.391	0.476	0.291	0.099
	750	4492.9	**1.64**	1.465	0.245	0.3595	0.22	0.0735
	1000	2080.65	**1.56**	1.24	0.1665	0.1665	0.107	0.0365
Rep 2	Control	2280.45	**1.54**	1.33	0.137	0.182	0.1185	0.029
	250	2977.6	**1.63**	1.24	0.1905	0.238	0.1455	0.0515
	500	1093.55	**1.49**	0.89	0.0985	0.0875	0.059	0.0265
	750	5319.1	**1.58**	1.47	0.284	0.4255	0.267	0.104
	1000	4638.9	**1.75**	1.675	0.221	0.371	0.212	0.0265
Rep 3	Control	2113.75	**1.73**	1.775	0.094	0.169	0.097	0.0235
	250	2248.7	**1.44**	1.3	0.141	0.1795	0.1245	0.0635
	500	2623.85	**1.62**	1.265	0.1635	0.2095	0.128	0.049
	750	3167.8	**1.63**	1.235	0.236	0.2535	0.1555	0.057
	1000	4027.05	**1.70**	1.755	0.1835	0.372	0.1795	0.052

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Table 4.5 Spectrophotometer data of extracted DNA from *A. gracilis*

	Sample (ppm)	Concentration (ng/ µl)	A260/ A280	A260/ A230	A230	A260	A280	A340
Rep1	Control	217.25	**1.82**	1.05	0.0165	0.0175	0.0415	0.0005
	100	390	**2.02**	1.83	0.0135	0.024	0.0105	0
	200	415.8	**1.78**	1.215	0.032	0.0375	0.022	0.0005
	300	705.75	**1.78**	1.565	0.029	0.042	0.023	0.0015
	400	370.3	**1.93**	1.28	0.0295	0.0295	0.0155	0.002
Rep 2	Control	644.8	**1.58**	1.18	0.028	0.0515	0.033	0.007
	100	811.55	**1.89**	1.38	0.043	0.067	0.0375	0.01
	200	727.6	**1.72**	1.515	0.049	0.0505	0.0295	0.0025
	300	702.2	**1.73**	1.26	0.0405	0.0575	0.0315	0.004
	400	827.75	**1.8**	1.46	0.04	0.0535	0.032	0.003
Rep 3	Control	430.35	**1.37**	1.17	0.045	0.033	0.027	0.0025
	100	600.25	**1.73**	1.125	0.037	0.048	0.028	0.013
	200	970.25	**1.67**	1.235	0.0475	0.0775	0.046	0.006
	300	785.45	**1.77**	1.195	0.0645	0.0625	0.0355	0.0055
	400	893.2	**1.84**	1.29	0.0575	0.0715	0.039	0.0015

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Table 4.6 Spectrophotometer data of extracted DNA from *A. spinosus*

	Sample (ppm)	Concentration (ng/ µl)	A260/ A280	A260/ A230	A230	A260	A280	A340
Rep1	Control	765.5	**1.71**	1.77	0.0345	0.0615	0.036	0.001
	100	389.9	**1.73**	0.725	0.0435	0.032	0.017	0.005
	200	245.85	**1.92**	0.85	0.0235	0.0195	0.01	0.0005
	300	1845.2	**1.21**	1.21	0.1265	0.1475	0.1235	0.103
	400	-	-	-	-	-	-	-
Rep 2	Control	248.85	**1.35**	0.99	0.02	0.019	0.0145	0.0035
	100	1053.6	**1.15**	0.99	0.0855	0.0845	0.073	0.0685
	200	349.75	**1.55**	0.95	0.0295	0.028	0.0225	0.0465
	300	248.65	**1.77**	0.845	0.0215	0.027	0.0115	0
	400	-	-	-	-	-	-	-
Rep 3	Control	431.4	**1.92**	1.45	0.034	0.0325	0.018	0
	100	285.95	**1.67**	0.67	0.034	0.0315	0.0135	0.001
	200	479.15	**1.74**	1	0.0385	0.0285	0.022	0.0015
	300	248.65	**1.77**	0.845	0.0215	0.027	0.0115	0
	400	-	-	-	-	-	-	-

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4.2.2. Assessment of DNA degradation by electrophoresis on 2% agarose gel

