

EFFECTS OF *MELIA AZEDARACH* METHANOL EXTRACT AND NUCLEOPOLYHEDROVIRUS (*SLNPV*) ON *SPODOPTERA LITTORALIS* (BOISD.)

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Abstract

Insecticidal activity of *Melia azedarach* methanol extract and nucleopolyhedrovirus (*SLNPV*) against *Spodoptera littoralis* larvae was evaluated under laboratory conditions. NPVs showed high significant toxicity on the 2nd and 4th instars larvae; while, the *Melia* extract was a relatively high toxic against the 2nd instar larvae and less effective against the 4th instar. The extract recorded strong reduction in larval weight as compared to the control. Generally, *Melia* extract potentiates the effect of *SLNPV* by producing mortality within a short time and negatively influences the larval growth and development which could result lesser feeding damage to the host plant.

Key words: *Melia azedarach*, Nucleopolyhedrovirus, Insecticidal Activity, *Spodoptera littoralis*.

INTRODUCTION

The Egyptian cotton leafworm, *Spodoptera littoralis*, is recorded in Africa, southern Europe, and the Middle East, where it is a particularly destructive pest of cotton and a wide range of economically important vegetables and ornamentals (Ellis, 2004).

Nucleopolyhedroviruses (NPVs), members of the family Baculoviridae, have received considerable attention as potential microbial insecticides, and some of the NPVs have been successfully used for the control of insect pests in agriculture (Moscardi, 1999). NPVs are safe and show selective biological control agents because of the little negative impact on non-target organisms and the environment.

The slow speed of action against target insects represents a serious disadvantage of NPVs as efficient insecticides, allowing the pests to infest the crops for considerable periods of time. Hence, knowledge of the effectiveness when combined with other insecticide especially plant-based insecticide is very much needed.

One member of the Meliaceae, known as Chinaberry or Persian lilac tree (*Melia azedarach*) is a deciduous tree that is native to North Western India and has long recognized for its insecticidal properties. The effectiveness of extracts from fruits and

leaves of *Melia azedarach* L. has been previously demonstrated against insects (Sharma *et al.*, 2012; Abou-Fakhr Hammad *et al.*, 2013; Farag *et al.*, 2011).

The Purpose of the present work was to study the insecticidal activity of *M. azedarach* methanol extract and NPVs against *S. littoralis*. The effectiveness of NPVs by binary mixture with strong antifeeding *Melia azedarach* methanol extract has been evaluated.

MATERIALS AND METHODS

Strain of cotton leafworm *S. littoralis*

The *S. littoralis* strain was obtained from the Faculty of Agriculture, Cairo University, Egypt, and was reared in the laboratory at the Pest Physiology Department, Plant Protection Research Institute, Agricultural Research Center, Giza, Egypt, as described by **El-Defrawi *et al.*, (1964)**, under constant laboratory conditions at $(25 \pm 1) ^\circ\text{C}$, (70 ± 5) % relative humidity and a photoperiod of 16: 8 h (L:D). Adults were fed with a 15% solution of honey. Filter paper was provided as an oviposition site, and it was renewed periodically.

Method of insect diet preparation

The following diet ingredients were added to 1Kg of kidney beans soaked overnight.

Bean	1000g
Yeast extract	120g
Ascorbic acid	10g
Sorbic acid	5g
Methyl hydroxyl benzoate	10ml
Agar	80g
Sterile water	900ml

The diet ingredients, except the agar and ascorbic acid, were blended in half volume of water. The agar was dissolved separately in the remaining water at 100°C . The agar solution was mixed with other ingredients and after cooling below 70°C , the ascorbic acid was added.

Preparation of NPVs

Virus propagation

The 3rd instar larvae of *S. littorals* were inoculated with *SINPV* isolate provided Insect Pathogen Unit, Plant Protection Research Institute, Agriculture Research Center by surface contamination of the artificial diet. The inoculated larvae were observed daily to identify the NPVs infected ones based on the sign and symptoms of disease.

