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Effects of Root Extract from Derris (*Derris elliptica* Benth) on Mortality and Detoxification Enzyme Levels in the Diamondback Moth Larvae (*Plutella xylostella* Linn.)

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ABSTRACT

Two types of ethanol extraction methods, Soxhlet and stirring soaking, were carried out. The rotenone content determined by the HPLC was 8.6 % w/w for the former method compared to 5.2% w/w for the latter one. Third instar larvae of the diamond back moth gave LD_{50} of 24.25 PPM and 89.07 PPM for the Soxhlet and stirring methods, respectively. Triphenyl phosphate (TPP) and Piperonylebutoxide (PB) reduced the LD_{50} upto ca. 4 folds and also significantly lowered the number of larvae in the field.
The optimal medium for the detoxification enzymes, esterase, GSH-S-transferses and monooxygenases, was found to contain 0.1 M phosphate buffer pH 7.5 with 10 mM glutathione (reduced forms), 1 mM EDTA and 50% w/w PVPP. Protein concentrations (BSA as a standard protein) between 50-100 mg protein/g larvae/ml extracted from 2 – 4 instar larvae were used for all enzyme assays.

Derris extracts induced ca. 10-20% of all enzyme activities. By adding TPP to the extracts, esterase activity was reduced by 20%. The coefficient of correlation, r^2 (mortality against esterase activity), was ca. 0.9. The addition of DEM showed a lower in r^2 value (0.62 – 0.77) (mortality against GSH-S-transferases activity). The highest fluctuation of r^2 (0.48 – 0.97) (mortality against monooxygenases activity) was observed by the addition of PB to both extracts.

Key words: derris extracts, detoxification enzymes, diamondback moth, *Derris elliptica* Benth, *Plutella xylostella* Linn.

INTRODUCTION

Many types of insecticides have been imported to control insect pests in Thailand. The improper use of insecticides by farmers may lead to pest resistance. Although many measures have been introduced to control insect pests, many problems, such as high cost of production and pollution, are among the controversial issues in vegetable producing areas (Waleeluck and Visetson, 1995). Of all insect pests of vegetables, Diamondback moth (*Plutella xylostella* Linn.) is one of the most serious pests found in Thailand. It is resistant to many insecticides in the central part of Thailand where insecticides are frequently used.

Thailand is one of the most diverse countries in terms of plants and animals in Southeast Asia. Some plants show high ability to adapt to and tolerate herbivores and their environment. The adapting ability derived from the production of special chemicals is called allelochemicals, which are parts of secondary plant substances (Yu and

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Hsu, 1985). Plant active ingredients show hormonal inhibition in insects such as azadirachtin from neem seed kernels (Sombatsiri *et al*. 1995). The others show repellent properties such as citronellal from citrus leaves (Visetson, 1996). Feeding inhibition from plant substances such as eupathal in Siam weed and nimbidin from neem seed kernels were carried out (Lange and Schmutterer, 1982). As a result, Thailand will be the one of many Southeast Asian countries where plant extracts are increasingly important as pest control alternatives.

Derris (*Derris elliptica* Benth) is another pesticide alternative. Thai farmers used to grow it widely in the eastern part of Thailand. It is called "Lotin". They used it to kill some insect pests especially in the Order Homoptera. Derris has not appeared on the insecticide market for two decades because more highly effect, synthetic pesticides have been introduced. However, with the development of resistant insects, the threat of contaminated food and high production cost problems, derris came back again in 1995.

Derris belongs to the family Leguminosae. It's principal secondary plant substance is called rotenone (1,2, 12a-tetrahydro-8,9-dimethoxy-2 (1-menthylethenyl-(1) benzopyrano (2,4-b) furo (2,3-h) (1) benzophyran-6 (6H)-one with M.W. at 394.41). Its mechanism of action in higher organisms is on interference with the electron transport chain at the inner mitochondrial membrane. It has low toxicity to mammals, but is extremely toxic to fish (Matsumura, 1975). The detoxification enzyme mechanisms in insects have not been investigated.

