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The Feeding Response of *Epilachna indica* (Coleoptera: Coccinellidae: Epilachninae) Towards Extracts of *Azadirachta indica*

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Abstract: A study was conducted to observe the antifeedant properties of *Azadirachta indica* towards the pest of eggplant, *Epilachna indica* (Coleoptera: Coccinellidae: Epilachninae) in a farm at Unit of Landscape, University of Malaya. Eight eggplants of *Solanum melongena* tree were planted in a gardening pot and placed into a big rearing cage for the study on the life cycle of *E. indica*. Methanolic extraction method was used to extract the antifeedant properties from *A. indica* leaves. A dual choice feeding bioassay was conducted using agar as the feeding substrate in the feeding experiment. Different treatments were placed in two of the equal compartments in petri dishes. One of the agar compartments was treated with leaves extract or synthetic neem compound and the other half of the agar was treated with methanol as control. Analysis using High Performance Liquid Chromatography identified Azadirachtin as one of the chemical components that has the antifeeding property. Synthetic Azadirachtin compound in 50, 100 and 200 ppm concentration was bioassayed to determine the minimum concentration that can cause optimal antifeedant effect on *E. indica*. There was significant difference (ANOVA) in antifeeding response between 50 and 100 ppm concentration but a concentration of 100 and 200 ppm exhibited similar response. It was found that a 100 ppm concentration of Azadirachtin was the minimum concentration that can cause optimal antifeedant effect on *E. indica*.

Key words: *Solanum melongena*, *Epilachna indica*, *Azadirachta indica*, Azadirachtin, biopesticide

INTRODUCTION

The twelve spotted lady bird beetle *Epilachna indica* (Coleoptera: Coccinellidae: Epilachninae) (Fig. 1) is one of the pests for eggplant, *Solanum melongena*.

Extract of neem fruit, seed, seed kernels, twigs, stem bark and root bark have been shown to possess insect antifeedant, insecticidal, insect growth disrupting, nematicidal, fungicidal (Jacobson, 1989; Randhawa and Parmar, 1993; Schmutterer *et al*., 1981; Schmutterer and Asher, 1984, 1987) bactericidal (Ara *et al*., 1989d), anti-inflammatory (Dhawan and Patnaik, 1993) and (Fujiwara *et al*., 1984), antitumor (Fujiwara *et al*., 1984), immunostimulating (Van Der Nat *et al*., 1991) and other (Randhawa and Parmar, 1993) activities. More than 100 compounds have been isolated from various part of the tree and several reviews on constituents of neem (Champagne *et al*., 1992; Devakumar and Dev, 1993; Jones *et al*., 1989; Koul *et al*., 1990; Lee *et al*., 1991; Siddiqui *et al*., 1986b; Taylor, 1984; Warthen, 1979) have been published. However, only relatively few pure compounds were tested for biological activity. Most of the active compounds belong to the group of tetranortriterpenoids, but biologically active diterpenoids, triterpenoids, pentanortriterpenoids and a small number of nonterpenoidal ingredients have also been isolated.

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Fig. 1: Ladybird beetle, *Epilachna indica*

The objective of this study was to investigate the effect of *Azadirachta indica* extracts towards adult *Epilachna indica* (Coleoptera: Coccinellidae: Epilachnae) and the optimal concentration that can cause antifeedant behaviour upon feeding on treated agar.

MATERIALS AND METHODS

Life Cycle Study of *E. Indica* in Outdoor Cage

Twenty pairs of ladybirds were collected from Malaysian Agriculture Research Development Institute (MARDI) and reared in a cage of 3×1×3 m containing *S. melongena* plant of 2 months old. Time taken for every pair of beetles to lay eggs was recorded. The eggs were then transferred to new cages containing *S. melongena* of 2 months old. Each cage was checked daily in order to record the time taken for eggs to hatch. Twenty newly hatched larvae were collected and reared in new cage. The time taken for larvae to pupate was also recorded. Newly developed pupae were identified from the *S. melongena* plant and their location was marked. Duration of time for pupae to become adult ladybird beetles was recorded (Table 1).

Beetles

Wild adult beetles were collected from a farm of Malaysian Agriculture Research Development Institute (MARDI) at Jalan Kebun in order to start a colony of *E. indica* for future experiments. The beetles were maintained on 2 months old *S. melongena* plants placed in a rearing cage of size 3×1×3 m. Adult beetles of the same age were used in all experiments. Beetles to be used in feeding bioassay were only fed with water for three days before used.

Plant Material

The leaves of the neem tree *Azadirachta indica* (6 years old of 10 meter height) were collected from Section 12, Petaling Jaya, Selangor, Malaysia. One hundred gram (dried weight) of leaves were soaked in 1000 mL methanol for 2 h. The mixture was then filtered and the filtrate was concentrated into 20 mL using a rotary evaporator at 40°C. This concentrate was used as stock extract.

