BIOLOGICAL CONTROL OF ORIENTAL FRUIT FLIES (*Bactrocera dorsalis* (Hendel)) BY THE EXTRACTS OF NEEM, SUGAR APPLE AND MINTWEED

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การควบคุมแมลงวันผลไม้ (*Bactrocera dorsalis* (Hendel)) โดยชีววิธี ด้วยสารสกัดจากสะเดา น้อยหน่า และแมงลักคา

นายพงษ์นรินทร์ ชื่นวงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาสิ่งแวดล้อม
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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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พงษ์นรินทร์ ชื่นวงศ์ : การควบคุมแมลงวันผลไม้ (*Bactrocera dorsalis* (Hendel)) โดยชีววิธี ด้วยสารสกัดจาก สะเดา น้อยหน่า และแมงลักคา (BIOLOGICAL CONTROL OF ORIENTAL FRUIT FLIES (*Bactrocera dorsalis* (Hendel)) BY THE EXTRACTS OF NEEM, SUGAR APPLE AND MINTWEED). อ. ที่ปรึกษา : รศ. ดร. กรกช อินทราพิเชฐ, 103 หน้า

ศึกษาการควบคุมแมลงวันผลไม้โดยชีววิธีจากสารสกัดเดี่ยวจากพืช 3 พืช คือ สะเดา น้อยหน่า และแมงลักคา และสารผสมของพืชเหล่านี้ พบว่าสารสกัดด้วยเอธานอลจากใบสะเดา มีสารประกอบฟีโนลิกสูงที่สุด สารสกัดด้วยเอธานอลจากเมล็ดแมงลักคา มีคุณสมบัติแอนตี้ออกซิแดนท์แอกติวิตีสูงที่สุด ผลการแยกสารสกัดด้วยทินเลเยอร์โครมาโตกราฟี (TLC) ช่วยยืนยันได้ว่าพืชแต่ละชนิดสร้างสารประกอบทางเคมีที่แตกต่างกันเป็นผลให้มีแอนตี้ออกซิแดนท์แอกติวิตีแตกต่างกันด้วย ความสามารถในการไล่แมลงวันผลไม้ของสารสกัดจากพืชชนิดเดียว และสารสกัดผสมของพืช โดยใช้อุปกรณ์โอลแฟคโตมิเตอร์ ชี้ให้เห็นว่าสารสกัดจากพืชชนิดเดียวที่มีฤทธิ์ในการไล่แมลงวันผลไม้ดีที่สุดคือสารสกัดด้วยเอธานอลจากเมล็ดแมงลักคา สารสกัดด้วยเอธานอล จากทุกพืช จะให้ผลต่อการไล่แมลงวันผลไม้ ได้ดีกว่าสารสกัดด้วยน้ำ สารสกัดผสมระหว่างสารสกัดด้วยน้ำจากใบสะเดากับสารสกัดด้วยน้ำจากใบน้อยหน่าให้ฤทธิ์ในการไล่แมลงวันผลไม้ได้ดีกว่าสารผสมของสารสกัดอื่นๆ และดีกว่าสารสกัดด้วยเอธานอลจากพืชทั้ง 3 ชนิด พบว่าพิษของสารสกัดผสมสูงกว่าสารสกัดเดี่ยว โดยเฉพาะสารสกัดด้วยเอธานอลจากใบสะเดาผสมกับสารสกัดด้วยเอธานอลจากใบแมงลักคา ควบคุมแมลงวันผลไม้ได้มากที่สุด สารสกัดผสมมีความเป็นพิษเสริมหรือลดค่า LD_{50} อย่างมีนัยสำคัญ การสกัดด้วยเอธานอลทั้งในสารสกัดเดี่ยว และสารสกัดผสม ให้ค่า LD_{50} ต่ำกว่าการสกัดด้วยน้ำ นอกจากนั้นสารสกัดที่มีฤทธิ์ในการฆ่าแมลงวันผลไม้ได้ทุกๆ ระยะเมื่อมีแมงลักคาเป็นส่วนประกอบ ผลการทดลองนี้สอดคล้องเป็นอย่างดีกับการทดสอบพิษด้วย BSLA จึงสรุปได้ว่าในสารสกัดจาก 3 พืชที่ศึกษานี้ สารสกัดแมงลักคา มีประสิทธิภาพต่อการควบคุมแมลงวันผลไม้ได้ดีที่สุด

PONGANARIN CHUENWONG: BIOLOGICAL CONTROL OF ORIENTAL FRUIT FLIES (*Bactrocera dorsalis* (Hendel)) BY THE EXTRACTS OF NEEM, SUGAR APPLE AND MINTWEED
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ORIENTAL FRUIT FLIES / BIOLOGICAL CONTROL / TOTAL PHENOLIC COMPOUNDS / ANTIOXIDANT / RADICAL SCAVENGER / CYTOTOXICITY / THIN LAYER CHROMATOGRAPHY / NEEM / SUGAR APPLE / MINTWEED

Biological control of oriental fruit flies using plant extracts, neem, sugar apple, mintweed, and their combinations was performed in this study. The neem leaf-ethanol extract had the highest total phenolic content while mintweed seed-ethanol extract had the lowest total phenolic content. Thin Layer Chromatography (TLC) results confirmed the differences of phytochemical compositions with differences in polarity as well as antioxidant activity. Repellent activities of single plant extracts and two-plant combination extracts were elucidated using an olfactometer. Results indicated that the highest repellent activity of single plant extract was mintweed seed-ethanol extract. In the case of single plant extracts, repellent activities of all ethanolic extracts were higher than those of all water extracts . It was found that combination of neem leaf-water extract and sugar apple leaf-water extract gave the highest repellent activity than the other combination extracts. The combinations of water extracts of all plant extracts showed higher repellent activities than ethanolic extracts. However, neem leaf-ethanol extract combined with mintweed leaf-ethanol extract gave the highest cytotoxicity. LC_{50} values implied that two-plant combination extracts exhibited

synergistic effect. In addition, ethanol extractions of both single extract and plant combination extracts obviously showed lower LC_{50} than water extractions. The mintweed extracts had greatest insecticidal activity (LD_{50}) of the extraction against all stages of oriental fruit flies. This result is well in agreement with the cytotoxicity assayed by BSLA. It can be concluded that mintweed is the most insecticide as the effective botanical products for controlling oriental fruit flies.

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CHAPTER I

INTRODUCTION

1.1 The general background of insects

Insects are the largest group of animals on earth. They are found in soil, hot spring water, snow, air, and on or inside of plants and animals. The large number of insects can be divided into three categories according to their importance to people as followings (UAB "Kauno profilaktines dezinfekcijos stotis, On-line, 2003):

1.1.1 Species of ecologically important insects

About 99% of insects are ecological inportance. They do not directly help or harm people, but they are essential in the food web. They are food for birds, fishes, mammals, reptiles, amphibians, aquatic lives, and other insects. Some remove animal wastes and dead plants and animals, which return the nutrients to the environment. Some are considered beautiful.

1.1.2 Beneficial insects

Some insects are beneficial as predators and parasites that feed on harmful insects, mites, and weeds. Examples are ladybird beetles, ground beetles, tachinid flies, praying mantids, and many tiny parasitic wasps. Some are pollinating insects, such as bumblebees and honeybees, some moths, butterflies, and beetles. Without pollinators, many kinds of plants could not reproduce nor widely distribute. Honey from honeybees is food for people. Secretions from some insects are made into dyes and paints. Silk is the product of insect, from the cocoons of silkworms.

1.1.3 Destructive insects

Destructive insects are also important, although they are the fewest species. They feed on, cause injury to, or transmit disease to people, animals, and plants, for example, aphids, beetles, fleas, mosquitoes, caterpillars, and termites.

1.2 Oriental fruit fly (Diptera: Tephritidae) (Sinclair, 2000)

Scientific name: *Bactrocera dorsalis* Hendel

Synonymy: *Dacus dorsalis* Hendel, *Chaetodacus ferrugineus* var. *okinawanus* Shiraki, *Musca ferruginea* Fabricius.

Common name: Oriental fruit fly.

The adult female and male oriental fruit files were represented in Figure 1. The adult oriental fruit fly is larger than a housefly about 8 mm in length. The body color is variable but generally bright yellow with a dark “T” shaped marking on the abdomen. The wings are clear. The female has a pointed slender ovipositor to deposit eggs under the skin of host fruit. Eggs are minute cylinders laid in batches. The larvae are creamy-white, legless, and may attain a length of 10 mm inside host fruit.

Figure 1.1 Adult female and male oriental fruit flies.

1.2.1 Life cycle of the fruit fly

Fruit fly life cycle is complete metamorphosis as shown in Figure 2. The eggs, difficult to see with the naked eyes, are deposited near the surface of fermenting fruit or organic matter. A pair of filaments that attache to the eggs protrudes above the surface of the liquid. The female fruit fly lays about 500 eggs. The larvae emerge within about 30 hours afterward and feed near the surface of the fermened material. The larvae feed for five to six days then crawl to drier areas of the food source or even out of the food source to pupate. The larvae transform into the pupa in a puparium, which bears a conspicuous pair of filaments on the anterior end. The adult fruit flies emerge several days later. The newly emerged fruit flies expose to light and become sexually active in about two days. The adults mate more than once. Under ideal conditions, the life cycle from egg to adult can complete in as little as eight days. The complete metamorphosis of all stages of the fruit fly on moist, decaying (or fermenting) organic materials is a key point of attention in inspection and elimination of fruit fly infestations. The fruit fly breeds in and feeds on ripened fruits and vegetables, as well as in moistly decayed organic matter. In vinegar-producing plants, the fruit fly is responsible for infecting tanks of vinegar with a tiny nematode called the vinegar worm (Professional Pest Control Products, On-line, 2004).

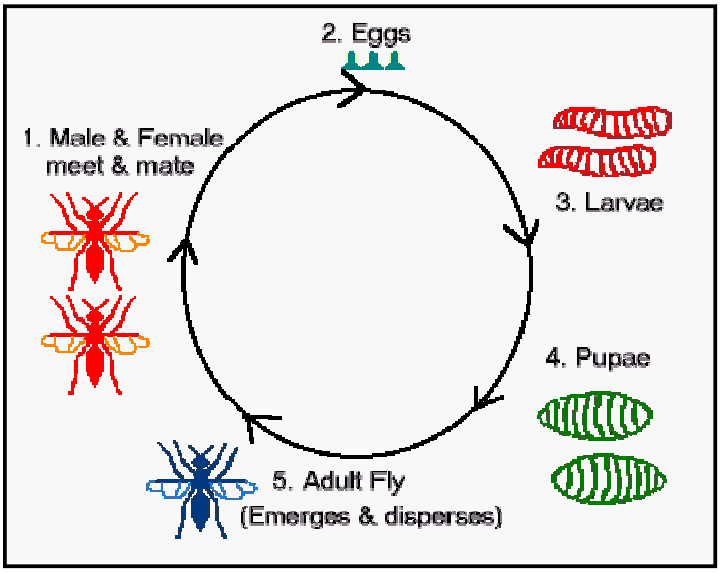

Figure 1.2 Life cycle of the fruit flies.

Egg and pupal stages of insects are generally difficult to control, because they are inactive. They do not feed or move. Also, they are often in hard-to-reach areas such as under the ground, in cocoons, in cracks and crevices (Allwood and Drew, 1997). In the late instar and adult may be controlled with moderate success, because of their size, resistant capacity to pesticides, or they may already laid eggs for the next generation. The best control of insects can be achieved at the early larval or nymphal stages, when the insects are small, active, and vulnerable.

1.2.2 Fruit flies in Thailand and their economic importance

Thailand grows numerous tropical fruits, such as mangoes, mangosteens, bananas, longans, oranges, rambutans, sugar apples, lichees, etc. Besides, domestic consumption, fruits export cost over a hundred million baht per year (รัตนาภรณ์ พรหม

ศรัทธา และคณะ, 2544). However, many fruit crops are severely damaged by oriental fruit flies (*Bactrocera dorsalis* Hendel) which highly affect Thailand fruit export.

Fruit flies are considered as the most damaging insect pests to fresh fruit and fruiting vegetables all over the world. They are found in tropical, sub-tropical and temperate regions. Although many species are not of economic importance where they feed on wild fruits, but the pest species have successfully adapted their life cycle to most cultivated fruits. Fruit fly eggs are laid into unripe or ripening fruit where the larvae develop and feed on the pulp of the fruits. Infested fruits are spoiled quickly and often fallen to the ground prior to ripening. Without control measurement, growers can lose the entire crops. The use of classical biological controls such as the introduction of parasites and predators has so far been unsuccessful, due to the rapid breeding of the insect pest in a highly perishable host. (Darryl, On-line, 1997)

Generally, the oriental fruit flies are able to reproduce in most environments and climates. The more spread of the flies the more synthetic insecticide is used. The accumulation of insecticides in fruits, crop productions and environment causes problem in agro-ecosystem which lead to health problems of human beings. In order to maintain the quality of fruit productions and the market share of export, many restrictions are required by exporters to eliminate the oriental fruit flies as well as other insects. Various methods such as irradiation and synthetic insecticides are utilized to control the oriental fruit flies (ชุมพล กันทะ, 2533). In many countries, quarantine laws restrict to prevent spreading of the fly. However, each prevention method affects quality, storage period and costs of fruit productions as well as

farmers' health and environment. Biological control of oriental fruit flies by plants seems to be a promising approach.

1.3 Pets control strategy

The control of insect pests may involve in one of the three basic pest control objectives. First, control is usually aimed at suppression of pests to the point that the presence or damage level is acceptable. Second, prevention and eradication are useful only in relatively small confined areas or in programs designed to keep foreign pests out of a new area. Third, successful control of insects and insect-like pests is obtained by thorough studies on, feeding habits and life cycle must thoroughly be studied. Environmental conditions, such as humidity, temperature, and availability of food, can affect the length of life cycle by altering the growth rate of insects. A favorable environment, usually warm and humid, can shorten the time period of development from eggs to adults. Therefore, carefully monitoring pest populations and taking management action at an appropriate time are most likely to succeed. It is particularly useful to know the life cycle stages of which the pests are most vulnerable. The best control of insects can be achieved at the early larval or nymphal stages, when the insects are small, active, and vulnerable.

During the past decade, extensive uses of chemically synthetic insecticides have resulted in environmental pollution and in the development of physiological resistance in major vector species. It is an essence to search and develop biological control as an alternative method with low cost for environmental safety. It can be used with minimal care by individuals and communities in any situations. Secondary plant metabolites, those are not to primarily believe for sustaining the life of an organism, have been an importance and growing area of research in recent years. Especially, the

interactions involving chemicals metabolized by plants, insect herbivores, parasitoids of herbivores, and plant pathogens have been focused extensively.

Because of this focus is attributed to the expanding awareness and need to prevail upon biological control to keep plant pets in environment (Loretta, On-line, 2000). Phytochemicals process a wide spectrum of biological properties. They may act as insect antifeedants, repellents, growth inhibitors, attractants, chemosterilants, or insecticides. In addition, they are biodegradable. Therefore, phytochemicals will be potential and economic pesticides to replace synthetic insecticides.

There are some reports show that plant products have insecticidal, growth inhibition and repellent activities against insects (Chari, M.S., 1996; Sehmutterer, H., 1995).

1.4 Research objectives

This research aims to investigate the biological control of oriental fruit fly (*Bactrocera dorsalis* Hendel) by the extracts of plants collected on SUT campus and its vicinity. Three plants selected were neem, sugar apple, and mintweed (Figures 3, 4, and 5 respectively). The extracts were evaluated for total phenolic compounds and antioxidant activity. The objectives of this study were to investigate some phytochemical properties of leaves and seeds of water- and ethanol- extracts of neem, sugar apple and mintweed. Moreover, it intended to elucidate the effects of the extracts on biological control of fruit flies, and to examine the combination effects of the extracts on the control.

Figure 1.3 Neem (Sadao) (*Azadirachta indica* Juss).

Figure 1.4 Sugar Apple (Noi-Na) (*Annona squamosa* Linn).

Figure 1.5 Mintweed (Maenglukka) (*Hyptis suaveolens* L. Poit).

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CHAPTER II

EVALUATION OF PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITIES OF NEEM, SUGAR APPLE, AND MINTWEED EXTRACTS

2.1 Abstract

Some phytochemical properties, the total phenolic compounds, and radical scavenging capacity of mintweed, neem, and sugar apple crude extract were investigated in order to determine their natural potential. Total phenolic compounds were evaluated according to Folin-Ciocalteu method. The neem leaf-ethanol extract had the highest total phenolic content (337.8 ± 18.2 mgGAE/L) while mintweed seed-ethanol extract had the lowest total phenolic content (179.4 ± 6.2 mgGAE/L). Radical scavenging activity determined from IC_{50} by DPPH-radical scavenging method. The mintweed seed-ethanol extract showed the highest activity (155.5 ± 3.2 ppm), whereas the lowest activity of radical scavenging was observed in mintweed leaf-water extract (288.9 ± 6.2 ppm). Additionally, it revealed that solvents in extraction affected the amount of total phenolic content and IC_{50}. This may be due to each plant produces different phytochemical compositions with difference in polarity as well as antioxidant activity. According to correlation coefficient, there was positive correlation between total phenolic contents IC_{50} values of most extracts excepting negative correlation sugar apple leaf extracts using water and ethanol as a solvents

and mintweed seed-ethanol extract. Antioxidant activities of all extracts but not of sugar apple extracts and mintweed seed-ethanol extract were directly correlated to the amount of total phenolic compounds. Thin layer chromatograghy (TLC) of the plant extracts was determined using the different solvent systems. N-buthanol : glacial acetic acid : water (40 : 10 : 50) system seem to be most suitable mixture solvent for separating compounds of plant extracts in this study. Because all of plant extracts obtained from this solvent system showed more than bands and R_f values.

2.2 Introduction

Reactive oxygen species (ROS) including free radicals such as superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen ($^1O_2$) and non-free radical species such as hydrogen peroxide (H_2O_2) are various forms of activated oxygen and often generated by oxidation product of biological reactions or exogenous factors (Ceruitti 1991; Yildirim et al., 2001; Gülcin et al., 2002b). ROS have aroused significant interest among scientists in the past decades. Their broad range of effects in biological and medicinal systems has drawn the attention of many experimental works (Buyukokuroglu et al., 2001; Gülcin et al., 2002a).