The aims of this research are to investigate the toxicity of rotenone extracts (LD_{50}) and synergistic effects as well as the activity of detoxification enzymes, namely general esterases, glutathione-S- transferases and monooxygenases, in diamondback moth larvae after exposure to the extracts. Their results could be beneficial for farmers as well as businessmen who are concerned of the environmental deterioration.

MATERIALS AND METHODS

Insect larvae and plant samples

Diamondback moth larvae were collected from the vegetable producing area in Kanchanaburi province, 150 km west of Bangkok. Larvae were reared under the laboratory at 23 ± 2°C following the method of Leckprayun *et al*. (1999). Two-year old derris root was collected from Chonburi province, 150 km east of Bangkok. Only the diameter size of roots less than 1 cm were used. They were oven- dried at 45°C for 7 days and then ground to powder.

Plant extraction and efficacy tests

Two types of ethanolic extractions; the Soxhlet extraction at 70°C and stirring soaking at room temperature were administered for 8 hours. The crude extracts then were evaporated and analyzed for the rotenone content by Thin Layer Chromatography and then HPLC-UV detector using methods modified from Pitiyon and Sangwanit (1997). The extracts were diluted into various concentrations and were conducted toxicity test for 10 – 90% mortality of 3^{th}- instar larvae of diamondback moths. Three replicates comprised 20 larvae in each replicate. A 5% emulsifier, triton X-100, was mixed into each concentration before the trials commenced. Following Raffa and Priester (1985), the synergists, triphenyl phosphate (TPP), diethyl maleate (DEM) and piperonyl butoxide (PB) were used in separate experiments. A no-choice leaf dipping method using a leaf circle disk of Chinese kale with a diameter of 5 cm was placed to feed the larvae. Mortality was checked after 24 hours exposure. A Completely Randomized design with 3 replicates was used. All experiments were run at 23 ± 2°C. In case of control mortality, Abbott's formula (Matsumura,1975) was employed. LD_{50} were calculated by using regression equation with cypermethrin as a control.

Detoxification enzyme assays

The surviving larvae from the treatment were in vitro assays to optimize enzyme activity of esterases, GSH-S-transferases and monooxygenases activities following the methods of Visetson (1991) by using paranitrophenyl acetate (PNPA) , diethyl maleate (DEM) and aldrin for their substrates, respectively. The enzyme optimization included some stabilized chemicals in extracting buffers such as polyvinyl polypyrrolidine (PVPP), glutathione, ethylenediaminetetraacetic acid tetra sodium salt (EDTA), phenylmethylsulfonyl flouride (PMSF) and bovine serum albumin (BSA) and pH were carried out. The larval stages were also tested for their optimal activities. Protein measurement followed the method of Lowry *et al*., (1951) and BSA was used as a standard protein. The best condition was used to quantify all enzyme activity.

The coefficient of determination (r^2) was determined for both insect's larval mortality and enzyme activity. Synergist ratios (SR) and change factors (CF) were quantified to measure the effectiveness of synergists and changes in enzyme levels, respectively.

Field experiments

Field trials using Chinese kale were separately undertaken to confirm the laboratory results. The experimental design was allocated in a Randomized Complete Block design with 3 replicates. Plot size was given as 6×2 square meters. Spraying was done once a week by beginning on 10-day-old Chinese kale. There were 7 sprays for the whole experiment. Larval numbers were regularly checked at fixed points before and after spraying for 24 hours. DMRT was employed for mean comparisons with probability > 95% following Finney (1964).