Table 1: Period of the ladybird beetles' life cycle at Somme Estate, Serdang, Kedah

The period for different stages	Mating pair N = 25																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Total	Mean±SD
Time taken for laying eggs (day)	7	6	8	7	6	7	8	7	7	6	7	7	7	6	7	8	7	7	6	8	139	6.95±0.67
Time taken for the eggs to hatch (day)	4	5	3	5	3	4	4	3	5	5	3	4	5	3	4	3	5	4	5	4	81	4.05±0.80
Period of the larvae stage (day)	12	13	13	12	14	12	13	13	12	14	13	13	12	14	13	14	12	13	12	13	257	12.85±0.73
Period of the pupae stage (day)	5	6	6	5	5	6	5	6	5	5	5	6	6	5	6	5	6	6	6	5	110	5.50±0.50
Total days of the life cycle	28	30	30	29	28	29	30	29	29	30	28	30	30	28	30	30	30	30	29	30	587	29.35±0.79

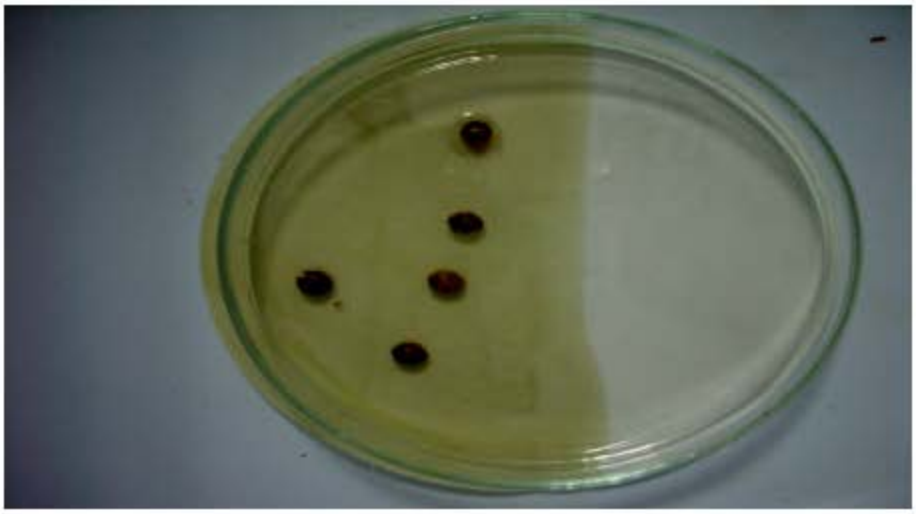

Fig. 2: Feeding bioassay in agar

Dual Choice Feeding Bioassay

Five starved adult beetles were placed in a petri dish (Fig. 2) containing methanolic crude extract in 2% agar in one half of compartment. The other half compartment contained 2% agar as control. The petri dishes were covered with a nylon mesh of aperture 0.5 mm. After every 24 h for three consecutive days, the numbers of triangular bite marks made by the beetles were examined under binocular microscope. Ten petri dish were given the treatment above. Ten fractions were collected from 2 mL methanolic extract isolated by column chromatography with silica gel 60 (230-400 mesh). The agar were also treated with fractions from column chromatography then subjected to the same behaviour bioassay as above.

Thin Layer Chromatography Analysis (TLC)

Five microliter of the fraction which gave positive anti feeding response in the bioassay was analysed with Thin Layer Chromatography (TLC) plate silica gel F254 using diethyl ether and methanol at 70:30 solvent mixtures. The separation that occurred on the TLC plate was observed under UVGL-58 UV light of short wave 254 nm long^{-1} wave 366 nm. Each compounds of different Rf was subjected to the same bioassay as in crude and fractions.

High Performance Liquid Chromatography Analysis (HPLC)

The fractions that gave positive result from bioassay were analyzed using HPLC to identify the active components in them. Analyses were performed on a Shimadzu HPLC model that build in combination of LC-10AT pump, fitted with ODS Hypersil C_{18} column (250×4.6 mm I.D.). The injection system (Rheodyne) used was 20 μL sample loop. Detection was done by a SPD-MIOA Variable Wavelength Detector at wavelength of 220 nm. KT- 25S degassing device (Shodex degasser, Tokyo, Japan) was used to degas the solvents. Mobile phase consisted of an isocratic mixture of acetonitrile-water (43:57) at a flow rate of 1.5 mL min^{-1}. The peaks were confirmed by comparing with standards of pure Azadirachtin. Five Azadirachtin solutions at concentrations ranging from 0.01, 0.05, 0.1, 0.125 and 0.25 mg mL^{-1} were used for analysis. Each concentration of standard Azadirachtin were injected 3 times into HPLC and peak area responses were obtained. The calibration curve for Azadirachtin was prepared by plotting concentration of Azadirachtin versus peak area (average of three runs). Fraction 3 from TLC which had evoked positive antifeedant response from the beetles, with known amounts of standard Azadirachtin was analyzed.

