Toxicity of Seed oil of *Azadirachta indica*, *Calophyllum inophyllum* and their Mixture against *Crocidolomia pavonana* Larvae

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Abstract

Crocidolomia pavonana is one of the problems encountered in the cultivation of cabbage. This insect causes significant yield losses. Therefore, it is necessary to have control actions such as by using a bioinsecticide. Azadirachta indica and Calophyllum inophyllum are known to have chemical compounds that have potency to be developed as bioinsecticides. The reserch aimed to determine insecticidal toxicity of A. indica and C. inophyllum seed oil and their mixture to C. pavonana. The treatments tested were control, A. indica (v), C. inophyllum (v), A. indica: C. inophyllum(1:1) v/v, A. indica : C. inophyllum(1:2) v/v, A. indica : C. inophyllum(2:1) v/v, A. indica : C. inophyllum(1:3) v/v and A. indica : C. inophyllum(3:1) v/v. Each treatment was tested at concentrations of 0.05% (v/v) and 0.1% (v/v).

The experiment was arranged in a randomized block design with 4 replications. Toxicity testing was performed using a leaf-residual feeding method on the instar II-IV of C. pavonana larvae. The results of the research showed that the most toxic was A.indica seed oil and then mixture of A. indica and C. innophillum seed oil at the ratio of 3 : 1. Besides having toxic effect, A. indica seed oil has the highest growth inhibitory activity, reduced food consumption and weight of C. pavonana larvae compared with the C.inophyllum seed oil and the mixture of both seed oils.

Keywords: Toxicity, Neem seed oil, Nyamplung seed oil, *Crocidolomia pavonana.*

Introduction

Crocidolomia pavonana and *Plutella xylostella* are the main pests in cabbage that cause yield loss until 100% if no control action is taken[4,8,16]. Usually, the farmers control this pest by using synthetic insecticides[15]. Unfortunately, unwise use of synthetic insecticides can harm human health and pollute the environment. Therefore, the use of synthetic insecticides should be reduced immediately and should switch to bioinsecticides that have relatively cheaper prices and are safe for health and the environment[3,17]. The use of bioinsecticides can reduce environmental pollution and the price is relatively cheaper than synthetic insecticides[17].

Neem (*Azadirachta indica*) and nyamplung (*Calophyllum inophyllum*) are two examples of plants used for bioinsecticides. Parts of *A. indica* plants can be used as insecticides such as leaves and seeds. Some of the compounds contained in *A. indica* plants are azadirachtin, meliantriol, azadiron, salanin, nimbin and nimbidin[18]. Azadirachtin is the main active compound from seed of *A. indica. A. indica* seeds oil is considered effective enough to control many pests that damage plants[21]. Similarly, the seed oil of *C. inophyllum* can be used as a bioinsecticide[12]. According to Tukimin and Heri[22], oil from *C. inophyllum* seeds had activity as an antifeedant, repellent and larvicidal. *C. inophyllum* seed oil contains fatty acids such as oleic acid, linoleic acid, stearic acid, palmitic acid, stigmasterol and β stigmasterol which are both antioxidant and cytoprotective[20].

Many researches on bioinsecticides are done in a single form. However, single bioinsecticide was often insufficient, such as plant or a part of the plant is not always available every season in the nature, the complexity of pests that attack plants and so on. Therefore, a mixture of insecticides is required[7]. The use of mixed insecticides can improve effectiveness, reduce the number of insecticides and suppress the emergence of resistant pests[6]. The use of bioinsecticide made from two or more kind of plants can be more efficient in using raw materials of bioinsecticide. Plant material needed can be reduced and can be added with other plant materials[19]. The use of mixture insecticides can inhibit the occurrence of insect resistance. The mixing of botanical insecticides is usually done at lower doses for each component, causing more efficient and synergistic results[7].

Material and Methods

The experiment was carried out at Pesticide and Environment Toxicology Laboratory, Department of Plant Pests and Diseases, Faculty of Agriculture, Universitas Padjadjaran, Jatinangor, West Java, Indonesia.

Planting of Broccoli Plant: Broccoli plant (*Brassica olearacea* var italica) was used as the feed of *C. pavonana* larvae. Broccoli planting was done on mixture of soil with manure (2:1). Maintenance of broccoli plants included watering, weeding and mechanical pest exclusion. The leaves from age of broccoli two month were used for feeding *C. pavonana* larvae.