RESULTS AND DISCUSSIONS

Extraction and rotenone content

The Soxhlet extraction and stirring soaking methods showed ca. 8.6 and 5.2% w/w of rotenone respectively. The former method gave a 1.6 fold higher amount of rotenone than the latter which indicated that the high temperature during Soxhlet extraction had a greater influence on rotenone solubility in ethanol than did the stirring soaking method. This result was similar to that found for the extraction of azadirachtin from neem seed kernels (Visetson, 1994) and euphathal and α-pinine extracted from Siam weed (Leckprayun *et al*. 1999). However, temperatures above 70°C may cause decomposition of plant active ingredients (Visetson and Chuchoui, 1999). Although the use of chloroform as a solvent (Pitiyon and Sangwanit, 1997) resulted in a higher amount of rotenone being extracted; the chloroform was proved to be dangerous to human health. Ethanol, therefore, is a more suitable solvent for rotenone extraction for Thai farmers.

The LD_{50} values obtained from the Soxhlet and stirring soaking methods without any synergists were 24.25 PPM and 89.07 PPM, respectively (Table 1). These results also indicated that higher temperature of Soxhlet extraction yielded a higher quality of rotenone extracted than the stirring soaking method.

The addition of the synergists, TPP and PB, showed synergist ratio (SR) of ca. 3 fold, whereas addition of DEM showed the SR of ca. 1.0 (Table 1). The results indicated that esterase and monooxygenase played major roles in detoxifying rotenone (Table 3). These indications differ to those from experiments with other plant extracts. Leckprayun *et al*. (1999) indicated no correlation with these enzymes in the detoxification of Siam weed extracts. Although the inefficient of DEM showing increased SR in these experiments that did not mean GSH –S-transferases were unimportant in rotenone detoxification. Rotenone might be broken down in the phase I reaction (energy requiring reaction) before conjugation took place in phase II (glutathione conjugating reaction), indicating the increase of SR by DEM. This interpretation was

supported by the results of field trial. Field applications revealed that the addition of TPP and PB to the extracts was found to lower the larval number to levels similar to the control plot treated with cypermethrin. It was also noticed that the addition of DEM to both extracts did not give any significant change in larval number (P>0.05) (Table 2).

The homogenizing medium, composed of 0.1 M phosphate buffer (pH 7.5) with 10 mM glutathione (reduced form), 1 mM EDTA and 50% PVPP (w/w) was suitable for detoxification enzyme extraction in this study. Although further addition of PMSF and BSA gave higher monooxygenase activity, but this reduced GSH-S-transferase activity (Figure 1a). The compounds might help stabilizing pH of monooxygenase (Powis and Schenkman, 1977), but stable pH may not be sufficient to enhance GSH-S-transferase levels. The homogenizing media without PVPP showed very little enzyme activity indicating quinone to be produced whilst extraction was in process. The inhibition of GSH-S-transferase activity by quinone was known by a number of research workers (Kotze and Rose, 1989).

Enzyme activity was quantified the highest at 3rd instar larvae and the least activity was detected in pupae (Figure 1b). Furthermore, pH 7.5 showed optimum activity occurring for all enzyme systems with little variation (Figure 1c). This pH corresponded with that determined by the previously

Table 1 Comparisons of linear regression on LD_{50} values and synergistic ratio (SR) among different synergist treatments of derris extracts on larvae of diamond back moth under the laboratory condition[1]

Synergists added[2]	Soxhlet (LD_{50})[SR][3,4]	Stirring-soaking (LD_{50})[5][SR]
None	Y = 20.89 + 1.2X (24.25)	Y = 14.37 + 0.4X (89.07)
TPP[5]	Y = 34.77 + 2.3X (6.62) [3.66]	Y = 22.97 + 1.2X (22.52)[3.95]
DEM	Y = 18.01 + 1.2X (26.65)[0.90]	Y = 7.07 + 0.4X (107.32)[0.83]
PB	Y = 37.85 + 1.2X (10.12)[2.39]	Y = 16.23 + 1.3X (25.97)[3.43]

[1] n = 3, 20 of 3th instar each batch, assay at 23 ± 2°C with leaf dipping method, 24 hrs. check.
[2] TPP , DEM and PB stand for triphenyl phosphate, diethyl maleate and piperonyl butoxide, respectively.
[3] Y and X represents % mortality and concentration, respectively.
[4] SR = LD_{50} none/ LD_{50} with synergists.
[5] Linear regression for cypermethrin showed LD50 = 17.2 ppm (Y = 13.89 + 2.1X)

Table 2 Mean ±SD of diamond back moth lavae found on the leaves of Chinese kale after application of the different derris extracts and cypermethrin.