Antioxidants are molecules that can neutralize free radicals by accepting or donating an electron to eliminate the unpaired condition. Typically this means that the antioxidant molecule becomes a free radical in the process of neutralizing from a free radical molecule to a non-free-radical molecule. The antioxidant molecule will usually be a much less reactive free radical than the neutralized free radical. The antioxidant molecule may be very large (allowing it to "dilute" the unpaired electron), it may be readily neutralized by another antioxidant and/or it may have some other

mechanisms for terminating its free radical condition (Best, On-line 2006). Antioxidants can be classified according to their protective properties at different stages of the oxidation process or other different mechanisms. They are divided into two main types, primary and secondary antioxidants. Primary antioxidants can inhibit or retard oxidation by scavenging free radicals. They convert hydrogen atoms or electrons to be more stable products. Secondary antioxidant function by many mechanisms, including binding of metal ions, scavenging oxygen, converting hydroperoxides to non-radical species, absorbing UV radiation or deactivating singlet oxygen (Gordon, 1990, 2001).

Phytochemicals or plant secondary metabolites are believed in sustaining the life of organisms, have been importance for health and growing area of research in recent years. Phytochemicals process a wide spectrum of biological properties. Besides acting as antioxidants, they can be insect antifeedants, repellents, growth factors, growth inhibitors, attractants, chemosterilants, or insecticides. They are usually biodegradable. They offer great potential and economic pesticides to replace synthetic insecticides.

Neem (*Azadirachta indica* Juss.; Thai local name “Sadao” สะเดา) is a large, evergreen, hardy tree, and native to the Indian sub-continent (Chari, 1996; Gajalakshmi, 2002). Its leaves and fruits are known to possess fungicidal, and nematicidal properties (Schmutterer, 1995; Praveen and Alam, 1996; Pundt, 2000). Recently, neem has drawn a global attention due to its potential as a source of natural drugs and also environmental friendly pesticides (Schmutterer, 1995; Agarwal, 1996; Alam, 1996; Randhaawa and Parmar, 1996; Mulla and Su, 1999; Joshi and Lockwood, 2000; Daniel, 2000; Kumar, 2002).

It was reported that six phenolic compounds isolated and identified in both neem bark and leaves by HPLC were gallic acid, benzoic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, vanillic acid, and *trans*-cinamic acid. Ferulic acid was only found in the bark. Concentration of these phenolic compounds in bark was higher than in the leaves as shown in Table 2.1 (Xuan et al., 2004).

Table 2.1 The concentration of phenolic compounds identified by HPLC from the bark and leaves of neem (Xuan et al., 2004).

Chemicals	**Concentration (mg/g)**	
	Bark	**Leaves**
Gallic acid	8.91 ± 0.07	0.92 ± 0.20
p-Coumaric acid	5.01 ± 0.01	1.10 ± 0.06
p-Hydroxybenzoic acid	1.03 ± 0.02	0.79 ± 0.15
Vanillic acid	1.10 ± 0.03	0.33 ± 0.01
Benzoic acid	2.95 ± 0.04	0.86 ± 0.07
Ferulic acid	0.87 ± 0.22	-
trans-Cinamic acid	3.49 ± 0.31	0.65 ± 0.05

Neem is highly toxic to phytophagous mite (*Tetramychus cinnabrinus*), but not to predacious mite (*Phytoseiulus perimilis*), and predatory spider (*Chiracanthium mildei*) (Mansour et al., 1997). The active ingredient in neem is azadirachtin that act as an insecticide (Wewetzer, 1998). Azadirachtin is able to inhibit feeding activity of common cockchafer (*Melolontha melolontha*) and egg production of female cockchafer (Malinowski et al., 2000). It was able to reduce fleas *Ctenocephalides felis*

in the dog and cat (Guerrini and Kriticos, 1998). It was reported that diethyltoluamide (Deet) with citronella potentiated the effect of azadirachtin on *C. felis*). Neem oil affected the feeding activity of the large pine weevil (*Hylobius abietis*) in a commercial conifer plantation (Thacker et al., 2003). This indicates that the neem oil had a significant deterrent effect on weevil feeding. The pine trees treated with undiluted neem oil remained unaffected by the resident weevil population. The data suggest that neem extracts may play a role in protecting seeding trees from pine weevil during the first year of growth in the field.

Sugar apple (*Annona. squamosa* L.; Thai local name "Noi-Na" น้อยหน่า), native to West Indies, is cultivated throughout Thailand. The plant is attributed to medicinal properties which include antifertility and anti-tumor activities in mice and rats (Rao et al., 1979; Asolkar et al., 1992). Seed extract of *A. reticulata* and *A. squamosa* in ethanol and methanol can cause 100% mortality of pulse beetles (*Callosobruchus chinensis*) (Al-Lawati et al., 2002). The chemical composition of fruit pulp of *A. squamosa* was identified by Andrade et al. (2001). It contained of the diterpenoid compound kaur-16-en-18-oic acid (0.25%) in a considerable amount of dry mass, α-pinene (25.3%), sabinene (22.7%) and limonene (10.1%). The petroleum ether extract from the leaves of *A. squamosa* yielded n-alkanes, n-alkanols, 16-hentriacontanone, and sterols. These compounds were studied for antibacterial activity against gram-positive bacteria of *Staphylococcus aureus*, *Staphylococcus albus*, and *Streptocorus viridans* and gram-negative bacteria of *Escherichia coli*, *Pseudomonas pvocyanea*, and Klebsiella. The results indicated that among the compounds isolated from these plants, 16-hentriacontanone and sterols, exhibited antibacterial properties stronger than the n-alkanes (Sharma, 1993).

Mintweed (*Hyptis suaveolens* (L.); common names are Chan, Wild spikenard; and Thai local name "Maenglukka" แมงลักคา), widely distributed in the tropical and subtropics including in the Northeast of Thailand. It possesses some medicinal properties and is frequently used in the treatment of gastrointestinal infection, cramps and pain as well as in the treatment of skin infections (Wulff, 1987). It was found that *Hyptis suaveolens* (Labiatae) had strong toxicity against fungi *Pythium aphanidermatum* and *P. debaryanum*. The results showed the essential oil of *H. suaveolens* could control damping-off disease of tomato (*Lycopersicon esculentum*) infected with *P. aphanidermatum* and *P. debaryanum* (Pandey et al., 1994). Mintweed oil showed selective fungitoxicity but was not phytotoxic. However, soil amendments with leaves of these fungitoxic plants increased the saprophytic fungal community. The essential oil of *H. suaveolens* leaves showed antibacterial activity against gram-negative bacteria (Asekun et al., 1999). *H. suaveolens* extract can also control insect pest; *Aphis gossypil* Glov., and *Orthaga* sp. (กนก อุไรสกุล, 2540), and American ballworms (*Heliothis armigera* Hubn.) (รัชดาภรณ์ พิทักษ์ธรรม, 2544). In addition, many species of *Hyptis* are used against pest and other pest insects in stored product and mosquito control (Palsson and Jaeson, 1999). The leaves of those species are also largely used as potent insect repellents by native populations of many parts of the world (Aycard et al., 1993; Pereda-Mianda and Delgado, 1990).

H. suaveolens extract was identified by GC-MS, twenty three compounds were found as shown in Table 2.2 and some chemical structures are in Figure 1 (Preezada, 1997). Campos et al. (2001) reported that the principal constituents in the

essential oil of *H. suaveolens* are sabinene, limonene, biclyclogermacrene, β-phellandrene and 1, 8-cineole.

Table 2.2 The chemical compositions of the essential oil from *Hyptis suaveolens* (Preezada, 1997).

Component	Percentage (%)
α-Thujene	0.3
α-Pinene	2.5
Camphene	0.02
Sabinene	3.9
β-Pinene	4.2
Myrcene	0.6
α-Phellandrene	2.0
1,8-Cineole	32
γ-Terpinene	0.7
α-Terpinolene	0.3
Linalool	0.06
Fenchol	0.3
4-Terpinenol	2.3
α-Terpineol	0.2
Eugenol	1.2
α-Copaene	1.8
β-Elemene	1.0
β-Caryophyllene	2.9
α-Humulene	1.6
α-Bergamotene	2.0
Aromadendrene	0.5
γ-cadinene	0.1
δ-cadinene	0.5

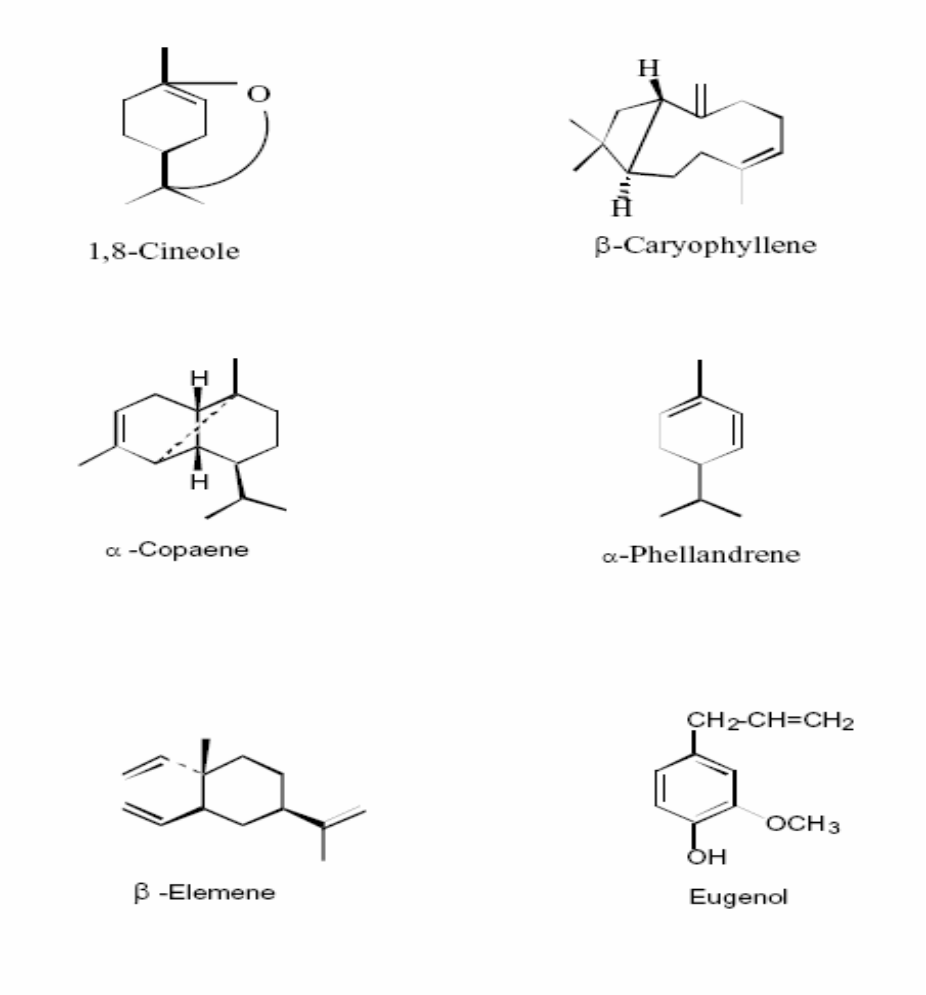


Figure 2.1 Some chemical components found in *Hyptis suaveolens* extracts, 1-8-cineole, β-caryophyllene, α-copaene, α-phellandrene, β-elemene, and eugenol (Preezada, 1997).

Thin layer chromatography (TLC) is widely used as a standard technique for rapid separation and qualitative analysis of a mixture of chemical compounds. Its sensitivity is high which allows separation of less than microgram amounts of material. Silica gel on a support material such as glass or aluminum is most widely employed. In principle, the components will differ in solubility and in the strength of

their adsorption to the adsorbent. With silica gel, the dominant interaction force between the adsorbent and the components to be separated are dipole-dipole type. Highly polar molecules fairly strong interact with the polar Si-O bonds of these adsorbent and tend to stick or adsorb onto the fine particles of the adsorbent, whereas weakly polar molecules are held less tightly. Thus, weakly polar molecules generally tend to move through the adsorbent more rapidly than the polar species (CUBoulder Organic Chemistry Undergraduate Course, On-line, 2007). The different components in the mixture move up the plate at different rates due to differences in their partitioning behavior between the mobile liquid phase and stationary phase. The R_f value for each spot was calculated as following:

$$R_f = \frac{\text{Distance from start to center of sustance spot (cm)}}{\text{Distance from start to solvent front (cm)}}$$

R_f stands for "ratio of front" and is characteristics of any given compound on the same stationary phase using the same mobile phase for development of the plate.

The purposes of this study were to examine the total phenolic compounds, antioxidant properties and TLC fingerprints of neem, sugar apple and mintweed extracts prior to investigate the efficacy them as biological control reagents for oriental fruit flies.

2.3 Materials and methods

2.3.1 Materials

(2, 2 diphenyl-1-picrylhydrazyl) and gallic acid were purchased from Sigma

(St Louise, MO, U.S.A.). Methanol, absolute ethanol (95%), chloroform, ethyl acetate, gacial acetic acid, n-buthanol, and Folin-Ciocalteu's reagent were purchased from Carlo Erba Reagents (Strada Rivoltana, SpA). Aluminium sheets pre-coated with a 0.25-mm layer of silica gel $60F_{254}$ providing are purchased from Merck (Germany).

2.3.2 Sample preparation

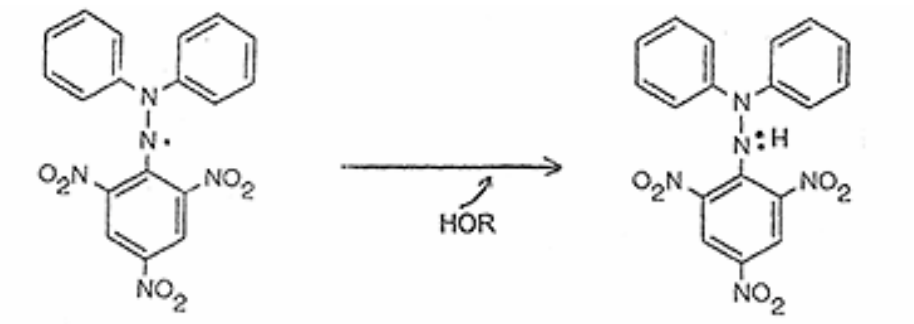
Leaves of neem, sugar apple, mintweed, and seeds of mintweed were collected at Suranaree University of Technology (SUT) campus and its vicinity. The leaves were cleansed, chopped, and dried at 45°C for 2 days. The seeds were soaked in water, the mucilage was gotten rid by squeezing against a stainless steal strainer and then dried. Dried leaves and seeds were ground into fine powder. Ten grams of powder was extracted in 140 mL of water or 95% ethanol for 12 hours by using universal extraction (Buchi model B811, Germany). The crude extract was evaporated and then dried at -54ºC and stored at -20ºC for further studies. The dried extract was dissolved in its original solvent and kept at 4ºC during study.

2.3.3 Determination of total phenolic compounds

Total phenolic compounds were measured according to Folin-Ciocalteu method (Matthaus et al., 2002). Gallic acid was used as standard. Crude extract of 10 μL was introduced into a test tube, 2 mL of 2% of sodium carbonate was added and incubated for 2 min. Hundred microliters of Folin-Ciocalteu's reagent (Foiln:Methanol, 1:1 vol:vol) was added. The absorbance of blue colored solution was measured after incubated the sample for 30 min at 750 nm. The total phenolic compounds of the sample were calculated using pure gallic acid as a standard curve. All determinations were performed in triplicate and expressed as milligram of gallic equivalent (GAE) per liter of sample.

2.3.4 Determination of antioxidant activity

Antioxidant property was determined by using 2, 2 diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams et al., 1995). Antioxidant activity, measured as % radical scavenging mainly depended on the dissociation of hydrogen radical from phenolic compounds to form a stable compound with DPPH radical. The higher % radical scavenging, the higher stable compounds are formed as shown in the following (Franco, On-line, 2006):



DPPH Radical (Violet) (DPPH):H (Pale orange)

Radical scavenger ability of plant extracts at various concentrations (31.5, 62.5, 125, 250, 500 and 1000 ppm) were investigated. Crude extract of 0.5 mL was added to 1 mL of 0.2 mmol methanolic DPPH solution. The tube was incubated for 30 min in the dark. Then, the absorbance at 517 nm was measured against a blank of pure methanol as control. Percent radical scavenger was calculated from the following equation.

$$\% \text{ Radical Scavenging} = \left(1 - \frac{A_{SAMPLE}}{A_{CONTROL}}\right) x\, 100$$

When A_{SAMPLE} = Absorbance of the mixture of extract solution and DPPH

$A_{CONTROL}$ = Absorbance of the mixture of solute and DPPH

Antioxidant activity of the sample was defined as the amount of antioxidant necessary to reduce the initial DPPH concentration by 50% (Inhibitory concentration), defined as IC_{50}

2.3.5 Thin layer chromatography (TLC) fingerprints of plant extracts

In this study TLC was used to obtain the fingerprinting of all plant extracts in order to figure out the differences of their components. TLC was performed on 2.5 × 7 cm alumina sheets pre-coated with a 0.25-mm layer of silica gel 60 F_{254}. Five miroliters of extracts were spotted onto the TLC plate, using a capillary, at about 1 cm above the edges of the bottom plate. The spotted plate was placed in a 125-mL beaker containing 10 mL of solvent systems as mobile phases. Ethyl acetate : methanol : water (100 : 13.5 : 10 vol:vol), n-buthanol : glacial acetic : water (40 : 10 : 50 vol:vol), and chloroform : methanol : glacial acetic acid (47.5 : 47.5 : 5 vol:vol), respectively (Wagner and Bladt, 1995). The chromatography chamber was closed to prevent evaporation. At the end of the chromatography, the plate was removed, dried, and then detected under UV light (254 nm).

2.3.6 Data analysis

Data from all experiments were analyzed by t-test using Program SPSS for Window V.10. All analyzes were done at the 95% confident level.

