Pak. J. Bot., 40(5): 1983-1992, 2008.

ANTIFEEDANT ACTIVITIES OF SECONDARY METABOLITES FROM *AJUGA NIPPONENSIS* AGAINST *PLUTELLA XYLOSTELLA*

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Abstract

Antifeedant activities of seven chemicals isolated from *Ajuga nipponensis* were examined in a bioassay against 3rd instar larvae of *Plutella xylostella*. Five compounds (luteolin, stigmasterol, acacetin, 20-hydroxyecdysone and fraction 1) showed significant antifeedant activities, with the AFI_{50} values of 1935.02, 2515.94, 6589.58, 287.58, 2171.31µg/ml after 24h and 1853.20, 3812.24, 2581.43, 103.42, 1429.75µg/ml respectively after 48h of treatment. The synergistic effects of mixtures of secondary compounds were studied and antifeedant index was directly related to the component and the proportion of the mixture i.e., no synergism existed between luteolin and 20-hydroxyecdysone mixture, but the values of CTC for fraction 1, stigmasterol, acacetin, luteolin and 20-hydroxyecdysone mixture were up to 627.07 and 111.72 after 24 and 48h, respectively. In addition, only apigenin showed effectiveness against Diamondback moth, with a high mortality percentage and low consumed leaf area when mixed with avermectins.

Introduction

Diamondback moth (*Plutella xylostella* L., DBM) is a major pest of cabbage, broccoli, and canola. Every year, farmers spend more than $1 billion to control this pest worldwide, primarily by using chemical insecticides (Talekar & Shelton, 1993; Tabashnik, 1994). Consequently, more target-selective and biodegradable compounds are needed to replace the environmentally persistent chemicals with broad-spectrum toxicity that are being phased out (Alkofahi *et al*., 1989). Alternative sources of potentially suitable insecticides include botanical insecticides, antifeedants and insect growth regulators of their natural origin having non-neurotoxic modes of action, and low environmental persistence (Arnason *et al*., 1992; Isman, 1994; Isman, 2006). Plant secondary compounds have been the subject of thorough investigation for the last 30 years as an effort to discover new sources of botanical insecticides and antifeedants. Among the plant families studied, Meliaceae, Rutaceae, Asteraceae, Labiateae, Piperaceae and Annonaceae are perhaps the most promising ones (Schoonhoven, 1982; Jacobson, 1989; Isman, 1995). Meliaceae and Rutacae have received much attention owing to the presence of triterpenoids called limonoid (Connolly, 1983). Azadirachtin, a limonoid from seeds of the neem tree (*Azadirachta indica*, Meliaceae), possesses strong antifeedant and growth inhibitory effects against various insect pests (Isman, 1997).

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Complex mixtures of secondary compounds in plant extracts contribute to a great deal for synergism, which enhances the joint action of active compounds against insect (Bernard & Philogène, 1993) and reduces the rate of resistance development (Feng & Isman, 1995; Isman *et al*., 1997). Mixtures of psoralen with xanthotoxin or bergapten resulted in reduced mortality of *Spodoptera exigua* reared on artificial diets mixed with linear and angular furanocoumarins and then exposed to UVB radiation, suggesting an antagonistic effect (Diawara *et al*., 1993). In contrast, mixtures of similar composition, but at lower combined concentration where psoralen was the minor component, caused greater mortality in *S. exigua* than the sum of individual linear or angular furanocoumarins, suggesting a synergistic effect of psoralen (Brewer *et al*., 1995). Synergism has been observed in mixtures of xanthotoxin with nonfuranocoumarins myristicin, safrole and fagaramide (Berenbaum & Neal, 1985; Neal, 1989).

Ajuga (Labiatae) a cosmopolitan plant genus well known for its ecdysteroids has more than 150 varieties and subspecies with more abundance in China, Japan, Korea as well as in Europe (Darvas, 1991). In China, the genus *Ajuga* is represented by 35 species, out of which some are used as traditional medicine for the treatments of healing of wounds, detumescence and high fever (Liu & Shir, 2001). It has been reported that *A. nipponensis* contains phytoecdysteroids, such as 20-hydroxyecdysone, cyasterone (Zen *et al.,* 2001) and so on with toxic, antifeeding, growth and development inhibitive activities against some insects i.e., DBM and termite (Liu & Shir, 2001; Qiu & Zhao, 1994; Josep *et al.,* 2007).

