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TITLE: Bio-efficacy of allelochemicals from *Wedelia trilobata* (L.) Hitch (Asteraceae) against *Plutella xylostella* (Lepidoptera: Plutellidae)

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THESIS

BIO-EFFICACY OF ALLELOCHEMICALS FROM *WEDELIA TRILOBATA* (L.) HITCHC (ASTERACEAE) AGAINST *PLUTELLA XYLOSTELLA* (LEPIDOPTERA: PLUTELLIDAE)

PUNTIPA JUNHIRUN

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Puntipa Junhirun 2013: Bio-efficacy of allelochemicals from *Wedelia trilobata* (L.) Hitchc (Asteraceae) against *Plutella xylostella* (Lepidoptera: Plutellidae). Master of Science (Biology), Major Field: Biology, Department of Zoology. Thesis Advisor: Assistant Professor Vasakorn Bullangpoti, Ph.D. 73 pages.

This research prospective was investigated efficacy of *Wedelia trilobata* (L.) Hitchc (Asteraceae) (BK 064385) to control *Plutella xylostella* (Lepidoptera: Plutellidae). *W. trilobata* dried leaves were extracted with sequential polarity solvent; hexane, dichloromethane, ethyl acetate and ethanol, respectively by using Soxhlet's apparatus. The topical application assay was used to determine toxicity on 2nd instars larvae of *P. xylostella*. Ethyl acetate crude extract was the most effective as the results presented yield was 0.2130% w/w, LD_{50} value at 24 hours was 358.39 ppm and LC_{50} value at 48 hours was 316.82 ppm. The alkanes mixture were consisted of Nonacosane 31.02%, Hexacosane 16.70%, Heptacosane 15.77%, Pentacosane 13.45%, Octacosane 10.45%, Tetracosane 4.20% and Docosane 1.42%. The mortality percentages were 44.16% and 66.67% after exposed with 400 ppm of alkanes mixture 24 hours and 48 hours, respectively. Mode of action of insect enzyme activity showed carboxylesterase was inhibited by ethyl acetate crude extract and acetylcholinesterase was inhibited by alkanes mixture after exposed 24 hours.

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Student's signature Thesis Advisor's signature

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Puntipa Junhirun

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LIST OF ABBREVIATIONS

α-	=	Alpha-
β-	=	Beta-
BHT	=	Butylated hydroxytoluene
cm	=	Centimeter
°C	=	Degree Celsius
CH_2Cl_2	=	Diochloromethane
EtOAc	=	Ethylacetate
EtOH	=	Ethanol
MeOH	=	Methanol
g	=	Gram
hr	=	Hours
LD_{50}	=	Median lethal dose
kg	=	Kilogram
µg	=	Microgram
µl	=	Microliter
mg	=	Milligram
min	=	Minute
mM	=	Millimolar
mol	=	Mole
nm	=	Nanometer
%	=	Percent
rpm	=	Revolutions per minute
ppm	=	Parts per million

BIO-EFFICACY OF ALLELOCHEMICALS FROM *WEDELIA TRILOBATA* (L.) HITCHC (ASTERACEAE) AGAINST *PLUTELLA XYLOSTELLA* (LEPIDOPTERA: PLUTELLIDAE)

INTRODUCTION

The diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae), is a serious pest of cruciferous crops reported for more than 128 countries (Saeed *et al*., 2010) including Thailand (Harcourt, 1956). *P. xylostella* is known for its ability to use alternative hosts like weeds, has dispersal ability and capacity to develop resistance against wide range of insecticides such as pyrethroids and nornicotenoids (Vanichpakorn *et al*., 2010) against permethrin in Taiwan (Cheng, 1981), against DDT, diazinon, fenvalerate and permethrin in Hawaii (Tabashnik *et al*., 1987). In Thailand, they had also shown resistance against phenthoate, prothiophos, pyraclofos, mevinphos, synthetic-pyrethroids and insect growth regulators like chlorfluazuron, teflubenzuron, methoprene, and NK-081 (Rushtapakornchai *et al*., 1990). This resistance develops through different physiological mechanisms, reduction in chemical penetration, decreased nerve sensitivity and degradative metabolism involving enzymes such as glutathione-S-transferase (GSH), microsomal P-450 monooxygenase and carboxylesterases (Mohan *et al*., 2003).

Indiscriminate use insecticides have resulted in resistance, decreased production and adverse environmental impacts. Moreover, environment friendly alternative methods could be solution in Integrated Pest Management (IPM) systems such as botanical insecticides has low rates and biodegradable. Some studies has clearly demonstrated that botanical products like *Veratrum nigrum* extracts (Vanichpakorn *et al*., 2010), *Syzygium aromaticum* flower extracts, *Azadirachta indica* seed extracts (Schmutterer, 1990) and *Acalypha fruticosa* Forssk extract can control *P. xylostella* (Kamaraj *et al*., 2008).

In view of the potential of plant products to control *P. xylostella* to a reasonable extent; present study focuses on bioefficacy of *Wedelia trilobata* (L.) Hitch and allelochemicals against various stages of *P. xylostella*. *W. trilobata* belongs to the family Asteraceae which thrives very well in hot and warm climates. It has least role to play in agriculture and economy, grows rapidly and difficult to eradicate therefore, a suitable source for the development of an insecticidal product.

There are studies available, which show that *W. trilobata* contains several active compounds that can reduce pest attack. For instance, kauranes are molluscicidal against golden apple snail (Rezende *et al.*, 2000) and anti-feedant for cotton boll weevils (Howard *et al.*, 1990). They are also intermediate in the biosynthesis of the gibberellins, which are plant growth hormones that inhibit growth of weeds (Michael *et al.*, 2009) and some fungal metabolites of diterpene alkaloids (Ghisalberti, 1997). *W. trilobata* is also known as source of herbal drugs that act as antimicrobial, antiparasitic, anti-HIV and anti-inflammatory products (Taddei and Rosas-Romero, 1999).

Although various reports suggest the efficacy of this plant against variety of pests, there is no report bioefficacy of *W. trilobata* against *P. xylostella* and its study would help in developing a safer botanical pesticide against diamondback moths. Therefore, *W. trilobata* study is being taken up with following objectives.

OBJECTIVES

1. To study the bio-efficacy of *Wedelia trilobata* (L.) Hitch extracts against diamondback moth, *P. xylostella*.

2. To isolate active ingredients from *W. trilobata* and study their efficacy comparative to extracts against *P. xylostella*.

3. To study the possible mode-of-action at biochemical level.

LITERATURE REVIEW

1. Diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae)

P. xylostella is a common Lepidoptera pest of Brassica family including cabbage, collards, turnip greens, mustard greens, broccoli, cauliflower, etc. This insect has long history of developing resistance to insecticides, beginning with DDT in 1953. Subsequent studies have also shown resistance against many commercial insecticides; carbamate, carbofuran, synthetic pyrethroid, and benzoylphenyl urea (Cheng, 1981). The more effective new insecticides relate to more rapidly selection for resistance seems to occur.

In Thailand, *P. xylostella* disperse well in February to April which is the most optimum climate for their host plants. *P. xylostella* is serious pest of cruciferous crops in Thailand for a long time and only choice for control of this pest is using of organochlorine, organophosphate, carbamates, pyrethroid, insect growth regulators, abamectin, pyrazole, oxadiazine, and neonicotinoid (Vanichpakorn *et al*., 2010).

1.1 Biology and Life cycle of *P. xylostella* (HDRA, 2000)

Insect taxonomy of *P. xylostella*

Phylum Arthopada
Class Insecta
Order Lepidoptera
Family Plutellidae
Genus *Plutella*
Species *P. xylostella*

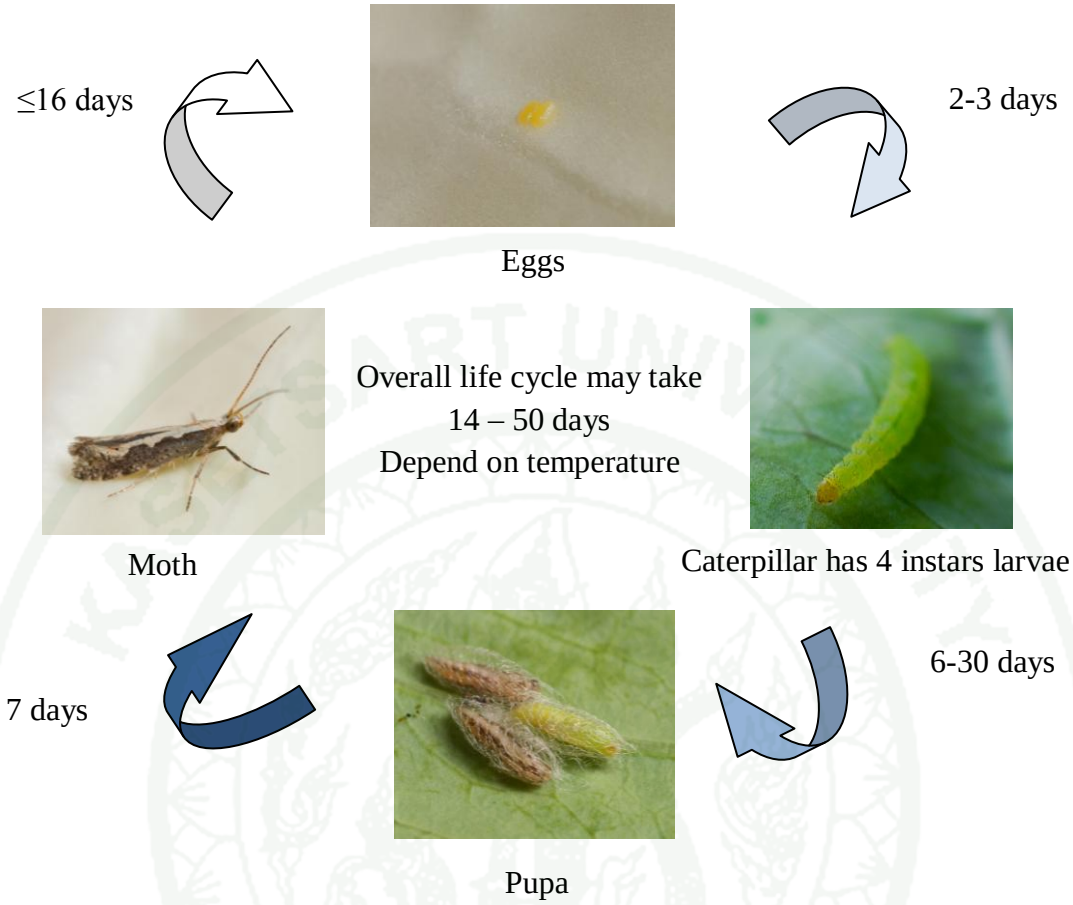


Figure 1 Life cycle of diamondback moth

Life cycle: The female moth lays pale-yellow eggs on the top of the leaf. Females may deposit 250 to 300 eggs in life cycle but average total egg production is probably 150 eggs. After 3 to 8 days, caterpillars hatch, crawl to underside of leaf till development is completed and pupae are formed. The adult moths emerge one week later. They might have 10 generations per year. Population can increase rapidly at temperatures above 26°C (Figure 1).

Eggs are less than 1 mm diameter, yellowish, flat and oval in shape. They are laid individually or in groups of 2 to 3 along the veins on the upper and lower leaf surface. The eggs hatch in 3 to 8 days depending on the environmental conditions.

Caterpillars are pale yellowish-green to green covered with fine tiny scattered, erect hair. Mature caterpillars are cigar-shaped and about 12 mm long. They have chewing mouthparts. The caterpillars go through four instars and normally complete development within 6 days however in adverse condition it may extend to 30 days. *P.xylostella* caterpillars are easily identified because they wriggle violently when disturbed, drop from the plant suspended by a silken thread and finally climb their way back up and continue feeding.

Pupae are 5 to 6 mm in length. Pupae are initially light green and turn brown as the adult moths become visible through the pupa. They are covered with a loosely spun net-like cocoon that is attached to the leaves, stems or seedpods of the host plant. The moths emerge within 7 days after pupation depending on the environmental conditions.

Adult moths are small approximately 8 to 9 mm long with a wingspan of 12 to 15 mm. They have diamond-shaped markings on back when wings are folded, which gives the common name “Diamondback moth”. The moths fold their wings over the abdomen when resting. The wing tips are fringed with long hair. Adult females can lay an average of 150 eggs during their lifespan (16 days). Moths lay eggs at night. Most of eggs are laid in the first night after emergence and subsequently continue up to 10 days.

1.2 *P. xylostella* management using botanical pesticide

Botanical products have been evaluated for their toxicity (Table 1), antifeedant and larvicidal activity against *P. xylostella* such as *Azadirachta indica* seed extract (Robert and Denis, 1993), *Derris elliptica* Benth root extract (Visetson and Milne, 2001), *Annona squamosa* seed extract (Andrey and Isman, 2004 and Dadang *et al*., 2009), *Syzygium aromaticum* flower extracts (Kumnuan, 2006), *Piper sarmentosum* Roxburgh leaves extract (Orratai, 2008) and *Mammea americana* seed extract (Kritchaya *et al*., 2011).

Table 1 The median lethal dose (LD_{50}) of plants extracts on *P. xylostella* larvae in toxicity bioassay.

Scientific name	Common name	24h LD_{50} value (ppm)
Azadirachta indica	Neem	0.54
Derris elliptica	Derris	24.25
Annona squamosa	Annona	0.10
Syzygium aromaticum	Clove	1.09
Piper sarmentosum	Wild betal Leaf bush	4.34
Mammea americana	Mammey	5.90

2. *Wedelia trilobata* (L.) Hitch

2.1 Plant taxonomy

Wedelia trilobata (L.) Hitch is a member of the family Asteraceae (formerly Compositae), the sunflower family (Figure 2). Its common names include "wedelia" and "trailing daisy", although some people mistakenly call it "Singapore daisy". In a wider sense the genus *Wedelia* was named in honor of Georg Wolfgang Wedel (1645-1721), Professor of Botany at Jena, Germany (Garcia *et al*., 1975).

Plant taxonomy (Thaman, 1999; Watson and Dallwitz, 2000)

Division Magnoliophyta
Class Magnoliopsida
Subclass Asteridae
Order Asterales
Family Asteraceae (Compositae)
Genus *Wedelia*
Species *W. trilobata*

Figure 2 *Wedelia trilobata* (L.) Hitch

2.2 Propagation and Beneficial

W. trilobata thrive well in both sun and shade, but best flowering is seen when plants get enough sun. It grows well in any soil when provided with ample moisture and fertilizer. *W. trilobata* does well over a range of soil pH, but pH 5.5 to 7.5 is preferred. *W. trilobata* is one of the most common and dependable groundcovers in Hawaii. It has excellent erosion control on slopes and banks because it roots where the stem comes in contact with the soil. It may be growed under trees, around rocks and ponds, and as a covering in parkways. It grows well when allowed to trail over planters walls or is used as a basket plant. The plant has medium tolerance to drought (Heansley, 1997).