Because nucleic acids are negatively charged ions at neutral or basic pH in an aqueous environment, they can be mobilized by an electric field. Gel electrophoresis is a technique used to separate molecules on the basis of size, using this charge. The nucleic acids can be separated as whole chromosomes or fragments. The nucleic acids are loaded into a slot near the negative electrode of a semisolid, porous gel matrix and pulled toward the positive electrode at the opposite end of the gel. Smaller molecules move through the pores in the gel faster than larger molecules; this difference in the rate of migration separates the fragments on the basis of size. There are molecular weight standard samples that can be run alongside the molecules to provide a size comparison. Nucleic acids in a gel

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matrix can be observed using various fluorescent or colored dyes. Distinct nucleic acid fragments appear as bands at specific distances from the top of the gel (the negative electrode end) on the basis of their size. A mixture of genomic DNA fragments of varying sizes appear as a long smear, whereas uncut genomic DNA is usually too large to run through the gel and forms a single large band at the top of the gel.

Figure 4.4 showed good quality and normalized DNA of *A. tricolor* in lane 2, 8, 14 and 20 whereas degraded genomic DNA in other lanes such as: 3, 4, 5, 6, 9, 10, 11, 12, 15, 16, 17, 18, 21, 22, 23, 24. At higher concentration of *T. erecta* in SC formulation from 250 ppm to 1000 ppm, genomic DNA show more degraded (genomic DNA fragments of varying sizes appear as a long smear)

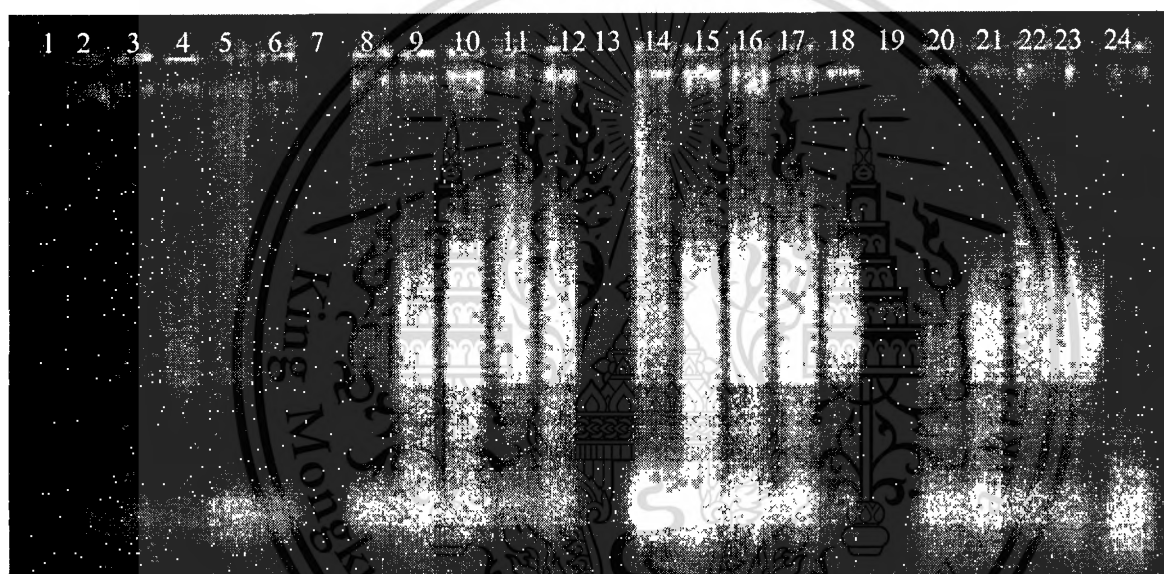

Figure 4.4 Electrophoresis of DNA extracted from *A. tricolor* treated with different concentration of *T. erecta*; lane 1, 7, 13,19: DNA ladder 1kb, lane 2, 8,14, 20: control, lane 3, 9, 15, 21: 250 ppm, lane 4, 10, 16, 22: 500 ppm, lane 5, 11, 17, 23: 750 ppm, lane 6, 12, 18, 24: 1000 ppm

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Figure 4.5 showed good quality and normalized DNA of *A. gracilis* in lane 2, 8, 14 (control) whereas degraded genomic DNA of *A. gracilis* in other lanes (except marker lanes: 1, 7, 13). By increasing the dose of application from 200 - 400 ppm, genomic DNA of *A. gracilis* showed more degraded (genomic DNA fragments of small sizes appear as a longer smear). This suggests that at concentration less than complete inhibition concentration of *T. erecta* leaf extract (1000 ppm) DNA genomic of *A. gracilis* was broken and degraded. This is also molecular mechanisms of allelopathic potential of *T. erecta* leaf extract on DNA degradation of *Amaranthus* spp.