2.4 Results and discussion

2.4.1 The total phenolic compounds

The phenolic compounds are very important constituents in plants because of their scavenging ability of their hydroxyl groups (Hatano et al., 1989), and preventing decomposition of hydroperoxides into free radicals (Gordon, 2001). The Folin-Ciocalteu method is a rapid and widely used assay to investigate the total phenolic content. However, it is known that different phenolic compounds have different responses in the Folin-Ciocalteu method (Kähkonen et al., 1999). Moreover, the total contents of different phenolic compound types and of different parts of plants are different.

Total phenolic compounds of plant extracts, using water and ethanol as solvents were shown in Table 2.3 The total phenolic content of plant extracts were ranged from the highest to the lowest values as following: neem leaf-ethanol extract was 337.8 ± 18.2 mgGAE/L, sugar apple leaf-water extract was 308.8 ± 20.2 mgGAE/L, neem leaf-water extract was 297.0 ± 14.0 mgGAE/L, sugar apple leaf-ethanol extract was 260.8 ± 14.1 mgGAE/L, mintweed seed-water extract was 254.0 ± 16.4 mgGAE/L, mintweed leaf-water extract was 251.2 ± 14.8 mgGAE/L, mintweed leaf-ethanol extract was 244.6 ± 12.5 mgGAE/L, and mintweed seed-ethanol extract was 179.4 ± 6.2 mgGAE/L.

In comparison, total phenolic compounds from water and ethanol extractions of each plant was determined using t-test. In the case of Mintweed seed extracts, there was significant difference ($P < 0.05$) between water and ethanol extractions. The total phenolic content of neem leaf-ethanol extract (337.8 ± 18.2 mgGAE/L) was about 1.14 fold higher than that of neem leaf-water extract (297.0 ± 14.0 mgGAE/L). Even

though sugar apple water-extract (308.8 ± 20.2 mgGAE/L) had greater total phenolic content than sugar apple-ethanol extract (260.8 ± 14.1 mgGAE/L) around 1.18 fold. There was no significant difference in sugar apple extracts using water and ethanol as solvents. Mintweed leaf-water extract (251.2 ± 14.3 mgGAE/L) and mintweed leaf-ethanol extract (244.6 ± 12.5 mgGAE/L) showed slightly different in total phenolic contents. The t-test analysis indicated that the total phenolic compounds from water and ethanol extractions of mintweed leaf (2610.8 ± 14.1 mgGAE/L v.s. 251.2 ± 14.8 mgGAE/L) had no significantly difference. The water extraction of mintweed seed increased the total phenolic content (254.0 ± 16.4mgGAE/L) about 1.42 fold as compared to ethanol extraction of minweed seed (179.4 ± 6.2 mgGAE/L). It was significant difference ($P < 0.05$) between mintweed seed extract between water and ethanol solvents. This was in agreement with the finding that higher extraction yields of phenolic compounds were obtained with an increase in polarity of the solvent (Goli et al., 2005)

Table 2.3 Total phenolic compounds of plant extracts using water and ethanol as solvents.

Plant	Total Phenolic Compound (mgGAE/L) (N=5)		
	Water extracts	**Ethanol extracts**	**P-value**
Neem leaf	297.0 ± 14.0	337.8 ± 18.2	0.320
Sugar apple leaf	308.8 ± 20.2	260.8 ± 14.1	0.145
Mintweed leaf	251.2 ± 14.8	244.6 ± 12.5	0.660
Mintweed seed	254 .0± 16.4	179.4 ± 6.2	0.010*

The evaluation of total phenolic compounds and free radical scavenging capacity from ethanolic extract from various parts of 26 Thai indigenous plants was reported (Maisuthisakul et al., 2007). The extracts of berries used in wine production were found to have a higher antiradical activity than those of the other herbs and vegetables. Whereas chewing plants with astringent taste had a higher level of total phenolic content and flavonoids content. The correlation coefficients exhibited a high positive relationship between total phenolic and flavonoid contents in the plant extracts and antiradical activity.

2.4.2 Antioxidant activity

The antioxidant activity is measured as chemical substances that removes or inactivates unstable radicals. 2, 2 diphenyl-1-picrylhydrazyl (DPPH) method is a rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts (Koleva et al., 2002). $DPPH^{\bullet}$ is a stable free radical, accepts electrons or hydrogen radicals and becomes a stable diamagnetic molecule (Soares et al., 1997). The radical scavenging activity of plant extracts are evaluated as effectiveness at fifty

percent of inhibitory contents or IC_{50} values. The IC_{50} values of the different plant extracts in this study were shown in Figurs 2.2-2.5 and were summarized in Table 2.4

The fifty percent of radical scavenger or IC_{50} of neem leaf-water extract was 173.0 ± 3.2 ppm which was about 0.82 fold, lower than that of neem leaf-ethanol extract (211.5 ± 4.6 ppm) shown in Figure 2.2 It was suggested that water extraction of neem leaf exhibited more efficiency ($P < 0.01$) than ethanol extraction. In case of sugar apple leaf extract shown in Figure 2.3, IC_{50} of water extraction (163.5 ± 4.0 ppm) gave approximately 0.75 fold lower than that of ethanol extraction (218.6 ± 1.6 ppm). This implied that water extraction of sugar apple leaf had also higher effectiveness ($P < 0.01$) than ethanol extraction.

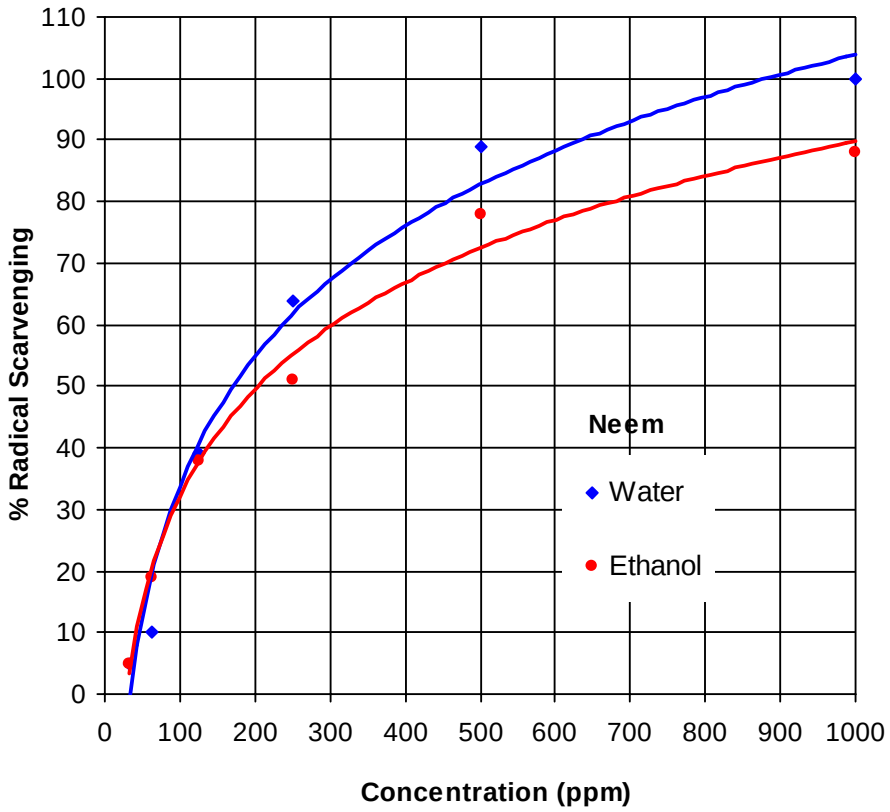


Figure 2.2 Radical scavenging of neem leaf extracts using water and ethanol as solvents. The IC_{50} of water extract was 172.99 ± 4.53 ppm and of ethanolic extract was 211.53 ± 8.61 ppm (n = 5)

In contrast, mintweed leaf-ethanol extract showed lower IC_{50} (226.4 ± 2.8 ppm) as compared to IC_{50} of mintweed leaf-water extract (288.92 ± 13.9 ppm) (Figure 2.4). It can be concluded that mintweed leaf-ethanol extract exhibited about 0.78 fold greater efficiency than mintweed leaf-water extract ($P < 0.01$). In addition, there was no significant difference between water extraction and ethanol extraction of mintweed seed extracts. IC_{50} of mintweed seed-water extract and mintweed seed-ethanol extract were 156.4 ± 1.8 ppm and 155.5 ± 3.2 ppm, respectively. In this study, the lowest IC_{50}

of radical scavenging (156.4 ± 1.8ppm) was obtained from mintweed seed-ethanol extract whereas mintweed leaf-water extract showed the highest IC_{50} (288.9 ± 6.2 ppm).

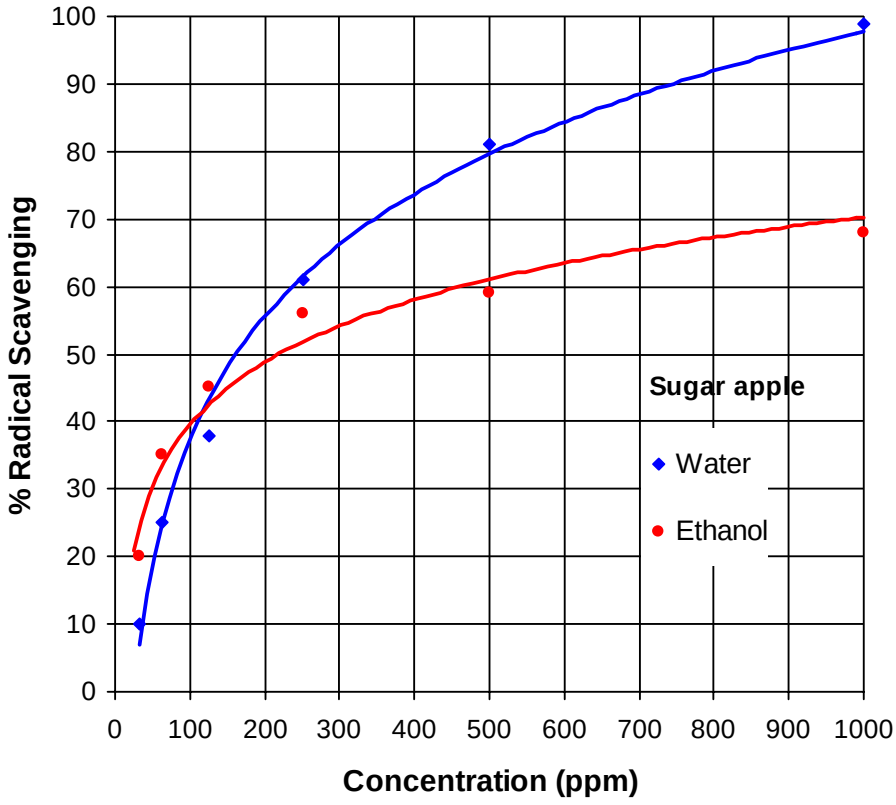


Figure 2.3 Radical scavenging of sugar apple leaf extracts using water and ethanol as solvents. The IC_{50} of water extract was 163.55 ± 8.99 ppm and of ethanolic extract was 218.62 ± 3.64 ppm (n = 5)

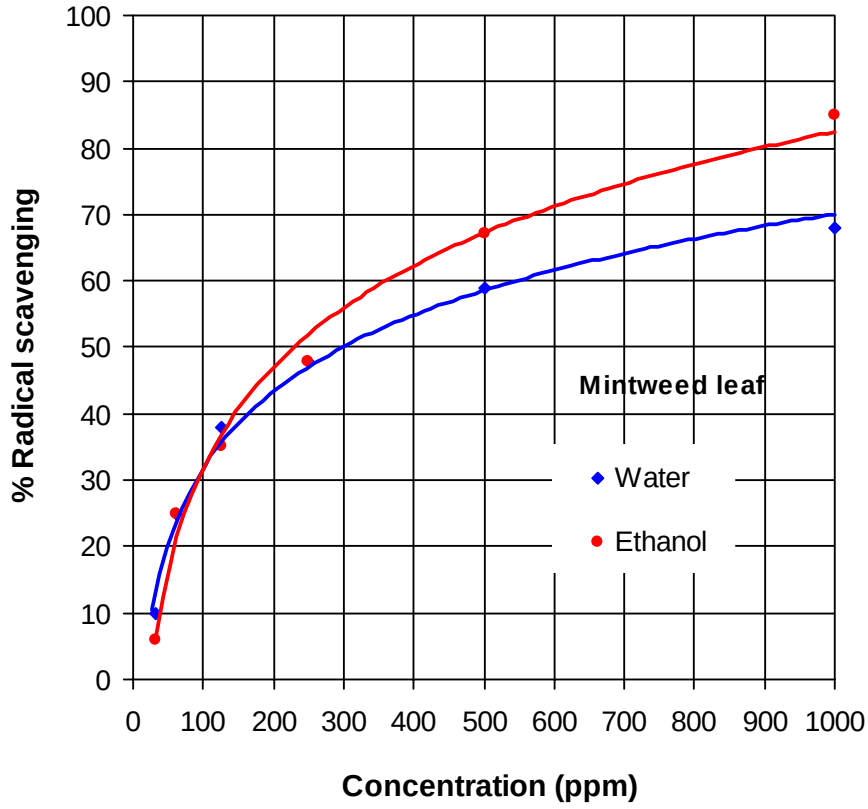


Figure 2.4 Radical scavenging of mintweed leaf extracts using water and ethanol as solvents. The IC_{50} of water extract was 288.92 ± 13.91 ppm and of ethanolic extract was 226.39 ± 6.22 ppm (n = 5)

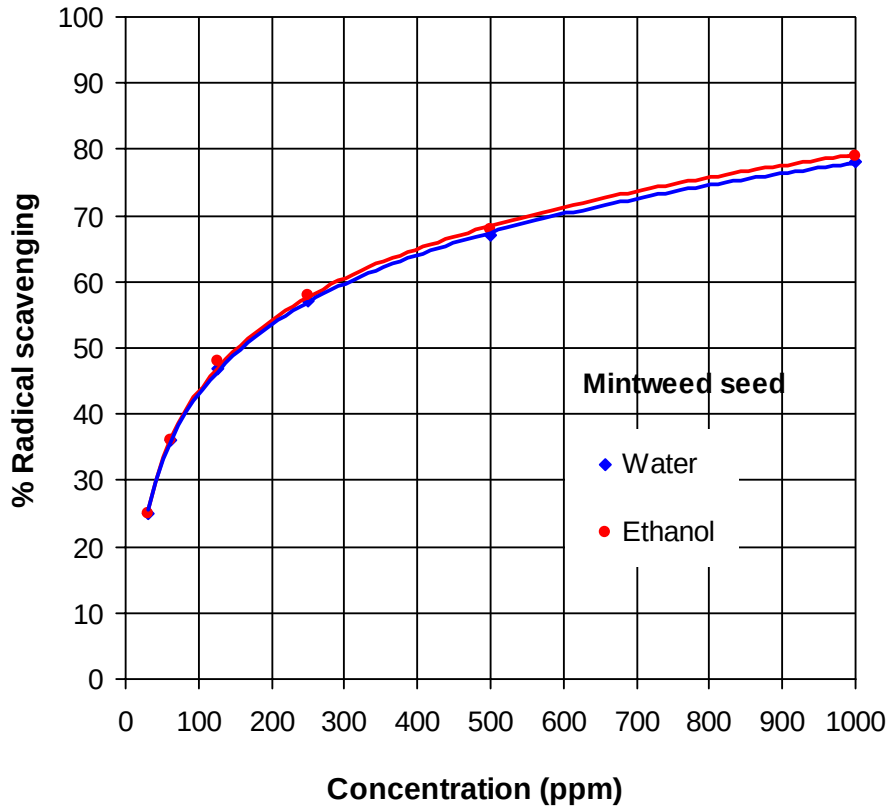


Figure 2.5 Radical scavenging of mintweed seed extracts using water and ethanol as solvents. The IC_{50} of water extract was 156.44 ± 3.99 ppm and of ethanolic extract was 155.48 ± 7.06 ppm (n = 5)

Table 2.4 The effectiveness of radical scavenging of neem, sugar apple, and mintweed extracts using water and ethanol as solvents and expressing as IC_{50} values (n = 5).

Plant	IC_{50} (ppm) (N = 5)		
	Water extracts	**Ethanol extracts**	**P-value**
Neem leaf	173.0 ± 3.2	211.5 ± 4.6	0.000**
Sugar apple leaf	163.5 ± 4.0	218.6 ± 1.6	0.000**
Mintweed leaf	288.9 ± 6.2	226.4 ± 2.8	0.001**
Mintweed seed	156.4 ± 1.8	155.5 ± 3.2	0.809

The correlation between the amounts of total phenolic compounds and the antioxidant activity of the plant extracts is expressed as correlation coefficient presented in Table 2.5. For neem extracts, the correlation coefficient of neem leaf-water extract was 0.817 and of neem leaf-ethanol extract was 0.621. That is increasing of total phenolic compounds in the extracts caused an increased of IC_{50} values, meaning that the effectiveness of radical scavenging was a reverse relationship to the amounts of total phenolic compound in the extract. On the other hand, the water extract of neem had better antioxidant activity than the ethanolic extract.

For the extracts of sugar apple leaves, the correlation coefficient was -0.04 and -0.082 for water and ethanolic extracts respectively. It means that the antioxidant activities of both types of extracts were increased along with an increase of total phenolic compounds. In the case of mintweed extracts, the correlation coefficient of total phenolic compounds and IC_{50} values of antioxidant activities of mintweed extracts by water and ethanol were indirect relation, except the extract of mintweed

seeds by ethanol. The correlation of mintweed leaf-water extract was 0.833, of mintweed leaf-ethanol extract was 0.974 ($P < 0.01$) and of mintweed seed-water extract was 0.622. This indicated that the IC_{50} values increased as the total phenolic compound increased or the effectiveness of antioxidants was low. The correlation of the Mintweed seed-ethanol extract was -0.741, meaning that the effectiveness of antioxidants increased (low figure of IC_{50} value) when total phenolic compound increased.

Therefore, it is concluded that the total phenolic compounds extracted from neem and mintweed leaves showed inverse proportion with the effectiveness of their antioxidant activities. The extracts of sugar apple by both water and ethanol extractions and of mintweed seeds by ethanol extraction had more effectiveness of antioxidant activities along with the amounts of total phenolic compounds.

Table 2.5 T-test of plant extracts using water and ethanol as solvents for determining total phenolic content and antioxidant acitivity (IC_{50}) of the extracts .

