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NAME: Miss Nutchaya Kumrungsee

THIS THESIS HAS BEEN ACCEPTED BY

(Assistant Professor Vasakorn Bullangpoti, Ph.D.) **THESIS ADVISOR**

(Assistant Professor Wanchai Pluemphanuphat, Ph.D.) **THESIS CO-ADVISOR**

(Mr. Opender Koul, Ph.D.) **THESIS CO-ADVISOR**

(Associate Professor Monchan Maketon, Ph.D.) **DEPARTMENT HEAD**

APPROVED BY THE GRADUATE SCHOOL ON _____

(Associate Professor Gunjana Theeragool, D.Agr.) **DEAN**

THESIS

BIO-EFFICACY AND MODE-OF-ACTION OF SOME ESSENTIAL OIL COMPOUNDS AND THEIR BINARY MIXTURES AGAINST LEPIDOPTERAN SPECIES

NUTCHAYA KUMRUNGSEE

A Thesis Submitted in Partial Fulfillment
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Nutchaya Kumrungsee 2014: Bio-efficacy and Mode-of-action of Some Essential Oil Compounds and Their Binary Mixtures Against Lepidopteran Species. Doctor of Philosophy (Zoology), Major Field: Zoology, Department of Zoology. Thesis Advisor: Assistant Professor Vasakorn Bullangpoti, Ph.D. 83 pages.

Present work determines the bio-efficacy of thymol, 1,8 cineole, pulegone and linalool and their impact on detoxification enzymes of *Plutella xylostella* (Lepidopteran : Plutellidae) The activity was also determined against *Spodoptera litturalis* larvae. The compounds were applied topically to *P. xylostella* 3rd instars. Thymol was most active compound with 24 hours LD_{50} of 220 ± 22.87 ppm. The effect on feeding behavior was observed using leaf disc no-choice assay. 1,8 cineole exhibited the best antifeedant action with an FI_{50} of 339.5 ppm compared to least active linalool with and FI_{50} of 1,598.02 ppm 6 hours post-treatment. Antifeedant activity of pulegone (FI_{50} of 556.5 ppm) was significantly closer to 1,8 cineole. In case of *S. littoralis* oral administration effects were prominent only in case of thymol treated insects. After treating larvae with binary mixtures significant synergism was observed in pulegone and thymol combinations. Detoxification enzymes like α-esterase, β-esterase, glutathione-s-transferase and acetylcholinesterase were induced in both *in vitro* and *in vivo* assay. The results obtained in present study suggest that all the 4 evaluated compounds have potential to control lepidopteran larvae if a strategized approach of application is used

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Nutchaya Kumrungsee

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LIST OF ABBREVIATIONS

DBM	=	Diamondback moth
mm	=	Millimetre
°C	=	Degree celcius
µL	=	Microliter
mg	=	Milligram
ml	=	Milliliter
µg	=	Microgram
LC_{50}	=	Median lethal concentration
kg	=	Kilogram
P-450	=	Mixed function oxidase
JH	=	Juvenile hormone
JHE	=	Juvenile hormone esterase
GST	=	Glutathione-s-transferase
DNA	=	Deoxyribonucleic acid
ACh	=	Acetylcholine
His	=	Histidine
Ser	=	Serine
Glu	=	Glutamine
OPs	=	Organophosphates
MCs	=	Methylcarbamates
alpha-NA	=	1- naphthyl acetate
beta-NA	=	2- Naphthyl acetate
p-NPA	=	Paranitrophenylacetate
SDS	=	Sodium dodecyl sulfonate
cDNB	=	1- Chloro -2, 4- dinitrobenzene
DCNB	=	1, 2- dichloro- 4,nitrobenzene
CBD	=	Complete block design
ppm	=	Part per million
mM	=	Milli molar
PBK	=	Phosphate buffer

LIST OF ABBREVIATIONS (Continued)

nm	=	Nanometre
TpS	=	Tampon substrate
DTNB	=	2-nitrobenzoic acid
EDTA	=	Ethylenediaminetetraacetic acid
AsCh	=	Acetylthiocholineiodide
TpI	=	Tampon inhibitors
M	=	Molar

BIO–FFICACY AND MODE-OF-ACTION OF SOME ESSENTIAL OIL COMPOUNDS AND THEIR BINARY MIXTURES AGAINST LEPIDOPTERAN SPECIES

INTRODUCTION

Thailand, being an agriculture oriented country, has in recent past suffered because of resistance and various environmental impacts caused by discriminate use of conventional chemical insecticides. This has made necessary to look for safer measures and alternative pest control methods that could be used in Integrated Pest Management (IPM) systems. Today, IPM has to face up to the economic and ecological consequences of the use of pest control measures. Fifty years of sustained struggle against harmful insects using synthetic and oil-derivative molecules has produced perverse secondary effects (mammalian toxicity, insect resistance and ecological hazards). The diversification of the approaches inherent in IPM is necessary for better environmental protection. Among the alternative strategies, the use of plants, insecticidal allelochemicals appears to be promising. Aromatic plants, and their essential oils, are among the most efficient botanicals. Their activities are manifold. They induce fumigant and topical toxicity as well as antifeedant or repellent effects. They are toxic to adults but also inhibit reproduction. Although mechanisms depend on phytochemical patterns and are not yet well known, this widespread range of activities is more and more being considered for both industrial and household uses: essential oils are presently regarded as a new class of ecological products for controlling insect pests (Catherine, 1997).

Most pesticidal essential oil compounds are generally composed of complex mixtures of monoterpenes, biogenetically related phenols, and sesquiterpenes. It is important to note that the composition of these oils can vary dramatically, even within species. Therefore, it is not only the plant biodiversity that needs to be looked into as bio-resource for their potential but the diversity of modes-of-action of such products is also equally important (Koul *et al*., 2008). The rapid action against some pests is indicative of a neurotoxic mode of action. Some of the compounds have also made a commercial impact during the past decade like eugenol and 2-phenethylpropionate

(from EcoSMART); rosemary oil and 1,8-cineole (from EcoTrol). Interestingly many monoterpenoids, phenylpropanoids, and alcohols from essential oils are responsible for ovicidal activity or altering the oviposition potential of insects (Singh *et al*., 2010).

The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae), is a worldwide insect pest that attacks crucifers, particularly cabbage, broccoli and cauliflower. DBM larvae feeding may cause serious damage to plant foliage and resultin significant losses to the production of these economically important vegetables. In some places, DBM is responsible for more than 90% of crop losses. The global importance of DBM is reflected in the estimated pest management cost of US $1.0 billion annually (Weiyi *et al*., 2012). Under the high selection, the pests insect often develop high levels of insecticide resistance and *P. xylostella* is one of the leaders among insect pests that are very difficult to control. Among the best known essential oils with bioactivity against insects and other pests are clove oil (eugenol), thyme oil (thymol, carvacrol), mint oil (menthol, pulegone), lemongrass oil (citronellal, citral), cinnamon oil (cinnamaldehyde), rosemary oil (1,8-cineole) and oil of oregano (carvacrol)., biological effect, pesticide (Isman and Machial., 2006). For example, Chang-Geun *et al* (2007) studied on fumigant toxicity of 66 plant essential oils control *P. xylostella* larvae.

In addition, *Spodopter littoralis* (Boisduval); the Egyptian cotton leafworm (Lepidoptera : Noctuidae) is an important and widespread pest of cultivated crops primarily in tropical and subtropical regions. Because of its polyphagy, this species causes economical yield losses on several crops (El-Sheikh and Aamir, 2011) . The damaged of *S. littoralis* was record on cotton, spinach, alfalfa, pepper, eggplant, tomato, lettuce, bean, strawberry and some ornamental crops, the main distribution has been recorded in Africa, the Middle East, in the Mediterranean, Asia, andEurope. The direct damage caused by reducing photosynthetic area, the occurrence of larvae, feeding damage and excrement reduce marketability of vegetables and ornamentals . Although, *S. littoralis* can developed to insecticide and difficult to control which residue of cultivated crops and resistance problems. Commonly, some studies using essential oil and extracts obtained from the seeds of *Angelica archangelica* were used

to determine the efficacy in terms of chronic and acute toxicity, antifeedancy and growth inhibition of *S. littoralis* larvae (Pavela and Vrchotova, 2013). Otherewise, BenFarhat-Touzri *et al* (2013) controlled which were used *Cry toxins of Bacillus thuringiensisare* effective insecticidal proteins, several agronomically important insects, Ahmed *et al* (2003), they studied the effective of limonoids from *Khaya senegalensis* for larval growth inhibition on *S. littoralis* by antifeeding.

However, there are no research on efficacy of 4 essential oil compounds (thymol, linalool, pulegone and 1,8 cineole) on *P. xylostella* and *S. littoralis* before. In view of this present program focused on the bio efficacy of essential oil compounds, to *P. xylostella* and *S. littoralis,* studying the mode-of-action of detoxification enzyme, carboxyesterases and glutathione-s-transferase including the neuron enzyme (acetylcholinesterase).

OBJECTIVES

1. To study the bioefficacy of some essential oil compounds against an economically important crop pest *P. xylostell* and *S. littoralis*.

2. To study the efficacy of binary mixtures of these compounds for stability and enhancement of activity on *P. xylostella*.

3. To study the possible mode-of-action at biochemical level against *P. xylostella*.

LITERATURE REVIEW

1. Insect used in experiment

1.1 The diamondback moth (DBM), *Plutella xylostella* (Lepidoptera : Plutellidae)

The diamondback moth (DBM), *Plutella xylostella* (Lepidoptera : Plutellidae) (Figure 1), is one of the most destructive insect pests of Brassica family worldwide and a serious pest of cauliflower, cabbage, broccoli, mustard, radish and turnip (Lingathurai *et al*., 2010). This pest may have originated from Europe or possibly South Africa. Whatever the origin of this pest, it has now recorded presence in 128 countries of the world (Saeed *et al*., 2010), including Thailand.

Figure 1 Second instar larvae of *P. xylostella.*

In Thailand, *P. xylostella* is prevalent from February to April when optimum climatic conditions and food plants are more readily available and the insect has been the most serious threat to cruciferous crops for many years (Prasongsap, 2001). Moreover, control of this pest is usually achieved through the application of synthetic insecticides, but their high cost, environmental contamination, development of resistance to chemicals, and pest resurgence have encouraged the search for alternatives more compatible with the environment.

The diamondback moth (DBM), *Plutella xylostella* (Lepidoptera:Plutellidae) is classified as;

Phylum Arthopoda
Class Insecta
Order Lepidoptera
Family Plutellidae
Genus *Plutella*
Species *Plutella xylostella*

Figure 2 Radish field that damaged by *P. xylostella.*

In recent years, scientists have evaluated botanical insecticide for the control of *P. xylostella* using *Melia azedarach* (Charleston *et al*., 2005), leaf extracts of *Momordica charantia* (Bing *et al*., 2008) and extracts from *Pseudolarix kaempferi* Gord. (Vanichpakorn *et al*., 2010). Yi *et al*. (2007) studied essential oil efficacy against *P. xylostella* by fumigant method as shown in Table 1. Another biological control approach has been the use of Ascoviruses (AVs), which are pathogenic to lepidopteran larvae, and most commonly attack species in the Noctuidae like

Heliothis virescens AV (HvAV-3e). Use of natural enemies for the control of *P. xylostella* is also well known and *Cotesia plutellae* is an endoparasitoid wasp that parasitizes the larvae of this insect (Bay and Kim, 2009). In addition, Species of natural enemies collected parasitizing or preying upon *Plutella xylostella* larvae in the field. *Conura pseudofulvovariegata* emerged from *P. xylostella* pupa; *Tetrastichus howardi* emerged from *P. xylostella* pupa; *Cheiracanthium inclusum* and *P. xylostella* pupa attacked by *C. inclusum*; *Podisus nigrispinus* nymph preying on larvae, and *Pheidole* sp. attacking larvae (Christian *et al*., 2010).

Table 1 Toxicity effect of linalool, thymol, 1,8-cineole and pulegone on insect pests.

Essential oil	Insect	Toxicity
Linalool	*Plutella xylostella*	10-20 µL/seedling (oviposition activity) (Zhang *et al*., 2004).
	Chilo partellus	0.16 mg/ml (Dietary bioassays) (Singh *et al*., 2010).
Thymol	*Plutella xylostella*	0.17 ug/mg body weight (Dietary bioassays) (Rejesus *et al*., 1999).
	Spodoptera litura	(25.4 µg/larva) (Dietary bioassays) (Laurin *et al*., 2001)
	Chilo partellus	0.11 mg/ml (Dietary bioassays) (Singh *et al*., 2010).
1,8-cineole	*Chilo partellus*	0.13 mg/ml (Dietary bioassays) (Singh *et al*., 2010).
Pulegone	*Musca domestica*	(LC_{50}=1.7 mg/dm^3(Fumigant bioassays)

Biology and Life cycle of *P. xylostella*

Egg: *P. xylostella* eggs are oval and flattened, and measure 0.44 mm long and 0.26 mm wide. Eggs are yellow or pale green in color, and are deposited singly or in small groups of two to eight eggs in depressions on the surface of foliage, or occasionally on other plant parts (Figure 3). Females may deposit 250 to 300 eggs

but average total egg production is probably 150 eggs. Development time averages 5-6 days.

Figure 3 *P. xylostella*'s egg colony.

Larvae: *P. xylostella* has four instars. Average and range of development time is about 3-7, 2-7, 2-8, and 2-10 days, respectively (Harcourt, 1957). Throughout their development, larvae remain quite small and active. If disturbed, they often wriggle violently, move backward, and spin down from the plant on a strand of silk. Overall length of each instar rarely exceeds 1.7, 3.5, 7.0, and 11.2 mm, respectively. Mean head capsule widths for these instars are about 0.16, 0.25, 0.37, and 0.61 mm. The larval body form tapers at both ends, and a pair of prolegs protrude from the posterior end, forming a distinctive "V". The larvae are colorless in the first instars, but there after are green. The body bears relatively few hair, which are short in length, and most are marked by the presence of small white patches. There are five pairs of pro-legs. Initially, the feeding habit of first instar larvae is leaf mining, although they are so small that the mines are difficult to notice. The larvae emerge from their mines at the conclusion of the first instars, molt beneath the leaf, and there after feed on the lower surface of the leaf. Their chewing results in irregular patches of damage, and the upper leaf epidermis is often left intact.

