ศักยภาพด้านอัลลิโลพาธิคของวัสดุเหลือทิ้งจากทานตะวัน *Helianthus annuus* L.

นางสาววิริยา หนูทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ALLELOPATHIC POTENTIAL OF LEFTOVER MATERIALS FROM SUNFLOWER *Helianthus annuus* L.

Miss Wiriya Noothong

A Thesis Submitted in Partial Fulfillment of the Requirements
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By	Miss Wiriya Noothong
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Thesis Advisor	Assistant Professor Warinthorn Chavasiri, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Associate Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Vudhichai Parasuk, Ph.D.)

.....Thesis Advisor
(Assistant Professor Warinthorn Chavasiri, Ph.D.)

.....Examiner
(Associate Professor Nattaya Ngamrojanavanich, Ph.D.)

.....External Examiner
(Pichittra Kaewsorn, Ph.D.)

วิริยา หนูทอง : ศักยภาพด้านอัลลิโลพาธิคของวัสดุเหลือทิ้งจากทานตะวัน *Helianthus annuus* L. (ALLELOPATHIC POTENTIAL OF LEFTOVER MATERIALS FROM SUNFLOWER *Helianthus annuus* L.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. วรินทร ชวศิริ, 93 หน้า.

ศึกษาการใช้ประโยชน์ของวัสดุเหลือทิ้งจากทานตะวันได้แก่ ใบ ลำต้น และราก ทางด้านอัลลิโลพาธีที่มีต่อการงอกและการเจริญเติบโตของไมยราบยักษ์ โดยสกัดทานตะวันแห้งส่วนใบ ลำต้น และ ราก ด้วย เฮกเซน ไดคลอโรมีเทน และ เมทานอล ทดสอบที่ความเข้มข้น 1, 2.5 และ 5 กรัมสมมูลของพืชแห้ง (gE) พบว่าสิ่งสกัดเมทานอลจากใบแสดงฤทธิ์ในการยับยั้งได้ดีที่สุด จึงได้นำสิ่งสกัดดังกล่าวมาทดสอบฤทธิ์ทางอัลลีโลพาธิกกับวัชพืชและพืชปลูก พบว่าที่ความเข้มข้น 1 gE สิ่งสกัดดังกล่าวแสดงผลยับยั้งการงอกของวัชพืชมากกว่าพืชปลูกแต่ไม่เลือกยับยั้งการเจริญเติบโตของพืชทั้งสองชนิด จึงนำสิ่งสกัดดังกล่าวและใบแห้งของทานตะวันมาทดสอบการควบคุมวัชพืชในกระถางด้วยวิธีใช้ก่อนงอก (pre-emergence) และหลังงอก (post-emergence) พบว่าสามารถยับยั้งการงอกและการเจริญเติบโตของวัชพืชทุกชนิดได้แต่ผลการยับยั้งที่ได้ต่ำกว่าการทดลองใน petri-dish เมื่อนำสิ่งสกัดดังกล่าวมาทดสอบการควบคุมหญ้าข้าวนกในการปลูกร่วมกับข้าว ด้วยวิธีใช้ก่อนและหลังงอก พบว่าการรดสิ่งสกัดก่อนวัชพืชงอกที่ความเข้มข้น 1 gE สามารถยับยั้งการงอกของหญ้าข้าวนกได้ 33% และไม่มีผลต่อการงอกของข้าวและการเจริญเติบโตทางลำต้นแต่มีผลยับยั้งความยาวราก 29% สำหรับการรดสิ่งสกัดหลังวัชพืชงอกพบว่า ที่ความเข้มข้น 1 gE สามารถทำให้หญ้าข้าวนกตายได้ 100% โดยที่ไม่มีผลกระทบต่อการเจริญเติบโตของข้าว นอกจากนี้ยังได้ทดสอบฤทธิ์ทางชีวภาพด้านอื่นๆ ได้แก่ การยับยั้งการกินของหนอนกระทู้ผัก การยับยั้งการเจริญเติบโตของแบคทีเรีย 6 ชนิด และรา 2 ชนิด พบว่าสิ่งสกัดไดคลอโรมีเทนจากรากทานตะวันมีฤทธิ์ยับยั้งการเจริญเติบโตของจุลินทรีย์ดีที่สุด โดยยับยั้ง *Rhizoctonia solani* 79% นอกจากนี้สิ่งสกัดไดคลอโรมีเทนจากรากที่ความเข้มข้น 1 gE ยังมีฤทธิ์เลือกยับยั้งเฉพาะความยาวลำต้นในบานชื่นและข้าว และยังส่งเสริมความยาวรากของพืชดังกล่าว จึงนำสิ่งสกัดไดคลอโรมีเทนจากรากมาแยกองค์ประกอบทางเคมี พบสาร 5 ชนิด ได้แก่ demethylencecalin, demethoxyencecalin, mokko lactone, ของผสมระหว่าง stigmasta-4,22-dien-3-one กับ stigmast-4-en-3-one และ ของผสมระหว่าง stigmasterol กับ β-sitosterol

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

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The allelopathic effects of the extracts of sunflower (*Helianthus annuus* L.) were evaluated on seed germination and growth inhibition of *Mimosa pigra* L. Dried leaves, stems and roots of sunflower were separately extracted by different solvents. The inhibition activity using three concentrations of 1.0, 2.5 and 5.0 gE was compared. The CH_3OH extract of the leaves inhibited seed germination and the growth of *M. pigra*. This extract was further evaluated on allelophatic effects of selected weeds and crops. At 1 gE, the extract showed selective inhibition on seed germination of weeds more than crops, but expressed as non-selective inhibition on shoot and root elongation of all tested plants. Further examinations on pot experiments as pre- and post-emergence weed controller were conducted. The use of dried leaves as pre-emergence, and that of the CH_3OH extract of the leaves as pre- and post-emergence inhibited seed germination and growth of all weeds, but less than those observed in petri-dish experiments. To control barnyard grass in rice cropping as pre- and post-emergence weed controller, at 1 gE of pre-emergence this extract inhibited barnyard grass germination 33% with no effect on the rice germination and shoot elongation, while inhibited 29% of root elongation. For post-emergence at 1 gE, the extract could completely inhibit the barnyard grass population with no side-effect on the growth of rice seedlings. In addition, all crude extracts were evaluated on other biological activities including antifeedant activity against common cutworm, antibacterial activity against 6 bacteria and 2 fungi. The CH_2Cl_2 extract of the roots gave the highest inhibition to *Rhizoctonia solani* (79%). This extract also expressed as selective inhibition on shoot elongation of zinnia and rice by stimulating their root elongation. The chemical composition of this fraction was investigated. Five substances: demethylencecalin, demethoxyencecalin, mokko lactone, a mixture of stigmasta-4,22-dien-3-one and stigmast-4-en-3-one, and a mixture of stigmasterol and β-sitosterol were identified based on spectroscopic evidence.

Field of Study: Biotechnology Student's Signature .....

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LIST OF SCHEMES

LIST OF ABBREVIATIONS

°C	=	degree Celsius
µL	=	microliter
^{13}C NMR	=	carbon-13 nuclear magnetic resonance
$CDCl_3$	=	deuterated chloroform
CH_2Cl_2	=	dichloromethane
CH_3OH	=	methanol
cm	=	centimeter
δ	=	chemical shift
DAT	=	day after treatment
EtOAc	=	ethyl acetate
g	=	gram
gE	=	gram dry weight equivalent
^{1}H NMR	=	proton nuclear magnetic resonance
h	=	hour
Hz	=	Hertz
J	=	coupling constant
kg	=	kilogram
L	=	liter
µL	=	microliter
m	=	meter
mL	=	milliliter
mm	=	millimeter

no.	=	number
PDA	=	Potato Dextrose Agar
ppm	=	part per million
R_f	=	retention factor
RT	=	room temperature
TLC	=	thin layer chromatography
UV	=	ultraviolet
w/v	=	weight by volume
w/w	=	weight by weight

CHAPTER I

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of important oilseed crops and widely grown in Thailand. Seed of sunflower can produce cooking oil, used in food industry or as ingredients in cosmetics. However, the leftover materials such as leaf, stem and root are not properly used. This leads to search for utilization of the leftover materials for agriculture management such as insect, pathogen especially weeds. Weeds are defined as the undesired plants growing in an area of cultivated crops and generally difficult to control. Synthetic herbicides have been used to solve this problem. Currently, the use of the synthetic herbicides is increasing rapidly. According to the report of the Department of agriculture in 2014, Thailand has imported herbicide 147,000 tons and trends to increase in 2015 [1]. The use of synthetic herbicide can increase the cost of production and may have side effects to the agro-ecosystem, environment and human health. These concerns are shifting attention to alternative weed management based on natural products. Allelopathy is one of those choices through natural products release from allelopathic plants that may help to reduce the use of synthetic herbicides, less pollution, safer agro-ecosystems, reduce cost and human health for sustainable agriculture.

1.1 Allelopathy

Allelopathy has been recognized as ecological phenomenon. The word allelopathy is derived from two Greek words including allelon, which means ‘each other’ and pathos which mean ‘to suffer’ [2]. Hans Molisch in 1937 defined the term allelopathy as stimulated or inhibitory biochemical interactions among all classes of plants as well as microorganisms [3]. In 1996, the International Allelopathy Society (IAS) defined allelopathy as ‘The science that studies any process involving secondary metabolites produced by plants, algae, bacteria and fungi that influence the growth and development of agricultural and biological system’ [4].

1.1.1 Allelopathic chemistry

The chemicals responsible for the phenomenon of allelopathy are generally referred to as phytotoxins or allelochemicals. Most of these chemicals are secondary metabolites which are produced as offshoots in the primary metabolic pathways of plants [5]. Allelochemicals can be classified into the following major categories: simple soluble organic acids, straight chain alcohols, aliphatic aldehydes and ketones, simple unsaturated lactones, long-chain fatty acids and polyacetylenes, naphthoquinones, anthraquinones and complex quinones, steroids and terpenoids (sesquiterpene lactones, diterpenes, and triterpenoids), simple phenols, benzoic acid and derivatives, cinnamic acid and derivatives, coumarins, flavonoids and tannins [6]. The biosynthetic pathways of the major allelopathic substances are shown in **Figure 1.1**.

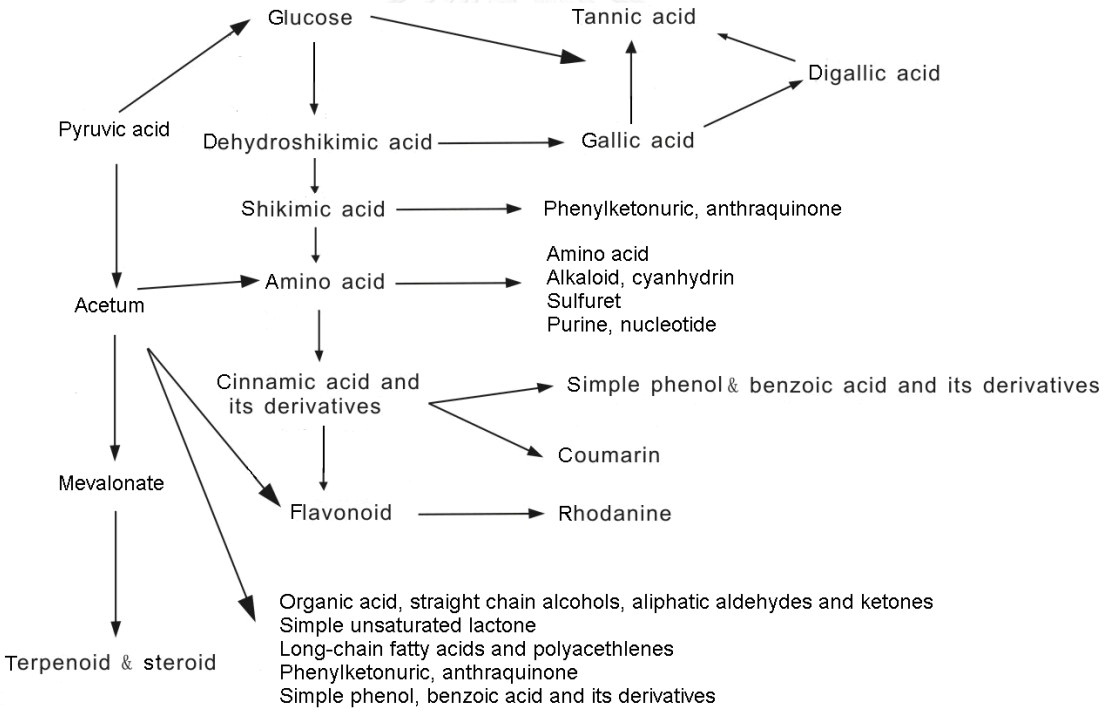


Figure 1.1 Biosynthetic pathways of major allelopathic substances [7]

1.1.2 Production of allelochemicals

Allelochemicals can be transferred from a donor plant to a receiver plant. Allelochemicals can be found in different concentrations in several parts of plants including leaves, stems, roots, rhizomes, seeds, flowers and even pollens, but roots, seeds, and leaves are the most common sources [6]. Quantities of allelochemicals produced can be greater under condition of mineral deficiency, drought stress, and cool temperatures as opposed to more optimal growing condition [8]. Allelochemicals can be released into the environment by a variety of mechanisms as presented in **Figure 1.2**.

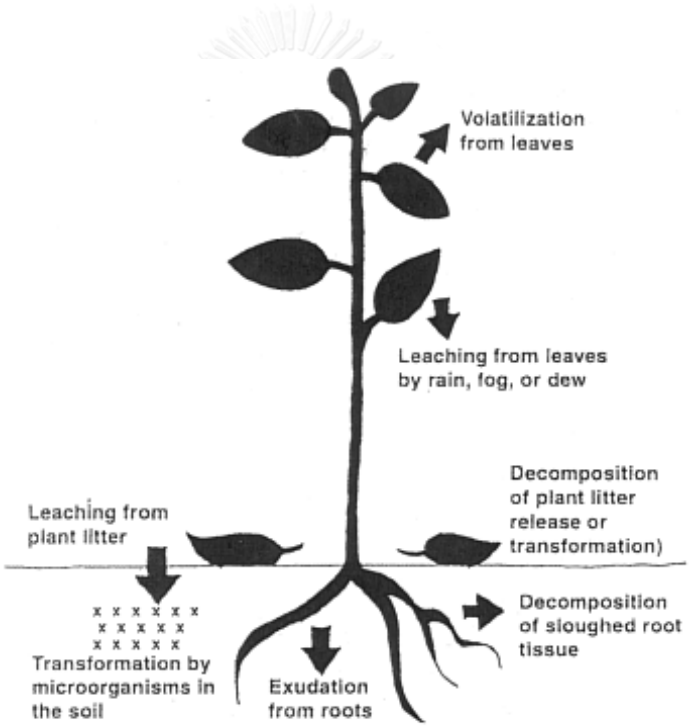


Figure 1.2 Allelochemicals released into the environment [9]

Volatilization from leaves

The plant releases a chemical in the form of a gas through small openings in their leaves. The volatile vapors may be adsorbed directly from atmosphere by plants which affected the growth of the receiver plants [10].

Leaching from leaves by rain, fog or dew and plant litter

Leaching takes place when plant drop leaves that contain allelochemicals in to the soil, either by the littering and decomposition of the leaves, or *via* runoff rain, fog, or dew that comes into contact with the leaves leading to the inhibition of growth and germination of other plants [11].

Exudation from root

Plants can release chemicals into the soil through their roots. The released chemicals are adsorbed by nearby plants. Exuding compounds are selectively toxic to other plants. Exudates usually are various phenolic compounds that tend to inhibit plant development [12].

Decomposition

The decomposition of plant residues can release a large amount of allelochemicals to the soil. The root of receiver plants may absorb decaying plant residues and are impacted by allelochemicals. Some decomposition products on plants can inhibit seed germination, stunted growth, and inhibition of the primary root system and increase in secondary roots, inadequate nutrient absorption [13].

1.1.3 Mode of action of allelochemicals

The mode of action of allelochemicals can broadly be divided into indirect and direct action. Indirect action may include the effects through alteration of soil property, its nutritional status and an altered population and/or activity of harmful/beneficial organisms like microorganisms, insect, nematodes, *etc.* This is relatively less studied aspect. On the other hand, the direct mode of action, which includes the effects of allelochemicals on various aspects of plant growth and metabolism, has received fairly wide attention [2].

The followings are some important site and processes known to be attacked or influenced by allelochemicals.

- Cytology and ultrastructure
- Phytohormones and their balance
- Membrane and its permeability

- Germination of pollens/spores
- Mineral uptake
- Stomatal movement, pigment synthesis and photosynthesis
- Respiration
- Protein synthesis
- Leghaemoglobin synthesis and nitrogen fixation
- Specific enzyme activity
- Conducting tissue
- Water relation of plants
- Genetic material

In nature, the action of allelochemicals seems to revolve round a finetuned regulatory process in which, perhaps, many compounds of the act together with one or more than one of the above processes in a simultaneous or sesquential manner [2]. Mode of action of some allelochemicals is similar to synthetic herbicides. These features have allowed them to be considered for possible use in weed management as bioherbicides.

1.1.4 Effect of allelochemicals

Allelochemicals have mostly negative effects on crop plants such as delay or complete inhibition of seed germination, reduced plant population, stunted and deformed roots and shoots, deranged nutrient absorption, lack of seedling vigour, reduced tillering, chlorosis, wilting, and increase susceptibility to disease. However, the main impacts of phytotoxins on crop plants are inhibition of nitrification and biological nitrogen fixation, predisposing the plants to diseases and inhibition or stimulation of germination, growth and yield [13].

1.2 Botanical description of sunflower

Sunflower or *Helianthus annuus* L. is cultivated primarily for its seeds. The name Helianthus, being derived from helios (the sun) and anthos (a flower), which it is popularly supposed has been given these flowers from a supposition that they follow the sun by day, always turning towards its direct rays [14]. Sunflower has been grown widely in the central part of Thailand for oil, seed meal and tourist attraction. Sunflower is one of a few plants that originated in North America and was probably first introduced in Europe through Spain, and spread throughout the Europe as a curiosity until it reached Russia where it was readily adapted [15].

Scientific classification

Kingdom: Plantae

Division: Angiospermae

Subdivision: Eudicots

Class: Asterids

Order: Asterales

Family: Asteraceae (Compositae)

Subfamily: Helianthoideae.

Roots

Strong taproot, with maturing plants developing a large fibrous and prolific lateral spread of surface roots.

Stems and Branching

The rough and hairy stem is branched in the upper part in wild plants but is usually unbranched in domesticated cultivars. Stem is usually round early season, angular and woody later in the season.

Leaves

Lowermost leaves mostly opposite along stem, upper leaves mostly alternate along stem. Leaf blades narrowly to usually broadly deltoid-ovate, lower ones often cordate, to subtruncate to broadly cuneate at base, 4-20 cm long or more, 3-15 cm wide or more, entire to margins minutely to coarsely serrate, apex acute to abruptly

acuminate; rough and pubescent, often 3-veined from the leaf base; long-petioled being often one-half as long to equaling blade.

Inflorescence

Large, composite heads, solitary at terminal end of peduncle or terminal on a branch, or axillary; composite disk usually 2-8 cm wide or more including rays; peduncles 2-20 cm long, densely hispid-scabrous. Receptacle low-convex, chaffy. Heads few to many.

Flowers

Ray flowers sterile, 1.5-4 cm long, ligules yellow. Disc flowers perfect, corolla lobes 5, 5-8 mm long, tubular, purple-brown to yellow; each floret subtended by a small firm, paleaceous bract attached to the receptacle, often 3- toothed. Pappus 2 readily deciduous, awn-like palea floret subtended by a small firm, paleaceous 2-3.5 mm. long

Fruits

Achenes 3-6 mm long or more, narrowly obovate to ovate, more or less 4 angled, somewhat compressed, glabrous to minutely puberulent especially at apex, gray to brown and occasionally mottled to striped [16, 17].

1.3 Chemical constituents studies on sunflower

Sunflower has been studied for its allelopathic potential. The chemicals studies show that sunflower is plant which a rich source of phenolic compounds and terpenoids, particularly sesquiterpene lactones, heliaspirones, annuionones, helibisabonols, helianuols, with a wide spectrum of biological activities including allelopathy. The first study on sunflower showed that sunflower extracts inhibited germination of growth of a variety of weed species [17, 18]. The cultivated sunflower is allelopathic and has activity against such troublesome weeds as morning glory, velvetleaf, pigweed, jimson weed, wild mustard, and others [19].

Heliannuols

A promising group of phenolic allelochemicals isolated from sunflower are novel sesquiterpenes, heliannuols (**Figure 1.3**). This functional group has long been associated with allelopathic activity. The heliannuols were isolated from the moderately polar active fraction of leaf aqueous extract of *H. annuus L.* var. SH-222 and VYP.[19-23]. The comparison of active heliannuols with the commercial herbicide Logran® showed that the most important effects with those caused by heliannuols A, C, H, I and K inhibiting germination of lettuce and caused by heliannuols C, G, H, I and K stimulating root growth of barley [24].

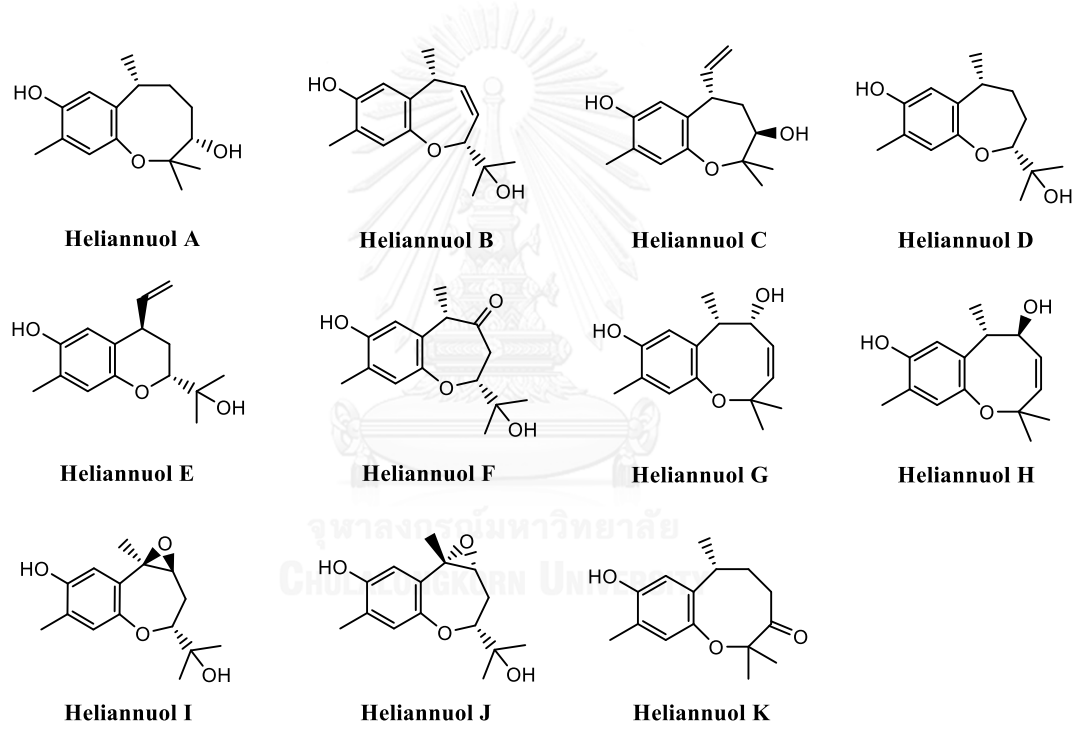


Figure 1.3 Heliannuols isolated from *H. annuus*

Heliespirone A (**Figure 1.4**), a novel allelopathic quinone spiroether, was also isolated from *H. annuus* and likely arises from a bisabolene precursor. Bisabolene quinones such as glandulones A-C (**Figure 1.4**) have previously been isolated from the noncapitate glandular trichomes of *H. annuus* [19].

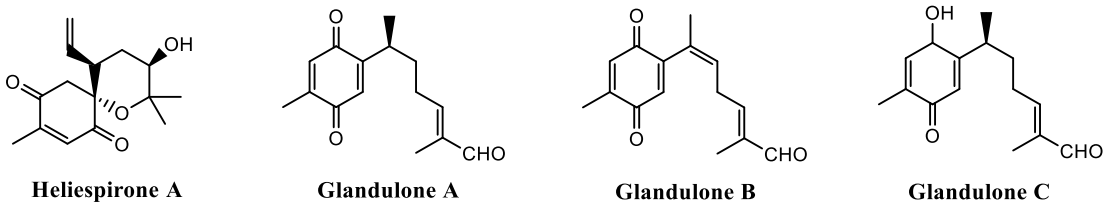


Figure 1.4 Heliannuols isolated from *H. annuus*

Sunflower terpenoids

Sesquiterpene lactones are common constituents of helianthus species. Annuolides A-G (**Figure 1.5**) are a family of guaianolides isolated from leaf aqueous extracts of cultivar sunflower that exhibit allelopathic activity. Helianthus cultivars have also yielded some interesting bisnorsesquiterpenes such as annuionones A-H and helinorbisabone (**Figure 1.6**). *Ohno et al.* studied the exudates of sunflower seeds during germination and isolated a stereoisomer of sundiversifolide, diversifolide (**Figure 1.7**). Bioassay showed that this compound inhibited shoot and root growth of cat's-eyes seedlings [19, 25].

