รายงานวิจัยฉบับสมบูรณ์

โครงการ ประสิทธิภาพและกลไกของสารกระดุมทองเลื้อยเพื่อการเกษตรอินทรีย์

Bioefficiency and its mode of action of *Wedelia trilobata* (L.) Hitchc extract for organic agriculture

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย และมหาวิทยาลัยเกษตรศาสตร์

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รหัสโครงการ RSA5880045

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Abstract

Project Code : RSA5880045

Project Title : Bioefficiency and its mode of action of *Wedelia trilobata* (L.) Hitchc extract for organic agriculture

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The antifeedant and contact toxicity of *Wedelia trilobata* (Asteraceae) extracts (Synnonyme:: *Sphagneticola trilobata)* and isolated alkane compounds were investigated. Leaves of *W. trilobata* were sequentially extracted with hexane, dichloromethane, ethyl acetate, and methanol. Each extract and the compounds isolated were evaluated against the third instars of *Spodoptera litura, Spodoptera exigua*, and *Plutella xylostella.* Ethyl acetate extract and isolated alkanes were feeding deterrents as well as contact toxins against all the three species evaluated (FI50 ~ 0.27- 2.34 mg/ml; LD50~0.88-4.2 µg/larvae for ethyl acetate extract, and FI50 ~0.06-4.35 mg/ml; LD50 ~ 0.72-3.54 Ethyl acetate extract for isolated alkane). Impact on detoxifying enzymes was variable. The ethyl acetate crude extract reduced carboxylesterase activity in *S. litura* and *P. xylostella* while in *S. exigua* the enzyme was induced. In contrast, glutathione-*S*-transferase activity was induced in *S. exigua* but no significant difference in *P. xylostella* and *S.litura* was observed. Our results suggest that the *S. trilobata* extracts have multiple biological activities that contribute to the toxicity in lepidopterans. Variable enzyme responses to the products evaluated in different lepidopteran species also confirm that some species specific inductions do occur, suggesting the possibility of resistance development in the future, which cannot be summarily ignored. However, for this detailed biochemical studies are required. Morover, there are no toxicity occur when applies the extract on guppies fish at dose 10,000 mg/L. The crude extract and their isolated compound also showed the synergistic effect to cypermethrin for feeding deterent in *S.litura.* Multiple bioefficacies of *S. trilobata* makes it a potential botanical for further exploitation on larger scale so that field potential can be established in any IPM system.

Key words: *Wedelia trilobata*, *Sphagneticola trilobata* , plant extract, Lepidopteran larvae

บทคัดย่อ

รหัสโครงการ: RSA5880045

ชื่อโครงการ: ประสิทธิภาพและกลไกของสารกระดุมทองเลื้อยเพื่อการเกษตรอินทรีย์

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ระยะเวลาโครงการ: 3 ปี

สารสกัดจากกระดุมทองเลื้อย หรือ *Wedelia trilobata* (Asteraceae) (Synnonyme :: Sphagneticola trilobata) ซึ่งประกอบด้วยสารประกอบกลุ่มอัลคาไลน์ที่สามารถกำจัดแมลงศัตรูกลุ่มหนอนผีเสื้อโดยวิธีการรับสัมผัส และมีฤทธิ์ในการยับยั้งการกิน ทั้งนี้ ใบของ ต้นกระดุมทองเลื้อยจะถูกสกัดด้วยสารละลายเฮกเซน ไดคลอโรมีเทน เอทิลอะซิตเตท และเมทานอล แล้วนำมาทดสอบกับหนอนกระทู้ผักวัยที่สาม (*Spodoptera litura*) หนอนกระทู้หอมวันสาม (*Spodoptera exigua*) และหนอนใยผักวัยสาม (*Plutella xylostella*) จากการศึกษาพบว่า สารสกัดหยาบที่สกัดโดยเอทิลอะซิเตตและสารกลุ่มอัลเคน มีความสามารถในการยับยั้งการกินและมีความเป็นพิษจากการรับสัมผัสสารกับหนอนทั้งสามชนิด (FI50 ~ 0.27-2.34 มก./มล. และ LD50 ~ 0.88-4.2 ไมโครกรัม / ตัวอ่อนสำหรับสารสกัดหยาบจากเอทิลอะซิเตท และ FI50 ~ 0.06-4.35 มก. /มล. และ LD50 ~ 0.72-3.54 สำหรับสารกลุ่มอัลเคน) นอกจากนี้พบว่า สารสกัดจากต้นกระดุมทองเลื้อยและสารเดี่ยวกลุ่มอัลเคนส่งผลต่อเอนไซม์กำจัดสารพิษ โดยสารสกัดหยาบเอทิลอะซิเตตลดปฏิกิริยาของคาร์บอกซิลเอสเทอรส ใน *S. litura* และ *P. xylostella* แต่กลับกระตุ้นปฏิริยาของเอนไซม์คาร์บอกซิลเอสเทอรสใน *S. exigua* ในขณะที่เอนไซทม์กลูต้าไธโอนเอสทรานสเฟอเรสกลับมีปฏิกิริยาสูงขึ้นใน *S. exigua* แต่ไม่พบความแตกต่างของปฏิริยาใน *P. xylostella* และ *S.litura* อย่างมีนัยสำคัญ ดังนั้น ผลการทดลองชี้ให้เห็นว่าสารสกัดกระดุมทองเลื้อยมีกิจกรรมทางชีวภาพหลายอย่างที่ส่งผลต่อความเป็นพิษของแมลงกลุ่มผีเสื้อ ซึ่งการเพิ่มขึ้นของปฏิริยาของเอนไซม์ทำลานพิษสามารถก่อให้เกิดการพัฒนาความต้านทานต่อไปในอนาคต นอกจากนี้ในการทดลองนี้ยังทดสอบในปลาหางนกยูง ซึ่งพบว่าสารสกัดจากกระดุมทองเลื้อยมีความเป็นพิษค่อนข้างต่ำเมื่อเทียบกับสารจากพืชชนิดอื่นเนื่องจากไม่พบอัตราการตายที่ระดับความเข้มข้นสูงกว่า 10,000 มก. / มล. รวมทั้งพบว่า สารสกัดหยาบและสารประกอบที่แยกได้จากกระดุมทองเลื้อยมีความสามารถในการเสริมฤทธิ์กับไซเปอร์เมทริน เมื่อผสมในอาหารกับหนอนกระทู้ผัก ลักษณะฤทธิ์ทางชีวภาพในการกำจัดแมลงที่หลากหลายของสารสกัดจากกระดุมทองเลื้อยนี้ จึงเป็นไปได้ว่า อาจจะสามารถใช้ในการพัฒนาเพื่อใช้ในการควบคุมแมลงศัตรูพืชด้วยระบบ IPM