Figure 4 Second instar larvae of *P. xylostella.*

Pupae: Pupation occurs in a loose silk cocoon (Figure 5), usually formed on the lower or outer leaves. In cauliflower and broccoli, pupation may occur in the florets. The yellowish pupa is 7 to 9 mm in length. The duration of the cocoon averages about 8.5 days (range 5 to 15 days).

Figure 5 Pupae stage of *P. xylostella*.

Adult: The adult is a small, slender, grayish-brown moth with pronounced antennae (Figure 6). It is about 6 mm long, and marked with a broad cream or light brown band along the back. The band is sometimes constricted to form one or more light-colored diamonds on the back, which is the basis for the common name of this insect. When viewed from the side, the tips of the wings can be seen to turn upward slightly. Adult males and females live about 12 and 16 days respectively, and females deposit eggs for about 10 days. The moths are weak fliers, usually flying within 2 meters of the ground, and for short distances. However, they are readily carried by the wind. The adult is the overwintering stage in temperate areas, but moths do not survive cold winters such as those found in most of Canada. They routinely re-invade these areas each spring, evidently aided by southernly winds (Harcourt, 1957).

Figure 6 Adult of *P. xylostella*

Life Cycle: Total development time from the egg to pupal stage averages 25 to 30 days, depending on the weather, with a range of about 17 to 51 days. The number of generations varies from 4 in cold climates, such as southern Canada, to perhaps 8 to 12 in the south. Overwintering survival is positively correlated with the abundance of snowfall in northern climates (Harcourt, 1957).

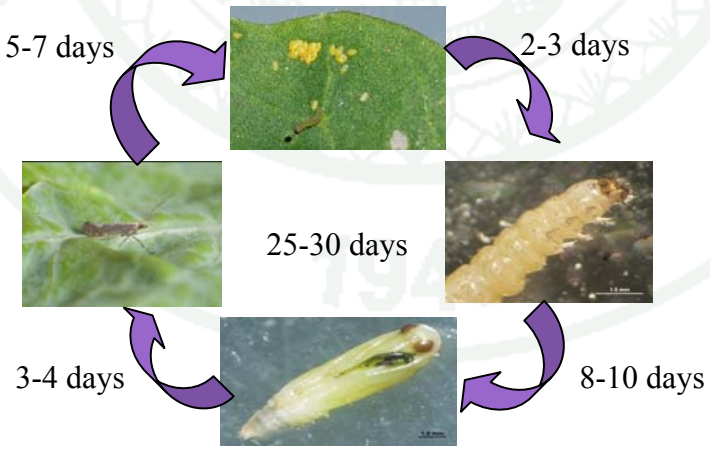

Figure 7 Life cycle of *P. xylostella*.

1.2 *Spodoptera littoralis*

The noctuid *Spodoptera littoralis* (Boisd.) is most important polyphagous pest, widely distributed all over the world. Larvae of this pest can feed on 90 economically important plant species belonging to 40 families and the rate of development has a strong nutritional component (Roman, 2010).

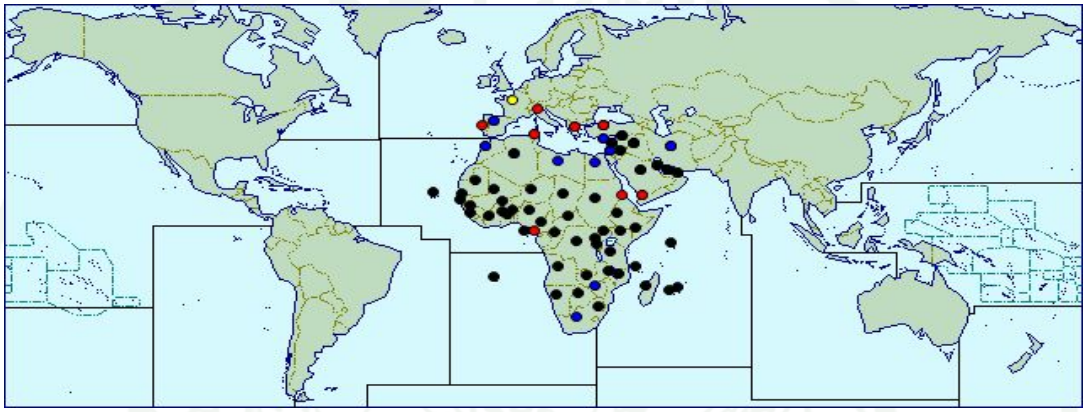

Figure 8 Distribution of *S. littoralis*.

Source: CIE (1967)

For control *S. littoralis* used of insecticide, natural product and natural enermies as chlorosan, feroban, cygron, engeo, and kingbo with 2nd and 4th instar larvae (Abd El-Mageed and Shehata, 2011) Roman and Nadezda (2013) were used the essential oil (EO) and extract obtained from the seeds of *Angelica archangelica* in terms of chronic and acute toxicity, antifeedancy and growth inhibition for control *Spodoptera littoralis* larvae. In addition, Tiago and Patricia (2005) control *S. littoralis* larvae by fungal pathogen, *Furia virescens* (Thaxter) Humber (Zygomycetes: Entomophthoraceae) and parasitoid, *Meteorus communis* (Cresson) (Hymenoptera: Braconidae).

Spodoptera littoralis (Boisd.) is classified as;

Phylum Arthopoda

Class Insecta

Order Lepidoptera

Family Noctuidae

Genus *Spodoptera*

Species *Spodoptera littoralis*

Biology and lifecycle of *Spodoptera littoralis*

Between 2 and 5 days after emergence, females lay 1000-2000 eggs in egg masses of 100-300 on the lower leaf surface of the host plant. The masses are covered by hair-like scales from the end of the insect's abdomen. Fecundity is adversely affected by high temperature and low humidity (about 960 eggs laid at 30°C and 90% RH and 145 eggs at 35°C and 30% RH). Newly laid eggs of one strain of *S. littoralis* were reported to survive exposure to 1°C for 8 days. Partially developed eggs survived longer than newly laid ones under equivalent conditions.

Figure 9 Eggs of *S. littoralis*.

Egg: The eggs hatch in about 4 days in warm conditions, or up to 11-12 days in winter. The larvae pass through six instars in 15-23 days at 25-26°C. At lower temperatures, for example *S. littoralis* on glasshouse chrysanthemums in Europe, larvae often go through an extra instar, and maturation may take up to 3 months.

Figure 10 *S. littoralis* larvae.

Larvae: The young larvae (first to third instar) feed in groups, leaving the opposite epidermis of the leaf intact. Later, the (4^{th} to 6^{th} instar) larvae disperse and spend the day in the ground under the host plant, feeding at night and early in the morning.

Figure 11 Pupae of *S. littoralis*.

Pupae: The pupal period is spent in earthen cells in the soil and lasts about 11-13 days at 25°C. Longevity of adults is about 4-10 days, being reduced by high temperature and low humidity. Thus, the life cycle can be completed in about 5 weeks. In Japan, four generations develop between May and October, while in the humid tropics there may be eight annual generations. In the seasonal tropics, several generations develop during the rainy season, while the dry season is survived in the pupal stage.

Figure 12 Adult of *S. littoralis*.

Adult: Moth, with grey-brown body, 15-20 mm long; wingspan 30-38 mm. The forewings are grey to reddish-brown with a strongly variegated pattern and paler lines along the veins (in males, bluish areas occur on the wing base and tip);

Figure 13 Life cycle of *S. littoralis*

Life cycle; Emergence of adult moths occurs at night and they have a life span of 5-10 days (Shalama and Shoukry, 1972). The reproductive capacity, egg facility and life span of moths are affected by the difference in ages between males and females. The highest ratio of egg fertility was obtained by mating between 4-day-old males with fresh females . There is also a correlation between the host plant and the longevity and fecundity of S. littoralis . The majority of adults mate on the first night of emergence, copulation lasting for 20 minutes to 2 hours (Nasr and Nassif, 1978) .

2. Essential oil compounds

Essential oils compound are defined as any volatile oil (s) that have strong aromatic components and that give distinctive odor, flavor or scent to a plant. These are the by-products of plant metabolism and are commonly referred to as volatile plant secondary metabolites. Essential oils are found in glandular hairs or secretary cavities of plant-cell wall and are present as droplets of fluid in the leaves, stems, bark, flowers, roots and/or fruits in different plants (Koul *et al*., 2008).

Essential oil constituents are primarily lipophilic compounds that act as toxins, feeding deterrents and oviposition deterrents to a wide variety of insect pests. Some essential oils and isolated compounds are known to effect *P. xylostella* larvae having significant repellent and oviposition deterrent effects (Zhang *et al*., 2004). In some preliminary studies, volatile compounds like ά-terpinene, limonene and linalool do have significant repellent and oviposition deterrent effects on the *P. xylostella* larvae . Most insecticides act by ingestion, by contact and/or viathe respiratory tract. According to the two asarone isomers isolated from the essential oil of *Acorus calamus* L. rhizomes (Araceae) against to *Peridroma saucia* have different modes of action. cis-Asarone is toxic in addition to having strong antifeedant activity, whereas the trans-isomer acts only as an antifeedant with no appreciable toxicity (Koul *et al*, 1990) .In all cases, penetration of the insecticide (or molecules acting as insecticides) through the insect cuticle act at the corresponding binding site (s), especially on the receptor proteins in target insects (neurological active compounds), e.g. the antagonist

of NMDA-sensitive glutamate receptors (Lahlou, 2004). The aforementione studies with insects convincingly demonstrate the fumigant toxicity of essential oils and their constituents. Knockdown activity and lethal toxicity via contact has been demonstrated in the American cockroach (*Periplaneta americana*) the German cockroach (*Blattella germanica*) and the housefly (*Musca domestica*).

Essential oil used in this experiment are;

2.1 Linalool

Linalool is the naturally occurring enantiomer of the monoterpene compound commonly found as a major volatile component of the essential oils in several aromatic plant species such as citrus fruits, cinnamon and rosewood (Peana *et al*., 2006)In addition, Linalool is a fragrance ingredient used in many fragrance compounds. It may be found in fragrances used in decorative cosmetics, fine fragrances, shampoos, toilet soaps and other toiletries as well as in non-cosmetic products such as household cleaners and detergents. Its worldwide use is in the region of >1000 metric tons per annum. Linalool with 1,8-cineole do effect various pests like *Chilo partellus* (Lepidoptera : Pyralidae) (Singh *et al*., 2009). However, oral studies in rats which showed the acute toxicity in ten Osborne–Mendel rats (5/sex) were dosed with linalool (Letizia *et al*., 2003). The rats were observed for mortality and/or systemic effects over a period of 14 days. The oral LD_{50} was calculated to be 2790 mg/kg (95% C.I.2440–3180 mg/kg). Deaths occurred between 4 and 18h.

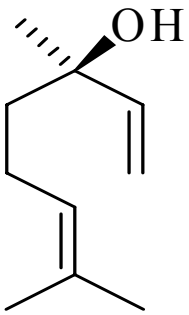

Figure 14 Chemical structure of linalool.

2.2 1,8-Cineole

1,8-cineole (cineole, eucalyptol), a monoterpene oxide present in many essential oils of plants including eucalyptus is traditionally used as a food flavoring agent, for treating symptoms of airway diseases exacerbated by infection and in aromatherapy as a skin stimulant in the form of skin baths. Nevertheless, cineole derived from sagebrush, eucalyptus genus, tea tree and bay leaves. Along with several other monoterpenes, it adversely affects the preferences of herbivores for sagebrush, but its mechanisms of action are not well known. In general, terpenes are toxic when consumed or dosed at too high concentrations. (Santos *et al*., 2004). In addition, 1,8-cineole has significant bioactivity as a mosquito feeding deterrent and ovipositional repellent and repellent and toxicant against stored-grain beetles. The use of terpenoids, including trace quantities of 1,8-cineole in the fecal shields of the tortoise beetle, *Paropsisterna tigrina* when fed 1,8-cineole-rich *Melaleuca* leaves raised the question: do other insect species metabolize 1,8-cineole in an identical manner with *P. tigrina*, frass analysis indicated that all terpenoids except 1,8-cineole were being excreted unchanged. In an attempt to acquire evidence more general for insects . Neverless, Lee *et al* (2002) use of 1,8-cineole as fumigant against *Tribolium castaneum* Herbst with an LD_{50} of 7.4 µl/l.

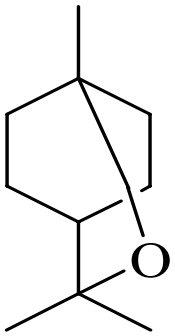

Figure 15 Chemical structure of 1,8 Cineole.

2.3 Thymol

Thymol is a monocyclic phenolic compound, the usual natural source being the essential oil of *Thymus vulgaris* (Lamiaceae) and thyme species. Its main therapeutic application is in dental preparations to kill odour-producing bacteria. It is also employed as a preservative on the strength of its antimicrobial and antioxidant properties. Expecially thymol has molluscicidal and insecticidal properties. In addition, reported that Thymol enhanced the GABA-dependent chloride currents in oocytes expressing various human GABAA receptor isoforms as well as the insect GABA receptor. Consistent with its action on mammalian GABAA receptors, thymol also potentiated the binding of tert-butyl bicycloorthobenzoate at this complex. Likewise, ivermectin increases chloride ion permeability in invertebrate muscle and nervous tissue through positive modulation of GABA-gated and glutamate-gated chloride channels, and paralytic effects have been reported in dipterans (Ranil *et al*., 2009). Some preliminary studies also show that thymol is toxic to third instars of *P. xylostella* (Rejesus *et al*., 1999) and has feeding deterrent properties as well (Yasmin, 2003).

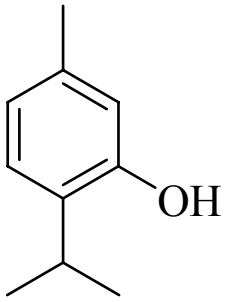

Figure 16 Chemical structure of Thymol.