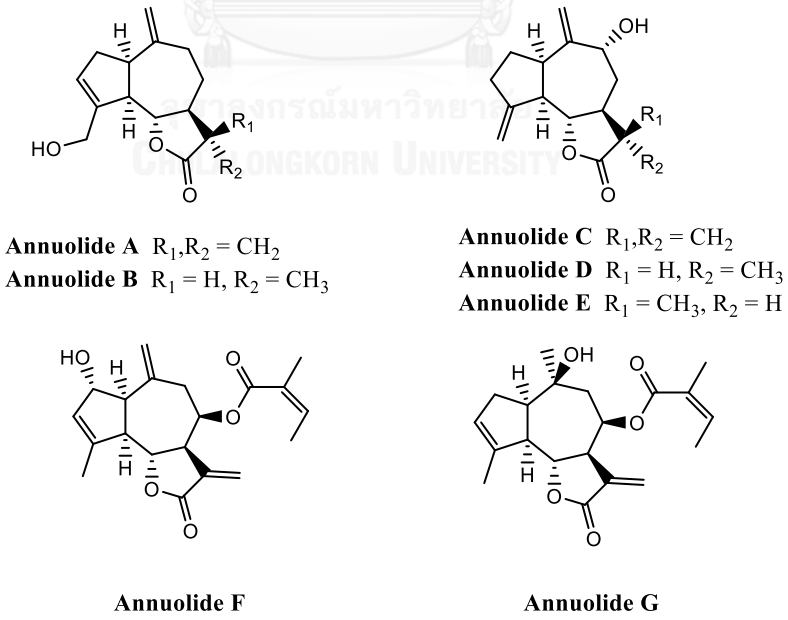


Figure 1.5 Guaianolides isolated from *H. annuus*

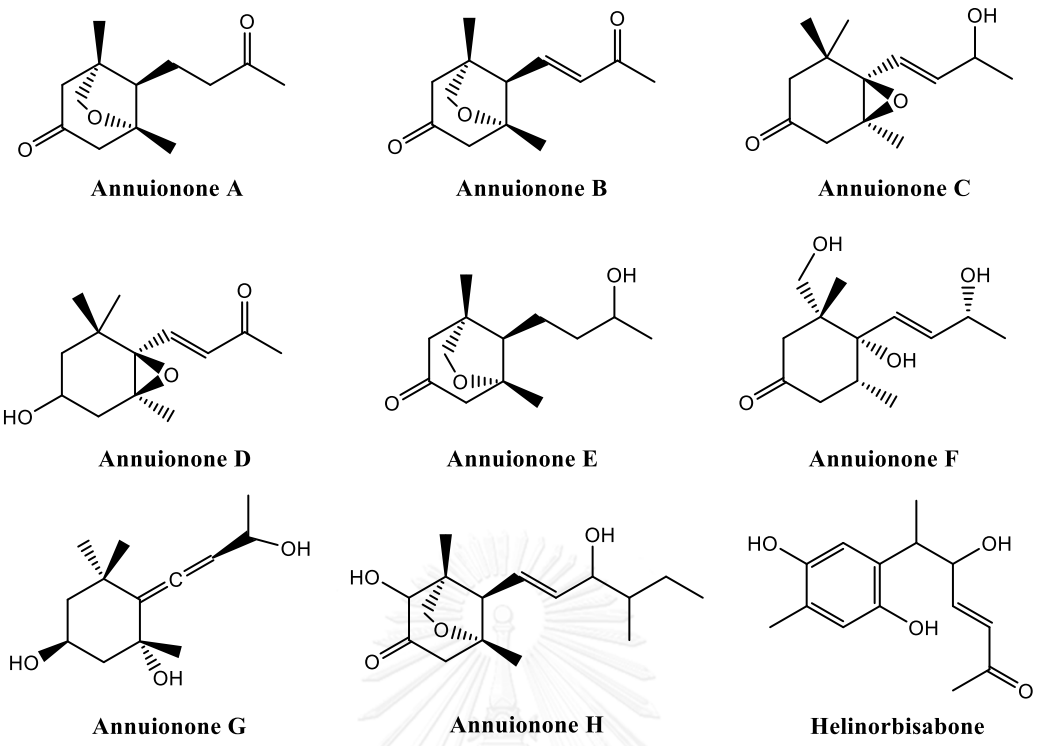


Figure 1.6 Bisnorsesquiterpenes isolated from *H. annuus*

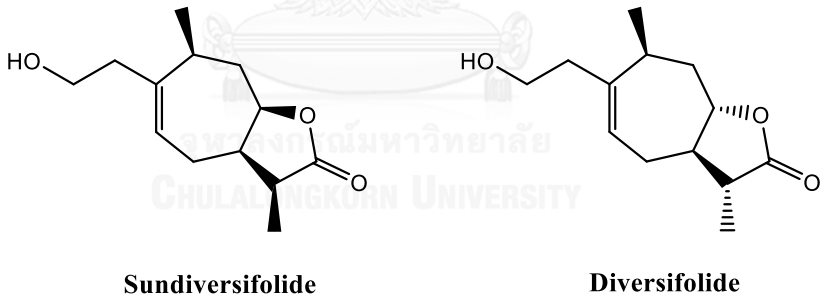


Figure 1.7 Metabolites isolated from the exudates of sunflower seeds.

Sunflower chalcones and flavonoids

Sunflower has also yielded chalcones and flavonoids in the search for allelochemicals. Chalcones kulkulkanin B and heliannone A were isolated along with flavonoids tambulin, and heliannones B and C from both *H. annuus* cultivar VYP and Peredovick (**Figure 1.8**). Bioassays indicated the flavonoids mainly affected shoot

growth of tomato and barley seedling, but kulkulkanin B and heliannone A affect germination [19].

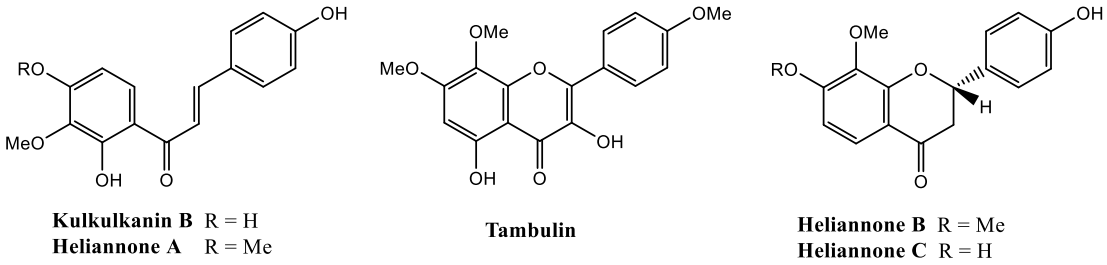


Figure 1.8 Flavonoids isolated from *H. annuus*

1.4 Knowledge about studied weeds and crop plants

In this research *Mimosa pigra* L. was selected for bioassay test. In addition, various weeds including prickly chaff-flower (*Achyranthes aspera* Linn.), barnyard grass (*Echinochloa crus-galli* (L.) P. Beauv.), swollenfinger grass (*Chloris barbata* L.) and crop plants such as Chinese kale (*Brassica alboglabra* L.H. Bailey) and water convolvulus (*Ipomoea aquatica* Forssk.), corn (*Zea mays* L.), rice (*Oryza sativa* L.) were chosen for allelopathic study.

1.4.1 *Mimosa pigra* L.

Family: Mimosaceae (Leguminosea)

Common name: giant mimosa, catclaw mimosa, giant sensitive plant, shrubby sensitive plant, thorny sensitive plant

Local name: ไมยราบยักษ์ (Mai Yah Laap Yak)

Botany description

Erect or scandent shrub, 2-4 m high, all parts armed with prickles. Leaves bipinnate; pinnae 10-15 pairs; leaflets 35-51 pairs per pinna, linear-oblong; rachis with straight thorns. Inflorescence pedunculate heads, in the axils of the upper leaves, calyx 1.5 cm across, many flowers. Flowers pink to purplish-red; calyx scarious, small; corolla

funnel-form; stamens 8; ovary densely velutinous. Pod clustered, oblong, beaked, densely scabrous, 4-6 cm long and 0.6-1 cm wide. Seed ovoid, brown and small.

Distribution

Mimosa pigra L. widely distributed throughout tropical regions. In Thailand, a common weed, in the north, found in the wetlands and roadsides. Flowering from November to June [26].

Figure 1.9 *Mimosa pigra* L. (Giant mimosa)
(Source: http://www.qsbg.org)

1.4.2 *Achyranthes aspera* L.

Family: Amaranthaceae

Common name: Prickly-chaffed flower, prickly-chaff flower, rough chaff tree

Local name: หญ้าพันงู (Ya pan ong)

Achyranthes aspera L. is an erect or procumbent, annual or perennial herb of about 1-2 meter in height, often with a woody base, stems angular, ribbed, simple or branched from the base, often with tinged purple color, branches terete or absolutely quadrangular, striate, pubescent, leaves opposite, elliptic or obovate, form an acute or obtuse base, acuminate or rounded at apex, flowers greenish white, numerous in axillary or terminal spikes up to 75 cm long, seeds subcylindric, truncate at the apex,

rounded at the base, reddish brown. Commonly found as a weed of waysides, on roadsides in tropical and warm regions [27].

Figure 1.10 *Achyranthes aspera* L. (Prickly-chaffed flower)
(Source: http://herbsdatabase.blogspot.com)

1.4.3 *Echinochloa crus-galli* (L.) P.Beauv.

Family: Poaceae

Common name: Barnyard grass, barnyard millet, chicken-panic grass

Local name: หญ้าข้าวนก (Ya Kao Nok)

Annual, erect, tufted or reclining at base up to 200 cm tall, stem culms rooting at lower nodes, cylindrical, without hairs, and filled with white spongy pith, leaf linear with a broad round base and narrow top, blade 10–40 cm long, ligule absent. *Echinochloa crus-galli* prefers moist to wet land easily grows in direct-seeded rice fields and wastelands. It is a common weed in swamps and aquatic places [28].

Figure 1.11 *Echinochloa crus-galli* L. Beauv. (Barnyard grass)

(Source: http://www.brrd.in.th/rkb/weed/index.php-file=content.php&id=1.htm)

1.4.4 *Chloris barbata* Sw.

Family: Poaceae

Common name: Swollen finger grass, finger grass, pea-cock plumegrass

Local name: หญ้ารังนก (Ya Rung Nok)

Tufted annual grass about 70 cm high, internodes are longer at the top and shorter at base; leaves lanceolate, narrowly linear, acuminate; spikes 6 cm long, floral glumes densely hair, awned, grains oblong. Frequently found along cultivated fields and in forest hilly areas [29].

Figure 1.12 *Chloris barbata* Sw. (Swollen finger grass)

(Source: http://www.brrd.in.th/rkb/weed/index.php-file=content.php&id=22.htm)

1.4.5 *Brassica oleracea* var. alboglabra

Family: Brassicaceae

Common name: Chinese kale, chinese Broccoli, Gai lan

Local name: คะน้า (Ka na)

A leafy green vegetable that belongs to the Brassica family. Perennial growing to 0.5 m at a fast rate. It is not frost tender. The flowers are hermaphrodite (have both male and female organs) and are pollinated by bees. The plant is self-fertile [30].

Figure 1.13 *Brassica oleracea* var. alboglabra (Chinese kale)
(Source: http://www.websanom.com/sanom_info_chinese_kale.php)

1.4.6 *Ipomoea aquatica* Forsk. var. reptans

Family: Convolvulaceae

Common name: Swamp morning glory, morning glory, water convolvulus

Local name: ผักบุ้ง (Pakbung)

An annual or perennial vine herb, stems prostrate or floating thick, herbaceous, 2-3 m long, rooting at the nodes. Leaves are alternate. Flower stalk arises from the leaf axil and bears 1 to several flowers [31].

Figure 1.14 *Ipomoea aquatica* Forsk. (Water convolvulus)

Source: http://overcomedisease.blogspot.com/2013_07_01_archive.html)

1.4.7 *Oryza sativa* L. cultivar riceberry

Family: Poaceae

Common name: Rice (Riceberry)

Local name: ข้าวไรซ์เบอรี่ (Kao Rice Berry)

Rice is a typical grass, forming a fibrous root system bearing erect culms and developing long flat leaves. It has a semi-aquatic lifestyle, requiring water particularly during the reproductive growth phase [32].

Figure 1.15 *Oryza sativa* L. cultivar riceberry (Rice)

1.4.8 *Zea mays* var. ceratina Kuleshov

Family: Poaceae

Common name: Waxy corn

Local name: ข้าวโพดข้าวเหนียว (Kao Pod Kao Neaw)

Corn is a robust annual grass, usually single-stemmed, occasionally tillering, with stout culm, sometimes stilt-rooted at the basal nodes, to 1-4 m high, even to 6 m, and 3-4 cm in diameter. The flowers are monoecious and pollinated by wind [33].

Figure 1.16 *Zea mays* var. ceratina Kuleshov (Corn)
(Source: http://nutrition.dld.go.th/Nutrition_Knowlage/ARTICLE/PRO1.HTM)

1.5 Objectives of this research

This research aims to utillize the extracts of leftover materials from *Helianthus annuus* L. and the objective of this research can be summarized as follows:

1. To study allelopathic effects of the extracts from *Helianthus annuus* L. on seed germination and the growth of weeds and crop plants.
2. To explore the biological activity of the extracts from *Helianthus annuus* L. to control pest and microorganism.
3. To identify substances from CH_2Cl_2 extract of the roots.

CHAPTER II

EXPERIMENTAL

2.1 Plant materials

Sunflower (*Helianthus annuus* L.) variety Pioneer-jumbo was collected from Lop Buri province, Thailand in January 2013. The plant was washed thoroughly using tap water, separated into leaves, stems and roots, dried under sunlight and finally ground into powder.

2.2 Model plants for bioassays

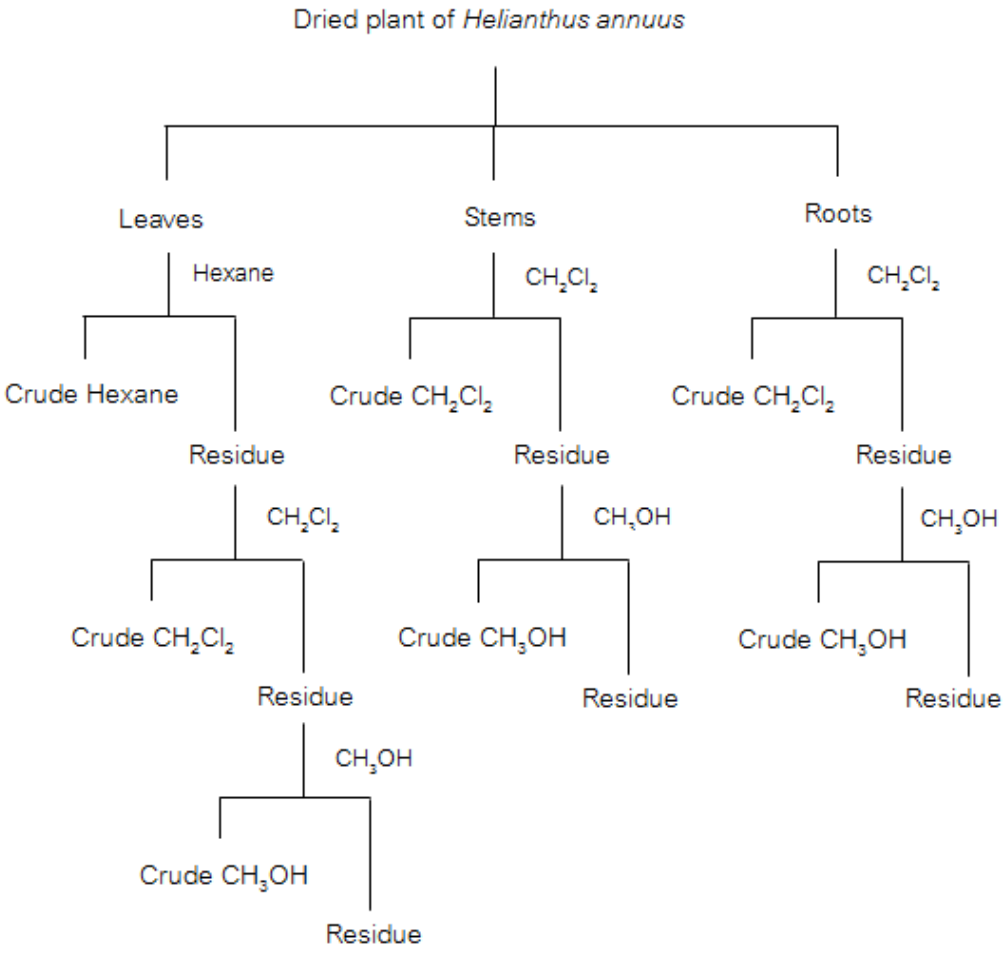
The seeds of Giant mimosa (*M. pigra*) were collected from Lop Buri province. The seeds were breaking dormancy prior to use by soaking in 70°C distilled water for 24 h to soften the seed coat. The seeds of other selected weeds including prickly Chaff flower (*A. aspera*), barnyard grass (*E. crus-galli*) and swollen finger grass (*C. barbata*) were collected from Prachin Buri province. All weed seeds were kept in 5 °C until use. The seeds of crop plants namely Chinese kale (*B. alboglabra*), water convolvulus (*I. aquatica*) and corn (*Z. mays*) were bought from Chua Youg Seng seed company Limited, rice (*O. sativa*) and mung bean (*V. radiata*) were collected from Lop Buri province, marigold (*T. erecta*) and zinnia (*Z. violacea*) were bought from Chia Tai company Limited.

2.3 Instrument and equipment

Thin layer chromatography (TLC) was performed on aluminum sheets precoated with silica gel (Merck's Kiesel gel 60 PF_{254}) and spots on the plate were observed under UV light. Column chromatography was performed on silica gel Merck Kieselgel 60 no. 7729 and 7734. The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker ACF 200 or Jeol JNMA 500 spectrometer using tetramethylsilane (TMS) as an internal reference.

2.4 Extraction procedure

One kg of ground leaves, stems and roots of sunflower was separately extracted by soaking with appropriate solvent. For leaves, the plant material was soaked in hexane for three days at room temperature (RT). The residue was repeatedly extracted by CH_2Cl_2 and CH_3OH, respectively for three times. For stems and roots, the soaking in CH_2Cl_2 for three days was first conducted and repeatedly extracted with CH_3OH for three times. The extract was filtered and evaporated with a rotatory evaporator at 40 °C. The extraction procedure for the plants was summarized as shown in **Scheme 2.1**.



Scheme 2.1 The extraction procedure of *H. annuus*

2.5 Experiment for bioassay

2.5.1 Allelopathic activity test

Seed germination inhibition test

The crude extract was dissolved in 3 mL of an appropriate solvent at different concentrations: 1.0, 2.5 and 5.0 g equivalent (gE), then poured into Petri-dishes (diameter 90 mm), each containing a filter paper. The equal amount of the same solvent to dissolve crude extract was used as control. Leave overnight to remove the solvent, then 5.0 mL of distilled water was added to each plate. The selected 50 seeds were placed per dish (25 seeds for *Z. mays* and *I. aquatica*). Then, Petri-dishes were closed and placed in growth chamber at 25 ℃, 12/12 light to observe the growth for 7 days. Each experiment was performed in three replications. The inhibition percentage was calculated as shown below.

$$\text{Germination Inhibition (\%)} = (C-T) \times 100 / C$$

Where T is germination number of treated

C is germination number of controlled

*Germination inhibition of 100% means completely inhibitory effect

Growth inhibition test

The crude extracts, 1.0, 2.5 and 5.0 gE were dissolved in 3 mL of an appropriate solvent and poured into Petri-dishes (diameter 90 mm), each containing a filter paper. The equal amount of the same solvent to dissolve crude extract was used as control. Leave overnight to remove the solvent, then 5.0 mL of distilled water was added to each plate. Six seedlings of selected seeds with radical root length 1-2 mm (seeds for bioassay were soaked for 12 h and germinated in Petri-dish one night before testing) were placed in each plate, 3 replications for each experiment. Petri-dishes were closed and kept in growth chamber at 25 ºC, 12/12 light. The root and shoot lengths were recorded at 7 days after transplanting. The inhibition percentage was calculated as shown below.

Growth Inhibition (%) = (C-T) ×100 / C

Where T is root or shoot length of treated

C is root or shoot length of controlled

*Germination inhibition of 100% means completely inhibitory effect

2.5.2 Pot experiments

Pre-emergence with dry leaf bioassay

Bioassays were conducted using dried sunflower leave powder incorporated with soil (a loam soil with organic matter) at 1, 5 and 10% per 100 g of soil, then filled in plastic pots (3 inches diameter). Ten selected seeds were sown at 1 cm soil depth. Control seeds were sown in soil without leave powder, 3 replications for each experiment. Pots were watered with tap water as needed to maintain adequate soil moisture. Seed germination was recorded after 7 days. The inhibition percentage was calculated as shown in 2.5.1.

Pre-emergence crude extract bioassay

Bioassays were conducted using the CH_3OH extract of the leaves dissolved in distilled water as following: plastic pots (3 inches diameter) were filled with 100 g of soil (a loam soil with organic matter). Pots were watering with 10 mL of the extract solution at concentrations: 1, 2.5 and 5 gE, incubated for 24 h. Ten selected seeds were sown at 1 cm soil depth. Control pots were watered with only tap water, 3 replications for each experiment. Pots were watered with tap water as needed to maintain adequate soil moisture. Seed germination was recorded after 7 days. The inhibition percentage was calculated as shown in 2.5.1.

Post-emergence with crude extract bioassay

Bioassays were conducted using the CH_3OH extract of the leaves dissolved in distilled water as following: plastic pots (3 inches diameter) were filled with 100 g of soil (a loam soil with organic matter). Ten selected seeds were sown at 1 cm soil depth. Control seeds were sown in soil without the extract solution, 3 replications for each

experiment. After seed emergence, seedlings were thinned to 5 plants per pot, then watered with 10 mL of the extract solution at concentrations: 1, 2.5 and 5 gE. Pots were watered with tap water as needed to maintain adequate soil moisture. Shoot and root lengths were recorded after 7 days. %inhibition was calculated as shown in 2.5.1.

2.5.3 Other bioassays

Antifeedant bioassay

The hexane, CH_2Cl_2 and CH_3OH extracts from different parts of sunflower were preliminarily evaluated for antifeedant activity as following: the extract was dissolved in an appropriate solvent at 0.25% w/w and mixed in artificial diet. Leave overnight to remove the solvent. The artificial diet was weighed and placed in 24 well-plate, then placed one of common cutworm (second instar larvae) on the artificial diet. Control experiment was conducted using artificial diet without the extract. Each experiment was performed for 30 replications. After 24 h, the artificial diet was weighed and measured %antifeedant by the following equation.

$$\%\text{Antifeedant} = [(1-T/C) \times 100]$$

Where T is the weight loss of artificial diet in treatment plate

C is the weight loss of artificial diet in control plate

Antimicrobial activity

The hexane, CH_2Cl_2 and CH_3OH extracts from different parts of sunflower were preliminarily evaluated for antibacterial activity using agar diffusion method at 10,000 ppm (45 µL/well) and agar incorporation method at 1,000 ppm for screening of antifungal activity. Each experiment was performed for three replications. The results were expressed as the clear zone (in mm) and %inhibition, respectively.

CHAPTER III

RESULTS AND DISCUSSION

The main objective of this research is to examine the allelopathic effect of the extracts from left over materials of sunflower (*Helianthus annuus* L.) including leaves, stems and roots on seed germination and growth inhibition *of M. pigra* and other weeds. The latter includes prickly chaff-flower (*A. aspera*), barnyard grass (*E. crus-galli*) and swollen finger grass (*C. barbata*) while selected crops were chinese kale (*B. alboglabra*), water convolvulus (*I. aquatica*), rice (*O. sativa*) and corn (*Z. mays*). In addition, other biological activities- antifeedant activity on common cutworm (*Spodoptera litura*) and antimicrobial activity were conducted and to identify the isolated substances from sunflower root.

3.1 The extraction of *Helianthus annuus*

Dried leaves (3 kg), stem (1 kg), and root (1 kg) were separately milled to fine powder and extracted by soaking in hexane, CH_2Cl_2, and CH_3OH, respectively followed **Scheme 2.1**, then filtrated the extract and evaporated by rotatory evaporator to obtain leave, stem, and root extracts. The summary of the extraction is show in **Table 3.1**.