คำสำคัญ: กระดุมทองเลื้อย , สารสกัดจากพืช, แมลงกลุ่มหนอนผีเสื้อกลางคืน

1. Introduction

Polyphagous lepidopterans like *Spodoptera litura, Spodoptera exigua* or *Plutella xylostella* are well known economically important insect pests that defoliate many economically important crops. As of today the major control method is the use of synthetic insecticides. However, their indiscriminate use has also led to development of multiple resistances (Ahmed and Irfanullah, 2007) and subsequent field control failures (Ahmad *et al*, 2008).

The commonly recommended approach is an integrated pest management for efficient, low-residue, and cost-effective management of these insect pests. In the management schemes use of botanical insecticides has gained momentum during last 2 decades. Several plant extracts, essential oils and isolated compounds have been evaluated for their activity against a variety of pests (Koul, 2005; Koul, 2016 and Koul *et al*, 2008). Botanical insecticides are considered to replace synthetic insecticides due to their rapid biodegradation and minimal toxicity to mammals. Neem has made substantial impact as a botanical insecticide globally (Schmutterer, 1990 and Koul and Wahab, 2004) and many other botanical products have been comprehensively documented recently (Koul, 2016) for their efficacy as insecticides.

One such plant material is *Sphagneticola trilobata* L. from the Asteraceae family that thrives well under tropical conditions of Thailand. It is on record that this plant has growth inhibitory properties, like against golden apple snail (Rezende *et al*, 2000). From the insecticidal point of view, the antifeedant activity against cotton boll weevils has been reported (Howard *et al*, 1990).

In addition, earlier studies showed that some plant extracts that could be alternative insect control products for farmers but at the same time, there are challenges for non-targets as well; therefore, there is need to understand how these compounds influence other non-target organisms, especially aquatic organisms. Thus, In this research, the studied on effect of *Sphagneticola trilobata* will analysed toxicity on guppies also.

2. Obejective

The objective of the present study was to determine the efficacy of this plant against lepidopterans in general; therefore, the detailed study was conducted against *S. litura, S. exigua and P. xylostella* larvae. The aim was to determine the efficacy of different extracts of this plant as feeding deterrent and/or contact toxin and establish a potential of *S. trilobata* as a biopesticide. An addition objective was to examine if these products interfere with detoxification enzymes of the insects.

Moreover, the acute toxicity of *S. trilobata extracts* and some essential oil compoounds which established as insecticidal compounds were studied to the insectivorous guppy *Poecilia reticulata*, the standard test species pursuant to American Public Health Association (APHA), the American Water Works Association (AWWA), and the Water Environment Federation (WEF). (1998) and Organisation for Economic Co-operation and Development (1993) under laboratory conditions.

3. Materials and methods

3.1 Insects

S. litura, S. exigua and P. xylostella larvae were obtained from a laboratory colony maintained in the Animal Toxicology and Physiology Specialty Research Unit, Department of Zoology, Faculty of Science, Kasetsart University. Larvae were reared in plastic boxes (15×25 cm) under controlled condition at 25±2^{0}C, 75% relative humidity and photoperiod of 16:8 (L:D). *S. litura* and *S. exigua* larvae were fed on artificial diet (the mixture of 240 g green bean, 25 g agar, 40 ml mixed vitamin solution, 5 g ascorbic acid, 40 ml amoxicillin solution, 3 g sorbic acid, 5 g methylparaben, 20 g yeast, 4 ml of 40% formalin and 1.41 L water) and *P. xylostella* larvae were fed on kale leaves organically grown by us in the campus. After pupation, pupae were transferred to pupation boxes lined with 2-3 layers of moist sterile filter paper. After moths emerged, they were moved to net cages, fed with 10% sugar solution and allowed to oviposit on folded filter papers in moth cages. The population obtained from this culture (third generation) was used for the study and third instars were evaluated for their bioefficacy.

All experimental procedures in this research were performed with the approval of an appropriate animal Ethics Committee of Kasetsart University, Thailand under the references number OACKU01059.

3.2 Fish

Both male and female adult guppy, *Poecilia reticulata* (2 months old) with a mean weight of 0.20 g and a mean total length of 2.50 cm were obtained from a local breeder in Bangkok, Thailand and brought to the laboratory within 30 min in plastic bags with sufficient air. The plastic bags were placed in the maintenance aquarium for about 30–35 min for acclimatization. Then the bags were cut open and the fish were allowed to swim into the aquarium filled with dechlorinated tap water and allowed to acclimatize to laboratory conditions (26.0 ± 0.8°C, 78 ± 2% RH, pH = 7.0). Test chambers were glass aquaria of 10 L capacity. Healthy fish were chosen for the experiments with the lengths and weights of the fish in the range 3.8–5.1 cm and 15–25 g, respectively. The procedure was performed with the approval of an appropriate Ethics Committee of Kasetsart University, Thailand under the reference number ACKU03858.