Rearing of Crocidolomia pavonana: Insect test used was *C. pavonana*larvae already reared in Laboratory of Pesticide and Environmental Toxicology, Department of Plant Pests and Diseases, Faculty of Agriculture Universitas Padjadjaran. The colony has been reared as described by Prijono and Hassan[10]. The larvae were fed with pesticide-free broccoli leaves and the adults were fed 10% honey solution in cotton swab.

***A. indica* and *C. inophyllum* Seed Oil:** The seed oil of *A. indica* and *C. inophyllum* was obtained from The East Java, Indonesia. The oil was then stored in the refrigerator at 4°C until prior to use.

Experiment: The treatments used in the toxicity test of *A. indica* and *C. Inophyllum* seed oil and mixture were a) Control (without seed oils treatment V/V), b) *A. indica* (v), c) *C. inophyllum* (v), d) *A. indica* : *C. inophyllum*(1:1) v/v, e) *A. indica* : *C. inophyllum*(2:1) v/v, g)*A. indica* : *C. inophyllum*(1:3) v/v and h) *A. indica* : *C. inophyllum*(3:1) v/v.

Each seed oil and the mixture of *A. indica* and *C. inophyllum* were tested at the concentrations of 0.05% and 0.1%. To obtain a solution of the desired concentration, *A. indica* seed oil or *C. inophyllum* was dissolved in the aquadest containing Tween-80 as much as 0.24%, Span-80 as much as 0.06 %. Preparation of oil solution with the concentration of 0.05%-0.1% was done by dissolving oil as much as 0.05 mL - 0.1 mL and the dilution solution up to 100 mL volume. The preparation of other solutions was carried out in the same manner. Water containing Tween-80 and Span-80 at concentration of 0.24 % and 0.06 % respectively was used as control treatment. All solutions for the treatment were mixed with the composition as above. The treatment differentiator was the oil ratio used for the experiments.

Bioassay was conducted by using feeding method. Pieces of broccoli leaves (4 cm x 4 cm) were dipped into the oil mixture according to the concentration. The leaf was dipped for 20 seconds until the solution was wetting the entire surface of the leaf evenly. Then, the leaf was air-dried on the filter paper. Two pieces of treated leaves were put into a Petri dish lined with filter paper. Thereafter, ten instar II of *C. pavonana* larvae were put into a Petri dish using a fine brush.

Feeding period treatment was done for 48 hours. Furthermore, larvae were feed fresh leaf without treatment until they reach instar IV larvae. Observations were made by counting the number of dead larvae, larval development, the area of feed consumed and the weight of instar IV larvae. The observation of mortality of *C. pavonana* larvae was carried out from day one after application of the initial instar II larvae to the initial instar IV larvae with a 24-hour observation interval. The mortality of larvae was calculated in the percentage of death by the following formula:

$$\text{Mortality}(\%) = \frac{\sum \text{dead larva}}{\sum \text{Larvae tested}} \text{ x100\%}$$

The observation of development time was started from 1 day after application at larvae instar II to instar IV larvae at 24-hour observation interval. Observations were made by recording the time taken for the larvae to instar III and instar IV.

Observation of feed consumption was done by measuring the leaf area eaten by larvae using transparent millimeter block. Next, the leaf area was calculated as the percentage with the following formula:

$$\text{Feed Consumption}(\%) = \frac{\sum \text{Leaf area eaten}}{\sum \text{Total leaf area}} \times 100\%$$

Observation of larvae weight was performed on larvae reaching the instar IV. The instar IV larvae were dried by the oven at 95°C for 24 hours to obtain the dry weight of the larvae, then weighed using an analytic scale.

The study was arranged in a randomized block design (RBD) with 4 replications. Each treatment used a concentration of 0.05% and 0.1% and every treatment used 40 second instar of *C. pavonana* larvae.

Data obtained from observation were analyzed by using analysis of variance. If the result was significant, data were then analyzed with Duncan Test by using SPSS 17 program.