Table 3.1 Weight and %yield of the crude extracts of *H. annuus*

Part	Solvent	Weight (g)	%Yield	Remark
Leaves 3 kg	Hexane	97.2	3.24	Yellow liquid
	CH_2Cl_2	42.9	1.43	Green solid
	CH_3OH	204.2	6.81	Dark brown liquid
Stem 1 kg	CH_2Cl_2	17.4	1.75	Yellow liquid
	CH_3OH	68.3	6.83	Brown liquid
Root 1 kg	CH_2Cl_2	9.4	0.94	Yellow liquid
	CH_3OH	25.8	2.59	Brown liquid

3.2 Bioassay results

3.2.1 Germination inhibition of *M. pigra*

Each crude extract including hexane, CH_2Cl_2 and CH_3OH was assayed for seed germination inhibition on *M. pigra* at three different concentrations (1, 2.5, and 5 gE) compared with the control to observe % germination inhibition. The results are summarized as shown in **Figures 3.1** and **3.2**.

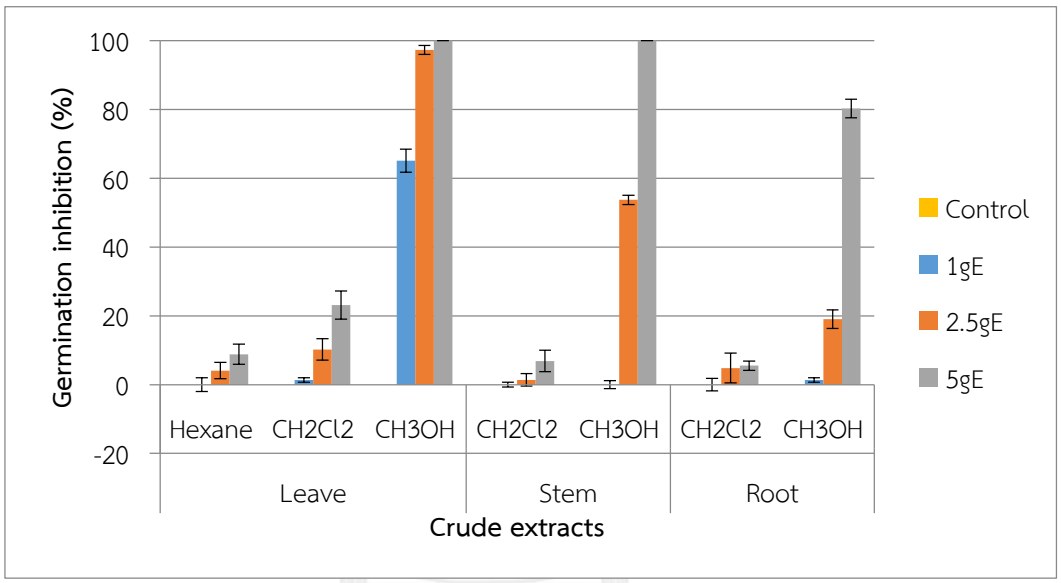


Figure 3.1 The seed germination inhibition effects of crude extracts from *H. annuus* on *M. pigra* L.

As control experiments, the same solvents that used to dissolve the extracts were left overnight. The germination results showed no significant compared with using only water. This indicated that the used solvents expressed no side-effect on seed germination of tested plants. **Figures 3.1** shows that the CH_3OH extract of the leaves revealed significant higher inhibition on seed germination of *M. pigra* than the control which displayed the inhibition of 65 and 97% at 1 and 2.5 gE and completely inhibited at 5 gE. The leave extracts gave stronger inhibitory effect against *M. pigra* than those of stems and roots. Anywise, the CH_3OH extract of the stems could completely inhibit at 5 gE. The CH_3OH extract was found to exhibit this activity more than the CH_2Cl_2 and hexane extracts and the inhibition effect was increased by increasing the concentration.

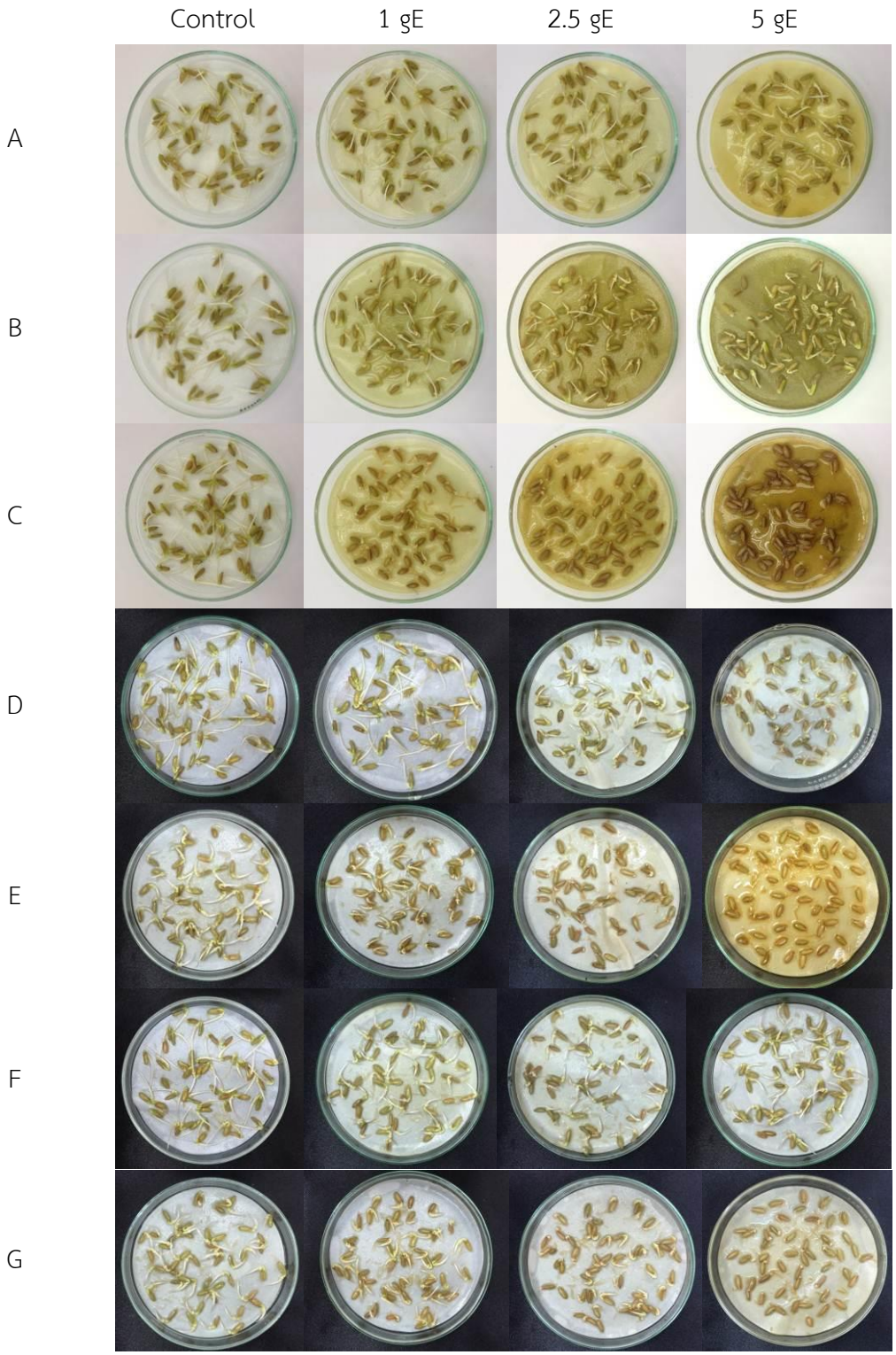


Figure 3.2 The effects of crude extracts of *H. annuus*; leave hexane (A), leave CH_2Cl_2 (B), leave CH_3OH (C), stem CH_2Cl_2 (D), stem CH_3OH (E), root CH_2Cl_2 (F), root CH_3OH (G) on seed germination of *M. pigra* at 2 DAT.

3.2.2 Growth inhibition of *M. pigra*

All crude extracts from *H. annuus* were assayed on the growth of *M. pigra* at three different concentrations (1, 2.5, and 5 gE) compared with the control to observe %shoot and root elongation inhibition. The results are shown and summarized in **Figures 3.3-3.5**.

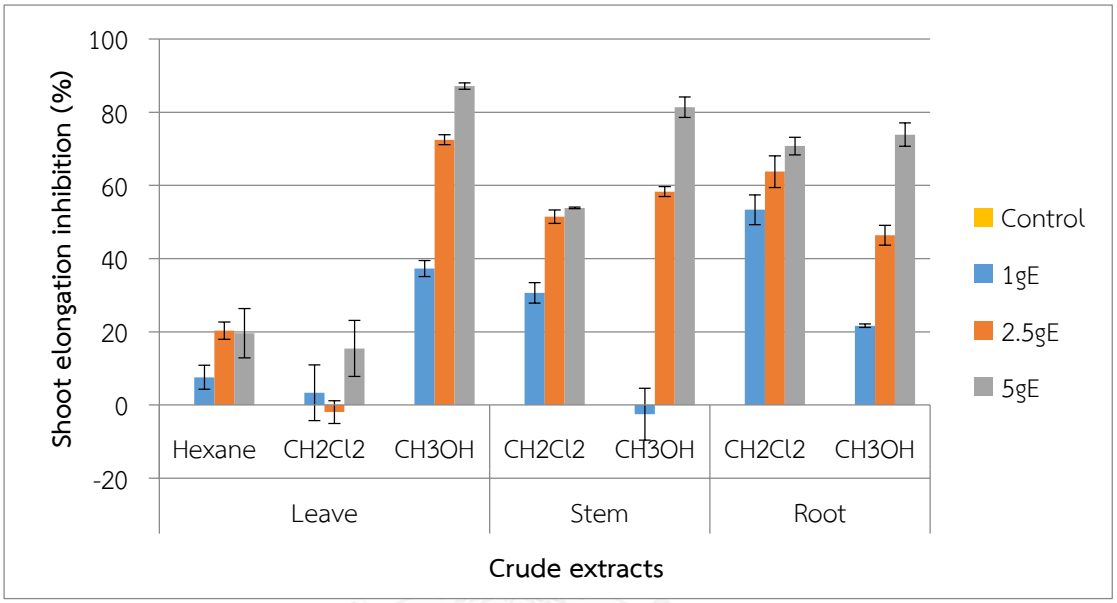


Figure 3.3 The shoot elongation inhibition of crude extracts from *H. annuus* on *M. pigra*.

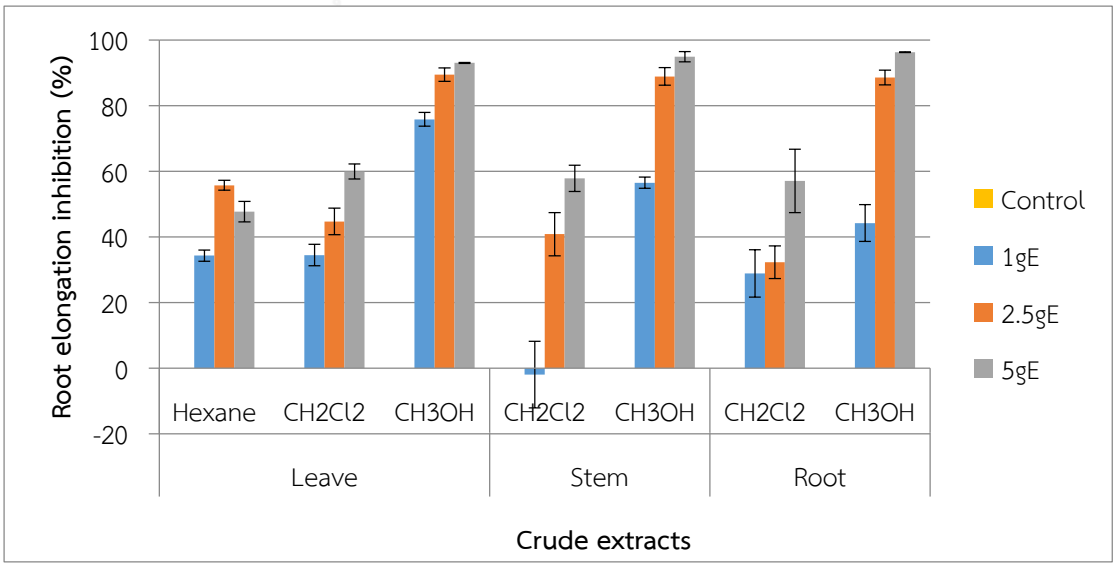


Figure 3.4 The root elongation inhibition effects of crude extracts from *H. annuus* on *M. pigra*.

From the shoot elongation inhibition results, the CH_3OH extract of the leave exhibited significant inhibition and expressed the highest inhibitory effect against *M. pigra* with 37, 73 and 87% at 1, 2.5 and 5 gE, respectively. On the other hand, the CH_2Cl_2 extract of the root also expressed high inhibition effect with 53, 64 and 71% at the same concentrations used. The inhibition of *M. pigra* root was sensitive than that of the shoot. That was because the roots were directly contacted to the tested extracts while the shoot growth is contributed from the root and attributed to accumulated food from seeds. The CH_3OH leave extract displayed the best effect to reduce the root elongation which 76, 89 and 93% at 1, 2.5 and 5 gE, respectively. The inhibition more than 80% could be observed from the CH_3OH extracts of all parts at ≥ 2.5 gE.

According to the above results, the allelopathic effects of *H. annuus* on seed germination and growth inhibition of *M. pigra* revealed that the CH_3OH extract of *H. annuus* gave the highest activity than those of CH_2Cl_2 and hexane extracts. The leave extract gave stronger inhibition than those extracts derived from stem and root.

Faezad *et al.* (2014) reported that different concentrations of the CH_3OH extract had various inhibitory impacts on the growth of target plant. It could be a reflection of plant growth inhibitor concentration being released by plant tissue [34]. Hanvongsa (1999) reported the allelopathic activity of the leave extract of sunflower inhibited seed germination and growth 5 crop species: *S. bicolor*, *Z. mays*, *H. annuus*, *G. max*, *V. radiate* and 4 weed species: *T. portulacastrum*, *A. spinosus*, *E.heterophylla* and *D. ciliaris* [35]. Similarly in 2011, Asgharipour and Majid reported that among sunflower aqueous extracts (root, stem and leave), the leave extracts revealed more allelopathic effect than the root and stem extracts on seed germination of amaranth and nutsedge [36].

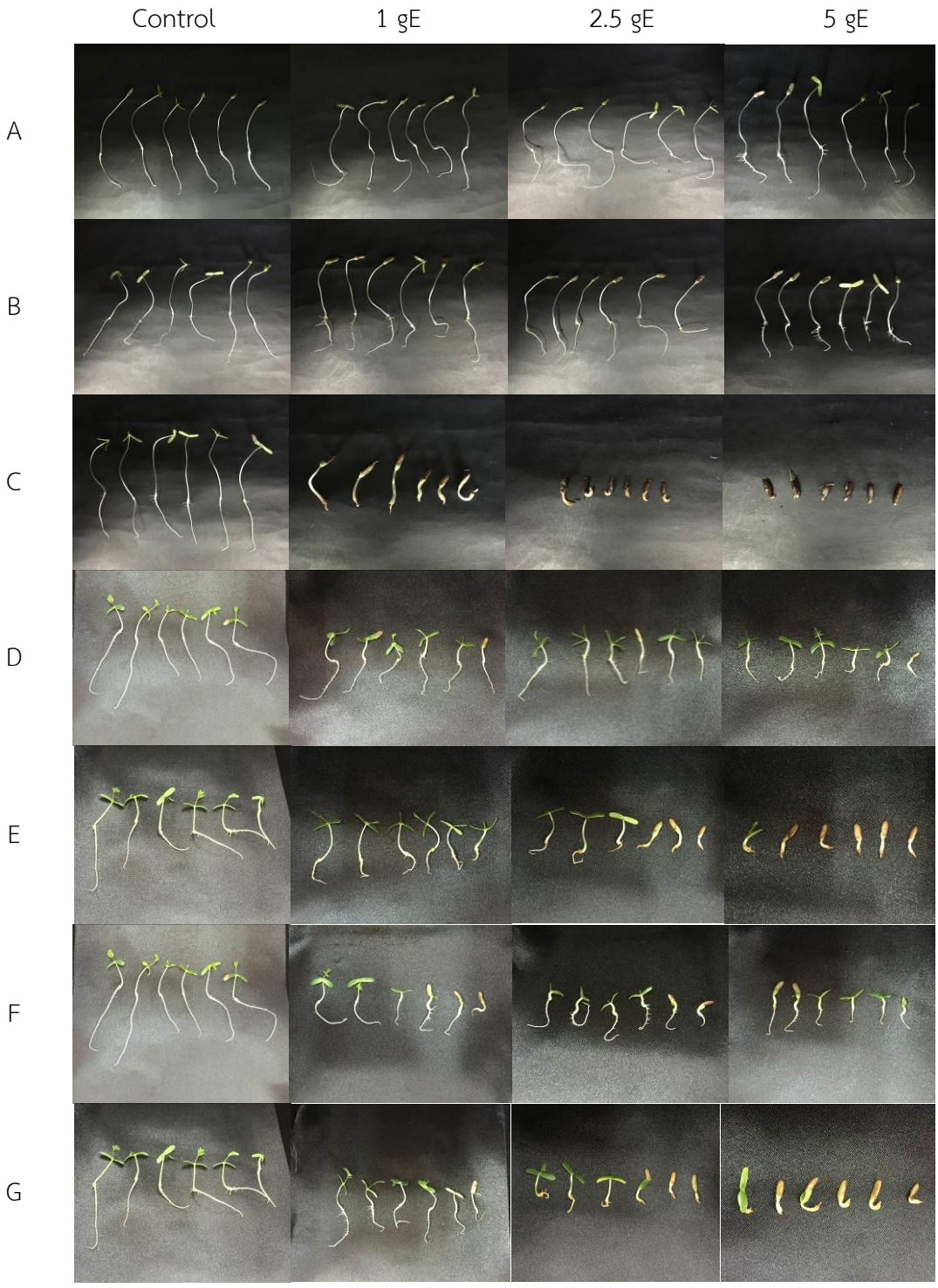


Figure 3.5 The effect of crude extracts of *H. annuus*; leave hexane (A), leaves CH_2Cl_2 (B), leave CH_3OH (C), stem CH_2Cl_2 (D), stem CH_3OH (E), root CH_2Cl_2 (F), root CH_3OH (G) on shoot and root elongation of *M. pigra* at 7 DAT.

From allelopathic test on *M. pigra*, the germination and growth inhibition results indicated that the CH_3OH extract of the leaves displayed the highest allelopathic activity. Therefore, the CH_3OH extract of the leaves was selected for further investigation on seed germination and growth inhibition against other weeds and crops as mentioned in experimental part.

Additionally, the CH_2Cl_2 extract from sunflower root showed strong effect on shoot elongation inhibition of *M. pigra* at ≥ 1 gE concentration, while the root elongation expressed low activity. These results suggested that the extract be a possible candidate to use as selective inhibition on shoot for some plants that need to control the shoot elongation such as flowers for compact and cereal crops for preventing the stem broken by wind. Therefore, the CH_2Cl_2 extract of the root at 1 gE concentration was selected for further investigation on growth inhibition with flowers including marigold (*T. erecta*), zinnia (*Z. violacea*) and cereal crops including mung bean (*V. radiata*) and rice (*O. sativa*) to expect shoot elongation inhibition and ineffective or stimulate on root elongation.

3.2.3 The effect of the CH_3OH extract of the leaves on selected weeds and crops

The CH_3OH extract of the leaves at 1 gE was assayed for seed germination and growth inhibition of above mentioned weeds and crops compared with the control to measure %germination inhibition, shoot and root elongation inhibition. The results are summarized as presented in **Figures 3.6-3.10**.

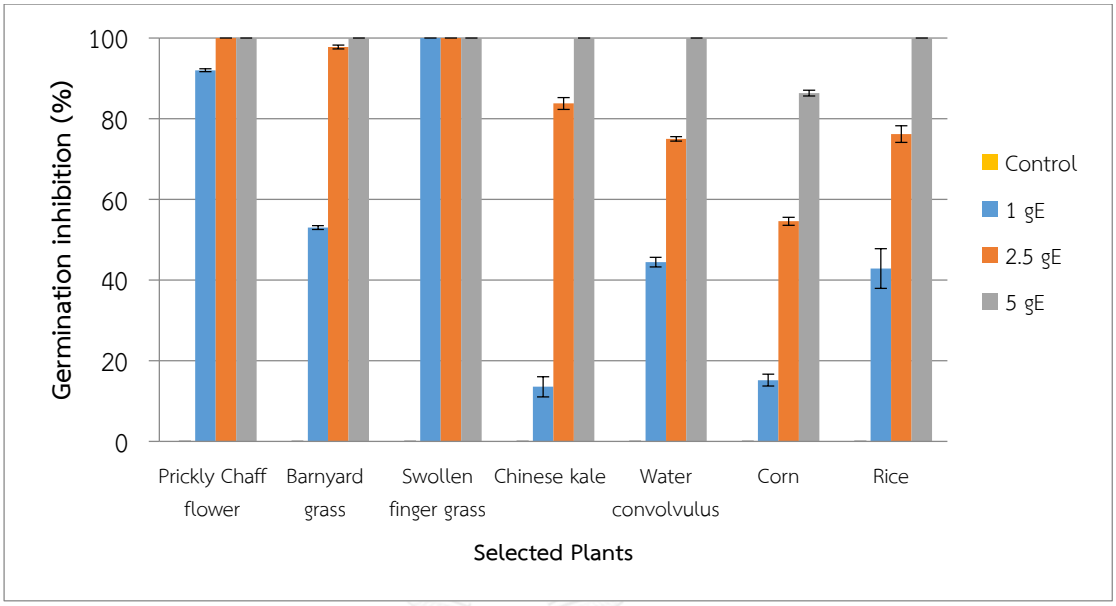


Figure 3.6 The seed germination inhibition of the CH_3OH extracts of the leaves on weeds and crops

The effect of the CH_3OH extract of the leaves at 1 gE on seed germination inhibition of weeds and crops showed that swollen finger grass was the most effective plant and completely inhibited at ≥ 1 gE. Prickly chaff-flower, barnyard grass, water convolvulus, rice, Chinese kale and corn displayed %inhibition of 92, 53, 44, 43, 14 and 15, respectively at 1 gE. This allelopathic effect was further enhanced with increasing in the extract concentration. From the above results, the extract could inhibit seed germination of weeds more than crops (See also Table A4. in Appendices). Particularly, at 1 gE of the extract presented %germination inhibition of weeds more than 50% while lower than 50% in crops. Additionally, the CH_3OH extract of the leaves at 5 gE could completely inhibit seed germination of all selected plants except corn (with %inhibition of 86).