Feeding Bioassay Using Synthetic Compound (SC) of Azadirachtin

Synthetic neem compound, Azadirachtin supplied by Sigma-Aldrich was prepared in concentration of 50, 100 and 200 ppm with methanol. One half of the agar compartment was treated with 50 ppm and the other half of the agar compartment was treated with methanol as control. Each different concentration was subjected to the same bioassay method as in crude and fractions.

RESULTS

Life Cycle Study of *Epilachna indica* in Outdoor Cage

In this study, it was observed that the life cycle of *Epilachna indica* has four distinct life stages; egg, larva, pupa and adult. A male beetle finds a female beetle by using antennae to locate its mate. A single female beetle laid 10 to 15 eggs on the underside of a leaf after 6.95±0.67 days of mating. The eggs were tiny, elongated and yellow jellybeans like which hatched in about 4.05±0.80 days and larvae begin searching on plants. The larvae are white when they hatch, but soon turn black. Larvae were spindle-shaped, wrinkled and have short antennae. As the larva grows it sheds its skin four times before it is fully grown after feeding for 12.85±0.73 days. The larva attached its rear end to the back of a leaf with a sticky liquid and for the final time sheded its skin to reveal the soft orange colored skin. During the immobile pupa stage, the wings and other adult body parts developed. Pupation lasted 5.50±0.50 days until the adult beetles emerged from the pupa case. The time for development from an egg to an adult in this study was about 29.35±0.79 days.

Figure 3 shows the life cycle of the ladybird beetle at Somme Estate in October 2003. After the mating of 20 pairs, 211 eggs (100%) were laid. Every *Epilachna indica* laid 10 to 15 eggs. From the 211 eggs, 105 eggs (49.8%) were hatched to become larvae, 58 eggs (27.5%) did not hatch and the rest of 48 eggs (22.7%) were missed. Out of the living larvae, 83 larvae (39.4%) lived to become pupae and 22 larvae (10.4%) died in the process of becoming pupae. Among the surviving pupae, 62 pupae (29.4%) lived and become adult beetles and 21 pupae (10%) died before they became adult beetles. According to this study, 62 new born adult beetles (29.4%) were produced from the mating of 20 pairs *Epilachna indica*.

Figure 4 shows the life cycle of ladybird beetles, *Epilachna indica* at Somme estate during October 2003.

Feeding Bioassay Using Fraction from Column Chromatography

Table 2 shows the number of bites by ladybird beetles in fraction 1, 2 and 3. Table 3 shows the number of bites by ladybird beetles in fraction 4, 5 and 6. Table 4 shows the number of bites by ladybird beetles in fraction 7 and 8. Table 5 shows the number of bites by ladybird

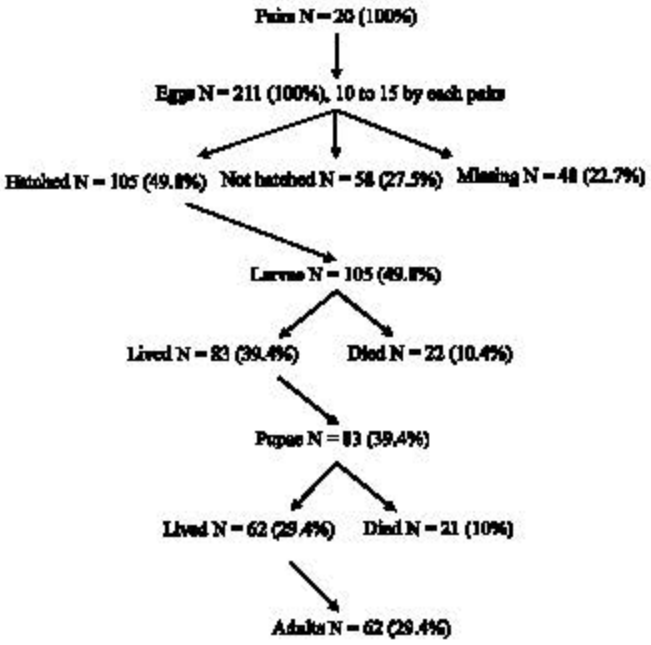

Fig. 3: Life cycle sequence of ladybird beetles, *Epilahna indica* at Somme estate during October 2003