3.3 Plant material

S. trilobata leaves, which grows as a weed, were collected from Kasetsart University, Thailand in July 2011. The leaves were washed with water and shade dried at ambient temperature for three days. Dried leaves were powdered for disintegration using agrinder (WF-10, Thailand) and stored in zip-lock bags in refrigerator at 4^{0}C to prevent sample contamination. A voucher plant specimen (BK 064385) was preserved in Princess Sirindhorn Plant Herbarium of Plant Varieties Protection Division, Department of Agriculture, Thailand.

3.4 Extraction and isolation

The dried leaf powder of *S. trilobata* (200 g) was extracted by a Soxhlet's apparatus using 33 g per thimble. Extraction solvents used were hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH). The extraction was conducted in 1 L of each solvent for 8 h/solvent. Each crude extract was filtered and dried using a rotary evaporator (IKA®RV10 basic, Thailand) and stored at 4^0C until further use in the experiments.

As ethyl acetate extract was determined as highly active compared to other extracts both as antifeedant and contact toxin; this extract was processed further to isolate active compounds. The crude extract was fractionated using vacuum silica gel column chromatography (Kiesel gel 60G, Merck, Thailand) and eluted with a gradient of gradually increasing polarity (5% increments) of hexane-EtOAc and EtOAc-methanol, respectively. All fractions were subjected to thin-layer chromatography (TLC), and those with similar components were combined. Using this procedure, five fractions were obtained. Active fraction 1 (100% Hexane-55% EtOAc-Hexane) was subjected to silica gel column chromatography (Kiesel gel 60G) and eluted with hexane-EtOAc (9:1) to obtain sub-fraction 1–1 as a white solid (2.45%), which was subsequently subjected to structural analysis using ^{1}H NMR and GC–MS. Sub-fraction 1–1 was identified as a mixture of long chain alkanes. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.37–1.15 (brm), 0.95–0.76 (brm). GC–MS analysis data of sub-fraction 1–1 showed the compounds were alkanes.

3.5 Essential oil compounds

1,8-cineole and thymol were obtained commercially (97–99 % purity) from Sigma- Aldrich (St. Louis, USA) and were evaluated individually to determine their efficacy levels against guppy. Compounds were dissolved in 0.5% triton X100 in acetone (AR grade). The dosing volume never exceeded 1 mL in the test aquarium (0–50 mg/L for thymol and 0–10,000 mg/L for 1,8-cineole).

3.6 Antifeedant assay

The no-choice procedure (Singh *et al*, 2009) was followed using 4 hours starved third instars of *S. litura, S. exigua* and *P. xylostella*. The duration of experiments was 4 hours to observe the behaviour of the insects. The kale leaf discs of 9 cm^2 were used for the study. The extracts and active compounds were dissolved in acetone (AR grade) and various concentartions prepared were in the range of 0-20 mg/ml. The test materials were painted with 1 ml of solution directly onto the two sides of leaf discs (4 cm^2) and then dried at room temperature for 2 hours. The leaf discs were placed over a moistened filter paper in Petri dishes of 15 × 100 mm size. Each arena contained one leaf disc and one larva. For each treatment concentration and control group 10 replicates were used. The consumed area of the leaf disc was determined by leaf area meter (WinDias3, Delta T Device, UK).

The antifeedant index was calculated as

Antifeedant (%) = [(C – T)/C] x 100

Where C = the consumed leaf area of control discs

T = the consumed leaf area of treated discs

The feeding deterence index was finally converted to the median feeding deterrence (FI_{50}) that was calculated by using antifeedant percentage of each concentration with Probit analysis (StatPlus Program for Mac 2017).

3.7 Contact toxicity

Acute contact toxicity of each crude extracts and alkane compounds was determined by topical application to third instars *S. litura, S. exigua* and *P. xylostella*. Plant extracts were evaluated in the range of 0-50 µg/larvae and alkane compounds in the range of 0-20 µg/larvae using acetone as a carrier such that each larva received 2 µl of solution per treatment. Acetone alone was used in controls as this solvent is safe and quick to evaporate. Each larva was placed in the Petri dish lined with filter paper at the bottom. The larvae were treated topically using a 1 ml microapplicator (Model PB-600, PAT. 3161323 ,Hamilton company, Switzerland) by dropping 2 µl dose on to the thorax of each insect based on microlitter scale of the syringe. The treated larvae were provided a fresh kale leaf disc or artificial diet as required for the feeding of insects. In each experiment, 30 larvae/treatment in five replicates were used (n = 150 per treatment). After treatment, treated larvae were placed in a sealed plastic tray (30 x 20 x 10 cm) lined over with moistened filter paper for 24 h under controlled conditions in a rearing room. The trays were covered with a black cover as *S. litura*, *S. exigua* and *P. xylostella* exhibit positive phototropism. Mortality was assessed after 24 h. The median lethal dosewas calculated by using Probit analysis (StatPlus Program for Mac 2017).

3.8 Detoxification enzyme assays

Insect's enzyme activities were determined with two assays including carboxylesterase activity (CE), and glutathione-*S*-transferase (GST) activity. *In-vivo* assays were done for 20 surviving third instars of *S. litura, S. exigua* and *P. xylostella* after they were treated for 24 hours with *S. trilobata* extracts and its pure compounds at LD_{50} dose and compared with control group (treated with acetone only) by using microplate reader technique. Twenty larvae were pooled and homogenized in 0.5 mL buffer (100 mM Potassium phosphate buffer, pH 7.2 and 1% Triton-X-100) for 5 replicates of each homogenate. The homogenate was centrifuged at 10 000 × *g* for 15 min at 4°C, and the supernatant was used as an enzyme source. All enzymes analyses were done in 5 biological replicates. For both enzyme assays positive controls were also used. For CE, triphenyl phosphate and for GST diethyl maleate were evaluated

Carboxylesterase (CE) activity was determined by modified method of Bullangpoti *et al* (2012). Enzyme solution (40 µl) was mixed with *p*- nitrophenylacetate (pNPA) (40 µl; 10 mM in DMSO) and potassium phosphate buffer (200 µl; 50 mM, pH 7.4). Enzyme activity was measured at 410 nm and 37°C for 90 s with the microplate reader in the kinetic mode. The activity of CE was determined by using the extinction coefficient of 176.4705 for pNPA.