Virus occlusion bodies purification

The method of OBs purification was done as (Sudhakar *et al.*, 1997) with some modification. The number of OBs was counted by using Neubaur Hemocytometer to determine the concentration of OBs/ml. Five concentrations were prepared from the viral OBs mother suspension by serial dilution to be used in bioassay.

Plant material

The ripe fruits of *M. azedarach* were collected from El-Menoufia, Egypt, in November 2012.

Preparation of crude methanol extract

The ripe fruits of *M. azedarach* were crushed to fine particles and shade-dried at room temperature. In a 500-ml flask, 70g of crushed and dried fruits in 200 ml of methanol were stirred for 3 h. After leaving the methanol solution overnight, it was filtered through Whatman No. 40 filter paper. The solid filtration residue was extracted again following an identical procedure, and the two filtrates were mixed. The solvent was removed using a rotary evaporator, and a dark red residue was obtained (14.3g/70g plant). Series of concentrations of *M. azedarach* extract were carried out (1.25, 2.50, 5.00, 10.00, and 20.00g extract/100g diet).

Bioassays

Insecticidal activity of virus

Laboratory bioassay tests were conducted using diet surface treatment procedure (Addy, 1969). The *SINPVs* was tested against the 2nd and 4th instars larvae. Bioassay was performed using five concentrations of the isolated virus (10^9 , 10^8 , 10^7 , 10^6 , and 10^5). The tested larvae were starved for 4 h prior to feeding viruses. Bioassays were carried out in plastic cups containing semi-synthetic diet described above. 250 μ l of the viral suspensions were dispersed on the diet using micropipette. Forty larvae were used for each concentration of virus and control. Larvae of the 2nd or 4th instar were allowed to feed on treated diet for 48 h, then, larvae were fed on untreated diet for 5 days. The control larvae were fed on diet treated with distilled water. All treatments were kept at $25 \pm 2^\circ\text{C}$. Mortality was recorded after 3, 5, and 7 days post infection.

Insecticidal activity of methanol extract

Laboratory bioassay tests were conducted using diet surface treatment procedure (Addy, 1969). The methanol extract was tested against 2nd or 4th instars larvae. Bioassay was performed using five concentrations of the extract (20, 10, 5, 2.5, 1.25 g extract/100g diet). Tested larvae were starved for 4 h prior to feeding extract. Bioassays were carried out in plastic cups containing semi-synthetic 5 gm diet. Forty larvae were used for each concentration of extract and control. Larvae of 2nd or 4th instar were allowed to feed on treated diet for 48 h, then, larvae were fed on untreated diet for 5 days. The control larvae were fed on pure diet. All treatments were kept at 25 ± 2°C. Mortality was recorded after 3, 5, and 7 days post treatment and larval weight was recorded on the 7th day post treatment.

Combination treatment

LC₅₀ values recorded after 7 days post treatment were used in combination treatment against the 2nd and 4th instars larvae.

Statistical analysis

The significant differences were calculated by ANOVA and Duncan's multiple range tests (ANOVA of arcsine square root transformed percentages). Differences between the treatments were determined by Tukey's multiple range test ($P < 0.05$) (Snedecor and Cochran, 1989). The LC₅₀ values were determined by using Finney (1952) and corrected according to Abbott's formula (Abbott, 1925).

RESULTS AND DISCUSSION

As shown in Table 1 the percentages of larval weight reduction of the *Melia* extract against the 2nd and 4th instars *S. littoralis* larvae increased gradually with increasing concentrations. Generally the highest decrease in larval body weight was recorded at a concentration of 20g extract/100g diet with the 4th instar larvae. Larval weight reduction percentages were 35.66, 38.69, 40.57, 42.63, 47.36% and 48.12, 56.93, 58.42, 60.23, 64.71% for the 2nd and 4th instars, at 1.25, 2.50, 5.00, 10.00, 20.00 g/100 g diet, respectively.