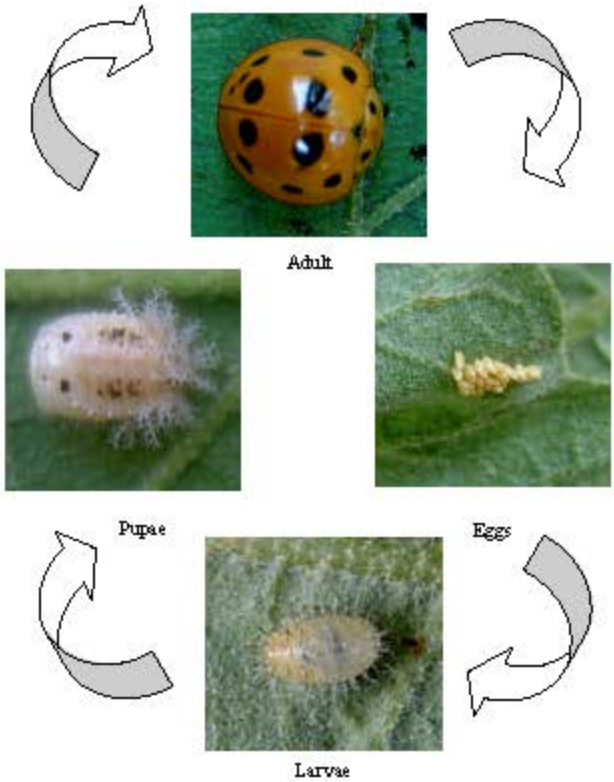

Fig. 4: Life cycle of ladybird beetles, *Epilahna indica* at Somme estate during October 2003

Table 2: The No. of bites by ladybird beetles in fractions 1, 2 and 3

			No. of bites by ladybird beetles													
Fractions		Hours	1	2	3	4	5	6	7	8	9	10	Total	Mean ±SD	p-value	Effect size	Power (%)
1	Treated agar with fraction 1	24	29	32	31	35	34	30	33	30	31	29	314	31.4±1.96	<0.001	4.897	>99.99
	Untreated agar (control)		39	42	40	43	38	40	41	39	42	40	404	40.4±1.50			
	Treated agar with fraction 1	48	56	60	54	63	59	62	55	59	61	57	586	58.6±2.87	<0.001	6.650	>99.99
	Untreated agar (control)		74	80	73	79	75	77	76	79	80	78	771	77.1±2.39			
	Treated agar with fraction 1	72	110	105	114	103	115	107	109	102	118	112	1095	109.5±5.04	0.002	1.471	87.50
	Untreated agar (control)		119	117	108	115	120	121	111	116	118	124	1169	116.9±4.48			
2	Treated agar with fraction 2	24	34	31	34	33	31	29	31	33	34	35	325	32.5±1.80	<0.001	3.716	>99.99
	Untreated agar (control)		36	38	42	41	40	42	38	41	39	42	399	39.9±1.97			
	Treated agar with fraction 2	48	54	57	60	58	55	64	58	61	60	58	585	58.5±2.77	<0.001	5.455	>99.99
	Untreated agar (control)		79	75	77	70	78	72	76	82	74	78	761	76.1±3.33			
	Treated agar with fraction 2	72	110	118	102	105	108	110	107	112	101	116	1089	108.9±5.24	0.001	1.565	91.10
	Untreated agar (control)		122	119	112	114	117	121	115	116	109	122	1167	116.7±4.15			
3	Treated agar with fraction 3	24	6	7	3	8	7	4	5	6	8	4	58	5.8±1.66	<0.001	15.628	>99.99
	Untreated agar (control)		40	43	36	39	37	41	43	40	42	37	398	39.8±2.40			
	Treated agar with fraction 3	48	12	11	14	16	12	11	15	13	10	12	126	12.6±1.80	<0.001	25.572	>99.99
	Untreated agar (control)		78	76	74	73	75	78	70	77	74	80	755	75.5±2.77			
	Treated agar with fraction 3	72	19	17	14	22	16	15	20	14	22	19	178	17.8±6.19	<0.001	21.624	>99.99
	Untreated agar (control)		117	108	113	120	123	118	110	108	113	122	1152	115.2±5.31			

beetles in fraction 9 and 10. Total number of bites was analysed using ANOVA. Power of the test was more than 80% showing that 10 replicates are sufficient to show the difference between treated and untreated (control). Effect Size (ES) of the test was more than 0.14, showing that there was a clear difference between the treated and untreated (control). Probability value (p-value) was less than 0.05, meaning that there is a significant difference between control and treatment. Regardless of time there is a significant difference between the treated agar and untreated agar. Based on observation from Table 2-5 fraction 3 was considered as giving the positive results in antifeeding bioassay as it gave maximum differences between treated agar and untreated agar (control). The other fractions (fraction 1, 2, 4, 5, 6, 7, 8, 9 and 10) show negative results in antifeeding bioassay. Fraction 3 was then analysed with Thin Layer Chromatography.