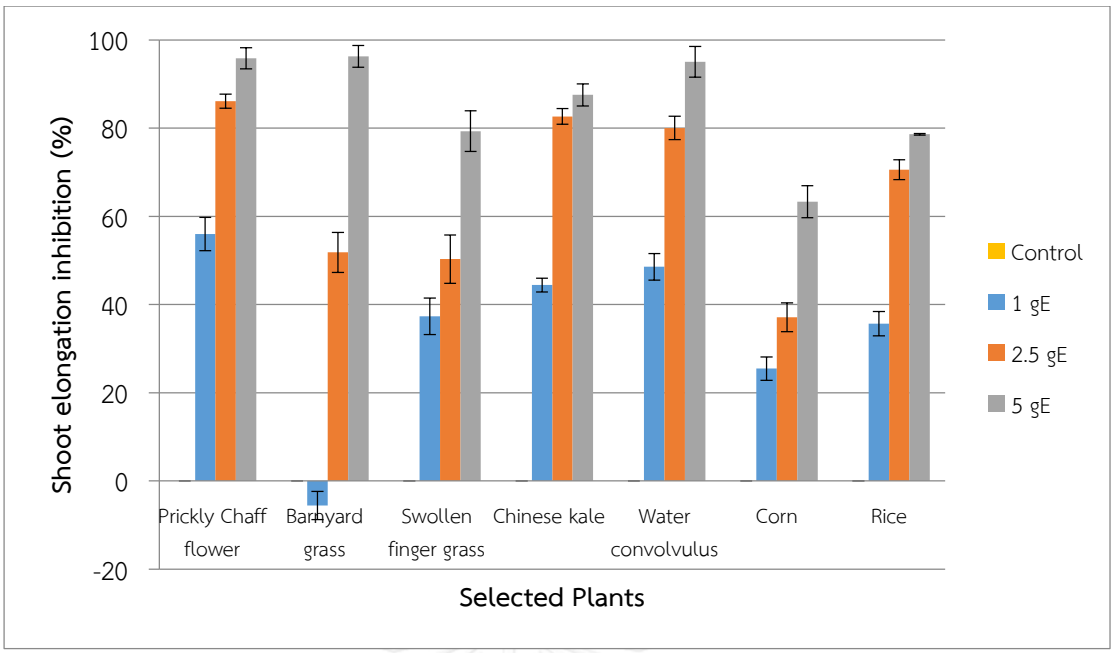


Figure 3.7 The shoot elongation inhibition of the CH_3OH extracts of the leaves on weeds and crops

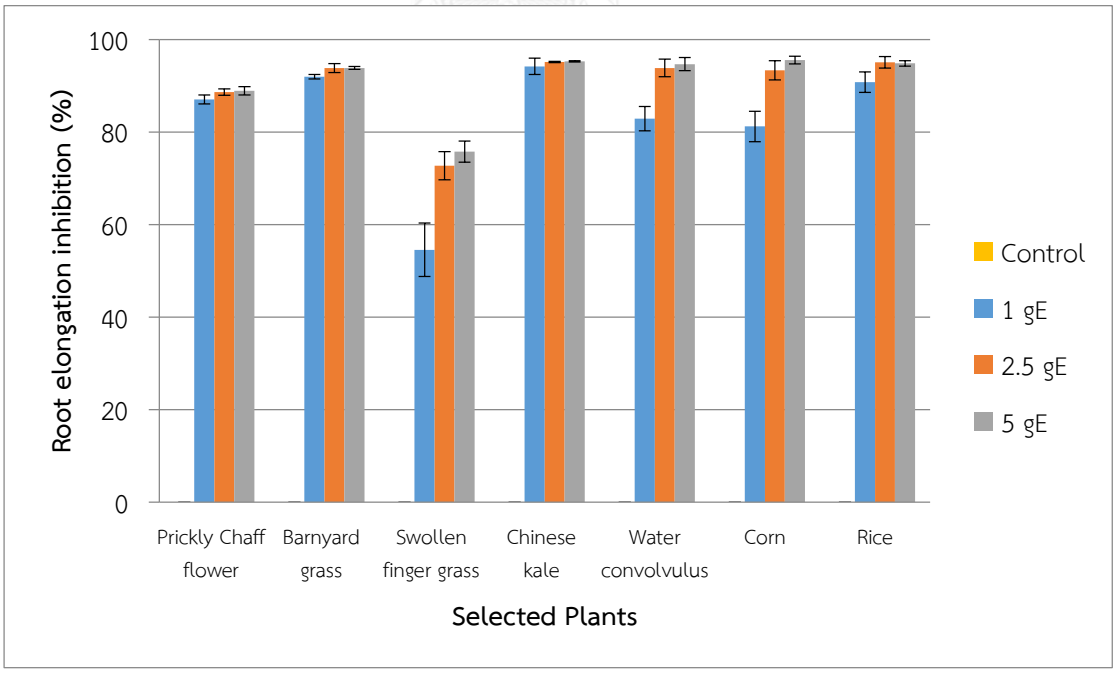


Figure 3.8 The root elongation inhibition of the CH_3OH extracts of the leaves on weeds and crops

The effects of the CH_3OH extract of the leave at three different concentrations on shoot elongation inhibition exhibited the most effectiveness on barnyard grass then prickly chaff-flower, water convolvulus, Chinese kale, swollen finger grass, rice, and corn. %Inhibition at 5 gE could be arranged as 96, 96, 95, 88, 79, 79, 63%, respectively. These inhibitory effects were enhanced by increasing the concentration of the extract. However, the use of 1 gE gave stimulated effect on shoot elongation of barnyard grass. The above results suggested that the extract be non-selective inhibition on shoot elongation between weeds and crops (See also Table A5. in Appendices).

The effect of the CH_3OH extract of the leave at 1.0, 2.5 and 5.0 gE on root elongation inhibition showed that the CH_3OH leave extract could inhibit the root elongation of all plants more than 80% at ≥ 2.5 gE except for swollen finger grass. The roots of selected plants had more effective than shoot because the roots were directly contacted with the extract. These results indicated that the extract was non-selective inhibition on root elongation between weeds and crops (See also Table A6. in Appendices).

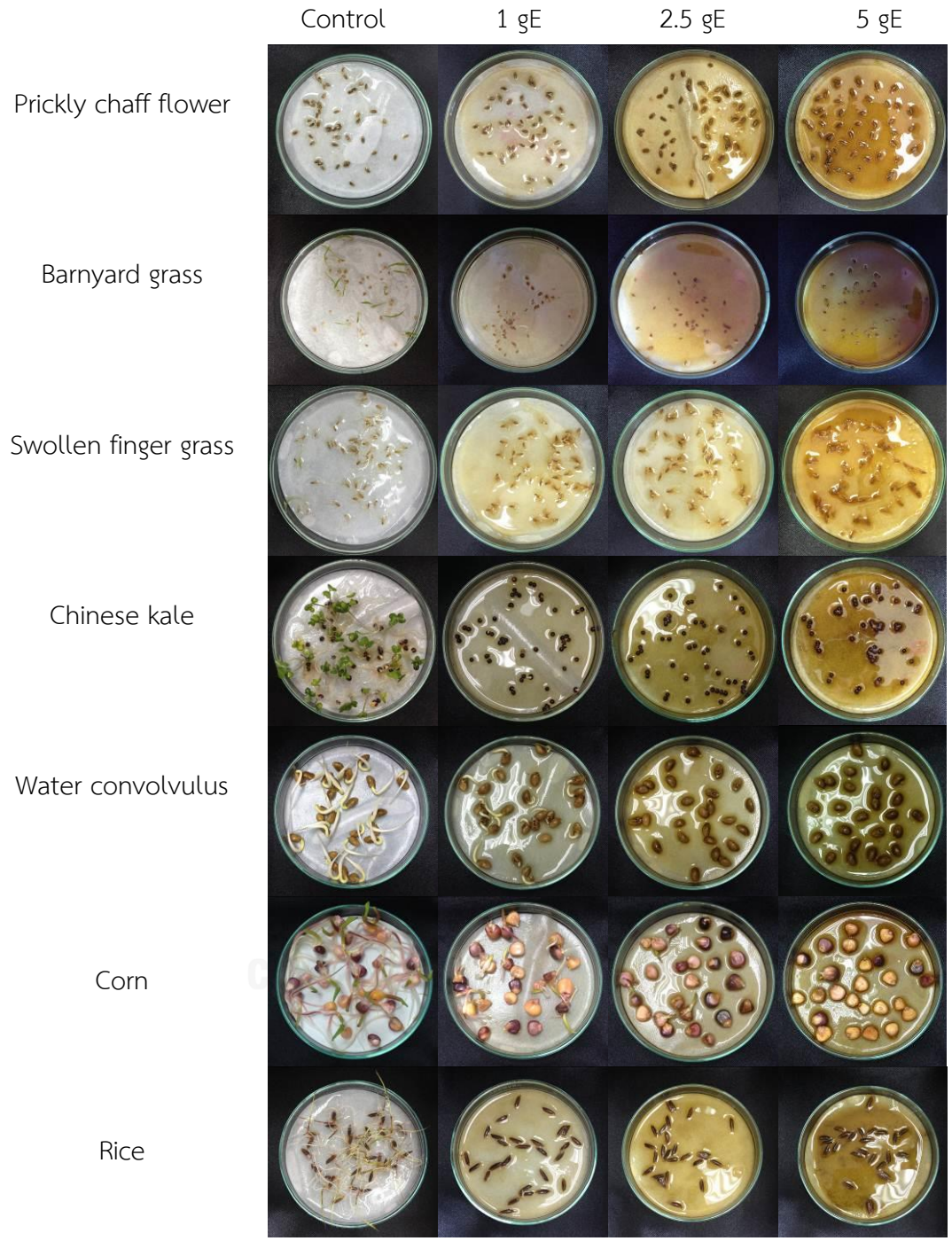


Figure 3.9 The effects of the CH_3OH extracts of the leaves on seed germination of weeds; prickly chaff flower (A), barnyard grass (B), swollen finger grass (C) and crops; Chinese kale (D), water convolvulus (E), corn (F), rice (G) at 7 DAT.

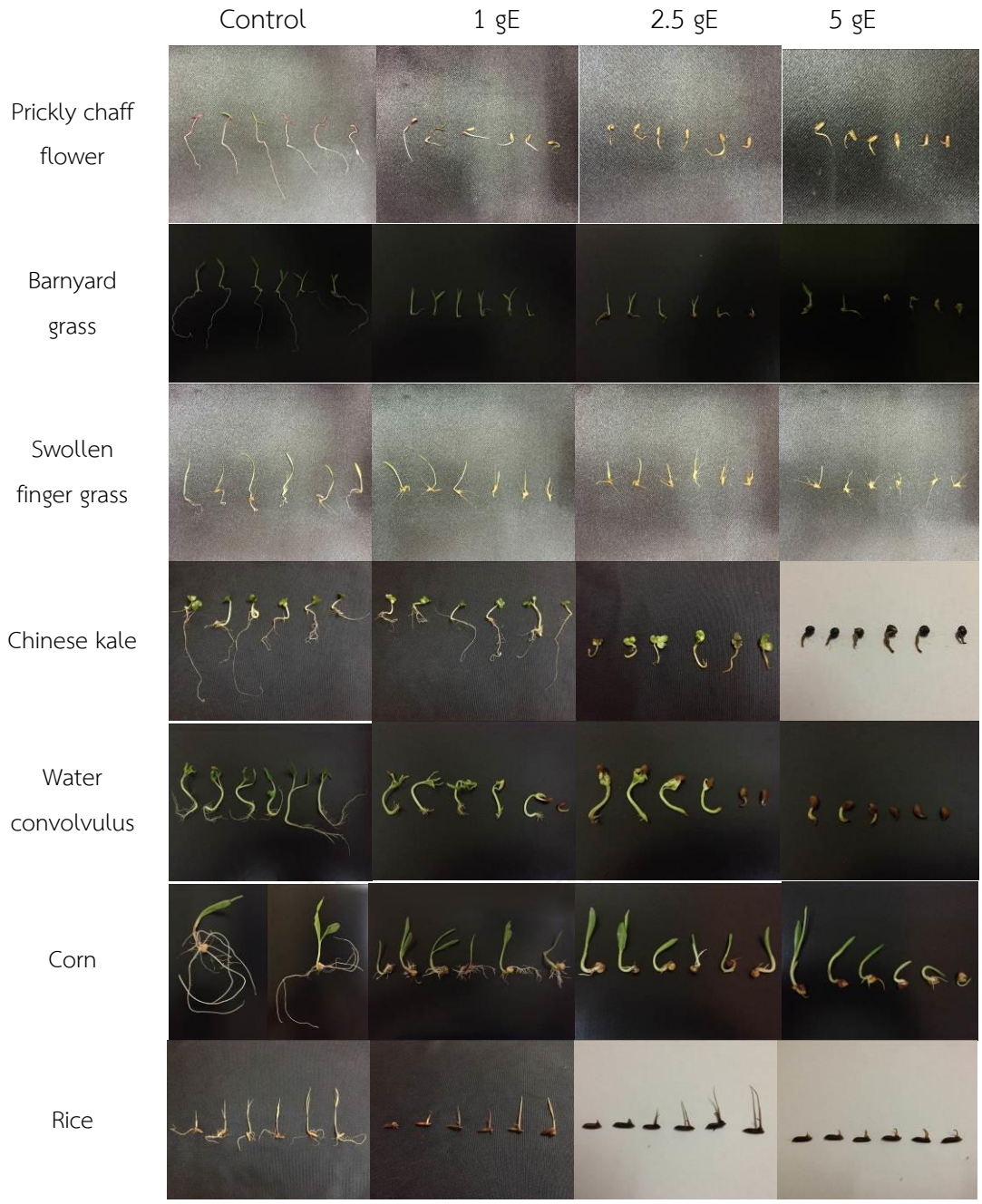


Figure 3.10 The effects of the CH_3OH extracts of the leaves on shoot and root elongation of weeds and crops at 7 DAT.

The effect of the CH_3OH extract of the leaves on seed germination and growth inhibition of weeds and crops could be demonstrated that the extract at 1 gE displayed selective inhibition on seed germination of weed more than crops while the extract was non-selective on shoot and root elongation between weeds and crops. Hanvongsa (1999) reported that the sunflower extract inhibited seed germination of crops lower than weeds, but similarly inhibited on the growth of weeds and crops. Both weeds and crops presented the growth of plants were sensitive to sunflower extract than the germination [35]. According to the above results, the CH_3OH leave extracts possessed allelopathic potential and it was a possible candidate to use as pre-emergence weed controller. However, the use of this extract in high concentration may possess as non-selective weed controller. Therefore, this extract needed for further examination in pot experiments using as pre-emergence and post-emergence controller.

3.3 Pot experiment

From previous studies, the CH_3OH extract from sunflower leave gave strong allelopathic effect. This extract was further assayed for pot experiment regarding as pre- and post-emergence weed controlling agent. The weeds studied including prickly chaff-flower (*A. aspera*), barnyard grass (*E. crus-galli*) and swollen finger grass (*C. barbata*). In addition, barnyard grass and rice were assayed together for the application in terms of pre- and post-emergence weed controller. On the other hand, dried leaves of sunflower were assayed in pot experiment as pre-emergence to explore for its application on the same weeds.

3.3.1 Effect of dried leaves as pre-emergent weed controller

Dried sunflower leaves were mixed at 1, 5, and 10% per 100 g of soil, respectively. Four selected weed seeds including giant mimosa (*M. pigra*), prickly chaff-flower (*A. aspera*), barnyard grass (*E. crus-galli*) and swollen finger grass (*C. barbata*) were planted to observe germination inhibition activity compared with the control. The results are summarized in **Figures 3.11** and **3.12.**

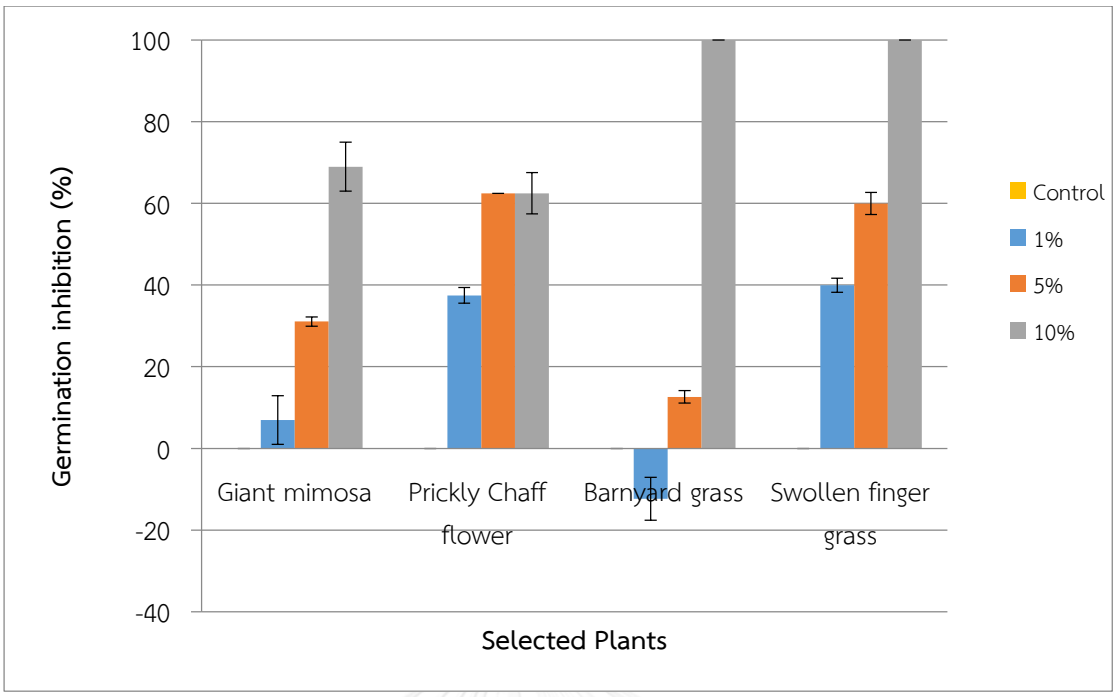


Figure 3.11 The seed germination inhibition of dried leaves as pre-emergent weed controller

The dried sunflower leaves inhibited seed germination of all weeds except for barnyard grass at 1% of dried leaves content. In case of the mean inhibition of all concentrations presented that swollen finger grass was the most sensitive plant on seed germination inhibition, followed by prickly chaff flower, giant mimosa and barnyard grass, respectively (See also Table A7. in Appendices). However, the use of 10% dried leaves could completely inhibit seed germination of barnyard grass and swollen finger grass.

The above results presented that the dried leaves of *H. annuus* showed low inhibition at 1 to 5% of dried leave content on weeds. The strong effects could be observed for dried leaves content up to 10%. However, the use of dried leaves as pre-emergence by incorporating with soil or plant litter might control weeds germination; nonetheless, it should be used with more content for more inhibition.

Figure 3.12 Effect of dried leaves as pre-emergent weed controller on seed germination inhibition of weeds at 7 DAT.

3.3.2 Effect of the CH_3OH extract of the leaves as pre-emergent weed controller

The CH_3OH extract of sunflower leaves at 1, 2.5 and 5 gE in soil 100 g was tested in pot as pre-emergence on 4 selected weeds to observe seed germination inhibition compared with the control. The results are presented in **Figures 3.13** and **3.14.**

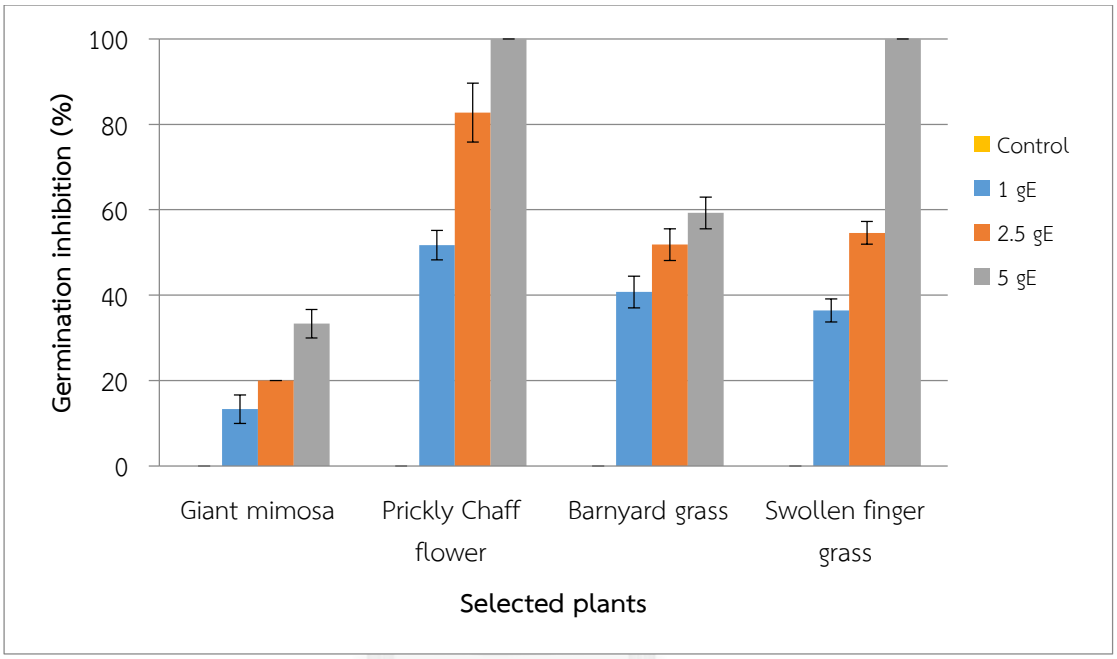


Figure 3.13 The seed germination inhibition of the CH_3OH extract of *H. annuus* leaves as pre-emergent controller on weeds

The CH_3OH extract of the leaves could inhibit seed germination of all weeds. Prickly chaff flower and swollen finger grass showed the strongest inhibition followed by barnyard grass, giant mimosa respectively. At 5 gE, completely inhibition for prickly chaff flower and swollen finger grass could be visualized. These pot experiment results gave the similar trend to petri-dish experiment.

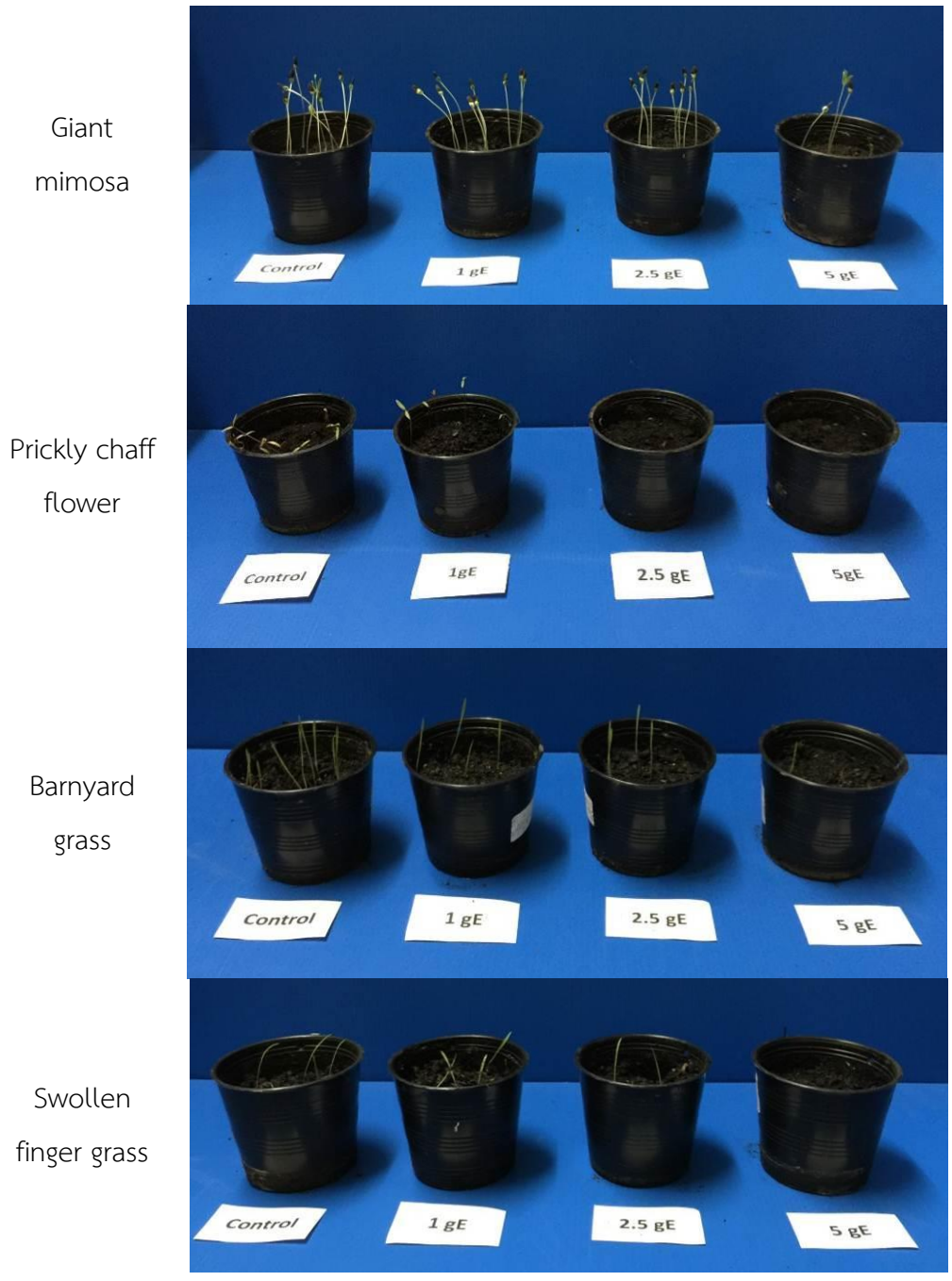


Figure 3.14 Effect of the CH_3OH extract of *H. annuus* leaves as pre-emergent control on seed germination inhibition of weeds at 7 DAT.