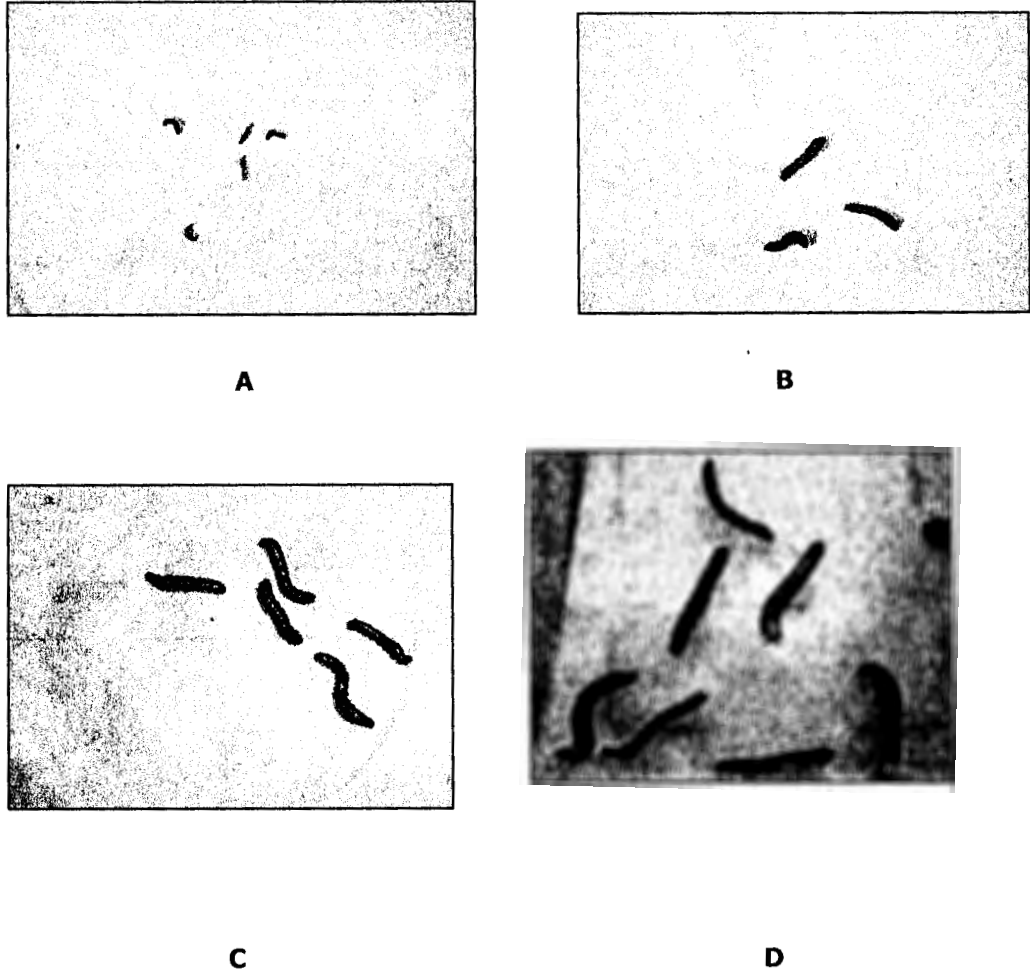
Table 1. Effects of methanol extract of *M. azedarach* on mean larval weight and weight reduction of the 2nd and 4th instars larvae of *S. littoralis*.