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This was similar with DNA degradation of *A. spinosus* showed in Figure 4.6. Lane 2 (control) genomic DNA showed clear band (normalized DNA) whereas lane 3, 4, 5 showed genomic DNA degradation.

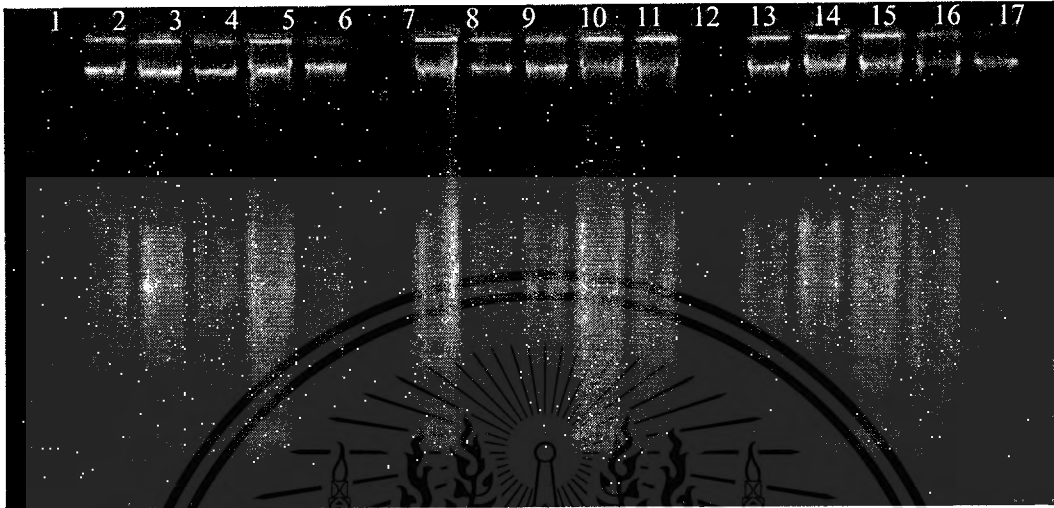

Figure 4.5 Electrophoresis of DNA extracted from *A. gracilis* treated with different concentration of *T. erecta*; lane 1, 7, 13: DNA ladder 1kb, lane 2, 8, 14: control; lane 3, 9, 15: 100 ppm; lane 4, 10, 16: 200 ppm; lane 5, 11, 17: 300 ppm; lane 6, 12, 18: 400 ppm

Figure 4.6 Electrophoresis of DNA extracted from *A. spinosus* treated with different concentration of *T. erecta*; lane 1: DNA ladder 1kb; lane 2, 6: control; lane 3: 100 ppm; lane 4: 200 ppm; lane 5: 300 ppm

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Comparison of DNA degradation of *A. tricolor*, *A. spinosus* and *A. gracilis*

The genomic DNA was degraded by being treated with concentration application of *T. erecta* in SC formulation from 100 ppm to 1000 ppm on 3 species of amaranth in comparison with control. The genomic DNA of 3 amaranth species showed different degradation according to each species. Genomic DNA extracted from *A. spinosus* showed the most sensitively degraded, following by *A. gracilis* and *A. tricolor*.

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CHAPTER 5

CONCLUSIONS AND SUGGESTIONS

5.1 Conclusions

At the concentration is lower than the concentration of SC formulation that completely inhibited seed germination and seedling growth of *Amaranthus* spp. (less than 1000 ppm) still contains inhibition on germination and seedling growth.

DNA extracted from *Amaranthus* spp. treated with leaf extract in SC formulation from *T. erecta* by concentration of 100 - 1000 ppm showed different genomic DNA degradation. Genomic DNA extracted from *A. spinosus* showed the most sensitively degraded, followed by *A. gracilis* and *A. tricolor*.

5.2. Suggestions

5.2.1 Further studies about molecular and cytogenetic mechanisms of *T. erecta* leaf extract effects on *A. tricolor, A. spinosus* and *A. gracilis*: study on protein, DNA degradation in polyacrylamide electrophoresis, gene expression and cytotoxicity should be carried out.

5.2.2 Effects of *T. erecta* leaf extract on seed germination and seedling growth of 3 above amaranth species in green house should be carried out.

5.2.3 Effects of leaf extract of *T. erecta* on other bioassays should also be carried out.

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