การควบคุมยุงลาย โดยชีววิธีด้วยสารสกัดจากเมล็ดแมงลักคา มันแกว และ ขึ้นฉ่าย

นางสาวบุษรา ยงคำชา

BIOLOGICAL CONTROL OF DENGUE FEVER MOSQUITOES

(*AEDES AEGYPTI* L.) BY MINTWEED (*HYPTIS SUAVEOLENS*

(L.) POIT), YAM BEAN (*PACHYRHIZUS EROSUS* L.), AND

CELERY (*APIUM GRAVEOLENS* L.) SEED EXTRACTS

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BIOLOGICAL CONTROL OF DENGUE FEVER MOSQUITOES (*AEDES AEGYPTI* L.) BY MINTWEED (*HYPTIS SUAVEOLENS* (L.) POIT), YAM BEAN (*PACHYRHIZUS EROSUS* L.), AND CELERY (*APIUM GRAVEOLENS* L.) SEED EXTRACTS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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บุษรา ยงคำชา : การควบคุมยุงลายโดยชีววิธีด้วยสารสกัดจากเมล็ดแมงลักคา มันแกว และ ขึ้นฉ่าย (BIOLOGICAL CONTROL OF DENGUE FEVER MOSQUITOES (*AEDES AEGYPTI* L.) BY MINTWEED (*HYPTIS SUAVEOLENS* (L.) POIT), YAM BEAN (*PACHYRHIZUS EROSUS* L.), AND CELERY (*APIUM GRAVEOLENS* L.) SEED EXTRACTS). อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.กรกช อินทราพิเชฐ, 130 หน้า.

การศึกษาพฤกษเคมีและความเป็นพิษของสารสกัดเมล็ดของแมงลักคา มันแกว และขึ้นฉ่าย ในลูกน้ำและตัวเต็มวัยของยุงลาย และศึกษาประสิทธิภาพของสารสกัดที่มีต่อไซโทโครม c ออกซิ เดสของไมโทคอนเดรียยุงลาย พบว่าปริมาณสารประกอบฟีนอลิกทั้งหมดของสารสกัดด้วยเอทา นอลของเมล็ดแมงลักคา, ขึ้นฉ่าย และมันแกว คือ 275.91 ± 0.68, 246.64 ± 0.66 และ 108.34 ± 0.67 mg GAE/g และสารสกัดด้วยน้ำของเมล็ดแมงลักคา ขึ้นฉ่าย และมันแกว คือ 69.93 ± 0.48, 48.75 ± 0.37 และ 37.83 ± 1.31 mg GAE/g ตามลำดับ ปริมาณสารประกอบฟลาโวนอยด์ทั้งหมดที่พบในสาร สกัดเอทานอลของเมล็ดแมงลักคา ขึ้นฉ่าย และมันแกว คือ 196.21 ± 0.92, 185.43 ± 11.54 และ 95.16 ± 6.37 mg CE/g และในสารสกัดน้ำของเมล็ดแมงลักคา ขึ้นฉ่าย และมันแกว คือ 65.92 ± 2.88, 41.81 ± 15.86 และ 37.85 ± 1.31 mg CE/g ตามลำดับ การตรวจสอบสารประกอบทางพฤกษเคมีด้วย วิธีทินเลเยอร์โครมาโทกราฟีและยืนยันผล โดยใช้กรดวานิลีนซัลฟูริกและสารเคดเด เพื่อบ่งชี้ว่ามี สารกลุ่มเทอร์พีนและไซยาโนเจนนีติก กลัยโคไซด์ สารประกอบเหล่านี้ซึ่งเป็นองค์ประกอบหลักใน สารสกัดทำให้เกิดความเป็นพิษและการตายต่ออาร์ทีเมียร์ ลูกน้ำ และตัวเต็มวัยของยุงลายในช่วงที่ กว้าง ประสิทธิภาพของสารสกัดมีความแตกต่างกันค่อนข้างกว้างในระดับความเข้มข้นนาโนกรัมถึง มิลลิกรัม ขึ้นอยู่กับชนิดของพืช ตัวทำละลายที่ใช้สกัด และองค์ประกอบในพืช สารสกัดจากเมล็ด มันแกวมีประสิทธิภาพสูงสุดในทุกการทดลอง ค่า LC_{50} ที่ 24 ชม. สารสกัดด้วยเอทานอลและน้ำของ เมล็ดมันแกว คือ 0.02 ± 0.24 และ 257.11 ± 0.29 µg/ml นอกจากนั้นยังพบว่า สารสกัดด้วย เอทานอลของเมล็ดมันแกว มีประสิทธิภาพสูงสุดในการควบคุมลูกน้ำระยะที่ 2 ซึ่งมีค่า LC_{50} 16.22 ± 0.20 µg/ml ส่วนสารสกัดด้วยน้ำของเมล็ดขึ้นฉ่ายมีประสิทธิภาพต่ำสุดซึ่งมีค่า LC_{50} 25.23 ± 0.12 mg/ml สารสกัดที่พ่นเข้าไปในกรงสามารถควบคุมยุงในระยะตัวเต็มวัยได้ สารสกัด YSE/e มีความ เป็นพิษสูงสุดต่อยุงตัวเต็มวัยซึ่งมีค่า LC_{50} 91.41 µg/ml และ สารสกัดด้วยน้ำของเมล็ดขึ้นฉ่ายมีความ เป็นพิษต่ำสุดซึ่งมีค่า LC_{50} 109.03 ± 0.17 mg/ml การทดสอบสารสกัดผสมพืช 2 ชนิด (v/v; 1 : 1) ใน

การควบคุมลูกน้ำระยะที่ 2 และตัวเต็มวัย พบว่า สารสกัดของมันแกว เมื่อนำมารวมกับสารอื่น สามารถเพิ่มอัตราการตายให้กับพืชชนิดอื่นได้ การใช้สารสกัดด้วยเอทานอลผสมระหว่างมันแกว และแมงลักคา ชักนำให้เกิดประสิทธิภาพสูงสุด ซึ่งมีค่า LC_{50} 11.79 ± 0.15μg/ml ในการควบคุม ลูกน้ำระยะที่ 2 และ มีค่า LC_{50} 23.82 ± 0.20 μg/ml ในการควบคุมตัวเต็มวัย สารสกัดด้วยน้ำผสม ระหว่างแมงลักคาและขึ้นฉ่ายมีฤทธิ์ต่ำสุดซึ่งมีค่า LC_{50} 12.32 ± 0.23 mg/ml นอกจากนั้นยังศึกษา ประสิทธิภาพของสารสกัดต่อไซโทโครม c ออกซิเดสที่สกัดจากไมโตคอนเดรียของลูกน้ำและ ตัวเต็มวัยของยุงลาย พบว่า สารสกัดด้วยเอทานอลของมันแกว มีศักยภาพในการยับยั้งไซโทโครม c ออกซิเดส สูงสุด คือ 65.24% ในลูกน้ำ และ 59.08% ในตัวเต็มวัย ตามลำดับ สารสกัดด้วยน้ำจาก เมล็ดขึ้นฉ่ายมีฤทธิ์ต่ำมาก ความสามารถของสารสกัดผสมในการยับยั้งไซโทโครม c ออกซิเดส ขึ้นอยู่ กับสารสกัดด้วยเอทานอลของมันแกว แสดงผลการเสริมฤทธิ์ต่อการยับยั้งไซโทโครม c ออกซิเดส ให้กับสารผสม การใช้สารสกัดด้วยเอทานอลผสมระหว่างมันแกวและแมงลักคา ชักนำให้เกิดการ ยับยั้งไซโทโครม c ออกซิเดส ได้สูงสุด 79.54% ในลูกน้ำ และ 70.62% ในยุงตัวเต็มวัย ส่วนสารสกัด จากพืชของขึ้นฉ่าย ยับยั้งไซโทโครม c ออกซิเดสได้น้อยที่สุด ดังนั้น สามารถสรุปได้ว่า สารสกัด เมล็ดมันแกวเป็นสารที่มีฤทธิ์สูงในการควบคุมยุงโดยวิธีทางชีวภาพ

สาขาวิชาชีววิทยา   ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2553   ลายมือชื่ออาจารย์ที่ปรึกษา_____

                                       ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

BUTSARA YONGKHAMCHA : BIOLOGICAL CONTROL OF DENGUE FEVER MOSQUITOES (*AEDES AEGYPTI* L.) BY MINTWEED (*HYPTIS SUAVEOLENS* (L.) POIT), YAM BEAN (*PACHYRHIZUS EROSUS* L.), AND CELERY (*APIUM GRAVEOLENS* L.) SEED EXTRACTS. THESIS ADVISOR : ASSOC. PROF. KORAKOD INDRAPICHATE, Ph.D. 130 PP.

BIOLOGICAL CONTROL/DENGUE FEVER MOSQUITOES (*AEDES AEGYPTI* L.)/TOTAL PHENOLIC COMPOUNDS/TOTAL FLAVONOID CONTENTS/THIN LAYER CHROMATOGRAPHY/CYTOTOXICITY/MINTWEED/YAM BEAN/CELERY

Phytochemicals and toxic activities of the seed extracts of mintweed (*Hyptis suaveolens,* MSE), yam bean (*Pachyrhizus erosus,* YSE), and celery (*Apium graveolens,* CSE) in larvae and adults of *Aedes aegypti* were studied. The activity of the extracts on the cytochrome c oxidase (COX) of mitochondria in the mosquitoes was also investigated. The contents of total phenolic compounds MSE/e, CSE/e, and YSE/e were 275.91 ± 0.68, 246.64 ± 0.66, and 108.34 ± 0.67 mg (catechin equivalent : CE) CE/g and in MSE/w, CSE/w, and YSE/w were 69.93 ± 0.48, 48.75 ± 0.37 mg (gallic acid equivalent : GAE) GAE/g, and 37.85 ± 1.31 mg GAE/g, respectively. The amounts of total flavonoids in MSE/e, CSE/e, and YSE/e were 196.21 ± 0.92, 185.43 ± 11.54, and 95.16 ± 6.37 mg CE/g and in MSE/w, CSE/w, and YSE/w were 65.92 ± 2.88, 41.81 ± 15.86 mg CE/g, and 37.85 ± 1.31 mg CE/g, respectively. Thin layer chromatographs of the extracts, identified by Vanillin-sulphuric reagent and Kedde reagent, indicated the presence of terpenes and cyanogenic glycosides. These compounds could be the major constituents, including their quantity, in the extracts that cause very wide range of toxicity and death to the brine shrimps (*Artemia salina* L.) in cytotoxicity tests and to the larvae and the adults of *Ae. aegypti*. The magnitude effect of the extracts varied broadly from nano-to milligram level, which possibly

depended on plant types, extracted solvents, and constituents. The YSEs were the most potent for all treatments. The median lethal concentration (LC_{50}) at 24 h of the YSE/e and the YSE/w on brine shrimps was 0.02 ± 0.24 µg/ml and 257.11 ± 0.29 µg/ml. The YSE/e was the most potent in controlling the 2nd instar larvae with LC_{50} 16.22 ± 0.20 µg/ml and the CSE/w was the least with LC_{50} 25.23 ± 0.12 mg/ml. The extract spraying into the adult rearing case was able to control the mosquitoes. The most toxic effect was produced by the YSE/e with LC_{50} 91.41 µg/ml and the least effect was by the CSE/w with LC_{50} 109.03 ± 0.17 mg/ml. The combination of two extracts (v/v; 1 : 1) was tested for the control of the 2nd instar larvae and the adults. The YSEs synergistically enhanced the mortality to the other extracts. The combination of YSE/e and MSE/e induced highest efficacy with LC_{50} 11.79 ± 0.15 µg/ml in the control of the 2nd instar larvae and LC_{50} 23.82 ± 0.20 µg/ml on the adults. The MSE/w and CSE/w combination produced least LC_{50} 12.32 ± 0.23 mg/ml. The effects of the extracts on the COX in mitochondria of the mosquitoes were investigated. The YSE/e was the most potent inhibit 65.24 ± 3.59 and 59.08 ± 10.08 of % COX for the larvae and the adults, respectively. The celery seed extracted with water (CSE/w) had very low potential. The combination of the extracts inhibited COX was dependent on the YSE/e which synergistically enhanced the COX inhibitory effects in the mixture. The combination of YSE/e and MSE/e induced the highest % COX inhibition in larvae 79.54 ± 5.39, and in adults 70.62 ± 4.37. The CSEs were highly reduced the COX inhibition. Therefore, it could be concluded that the yam bean seed extracts was likely to be the most potent insecticidal agent for the biological control of mosquitoes.

School of Biology   Student's Signature _____

Academic Year 2010   Advisor's Signature _____

                                                 Co-advisor's Signature_____

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

1.1 Background

Aedes aegypti L. widely distributes in the tropical and subtropical zones of the world (Otero, Solari and Schweigmann, 2006). It is the most dominantly domestic vectors carrying viruses that causes dengue, hemorrhagic, yellow, and chikungunya fevers in human resulting to death of millions people annually (Michael, Arthur, Rosser and Neil, 2004). Dengue hemorrhagic fever (DHF) is also one of the deadly diseases in Thailand, particularly in children. The mosquitoes are abundantly peridomistic, breeding, and habitating in hot and humid areas, particularly in towns and cities (Michael, Arthur, Rosser and Neil, 2004). The emergence of DHF can be reduced by intervening the life cycle of *Ae. aegypti*. The control of DHF vector mosquitoes efficiently uses synthetic chemicals. However, they seriously cause environmental pollution and are toxic to health problems of both human beings and animals (Forget, Goodman and Villiers, 1993; National Research Council, 2000). Recently, investigation for various biological agents in the control of the DHF vector mosquitoes performed on the basis of safety, economy, and efficiency under controllable conditions (Brattsten, Hamilton and Sutherland, 2007). The use of plant products is able to reduce the use of synthetically chemical insecticides and avoid the problem of insect resistance (Thomas and Callaghan, 1999). Plants produce a number of phytochemicals, such as phenolics, tannins, essential oils and terpenes, as their

CHAPTER II

SOME PHYTOCHEMICAL PROPERTIES OF

MINTWEED, YAM BEAN, AND CELERY

SEED EXTRACTS

2.1 Abstract

Mintweed (*Hyptis suaveolens* (L.) Poit, MSE), yam bean (*Pachyrhizus erosus* L., YSE), and celery (*Apium graveolens* L., CSE) seed were extract in 70% ethanol and water. The extracts were subjected to be quantified and determined for some phytochemical properties. Total phenolic compounds (TPC) in the extracts were measured by Folin-Ciocalteu method and total flavonoid contents (TFC) were quantified by $AlCl_3$ Method. The contents of TPC in MSE/e, CSE/e, and YSE/e were 275.91, 246.64, and 108.34 (catechin equivalent, CE) mg CE/g and in MSE/w, CSE/w, and YSE/w were 69.93, 48.75 (gallic acid equivalent, GAE) mg GAE/g, and 37.85 mg GAE/g, respectively. The amounts of TFC in MSE/e, CSE/e, and YSE/e were 196.21, 185.43, and 95.16 mg CE/g and in MSE/w, CSE/w, and YSE/w were 65.92, 41.81 mg CE/g, and 37.85 mg CE/g, respectively. TPC and TFC of ethanol extracts were significantly higher than that of with water, ($P<0.05$). In particular, TPC and TFC of mintweed seed extracts were the highest among the others. The major constituents of the extracts were analyzed by thin layer chromatography (TLC). The mobile phase system for separation of MSEs was ethyl acetate : toluene : hexane (1 : 3 : 1) and

toluene : chloroform : ethanol (9 : 3 : 4); for YSEs was toluene : ethyl acetate : ethanol (6 : 5 : 3) and ethyl acetate : toluene : acetic acid (1 : 9 : 1); and for CSEs were toluene : chloroform : ethanol (5 : 7 : 2) and methanol : ethyl acetate : water (10 : 5 : 6). The TLC spots were likely to be terpenes (Tp) and cyanogenic glycosides (Cg) as detected by Vanillin-sulphuric and Kedde reagents, respectively.

2.2 Introduction

Plant seed extracts are well known as one of the highest sources of phytochemicals which are secondary metabolites. The diversity of plant secondary metabolites have been identified. The chemical structures of more than 40,000 different terpenes, 20,000 phenolics and 5,000 alkalloids have been known (Lewinsohn and Gijzen, 2009). Phenolic compounds are the largest subgroup of phytochemicals found most common in plants (Balasundram, Sundram and Samman, 2006). The main classes of dietary phenolics are flavonoids, phenolic acids, and polyphenols, which are commonly known as tannins. Flavonoids are derived primarily from the skins and seeds of fruits, and less frequently found from the stems (Jackson, 2000). Flavonoids exist either free monomers or polymerized with sugars to form glycosides. The most common flavonoids in wine are flavan-3-ols (catechin, epicatechin, tannins), flavonols (quercetin, kaempferol, myricetin), and anthocyanins (cyanin). The basic structures of phenolic compounds and flavonoids are shown in Figure 2.1 and Table 2.1. Each plant synthesizes different individual phytochemicals. Numerous compounds are synthesized by amazingly diverse network of metabolic pathways (Leland *et al.*, 2006) because of their bitter, astringent properties (King and Young, 1999; Apak *et al.*, 2007).

Simple phenols   Hydroxybenzoic acids (e.g., gallic acid)

Basic flavonoid structure   Flavanols (flavan-3-ols) (e.g., (+)-catechin)
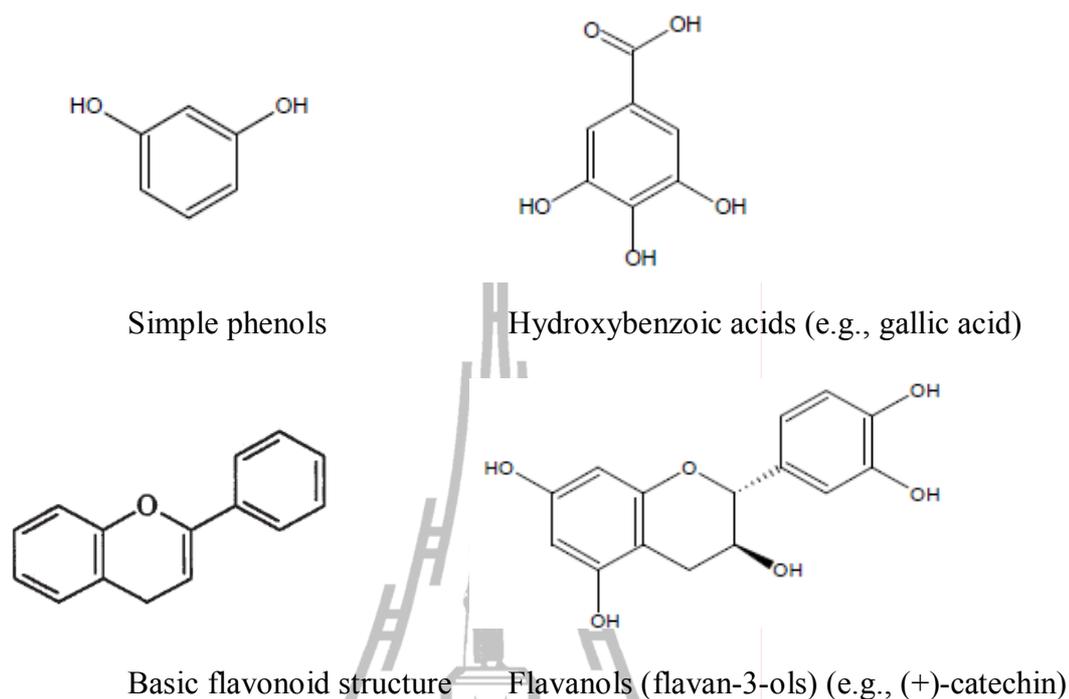
Figure 2.1 Basic structures of phenolic compounds and flavonoids (Apak *et al*., 2007;

King and Young, 1999).

Many phytochemicals are associated with human health benefits. Some reports have explained that phytochemicals play a major role in protecting themselves against herbivorous insects. In addition, their functions are related to disease resistance in plants and lipid oxidation prevention in living cells, including antitumor, antivirus, antibiotic activities and insecticidal properties (Tanprasit, 2005; Woo, Jeong and Hawes, 2005; Chuenwong, 2006; Apak *et al*., 2007; Okwu and Ukanwa, 2010).

It is generally evident that the secondary metabolites are synthesized by plants to act as deterrents to animals. Lemon essential oil is distilled from the peels of *Citrus limonum*. It has a light yellow color and lemon aroma characteristics. Lemon essential oil contains several terpenes and geraniol, which have been shown to attract thrips,

fungi, gnats, mealybugs, scales, and beetles (Koul, Wilia and Dhaliwal, 2008). Rotenone from *Derris elliptica* was reported that it processed defensive activity against arthropods and pathogens (Sae-Yun, Ovatlarnporn, Itharat and Wiwattanapatapee, 2006). Numerous alkaloids were from several species of annona and neolignans from *Piper decurrens* (Chauret *et al*., 1996).

2.2.1 Classification and bioavailability of phenolic compounds

In certain plants, one main essential oil constituent will contain a cocktail of various terpenes. In *Ocimum basilicum* (basil), for example, methyl chavicol makes up 75% in the oil. Asarone of 70-80% are found in *Acorus calamus* rhizomes and linalool of 50-60% occurs in coriander seed and leaf oils (Lawrence and Reynolds, 2001). Most essential oils comprise of monoterpenes-compounds that contain 10 carbon atoms often arranged in a ring or in acyclic form, as well as sesquiterpenes which are hydrocarbons comprising of 15 carbon atoms (Table 2.1). Higher terpenes may also be present as minor constituents. The most predominant groups are cyclic compounds with saturated or unsaturated hexacyclic or an aromatic system, bicyclic (1,8-cineole) and acyclic (linalool, citronellal) (Koul, Wilia and Dhaliwal, 2008).

Table 2.1 The important classes of total phenolic compounds in plants.

Number of C - atoms	Basic skeleton	Class
6	C_6	simple phenols, benzoquinones
7	$C_6 - C_1$	phenolic acids
8	$C_6 - C_2$	acetophenone, phenylacetic acid
9	$C_6 - C_3$	hydroxycinnamic acid, polypropene, coumarin, isocoumarin
10	$C_6 - C_4$	naphtoquinone
13	$C_6 - C_1 - C_6$	xanthone
14	$C_6 - C_2 - C_6$	stilbene, anthrachinone
15	$C_6 - C_3 - C_6$	flavonoids, isoflavonoids
18	$(C_6 - C_3)_2$	lignans, neolignans
30	$(C_6 - C_3 - C_6)_2$	biflavonoids
N	$(C_6-C_3)_n$	lignins
	$(C_6)_n$	catecholmelanine
	$(C_6 - C_3 - C_6)_n$	(condensed tannins)

Recently, the bioactivity of the flavonoids from higher plants is of interest, at least in part, in the potential health benefits (Rice-Evans, Miller and Paganga, 1996). Flavonoids are plant pigments that are synthesized from phenylalanine, generally display marvelous colors known from flower petals, mostly emit brilliant fluorescence when they are excited by UV light, and are ubiquitous to green plant cells. They regulate plant growth by inhibition of the exocytosis of the auxin indolyl acetic acid, as well as by induction of gene expression. Flavonoids influence other biological cells in numerous ways as well.

Havsteen (2002) described that the flavonoids are very reactive compounds. They can enter into almost any type of reaction known to organic chemistry, e.g., oxidation-reduction reactions, carbonyl reaction, acid-base reactions, free-radical reaction, hydrophobic interactions, tautomery, and isomerisations. The substituents of flavonoids may also exert their influence by electronic induction, hyperconjugation, resonance, steric hindrance, and complexity with heavy metal ions. The basis of the great variability of the flavonoids difference in the ring structure of the aglycone and in its state of oxidation/reduction; differences in the extent of hydroxylation of the aglycone and in the positions of the hydroxyl groups; and differences in the derivation of the hydroxyl groups, e.g., with methyl groups, carbohydrates, or isoprenoids.
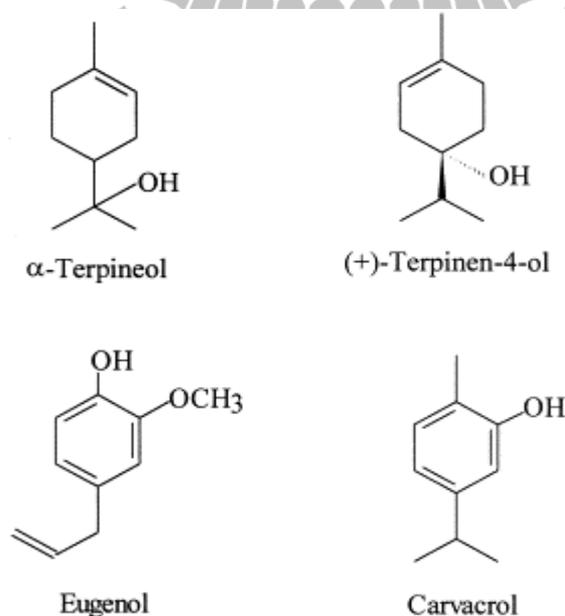
Figure 2.2 Chemical structures of some monoterpenes and related phenols with insecticidal activities (Isman, 2000).

Table 2.2 Chemical compositions in the essential oil of *Hyptis suaveolens* (Preezada, 1997).

Component	Percentage (%)	Component	Percentage (%)
1,8-Cineole	32.0	γ-Terpinene	0.7
β-Pinene	4.2	Myrcene	0.6
Sabinene	3.9	Aromadendrene	0.5
β-Caryophyllene	2.9	δ-Cadinene	0.5
α-Pinene	2.5	Fenchol	0.3
4-Terpinenol	2.3	α-Terpinolene	0.3
α-Phellandrene	2.0	α-Thujene	0.3
α-Bergamotene	2.0	α-Terpineol	0.2
α-Copaene	1.8	γ-Cadinene	0.1
α-Humulene	1.6	Linalool	0.06
Eugenol	1.2	Camphene	0.02
β-Elemene	1.0		

Some secondary metabolites in mintweed (*Hyptis suaveolens* (L.) Poit) extract were identified by gas chromatography-mass spectrometry (GC-MS). Twenty three compounds were found. The principal constituents in the essential oil of *H. suaveolens* are sabinene, limonene, biclyclogermacrene, β-phellandrene and 1, 8-cineole (Table 2.2) and some chemical structures are in Figure 2.2 (Preezada, 1997; Isman, 2000; Campos, Heleno, Tomas and Suzana, 2001).

The essential oil in leaves of *H. suaveolens*, grown in Nigeria, was analyzed by gas chromatography (GC) and GC-MS. There are 49 components which were detected. The dominant components were sabinene (16.5%), trans-α-bergamotene and β-caryophyllene (19.8%), terpinen-4-ol (9.6%) and β-pinene (8.6%) (Asekun and Ekundayo, 2000). The essential oils of *H. suaveolens* in vegetative and

fruiting stage collected from seven localities from Brazilian Cerrado were investigated by GC-MS. Sabinene, 1,8-cineole, spathulenol, (E)-caryophyllene and biclyclogermacrene were the principal constituents (Table 2.2) (Preezada, 1997). There are three distinguish groups in the essential oils which are α-terpinene/ terpin-4-ol/globulol/*epi-a*-cadinol, *o*-ocymene and β-ourbonene/germacrene B/(E)-caryophyllene. Geographical variation in essential oil composition indicated that sesquiterpenes are mainly produced in plants grown at lower latitudes and altitudes (Campos, Heleno, Tomas and Suzana, 2001).