2.4. Pulegone

Pulegone is a monoterpene ketone present in essential oils from many mint species, *Hedeoma pulegoides* and *Mentha pulegium*, both commonly called pennyroyal, contain essential oils, which are chiefly pulegone. Pennyroyal oil has been used as a flavoring agent in foods and beverages, as well as a component in fragrance products and flea repellents. Pennyroyal herb has also been used for the purpose of inducing menstruation and abortion. However, high doses of pennyroyal oil have sometimes been taken in attempted abortion and have resulted in central nervous system toxicity, gastritis, hepatic and renal failure, pulmonary toxicity, and death. Pulegone was found to constitute greater than 80% of the terpenes in pennyroyal oils that were obtained from health food stores and was found to be both hepatotoxic and pneumotoxic in mice (Ling-jen *et al*., 2001).

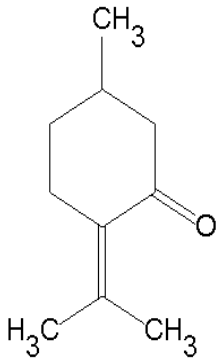

Figure 17 Chemical structure of Pulegone.

Pugelone repellent to birds, including the common starling *Sturnus vulgaris*, red-winged blackbird Agelaius phoeniceus, and Northern bobwhite *Colinus virginianus*. It is also toxic to the German cockroach *Blatella germanica*, *Musca domestica*, and storage pests (rice weevil *Sitophilus oryzae*, red flour beetle *Tribolium castaneum*, and the sawtoothed grain beetle *Oryzaephilus surinamensis*) (Katarzyna *et al*., 2008).

3. Detoxification enzyme

3.1 Carboxylesterase

Esterases are hydrolytic enzymes that cleave ester bonds in a diversity of biomolecules. Many insect esterases have well-defined biological functions, such as those involved in xenobiotic, lipid, acetylcholine, and Juvenile hormone metabolism. Carboxylesterases are a multifunctional superfamily ubiquitous in all living organisms, including insects and other animals, plants and microbes. Carboxylesterases have been the subject of intense research, in terms of their catalytic mechanism, molecular evolution and developmental regulation. Based on sequence similarity and substrate specificity, insect carboxylesterases genes can be subdivided into eight subfamilies: α-esterases, β-esterases, juvenile hormone esterases, gliotactins, acetylcholinesterases, neurotactins, neuroligins, and glutactin class. Juvenile hormone esterases, α-esterases, β-esterases, and acetylcholinesterases account for the majority of the catalytically active carbox. However, the physiological role of the vast majority of insect carboxylesterase is unknown . Insect carboxylesterase can be divided into three major classes (intracellular catalytic, secreted catalytic, and neurodevelopmental classes based on the topology of a phylogenetic tree of known carboxylesterase. Juvenile hormone esterase (JHE) integument esterase, β-esterase, and glutactin belong to the secreted catalytic class. Juvenile hormone esterase (JHE), is a carboxylesterase that has attracted great interest regarding its critical role in regulating larval to adult transition in insects and other arthropods (Kontogiannatos *et al*., 2011). In case of *P. xylostella*, Mohan and Gujar (2003) have studied effects of fenvalerate, fipronil, flufenoxuron in relation to carboxylesterase using cabbage leaf disc feeding bioassays

and all chemicals showed the highest level of carboxylesterase compared with susceptible strain.

3.2. Glutathione-S-transferase

Glutathione-S-transferases (GST) are enzymes that catalyze the detoxification of xenobiotics and endogenous substances by conjugating with reduced glutathione (GSH). There have been known to date seven classes of mammalian glutathione-s-transferase, Alpha, Mu, Pi, Sigma, Theta, Zeta, and Omega differing in amino acid sequence; these show the identities of about 50% within a class and less than 30% between different classes. In the case of insects, glutathione-s-transferase have been identified from various species mainly from the viewpoint of insecticide metabolism, and compared for their genomic sequences in the light of the sequence databases determined recently for insects. As a result, six different glutathione-s-transferase classes, designated Delta, Sigma, Epsilon, Theta, Omega, and Zeta, have been found in dipteran insects. It was notable that 12 Delta-, two Sigma-, eight Epsilon-, two Theta-, one Omega-, and one Zeta-class glutathione-s-transferase were found in *Anopheles gambiaem* whereas 11 Delta-, one Sigma-, 10 Epsilon-, four Theta-, four Omega-, and two Zeta-class glutathione-s-transferase were detected in *Drosophila melanogaster* (Kohji *et al*., 2008). Iason *et al* (2001) have been reported that high levels of GST with high resistance to pyrethroids do exist for *Spodoptera littoralis*, *Tribolium castaneum*, and *Aedes aegypti*. Induction of glutathione-s-transferase by pyrethroids has also been reported for the honey bee, *Spodoptera frugiperda* and German cockroach. However, glutathione-s-transferase are not thought to be involved in the direct detoxification of pyrethroids, but only as binding proteins. Although, It is well known that glutathione-s-transferase confer resistance to organochlorine, organophosphorous and pyrethroid insecticides. Glutathione-s-transferase catalyze the conjugation of reduced glutathione (GSH) with exogenous and endogenous toxic compounds or their metabolites, rendering them more water soluble, less toxic, and easier to excrete. It is well known that higher GST-3 gene expression do occur in resistant strains of *P. xylostella* (Sonoda *et al*, 2005). There is

also a correlation between high levels of GST with high resistance in various insects (Kostaropoulos *et al*., 2001).

Mode of action; In a glutathione-s-transferase catalysed conjugation reaction, one molecule of reduced GSH and one molecule of a second substrate are combined to form a thioester. The reaction proceeds via substrate binding, the activation of the thiol group of GSH and subsequent nucleophilic attack by the anionic GSH on the bound hydrophobic compound This conjugation neutralizes the electrophilic sites of thelipophilic substrate and protects the cellular components, especially the nucleophilic oxygen and nitrogen of DNA from electrophilic attack of nucleophiles. Conjugation alsorenders the product more water soluble and therefore more readily excretable from the cell. Glutathione-s-transferase have a high affinity towards GSH and because this tripiptide is present at high intracellular concentrations the GSH binding site of glutathione-s-transferase may always be occupied. The 'active site residue' in the N-terminal domain interacts with and activates the sulphydryl group of glutathione. In most mammalian glutathione-s-transferase the active site residue is a tyrosine but in the Delta and Epsilon insect glutathione-s-transferase classes this role performed by a serine residue (Enayati *et al*., 2005).

3.3 Neuron enzyme

Acetylcholinesterase is one of a key enzyme in the nervous system, terminating nerve impulses by catalysing the hydrolysis of the neurotransmitter acetylcholine. In insects, Acetylcholinesterase is the only cholinesterase and possesses a substrate specificity that is intermediate between that of vertebrate acetylcholinesterases and butyrylcholinesterases. In contrast to vertebrate cholinesterases which display a variety of molecular forms, the predominant form of acetylcholinesterase in insects is a globular amphiphilic dimer bound to membranes by a glycolipid anchor at the C-terminal of each catalytic subunit. acetylcholinesterase is the major target for organophosphate and carbamate insecticides, which inhibit enzyme activity by covalently phosphorylating or carbamylating the serine residue within the active site gorge. Inhibition of acetylcholinesterase by insecticides causes

excessive excitement in nerves, a blockage of neurotransmission and death of insects (Yu-Xin *et al*., 2007). Luo-gen *et al*, (2008) studies the combinative rate measurement of α-bungarotoxin and was applied in the analysis of the relation between nerve acetylcholine receptor and three types of insecticide resistance in *P. xylostella*, in the hypo-resistant strain and in the cartap-resistant strain. The nerve acetylcholine receptor showed the remarkable insensitivity to dimehypo and cartap, of which the binding rate to ligand was approximately 66 and 60%, respectively, of the susceptible strain. Acetylcholinesterase resistance to organophosphates have also been reported of insect such as potato aphid, *Myzus persicae* (Nabeshima *et al*., 2003).

Mode of action; Acetylcholine (ACh) is the principal excitatory neurotransmitter of the central and peripheral nervous systems of mammals but only the central nervous system of insects. Acetylcholinesterase inhibitors cause ACh to accumulate resulting in excessive stimulation of cholinergic receptors. Insect and mammalian acetylcholinesterase have an active center gorge for Ach hydrolysis (Figure 19) consisting at its base of a catalytic triad (His, Ser, Glu), choline subsite (Trp), acyl pocket and a peripheral site at the rim. Organophosphates (OPs) phosphorylate at serine for essentially irreversible inhibition (in the absence of a reactivator such as pralidoxime which acts as and antidote in mammals) whereas methylcarbamates (MCs) act as both competitive acetylcholinesterase inhibitors and methylcarbamoylating agents. Resistance in some cases is associated with multiple mutations in the active site, conferring cross resistance to many OPs and MCs (Casida and Quistad, 2004).

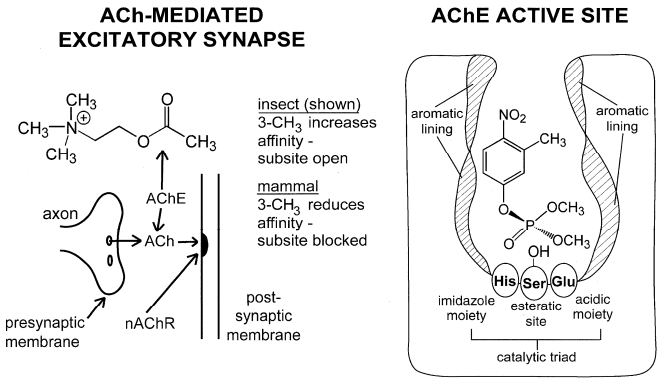

Figure 18 Acetylcholinesterase hydrolyzes acetylcholine and is inhibited by organophosphates such as fenitrooxon with subsite specificity between insects and mammals conferred in this case by the aryl methyl substituent

MATERIALS AND METHODS

Materials

1. Materials for rearing insect

1.1. *P. xylostella*

1.1.1 10% Sugar solution
1.1.2 Plastic boxes (20 x 10 x 4 cm)
1.1.3 Insect cage (45 x 45 x 45 cm)
1.1.4 Organic kale leaves

1.2. *Spodoptera littoralis*

1.2.1 Bean flour
1.2.2 Agar (Agar-Agar bakteriologisch)
1.2.3 Ascobic Acid
1.2.4 4-Ethylbenzoic acid
1.2.5 Sunflower oil
1.2.6 Vitamin E
1.2.7 3.7 % Formaldehyde

2. Materials for detoxification enzyme analysis

2.1. Material for general enzyme

2.1.1 Spectrophotometer (Hitachi ; U200)
2.2.2 Refrigerator Centrifuge (Hettich ; Universal 16 R)
2.2.3 Homogenizer (OMNI)
2.2.4 pH meter (Sartorius ; PP25)
2.2.5 Microplate reader (Biotek power wave XS)

2.2.6 Potassium phosphate buffer (KH_2PO_4) (pH 7.2)

2.2 . Materials for carboxylesterase analysis (*in vitro* / *in vivo*)

2.2.1 1- naphthyl acetate (alpha-NA) (Sigma®)
2.2.2 2- Naphthyl acetate (beta-NA) (Sigma®)
2.2.3 Fast garnet solution (Sigma®)
2.2.4 4′-Amino-2,3′-dimethylazobenzene, o-Aminoazotoluene (Sigma®)
2.2.5 Alpha-naphthol (Sigma®)
2.2.6 Beta naphthol (Sigma®)
2.2.7 Paranitrophenylacetate (p-NPA) (Sigma®)
2.2.8 Sodium dodecyl sulfonate (SDS) (Sigma®)
2.2.9 DMSO (Sigma®)

2.3. Materials for glutathione-s-transferase analysis (*in vitro* / *in vivo*)

2.3.1 cDNB (1- Chloro -2, 4- dinitrobenzene) (Sigma®)
2.3.2 Glutathione reduced form (Sigma®)
2.3.3 Ethanol

3. Materials for neuron enzyme (Acetylcholinesterase)

3.1 DCNB (1, 2- dichloro- 4,nitrobenzene) (Fluka)
3.2 Triton X-100 (Sigma)
3.3 Acetyl-cholineiodide (Sigma®)

4. Materials for toxicity test (topical application method / antifeedant)

4.1 Micro applicator
4.2 Petridish plate (Ø 7.5 cm.)
4.3 Filter paper (Whatman®)
4.4 Acetone
4.5 Leave area meter (WinDias3; Delta-T Devices)

5. Essential oils

5.1 Linalool (Fluka®)

5.2 1,8-cineole (Fluka®)

5.3 Thymol (Aldrich®)

5.4 Pulegone (Sigma®)

Methods

1. Rearing of insect

1.1. Rearing method of *P. xylostella*

P. xylostella rearing method were modified from Vanichpakorn *et al* (2010). Insects colony to be used were from radish farm at Nontaburi province (40 kms from Bangkok, Thailand) and subsequently reared in a insect rearing room at Zoology Department, Faculty of Science Thailand at 27 °C, 75±10% RH,with a 16 L:8D photoperiod. The larvae were fed on non pesticide kale leaves and adults on 10 % honey solution, eggs colony were collected daily and kept in the plastic boxes (20 x 10 x 4 cm). Second instar and third instar of the larvae they rapidly spread in the field crops were used in the experiments (Robert *et al*., 2006).

Figure 19 Radish farm for collected the colony of *P. xylostella*.

Figure 20 The cages for rearing *P. xylostella* adults were fed with 10% honey solution.

Figure 21 Rearing of *P. xylostella* larvae were fed on kale leaves.

1.2 . Rearing method of *S. littoralis*

S. littoralis were reared on artificial diet, The artificial diet were prepared from aboratory of Maxplank Institue for Chemical Ecology, Germany. A bean flour mixed with ascobic acid, 4-Ethylbenzoic acid, sunflower oil, vitamin E and 3.7% formaldehyde exclude agar-agar bakterilogisch, which dissvoled in distill water and

heating, then agar solution were mixed with othere compounds, after that artificial diet dried at room temperature. A piece of diet were placed in *S. littoralis* rearing boxes and it changed every 2 or 3 days, kept on growth chamber with the similary *P. xylostella* conditions. laboratory of Maxplank Institue for Chemical Ecology, Germany.