Table 3: The number of bites by ladybird beetles in fractions 4, 5 and 6

			No. of bites by ladybird beetles														
Fractions		Hours	1	2	3	4	5	6	7	8	9	10	Total	Mean ±SD	p-value	Effect size	Power (%)
4	Treated agar with Fraction 4	24	31	34	31	29	30	33	30	31	29	32	310	31.0±1.55	<0.001	4.398	>99.99
	Untreated agar (control)		39	37	40	38	43	41	36	41	38	40	393	39.3±2.00			
	Treated agar with fraction 4	48	54	58	63	62	56	61	57	60	55	61	587	58.7±2.97	<0.001	5.442	>99.99
	Untreated agar (control)		72	79	74	73	77	75	78	82	73	75	758	75.8±2.99			
	Treated agar with fraction 4	72	114	119	104	108	106	111	104	112	110	113	1101	110.1±4.50	0.009	1.161	68.7.0
	Untreated agar (control)		121	118	107	110	117	123	115	118	110	121	1160	106.0±5.12			
5	Treated agar with fraction 5	24	33	36	29	32	30	36	32	30	28	32	318	31.8±2.56	<0.001	2.999	100.00
	Untreated agar (control)		38	36	43	40	42	39	37	45	44	40	404	40.4±2.87			
	Treated agar with fraction 5	48	64	74	70	69	67	76	71	63	69	70	693	69.3±3.80	<0.001	1.841	97.10
	Untreated agar (control)		76	74	80	70	81	75	80	73	79	75	763	76.3±3.41			
	Treated agar with fraction 5	72	109	105	99	106	104	103	101	109	100	108	1044	104.4±3.47	0.012	1.095	63.40
	Untreated agar (control)		110	119	103	110	106	109	103	112	106	114	1092	109.2±4.75			
6	Treated agar with fraction 6	24	35	33	31	35	32	36	30	37	31	29	329	32.9±2.59	<0.001	2.915	>99.99
	Untreated agar (control)		42	40	39	37	40	43	42	40	37	39	399	39.9±1.92			
	Treated agar with fraction 6	48	54	62	55	60	59	57	61	58	55	63	584	58.4±2.97	<0.001	6.511	>99.99
	Untreated agar (control)		75	80	77	76	83	78	75	79	74	78	775	77.5±2.58			
	Treated agar with fraction 6	72	101	114	110	108	115	103	109	114	119	104	1097	109.7±5.55	0.005	1.307	78.90
	Untreated agar (control)		120	112	114	117	125	121	117	108	115	118	1167	116.7±4.56			

Thin Layer Chromatography Analysis (TLC)

Fraction 3 was analysed using Thin Layer Chromatography plate. Three spots were observed for fraction 3. The retention time (Rf) of the three spots from fraction 3 was 0.44, 0.77 and 0.94. On the basis of number of bites by ladybird beetle after every 24 h for 3 days in 10 replicates (Table 6) fraction spot 2 was considered most promising and was therefore further analysed using High Pressure Liquid Chromatography Analysis (HPLC).

Table 4: The number of bites by ladybird beetles in fractions 7 and 8

			No. of bites by ladybird beetles													
Fractions		Hours	1	2	3	4	5	6	7	8	9	10	Total	Mean ±SD	p-value	Effect size	Power (%)
7	Treated agar with fraction 7	24	32	30	35	34	30	32	30	32	31	33	319	31.9±1.64	<0.001	4.604	>99.99
	Untreated agar (control)		38	39	41	40	44	41	39	42	38	40	402	40.2±1.78			
	Treated agar with fraction 7	48	53	56	62	60	63	55	61	58	60	57	585	58.5±3.07	<0.001	6.485	>99.99
	Untreated agar (control)		75	78	73	77	75	78	80	76	76	74	762	76.2±1.99			
	Treated agar with fraction 7	72	114	118	103	110	105	112	103	116	104	114	1099	109.9±5.43	0.020	0.991	54.50
	Untreated agar (control)		109	114	117	108	119	122	116	114	112	119	1150	115.0±4.27			
8	Treated agar with fraction 8	24	34	35	33	30	31	36	29	32	34	30	324	32.4±2.24	<0.001	3.377	>99.99
	Untreated agar (control)		36	39	41	42	40	39	38	43	41	43	402	40.2±2.14			
	Treated agar with fraction 8	48	63	73	71	65	67	70	72	64	69	70	684	68.4±3.29	<0.001	2.524	>99.99
	Untreated agar (control)		73	75	78	73	81	74	80	75	77	78	764	76.4±2.69			
	Treated agar with fraction 8	72	116	102	113	105	104	112	105	107	102	118	1084	108.4±5.57	<0.001	3.352	>99.99
	Untreated agar (control)		126	135	130	122	130	129	135	132	118	122	1279	127.9±5.47			

Table 5: The number of bites by ladybird beetles in fractions 9 and 10

			No. of bites by ladybird beetles													
Fractions		Hours	1	2	3	4	5	6	7	8	9	10	Total	Mean ±SD	p-value	Effect size	Power (%)
9	Treated agar with fraction 9	24	29	35	32	40	37	30	33	39	40	35	350	35.0±3.79	<0.001	1.558	90.8
	Untreated agar (control)		37	42	39	38	41	44	41	40	42	37	401	40.1±2.21			
	Treated agar with fraction 9	48	52	57	61	64	51	59	65	60	57	64	590	59.0±4.60	<0.001	4.412	>999
	Untreated agar (control)		75	76	73	77	74	80	74	75	77	81	762	76.2±2.48			
	Treated agar with fraction 9	72	115	103	107	102	114	103	101	100	105	116	1066	106.6±5.82	<0.001	1.851	97.2
	Untreated agar (control)		122	112	114	117	124	121	117	108	114	121	1170	117.0±4.80			
10	Treated agar with fraction 10	24	32	28	35	30	33	29	30	32	31	33	313	31.3±2.00	<0.001	3.958	>999
	Untreated agar (control)		42	39	46	42	44	40	37	42	38	40	410	41.0±2.61			
	Treated agar with fraction 10	48	53	57	66	60	54	66	55	60	59	57	587	58.7±4.29	<0.001	4.628	>999