Yam bean (*Pachyrhizus erosus* L.) is widely grown in the Northeast of Thailand. Yam bean seeds contain rotenone, cyanogenic glycosides, pachyrrhizine, pachyrrhizone, 12-(A)-hydropachyrrhizone, dolineone, dehydropachyrrhizone, erosone, neodehydrorautenone, erosenone, erosenin, 12-(A)-hydroxylinenone, and pachysaponin A & B (Figure 2.3) (Narongchai, Narongchai and Thampituk, 2005).
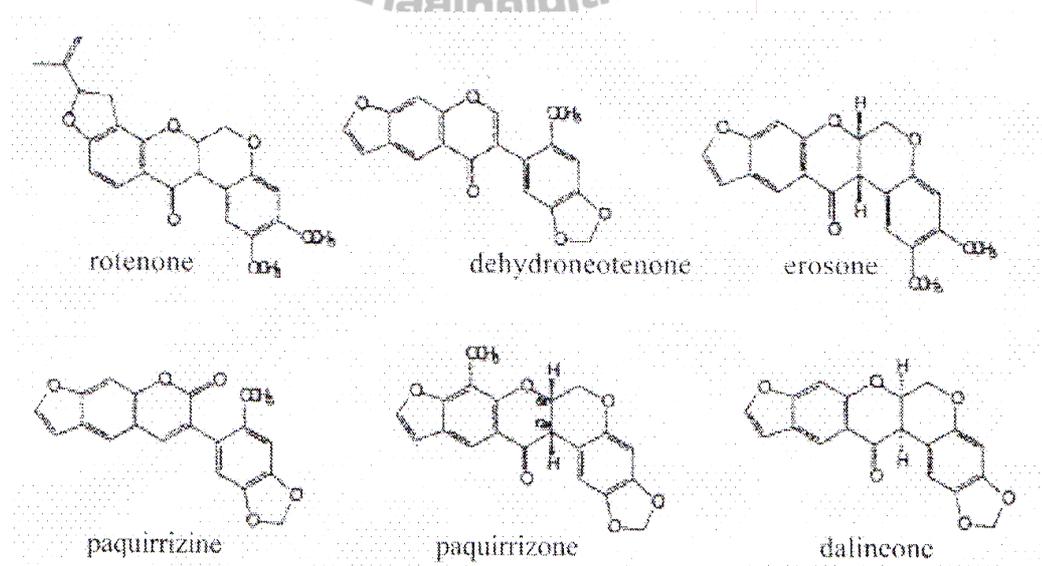
Figure 2.3 Chemical structure of constituents isolated from *P. erosus* (Laura, Silvia, Leticia and Francisco, 2004).

The modern and ancient native medical applications of selected plants quoted in these ethnohistorical sources are revisited and discussed under the current chemical and biological knowledge. These compounds elicit a broad spectrum of activities including acaricidal, antibiotic, anti-inflammatory, antioxidant, antifungal, antisecretory, antiserotonergic, choleretic, cytotoxic, herbicidal, insecticide, molluscicidal, spasmogenic, spasmolytic, and trypanosomal (BÉjar, Reyes-Shilpa and JimÉnez-Estrada, 2000).

Celery (*Apium graveolens* L.) seeds are traditionally used in remedy of urinary tract infections and reduce the degeneration of joints (Boiling, 2006). In addition, they are used as insect larvicides and repellants, including adult mosquito killers (Choochote *et al.*, 2004). The celery seeds contain a variety of bioactive constituents such as phthalides, coumarins, flavonoids, sesquiterpenoids, and aromatic glucosides (Figure 2.4) (Zhou *et al.*, 2009). The crude alcohol extract of celery seeds was fractionated by organic solvent separation, column chromatography (CC) and high-performance liquid chromatography (HPLC). Fractions were assayed for antimicrobial activity against the gastric pathogen, *Helicobacter pylori* and other bacteria (Zhou *et al.*, 2009), mosquitoes (Rafikali and Muraleedharan, 2001). Since the isolation of sedanolide and several other phthalides such as sedanenolide, 3-*n*-butylphthalide, cnidilide, neocnidilide, ligustilide, 3-isobutylidene-3a, and 4-dihydrophthalide have been reported from celery seed oil (Rafikali and Muraleedharan, 2001). The methanol extract of *Apium graveolens* seeds was investigated for bioactive compounds and resulted in the isolation and characterization of mosquitocidal, nematicidal, and antifungal compounds, sedanolide, senkyunolide-

N, and senkyunolide-J (Rafikali and Muraleedharan, 2001). The celery seeds have shown effects on treating bronchitis asthma, liver, and spleen diseases.
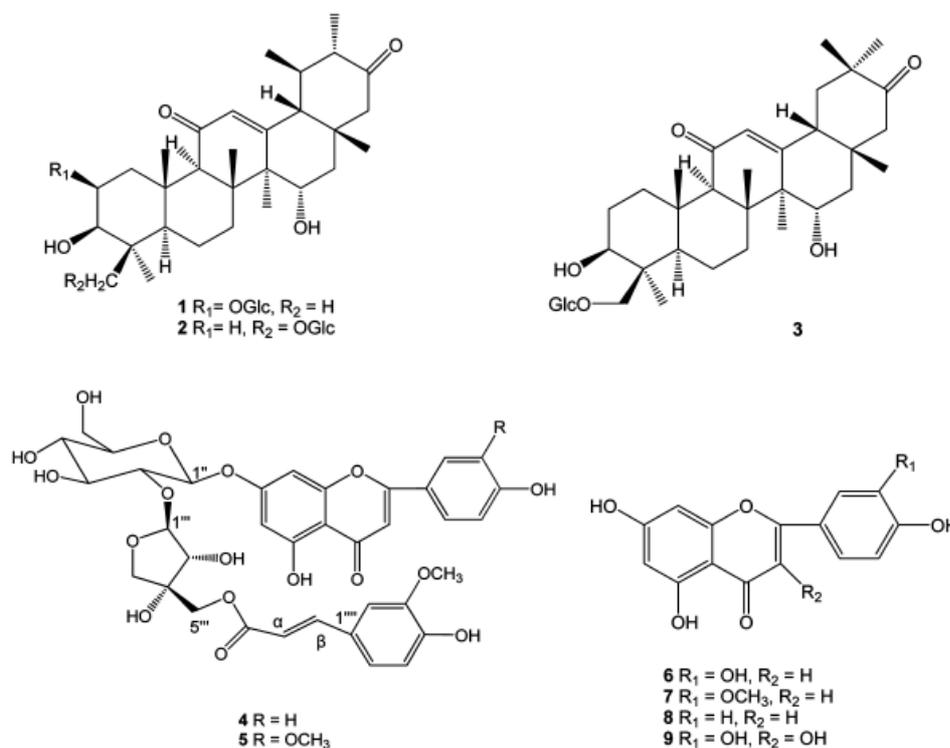
Figure 2.4 Some chemical compound found in celery (*A. graveolens*). The structures of the compounds were 11,21-dioxo-2β,3β,15α-trihydroxyurs-12-ene-2-O-β-d-glucopyranoside(1),11,21-dioxo-3β,15α,24-trihydroxyurs-12-ene-24-O-β-d-glucopyranoside (2), and 11,21-dioxo-3β,15α,24-trihydroxyolean-12-ene-24-O-β-d-glucopyranoside (3), and two new flavonoids, apigenin-7-O-[2″-O-(5‴-O-feruloyl)-β-d-apiofuranosyl]-β-d-glucopyranoside (4), chryso eriol-7-O- [2″-O-(5‴-O-feruloyl) -β-d-apiofuranosyl]-β-d-glucopyranoside (5), Luteolin (6), Isorhamnetin (7), Apigenin (8) and Quercetin (9) (Zhou *et al.*, 2009).

This study was aimed to investigate some phytochemicals in the water and ethanol extracts of mintweed, yam bean and celery seeds in order to determine their natural potential in biological control of mosquitoes. Some major chemical compounds of three seed extracts were identified by thin-layer chromatography (TLC).

2.3 Materials and methods

2.3.1 Chemicals

Gallic acid, sodium nitrite, sodium carbonate, aluminium trichloride, sodium hydroxide and catechin were obtained from Sigma (St. Louise, MO, U.S.A.). Absolute methanol, absolute ethanol and Folin-Ciocalteu's reagent were purchased from Carlo Erba Reagents (Strada Rivoltana, Spain). TLC plate of silica gel, 60 F-254 (thick 0.2 mm, 20 x 20 cm^2) was supplied by Merck (Darm-Stadt, Germany).

2.3.2 Seed collection and extraction

Mintweed seeds were collected at Suranaree University of Technology campus. Yam bean seeds were purchased from a farmer at a local farm in Som Had, Borabu, Mahasarakham. Celery seeds were obtained from a local market in Nakhon Ratchasima. The mintweed seeds were soaked in water and their mucilage was removed by squeezing against a stainless steel strainer. All seeds were cleaned, air dried and ground to powder. Ten grams of seed powder in a cellulose extraction thimble (Whatman International Ltd., Maidstone, England) were extracted in Soxhlet extraction apparatus (Buchi model B811, Germany) in 150 ml of water or 70% ethanol. The seed extracts were evaporated, dried and stored at -20 ^{o}C until used.

2.3.3 Total phenolic compounds (TPCs) measurement

The phenolic compounds are very important constituents in plants because of scavenging ability of the hydroxyl groups and preventing decomposition of hydroxyl peroxides into free radicals (Hatano *et al*., 1989; Gordon, 2001). The Folin-Ciocalteu method is a rapid and widely used assay to investigate the total phenolic compound content. Total phenolic compounds were measured according to Folin-Ciocalteu method (Matthaus, 2002). Gallic acid was used as a standard. A hundred microliter of samples was mixed with 2 ml of 2% Sodium carbonate and incubated for 2 min. A hundred microliter of Folin-Ciocalteu's reagent (Folin : Methanol, 1 : 1, v/v) was added, incubated for 30 min and measured at A_{750} nm. The amount of total phenolic compounds was expressed as gallic acid equivalent (mg GAE/g). The samples were assayed in triplicate.

2.3.4 Total flavonoid content (TFC) measurement

Total flavonoid content was measured by aluminum chloride colorimetric method (Chang, Yang, Wen and Chern, 2002). Catechin standard was dissolved in 70% ethanol. The catechin solution of 0.10 mg/ml was used to set up the standard curve. The sample of 250 µl was mixed with 75 µl of sodium nitrite, 1,250 µl of dH_2O. After incubation at room temperature for 6 min, 150 µl of 10% aluminum chloride was added and continued the incubation for 5 min and then 500 µl of sodium hydroxide was added. Distilled water of 275 µl was filled to adjust the net volume. The absorbance of the reaction mixture was measured at A_{510} nm. The samples were assayed in triplicate and the amount of the flavonoid content was expressed as catechin equivalent (mg CE/g).

2.3.5 Thin layer chromatography (TLC)

TLC method is commonly used in identical phytochemicals. It is rapid, simple, and versatile analysis. It is amenability to detection reagents and possible to of run several samples at a time (Andersen and Markham, 2006). The seed extracts of 0.50 µl were diluted in distilled water, spotted on a TLC plate, silica gel 60 F_{254} (2 x 7.4 cm^2) and then place in the solvent. The TLC system for mintweed seed extraction used the mobile phase systems of ethyl acetate : toluene : hexane (1 : 3 : 1) and plattoluene : chloroform : ethanol (9 : 3 : 4). The TLC system for yam bean seed extraction used the mobile phase systems of toluene : ethyl acetate : ethanol (6 : 5 : 3) and ethyl acetate : toluene : acetic acid (1 : 9 : 1). The TLC system for celery seed extraction used the mobile phase systems of toluene : chloroform : ethanol (5 : 7 : 2) and methanol : ethyl acetate : water (10 : 5 : 6). The TLC was run until the front line was about 6.4 mm below the edge of the plate. The TLC plate was air dried and developed with different solvent compositions. The plate was visualized under UV lamp at 254 nm. The migration of compounds in a given TLC system was described by R_f value as the following. To identify some major compounds in the extracts, the TLC gels were sprayed with Vanillin-sulphuric acid reagent and Kedde reagent. The Vanillin-sulphuric acid reagent (VS) produced pink-red and blue spots, while Kedde reagent produced violet-blue, red-violet and brown spots. The TLC plates then were heated at 100 ºC until color appeared (Cannell, 1998).

$$R_f = \frac{\text{Distance traveled by the center of substance spot from the origin (cm)}}{\text{Distance traveled by the solvent from the origin (cm)}}$$

where : R_f stands for ratio of front and its characteristics of any given compounds on each stationary phase using the appropriate mobile phase for the development of the plate.

2.3.6 Data analysis

Data from all experiments were analyzed with a two-way analysis of variance (ANOVA) using program Statistical Package for the Social Sciences (SPSS) program for Windows v.17.0. All analyzes were at 95% confident level.

2.4 Results

2.4.1 Total phenolic compounds (TPCs)

It was observed that different plants contained different amount of total phenolic compounds (TPCs) (Kähkonen *et al*., 1999). The TPCs were also different in plant types and parts. It appeared that the ethanol extracts of all seeds contained higher total phenolic compound content than water extracts (Figure 2.5).
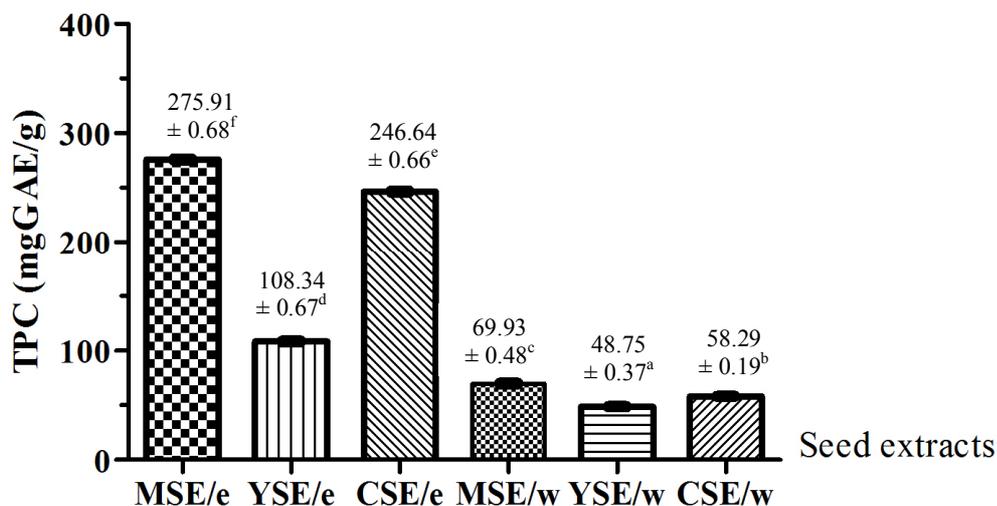
Figure 2.5 Total phenolic compounds of seed ethanol extracts of mintweed (MSE/e), yam bean (YSE/e), and celery (CSE/e) and of seed water extracts (MSE/w, YSE/w, and CSE/w).

The mintweed seed ethanol extract (MSE/e) processed had the highest TPCs of 275.91 mg GAE/g. The celery seed ethanol extract (CSE/e) contained the second most TPCs of 246.64 mg GAE/g. The yam bean seed ethanol extract (YSE/e) had the least amount of TPCs of 108.34 mg GAE/g. The amount of total phenolic compounds of seed water extracts was ranged from mintweed, celery, and yam bean which was 69.93, 58.29, and 48.75 mg GAE/g, respectively.

2.4.2 Total flavonoids contents (TFCs)

The total flavonoids contents of ethanol extracts of all plant seeds were higher than of water extracts. The TFCs in seed extracts were MSE/e>CSE/e>YSE/e and MSE/w>CSE/w>YSE/w (Figure 2.6). The amount of TFCs in MSE/e, CSE/e, and

YSE/e was 196.21, 185.43, and 95.16 mg CE/g; and in MSE/w, CSE/w, and YSE/w was 65.92, 41.81 and 37.85 mg CE/g dried weight, respectively.
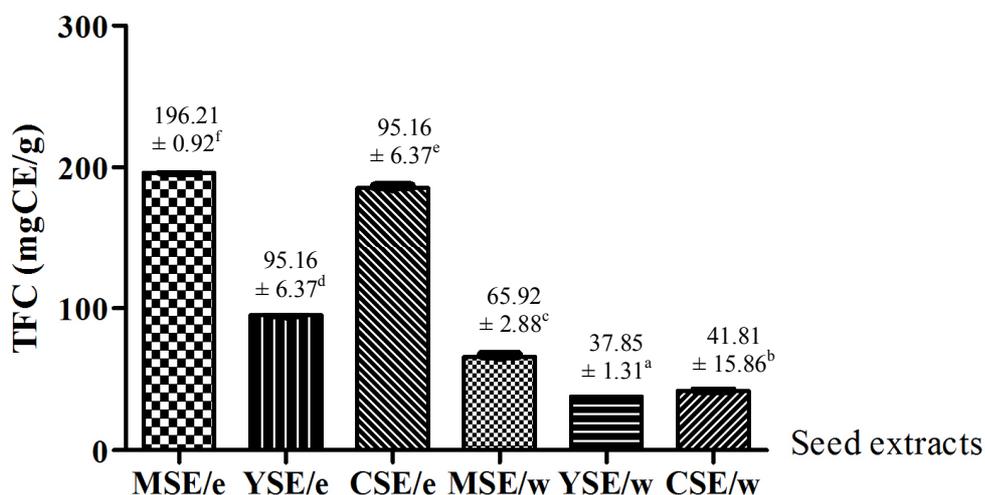
Figure 2.6 Total flavonoid contents of mintweed, yam bean, and celery seed ethanol extracts (MSE/e, YSE/e, and CSE/e); and seed water extracts (MSE/w, YSE/w, and CSE/w).

The quantities of the TPCs and the TFCs were compared. The TPCs of seed ethanol extracts were higher than of seed water extracts (Figure 2.7 and Table 2.3). The TPC of MSE/e was 1.4 fold higher than the TFC of MSE/e. Similarly, the TPC of CSE/e was 1.3 fold higher than the TFC of CSE/e. The rests were not much different.

The proportions TFCs/TPCs of seed ethanol extracts are presented in the Table 2.3. The MSE/e has also the highest TFC and TPC with ratio 0.71, followed by YSE/e and CSE/e have TFC and TPC with ratio 0.88 and 0.75, respectively. The MSE/w had the highest amount of TFC and TPC with ratio 0.94. The YSE/w showed

the amount of TFC and TPC greater than CSE/w which TFC/TPC ratio were 0.72, and 0.78.

In YSE/w the TFCs (37.85 mg CE/g) had a smallest value of all, and MSE/e (196.21 mg CE/g) had a greatest value of all with ratio 0.78 and 0.71, respectively; the ratio of YSE/w was slightly more than MSE/e.
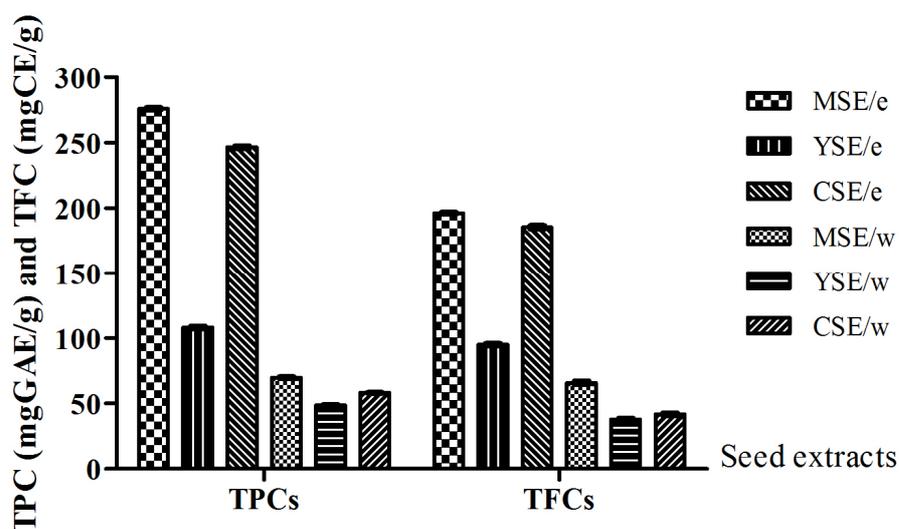
Figure 2.7 Comparison of the amounts of total phenolic compounds and total flavonoid contents among mintweed, yam bean, and celery seed ethanol extracts (MSE/e, YSE/e, and CSE/e) and seed water extracts (MSE/w, YSE/w, and CSE/w).

Table 2.3 The total phenolic compounds of the seed extracts of mintweed, yam bean, and celery.

Seed extracts	TPC mg GAE/g	TFC mg CE/g	TFC/TPC	P-value
MSE/e	275.91 ± 0.68^f	196.21 ± 0.92^f	0.71	0.00
YSE/e	108.34 ± 0.67^d	95.16 ± 6.37^d	0.88	0.00
CSE/e	246.64 ± 0.66^e	185.43 ± 11.54^e	0.75	0.00
MSE/w	69.93 ± 0.48^c	65.92 ± 2.88^c	0.94	0.00
YSE/w	48.75 ± 0.37^a	37.85 ± 1.31^a	0.78	0.00
CSE/w	58.29 ± 0.19^b	41.81 ± 15.86^b	0.72	0.00

Values are the mean of 6 observations, within a column followed by different letters are significantly different by Duncann's New Multiple Range Test, $P < 0.05$.

2.4.3 Thin layer chromatography (TLC) analysis

The TLC fingerprint of all seed extracts were analyzed in order to obtain its subtype compounds (Figures 2.8, 2.9 and 2.10). Vanillin-sulphuric spray gave pink, red and blue under spotlight, which indicated the presence of terpenes. TLC plate sprayed with Kedde reagent produced violet-blue, red-violet and brown spots under UV lamp. It indicated the presence of cyanogenic glycosides. The R_f values of TLC spots were 0.23-1.02 for all solvent systems, which were almost identical to the R_f value for standard compound (Table 2.4). However, there were other appropriate R_f and solvent systems, indicating the differences of the components. Moreover, the TPCs (blue) and TFCs (slight pink) measurement showed alteration of the color in wavelength and intensity of light through spectrophotometry. The detected coloration revealed by the solvent differed in extraction affected the presence of TPCs and TFCs, including functionality and secondary metabolite of natural products.
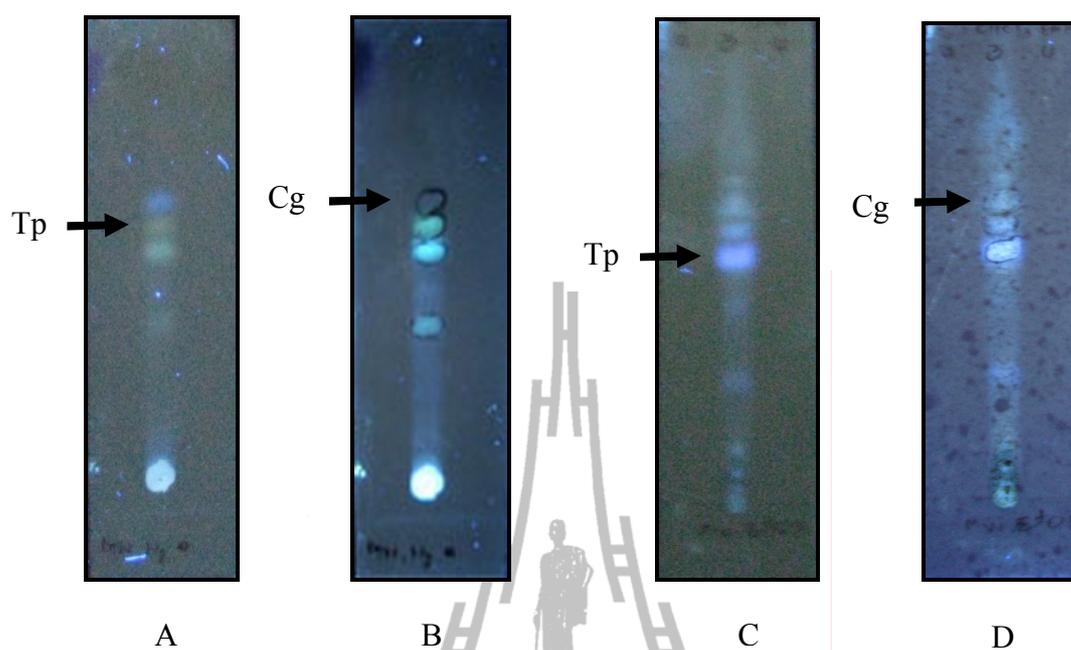
Figure 2.8 TLC chromatographs of mintweed water (A, B) and ethanol (C, D) seed extracts. The mobile phase systems were ethyl acetate : toluene : Hexane (1 : 3 : 1) (A, B) and toluene : chloroform : ethanol (9 : 3 : 4) (C, D). The TLC spots were likely to be terpenes (Tp) and cyanogenic glycosides (Cg) which A and C were analyzed by Vanillin-sulphuric acid reagent and B and D were analyzed by Kedde reagent.
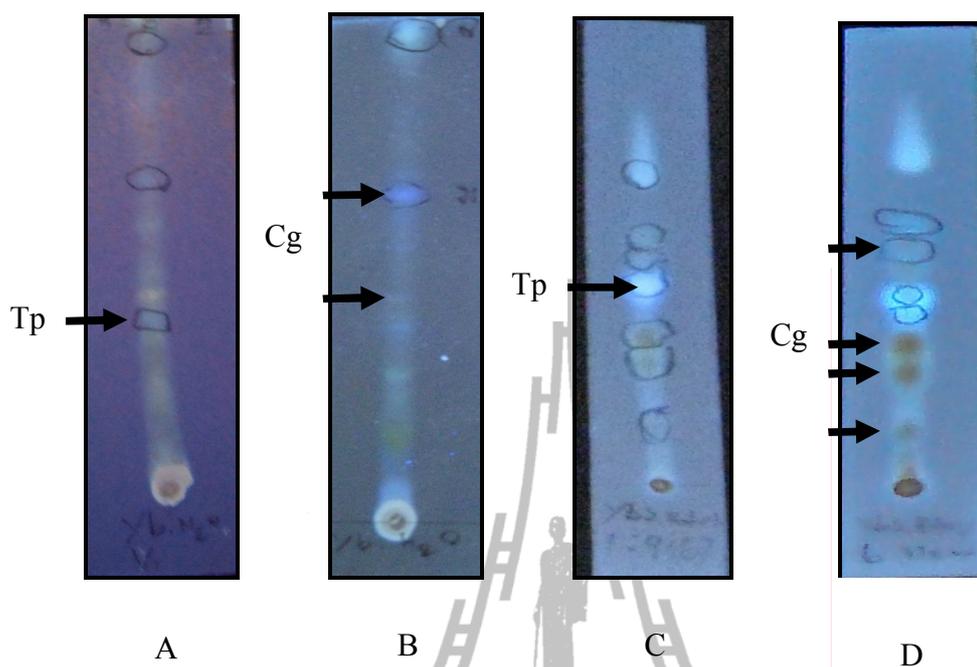
Figure 2.9 TLC chromatographs of yam bean water (A, B) and ethanol (C, D) seed

extracts. The mobile phase systems of toluene : ethyl acetate : ethanol (6 :

5 : 3) (A, B) and ethyl acetate : toluene : acetic acid (1 : 9 : 1) (C, D). The

TLC spots were likely to be terpenes (Tp) and cyanogenic glycosides (Cg)

which A and C were analyzed by Vanillin-sulphuric acid reagent and B

and D were analyzed by Kedde reagent.
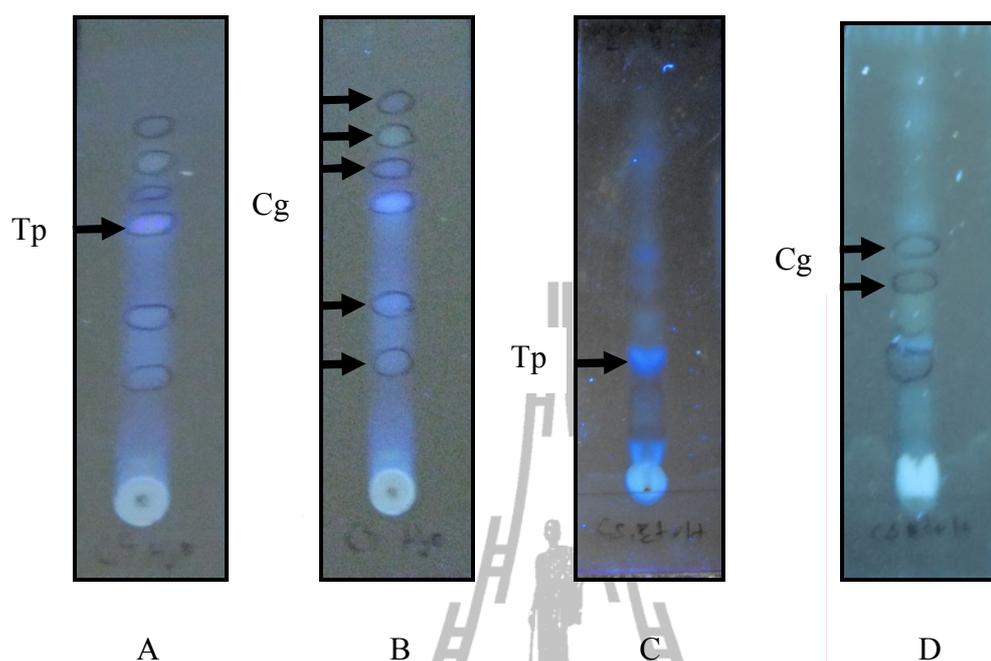
Figure 2.10 TLC chromatographs of celery water (A, B) and ethanol (C, D) seed extracts. The mobile phase systems of toluene : chloroform : ethanol (5 : 7 : 2), (A, B) and methanol : ethyl acetate : water (10 : 5 : 6) (C, D). The TLC spots were likely to be terpenes (Tp) and cyanogenic glycosides (Cg) which A and C were analyzed by Vanillin-sulphuric acid reagent and B and D were analyzed by Kedde reagent.