10% Synergists added[1]	10% cypermethrin	Extract I[2]	Extract II
None	25.23 ± 6.12 b[3]	30.16 ± 1.23 b	49.16 ± 4.17 c[3]
TPP	10.33 ± 3.17a	15.12 ± 2.61 a	28.16 ± 2.17 a
DEM	23.45 ± 4.35b	30.13 ± 2.74 b	39.21 ± 1.89 c
PB	14.43 ± 5.13a	18.27 ± 3.32 a	29.23 ± 2.32 a

[1] TPP, DEM and PB stand for triphenyl phosphate, diethyl maleate and piperonyl butoxide, respectively. SD is a standard deviation.
[2] Extracts I and II derived from soxhlet extraction and stirring soaking method, respectively.
[3] Means following by the same letter in the same collumn are not significantly different, P>0.05, DMRT

work of Visetson (1991) on the red-rust flour beetles. Protein contents ranging from 50-70 mg protein/g larvae/ml resulted in a little variation of activities (CV<20%) (Figure 1d). These results are similar to those obtained for the Australian blow fly larvae (Kotze and Rose, 1989) the American ball worm (Rose, 1985) and fall armyworm (Yu, 1984).

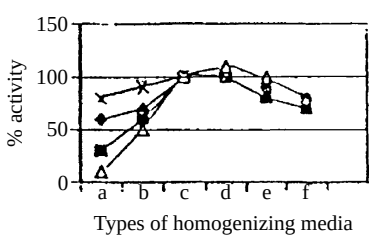
With the Soxhlet method, all CFs (controled enzyme activity versus treated enzyme activity) were less than 1 (Table 3). After addition of DEM into the Soxhlet extracts the CFs ratio found to increase to 2.61. Additions of PB resulting in CFs of ca. 2.05 indicated that the inhibition of monooxygenase occurred. On the other hand, the addition of synergists did not produce any effect on the efficacy of the stirring soaking extract.

In short, synergists actively inhibited the enzymes proportionately. The results showed that the specificity of TPP was as an esterase inhibitor (r^2 = 0.88-0.97). DEM and PB fluctuated (r^2 = 0.68 - 0.70 and r^2 = 0.36 – 0.98, respectively) in inhibiting GSH-S-transferases and monooxygenases, respectively. The correlation between the synergist mortality and enzyme activity resulted from addition of PB and monooxygenases activity may be the indication of nonspecificity of PB (Collins, 1990). The synergist was reported to be an esterase inhibitor as well. However, before certain conclusion can be made, an experiment using purified enzymes should be conducted for further verification.

CONCLUSION

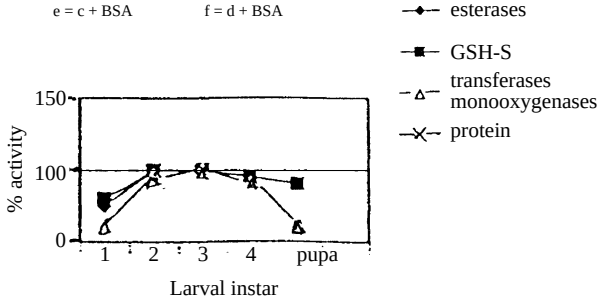
The highest rotenone content determined using HPLC showed 8.6 % w/w yield for the Soxhlet's method. Third instar larvae of the diamond back moth gave LD_{50} of 24.25 PPM and 89.07 PPM for the Soxhlet and stirring methods, respectively. The addition of Triphenyl phosphate (TPP) and Piperonylebutoxide (PB) found reduced the LD_{50}significantly.