The method for determining glutathione-*S*-transferase (GST) activity were modifeid from Oppenoorth *et al* (1979). The reaction solution contained 100 µl of enzyme solution, 200 µl of 50 mM potassium phosphate buffer (pH 7.3) and 10 µl of 150 mM 1-chloro-2,4-dinitrobenzene (CDNB). Optical density was recorded at

intervals of 30 s for 3 min at 37°C and 340 nm with a microplate reader. The GST activity was determined from the extinction coefficient of 0.0096 for CDNB.

Total protein content of each fraction used as enzyme source was determined by the Bradford method (Bradford, 1976) before measuring enzyme activities. All the stastistic analysis was done by using StatPlus Program for Mac version 2017 (AnalystSoft Inc., Canada).

3.9 Acute toxicity bioassay for guppies

A standard bioassay was followed (American Public Health Association (APHA), the American Water Works Association (AWWA), and the Water Environment Federation (WEF), 1998; Organisation for Economic Co-operation and Development (OECD), 1993). The procedure was performed with the approval of an appropriate Ethics Committee of Kasetsart University, Thailand under the reference number ACKU03858. Test chambers were filled with 5 L of non-chlorine water (drinking water). The water temperature was 26 ± 1°C and the dissolved oxygen level was 7.2–7.9 mg/L. All determinations were repeated five times for each concentration. Groups of experimental animals, each consisting of 10 individuals, were selected at random and placed into aerated aquaria. After 24 hr of adaptation, 1 mL of different concentrations of essential oil compounds were added to the experimental aquaria making total concentrations of 0–20 mg/L for thymol and 0–10,000 mg/L for 1,8-cineole. A control group was provided with 1 mL of 0.5% triton X100 in acetone (AR grade) in a test aquarium. The fish were not fed for the duration of the experiment. Mortality was recorded 24 hr after the start of the tests. Dead individuals were removed immediately. Behavioral changes were followed closely. The median lethal concentration (LC_{50}) and 95% confidence limits were calculated using the Statplus for Mac software program (AnalystSoft Inc., Walnut, Canada).

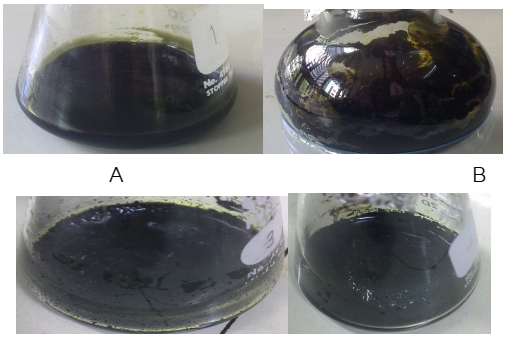
3.10 Synergistic effect studies

Cypermethrin (25% EC) was purchased from Fluka. For combination-based studies, LD50 value of cypermethrin and LD50 value of best control efficiency of crude extract and isolated compounds were prepared in a ratio of 1 : 1. Antifeedant efficacy of eachformulation was estimated as above, formulation was estimated as above, and the feeding deterence index was finally converted to the median feeding deterrence (FI_{50}) that was calculated by using antifeedant percentage of each concentration with Probit analysis (StatPlus Program for Mac 2017).

4. Results

4.1 Yield of extracts

All extracts obtained were dark green viscous semisolids. The percent yields were calculated by comparing the mass of crude extracts to the amount of fresh young leaves. The yield from hexane, dichloromethane, ethyl acetate and methanol extraction were 3.59, 1.02, 1.14 and 5.02% w/w.



A B

C D

Figure 1 Characteristic of crude extract; A=Hexane crude extract: B=Dichloromethane crude extract: C=Ethyl acetate crude extract: D=Ethanol crude extract

4.2 Antifeedant and contact toxicity result

When compared to controls, reduced food intake was observed in all plant extract treated leaf discs consumed by the three species. The highest antifeedant efficacy was observed in the ethyl acetate extract (Table 1).

Table 1. Median feeding deterrence index (FI_{50}: mg/ml)[1] and 95% confidence interval due to *S. triolobata* extracts in third instar *S. litura*, *S.exigua* and *P. xylostella* larvae.

	S. litura	*S.exigua*	*P. xylostella*
Hexane crude extract	9.64a	5.25a	0.60a
	(9.07- 10.21)	(4.9-5.6)	(0.56-0.64)
DCM crude extract	7.13b	4.19b	0.49b
	(6.8 – 7.82)	(3.52-4.86)	(0.42-0.56)
EtOAc crude extract	1.74c	2.34c	0.27d
	(1.46 – 2.02)	(2.17-2.51)	(0.24-0.30)
MeOH crude extract	9.47a	5.13a	0.33c
	(8.67-10.27)	(4.76-5.5)	(0.29-0.37)

[1] Values with the same letter are not significantly different at $P>0.05$ (Tukey's test)

For contact toxicity, ethyl acetate extract was most toxic (LD_{50} = 3.24 µg/larvae) as compared to other 3 extracts (Table 2). Methanol extract was least toxic with an LD_{50} of 35.94 µg/larvae against *S. litura* larvae. Though Ethyl acetate extract was a general toxin against all the 3 species evaluated but least LD_{50} was recorded for *P. xylostella* (0.88 µg/larvae) and highest for *S. exigua* (4.20 µg/larvae) (Table 2).