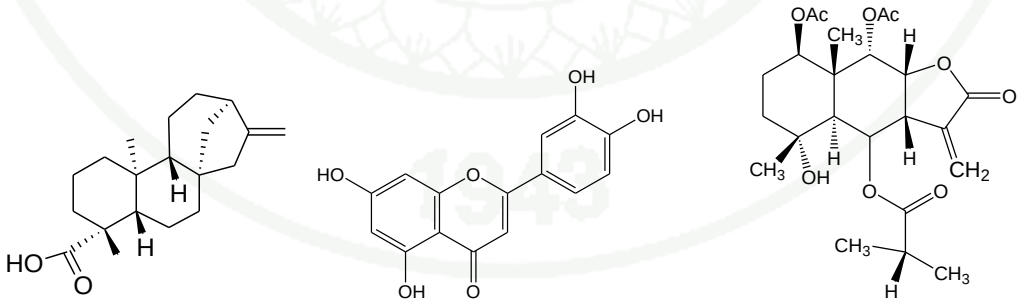
2.3 Characteristics

W. trilobata is now cultivated as an ornamental groundcover in many areas of the tropics and subtropics. It is a creeping, mat-forming perennial herb with fast-growing rounded stems up to 40 cm long or longer that root at the nodes and grow upwards (ascend) when flowering (Rauch, 1979). When young, the stems are coarsely hairy. The leaves are opposite and simple, the blade ovate is, 2 to 9 cm long and 2 to 5 cm wide, acute at the apex and winged and sessile at the base. They are attractive, bright shiny green, somewhat fleshy, and irregularly toothed on the margins, commonly with three shallow lobes (hence the name *trilobata*). The single attractive bright-yellow daisy- like flower is borne on the end of terminal and axillary stalks (peduncles) 2 to 9 cm long, with 2 to 4 series of bracts forming the involucres at the base of the flower. The ray florets, mostly 8 to 13, are 6 to 15 mm long having 1- to 3-teeth and are pistillate. The numerous disc florets are tubular, 4 to 5 mm long, and mixed with chaffy bracts. The ray and disc florets are both yellow. The fruit is a 2- to 4-angled achene with short and narrow pappus scales on the top (Whistler, 1995).

2.4 Chemical composition and bioactivity

Many compounds have been isolated from *W. trilobata* (Taddei and Romero, 1999). However, leaves and stems contain many compounds such as, Kaurenoic acid (Ent-16-kauren-19-oic acid) (Figure 3 A) which has interesting biological properties including analgesic (Block *et al.*, 1998), antifungal (Sartori *et al.*, 2003) smooth muscle relaxation (Alencar *et al.*, 2003), and hypoglycemic effect on diabetic rats (Bresciani, 2004). Moreover, derivative of Kaurane is intermediate of gibberellins; the plant growth hormones (Ghisalberti, 1997), which is antimicrobial, antiparasitic, anti inflammatory and insect antifeedant (Rezende *et al.*, 2000). Luteolin bioflavonoid (2-(3, 4-Dihydroxyphenyl)-5, 7- dihydroxy-4-chromenone) (Figure 3 B) is anti-allergic, anti-inflammatory, and smooth muscle-relaxant. It also prevents many respiratory disorders, including asthmatic conditions and chronic bronchitis (Wang, 2000). Antitumor, mutagenic and antioxidant effects, depressant action on smooth muscles and a stimulant action on isolated guinea pig heart are also well known (Block *et al.*, 1998).

Recently new compound has been isolated from flowers of this species; Trilobolide-6-O-isobutyrate (Figure 3 C) which is sesquiterpene lactones did not describe on biological activity yet (Huang, 2003; Maldini *et al*., 2009).



Kaurenoic acid Luteolin Trilobolide-6-Oisobutyrate

A B C

Figure 3 Structure compounds of *W. trilobata*

Source: Huang (2003); Maldini et al. (2009)

3. Mode of action study of insect enzyme activity

3.1 Carboxylesterase activity

Carboxylesterase (CEs) metabolism plays important role in *P. xylostella* resistance. It can be utilized for enhancing toxicity of insecticides and pesticides such as malathion and permethrin by using corresponding alcohol and carboxylic acid, hydrolysis (Figure 4) of tri-acylglycerols and can trans-esterify fatty acids to fatty acid ethyl esters of variety of esterified drugs like meperidine, cefuroximine axetil, cefpodoxime proxetil, cocaine and heroin. CEs of insects are located in cytosol, microsomes; mitochondria and nuclei (Bullangpoti, 2007). Increase of CEs is used for devising biochemical diagnostic methods of detection of insecticide resistance (Brown and Brogdon, 1987). The role of CEs as biochemical diagnose tool for benzoate and lambdacyhalothrin insecticide resistance in *P. xylostella* has reported higher level activity of CEs (Botwe *et al*., 2012).

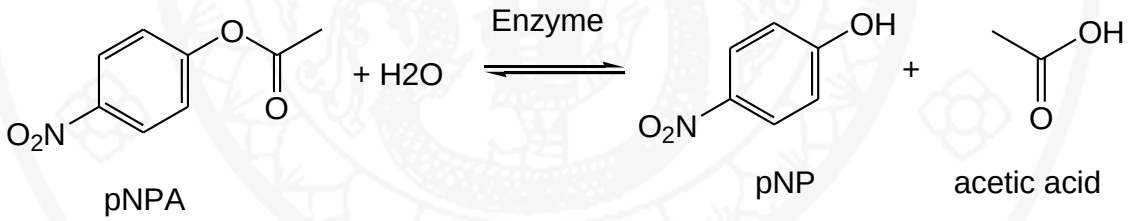


Figure 4 Reaction of pNPA assay

Source: Ganske (2009)

3.2 Glutathione-S-transferase (GST) activity

Glutathione-S-transferase plays important role in detoxification of organophosphate and oganochlorine by catalyzes the formation of thiol group (Figure 5) of glutathione to electrophilic xenobiotics which provides ability to scavenge toxic compound like oxidative stress. The role of GST as biochemical diagnose tool for methyl parathion resistance in *P. xylostella* has reported increased expression of this GST *isozyme*. (Huey *et al*., 1998)

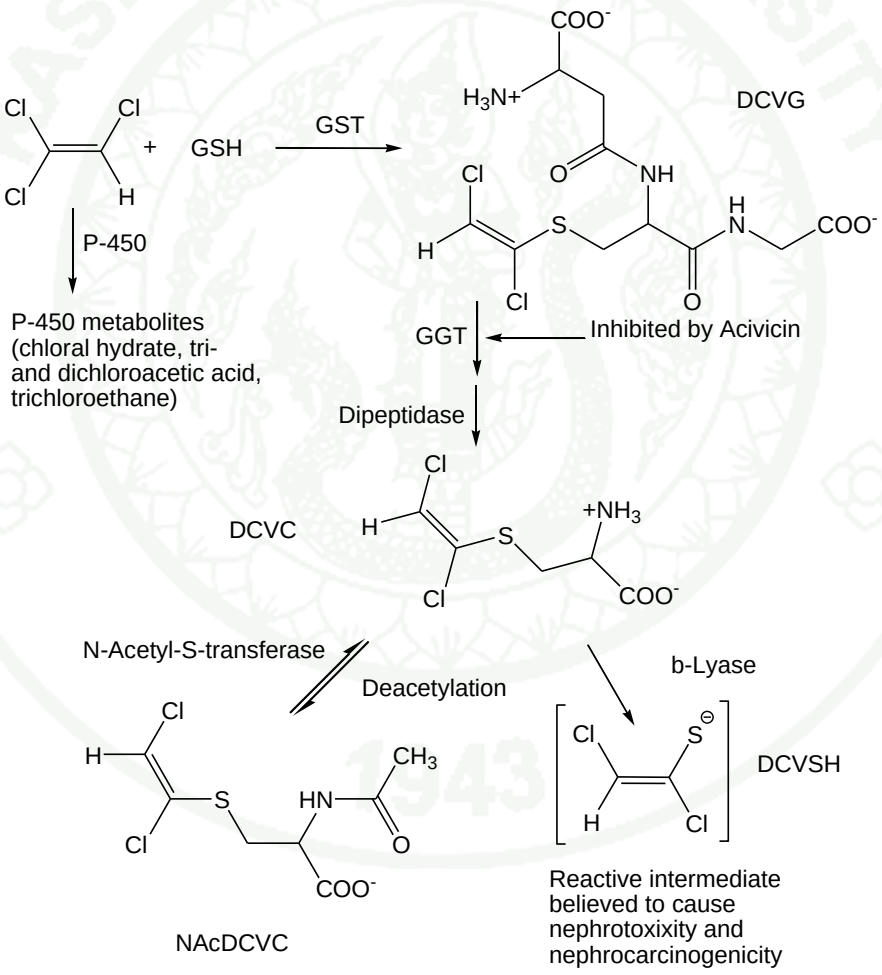


Figure 5 Scheme GSH-conjugation pathway

Source: Brian (2000)

3.3 Acetylcholinesterase activity

Acetylcholinesterase (AchE) is significant factor to resist organophosphate and carbamate compounds. This enzyme plays resistant role to insecticide by phosphorylation (Figure 6) and decrease activity. Cause the accumulation of acetylcholine reducing sensitivity of synapses, stimulate neurotransmission of central nervous system and peripheral nervous system occurring changing behavior in insect call chemical avoidance. The role of AchE as biochemical diagnose tool for insecticide resistance in *P. xylostella* has reported of prothiofos inhibition AChE for paraoxon (Ji Hyung Baek *et al*., 2005).

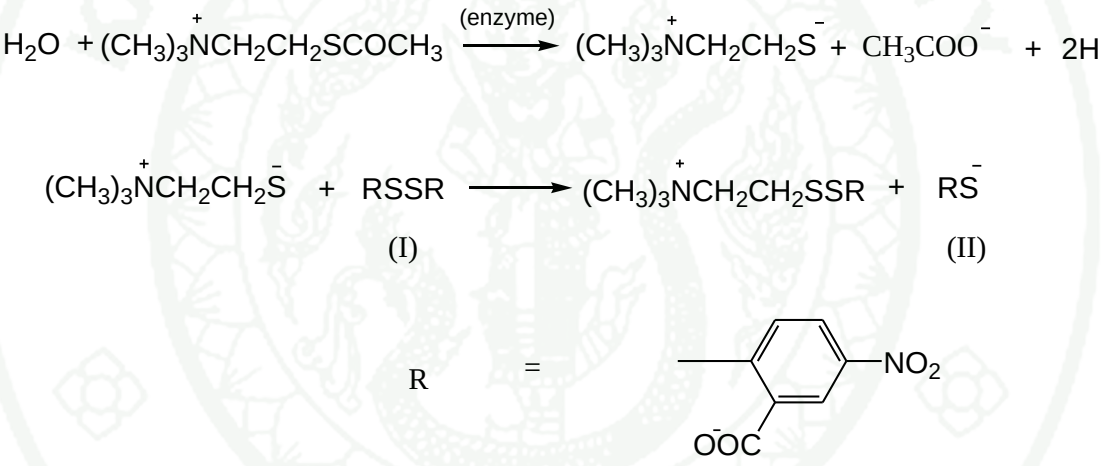


Figure 6 Reaction of acetylcholine assay

Source: Geoge (1960)

3.4 General esterase activity

Esterase activity variation has been associated with organophosphate, pyrethroid and chitin insecticide resistance protecting the target site by catalyzing and hydrolysis of insecticide (Figure 7) or scavenging action like alternative target esterase have wide range specific with substances with ester group. In insects, esterase enzymes are accumulated in adipose cells and intestine (Bullangpoti, 2007). Normally founds in cytosol, mitochondria and nuclei. The role of esterase as biochemical diagnose tool for malathion resistance in *P. xylostella* has reported significant higher frequency of esterase in zymogram study using 1-naphthyl acetate (William and Sin, 2000).

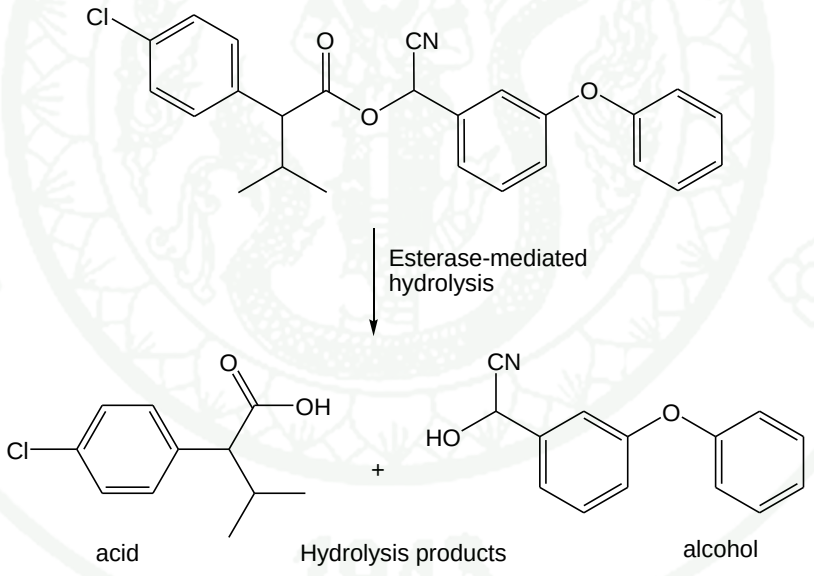


Figure 7 Esterase detoxification mechanisms by hydrolyzed pyrethroid esfenvalerate to acid and alcohol

Source: Craig (2005)

MATERIALS AND METHODS

Materials

1. 96-well plate (Axygentechnology)
2. Blender (Moulinex)
3. Cages (24x24x24 inch)
4. Cellulose extraction thimble 28x100 mm (Whatman®)
5. Centrifuge (Hettichi, Universal 32)
6. Feeding boxes (20x10x4 cm)
7. Filter paper no.1 (Whatman®)
8. Freeze dryer (Flexi Dry™, µp)
9. Glass column (10x10 cm)
10. Hot air oven (Memmert-600)
11. Micropipette 0.5-10, 10-100 and 100-1,00 µl (DENVILLE, XL3000i™)
12. Microplate reader (Biotek, Powerwave 340)
13. pH meter (Cyberscan, 500^{PH} Eutech)
14. -25°C Refrigerator (Brandt)
15. Rotary evaporator (Buchi,Vacuum controller V-850)
16. Soxhlet apparatus
17. TLC plates (20x20cm)
18. Vacuum pump (Büchner)
19. Vortex (VELP Scientifica)
20. Weighing apparatus (Mettler- Toiedo)

Chemicals

1. 1-Chloro-2, 4-dinitrobenzene (CDNB) (ALDRICH)
2. 5,5'- Dithiobis (2-nitro-benzoic acid) (DTNB) (Sigma®)
3. Acetone (J.T. Baker)
4. Acetyl cholinesterase (AchE) (Sigma®)
5. Bovinserum albumin (BSA) (Sigma®)
6. Bradford solution (Sigma®)
7. Chloroform-D1 ($CDCl_3$)
8. Dichloromethane (CH_2Cl_2) (J.T. Baker)
9. Dimethylsulfoxide (DMSO) (Amresco®)
10. Disodium hydrogen phosphate (Na_2HPO_4) (Sigma®)
11. Distilled water (GIBCO™)
12. Ethanol (EtOH) (Mallinckrodt)
13. Ethyl acetate (EtOAc) (LAB-Scan)
14. Ethylenediaminetetraacetic acid (EDTA) (UNIVAR)
15. Fast Garnet GBC base (Sigma®)
16. Glycerin (Sigma®)
17. Hexane (MERCK)
18. Hydrochloric acid (HCl) (Sigma®)
19. L-Glutathione reduced (GST) (Sigma®)
20. Methanol (MeOH) (Mallinckrodt)
21. Paranitrophenyl acetate (pNPA) (Sigma®)
22. Polyvinyl poly pyrrolidone (PVPP) (ALDRICH)
23. Potassium phosphate (KH_2PO_4) (UNIVAR)
24. Silica gel no. 1.07731 and 1.07734 (Whatman®)
25. Sodium chloride (NaCl) (Sigma®)
26. Sodium dodecyl sulfate (SDS) (Fluka)
27. Sodium hydroxide (NaOH) (J.T. Baker)
28. Sodium sulfate anhydrous(Garloerba)
29. Tris (hydroxymethyl) aminomethane (Tris-HCl) (Sigma®)
30. Triton X-100 (Sigma®)

Methods

1. Insect rearing

P. xylostella (Figure 8) were obtained from organic farm at Nonthaburi province, Thailand. *P. xylostella* were reared in insect rearing room of Zoology Department of Zoology, Faculty of Science, Kasetsart University. Fresh Kale leaves were fed for *P. xylostella* on tissue papers to decreased humidity in a cage. Kale leaves and tissue papers were replaced daily; cages were cleaned by detergent every week in order to protected fungal infection. Pupae were observed and carefully separated into net cage; moth fed with sugar solution. Fresh kale leaves or cabbage leaves were kept in net cage for Moth oviposition. *P. xylostella* were maintained in laboratory at 25±2°C, 70% RH and a photoperiod of 12: 12 (L: D) hours without exposure to any xenobiotic. *P. xylostella* 2^{nd} instar larvae were used CRD for the toxicity assay.