Table 5: Continue

Fractions		Hours	No. of bites by ladybird beetles 1	2	3	4	5	6	7	8	9	10	Total	Mean ±SD	p-value	Effect size	Power (%)
	Untreated agar (control)		77	74	75	72	79	74	75	81	76	75	758	75.8±2.48			
	Treated agar with fraction 10	72	113	117	103	105	108	111	106	112	105	114	1094	109.4±4.41	0.002	1.448	86.5
	Untreated agar (control)		118	113	106	119	117	125	122	115	112	121	1168	116.8±5.25			

Table 6: The number of bites by ladybird beetle for every 24 h in 10 replicates

Spot		Hours	No. of bites by ladybird beetles 1	2	3	4	5	6	7	8	9	10	Total	Mean ±SD	p-value	Effect size	Power (%)
1	Treated agar with spot 1	24	33	35	32	31	30	35	30	31	29	33	322	32.2±2.079	<0.001	3.951	>99.99
	Untreated agar (control)		39	37	40	42	41	37	39	41	43	40	399	39.9±1.969			
	Treated agar with spot 1	48	61	73	70	67	69	74	70	63	67	73	687	68.7±4.296	<0.001	2.024	98.70
	Untreated agar (control)		73	76	79	72	80	73	81	74	78	78	764	76.4±3.239			
	Treated agar with spot 1	72	115	104	115	106	105	111	106	108	103	118	1091	109.1±5.301	<0.001	3.509	>99.99
	Untreated agar (control)		124	130	134	121	131	128	136	134	120	125	1283	128.3±5.638			
2	Treated agar with spot 2	24	4	6	4	7	5	4	6	7	5	7	55	5.5±1.269	<0.001	23.740	>99.99
	Untreated agar (control)		39	41	38	39	42	43	42	41	40	39	404	40.4±1.647			
	Treated agar with spot 2	48	12	13	15	13	11	14	12	10	11	12	123	12.3±1.494	<0.001	35.456	>99.99
	Untreated agar (control)		77	75	74	76	75	78	73	77	75	80	760	76.0±2.055			
	Treated agar with spot 2	72	18	19	15	20	16	17	19	15	21	17	177	17.7±2.058	<0.001	27.503	>99.99
	Untreated agar (control)		120	110	115	119	123	120	116	109	115	121	1168	116.8±4.662			
3	Treated agar with spot 3	24	31	32	34	33	31	30	31	33	30	33	318	31.8±1.398	<0.001	5.402	>99.99
	Untreated agar (control)		41	38	40	41	43	42	38	40	39	39	401	40.1±1.663			
	Treated agar with spot 3	48	55	59	60	62	55	63	58	61	57	59	589	58.9±2.726	<0.001	7.137	>99.99
	Untreated agar (control)		78	75	77	73	78	75	76	80	74	77	763	76.3±2.111			
	Treated agar with spot 3	72	113	118	102	109	107	110	105	114	103	114	1095	109.5±5.276	0.002	1.466	87.30
	Untreated agar (control)		119	115	108	117	119	124	118	116	111	121	1168	116.8±4.662			

Table 7: The number of bites by ladybird beetle for every 24 h towards Synthetic Compound (SC)