3.3.3 Effect of the CH_3OH extract of the leaves as post-emergent weed controller

The CH_3OH extract of the leaves at 1, 2.5 and 5 gE were examined as post-emergent weed controller by pot assay on the same four selected weeds. The shoot and root elongation inhibitions were compared with the control. The results are displayed in **Figures 3.15-3.17.**

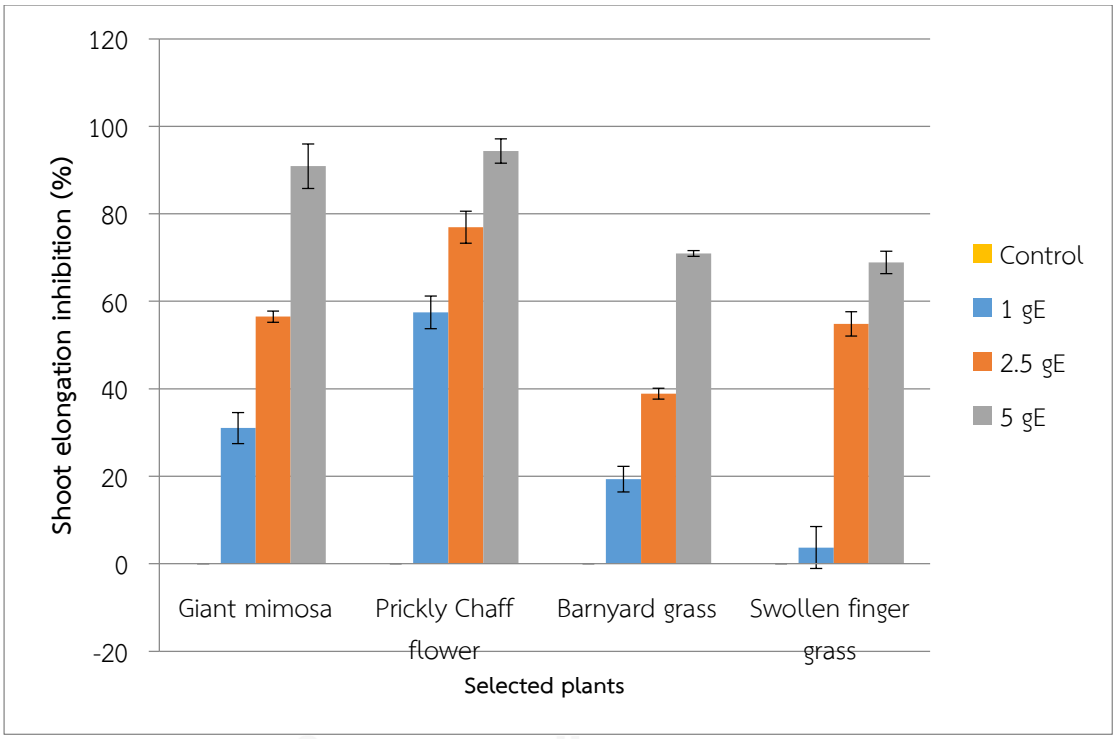


Figure 3.15 The shoot elongation inhibition of the CH_3OH extract of the leaves as post-emergent controller on weeds

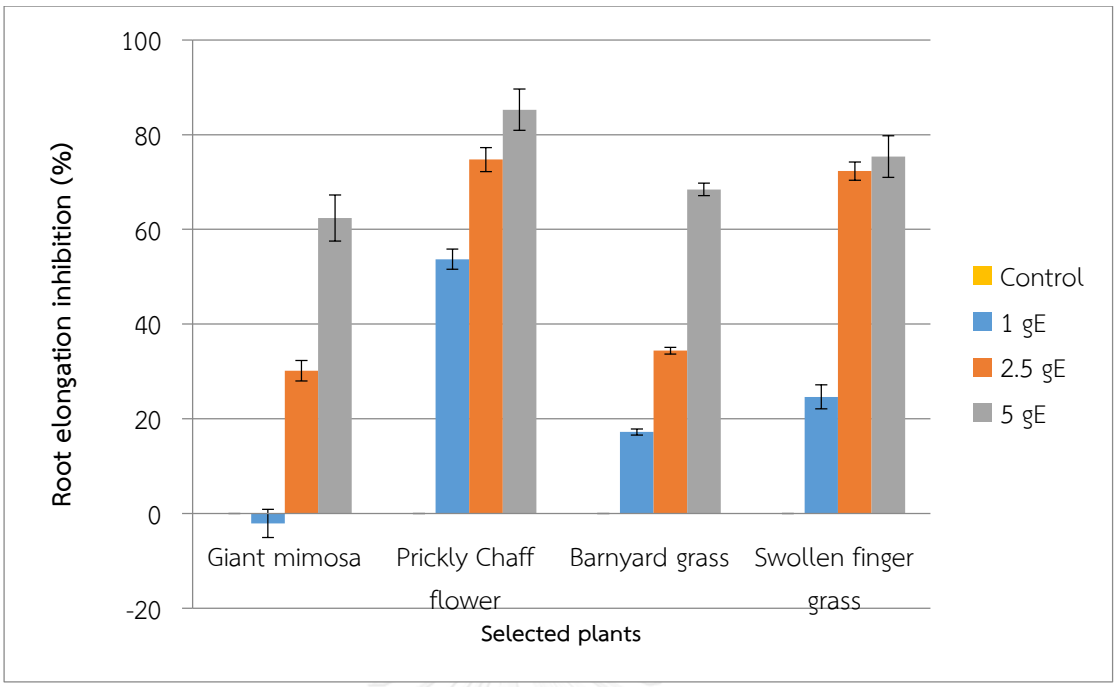


Figure 3.16 The root elongation inhibition of the CH_3OH extract of the leaves as post-emergent controller on weeds

The highest inhibition effect on the shoot elongation could clearly be observed from prickly chaff flower, followed by giant mimosa, barnyard grass and swollen finger grass, respectively. In case of monocotyledon and dicotyledon plants, it might be considered that the extract gave the trend to inhibit the shoot elongation of dicotyledon plants (giant mimosa and prickly chaff flower) more than monocotyledon (barnyard grass and swollen finger grass) (see Table A9 in Appendices). The highest inhibition effect on the root elongation could be seen from prickly chaff flower, followed by swollen finger grass, barnyard grass and giant mimosa, respectively (see Table A10 in Appendices). From the above results, it was suggested that the root elongation of the plants be sensitively more than shoot elongation and the inhibition effect was increased when the concentration increased.

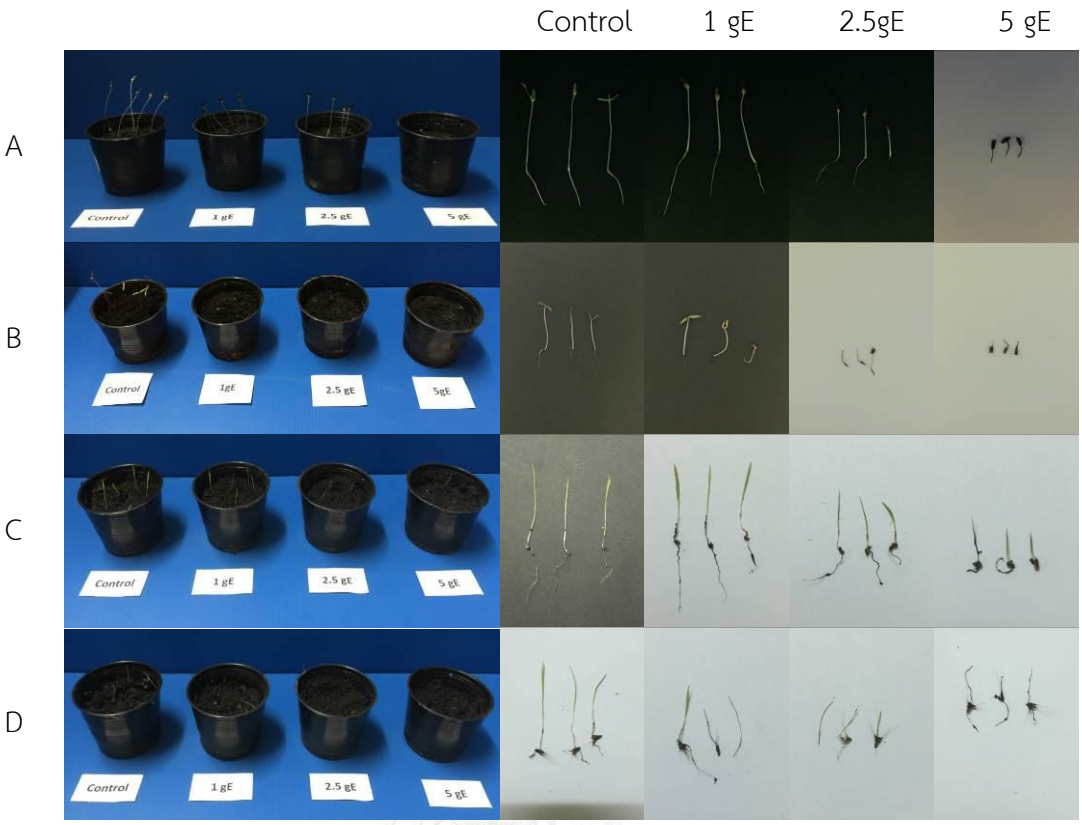


Figure 3.17 Effects of the CH_3OH extract of *H. annuus* leaves as post-emergent weed controller on root elongation inhibition of giant mimosa (A), prickly chaff flower (B), barnyard grass (C), swollen finger grass (D) at 7 DAT.

From the pot experiment result, it was revealed that the obtained results gave the similar trend to those of petri-dish. However, less effects of the CH_3OH extract of the leaves in the former case could be visualized than in the latter case. These might be because the seed/root of tested plants in petri-dish experiment were contacted and absorbed the extract directly, thus this caused strong inhibition. While in pot experiment, the extracts were treated into soil, the seed/root of tested plant might be indirectly absorbed the extract and the extracts were diluted after watering. It was thus toxic to plant in short time showing low inhibition in pot experiment. By the way, the use of the CH_3OH extract of the leaves should be studied more about its concentration towards various crops. The outcome should be able to use for selective inhibition and should be studied the effects in long term to consider the growth and productivity of the plant.

3.3.4 Effects of using the CH_3OH extract of the leaves as pre- and post- emergence for controlling barnyard grass in rice cropping

The CH_3OH extracts of the leaves at 1 and 2.5 gE were assayed as pre-emergent weed control on barnyard grass. Three days after treated, rice seeds were sown for assay side-effect on the growth of rice seedling at 7 days after sowing. On the other hand this extract was assayed as post-emergence weed control for the growth of barnyard grass and side-effect on rice seedling. %Seed germination and growth inhibition are shown and summarized in **Figures 3.18-3.20.**

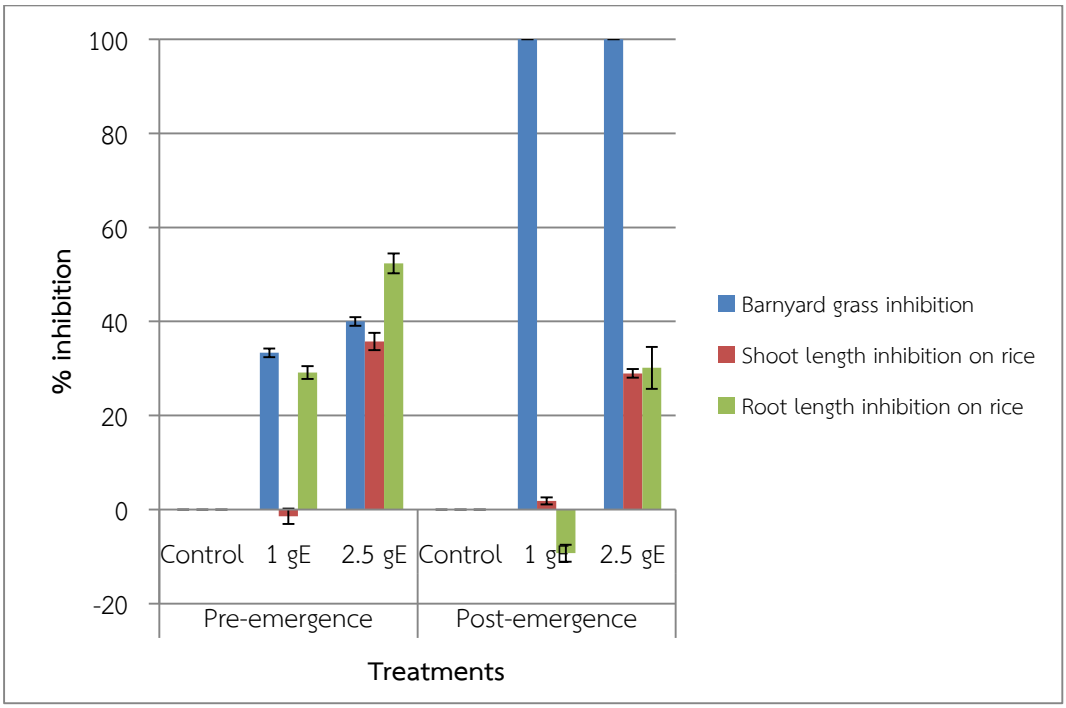
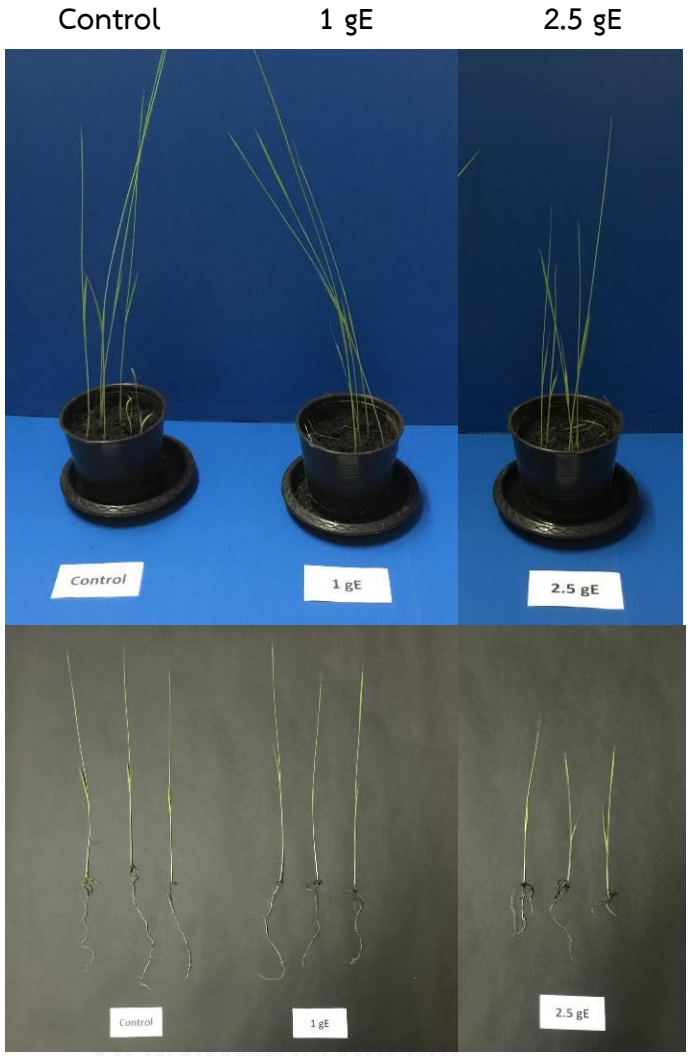


Figure 3.18 Barnyard grass inhibition and the growth inhibition of rice seedling of using CH_3OH extract of the leaves as pre- and post-emergent weed controller

Figure 3.19 Effect of using the CH_3OH extract of the leaves as pre-emergent weed controller on barnyard grass and rice; (A) growth of rice seedlings (B), growth of barnyard grass seedling at 7 DAT.



A

Figure 3.20 Effect of using the CH_3OH extract of the leaves as post-emergent weed controller on barnyard grass and rice; (A) growth of rice seedlings at 7 DAT.

The above results revealed that the use of 1 and 2.5 gE of the CH_3OH extract of the leaves as pre-emergence exhibited significant seed germination inhibition on barnyard grass compared with the control. The inhibitions were 33 and 40% at 1 and 2.5 gE, respectively and germinated seeds were inhibited the shoot and root growth by the extract. After pre-emergence, next 3 days rice seeds were sown and left for germination for 7 days. For 1 and 2.5 gE treatment, rice seeds could still completely germinate similar to that of control. The same trend could be seen from the result that 1 gE of the extract did not affect on the shoot length of rice seedlings compared

with the control. However, the roots of these seedlings were inhibited 29%. In addition, at 2.5 gE the extract could reduce shoot and root length of rice seedlings 36 and 52%, respectively.

In the case of using 1 and 2.5 gE as post-emergence, it was revealed that for both concentrations the extract became toxic to barnyard grass seedlings and completely died in 7 days. On the growth of rice seedlings, 1 gE did not affect on the shoot and root lengths while the use of 2.5 gE inhibited the shoot and root length 29 and 30%, respectively.

Barnyard grass is the most problematic weed in rice production, causing yield reduction by competing with the crops for light, nutrient, and moisture [37]. From the above results, it was demonstrated that the use of the CH_3OH extract of *H. annuus* leaves at 1 gE could control barnyard grass in rice cropping which post-emergent control. Thus, this was suitable for controlling weeds with no side-effects to crop. Although the use of 1 gE of the extract as pre-emergence inhibited the roots of rice seedlings, it might be ineffective to rice if use longer times before planting.

3.4 The effect of the CH_2Cl_2 extract of the root on the growth inhibition of selected plants

The CH_2Cl_2 extract of the root at 1 gE was bioassayed on growth inhibition of flowers including marigold (*T. erecta*), zinnia (*Z. violacea*) and cereal crops including mung bean (*V. radiata*) and rice (*O. sativa*). This experiment expected to observe the shoot elongation inhibition and ineffective or stimulate on root elongation. The results of shoot and root elongation inhibition are presented in **Figures 3.21** and **3.22**.

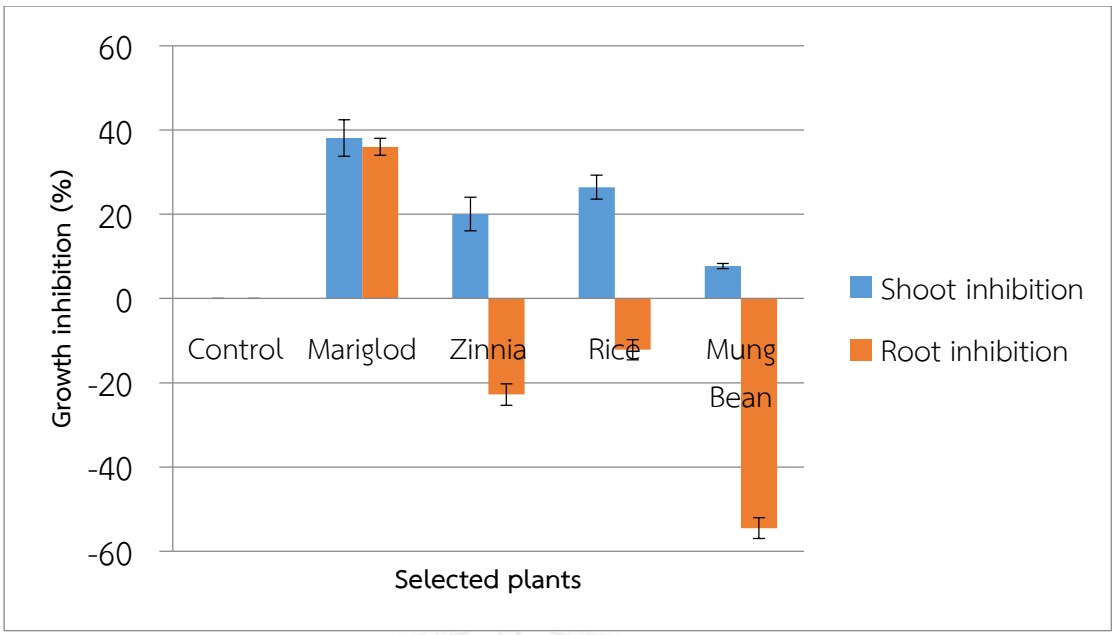


Figure 3.21 The shoot and root elongation inhibition of the CH_2Cl_2 extract of the root on selected plants

The effect of the CH_2Cl_2 extract of the roots at 1 gE on the growth of selected plant showed that the extract could inhibit shoot elongation in marigold, rice, zinnia and mung bean 38, 26, 20 and 8%, respectively. The root elongation was stimulated on mung bean (-55%), zinnia (-23%) and rice (-12%) except for marigold which inhibited the root elongation (36%). Allelopathic activity can be promoted the growth of plant at low concentration; however, suppress the growth if applied at high concentration [38]. These stimulated effects on root might be beneficial to the selected plants because the root can absorb nutrient and water better and the plant can tolerate drought well. From these results, it was suggested that the extract at 1 gE be selective inhibition on shoot elongation of rice, mung bean and zinnia except for marigold. However, more study should be carried on for the use of the extract under this condition for other plants to search for appropriate concentrations that selective inhibition on shoot elongation and ineffective or stimulate in root elongation.

The use of plant growth regulators in agriculture has been widely used for controlling the crop growth. This manner was benefit for cereal crop, pot crop, fruit crop even in vegetable. Synthesized substances have been frequently used with better

effectiveness than natural substances [39]. From the above results, it might be possible to use this extract as natural plant growth regulator to reduce the use of synthesized compounds for safer agro-ecosystems, reduced cost and sustainable agriculture.

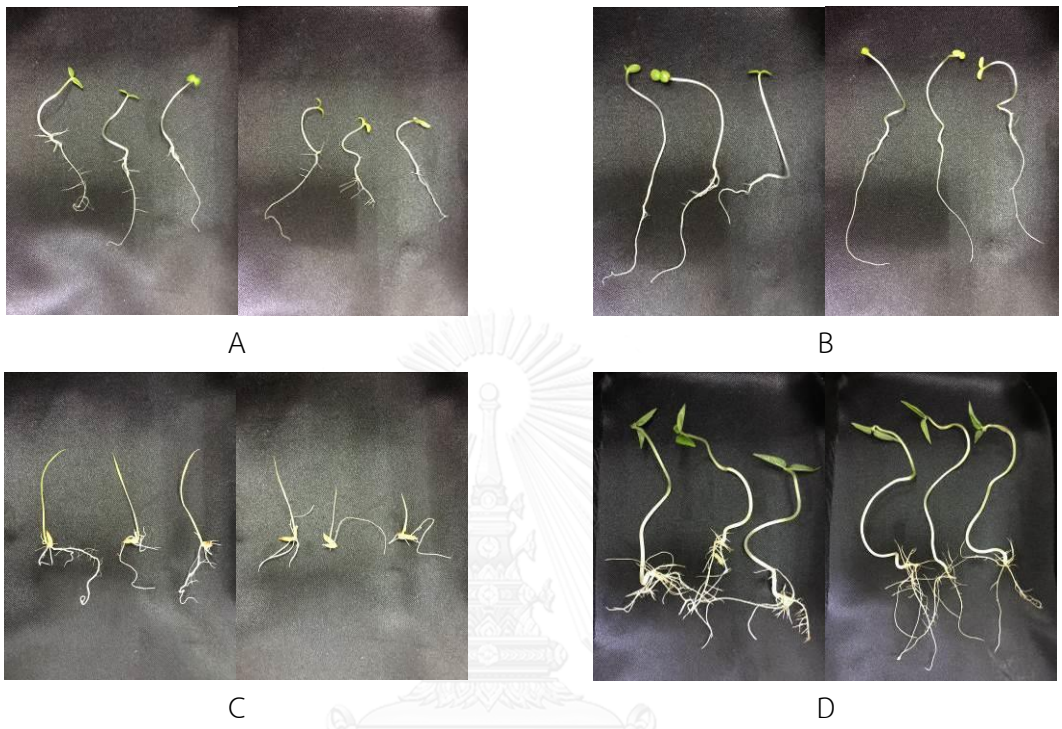


Figure 3.22 Effect of the CH_2Cl_2 extract of the root on shoot and root elongation of marigold (A), zinnia (B), rice (C) and mung bean (D) at 7 DAT.

3.5 Bioassay results of other biological activities

Various crude extracts of *H. annuus* were screened for other activities for more utilization including antifeedant activity on common cutworm (*Spodoptera litura* Fabricius): the insect damage flower and leaves of plants in Brassicaceae family; antibacterial activity on gram positive bacteria including *Staphylococcus aureus* ATCC 25923 which was pathogenic bacteria causing food poisoning in human, *Streptococcus mutans* ATCC 25175 and *Streptococcus sobrinus* KCCM 11898: the oral cavity bacteria in human beings. A selected gram negative bacterium, *Escherichia coli* ATCC 553 which was pathogenic bacteria causing food poisoning. In addition, two plant pathogenic bacteria, namely *Xanthomonas oryzae* pv. oryzae TB0006 and pv. oryzicola TS8203

causing bacterial leaf blight and bacterial leaf streak disease in rice and antifungal activity on plant pathology fungal of seed rot, root rot (*Rhizoctonia solani* DOAC 1406) and damping off, root rot and stem rot (*Phytophthora parasitica* DOAC 2052).

3.5.1 Antifeedant activity on common cutworm (*Spodoptera litura*)

All crude extracts including hexane, CH_2Cl_2 and CH_3OH from *H. annuus* leaves, stems and roots at 0.25% w/w were tested on antifeedant activity on two-stage larvae of common cutworms. The results are summarized in **Table 3.2**.

Table 3.2 Antifeedant activity of crude extracts from *H. annuus* on common cutworm

Parts	% Antifeedant		
	Hexane extract	CH_2Cl_2 extract	CH_3OH extract
Leave	63.01±8.86	55.86±10.28	55.28±11.15
Stem	-	25.34±10.27	36.43±10.31
Root	-	55.94±10.48	51.32±7.73

Values are given as mean ± SD of duplication experiment

From the above results, it was revealed that the leave extract gave better result than those of root and stem. The hexane extract of the leaves provided the highest effect on feeding deterrence of *S. litura* 63%. The leave and root extracts showed similar inhibitory effect more than 50%, while the stem extract gave low activity. Plants in Asteraceae family are known for their content in diterpenes and sesquiterpenes. Sesquiterpenes have been reported to serve as toxic or feeding deterrents to herbivore insects. Among diterpenes, clerodanes are a large chemical group and a rich source of natural insect antifeedants and attractants [40]. Phumnuan and Teerarak (2012) reported antifeedant activity of *T. erecta* against Diamondback Moth Larvae (*P. xylostella*). The root and leave extracts at concentration of 8% (w/v) gave strongly effective for antifeedant, which showed percentage of leaf damage area as 100% within 24 h [41].