Plant Extracts	Total Phenolic Compound (mgGAE/L) (N = 5)	IC_{50} (ppm) (N = 5)	Correlation Coefficient (N=5)	P-value
Neem/H_2O	297.0±14.0	173.0±3.2	0.817	0.091
Neem/EtOH	337.8±18.2	211.5±4.6	0.621	0.263
Sugar apple/H_2O	308.8±20.2	163.5±4.0	-0.041	0.948
Sugar apple/EtOH	260.8±14.1	218.6±1.6	-0.082	0.895
Mintweed leaf/H_2O	251.2±14.8	288.9±6.2	0.833	0.080
Mintweed leaf/EtOH	244.6±12.5	226.4±2.8	0.974	0.005**
Mintweed seed/H_2O	254.0±16.4	156.4±1.8	0.622	0.263
Mintweed seed/EtOH	179.4±6.2	155.5±3.2	-0.741	0.152

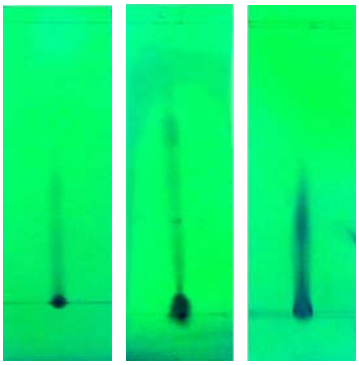
However, some authors found correlation between phenolic contents and antioxidant activity (Yen et al., 2002), whereas the others found no such relationship, since other compounds are responsible for the antioxidant activity (Bocco et al., 1998; Maillard and Berset, 1995; Heinomen et al., 1998; Kahkonen et al., 1999). The phenolic compounds may contribute directly to antioxidative action (Duh et al., 1999). Moreover, it was found that the solvent differences in extraction method affected to the amount of total phenolic compounds. This may be due to each plant produces different phenolic compounds with difference in polarity as well as antioxidant activity.

Antioxidant activity of Siamese neem (Sithisarn et al., 2005) was reported that leaf water extract, flower and stem bark ethanol extracts exhibited higher free radical scavenging effect on the DPPH assay with 50% scavenging activity at 26.5, 27.9 and 30.6 ppm, respectively

2.4.3 Thin Layer Chromatography (TLC) analysis

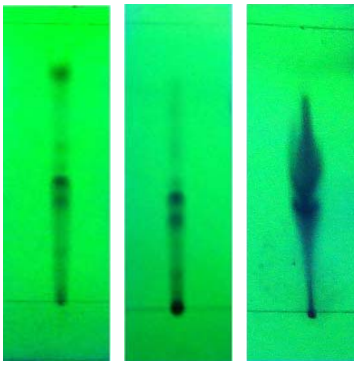
Thin layer chromatograghy (TLC) of the plant extracts was determined using the different solvent systems. The chromatograms were examined under UV light. The mobile phase systems used in this study were ethyl acetate : methanol : water (100 : 13.5 : 10); designated as system A, n-buthanol : glacial acetic acid : water (40 : 10 : 50); designated as system B, and chloroform : methanol : glacial acetic acid (47.5 : 47.5 : 5); designated as system C. The phytochemicals in the plant extracts were separated and compared according to the TLC mobile phase systems, demonstrated in Figures 2.6 - 2.13. The R_f values of the chromatograms are listed in Table 2.6.

Comparing among the solvent systems, the n-buthanol : glacial acetic acid : water (40 : 10 : 50) system seem to be most suitable mixture solvent for separating compounds of plant extracts in this study. Because all of plant extracts obtained from this solvent system showed more than bands and R_f values.



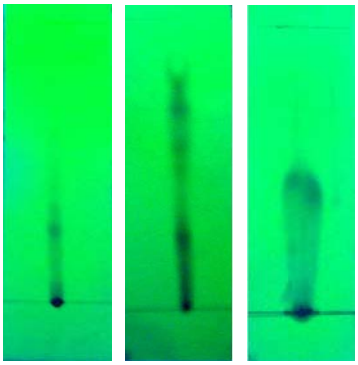
A **B** **C**

Figure 2.6 TLC of neem leaf-water extracts using mobile phase systems of A, ethyl acetate : methanol : water (100 : 13.5 : 10); B, n-buthanol : glacial acetic acid : water (40 : 10 : 50); C, chloroform : methanol : glacial acetic acid (47.5 : 47.5 : 5)



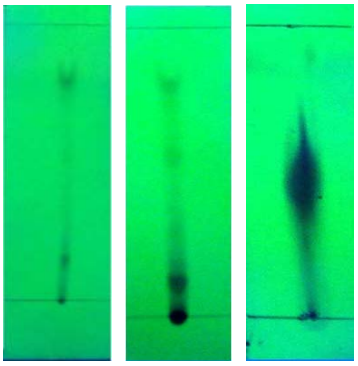
A **B** **C**

Figure 2.7 TLC of neem leaf-ethanol extracts using mobile phase systems of A, ethyl acetate : methanol : water (100 : 13.5 : 10); B, n-buthanol : glacial acetic acid : water (40 : 10 : 50); C, chloroform : methanol : glacial acetic acid (47.5 : 47.5 : 5)



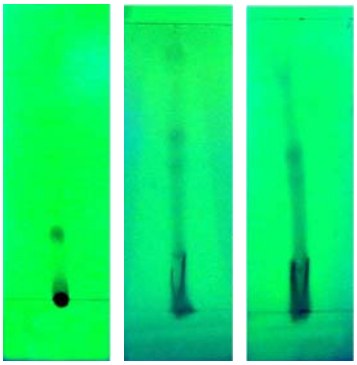
A **B** **C**

Figure 2.8 TLC of sugar apple leaf-water extracts using mobile phase systems of A, ethyl acetate : methanol : water (100 : 13.5 : 10); B, n-buthanol : glacial acetic acid : water (40 : 10 : 50); C, chloroform : methanol : glacial acetic acid (47.5 : 47.5 : 5)



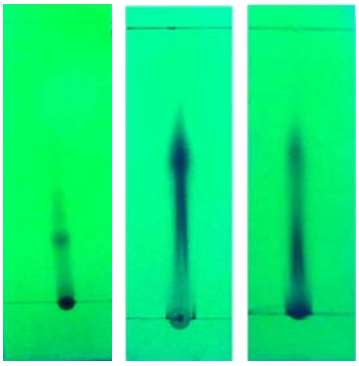
A **B** **C**

Figure 2.9 TLC of sugar apple leaf-ethanol extracts using mobile phase systems of A, ethyl acetate : methanol : water (100 : 13.5 : 10); B, n-buthanol : glacial acetic acid : water (40 : 10 : 50); C, chloroform : methanol : glacial acetic acid (47.5 : 47.5 : 5)



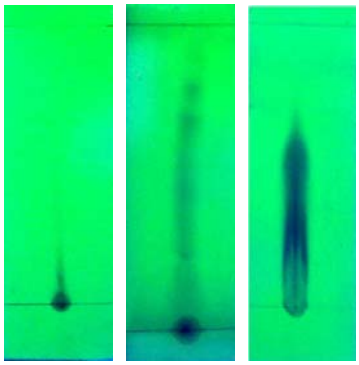
A **B** **C**

Figure 2.10 TLC of mintweed leaf-water extracts using mobile phase systems of A, ethyl acetate : methanol : water (100 : 13.5 : 10); B, n-buthanol : glacial acetic acid : water (40 : 10 : 50); C, chloroform : methanol : glacial acetic acid (47.5 : 47.5 : 5)



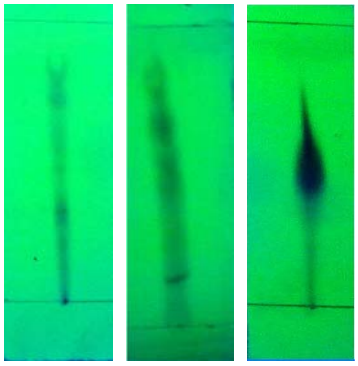
A **B** **C**

Figure 2.11 TLC of mintweed leaf-ethanol extracts using mobile phase systems of A, ethyl acetate : methanol : water (100 : 13.5 : 10); B, n-buthanol : glacial acetic acid : water (40 : 10 : 50); C, chloroform : methanol : glacial acetic acid (47.5 : 47.5 : 5)



A **B** **C**

Figure 2.12 TLC of mintweed seed-water extracts using mobile phase systems of A, ethyl acetate : methanol : water (100 : 13.5 : 10); B, n-buthanol : glacial acetic acid : water (40 : 10 : 50); C, chloroform : methanol : glacial acetic acid (47.5 : 47.5 : 5)



A **B** **C**

Figure 2.13 TLC of mintweed seed-ethanol extracts using mobile phase systems of A, ethyl acetate : methanol : water (100 : 13.5 : 10); B, n-buthanol : glacial acetic acid : water (40 : 10 : 50); C, chloroform : methanol : glacial acetic acid (47.5 : 47.5 : 5)

Table 2.6 Comparison of R_f values of neem, sugar apple, and mintweed extracts separated by thin layer chromatography. Three mobile phase systems were used, system A contained ethyl acetate : methanol : water (100 : 13.5 : 10), system B contained n-buthanol : glacial acetic acid : water (40 : 10 : 50), and system C contained chloroform : methanol : glacial acetic acid (47.5 : 47.5 : 5).

Mobile Phase System	R_f values							
	Neem leaf extract		Sugar apple leaf extract		Mintweed leaf extract		Mintweed seed extract	
	H_2O	EtOH	H_2O	EtOH	H_2O	EtOH	H_2O	EtOH
A	0.40(DB)	0.34(DB) 0.42(LY) 0.54(LG) 0.82(DG)	0.24(DB) 0.34(LB)	0.14(B) 0.80(LG)	0.24(B)	0.24(G)	0.10(DB)	0.34(DB) 0.44(LY) 0.56(LG) 0.74(G) 0.82(DG)
B	0.11(B) 0.24(Y) 0.44(Y) 0.61(LY)	0.31(Y) 0.47(G) 0.63(Y) 0.74(LY)	0.06(B) 0.14(B) 0.40(LB) 0.47(G) 0.58(Y)	0.19(B) 0.46(Y) 0.60(LY) 0.82(G)	0.15(B) 0.28(LB) 0.46(LY) 0.60(LY) 0.80(LY)	0.19(B) 0.42(LB) 0.51(Y) 0.64(LY) 0.81(LG)	0.12(B) 0.21(LB) 0.44(Y) 0.66(LY) 0.78(LY) 0.84(DY)	0.36(LY) 0.44(LY) 0.56(LY) 0.75(G)
C	0.09(LB) 0.31(G)	0.31(LB) 0.39(G) 0.49(LG) 0.66(Y)	0.63(LB)	0.38(B) 0.49(LB)	0.35(B) 0.56(LB)	0.14(B) 0.50(LB)	0.24(B) 0.40(LB) 0.50(LG)	0.51(G) 0.65(LB)

Note: DB = Dark brown, LB = Light brown, B = Brown, DG = Dark green, LG = Light Green, G = Green, LY =Light yellow, and Y= Yellow.

2.5 Conclusion

The present study showed that neem leaf ethanol extract contained the highest total phenolic content (337.8 ± 18.2 mgGAE/L). On the other hand, mintweed seed ethanol extract exhibited the greatest antioxidant activity (155.5 ± 3.2 ppm). The high radical scavenging activity of mintweed seed ethanol extract may be due to hydroxyl groups existing in the chemical structure of the phenolic compounds that could provide the necessary component as a radical scavenger. A potent scavenger of free radicals may serve as a possibly preventive intervention for diseases (Gyamfi et al., 1999). However, all of the plant extracts in this research exhibited antioxidant activity to some extent. Because, the different solvents in extraction method affected the amount of total phenolic compounds and antioxidant activities. Moreover, all plant extracts excluding water and ethanol sugar apple-leaf extracts showed positive correlation between total phenolic contents and antioxidant activities. The evaluation of the efficiency of solvent systems as a mobile phase in TLC experiment showed that n-buthanol : glacial acetic acid : water (40 : 10 : 50) system seemed to be most suitable solvent mixture for separating compounds of plant extracts in this study. These results may be suggested that each plant produces different phytochemical compositions with difference in polarity as well as antioxidant activities.

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CHAPTER III

CYTOTOXICITY AND BIOCONTROL OF ORIENTAL FRUIT FLIES (*Bactrocera dorsalis* (Hendel)) BY NEEM, SUGAR APPLE, AND MINTWEED EXTRACTS

3.1 Abstract

Ethanol and water extracts of neem, sugar apple, and mintweed were evaluated against oriental fruit flies, *Bactrocera dorsalis* Hendel. Repellent activity of single plant extract and two-plant combination extracts were elucidated using an olfactometer. Results indicated that the highest repellent activity of single plant extracts were mintweed seed-ethanol extract. In the case of single plant extracts, repellent activity of all ethanolic extracts was higher than those of all water extracts ($P < 0.05$), ($P< 0.01$). It was found that repellent activity of neem leaf-water extract with sugar apple leaf-water extract showed the highest repellent activity than the other types of two-plant combination extracts. Comparison of all the same type of two-plant combination extracts, water extracts showed higher repellent activity than ethanolic extracts. Cytotoxicity (LC_{50}) of single plant extract and two-plant combination extracts were investigated by Brine Shrimp Lethality Assay (BSLA). In comparison of cytotoxicities between single and plant combination extracts, results showed that cytotoxicities of all two-plant combination extracts were greater than those of single plant extracts. Especially, neem leaf-ethanol extract with mintweed leaf-ethanol

extract gave the highest cytotoxicity (LC_{50} was 0.07 ± 1.7E-02 ppm) (P< 0.01). Results also implied that two-plant combination extracts exhibited synergistic effect on LC_{50}. In addition, ethanol extraction of both single extract and plant combination extracts obviously showed lower LC_{50} than water extractions.

Insecticidal activity (LD_{50}) of single plant extract and two-plant combination extracts were investigated on biosis of oriental fruit flies i.e. eggs, lavae and adults. In comparison of cytotoxicities between single and plant combination extracts, it revealed that cytotoxicities of single extracts on eggs were lower than those of two-plant combination extracts. The cytotoxicity of single plant extract on eggs which gave LD_{50} lower than 3,000 ppm was mintweed leaf-water extract (2,920.3 ± 55.9 ppm). The two effective cytotoxicity of plant combination extracts on eggs which gave LD_{50} lower than 3,000 ppm were sugar apple leaf-water extract with mintweed leaf-water extract (2,902.4 ± 50.8 ppm) and sugar apple leaf-water extract wih mintweed seed-water extract (2,934.7 ± 54.9 ppm). Cytotoxicity on larvae using feeding treatment of single extracts was lower than those of two-plant combination extracts. The cytotoxicity of single plant extracts on larvae feeding treatment which gave LD_{50} lower than 3,000 ppm was sugar apple leaf-water extract (2,568.3 ± 47.5 ppm), mintweed leaf-ethanol extract (2,658.4 ± 132.4 ppm) and mintweed leaf-water extract (2,707.8 ± 120.3 ppm). The most two effective combination extracts which exhibited LD_{50} lower than 3,000 ppm were neem leaf-water extract with sugar apple leaf-water extract (2,595.1 ± 95.6 ppm) and sugar apple leaf-water extract with neemweed seed-water extract (2,700.4 ± 65.1 ppm). In conparision of cytotoxicity between single and two-plant combnation extracts on larvae using dipping treatment, the cytotoxicity of single plant extracts on larvae were higher than the two-plant

combination extracts. The cytotoxicity of single plant extracts on larvae dipping treatment which gave LD_{50} lower than 3,000 ppm was mintweed leaf-ethanol extract (2,651.0 ± 143.8 ppm) and sugar apple leaf-water extract (2,977.2 ± 67.1 ppm). The most three effective combination extracts which gave LD_{50} lower than 3,000 ppm were neem leaf-water extract with sugar apple leaf-water extract (2,673.8 ± 114.7 ppm), sugar apple leaf-ethanol extract with mintweed seed-ethanol extract (2,680.2 ± 121.0 ppm) and sugar apple leaf-water extract with mintweed seed-water extract (2,928.41 ± 45.62 ppm). In conparision of cytotoxicity between single and two-plant combination extracts on adult using feeding treatment, the single plant extracts was lower than the plant combination extracts. The cytotoxicity of single plant extracts on adult feeding treatment which gave LD_{50} lower than 3,000 ppm was mintweed seed-water extract (2,521.3 ± 83.8 ppm) and sugar apple leaf-water extract (2,710.9 ± 67.1 ppm). The most three effective combination extracts which gave LD_{50} lower than 3,000 ppm were sugar apple leaf-ethanol extract with mintweed seed-ethanol extract (2,568.3 ± 121.0 ppm), sugar apple leaf-water extract with mintweed leaf-water extract (2,783.3 ± 83.9 ppm) and sugar apple leaf-water extract with mintweed seed-water extract (2,784.4 ± 111.6 ppm).

3.2 Introduction

Thailand and many other countries have their national goals in plant protection which is to reduce the use of synthetic chemical pesticides. One approach toward this goal is to replace such chemicals with botanical-based insecticides. The azadirachtins, triterpenoids of neem, have been recognized for their insecticidal properties which are antifeedant, growth disruptant (Isman et al., 1990; Prijono and Hassan, 1993;

Villanueva-Jiménez et al., 2000), and reproductive effects on several insect species (Pathak and Krishna, 1986; Shimizu, 1988; Riba et al., 2003). Neem extracts have been developed as commercially available insecticides in several countries. However, biological control of many pests by neem still waits for research and proper application.

The Annonaceae is a large tropical plant family. Sugar apple (*Annona squamosa*) is one of this family. Phytochemical and, pharmacological studies of Annonaceous species were intensive in the last 15 years. The Annonaceous acetogenins were isolated and identified (Santos dos and Sant′Ana, 2001).