Table 2.4 Show evaluated R_f values and color of mintweed, yam bean, and celery seed

extracts, extracted with water and ethanol separated to mobile phase by TLC

method.

Seed extracts	R_f values and color components in seed extracts	
	Vanillin-sulphuric acid reagent	**Kedde reagent**
MSE/w	0.25 (ND) , 0.36 (LY), 0.41 (O), 0.61 (Bl)	0.20 (W), 0.30 (LG), 0.41 (LY), 0.45 (DBr)
MSE/e	0.63 (P), 0.72 (W), 0.75 (W)	0.64 (P), 0.70 (W) , 0.75 (W)
YSE/w	0.47 (DBl), 0.75 (Bl), 1.02 (Bl)	0.39 (LBl), 0.70 (BlV), 0.98 (Y)
YSE/e	0.12 (LBr), 0.23 (LBr), 0.31 (LBr), 0.40 (LBl), 0.50 (LBr), 0.57 (LBr), 0.72 (W)	0.12 (LV), 0.20 (DV), 0.33 (DV), 0.45 (LBl), 0.50 (DBl), 0.55 (LBr), 0.68 (LP)
CSE/w	0.23 (Bl), 0.36 (Bl), 0.55 (LBl), 0.61 (BlV), 0.67 (LG), 0.73 (BlV)	0.25 (Bl), 0.34 (Bl), 0.56 (P), 0.64 (DBlV), 0.70 (GBr), 0.75 (B)
CSE/e	0.23 (DBl), 0.28 (BlV), 0.38 (DBl), 0.45 (DV)	0.25 (DV), 0.30 (LBl), 0.41(DBr), 0.48 (LBr)

Note: color symbols were used; DBr = Dark brown, LBr = Light brown, Br = Brown, LG =
Light green, GBr = Green brown, G = Green, LY = Light yellow, Y = Yellow, DBl =
Dark blue, LBl = Light blue, Bl = Blue, DV= Dark violet, LV = Light violet, V = violet,
DBlV = Dark blue violet, BlV = Blue violet, O = Orange, and ND = Not detected.

2.5 Discussion

It is noticed that the different solvents in the extraction procedure affected the

contents and the subgroups of the extract constituents. This study showed that the

extracts of mintweed, yam bean, and celery seeds in 70% ethanol significantly

contained TPCs and TFCs higher than those extracted from water. Similarly, the TPCs

were higher than TFCs in all extracts. TLC was able to detect some phytochemical

constituents in the extracts to some certain extend. The major components of the extracts were analyzed by TLC. Vanillin-sulphuric reagent detected terpenes and Kedde reagent detected cardiac glycosides in the extracts of this study. It was in agreement with the universal spray that many terpenes gave red and blue colors and cyanogenic glycosides gave violet-blue, red-violet and brown (Cannell, 1998). It is suggested that plants produces different and several phytochemicals in their secondary metabolic pool. Measurement of color intensity or density can be achieved by summation of absorbance reading at 420 and 520 nm (Zoecklein, Fugelsang, Gump and Nury, 1995). Plants produce diverse chemical with respect to volatile monoterpene and phenylpropene content has been documented (Lewinsohn and Gijzen, 2009) in seeds by detecting TPCs and TFCs (Marinova, Ribarova and Atanassova, 2005; Yu, Zhou and Parry, 2005; Balasundram, Sundram and Samman, 2006). Yu, Zhou and Parry (2005) studied on cold-pressed black caraway, carrot, cranberry, and hemp seed oils extracted with methanol, the greatest TPC of 3.53 mg GAE/g was detected in the cold-pressed black caraway seed oil extract, while the lowest TPC of 0.44 mg GAE/g, was observed in the cold-pressed hemp seed oil extract. Similar to other previous reports on grape seed and skin, gallic acid, catechin (flavanols), and epicatechin were found mainly in the seeds (Yilmaz and Toledo, 2004). Phenolics consist of simple phenols and polyphenols (Marinnova, Ribarova and Atanassova, 2005). Polyphenolic substances are naturally presented in both vegetables and fruits, such as flavonoids which are the most diverse family of polyphenols (Andersen and Markham, 2006). Secondary plant metabolites can influence at critical points of biosynthetic pathways by stimulatory or inhibitory actions on enzyme activity, by changing in membrane permeability and substrate availability. Treutter (2006) showed strong evidences for a

role of flavonoids as multi-functions, which may influence plant physiology. Some may have toxic property which can use as insecticides (Shaalan, Canyon, Younes, Abdel-Wahab and Mansour, 2005). Some seed extracts interfere with the function of insect nervous systems and some have very low toxic effects on dogs, cats and humans. Some are toxic to most fish, birds, reptiles and amphibians (Varma and Dubey, 1998). In this study (chapter III), brine shrimps were used as an indicator for cytotoxicity test for the ecosystem. In brine shrimp lethality assay indicated that YSE/e was the highest toxic. It is in agreement with actions of rotenone and cyanogenic glycosides found in seeds (Hung *et al.*, 2007). TLC chromatograms indicated that YSE/e was very likely to process glycoside which, generally, it had hydrolysis process on hydrocyanic acid (HCN) or cyanide (CN) that caused the death of animals. YSE/e may be dissolved well with ethanol and sugar containing glycoside. It absorbed and distributed better than the other seed extracts. The CN absorbed into the body could destroy the cytochrome oxidase and enzyme in various tissues of insects.

2.6 Conclusion

It is concluded that the quantity of total phenolic compounds in three seed extracts of MSE, YSE, and CSE depends upon the extracted solvents. The MSE/e and the CSE/e contained high quantity of total phenolic compounds and flavonoids. Both YSE contained much low total phenolic compounds and flavonoids. However, all water seed extracts had low quantity of both total phenolic compounds and flavonoids. The major constituent compounds in all extracts separated by TLC were likely to be terpenes and cardiac glycosides which were known toxic to organisms. Therefore, the mintweed, yam bean, and celery seed extracts can be selective choices for further

study for the biological control of *Aedes aegypti*, the dengue hemorrhagic fever (DHF) carrier.

2.7 References

Andersen, O. M., and Markham, K. R. (2006). **Flavonoids; Chemistry, Biochemistry and Applications** (eds.). London, New York: Taylor and Francis Group.

Apak, R., Guclu, K., Demirata, B., Ozyurek, M., Celik, S. E., Bektasoglu, B., Berker, K. I., and Ozyurt, D. (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. **Molecules**. 12: 1496-1547.

Asekun, O. T., and Ekundayo, O. (2000). Essential oil constituents of *Hyptis suaveolens* (L.) Poit. (bush tea) leaves from Nigeria. **Journal of Essential Oil Research**. 12(2): 227-230.

Balasundram, N., Sundram, K., and Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. **Food Chemistry**. 99: 191-203.

BÉjar, E., Reyes-Shilpa, R., and JimÉnez-Estrada, M. (2000). Bioactive compounds from selected plants used in the XVI century Mexican traditional medicine. Abstract from: **Bioactive Natural Products (Part E)**. 24: 799-844.

Boiling, J. (2006). Celery and celery seed for urinary problems and gout. [On-line]. **Available**: http://everygreenherb.com/fromAtoZ/celery.html.

Campos, I. F. P., Heleno, D. F., Tomas, A. P., and Suzana, C. S. (2001). Chemical variability in essential oil of *Hyptis suaveolens*. **Phytochemistry**. 57: 733-736.

Cannell, R. J. P. (1998). **Natural Products Isolation**. New Jersey, The United States of America; Humana Press.

Chang, C. C., Yang, M. H., Wen, H. M., and Chern, J. C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. **Journal of Food Drug Analysis**. 10: 178-182.

Chauret, D. C., Bernard, C. B., Arnason, J. T., Durst, T., Krishnamurty, H. G., Sanchez-Vindas, P., Moreno, N., Roman, L. S., and Poveda, L. (1996). Insecticidal neolignans from *Piper decurrens*. **Journal Natural Products**. 59(2): 152-155.

Choochote,W., Tuetun, B., Kanjanapothi, D., Rattanachanpichai, E., Chaithong, U., Chaiwong, P., Jitpakdi, A., Tippawangkosol, P., Riyong, D., and Pitasawat, B. (2004). Potential of crude seed extract of celery, *Apium graveolens* L., against the mosquito *Aedes aegypti* L. **Journal of Vector Ecology**. 29(2): 340-346.

Chuenwong, P. (2006). **Biological control of oriental fruit flies (*Bactrocera dorsalis* (Hendel)) by the extracts of neem, sugar apple and mintweed**. Ph.D. Thesis, Suranaree University of Technology, Thailand.

Gordon, H. M. (2001). The development of oxidative rancidity in food. In Pokorny, J., Yanishlieva, N., and Gordon, H. M. (2001). **Antioxidants in food practical applications** (eds.) (pp. 7-21). Cambridge, United Kingdom.

Hatano, T., Edamatsu, R., Yasuhara, E., Mori, A., Fujita, Y., and Yasuhara, E. (1989). Effects of tannins and related polyphenols on superoxide anion radical and on DPPH radical. **Chemistry and Pharmacology Bulletin**. 37: 2016-2021.

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. **Pharmacol Therapeutics**. 96: 67-202.

Hung, Y. M., Hung, S. Y., Olson, K. R., Chou, K. J., Lin, S. L., Chung, H. M., Tung, C. N., and Chang, J. C. (2007). Yam bean seed poisoning mimicking cyanide intoxication. **Internal Medicine Journal**. 37: 130-132.

Isman, M. B. (2000). Plant essential oils for pest and disease management. **Crop Protection**. 19: 603-608.

Jackson, R. S. (2000). **Wine Science: Principle, practice, perception** (2nd ed.) (pp. 99-100). San Deigo, California, Academic.

Kähkonen, M. P., Hopia, A. I., Vuorela, H. J., Rauha, J. P., Pihlaja, K., Kujala, T. S., and Heinonen, M. (1999). Antioxidant activity of plant extracts containing phenolic compounds. **Journal of Agricultural and Food Chemistry**. 47: 3954-3962.

King, A., and Young, G. (1999). Characteristics and occurrence of phenolic phytochemicals. **Journal of the American Dietetic Association**. 99: 213-218.

Koul, O., Wilia, S., and Dhaliwal, G. S. (2008). Essential oils as green pesticides: Potential and constraints. **Biopesticides International**. 4(1): 63-84.

Laura, L. B. N., Silvia, B. B., Leticia, B. L., and Francisco, J. L. G. S. (2004). Antifungal activity of seed powders, extracts, and secondary metabolites of *Pachyrhizus erosus* (L.) Urban (Fabaceae) against three postharvest fungi. **Revista Maxicana de Fitopatologia**. 22(3): 356-361.

Lawrence, B. M., and Reynolds, R. J. (2001). Progress in essential oils. **Perfect Flavour**. 26: 44-52.

Leland, J. C., Ara, K., Peter, B. K., Sara, L. W., James, A. D., and Harry L. B. (2006). **Natural products from plants** (2nd eds.). London, United Kingdom: Taylor and Francis Group.

Lewinsohn, E., and Gijzen, M. (2009). Phytochemical diversity: The sounds of silent metabolism. **Plant Science**. 176(2): 161-169.

Marinova, D., Ribarova, F., and Atanassova, M., (2005). Total phenolics and total flavonoids in Bulgarian fruits and vegetables. **Journal of the University of Chemical Technology and Metallurgy**. 40(3): 255-260.

Matthaus, B. (2002). Antioxidant activity of extracts obtained from residues of different oilseeds. **Journal of Agricultural and Food Chemistry**. 50: 3444-3451.

Narongchai, P., Narongchai, S., and Thampituk, S. (2005). The first fatal case of yam bean and rotenone toxicity in Thailand. **Journal of the Medical Association of Thailand**. 88(7): 984-987.

Okwu, D. E., and Ukanwa, N. (2010). Isolation, characterization and antibacterial activity screening of anthocyanidine glycosides from *Alchornea cordifolia* (Schumach. and Thonn.) mull. Arg. leaves. **E-Journal of Chemistry**. 7(1): 41-48.

Peerzada, N. (1997). Chemical composition of the essential oil of *Hyptis suaveolens*. **Molecules**. 2(11): 165-168.

Rafikali, A. M., and Muraleedharan, G. N. (2001). Mosquitocidal, nematicidal, and antifungal compounds from *Apium graveolens* L. seeds. **Journal of Agricultural and Food Chemistry**. 49: 142-145.

Rice-Evans, C., Miller, N. J., and Paganga, G. (1996). Abstract from: Structure-antioxidant activity relationships of flavonoids and phenolic acids. **Free Radical Biology and Medicine**. 20(7): 933-956.

Sae-Yun, A., Ovatlarnporn, C., Itharat, A., and Wiwattanapatapee, R. (2006). Extraction of rotenone from *Derris elliptica* and *Derris malaccensis* by pressurized liquid extraction compared with maceration. **Journal of Chromatography A**. 1125: 172-176.

Shaalan, E. A. S., Canyon, D., Younes, M. W. F., Abdel-Wahab, H., and Mansour, A. H. (2005). Synergistic efficacy of botanical blends without synthetic insecticide against *Aedes aegypti* and *Culux annulirostris* mosquitoes. **Journal of Vector Ecology**. 30(2): 284-288.

Tanprasit, P. (2005). **Biological control of dengue fever mosquitoes (*Aedes aegypti* L.) using leaf extracts of chan (*Hyptis suaveolens* (L.) Poit) and hedge flower (*Lantana camara* L.)**. Master Thesis, Suranaree University of Technology, Thailand.

Treutter, D. (2006). Significance of flavonoids in plant resistance : A review. **Environmental Chemistry Letters**. 4: 147-157.

Varma, J., and Dubey, N. K. (1998). Perspectives of botanical and microbial products as pesticides of tomorrow. [On-line], **Available**: http://www.iisc.ernet.in/ currsci/jan25/articles22.htm.

Woo, H. H., Jeong, B. R., and Hawes, M. C. (2005). Flavonoids : From cell cycle regulation to biotechnology. **Biotechnology Letters**. 27(6): 365-374.

Yilmaz Y., and Toledo, R. T. (2004). Health aspects of functional grape seed constituents. **Trends in Food Science and Technology**. 154: 22-33.

Yu, L., Zhou, K., and Parry, J. (2005). Antioxidant properties of cold-pressed black carraway, carrot, cranberry, and hemp seed oils. **Food Chemistry**. 91: 723-729.

Zhou, K., Zhao, F., Liu, Z., Zhuang, Y., Chen, L., and Qiu, F. (2009). Triterpenoids and flavonoids from celery (*Apium graveolens*). **Journal of Natural Products**. 72: 1563-1567.

Zoecklein, B. W., Fugelsang, K. C., Gump, B. H., and Nury, F. S. (1995). **Wine Analysis and Production**. New York: Chapman and Hall.
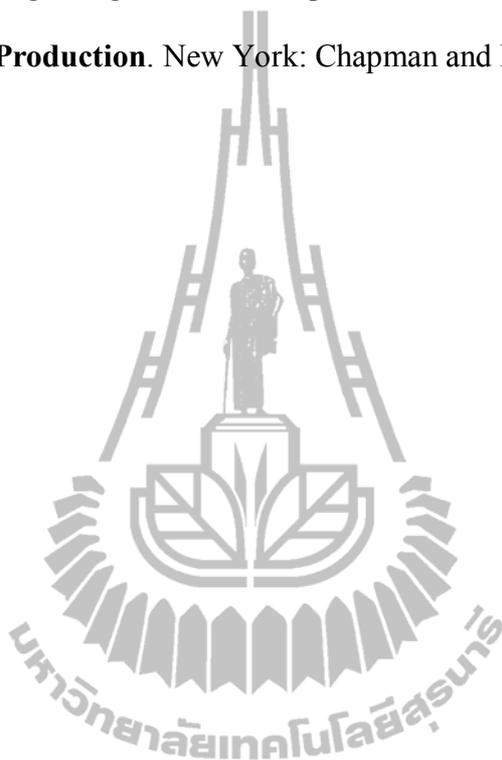
CHAPTER III

CYTOTOXICITY AND BIOLOGICAL CONTROL OF HEMORRHAGIC FEVER MOSQUITOES (*AEDES AEGYPTI* L.) BY MINT WEED (*HYPTIS SUAVEOLENS* (L.) POIT) YAM BEAN (*PACHYRHIZUS EROSUS* L.) AND CELERY (*APIUM GRAVEOLENS* L.) SEED EXTRACTS

3.1 Abstract

Mintweed (*Hyptis suaveolens* (L.) Poit) (MSE), yam bean (*Pachyrhizus erosus* L.) (YSE), and celery (*Apium graveolens* L.) (CSE) seeds, extracted in 70% ethanol and water, were investigated for the effects on the biological control of *Aedes aegypti* L. The activities of the extracts were dependent on the plant species and extracted solvents and very broad magnitude from nanogram to milligram level. The cytotoxicity of the extracts were observed by brine shrimp (*Artemia salina* L.) lethality assay (BSLA). The LC_{50} at 24 h of the YSE/e and the YSE/w on brine shrimps was 0.02 µg/ml and 257.11 µg/ml. The extract individuals and paired combinations (1 : 1, v/v) were applied to the 2nd instar larvae and the adult mosquitoes at 24 h The YSE/e was the most potent in controlling the 2nd instar larvae with LC_{50} 16.22 µg/ml and the CSE/w was the least with LC_{50} 25.23 mg/ml. The extract efficacy was ranged as YSE/e>YSE/w>MSE/e>CSE/e>MSE/w>CSE/w. The extract sprayed into the adult rearing case was able to control the mosquitoes. The most toxic

effect was produced by the YSE/e with LC_{50} 91.41 µg/ml and the least effect was by the CSE/w with LC_{50} 109.03 mg/ml. The efficacy was ranged as YSE/e>MSE/e> YSE/w>CSE/e>MSE/w>CSE/w. The effects of the seed extract combinations on the 2nd instar larvae and the adult mosquitoes were evaluated. The YSE enhanced the mortality to the other extracts. The combination of YSE/e and MSE/e induced highest efficacy with LC_{50} 11.79 µg/ml on the control of the 2nd instar larvae and LC_{50} 23.82 µg/ml on the adults. The MSE/w and CSE/w combination produced least LC_{50} 12.32 mg/ml. The efficacy of all combinations was ranged as MSE/e + YSE/e>CSE/e + MSE/e>YSE/e + CSE/e>YSE/w + CSE/w>MSE/w + YSE/w>CSE/w + MSE/w. Thus, these three seed plant extracts are possible candidates for the control of *Ae. aegypti*, the important dengue virus carrier of the dengue hemorrhagic fever, one of the deadly diseases in Thailand. However, a further research of the yam bean seed ethanol extract is essential for the safe application in controlling mosquitoes and other insect pests.

3.2 Introduction

Aedes aegypti L. is the important vector of dengue virus which transmits dengue hemorrhagic fever (DHF) to human beings. DHF is a serious deadly disease in most tropical regions. The outbreak of DHF repeatedly appears as a cyclic of every 2-3 years. In Thailand, DHF continues to be one of the major public concerns since its incidence had greatly increased from 46,829 to 76,059 cases during 2006-2008. (Martínez, Rojas, Valdés and Noa, 2005; WHO, 2008). There was a report that during January-July, 2010 the incident rate of Dengue fever was 71.5 per 100,000 and the mortality rate was 0.07 per 100,000 (Bureau of Epidemiology, Department of Disease

Control, Ministry of Public Health, Thailand, 2010). The re-emergence of DHF is primarily related to the *Ae. aegypti* reproduction. To avoid mosquito bite, mosquito nets are traditionally used. While to control or get rid of them physically, catch and kill or using some plant parts are normally practiced. Botanical insecticides are widely used in mosquito eradication, such as pyrethrins, carvacrol, quassia, nicotine, allicin, anabasine, azadirachtin, d-limonene and triptolide, before the introduction of synthetically organic insecticides of pyrethroids and organophosphates (Wood, 2010). However, the synthetic insecticides causes mosquito resistance, and adverse effects to non-target organisms and environment (Stenersen, 2004; Sommerville and Glasgow, 2006; Mongkalangoon, Grieco, Achee, Suwonkerd and Chareonviriyaphap, 2009). Therefore, natural products from plants and bacterium have been researched to overcome the mosquito population and sustain the environment. *Bacillus thuringiensis* which produces crystal endotoxin has been introduced in the control of insect pests, including mosquitoes (Lee *et al*., 2008). Natural plant products are degradable in a short period of time and there are no toxic residues left in the environment. New phytochemicals as alternatives for controlling *Ae. aegypti* have been searched (Chaiyasit *et al*., 2006; Gillij, Gleiser and Zygadlo, 2008; Silva *et al*., 2008).

A few studies on the effects of natural products extracted from plant seeds in the control of mosquitoes. *Hyptis suaveolens* (L.) Poit local name maeng luk kha, is an aromatic weed which possesses some medicinal properties used in the treatment of gastrointestinal infection, cramps and pain as well as in the treatment of skin infections (Wulff, 1987; Attawish *et al*., 2005). Its seeds had insecticidal activity. (Amusan, Idowu and Arowolo, 2005; Iloba and Ekrakene, 2006; Keita, Umoetok and Smith 2006; Niwatananun, Niwatananun, Lertprasertsook and Okonogi, 2006).

Apium graveolens L. local name khuen chaai, is an aromatic and spicy vegetable. Its seeds provide a remedy for urinary tract infections and degeneration of joints (Boiling, 2006). They repel insects, including larvae, and adult mosquitoes (Choochote *et al*., 2004). *Pachyrhizus erosus* L. local name mun kaew, is a tuber-root climbing plant. Its tubers are edible, but its seeds process potent antitumor activity (Morales-Arellano, Chagolla-López, Paredes-López and Barba, 2001; Hung *et al*., 2007) and toxic property to insects, fishes, rats, and fungi (Barrera-Necha *et al*., 2004; Stenersen, 2004). This study aimed to explore activity of the seed extracts of *H. suaveolens*, *P. erosus* and *A. graveolens* on the biological control of *Ae. aegypti* mosquitoes, emphasis on the second instar larvae and adults.

3.3 The general background of *Artemia salina* L. and its importance

Brine shrimp (*Artemia salina* L., Artemiidae) is a small marine crustacean. It is found worldwide such as inland salt lakes and pans, coastal lagoons, and salt works (Kaiser, Gordon and Paulet, 2006). Twenty-five geographical strains have been studied. The genetic variations demonstrate the differences in geographical origin among various strains such as *A. franciscana*, *A. tunisiana*, *A. urmiana*, *A. monica*, *A. persimilis*, and *A. parthenogenetica* (Mona, 2010). Artemia population distributes in lakes upon four factors, i.e., salinity, carbonate, oxygen, and carbon dioxide contents. These factors are related to the seasonal variations that lead to differences in tolerant ranges. The salinity ranges from 5% to 120% and the temperature ranges from 18 $^{\circ}$C to 34 $^{\circ}$C. (Vanhaecke, Siddall and Sorgeloos, 1984).

Hatching of Artemia in fresh artificial seawater takes 24 h at room temperature. The newly hatched, free-swimming, pink-coloured nauplii develop into larvae and

adults (Figure 3.1). However, Artemia has short life span (Artemia Reference Center, 2007). Artemia is used as a feed for the growth and development of fish and crustacean, because of its enriched nutrients.

Figure 3.1 Artemia cysts for hatching (Tropical company, 2007) and adults (Forum

Biologia Marina-Mare Mediterraneo, 2006).

Artemia cultivation is simple, rapid, and inexpensive, it is valued as a test organism in studies on cytotoxicity for various living and non-living subjects. The brine shrimp lethality assays were used to determine the cytotoxicity of many plant extracts before approaching other research aspects (Krishnaraju *et al*., 2005; Chuenwong, 2006).

3.4 Materials and methods

3.4.1 Materials

Mintweed (*Hyptis suaveolens*) seeds were collected at Suranaree University of Technology (SUT) campus. Yam bean (*Pachyrhizus erosus*) and celery (*Apium graveolens* L.) seeds were purchased from a local farm in Mahasarakham and

Nakhon Ratchasima provinces, Northeast Thailand. Dimethylsulfoxide (DMSO) was purchased from Merck (Darm-Stadt, Germany). Ethanol was from BDH Chemical Limited. (Poole, Dorset, BH15 1TD, England). *Aedes aegypti* eggs were a gift from Communicable Disease Control Centre Zone 6, Ministry of Public Health, Khon Kaen province, Thailand.