3.5.2 Antibacterial activity

The hexane, CH_2Cl_2 and CH_3OH extracts from different plant parts were tested for antibacterial activity using agar diffusion method against three gram-positive bacteria including *Staphylococcus aureus*, *Streptococcus mutans* and *S. sobrinus* and three gram-negative bacteria including *Escherichia coli*, *Xanthomonas oryzae* pv. oryzae and pv. oryzicola at 10,000 ppm. The diameter of the inhibition zone was evaluated as shown in **Table 3.3** and **Figure 3.23**.

Table 3.3 Antibacterial activity of *H. annuus* extracts at 10,000 ppm on the growth of selected bacteria

Parts	Inhibition zones diameter (mm) of crude extracts[a]					
	S. aureus	*S. mutans*	*S. sobrinus*	*E. coli*	*X. oryzae* pv. oryzae	*X. oryzae* pv. oryzicola
Leave hexane extract	NA	NA	8.0±1.4	NA	NA	NA
Leave CH_2Cl_2 extract	NA	NA	7.0±0.7	NA	NA	NA
Leave CH_3OH extract	NA	NA	7.5±0.7	NA	NA	NA
Stem CH_2Cl_2 extract	6.5±0.7	NA	8.0±0.1	NA	NA	NA
Stem CH_3OH extract	NA	NA	NA	NA	NA	NA
Root CH_2Cl_2 extract	7.0±1.4	NA	8.5±0.1	NA	NA	NA
Root CH_3OH extract	6.5±0.7	NA	7.0±0.1	NA	NA	NA

Values are given as mean ± SD of duplication experiment, NA = not active

[a]Diameter of inhibition zones of *H. annuus* extracts including diameter of agar well (6 mm)

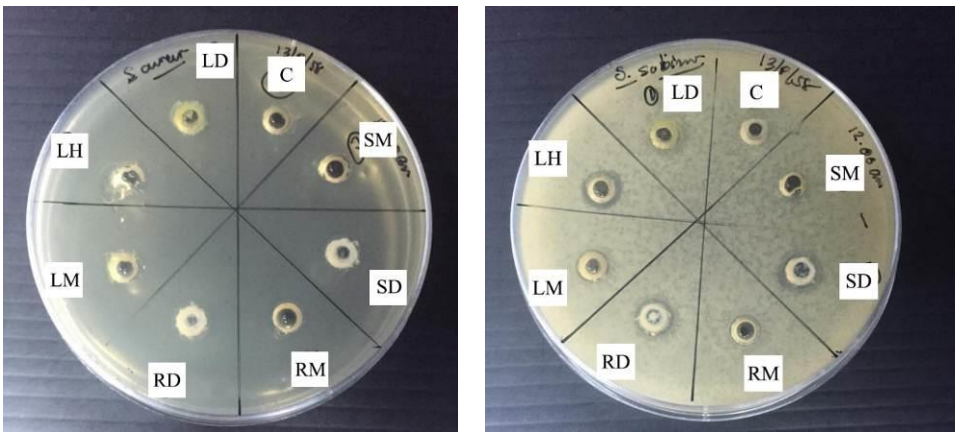


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Figure 3.23 Effect of crude extracts of *H. annuus*; leave hexane (LH), leave CH_2Cl_2 (LD), leave CH_3OH (LM), stem CH_2Cl_2 (SD), stem CH_3OH (SM), root CH_2Cl_2 (RD), root CH_3OH (RM) on antibacterial activity against *S. aureus* (I) and *S. sobrinus* (II) compared with control (C).

From the above results, the extracts of sunflower did not affect on gram negative bacteria, while presented inhibition effect in gram positive bacteria. Among the extracts studied, that derived from the roots gave 14% inhibition against *S. aureus*. For *S. sobrinus*, the CH_2Cl_2 extract of the root still revealed the strongest inhibition (29%), followed by the hexane extract of the leave (25%), the CH_2Cl_2 extract of the stem (25%), the CH_3OH extract of the leave (20%), the CH_2Cl_2 extract of the leave (14%), the CH_3OH extract of the root (14%), respectively while the CH_3OH extract of the stem was inactive on the growth inhibition of *S. sobrinus*. (see also Table A13 in Appendices). From literature review, the seeds and leaves of *H. annuus* were studied for antimicrobial activity. Active chemical compositions of the leaves extract were identified as *iso*-chlorogenic and chlorogenic acids which inhibited the growth of nitrogen fixing and nitrifying bacteria [42]. Aboki *et al.* (2012) reported that sunflower seed oil was effective on some microorganisms such as *S. aureus*, *E. coli*, *B. subtilis* and *C. albicans* [43]. Similarly in (2012) Subashini and Rakshitha reported that the seed extracts of sunflower showed high sensitivity to *S. typhi*, moderate sensitivity to *S. aureus* and *V. cholera* and less sensitivity to *B. subtilis* [44].

3.5.3 Antifungal activity

Each 1,000 ppm of crude sunflower extracts (hexane, CH_2Cl_2 and CH_3OH extracts) from different plant parts were assayed on PDA media and carrot agar for growth inhibition of *Rhizoctonia solani* and *Phytophthora parasitica* at final concentration of 1,000 ppm by agar incorporation method. The antifungal activity was depicted as shown in **Table 3.4** and **Figure 3.24**.

Table 3.4 Antifungal activity of *H. annuus* extracts on the growth of *R. solani* and *P. parasitica*

Parts	*R. solani*	*P. parasitica*
	%inhibition[a]	%inhibition[a]
Leave hexane extract	41.1±2.2	26.7±3.4
Leave CH_2Cl_2 extract	51.8±0.6	16.3±1.7
Leave CH_3OH extract	45.9±0.6	22.2±4.3
Stem CH_2Cl_2 extract	63.0±2.8	30.0±4.4
Stem CH_3OH extract	31.5±4.6	10.0±1.1
Root CH_2Cl_2 extract	78.9±1.1	33.7±0.6
Root CH_3OH extract	45.9±5.0	20.4±1.7

[a] Values, an average ± standard deviation of 3 replicates of the mean growth inhibition of fungi species

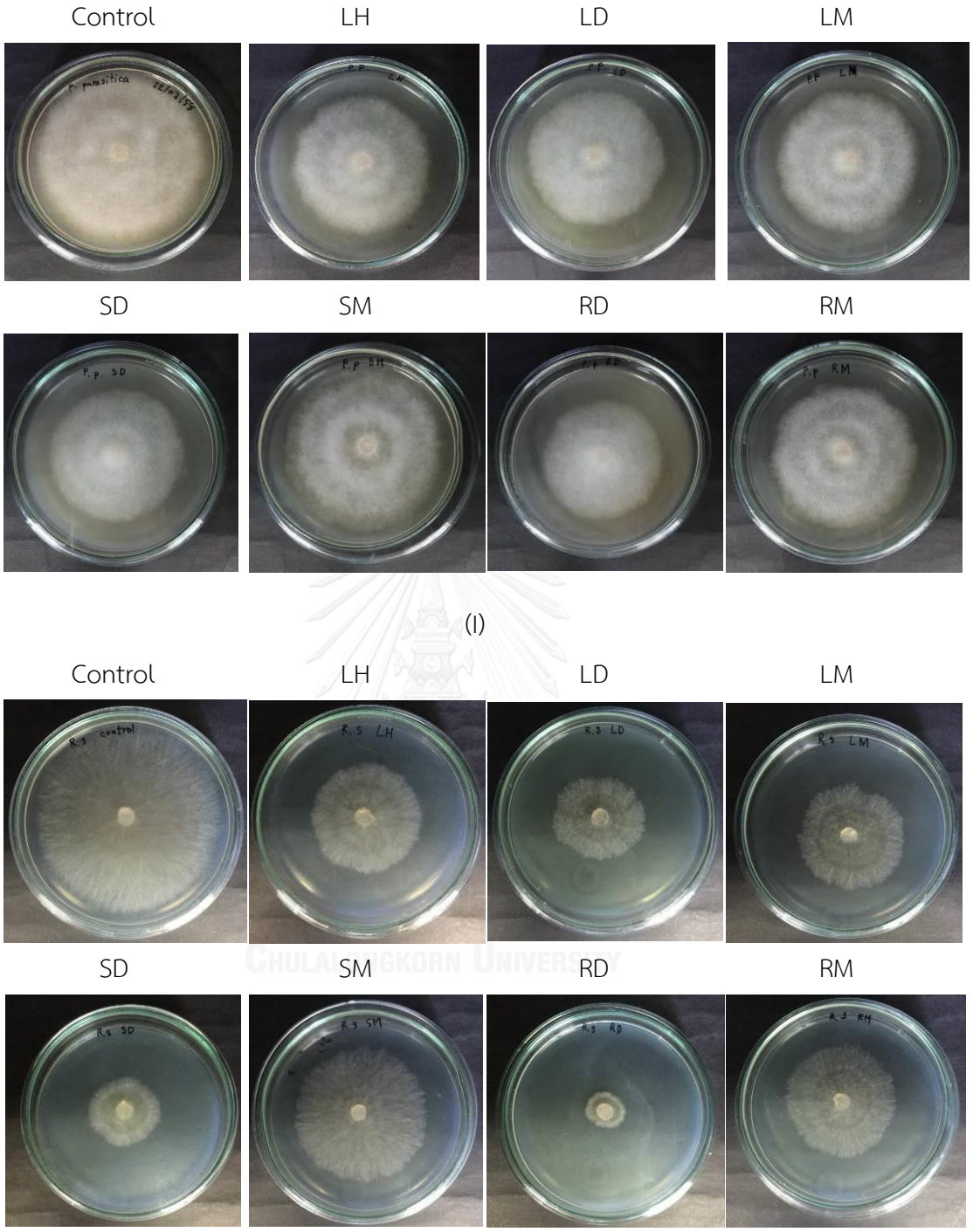


Figure 3.24 Effect of crude extracts of *H. annuus*; leaves hexane (LH), leaves CH_2Cl_2 (LD), leaves CH_3OH (LM), stem CH_2Cl_2 (SD), stem CH_3OH (SM), root CH_2Cl_2 (RD), root CH_3OH (RM) on antifungal activity of *P. parasitica* (I) and *R. solani* (II).

The results displayed that the CH_2Cl_2 extract of the roots exhibited the highest effective antifungal activity against *R. solani* (79% inhibition) more than the CH_2Cl_2 extract of the stems (63%), the CH_2Cl_2 extract of the leaves (52%), the CH_3OH extract of the leaves (46%), the CH_3OH extract of the roots (46%), the hexane extract of the leaves (41%) and the CH_3OH extract of the stems (32%), respectively. The CH_2Cl_2 extract of the roots also represented the highest activity against *P. parasitica* (33%), followed by the CH_2Cl_2 extract of the stems (30%), the hexane extracts of the leaves (27%), the CH_3OH extract of the leaves (22%), the CH_3OH extract of the roots (20%), the CH_2Cl_2 extract of the leaves (16%) and CH_3OH extract of the stems (10%), respectively. From the data present, the inhibition effect of the root extract displayed more potent activity than the stems and leaves. In addition the CH_2Cl_2 extracts presented more potent activity than those derived from CH_3OH and hexane fractions, except for the leave extract against *P. parasitica*. Yavuz and Arslan (2013) reported that the ethanol extract of sunflower leaves at 10% concentration (w/v) presented the highest antifungal effect on mycelial growth of *R. solani* [45]. Qasem and AbuBlan (1996) indicated that the difference in fungitoxicity of extracts of the same plant may also be due to the presence of inhibitors to the fungitoxic principles [46].

3.6 Separation of the CH_2Cl_2 extract of the root

Based on biological studies, the CH_2Cl_2 extract of *H. annuus* roots was interesting for high inhibition on plants shoot elongation and high toxicity on microorganisms. There were a few reports on their chemical composition. It is thus rationalized to search for chemical constituent of this extract.

3.6.1 Fractionation of the CH_2Cl_2 extract of the root

The CH_2Cl_2 extract of the roots 80 g was separated by quick column chromatography on silica gel (No.7729). The column was eluted using gradient solvent starting form hexane and increasing polarity by mixing with EtOAc and CH_3OH. Each

fraction was collected and combined according to the TLC results to obtain five fractions, HAR-D1 – HAR-D5. The results of fractionation are presented in **Table 3.5**.

Table 3.5 The fractionation the CH_2Cl_2 extract of the roots of *H. annuus* by quick column chromatography

Fraction Code.	Solvent system	Weight (g)	Remarks
HAR-D1	100% haxane	9.98	Yellow liquid
HAR-D2	5% EtOAc in hexane	28.71	Yellow oil, wax
HAR-D3	10 – 40% EtOAc in hexane	11.64	Yellow wax
HAR-D4	10% EtOAc in hexane	19.57	Dark green solid
HAR-D5	40% EtOAc in hexane - 5% CH_3OH in EtOAc	13.41	Dark green solid

Five fractions were obtained by quick column chromatography. HAR-D2 gave the highest yield as 28.71 g. The TLC possessed that the major constituents were mainly in HAR-D2. Thus these fractions were selected for further purification.

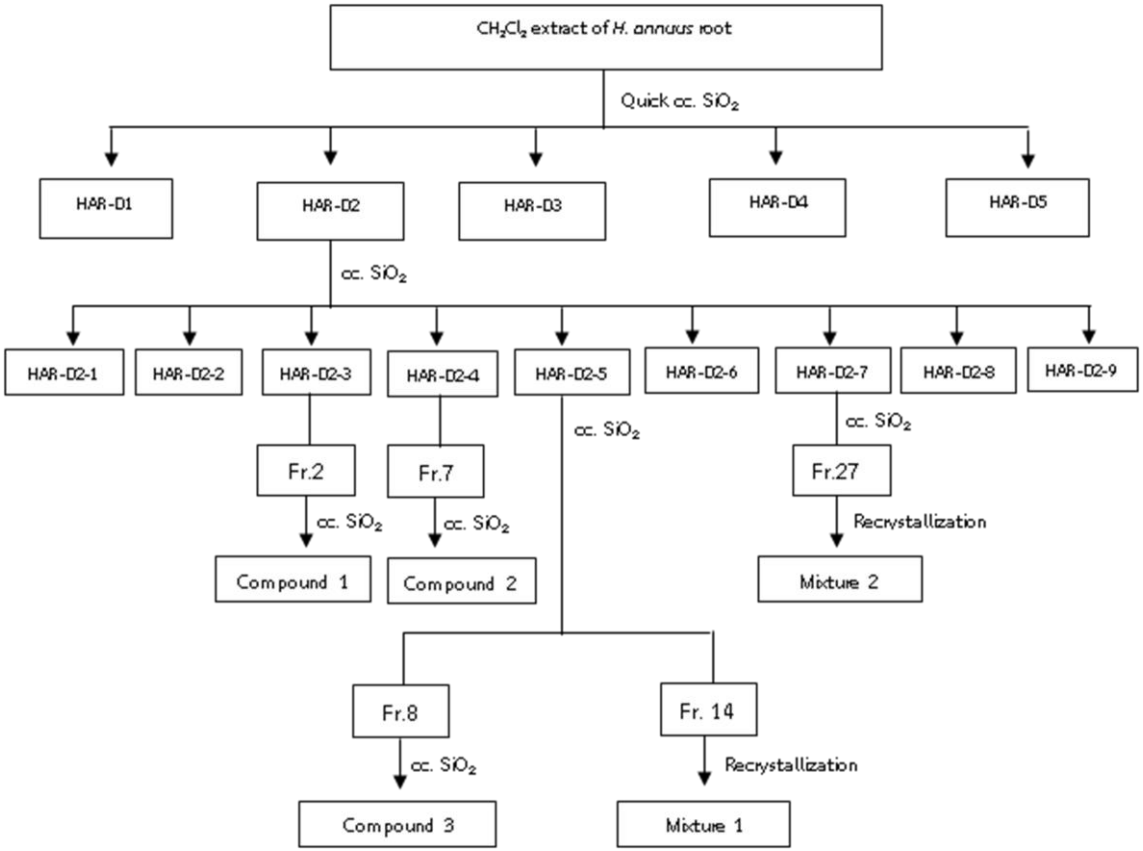
3.7 Separation and structural elucidation of isolated compounds

HAR-D2 (26 g) was further separated by column chromatography (Silica gel No. 7734) using hexane–EtOAc as eluents to yield 9 fractions (HAR-D2-1 to HAR-D2-9). The results of fractionation are displayed in **Table 3.6**.

Table 3.6 The fractionation of HAR-D2 by silica gel column

Fraction Code.	Solvent system	Weight (g)	Remarks
HAR-D2-1	100% haxane	0.14	Colorless liquid
HAR-D2-2	100% hexane – 5% EtOAc/hexane	2.75	Green solid
HAR-D2-3	5% EtOAc/hexane	3.41	Yellow solid
HAR-D2-4	5% EtOAc/hexane	2.14	Yellow liquid
HAR-D2-5	5% EtOAc/hexane	1.85	Yellow liquid
HAR-D2-6	5% EtOAc/hexane – 10% EtOAc/hexane	1.87	yellow liquid
HAR-D2-7	10% EtOAc/hexane	2.51	Yellow liquid
HAR-D2-8	10% EtOAc/hexane – 20%EtOAc/hexane	1.17	Dark yellow liquid
HAR-D2-9	20% EtOAc/hexane – 100%EtOAc	1.86	Red brown liquid

After further separation, 3 compounds and 2 mixtures were obtained. Compound **1** (234 mg) from HAR-D2-3, compound **2** (12 mg) from HAR-D2-4, compound **3** (16 mg), mixture **1** (105 mg) from HAR-D2-5, and mixture **2** (237 mg) from HAR-D2-7 were isolated. Compounds **1**, **2** and mixture **1** and **2** were determined as major compounds of the CH_2Cl_2 extract of the root. All isolated compounds were identified by comparison of their ^{1}H, ^{13}C NMR and TLC with the corresponding authentic samples or literature data. The isolation procedures are summarized as shown in **Scheme 3.1**.



Scheme 3.1 The isolation procedure of the CH_2Cl_2 extract of *H. annuus* roots

3.7.1 Compound 1: Demethylencecalin

Compound **1** was isolated by column chromatography to give yellow needle, 234 mg (0.9 %yield). This compound showed a single spot on TLC with R_f 0.60 (solvent: 100% CH_2Cl_2). The ^1H and ^{13}C NMR are shown in **Figures 3.25** and **3.26**.

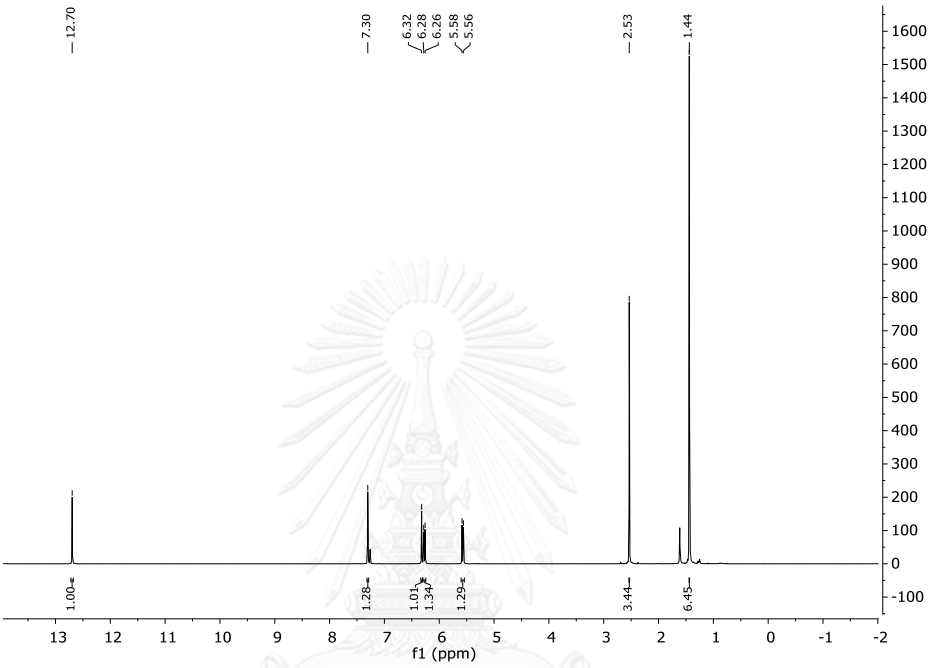


Figure 3.25 The ^1H NMR spectrum ($CDCl_3$) of compound **1**

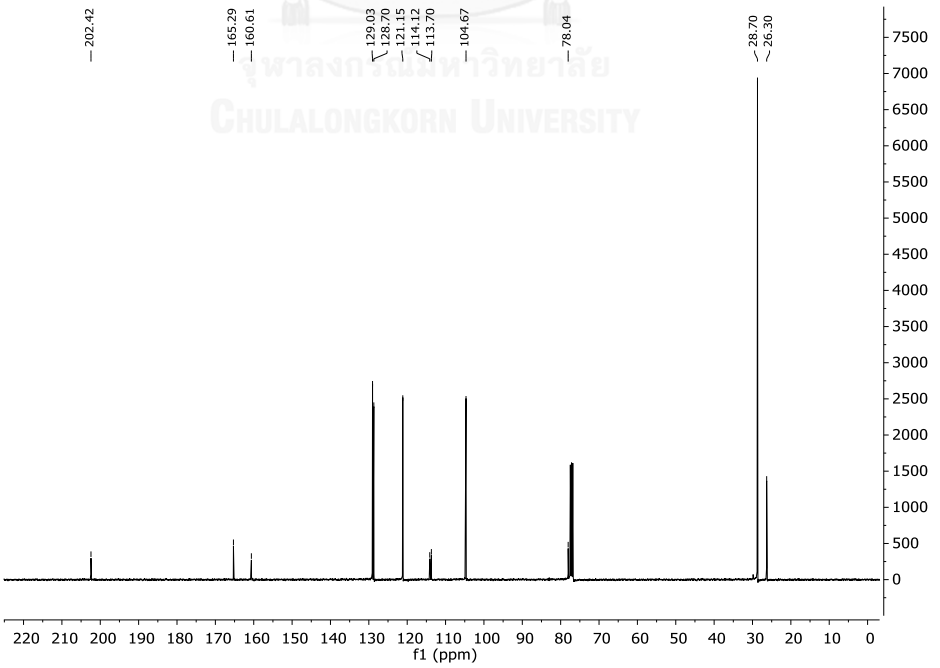
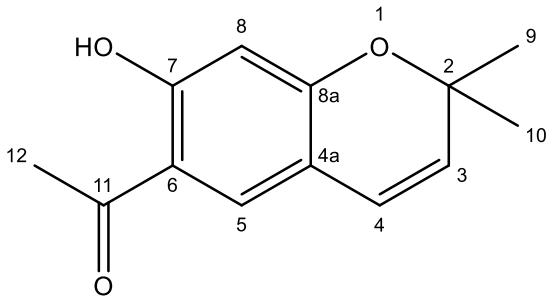


Figure 3.26 The ^{13}C NMR spectrum ($CDCl_3$) of compound **1**

By comparison of spectroscopic data of compound **1** with that published in the literature [47], this compound was designated as demethylencecalin. The ^{1}H and ^{13}C NMR ($CDCl_3$) NMR spectral assignments are compared as displayed in **Table 3.7**.

Table 3.7 The ^{1}H and ^{13}C spectral data assignment of demethylencecalin and compound **1** (in $CDCl_3$)

Position	Chemical shift (ppm)			
	Demethylencecalin		Compound **1**	
	^{1}H	^{13}C	^{1}H	^{13}C
2		77.9		78.0
3	5.58 (d, *J* = 9.9 Hz, 1H)	128.9	5.57 (d, *J* = 9.9 Hz, 1H)	129.0
4	6.28 (d, *J* = 9.9 Hz, 1H)	121.0	6.27 (d, *J* = 9.9 Hz, 1H)	121.2
4a		113.5		113.7
5	7.31 (s, 1H)	128.5	7.30 (s, 1H)	128.7
6		113.8		114.1
7		165.2		165.3
8	6.33 (s, 1H)	104.5	6.32 (s, 1H)	104.7
8a		160.4		160.6
9,10	1.44 (s, 6H)	28.6	1.44 (s, 6H)	28.7
11		202.3		202.4
12	2.54 (s, 3H)	26.1	2.53 (s, 3H)	26.3
OH	12.70 (s, 1H)		12.70 (s, 1H)	



Demethylencecalin

The biological activity of demethylencecalin has been addressed. Merrill (1989) reported that demethylencecalin from *C. solstitialis* L. retarded seed germination and reduced the growth of weed and crop plant seedling and increased adventitious root formation of mung bean cutting [48]. Castaneda, Gomez and Mata (1996) addressed that demethylencecalin from *H. quinquenervis* root showed marginal cytotoxicity against three human tumor cell lines and inhibited the radicle growth of *A. hypochondriacus* and *E. crusgalli* [49]. This compound has also been reported in *H. annuus*; nonetheless, this is the first time to report this compound in the root part.