Table 2 Toxicity value[1] (LD_{50} (CI95%[2]) (µg/larvae)) of *S. litura*, *S. exigua* and *P. xylostella* larvae after 24 hours of topical application by *S. triolobata* extracts.

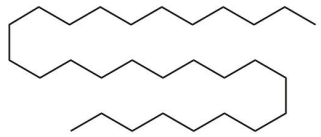
	LD_{50}	CI 95%	Slope	Chi^2
S. litura				
Hexane crude extract	15.32b	14.30-16.34	0.89±0.15	11.28
DCM crude extract	8.78c	7.48-10.08	0.96±0.08	1.41
EtOAc crude extract	3.24d	2.74-3.74	0.88±0.08	0.29
MeOH crude extract	35.94a	33.4-38.48	0.73±0.15	7.57
S. exigua				
Hexane crude extract	7.98b	7.77-8.28	1.07±0.50	9.77
DCM crude extract	14.26d	10.52-18.96	1.05±0.14	2.77
EtOAc crude extract	4.2a	1.84-6.92	1.20±0.41	1.30
MeOH crude extract	10.88c	6.98-17.16	0.85±0.12	3.92
P.xylostella				
Hexane crude extract	5.68a	4.50-6.86	2.31±0.09	18.09
DCM crude extract	1.11b	0.84-1.36	2.05±0.10	0.311
EtOAc crude extract	0.88b	0.66-1.10	2.06±0.11	0.24
MeOH crude extract	1.46b	1.08-1.84	1.76±0.11	0.35

[1] Values with the same letter are not significantly different at $P>0.05$ according to Tukey's Test. No mortality occurred in control group (treated with acetone only).

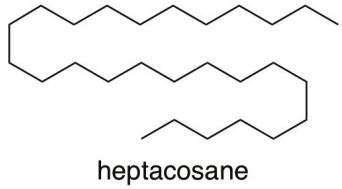
[2] CI95% = confidence interval of 95%

Though 6 alkanes were identified by GC-MS analysis, 3 major alkanes present in the extract (nonacosane, hexacosane and heptacosane) were confirmed by comparing with standards purchased from Sigma® and evaluated for both antifeedant and contact toxicity against all the three insects. Only 3 alkanes, nonacosane, hexacosane, heptacosane (Fig. 2) were tested because they constituted about 65% of the total active fraction. Other 3 compounds were in the range of 1 to 13% (Table 3).

(a)



(b)



(c)

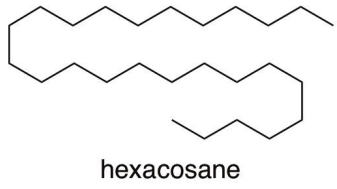


Figure 2 Structure of (a) Nonacosane (b) Heptacosane and (c) Hexacosane

Table 3 GC-MS analysis data of sub-fraction of ethyl acetate extract

Compound	Results		
	Retention time	Area (%)	Match (%)
Docosane	56.93	1.42	90
Tetracosane	58.70	4.20	98
Pentacosane	60.87	13.45	99
Hexacosane	63.62	16.70	96
Heptacosane	67.21	15.77	97
Nonacosane	78.01	31.02	98

Both contact toxicity and feeding deterrence for pure compounds were dose-dependent. Nonacosane was most toxic to *S. litura* (LD_{50} = 2.70 µg/larvae) and *P. xylostella* larvae (LD_{50} = 0.72 µg/larvae) compared to heptacosane and hexacosane (Table 4). However, in case of *S. exigua* hexacosane was most toxic (LD_{50} = 1.34 µg/larvae) compared to other two compounds (Table 4). Notwithsatanding, nonacosane was the best in inhibiting feeding in all the three species compared to other 2 compounds (Table 4).

Table 4 Feeding deterrence ($FI_{50,}$ mg/ml) [1] and contact toxicity ($LD_{50,}$ µg/larvae) [1] of alkane compounds from *S. triolobata* ethyl acetate extract against *S. litura*, *S. exigua* and *P. xylostella* larvae after 24 hour exposure.

	S. litura		*S.exigua*		*P. xylostella*	
	Antifeedant	Contact toxicity	Antifeedant	Contact toxicity	Antifeedant	Contact toxicity
Nonacosane	1.15±0.12c	2.7±0.62a	1.27±0.03c	3.54±0.18a	0.06±0.001c	0.72±0.08a
Heptacosane	4.35±0.58a	3.35±0.24a	2.62±0.06a	2.16±0.24b	0.25±0.01a	1.30±0.16b
Hexacosane	4.08±0.75a	3.02±0.18a	2.33±0.04b	1.34±0.18c	0.15±0.04b	0.92±0.18a

[1] In all experiments, values followed by the same letter are not significantly different at *P*>0.05 according to Tukey's Test. No mortality occurred in control group (treated with acetone only).

4.3 Detoxification enzyme assays

The carboxylesterase activity of *S. litura* and *P. xylostella* were inhibited by *S. trilobata* extracts *in-vivo*, specifically by ethyl acetate extract when compared with controls (the treated with acetone) ($P < 0.05$, Tukey's

test). The correlation factor suggested 1.11 and 3.14-fold decrease from control groups, respectively (Table 5). Heptacosane also showed significant decrease ($P < 0.05$, Tukey's test) with correlation factor range of about 3.27-folds for carboxylesterase in *P. xylostella.*

DCM, EtOAC and MeOH extract of *S. trilobata* and nonacosane had significant induction activity for glutathione-s -transferase in *S. exigua* with correlation factor in the range of 0.67-0.79 fold; whereas no siginificant difference for *P. xylostella* and *S.litura* were observed ($P > 0.05$, Tukey's test, Table 6).

Table 5 Carboxylesterase activity ± SE (nM p-nitrophenol/ min/ mg protein)[1] and correlation factor (CF)[2] of 24 hour survived larvae of *S. litura , S. exigua and P. xylostella* after treated with *S. triolobata* crude extract and their alkane compounds at FI_{50} level.