(A) (B) (C) (D)

Figure 8 (A) Eggs, (B) Larva, (C) Pupae and (D) Adult moth of *P. xylostella*

2. Plant material

W. trilobata (Figure 9) were collected from Kasetsart University, Thailand in July 2011 subsequently separated young leaves and rinsed by water and dried in shade for three days. Dried leaves had also been powdered and stored in zip-lock bags in refrigerator at 4°C to prevented sample contamination. A voucher specimen (BK 064385) had preserved in Princess Sirindhorn Plant Herbarium of Plant Varieties Protection Division, Department of Agriculture Thailand.

Figure 9 Sampling of *W. trilobata*

3. Extraction

The dried leaves powders of 200 g *W. trilobata* were extracted by a Soxhlet's apparatus (Figure 10) with the solvents in sequential order polarity solvents: hexane, dichloromethane, ethyl acetate and ethanol (Figure 11). Each crude extract was filtered with vacuum pump and dried by using rotary evaporator (Figure 12) and stored at 4^0C until further use in the experiments.

Figure 10 *W. trilobata* extraction by using Soxhlet's apparatus

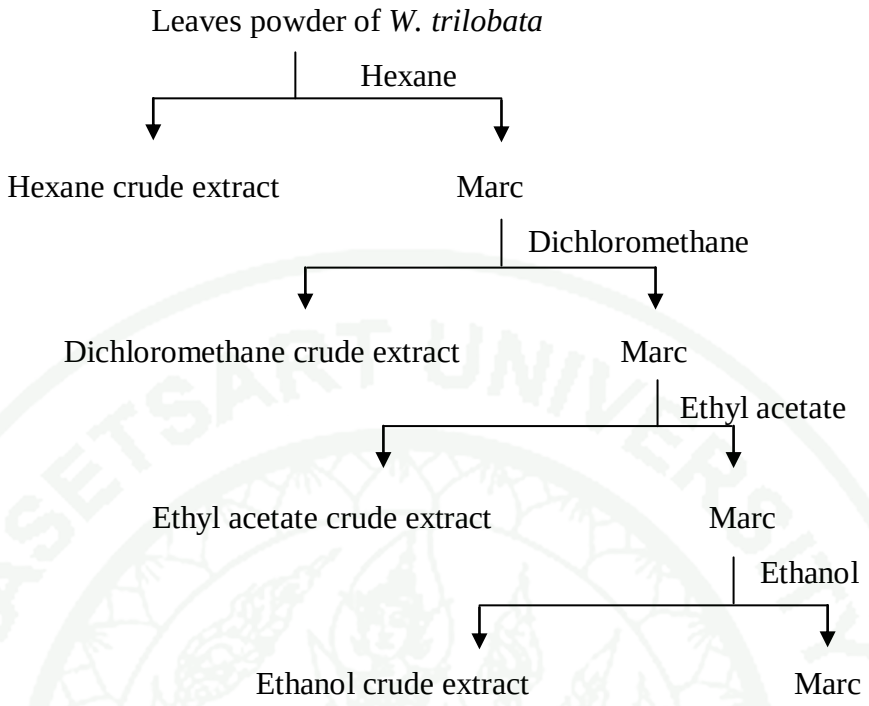


Figure 11 Extraction of *W. trilobata*

(A) (B)

Figure 12 Vacuum pump filter (A) and Evaporation (B)

4. Isolation

The highest efficacy of *W. trilobata* crude extracts were selected to isolate by using Quick Column Chromatography and Preparative Thin Layer Chromatography (PTLC). Quick Column Chromatography was first separated group of non-polarity compound, low-polarity and high-polarity. Ethyl acetate crude extract (7g.) was dissolved with minimum solvent of CH_2Cl_2 which easier evaporation and mixed with silica gel (no. 1.07731). Mixed silica gel was loaded into packed silica gel column. The solvents using in sequential order 100% hexane, 5% EtOAc: hexane - 100% EtOAc, 5% MeOH: EtOAc - 20% MeOH: 80% EtOAc and finally collected with 100% MeOH (Figure 13). Thin Layer Chromatography was used to screening check compounds in each fraction (Figure 14) with suitable solvent system i.e. hexane: EtOAc (3:1) and observed under UV (λ=254nm). Fractions were categorized by similar spots showed on TLC plates. Groups of fraction were tested efficacy on *P. xylostella*.

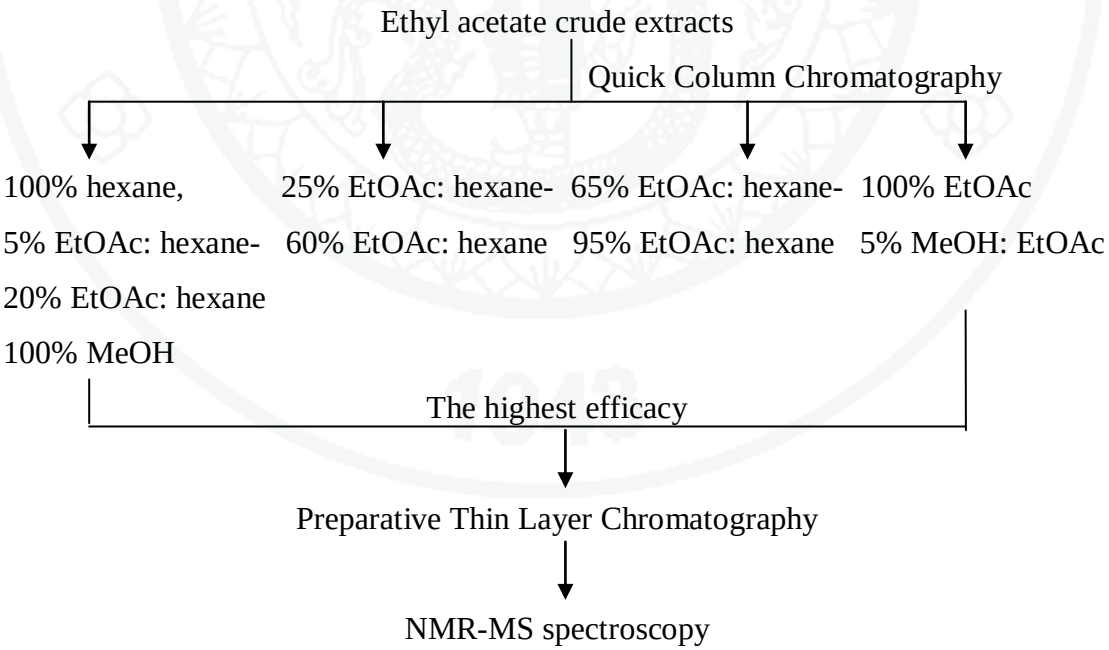
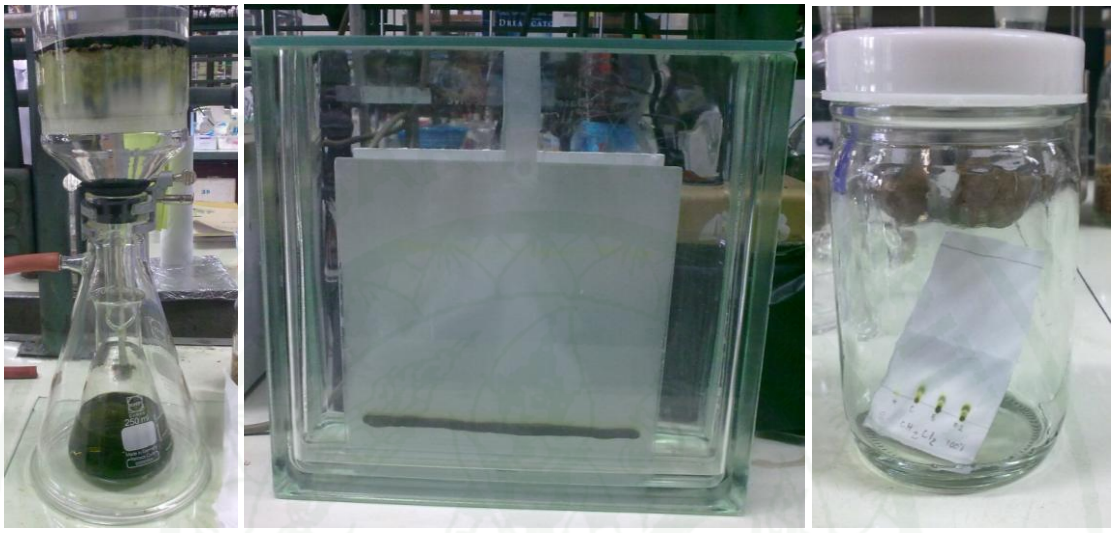


Figure 13 Isolation active compounds in *W. trilobata* Ethyl acetate crude extracts

The highest efficacy was purified by using PTLC with suitable solvent system. Isolated compound was analyzed by Nuclear Magnetic Resonance spectroscopy (NMR spectroscopy) and Mass Spectroscopy (MS) for compound elucidation.



(A) (B) (C)

Figure 14 (A) Quick Column Chromatography, (B) Preparative Thin Layer Chromatography and (C) Thin Layer Chromatography

5. Bio-efficacy against *P. xylostella*

Topical application test

The 2nd instars *P. xylostella* larvae were used to determine median lethal dose (LD_{50}) by topical method with CRD method. Nine concentrations (Figure 15) were prepared with acetone (AR grade). 2µl of each sample were applied using topical applicator on thorax of 2nd instars larvae. Each concentration was tested against 30 larvae in 3 replicates. Mortality were recorded after 24 and 48 hours after exposed. LD_{50} values were analyzed by Probit analysis using StatPlus. *P. xylostella* behavioral response such as paralysis and knock-down were recorded.

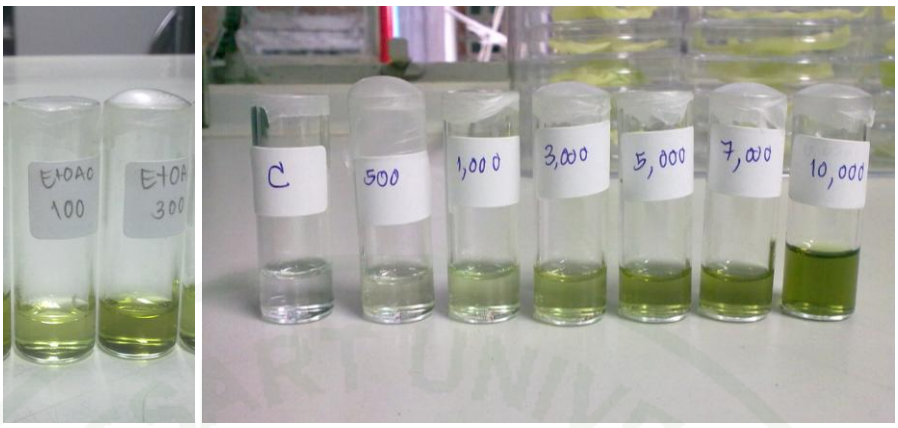


Figure 15 Prepared solution *W. trilobata* extracts

6. Mode of action study of 2nd instars larvae of *P. xylosell*a after treated with crude extracts and active compound of *W. trilobata*

6.1 Preparing insect for enzyme extraction

For *In-vivo* treatment assay, 2nd instars larvae of *P. xylosell*a were treated with leaves crude extracts and active pure compound of *W. trilobata* at LD_{50} value concentration and control group were treated with 95% acetone (10 larvae/ replication). After treated 24 hours, the survival of *P. xylostella* were used for enzyme extraction. For *In-vitro* assay control and treatment groups were used non-treated *P. xylosella* 2nd instars larvae.

6.2 Extraction of enzymes activities method

This method was modified from Feyereisen (2005), survival *P. xylostella* were placed micro tube and kept on ice. After that, grinded with homogenized buffer (0.1M photasium phosphase buffer mixed with 1mM EDTA at pH 8.0). Homogenates solutions were centrifuged at 4°C, 18,000 rpm for 5 minutes. Supernatant were separated and kept on ice and immediately use enzyme activity test.

6.3 Carboxyl esterase enzyme activity

The carboxylesterase activity of pNPA assay was modified method from Ganske (2009). For In-vivo assay, 50 mM phosphate buffer were mixed with supernatant. Then add 10 mM pNPA (paranitrophenyl acetate) and measured by kinetic mode at λ_{max} = 410nm, 37°C. For *In-vitro* assay, 50mM phosphate buffer were mixed with supernatant and treating compound then were incubated at 37°C for 30 minutes. Then added 10 mM pNPA and measured at λ_{max} = 410 nm, 37°C by kinetic mode using micro plate reader. The activity was described as changing of yellow color of paranitrophenol from hydrolysis of paranitrophenylacetate.