Results and Discussion

Mortality of *C. pavonana* larvae: The observed mortality of larvae from the examined treatment of *A. indica* seed oil, *C. inophyllum* seed oil and mixtures caused different mortality. In a single treatment, the highest mortality of *C. pavonana* was in *A. indica* treatment followed by the combination treatment of *A. indica* and *C. inophyllum* (3: 1). The lowest mortality was in Control. The larval mortality was started from 2 DAT (Day after treatment) and increased sharply at 3 and 4 DAT (figures 1 and 2).

In the graph of larval mortality, it is shown that the mortality of *C. pavonana* in *C. inophyllum* oil was lower than that of *A. indica* oil treatment. It can be seen from larval mortality at 8 DAT of *C. inophyllum* oil treatment at the concentration of 0.1% which caused larval mortality of only 12.5%, while the seed oil treatment of *A. indica* was 65%.

C. inophyllum oil treatment was not effective in controlling *C. pavonana* at a concentration of 0.05% -0.1% with the larval mortality of only 7.5% -12.5%. In the previous study, *C. inophyllum* at 0.1% concentration was able to inhibit the feeding activity of *Epilachna sparsa* larvae (Ladybug) of 58.32%[14]. Treatment of *A. indica* and *C. inophyllum* oil mixture (3:1) at the concentration of 0.05% -0.1% showed a high mortality of 60% -45%, starting from 2 DAT and continuing to increase to 6 DAT. For the mixture of *A. indica* oil and *C. inophyllum* with a ratio of 1:1, 1:2, 2:1 and 1:3, they did not show the effect of synergism, because the mortality rate was low and not significantly different from the control treatment. The treatments of *A. indica*, *C.*

inophyllum and the mixture of *C. pavonana* showed a significant difference effect to larval mortality at 5% level.

Azadirachta indica oil treatment was quite effective in causing the larvae mortality of *C. pavonana*. This effect is likely because *A. indica* seed oil contains azadirachtin compounds that are quite effective, although the bioactive compound has a slow effect on the test insects. It takes 7-10 days to kill the insects[1]. Azadirachtin compounds can act as repellent, inhibit growth, cause morphological abnormalities and can kill insects[13].

The death of the larvae after being treated shows a drying body, an abnormal size (smaller than the other larvae) and the body color becomes yellowish and finally blackish. The death of the larvae was caused by the starvation of the larvae due to the antifeedant effect of the seed oil. In this test, the mortality at the treatment of Azadirahtin was higher than that of the mixture. This is supposedly because of reduction in the toxicity of one of the extracts[2]. The mortality of the tested treatment was not so high, presumably because the concentrations given were quite low (0.05% and 0).

The larval mortality of both single and mixed treatments with different concentrations shows the same graphic trend. The highest mortality observed on the single treatment of *A. indica* oil. In this experiment, it can be stated that mixing of botanical insecticides cannot always increase its effectiveness.

Development Time of *C. pavonana:* The development time of larvae was influenced by the concentration of seed oil and the compounds contained in the botanical insecticide. The fastest larval development in the control treatment from instar II to instar IV were 5.98 and 6.03 days. The treatment of neem oil at the concentrations of 0.05% and 0.1% resulted in the delayed development of the larvae which was 9.64 and11.86 days respectively (Table 2). The mixed oil treatment of *A. indica* and *C. inophyllum* (3: 1) at concentrations of 0.05% and 0.1% also caused the delayed development of larvae (6.69 and 9.00 days respectively) which were not significantly different from the single treatment of *A. indica*. It can be seen that higher concentration can increase the inhibition of the growth of *C. pavonana* larvae.

Disturbance on length of developmental time of *C. pavonana* larvae was caused by inhibition of feeding activity or poisoning on hormone-producing organs that regulate insect development. According to Chapman[5] and Samsudin[13], the hormone ecdysone and 20-hydroxyecdysone is a hormone that belongs to insects in regulating the growth and changes the cuticle. If the hormone is disturbed, this will inhibit the process of larval development. In addition to hormonal disorders, physiological disorders can also inhibit the growth of larvae such as protease and invertase enzymes that can interfere with the food digestion of insects[9].