Figure 22 Rearing method of *S. littoralis* with artificial diet.

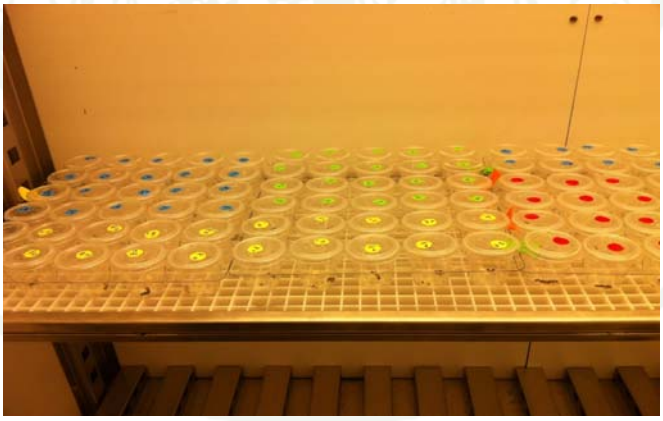

Figure 23 Growth chamber of *S. littoralis* at Maxplank Institue for Chemical Ecology, Germany.

2. Compounds

All essential oil compounds as thymol, 1,8 cineole, linalool and pulegone used in the study were from Fluka® and Aldrich® .

3. Toxicity bioassay

3.1. Topical application method

Modified method of Abro *et al* (1988) by using 3rd instar *P. xylostella* larvae . These procedure using complete block design (CBD). Larval mortality was recored at 24 and 48 hours after topical application of 1µl of essential oil (1,000-10,000 ppm) by micro-applicator (Figure 24). Control received only acetone (AR grade). The topical method were applied 3 replicates (30 larval / concentration). LD_{50} values were determined using Stat Plus Program by using Probit analysis.

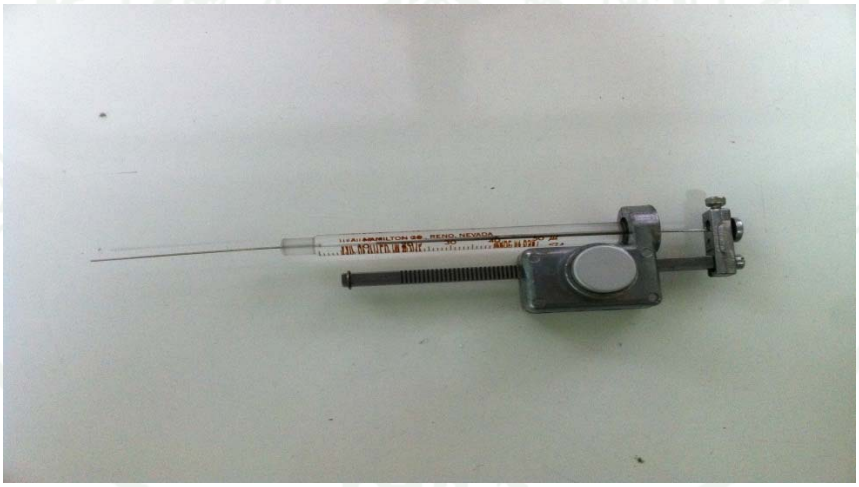

Figure 24 Micro-applicator.

3.2. Antifeeding method

The antifeeding procedure modified from Koul *et al* (2004) for choice and no choice. The experiment using 3rd instar larvae.

Choice-test ; Feeding choice-test were used essential oil compounds against to 3rd instar *P. xylostella* (Larvae were starved at 5 hours before feeding). The leaf disc with 2 cm^2 area were made from fresh kale leaves. Various of essential oil compounds (50-500 ppm) in acetone and it were applied on the two side of leaf disc (Each of 75 µl). Afterwards, the leaf disc was dried at the room temperature for solvent evaporated. There were treated leaf discs and control leaf discs in a Petri-dish (13.75 cm diameter). 3rd instar larvae of *P. xylostella* was placed in the center of Petri-dish contaning leaf disc (Treated leaf disc and control leaf disc), these test were replicated for 20 times per concentration. Feeding inhibition was calculated at 4 hours and 6 hours by the ratio difference of treated group and control with leaves area meter (WinDias3; Delta-T Devices).

Figure 25 Leave area meter (WinDias3; Delta-T Devices).

No-choice test; The leaf discs were treated as described in the feeding choice test. For no-choice test, various concentration of essential oil compounds as 1-4,000 ppm. The one leaf disc was placed in the center of a Petri-dish (7.30 cm diameter). Each Petri-dish containing 3rd instar *P. xylostella*, the experiment was replicated for 10 times per concentration. Antifeedant results were calculated at 4 hours and 6 hours by the ratio of the difference area between the treated group and the control group with leaves area meter (WinDias3; Delta-T Devices).

Percentage of antifeeding inhibition for both choice and no-choice experiment were calculated using formula from Koul (2008).

$$\frac{(C - T)}{(C + T)} \times 100$$

Whereas;

C = Consumption of control leaf disc

T = Consumption of treated leaf disc.

Figure 26 Preparing materials for antifeedant test.

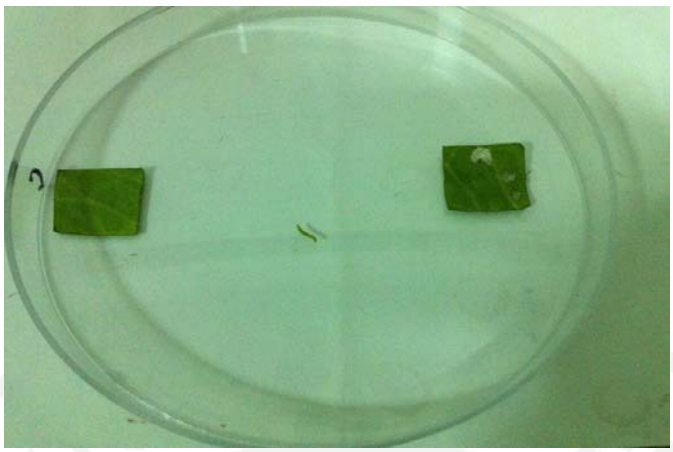

Figure 27 Choice test of Antifeedant.

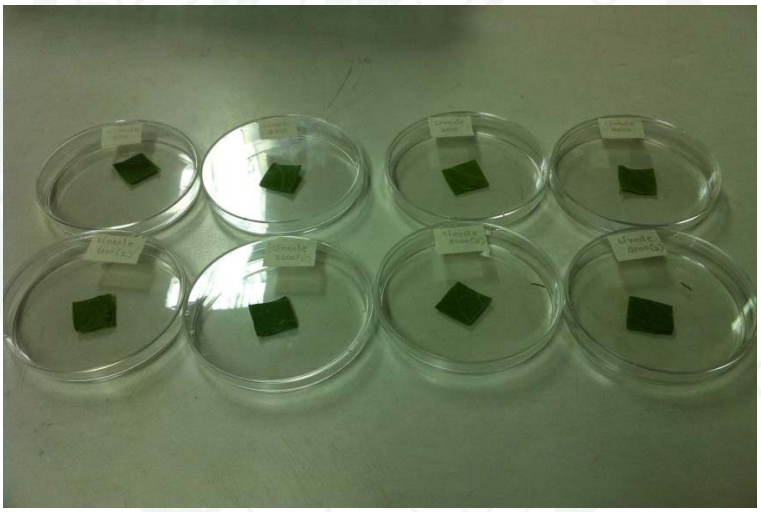

Figure 28 No-choice test of Antifeedant.

3.3. Oral toxicity test

Oral toxicity test of thymol was focused on 3rd instar *S. littoralis*, collected from bioassay laboratory of Max Planck Institue for Chemical Ecology , Germany (The experiment were done using Complete Block Design). The procedure of toxicity test was modified from El-Aswad *et al* (2003). Thymol was dissolved in acetone and mixed with artificial diet to provide a range of concentration, control diet mixed with acetone only. The carterpillars to weight before placed on a cups and kept diet to them, these procedure replicated 3 times; 20 carterpillars per concentrations

(100, 1,000, 2,000, 3000 and 4,000 ppm). The mortality was recorded every days for 7 days, whereas carterpillars weight were weighted every 3 days. The larvae were weight before experiment.

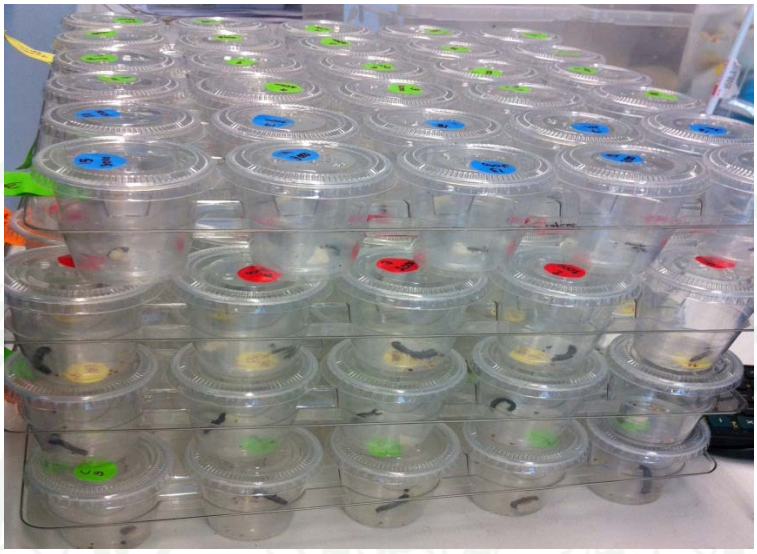

Figure 29 Oral toxicity test of *S. littoralis*.

3.4. Binary Mixtures

Acute toxicity of binary mixtures procedure modifield from Roman (2010), it was determined by topical application method to 2nd instar larvae *P. xylostella.*The essential oil compounds were combined in difference concentration for 3 replicates of 30 larvae were tested per dose. The motalities was calculated through Probit analysis program. Actual mortalities were compared to expected mortalities based on the formula.

$$E = O_a + O_b (1 - O_a)$$

Whereas;

E = Expected mortality

O_a = Observed mortalities of pure compounds at the given concentration

O_b = Observed mortalities of pure compounds at the given concentration

The effects of mixtures were designated antagonistic, additive or synergistic by analysis using X^2 comparisons;

$$X^2 = (O_m\text{-}E)^2 / E$$

O_m = Observed mortality from the binary mixture

X^2 = X^2 with df = 1 and α = 0.05 is 3.84

Remarks;

A pair with ÷2 values > 3.84 and having greater than expected mortality were considered to be synergistic (negativeness = antagonist effect), with ÷2 values < 3.84 representing additive effects.

4. Mode of action analysis

4.1. General enzyme extraction

General enzyme extraction was done using 100 mM Phosphate buffer pH 7.2, homogenized buffer (100 mM PBK + 0.5% triton X-100) and whole body of 24 hours survival treated (LD_{50}) *P. xylostella* . The homogenate were centrifuge at 12,000 rpm 4 Degree Celcius 5 minutes, all homogenized were done on ice and supernatant were used for enzyme source.

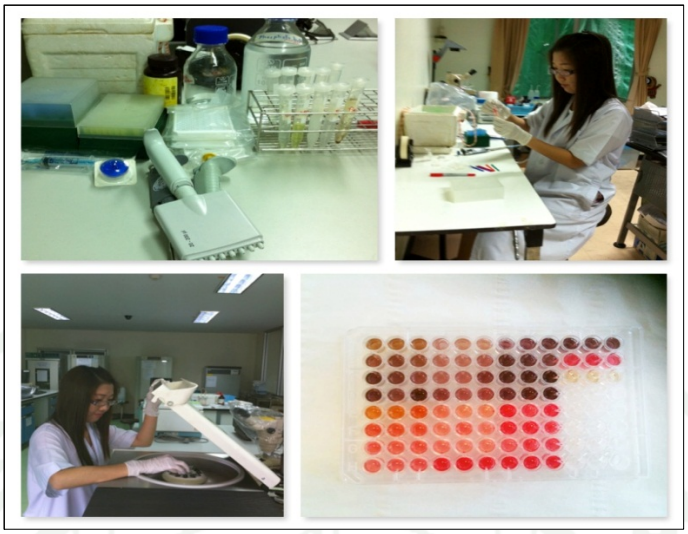

Figure 30 Detoxification enzyme analysis.

4.2. General esterase activity

In vivo: The activity was determined by the method of Darry *et al* (1990) with modifications. The reaction mixtures consitsted of crude enzyme from each treatment, 100 mM phosphate buffer (PBK) , pH 7.2, 30 mM of alpha or beta naphthylacetate and 5 mM fast garnet solution. The activity was measured with microplate reader at 505 nm and 527 nm for 1- naphthyl acetate (Alpha-NA) and 2-Naphthyl acetate (Beta-NA) respectively. The esterase activity was recorded as nM Napthol product/min/mg protein using Napthol as standard curve.

In vitro: The reaction of general esterase consisted of crude enzyme from control, 100 mM PBK, pH 7.2 and 17 µl testing compounds. The mixtures incubated 30 minutes at 30 ^{o}C, then add 30 mM alpha- or beta- naphthyl acetate and incubated at 30 ^{o}C for 30 minutes. Add 5 mM fast garnet solution, incubated at 30 ^{o}C analyzed activities using microplate reader (Alpha-NA read at 505 nm; Beta-NA: read at 527 nm) The activities of enzyme were recorded as nM Napthol product/ min/ mg protein using Napthol as standard curve.

4.3. Carboxylesterase

In vivo: For *in vivo* experiments, carboxylesterase was determined using the method of Han et al (1998). Enzyme solution (50 µl) was mixed with p-nitrophyenylacetate (pNPA) (50 µl, 0.12 M) and phosphate buffer (2.9 ml, 0.1 M, pH 7.5). Enzyme activity was measured at 400 nm and 25 ^{o}C for 3 minutes using 96-well microplate reader in the kinetic mode. The activity of carboxylesterase was determined at the extinction codfficient of 176.4705 for pNPA. Three biological replicates pre treatment were estimated.