3.4.2 Seed collection and extraction

H. suaveolens seeds were soaked in water and removed their mucilage before extraction. The naked seeds were cleaned, air dried and ground to powder. *P. erosus* seeds were purchased from the farmer at a local farm in Som Had, Borabu, Mahasarakham. *A. graveolens* seeds were obtained from the market in Nakhon Ratchasima. All seeds were cleaned, air dried and ground to powder. Ten grams of the seed powder in a cellulose extraction thimble (Whatman International Ltd., Maidstone, England) were extracted in 150 ml of water or 70% ethanol in Soxhlet extraction apparatus (Buchi model B811, Germany). The extracts were evaporated, dried and stored at -20 ^{o}C until used.

3.4.3 Mosquito rearing

Ae. aegypti was reared at Building F9, the Center for scientific and Technological Equipment, SUT by rearing standard techniques of World Health Organization (WHO). The eggs on a filter paper were placed on a pottery bowl containing water and the bowl was in a steel-net wire-cage ($50 \times 50 \times 50$ cm^3) and allowed to hatch at 26 ^{o}C and 75% relative humidity. The larvae were transferred into enamel trays ($30 \times 21 \times 5$ cm^3) and fed with ground rat food (Figure 3.2). The adults were maintained in a cage ($80 \times 80 \times 80$ cm^3), the males were fed with syrup containing vitamins and the females were fed with guinea pig blood.
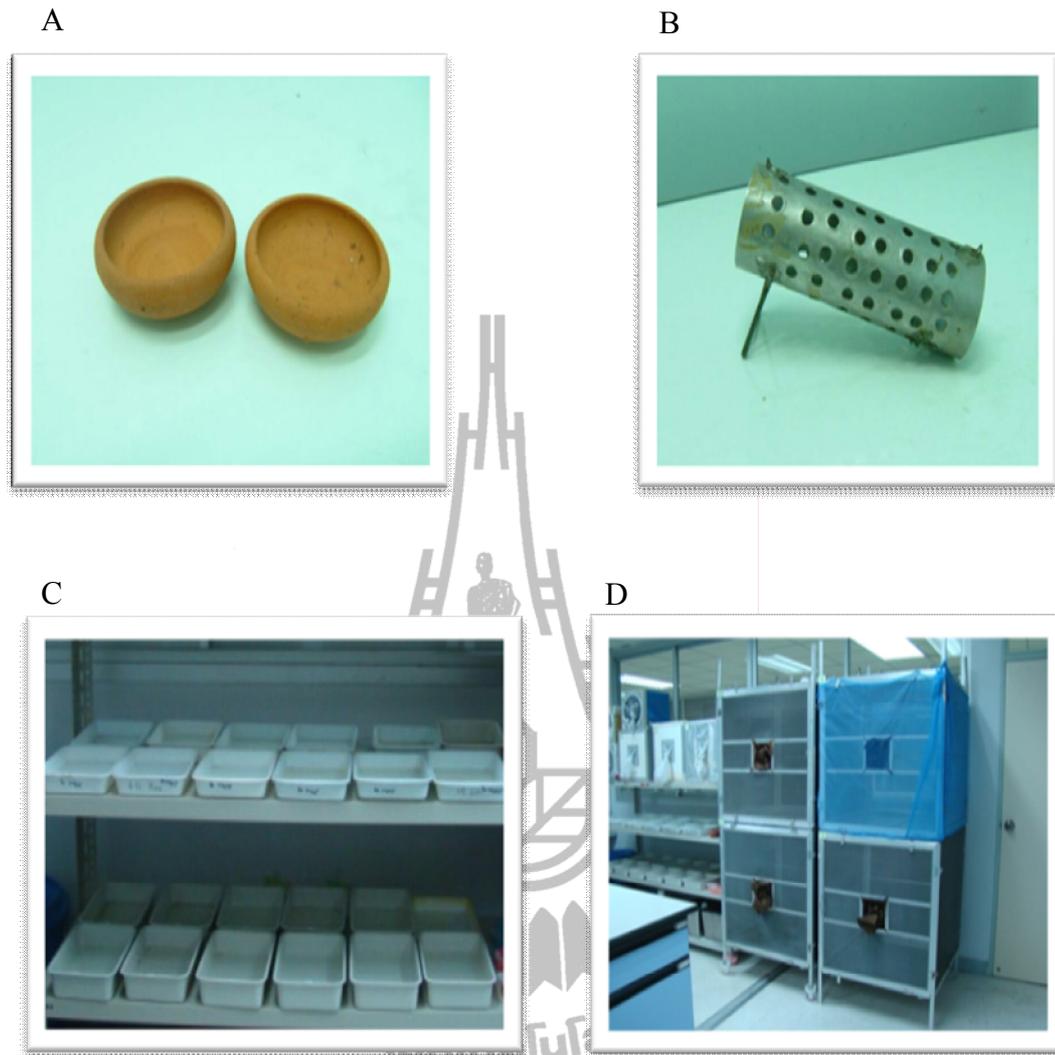
Figure 3.2 *Aedes aegypti* rearing: The mosquitoes eggs in pottery bowl (A), guinea pig

trap (B), larvae in enamel trays (C) and steel-wire net-cages (D).

3.4.4 Brine shrimp lethality assay (BSLA)

Brine shrimp (*Artemia salina* L.) eggs were hatched and reared in artificial seawater (120 g/l sea salt) under continuous light, at 25 $^{\circ}$C for 24 h (Briski, Van, Bossier and Sorgeloos, 2008). Ten nauplii were transferred into a 24-well plate containing 200 μl of artificial seawater. The seed extracts of 0-3,500 μg/ml were added and cultured for 12 h 0.1% DMSO v/v and 1 μg/ml pyrethroid were used as controls.

Number of dead (non-motile) nauplii were counted and calculated for mortality. The median lethal concentration (LC_{50}) was analyzed by Probit analysis.

3.4.5 Larval mortality test

The extract powder was dissolved in water or 0.1% DMSO v/v. Fifty number of 2nd instar larvae were gently transferred into 10 ml of water in a-20 ml vial and treated with 0-100 mg/ml extracts for 24 h Single or combination (1 : 1, v/v) of the extracts were tested. DMSO and 5 µg/ml pyrethroid were used as controls. The tests were performed in triplicate and was done twice. The dead larvae were counted, calculated for mortality and analyzed for LC_{50} values.

3.4.6 Adult mortality test

The extracts were dissolved in 0.1% DMSO. Fifty newly emerged mosquitoes were transferred into a steel-wire net cage ($30 \times 30 \times 30$ cm^3). The first spray was performed with 5 ml of various concentrations of the extracts through the cage door (Figure 3.3). The cage was covered with plastic sheets at all sides. After 12 h of the first spray, the second spray with 5 ml of the same extracts was followed. The dead mosquitoes were counted after 12 h of the second spray. Single or combination (1 : 1, v/v) of the extracts were applied. 0.1% DMSO and 20 µg/ml pyrethroid were used as controls. The tests were performed in triplicate and was done twice. The mortality was corrected by Abbott's formula. The mortality was calculated and analyzed for the LC_{50} by Probit analysis.

A

B
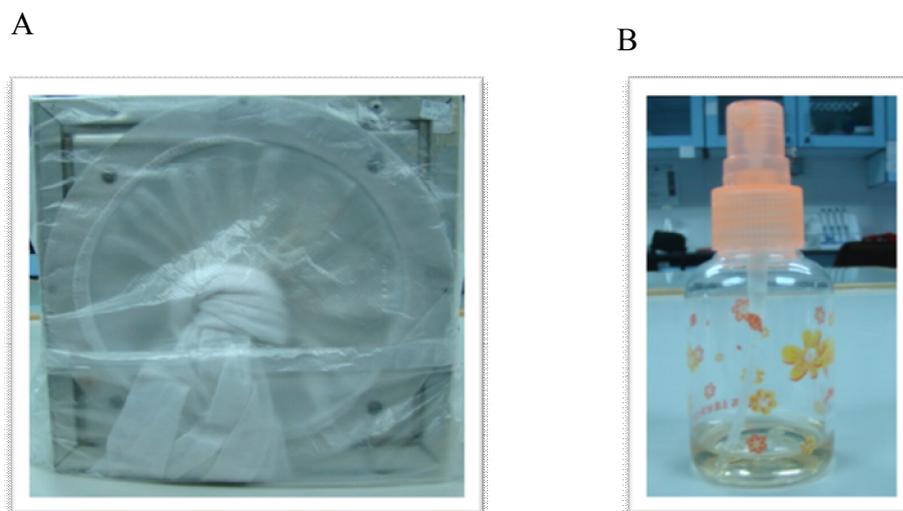
Figure 3.3 The steel-wire net-cage for adult spraying (A) and sprayer bottle (B).

3.4.7 Data analysis

All data were analysis of variance (ANOVA) and in completely randomized design (CRD) using Statistical Package for the Social Sciences (SPSS) program for Windows v.17. The means were compared using Duncan's Multiple Range Test (DMRT). The mortality was corrected by Abbott's formula (1925).

$$\% \text{ Mortality} = \frac{\% \text{ Test mortality-}\% \text{ Control mortality}}{100\text{-}\% \text{ Control mortality}} \times 100$$

The LC_{50} value was determined by Probit analysis (Finny, 1971).

3.5 Results

3.5.1 Cytotoxicity

The cytotoxicity of the seed extracts of mintweed, *Hyptis suaveolens* (MSE), yam bean, *Pachyrhizus erosus* (YSE), and celery, *Apium graveolens* (CSE) in

water and ethanol were observed by BSLA at 24 h. However, the toxicity of all the seeds extracts and solvents was not directly compared on its individual effects since the scales of effective concentrations and the magnitudes of activities were broadly different in solvent- and dose dependent manner. It appeared that all ethanol extracts were much more toxic than water extracts (Table 3.1). The YSE was the most toxic for both water and ethanol extracts. The YSE/e in particular causes 100% death of brine shrimps at the concentration of only 1 μg/ml, while the 1,000 μg/ml YSE/w, which was thousand fold of concentration, could only cause death by 85%. The MSE/w was required approximately 10 fold to obtain similar effect to the MSE/e. Even though, the highest activity was about 60-68% mortality with 1,500 and 1,000 μg/ml of the MSE/w and the MSE/e, respectively. The toxicity of CSEs on brine shrimps was much divert. The 3,000-μg/ml CSE/w caused 35% death, while the 100-μg/ml CSE/e caused 65% death.

Table 3.1 Cytotoxicity of seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE), determined by brine shrimp lethality assay (BSLA).

Conc. µg/ml	% Mortality Mean ± S.E.	LC$_{50}$ µg/ml, 24 h	Conc. µg/ml	% Mortality Mean ± S.E.	LC$_{50}$ µg/m, 24 h
MSE/w			MSE/e		
10	1.67 ± 0.21^a	1,478.91 ± 0.90	1	1.67 ± 0.17^a	506.23 ± 0.26
50	3.33 ± 0.33^a		10	5.00 ± 0.22^{ab}	
100	5.00 ± 0.22^a		50	11.67 ± 0.17^{bc}	
1,000	16.67 ± 0.49^b		100	15.00 ± 0.22^c	
1,500	60.00 ± 0.26^c		1,000	68.33 ± 0.60^d	
YSE/w			YSE/e		
1	3.33 ± 0.21^a	257.11 ± 0.29	0.001	10.00 ± 0.26^a	0.02 ± 0.24
10	11.67 ± 0.31^b		0.01	40.00 ± 0.26^b	
50	21.67 ± 0.40^c		0.1	78.33 ± 0.31^c	
100	25.00 ± 0.43^c		1	100.00 ± 0.00^d	
1,000	85.00 ± 0.22^d		10	100.00 ± 0.00^d	
CSE/w			CSE/e		
1,500	5.00 ± 0.22^{ab}	3,134.45 ± 0.80	1	3.33 ± 0.21^a	72.30 ± 0.75
2,000	11.67 ± 0.31^{bc}		10	15.00 ± 0.50^b	
2,500	21.67 ± 0.48^c		50	35.00 ± 0.76^c	
3,000	35.00 ± 0.43^d		100	65.00 ± 0.72^d	
3,500	73.33 ± 0.42^e		1,000	100.00 ± 0.00^e	
Pyr-1 µg/ml	100.00 ± 0.00^d	0.007 ± 0.18			
0.1%-DMSO	0.00	0.00			

Values are the mean of 6 observations, within a column followed by different letters are significantly different by Duncan multiple range test (DMRT) (P<0.05). Pyr = pyrethriod; n = 6.

The cytotoxicity of all extracts in brine shrimps could be comparable by the LC_{50} values. The toxicity of ethanol seed extracts, determined by LC_{50} at 24 h were YSE/e>CSE/e>MSE/e and of water extracts was YSE/w>MSE/w>CSE/w (Figure 3.4). The YSE/e processed highest cytotoxicity with LC_{50} of 0.02 µg/ml, while the YSE/w had LC_{50} of 257.11 µg/ml which was over 10^5 fold less toxic (Table 3.1). In addition, it was noticed that the YSE/e caused 100% mortality of brine shrimp within 12 h (data not shown). The CSE/e produced cytotoxicity with LC_{50} of 72 µg/ml which was 43 fold more toxic than the CSE/w which had LC_{50} of 3,135 µg/ml. The MSE/e cytotoxicity was approximately 3 fold of MSE/w. In summary, the cytotoxicity was YSE/e>CSE/e>YSE/w>MSE/w>MSE/w>CSE/w.
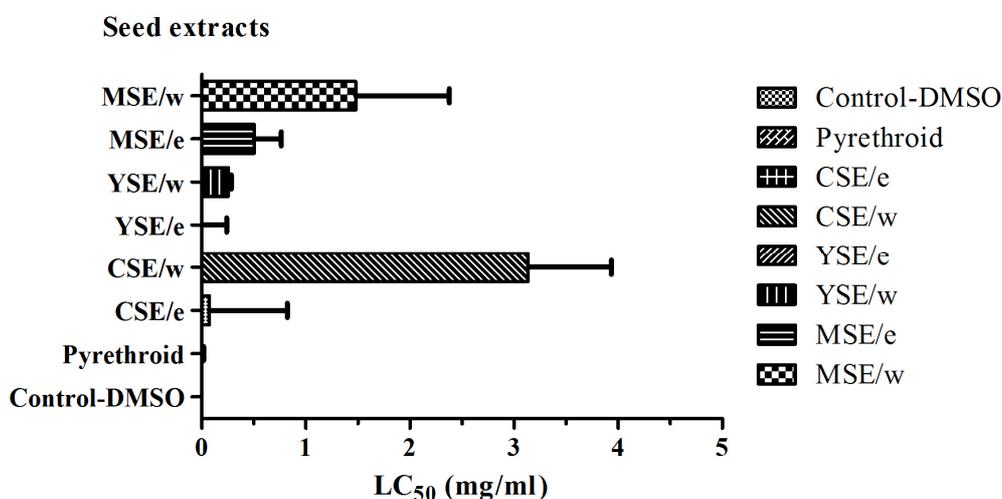
Figure 3.4 Cytotoxiciti LC_{50} values of seed extracts of *Hyptis suavelens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE), determined by brine shrimp lethality assay (BSLA).

3.5.2 Effects on mosquito larvae

The 2nd instar larvae of mosquitoes were treated with individual extracts of *H. suaveolens* (MSE), *P. erosus* (YSE) and *A. graveolens* (CSE) for 24 h. The effects of the extracts were dose dependent and again the magnitudes of doses were widely broad. The YSEs were most toxic to the mosquito larvae, YSE/e at 30 μg/ml caused 63% larval death and YSE/w at 1,000 μg/ml caused 70% (Table 3.2). The MSE/e was much higher toxic to the larvae; the same concentration at 8,000 μg/ml, the MSE/e produced 80% death, but the MSE/w produced only 14% death which was 5.7 fold lower. Similarly, the CSE/e at 8,000 μg/ml caused 68% larval death, while the CSE/w at the same dose caused 9% death. The CSE/e was 7.5 fold more toxic than the CSE/w.

The efficiency of the extracts induced by the larval mortality could be compared, by the LC_{50} values at 24 h It was clear that the LC_{50} values were so wide range depending on the plant seeds and the extracted solvents used. It was noticed that all ethanol extracts of plant seeds induced higher mortality of the mosquito larvae than the water extracts (Figure 3.5). The extracts of YSE produced the highest effects on the 2nd instar larvae. The LC_{50} value of YSE/e was 16.22 μg/ml and of YSE/w was 724.64 μg/ml (Table 3.2). It was noticed that the magnitudes of the other extracts were in milligram per milliliter level. The efficacy of MSEs was moderate. The LC_{50} of MSE/e was 1.41 mg/ml and of MSE/w was 16.65 mg/ml. The LC_{50} value of CSE/e was 3.7 mg/ml and of CSE/w was 25.23 mg/ml which was the least. Thus, the efficacy of the extracts on the mosquito larvae was ranged from YSE/e>YSE/w>MSE/e> CSE/e>MSE/w>CSE/w.

Table 3.2 Effects of the seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) on the 2nd instar larvae of *Aedes aegypti*.

Conc. µg/ml	% Mortality Mean ± S.E.	LC_{50} µg/ml, 24 h	Conc. µg/ml	% Mortality Mean ± S.E.	LC_{50} µg/ml, 24 h
MSE/w			MSE/e		
6,000	8.33 ± 0.32^a	$16,650 \pm 0.46$	800	40.33 ± 0.40^a	$1,412.53 \pm 0.82$
8,000	14.33 ± 0.30^b		1,000	47.00 ± 0.43^b	
0,000	34.33 ± 0.48^c		2,000	56.00 ± 0.37^c	
20,000	51.67 ± 0.60^d		4,000	61.00 ± 0.43^d	
30,000	95.33 ± 0.33^e		6,000	70.00 ± 0.26^e	
40,000	98.67 ± 0.21^f		8,000	79.67 ± 0.31^f	
YSE/w			YSE/e		
100	4.33 ± 0.31^a	724.64 ± 1.25	5	12.33 ± 0.40^a	16.22 ± 0.20
200	8.00 ± 0.37^b		10	41.67 ± 0.31^b	
400	28.00 ± 0.58^c		15	53.33 ± 0.56^c	
600	37.33 ± 0.49^d		20	58.67 ± 0.49^d	
800	50.67 ± 0.42^e		25	61.00 ± 0.42^e	
1,000	70.00 ± 0.58^f		30	63.00 ± 0.76^f	
CSE/w			CSE/e		
6,000	5.33 ± 0.21^a	$25,230.74 \pm 0.12$	800	14.67 ± 0.21^a	$3,778.39 \pm 0.23$
8,000	9.33 ± 0.33^b		1,000	29.00 ± 0.43^b	
10,000	21.00 ± 0.76^c		2,000	42.67 ± 0.67^c	
20,000	44.67 ± 0.66^d		4,000	49.00 ± 0.43^d	
30,000	59.00 ± 0.43^e		6,000	55.33 ± 0.33^e	
40,000	63.33 ± 0.49^f		8,000	67.67 ± 2.49^f	
0.1% DMSO	0.00	0.00	0.1% DMSO	0.00	0.00

Values are the mean of 6 observations, within a column followed by different letters are significant difference, analyzed by Duncan multiple range test (DMRT) ($P<0.05$), n = 6.

Seed extracts

Figure 3.5 Effects LC$_{50}$ values of the seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE), on the 2nd instar larvae of *Aedes aegypti*.

3.5.3 Effects on adult mosquitoes

The adult mosquitoes were twice sprayed with various concentrations of individual extracts of *H. suaveolens* (MSE), *P. erosus* (YSE) and *A. graveolens* (CSE) for 24 h. These toxic effects were similar to the effects on the larval treatments. The ethanol extracts were more potent than the water extracts. The YSE/e of 100 μg/ml induced 57.38% death of adult mosquitoes and the YSE/w of 1,000 μg/ml induced 55% death (Table 3.3). At 8 mg/ml, the MSE/e caused mosquito death 77.70% and the MSE/w caused only 54.85%. While at 80 mg/ml, the CSE/e induced 97.97% death and the CSE/w induced 44.15% death.

Table 3.3 Effects of the seed extract of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) twice sprays on the adults of *Aedes aegypti*, 12 and 24 h each.

Conc. µg/ml	% Mortality Mean ± S.E.	LC_{50} µg/ml, 24 h	Conc. µg/ml	% Mortality Mean ± S.E.	LC_{50} µg/ml, 24 h
MSE/w			MSE/e		
20,000	13.38 ± 0.48^a	$75,046.45 \pm 0.12$	1,000	26.69 ± 0.48^a	$2,952.70 \pm 0.11$
40,000	20.74 ± 0.62^b		2,000	41.22 ± 1.00^b	
60,000	29.77 ± 0.45^c		4,000	54.73 ± 0.42^c	
80,000	54.85 ± 0.43^d		6,000	58.45 ± 0.43^d	
100,000	68.56 ± 0.56^e		8,000	77.70 ± 0.52^e	
200,000	84.62 ± 0.49^f				
YSE/w			YSE/e		
2,000	18.45 ± 0.76^a	$9,600.51 \pm 0.25$	20	4.36 ± 0.43^a	91.41 ± 0.49
4,000	28.52 ± 0.43^b		40	10.74 ± 0.33^b	
6,000	40.27 ± 0.49^c		60	20.13 ± 0.56^c	
8,000	47.31 ± 0.47^d		80	46.64 ± 0.89^d	
10,000	55.03 ± 0.49^e		100	57.38 ± 0.48^e	
20,000	61.74 ± 0.26^f				
CSE/w			CSE/e		
20,000	4.02 ± 0.31^a	$109,030.36 \pm 0.17$	10,000	15.88 ± 0.40^a	$25,097.59 \pm 0.12$
40,000	12.38 ± 0.61^b		20,000	47.64 ± 0.91^b	
60,000	29.10 ± 0.56^c		40,000	61.49 ± 0.89^c	
80,000	44.15 ± 0.60^d		60,000	68.92 ± 0.42^d	
100,000	55.19 ± 0.76^e		80,000	97.97 ± 0.52^e	
200,000	63.55 ± 0.31^f				
0.1% DMSO	0.00	0.00	0.1% DMSO	0.00	0.00

Values are the mean of 6 observations, within a column followed by different letters are significant difference, analyzed by Duncan multiple range test (DMRT) ($P<0.05$), n = 6.

The toxic efficiency of all extracts on the adult mosquitoes could be compared by their LC_{50} values. The ethanol extracts of all plant seeds had higher efficacy than water extracts (Figure 3.6). The YSEs were the most effective in killing

the adult mosquitoes as compared among the same extracted solvents, while the CSEs were the least effective. The LC_{50} value of the YSE/e was 91.41 µg/ml and of the YSE/w was 9.6 mg/ml which was 11 fold less effective. The MSE/e exhibited LC_{50} of 2.95 mg/ml and the MSE/w exhibited 75.05 mg/ml which was 25 fold less effective. The CSEs was less effective when used as sprayers. LC_{50} of the CSE/e was 25.09 mg/ml and of the CSE/w was 109.03 mg/ml which 4 fold less effective. It was then concluded that the efficiency of all extract sprayers on the adult mosquitoes was ranged as YSE/e>MSE/e>YSE/w>CSE/e>MSE/w>CSE/w.

Figure 3.6 Effects LC_{50} values of the seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens*(CSE), on the adults of *Aedes aegypti*.

3.5.4 Effects of extract combinations larvae and adults

The effects of seed extract combinations (1 : 1, v/v) on the 2nd instar larvae and the adult mosquitoes were evaluated. The combination proportion was 1 : 1 (v/v) and made between the same extract solvent, but the concentration of each combination pair was arbitrarily selected as designated since the wide range of the magnitudes of the extracts' effects. It is obviously found that the ethanol seed extracts enhanced the effects of the water seed extracts on the control of the 2nd instar larvae (Table 3.4) as well as on the adult mosquitoes (Table 3.5).

The combination effects on the mortality of the 2nd larvae by the three extracts were exhibited in Table 3.4. The pair of MSE/e and YSE/e at 30 µg/ml was highly effective which induced 97.67% death of the larvae. The least effective pair was from the CSE/w and MSE/w which was only 60.33% at 10 mg/ml. It was noticed that the YSE/e in the combination could enhance the death of the larvae.

Table 3.4 Efficacy of the seed extracts combinations of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) on the 2nd instar larvae of *Aedes aegypti*.

Extract Combination (1 : 1, v/v) µg/ml	% Mortality Mean ± S.E.	LC$_{50}$ µg/ml, 24 h	Extract Combination (1 : 1, v/v) µg/ml	% Mortality Mean ± S.E.	LC$_{50}$ µg/ml, 24 h
MSE/w + YSE/w			MSE/e + YSE/e		
400	49.00 ± 0.43^a	225.43 ± 0.10	5	25.67 ± 0.31^a	11.79 ± 0.15
600	57.00 ± 1.00^b		10	53.00 ± 0.31^b	
800	67.67 ± 0.48^c		15	70.33 ± 0.56^c	
1,000	74.67 ± 0.33^d		20	95.67 ± 1.85^d	
2,000	80.00 ± 0.48^e		30	97.67 ± 0.40^e	
CSE/w + MSE/w			CSE/e + MSE/e		
2,000	36.00 ± 0.45^a	5,086.76 ± 0.10	40	21.67 ± 0.52^a	121.66 ± 0.10
4,000	43.33 ± 0.33^b		60	41.00 ± 0.43^b	
6,000	51.33 ± 0.67^c		80	50.00 ± 0.49^c	
8,000	56.00 ± 0.45^d		100	68.00 ± 0.84^d	
10,000	60.33 ± 0.40^e		200	77.33 ± 0.56^e	
YSE/w + CSE/w			YSE/e + CSE/e		
200	24.00 ± 0.52^a	930.65 ± 0.10	40	21.67 ± 0.91^a	82.04 ± 0.10
400	31.67 ± 0.31^b		60	41.00 ± 0.43^b	
600	36.00 ± 0.37^c		80	50.00 ± 0.37^c	
800	44.00 ± 0.37^d		100	68.00 ± 0.37^d	
1,000	55.33 ± 0.49^e		200	77.33 ± 0.42^e	
0.1% DMSO	0.00	0.00	0.1% DMSO	0.00	0.00

Values are the mean of 6 observations, within a column followed by different letters are significant difference, analyzed by Duncan multiple range test (DMRT) (P<0.05), n = 6.

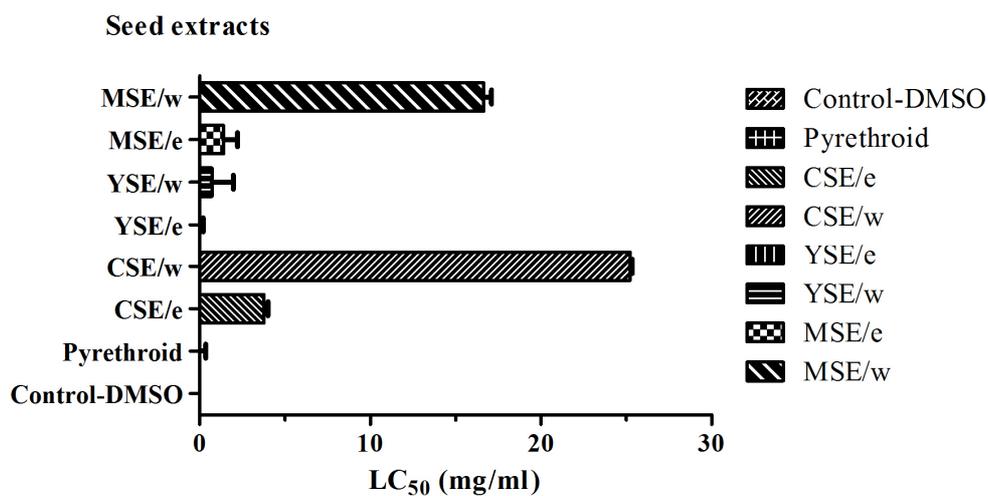**Table 3.5** Efficacy of the seed extracts combinations of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) on adults of *Aedes aegypti*.