Feed Consumption of *C. pavonana* larvae: Treatment of *A. indica* oil, *C. inophyllum* oil and mixtures affected food consumption by larvae. The results of observation showed that the consumption of *C. pavonana* larvae was significantly different between control and the treatments of *A. indica* oil and mixed oil *A. indica* and *C. inophyllum* (3: 1). There was no significant difference between the other treatments and control (table 3).

Low feed consumption indicates that antifeedant effect was effective in inhibiting larval consumption activity. *A. indica* treatment at the concentrations of 0,05% and0,1% caused leaf consumed 1,37% and 0,13% respectively. High feed consumption shown in control was 8.94% and 9.40%. Feed consumption in treatments at 0.05% concentration showed average percentage of feed consumption greater than that of 0.1% concentration. This shows that the higher is the concentration, the stronger is the effect in inhibiting larval feeding activity. However, in the treatment of *C. inophyllum* oil and the mixture of *A. indica* : *C. inophyllum* (1:1) at the concentration of 0.1%, there was a greater percentage than the 0.05% concentration. This was possibly because the larvae stop eating after ingesting an amount of inedible extract.

Observation on the behavior of larvae at the time of *A. indica* oil treatment and the mixed oil treatment of *A. indica* : *C. inophyllum* (3: 1) showed that the all larvae was moving away from the feed. At the treatment of mixed *A. indica* and *C. inophyllum* (2: 1), some larvae consumed feed and the other stayed away from feed. In other treatments, larvae tended to hide under the leaves and immediately consume them. The behavior of these larvae indicates the antifeedant effect on *C. pavonana.* In general, it can be said that all leaves that have been treated, still consumed larvae although only a little.

The Inhibition of larval feeding activity was caused by azadirachtin compound in the neem. These compounds can decrease the activity of larval feeding. According to Rafiq et al[11], azadirachtin has antifeedant effects against *Spodoptera litura*. Seed oil of *A. indica* has a strong smell that rejects insects to consume the feed that has been given treatment. The primary effect of azadirachtin compounds disrupts the perception of stimuli to eat, which is an antifeedant that produces a specific deterrent in the form of chemical receptors (chemoreceptor) in the mouth that works in relation with other chemical receptors[23].

Weight of *C. pavonana* larvae: The results showed that the influence of *A. indica* oil and *C. inophyllum* and its mixture was able to reduce the weight of *C. pavonana* larvae. The highest average larvae weight in control treatment was 0.0454 g and 0.0439 g, while the lowest larvae weight was in the treatment of *A. indica* oil at concentrations of 0.05% and 0.1% i.e. 0.0172 g and 0.0173 g respectively. The highest effect of combination treatment was on the treatment of *A. indica* and *C. inophyllum* mixture (3:1) at

concentrations of 0.05% and 0.1% i.e. 0.0237 g and 0.0277 g respectively. There was a significant difference between the treatment and control (table 4).

This study suggests that the compounds contained in the oils used do not kill directly but affect the weight of the larvae. The weight of larvae is also affected by feed consumption due to antifeedant effects of the oil.

C. pavonana larvae treated with *A. indica* oil has a smaller size than the control. The decrease in the weight of these larvae was because of the azadirachtin compounds contained in neem. This compound had antifeedant effects and decreased consumption of larval feed. Larval growth was hampered and larvae weight was low due to decreased feed consumption. This resulted in the deficiency of nutrient needed by the larvae to support their growth.