Treatment	*S.litura* (CF) [2]	Activity[3]	*S.exigua* (CF) [2]	Activity[3]	*P.xylostella* (CF) [2]	Activity[3]
Control[4]	1.13 ±0.08a	-	1.26±0.02a	-	0.51±0.37a	-
Hexane crude extract	1.15±0.05a (0.98)	*N.F*	1.49±0.05b (0.84)	Induction	0.33±0.07a (1.55)	*N.F*
DCM crude extract	1.14±0.05a (1.0)	*N.F*	1.44±0.06b (0.88)	Induction	0.20±0.01a (2.50)	*N.F*
EtOAc crude extract	1.03±0.10b (1.11)	Inhibition	1.53±0.04b (0.82)	Induction	0.16±0.02b (3.14)	Inhibition
MeOH crude extract	1.10±0.14a (1.04)	*N.F*	1.80±0.06c (0.7)	Induction	0.20±0.01a (2.56)	*N.F*
Nonacosane	1.09±0.08a (1.04)	*N.F*	1.65±0.09c (0.76)	Induction	0.21±0.01a (2.37)	*N.F*
Heptacosane	1.16±0.07a (0.98)	*N.F*	1.38±0.14a (0.91)	*N.F*	0.16±0.01b (3.19)	Inhibition
Hexacosane	1.27±0.17a (0.89)	*N.F*	1.38±0.01a (0.91)	*N.F*	0.20±0.01a (2.53)	*N.F*
Triphenyl phosphate	1.04±0.10b (1.11)	Inhibition	0.21±0.14b (6.00)	Inhibition	0.16±0.01b (3.19)	Inhibition

[1] In all experiments, values followed by the same letter within the same column is no significant level using Tukey's Test ($P>0.05$)

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

[3] N.F = No Effect

[4] Control = the treatment with acetone only

Table 6 Glutathione-*S*-transferase activity ± SE (CDNB conjugated product/ mg protein/min)[1] and correlation factor (CF)[2] of 24 hours survived larvae of *S. litura* , *S. exigua* and *P. xylostella* after treated with *S. triolobata* crude extract and their alkane compounds at FI_{50} level

Treatment	*S.litura* (CF) [2]	Activity[3]	*S.exigua* (CF) [2]	Activity[3]	*P. xylostella* (CF) [2]	Activity[3]
Control[4]	1.55±0.02a	-	0.65±0.02a	-	0.76± 0.11a	-
Hexane crude extract	1.94±0.06a (0.80)	*N.F*	0.78±0.09a (0.83)	*N.F*	0.71±0.09a (1.07)	*N.F*
DCM crude extract	2.45±0.08b (0.63)	Induction	0.96±0.06c (0.67)	Induction	0.75±0.08a (1.02)	*N.F*
EtOAc crude extract	1.79±0.04a (0.87)	*N.F*	0.85±0.05b (0.76)	Induction	0.76±0.14a (1.00)	*N.F*
MeOH crude extract	2.30±0.09b (0.67)	Induction	0.88±0.15b (0.74)	Induction	0.71±0.05a (1.07)	*N.F*
Nonacosane	0.96±0.08c (1.62)	Inhibiton	0.82±0.06b (0.79)	Induction	0.78±0.14a (0.98)	*N.F*
Heptacosane	0.84±0.14c (1.84)	Inhibiton	0.63±0.01a (1.03)	*N.F*	0.87±0.29a (0.87)	*N.F*
Hexacosane	1.24±0.22a (1.25)	*N.F*	0.62±0.03a (1.05)	*N.F*	0.81±0.18a (0.94)	*N.F*
Diethylmaleate	0.43±0.03d (1.08)	Inhibiton	0.56±0.06d (1.16)	Inhibiton	0.56±0.03b (1.36)	Inhibition

[1] In all experiments, values followed by the same letter within the same column is no significant level using Tukey's Test (P>0.05)

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

[3] *N.F* = No Effect

[4] Control = the treatment with acetone only

4.4 Toxicity of thymol and 1,8- cineole and crude extract on fish

The calculated 24-h acute LC_{50} values of thymol and 1,8-cineole for the female fish were 12.51 and 3,997.07 mg/L, respectively, and for males were 10.99 and 1,701.93 mg/L, respectively (Table 7); whereas, the toxicity of *S. triolobata* crude extract is higher than 10,000 ppm. No mortality occurred in the control group and at 10,000 mg/L of *S. triolobata* crude extract. The results showed that thymol was more toxic to fish

than 1,8-cineole and of *S. triolobata* crude extract. The mortality was dose dependent. However, there was no significant ($p > 0.05$), sex-dependent difference in toxicity for both compounds.

Observations were made of the behavioral responses of the guppies during the acute toxicity tests. The control group showed normal behavior during the test period. The changes in behavioral response started after dosing with all compound, especially thymol, where paralytic, erratic swimming, rapid gill movement and motionless stature of fish were observed. Fish were seen adhering to the bottom of aquaria at all treatment concentrations. At a dose of more than 10 mg/L of thymol, the paralysis occurred within the first hour of treatment in aquaria.

Table 7 Acute 24 hours toxicity of thymol and 1-8, cineole to both sexes of guppy fish, *Poecilia reticulata*

	Thymol		1-8 cineole	
	Female	Male	Female	Male
LC_{50} (mg/L) *,†	12.51[a]	10.99[a]	3,997.07[b]	1,701.93[b]
95% Confidence limits	6.86–19.02	5.70–13.94	2,722.93–4280.53	1,087.77–2766.35
LC_{90} (mg/L) *,†	23.11[a]	21.51[a]	7,385.35[b]	8,898.72[b]
95% Confidence limits	16.04–31.94	15.74–27.11	7,022.53–8,280.51	7,087.93–10,766.02
Slope ± SE	4.81 ± 2.05	4.40 ± 1.85	4.81 ± 2.05	4.40 ± 1.85
Intercept ± SE	-0.27 ± 0.15	0.42 ± 0.15	-12.31 ± 0.15	-11.08 ± 0.16
Chi square	0.29	0.40	0.09	0.47

* No mortality in control group and *S. triolobata* crude extract at dose higher than 10,000 mg/ml

† Median lethal concentration (LC_{50}) and 90 percentage lethal concentration (LC_{90}) values followed by a common, lowercase, superscript letter in the same raw are not significantly different at the 5% level using Duncan's multiple range test.