Extract Combi-nation (1:1, v/v) µg/ml	% Mortality Mean ± S.E.	LC$_{50}$ µg/ml, 24 h	Extract Combi-nation (1:1, v/v) µg/ml	% Mortality Mean ± S.E.	LC$_{50}$ µg/ml, 24 h
MSE/w + YSE/w			MSE/e + YSE/e		
400	7.33 ± 0.99[a]	1,637.74 ± 0.20	10	13.00 ± 1.13[b]	23.82 ± 0.20
600	12.33 ± 1.00[b]		15	33.33 ± 1.34[c]	
800	23.67 ± 0.96[c]		20	49.00 ± 0.86[d]	
1,000	36.67 ± 0.85[d]		30	57.00 ± 0.86[e]	
2,000	55.00 ± 1.52[e]		40	70.00 ± 1.16[f]	
CSE/w + MSE/w			CSE/e + MSE/e		
2,000	7.00 ± 0.86[a]	12,315.31 ± 0.23	40	1.67 ± 0.61[a]	275.18 ± 0.14
4,000	13.33 ± 1.01[b]		60	9.00 ± 1.13[b]	
6,000	22.67 ± 1.00[c]		80	21.67 ± 1.20[c]	
8,000	31.33 ± 1.12[d]		100	30.33 ± 0.96[d]	
10,000	54.33 ± 1.21[e]		200	44.00 ± 1.15[e]	
20,000	67.33 ± 1.24[f]		400	54.33 ± 1.41[e]	
YSE/w + CSE/w			YSE/e + CSE/e		
200	4.33 ± 0.96[a]	1,250.93 ± 0.22	40	1.33 ± 0.14[a]	318.64 ± 0.16
400	13.67 ± 0.96[b]		60	9.33 ± 1.00[b]	
600	20.00 ± 0.73[c]		80	16.67 ± 1.00[c]	
800	34.33 ± 1.20[d]		100	25.33 ± 1.34[d]	
1,000	49.00 ± 0.86[e]		200	33.33 ± 1.13[e]	
2,000	68.33 ± 2.60[f]		400	55.00 ± 0.45[e]	
0.1% DMSO	0.00	0.00	0.1% DMSO	0.00	0.00

Values are the mean of 6 observations, within a column followed by different letters are significant difference, analyzed by Duncan multiple range test (DMRT) ($P<0.05$), n = 6.

The efficacy of the extracts combinations on the 2nd instar larvae was much better in individual treatments. The efficacy was ranged as YSE/e + MSE/e>

YSE/e + CSE/e>CSE/e + MSE/e>YSE/w + MSE/w>YSE/w + CSE/w>CSE/w + MSE/w

(Figure 3.7). The LC_{50} values of the above mentioned pairs were 11.79, 82.04, 121.66,

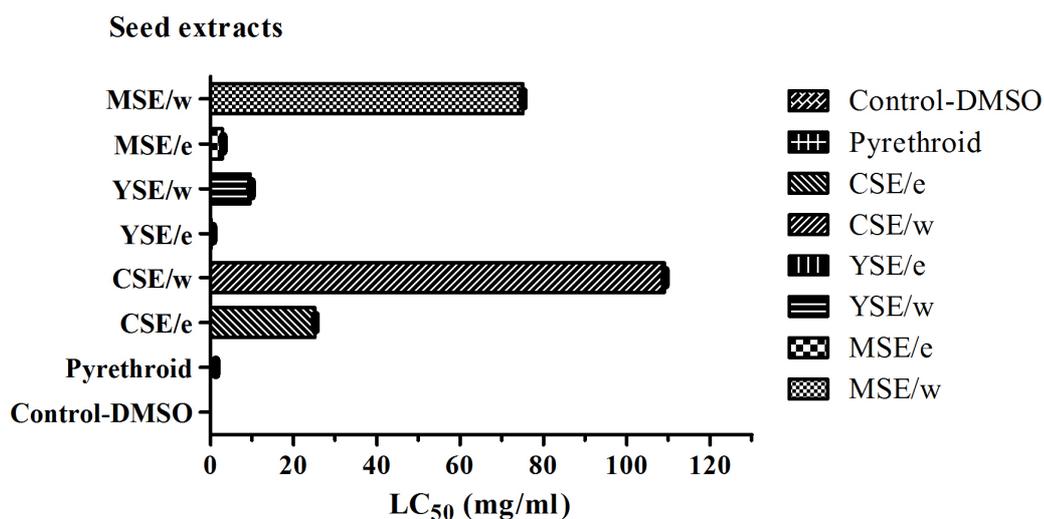225.43, 930.60 µg/ml and 5.09 mg/ml, respectively.
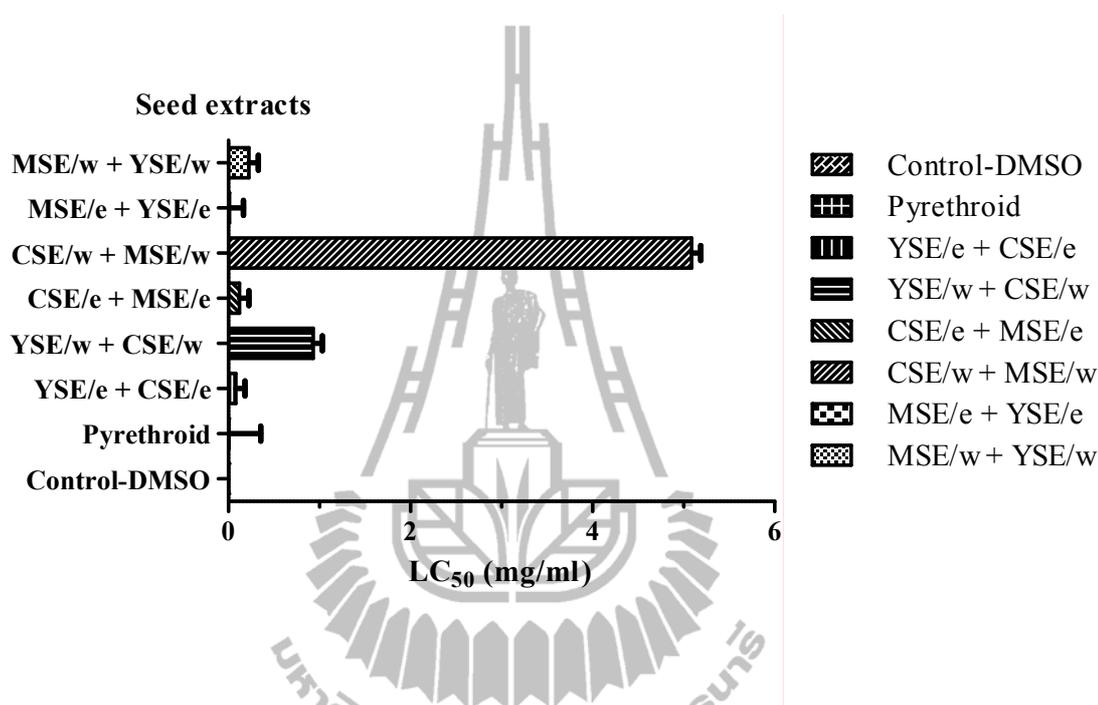
Figure 3.7 Effects LC_{50} values of the combination seed extracts of *Hyptis suaveolens*

(MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE), on the

2nd instar larvae of *Aedes aegypti*.

For the combination effects on the mortality of the adult mosquitoes, the

MSE/e also enhanced the mixtures of its pair. The combination of MSE/e and YSE/e at

40 µg/ml induced 70% mortality (Table 3.5). While 400 µg/ml of the combinations of

MSE/e and CSE/e; and of CSE/e and YSE/e caused 54.33 and 55.00%, respectively.

The combination between the water extracts had some what enhanced the mortality of

the adult mosquitoes. However, the YSE/w seemed to slightly enhance the death while

the MSE/w seemed to reduce the death. The combination of the YSE/w and MSE/w of

2 mg/ml caused 55% and of the YSE/w and CSE/w caused 68.33%. While to obtain approximately the same effect of death, combination of CSE/w and MSE/w required 20 mg/ml.

The efficacy of the combination of the extracts was comparably determined by the LC_{50} values. The combination of the MSE/e and YSE/e had the highest efficacy of LC_{50} of 23.82 µg/ml (Table 3.5). The mixture of the MSE/w and CSE/w had the least efficacy of 12 mg/ml. The efficacy of all combinations was ranged as MSE/e + YSE/e>MSE/e + CSE/e>CSE/e + HSE/e>CSE/w + YSE/w> MSE/w + YSE/w>MSE/w + SCE/w (Figure 3.8) and the LC_{50} values were 23.82, 275.18, 318.64 µg/ml, 1.25, 1.64, and 12.32 mg/ml, respectively.
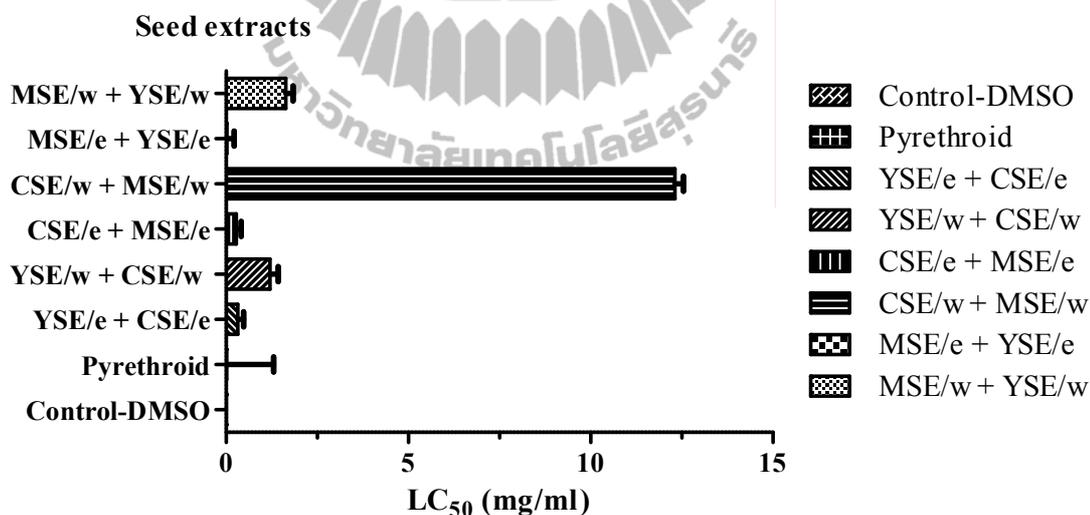
Figure 3.8 Effects LC_{50} values of the combination seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE), on the adults of *Aedes aegypti*.

3.6 Discussion

The biological control of *Ae. aegypti*, the important vector of the DHF, in Thailand is essential. Plant products are better alternative candidates for this purpose in order to avoid the development of insect resistance to synthetic insecticides and is also safe to the environment and human beings. In addition, plants are also locally available. This study demonstrated an evidence that three seed extracts of *H. suaveolens* (MSE), *P. erosus* (YSE) and *A. graveolens* (CSE) could effectively control the 2nd instar larvae and the adults of *Aedes aegypti*. There was evidence that *H. suaveolens* contained mainly *β*-caryophyllene, 1,8-cineole, sabinene, *β*-selinene limonene, biclyclogermacrene, *β*-phellandrene, which could cause death of both larvae and adult mosquitoes (Peerzada, 1997; Campos, Heleno, Tomas and Suzana, 2001). There was a report that ethanol extract of *H. suaveolens* leaves could be used as larvicidal on larvae of *Ae. aegypti* in Nigeria (Amusan, Idowu and Arowolo, 2005). In addition, petroleum ether extract of *H. suaveolens* seeds was found to use against Lepidoptera*, Plutell xylostella* (Keita, Umoetok and Smith, 2006). However, *H. suaveolens* extract at high dose, up to 500 mg/kg/day for 6 months, did not produce any toxic effect in rats (Attawish *et al.*, 2005; Niwatananun, Niwatananun, Lertprasertsook and Okonogi, 2006), i.e, it was safe to mammals. This was in agreement with our investigation on brine shrimps. The extracts of *A. graveolens* seeds (CSE) in this study showed larvacidal and adulticidal activities against *Ae. aegypti* to some certain extent. Possibly, its constituent of limonene, selinene, glucosides and flavonoid, which may not be that toxic to mosquitoes (Fehr, 1979; Garg, Gupta and Sharma, 1980). However, there was a report that the essential oil of *A. graveolens*

prepared from the whole plant was potent as a adulticide to *Ae. aegypti* (Chaiyasit *et al*., 2006).

It is clear stated that the ethanol extract of *P. erosus*, YSE/e, was the most toxic to the larvae and adults of *Ae. aegypti*. Possibly, the YSE/e contained high toxic products as it was reported that *P. erosus* seeds comprised of rotenone and cyanogenic glycosides which were known poisonous to human beings and animals (Narongchai, Narongchai and Thampituk, 2005; Hung *et al*., 2007). Rotenone is now widely used as insecticide in agriculture and home gardens, Particularly, in controlling aphids, thrips, lice, ticks, moths, beetles, spider mites, and mosquito larvae (Marrs and Ballantyne, 2004; Dayan, Cantrell and Duke, 2009). Rotenone caused death to insects by inhibited NADH oxidation in mitochondria leading to critically blocking respiration and nerve conduction (Marrs and Ballantyne, 2004). Having *P. erosus* seed extract in combination with the other seed extracts highly enhanced the insecticidal activities on *Ae. aegypti*. Therefore, the extracts of *H. suaveolens*, *P. erosus*, and *A. graveolens* could safely be used as botanical insecticides in controlling of *Ae. aegypti*.

3.7 Conclusion

It was then concluded that all seed extracts of mintweed, *Hyptis suaveolens* (MSE), yam bean, *Pachyrhizus erosus* (YSE) and celery, *Apium graveolens* (CSE) were high potent in controlling *Aedes aegypti* mosquitoes and their effects were dose dependent both in 2nd instar larvae and adults *Ae. aegypti* by death rate of 100%. The magnitudes of effective doses of the extracts of these three plant seeds were obviously much varied, ranging from 10^1 to 10^5 μg/ml. Both YSEs, water and ethanol extracts, were the most effective. In particular, the YSE/e was extremely potent against

both larvae and adult mosquitoes at 10^{-1} µg/ml level and synergized the other extracts in the dual combination tests. Adverse effects of the yam seed extracts when applied in water medium should be expected. Therefore, these three seed extracts are possibly applicable and further investigation for safe practices of the yam seed extracts, particularly extracted by ethanol in the future is needed in biological control of *Ae. aegypti*.

3.8 References

Amusan, A. A. S., Idowu, A. B., and Arowolo, F. S. (2005). Comparative toxicity effect of bush tea leaves (*Hyptis suaveolens*) and orange peel (*Citrus sinensis*) oil extract on larvae of the yellow fever mosquito *Aedes aegypti*. **Tanzania Health Research Bulletin**. 7(3): 174-178.

Artemia Reference Center. (2007). *Artemia salina*-brine shrimp-sea monkeys. [On-line]. **Available**: http://www.captain.at/artemia/.

Attawish, A., Chivapat, S., Chavalittumrong, P., Phadungpat, S., Bansiddhi, J., and Chaorai, B. (2005). Chronic toxicity study of *Hyptis suaveolens* (L.) Poit in rats. **Songklanakarin Journal of Science and Technology**. 27(5): 1028-1036.

Barrera-Necha, L. L., Bautista-Banos, S., Bravo-Luna, L., Garcia-Suarez, F. J., Alaez-Sololano, D., and Reyes-Shilpa, R. (2004). Antifungal activity seed powders, extracts, secondary metabolites *Pachyrhizus erosus* (L.) urban (Fabaceae) postharvest fungi. **Sociedad Mexicana de Fitopatologia**. 22(3): 356-361.

Boiling, J. (2006). Celery and celery seed for urinary problems and Gout. [On-line]. **Available**: http://everygreenherb.com/fromAtoZ/celery.html.

Briski, E., Van, S. G., Bossier, P., and Sorgeloos, P. (2008). Laboratory production of early hatching *Artemia* sp. cysts by selection. **Aquaculture**. 282: 19-25.

Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand. (2010). Situation of dengue infection in Thailand. [On-line]. **Available**: http://203.157.15.4/?page=h1n1.

Campos, I. F. P., Heleno, D. F., Tomas, A. P., and Suzana, C. S. (2001). Chemical variability in essential oil of *Hyptis suaveolens*. **Phytochemistry**. 57: 733-736.

Chaiyasit, D., Choochote, W., Rattanachanpichai, E., Chaithong, U., Chaiwong, P., Jitpakdi, A., Tippawangkosol, P., Riyong, D., and Pitasawat, B. (2006). Essential oils as potential adulticides against two populations of *Aedes aegypti*, the laboratory and natural field strains, in Chiang Mai province, Northern Thailand. **Parasitology Research**. 99(6): 715-721.

Choochote, W., Tuetun, B., Kanjanapothi, D., Rattanachanpichai, E., Chaithong, U., Chaiwong, P., Jitpakdi, A., Tippawangkosol, P., Riyong, D., and Pitasawat, B. (2004). Potential of crude seed extract of celery, *Apium graveolens* L., against the mosquito *Aedes aegypti* L. **Journal of Vector Ecology**. 29(2): 340-346.

Chuenwong, P. (2006). **Biological control of oriental fruit flies (*Bactrocera dorsalis* (Hendel)) by the extracts of neem, sugar apple and mintweed**. Ph.D. Thesis, Suranaree University of Technology, Thailand.

Dayan, F. E., Cantrell, C., and Duke, S. O. (2009). Natural products in crop protection. **Bioorganic and Medicinal Chemistry**. 17: 4022-4034.

Fehr, D. (1979). Study on the aroma substances of celery (*Apium graveolens* L.), part I. **Pharmazie**. 34: 658-662.

Finny, D. J. (1971). **Probit Analysis** (3rd ed.). England, Cambridge University Press.

Forum Biologia Marina - Mare Mediterraneo, (2006). Natura mediterranio. [On-line]. **Available**: http://www.naturamediterraneo.com/forum/topic.asp?TOPIC_ID= 11081.

Garg, S. K., Gupta S. R., and Sharma, N. D. (1980). Glucoside of *Apium graveolens*. **Planta Medica**. 38: 363-365.

Gillij, Y.G., Gleiser, R. M., and Zygadlo, J. A. (2008). Mosquito repellent activity of essential oils of aromatic plants growing in Argentina. **Bioresource Technology**. 99(7): 2507-2515.

Hung, Y. M., Hung, S. Y., Olson, K. R., Chou, K. J., Lin, S. L., Chung, H. M., Tung, C. N., and Chang, J. C. (2007). Yam bean seed poisoning mimicking cyanide intoxication. **Internal Medicine Journal**. 37: 130-132.

Iloba, B.N. and Ekrakene, T. (2006). Comparative assessment of insecticidal effect of *Azadirachta indica*, *Hyptis suaveolens* and *Ocimum gratissimum* on *Sitophilus zeamais* and *Callosobruchus maculatus*. **Journal of Biological Sciences**. 6(3): 626-630.

Kaiser, H., Gordon, A. K., and Paulet, T. G. (2006). Review of the African distribution of the brine shrimp genus *Artemia*. **South African Journal of Psychiatry, Water SA**. 32(4): 597-603.

Keita, S., Umoetok, S. B. A., and Smith, J. G. (2006). The insecticidal activity of petroleum ether extract of *Hyptis suaveolens* Poit (Labiatae) seeds on *Plutella xylostella* (L.) (Lepidoptera: Y ponomeutidae). **Agricultural Journal**. 1(1): 11-13.

Krishnaraju, A. V., Rao-Tayi, V. N., Sundararaju, D., Vanisree, M., Tsay, H. S., and Subbaraju, G. V. (2005). Assessment of bioactivity of Indian medicinal plant using brine shrimp (*Artemia salina*) lethality assay. **International Journal of Applied Science and Engineering**. 3: 125-134.

Lee, H. L., Chen, C. D., Mohd, M. S., Chiang, Y. F., Chooi, K. H., and Benjamin, S. (2008). Impact of larviciding with a *Bacillus thuringiensis israelensis* formulation, VECTOBAC WG®, on Dengue mosquito vectors in a Dengue endemic site in Selangor State, Malaysia. **The Southeast Asian Journal of Tropical Medicine and Public health**. 39(4): 601-609.

Marrs, T. C., and Ballantyne, B. (2004). **Pesticide Toxicology and International Regulation** (eds.). West Sussex, England, John Wiley and Sons.

Martínez, P. T. T., Rojas, I. L., Valdés, S. L., and Noa, R. R. (2005). Spatial vulnerability to Dengue: an application of the geographic information systems in Playa Municipality, city of Havana. **Medicc Review**; health and medical news of Cuba. 7(5). ISSN: 1527-3172.

Mona, M. E. G. (2010). Morphometric and molecular variability of three Artemia strains (El-Max and Wadi El-Natrun, Egypt and San Francisco Bay, U.S.A). **Journal of American Science**. 6(2): 98-107.

Mongkalangoon, P., Grieco, J. P., Achee, N. L., Suwonkerd, W., and Chareonviriyaphap, T. (2009). Irritability and repellency of synthetic pyrethroids on an *Aedes aegypti* population from Thailand. **Journal of Vector Ecology**. 34(1): 217-224.

Morales-Arellano, G. Y., Chagolla-López, A., Paredes-López, O., and Barba, R. A. P. (2001). Characterization of yam bean (*Pachyrhizus erosus*) proteins. **Journal of Agricultural and Food Chemistry**. 49(3): 1512-1516.

Narongchai, P., Narongchai, S., and Thampituk, S. (2005). The first fatal case of yam bean and rotenone toxicity in Thailand. **Journal of the Medical Association of Thailand**. 88(7): 984-987.

Niwatananun, K., Niwatananun, W., Lertprasertsook, L., and Okonogi, S. (2006). Repeated-dose dermal toxicity of topical formulation of *Hyptis suaveolens* oil. **Chiang Mai University Journal**. 5(3): 369-375.

Peerzada, N. (1997). Chemical composition of the essential oil of *Hyptis suaveolens*. **Molecules**. 2: 165-168.

Silva, W. J., Dória, G. A., Maia, R. T., Nunes, R. S., Carvalho, G. A., Blank, A. F., Alves, P. B., Marçal, R. M., and Cavalcanti, S. C. (2008). Effects of essential oils on *Aedes aegypti* larvae: Alternatives to environmentally safe insecticides. **Bioresource Technology**. 99(8): 3251-3255.

Sommerville, M., and Glasgow, B. (2006). Insect repellent based on a mixture of essential oils. **United States; Patent Application Publication**. US 20060 147562A1. [On-line]. **Available**: http://www.freepatentsonline.com/EP15580 87A1.html.

Stenersen, J. (2004). **Chemical pesticides: mode of action and toxicology**. Boca Raton, London; CRC Press.

Tropical company. (2007). *Artemia salina*. [On-line]. **Available**: http://www. tropical. com.pl/artemia-salina,d129,0,en.html.

Vanhaecke, P., Siddall, S. E., and Sorgeloos, P. (1984). International study on Artemia. XXXII. Combined effects of temperature and salinity on the survival of Artemia of various geographical origin. **Journal of Experimental Marine Biology and Ecology**. 80(3): 259-275.

Wood, A. (2010). Compendium of pesticide common names: Insecticides. [On-line]. **Available**: http://www.alanwood.net/pesticides/index.html.

World Health Organization (2008). Dengue status in south east Asia region: An epidemiological perspective [On-line]. **Available**: http://www.searo.who.int/ LinkFiles/Dengue_dengue-SEAR-2008.pdf.

Wulff, R. (1987). Effect of irradiance, temperature, and water status on growth and photosynthetic capacity in *Hyptis suaveolens*. **Journal of Botany**. 65: 2501-2506.
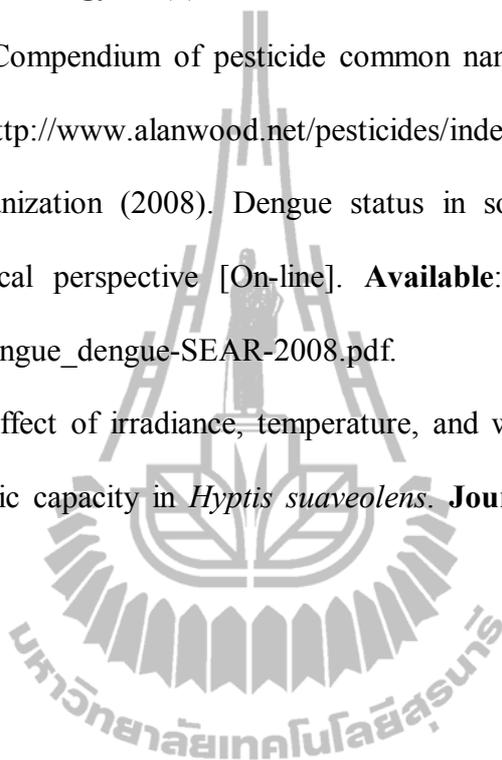
CHAPTER IV

EFFECTS OF MINTWEED (*HYPTIS SUAVEOLENS* (L.) POIT), YAM BEAN (*PACHYRHIZUS EROSUS* L.), AND CELERY (*APIUM GRAVEOLENS* L.) SEED EXTRACTS ON CYTOCHROME C OXIDASE IN *AEDES AEGYPTI* L.

4.1 Abstract

The seed extracts of mintweed (*Hyptis suaveolens* (L.) Poit) (MSE), yam bean (*Pachyrhizus erosus* L.) (YSE), and celery (*Apium graveolens* L.) (CSE) caused death to both larvae and adults of *Aedes aegypti* L. the dengue hemorrhagic fever vector. This is the first observation that these seed extracts inhibited the activity of cytochrome c oxidase (COX), one of the complexes for electron transport chain in mitochondria. The effect of the extracts on the COX activity was dependent on the seed types and the extracted solvents. The yam bean seed extracted with ethanol (YSE/e) was the most potent inhibit at 65.24% and at 59.08% of COX for the larvae and the adults, respectively. The celery seed extracted with water (CSE/w) had very low potential. The inhibitory potential of all individual extracts on COX of both the larvae was ranged as YSE/e>YSE/w>MSE/e> CSE/e>MSE/w>CSE/w. The inhibitory potential of all individual extracts on COX of both the adult was ranged as YSE/e>MSE/e>YSE/w>CSE/e>MSE/w>CSE/w. The combination of the extracts inhibited COX was dependent on the YSE/e which synergistically enhanced the COX inhibitory effects in the mixture. The combination of YSE/e and MSE/e induced highest COX inhibition in larvae at 79.54%,

and in adults at 70.62%. The CSEs were highly reduce the COX inhibition. The efficacy of the combination on larvae was ranged as MSE/e + YSE/e>YSE/ + CSE/e>CSE/e + MSE/e>MSE/w + YSE/w>YSE/w + CSE/w>CSE/w + MSE/w. The efficacy of the combination on adult was ranged as MSE/e + YSE/e>CSE/e + MSE/e>YSE/e + CSE/e>MSE/w + YSE/w>YSE/w + CSE/w>CSE/w + MSE/w. Therefore, it could be concluded that the yam bean seed extracts was likely to be the most potent insecticidal agent for the biological control of mosquitoes.