Adding of TPP and PB to the extracts also reduced esterase as well as monooxygenase activity.

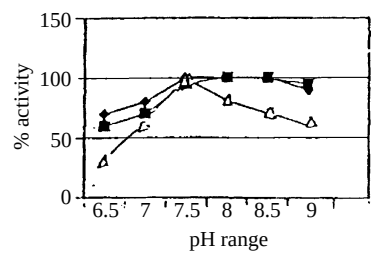


(a). Enzyme activity for different homogenizing media

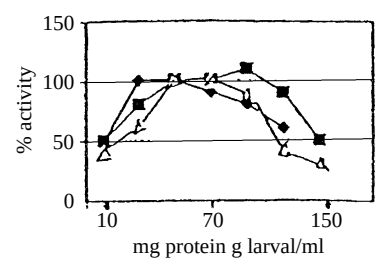
a = buffer alone b = buffer + PVPP + glutathione
c = b + EDTA d = c + PMSF
e = c + BSA f = d + BSA



(b). Enzyme activity of different larval instars and pupae



(c). Effects of assay pH on enzyme activity



(d). Effect of protein concentration on enzyme activity

Figure 1 Effects of homogenizing media (a), larval instars and pupae (b), pH (c) and protein concentration (d) on activity of detoxification enzymes.

Table 3 Mean ±SD activity of esterase, GSH-S-transferase and monooxygenase of diamondback moth larvae survived after being exposed 24 h to derris extracts and cypermethrin treated with synergists. (n = 3)

Type of pesticides	Synergist added	Enzyme activity (CF)[r^2] Gen. Esterases	GSH-S-transferases	monooxygenase
None	None	12.14 ± 2.23 a [1]	32.13 ± 2.46 b	4,320.12 ± 126.22b
Cypermethrin		15.21 ± 1.64 b	40.21 ± 6.41c	5,120.45 ± 123.31b
		(0.79)[0.98]	(0.79)[0.95]	(0.84)[0.90]
Extract I	None	16.42 ± 1.24b	40.16 ± 1.67c	4,100.43 ± 213.66b
		(0.73)[0.45]	(0.80)[0.58]	(1.05)[0.97]
	TPP	10.32 ± 1.23a	34.12 ± 1.42b	4,321.45 ± 126.77b
		(1.18)[0.97]	(0.94)[0.92]	(0.99)[0.68]
	DEM	15.12 ± 1.41b	12.33 ± 1.46a	4,273.78 ± 177.09b
		(0.80)[0.68]	(2.61)[0.62]	(1.01)[0.94]
	PB	10.23 ± 1.41a	33.14 ± 1.24b	2,100.87 ± 231.60a
		(1.19)[0.98]	(0.97)[0.95]	(2.05)[0.48]
Extract II	None	16.12 ± 1.76b	40.16 ± 1.67c	4,100.75 ± 213.58b
		(0.75)[0.77]	(0.80)[0.57]	(1.05)[0.71]
	TPP	12.14 ± 2.16a	32.16 ± 1.72b	4,000.67 ± 161.96b
		(1.00)[0.88]	(0.99)[0.89]	(1.08)[0.14]
	DEM	13.14 ± 1.23a	30.12 ± 1.47b	4,121.54 ± 123.89b
		(0.92)[0.70]	(1.07)[0.77]	(1.05)[0.93]
	PB	13.12 ± 1.42a	31.16 ± 1.26b	3,214.67 ± 162.56a
		(0.92)[0.36]	(1.03)[0.66]	(1.34)[0.83]

[1] Means followed by the same letters within the same collumn are not significantly different, P>0.05, DMRT

Further steps of complete enzyme purification data including of isoelectric emphasizing on the methods from both controlled and induced forms in the larvae must be conducted. These steps will elucidate the unbiased mechanisms and be important steps to modify the synergists as well as the structure of rotenone in perfect binding at the exact biding side of the enzymes leading of better rotenone formulations in the future.

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