6.4 Acetylcholinesterase activity

Acetylcholinesterase activity method was modified from Ellman (1959). For In-vivo assay, 100mM Potassium phosphate buffer were mixed with supernatant 50µl and incubated at 30°C for 30 minutes. Then added Tps (Tampon substrate) and measured at λ_{max} = 412nm by kinetic mode. For In-vitro assay, 100 mM Potassium phosphate buffer were mixed with treated compound and incubated at 30°C for 30 minutes. Then added supernatant and incubated at 37°C for 30 minutes. After that added Tps (Tampon substrate) and measured. The activity was described as changing of yellow color generated by reaction of DTNB.

6.5 Glutathione-S-transferase (GST) activity

Glutathione-S-transferase activity of 1- chloro-2, 4'- dinitrobenzene (CDNB) method was follow by Spectrophotometric method. For In-vivo assay, 50 mM phosphate buffer were mixed with glutathion solution, supernatant and CDNB. Then measured at λ_{max} = 340nm using micro plate reader. For In-vitro assay, 50 mM phosphate buffer were mixed with glutathion solutions and supernatant then were incubated at 30°C for 30 minutes and added CDNB. Then measured at λ_{max} = 340nm. The activity was described as changing absorbance of CDNB.

6.6 General esterase activity of 1- naphthyl acetate or 2- naphthyl acetate

The General esterase activity of α-NA and β-NA were modified from Dary *et al.* (1990). For In-vivo assay 10 mM Potassium phosphate buffer were mixed with supernatant. Then, added of α-NA or β-NA and incubated at 30°C for 30 minutes. After that added Fast Granet solution and incubated at 30°C for 5 minutes and measured at λ_{max} = 505nm (α-NA) and 527nm (β-NA). For In-vitro assay 10 mM Potassium phosphate buffer were mixed with supernatant and testing compound, incubated at 30°C for 30 minutes. Then added α-NA or β-NA and incubated at 30°C for 30 minutes. Added of Fast Granet solution and incubated at 30°C for 5 minutes before measurement.The activity were described as changing of α-NA and β-NA.

7. Statistical analysis

The data of report were expressed as mean±SD. Homogeneity of variances were calculated by Levene's test with One-way ANOVA. Differences results significant calculated by using Duncan's multiple range test (DMRT) and analyses median lethal dose (LD_{50}) with StatPlus 2008 before created graph with Sigma Plot 11.0.

RESULTS

1. Bio-efficacy of *W. trilobata* crude extracts

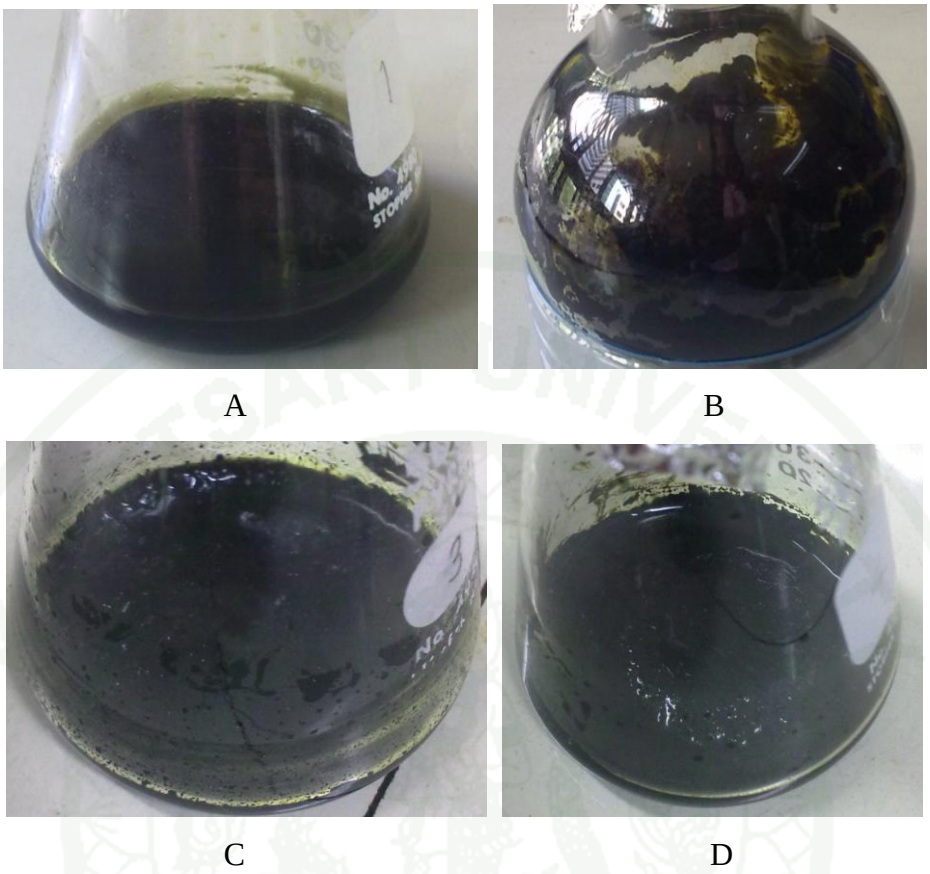
1.1 Physical properties and extraction yields of *W. trilobata* extracts

W. trilobata dried leaves were extracted by using Soxhlet apparatus as sequential solvent polarity. Then, extracted solutions were dried by rotary evaporator. All extracts were dark green color, high viscosity, bad odor and semisolid properties (Table 2, Figure 16). The percent yields were calculated by comparing the mass of crude extracts to the amount of fresh young leaves. The %yield of hexane, dichloromethane, ethyl acetate and ethanol crude extracts are 0.6330%, 0.1906%, 0.2130% and 0.9422%, respectively. (Table 2)

Table 2 Information of *W. trilobata* extracts

Solvent	%Yield (w/w) [(1)]	Appearance
Hexane	0.6330[b]	dark green viscous semisolid
Dichloromethane	0.1906[d]	dark green viscous semisolid
Ethyl acetate	0.2130[c]	dark green viscous semisolid
Ethanol	0.9422[a]	dark green viscous semisolid

[(1)] For all experiments followed by a common letter within the same column are not significantly level using Duncan's Multiple Range Test (P>0.05)



A B

C D

Figure 16 *W. trilobata* crude extracts; (A) Hexane crude extract
(B) Dichloromethane crude extract
(C) Ethyl acetate crude extract
(D) Ethanol crude extract

1.2 Topical application test against *P. xylostella* 2nd instars larvae

1.2.1 Toxicity of *W. trilobata* Hexane crude extracts

The 24 hours mortality percentage of 2nd instars *P. xylostella* after topical application with *W. trilobata* hexane crude extracts were started 100% mortality when the dose was up to 10,000 ppm (Figure 17). The mortality percentage values showed significant increase (P>0.05) from Duncan's Multiple Range Test inorder to dose. LD_{50} values at 24 hours of *P. xylostella* 2nd instars after topical application test with *W. trilobata* hexane crude extracts was 3,350.29±269.96 ppm (Figure 17).

After 48 hours treated in same concentration mortality percentage slightly increased but not significant for time dependant (Figure 17). The mortality percentage values showed significant increase correlation with increasing concentration from 500 ppm to 10,000 ppm at P>0.05 from Duncan's Multiple Range Test. LD_{50} values at 48 hours of instars *P. xylostella* 2nd after topical application test with *W. trilobata* hexane crude extracts was 3,347.46±312.30 ppm (Figure 17).

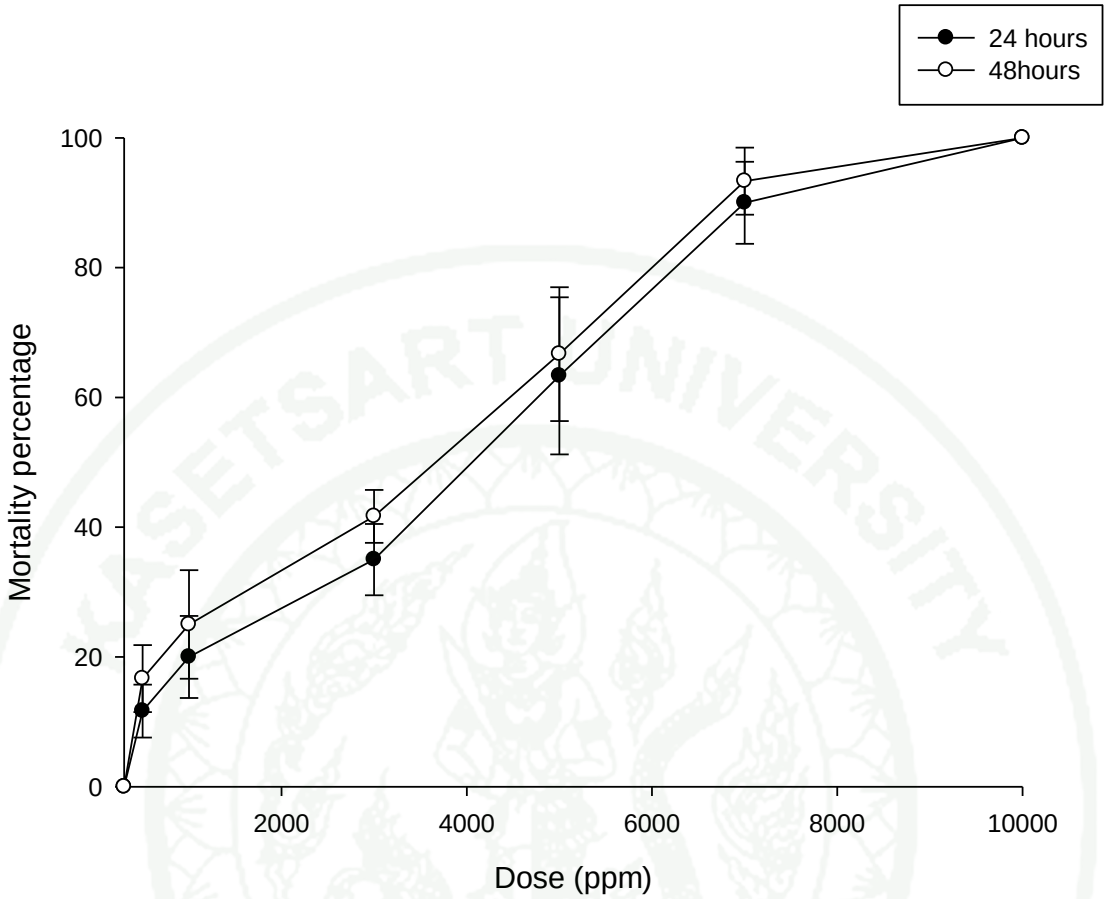


Figure 17 Mortality percentage of instars *P. xylostella* 2nd after topical application test with *W. trilobata* hexane crude extracts after 24 and 48 hours

1.2.2 Toxicity of *W. trilobata* Dichrolomethane crude extract

The 24 hours mortality percentage of 2nd instars *P. xylostella* larvae after topical application with *W. trilobata* dichrolomethane crude extracts were also lead 100% mortality when the dose was up to 10,000 ppm (Figure 18). The mortality percentage values showed significant increase at P>0.05 from Duncan's Multiple Range Test. LD_{50} values at 24 hours of 2nd instars *P. xylostella* after topical application test with *W. trilobata* Dichrolomethane crude extracts was 464.19±92.84 ppm.

After 48 hours treated in same concentration mortality percentage slightly increased which no significant different for time dependent (Figure 18). The mortality percentage values showed significant increase correlation with increasing concentration from 100 ppm to 5,000 ppm at P>0.05 from Duncan's Multiple Range Test. LD_{50} values at 48 hours of 2nd instars *P. xylostella* after topical application test with *W. trilobata* dichrolomethane crude extract was 508.04±86.78 ppm.

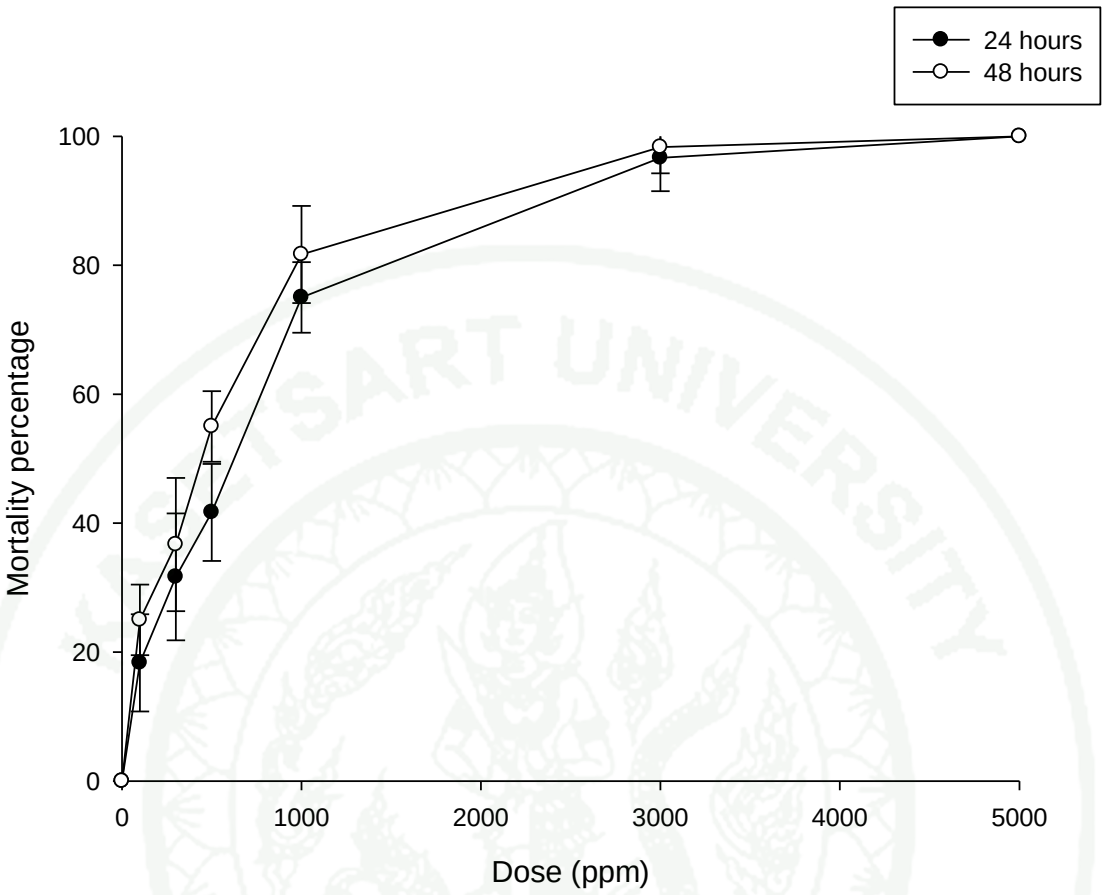


Figure 18 Mortality percentage of 2nd instars *P. xylostella* after topical application test with *W. trilobata* dichrolomethane crude extracts after 24 and 48 hours

1.2.3 Toxicity of *W. trilobata* ethyl acetate crude extract

The 24 hours mortality percentage of 2nd instars *P. xylostella* larvae after topical application with *W. trilobata* ethyl acetate crude extracts were also lead 100% mortality when the dose was up to 10,000 ppm (Figure 19). The mortality percentage values showed significant increase at P>0.05 from Duncan's Multiple Range Test. LD_{50} values at 24 hours of 2nd instars *P. xylostella* after topical application test with *W. trilobata* ethyl acetate crude extract was 358.39±40.52 ppm.