In vitro: For *in vitro* inhibition experiments, 50 µl of enzyme was preincubated for 30 minitues at 30 ^{o}C with 50 µl of test compounds or acetone for control, and then 50 µl of p-nitrophenylacetate (pNPA, 0.12 M) was added along with phosphate buffer (2.9 ml, 0.1 M; pH 7.5). The rest of the procedure followed was similar to the *in vivo* experiment.

4.4. Glutathione-*S*-transferase activity

Glutathione-*S*-transferase activity was modified from the procedure of Kamiya biochemical company.

In vivo: The mixtures contaning 50 mM phosphate buffer (pH 7.2),. Add GST solution, enzyme (only sample), 1-chloro-2,4'-dinitrobenzene (CDNB). The mixture was measured activity at waverange 340 nm by microplate reader as showed CDNB conjugated with GST min/mg protein.

In vitro: The mixutes consisting 50 mM phosphate buffer (pH 7.2), GST solution and 100 µl supernatant which continue incubated 30 minutes at room temperature. Finally, add CDNB soulution and detected the activities were absorbance at 340 nm using microplate reader.

4.5. Acetylcholinesterases

Enzyme acitvity was modified method of Ellman (1959).

In vivo: The mixture consists of 100 mM PBK (pH 7.2), 50 µl supernatant (PBK = Blank), which incubated 30 minutes at 30 ^{o}C. Add 50 µl substrate solution, it contains 10 mM 5, 5'-Dithio-bis (2-nitrobenzoic acid ; DTNB) in 0.1M phosphate buffer (PBK), 0.1M ethylenediaminetetraacetic acid (EDTA), 100 mM acetylthiocholineiodide (AsCh) and 100 mM PBK, pH 7.2 46 microliter. Analyze kinetic mode at 412 nm for 10 minutes (every 2 seconds) via microplate reader.

In vitro: The acitivities of enzyme contains 100 mM PBK (pH 7.2), inhibitors solution, essential oil compounds, 100 mM PBK (pH 7.2), then incubated at 30 ^{o}C for 30 minutes. Add supernatant and incubated with the same conditions, finally add substrate suloution. Enzyme acitvities were measured with the similar conditions of *in vivo* assay.

RESULTS AND DISCUSSION

Results

1. Bioefficacy of some essential oil compounds against an economically important crop pest *P. xylostella* and *S. littoralis*.

1.1 Bioefficacy of essential oil compounds on *P. xylostella* testing by topical application method.

Toxicity of essential oil compounds were treated by topical application method on 3rd larvae *P. xylostella*. Thymol and 1,8-cineole were dissolved in acetone for various concentration (acetone : control), Acute toxicity were observed at 24 and 48 hours after exposed, each LD_{50} and LD_{90} calculated using Stat Plus program, the results showed differently significant of control group and treated group of thymol, 1,8 cineole showed differently significant based on Turkey's test. After treated thymol showed LD_{50} and LD_{90} at 24 and 48 hours exposed 220.89 ± 22.87, 1,230.19 ± 117.183 ppm respcectively, 1,8-cineole showed strong effective at 24 and 48 hours at LD_{50}, LD_{90} values are 417.24 ± 82.06 ppm, 866.88-1,918.84 ppm ($p < 0.05$) (Table 2). For linalool, after treated on 3rd *P. xylostella* which showed the highest percentage of mortality at 24 and 48 as 16.67±11.55 ppm. In addition, pulegone showed LD_{50}, LD_{90} values are 20.00±00.00 ppm and 23.33±5.80 ppm after exposed (Table 3).

The relative toxicity of essential oil compounds in Figure 31and 32, whereas the relation between concentration and percentage of mortality of larvae, the concentration contained 100 ppm-10,000 ppm shows that essential oil of thymol had the highest toxicity at 24 and 48 hours after exposure. The lower ralative toxicity as shows of pulegone and linalool, percentage of mortality more than 3,000 ppm ($p < 0.05$).

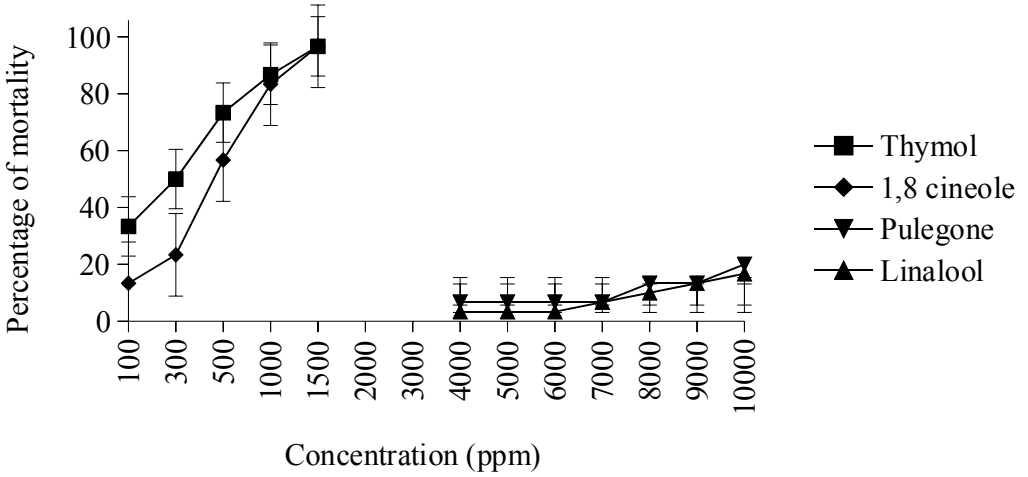

Figure 31 Percentage of mortality of thymol, 1,8 cineole, linalool and pulegone after treated with 3rd instaras larvae *P. xylostella* via topical application method at 24 hours.

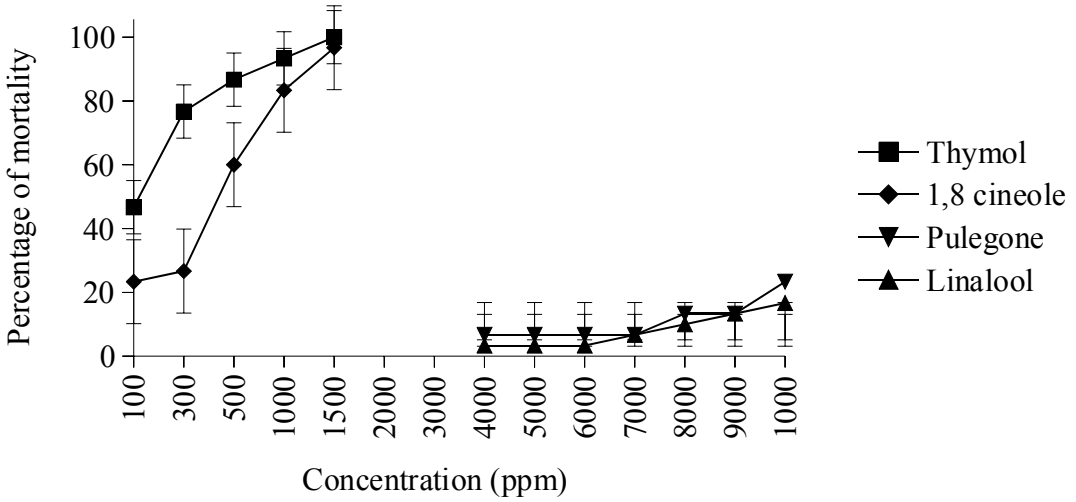

Figure 32 Percentage of mortality of thymol, 1,8 cineole, linalool and pulegone after treated with 3rd instars larvae *P. xylostella* via topical application method at 48 hours.

Table 2 Efficacy of 1,8 cineole and thymol to 3rd instar larvae *P. xylostella* after 24 and 48 hours after exposed (topical application method).

Concentration (ppm)	Essential oil Percentage of mortality,Mean(2) ± SD			
	1,8 cineole		Thymol	
	24 hrs	48 hrs	24 hrs	48 hrs
0(1)	0.00± 0.00^{f}	0.00± 0.00^{f}	0.00± 0.00^{f}	0.00± 0.00^{f}
100	13.33±5.80^{e}	23.33±15.30^{e}	33.33±11.55^{e}	53.33±11.55^{e}
300	23.33±5.80^{d}	26.67±5.80^{d}	50.00±10.00^{d}	76.67±15.30^{d}
500	56.67±15.30^{c}	60.00±10.00^{c}	73.33±11.55^{c}	86.67±11.55^{c}
1,000	83.33±5.80^{b}	83.33±5.80^{b}	86.67±23.10^{b}	93.33±11.55^{b}
1,500	96.67±5.80 ^{a}	96.67±5.80 ^{a}	96.67±5.80 ^{a}	100.00±0.00 ^{a}
LD_{50}	564.55	500.66	284.44	98.35

(1) Control treatment: acetone

(2) Means ± SD, all experiments followed by different letters within the same row are significantly different at $P<0.05$, DMRT.

Table 3 Efficacy of Linalool and Pulegone to 3rd instars larvae *P. xylostella* after 24 and 48 hours after exposed (topical application method).

Concentration (ppm)	Essential oil Percentage of mortality, Mean[2] ± SD			
	Linalool		Pulegone	
	24 hrs	48 hrs	24 hrs	48 hrs
0[1]	0.00±0.00[(j)]	0.00±0.00[(j)]	0.00±0.00[(j)]	0.00±0.00[(j)]
1,000	0.00±0.00[(j)]	0.00±0.00[(j)]	0.00±0.00[(j)]	0.00±0.00[(j)]
2,000	0.00±0.00[(i)]	0.00±0.00[(i)]	0.00±0.00[(i)]	0.00±0.00[(i)]
3,000	0.00±0.00[(h)]	0.00±0.00[(h)]	0.00±0.00[(h)]	0.00±0.00[(h)]
4,000	3.33±5.80[(g)]	3.33±5.80[(g)]	6.67±11.55[(g)]	6.67±11.55[(g)]
5,000	3.33±5.80[(f)]	3.33±5.80[(g)]	6.67±11.55[(g)]	6.67±11.55[(g)]
6,000	3.33±5.80[(e)]	3.33±5.80[(g)]	6.67±11.55[(g)]	6.67±11.55[(g)]
7,000	6.67±5.80[(d)]	6.67±5.80[(d)]	6.67±11.55[(g)]	6.67±11.55[(g)]
8,000	10.00±10.00[(c)]	10.00±10.00[(c)]	13.33±11.55[(c)]	13.33±11.55[(c)]
9,000	13.33±05.80[(b)]	13.33±05.80[(b)]	13.33±11.55[(c)]	13.33±11.55[(c)]
10,000	16.67±11.55[(a)]	16.67±11.55[(a)]	20.00±00.00[(a)]	23.33±5.80[(a)]

[1] Control treatment: acetone

[2] Means ± SD, all experiments followed by different letters within the same row are significantly different at P<0.05, DMRT.

1.2. Bioefficacy of feeding inhibition

No-choice; The antifeedant activities of thymol, 1,8 cineole, linalool and pulegone againt to 3rd *P. xylostella,* feeding inhibitions were showed (Table 4). Thymol showed significant difference in antifeedant at the highest dose at 4,000 ppm compared to control ($p < 0.05$). Pulegone showed the less effective as 49.27 % at 1 ppm, out of all showed feeding inhibition in rage 50%-98% were found in 1,8 cineole and linalool. Various concentration of 1 ppm-4,000 ppm respectively (Figure 33, 34) at 4 hours and 6 hours. The results showed strong feeding inhibition at the lower dose to the higher dose over 50 % except pulegone (46.70% at 1 ppm) when compared with control group ($p < 0.05$).

Choice test; all of compounds showed feeding inhibition as 100%, at the highest dose (500 ppm) showed non-selective antifeedant ($p < 0.05$). The activities of essential oil compounds againt to 3rd *P. xylostella* were showed (Table 5). Generally, choice-test of antifeedant showed the percentage of feeding inhibition in range 10%-20% at the low concentration (50 ppm). Feeding inhibition at 6 hours, various concentration of essential oil compounds as 50 ppm – 500 ppm, at concentration of 500 ppm the antifeedant index was significantly higher than at 50 ppm ($P < 0.05$). Generally, percentage of feeding inhibition of choice-test with leaf disc showed the highest effective relation with the hight concentration (4,000 ppm) (Figure 35, 36).

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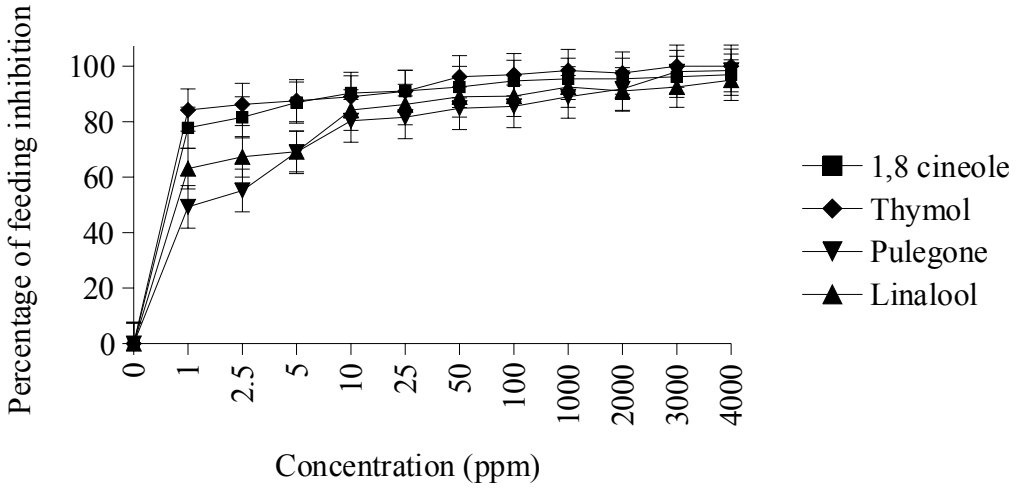

Figure 33 Feeding inhibition of essential oil compounds of no-choice against to 3rd instar larvae *P. xylostella* at 4 hours

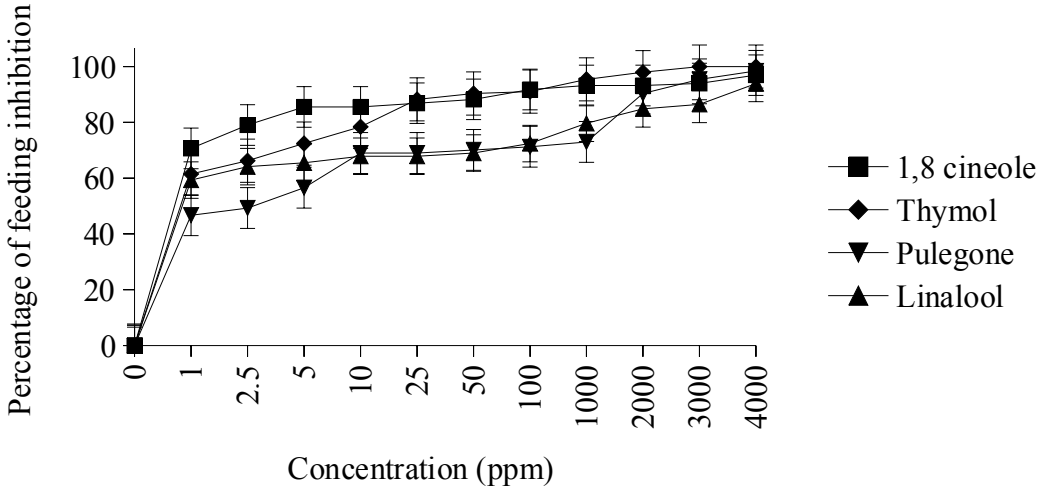

Figure 34 Feeding inhibition of essential oil compounds of no-choice against to 3rd instar larvae *P. xylostella* at 6 hours.