The avermectins are a group of broad-spectrum pesticides with activity against a variety of arthropods (Strong & Brown, 1987). Avermectin has shown high efficiency in controlling vegetable pests like *P. xylostella* L. and arthropods (Sengonca & Liu, 2002). Furthermore, it has been successfully used as an essential part of integrated cabbage pest management in cabbage fields in Fuzhou, China (Sengonca & Liu, 2003), where it showed high efficacy in reducing pest species abundance while very low harmfulness to their natural enemies that caused higher overlaps between the predators and their preys as compared to methomyl.

A generalist herbivore like DBM is likely to be affected by some other secondary chemical compounds or its mixture isolated from *A. nipponensis* in the diet and might be sensitive to possible synergistic antifeedant effects of *A. nipponensis* and Avermectins. The plants contain several different secondary metabolites whose possible synergistic interactions are needed to be worked out. Keeping in view all the facts, the current study was planned to determine the feeding deterrence effect of the isolated chemical compounds from *A. nipponensis* individually and as well as in mixtures against *P. xylostella*.

Materials and Methods

Insects: *P. xylostella* was maintained on the *Brassica campestris* L., leaves in greenhouse of Engineering Research Center of Biological Control, South China Agricultural University. Plants were grown in plastic pots having a diameter of 15-cm. Sufficient slow release fertilizer (N: P: K=13:7:15, Shenzhen Batian ecotypic engineering Co., LTD. Xili Shenzhen China) was added as required to maintain normal plant growth. DBM culture was maintained on *B. campestris* for several generations under laboratory conditions before they were used in these studies.

Plant extracts and insecticide: Secondary compounds (Fig. 1 and Table 1) isolated from EtOAc extract of *A. nipponensis* (Stigmastrol, acacetin, Ajugacumbins B, Apigenin, luteolin and 20-Hydroxyecdysone) were isolated and identified (Talpetch *et al*., 1983; Min *et al*., 1989; Liu & Li 1998; Shen *et al*., 2004 and Zou *et al*., 2006). 0.6% Avermectins EC (B1a>90%, B1b<5%) was purchased from Huaxing Pesticide Co. Ltd, Zhejiang.

Antifeedant assay: The antifeedant activities of the fraction or pure compounds and their mixture were tested against the 3rd instar larvae of *P. xylostella* by using non-choice leaf disc (1.8cm, diameter) methods. The stock solutions were prepared in acetone or methanol. A series of concentrations for each compound and their mixtures were prepared by serial dilutions with 0.3% Tween-80. Late 2nd instar larvae of DBM were fed on *B. campestris* leaves overnight. Newly moulted 3rd instar larvae were selected and starved for 2 h. *B. campestris* leaves were washed with distilled water and leaf discs (Ø1.5 cm) were cut after drying. The leaf discs of treatment groups were immersed in the test solution for 10 seconds and were allowed to dry at room temperature. The leaf discs of control groups were also treated with the solvent as used in treated groups. Two treated discs were placed in a Petri dish (9cm) having a piece of moistened cotton pad. One larva was added to each Petri dish, and each treatment was having 30 larvae. All the treatments and control were replicated three times. After 24 and 48h of treatments, the larvae were removed from the Petri dish using a fine camel hair brush. Leaf discs were photographed using an IS-500 Digital Imaging System (Alpha Innotech Corporation) and the leaf area eaten was determined by using Scion Image software for Windows 95.

Data analysis: The antifeedant activity of compounds was calculated (Abivardi & Benz, 1984) using an antifeedant index (AFI).

$$AFI = (1 - T / C) \times 100$$

where C is the leaf area consumed in control and T is the leaf area consumed in treatments. AFI_{50} (effective concentration for 50% antifeedant activity of compound relative to the control) was calculated based on AFI values at each dose using a simple linear regression model with log transformed doses (inverse prediction).

The theoretical AFI_{50} of mixture = the AFI_{50} of A × the proportion of A in the mixture + the AFI_{50} of B × the proportion of B in the mixture.

$$\text{The actual } AFI_{50} \text{ of mixture} = \frac{\text{The } AFI_{50} \text{ of standard chemical}}{\text{The } AFI_{50} \text{ of the mixture chemical}} \times 100$$

$$\text{The co-toxicity coefficient (CTC) of mixture} = \frac{\text{The actual } AFI_{50} \text{ of mixture}}{\text{Theoretical } AFI_{50} \text{ of mixture}} \times 100$$

Antifeedant index, percent mortality, and leaf area consumed were analyzed by using one-way analysis of variance (ANOVA). Mean values were separated by Duncan's Multiple Range Test (DMRT) when *F*- value was significant (Anon., 1988).