Treatment	Mean larval weight (mg) and weight reduction (%)			
	2 nd instar		4 th instar	
	M. W. [mg]	W. R. (%)	M. W. [mg]	W. R. (%)
Control	11.19	0.00	69	0.00
1.25 [g/100g diet]	7.20	35.66 ^e	35.80	48.12 ^e
2.50 [g/100g diet]	6.86	38.69 ^d	29.72	56.93 ^d
5.00 [g/100g diet]	6.65	40.57 ^c	28.69	58.42 ^c
10.00 [g/100g diet]	6.42	42.63 ^b	27.44	60.23 ^b
20.00 [g/100g diet]	5.89	47.36 ^a	24.35	64.71 ^a
<i>F</i>	449.54***		12693.23***	
LSD	0.65		0.17	

Values in the same column are all significantly different from each other (ANOVA, Duncan's multiple range test, $P < 0.05$).

Weight reduction% = [(M.W. Control - M. W. Treatment) / M. W. Control] x 100.

As shown in Fig 1, *M. azedarach* methanol extract represents antifeeding and insecticidal activity with a remarkable retardation in larval development and significant differences in weight between the treated larvae (Fig 1- A and C) and untreated ones (Fig 1- B and D).



A and C: - treated 2nd and 4th instar larvae of *S. littoralis*
 B and D: - untreated 2nd and 4th instar larvae of *S. littoralis*

Fig 1. Feeding on *Melia azedarach* methanol extract caused a strong inhibition of larval growth in *S. littoralis* (A and C) compared to control (B and D). The photos were taken after 7 days post treatment.

Larvae of *Spodoptera frugiperda* allowed to feed until pupation on an artificial diet to which a methanolic extract of *M. azedarach* had been added, food intake was low, growth retarded, and larval development prolonged (Breuer and Schmidt, 1990). The antifeedant and growth inhibitory activities of *M. azedarach* methanol extract to *Helicoverpa armigera* were investigated by Nathala and Dhingra (2005).

Data represented in Table 2 revealed that methanol extract showed significant toxic effect on *S. littoralis* larvae. The extract was slightly less effective against the 4th instar larvae than the 2nd instar at all concentrations. The extract recorded 47.5%

mortality against the 4th instar of *S. littoralis* at the highest concentration, while the extract exhibited high significant toxic effect against the 2nd instar; the highest concentration of 20g extract/100g diet represents 70% mortality.

The insecticidal activity of *M. azedarach* seeds was previously reported by Chiu (1987) who found that a 1% methanol extract of seed kernels of *M. azedarach* caused 80% inhibition of feeding in 1st and 2nd instar larvae of the noctuid *Mythimna separate*. Methanol extract of *M. azedarach* (12.5 and 25%) caused 100% mortality of *Aphis fabae* within 96 h (Dimetry and Schmidt, 1991). Methanol extract of *M. azedarach* fruits had some antifeedant activity against larvae of *S. littoralis* and *Agrotis ipsilon*. The percentage of mortality increased with application of higher concentrations of *Melia* extract in both species (Schmidt *et al.*, 1997).

Table 2. Toxic effect of methanol extract of *M. azedarach* and LC₅₀ against 2nd and 4th instars larvae of *S. littoralis* after 3, 5, and 7 days post treatment.

Treatment	mortality (%) of the 2 nd instar			mortality (%) of the 4 th instar		
	3 days	5 days	7 days	3 days	5 days	7 days
Control	0.00	0.00	0.00	0.00	0.00	0.00
1.25 [g/100g diet]	7.50 ^e	12.50 ^d	17.50 ^e	0.00 ^d	0.00 ^e	10.00 ^e
2.50 [g/100g diet]	22.50 ^d	32.50 ^c	40.00 ^d	0.00 ^d	7.5 ^d	15.00 ^d
5.00 [g/100g diet]	25.00 ^c	37.50 ^b	45.00 ^c	10 ^c	17.5 ^c	30.00 ^c
10.00 [g/100g diet]	30.00 ^b	37.50 ^b	62.50 ^b	15 ^b	25 ^b	37.50 ^b
20.00 [g/100g diet]	35.00 ^a	42.50 ^a	70.00 ^a	25 ^a	32.5 ^a	47.50 ^a
LC ₅₀	56.22	28.44	5.94	45.4	36.18	20.82
<i>F</i>	240.27	315.37	5037.20	6750	20475	2647.50
LSD	2.11	2.08	0.91	0.41	0.90	0.91

Values in the same column are all significantly different from each other (ANOVA, Duncan's multiple range test, $P < 0.05$).