3.7.2 Compound 2: Demethoxyencecalin

Compound **2** (12 mg) was isolated by column chromatography from HAR-D2-4 to give yellow gum. It showed a single spot on TLC with R_f 0.48 (solvent: 100% CH_2Cl_2). The ^1H and ^{13}C NMR ($CDCl_3$) are shown in **Figures 3.27** and **3.28**.

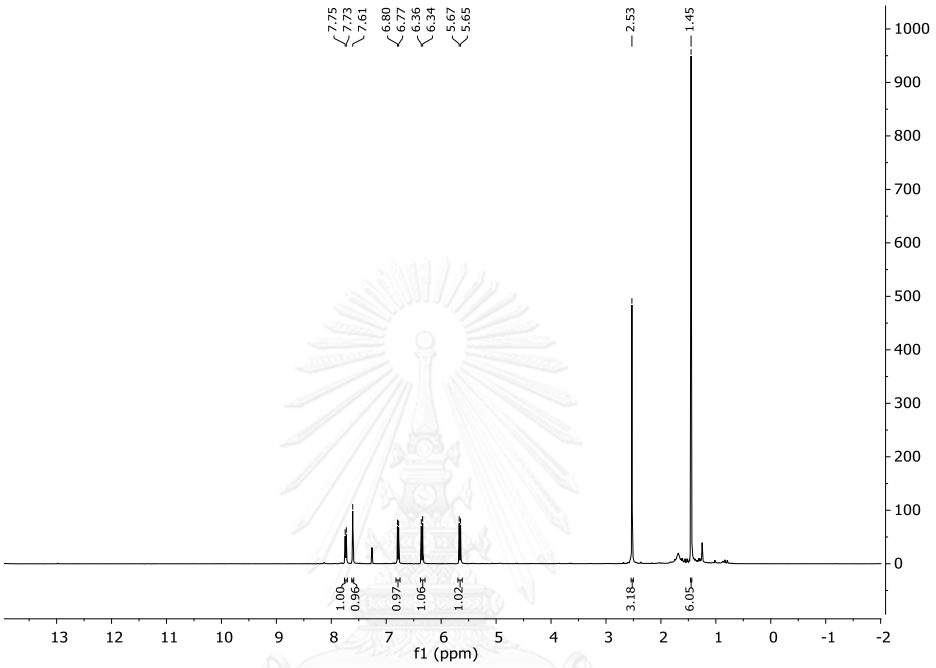


Figure 3.27 The ^1H NMR spectrum ($CDCl_3$) of compound **2**

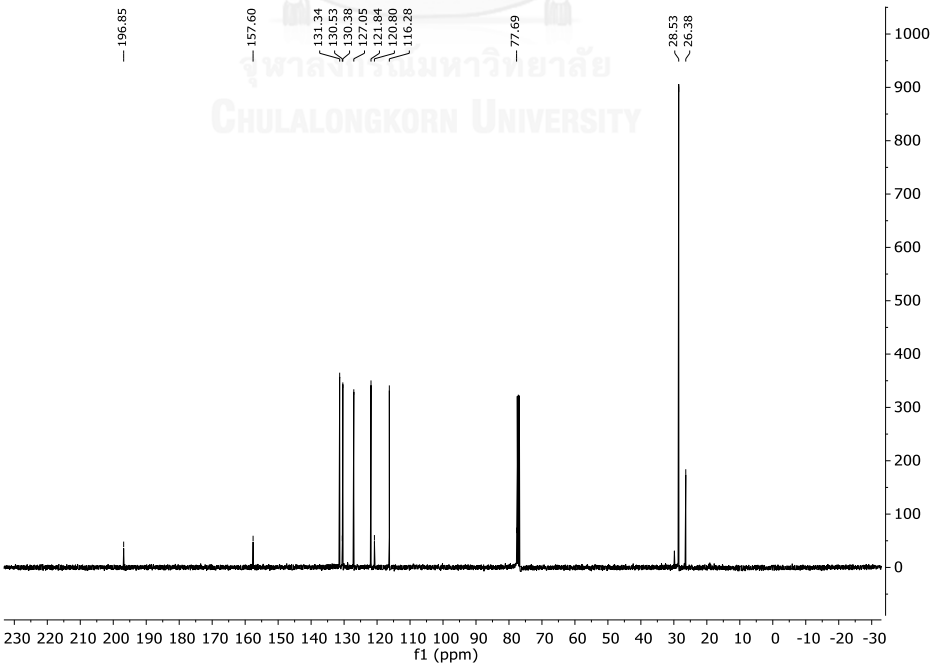
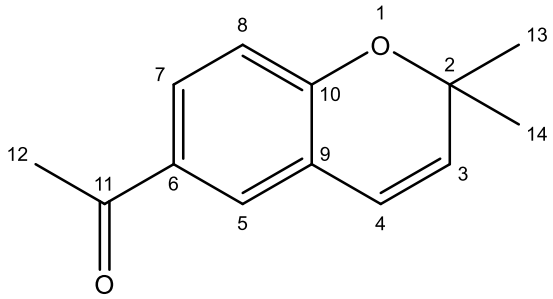


Figure 3.28 The ^{13}C NMR spectrum ($CDCl_3$) of compound **2**

The spectroscopic data of compound **2** was compared with the published in the literature [50]. Compound **2** was designated as demethoxyencecalin. The ^{1}H and ^{13}C NMR ($CDCl_3$) spectral assignment are compared as presented in **Table 3.8.**

Table 3.8 The ^{1}H and ^{13}C spectral data assignment of demethoxyencecalin and compound **2** (in $CDCl_3$)

Position	Chemical shift (ppm)			
	Demethoxyencecalin		Compound **2**	
	^{1}H	^{13}C	^{1}H	^{13}C
2		77.6		77.7
3	5.67 (d, 1H)	127.0	5.66 (d, *J* = 9.9 Hz, 1H)	127.1
4	6.36 (d, 1H)	121.7	6.35 (d, *J* = 9.9 Hz, 1H)	121.8
5	7.62 (d, 1H)	130.3	7.61 (s, 1H)	130.4
6		130.5		130.5
7	7.74 (dd, 1H)	131.2	7.74 (d, *J* = 8.4 Hz, 1H)	131.3
8	6.79 (d, 1H)	116.6	6.79 (d, *J* = 8.4 Hz, 1H)	116.3
9		120.7		120.8
10		157.4		157.6
11	2.52 (s, 3H)	196.5	2.53 (s, 3H)	196.9
12		26.2		26.4
13,14	1.45 (s, 6H)	28.4	1.45 (s, 6H)	28.5



Demethoxyencecalin

From reported literatures, Satoh *et al*. (1995) reported that demethylencecalin and demethoxyencecalin from sunflower receptacles expressed antifungal activity on *Pyricularia oryzae* [51]. Similarly in 2007, Prats *et al.* reported demethoxyencecalin from sunflower bracts revealed antifungal activity on *Sclerotinia sclerotiorum* [52].

3.7.3 Compound 3: 10(14)-Guaiadien-12,6-olide (Mokko lactone)

This compound was isolated from HAR-D2-5 by column chromatography, compound **3** (16 mg), white powder was obtained. It revealed a single spot on TLC with R_f 0.50 (solvent: 100% CH_2Cl_2). The ^1H and ^{13}C NMR spectrum ($CDCl_3$) are displayed in **Figure 3.29** and **3.30**.

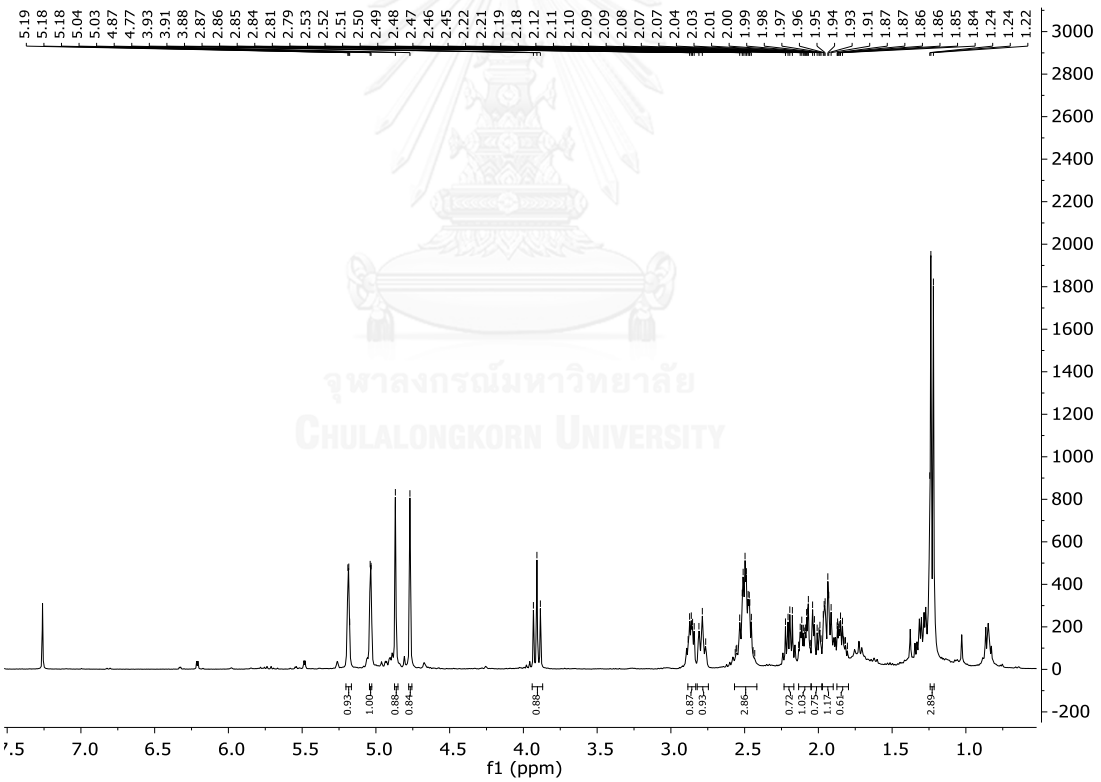


Figure 3.29 The ^1H NMR spectrum ($CDCl_3$) of compound **3**

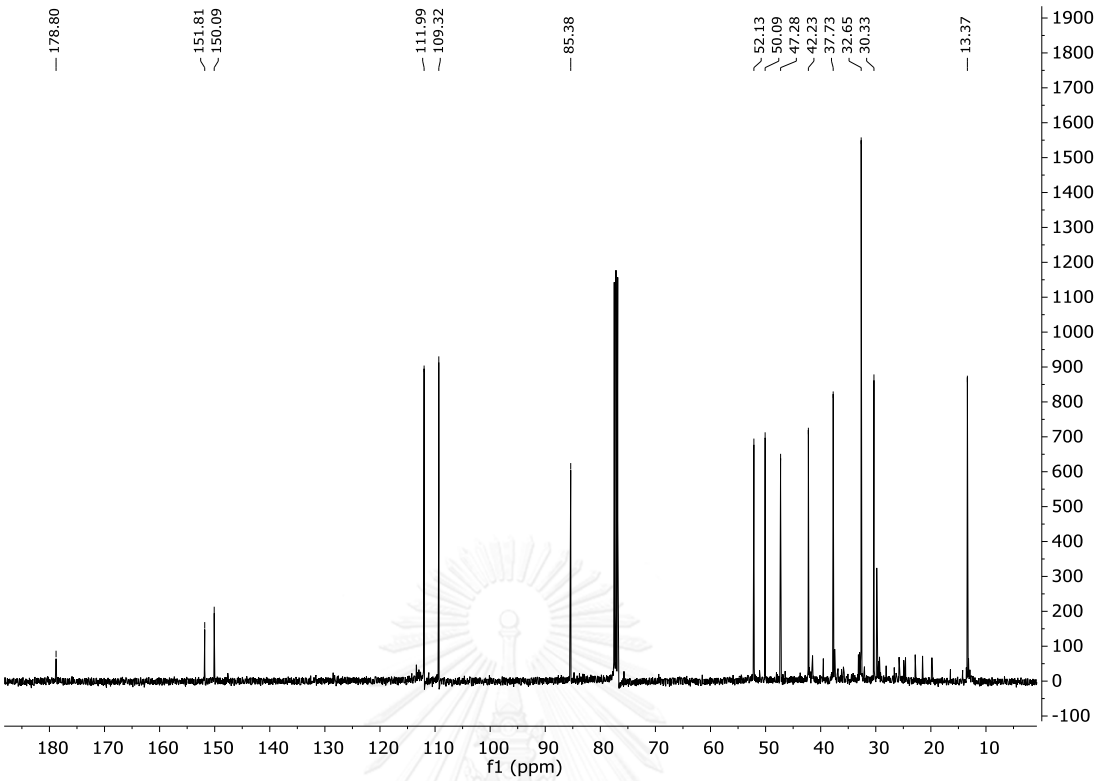
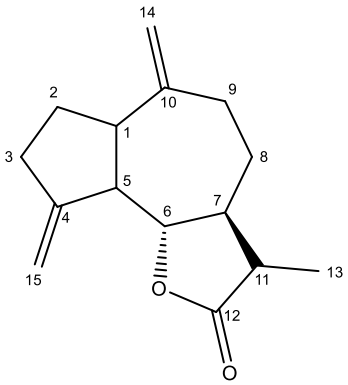


Figure 3.30 The ^{13}C NMR spectrum ($CDCl_3$) of compound **3**

Compound **3** was designated as 10(14)-guaiadien-12,6-olide (Mokko lactone) by comparison of ^{1}H and ^{13}C NMR spectral data with those previously published [53]. The comparative study on spectroscopic data are presented in **Table 3.9**.

Table 3.9 The ^{1}H and ^{13}C spectral data assignment of mokko lactone and compound **3** (in $CDCl_3$)

Position	Chemical shift (ppm)			
	Mokko lactone		Compound **3**	
	^{1}H	^{13}C	^{1}H	^{13}C
1	2.89 (dt, *J* = 8.1,4.5 Hz, 1H)	47.3	2.86 (dt, *J* = 7.9, 4.2 Hz, 1H)	47.3
2	1.95 (m, 1H); 1.87 (m, 1H)	30.5	1.94 (m, 1H); 1.85 (m, 1H)	30.3
3	2.49 (m, 3H)	32.8	2.49 (m, 3H)	32.7
4		152.0		151.8
5	2.81 (dd, *J* = 9.5,8.1 Hz, 1H)	52.2	2.79 (t, *J* = 9.2 Hz, 1H)	52.1
6	3.93 (t, *J* = 9.5 Hz, 1H)	85.6	3.91 (t, *J* = 9.5 Hz, 1H)	85.4
7	2.12 (m, 1H)	42.3	2.09 (m, 1H)	42.2
8	1.94 (m, 1H); 1.32 (m, 1H)	32.8	1.94 (m, 1H); 1.32 (m, 1H)	32.7
9	2.22 (dd, *J* = 12.0,7.1 Hz, 1H); 2.05 (dt, *J* = 12.0,5.1 Hz, 1H)	37.9	2.20 (dd, *J* = 11.8, 7.0 Hz, 1H); 2.01 (m, 1H)	37.7
10		150.2		150.1
11		50.1		50.1
12		179.0		178.8
13	1.25 (d, *J* = 6.8 Hz, 3H)	13.5	1.23 (d, *J* = 6.9 Hz, 3H)	13.4
14		112.1		112.0
15		109.5		109.3
H-14a	4.89 (br.s, 1H)		4.87 (br.s, 1H)	
H-14b	4.79 (br.s, 1H)		4.77 (br.s, 1H)	
H-15-a	5.21 (d, *J* = 2.1 Hz, 1H)		5.19 (d, *J* = 2.5 Hz, 1H)	
H-15b	5.06 (d, *J* = 2.1 Hz, 1H)		5.04 (d, *J* = 2.4 Hz, 1H)	



10(14)-Guaiadien-12,6-olide (Mokko lactone)

Mokko lactone has been reported on pharmacological activities including anticancer or antitumor promoters. Yun *et al.* (2004) reported the effect of mokko lactone isolated from the roots of *Saussurea lappa* (Asteraceae). This compound exhibited cytotoxic to HL-60 cells [54]. It should be noted that this is the first time for the report of the isolation of this compound from *H. annuus*.

3.7.4 Mixture 1: Stigmasta-4,22-dien-3-one and stigmast-4-en-3-one

Mixture **1** was collected from HAR-D2-5 and recrystallized from CH_3OH to give white powder, 105 mg. This mixture showed a single spot on TLC with R_f 0.24 (solvent: 100% CH_2Cl_2). The ^1H and ^{13}C NMR spectrum ($CDCl_3$) are presented in **Figures 3.31** and **3.32**.

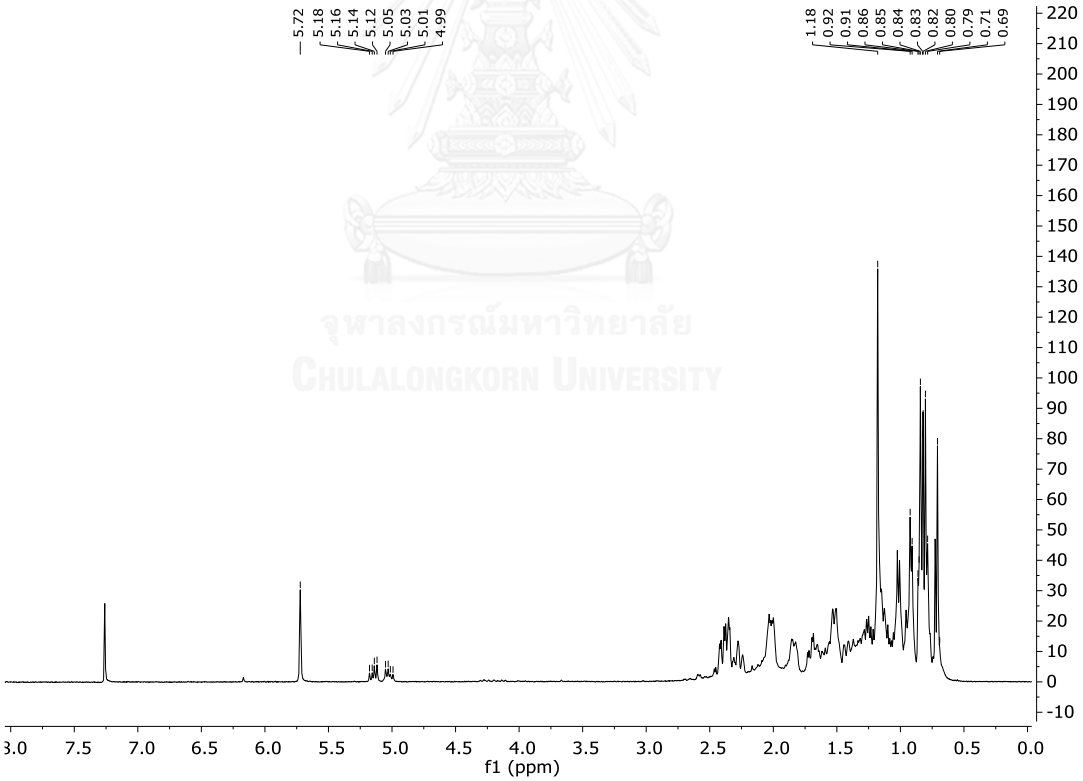


Figure 3.31 The ^1H NMR spectrum ($CDCl_3$) of mixture **1**

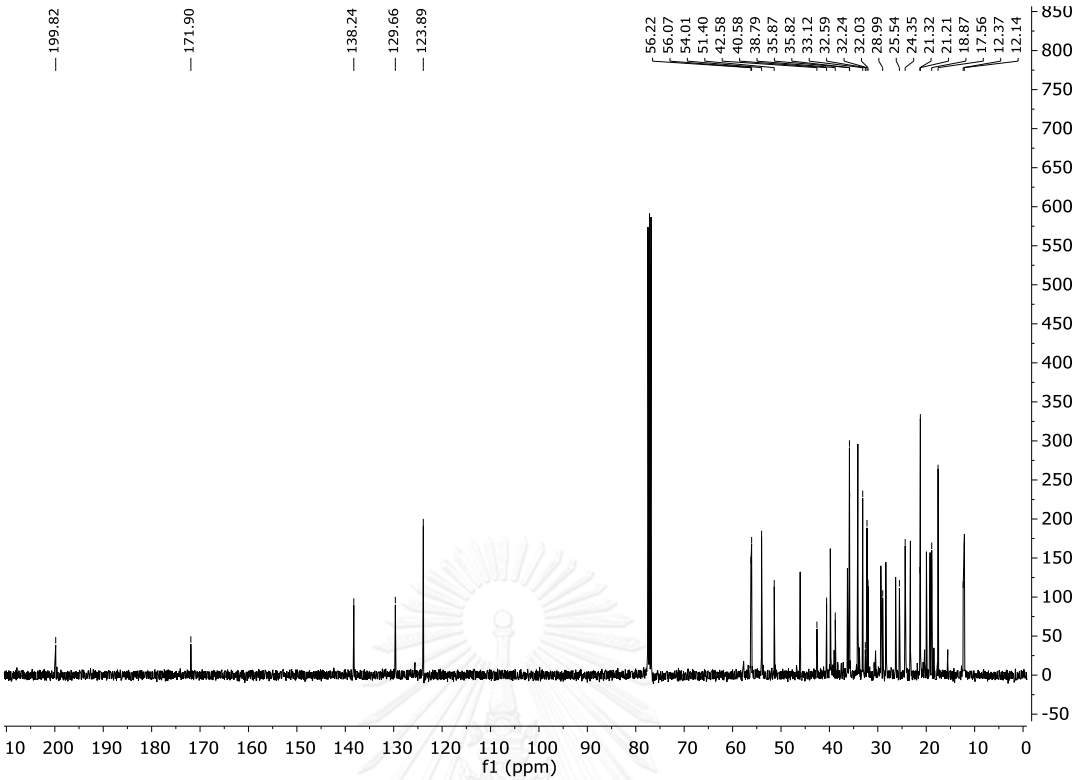
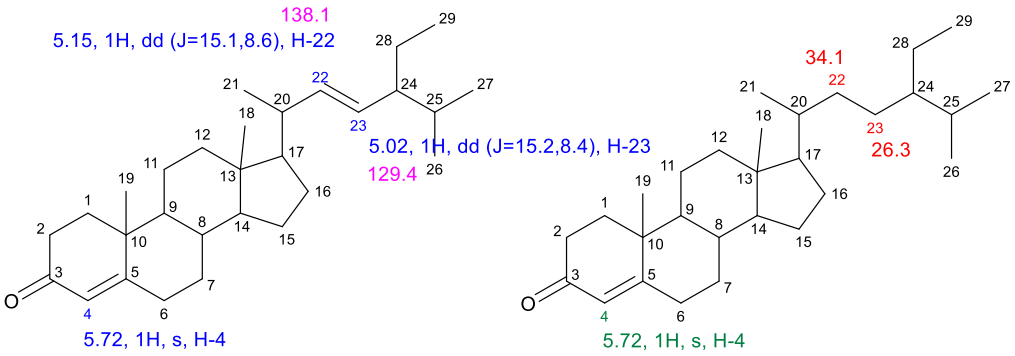


Figure 3.32 The ^{13}C NMR spectrum ($CDCl_3$) of mixture **1**

This substance was verified by comparison their spectroscopic data with those published [55, 56]. It was designated as a mixture of stigmasta-4,22-dien-3-one and stigmast-4-en-3-one. Two-proton signals could be visualized at δ_H 5.72 ppm. These could be assigned for the H-4 of stigmasta-4,22-dien-3-one and stigmast-4-en-3-one. In addition, the appearance of the carbon signal of this substance was not a sharp peak. This implied that this mixture should compose of at least two compounds. The occurrence of a mixture of stigmasta-4,22-dien-3-one and stigmast-4-en-3-one was the first time for reporting from *H. annuus*. The comparison of the ^{1}H and ^{13}C NMR spectral assignments is tabulated in **Table 3.10**.