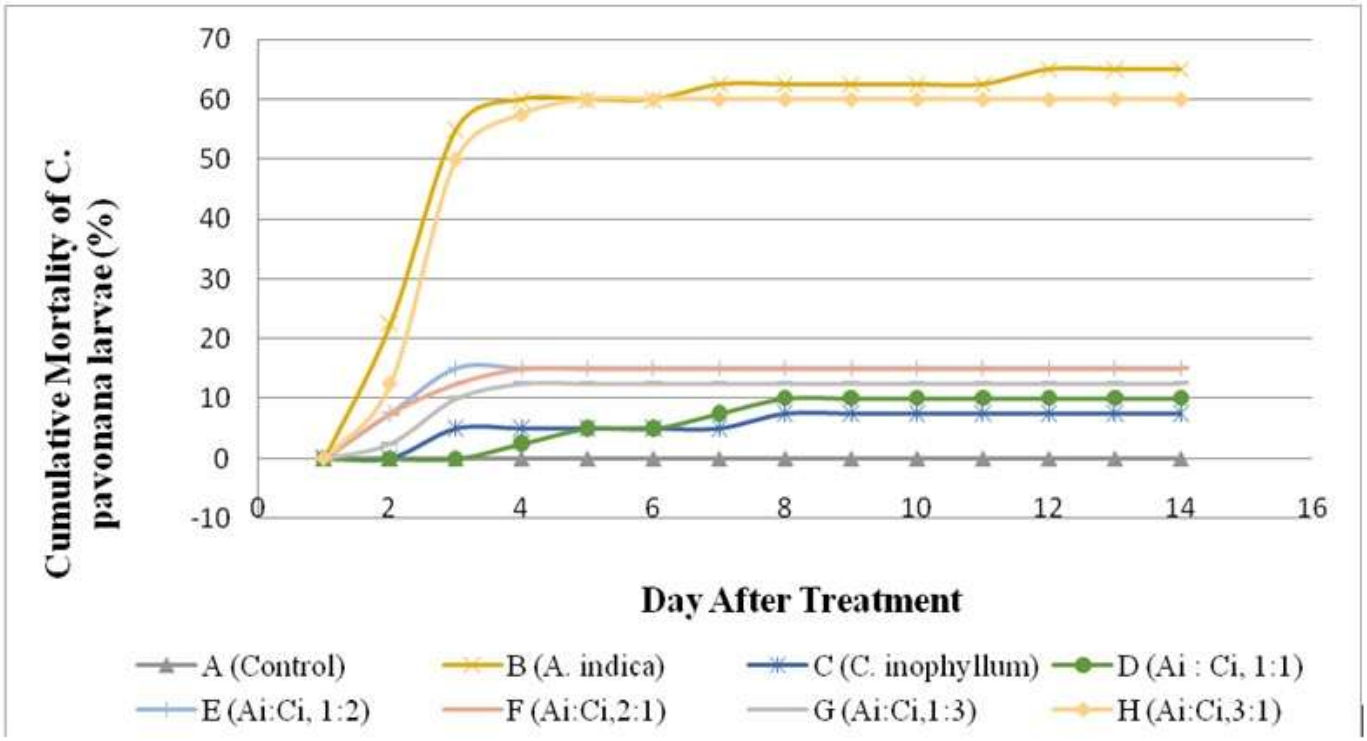

Figure 1: Mortality of *C. pavonana* larvae Instar II- IV at concentrations of 0.05%