4.5 Possiblity of extract to synergist cypermethrin for feeding deterent

The results showed all isolated alkane compounds and *S. trilobata* ethylacetate crude extract seem to have abibity as synegistic effect with cypermethrin for feeding deterent on *S.litura* (Table8) and it is possible that all isolated alkane compound can reduce the use of agricultural chemicals.

Table 8 Synergistic effect of *S. trilobata* on the antifeedant of cypermethrin to *S. litura* larvae

Compound	%FI ± SD (%)[a] (PPM)	SF[b]
Cypermethrin	18.24 ± 0.57[a]	-
Cypermethrin + *S. trilobata* ethylacetate crude extract	42.66 ± 0.69[b]	2.33
Cypermethrin + Hexacosane	58.26 ± 0.22[c]	3.24
Cypermethrin + Heptacosane	46.03 ± 0.31[d]	2.56
Cypermethrin + Nonacosane	47.41 ± 0.30[e]	2.63

[a] Values with the same letter are not significantly different at $P<0.05$ according to Tukey.

[b] Synergistic ratio (SR) = [%FI of cypermethrin alone/ (%FI of cypermethrin + compound)]

5. Discussion and conclusion

Botanical pesticides are often tried as suitable alternative to conventional pesticides of plant protection as they are considered to incur minimum negative risks (Isman and Machial, 2006 and Pavela, 2015). Accordingly, our objective was to study *S. trilobata* crude extracts and alkanes obtained from the plant, specifically as this species was easily available in hot and warm climate of Thailand. The extracts and alkanes were evaluated against lepidopteran larvae like *S. litura, S. exigua and P. xylostella* which are economically very important pests of various crops in Thailand.

On comparing the yield of extracts in various solvents, methanol extracted the highest amount (5.02%, w/w) followed by hexane, ethyl acetate and dichloromethane, respectively. It appears to be a generalist phenomenon that polar solvents give maximum yields of extracts as compared to non-polar solvents. For instance, *Coffea arabica* parchment (Phankaen *et al*, 2017), *Bauhinia scandens* var. *horsfieldii* (Poonsri *et al*, 2015), and *Andrographis paniculata* leaves (Kumoro *et al*, 2009) gave highest yield when extracted with methanol, ethanol or aqueous acetone. However, there does not seem to be sequential solvent specificity on the amount of extract obtained in less polar solvents. For instance, amount of extract obtained from *S. trilobata* in hexane was higher than the yield obtained in ethyl acetate and dichloromethane.

The feeding deterrent and insecticidal activity of several plants is well known and comprehensively documented (Koul, 2005; Koul, 2016 and Koul *et al*, 2008) and there are still many number of plant species that need to be screened and evaluated. Even the quantification of various anti-insect activities of botanicals is of great importance in the field of insect pest management (Kamaraj *et al,* 2008; Pavunraj *et al,* 2012; Lingathurai *et al,* 2011; Ling *et al*, 2008; Perera *et al*, 2000; Arivoli and Tennyson, 2012 and Nebapure *et al*, 2016). Accordingly, in the absence of quantitative research on *S. trilobata* against economically important

lepidoperans like *S. litura, S. exigua* and *P. xylostella*, the present study was undertaken. It was interesting to note from our present study that several solvent extracts from *S. trilobata*, especially ethyl acetate extract was effective as feeding deterrent and also showed contact toxicity against all three species evaluated. Ethyl acetate extract was composed of several alkanes; the fraction that possessed both the activities. Nonacosane showed the highest antifeedant activity in comparison to other alkane compounds against all three species (Table 4). However, additive or synergistic effect among alkane fractions could not be summarily excluded as compound mixtures of allelochemicals might be more effective as antifeedants or toxins.

While the extracts and alkanes evaluated did not show any knockdown effect in *S. litura, S. exigua* and *P. xylostella*, similar effects have been reported by us earlier due to alkanes isolated from *Bauhinia scandens* against *P. xylostella* larvae (Poonsri *et al*, 2015). Earlier the alkane like nonacosane-10-ol has been reported as nematicidal as well against *M. incognita* (Naz *et al,* 2013).

Our results also suggest that toxicity and antifeedant action of ethyl acetate extract was higher in *P. xylostella* than other two *Spodoptera* species. However, feeding deterrence was more prominent in *S. litura* than in *S. exigua* and same was true for ethyl acetate extract toxicity as well. This shows that different species respond differently to the extracts or the compounds isolated. This is not specific to *S. trilobata* but recorded in other studies as well (Feyereisen, 2012; Oakeshott *et al,* 2005; Ranson and Hemingway, 2005; Wright *et al,* 2000; Rachokarn *et al,* 2008; Sukhirun *et al,* 2011 and Feng *et al,* 1995). The obvious reason for this is the response of detoxification enzyme mechanism of each species. It is well known that herbivorous insects use detoxification enzymes, including carboxylesterase and glutathione-*S*-transferases, to metabolize otherwise deleterious plant secondary metabolites (Feyereisen, 2012; Oakeshott *et al,* 2005 and Ranson and Hemingway, 2005). These two detoxification enzymes normally play an important role in allelochemical metabolism and resistance (Oakeshott *et al,* 2005; Ranson and Hemingway, 2005 and Wright *et al,* 2000). The esterases are associated with pyrethroid and organophosphate detoxification in insects and carboxylesterase is an important enzyme under esterase family. Glutathione- *S*-transferase is specifically involved in organophosphate metabolism *via* conjugation.