Oil extracts from plants have been extensively used for crop protection (Singh et al., 1978; Dabiré, 1993; Rajapakse and van Emden, 1997) and plant-based insecticides (Arnason et al., 1989) in tropical countries. Aromatic plant species, particularly the family Labiatae (Lamiaceae), are among the most widely used in insect pest control (Lambert et al., 1985; Morton, 1981; Shaaya et al., 1997; Lawrence, 1988). Mintweed (*Hyptis suavens*) is a well known example in the family Labiates. It has been reported that *H. suaveolens* extract exhibited synergistic effect on the mortalilty of *Aedes aegypti* larvae and adults when presented in the combination extracts between *H. suaveolens* and *L. camara* (Tanprasit, 2005). Four species of Lamiaceae, namely, *Mentha cordifolia* Opiz ex Fresen, *Ocimum basilicum* L., *Forma citratum* Back, and *H. suaveolens* (L.) were examined individually for the antibacterial study and synergistic effect against drugs-susceptible and drugs-resistant clinical isolates of bacteria (Chitsomboon et al., 2003). *H. suaveolens* individually displayed the best antibacterial activity against Methicilin-resistant Staphylococcus aureus. *H. suaveolens* in combination with *O. basilicum* showed synergistic effect

against Ciprofloxacin-resistant *Pseudomonas aeruginosa*.This study, therefore, aimed to investigate the cytotoxicity of neem, sugar apple and mintweed extracts and their biological control of oriental fruit flies. Brine Shrimp Lethality Assay (BSLA) was used for cytotoxicity test prior to the insect control tests.

3.3 Material and methods

3.3.1 Materials

Ethanol (95%) and hydrochloric acid (HCl) were purchased from Carlo Erba Reagents (Strada Rivoltana, SpA). Sodium benzoate was supplied Sigma Chemical Co. Yeast hydrolysate was kindly provided from “Radiation Entomology Group”, the Irradiation for Agriculture Research Program, Office of Atoms for Peace (OAP), Ministry of Science and Technology, Bangkok, Thailand. Other chemicals and materials were indicated in the procedures.

3.3.2 Sample collection and extract preparation

Leaves of mintweed, neem, sugar apple, and mintweed seeds were collected at Suranaree University of Technology (SUT) campus and its vicinity. The leaves were cleansed, chopped, and dried at 45°C for 2 days. The mintweed seeds were soaked in water and the mucilage was then gotten rid by squeezing against a stainless steal strainer and dried. Dried leaves and seeds were ground into fine powder. Ten grams of powder in 140 mL water or ethanol were extracted in an extractor (Universal extraction: Buchi model B811, Germany) at 100ºC for 12 hours. The crude extract was evaporated and then dried at -54ºC and stored at -20ºC for further studies. The dried extract was dissolved in its original solvent and kept at 4ºC during study.

3.3.3 Cytotoxicity test by brine shrimp lethality assay (BSLA)

The brine shrimp lethality assay is considered a useful tool for preliminary assessment of cytotoxicity. *Artemia salina* (Sanders™ Great Salt Lake, Brine Shrimp Company L.C., U.S.A.) was used since its response to the bioactive agents similar to that of mammalians' (Michael et al., 1956). In this study, microwell cytotoxicity was conducted (Solis et al., 1993). LC_{50} values were calculated using Probit Analysis (Finney, 1971). Brine shrimp (*A. salina*) were hatched in artificial seawater (preparing from sea salt 120 g/L). Two unequal compartments plastic chamber with several holes on the divider was used for hatching. The eggs (1 g per seawater 500 mL) were sprinkled into the larger compartment which was darkening while the smaller compartment was illuminated under constant temperature at 25°C and light. After 24 hours, nauplii (larvae) were collected by micropipette from the lighted side whereas their shells were left in another side. Ten nauplii were placed in each microwell which contained 100 μL of artificial seawater. In each experiment, 4 mg of the plant extract was added to 80 μL Dimethyl Sulfoxide (DMSO) and 3,120 μL of artificial seawater which designated as about 1,000 ppm of extract stock solution. Serial dilutions (10-1,000 ppm) of extract stock solution with 3,600 μL of seawater were made in 24-well microplate in a set of six replications per dose. Experiments were conducted along with control solution of 400 μL (DMSO 80 μL in seawater 3600 μL). The numbers of dead (non-motile) nauplii in each well were counted in the period of 24 hours.

3.3.4. Lethality concentration determination

The percentage lethality was determined by comparing the mean dead nauplii of the test and the control wells. Analysis of the data was performed by probit analysis to determine the lethal concentration to half of the test organisms (LC_{50}).

3.3.5 Oriental fruit fly rearing

Pupae of oriental fruit flies (*B. dorsalis)* were kindly supported from Radiation Entomology Group, The Irradiation for Agriculture Research Program, Office of Atoms for Peace (OAP), Ministry of Science and Technology, Bangkok, Thailand. The pupae were placed in a wire-net cage (Figure 3.1). After 10-15 days, the adult flies emerged and then took a further week to 10 days to reach sexual maturity. The adult fruit flies can survive on sugar and water alone and this should be supplied in the cages as the adult emerged. Water was supplied using an agar. Sugar with yeast hydrolysated (1 : 1 wt/wt) was used for sugar source. The female flies were allowed to lay eggs in an artificial dome (Figure 3.2). Eggs were collected by washing out the dome with water and then dispensed onto the artificial food containing, 300 g wheat germ, 120 g sugar, 40 g yeast hydrolysate, 1 g sodium benzoate, 1 mL HCl in 500 mL water (Table 3.1 and Figure 3.3) in order to ensure an excess food for larvae. After hatching within 24-48 hours, the larvae were moved to grow and develop in wood chip trays. The larvae transformed into the pupae with 12-24 hours. Then, the new generation of the fruit flies started again.

Figure 3.1 The wire-net cage for oriental fruit fly rearing.

Figure 3.2 The artificial egging devicean (artificial dome).

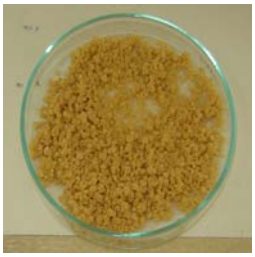

Figure 3.3 The artificial food for oriental fruit flies.

Table 3.1 The formulation of artificial food for feeding oriental fruit flies.

Ingredient	Content
Wheat germ	300 g
Sugar	120 g
Yeast hydrolysate	40 g
Sodium benzoate	1 g
HCl	1 mL
Distilled water	500 mL

3.3.6 Repellent test

The repellent action of plants was tested in an olfactometer (Figure 3.4), which was a rectangular plastic box, dimension of 75 × 10 × 10 cm^2 (L × W × H) with a square hole of 10 × 10 cm in the middle.

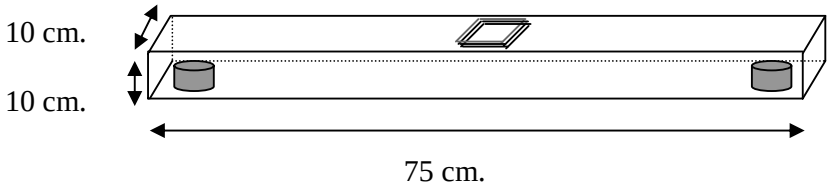

Figure 3.4 An olfactometer set-up. An individual female fly was introduced in the center. The treatment and control artificial food were positioned at either end of the device.

One end of the olfactometer was marked as a control end and the other was a treatment end. The control end of the tube, a 10-mL beaker containing sugar source which comprised of 1 g sugar, 1 g yeast hydrolysate and 10 μL methyeuginol was placed. The treatment end, a 10-mL beaker contained one gram of plant extracts and sugar source was settled. The hole in the middle was covered with a gauze and the ends of the tube were sealed. One freshly female and one male oriental fruit fly was released one by one through the hole in the middle of the tube. The fly's behavior was continuously observed for 1 hour. All repellent tests were repeated for 30 pairs of the flies.

3.3.7 The effects of plant extracts on the biosis of oriental fruit flies

The insecticidal activity of plant extracts was studied by direct contact application (dipping or eating). Four concentrations of plant extracts, 2,500, 5,000, 7,500, and 10,000 ppm were prepared by diluting crude extracts with distilled water

or ethanol depending on the original solvents. Each treatment of plant extracts on the biosis of flies was done as the followings:

Eggs: Thirty eggs were placed at the center of a dark paper piece with a dimension of $1 \times 1 \times 1$ cm^3. A hundred microlitre of plant extracts at various concentrations were dropped onto the cluster of eggs. The pieces of paper with treated eggs were then placed in a 50-mL beaker which contained the artificial food. Sealed the beaker and observed the non-hatching in the beaker after 24 hours.

Larvae (Feeding): Thirty second instar larvae of flies, which hatched from eggs for 7 days, were placed into the 50-mL beaker which contained the mixture of plant extracts at various concentrations and artificial food (1 : 1 wt/wt). Sealed the beaker and counted numbers of dead larvae after 24 hours.

Larvae (Dipping): Thirty second instar larvae of flies, which hatched from eggs for 7 days, were dipped into the plant extracts at various concentrations for 3 seconds. Treated larvae were put in a 50-mL beaker and then the beaker was sealed. Observed and counted numbers of dead larvae after 24 hours.

Adults: Thirty adult flies, which emerged from pupae for 10-15 days, were placed in a small cage with dimension of $20 \times 20 \times 20$ cm^2. Sugar with yeast hydrolysated (1 : 1 wt/wt) was mixed with plant extracts at various concentrations (1 : 1 wt/wt). The mixture of sugar source for files was then put in the cage. Observed and counted the numbers of dead adults after 24 hours.

The effects of single and combination of the two plant extracts on eggs, larvae and adults of fruit flies was conducted. In each treatment, 30 samples per stage were used. Cabamate was used as a positive control group, whereas water and ethanol was used as normal control groups. The treatments of single and combination extracts were performed

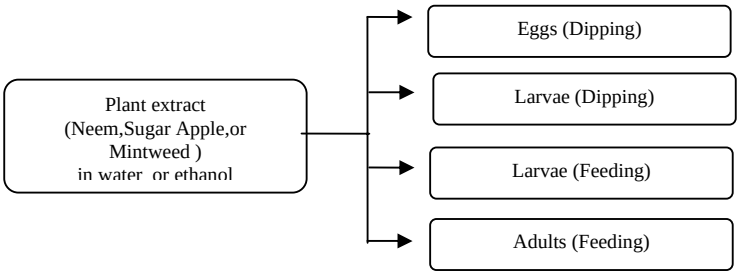

Chart 3.1 Single plants extract treatments on biosis of oriental fruit flies.

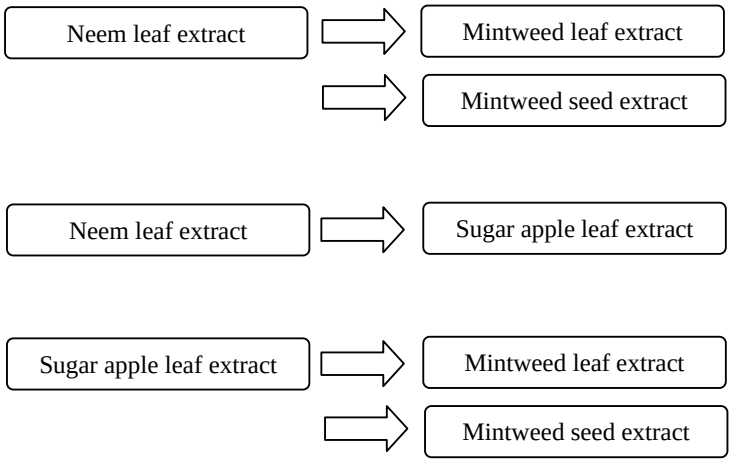

Chart 3.2 Five pairs of combination treatments of plant extracts on biosis of oriental fruit flies.

3.3.8 Data analysis

The percentage of mortality of each stage of oriental fruit flies was evaluated by using program Statistic Package for the Social Science (SPSS). LC_{50} and LD_{50} were analyzed by using Probit analysis. In addition, the comparison of the relationships among-subject affects the type of extracts, and the extraction methods were determined by using t-Test of the variance. All analyzes were done at the 95% confident level.

3.4 Results and discussion

3.4.1 Repellent Test

Percentage of repellence of single and two-plant combination extracts on oriental fruit flies was listed in Table 3.2 and Table 3.3. Percentage of repellence of mintweed seed leaf-ethanol extract was 74.0 ± 5.0 ($P < 0.05$), of mintweed leaf-ethanol extract was 73.3 ± 1.5, ($P < 0.01$) of sugar apple leaf-ethanol extract was 70.0 1.4($P < 0.05$), of neem leaf-ethanol extract was 69.3 ± 2.0 ($P < 0.01$), of sugar apple leaf-water was 61.21 ± 2.1 ($P < 0.05$), of mintweed seed-water extract was 50.0 ± 2.4 ($P < 0.05$), of neem leaf-water extract was 46.4 ± 2.6 ($P < 0.01$), and of mintweed seed-water extract was 33.3 ± 2.2 ($P < 0.01$), respectively. Results indicated that repellent activity of single plant extracts were ranged from the highest to the lowest as following: mintweed seed-ethanol extract > mintweed leaf-ethanol extract > sugar apple leaf-ethanol extract > neem leaf-ethanol extract > sugar apple leaf-water extract > mintweed seed-water extract > neem leaf-water extract > mintweed leaf-water extract, respectively. Moreover, repellent activity of all ethanolic extracts was higher than those of all water extracts .

In the case of two-plant extract combinations (Table 3.3), Percentage of repellence of neem leaf-water extract with sugar apple leaf-water extract was 78.0 ± 2.6 ($P < 0.05$). Percentage of repellence of neem leaf-water extract with mintweed seed-water extract was 76.7 ± 2.5 ($P < 0.01$) Percentage of repellence of neem leaf-water extract with mintweed leaf-water extract was 73.3 ± 2.2. Percentage of repellence of sugar apple leaf-water extract with mintweed seed-water extract was 66.2 ± 3.6. Percentage of repellence of sugar apple leaf-ethanol extract with mintweed seed-ethanol extract was 61.2 ± 3.2. Percentage of repellence of neem leaf-ethanol

extract with mintweed leaf-ethanol extract was 60.0 ± 4.1. Percentage of repellence of neem leaf-ethanol extract with sugar apple leaf-ethanol extract was 56.7 ± 2.6($P < 0.05$). Percentage of repellence of sugar apple leaf-water extract with mintweed leaf-water extract was 55.2 ± 2.0. Percentage of repellence of sugar apple leaf-ethanol extract with mintweed leaf-ethanol extract was 50.0 ± 5.4 and percentage of repellence of neem leaf-ethanol extract with mintweed seed-ethanol extract was 33.3 ± 3.3($P < 0.01$). It was suggested that repellence activity of neem leaf-water extract with sugar apple leaf-water extract > neem leaf-water extract with mintweed seed-water extract > neem leaf-water extract with mintweed leaf-water extract > sugar apple leaf-water extract with mintweed seed-water extract > sugar apple leaf-ethanol extract with mintweed seed-ethanol extract > neem leaf-ethanol extract with mintweed leaf-ethanol extract > neem leaf-ethanol extract with sugar apple leaf-ethanol extract > sugar apple leaf-water extract with mintweed leaf-water extract > sugar apple leaf-ethanol extract with mintweed leaf-ethanol extract > neem leaf-ethanol extract with mintweed seed-ethanol extract, respectively. When compared all same type of two-plant combination extracts, water extracts showed higher repellence .

In comparison of repellent activity between single and plant combination extracts (Table 3.4) it was found that neem leaf-water extract gave lower repellence activity than neem combination extracts using water as a solvent. On the other hand, neem leaf-ethanol extract showed higher repellence activity than neem combination extracts using ethanol as a solvent. Similary, sugar apple leaf-ethanol extract exhibited higher repellent activity than sugar apple combination extracts using ethanol as a solvent. Sugar apple leaf-water extract had higher repellent activity than sugar

apple leaf-water extract with mintweed leaf-water extract, but had lower repellent activity than sugar apple leaf-water extract with mintweed seed-water extract.

Table 3.2 Percentage of repellence of single plant extracts on oriental fruit flies.

Plant Part	Percentage of Repellence (N=6)		
	Water extracts	**Ethanol extracts**	**P-value**
Neem leaf	46.4 ± 2.6	69.3 ± 2.0	0.002**
Sugar apple leaf	61.2 ± 2.1	70.0 ± 1.4	0.024*
Mintweed leaf	33.3 ± 2.2	73.3 ± 1.5	0.000**
Mintweed seed	50.0 ± 2.4	74.0 ± 5.0	0.031*

Seyoum et al. (2002) reported that *Azadirachta indica* A. Juss and *Hyptis suaveolens* Poit. did not significantly repel mosquitoes which was a malaria vector *Anopheles gambiae sensu stricto* in semi-field experimental hut trials. However, Khaire et al. (1993) reported that treating pigeon pea seeds with neem oil showed significant repellent action against egg laying by adult *Callosobruchus chinensis* beetles for up to 100 days after treatment. Pandey et al. (1986) also found that plant extracts of neem leaves and twigs gave a high repellent action against *C. chinensis*. Al Lawati et al (2002) was found that legume seeds treated with extracts of *Annona squamosa* were not repellent, rather the beetles *C. chinensis* were attracted to them.

Table 3.3 Percentage of repellence of two-plant combination extracts on oriental fruit flies.