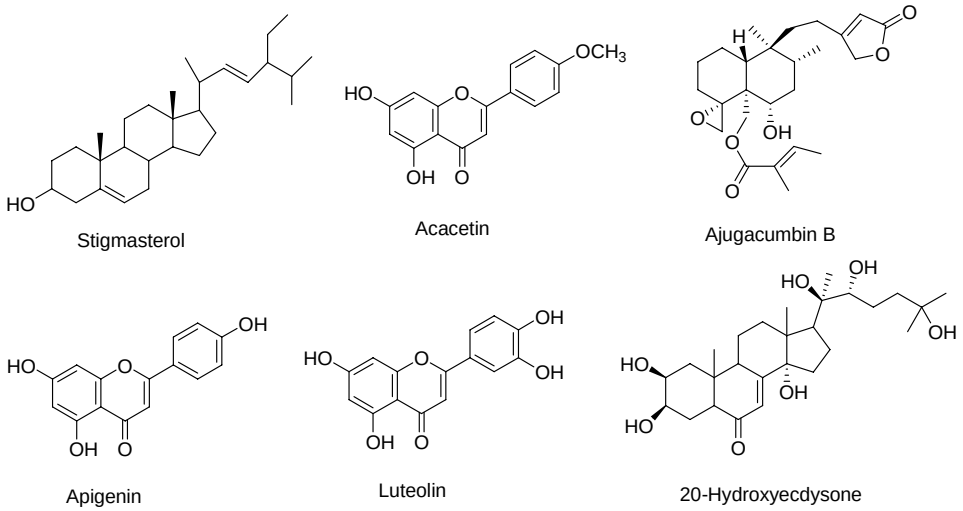

Fig. 1. Structures of secondary compounds from *Ajuga nipponensis* EtOAc extract

Results

Antifeedant activities of the individual compounds: The antifeedant activity of the 7 secondary chemical compounds isolated from EtOAc extract of *Ajuga* against *P. xylostella* showed significant differences after 24 and 48h of treatment (Table 2). After 24h of the chemical treatment, 20-hydoxyecdysone showed highest antifeedant activity with AFI value of 89.73, whereas luteolin, stigmasterol and fraction 1 were significantly similar to each other. The data revealed that the indices of luteolin, stigmasterol and acacetin decreased as the duration increased and in comparison to that the other chemicals revealed a minute increase as the time extended, in contrast to this apigenin showed a strong attraction to DBM with AFI value of - 46.8.

Table 1. Fraction-1 and their chemical percentages isolated from *Ajuga nipponensis* etoac extract.

Retention time (min)	Formula	Compounds	Relative content□%□
15.78	$C_{18}H_{36}O$	6,10,14-trimethyl-2-pentadecanone	13.43
16.88	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester	29.16
17.36	$C_{16}H_{32}O_2$	Hexadecanoic acid	6.78
18.05	$C_{18}H_{36}O_2$	Hexadecanoic acid, ethyl ester	5.98
20.07	$C_{19}H_{34}O_2$	(Z, Z)-9,12-octadecadienoic acid methyl ester	9.03
20.15	$C_{19}H_{32}O_2$	(Z, Z, Z)-9,12,15-octadecadienoic acid methyl ester	4.04
20.29	$C_{19}H_{36}O_2$	(Z)-9- octadecadienoic acid methyl ester	7.50
21.00	$C_{19}H_{38}O_2$	Octadecanoic acid methyl ester	5.03
21.65	$C_{19}H_{36}O$	2-Methyl-Z, Z-3,13-octadecadienol	3.32

Table 2. Antifeedant activities of the individual compounds against *Plutella xylostella*.

Individual compounds (2000µg/ml)	Antifeedant index (%) (AFI±SE)[#]	
	24h	48h
Luteolin	61.13 ± 2.08 b	56.67 ± 3.04 b
Stigmasterol	44.26 ± 2.12 bc	27.78 ± 3.07 c
Acacetin	39.73 ± 2.11 c	36.27 ± 2.94 bc
20-Hydroxyecdysone	89.73 ± 4.57 a	92.65 ± 2.25 a
Fraction 1	49.28 ± 2.46 b	55.80 ± 1.98 b
Ajugacumbins B	6.24 ± 4.38 d	21.97 ± 3.16 c
Apigenin	- 46.80 ± 2.44 e	- 19.35 ± 2.32 d
F, df, P	59.7, 6, 0.0043	93.1, 6, 0.0012

[#]Antifeedant index percentages within a column followed by different letters are significantly different at $P = 0.05$ by F test (DMRT).