Table 3.10 The ^{1}H and ^{13}C spectral data assignment of stigmasta-4,22-dien-3-one, stigmast-4-en-3-one and mixture **1** (in $CDCl_3$)

Position	Chemical shift (ppm)					
	Stigmasta-4,22-dien-3-one		Stigmast-4-en-3-one		Mixture **1**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1		35.7		35.9		35.9
2		33.0		34.1		32.6
3		199.7		199.9		199.8
4	5.74 (s, 1H)	123.7	5.74 (br.s, 1H)	124.0	5.72 (s, 2H)	123.9
5		171.7		171.9		171.9
6		32.9		33.2		33.1
7		32.0		32.3		32.2
8		35.7		35.8		35.9
9		53.8		54.0		54.0
10		38.6		38.8		38.8
11		21.0		21.2		21.2
12		35.6		39.8		35.8
13		42.3		42.6		42.6
14		55.9		56.1		56.1
15		24.2		24.4		24.3
16		28.8		28.4		29.0
17		56.0		56.2		56.2
18	0.71 (s, 3H)	12.1	0.73 (s, 3H)	12.2	0.71 (s, 3H)	12.1
19	1.18 (s, 3H)	17.4	1.20 (s, 3H)	17.6	1.18 (s, 3H)	17.6
20		40.4		36.3		40.6
21	0.91 (d, *J* = 6.8 Hz, 3H)	21.1	0.93 (d, *J* = 6.5 Hz, 3H)	18.9	0.92 (d, *J* = 6.3 Hz, 3H)	21.3
22	5.01 (dd, *J* = 15.2, 8.8 Hz, 1H)	138.1		34.2	5.02 (dd, *J* = 15.2, 8.4 Hz, 1H)	138.2
23	5.14 (dd, *J* = 15.2, 8.8 Hz, 1H)	129.4		26.3	5.15 (dd, *J* = 15.2, 8.6 Hz, 1H)	129.7
24		51.2		46.0		51.4
25		31.8		29.4		32.0
26	0.79 (d, *J* = 6.4 Hz, 3H)	18.9	0.84 (d, *J* = 6.5 Hz, 3H)	20.0	0.79 (d, *J* = 6.9 Hz, 3H)	18.9
27	0.82 (d, *J* = 6.4 Hz, 3H)	21.0	0.83 (d, *J* = 6.5 Hz, 3H)	19.2	0.82 (s, 3H)	21.2
28		25.4		23.3		25.5
29	0.85 (t, *J* = 6.8 Hz, 3H)	12.2	0.85 (t, *J* = 7.5 Hz, 3H)	12.1	0.85 (d, *J* = 7.4 Hz, 3H)	12.4



Stigmasta-4,22-dien-3-one **Stigmast-4 en-3-one**

3.7.5 Mixture 2: Stigmasterol and beta-sitosterol

Mixture **2** (237 mg) was isolated by column chromatography from HAR-D2-5 and recrystallized with CH_3OH to give white needle crystal. TLC showed a single spot with R_f 0.18 (solvent: 100% CH_2Cl_2). **Figures 3.33** and **3.34** present the ^1H and ^{13}C NMR spectra of mixture **2**.

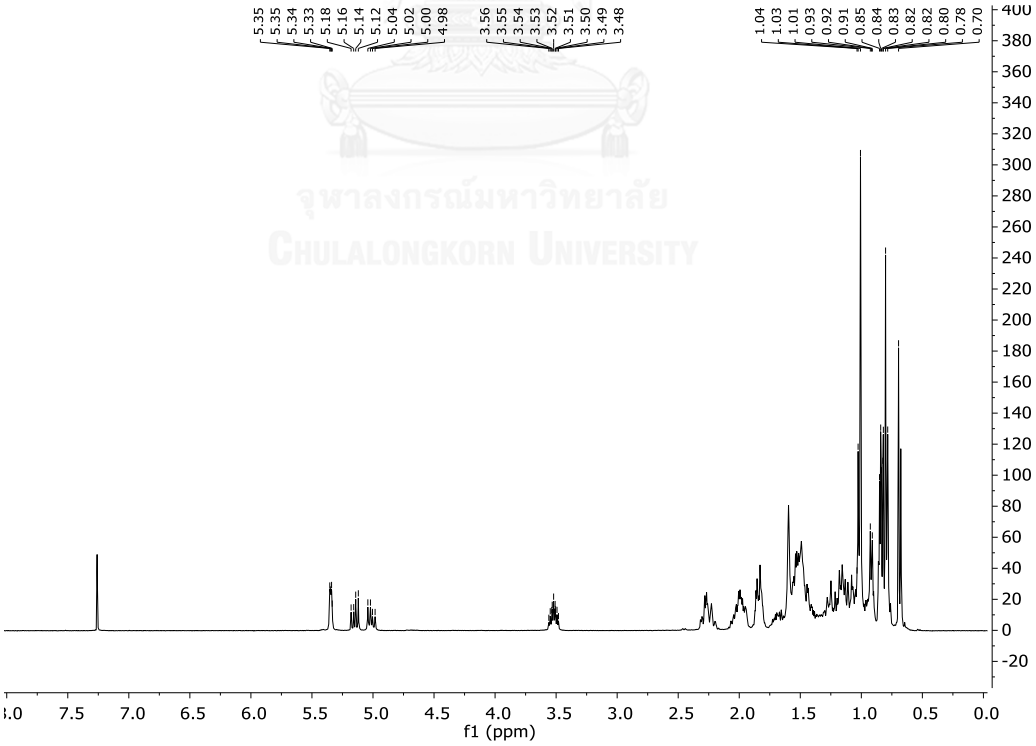


Figure 3.33 The ^1H NMR spectrum ($CDCl_3$) of mixture **2**

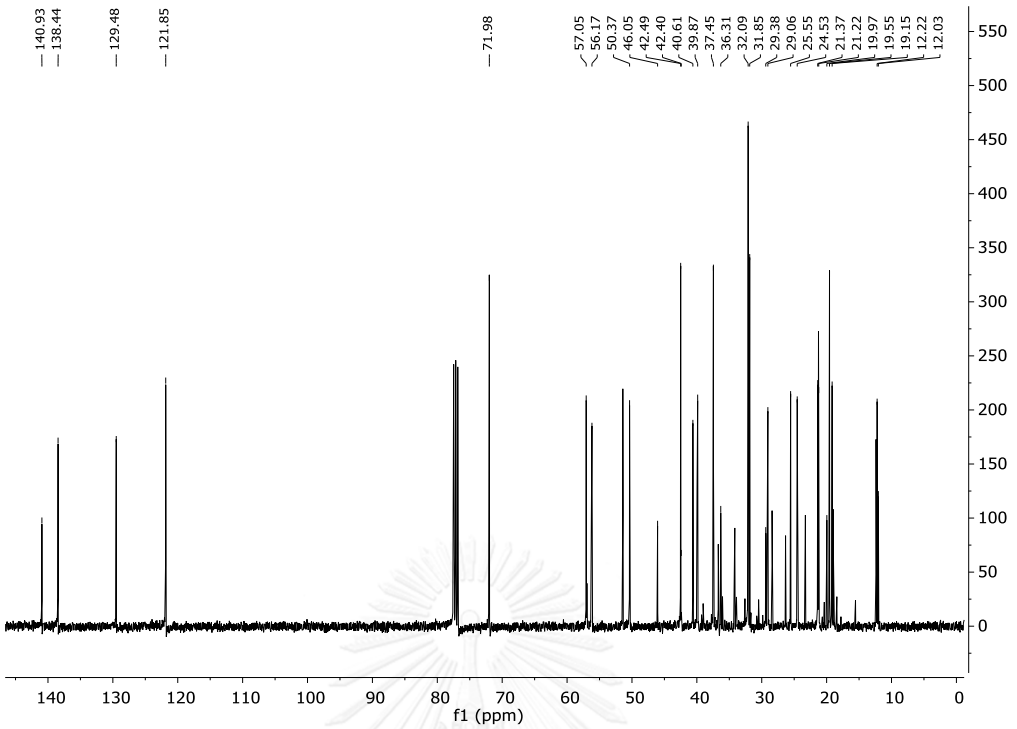
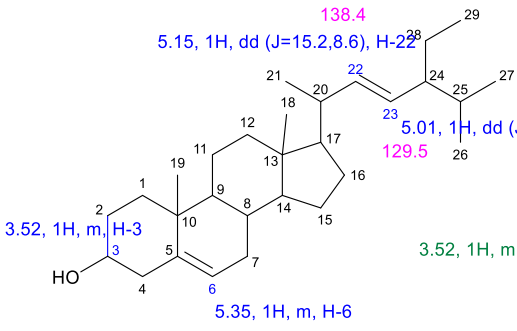


Figure 3.34 The ^{13}C NMR spectrum ($CDCl_3$) of mixture **2**

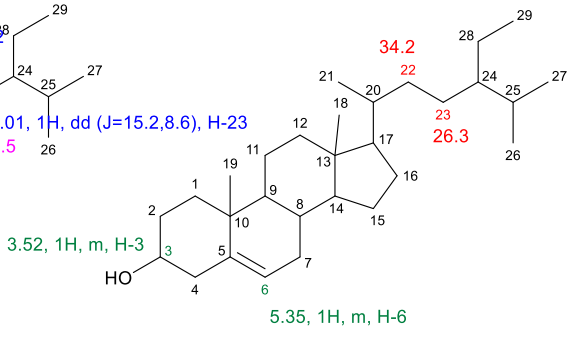
By comparison of spectroscopic data of mixture **2** with the reported data [57], this substance was designated as a mixture of stigmasterol and β-sitosterol. From the ^{1}H spectrum, according to the signal integration there were two protons at both δ_H 3.52 and 5.35 ppm. These could be assigned for the H-3 and H-6 of stigmasterol and β-sitosterol [57]. The carbon signal at δ_C 72.0 ppm was not shown as a sharp peak. This implied that this mixture may contain at least two compounds. According to the literature, steroids have been detected in various parts especially in seeds of *Helianthus* species, such as campesterol, β-sitosterol and stigmasterol [58]. Generally, the steroid in nature was occurred as a mixture. The ^{1}H and ^{13}C NMR spectral assignment are tabulated as shown in **Table 3.11**.

Table 3.11 The ^{1}H and ^{13}C spectral data assignment of stigmasterol, β-sitosterol and mixture **2** (in $CDCl_3$)

Position	Chemical shift (ppm)					
	Stigmasterol		β-sitosterol		Mixture **2**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1		37.5		37.5		37.4
2		32.1		31.9		32.1
3	3.51 (tdd, *J* = 4.5, 4.2, 3.8 Hz, 1H)	72.1	3.53 (tdd, J = 4.5, 4.2, 3.8 Hz, 1H)	72.0	3.52 (m, 2H)	72.0
4		42.4		42.5		42.5
5		141.1		140.9		140.9
6	5.31 (t, *J* = 6.1 Hz, 1H)	121.8	5.36 (t, *J* = 6.4 Hz, 1H)	121.9	5.35 (m, 2H)	121.9
7		31.8		32.1		31.9
8		31.8		32.1		31.9
9		50.2		50.3		50.4
10		36.6		36.7		36.3
11		21.5		21.3		21.2
12		39.9		39.9		39.9
13		42.4		42.6		42.4
14		56.8		56.9		57.1
15		24.4		26.3		24.5
16		29.3		28.5		29.1
17		56.2		56.3		56.2
18		40.6		36.3		40.6
19	0.91 (d, *J* = 6.2 Hz, 3H)	21.7	0.93 (d, *J* = 6.5 Hz, 3H)	19.2	0.92 (m, 3H)	21.4
20	4.98 (m, 1H)	138.7		34.2	5.01 (dd, J = 15.2,8.6 Hz, 1H)	138.4
21	5.14 (m, 1H)	129.6		26.3	5.15 (dd, J = 15.2,8.6 Hz, 1H)	129.5
22		46.1		46.1		46.1
23		25.4		23.3		25.6
24	0.83 (t, *J* = 7.1 Hz, 3H)	12.1	0.84 (t, 3H, *J* = 7.2 Hz)	12.2	0.83 (m, 3H)	12.0
25		29.6		29.4		29.4
26	0.82 (d, *J* = 6.6 Hz, 3H)	20.2	0.83 (d, *J* = 6.4 Hz, 3H)	20.1	0.82 (d, J = 6.6 Hz, 3H)	20.0
27	0.80 (d, *J* = 6.6 Hz, 3H)	19.8	0.81 (d, *J* = 6.4 Hz, 3H)	19.6	0.79 (d, J = 7.0 Hz, 3H)	19.6
28	0.71 (s, 3H)	18.9	0.68 (s, 3H)	19.0	0.70 (s, 3H)	19.2
29	1.03 (s, 3H)	12.2	1.01 (s, 3H)	12.0	1.01 (s, 3H)	12.2



Stigmasterol



β-Sitosterol

From the above results, the major compound, demethylencecalin was reported for the first time in *H. annuus* root and possessed allelopathic and antifungal activity. It might express as a main active compound from this root extract. In addition, other two isolated compounds: mokko lactone and a mixture of stigmasta-4,22-dien-3-one and stigmast-4-en-3-one were reported for the first time in *H. annuus*.

CHAPTER IV
CONCLUSION

The extracts of leaves, stems and roots (hexane, CH_2Cl_2 and CH_3OH) were investigated on seed germination and growth inhibition of *M. pigra* at 1, 2.5 and 5 gE. The CH_3OH of the leaves revealed the highest seed germination and growth inhibition effect more than other parts. This extract also exhibited the seed germination and the growth inhibition of weeds and crops. With 1 gE of the extract, the inhibition of seed germination of weeds more than crops were noticed. However, the use of this extract in high concentration may possess as non-selective weed controller.

The CH_3OH extract of the leaves was assayed for pot experiment regarding as pre- and post-emergent weed controller. The results of using dried leaves and the CH_3OH extract of the leaves as pre- and post-emergence weed controller gave the similar trend to those of petri-dish; however, with a bit less effective. In addition, this extract was further assayed for controlling barnyard grass in rice cropping by using pre- and post-emergent weed controller. 1 gE of post-emergent controller was disclosed to be a proper concentration to completely control barnyard grass population with no side-effect to the growth of rice seedlings. However, it should be explored more about the concentration towards various crops. This present outcome should be able to use for selective inhibition and further studied on the effects in long term to consider the growth and productivity of the plant.

The effect of the CH_2Cl_2 extract of the root on the growth inhibition of flowers and cereal crops at 1 gE showed the selective shoot elongation inhibition on zinnia and rice and expressed stimulated effect on the root elongation. It might be possible to use this extract as natural plant growth regulator to reduce the use of synthesized compounds.

All crude extracts were conducted on other biological activities including antifeedant activity, antibacterial and antifungal. The leave and root extracts at 0.25% w/w gave the highest antifeedant activity on common cutworm. For antibacterial and antifungal activity, the CH_2Cl_2 extract of the roots displayed the highest inhibitory effect

against the selective microorganism. *R. solani* was the most sensitive to this extract. This might be demonstrated that this extract contained some antifeedant and antifungal substances.

The chemical composition of this fraction was further investigated. Five substances: demethylencecalin, demethoxyencecalin, mokko lactone, a mixture of stigmasta-4,22-dien-3-one and stigmast-4-en-3-one, and a mixture of stigmasterol and β-sitosterol were identified based on spectroscopic evidence.

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APPENDIX

APPENDIX A

Table A1. The seed germination inhibition of crude extracts from *H. annuus* on *M. pigra* L.

Crude extracts		% Germination inhibition			Mean
		Concentration			
		1gE	2.5gE	5gE	
Leave	Hexane	0.00	4.08	8.84	4.31 d
	CH_2Cl_2	1.36	10.2	23.13	11.56 d
	CH_3OH	65.1	97.31	100	87.47 a
Shoot	CH_2Cl_2	0.00	1.37	6.89	2.75 d
	CH_3OH	0.00	53.74	100	51.25 b
Root	CH_2Cl_2	0.00	4.82	5.51	3.44 d
	CH_3OH	1.36	19.05	80.27	33.56 c
Control		0.00	0.00	0.00	0.00 d

[a] **Different letters in a column indicate values significantly different at the 0.05 level according to DMRT.**

Table A2. The shoot elongation inhibition of crude extracts from *H. annuus* on *M. pigra* L.

Crude extracts		% Shoot elongation inhibition			Mean
		Concentration			
		1gE	2.5gE	5gE	
Leave	Hexane	7.56	20.35	19.65	15.85 c
	CH_2Cl_2	3.33	-1.92	15.44	5.62 c
	CH_3OH	37.33	72.5	87.17	65.67 a
Shoot	CH_2Cl_2	30.67	51.45	53.86	45.33 b
	CH_3OH	-2.50	58.33	81.39	45.74 b
Root	CH_2Cl_2	53.38	63.77	70.77	62.64 a
	CH_3OH	21.67	46.38	73.89	47.31 b
Control		0.00	0.00	0.00	0.00 c

[a] **Different letters in a column indicate values significantly different at the 0.05 level according to DMRT.**

Table A3. The root elongation inhibition of crude extracts from *H. annuus on M. pigra* L.

Crude extracts		% Root elongation inhibition			Mean
		Concentration			
		1gE	2.5gE	5gE	
Leave	Hexane	34.29	55.72	47.70	45.90 b
	CH_2Cl_2	34.44	44.68	59.92	46.35 b
	CH_3OH	75.79	89.44	93.02	86.08 a
Shoot	CH_2Cl_2	-1.95	40.84	57.81	32.23 c
	CH_3OH	56.50	88.89	94.91	80.10 a
Root	CH_2Cl_2	28.83	32.28	57.06	39.39 bc
	CH_3OH	44.21	88.54	96.24	76.33 a
Control		0.00	0.00	0.00	0.00 d

[a] **Different letters in a column indicate values significantly different at the 0.05 level according to DMRT.**

Table A4. The seed germination inhibition of the CH_3OH extract of the leaves on weeds and crops

Selected plants	% Germination inhibition			Mean
	Concentration			
	1gE	2.5gE	5gE	
Prickly Chaff flower	92	100	100	97.33 ab
Barnyard grass	52.99	97.76	100	83.58 bc
Swollen finger grass	100	100	100	100 a
Chinese kale	13.51	83.78	100	65.76 de
Water convolvulus	44.44	75	100	73.15 cd
Corn	15.15	54.55	86.36	52.02 e
Rice	42.86	76.19	100	73.02 cd
Control	0.00	0.00	0.00	0.00 f

[a] **Different letters in a column indicate values significantly different at the 0.05 level according to DMRT.**

Table A5. The shoot elongation inhibition of the CH_3OH extract of the leaves on weeds and crops

Selected plants	% Shoot elongation inhibition			Mean
	Concentration			
	1gE	2.5gE	5gE	
Prickly Chaff flower	56.02	86.11	95.83	79.32 a
Barnyard grass	-5.56	51.85	96.30	47.53 c
Swollen finger grass	37.35	50.31	79.32	55.66 bc
Chinese kale	44.44	82.66	87.55	71.55 ab
Water convolvulus	48.56	80.04	95.06	74.55 ab
Corn	25.49	37.13	63.33	41.98 c
Rice	35.69	70.59	78.63	61.63 abc
Control	0.00	0.00	0.00	0.00 d

[a] **Different letters in a column indicate values significantly different at the 0.05 level according to DMRT.**

Table A6. The root elongation inhibition of the CH_3OH extract of the leaves on weeds and crops

Selected plants	% Root elongation inhibition			Mean
	Concentration			
	1gE	2.5gE	5gE	
Prickly Chaff flower	87.04	88.62	88.89	88.18 a
Barnyard grass	91.97	93.83	93.83	93.21 a
Swollen finger grass	54.55	72.73	75.76	67.68 b
Chinese kale	94.18	95.12	95.27	94.86 a
Water convolvulus	82.86	93.85	94.68	90.46 a
Corn	81.20	93.33	95.53	90.02 a
Rice	90.76	95.05	94.84	93.55 a
Control	0.00	0.00	0.00	0.00 c

[a] **Different letters in a column indicate values significantly different at the 0.05 level according to DMRT.**

Table A7. The germination inhibition of dried leaves as pre-emergent controller on weeds

Selected plants	% Germination inhibition			Mean
	Concentration			
	1gE	2.5gE	5gE	
Giant mimosa	6.93	31.06	68.98	35.65 ab
Prickly Chaff flower	37.46	62.48	62.48	54.14 a
Barnyard grass	-12.36	12.61	100	33.42 ab
Swollen finger grass	39.94	59.96	100	66.63 a
Control	0.00	0.00	0.00	0.00 b

[a] **Different letters in a column indicate values significantly different at the 0.05 level according to DMRT.**

Table A8. The germination inhibition of the CH_3OH extract of the leaves as pre-emergent controller on weeds

Selected plants	% Germination inhibition			Mean
	Concentration			
	1gE	2.5gE	5gE	
Giant mimosa	13.33	20.00	33.33	22.22 c
Prickly Chaff flower	51.74	82.77	100	78.17 a
Barnyard grass	40.74	51.85	59.26	50.62 b
Swollen finger grass	36.42	54.59	100	63.67 ab
Control	0.00	0.00	0.00	0.00 d

[a] **Different letters in a column indicate values significantly different at the 0.05 level according to DMRT.**

Table A9. The shoot elongation inhibition of the CH_3OH extract of the leaves as post-emergent controller on weeds

Selected plants	% Shoot elongation inhibition			Mean
	Concentration			
	1gE	2.5gE	5gE	
Giant mimosa	31.03	56.49	90.91	59.48 b
Prickly Chaff flower	57.44	76.92	94.36	76.24 a
Barnyard grass	19.33	38.89	70.93	43.05 c
Swollen finger grass	3.70	54.81	68.89	42.47 c
Control	0.00	0.00	0.00	0.00 d

[a] **Different letters in a column indicate values significantly different at the 0.05 level according to DMRT.**

Table A10. The root elongation inhibition of the CH_3OH extract of the leaves as post-emergent controller on weeds

Selected plants	% Root elongation inhibition			Mean
	Concentration			
	1gE	2.5gE	5gE	
Giant mimosa	-2.13	30.13	62.40	30.13 c
Prickly Chaff flower	53.68	74.74	85.26	71.23 a
Barnyard grass	17.20	34.39	68.42	40.00 bc
Swollen finger grass	24.62	72.31	75.38	57.44 ab
Control	0.00	0.00	0.00	0.00 d

[a] **Different letters in a column indicate values significantly different at the 0.05 level according to DMRT.**

Table A11. Barnyard grass inhibition and the growth inhibition of rice seedling of using the CH_3OH extract of the leaves as pre- and post-emergent weed controller

Treatments		% Barnyard grass inhibition	% Shoot length inhibition on rice	% Root length inhibition on rice
Pre-emergence	Control	0.00 c	0.00 c	0.00 c
	1 gE	33.33 b	-1.44 c	29.16 b
	2.5 gE	40.00 b	35.74 a	52.35 a
Post-emergence	Control	0.00 c	0.00 c	0.00 c
	1 gE	100 a	1.84 c	-9.28 c
	2.5 gE	100 a	28.96 b	30.13 b

[a] **Different letters in a column indicate values significantly difference at 0.05 level determined by one-way ANOVA followed DMRT.**

Table A12. The shoot and root elongation inhibition of the CH_2Cl_2 extract of the roots on selected plants

Selected plants	% Shoot length inhibition	% Root length inhibition
Marigold	38.14 a	36.02 a
Zinnia	20.05 bc	-22.80 b
Rice	26.45 ab	-12.15 b
Mung bean	7.71 cd	-54.51 c
Control	0.00 d	0.00 b

[a] **Different letters in a column indicate values significantly different at 0.05 level determined by one-way ANOVA followed DMRT.**

Table A13. The inhibition rate of *H. annuus* extracts against two pathogenic bacteria

Crude extracts		% Inhibition rate[a]	
		S. aureus	S. sobrinus
Leave	Hexane	ND	25.0±0.1
	CH_2Cl_2	ND	14.3±0.2
	CH_3OH	ND	20.0±0.4
Shoot	CH_2Cl_2	7.7±0.1	25.0±0.2
	CH_3OH	ND	ND
Root	CH_2Cl_2	14.3±0.2	29.4±0.3
	CH_3OH	7.7±0.6	14.3±0.7

Values are given as mean ± SD of duplication experiment, ND is not detected

[a]The inhibition rate (%) = [(the inhibition zone diameters-agar well diameter)/ the inhibition zone diameters]*100%

APPENDIX B

Artificial diet for common cutworm culture

Soaked mung bean	150 g
Dried Brewer's yeast	10 g
Methyl parahydroxy benzoic acid	2.5 g
Sorbic acid	1.5 g
Ascorbic acid	3 g
Casein	3 g
Choline chloride	0.5 g
Agar	14 g
40% Formalin	2.0 ml
Vitamin stock	10 ml
Distilled water	750 ml

Vitamin stock preparation

Niacin	6 g
Calcium panthothenate	6 g
Thiamine (B1)	3 g
Riboflavin (B2)	3 g
Pyridoxin monohydrochloride	1.5 g
Folic acid	1.5 g
Biotin	120 mg
Vitamin B12 (Cyanocobalamin)	12.0 mg
Inositol	10 g
Choline chloride	25 g
Distilled water	1 L

Combine all ingredients with 350 mL of distilled water except vitamin stock and formalin, then poured into blender and mixed for 10 min while pour the agar that already mixed and heat with 400 mL of distilled water stirred and poured vitamin stock

and formalin, mixed again then pour the artificial diet into plastic box. Leave until it set and keep in the refrigerator.

Media for microbial culture

1. Nutrient broth and agar (NB and NA)

Beef extract	3 g
Peptone	5 g
Agar	15 g (for NA)

Dissolved in distilled water up to 1 L and autoclaved at 121 °C, 15 psi for 15 min.

2. Potato dextrose agar (PDA)

Potato starch	4 g
Dextrose	20 g
Agar	15 g

Dissolved in distilled water up to 1 L and autoclaved at 121 °C, 15 psi for 15 min.

3. Carrot agar

Carrot	200 g
Agar	15 g

Dissolved in distilled water up to 1 L and autoclaved at 121 °C, 15 psi for 15 min.

VITA

Miss Wiriya Noothong was born on February 5, 1989 in Suphanburi province, Thailand. She graduated with Degree of Bachelor of Science (Agriculture) from the Faculty of Agriculture, Kasetsart University in 2011. She graduated in master degree of Science in Biotechnology in 2015 from program in Biotechnology Faculty of Science, Chulalongkorn University.