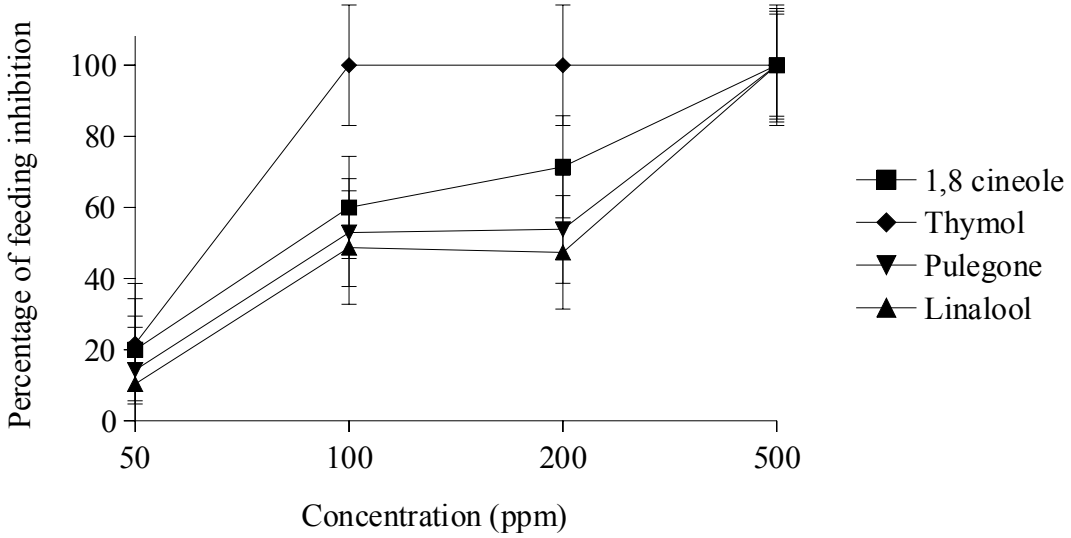

Figure 35 Feeding inhibition of essential oil compounds of choice-test againt to 3rd instar larvae *P. xylostella* at 4 hours.

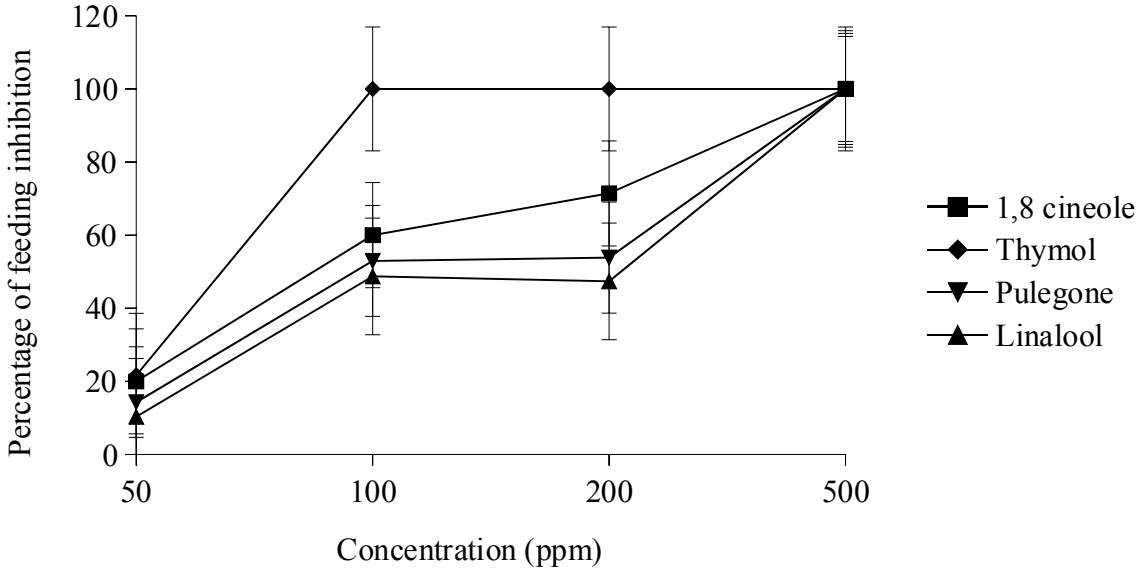

Figure 36 Feeding inhibition of essential oil compounds of choice-test againt to 3rd instar larvae *P. xylostella* at 6 hours.

Table 4 Feeding inhibition of essential oil compounds to 3rd instar larvae of *P. xylostella* for no-choice test after application at 4 hours and 6 hours.

Dose (ppm)	Feeding inhibition Percentage			
	1,8-cineole		Thymol	
	4 hrs	6 hrs	4 hrs	6 hrs
0(1)	0.00	0.00	0.00	0.00
1,000	77.78	70.67	84.17	61.50
2,000	81.56	79.02	86.18	66.23
3,000	86.86	85.51	87.55	72.40
4,000	90.33	85.51	88.93	78.40
5,000	91.04	86.86	90.94	88.24
6,000	92.50	88.24	96.17	90.34
7,000	94.68	91.76	96.92	91.04
8,000	95.42	93.21	98.45	95.42
9,000	95.42	93.21	97.53	97.99
10,000	96.17	93.94	100.00	100.00
FI_{50}	3,639.40±287.91	339.44±102.28	2,786.31±143.70	1,230.52±88.79

Table 5 Feeding inhibition of essential oil compounds to 3rd instar larvae of *P. xylostella* for no-choice test after application at 4 hours and 6 hours.

Dose (ppm)	Feeding inhibition Percentage			
	Pulegone		Linalool	
	4 hrs	6 hrs	4 hrs	6 hrs
0	0.00	0.00	0.00	0.00
1	49.27	46.70	63.06	59.30
2.5	55.15	49.27	67.32	64.10
5	68.98	56.57	69.20	65.48
10	80.28	68.98	84.17	67.87
25	81.56	68.98	86.18	67.87
50	84.84	70.10	88.93	68.98
100	85.51	71.24	89.21	72.40
1,000	88.93	72.97	92.48	79.65
2,000	91.76	90.33	91.04	84.84
3,000	97.99	95.42	92.48	86.45
4,000	98.45	98.45	94.97	93.93
FI_{50}±SD	1,329.61±112.04	556.50±102.28	3,313.17±191.64	1,598.02±163.81

Table 6 Feeding area and percent feeding area of essential oil compounds 3rd instar larvae of *P. xylostella* for choice test after application at 4 and 6 hours

Dose (ppm)	1,8 cineole				Thymol			
	4 hours		6 hours		4 hours		6 hours	
	C	T	C	T	C	T	C	T
50	0.25	0.20	0.30	0.20	0.50	0.33	0.73	0.47
%FI	11.11		20.00		20.48		21.67	
100	0.40	0.00	0.80	0.20	0.43	0.00	0.76	0.00
%FI	100.00		60.00		100.00		100.00	
200	0.40	0.00	0.1	0.1	0.63	0.00	1.10	0.00
%FI	100.00		71.43		100.00		100.00	
500	0.40	0.00	0.00	0.00	0.63	0.00	1.23	0.00
%FI	100.00		100.00		100.00		100.00	
FI_{50}±SD	57.71±17.24		115.68±10.87		50.10 ±17.63		48.15±17.72	

Remarks: C = Control , T= Treatment

Table 7 Feeding area and percent feeding area of essential oil compounds 3rd instar larvae of *P. xylostella* for choice test after application at 4 and 6 hours.

Dose (ppm)	Pulegone				Linalool			
	4 hours		6 hours		4 hours		6 hours	
	C	T	C	T	C	T	C	T
50	0.40	0.30	0.45	035	2.00	1.35	1.60	1.30
%FI	12.50		14.29		19.40		10.34	
100	0.45	0.20	0.65	0.20	1.30	0.40	1.45	0.50
%FI	38.46		52.94		48.72		38.46	
200	0.30	0.10	0.50	0.15	1.05	0.00	1.40	0.50
%FI	50.00		53.85		100.00		47.37	
500	0.20	0.00	0.25	0.00	0.40	0.00	0.45	0.45
%FI	100.00		100.00		100.00		100.00	
FI_{50}±SD	174.28±10.17		152.53±10.94		101.78±11.40		167.88±10.68	

Remarks: C = Control , T= Treatment

1.3. The efficacy of thymol against to *S. littoralis*

Oral toxicity of thymol on *S. littoralis*, various concentrations as 100 ppm-4,000 ppm were mixed with artificial diet and weight every 2 days. Moreover, the *S. littoralis* weight was increased after feeding the hight concentration of artificial diet. (Figure 37). Percentage of mortality checked every day for 8 days. Thymol showed the lower effective to 3rd instar larvae *P. xylostella* less than 40 % (Figure 38).

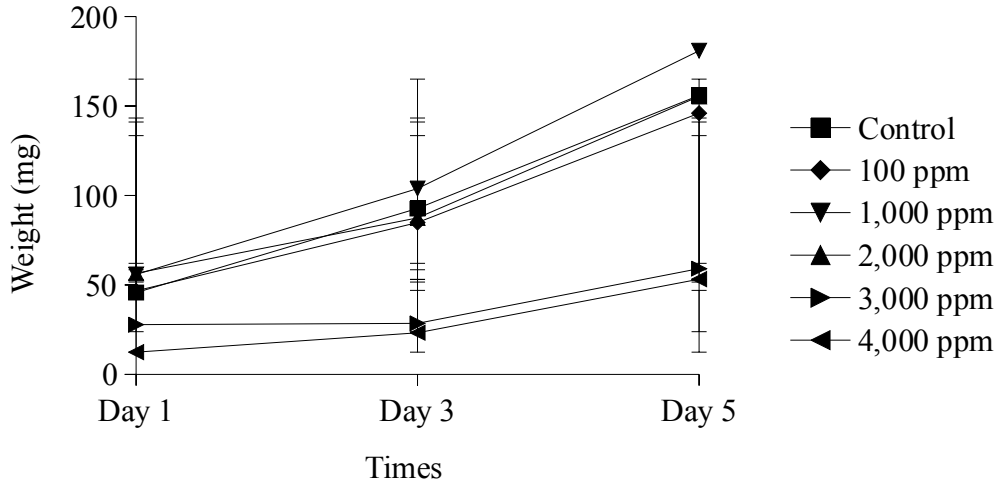

Figure 37 Weight (mg) of *S. littoralis* after feeding thymol with artificial diet

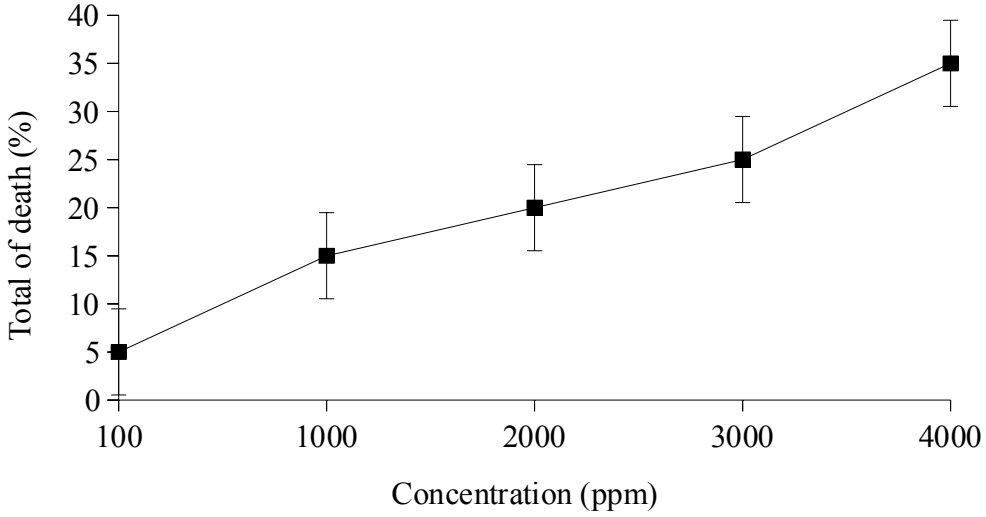

Figure 38 Total death of third instar *S. littoralis* via oral toxicity test treated by thymol for 8 days.

2. The efficacy of Binary mixtures of essential oil compounds for enhancement of activity.

Binary mixtures of essential oil compounds applied by topical application method to 3rd instar larvae *P. xylostella.*The most importance synergism was found in thymol in the mixture with pulegone and 1,8 cineole in the mixture with pulegone. Out of the 15 mixtures tested, 6 showed synergistic effect, 9 showed antagonistic effect just like thymol in the mixtures with 1,8 cineole and 1,8 cineole with linalool ($P<0.05$) (Table 8).