Table 3. The AFI_{50} of individual chemicals against *Plutella xylostella*[#].

Chemicals	AFI_{50} (µg/ml)		Confidence limits		Slope	r^2
			Lower	Upper		
Luteolin	24h	1935.02	1251.75	2991.27	0.7157	0.9400
	48h	1853.20	1249.50	2748.57	0.7462	0.9415
Stigmasterol	24h	2515.94	2029.31	3119.27	2.0938	0.9871
	48h	3812.24	2650.36	5483.46	1.8827	0.9827
Acacetin	24h	6589.58	1049.30	11382.59	0.4259	0.9505
	48h	2581.43	1772.55	3759.43	1.1044	0.9463
20-Hydroxyecdysone	24h	287.58	157.33	525.65	1.3467	0.9829
	48h	103.42	27.66	386.67	1.0901	0.9894
Fraction 1	24h	2171.31	1696.64	2778.78	1.4611	0.9680
	48h	1429.75	1203.11	1699.09	1.5303	0.9449

[#]The series concentrations for each chemical were 2500µg/ml, 2000µg/ml, 1500µg/ml, 1000µg/ml and 500µg/ml used for testing the AFI_{50} values.

AFI_{50} values as calculated by the algebraic sum of individual activities represented by their particular best-fit equation are given in Table 3. Both 20-hydroxyecdysone and luteolin showed a strong antifeedant activity against *P. xylostella* after 24h. The lowest AFI_{50} value (287.58µg/ml) was observed for 20-hydroxyecdysone after 24h, while the highest AFI_{50} value of 6589.58 µg/ml was shown by acacetin. After 48h of treatment, the lowest AFI_{50} value of 103.42µg/ml for 20-hydroxyecdysone was recorded, and with the highest AFI_{50} value of 3812.24 µg/ml for stigmasterol. In comparison to the AFI_{50} values of the application of the chemicals after 24h and 48h, the values of luteolin, acacetin, 20-hydroxyecdysone and fraction 1 increased with the treated time last on.

Antifeedant activities of binary mixtures: Antifeedant activities of the secondary compound mixture at the same proportion against *P. xylostella* revealed that the value of antifeedant indices were significantly similar for the mixtures mixed between 20-Hydroxyecdysone and Fraction 1, Luteolin, Stigmasterol, respectively, after 24 and 48h

(Table 4). The value of antifeedant index showed non-significant differences among the mixtures of fraction 1, luteolin and stigmasterol mixed with each other, the values of antifeedant indices after 24h were higher than that of after 48h. The results showed that component of mixture had significant effect on the antifeedant activities, and the optimal mixture was fraction 1 and 20-hydroxyecdysone with the highest antifeedant index of 85.80 and 91.74 after treatment of 24h and 48h. The CTC values appeared to be less than 100 after the treatment (Table 5), this indicted that no synergism effect existed between fraction 1 and 20-hydroxyecdysone mixture.

Antifeedant activities of mixtures against *Plutella xylostella*: Antifeedant activities of different secondary compounds mixture at the same and different proportions against *P. xylostella* indicated that the component of the mixture was directly related to the antifeedant activity when they were at the same proportion (Table 6). At the same time, the data revealed that chemical proportion had significant effect on the antifeedant activities. In contrast to this the mixtures at same proportion and at different proportion (as per percentage in the plant) showed distinctive antifeedant index. The optimal mixture was fraction 1, stigmasterol, acacetin, luteolin and 20-hydroxyecdysone with the highest antifeedant index of 80.59 and 65.13 after treatment of 24h and 48h. The increased value of CTC up to 627.07 and 111.72 is a positive indication that synergistic effect existed among the compounds mixture.

Mortality and consumed leaf area of *Apigenin* and avermectins mixture against *Plutella xylostella*: Percent mortality of DBM and the leaf area consumed after the application of apigenin and avermectins mixture showed that the mortalities were directly related to the proportion of the mixtures i.e., as the proportion of apigenin increased the mortality of DBM also increased and retained an accumulative effect for an extended period (Table 7). The values of morality revealed no significant difference between the treatments (1000: 77.4) and (2000: 77.4) after treated 48h, with the highest mortality of 94.44. In contrast to this consumed areas were very small at the proportion of (1000: 77.4) and (2000: 77.4) compared to other treatments and the consumed areas increased just a little as the time duration increased.