4.2 Introduction

Mitochondria are the site of oxidative metabolism within the cell which involved in electron transport and oxidative phosphorylation (Voet, Voet and Pratt, 2008). During electron transport system (ETS), the electrons from nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$) are passed through the system of chain transport electrons which are complex I, II, III and IV as shown in Figure 4.1. Finally, the adenosine triphosphate (ATP) is the end product in the aerobic respiratory system (Paula, Sucheta, Szundi and Einarsdóttir, 1999). ETS contains several enzymes, various coenzymes and arrangements as a multi-enzyme complex in the inner membrane of the mitochondria. There are daily network served together in the electron transport. Reactions that occur in each step of the oxidation-reduction by transmitting electron reduced coenzymes where each type of enzyme when the electron is reduced, enzyme activity has been changed to its status to oxidize co-enzyme.
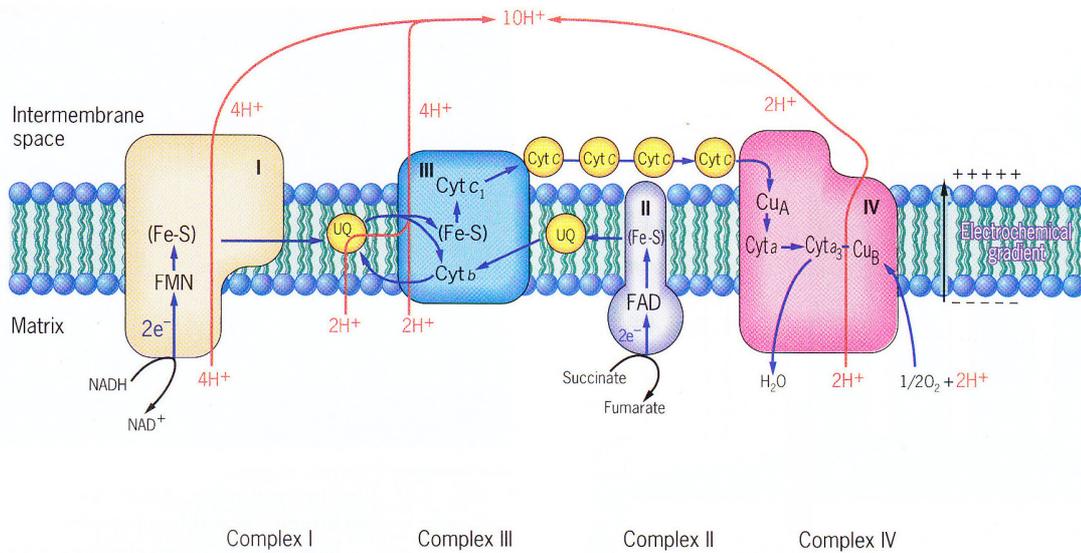
Figure 4.1 The electron-transport chain of the inner mitochondrial membrane. Complex I
= NADH Dehydrogenase, Complex II = Succinate-CoQ Reductase, Complex
III = CoQ-cyt c Reductase and Complex IV = Cytochrome C Oxidase (Karp,
2002).

Cytochrome c oxidase (Complex IV) is a membrane-bound enzyme that catalyzes
the four-electron reduction of oxygen to water as given in Equation 1.

$$4 \text{ ferrocytochrome c }^{(2+)} + 4H^+ + O_2 \longrightarrow 4 \text{ ferriccytochrome c }^{(3+)} + 2H_2O$$
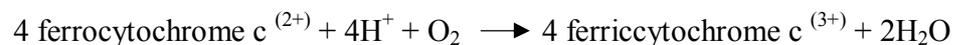
This highly exergonic reaction drives proton moving across the membrane into the
intermembrane space, creating a transmembrane proton electrochemical gradient, which
is used in the production of ATP (Brändén *et al.*, 2001).

In *Drosophila*, the protein ensures the proper formation and function of the
respiratory system (Hoch, 2007). It is suggested that the potassium activated ATPase in
the flabellum of the fly is an integral constituent of the electrogenic potassium pump,

which may be important for the generation of receptor (Wieczorek, 1982). Animals that live under aerobic conditions consume large amounts of O_2, which is mainly used to sustain the production of ATP in the respiratory chain of the mitochondria (Burmester and Hankeln, 2004). Chamberlin (2006) reported that the midgut of the tobacco hornworm (*Manduca sexta*) is a highly aerobic tissue that is destroyed by programmed cell death during larval-pupal metamorphosis. There were no developmental changes in the activity of complex I or III, activities of complexes II and IV (COX).

The synthesis of ATP is a complex process carried out by the mitochondrial respiratory electron transport chain involving a series of membrane complexes. Cyanide and azide are well known as inhibitors-effect of cytochrome c oxidase (COX) by interfering with the generation of ATP (Wallace and Starkov, 2000). Pesticides can disrupt many sites by inhibiting the activity of mitochondrial function and block the oxidative phosphorylation system. Naturally, some certain plant products have occurring toxic compound. There was a report that cyanide-resistance respiration is widespread in higher plants and organism (Solomos, 1977). However, the ADP and ATP conversion in mitochondria can be denied (ATP disappearing) when the animals take some toxic plants. Caffeine, ephedrine, cannabinoids, opioids and reserpine were possed to central nervous system (CNS) stimulant properties, for the memory effects in animals (Carlini, 2003). Prates, Santos, Waquil, Fabris and Oliveira (1998) found that Brazilian flora contained terpenoids, 1,8-cineole and limonene, which had lethal effects on rice weevil caused by their influence on the respiratory and digestive systems. Lee, Lee, Choi and Park (2001) showed that menthone, limonene and linalool isolated from *Mentha arvensis* L. had toxic activity against rice weevil. Plant monoterpenes, cineole, fenchrone and pulegone were demonstrated to successfully control pests by fumigation as they are highly volatile (Lee, Peterson and Coats, 2003).

Hyptis species were reported to contain hydrobenzoic and hydrocinnamic acids. Mintweed, *Hyptis suaveolens*, was found containing strong toxicity against fungi *Pythium aphanidermatum P. debaryanum, Rhizoctonia solani* (Schulz and Karl, 1980; Pandey and Dubey, 1991), 1,8-cineole, β-caryophyllene sabinene, limonene, biclyclogermacrene, and β-phellandrene (Peerzada, 1997; Campos, Heleno, Tomas and Suzana, 2001). *H. suaveolens* products were also able to control nematodes and insects (Oyedunmade, 1998; Chuenwong, 2006; Musa, Dike and Onu, 2009). The methanol extracts of mintweed seeds and leaves were compared with the seed powder for their relative effectiveness against Khapra beetle, *Trogoderma granarium*, in stored groundnuts (Musa, Dike and Onu, 2009).

Yam bean, *Pachyrhizus erosus*, seeds contain cyanogenic glycosides, pachyrrhizine, pachyrrhizone, 12-(A)-hydropachyrrhizone, dolineone, dehydropachyrrhizone, erosone, neodehydrorautenone, erosenone, erosenin, 12-(A)-hydroxylinenone, and pachysaponin A&B (Narongchai, Narongchai and Thampituk, 2005), rotenone and cyanogenic glycosides (Hung *et al.*, 2007). They showed insecticidal activity against insects (Li, Wei, Xu, Huang and Yao, 2009).

Celery, *Apium graveolens* extracts process medicinal properties, traditionally used as remedy for ashma and bronchitis and combined with other herbs to reduce blood pressure (Satyavati and Raina, 1976; Chevalier, 1998). *A. graveolens*, seeds are known to contain 3-n-butylphthalide derivatives with fragrance characteristics. They also contain nine coumarin derivatives and a phenylpropanoid which processed biological activities (Kitajima, Ishikawa and Satoh, 2003; Maruyama *et al.*, 2009). Nitrogenous compounds in celery seed essential oil were reported to have effects on the central nervous system, and anti-inflammatory activity (Kulshrestha, Saxena and Kohli, 1967; Satyavati and Raina, 1976; Momiin and Nair, 2002).

Interestingly, seed extracts of mintweed (MSE), yam bean (YSE) and celery (CSE) which contain terpenoids, rotenone and cyanogenic glycosides (Peerzada, 1997; Campos, Heleno, Tomas and Suzana, 2001; Kitajima, Ishikawa and Satoh, 2003; Keita, Umoetok and Smith, 2006) demonstrated that they process an insecticidal property leading death of *Aedes aegypti* mosquitoes, both larvae and adults (see Chapter III). Thus in this research, the cause of death at cellular mechanism, emphasis on the cytochrome c oxidase activity in mitochondria of both mosquito larvae and adults was investigated in order to support the efficiency of these plant seed.

4.3 Materials and methods

4.3.1 Materials

Cytochrome c (horse heart), *n*-dodecyl-β-D-maltoside, Brij 58$^®$, sodium dithionite, K_2HPO_4, KH_2PO_4, sucrose, disodium EDTA, magnesium sulfate, Tris buffer and bovine serum albumin were purchased from Sigma-Aldrich-Fluka, Milwaukee, WI). Potassium hexacyanoferrate (III) was purchased from Fisher Scientific, Suwanee, Georgia, the United States of America. Dimethylsulfoxide (DMSO) was purchased from Merck (Darm-Stadt, Germany). All chemicals were analytically graded.

4.3.2 Collection for samples and preparation for seed extract

Mintweed (*Hyptis suaveolens*) seeds were collected at Suranaree University of Technology (SUT) campus. Yam bean (*Pachyrhizus erosus*) and celery (*Apium graveolens* L.) seeds were purchased from a local farm in Mahasarakham and Nakhon Ratchasima provinces, Northeast Thailand.

H. suaveolens seeds were soaked in water and removed their mucilage before extraction. The naked seeds were cleaned, air dried and ground to powder.

P. erosus seeds were purchased from the farmer at a local farm in Som Had, Borabu, Mahasarakham. *A. graveolens* seeds were obtained from the market in Nakhon Ratchasima. All seeds were cleaned, air dried and ground to powder. Ten grams of the seed powder in a cellulose extraction thimble (Whatman International Ltd., Maidstone, England) were extracted in 150 ml of water or 70% ethanol in Soxhlet extraction apparatus (Buchi model B 811, Germany). The extracts were evaporated, dried and stored at -20 ^{o}C until used.

Single or combination (1 : 1, v/v) of the extracts were applied on larvae and adults *Ae. aegypti*. The seed extracts were dissolved in DMSO 0.1%. All experiments were carried out in triplicate.

4.3.3 Mosquito rearing

The *Aedes aegypti* eggs were obtained from Department of Disease Control Ministry of Public Health, Thailand and reared in the Building F9, the Center for Scientific and Technological Equipment, Suranaree University of Technology (SUT) by standard techniques of World Health Organization (WHO).

The female mosquitoes laid eggs on a filter paper, and the eggs were allowed to hatch in an enamel deep plate containing water and allowed to be developed to 2nd instar larvae. The 2nd instar larvae were reared on enamel given on a filter paper to sample collection. The mixed-sex adults which emerged within 2 days to use in all experiments, were kept -80 ^{o}C until used.

4.3.4 Isolation for mosquito mitochondria

Mitochondrial isolation modified the procedures described by Haritos and Dojchinov (2003) and Song and Scharf (2009). Six grams of death larval and adult mosquitoes were manually homogenized 10 ml 0.05 M Tris buffer (pH 7.4) containing 0.25 M sucrose, 0.001 M disodium Ethylenediaminetetraacetic acid (EDTA), 0.005 M

magnesium sulfate, and 2 g L^{-1} bovine serum albumin (BSA) on ice. The homogenate were centrifuged at 300 × g for 1 h at 4 °C to pellet the cellular debris. The supernatant was removed and transferred to a fresh tube and re-centrifuged, stepwise, at 500 × g for 30 min, at 5,000 × g for 20 min, at 10,000 × g for 10 min, and at 15,000 × g for 10 min at 4 °C. The supernatant was discarded. The pellet containing mitochondria was collected, gently rinsed and resuspended in 1 ml phosphate buffer. The suspension was centrifuged 15,000 × g for 10 min at 4 °C. The pellet was resuspended in volume 1.5 ml buffer. The larval supernatant had a black color and the adult supernatant had a bright brown color.

4.3.5 Cytochrome c oxidase inhibition

The effects of seed extracts of mintweed (MSE), yam bean (YSE) and celery (CSE) on cytochrome c oxidase were performed by following the methods of Haritos and Dojchinov (2003) and Song and Scharf (2009) with some modifications.

Cytochrome c (horse heart) standard was reduced by a tiny crystal sodium dithionite (added immediately before use) (Paula, Sucheta, Szundi and Einarsdóttir, 1999). The cytochrome c standard from horse heart was dissolved in phosphate buffer and used for measurement of cytochrome c activity (Song and Scharf, 2009). One microgram per milliliter reduced cytochrome c (36 mM final concentration, as a substrate). The reaction buffer containing 0.0062 M K$_2$HPO$_4$ + 0.0338 M KH$_2$PO$_4$, pH 6.2) was prepared in a 1.0 ml volume with 0.25 M sucrose and 0.05% (w/v) lauryl maltoside.

The extract sample concentration was selected base on one of the insecticidal activity approximately 1000 μg/ml. The seed extracts was then diluted to obtain the final concentrations of 100, 300, 500, and 1000 μg/ml in a reaction well. The reduced cytochrome c was put into a 96-well plate, 20 μl/well, containing 20 μl reaction buffer. The extract sample at the maximum concentration 1,000 μg/ml (40 μl)

were added. The mitochondrial homogenate (20 μl, containing COX) was added last. The COX activity was immediately measured in spectrophotometer (Enzyme-Linked Immunosorbent Assay : ELISA microplate reader (Benchmark ^{plusTm}, Bio Rad)) at 550 and 565 nm for 5 minutes at the most. Cyanide (0.003 mg/ml) and DMSO 0.1% was used to control. The percentage of inhibition was calculated as the following equation.

$$\% \text{ Inhibition} = \left[\left(\frac{\text{Absorbance of the sample}}{\text{Absorbance of reduced cytochrome c}}\right) \times 100\right]$$

The resulting A_{550}/A_{565} ratio of this mixed solution was between 6 and 8, as expected (Song and Scharf, 2009).

4.3.6 Statistical analysis

The variances were calculated with one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) statistical software.

4.4 Results

4.4.1 Effects of seed extracts on cytochrome c oxidase activity

The spectral profile of reduced-oxidize cytochrome c from horse heart was shown in Figure 4.2. The reduced cytochrome c was obtained by adding a grain of sodium dithionite into the solution system. It is typical to determine the extent of reduction of cytochrome c by measuring the difference in absorbance at 550 nm and 565 nm (Figure 4.2). The difference in absorbance is denoted absorbance of COX, were expressed as percent inhibition (% COX). The effect of an individual seed extracts and

the effect of combining of MSE, YSE and CSE on COX in mitochondria of the 2nd instar larvae and the adults of *Ae. aegypti* were carried out.
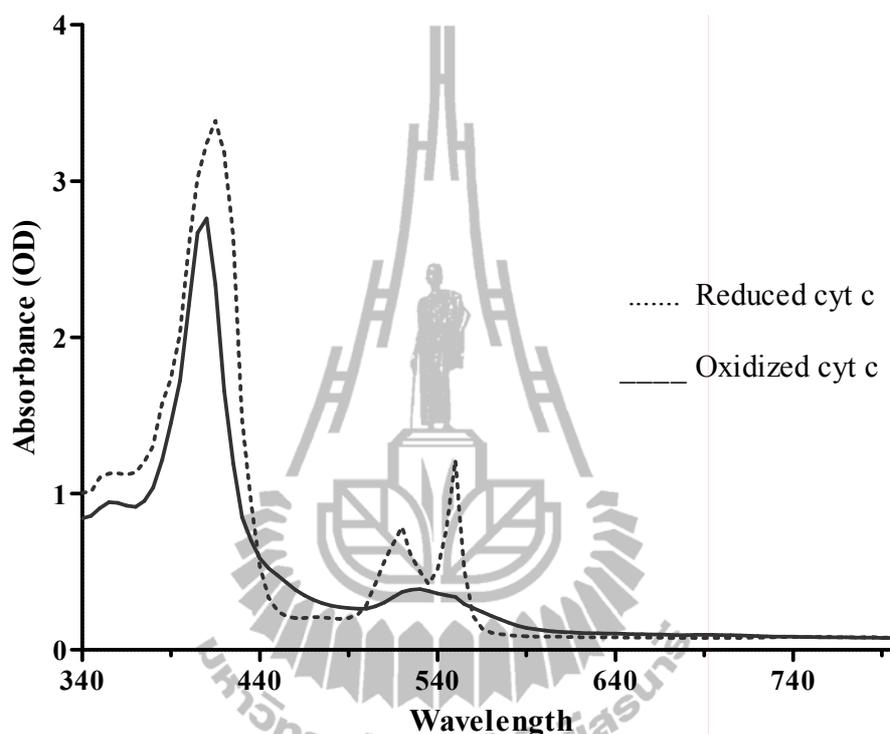
Figure 4.2 The spectral profile of reduced-oxidized cytochrome c from horse heart.

4.4.2 Effects of individual seed extracts on cytochrome c oxidase in the 2nd instar larvae and the adult mosquitoes

The effects of a single seed extracts of MSE, YSE and CSE on COX in mitochondria of *Ae. aegypti* the 2nd instar larvae and the adults were conducted. Tables 4.1 and 4.3 demonstrated that the effects of all extracts on the mosquito larvae and adults were done with the same pattern, but the magnitude on the larvae were slightly higher. The potency of single seed extracts on COX inhibition, treated on either larval or adult

mosquitoes, was ranged as YSE/e>YSE/w>MSE/e>CSE/e>MSE/w>CSE/w (Figure 4.3). The action spectrum of reduced cytochrome c on the 2nd instar larvae and adults of *Ae. aegypti* mitochondria in the presence of the water and ethanol seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) at maximum concentration of 1000 µg/ml (Figures 4.4 and 4.5).

Table 4.1 Inhibition activity of cytochrome c oxidase on the 2nd instar larvae of *Aedes aegypti* using the seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE).

Seed extracts	Conc. (µg/ml)	% COX inhibition (Mean ± S.E.)
MSE/w	100	15.68 ± 0.45^{ab}
	300	18.62 ± 0.64^{abc}
	500	24.04 ± 0.54^{de}
	1,000	28.93 ± 5.00^{f}
YSE/w	100	26.89 ± 6.13^{ef}
	300	38.30 ± 1.94^{i}
	500	45.24 ± 3.53^{j}
	1,000	53.01 ± 5.75^{k}
CSE/w	100	14.50 ± 1.61^{a}
	300	16.82 ± 0.50^{ab}
	500	19.91 ± 0.38^{bcd}
	1,000	21.39 ± 6.53^{cd}
MSE/e	100	27.11 ± 0.48^{ef}
	300	34.36 ± 3.29^{h}
	500	40.37 ± 1.30^{i}
	1,000	45.46 ± 5.80^{j}
YSE/e	100	55.48 ± 3.32^{kl}
	300	57.50 ± 4.20^{lm}
	500	60.94 ± 4.10^{m}
	1,000	65.24 ± 3.59^{o}
CSE/e	100	18.09 ± 0.48^{abc}
	300	21.56 ± 0.50^{cd}
	500	29.39 ± 1.73^{fg}
	1,000	33.22 ± 4.08^{gh}
Cyanide	0.003 (mg/ml)	84.06 ± 1.99

Values are the mean of 6 observations, within a column followed by different letters are significant difference, analyzed by Duncan multiple range test (DMRT) ($P<0.05$), n = 6.
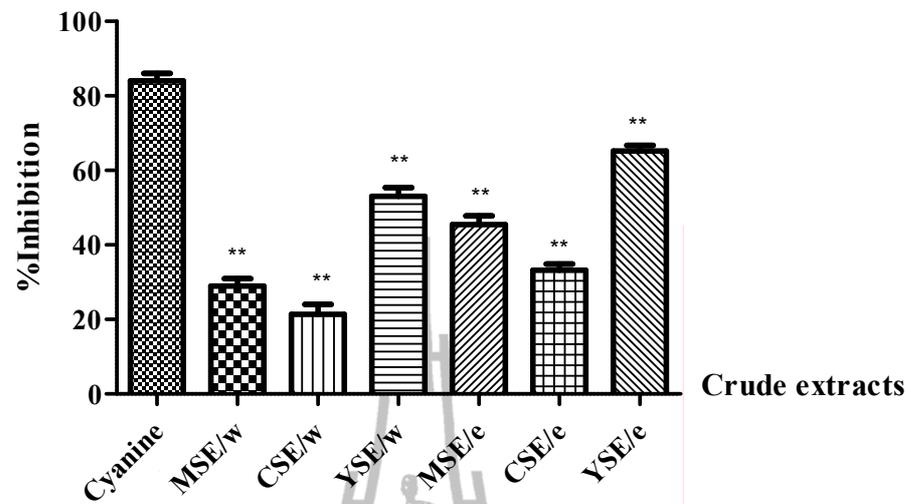
Figure 4.3 Efficiency of the individual seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) on the cytochrome c oxidase activity in mitochondria of the 2nd instar larvae of *Aedes aegypti* at maximum concentration of 1000 μg/ml, **P<0.01.

Figure 4.4 The action spectrum of reduced cytochrome c on the 2nd instar larvae of *Aedes aegypti* mitochondria in the presence of the water seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) at concentration of 1000 µg/ml.

Figure 4.5 The action spectrum of reduced cytochrome c on the 2nd instar larvae of *Aedes aegypti* mitochondria in the presence of the ethanol seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) at maximum concentration of 1000 µg/ml.

The effects of single seed extracts of MSE, YSE and CSE on COX in mitochondria of *Ae. aegypti* the adults were conducted. The potency of single seed extracts on COX inhibition, treated on adult mosquitoes, was ranged as YSE/e>MSE/e>YSE/w>CSE/e>MSE/w>CSE/w (Table 4.2 and Figure 4.6). The action spectrum of reduced cytochrome c on the 2nd instar larvae and adults of *Ae. aegypti* mitochondria in the presence of the water and ethanol seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) at maximum concentration of 1000 µg/ml (Figures 4.7 and 4.8).
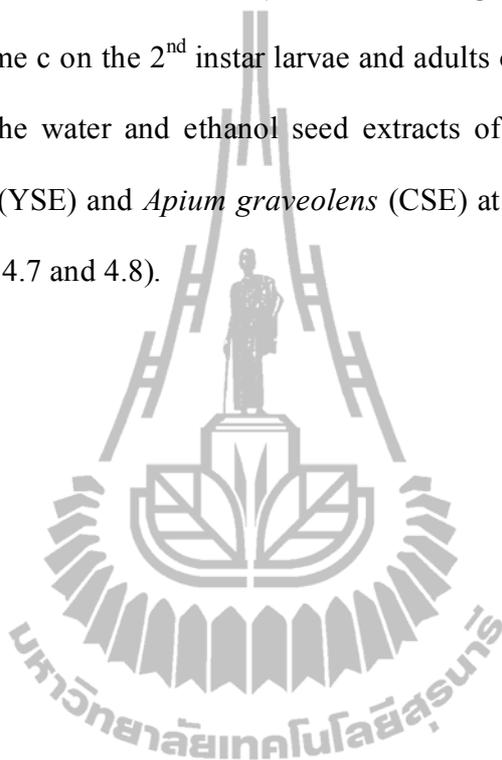
Table 4.2 Inhibition activity of cytochrome c oxidase on the adult of *Aedes aegypti* using the seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE).