Plant Part	Percentage of Repellence (N=6)		
	Water extracts	**Ethanol extracts**	**P- value**
Neem leaf+ Sugar apple leaf	78.0 ± 2.6	56.7 ± 2.6	0.029*
Neem leaf+ Mintweed leaf	73.3 ± 2.2	60.0 ± 4.1	0.133
Neem leaf + Mintweed seed	76.7 ± 2.5	33.3 ± 3.3	0.000**
Sugar apple leaf+ Mintweed leaf	55.2 ± 2.0	50.0 ± 5.4	0.937
Sugar apple leaf+ Mintweed seed	66.2 ± 3.6	61.2 ± 3.2	0.065

Table 3.4 Comparison percentage of repellence of single and two-plant combination extracts on biosis of adult oriental fruit flies

Plant Extracts	Percentage of Repellence (N = 6)
Neem leaf/H_2O	46.4 ± 2.6
Neem leaf/H_2O + Sugar apple leaf/H_2O	78.0 ± 2.6
Neem leaf/H_2O + Mintweed leaf/H_2O	73.3 ± 2.2
Neem leaf/H_2O + Mintweed seed/H_2O	76.7 ± 2.5
Neem leaf/EtOH	69.3 ± 2.0
Neem leaf/EtOH + Sugar apple leaf/EtOH	56.7 ± 2.6
Neem leaf/EtOH + Mintweed leaf/EtOH	60.0 ± 4.1
Neem leaf/EtOH + Mintweed seed/EtOH	33.3 ± 3.3
Sugar apple leaf/H_2O	61.3 ± 2.1
Sugar apple leaf/H_2O + Mintweed leaf/H_2O	55.2 ± 2.0
Sugar apple leaf/H_2O + Mintweed seed/H_2O	66.2 ± 3.6
Sugar apple leaf/EtOH	70.0 ± 1.4
Sugar apple leaf/EtOH + Mintweed leaf/EtOH	50.0 ± 5.4
Sugar apple leaf/EtOH + Mintweed seed/EtOH	61.2 ± 3.2

3.4.2 Cytotoxicity of neem, sugar apple, and mintweed extracts

LC_{50} of single plant extracts were listed in Table 3.5. LC_{50} of mintweed leaf-ethanol extract was 0.1 ± 1.2 ppm and of mintweed leaf-water extract was 0.9 ± 4.6E-02 ppm. LC_{50} of mintweed seed-water extract was 3.7 ± 0.2 ppm and of neem leaf-ethanol extract was 6.3 ± 0.6 ppm. LC_{50} of mintweed seed-ethanol extract was 6.4 ± 0.4 ppm and of sugar apple leaf-ethanol extract was 27.8 ± 1.5 ppm. LC_{50} of

neem leaf-water extract was 48.3 ± 2.8 ppm and of sugar apple leaf-water extract was 115.1 ± 4.3 ppm. Results suggested that cytotoxicity of single plant extracts were ranged from the highest to the lowest as following: mintweed leaf-ethanol extract > mintweed leaf-water extract > mintweed seed-water extract > neem leaf-ethanol extract > mintweed seed-ethanol extract > sugar apple leaf-ethanol extract > neem leaf-water extract > sugar apple leaf-water extract, respectively. Moreover, cytotoxicity of all ethanolic extracts was higher than those of all water extracts ($P < 0.01$).

In the case of two-plant extract combinations (Table 3.6), LC_{50} of neem leaf-ethanol extract with mintweed leaf-ethanol extract was 0.07 ± 1.7E-02 ppm. LC_{50} of sugar apple leaf-ethanol extract with mintweed leaf-ethanol extract was 0.7 ± 5.8E-02 ppm. LC_{50} of neem leaf-ethanol extract with sugar apple leaf-ethanol extract was 1.5 ± 0.1 ppm. LC_{50} of neem leaf leaf-ethanol extract with mintweed seed-ethanol extract was 4.7 ± 0.2 ppm. LC_{50} of sugar apple leaf-ethanol extract with mintweed seed-ethanol extract was 5.2 ± 1.2 ppm. LC_{50} of neem leaf-water extract with mintweed leaf-water extract was 7.3 ± 0.4 ppm. LC_{50} of sugar apple leaf-water extract with mintweed leaf-water extract was 8.4 ± 0.3 ppm. LC_{50} of sugar apple leaf-water extract with mintweed seed-water extract was 8.9 ± 0.4 ppm. LC_{50} of neem leaf-water extract with mintweed leaf-water extract was 11.0 ± 0.4 ppm. LC_{50} of neem leaf-water extract with mintweed seed-water extract was 11.0 ± 0.7 ppm. It can be concluded that cytotoxicity of neem leaf-ethanol extract with mintweed

leaf-ethanol extract > sugar apple leaf-ethanol extract with mintweed leaf-ethanol extract > neem leaf-ethanol extract with sugar apple leaf-ethanol extract > neem leaf leaf-ethanol extract with mintweed seed-ethanol extract > sugar apple leaf-ethanol

extract with mintweed seed-ethanol extract > neem leaf-water extract with mintweed leaf-water extract > sugar apple leaf-water extract with mintweed leaf-water extract > sugar apple leaf-water extract with mintweed seed-water extract > neem leaf-water extract with sugar apple leaf-water extract > neem leaf-water extract with mintweed seed-water extract, respectively. When compared all same type of two-plant combination extracts, ethanolic extracts showed higher cytotoxicity (lower LC_{50}) than water extracts ($P < 0.01$).

Table 3.5 LC_{50} of single plant extracts in Brine Shrimp Lethality Assay.

Plant Part	**LC_{50} (ppm)**		
	Water extracts	**Ethanol extracts**	**P-value**
Neem leaf	48.3 ± 2.8	6.3 ± 0.6	0.000**
Sugar apple leaf	115.1 ± 4.3	27.8 ± 1.5	0.000**
Mintweed leaf	0.9 ± 4.6E-02	0.1 ± 1.2E-02	0.000**
Mintweed seed	3.7 ± 0.2	6.4 ± 0.4	0.004**

Table 3.6 LC_{50} of two-plant extract combinations on brine shrimp lethality assay.

Plant Part	LC_{50} (ppm)		
	Water extracts	**Ethanol extracts**	**P-value.**
Neem leaf+ Sugar apple leaf	7.3 ± 0.4	0.07 ± 1.7E-02	0.000**
Neem leaf+ Mintweed leaf	11.0 ± 0.4	1.5 ± 0.1	0.000**
Neem leaf + Mintweed seed	11.0 ± 0.7	4.7 ± 0.2	0.000**
Sugar apple leaf+ Mintweed leaf	8.4 ± 0.3	0.7 ± 5.8E-02	0.000**
Sugar apple leaf+ Mintweed seed	8.9 ± 0.4	5.2 ± 1.2	0.002**

In comparison of cytotoxicities between single and plant combination extracts (Table 3.7) it was found that cytotoxicities of all two-plant combination extracts were greater than those of single plant extracts. This result implied that two-plant combination extracts exhibited synergistic effect on LD_{50}. In addition, ethanol extractions of both single extract and plant combination extracts obviously showed lower LC_{50} than water extractions

Table 3.7 Comparison of cytotoxicity effect of plant extracts, single and combination by Brine Shrimp Lethality Assay.

Plant Extracts	LC_{50} (ppm)
Neem leaf/H_2O	48.3 ± 2.8
Neem leaf/H_2O + Sugar apple leaf/H_2O	7.3 ± 0.4
Neem leaf/H_2O + Mintweed leaf/H_2O	11.0 ± 0.4
Neem leaf/H_2O + Mintweed seed/H_2O	11.0 ± 0.7
Neem leaf/EtOH	6.3 ± 0.6
Neem leaf/EtOH + Sugar apple leaf/EtOH	0.07 ± 1.7E-02
Neem leaf/EtOH + Mintweed leaf/EtOH	1.5 ± 0.1
Neem leaf/EtOH + Mintweed seed/EtOH	4.7 ± 0.2
Sugar apple leaf/H_2O	115.1 ± 4.3
Sugar apple leaf/H_2O + Mintweed leaf/H_2O	8.4 ± 0.3
Sugar apple leaf/H_2O + Mintweed seed/H_2O	8.9 ± 0.4
Sugar apple leaf/EtOH	27.8 ± 1.5
Sugar apple leaf/EtOH + Mintweed leaf/EtOH	0.7 ± 5.8E-02
Sugar apple leaf/EtOH + Mintweed seed/EtOH	5.2 ± 1.2

3.4.3 Insecticidal activity of neem, sugar apple, and mintweed extracts on biosis of oriental fruit flies

3.4.3.1 Eggs

Tables 3.8 and 3.9 showed percentage of non-hatching eggs at various concentrations and LD_{50} of single and two-plant combination extracts on biosis of oriental fruit flies. The data indicated that there was a progressive increase in percentage of non-hatching of eggs with the increase in concentration of both single

and two-plant combination extracts. LD_{50} of mintweed seed-ethanol extract, mintweed seed-water extract, sugar apple leaf-water extract and mintweed leaf-ethanol extract can not de figured out their LD_{50}, because 50% of mortality were out of range of the designated concentrations. LD_{50} of mintweed leaf-water extract was 2,920.3 ± 55.9 ppm and of neem leaf-water extract was 3,353.4 ± 157.0 ppm. LC_{50} of neem leaf-ethanol extract was 3,625.1 ± 162.4 ppm and of sugar apple leaf-ethanol extract was 4,815.3 ± 172.2 ppm. Results suggested that cytotoxicity of single plant extracts on eggs were ranged from the highest to the lowest as following: mintweed leaf-water extract > neem leaf-water extract > neem leaf-ethanol extract > sugar apple leaf-ethanol extract, respectively. Moreover, most wather extracts had higher cytotoxicity on eggs than ethanolic extracts, excepting mintweed leaf-water extracts and neem leaf-water extracts .

Table 3.8 Percentage of non-hatching eggs at various concentrations and LD_{50} of single plant extracts on biosis of oriental fruit fly eggs.

Plant Extracts	%Non-Hatching Eggs Concentration (ppm)				
	2,500	**5,000**	**7,500**	**10,000**	**LD_{50}**
Neem leaf /H_2O	44 ± 19	56 ± 6	72 ± 5	80 ± 15	3,353.4 ± 157.0
Neem leaf /EtOH	43 ± 8	51 ± 2	64 ± 5	77 ± 5	3,625.1 ± 162.4
Sugar apple leaf /H_2O	58 ± 4	61 ± 2	66 ± 11	74 ± 7	off range
Sugar apple leaf/EtOH	37± 3	47 ± 3	58 ± 5	71 ± 12	4,815.3 ± 172.2
Mintweed leaf/H_2O	50 ± 3.	58 ± 2	66 ± 2	86 ± 11	2,920.3 ± 55.9
Mintweed leaf /EtOH	59 ± 5	66 ± 2	77 ± 7	87 ± 7	off range
Mintweed seed /H_2O	64 ± 4	68 ± 4	76 ± 2	86 ± 7	off range
Mintweed seed /EtOH	69 ± 1	74 ± 2	79 ± 7	86 ± 6	off range

In the case of plant combination extracts, LD_{50} of sugar apple leaf-ethanol extract with mintweed leaf-ethanol extract, sugar apple leaf-ethanol extract with neem leaf-ethanol extract, neem leaf-ethanol extract with mintweed leaf-ethanol extract, sugar apple leaf-ethanol extract with mintweed seed-ethanol extract, neem leaf-ethanol extract with mintweed seed-ethanol extract, neem leaf-water extract with mintweed leaf-water extract and sugar apple leaf-water extract with neem leaf-water extract can not be figured out their LD_{50}, because 50% of mortality were out of range of the designated concentrations. LD_{50} of sugar apple leaf-water extract with mintweed leaf-water extract was 2,902.4 ± 50.8 ppm. LD_{50} of sugar apple leaf-water extract with mintweed seed-water extract was 2,934.7 ± 54.9 ppm. LD_{50} of neem leaf-

water extract with mintweed seed-water extract was 3,277.7 ± 173.0 ppm. It was suggested that cytotoxicity on eggs of sugar apple leaf-water extract with mintweed leaf-water extract > sugar apple leaf-water extract with mintweed seed-water extract > neem leaf-water extract with mintweed seed-water extract, respectively. As compared to all same type of two-plant combination extracts, water extracts had higher cytotoxicity on eggs (lower LD_{50}) than ethanolic extracts.

In comparison of cytotoxicities between single and plant combination extracts (Table 3.10) it revealed that cytotoxicities on eggs of single extracts were lower than those of two-plant combination extracts. LD_{50} of most plant extracts were approximately ranged from 2,500-4,800 ppm. However, there were only four types of plant combination extracts gave LD_{50} lower than 3,000 ppm i.e. sugar apple leaf-waterl extract with mintweed leaf-water extract (2902.4 ± 50.8 ppm), sugar apple leaf-weter extract with mintweed seed-weter extract (2934.7 ± 54.9 ppm),

Table 3.9 Percentage of non-hatching eggs at various concentrations and LD_{50} of two-plant combination extracts on biosis of oriental fruit fly eggs.

Plant Extract Combinations	%Non-Hatching of Eggs Concentration (ppm)				
	2,500	**5,000**	**7,500**	**10,000**	**LD_{50}**
Neem leaf/H_2O + Sugar apple leaf/H_2O	54 ± 1	61± 4	71 ± 4	78 ± 13	off range
Neem leaf/EtOH + Sugar apple leaf/EtOH	72 ± 2	76 ± 3	85 ± 2	90 ± 2	off range
Neem leaf/H_2O + Mintweed leaf/H_2O	64 ± 4	65 ± 4	75 ± 34	85 ± 2	off range
Neem leaf/EtOH + Mintweed leaf/EtOH	69 ± 6	76 ± 3	82 ± 2	88 ± 2	off range
Neem leaf/H_2O + Mintweed seed/H_2O	49 ± 2	57 ± 8	62 ± 4	82 ± 1	3,277.7 ± 173.0
Neem leaf/EtOH + Mintweed seed/EtOH	67 ± 5	70 ± 6	80 ± 2	88 ± 4	off range
Sugar apple leaf/H_2O + Mintweed leaf /H_2O	50 ± 9	58 ± 2	61 ± 5	76 ± 4	2,902.4 ± 50.8
Sugar apple leaf/EtOH + Mintweed leaf/EtOH	72 ± 2	76 ± 3	83 ± 4	88 ± 4	off range
Sugar apple leaf/H_2O + Mintweed seed/H_2O	50 ± 9	58 ± 2	64 ± 4	83 ± 6	2,934.7 ± 54.9
Sugar apple leaf/EtOH + Mintweed seed/EtOH	67 ± 5	69 ± 6	77 ± 7	87 ± 7	off range

Table 3.10 Comparison LD_{50} of single and two-plant combination extracts on biosis of oriental fruit fly eggs.

Plant Extracts	LD_{50} (ppm) of Eggs
Neem leaf/H_2O	3,353.4 ± 157.0
Neem leaf/H_2O + Sugar apple leaf/H_2O	off range
Neem leaf/H_2O + Mintweed leaf/H_2O	off range
Neem leaf/H_2O + Mintweed seed/H_2O	3,277.7 ± 173.0
Neem leaf /EtOH	3,625.1 ± 162.4
Neem leaf / EtOH + Sugar apple leaf / EtOH	off range
Neem leaf/EtOH + Mintweed leaf/EtOH	off range
Neem leaf/ EtOH + Mintweed seed/EtOH	off range
Sugar apple leaf/H_2O	off range
Sugar apple leaf/H_2O + Mintweed leaf/H_2O	2,902.4 ± 50.8
Sugar apple leaf/H_2O + Mintweed seed/H_2O	2,934.7 ± 54.9
Sugar apple leaf/EtOH	4,815.3 ± 172.2
Sugar apple leaf/EtOH + Mintweed leaf/EtOH	off range
Sugar apple leaf/EtOH + Mintweed seed/EtOH	off range

3.4.3.2 Lavae (feeding treatment)

Tables 3.11 and 3.12 displayed percentage of mortality of larvae using eating treatment at various concentrations and LD_{50} of single and two-plant combination extracts on biosis of oriental fruit flies. The data showed that there was a progressive increase in percentage of mortality of larvae with the increase in concentration of both single and two-plant combination extracts. LD_{50} of mintweed

seed-ethanol extract, mintweed seed-water extract and neem leaf-water extract can not be figured out their LD_{50}, because 50% of mortality were out of range of the designated concentrations. LD_{50} of sugar apple leaf-ethanol extract was 2,568.3 ± 47.5 ppm. LD_{50} of mintweed leaf-water extract was 2,658.4 ± 132.4 ppm and of mintweed leaf-water extract was 2,707.8 ± 120.3 ppm. LD_{50} of neem leaf-ethanol extract was 3,621.7 ± 102.4 ppm and of sugar apple leaf-ethanol extract was 5,088.8 ± 125.3 ppm. Results suggested that cytotoxicity of single plant extracts on larvae were ranged from the highest to the lowest as following: sugar apple leaf-water extract > mintweed leaf-ethanol extract > mintweed leaf-water extract > neem leaf-ethanol extract > sugar apple leaf-ethanol extract, respectively. In addition, most water extracts had higher cytotoxicity on larvae than ethanolic extracts, excepting sugar apple leaf-ethanol and water extracts ($P<0.01$), mintweed leaf-ethanolic and water extracts ($P<0.05$). This observation was similar to cytotoxicity of single plant extracts on eggs.

In the case of plant combination extracts, LD_{50} of neem leaf-ethanol extract with mintweed leaf-ethanol extract, neem leaf-water extract with mintweed leaf-water extract, neem leaf-ethanol extract with sugar apple leaf-ethanol extract, sugar apple leaf-ethanol extract with mintweed seed-ethanol extract, neem leaf-ethanol extract with mintweed seed-ethanol extrac, sugar apple leaf-water extract with mintweed leaf-water extract and sugar apple leaf-water extract with mintweed leaf-water extract can not be figured out their LD_{50}, because 50% of mortality were out of range of the designated concentrations. LD_{50} of sugar apple leaf-water extract with neem leaf-water extract was 2,595.1 ± 95.6 ppm. LD_{50} of sugar apple leaf-water extract with mintweed seed-water extract was 2,700.4 ± 65.1 ppm. LD_{50} of neem

leaf-water extract with mintweed seed-water extract was 3,062.3 ± 81.8 ppm. It implied that cytotoxicity on larvae using eating treatment of neem leaf-water extract with sugar apple leaf-water extract > sugar apple leaf-water extract with mintweed seed-water extract > neem leaf-water extract with mintweed seed-water extract, respectively. When compared all same type of two-plant combination extracts, water extracts had higher cytotoxicity on larvea (lower LD_{50}) than ethanolic extracts excepting neem leaf-water extract with sugar apple leaf-water extract (2595.1 ± 95.6 ppm) and sugar apple leaf-water extract with mintweed seed-water extract (2700.4 ± 65.1 ppm)

In comparison of cytotoxicities between single and plant combination extracts (Table 3.13) it was found that cytotoxicities on larvae using eating treatment of single extracts were lower than those of two-plant combination extracts. LD_{50} of all plant extracts were about 2,500-5,000 ppm. However, there were only three types of plant combination extracts gave LD_{50} lower than 3,000 ppm i.e. sugar apple leaf-weter extract (2,568.3 ± 47.5 ppm), neem leaf-water extract with sugar apple leaf-water extract (2,595.1 ± 95.6 ppm), and sugar apple leaf-water extract with mintweed seed-water extract (2,700.4 ± 65.1 ppm). There were some single plant extracts exhibited lower LD_{50} than two-plant combination extracts. These observations were found in neem leaf-water extract compared to sugar apple leaf-water extract with neem leaf-water extract, neem leaf water extract compared to neem leaf -water extract with mintweed seed-water extract, and were sugar apple leaf-water extract compared to sugar apple leaf-water extract with mintweed seed-water extract.