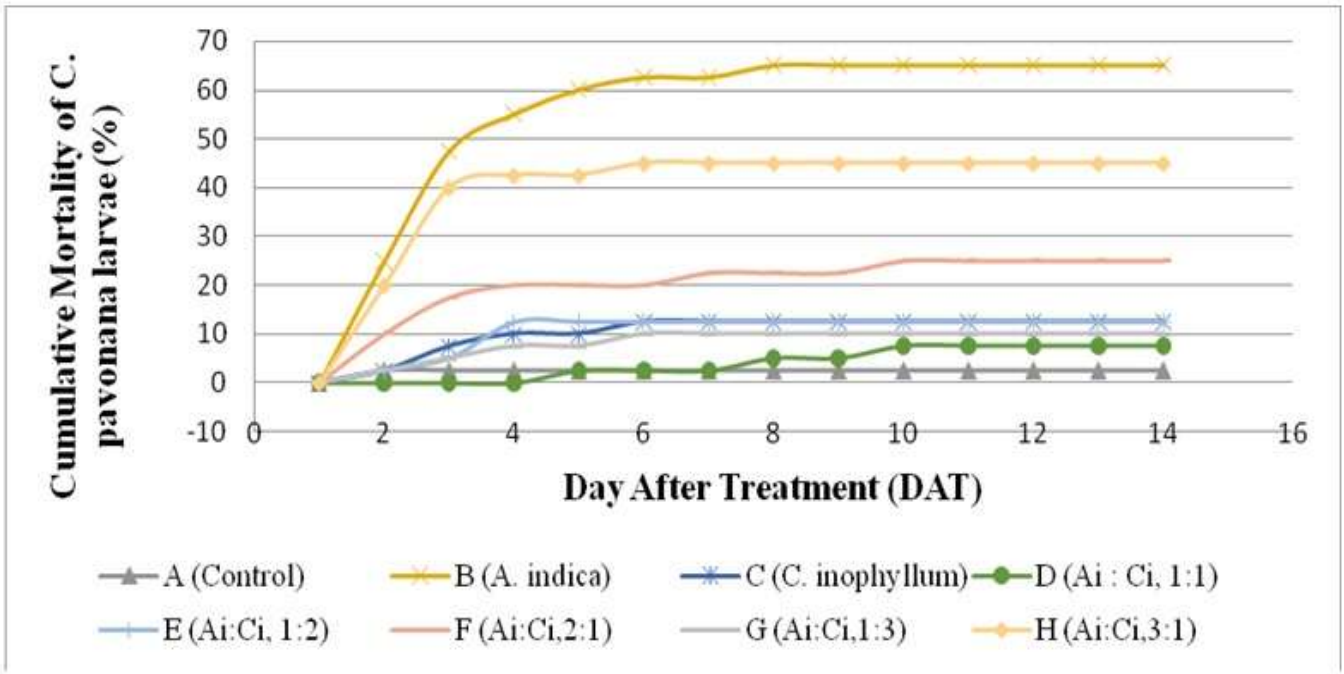

Figure 2: Mortality of *C. pavonana* larvae Instar II- IVat concentrations of 0.1%

Table 1
Mortality of *C. pavonana* larvae (%)

Treatment	Average larval mortality at test concentrations (x± SD) (%)					
	0,05%			0,1%		
	Day 3th	Day 8th	Day 14th	Day 3th	Day 8th	Day 14th
A (Control)	0.0 ± 0.00 a	0.0 ± 0.00 a	0.0±0.00 a	2,5± 5.00 a	2.5± 5.00 a	2.5± 5.00 a
B (*A. indica*)	55.0±34.16 b	62.5± 29.86 b	65.0 ± 33.16 b	47.5 ± 28.72 c	65.0 ± 35.12 c	65.0± 35.12 c
C (*C. inophyllum*)	5.0±5.77 a	7.5± 5.00 a	7.5± 5.00a	7.5± 9.57 a	12.5± 9.57 a	12.5±9.57 a
D (Ai : Ci, 1:1)	0.0 ±0.00 a	10.0± 8.16a	10.0± 8.16 a	0.0±0.00 a	5.0 ± 5.77 a	7.5± 5.00 a
E (Ai:Ci, 1:2)	15.0 ± 23.80 a	15.0 ± 23.80 a	15.0 ± 23.80 a	5.0 ± 10.00 a	12.5 ± 18.93 a	12.5± 18.93 a
F (Ai:Ci,2:1)	12.5 ± 12.58 a	15.0 ± 10.00 a	15.0 ± 10.00 a	17.5± 22.17 ab	22.5± 18.93 ab	25.0±17.32 ab
G (Ai:Ci,1:3)	10.0 ± 20.00 a	12.,5± 25.00 a	12.5 ± 25.00 a	5.0 ± 10.00 a	10.0± 8.16 a	10.0±8.16 a
H (Ai:Ci,3:1)	50.0 ± 35.59 b	60.0 ± 40.82 b	60.0 ± 40.82 b	40.0± 35.59 bc	45.0± 33.17 bc	45.0±33.16 bc

Explanation: The average number followed by the same letter in the same column is not significantly different according to Duncan Test at a 5% level.
x: Average mortality of larvae (%)
SD: Standar deviation