Seed extracts	Conc. (µg/ml)	% COX inhibition (Mean ± S.E.)
MSE/w	100	13.62 ± 0.43^a
	300	15.42 ± 0.27^{ab}
	500	18.25 ± 1.53^{abc}
	1,000	23.20 ± 10.00^{cde}
YSE/w	100	18.46 ± 0.67^{abc}
	300	26.82 ± 8.40^{def}
	500	36.43 ± 4.07^{hi}
	1,000	41.93 ± 8.84^i
CSE/w	100	12.72 ± 1.93^a
	300	14.74 ± 0.93^a
	500	15.90 ± 0.57^{ab}
	1,000	17.83 ± 5.60^{abc}
MSE/e	100	29.47 ± 1.94^{fg}
	300	32.88 ± 4.03^{gh}
	500	38.38 ± 1.79^{hi}
	1,000	50.18 ± 5.51^j
YSE/e	100	50.86 ± 3.71^J
	300	53.40 ± 4.54^{jk}
	500	57.21 ± 2.37^k
	1,000	59.08 ± 10.08^k
CSE/e	100	14.12 ± 0.51^a
	300	17.57 ± 5.63^{abc}
	500	21.23 ± 1.49^{bcd}
	1,000	28.77 ± 3.77^{efg}
Cyanide	0.003 (mg/ml)	84.06 ± 1.99

Values are the mean of 6 observations, within a column followed by different letters are significant difference, analyzed by Duncan multiple range test (DMRT) ($P<0.05$), n = 6.
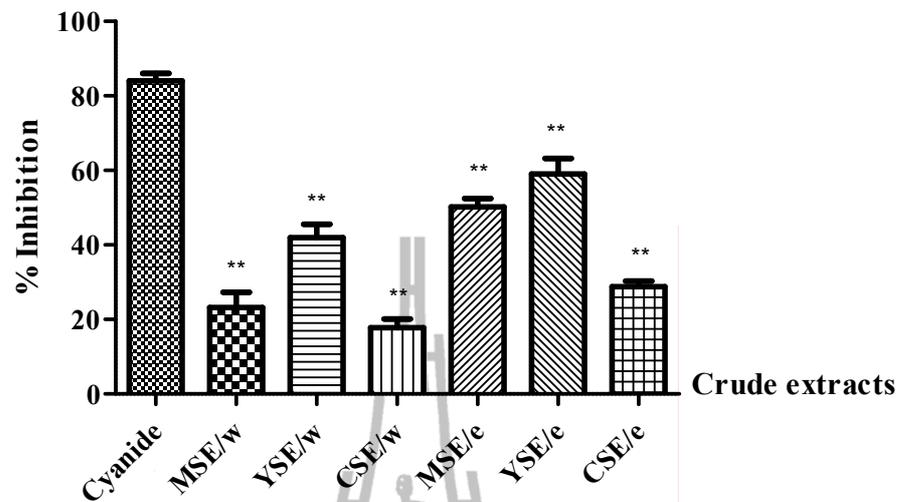
Figure 4.6 Efficiency of the individual seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) on the COX activity in mitochondria of the adult of *Aedes aegypti* at maximum concentration of 1000 µg/ml, **P<0.01.
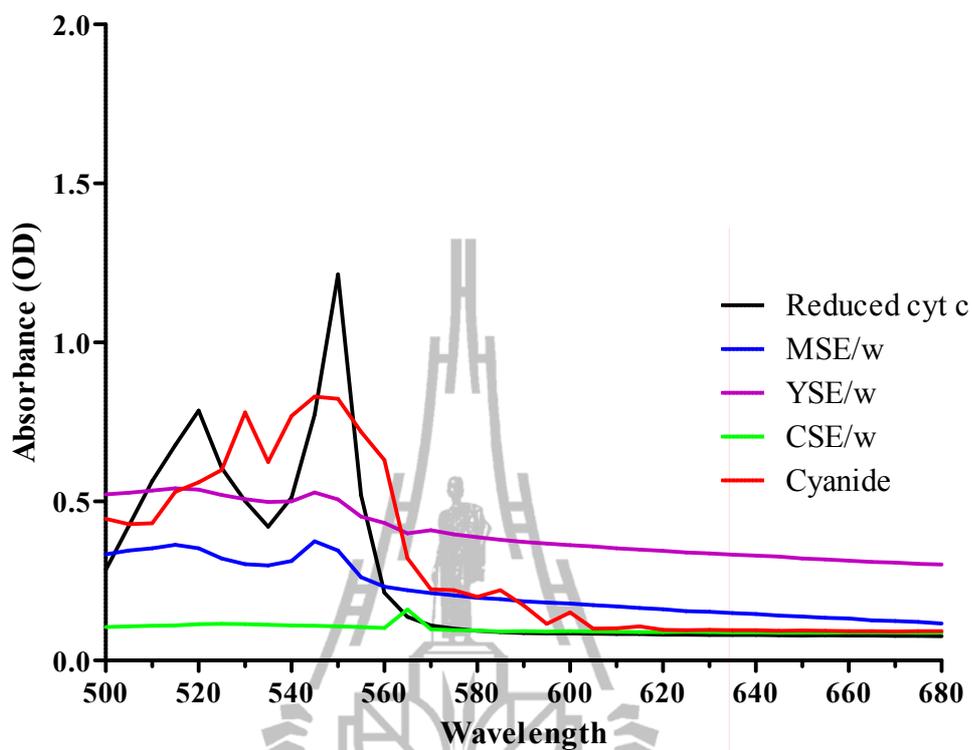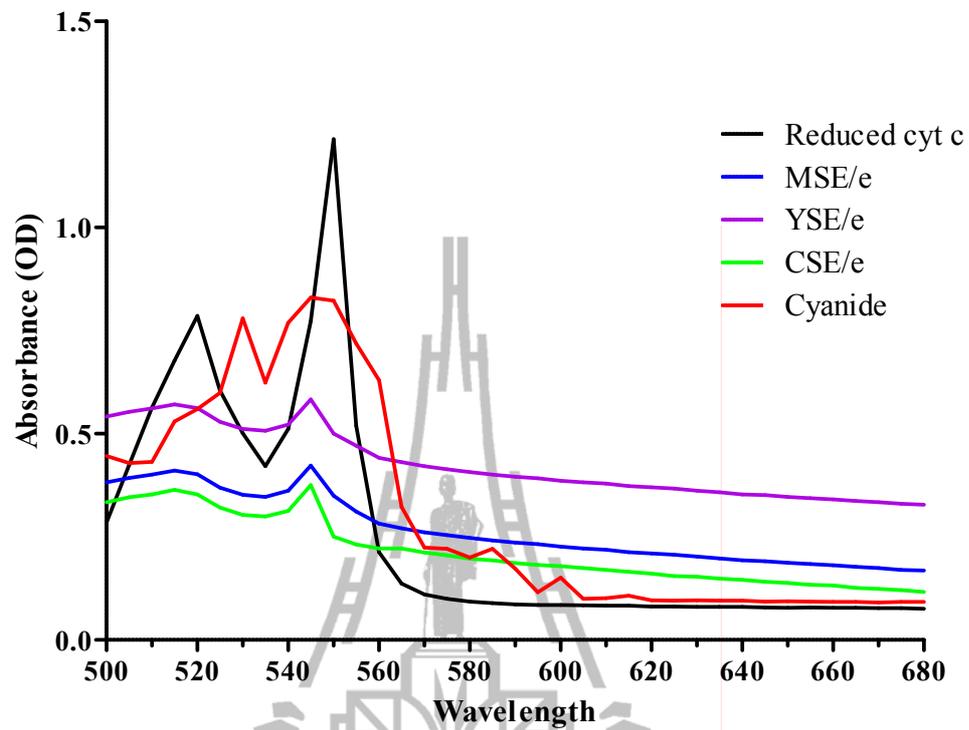
Figure 4.7 The action spectrum of reduced cytochrome c on the adult of *Aedes aegypti* mitochondria in the presence of the water seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) at maximum concentration of 1000 µg/ml.

**Figure 4.8** The action spectrum of reduced cytochrome c on adult of *Aedes aegypti* mitochondria in the presence of the ethanol seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) at maximum concentration of 1000 μg/ml.

4.4.3 Effects of extract combinations on cytochrome c oxidase in the 2nd instar larvae and the adult mosquitoes

The paired combination of the extracts (1 : 1, v/v) effects on the cytochrome c oxidase activity in the 2nd instar larvae and the adult mosquitoes were investigated. It was obvioused that the larvae were prone to induced death by the extract combination and the YSEs enhanced the other extracts (Table 4.3). The COX inhibition was ranged as MSE/e + YSE/e>YSE/e + CSE/e>CSE/e + MSE/e>MSE/w + YSE/w>YSE/w + CSE/w> CSE/w + MSE/w (Figure 4.9). The action spectrum of reduced cytochrome c on t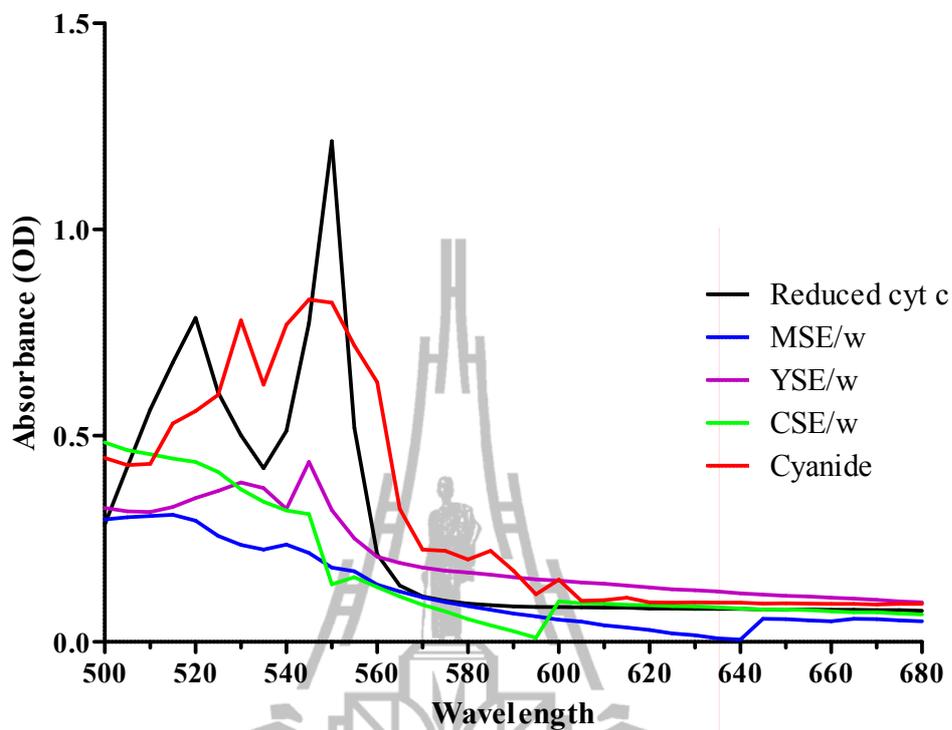he 2nd instar larvae and adults of *Ae. aegypti* mitochondria in the presence of water and ethanol seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) at maximum concentration of 1000 µg/ml (Figures 4.10 and 4.11).
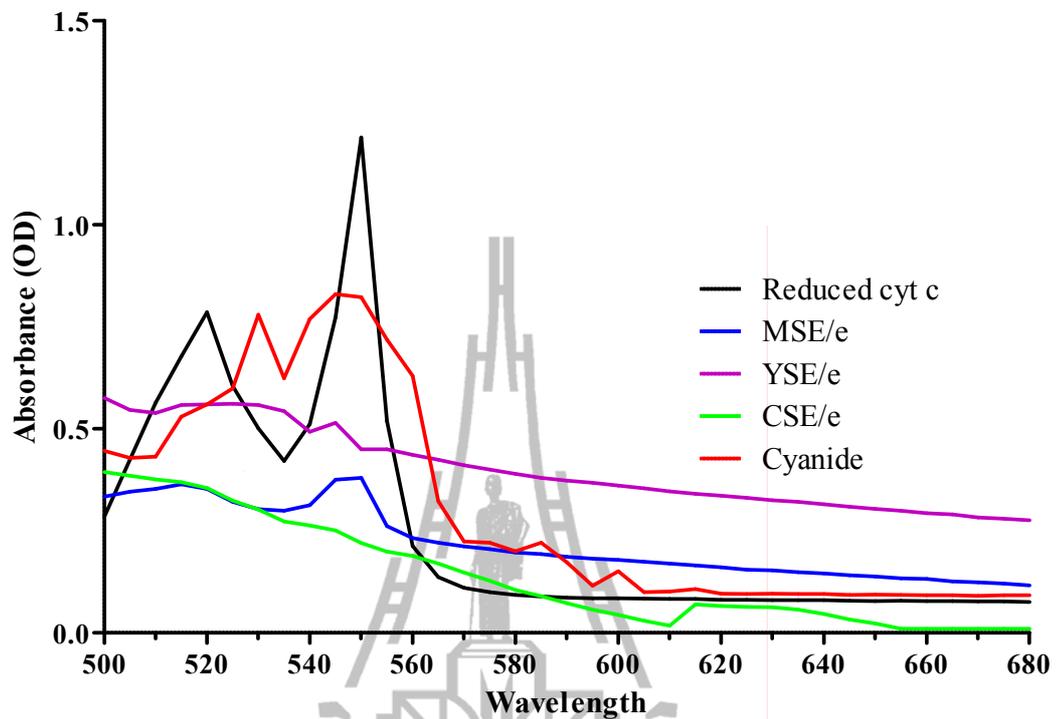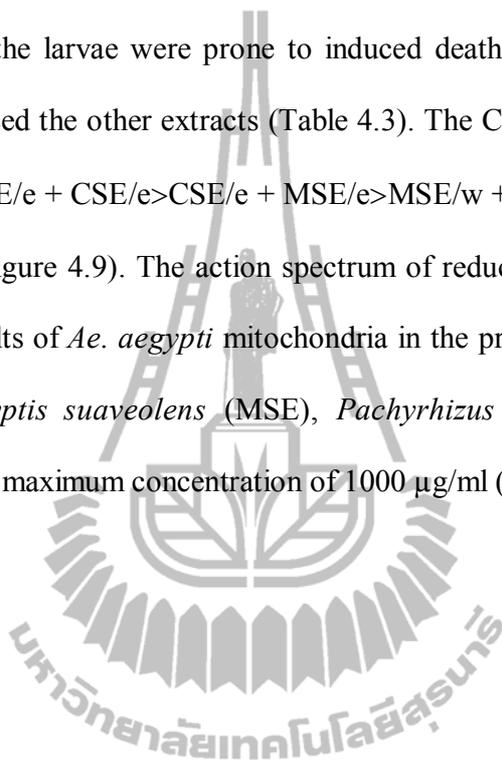
Table 4.3 Inhibition activity of cytochrome c oxidase on the 2nd instar larvae of *Aedes aegypti* using the seed extracts combination of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE).

Seed extracts	Conc. (μg/ml)	% COX inhibition (Mean ± S.E.)
MSE/w + YSE/w	100	44.06 ± 3.51^{de}
	300	51.89 ± 6.47^{f}
	500	55.33 ± 2.31^{fg}
	1,000	63.68 ± 5.84^{ij}
CSE/w + MSE/w	100	20.33 ± 3.57^{a}
	300	32.79 ± 3.10^{4b}
	500	39.94 ± 1.70^{cd}
	1,000	45.59 ± 4.49^{e}
YSE/w + CSE/w	100	22.02 ± 6.03^{a}
	300	35.93 ± 5.81^{bc}
	500	46.03 ± 3.15^{e}
	1,000	61.61 ± 5.53^{ij}
MSE/e + YSE/e	100	59.44 ± 3.70^{ghij}
	300	68.71 ± 2.25^{kl}
	500	71.25 ± 3.29^{l}
	1,000	79.54 ± 5.39^{m}
CSE/e + MSE/e	100	53.08 ± 2.24^{f}
	300	58.56 ± 2.24^{ghi}
	500	64.06 ± 1.39^{jk}
	1,000	69.92 ± 3.93^{l}
YSE/e + CSE/e	100	55.65 ± 2.49^{fgh}
	300	60.77 ± 4.74^{hij}
	500	69.75 ± 3.36^{l}
	1,000	76.39 ± 6.24^{m}
Cyanide	0.003 (mg/ml)	84.06 ± 1.99

Values are the mean of 6 observations, within a column followed by different letters are significant difference, analyzed by Duncan multiple range test (DMRT) (P<0.05), n = 6.

Figure 4.9 Efficiency of the seed extract combinations of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) on the COX activity in mitochondria of the larvae of *Aedes aegypti* at concentration of 1000 µg/ml, *P<0.05, **P<0.01, ns = not significant.

Figure 4.10 The action spectrum of reduced cytochrome c on the 2nd instar larvae of *Aedes aegypti* mitochondria in the presence of water seed extracts combinations of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) at maximum concentration of 1000 µg/ml.

Figure 4.11 The action spectrum of reduced cytochrome c on the 2nd instar larvae of *Aedes aegypti* mitochondria in the presence of combined ethanol seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) at maximum concentration of 1000 µg/ml.

The paired combination of the extracts (1 : 1, v/v) effects on the cytochrome c oxidase activity in the adult mosquitoes were investigated. It was obvioused that the adult were prone to induced death. The extract combination and the YSEs enhanced the other extracts (Table 4.3 and Table 4.4). The COX inhibition was ranged as MSE/e + YSE/e>CSE/e + MSE/e>YSE/e + CSE/e>MSE/w + YSE/w>YSE/w + CSE/w>CSE/w + MSE/w (Figure 4.12). The action spectrum of reduced cytochrome c on the adults of *Ae. aegypti* mitochondria in the presence of water and ethanol seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) at maximum concentration of 1000 µg/ml (Figures 4.13 and 4.14).
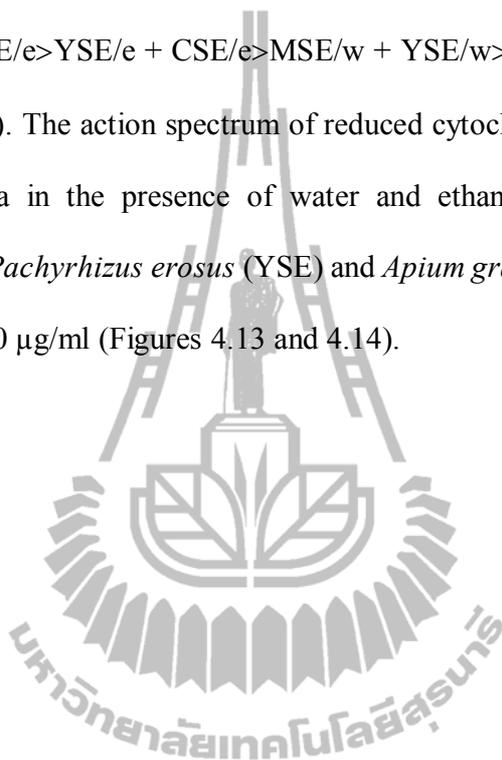
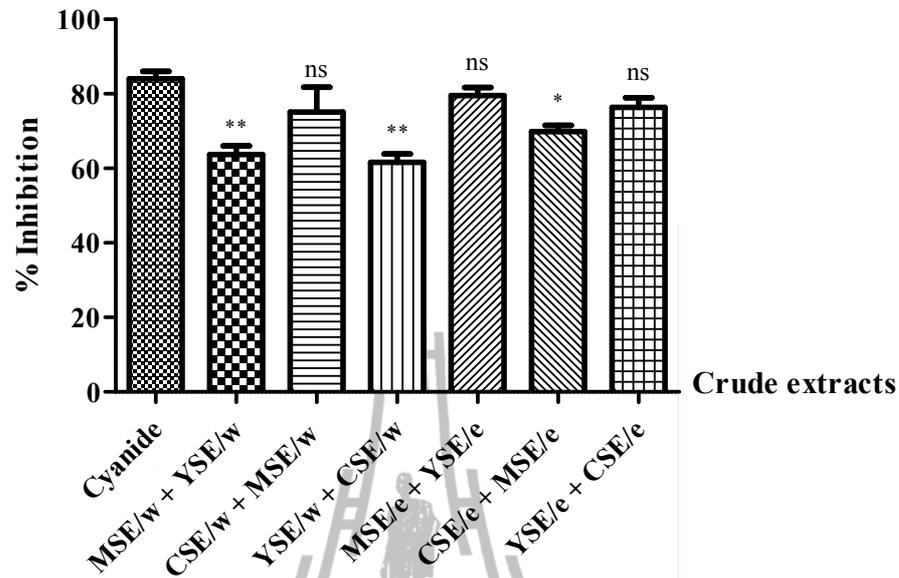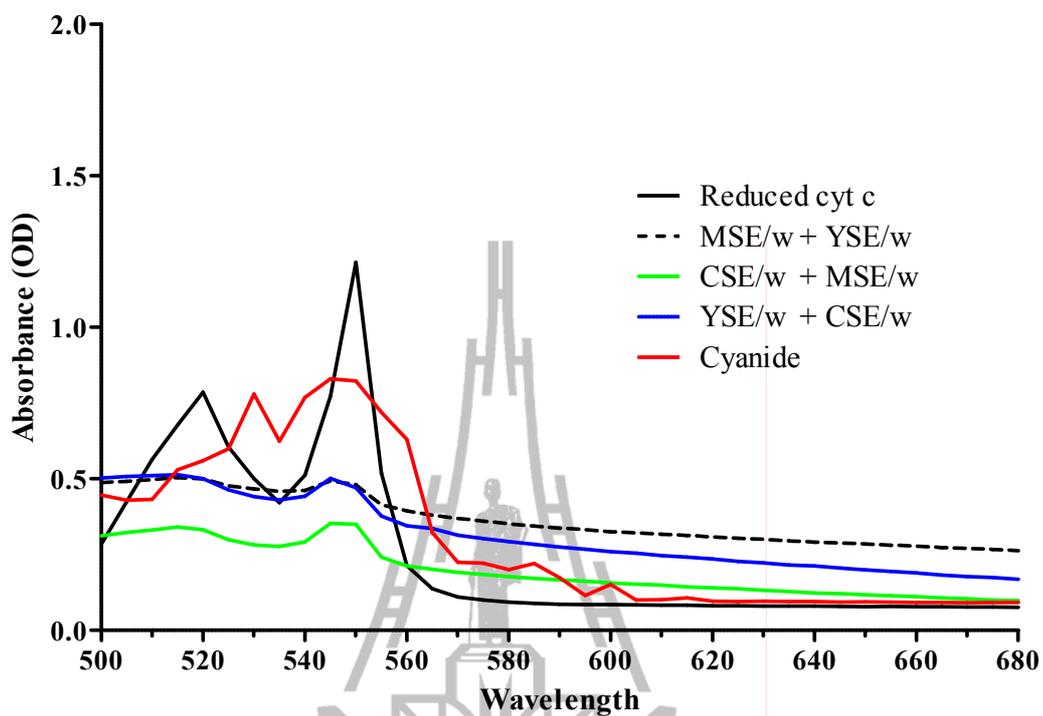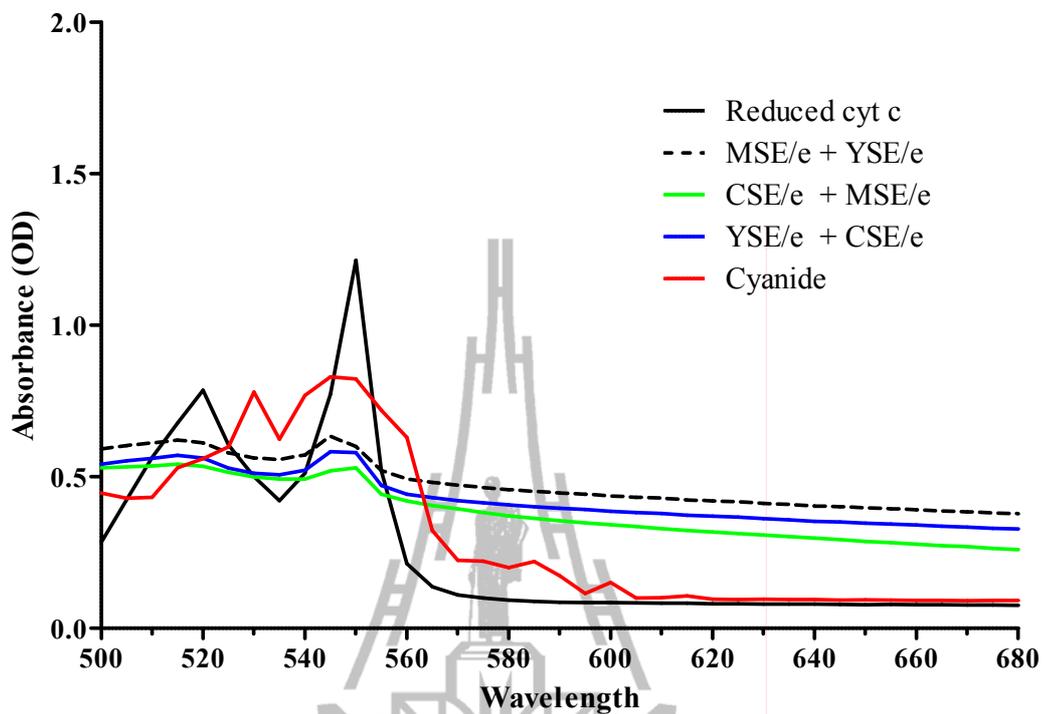**Table 4.4** Inhibition activity of cytochrome c oxidase on the adult of *Aedes aegypti* using the seed extracts combination of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE).

Seed extracts	Conc. (μg/ml)	% COX inhibition (Mean ± S.E.)
MSE/w + YSE/w	100	18.71 ± 3.25^b
	300	29.23 ± 4.00^{de}
	500	45.20 ± 2.71^g
	1,000	52.30 ± 2.90^{hi}
CSE/w + MSE/w	100	20.00 ± 2.87^b
	300	25.62 ± 2.99^{cd}
	500	32.09 ± 3.13^e
	1,000	45.39 ± 3.66^g
YSE/w + CSE/w	100	12.57 ± 1.39^a
	300	21.29 ± 4.95^{bc}
	500	30.53 ± 3.25^e
	1,000	36.71 ± 3.69^f
MSE/e + YSE/e	100	56.25 ± 2.50^{ij}
	300	60.55 ± 5.04^j
	500	65.57 ± 6.88^k
	1,000	70.62 ± 4.37^l
CSE/e + MSE/e	100	44.47 ± 3.06^g
	300	52.39 ± 2.10^{hi}
	500	56.45 ± 3.08^{ij}
	1,000	67.45 ± 5.08^{kl}
YSE/e + CSE/e	100	42.92 ± 3.00^g
	300	50.40 ± 5.62^h
	500	55.12 ± 3.09^i
	1,000	59.86 ± 3.68^j
Cyanide	0.003 (mg/ml)	84.06 ± 1.99

Values are the mean of 6 observations, within a column followed by different letters are significant difference, analyzed by Duncan multiple range test (DMRT) ($P<0.05$), n = 6.
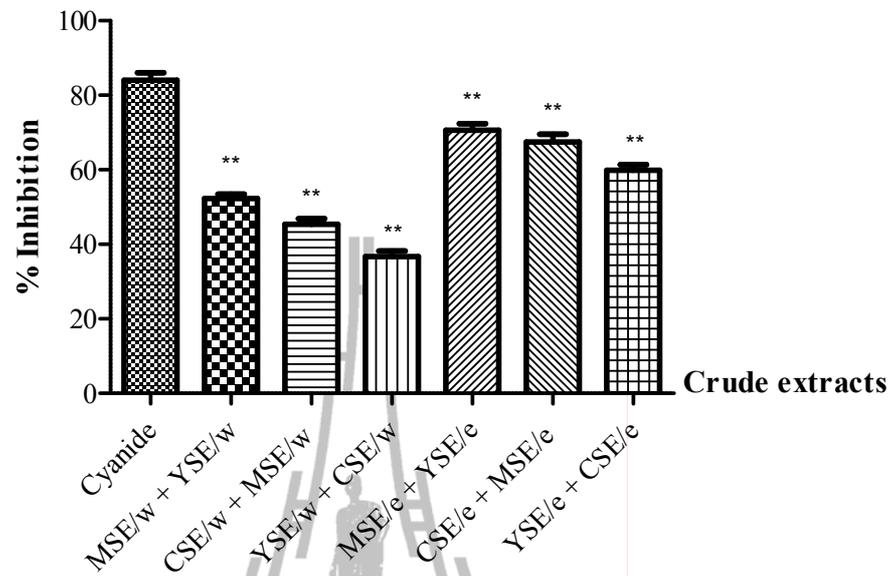
Figure 4.12 Efficiency of the seed extract combinations of *Hyptis suaveolens* (MSE),

Pachyrhizus erosus (YSE) and *Apium graveolens* (CSE) on the COX

activity in mitochondria of the adult of *Aedes aegypti* at concentration of

1000 μg/ml, **P<0.01.
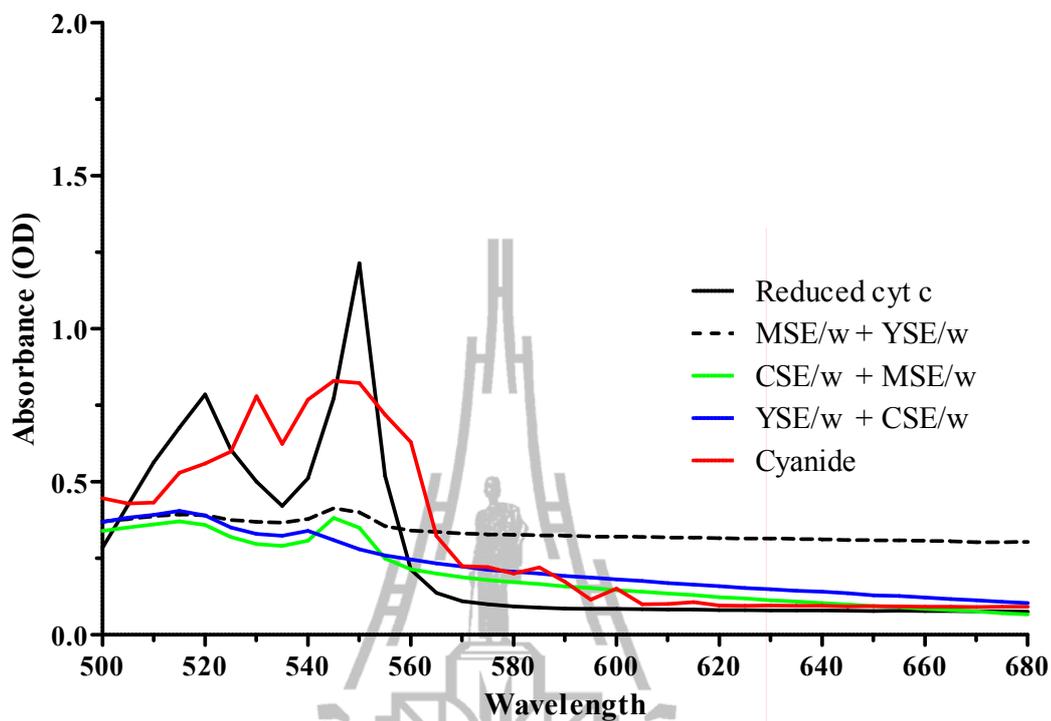
Figure 4.13 The action spectrum of reduced cytochrome c on the adult of *Aedes aegypti* mitochondria in the presence of combined water seed extracts of *Hyptis suaveolens* (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE) at concentration of 1000 µg/ml.
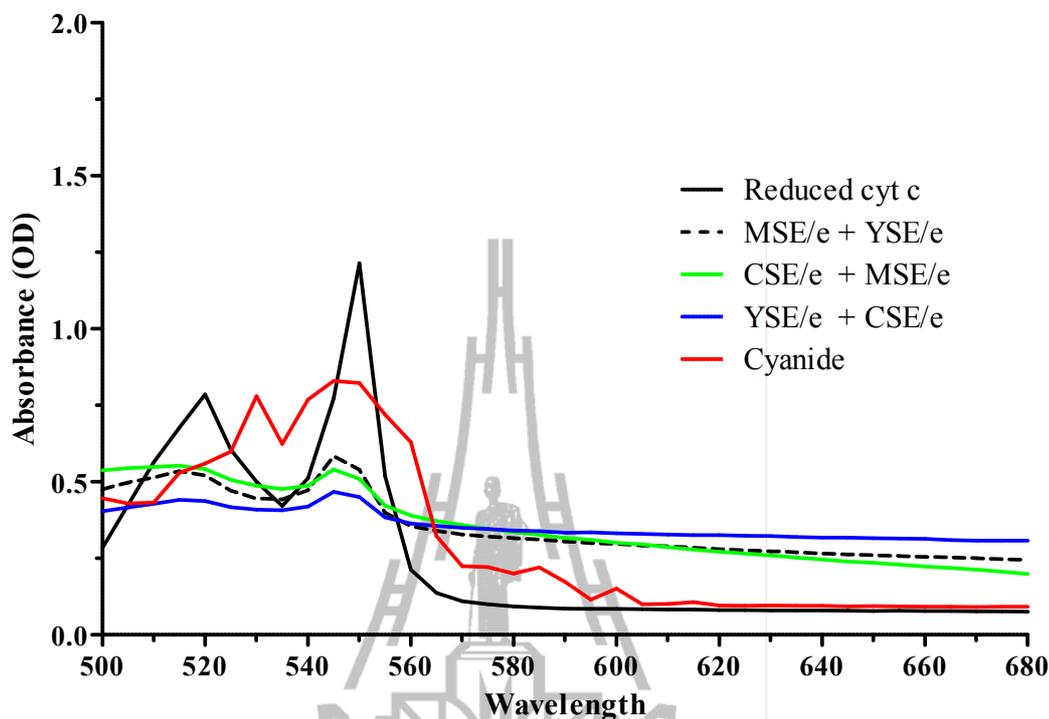
**Figure 4.14** The action spectrum of reduced cytochrome c on the adult of *Aedes aegypti*
mitochondria in the presence of combined ethanol seed extracts of *Hyptis*
suaveolens (MSE), *Pachyrhizus erosus* (YSE) and *Apium graveolens* (CSE)
at concentration of 1000 μg/ml.