Table 3.11 Percentage of mortality of larvae at various concentrations and LD_{50} of single plant extracts on biosis of oriental fruit fly larvae using feeding treatment.

Plant Extracts	**%Mortality of Larvae (Feeding)**				
	Concentration (ppm)				
	2,500	**5,000**	**7,500**	**10,000**	**LD_{50}**
Neem leaf/H_2O	45 ± 12	56 ± 5	66 ± 6	75 ± 10	off range
Neem leaf/EtOH	41 ± 7	48. ± 2	61 ± 2	74 ± 4	3,621.7 ± 101.4
Sugar apple leaf /H_2O	64 ± 4	72 ± 2	79 ± 1	82 ± 7	2,568.3 ± 47.5 a
Sugar apple leaf/EtOH	48 ± 3	59 ± 2	66 ± 5	74 ± 3	5,088.8 ± 125.3 a
Mintweed leaf/H_2O	47 ± 2	56 ± 3	65 ± 2	79 ± 7	2,707.8 ± 120.3 b
Mintweed leaf/EtOH	50 ± 4	62 ± 5	74 ± 4	82 ± 4	2,658.4 ± 132.4 b
Mintweed seed/H_2O	53 ± 3	62 ± 2	76 ± 2	79 ± 5	off range
Mintweed seed/EtOH	37 ± 3	45 ± 2	54 ± 2	63 ± 3	off range

a = significant difference at $P<0.01$
b = significant difference at $P<0.05$

Table 3.12 Percentage of mortality of larvae at various concentrations and LD_{50} of two-plant combination extracts on biosis of oriental fruit fly larvae using feeding treatment.

Plant Extract Combinations	**%Mortality of Larvae (Feeding) Concentration (ppm)**				
	2,500	**5,000**	**7,500**	**10,000**	**LD_{50}**
Neem leaf/H_2O + Sugar apple leaf/H_2O	47 ± 2	61 ± 4	64 ± 4	67 ± 8	2,595.1 ± 95.6
Neem leaf/EtOH + Sugar apple leaf/EtOH	58 ± 2	75 ± 2	83 ± 4	87 ± 5	off range
Neem leaf/H_2O + Mintweed leaf/H_2O	56 ± 8	65 ± 4	74 ± 4	85 ± 2	off range
Neem leaf/EtOH + Mintweed leaf/EtOH	69 ± 4	76 ± 5	82 ± 2	86 ± 2	off range
Neem leaf/H_2O + Mintweed seed/H_2O	48 ± 2	56 ± 5	62 ± 4	74 ± 5	3,062.3 ± 81.8
Neem leaf/EtOH + Mintweed seed/EtOH	64 ± 2	66 ± 7	79 ± 2	86 ± 4	off range
Sugar apple leaf/H_2O+ Mintweed leaf /H_2O	48 ± 2	58 ± 2	61 ± 5	74 ± 2	off range
Sugar apple leaf/EtOH + Mintweed leaf/EtOH	67.07 ± 7.69	76.33 ± 3.33	81.44 ± 1.92	85.78 ± 8.26	off range
Sugar apple leaf/H_2O + Mintweed seed/H_2O	48 ± 2	58 ± 2	64 ± 4	75 ± 4	2,700.4 ± 65.1
Sugar apple leaf/EtOH + Mintweed eed/EtOH	50 ± 5	63 ± 6	77 ± 7	84 ± 4	off range

Table 3.13 Comparison LD_{50} of single and two-plant combination extracts on biosis of oriental fruit fly larvae using feedting treatment.

Plant Extracts	LD_{50} (ppm)
Neem leaf/H_2O	off range
Neem leaf/H_2O + Sugar apple leaf/H_2O	2,595.1 ± 95.6
Neem leaf/H_2O + Mintweed leaf/H_2O	off range
Neem leaf/H_2O + Mintweed seed/H_2O	3,062.3 ± 81.8
Neem leaf /EtOH	3,621.7 ± 101.4
Neem leaf / EtOH + Sugar apple leaf / EtOH	off range
Neem leaf/EtOH + Mintweed leaf/EtOH	off range
Neem leaf/ EtOH + Mintweed seed/EtOH	off range
Sugar apple leaf/H_2O	2,568.3 ± 47.5
Sugar apple leaf/H_2O + Mintweed leaf/H_2O	off range
Sugar apple leaf/H_2O + Mintweed seed/H_2O	2,700.4 ± 65.1
Sugar apple leaf/EtOH	5,088.8 ± 125.3
Sugar apple leaf/EtOH + Mintweed leaf/EtOH	off range
Sugar apple leaf/EtOH + Mintweed seed/EtOH	off range

3.4.3.3 Lavae (dipping treatment)

Tables 3.14 and 3.15 summarized percentage of mortality of larvae using dipping treatment at various concentrations and LD_{50} of single and two-plant combination extracts on biosis of oriental fruit flies. The data showed that there was a progressive increase in percentage of mortality of larvae with the increase in concentration of both single and two-plant combination extracts. LD_{50} of mintweed

seed-ethanol extract and mintweed seed-water extract can not be figured out their LD_{50}, because 50% of mortality was out of range of the designated concentrations. LD_{50} of mintweed leaf-water extract was 2,651.0 ± 143.8 ppm and of sugar apple leaf-ethanol extract was 2,977.2 ± 67.1 ppm. LD_{50} of mintweed leaf-water extract was 3,222.9 ± 152.4 ppm and of neem leaf-water extract was 3,257.0 ± 190.8 ppm. LC_{50} of neem leaf-ethanol extract was 4,301.0± 259.8 ppm and of sugar apple leaf-ethanol extract was 5,429.5 ± 110.4 ppm. Results suggested that cytotoxicity of single plant extracts on larvae were ranged from the highest to the lowest as following: mintweed leaf-ethanol extract > sugar apple leaf-water extract > mintweed leaf-waterl extract > neem leaf-water extract > neem leaf-ethanol extract > sugar apple leaf-ethanol extract, respectively. Additionally, most water extracts had higher cytotoxicity on larvae than ethanolic extracts, excepting mintweed leaf-ethanol extract and sugar apple leaf-water extracts ($P < 0.01$). This observation was similar to cytotoxicity of single plant extracts on eggs and larvae using eating treatment.

In the case of plant combination extracts, LD_{50} of neem leaf-ethanol extract with mintweed leaf-ethanol extract, sugar apple leaf-ethanol extract with mintweed leaf-ethanol extract, mintweed seed-ethanol extract with neem leaf-ethanol extract, sugar apple leaf-ethanol extract with neem leaf-ethanol extract and neem leaf-water extract with mintweed leaf-water extract can not be figured out their LD_{50}, because 50% of mortality were out of range of the designated concentrations. LD_{50} of sugar apple leaf-water extract with mintweed leaf-water extract was 2,673.8 ± 114.7 ppm. LD_{50} of sugar apple leaf-ethanol extract with mintweed seed-ethanol extract was 2,680.2 ± 121.0 ppm. LD_{50} of sugar apple leaf-water extract with mintweed seed-water extract was 2,928.4 ± 111.6 ppm. LD_{50} of sugar apple leaf-water

extract with mintweed leaf-water extract was 3,029.4 ± 83.9 ppm. LD_{50} of neem leaf-water extract with mintweed seed-water extract was 3,135.0 ± 106.6 ppm. It indicated that cytotoxicity on larvae using dipping treatment of sugar apple leaf-water extract with neem leaf-water extract > sugar apple leaf-ethanol extract with mintweed seed-ethanol extract > sugar apple leaf-water extract with mintweed seed-water extract > sugar apple leaf-water extract with mintweed leaf-water extract > neem leaf-water extract with mintweed seed-water extract, respectively. When compared all same type of two-plant combination extracts, weter extracts had higher cytotoxicity on larvae (lower LD_{50}) than ethanolic extracts. However, there was no significant difference between water and ethanolic extractions of plan combination extracts of sugar apple leaf with mintweed seed.

In comparison of cytotoxicities between single and plant combination extracts on larvae using dipping treatment (Table 3.16), LD_{50} of all plant extracts approximately ranged from 2,500-5,400 ppm. However, there were only two types of plant combination extracts gave LD_{50} lower than 3,000 ppm i.e. neem leaf-water extract with sugar apple leaf-water extract (2,673.8 ± 114.7 ppm), and sugar apple leaf-ethanol extract with mintweed seed-ethanol extract (2,680.2 ± 121.0 ppm) and sugar apple leaf-water extract with mintweed seed-water extract (2,928.4 ± 111.6 ppm). Furthermore, there was only single plant extracts gave lower LD_{50} than two-plant combination extracts i.e. sugar apple leaf-water extract compared to sugar apple leaf-water extract with mintweed leaf-water extract.

Table 3.14 Percentage of mortality of larvae at various concentrations and LD_{50} of single plant extracts on biosis of oriental fruit fly larvae using dipping treatment.

Plant Extracts	**%Mortality of Larvae (Dipping)**				
	Concentration (ppm)				
	2,500	**5,000**	**7,500**	**10,000**	**LD_{50}**
Neem leaf/H_2O	38 ± 8	52 ± 2	63 ± 3	70 ± 9	3,257.0 ± 190.8
Neem leaf/EtOH	31 ± 6	50 ± 5	64 ± 5	74 ± 4	4,301.0 ± 259.8
Sugar apple leaf /H_2O	63 ± 3	70 ± 7	77 ± 13	81 ± 9	2,977.2 ± 67.1 a
Sugar apple leaf/EtOH	50 ± 11	60 ± 7	68 ± 3	76 ± 6	5,429.5 ± 110.4 a
Mintweed leaf/H_2O	46 ± 4	58 ± 3	68 ± 3	78 ± 5	3,222.9 ± 152.4
Mintweed leaf/EtOH	43 ± 4	60 ± 3	71 ± 6	74 ± 7	2,651.0 ± 143.8
Mintweed seed/H_2O	52 ± 2	61 ± 3	72 ± 6	79 ± 3	off range
Mintweed seed/EtOH	36 ± 4	46 ± 4	54 ± 4	70 ± 6	off range

a = significant difference at $P<0.01$

Table 3.15 Percentage of mortality of larvae and LD_{50} of two-plant combination extracts on biosis of oriental fruit fly larvae using dipping treatment.

Plant Extract Combinations	%Mortality of Larvae (Dipping) Concentration (ppm)				
	2,500	**5,000**	**7,500**	**10,000**	**LD_{50}**
Neem leaf/H_2O + Sugar apple leaf/H_2O	46 ± 2	60 ± 3	63 ± 3	69 ± 7	2,673.8 ± 114.7
Neem leaf/EtOH + Sugar apple leaf/EtOH	60 ± 3	78 ± 6	84 ± 2	90 ± 3	off range
Neem leaf/H_2O + Mintweed leaf/H_2O	58 ± 7	64 ± 2	74 ± 2	86 ± 2	off range
Neem leaf/EtOH + Mintweed leaf/EtOH	72 ± 2	79 ± 3	84 ± 2	91 ± 5	off range
Neem leaf/H_2O + Mintweed seed/H_2O	47 ± 3	58 ± 2	63 ± 4	74 ± 5	3,135.0 ± 106.6
Neem leaf/EtOH + Mintweed seed/EtOH	66 ± 2	69 ± 4	79 ± 3	89 ± 4	off range
Sugar apple leaf/H_2O + Mintweed leaf /H_2O	49 ± 4	59 ± 3	60 ± 6	71 ± 4	3,029.4 ± 83.9
Sugar apple leaf/EtOH + Mintweed leaf/EtOH	69 ± 5	78 ± 4	82 ± 3	89 ± 4	off range
Sugar apple leaf/H_2O + Mintweed seed/H_2O	49 ± 2	59 ± 2	68 ± 7	78 ± 4	2,928.4 ± 111.6
Sugar apple leaf/EtOH + Mintweed seed/EtOH	52 ± 7	64 ± 7	81 ± 4	89 ± 2	2,680.2 ± 121.0

Table 3.16 Comparison LD_{50} of single and two-plant combination extracts on biosis of oriental fruit fly larvae using dipping treatment.

Plant Extracts	LD_{50} (ppm)
Neem leaf/H_2O	3,257.0 ± 190.8
Neem leaf/H_2O + Sugar apple leaf/H_2O	2,673.8 ± 114.7
Neem leaf/H_2O + Mintweed leaf/H_2O	off range
Neem leaf/H_2O + Mintweed seed/H_2O	3,135.0 ± 106.6
Neem leaf /EtOH	4,301.0 ± 259.8
Neem leaf / EtOH + Sugar apple leaf / EtOH	off range
Neem leaf/EtOH + Mintweed leaf/EtOH	off range
Neem leaf/ EtOH + Mintweed seed/EtOH	off range
Sugar apple leaf/H_2O	2,977.2 ± 67.1
Sugar apple leaf/H_2O + Mintweed leaf/H_2O	3,029.4 ± 83.9
Sugar apple leaf/H_2O + Mintweed seed/H_2O	2,928.4 ± 111.6
Sugar apple leaf/EtOH	5,429.5 ± 110.4
Sugar apple leaf/EtOH + Mintweed leaf/EtOH	off range
Sugar apple leaf/EtOH + Mintweed seed/EtOH	2,680.2 ± 121.0

3.4.3.4 Adults

Tables 3.17 and 3.18 presented percentage of mortality of adults at various concentrations and LD_{50} of single and two-plant combination extracts on biosis of oriental fruit flies. The data revealed that there was a progressive increase in percentage of mortality of adults with the increase in concentration of both single and

two-plant combination extracts. LD_{50} of mintweed seed-ethanol extract can not be figured out their LD_{50}, because 50% of mortality were out of range of the designated concentrations. LD_{50} of mintweed seed-water extract was 2,521.3 ± 83.8 ppm. LD_{50} of sugar apple leaf-water extract was 2,710.8 ± 143.8 ppm and of mintweed leaf-ethanol extract was 3,295.8 ± 143.8 ppm. LD_{50} of mintweed leaf-water extract was 3,347.8 ± 152.4 ppm and of neem leaf-water extract was 4,239.1 ± 190.8 ppm. LC_{50} of neem leaf-ethanol extract was 4,810.9 ± 259.8 ppm and of sugar apple leaf-ethanol extract was 5,217.2 ± 110.4 ppm. Results indicated that that cytotoxicity of single plant extracts on larvae were ranged from the highest to the lowest as following: mintweed seed-water extract > sugar apple leaf-ethanol extract > mintweed leaf-ethanol extract > mintweed leaf-water extract > neem leaf-water extract > neem leaf-ethanol extract > sugar apple leaf-ethanol extract, respectively. Moreover, most water extracts had higher cytotoxicity on adults than water extracts, excepting neem leaf-ethanol and water extract ($P < 0.01$) and sugar apple leaf-ethanol and water extracts ($P < 0.05$). This observation was similar to cytotoxicity of single plant extracts on eggs and larvae using eating and dipping treatments.

In the case of plant combination extracts, LD_{50} of neem leaf-ethanol extract with mintweed leaf-ethanol extract, sugar apple leaf-ethanol extract with mintweed leaf-ethanol extract, neem leaf-ethanol extract with mintweed seed-ethanol extract, neem leaf-ethanol extract with sugar apple leaf-ethanol extract and neem leaf-water extract with mintweed leaf-water extract can not be figured out their LD_{50}, because 50% of mortality were out of range of the designated concentrations. LD_{50} of sugar apple leaf-ethanol extract with mintweed seed-ethanol extract was 2,568.3 ± 121.0 ppm. LD_{50} of sugar apple leaf-water extract with mintweed leaf-water extract

was 2,783.3 ± 83.9 ppm. LD_{50} of sugar apple leaf-water extract with mintweed seed-water extract was 2,784.4 ± 111.6 ppm. LD_{50} of sugar apple leaf-water extract with neem leaf-water extract was 3,142.1 ± 114.7 ppm. LD_{50} of neem leaf-water extract with mintweed seed-water extract was 3,193.9 ± 106.6 ppm. It was concluded that cytotoxicity on adults of sugar apple leaf-water extract with mintweed seed-water extract > sugar apple leaf-water extract with mintweed leaf-water extract > sugar apple leaf-water extract with mintweed seed-water extract > sugar apple leaf-water extract with neem leaf-water extract > neem leaf-water extract with mintweed seed-water extract, respectively. When compared all same type of two-plant combination extracts, water extracts had higher cytotoxicity on adults (lower LD_{50}) than ethanolic extracts. However, there was no significant difference between water and ethanolic extractions of plan combination extracts of sugar apple leaf with mintweed seed and neem leaf with mintweed leaf.

In comparison of cytotoxicities between single and plant combination extracts on adults (Table 3.19), LD_{50} of all plant extracts were in a range of 2,500-5,200 ppm. However, there were only two types of plant combination extracts gave LD_{50} lower than 3,000 ppm i.e. sugar apple leaf-ethanol extract with mintweed seed-ethanol extract (2,568.3 ± 121.0 ppm), and sugar apple leaf-water extract (2,710.9 ± 67.1 ppm). Furthermore, there was only single plant extracts gave lower LD_{50} than two-plant combination extracts i.e. sugar apple leaf-water extract compared to sugar apple leaf-water extract with mintweed leaf-water extract, and compared to sugar apple leaf-water extract with mintweed seed-water extract.

Table 3.17 Percentage of mortality of adults at various concentrations and LD_{50} of single plant extracts on biosis of oriental fruit fly adults.