Data presented in Table 3 revealed that the mortality percentages of the two tested instars larvae treated with *SINPVs* were increased with increasing the concentrations and time elapsed post treatment. The highest concentration of the *NPVs* (10⁹ OBs/ml) showed 85 and 75% mortality against the 2nd and 4th instars, respectively after 7 days. The 4th instar larvae showed to be less susceptible to the virus than the 2nd instar.

Table 3. Toxic effect of virus and LC₅₀ against the 2nd and 4th instars larvae of *S. littoralis* after 3, 5, and 7 days post treatment.

Treatment	mortality (%) of the 2 nd instar			mortality (%) of the 4 th instar		
	3 days	5 days	7 days	3 days	5 days	7 days
Control	0.00	0.00	0.00	0.00	0.00	0.00
10 ⁵ [OBs/ml]	37.5 ^c	50 ^e	65 ^d	32.5 ^b	47.5 ^d	50 ^e
10 ⁶ [OBs/ml]	42.5 ^b	57.5 ^d	75 ^c	32.5 ^b	47.5 ^d	57.5 ^d
10 ⁷ [OBs/ml]	45 ^b	62.5 ^c	75 ^c	35 ^b	50 ^c	62.5 ^c
10 ⁸ [OBs/ml]	45 ^b	67.5 ^b	80 ^b	35 ^b	55 ^b	65 ^b
10 ⁹ [OBs/ml]	50 ^a	70 ^a	85 ^a	40 ^a	60 ^a	75 ^a
LC ₅₀	1.47x10 ⁹	5.57x10 ⁴	124.96	1.5x10 ¹⁵	2.41x10 ⁶	9.15x10 ⁴
<i>F</i>	16.5	154.5	26.40	11.25	70.5	205.5
LSD	3.52	2.03	4.55	2.88	2.03	2.03

Values in the same column with the same letter are not significantly different from each other (ANOVA, Duncan's multiple range tests, $P < 0.05$).

The symptoms of viral infection can be classified into three stages:-

Initial stage of infection Fig (2-A):- In the initial stage of infection there was changing in the activity of infected larvae comparing to healthy one in which infected larvae show reduced motor function, their feeding begins to slow and virtually ceases, milky color seen on the cuticle which appear pale and thin.

Intermediate stage of infection Fig (2-B):- In this stage the invasion of viral occlusion bodies spread the infection throughout the whole host and damage all host cells and the larval body begins to appear swollen due to the complete destruction of all internal tissues organs.

Final stage of infection Fig (2-C):- in the final stage the host becomes packed with viral occlusion bodies and fragile epidermis ruptures releasing millions of viral occlusion bodies back into the environment to spread the infection to another host.

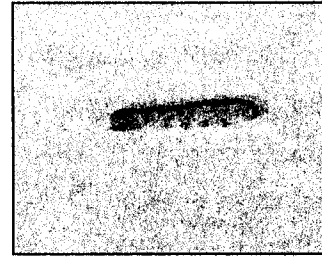


Fig (2-A)

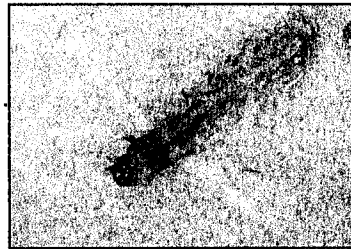


Fig (2-B)

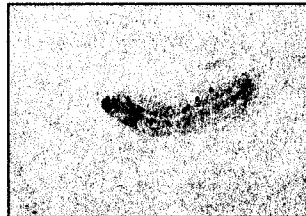


Fig (2-C)

Fig (2-A):- Slow motion larvae and cuticle showing milky color

Fig (2-B):- Swollen of larval body due to invasion of viral occlusion bodies

Fig (2-C):- liquefied larval body

The results of the present study showed that, high concentrations of *NPVs* caused a high mortality rate; this conclusion is parallel to that found by Duan and Otvos (2001) who reported that mortality was higher when younger larvae of *Choristonura fumiferana* were used.