After 48 hours treated in same concentration mortality percentage slightly increased which no significant different for time dependent (Figure 19). The mortality percentage values showed significant increase correlation with increasing concentration from 100 ppm to 5,000 ppm at P>0.05 from Duncan's Multiple Range Test. LD_{50} values at 48 hours of 2nd instars *P. xylostella* after topical application test with *W. trilobata* ethyl acetate crude extracts was 316.82±32.30 ppm.

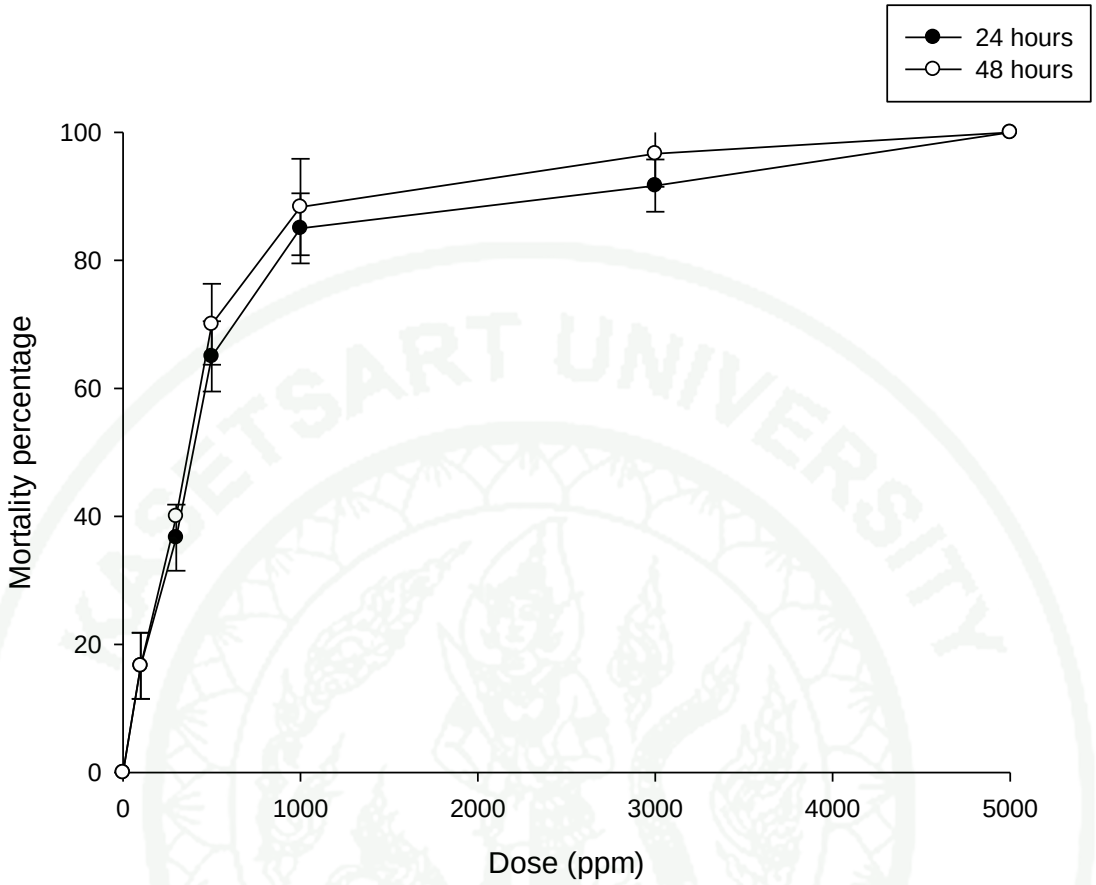


Figure 19 Mortality percentage of 2nd instars *P. xylostella* after topical application test with *W. trilobata* ethyl acetate crude extracts after 24 and 48 hours

1.2.4 Toxicity of *W. trilobata* Ethanol crude extracts

The 24 hours mortality percentage of 2nd instars *P. xylostella* after topical application with *W. trilobata* ethanol crude extracts were also lead 100% mortality when the dose was up to 10,000 ppm (Figure 20). The mortality percentage values showed significant increase at P>0.05 from Duncan's Multiple Range Test. LD_{50} values at 24 hours of 2nd instars *P. xylostella* after topical application test with *W. trilobata* ethanol crude extracts was 358.39±40.52 ppm.

After 48 hours treated in same concentration, mortality percentage slightly increased which no significant differant as time dependent (Figure 20). The mortality percentage values showed significant increase correlation with increasing concentration from 100 ppm to7,000 ppm at P>0.05 from Duncan's Multiple Range Test. LD_{50} values at 48 hours of 2nd instars *P. xylostella* after topical application test with *W. trilobata* ethanol crude extracts was 316.82±32.30 ppm.

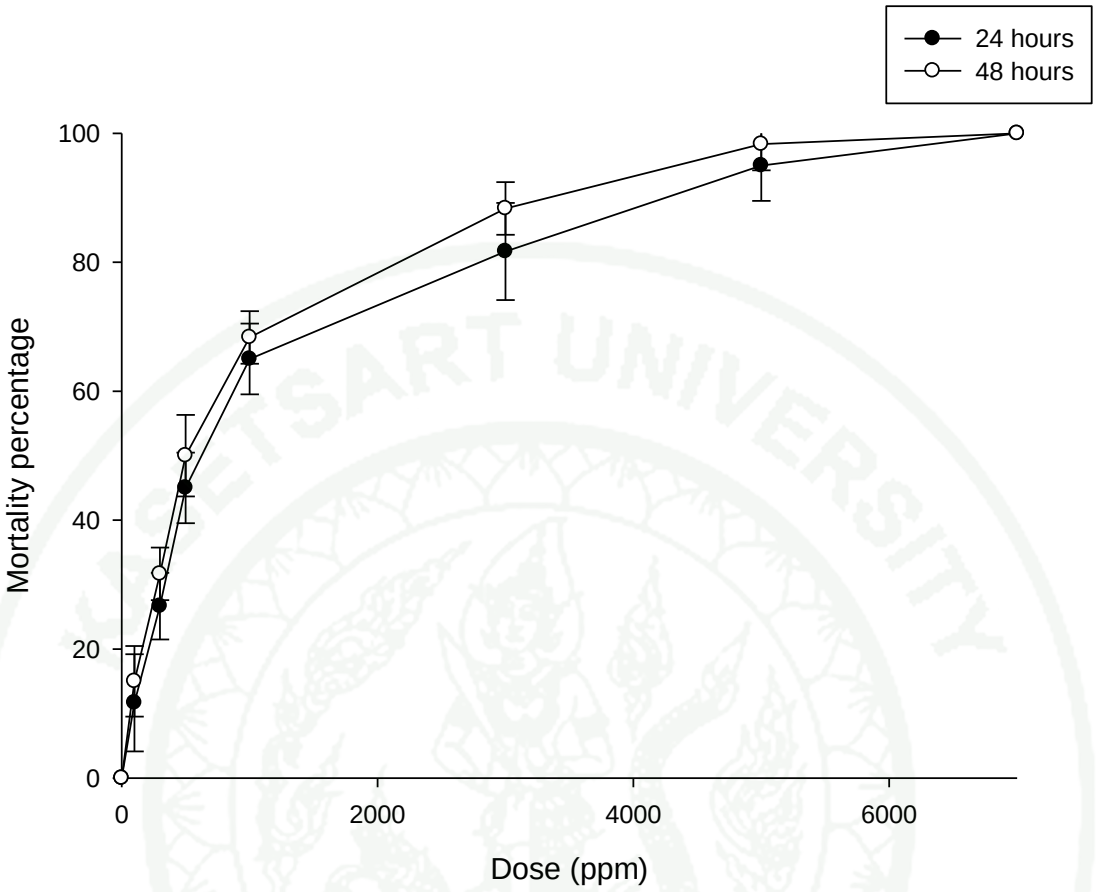


Figure 20 Mortality percentage of 2nd instars *P. xylostella* after topical application test with *W. trilobata* Ethanol crude extracts after 24 and 48 hours

Comparing LD_{50} value by topical application on *P. xylostella* 2nd insters larvae of *W. trilobata* four crude extracts; hexane, dichrolomethane, ethyl acetate and ethanol. The LD_{50} values after 24 hours exposed were 3,350.29, 464.19, 358.39 and 653.55 ppm. For 48 hours after exposed, the LD_{50} values were 3,347.46, 508.04, 316.82 and 516.95 ppm, respectively. Ethyl acetate crude extract show the most control efficiency against 2nd instar *P. xylostella* (Figure 21 and 22).

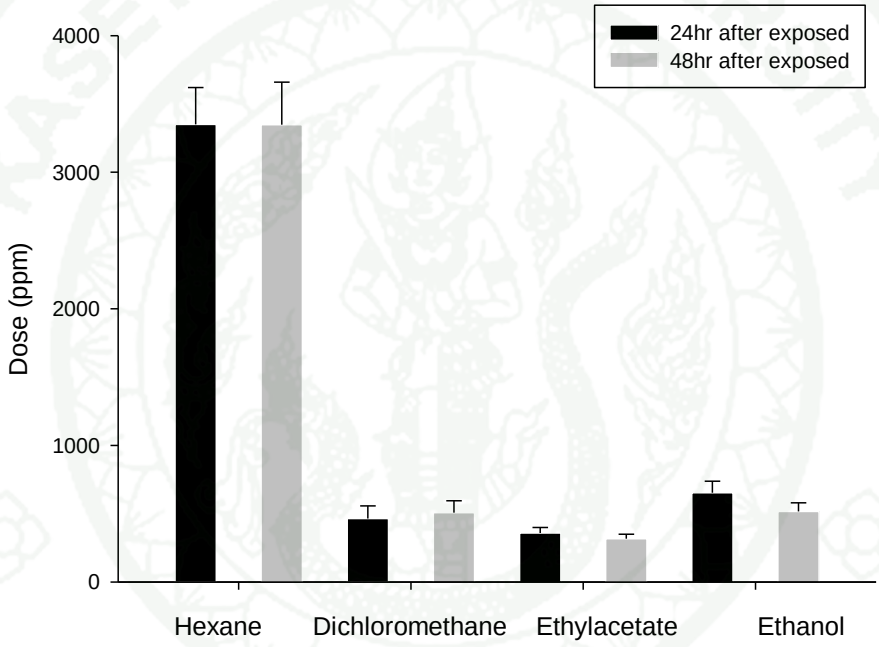
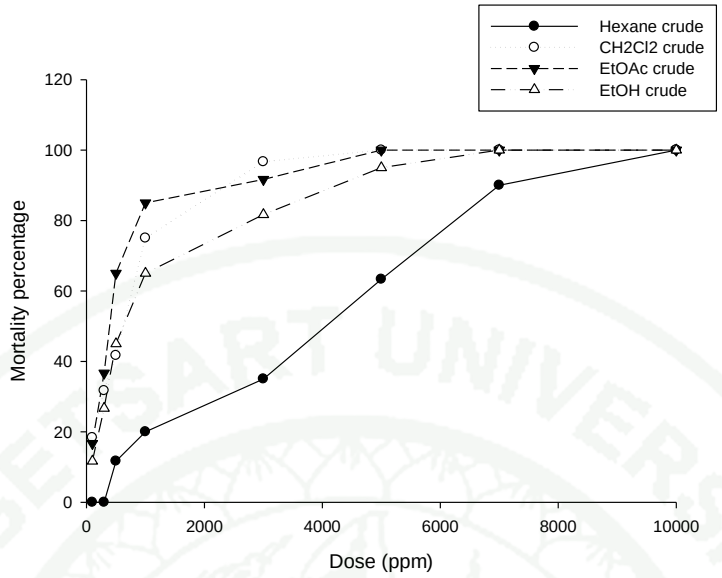
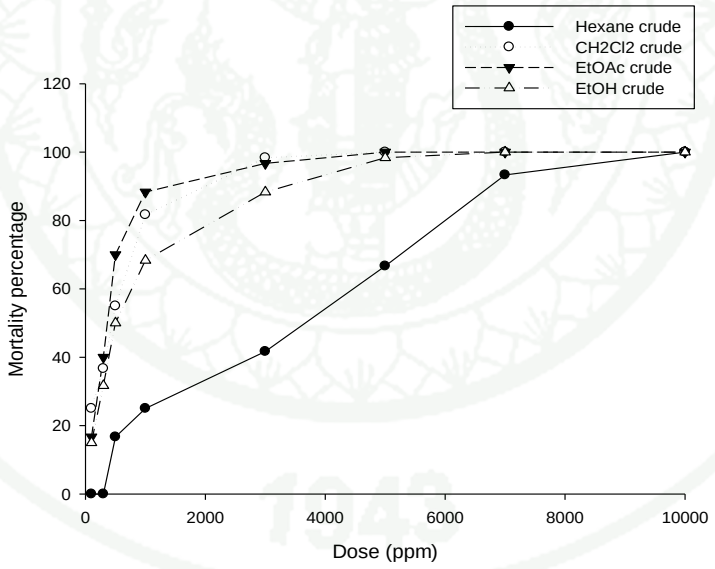


Figure 21 Compared LD_{50} values of *W. trilobata* leaves extracts by topical application test on 2nd instars *P. xylostella*



A



B

Figure 22 Graph comparing mortality percentage of *P. xylostella* topical test with hexane, dichloromethane, ethyl acetate and ethanol *W. trilobata* crude extracts after 24 hours (A) and 48 hours (B)