Plant Extracts	**%Mortality of Adults**				
	Concentration (ppm)				
	2,500	**5,000**	**7,500**	**10,000**	**LD_{50}**
Neem leaf/H_2O	43 ± 9	64 ± 4	70 ± 3	77 ± 8	4,239.1 ± 190.8 a
Neem leaf/EtOH	46 ± 2	52 ± 4	62 ± 4	78 ± 3	4,810.9 ± 259.8 a
Sugar apple leaf /H_2O	64 ± 4	74 ± 2	80 ± 7	84 ± 2	2,710.9 ± 67.1 b
Sugar apple leaf/EtOH	51 ± 7	61 ± 5	68 ± 4	78 ± 7	5,217.2 ± 110.4 b
Mintweed leaf/H_2O	50 ± 4	61 ± 4	67 ± 4	82 ± 5	3,347.8 ± 152.4
Mintweed leaf/EtOH	52 ± 4	66 ± 9	77 ± 7	84 ± 4	3,295.8 ± 143.8
Mintweed seed/H_2O	56 ± 7	63 ± 4	67 ± 8	82 ± 3	2,521.3 ± 83.8
Mintweed seed/EtOH	38 ± 5	48 ± 3	56 ± 3	67 ± 5	off range

a = significant difference at $P<0.01$
d = significant difference at $P<0.01$

Table 3.18 Percentage of mortality of adults and LD_{50} of two-plant combination extracts on biosis of oriental fruit fly adults.

Plant Extract Combinations	%Mortality of Adults Concentration (ppm)				
	2,500	**5,000**	**7,500**	**10,000**	**LD_{50}**
Neem leaf/H_2O + Sugar apple leaf/H_2O	47 ± 4	67 ± 6	68 ± 8	71 ± 7	3,142.1 ± 114.7
Neem leaf/EtOH + Sugar apple leaf/EtOH	59 ± 2	79 ± 4	91 ± 5	96 ± 4	off range
Neem leaf/H_2O + Mintweed leaf/H_2O	61 ± 2	69 ± 4	76 ± 4	88 ± 3	off range
Neem leaf/EtOH + Mintweed leaf/EtOH	58 ± 4	78 ± 2	81 ± 3	84 ± 2	off range
Neem leaf/H_2O + Mintweed seed/H_2O	48 ± 2	58 ± 2	64 ± 4	76 ± 4	3,193.9 ± 106.6
Neem leaf/EtOH + Mintweed seed/EtOH	53 ± 5	71 ± 4	81 ± 3	87 ± 3	off range
Sugar apple leaf/H_2O + Mintweed leaf /H_2O	52 ± 3	60 ± 3	67 ± 7	74 ± 4	2,783.3 ± 83.9
Sugar apple leaf/EtOH + Mintweed leaf/EtOH	52 ± 6	73 ± 4	86 ± 3	90 ± 3	off range
Sugar apple leaf/H_2O + Mintweed seed/H_2O	51 ± 2	59 ± 2	67 ± 7	79± 5	2,784.4 ± 111.6
Sugar apple leaf/EtOH + Mintweed seed/EtOH	56 ± 6	66 ± 3	81 ± 4	89 ± 3	2,568.3 ± 121.0

Table 3.19 Comparison LD_{50} of single and two-plant combination extracts on biosis of oriental fruit fly adults

Plant Extracts	LD_{50} (ppm)
Neem leaf/H_2O	4,239.1 ± 190.8
Neem leaf/H_2O + Sugar apple leaf/H_2O	3,142.1 ± 114.7
Neem leaf/H_2O + Mintweed leaf/H_2O	off range
Neem leaf/H_2O + Mintweed seed/H_2O	3,193.9 ± 106.6
Neem leaf/EtOH	4,810.9 ± 259.8
Neem leaf/EtOH + Sugar apple leaf/EtOH	off range
Neem leaf/EtOH + Mintweed leaf/EtOH	off range
Neem leaf/EtOH + Mintweed seed/EtOH	off range
Sugar apple leaf/H_2O	2,710.9 ± 67.1
Sugar apple leaf/H_2O + Mintweed leaf/H_2O	2,783.3 ± 83.9
Sugar apple leaf/H_2O + Mintweed seed/H_2O	2,784.4 ± 111.6
Sugar apple leaf/EtOH	5,217.2 ± 110.4
Sugar apple leaf/EtOH + Mintweed leaf/EtOH	off range
Sugar apple leaf/EtOH + Mintweed seed/EtOH	2,568.3 ± 121.0

Insecticidal activity (LD_{50}) of all extracts on oriental fruit fly was summarized in Table 3.20. Most effective cytotoxicity of the extracts against each stages of fruit fly was mintweed leaf-water extract and sugar apple leaf-water extract, which its LD_{50} was lower than 3,000 ppm for every stage of flies. It was noticed that the higher cytotoxicity of the extracts against all stages of oriental fruit fly comprised

of mintweed. This result gave well agreement with the cytotoxicity by BSLA as previously discussed in section 3.4.2. In addition, when compared between the eating and dipping treatments in larvae, eating treatment of most extracts to the fruit flies larvae showed higher cytotoxicity activity than those of dipping treatment. However, the reverse results were found in six extracts i.e., mintweed leaf-ethanol extract, mintweed seed-ethanol extract, neem leaf with mintweed seed-ethanol extract, sugar apple leaf-ethanol extract with mintweed leaf-ethanol extract, neem leaf-ethanol with sugar apple leaf-ethanol extract, and neem leaf-ethanol extract with mintweed leaf ethanol extract. In overall, in order to further investigaticns on the LD50 of range regarding cytotoxicity, thes with less 2,500 ppm of concentration should be more conducted with further researches.

Table 3.20 Summary the insecticidal activity (LD_{50}) of all plant extracts on oriental fruit flies.

Plants Extracts	LD_{50} (ppm)			
	Eggs	**Larvae (Feeding)**	**Larvae (Dipping)**	**Adults**
Neem leaf/H_2O	3,353.4 ± 157.0	off range	3,257.0 ± 190.8	4,239.1 ± 190.8
Neem leaf/EtOH	3,625.1 ± 162.4	3,621.7 ± 101.4	4,301.0± 259.8	4,810.9 ± 259.8
Sugar apple leaf/H_2O	off range	2,568.3 ± 47.5	2,977.2 ± 67.1	2,710.9 ± 67.1
Sugar apple leaf/EtOH	4,815.3 ± 172.2	5,088.8 ± 125.3	5,429.5 ± 110.4	5,217.2 ± 110.4
Mintweed leaf/H_2O	2,920.3 ± 55.9	2,707.8 ± 120.3	3,222.9 ± 152.4	3,347.8 ± 152.4
Mintweed leaf/EtOH	off range	2,658.4 ± 132.4	2,651.0 ± 143.8	3,295.8 ± 143.8
Mintweed seed leaf/H_2O	off range	off range	off range	2,521.3 ± 83.8
Mintweed seed/EtOH	off range	off range	off range	off range
Neem leaf/H_2O + Sugar apple leaf/H_2O	off range	2,595.1 ± 95.6	2,673.8 ± 114.7	3,142.1 ± 114.7
Neem leaf/EtOH + Sugar apple leaf/EtOH	off range	off range	off range	off range
Neem leaf/H_2O + Mintweed leaf/H_2O	off range	off range	off range	off range
Neem leaf/EtOH + Mintweed leaf/EtOH	off range	off range	off range	off range
Neem leaf/H_2O + Mintweed seed/H_2O	3,277.7 ± 173.0	3,062.3 ± 81.8	3,135.0± 106.6	3,193.9 ± 106.6
Neem leaf/EtOH + Mintweed seed/EtOH	off range	off range	off range	off range
Sugar apple leaf/H_2O + Mintweed leaf /H_2O	2,902.4 ± 50.8	off range	3,029.4 ± 83.9	2,783.3 ± 83.9
Sugar apple leaf/EtOH + Mintweed leaf/EtOH	off range	off range	off range	off range
Sugar apple leaf/H_2O + Mintweed seed/H_2O	2,934.7 ± 54.9	2,700.4 ± 65.1	2,928.4 ± 111.6	2,784.4 ± 111.6
Sugar apple leaf/EtOH + Mintweed seed/EtOH	off range	off range	2,680.2 ± 121.0	2,568.3 ± 121.0

It was reported that insects from different orders differ markedly in their behavior reponses to azadirachtin in neem as show in Table 3.21 (Mordue (Luntz) and Nisbet, 2000)

Table 3.21 Behavioural sensitivity of insects to azadirachtin: the effective dose (ED_{50}) which causes 50% inhibition of feeding (Mordue (Luntz) and Nisbet, 2000).

Insect order	**ED_{50} (ppm)**
Lepidoptera	< 0.001-50
Coleoptera	100-500
Hemiptera	100-500
Hymenoptera	100-500
Orthoptera	0.001- > 1000

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It was reported that the extract of *Annona squamosa* were highly effective as antifeedant and as growth regulators, no mention is made of theboviposition deterrent effects (Islam, 1987). *A. squamosa* extracted in methanol also siginificantly reduced the level of adult emergence from pulse beetle (*Callosobruchus chinensis*) egg laid. The mortality of beetles released on grain treated with *A. squamosa* was 100% within 2 and 6 days in ethanol- and methanol- based extracts, respetively (Al-Lawati et al., 2002). Other interesting study of Annona extract found that the testing of Annona suspension at 100 ppm for controlling broad mite showed the efficiency of 100 ppm. Annona could kill 100% of eggs and larvae of broad mite, 80% of adult in the laboratory room. The population of broad mite in the chilli tree where decreased by Annona suspension. Moreover, suspension could kill of 93.9% of Eryophyid mites, 50% of *Scirtothrips dorsallis*, 80% of *Aphis gossypii* and also inhibited destruction of

mealy bug, but it could not harm *Amblyseius longicaudatus* (predaceous mite) at eggs and adult stages (กนก อุไรสกุล, 2540).

Furthermore, the essential oil of *Hyptis suaveolens* leaves showed antibacterial activity at 5 mg/ml concentration against two gram-positive and four gram-negative bacteria (Asekun et al., 1999). A novel trypsin inhibitor purified from chan seed (*H. suaveolens*:Lamiaceae) was reported (Aguirre et al., 2004). Its inhibitory activity was potent toward to all typsin-like proteases extracted from the gut of the insect *Prostephanus truncatus* (Coleoptera: Bostricidae), a very important pest of maize. This activity was highly specific, because among proteases from seven different insects, only those from *P. truncates* and *Manduca sexta* (Lepidoptera: Sphingidae) were inhibited. This inhibitor has potential to enhance the defense mechanism of maize against the attack of *P. truncates*.

3.5 Conclusion

Repellence was evaluated by observing the behavior of both male and female adults flies exposed to treated artificial food with the extracts in a linear olfactometer. Most three effective repellency of the single plant extracts on fruit flies which gave percentage of repellence more than 70% were mintweed seed-ethanol extract, mintweed leaf-water extract, and sugar apple leaf-water extract (70.00 ± 3.48%). In the case of two-plant combinations extracts, most three effective repellency on fruit flies were neem leaf-water extract with sugar apple leaf-water extract, neem leaf-water extract with mintweed seed-water extract, and neem leaf-water extract with mintweed leaf-water extract. This two-plant combination extracts exhibited percentage of repellence more than 70%.

LC_{50} of single plant and combined plant extracts determined by BSLA was shown that most four effective cytotoxicity of the extracts contained mintweed both of its leaf and seed. This implied that for the given plants, mintweed may produce the effective botanical products for controlling oriental fruit flies. In addition, it was found that the greatest cytotoxicity of the combination extracts against all stages of oriental fruit flies comprised of mintweed. This result was well in agreement with the cytotoxicity. When compared between the feeding and dipping treatments in larvae, it was observed that eating treatment of most extracts to the fruit fly larvae had higher cytotoxicity activity than those of dipping treatment. However, the reverse results were found in six extracts i.e., mintweed leaf-ethanol extract, mintweed seed-ethanol extract, neem leaf with mintweed seed-ethanol extract, sugar apple leaf-ethanol extract with mintweed leaf-ethanol extract, neem leaf-ethanol with sugar apple leaf-ethanol extract, and neem leaf-ethanol extract with mintweed leaf ethanol extract.

3.5 References

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CHAPTER IV

CONCLUSIONS

The demand for pesticidal compound to control plant pests and weeds has created an agro-chemical business, worldwide. The synthetic pesticides have facilitated gaining in agricultural productions, but these compounds frequently have posed serious problems to health and environmental safety. Newer, more selective, and biodegradable compounds must replace these toxic and persistent chemicals of the present and immediate past. One of the world's most important and widespread pests is the oriental fruit flies (*Bactrocera dorsalis*). Economic loss caused by this species is extensive in Thailand and other Asian countries. Therefore, neem (*Azadirachta indica*), sugar apple (*Annona squamosa*), and mintwed (*Hyptis suaveolens*) are proposed for biological control oriental fruit flies. Leaves of neem, sugar apple, mintweed, and seeds of mintweed were extracted by water and ethanol using universal extraction. Phytochemical properties, total phenolic compounds and antioxidant activity (IC_{50}) of neem, sugar apple, and mintweed were investigated in order to determine their natural potential. Preliminary study on thin layer chromatography (TLC) technique was utilized for separating compounds of plant extracts. Repellent activity of single plant extract and two-plant combination extracts were elucidated using an olfactometer. Cytotoxicity (LC_{50}) of single plant extract and two-plant combination extracts were elucidated by brine shrimp lethality assay

(BSLA). Insecticidal activity (LD_{50}) of single plant extract and two-plant combination extracts were investigated on biosis of oriental fruit flies i.e. eggs, lavae and adults.

Neem leaf-ethanol extract had the highest total phenolic content (337.8 ± 18.2 mgGAE/L) while mintweed seed-ethanol extract had the lowest total phenolic content (179.4 ± 6.2 mgGAE/L). Mintweed seed-ethanol extract showed the highest activity (155.5 ± 3.2 ppm), whereas the lowest activity of radical scavenging was observed in mintweed leaf- water extract (288.9 ± 6.2 ppm). However, all of the plant extracts in this research exhibited different extent of antioxidant activity. Because, the solvent differences in extraction method affected the amount of total phenolic compounds and antioxidant activities. Antioxidant activities of all extracts but not of sugar apple extracts and mintweed seed-ethanol extract were directly correlated (positive correlation) to the amount of total phenolic compounds. The evaluation on the efficiency of solvent systems as a mobile phase in TLC experiment showed that n-buthanol : glacial acetic acid : water (40 : 10 : 50) system seem to be most suitable mixture solvent for separating compounds of plant extracts in this study. These results may be suggested that each plant produces different phytochemical compositions with difference in polarity as well as antioxidant activity.

The highest repellent activity of single plant extracts was mintweed seed-ethanol extract (74.0 ± 5.0%). Neem leaf-water extract with sugar apple leaf-water extract (78.0 ± 2.6%) gave the highest repellent activity than the other type of two-plant combination extracts. In the case of single plant extracts, repellent activity of all ethanolic extracts was higher than those of all water extracts ($P < 0.01$), ($P<0.05$). In contrast, water extracts showed higher repellent activity than ethanolic extracts compared with all same types of two-plant combination extracts. Cytotoxicities of all

two-plant combination extracts were greater than those of single plant extracts. Especially, neem leaf-ethanol extract with mintweed leaf-ethanol extract gave the highest cytotoxicity (LC_{50} was 0.1 ± 1.2E-02 ppm). Results also implied that two-plant combination extracts exhibited synergistic effect on LC_{50}. In addition, ethanol extraction of both single extract and plant combination extracts obviously showed lower LC_{50} than water extractions.

In the case of eggs, most four effective cytotoxicity of plant combination extracts on eggs which gave LD_{50} lower than 3,000 ppm were mintweed leaf-water extract (2,920.3 ± 55.9ppm), sugar apple leaf-water extract wit mintweed leaf-water extract (2,902.4 ± 50.8 ppm) and sugar apple leaf-water extract wit mintweed seed-water extract (2,934.7 ± 54.9 ppm). In addition, cytotoxicities of single extracts on eggs were lower than those of two-plant combination extracts. Cytotoxicities on larvae using eating treatment of single extracts were lower than those of two-plant combination extracts. Most three effective types of plant combination extracts which exhibited LD_{50} lower than 3,000 ppm were sugar apple leaf-ethanol extract (2,568.3 ± 47.5 ppm), mintweed leaf-water extract (2,658.4 ± 132.4 ppm), and mintweed leaf-water extract (2,707.8 ± 120.3 ppm), sugar apple leaf-water extract wit neem leaf-water extract (2,595.1 ± 95.6 ppm), sugar apple leaf-water extract wit mintweed seed-water extract (2,700.4 ± 65.1 ppm). In comparison of cytotoxicities between single and plant combination extracts on larvae using dipping treatment, most two effective types of plant combination extracts which gave LD_{50} lower than 3,000 ppm were mintweed leaf-water extract (2,651.0 ± 143.8 ppm),), sugar apple leaf-ethanol extract (2,977.2 ± 67.1 ppm), sugar apple leaf-water extract with mintweed leaf-water extract (2,673.8 ± 114.7 ppm), sugar apple leaf-ethanol extract with mintweed seed-ethanol

extract (2,680.2 ± 121.0 ppm) and sugar apple leaf-water extract with mintweed seed-water extract (2,928.4 ± 45.62 ppm). In comparison of cytotoxicities between single and plant combination extracts on adults, most two effective types of plant combination extracts which gave LD_{50} lower than 3,000 ppm were mintweed seed-water extract (2,521.3 ± 83.8 ppm), sugar apple leaf-water extract (2,710.9 ± 67.1 ppm), sugar apple leaf-ethanol extract with mintweed seed-ethanol extract (2,568.3 ± 121.0 ppm), sugar apple leaf-water extract with mintweed leaf-water extract (2,783.3 ± 83.9 ppm), and sugar apple leaf-water extract with mintweed seed-water extract (2,784.4 ± 111.6 ppm)

In summary, the most effective cytotoxicity of the extracts against each stage of fruit flies was sugar apple leaf-water extract, which its LD_{50} was lower than 3,000 ppm for every stage of flies. Moreover, the high cytotoxicity of the two-plant combinations extracts against all stages of oriental fruit fly comprised of sugar apple. This result gave well agreement with the cytotoxicity. Additionally, sugar apple can be used in combination with mintweed or neem in order to enhance the cytotoxicity of the plant combinations with synergistic effectiveness. However, the biological control of oriental fruit flies by some combinations of plant extracts was off range of the designated concentrations in this experiment. This can imply that these combinations are very potent in controlling oriental fruit flies. They are necessary to be further investigated with very low ranges of test concentrations. The expected results could be addition or synergistic effects of toxicity (LD_{50}) on the oriental fruit flies. They seem to be good plant based insecticides.

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