Table 4. Antifeedant activities of binary mixtures against *Plutella xylostella*.

Mixture of secondary compounds (2000µg/ml)	Antifeedant index (%) (AFI ± SE)[#]	
	24h	48h
Fraction 1 + 20-Hydroxyecdysone (1:1)[$]	85.80 ± 2.15 a	91.74 ± 3.11 a
Luteolin + 20-Hydroxyecdysone (1:1)	81.60 ± 1.01 a	88.02 ± 2.52 a
Stigmasterol + 20-Hydroxyecdysone(1:1)	80.44 ± 2.58 a	87.46 ± 0.56 a
Fraction 1 + Luteolin (1:1)	63.20 ± 4.22 b	60.60 ± 2.94 b
Fraction 1 + Stigmasterol (1:1)	61.30 ± 2.57 b	57.60 ± 1.13 b
Stigmasterol + Luteolin (1:1)	60.88 ± 2.65 b	54.13 ± 2.57 b
F, df, P	123.1, 6, 0.0004	107.9, 6, 0.0001

[#]Data within a column followed by different letters are significantly different at $P = 0.05$ by F test (DMRT). [$]The proportion of each second compounds was 1:1 in the mixture.

Table 5. The AFI_{50} of mixtures against *Plutella xylostella* [#].

Mixture	Time	AFI_{50} (μg/ml)	Slope	r^2	Confidence limits		CTC
					Lower	Upper	
Fr and Hy	24h	710.93	1.7369	0.9469	596.73	846.98	70.43
	48h	596.27	1.6304	0.9699	505.59	703.20	32.85
Fr, St, Ac, Lu and Hy	24h	159.35	1.0770	0.9537	89.59	283.43	627.07
	48h	387.21	1.2127	0.9539	304.35	492.64	111.72

Note: Fr, St, Ac, Aj, Ap, Hy and Lu means the second compounds of fraction 1, stigmasterol, acacetin, ajugacumbins B, apigenin, 20-hydroxyecdysone, and luteolin, respectively.
[#] The series concentrations for each mixture were 1000μg/ml, 800μg/ml, 600μg/ml, 400μg/ml and 200μg/ml used for testing the AFI_{50} values.

Table 6. Antifeedant activities of mixture at different secondary compounds and at different proportion against *Plutella xylostella*.

Mixture of secondary compounds (800μg/ml)	Antifeedant index (AFI ± SE)	
	24h	48h
Fr, St, Lu and Hy (1:1:1:1) [$]	43.53 ± 7.98 c	38.67 ± 1.87 b
Fr, St, Ac, Lu and Hy (1:1:1:1:1)	80.59 ± 8.84 a	65.13 ± 8.28 a
Fr, St, Ac, Aj, Lu and Hy (1:1:1:1:1:1)	66.81 ± 6.77 b	64.69 ± 7.80 a
Fr, St, Ac, Ap, Lu and Hy (1:1:1:1:1:1)	52.59 ± 3.76 b	39.91 ± 3.45 b
Fr, St, Ac, Aj, Ap, Lu and Hy (1:1:1:1:1:1:1)	58.62 ± 7.87 b	62.28 ± 4.96 a
F, df, P	47.38, 6, 0.0004	205.36, 6, 0.0015
St, Ac, Aj, Ap and Lu (1:1:1:1:1)	42.24 ± 5.40 b	36.84 ± 8.46 b
St, Ac, Aj, Ap and Lu (as per percentage in the plant)	14.66 ± 1.74 c	12.06 ± 5.08 c
Fr, St, Ac, Aj, Ap, Lu and Hy (1:1:1:1:1:1:1)	58.62 ± 7.87 a	62.28 ± 4.96 a
Fr, St, Ac, Aj, Ap, Lu and Hy (as per percentage in the plant)	28.27 ± 7.47 c	22.40 ± 3.61 bc
F, df, P	117.1, 6, 0.0002	95.45, 6, <0.0001

[$]The data in bracket were the proportion of each second compounds in the mixture.

Table 7. Mortality and consumed leaf area of apigenin and avermectins against *Plutella xylostella*.