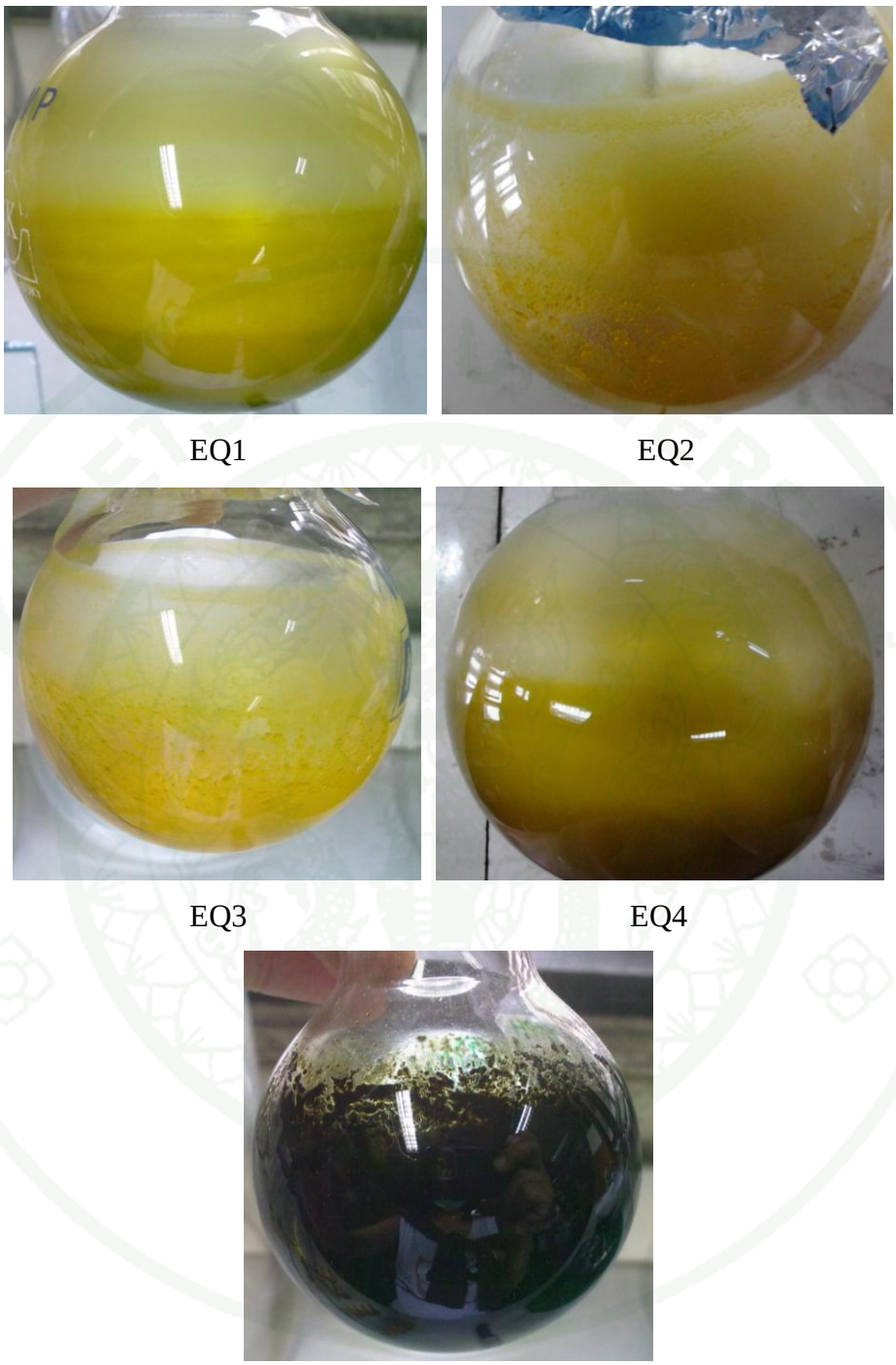
2. Bio-efficacy of alkanes mixture in *W. trilobata* crude extracts

2.1 Physical properties and extraction yields of crude extract isolated from Quick Column Chromatography

Dried *W. trilobata* leaves (651.43 g.) was extracted with sequential solvents; hexane, CH_2Cl_2, EtOAc and EtOH. After dried by Rotary evaporator, four crude extract was tested toxicity on *P. xylostella*. The most efficacy EtOAc crude extract was further isolated allelochemical compounds by Quick Column Chromatography. Mobile phase solvents were 100% Hexane, 5% EtOAc: Hexane-100% EtOAc and 5% MeOH: EtOAc -100% MeOH. EtOAc crude extracts 8.27 g. There were collected 5 fractions; EQ1, EQ2, EQ3, EQ4 and EQ5 %yield were 5.80, 26.11, 9.19, 10.16 and 30.69, respectively (Table 3, Figure 23).

Table 3 Information of crude extracts isolated from *W. trilobata* ethyl acetate crude extracts by Quick Column Chromatography

Fraction	Solvent%	Yield (w/w)	Appearance
EQ1	100% Hexane-55% EtOAc: Hexane	5.80	dark yellow viscous semisolid
EQ2	25% EtOAc: Hexane-60% EtOAc: Hexane	26.11	dark yellow viscous semisolid
EQ3	65 % EtOAc: Hexane-70% EtOAc: Hexane	9.19	yellow viscous semisolid
EQ4	75% EtOAc: Hexane-100% EtOAc	10.16	brown viscous semisolid
EQ5	5% MeOH: EtOAc – 100% MeOH	30.69	dark green viscous semisolid



EQ1 EQ2

EQ3 EQ4

EQ5

Figure 23 Crude extracts isolated from *W. trilobata* ethyl acetate crude extracts by Quick Column Chromatography fraction EQ1- EQ5.

2.2 Topical application test of crude extracts isolated from Quick Column Chromatography

Five fractions of *W. trilobata* ethylactate crude extracts from Quick Column Chromatography were prepared for topical application test at 400 ppm follow LD_{50} value of ethyl acetate crude extracts to find the most effective fraction in order to isolate allelochemical compounds which have ability to against *P. xylostella* 2nd instars larvae. From the result, fraction EQ1 was the highest mortality percentage at 24 hours (43.33±5.77%) and 48 hours (66.67±11.55%), related to result LD_{50} of ethyl acetate crude extracts at 400 ppm (Table 4).

Table 4 Mortality percentage of *P. xylostella* 2nd instars larvae after topical application with 400 ppm crude extracts isolated from *W. trilobata* Ethylactate crude extracts by Quick Column Chromatography.

Fraction	Mortality percentage(2)	
	24h	48h
Control(1)	0.00±0.00[a]	0.00±0.00[a]
EQ1	43.33±5.77[b]	66.67±11.55[b]
EQ2	13.33±5.77[c]	33.33±11.55[c]
EQ3	23.33±5.77[d]	36.67±15.28[d]
EQ4	20.00±0.00[e]	40.00±10.00[e]
EQ5	16.67±11.55[f]	33.33±5.77[f]

(1) Control group = Acetone (AR grade)

(2) Mean±SD, 3 replications, n=30. For all experiments followed by a common letter within the same column are not significantly level using Duncan's Multiple Range Test (P>0.05)

2.3 Physical properties and extraction yields of crude extract isolated from Column chromatography

From Quick Column Chromatography, all crude extracts were checked with TLC. Fraction EQ1, crude extracts of the highest efficacy shared the same composition compounds with crude extracts of fraction EQ2. Thus, fraction EQ1 were used for isolated allelochemical compounds by Column chromatography by using 100% hexane mobile phase solvents. Four fractions were collected; EQ1C1, EQ1C2, EQ1C3 and EQ1C4, which %yield were 1.82, 2.64, 26.12 and 54.15 respectively (Table 5). After evaporated, crude extracts of fraction EQ1C1 showed pale yellow color (Figure 24) brighter than fraction EQ1 and not shared same composition compounds after checked with TLC.

Table 5 Information of crude extracts isolated from *W. trilobata* ethyl acetate crude extract by Column chromatography

Fraction	% Yield (w/w)	Appearance
EQ1C1	1.82	Pale yellow
EQ1C2	2.64	Dark yellow
EQ1C3	26.12	Dark green
EQ1C4	54.15	Dark green

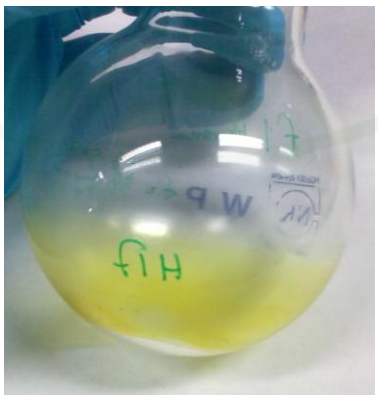


Figure 24 Crude extracts isolated from *W. trilobata* ethyl acetate crude extracts by Column chromatography fraction EQ1C1

2.4 Physical properties and extraction yields of crude extract isolated from Preparative Thin Layer Chromatography

After checked with TLC, fraction EQ1C1 had the lowest impurity. Preparative Thin Layer Chromatography was used to separate impure compound. Mobile phase solvents were 90% Hexane: 10% EtOAc. Crude extract of fraction EQ1C1 (100 mg) were collected 4 fractions; EQ1C1P1, EQ1C1P2, EQ1C1P3 and EQ1C1P4. % yield were 11.64, 2.45, 12.64 and 27.91 respectively. All fractions had same appearance of pale yellow color but on PTLC, they showed different colors (Table 6, Figure 25). Fraction EQ1C1P3 and EQ1C1P4 had difference composition compounds. So, only fractions EQ1C1P1, EQ1C1P2 and EQ1C1P3 were tested toxicity by topical application on *P. xylostella* 2nd instars larvae.

Table 6 Information of crude extracts isolated from Preparative Thin Layer Chromatography

Fraction	% Yield (w/w)	Appearance on TLC
EQ1C1P1	12.64	Yellow
EQ1C1P2	11.64	Pear yellow
EQ1C1P3	2.45	Dark green
EQ1C1P4	27.91	Dark green

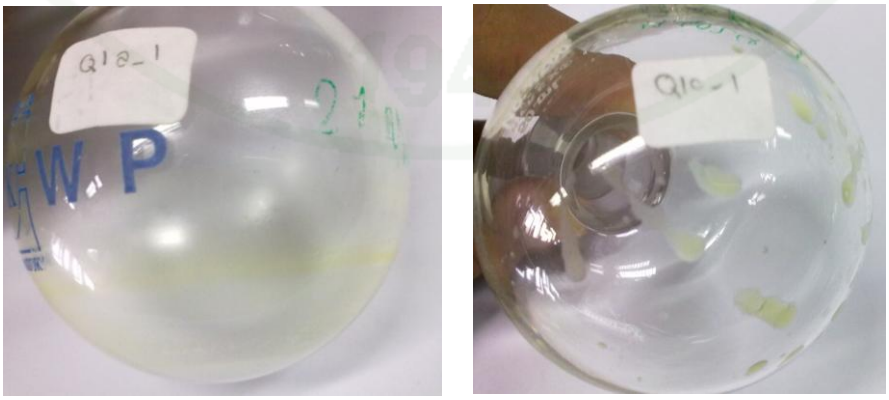


Figure 25 Fraction EQ1C1P1 and EQ1C1P3 from *W. trilobata* ethyl acetate crude extracts by Preparative Thin Layer Chromatography

2.5 Topical application test of crude extracts isolated from Preparative Thin Layer Chromatography

Crude extracts 3 fractions from Preparative Thin Layer Chromatography were prepared for topical application test at 400 ppm follow LD_{50} value of EtOAc crude extracts to find allelochemical compounds which have ability to against *P. xylostella*. From the result, mortality percentage at 24 hours of control, fraction EQ1C1P1, EQ1C1P2 and EQ1C1P3 showed 0±0, 15.00±12.91, 22.50±22.17 and 20.00±8.16, respectively. After 48 hours, mortality percentage increased to 0±0, 45.00±23.80, 37.50±37.74 and 30.00±8.16, respectively (Table 7). Crude extracts from fraction EQ1C1P2 showed the highest mortality percentage at 24 hours but after 48 hours, fraction EQ1C1P1 was the highest mortality percentage.

Table 7 Mortality percentage of *P. xylostella* 2nd instars larvae after topical application with 400 ppm of crude extracts isolated from Preparative Thin Layer Chromatography 24 and 48 hours

Fraction	Percentage of mortality (2)	
	24hrs	48hrs
Control(1)	0.00±0.00[a]	0.00±0.00[a]
EQ1C1P1	15.00±12.91[a]	45.00±23.80[b]
EQ1C1P2	22.50±22.17[a]	37.50±37.74[b]
EQ1C1P3	20.00±8.16[a]	30.00±8.16[b]

(1) Control group = Acetone (AR grade)

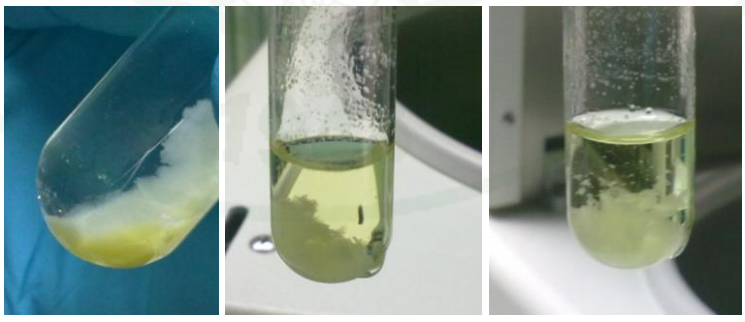
(2) Mean±SD, 4 replications, n=10. For all experiments followed by common letters within the same row is no significantly level using Duncan's Multiple Range Test (P>0.05)

2.6 Physical properties and extraction yields of white flake wax from Re-crystallization

After analysis by ^{1}H-NMR, crude extracts fraction EQ1C1P1 spectrum showed characteristic of aliphatic hydrocarbon but had impurity. So, Re-crystallization was used to eliminate impure compound in this experiment; fraction EQ1C1P1 were dissolved with minimum scale of CH_2Cl_2 in test tube then added with MeOH. After that white flake wax were occurred in yellow solution. Separated yellow solution in to another tube and rinsed by MeOH until white fluffs were cleaned. Filtered, dried and weighted for compare percent yield. EQ1C1P1R3 was occurred the highest percent yield 23.21168% (Table 8) and occurred white flake wax different from EQ1C1P1R1 and EQ1C1P1R2 that occurred impurity of yellow compound sticky on white scale (Figure 26).

Table 8 Information of white flake wax in *W. trilobata* from Re- crystallization

Methods	%Yield (w/w)	Appearance
EQ1C1P1R1	8.537439	Pale yellow flake
EQ1C1P1R2	18.35974	White flake wax
EQ1C1P1R3	23.21168	White flake wax



(A) (B) (C)

Figure 26 White flake wax isolated from Re-crystallization; (A) EQ1C1P1R1, (B) EQ1C1P1R2 (C) EQ1C1P1R3

2.7 Chemical Structure Elucidation

The highest efficacy, ethyl acetate crude extracts of *W. trilobata* dried leaves was isolated by Quick Column Chromatography. Then, the highest efficacy fraction was isolated again by Column Chromatography. After that, the highest efficacy fraction from Column Chromatography was screening check purity of compound by Thin Layer Chromatography with appropriate solvent system and observed under UV light at λ_{max}= 254 nm. Fraction mixture was isolated by Preparative Thin Layer Chromatography, Re-crystallization and corroborated efficacy test by topical application on *P. xylostella* 2nd instars larvae. Finally, fraction mixture (EQ1C1P1R3) were analyses by Nuclear Magnetic Resonance, Elemental Analysis, Infrared Spectroscopy, Gas Chromatography- Mass Spectrometry and elucidated by compared with the literature data.

The fraction EQ1C1P1R3 were alkanes mixture, long-chain saturated aliphatic hydrocarbon which consist of Nonacosane 31.02%, Hexacosane 16.70%, Heptacosane 15.77%, Pentacosane 13.45%, Octacosane 10.45%, Tetracosane 4.20% and Docosane 1.42%. It had white flake wax properties (Figure 27).

Figure 27 Physical properties of alkanes mixture

2.8 Topical application test of alkanes mixture

After analysis by ^{1}H-NMR white flake wax from fraction EQ1C1P1R3 had the lowest impurity spectrums. For corroborated the alkanes mixture had effect to 2nd instars larvae of *P. xylostella*. The alkanes mixture was prepared with Acetone (AR grade) at concentration 400 ppm follow LD_{50} value of ethyl acetate crude extract.