3. The possible mode-of-action at physiological level against *P. xylostella*.

The detoxification enzme and neuron enzyme analysis (*in vitro* and *in vivo)* were carried out 5 times to determine. The difference of enzyme activities was determined base on Turkey's test ($P<0.05$).

3.1. *In vitro*

Assays were conducted for thymol and 1,8 cineole in order to determine the detoxification ability of insect enzymes. Experiments revealed that carboxylesterase, esterase and glutathione-s-transferase activity of *P. xylostella* increased, whereas there was no significan ($P=0.05$, $F=0.45$, $df=3$) difference in the activity of acetylcholinesterase activity between treated and control insects in vitro (Table 7).

Acetylcholine; The acetylcholine activities in 3rd *P. xylostella* after treated with thymol and 1,8 cineole was significantly hight ($p<0.05$) with CF as 0.94 fold (Table 7).

3.2 . *In vivo*

A highly significant difference between control and treated groups was seen in glutathione-s-transferase activity ($p<0.05$, $F=0.60$, $df=3$). *In vivo*, however,

there was a significant increase in glutathione-s-transferase and acetylcholinesterase activity in thymol and 1,8 cineole-treated larvae (Table 8). There was no affect on esterase *in vivo***.**

Table 8 Relative toxicity due to binary mixtures of essential oil compounds to 3rd instars of *P. xylostella* and measures of interactions

.

Compound A	Compound B	Dose (µg/ larva)	Larval mortality (%)					
			Pure compounds		Binary mixtures			
			Observe A	Observe B	Expected	Observe	x^2	Effect
Thymol	1,8-cineole	0.14 + 0.38	20.00	20.00	36.00	10.00	-	Antagonist
Thymol	1,8-cineole	0.14+0.50	20.00	30.00	44.00	20.00	-	Antagonist
Thymol	1,8-cineole	0.22+0.38	30.00	20.00	44.00	10.00	-	Antagonist
Thymol	Linalool	0.14 + 16.00	20.00	10.00	28.00	0.00	-	Antagonist
Thymol	Linalool	0.18+16.00	25.00	10.00	32.50	20.00	-	Antagonist
Thymol	Linalool	0.14 +20.00	30.00	16.67	41.67	20.00	-	Antagonist
Thymol	Pulegone	0.14 + 18.00	20.00	13.33	30.64	50.00	12.23	Synergy
Thymol	Pulegone	0.14+20.00	20.00	16.67	33.34	60.00	21.31	Synergy
Thymol	Pulegone	0.22 + 20.00	30.00	16.67	41.67	60.00	8.06	Synergy
1,8-cineole	Linalool	0.44+16.00	25.00	10.00	32.50	20.00	-	Antagonist
1,8-cineole	Linalool	0.44 +18.00	25.00	13.33	35.00	20.00	-	Antagonist
1,8-cineole	Linalool	0.50+18.00	30.00	13.33	39.33	30.00	-	Antagonist
1,8-cineole	Pulegone	0.44+20.00	25.00	16.67	37.50	60.00	13.50	Synergy

Table 8 (Continued)

Compound A	Compound B	Dose (µg/ larva)	Larval mortality (%)					
			Pure compounds		Binary mixtures			
			Observe A	Observe B	Expected	Observe	x^2	Effect
1,8-cineole	Pulegone	0.50+16.00	30.00	10.00	37.00	60.00	14.30	Synergy
1,8-cineole	Pulegone	0.50 +20.00	30.00	16.67	41.66	90.00	56.09	Synergy

Table 9 Mode of action study of different essential oil compounds (µg/larva) to 3rd instars of *P. xylostella in vitro*.

	Dose (ppm)	CE	EST (NA1)	EST (NA2)	GST	AChE
Control	0.00	2.20 ± 0.06[a]	0.03 ± 0.01[a]	0.03 ± 0.01[a]	0.29 ± 0.01[a]	0.15 ± 0.09[a]
Thymol	220.89	3.02 ± 0.16[c]	0.06 ± 0.02[c]	0.05 ± 0.01[b]	0.56 ± 0.10[c]	0.16 ± 0.01[b]
(CF)	(0.00)	(0.73)	(0.50)	(0.60)	(0.52)	(0.94)
1,8-cineole	417.24	2.88 ± 0.33[b]	0.04 ± 0.01[b]	0.05 ± 0.01[b]	0.40 ± 0.07[b]	0.16 ± 0.01[b]
(CF)	(0.00)	(0.76)	(0.75)	(0.60)	(0.73)	(0.94)

Remark: Means within a column followed by same letter are not significantly different based on Tukey's test ($P < 0.05$).

CI = confidence interval.

CF is a correction factor = enzyme activity of control) / (enzyme activity of treatment)

the Unit of Carboxylesterase (CE) is nM p-nitrophenol/min/ mg protein

the Unit of Esterase (EST) (NA1) is µM 1-naphthol/min/ mg protein

the Unit of Esterase (EST) (NA1) is µM 2-naphthol/min/ mg protein

the Unit of Glutathione-s-transferase (GST) is nM glutathione conjugated product/min/ mg protein

the Unit of Acetylcholinesterase (AChE) is µM/min/ mg protein

Table 10 Mode of action study of different essential oil compounds (µg/larva) to 3rd instars of *P. xylostella in vivo*

	Dose (ppm)	CE	EST (NA1)	EST (NA2)	GST	AChE
Control	0.00	3.89 ± 0.04[a]	0.06 ± 0.01[a]	0.04 ± 0.01[a]	1.15 ± 0.13[a]	0.31 ± 0.01[a]
Thymol	220.89	3.97 ± 0.06[b]	0.06 ± 0.01[a]	0.05 ± 0.01[b]	1.32 ± 0.32[c]	0.50 ± 0.12[c]
(CF)	(0.00)	(0.98)	(1.00)	(1.25)	(1.15)	(0.62)
1,8-cineole	417.24	4.01 ±0.05[c]	0.06 ± 0.02[a]	0.05 ± 0.01[b]	1.25 ± 0.43[b]	0.39 ± 0.13[b]
(CF)	(0.00)	(0.97)	(1.00)	(1.25)	(1.09)	(0.79)

Remark: Means within a column followed by same letter are not significantly different based on Tukey's test (P<0.05).

CI = confidence interval.

CF is a correction factor = enzyme activity of control) / (enzyme activity of treatment)

the Unit of Carboxylesterase (CE) is nM p-nitrophenol/min/ mg protein

the Unit of Esterase (EST) (NA1) is µM 1-naphthol/min/ mg protein

the Unit of EsteraseEST (NA1) is µM 2-naphthol/min/ mg protein

the Unit of Glutathione-s-transferase (GST) is nM glutathione conjugated product/min/ mg protein

the Unit of Acetylcholinesterase (AChE) is µM/min/ mg protein

Discussion

1. Bioefficacy of some essential oil compounds against an economically important crop pest *P. xylostella*

1.1. Bioefficacy of pure compounds.

Thymol showed the highest activity than other compounds. Thymol is a monoterpenoid found in *Thymus vulgaris* L. (Lamiaceae) and exhibits antibacterial, antioxidant,6 molluscicidal, antifeedant and insecticidal activity. It showed strong insecticide acitivity, because it enhanced the GABA-dependent chloride currens in oocytes expressing various human GABA receptor isoforms as well as the insect GABA receptor (Ranil *et al*., 2009). Thymol also potentiated the binding of tert-butyl bicycloorthobezoate. Likewis, ivermectin increase chloride ion permeability in invertebrate muscle and nervous tissu through positive modulation of GABA-gated and glutamate-gated chloride channels, and paralytic effects have been reported in dipterans. Therefore, thymol showed the effect on nervous system in the insect, octopamine is a neurotransmitter and a neurohormone in insect, which regulates and desensitizes sensory inputs, excites nerves and maintains rhythmic and more complex processes such as learning and memory. Thus, thymol is a high toxic to *P. xylostella* than other essential oil compounds. (Santos *et al*., 2004). However, the toxic monoterpene was reported on microbial membrane structure and lipophilic character, it will preferentially partition from an aqueous phase into membrane structured results in membrane expansion, increased membrane fluidity and permeability, disturbance of membrane-embedded proteins, inhibition of respiration, and alteration of ion transport processes (Domenico *et al*.2005).

In addition, 1,8 cineole adversely affects the preferences of herbivores for sagebrush, and to our experiment insect *P. xylostella*. In general, terpene are toxic when consumed or dosed at too high concentrations No carcinogenicity, genotoxicity, or reproductive or developmental toxicity has been reported up until now and subacute nephrotoxic and hepatotoxic effects in animal experiments appeared only after the application of high doses , in accordance with a rather high acute oral LD_{50}

in rats of 2.5 g/kg bodyweight (Frauke and Andrea, 2013). In the insect, 1,8-cineole produced 100% contact toxicity in *C. maculatus, R. dominica* and *S. oryzae* adults at the highest dose of 0.1 ml/insect. The LD_{50} values in the topical application assay were 0.03, 0.04 and 0.04 ml/insect for *C. maculatus, R. dominica* and *S. oryzae* respectively (Kishan *et al*, 2001).

Linalool and pulegone were only moderately active against *P. xylostella,* exhibiting < 45% mortality even at a very high dose (10,000 ppm). This dosage variability is obvious from other studies, too; for example, citral from *Cymbopogon citratus* is toxic at 7.7 µg/larva (LD_{50} value by topical application) against *P. xylostella* (Dadang and Ohsawa, 2008) and thymol required for *Chilo partellus* is as high as 189.7 µg/larva (LD_{50}). Linalool being moderately active against *P. xylostella* suggests that the compounds can even behave defferently among lipdopterans. Linalool is individually efficacious along with 1,8 cineole at a similar level of treatment against *Chilo partellus*, causing extreme agitation (Singh *et al*., 2009). In fact, several essential oil compounds have been reported to block octopamine, neurotransmitter unique to insects that function similarly to epinephrine (adrenaline) and norepinephrine found in vertebrates (Hummelbrunner and Isman, 2001).

Although acute toxicity of the compounds evaluated in this study is a first report on their activity against this pest, many known studies are based on intact oils against *P. xylostella* larvae and *Cotesia glomerata* adults was examined using a vapor-phase toxicity bioassay and compared with that of dichlorvos. Data from these studies suggest that responses varied according to the oil and insect species used (Yi *et al*., 2007). These studies also suggest the merit of further study of essential oils as potential fumigants to control *P. xylostella* in greenhouses. Repellent and oviposition deterrent activities of limonene, α - terpinene, linalool ane verbenone on the diamondback moth were investigated in screen houses where α - terpinene, limonene and linalool had significant effects as repellents and oviposition deterrents, but verbenone and β -caryophylene were not active (Zhang *et al*., 2004). This suggests that linalool has a species-specific action and this may not necessarily be the toxic

action but purely an impact on feeding and reproductive behavior. Our studies have shown that linalool is moderately toxic to *P. xylostella,* but has a significant effects on the behavioral responses of this species (author's unpublished data). Recently, the activity of an essential oil from rhizome extracts of *Acorus calamus* against *P. xylostella* has been reported, showing the toxicity of this oil by feeding and contact methods but at very high concentrations (LC_{50} = 1.33%) (Nitbani *et al*., 2012). However, it would be appropriate to evaluate β -asarone, the established active ingredient in this plant species (Saxena *et al*., 1997; Koul *et al*., 1990).

1.2. Antifeeding effect

The concept of using insect antifeedants as crop protectants is intuitively attractive. Pest management in agriculture, forestry and managed landscapes has often relied on toxic, broad-spectrum insecticides with negative impacts on natural enemies, pollinators and other non-target organisms. And continuous use of specific insecticides has frequently resulted in the development of resistance in the very pests targeted for population suppression. Insect-plant chemical interactions in nature are usually very subtle. Most plant defensive chemicals discourage insect herbivory, either by deterring feeding and oviposition or by impairing larval growth, rather than by killing insects outright. (Isman, 2002).

Choice-test; The essential oil compounds as thymol, pulegone, linalool and 1,8 cineole had strong effective but not kill insects directly. (Luz *et al*., 2010). No-choice test; Pulegone showed more activities than other compounds as 100 % FI at 4 and 6 hours. In contrast, Akhtar and Isman (2004) studied antifeedant and growth inhibitory effects of thymol to 3rd *P. xylostella*, using leaf disc choice that showed feeding deterrents as 20.7 μg/cm^2 From our studies, at 4 hours of no-choice test showed strong $\%FI_{50}$ more than 6 hours may be depend on the periods of biodegradation and temperature conditions. Combined considering the results of choice-test and no-choice test feeding studies were conducted to increase efficacy compounds.

1.3 Efficacy of thymol against to *S. littoralis*.

Oral toxicity of thymol to 3rd *S. littoralis* as showed in Figure 38 , at high concentrations (3,000 ppm) showed the least percentage of mortality. Roman (2005) focused on thirty-four essential oils were tested for insecticidal activity (fumigation or topical application) against larvae *Spodoptera littoralis*. Twenty essential oils applied by fumigation were highly toxic to the third instar of *S. littoralis* larvae. Two essential oils *Nepeta cataria* and *Thuja occidentalis* were highly toxic, with LC_{50} 10.0 ml/m^3. Five essential oils *Salvia sclarea*, *Thymus mastichina*, *Origanum majorana*, *Pogostemon cablin* and *Mentha pulegium* were toxic with LC_{50} between 10.1 and 20.0 l ml/$m^{3.}$ Twentythree essential oils were highly toxic to the third instar of *S. littoralis* larvae after topical application. Eight essential oils *Mentha citrata*, *N. cataria*, *S. sclarea*, *O. vulgare*, *O. compactum*, *Melissa officinalis*, *T. mastichina*, and *Lavandula angustifolia* were highly toxic with LD_{50} < 0.05 µl/larvae.
Thus, thymol showed the negative results to *P. xylostella* when compared with another compounds. Combined considering thymol with another may be showed the additive effect or synergistic.