The present *in-vivo* experiments (Tables 5) showed that ethyl acetate crude extract and heptacosane had potential to inhibit carboxylesterase activity, which causes potential mortality of the *S.litura* and *P.yxlostella* larvae, which was similar to the positive controls used. However, ethyl acetate crude extract induced glutathione-*S*-transferase and carboxylesterase activity in *Spodoptera exigua*. Overall, the present results confirm that inhibition of carboxylesterases by botanical products is possible as is evident from some earlier studies as well. For instance, *Melia azedarach* and *Amaranthus viridis* against *Spodoptera exigua* (Hubner)(Rachokarn *et al,* 2008), *Jatropha grosspifolia* against *Spodoptera frugiperda* (Singh *et al,* 2009), *Alpinia galanga* against *Bactrocera dorsalis* (Sukhirun *et al,* 2011), *Melia toosendan* Sieb. et Zucc. Pron. against *Spodoptera litura* F. and *Melanoplus sanguinipes* F. are known to inhibit these enzymes (Feng *et al,* 1995). Our results suggest that the *S. trilobata* extract may have multiple biological activities that contribute to the toxicity in lepidopterans. As Table 5 and 6, Enzyme response for *S. exigua* shown in tables 5 and 6 do indicate the induction of enzymes, however, it may negligible since CF values were less than 1.0, however; induction of glutathione- *S*-transferase cannot be summarily ignored because indicates to the possibility of resistance

development in the future. This of course requires a detailed biochemical study on few generations of the insect pests to establish the observations made.

Also, variations in enzymatic activities may be appreciable from intrapopulation variability sampled by chance in the survived instars as they were used from pooled set of larvae and the whole body crude homogenates were processed. This variable could be eventually missed. Moreover, *S. litura* and *S. exigua* larvae were reared on artificial diet whereas *P. xylostella* instars on kale leaves. The effect of pre-adapted growth to the artificial diet might also condition the detoxifying repertoire and then the response to allelochemicals can cause more tolerance. In conclusion, the results demonstrate the potential of extracts and alkane compounds of *S. trilobata* as botanical insecticides against *S. litura, S. exigua* and *P. xylostella.* These extracts also inhibit several enzyme activities that are important to insect survival and may constitute a useful alternative approach for integrated pest management.

Thymol and 1,8-cineole are now well known biopesticides from plant essential oils and have been shown to be toxic to many insects (Corbet et al., 1995; Sfara et al., 2009; Jean-Luc et al., 2014; Kumrungsee et al., 2014; Yotavongse et al., 2015). However, there are no reports that describe the toxicity of these two compounds to non-target animals like insectivorous fish. Thus, this project would like to compare toxicity on guppies with *S. trilobata* extract . Therefore, it was interesting to study in-depth the acute toxicity to this economically important fish species. The 24 hr LC_{50} values for females of thymol and 1,8-cineole for *P. reticulata* were 12.51 and 3,997.07 mg/L, respectively; whereas for males they were 10.99 and 1,701.93 mg/L, respectively; whereas , no mortality occur for *S. trilobata* extract although treated at high dose more than 10,000 mg/L. The results suggested that *S. trilobata* extract was quite safe to guppy fish.

The present studies revealed a synergistic activity of *S. trilobata* extract and their isolated alkane compounds towards the insecticide cypermethrin, a photostable synthetic pyrethroid. The Feeding value of cypermethrin against S. litura larvae was increase feeding deterent about 2.33-3.44 folds or increase to feeding deterene efficiency to about 30-40%. This result is similar to those obtained in studies on botanicals as synergists for synthetic chemicals such as *Melia azedarach* and *Jatropha gosyypifolia* extract against *Spodoptera frugiperda* (Bullangpoti *et al,* 2012). Thus, *S. trilobata* extract and/or their isolated alkane compoounds may provide alternative synergist compounds at a cheaper price (the extracts cost less than $US 0.5 /1kg as it is a weed) and may contribute to reducing the use of the synthetic insecticide cypermethrin

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Output

1. **Publications:**

- Puntipa Junhirun, Wanchai Pluempanupat, Thitaree Yooboon, Torranis Ruttanaphan, Opender Koul, **Vasakorn Bullangpoti**; The Study of Isolated Alkane Compounds and Crude Extracts From *Sphagneticola trilobata* (Asterales: Asteraceae) as a Candidate Botanical Insecticide for Lepidopteran Larvae, *Journal of Economic Entomology*, , toy246, https://doi.org/10.1093/jee/toy246 **(PUBLISHED: Corresponding author)**

- **Vasakorn Bullangpoti**, Warasinee Mujchariyakul,Nutthalak Laksanavilat, Puntipa Junhirun. 2018. Acute toxicity of essential oil compounds (thymol and 1,8-cineole) to insectivorous guppy, *Poecilia reticulata* Peters, 1859. Agriculture and Natural Resources. https://doi.org/10.1016/j.anres.2018.06.011 **(PUBLISHED: Corresponding author)**

- Thitaree Yooboon, Anchulee Pengsook, Atcharee Ratwatthananon, Wanchai Pluempanupat, and **Vasakorn Bullangpoti.** 2018. The Development of a Plant-Based Extract Mixture for Control of *Spodoptera litura* (Lepidoptera: Noctuidae) **(SUBMITTED: Corresponding author)**

2. **Other:**

Produce 2 Ph.D. students and 2 undergraduate students in agricutural science studies area (Insect toxicology)

Recommendation

the persistanc of extracts under various environmental condition and side effect to some non-target species, especially beneficial insects should remain the questions of concern and subject of future studies.