The results of mortality percentage of alkanes mixture at 400 ppm was 44.16±10.92 and 66.67±1.15 at 24 and 48 hours after exposed (Table 9). The toxicity showed relative close to crude extract from Quick Column Chromatography (Table 4) and ethyl acetate crude extracts (Figure 21).

Table 9 Mortality percentage of *P. xylostella* 2nd instars larvae after topical application with alkanes mixture 400 ppm after 24 and 48 hours

Compound	Percentage of mortality[(2)]	
	24hrs	48hrs
Control[(1)]	0.00±0.00^{a}	0.00±0.00^{a}
Alkanes mixture	44.16±10.92^{a}	66.67±1.15^{b}

[(1)] Control group = Acetone (AR grade)

[(2)] Mean±SD, 4 replications, n=30. For all experiments followed by common letters within the same row is no significantly level using Duncan's Multiple Range Test (P>0.05)

3. Mode of action study

There were four methods using to determine insects enzyme activity including carboxylesterase activity, acetylcholinesterase activity, glutathione-S-transferase activity and general esterase activity by using microplate reader techniques. Two assay were compared 1) *In-vivo* assay determined variation amount of enzyme in survival 2nd instars *P. xylostella* after treated for 24 hours with *W. trilobata* leaves ethyl acetate crude extract comparing with Control group group which treated with acetone (AR grade). 2) *In-vitro* assay determined variation amount of enzyme in non-treated 2nd instars *P. xylostella* against *W. trilobata* leaves ethyl acetate crude extract.

3.1 Enzyme activity of 2nd instars *P. xylostella* after treated with *W. trilobata* ethyl acetate crude extracts 24 hours

3.1.1 Carboxyl esterase enzyme activity after treated with *W. trilobata* ethyl acetate crude extracts

Both *In-vitro* and *In-vivo* results of Carboxylesterase activity showed enzyme was inhibited. The both results showed significant decrease from control at P< 0.05 by Duncan's Multiple Range Test. The correlation factor shows *In-vivo* treatment group was decreased 48.65 times from control group and *In-vitro* treatment group also decreased from control group 6.13 times (Table 10).

Table 10 Carboxylesterase activity(1) (nM paranitrophenol/ mg protein/min) of 2nd instars larvae of *P. xylostella* after treated with 400ppm *W. trilobata* ethyl acetate crude extracts

	Control	Treatment	CF(2)
In-vivo	3.07±0.15[a]	0.07±0.00[b]	48.69
In-vitro	2.60±0.10[a]	0.43±0.08[b]	6.13

(1) Means ± SD, 5 replicates, n = 10 larvae of 2nd instars, 24 hours treated. For all experiments followed by common letters within the same row is no significant level using Duncan's Multiple Rang Test (P< 0.05)

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

3.1.2 Acetylcholinesterase activity after treated with *W. trilobata* ethyl acetate crude extracts

The *In-vitro* result of Acetylcholinesterase showed slantly inhibited whereas *In-vitro* result seems slightly increased (Table 11) both treatment activity results showed not significant from control at P<0.05 by Duncan's Multiple Rang Test. The correlation factor, *In-vivo* treatment group was increased 0.94 times from control group and *In-vitro* treatment group decreased from control group 1.08 times (Table 11).

Table 11 Acetylcholinesterase activity [(1)] (acetylcholinesterase activity/ mg protein/min) of 2 [nd] instars larvae of *P. xylostella* after treated with 400ppm *W. trilobata* ethyl acetate crude extracts

	Control	Treatment	CF[(2)]
In-vivo	0.22±0.01[a]	0.24±0.01[a]	0.94
In-vitro	0.30±0.01[a]	0.27±0.00[a]	1.08

[(1)] Means ± SD, 5 replicates, n = 10 larvae of 2[nd] instars, 24 hours treated

For all experiments followed by common letters within the same row is no significant level using Duncan's Multiple Rang Test (P< 0.05)

[(2)] CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).

3.1.3 Glutathione-S-transferase (GST) activity after treated with *W. trilobata* ethyl acetate crude extracts

The Glutathione-S-transferase activity results of *W. trilobata* ethyl acetate crude in both *In-vivo* and *In-vitro* assay showed the enzyme was inhibited (Table 12). *In-vitro* assay results showed significant decrease from control at $P< 0.05$ by Duncan's Multiple Rang Test. The correlation factor shows *In-vivo* treatment group was decreased 1.06 times from control group and *In-vitro* treatment group also decreased from control group 9.35 times (Table 12).

Table 12 Glutathione-S-transferase activity[(1)] (CDNB conjugated product/ mg protein/min) of 2nd instars larvae *of P. xylostella* after treated with 400ppm *W. trilobata* ethyl acetate crude extracts

	Control	Treatment	CF[(2)]
In-vivo	0.78±0.02[a]	0.73±0.53[a]	1.06
In-vitro	0.84±0.14[a]	0.09±0.00[b]	9.35

[(1)] Means ± SD, 5 replicates, n = 10 larvae of 2nd instars, 24 hours treated
For all experiments followed by common letters within the same row is no significant level using Duncan's Multiple Rang Test ($P< 0.05$)
[(2)] CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).

3.1.4 General esterase activity (α-NA and β-NA) after treated with *W. trilobata* ethyl acetate crude extracts

The general esterase activity results of *W. trilobata* ethyl acetate crude extracts in both *In-vivo* and *In-vivo* assay of α-NA and β-NA assay were increased (Table 13). All results showed tend to be increase from control but not significant at P< 0.05 by Duncan's Multiple Rang Test. The correlation factor shows *In-vivo* groups of α-NA and β-NA were increased from control groups 0.85 and 0.96 times respectively and *In-vitro* treatment of α-NA and β-NA increased from control groups 0.72 and 0.51 times, respectively (Table 13).

Table 13 General esterase activity[(1)] (nM naphol product/ mg protein/min) of 2nd instars larvae of *P. xylostella* after treated with 400ppm *W. trilobata* ethyl acetate crude extracts

	α-NA activity			β-NA activity		
	Control	Treatment	CF[(2)]	Control	Treatment	CF[(2)]
In-vivo	0.07±0.01[a]	0.08±0.02[a]	0.85	0.09±0.01[a]	0.10±0.01[a]	0.96
In-vitro	0.06±0.01[a]	0.08±0.01[a]	0.72	0.05±0.01[a]	0.09±0.15[a]	0.51

[(1)] Means ± SD, 5 replicates, n = 10 larvae of 2nd instars, 24 hours treated
For all experiments followed by common letters within the same row is no significant level using Duncan's Multiple Rang Test (P< 0.05)
[(2)] CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).

3.2 Enzyme activity of 2nd instars *P. xylostella* after treated with alkanes mixture 24 hours

3.2.1 Carboxylesterase enzyme activity after treated with alkanes mixture

The Carboxylesterase enzyme activity results of alkanes mixture in both *In-vivo* and *In-vivo* assay treatment were not differenced from control. The *In-vivo* results showed tend to be decreased from control but not significant at P< 0.05 by Duncan's Multiple Rang Test. The correlation factor comparing between treatment and control group of *In-vivo* assay showed 0.95 times and *In-vitro* assay showed 1.28 times (Table 14).

Table 14 Carboxylesterase activity(1) (nM paranitrophenol/ mg protein/min) of 2nd instars larvae of *P. xylostella* after treated with 400ppm alkanes mixture

	Control	Treatment	CF(2)
In-vivo	0.03±0.00[a]	0.04±0.00[a]	0.95
In-vitro	0.03±0.00[a]	0.03±0.00[a]	0.92

(1) Means ± SD, 5 replicates, n = 10 larvae of 2nd instars, 24 hours treated
For all experiments followed by common letters within the same row is no significant level using Duncan's Multiple Rang Test (P< 0.05)
(2) CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).

3.2.2 Acetylcholinesterase enzyme activity after treated with alkanes mixture

Both *In-vitro* and *In-vivo* results of the Acetylcholinesterase enzyme activity showed enzyme was inhibited (Table 15). The both results showed significant decrease from control at P< 0.05 by Duncan's Multiple Rang Test. The correlation factor shows *In-vivo* treatment group was decreased 1.10 times from control group and *In-vitro* treatment group also decreased from control group 1.40 times (Table 15).

Table 15 Acetylcholinesterase activity[(1)] (acetylcholinesterase activity/ mg protein/ min) of 2nd instars larvae of *P. xylostella* after treated with 400ppm alkanes mixture

	Control	Treatment	CF[(2)]
In-vivo	0.94±0.20[a]	0.86±0.02[b]	1.10
In-vitro	0.84±0.16[a]	0.61±0.00[b]	1.40

[(1)] Means ± SD, 5 replicates, n = 10 larvae of 2nd instars, 24 hours treated
For all experiments followed by common letters within the same row is no significant level using Duncan's Multiple Rang Test (P< 0.05)
[(2)] CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).

3.2.3 Glutathione-S-transferase (GST) enzyme activity after treated with alkanes mixture

The Glutathione-S-transferase activity results of alkanes mixture in *In-vitro* assay showed enzyme was inhibited. The *In-vivo* treatments showed tend to be decreased from control but not significant although *In-vitro* result showed significant decrease from control at $P< 0.05$ by Duncan's Multiple Rang Test. The correlation factor shows *In-vivo* treatment was decreased 1.02 times from control group and *In-vitro* treatment decreased from control group 1.43 times (Table 16).

Table 16 Glutathione-S-transferase activity[(1)] (CDNB conjugated product/ mg protein/ min) of 2nd instars larvae of *P. xylostella* after treated with 400 ppm alkanes mixture

	Control	Treatment	CF[(2)]
In-vivo	0.72 ± 0.17^{a}	0.71 ± 0.17^{a}	1.02
In-vitro	1.01 ± 0.07^{a}	0.71 ± 0.18^{b}	1.43

[(1)] Means ± SD, 5 replicates, n = 10 larvae of 2nd instars, 24 hours treated
For all experiments followed by common letters within the same row is no significant level using Duncan's Multiple Rang Test ($P< 0.05$)
[(2)] CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).

3.2.4 General esterase (1-naphthyl acetate and 2-naphthyl acetate) enzyme activity after treated with alkanes mixture

The general esterase activity results of alkanes mixture in both *In-vivo* and *In-vitro* assay of α-NA and β-NA assays treatments were not differenced from control (Table 17). All results showed not significant from control at P< 0.05 by Duncan's Multiple Rang Test. The correlation factor shows *In-vivo* groups of α-NA and β-NA bared increased from control groups 1.01 and 1.03times respectively and *In-vitro* treatment of α-NA and β-NA bared increased from control groups 1.00 and 0.95 times, respectively (Table 17).

Table 17 General Esterase activity [(1)] (α-NA and β-NA activity/ mg protein/min) of 2nd instars larvae of *P. xylostella* after treated with 400ppm alkanes mixture

P. xylostella	α-NA activity			β-NA activity		
	Control	Treatment	CF[(2)]	Control	Treatment	CF[(2)]
In-vivo	0.03±0.00[a]	0.03±0.00[a]	1.01	0.03±0.00[a]	0.03±0.00[a]	1.03
In-vitro	0.03±0.00[a]	0.03±0.00[a]	1.00	0.03±0.00[a]	0.03±0.00[a]	0.95

[(1)] Means ± SD, 5 replicates, n = 10 larvae of 2nd instars, 24 hours treated
For all experiments followed by common letters within the same row is no significant level using Duncan's Multiple Rang Test (P< 0.05)
[(2)] CF is a correction factor = (enzyme activity of control)/ (enzyme activity of treatment).

DISCUSSION

1. Bi-oefficacy of *W. trilobata* crude extracts

Wedelia genus has a few reports in pest management. *Wedelia biflora* which have chemical closely relating to *W. trilobata* (Bohlmann *et al.,* 1981) showed anti-feedant activity against Cotton Boll Weevil 90% by 24- Ethylcoprostanone and 83% by *ent*- Kauradienoic acid (Miles *et al.,* 1990) and ground cover of *W. trilobata* is major factor control thrips palmi Karny (Frants and Mellinger, 1997).

According to the result toxicity of *W. trilobata* crude extracts can be order from highest to lowest efficacy against *P. xylostella* after exposed by topical application test at LD_{50} values at ethyl acetate, dichloromethane, ethanol and hexane crude extract, respectively. LD_{50} values at 24 hours after exposed were 358.39, 464.19, 653.55 and 3,350.29 ppm, respectively and 48 hours LD_{50} values were 316.82, 508.04, 516.95 and 3,347 ppm, respectively (Figure 21). The mortality did not show significant time dependent. The mortality percentage increased correlatively the higher concentration that *P. xylostella* probably not enhance detoxification enzyme at higher concentration or other detoxification mechanism for eliminate toxicity and cause death.

Comparing to previous research, ethyl acetate crude extract show lower efficacy against *P. xylostella* such as *Azadirachta indica* seed extract (LD_{50} value ≈ 0.54 ppm; Robert and Denis, 1993), *Derris elliptica* Benth root extract (LD_{50} value ≈ 24.25 ppm; Visetson and Milne, 2001) *Annona squamosa* seed extract (LD_{50} value ≈ 0.10 ppm; Andrey and Isman, 2004) *Syzygium aromaticum* flower extracts (LD_{50} value ≈ 1.09 ppm; Kumnuan, 2006), *Piper sarmentosum* Roxburgh leaves extract (LD_{50} value ≈ 4.34 ppm; Orratai, 2008), and *Mammea americana* seed extract (LD_{50} value ≈ 5.90 ppm; Kritchaya *et al*., 2011).

While *P. xylostella exposed to W. trilobata* crude extracts, they change behavior to avoid extracts by moving immediately and stop feeding within 0- 2 hours

related to behavior avoidance to decrease expose to chemical (Bullangpoti, 2007) After that, paralyzed and died within 4-48 hours.

Nevertheless, *W. trilobata* has lower efficacy than previous report extracts but we can recommend agriculturist use *W. trilobata* extract to control pest because it is invasive plant which rapidly growth, valueless and recently well known as herbal use that no report side effect on human (Heansley, 1997).

2. Bio-efficacy of alkanes mixture in *W. trilobata* crude extracts

After exposed to alkanes mixture by topical on *P. xylostella*, treated larvae immediatly decrease moving and stop feeding, then paralyzed and died. This result is same as when apply alkane alone which can significantly reduce movement rate of *Spodoptera fugiperda*, *Tenebrio molitor* and *Drosophila melanogaster* during treatment contract (Spencer *et al*., 1999) indoxacarb against *P. xylostella* with the appearance of neurotoxic symptom (Wing *et al*., 2000).

Mortality percentage of *P. xylostella* after treated with 400 ppm alkanes mixture at 24 hours and 48 hours were 44.16% and 66.17%, respectively. Alkane mixture shown lower efficacy than long chain *n*- alkanes compounds against *S. frugiperda* ($LD_{50} \approx 3.89$ ppm), *T. molitor* ($LD_{50} \approx 5.2$ ppm) and *D. melanogaster* ($LD_{50} \approx 3.23$ ppm), respectively (Cespedes *et al*., 2013). Moreover, this compound show lower efficacy than indoxacarb and chlorantraniliprole, commercial insecticide for control several lepidopteran that reported LD_{50} value against *P. xylostella* was 18.53 ppm (Nehare *et al.*, 2010) and 0.015 ppm (Silva *et al.*, 2012), respectively.