4.5 Discussion

The effects of mintweed (*Hyptis suaveolens*, MSE), yam bean (*Pachyrhizus*
erosus, YSE) and celery (*Apium graveolens*, CSE) on the cytochrome c oxidase activities
of both *Ae. aegypti* larvae and adults were investigated. This could support the actions at
cellular level of these plant seed extracts which caused death to both larvae and the adults
of the mosquitoes. The ethanol seed extracts were more potent than the water extracts to
both the 2nd instar larvae and the adults. In particular, the yam bean ethanol extracts were
likely to synergistically enhance the seed extracts. Even though, the larvae were more

susceptible to the extracts than the adults. It could imply that these plant seeds may contained some phytochemicals those could interfere the cellular function at the electron transport chain in the mitochondria. There are very slim evidence of any plant extracts on the activity of cytochrome c oxidase in *Ae. aegypti*. However, there were reports that the seed extracts of mintweed, yam bean, and celery abundantly contained terpenes, 2a-hydoxyrotenone, rotenone, cyanogenic glycosides, pachyrrhizine, pachyrrhizone, dolineone, 12-(A)-hydropachyrrhizone, dehydropachyrrhizone, erosone, phthalides, coumarins, flavonoids, sesquiterpenoids, and aromatic glucosides which affected the respiratory system of organisms (Preezada, 1997; Isman, 2000; Campos, Heleno, Tomas and Suzana, 2001; Laura, Silvia, Leticia and Francisco, 2004; Narongchai, Narongchai, and Thampituk, 2005; Hung *et al.*, 2007; Li, Wei, Xu, Huag and Yao, 2009; Zhou *et al.*, 2009). The ethanol extracts of *Citrus sinensis* peels and *Hyptis suaveolens* leaves were found to be toxic to the *Aedes aegypti* larvae. The *H. suaveolens* extract caused death to this mosquito up to 80% at 0.3-0.9 µg/ml (Ausan, Idowu and Arowolo, 2005). The petroleum ether extract of *H. suaveolens* seed showed acute toxicity on the second instar larva of diamond back moth, *Plutella xylostella* (Keita, Umoetok and Smith, 2006). The toxicity of yam bean seeds is usually attributed to rotenone and rotenoid compounds in their extract. Rotenone is a potent inhibitor of mitochondrial electron transport. Hung *et al.* (2007) reported that rotenone and cyanogenic glycosides caused metabolic acidosis through disruption of oxygen transport. However, treatment with cyanide antidote agents such as nitrite and thiosulfate was not effective remedy for rotenone poisoning. Rotenone caused death to insects by inhibited NADH oxidation in mitochondria leading to critically blocking respiration and nerve conduction (Marrs and Ballantyne, 2004). Nitrogenous compounds in celery seed essential oil have been reported to have effects on the central nervous system (Momiin and Nair, 2002).

Choochote *et al*. (2004) reported that crude seed extract of celery was investigated for anti-mosquito potency including larvicidal, adulticidal, and repellent activities, which the extract has probably toxic effect on the nervous system of mosquitoes. These findings agreed with our observation which demonstrated that the extracts of mintweed, yam bean, and celery seeds could control both larval and adult mosquitoes through the inhibition of cytochrome c oxidase activity of the electron transport chain in cellular respiration. Our study is the first investigation of plant seed extracts in controlling mosquitoes by interfering with the cellular respiration at the electron transport chain in mitochondria.

Toxic agents are classified by their target organs (liver, kidney, hematopoietic system, etc.), their use (pesticide, solvent, food additive, etc.) and their sources (animal and plant toxins) (Klaassen, 2001). Organic insecticides comprise the largest numbers of pesticides available for use today. Natural insecticides such as nicotine, pyrethrum and neem extracts are made by plants as defenses against insects. These mechanisms can be altered target sites in the nervous and metabolic systems. Involving insecticides have a long time in the degradation mechanisms (Klaassen, 2001). Many scientists are interested in discovering herb plants that are not yet efficiently-useful. Utilizing study on the qualification of a substance as an insecticide, repellent, attractant, sterilant, pheromone, and antifeedant. Plant insecticides are pesticides active substances with insect for instance nicotine, alkaloids, sulphate, rotenone, azadirachtin, helleborn, ryania, sabadilla, and pyrethrum. The toxic properties of insecticides derived from plant source caused significant inhibition of COX activity (Marrs and Ballantyne, 2004).

Similarly, Song and Scharf (2009) was first investigated COX inhibition in *Drosophila melanogaster* mitochondria by hydramethylnon, There are two insecticidal materials, hydramethylnon and sodium cyanide caused significant inhibition of COX

activity. Moreover, pattern of substances have toxic properties are ester group insecticides for instance organophosphate, carbamate and pyrethroid (Liu, Zhu, Xu, Julia and Gao, 2006).

4.6 Conclusion

In conclusion, the individual of seed extracts both larval and adult were the most potent gather COX inhibition at 65.24% and 59.08%, respectively. The combination of YSE/e and MSE/e induced highest COX inhibition in larvae and in adults 64.59%, LC_{50} 23.82 µg/ml. The combination of MSE/e and YSE/e inhibited COX activity were 79.54% in larvae and 70.62% in the adults. The seed extracts was disrupting COX that inducing the cause of death and toxicity in other systems. Therefore, this result may suggest that the ethanol extracts of the species possess compounds with strong anti mosquito properties more than the water extracts, which can be used as new agents for the biological control of *Ae. aegypti*.

4.7 References

Ausan, A. A. S., Idowu, A. B., and Arowolo, F. S. (2005). Comparative toxicity effect of bush tea leaves (*Hyptis suaveolens*) and orange peel (*Citrus sinensis*) oil extract on larvae of the yellow fever mosquito *Aedes aegypti*. **Tanzania Health Research Bulletin**. 7(3): 174-178.

Brändén, M., Sigurdson, H., Namslauer, A., Gennis, R. B., Ädelroth, P., and Brzezinski, P. (2001). On the role of the K-proton transfer pathway in cytochrome c oxidase. **Proceeding of the Natural Academy of Sciences of the United States of America**. 98(9): 5013-5018.

Burmester, T., and Hankeln, T. (2004). Neuroglobin: A respiratory protein of the nervous system. **News in Physiological Sciences**. 19: 110-113.

Campos, I. F. P., Heleno, D. F., Tomas, A. P., and Suzana, C. S. (2001). Chemical variability in essential oil of *Hyptis suaveolens*. **Phytochemistry**. 57: 733-736.

Carlini, E. A. (2003). Plants and the central nervous system. **Pharmacology Biochemistry and Behavior**. 75: 501-512.

Chamberlin, M. E. (2006). Changes in mitochondrial electron transport chain activity during insect metamorphosis. **American Journal of Physiology-Regulatory, Integrative and Comparative Physiology**. 292(2): R1016-R1022.

Chevallier, A. (1998). **The Encyclopedia of medicinal plants** (p 217). New York; Dorling Kindersley.

Choochote, W., Tuetun, B., Kanjanapothi, D., Rattanachanpichai, E., Chaithong, U., Chaiwong, P., Jitpakdi, A., Tippawangkosol, P., Riyong, D., and Pitasawat, B. (2004). Potential of crude seed extract of celery, *Apium graveolens* L., against the mosquito *Aedes aegypti* L. **Journal of Vector Ecology**. 29(2): 340-346.

Chuenwong, P. (2006). **Biological control of oriental fruit flies (*Bactrocera dorsalis* (Hendel)) by the extracts of neem, sugar apple and mintweed**. Ph.D. Thesis, Suranaree University of Technology, Thailand.

Haritos, V. S., and Dojchinov, G. (2003). Cytochrome c oxidase inhibition in the rice weevil *Sitophilus oryzae* by formate, the toxic metabolite of alkyl formates. **Comparative Biochemistry and Physiology Part C**. 136(2): 135-143.

Hoch, M. (2007). Wurst ensures that the respiratory system works; Protein could be a new starting point for drug development [On-line]. **Available**: http://www.eurekalert. org/pub_releases/2007-06/uob-et061307.php.

Hung, Y. M., Hung, S. Y., Olson, K. R., Chou, K. J., Lin, S. L., Chung, H. M., Tung, C. N., and Chang, J. C. (2007). Yam bean seed poisoning mimicking cyanide intoxication. **Internal Medicine Journal**. 37: 130-132.

Isman, M. B. (2000). Plant essential oils for pest and disease management. **Crop Protection**. 19: 603-608.

Karp, G. (2002). **Cell and molecular biology; concepts and experiments** (4th ed.). Kingston, Rhode Island, The United States of America; John Wiley and Sons.

Keita, S., Umoetok, S. B. A., and Smith, J. G. (2006). The insecticidal activity of petroleum ether extract of *Hyptis suaeolens* Poit (Labiatae) seeds on *Plutella xylostella* (L.) (Lepidoptera: Y ponomeutidae). **Agricultural Journal**. 1(1): 11-13.

Kitajima, J., Ishikawa, T., and Satoh, M. (2003). Polar constituents of celery seeds. **Phytochemistry**. 64: 1003-1011.

Klaassen, C. D. (2001). **Casarett and doull's toxicology the basic science of poisons** (6th ed.) (pp.1-1,275). New York; The McGraw-Hill.

Kulshrestha, V. K., Saxena, R. C., and Kohli, R. P. (1967). Some central effects of *Apium graveolens* (Linn.). **Indian Journal of Physiology and Pharmacology**. 55(10): 1099-1102.

Laura, L. B. N., Silvia, B. B., Leticia, B. L., and Francisco, J. L. G. S. (2004). Antifungal activity of seed powders, extracts, and secondary metabolites of *Pachyrhizus erosus* (L.) Urban (Fabaceae) against three postharvest fungi. **Revista Maxicana de Fitopatologia**. 22(3): 356-361.

Lee, S. E., Lee, B. H., Choi, W. S., and Park, B. S. (2001). Fumigant toxicity of volatile natural products from Korean spices and medicinal plants towards the rice weevil, *Sitophilus oryzae* (L.). **Pest Management Science**. 57: 548-553.

Lee, S. E., Peterson, C. J., and Coats, J. R. (2003). Fumigation toxicity of monoterpenoids to several stored product insects. **Journal of Stored Products Research**. 39: 77-85.

Li, Y. Z., Wei, X. Y., Xu, H. H., Huang, X. Q., and Yao, Z. W., (2009). Insecticidal ingredients from yam bean (*Pachyrrhizus erosus*) seeds and their insecticidal toxicity. **Acta Entomologica Sinica**. 52(5): 514-521.

Liu, N. N., Zhu, F., Xu, Q., Julia W. P., and Gao, X. W. (2006). Behavioral change, physiological modification, and metabolic detoxification: mechanism of insecticide resistance. **Acta Entomologica Sinica**. 49(4): 671-679.

Marrs, T. C., and Ballantyne, B. (2004). **Herbicides in pesticide toxicology and international regulation**. Chichester; John Wiley and Sons.

Maruyama, T., Abbaskhan, A., Choudhary, I. M., Tsuda, Y., Goda, Y., Farille, M., and Reduron, J. P. (2009). Botanical origin of Indian celery seed (fruit). **Journal of Natural Medicines**. 63(3): 248-253.

Momin, R. A., and Nair, M. G. (2002). Antioxidant, cyclooxygenase and topoisomerase inhibitory compounds form *A. graveolens* seeds. **Phytochemistry**. 9: 312-318.

Musa, A. K., Dike, M. C., and Onu, I. (2009). Evaluation of nitta (*Hyptis suaveolens* Poit.) seed and leaf extracts and seed powder for the control of *Trogoderma granarium* everts (Coleoptera: Dermestidae) in stored groundnut. **American-Eurasian Journal of Agronomy**. 2(3): 176-179.

Narongchai, P., Narongchai, S., and Thampituk, S. (2005). The first fatal case of yam bean and rotenone toxicity in Thailand. **Journal of the Medical Association of Thailand**. 88(7): 984-986.

Oyedunmade, E. E. A. (1998). Control of nematode pests of cowpea with Mocap (Ethoprop), leaf residues of neem (*Azadirachta indica*, rattle weed (*Crotalaria retusa*) and nitta (*Hyptis suaveolens*). **Centrepoint Science Edition**. 8(1): 57-63.

Pandey, V. N., and Dubey, N. K. (1994). Antifungal potential of leaves and essential oils from higher plants against soil phytopathogens. **Soil Biology and Biochemistry**. 26(10): 1417-1421.

Paula, S., Sucheta, A., Szundi, I., and Einarsdóttir, O. (1999). Proton and electron transfer during the reduction of molecular oxygen by fully reduced cytochrome c oxidase: a flow-flash investigation using optical multichannel detection. **Biochemistry**. 38: 3025-3033.

Peerzada, N. (1997). Chemical composition of the essential oil of *Hyptis suaveolens*. **Molecules**. 2: 165-168.

Prates, H. T., Santos, J. P., Waquil, J. M., Fabris, J. D., and Oliveira, A. B. (1998). The potential use of plant substances extracted from Brasilian flora to control stored grain pest. In Zuxun, J., Quan, L., Yongsheng, L., Xianchang, T., and Liangua, G., (eds.) (1998). **Proceedings of the 7th International Conference on Stored-Product Protection** (pp 820-825). Beijing, China: Sichuan House of Science and Technology.

Satyavati, G. V., and Raina, M. K. (eds.) (1976). **Medicinal plants of India** (pp. 312-317). New Delhi; Indian Council of Medical Research.

Schulz, J. M., and Karl, H. (1980). Occurrence of hydroxyl benzoic acids and hydroxyl cinnamic acids in species of *Hyptis*-4. **Phendic Zlebensc-Zlebenscmunters-Forsch**. 17(3): 193-199.

Solomos, T. (1977). Cyanide-resistant respiration in higher plants. **Annual Review of Plants Physiology**. 28: 279-297.

Song, C., and Scharf, M. E. (2009**)**. Mitochondrial impacts of insecticidal formate esters in insecticide resistant and susceptible *Drosophila melanogaster*. **Pest Management Science**. 65: 697-703.

Wallace, K. B., and Starkov A. A. (2000). Mitochondrial targets of drug toxicity. **Annual Review of Pharmacology and Toxicology**. 40: 353-388.

Wieczorek, H. (1982). A biochemical approach to the electrogenic potassium pump of insect sensilla: potassium sensitive ATP ases in the labellum of the fly. **Journal of Comparative Physiology**. 148: 303-311.

Voet, D. J., Voet, J. G., and Pratt, C. (2008). **Principles of biochemistry** (3rd ed.) (pp. 534). New Jersey, The United States of America; John Wiley and Sons.

Zhou, K., Zhou, F., Liu, Z., Zhuang, Y., Chen, L., and Qiu, F. (2009). Triterpenoids and flavonoids from celery. **Journal of Natural Products**. 72(9): 1563-1567.
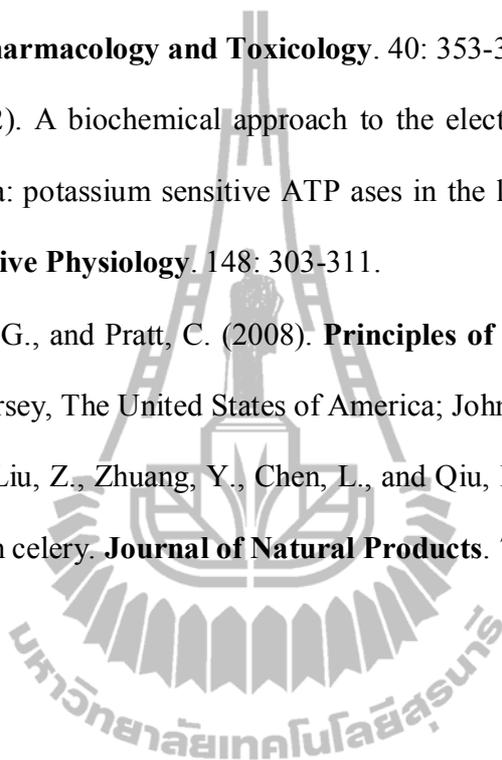
CHAPTER V

CONCLUSION

Aedes aegypti is well known as the main vector of dengue viruses that cause dengue hemorrhagic fever in the population of Thailand and other tropical countries. The eradication of the mosquitoes has been practiced for many decades by synthesis insecticides. It may reduce the risk of the disease, but in fact it causes environmental problems by inducing insect resistances and human health impairment by the chemicals. Therefore, to cope with these problems, the need to search for new selective and biodegradable insecticides from natural products is required. In the present study, the seed extracts of mintweed (*Hyptis suaveolens*), yam bean (*Pachyrhizus erosus*) and celery (*Apium graveolens*) were investigated for the biological control of *Ae. aegypti*, both larvae and adults. To summarized, the seed extracts performed in ethanol and water contained high phenolic compounds and flavonoids. The contents of TPC in MSE/e, CSE/e, and YSE/e were 275.91, 246.64, and 108.34 mg CE/g and in MSE/w, CSE/w, and YSE/w were 69.93, 48.75 mg GAE/g, and 37.85 mg GAE/g, respectively. The amounts of TFC in MSE/e, CSE/e, and YSE/e were 196.21, 185.43, and 95.16 mg CE/g and in MSE/w, CSE/w, and YSE/w were 65.92, 41.81 mg CE/g, and 37.85 mg CE/g, respectively. Thin layer chromatographic analysis of the extracts indicated that they were likely to be terpenes and cyanogenic glycosides as detected by Vanillin-sulphuric reagent and Kedde reagent, respectively. These compounds could be the important chemicals that caused the death of both larval and adult mosquitoes. The toxic activities of the extracts

were dependent on the plant species and extracted solvents and very broad magnitude from nanogram to milligram, it may because of their different components.

The yam bean seed extracts (YSEs) were the most cytotoxicity against brine shrimps (*Artemia salina*). The LC_{50} at 24 h of YSE/e was 0.02 µg/ml and of YSE/w was 257.11 µg/ml, assayed by the brine shrimp lethality assay (BSLA). The YSE/e was the most potent in controlling the 2nd instar larvae and the CSE/w was the least one. The extract efficacy on the 2nd instar larvae mortality was ranged as YSE/e>YSE/w>MSE/e> CSE/e>MSE/w>CSE/w with LC_{50} 16.22, 724.64 µg/ml, 1.40, 3.78, 16.65, and 25.23 mg/ml, respectively. By spraying in the adult habitat, the YSE/e accessed most toxic effect on the adult mosquitoes and the least spray effect was by the CSE/w. The efficacy on mortality was ranged as YSE/e>MSE/e>YSE/w>CSE/e>MSE/w>CSE/w with LC_{50} 91.41 µg/ml, 2.95, 9.60, 75.05, and 109.03 mg/ml, respectively.

The combinations (1 : 1, v/v) of the seed extract were more better in controlling both the 2nd instar larvae and adult mosquitoes. The YSE synergistically enhanced the mortality of the mosquitoes to the other extracts. The highest efficacy on the 2nd instar larvae and on the adult mosquitoes was the combination of MSE/e and YSE/e. The efficacy on the mortality of all combinations on the 2nd instar larvae was ranged as MSE/e + YSE/e>YSE/e + CSE/e>CSE/e + MSE/e>YSE/w + MSE/w>YSE/w + CSE/w> CSE/w + MSE/w with LC_{50} 11.79, 82.04, 121.66, 225.43, 930.6 µg/ml and 5.09 mg/ml, respectively. The efficacy on the adult mortality was ranged as MSE/e + YSE/e>CSE/e + MSE/e>CSE/e + YSE/e>CSE/w + YSE/w>MSE/w + YSE/w>CSE/w + MSE/w with LC_{50} 23.82, 275.18, 318.64 µg/ml, 1.25, 1.64, and 12.32 mg/ml, respectively. Thus, it could be assumed that the yam bean seeds ethanol extract was a synergistic agent

enhancing the other seed extracts in the biological control of both larvae and adults of the *Ae. aegypti* mosquitoes.

The actions of these seed extracts on the electron transport complex III, cytochrome c oxidase (COX), in the inner membrane of mitochondria were investigated in both larvae and adults of *Ae. aegypti*. The effect of the extracts on the COX activity was also dependent on the seed types and the extracted solvents. The yam bean seed extracted with ethanol (YSE/e) was the most potent. The inhibitory potential of all individual extracts on COX of the larvae was ranged as YSE/e>YSE/w>MSE/e> CSE/e>MSE/w> CSE/w with % COX inhibition 65.24%, 53.01%, 45.46%, 33.22%, 28.93, and 21.39%, respectively. The potency of individual seed extracts on COX inhibition, treated on the adult mosquitoes, was ranged as YSE/e>MSE/e>YSE/w> CSE/e>MSE/w>CSE/w with % COX inhibition 59.08%, 50.18%, 41.93%, 28.77%, 23.20%, and 17.83%, respectively. All extracts on the mosquito larvae and adults were of same the pattern, but the magnitude on the larvae was slightly higher.

The COX inhibition by the combinations of the extracts on larvae was ranged as MSE/e + YSE/e>YSE/e + CSE/e>CSE/e + MSE/e>MSE/w + YSE/w>YSE/w + CSE/w> CSE/w + MSE/w with % COX inhibition 79.54%, 76.39%, 69.92%, 63.68%, 61.61% and 45.59%, respectively.

The COX inhibition by the combinations of the extracts on adult was ranged as MSE/e + YSE/e>CSE/e + MSE/e>YSE/e + CSE/e>MSE/w + YSE/w>YSE/w + CSE/w> CSE/w + MSE/w with % COX inhibition 70.62%, 67.45%, 59.86%, 52.30%, 45.39%, and 36.71%, respectively. When compared all types of the COX inhibition by the combination above larvae and adults, MSE/e + YSE/e showed the highest % COX inhibition from all the combinations.

In conclusion, the yam bean (*Pachyrhizus erosus*) seed ethanol and water extracts were high in toxic to control in control *Ae. aegypti*, the dengue hemorrhagic fever vector, both larvae and adults. The other extracts showed their toxicity against the mosquitoes to some certain extent. The extracts of mintweed seed were moderately toxic and of celery were mild. It is noted that the efficacy of the extracts depend upon the constituents, which were terpenes and cyanogenic glycosides detected. The yam bean seed extracts could be used as selective insecticide in controlling the mosquitoes, but with caution when applying them in aquatic environment.
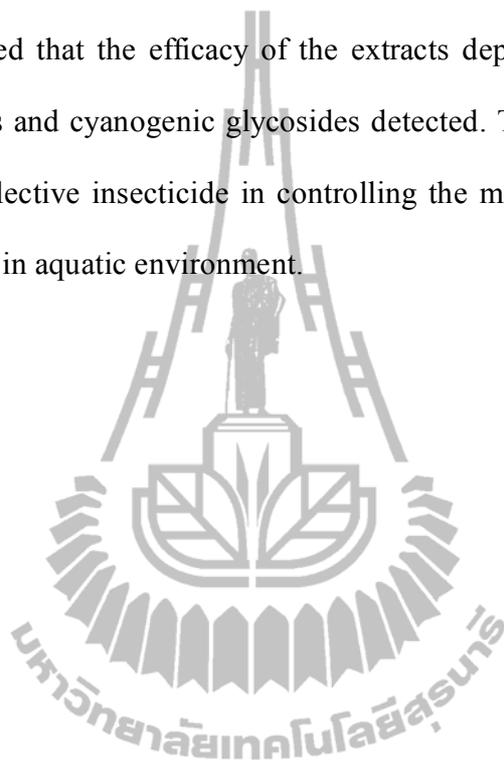
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