The proportion of Apigenin and Avermectins (μg/ml)	Percent mortality (%) (M ± SE)[#]		Consumed area (M±SE)[#] (mm^2)	
	24h	48h	24h	48h
0: 77.4	36.11 ± 2.78 b	69.44 ± 2.45 c	10.30 ± 0.18 b	23.63 ± 0.17 b
1000: 77.4	36.11 ± 2.78 b	91.67 ± 4.81 a	6.02 ± 4.89 c	9.18 ± 0.58 d
2000: 77.4	63.88 ± 7.35 a	94.44 ± 2.78 a	7.53 ± 0.44 c	8.26 ± 0.45 d
1000: 38.7	11.11 ± 2.78 c	86.11 ± 2.78 b	11.49 ± 0.30 b	15.73 ± 0.21 c
Control	0.00 ± 0.00 d	0.00 ± 0.00 d	32.57 ± 1.69 a	118.58 ± 1.43 a
F, df, P	65.7, 6, <0.0001	135.4, 6, 0.0007	73.9, 6, 0.0013	51.9, 6, 0.0034

[#]Data within a column followed by different letters are significantly different at $P = 0.05$ by F test (DMRT).

Discussion

20-hydroxyecdysone, known for its activity as an insect molting hormone is widely used in sericulture industry in China (Chou & Lu, 1980). However, it also possesses some toxic characteristics such as antifeedant, growth and development inhibitive activities against some insects (Francisco & Josep, 1993). It has been reported that 20-hydroxyecdysone was only affective when applied in the last stages of insect development (Francisco & Josep, 1993). But some insects, for example, the economic pests tobacco budworm (*Helicoverpa virescens*), cotton bollworm (*H. armigera*) and Egyptian cotton leaf worm (*Spodoptera littoralis*), which appear to have developed effective detoxification mechanisms for ingested phytoecdysteroid and are able to consume diets containing high dosage of 20-hydroxyecdysone without any adverse effect

on growth and development (Michaela *et al.,* 1996). However, in our findings, remarkable antifeedant activities of 20-hydroxyecdysone were observed against 3rd instar of DBM with AFI_{50} 287.58 µg/ml and 103.42 µg/ml after 24h and 48h, respectively.

The finding that 20-hydroxyecdysone alone possessed a higher feeding deterrence effect against DBM than 20-hydroxyecdysone and Fraction 1 mixture indicating a moderate antagonism. Similar results were seen with xanthotoxin in complex combinations against *Spodoptera littoralis* and *Pastinaca sativa* (Maria *et al*., 2002; Berenbaum *et al.,* 1991). In contrast to this our research findings revealed that the CTC value of mixture of fraction 1, stigmasterol, acacetin, luteolin and 20-hydroxyecdysone were up to 627.07. These results showed a high level of antifeedant activities indicating that high activity antifeedants can only be synthesized if proportion of the mixture can be adjusted. As not only the presence of components but also the concentration and, more importantly, the proportions of these compounds appear to be essential in the synergistic effect (Maria *et al*., 2002). It is necessary to determine the relative amounts of these materials quantitatively in a wide range of natural systems that find protection by use of these isolates. A lot of work on the mechanism of single secondary compound on insect have been reported i.e., 20-hydroxyecdysone played a dual role in the control of protein synthesis related to DNA puff activity in the anterior region of salivary glands (Carvalho *et al.,* 2000) and hormones might play a genetic control role in energy metabolism *via* nuclear receptors (Wahli, 2000).

Usually some secondary compounds isolated from plant show no bioactivities or attractiveness to insects, a feasible way to explore its potential is to mix these chemicals with some insecticide. Apigenin showed a strong attraction to DBM in this experiment, and resistance to avermectin was over 812-folds in *Plutella xylostella* population compared with the unselected parents (Li *et al.,* 2000). In order to delay the resistance to avermectins in DBM, avermectin was chosen for the blend containing the feeding stimulant apigenin. The result show that the high mortality of insect was caused by the mixture of apigenin and avermectins, at same time, a little leaf area was consumed. Mainly for the apigenin induce DBM to consume much leaves with avermectins in a short time, and the avermectins in body attack the target site to cause DBM die. These research findings suggest further work is needed to reveal the complexity of physiological activity exerted by mixture of secondary compound on insect.

Acknowledgements

This research was funded by grants from the National Basic Research Program (called 973 Program) (No.2006CB102005), South China Agricultural President Foundation (No. 2006K0047).

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(Received for publication 4 February 2008)