Although the alkane mixture (nonacosane) has no report in toxicological information (TCI Americana material safety data sheet) but their oil compound have been use as pesticides for centuries and it well known as ingredient base of commercial synthetic insecticide and fungicide (Bogran *et al*., 1914) which carrier active compound move though into insect cuticle. Furthermore alkanes has been

found in many insecticides including moth insecticide (Jurenka and Subchev, 2000; Subchev and Jurenka, 2001).

Morover, alkanes mixture could be considered to use as bio-insecticide in class of oil pesticide which low toxicity to human, active for a short time, insect and mites have not developed resistance (Bogran *et al*., 1914).

3. Mode of action study from ethyl acetate *W. trilobata extract*

According to detoxification enzyme activity results of *P. xylostella* after topical 24 hours carboxylesterase activity was significant inhibited by *W. trilobata* ethylacetate crude extract (Table 10) for both of *In-vivo* and *In-vitro* assay of this result has tended to be decrease related to carboxylesterase for malathion on *P. xylostella* (Maa and Liao, 2000). Glutathion-s-transferase activity of *In- vitro* assay was significant inhibited by ethylacetate crude extract; whereas *In-vivo* assay was not significant inhibited. The *In-vitro* result is same as described by Kao *et al*. (1989) that enzymes such as glutathione-S-transferase was degraded by parathion in *P. xylostella*. For acetylcholineesterase and general esterase activity, both *In-vivo* and *In-vitro* assay results, treated group were not different from control.

Although, alkane mixture showed significant inhibited acetylcholinesterase (AchE) *In-vitro* assay; whereas the *In-vivo* assay AChE was induce but not significant. AChE is the important enzyme in neurotransmitter nervous system that hydrolyzing acetylcholine (Ach) to choline and acetate. Decreasing of AChE occurred muscle constriction, paralysis and die of *P. xylostella* after exposed following decrease nerve sensitivity (Hama *et al*., 1987) related to behavior avoidant of *P. xylostella* after exposed which constriction, paralysis and die. Alkane mixture not showed significant in carboxylesterase esterase, general esterase and glutathione-s-transferase enzyme activity correlated to indoxacarb resistance in *P. xylostella* that not associated with esterase and glutathione-s-transferase (Toshio *et al*., 2004).

Toxicity mechanism of alkanes could be effectively of hydrophobic environmental presents the action of morphogenesis enzyme of insect which require an aqumeous medium for its action (Jung and Deetz, 1993) or inhibition of proteinase and other polyphenol oxidases that could bind to alkanes (Cespedes *et al.*, 2013). Moreover alkane could produce white substance accumulation (deposits) invacuoles (Delgado *et al.*, 2011).

CONCLUSION AND RECOMMENDATIONS

Conclusion

The results of for *Wedelia trilobata* crudes extracts showed % extraction yield result of hexane, dichloromethane, ethyl acetate and ethanol extraction as 0.6330%, 0.1906%, 0.2130% and 0.9422% respectively. LD_{50} value of 2nd instars *P. xylostella* larvae after topical application with hexane, dichrolomethane, ethylacetate and ethanol after exposed 24 hours showed 3,350.29, 464.19, 358.39 and 653.55 ppm, after 48 hours showed 3,347.46, 508.04, 316.82 and 516.95 ppm, respectively.

The results of alkanes mixture which could be a consist of 31.02% of Nonacosane compounds, 16.70% Hexacosane, 15.77% Heptacosane, 13.45% Pentacosane, 10.45% Octacosane, 4.20% Tetracosane, 1.42% Docosane and 0.95% Phenol, 2,2'-methyllenebis [6-(1,1-dimethylethyl)-4-methyl] showed percentage mortality of 2nd instars *P. xylostella* larvae after 24 hours topical application at 400 ppm as 44.16% and 48 hours as 66.67%.

Mode of action study of insect enzyme activity after exposed 24 hours of ethylacetate crude extract showed inhibition on carboxylesterase activity and *In-vitro* assay of gluutathiol-s-transferase but acetylcholinesterase and general esterase (α-NA and β-NA) were not significantly difference from control. Alkanes mixture showed inhibition on acetylcholinesterase activity and *In-vitro* assay of Gluutathiol-s-transferase but carboxylesterase and general esterase (α-NA and β-NA) were not significantly difference from control.

Recommendations

1. Isolate other active compounds in *Wedelia trilobata* must be done.

2. It should be studied and analysis in case of using in the real field.

3. The active compound remaining chemical toxicity which directly effects on environmental especially benefit insects must be tested.

4. The stability and degradable times of compound must be analysis to further developing into the commercial product.

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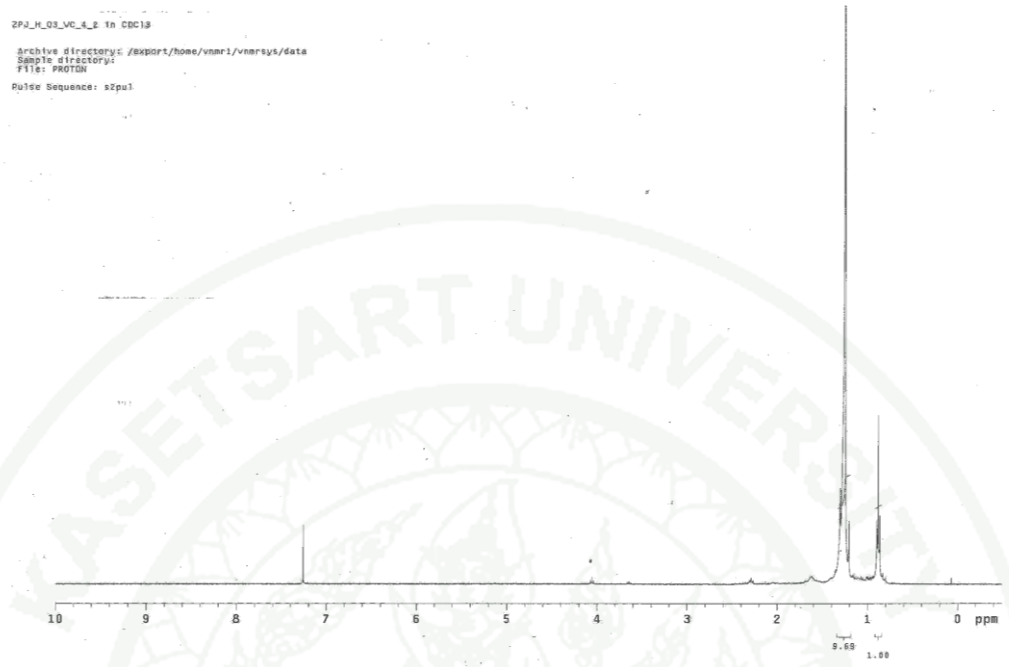
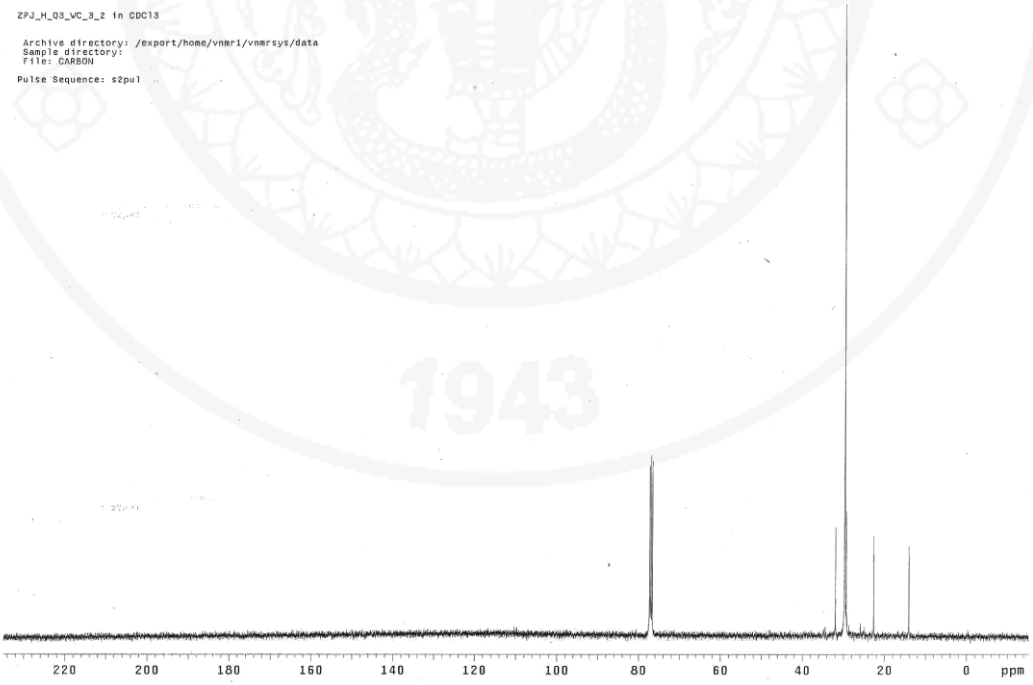
APPENDIX

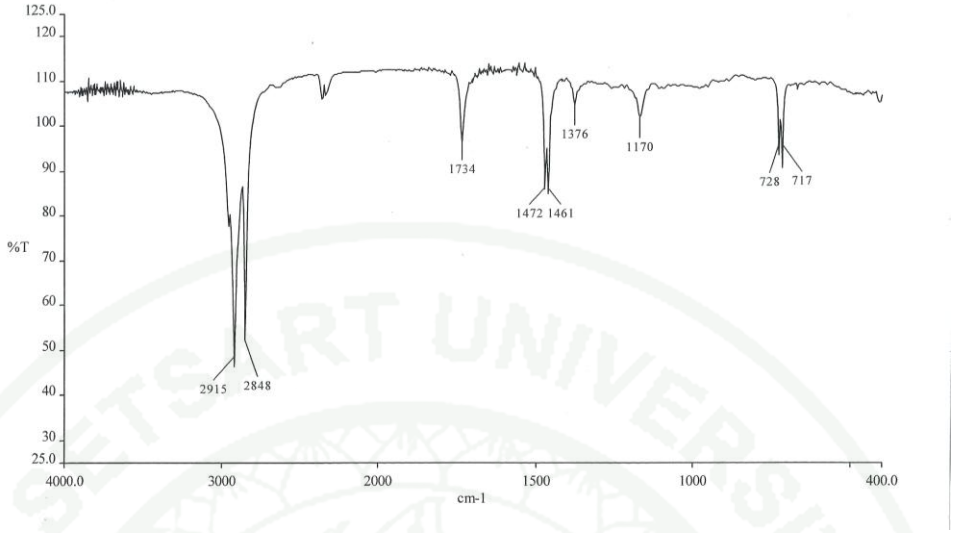
Results of alkanes mixture



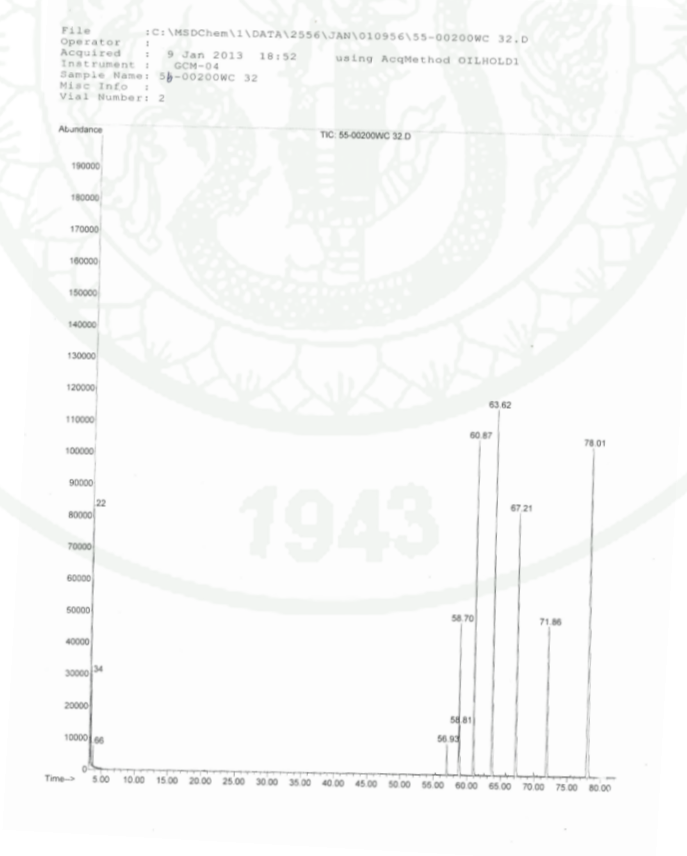
Appendix Figure 1 ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of alkanes mixture



Appendix Figure 2 ^{13}C-NMR (100 MHz, CDCl3) spectrum of alkanes mixture



Appendix Figure 3 IR spectrum of alkanes mixture



Appendix Figure 4 GC-MS spectrum of alkanes mixture

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รายละเอียดตัวอย่าง	ZPJ_H_Q3_WC_3_2
รหัสตัวอย่าง	56/00209-001
ลักษณะและสภาพตัวอย่าง	ประเภทตัวอย่าง : สารสกัด ภาชนะบรรจุ : หลอดแก้ว, จำนวน : 1 หลอด, น้ำหนัก/ปริมาตร : 3 มิลลิกรัม. อุณหภูมิ : อุณหภูมิห้อง, สภาพตัวอย่างปกติ
วันที่รับตัวอย่าง	04 มกราคม 2556
วันที่ทดสอบ	10 มกราคม 2556 - 14 มกราคม 2556

ผลการทดสอบ

รายการทดสอบ	ผลการทดสอบ			วิธีทดสอบอ้างอิง
	RT	%Area	%Match	
Docosane	56.93	1.42	90	In-house method based on GC-MS
Tetracosane	58.70	4.20	98	
Phenol, 2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl	58.81	0.59	96	
Pentacosane	60.87	13.45	99	
Hexacosane	63.62	16.70	96	
Heptacosane	67.21	15.77	97	
Octacosane	71.86	10.45	97	
Nonacosane	78.01	31.02	98	

อนุมัติผลโดย
(นายสมชาติ ศิริเรือง)
ลงนามแทนผู้อำนวยการห้องปฏิบัติการ
สาขากรุงเทพฯ

Appendix Figure 5 GC-MS data of alkanes mixture

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3. Staffs for "Second International symposium of BioPesticide and Ecotoxicological Network" (2nd-ISBioPEN) September 24th-25th, 2012 at Bangkok, Thailand.
4. Participated "Secondary Metabolites: From Extraction to structure elucidation" workshop November 12nd-16th, 2012 at Department of Biochemistry, Kasetsart University, Bangkok, Thailand.