2. The efficacy of Binary mixtures of these compounds for stability and enhancement of activity

Synergistic effects of complex mixtures are thought to be important in plant defenses against herbivore insects. Plants usually present defenses as a suite of compounds, not as individual ones, and it is thought that the minor constituents may act as synergists, enhancing the effect of the major constituents through a variety of mechanisms. It is frequently noted that the
“original” complex essential oils are considerably more efficacious than the pure compounds derived from them (Roman, 2010). After applied binary mixtures of essential oil compounds to 3rd larvae *P. xylostella* using topical application method the results as showed Table 8.

It is often observed that complex essential oil compounds are more efficacious than pure compounds (Don-Pedro, 1996; Ho *et al*., 1997; Hori, 1998). In our study, both active compounds (thymol + 1,8 cineole) were antagonistic in a mixture. However, linalool and pulegone synergized the activity of both thymol and 1,8 cineole. Notwithstanding their individual activities, linalool and pulegone were also antagonistic in a mixture. These data confirm earlier findings where linalool was shown to be synergistic when combined with 1,8-cineole or terpineol against *C. partellus* (Singh *et al*., 2009), which was not the case in earlier reports of the activity of these compounds, which exhibited only additive effects against *S. litura* larvae (Hummelbrunner and Isman, 2001). Given these finding, som candidate compounds could be selected to develop unique mixtures that could serve as acute toxicants, such as thymol (27%) in *Origanum compactum* Benth (Lamiaceae), linalool (68%) in *coriandrum sativum* L. (Apiaceae) and 1,8 cineole (36.2%) in Eucalyptus (Myrtaceae) (Bakkali et al., 2008). Thus, combinations of compounds are more desirable because the insecticidal spectrum of some binary mixtures is increased. This is shown in our studies where pulegone increase the activity of thymol or 1,8 cineole by about twofold. Thymol and 1,8 cineole are antagonistic to each other, though individualy they are the most active compounds againsts *P. xylostella* larvae, which suggests that both of these compounds may be competing for the same receptor site. Nonetheless, the four compounds evaluated here represent different chemical classes, such as a phenol (thymol), a cyclic ether (1,8 cineole), an alcohol (linalool) and a ketone (pulegone); it is obvious that all four show variability and overlap of activities in the present case as well as in other studies available in the literature (Hummelbrunner and Isman 2001; Koul *et al*., 2008); therefore, no generalizations can be made on the structure and activity of these compounds.

3. The possible mode-of-action on *P. xylostella*.

Several essential oil compounds have been demonstrated to block octopamine, a neurotransmitter unique to insects that functions similarly to epinephrine (adrenaline) and norepinephrine found in vertebrates.Because it is unique to insects, the octopaminergic system is of considerable interest as a target site for control agents.

Octopamine agonists and antagonists act as antifeedants and can have profound adverse effects on insect behavior, with symptoms including knockdown, agitation, hyperactivity, tremors, forced diuresis, convulsions, and death (Hummelbrunner and Isman, 2001).

3.1. Detoxification enzyme.

P. xylostella possesses a versatile and multiple resistance mechanisms to overcome toxicity of insecticides. The faster degradation of insecticides by metabolic enzymes is one such mechanism commonly associated with insecticide resistance. The involvement of glutathione-S-transferase and carboxylesterase in insecticide resistance has been reported in insecticide-resistant strains of many insect species (Mohan and Gujar, 2002). Similar with these research, significant results showed induced level of enzyme. For neuron enzyme as acetylcholine esterase showed increasing level when compaired with control group, Acetylcholinesterase is a key enzyme in the nervous system, terminating nerve impulses by catalysing the hydrolysis of the neurotransmitter acetylcholine. In insects, AChE is the only cholinesterase and possesses a substrate species that is intermediate between that of vertebrate acetylcholinesterases and butyrylcholinesterases In contrast to vertebrate cholinesterases which display a variety of molecular form, the predominant form of AChE in insects is a globular amphiphilic dimer bound to membranes by a glycolipid anchor at the C-terminal of each catalytic subunit (Chai *et al*., 2007).

In these studied, detoxification enzyme of 3rd instars *P. xylostella* after treated with thymol and 1,8 cineole were increased that means which are often more tolerant of insecticides, and thus eliminating enzyme induction would reduce insecticide application rates. As a result, this would decrease insecticide selection pressure in the field and hence delay the development of insecticide resistance. In this respect, various field manipulations of enzyme induction, as listed below, should be important considerations for managing insecticide resistance: (a) treat when larvae are young, because these tend to have lower detoxifying enzyme activities and induction, and thus would probably be more sensitive to insecticides; (b) rotate inductive and

non-inductive plant species if necessary in order to avoid constant enzyme induction in polyphagous insects; (c) adjust insecticide application rates depending on plant and insect species because of their differences in enzyme inducibility; (d) breed plant varieties devoid of inducing allelochemicals; (e) use insecticide/synergist combinations to eliminate enzyme induction; (f) breed plant varieties containing inhibitors of detoxification enzymes as antagonists of enzyme induction; and breed plant varieties containing inducers of organophosphate activation enzymes (microsomal desulfurase and sulfoxidase) to enhance insecticide toxicity (Yu and Hsu, 1993).

In vitro

Carboxylesterase; The increase in carboxylesterase was significant in the survival larvae homogenates of treated group, carboxylesterase, esterase (NA1) and esterase (NA2), which were induced the level of enzyme as 0.73, 0.50, 0.60 fold when compared with the control group. As a results, survival 3rd *P. xylostella* showed the resistance effect to thymol and 1,8 cineole including the faster degradation of insecticides by metabolic enzymes is one such mechanism commonly associated with insecticide resistance (Mohan and Gujar, 2003). Moreover, insect carboxylesterase can be divided into three major classes (intracellular catalytic, secreted catalytic, and neurodevelopmental classes) based on the topology of a phylogenetic tree of known carboxylesterase. Juvenile hormone esterase (JHE) integument esterase, **b**-esterase, and glutactin belong to the secreted catalytic class. Juvenile hormone esterase (JHE), is a carboxylesterase that has attracted great interest regarding its critical role in regulating larval to adult transition in insects and other arthropods. JHE hydrolyzes the key developmental and reproductive hormone, juvenile hormone (JH), and partially regulates its titer. Juvenile hormone (JH) plays a major role in the control of growth,development, metamorphosis, diapause and reproduction in insects (Kontogiannatos *et al*., 2011).

Glutathione-S-transferase; Glutathione-S-transferase (GST) of the 3rd instars larve *P. xylostella* showed induce by thymol and 1,8 cineole (CF as 0.52 and 0.73

fold), compared with control group, so these assay similary with the studied of Balabaskaran *et al* (1989), GST was present in all the developmental stages of *P. xylostella*. The enzyme levels increased rapidly and reached a maximum at the pupal stage and then declined towards adulthood, moreover the resistant strain was found to contain between 3-4 times more glutathione-s-transferase the susceptible strain, it is well known that higher GST do occur in resistance strains of of *P. xylostella.* From previous study of Maheswaran and Lgnacimuthu (2012) using the oils of neem (*Azadirachta indica*), karanj (*Pongamia glabra*) tested for larvicidal, ovicidal and oviposition deterrent activities against *Aedes aegypti* and *Aedes albopictus ,* showed similary investigate of enzyme it was 10.4814□±□0.23 and 11.4811□±0.21μmol/min/mg. GST are on of the most general and efficient xenobiotic detoxication systems in all animals. In insects, GST have been induced and is becoming recognized for their importance in the metabolic detoxication of insecticides, of allelochemicals from host plants, in protecting insects from the toxic effects of active oxygen species and for the practical role of GST induction in turning on the detoxifying enzymes enhancing the defense machinery, speeding the development of resistance and causing cross-tolerance to other pesticides (Sívori *et al*., 1997).

Acetylcholinesterase; Acetylcholinesterase is a key enzyme in the nervous system, terminating nerve impulses by catalysing the hydrolysis of the neurotransmitter acetylcholine. In insects, AChE is the only cholinesterase and possesses a substrate specificity that is intermediate between that of vertebrate acetylcholinesterases and butyrylcholinesterases (Chai *et al*., 2007). The levels of enzyme in these experiment were increased as 0.94 fold to both of thymol and 1,8 cineole, the result as showed important role to be resistance, similary with the experiments of Mona (2011), who studied the bioactivities of marjoram essential oil against immature stages and adults of potato tuber moth *Phthorimaea operculella* using fumigant bioassay. Insignificant increases were found in the activities of acetylcholinesterase.

In vivo

Carboxylesterase; Thymol and 1,8 cineole induced the level of carboxylesterase in 3rd *P. xylostella* similar with *in vitro* assay, which showed the activity as 0.98, 1.00 and 1.25 fold respectively of carboxylesterase, esterase (NA1) and esterase (NA2). Therefore, thymol and 1,8 cineole had a fast degradation and *P. xylostella* will be resistance to them. Scarcella *et al* (2012), they studied the activity of carboxylesterase (*in vivo*) of *Fasciola hepatica* exposed to triclabendazole and observed a statistically significant increase in enzyme activity at 24 and 48 h post treatment ($P < 0.01$ and $P < 0.001$, respectively). In contrast, Sarita *et al* (2010) studied the effect of indoxacarb and treated to *P. xylostella* for 10 generations to develop a resistant strain and biochemical analysis of indoxacarb resistance in different tissues of *P. xylostella* was carried out. As a results, found maximum esterase activity in gut homogenates of indoxacarb resistant strains followed by whole body and cuticle homogenates. In gut homogenates of indoxacarb resistant strains, maximum increase in esterases was found as compared to the unselected strain.

Glutathione-s-transferase; For survival 3rd *P. xylostella* after exposed via topical application method, which showed the activities increasing was 1.15 fold and 1.09 fold of thymol and 1,8 cineole. The high level of enzyme indicating that *P. xylostella* could be development resistance as same as *in vitro* assay. In the other hand, Iason *et al* (2001) have been reported that high levels of glutathione-s-transferase with high resistance to pyrethroids do exist for *Spodoptera littoralis*, *Tribolium castaneum*, and *Aedes aegypti*. Induction of glutathione-s-transferase by pyrethroids has also been reported for the honey bee, *Spodoptera frugiperda* and German cockroach. Moreover, glutathione-s-transferase can metabolize insecticides by facilitating their reductive dehydrochlorination or by conjugation reactions with reduced glutathione, to produce water-soluble metabolites that are more readily excreted. In addition, they contribute to the removal of toxic oxygen free radical species produced through the action of pesticides (Enayati *et al*, 2005).

Acetylcholinesterase; The activities of this enzyme increasing after exposed as 0.62 and 0.79 fold of thymol and 1,8 cineole when compared with the susceptible group. Acetylcholine is a key enzyme responsible forterminating the nerve impulse by catalyzing the hydrolysis of neurotransmitters in the nervous system. In addition, Rameshwar (2010) found that several essential oils from aromatic plants, monoterpenes and natural products have been shown as inhibitors of AChE against different insect species. Essential oil of *Zingiber officinale* was found to alter the behaviour and memory in cholinergic system, while linalool was identified as an inhibitor of acetylcholinesterase. The alkaloids viz. berberine, palmatine and sanguinarine, are toxic to insects and vertebrates.

CONCLUSION AND RECCOMMENDATIONS

Conclusion

This research, focused on the effect of essential oil compounds, thymol, 1,8 cineole, linalool and pulegone against to 3rd instar larvae *P. xylostella* using topical application method including antifeedant method (choice and no-choice test) using leaf disc assay. To study the efficacy of thymol on 3rd *S. littoralis* by oral toxicity test. In addition, analyzing on the mode of action of detoxification enzyme viz, carboxylesterase, glutathione-s-transferase and neuron enzyme (acetylcholinesterase).

For topical application method on 3rd *P. xylostella* treated with thymol which showed the highest efficacy, $LD_{50,}$ as 284.44 ppm at 24 hours. The effective of 1,8-cineole was showed LD_{50} values as 564.55 ppm after 24 hours. Moreover, linalool showed the 16 percentage of mortality at 24 hours at dose 10,000 ppm whereas, pulegone shows 20% mortality at the same dose (10,000) ppm.

Antifeedant of no-choice test, Pulegone had the highest effective compared to other compounds as FI_{50} was 1,329.61 (ppm) after 4 hours. For choice test, all of compounds showed feeding inhibition as 100%, at the highest dose (500 ppm). Which thymol showed the highest efficacy 24 hours, FI_{50} was 50.10 ppm.

Oral toxicity of thymol against to 3rd *S. littoralis,* the larvae were fed with artificial diet mixtures with thymol for 5 days. As a result, the weight were decreased when treated with the highest dose.

For binary mixtures, The most importance synergism was found in thymol in the mixture with pulegone and 1,8 cineole in the mixture with pulegone. Out of the 15 mixtures tested, 6 showed synergistic effect, 9 showed antagonistic effect just like thymol in the mixtures with 1,8 cineole and 1,8 cineole with linalool.

The mode of action of detoxification enzyme, all detoxification enzymes ane neuron enzyme were induced for both *in vitro* and *in vivo* assay.

Recommendations

1. The binary mixtures of essential oil compounds should be done with antifeedant method, Since every plant species containing a difference composition or mixture based on essential oil, So individual substances contained in oils can show significant mutual synergistic support of their biological effect.

2. We should be study the effect of juvenile hormone after expose, which play important roles in the regulation of physiological processes such as molting, metamorphosis, reproductive maturation and pheromone biosynthesis.

3. Detoxification enzyme, we should be done cytochrome P450. They can oxidize wildly substancesn extremely important metabolic system because of their involvement in regulating the endogenous compounds and xenobiotics such as pesticide drug plant toxins.

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CURRICULUM VITAE

NAME : Ms. Nutchaya Kumrungsee

BIRTH DATE : February 6, 1985

BIRTH PLACE : Nan, Thailand

EDUCATION :

YEAR	INSTITUTE	DEGREE/DIPLOMA
2006	Chiangrai Rajabhat Univ.	B.Sc. (Environmental Science)
2010	Kasetsart Univ.	M.Sc. (Zoology)

POSITION/TITLE :-

WORK PLACE : Faculty of Science, Kasetsart University

SCHOLARSHIP/AWAEDS : Fund from Faculty of Science Kasetsart University (BRC) 2013

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