Concentrations		Hours	No. of bites by ladybird beetles 1	2	3	4	5	6	7	8	9	10	Total	Mean ±SD	p-value	Effect size	Power (%)
50 ppm	Treated agar with 50 ppm	24	30	34	36	30	28	37	33	29	35	27	319	31.9±3.542	<0.001	1.909	97.80
	Untreated agar (control)		37	39	40	36	34	35	41	36	42	39	379	37.9±2.685			
	Treated agar with 50 ppm	48	67	70	69	65	72	74	77	66	69	75	707	70.7±4.006	0.006	1.244	74.80
	Untreated agar (control)		70	75	77	69	79	76	81	73	74	80	754	75.4±4.033			
	Treated agar with 50 ppm	72	117	106	119	104	108	115	107	102	104	114	1096	109.6±6.096	<0.001	3.609	>99.99
	Untreated agar (control)		120	126	132	125	130	129	128	131	126	128	1275	127.5±3.472			
100 ppm	Treated agar with 100 ppm	24	5	7	6	5	6	4	8	6	4	8	59	5.9±1.449	<0.001	10.877	>99.99
	Untreated agar (control)		40	37	43	35	46	33	41	44	38	43	400	40.0±4.190			
	Treated agar with 100 ppm	48	13	15	13	11	14	12	16	14	17	15	140	14.0±1.826	<0.001	18.520	>99.99
	Untreated agar (control)		78	70	75	79	72	77	70	76	74	84	755	75.5±4.327			
	Treated agar with 100 ppm	72	20	17	16	22	17	19	21	19	25	16	192	19.2±2.898	<0.001	18.961	>99.99
	Untreated agar (control)		125	105	117	121	127	122	114	112	116	124	1183	118.3±6.800			
200 ppm	Treated agar with 200 ppm	24	6	9	7	10	5	4	8	10	5	7	71	7.1±2.132	<0.001	9.546	>99.99
	Untreated agar (control)		39	35	43	48	41	35	39	46	37	44	407	40.7±4.498			
	Treated agar with 200 ppm	48	18	20	15	12	16	19	21	19	17	13	170	17.0±2.981	<0.001	15.492	>99.99
	Untreated agar (control)		76	72	79	74	80	73	82	85	71	78	770	77.0±4.595			
	Treated agar with 200 ppm	72	21	25	19	18	20	26	28	24	22	20	223	22.3±3.302	<0.001	18.097	>99.99
	Untreated agar (control)		117	110	121	116	124	128	120	106	113	122	1177	117.7±6.684			

Feeding Bioassay Using Synthetic Compound Azadirachtin

Table 7 represents the number of bites by ladybird beetle for every 24 h towards Synthetic Compound (SC) of Azadirachtin. Total numbers of bites from Table 7 were analysed using ANOVA. Feeding Bioassay using 50 ppm concentration of synthetic Compound revealed negative antifeedant activity while 100 and 200 ppm showed positive antifeedant response. Further, 100 ppm concentration of synthetic compound was considered as minimum concentration that could cause optimal antifeedant effect towards *Epilachna indica*.

High Pressure Liquid Chromatography Analysis (HPLC)

Major organic compound that was found in fraction 3 by HPLC was Azadirachtin as shown in result. Table 8 represents area for 5 concentrations to plot standard curve. Azadirachtin was resolved as single peak in all samples analyzed with no interference from other compounds. The identity of the Azadirachtin peak was confirmed by determination of retention time and by spiking with standard Azadirachtin. A calibration curve was derived from three injections of six concentrations of Azadirachtin. Linearity was found in the range and it has a good reproducibility and accuracy (Fig. 5). The following regression equation was obtained y = 1571.9; x-78.784, where y is the peak area and x is the concentration of Azadirachtin. The correlation coefficient of the calibration graph was ≥ 0.9821.

Table 8: Area for 5 concentrations to plot standard curve

Concentrations (mg mL^{-1})	Area			
	Aza a	Aza b	Aza c	Mean
0.01	339096	387187	361240	362507.7
0.05	461976	449422	350900	420766.0
0.1	1179410	1137139	1072783	1129777.0
0.125	742958	702578	666165	703900.3
0.25	2046775	2083991	1945457	2025408.0

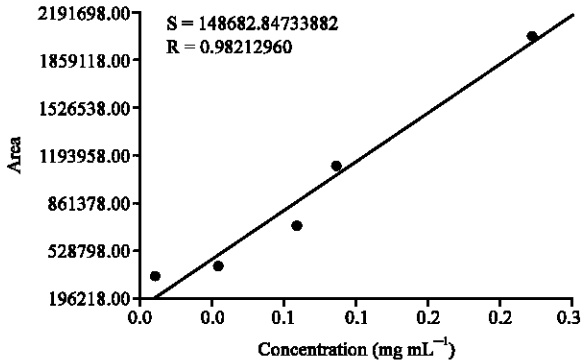

Fig. 5: The linear relationship between the area and concentration of Azadirachtin

DISCUSSION

In this study, female ladybird beetle *Epilachna indica* laid eggs 6.95±0.67 days after mating at environmental at temperature of 30 to 32°C. The eggs hatched in about 4.05±0.80 days to become larvae. Larvae fed for 12.85±0.73 days before changing to immobile pupa stage. Pupation lasted 5.50±0.50 days until the adult beetles emerged from the pupa case. The time for development from an egg to an adult is about 29.35±0.79 days whereas Tung (1983) reported that the life cycle of *E. indica* is about a month and *Epilachna sparsa* has a life cycle of 22 to 27 days (Khoo *et al*., 1991).

Since only 62 newborn adult *E. indica* were produced from laboratory culture it was not enough for feeding experiment thus adults were used to establish laboratory colony. All experiments in this study used adult captured from wild from the *S. melongena* farm at Jalan Kebun. Methanolic extraction method was used in experiment was similar to Schlüter and Seifert (1988) who also used methanolic extraction method in studying the Mexican bean beetle, *Epilachna varivesties*.