Table 2
Length of development time of *C. pavonana* larvae

Treatment	Lenght of larval development at test concentrations (x ± SD) (Day)							
	0,05%				0,1%			
	N	Instar II-III	N	Instar II-IV	N	Instar II-III	N	Instar II-IV
A (Control)	40	2.70 ± 0.939	40	5.98 ± 0.861	39	2.56 ± 0.502	39	6.03 ± 1.063
B (*A. indica*)	15	5.53 ± 2.850	14	9.64 ± 2.707	15	7.40 ± 1.724	14	11.86 ± 0.662
C (*C. inophyllum*)	38	3.68 ± 0.739	37	6.57 ± 0.648	36	2.92 ± 0.554	35	6.11 ± 0.529
D (Ai : Ci, 1:1)	38	3.03 ± 1.103	36	6.31 ± 1.214	40	3.00 ± 0.751	37	6.73 ± 1.835
E (Ai:Ci, 1:2)	34	2.79 ± 0.729	34	6.03 ± 0.869	35	2.97 ± 0.923	35	6.46 ± 1.197
F (Ai:Ci,2:1)	35	2.97 ± 0.618	34	6.,12 ± 1.149	32	3.69 ± 0.821	30	8.60 ± 1.792
G (Ai:Ci,1:3)	35	2.86 ± 0.974	35	6.00 ± 1.029	37	2.95 ± 0.911	36	6.47 ± 1.698
H (Ai:Ci,3:1)	16	4.06 ± 1.652	16	6.69 ± 1.579	22	4.23 ± 2.439	22	9.00 ± 2.309

Explanation:The average number followed by the same letter in the same column is not significantly different according to Duncan Testat a 5% level.
x : Long larval development (Day); SD : Standard deviation; N: The number of alive larvae

Table 3
Leaf area consumed by *C. pavonana* larvae

Treatment	The leaf area consumed by larvae at test concentration (x ± SD) (%)	
	0.05%	0.1%
A (Control)	8.94 ± 4.130 b	9.40 ± 2.183 d
B (*A. indica*)	1.37 ± 1.428 a	0.13 ± 0.000 a
C (*C. inophyllum*)	6.73 ± 2.931 b	7.17 ± 1.794 d
D (Ai : Ci, 1:1)	8.30 ± 3.503 b	9.06 ± 2.399 d
E (Ai:Ci, 1:2)	7.29 ± 3.560 b	5.77 ± 0.507 bc
F (Ai:Ci,2:1)	5.41 ± 4.124 ab	2.17 ± 1.955 abc
G (Ai:Ci,1:3)	8.19 ± 4.254 b	6.38 ± 5.005 cd
H (Ai:Ci,3:1)	1.87 ± 2.439 a	1.36 ± 1.784 ab

Explanation: The average number followed by the same letter in the same column is not significantly different according to Duncan Test at a 5% level.
x : average of the leaf area consumed (%); SD: Standar deviasi; Ai : *A. indica;* Ci : *C. inophyllum*

Table 4
Average dry weight of *C. Pavonana* instar IV larvae

Treatment	The dry weight of the larvae at the test concentration (x ± SD) (g)			
	0.05%	**N**	**0.1%**	**N**
A (Control)	0.0454 ± 0.00120 c	40	0.0439 ± 000158 b	39
B (*A. indica*)	0.0172 ± 0.00122 a	14	0.0173 ± 0.00227 a	14
C (*C. inophyllum*)	0.0425 ± 0.00151 c	37	0.0414 ± 0.00134 b	35
D (Ai : Ci, 1:1)	0.0378 ± 0.00105 c	36	0.0357 ± 0.00158 b	37
E (Ai:Ci, 1:2)	0.0391 ± 0.00153 c	34	0.0299 ± 0.00096 b	35
F (Ai:Ci,2:1)	0.0350 ± 0.00147 bc	34	0.0312 ± 0.00166 b	30
G (Ai:Ci,1:3)	0.0347±0.0095 bc	35	0.0361 ± 0.00158 b	36
H (Ai:Ci,3:1)	0.0237 ± 0.00192 ab	16	0.0277 ± 0.00162 ab	22

Explanation: The average number followed by the same letter in the same column is not significantly different according to Duncan Test at a 5% level.
x : average of the dry weight of the larvae (%); SD: Standar deviasi; Ai : *A. indica;* Ci: *C. inophyllum*

Conclusion

The treatment of *A. indica*seed oil resulted in higher mortality level than *C. inophyllum* seed oil against *C. pavonana* larvae. On the other hand, the treatment of *A. indica*and *C. inophyllum* seed oil mixture (3:1) had higher mortality effect against *C. pavonana* larvae than that of other composition of mixtures. The effect of each treatment was not only on larval mortality, but also on inhibition of feeding activity, length of larval development time and decrease of larvae weight. The effects of neem oil were caused by the activity of its bioactive compound i.e. azadirachtin which has hormonal disruption and antifeedant effects.

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