About 2500 plants species had one or more active feeding insects but only neem was found to be highly effective, non-toxic and environmentally friendly agent for controlling insects by acting as feeding inhibitor and growth regulator (Warthen, 1979) and neem was projected as the insecticide of the future for protection against field pests (Jotwani and Srivastra, 1981).

Various studies on neem extracts are known to affect various insects in certain ways. The neem extracts disrupt or inhibit the development of eggs, larvae, or pupae. This ensures that the pests do not develop in numbers. The neem extracts also block the molting of larvae or nymphs (Schmutterer and Asher, 1986). Similarly, Kubo and Klocke (1982) isolated and identified azadirachtin as an antifeedant while looking for limonoids as insect controlling agents. It was also observed that these limonoids prevented the completion of larval moulting by inhibiting the exuviae after the formation of new cuticle. These compounds did not kill the insects directly but lowered their growth rate and made them more vulnerable to other mortality factors. Jaipal *et al*. (1983) also noted juvenile hormone-like activities in the bark of neem and observed that the metamorphosis of the insect was inhibited to varying degrees by these.

This study successfully identified Azadirachtin as the antifeedant from *Azadirachta indica* which elicited antifeeding behaviour in *Epilachna indica* (Coleoptera: Coccinellidae). Eventhough Azadirachtin is not a new active compound since has already been found by Kubo and Klocke (1982), Jaipal *et al*. (1983), Swaminathan (1983), Freeman and Andow (1983), Jacobson (1986), Schmutterer and Asher (1986), Saxena (1987), Kareem *et al*. (1987), Singh and Singh (1988). However this is the first time Azadirachtin has been tested against *Epilachna indica*. The result of this study is significant for the field of pest management in Malaysia since *Epilachna indica* feed on several Solanaceae plants. The use of purified extract of neem was suggested for pest control. Swaminathan (1983) brought forward the potential of neem in pest control. Freeman and Andow (1983) described the role of neem as tree for protection of other plants as an insect feeding deterrent. Jacobson (1986) gave the details of its insecticidal activity. Schmutterer and Asher (1986) edited the proceedings of a conference which had research papers on the pesticidal activity of neem. Saxena (1987) brought forward the use of neem as an antifeedant in pest management in the tropics and recommended quality control and standardization of its biological properties for introduction on a commercial scale.

Qi *et al*. (2001) in their studies found that 50 and 200 ppm azadirachtin treatments had effects on Schneider, *Mallada signatus* (Neuroptera: Chrysophidae) pupal survival, with the 200 ppm treatment killing all individuals and about 50% being affected by the 50 ppm treatment. In contrast this study found that in *Epilachna indica*, 50 ppm concentration Synthetic Compound of Azadirachtin did not give positive antifeedant response unlike Qi *et al*. (2001). However at 100 and 200 ppm positive antifeedant response were seen in this study on *Epilachna indica* similar to the study on *Mallada signatus* (Qi *et al*., 2001). Both studies show that 200 ppm give maximum response whereas 100 ppm concentration is considered as minimum concentration that can cause optimal antifeedant effect on *Epilachna indica* this study.

Neem inhibited oviposition, larval development and feeding and greatly increased mortality of cabbage pest, *Mamestra brassicae* L. (Seljasen and Meadow, 2006). Neem limonoids azadirachtin, salannin, deacetylgedunin, gedunin, 17-hydroxyazadiradione and deacetylnimbin may be used in IPM programs for *Cnaphalocrocis medinalis,* rice leaffolder and should be evaluated for efficacy under field conditions (Nathan *et al*., 2005). The potential of neem to control two important pests, the coffee leaf miner (*Leucoptera coffeella*) and the coffee red mite (*Oligonychus ilicis*) occurring in coffee plantations was demonstrated by Venzon *et al*. (2005). In that study, neem was not lethal to an important predator commonly found in coffee agro ecosystems in Brazil. Neem extracts may have a role to play in protecting seedling trees from attack by pine weevil, *Hylobius abietis* L. during their first year of growth in the field (Thacker *et al*., 2003). Neem kernel water extract may be recommended for plant protection against third instar nymphs of *Jacobiasca lybica* in vegetables in the Sudan (El Shafie and Basedow, 2003).

The results of this study confirms that Azadirachtin has the potential for furthering the development of broader scale integrated pest management programs in controlling the ladybird beetle, *Epilachna indica* in small-scale plantations of *Solanum melongena* which is an important vegetable in Malaysia. This study is significant to agriculture in Malaysia especially to control pest of eggplant, *Epilachna indica* in view of using non-toxic natural product as biopesticide, which is safe for the environment and for human health. No other researchers have done studies on antifeedant properties of *Azadirachta indica* against pest of eggplant, *Epilachna indica* (Coleoptera: Coccinellidae). For future studies, the compound should be tested in the field on *S. melongena* plant to compliment results obtained in this study.

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