In this study bioassays carried out with the two larval instar tested revealed that the *S. littoralis* larvae showed symptoms during the first three days post-inoculation, these observations agree with Federici (1997) who confirmed that in typical *NPV* infections, such as the disease caused by the *NPV* of *Autographa californica* (Speyer.) (Lepidoptera: Noctuidae) (*AcMNPV*), *Trichoplusia ni* (Hubnar.) (Lepidoptera: Noctuidae) (*TnMNPV*) and *Helicoverpa zea* (Boddie.) (Lepidoptera: Noctuidae) (*HzMNPV*) there are few signs of disease during the first 3 days of infection.

On or near the 4th day, infected larvae begin to respond much more slowly than healthy larvae to stimuli such as touching Federici (1997). In our study, it was also noted that when healthy larvae were dropped from a low height, they collected themselves on their legs, but the infected larvae gave no such response and instead lay on their dorsal side. This situation can be related to the infection of the central nervous system and muscle cells. Thus, when a larva started to lose control of the central nervous system, it was not possible to respond to surroundings. By the 4th day of post-inoculation, infected larval cuticle showed pale and milky color and thinning. This symptom can be associated with the beginning of infection of hypodermal cells. The most typical symptoms are noted in larval stages where either whitening or yellowing of the gut and /or the remainder of the body organs is associated with infection and replication by Evans and Shapiro (1997).

Our results showed that the larvae became less active and generally lost its appetite, though some were known to continue to feed up to a few days before death. Thinner appearance of the cuticle can be associated with the activities of cathepsin and chitinase genes in the baculovirus genome. All these observations are in accordance to results reported by Tanada and Kaya (1993).

Following the 4th day, infected larvae showed swollen bodies. This could be due to the infection of nuclei and the hypertrophy that had occurred in the cell. Tanada and Kaya (1993) approved that the nucleus increased in size due to the baculovirus infection. In *NPV* infection, at day 4 or 5, the larvae will begin to appear swollen and the cuticle can appear flaccid (Federici, 1997). In the final stage of infection, OBs are formed and the nuclei are packed with OBs which causes the cellular hypertrophy and swollen appearance of the infected larvae Oecd (2002). Thus, the hypertrophy in the nucleus is an important sign of *NPV* infection and causes insect swollen.

In the baculovirus biological cycle, release of the polyhedra to the environment is the most important step. Release of the polyhedra to environment is only possible with the liquefaction of the host. The swollen of the cuticle is the beginning of the process that will bring the host to liquefaction. Both swollen of the cuticle and liquefaction are associated with the activities of cathepsin, proteinase and chitinase

genes of baculoviruses. These baculovirus genes have the principle role of damaging whole larval body.

The combination between the NPVs and the extract increase the effectiveness of both of them against *S. littoralis*. As shown in Table 4 the combination between the virus and the extract at the LC₅₀ concentrations gave 95% and 87.5% mortality for the 2nd and 4th instar, respectively after 7 days.

Table 4. The effects of combination between the virus and extract at the LC₅₀ concentrations against the 2nd and 4th instars larvae.

Combination treatment	2 nd instar			4 th instar		
	3 days	5 days	7 days	3 days	5 days	7 days
Mortality%	65	80	95	50	70	87.5
Reduction in wt %	-	-	95	-	-	52.05

The results of the present study showed that NPVs and methanol extract of *M. azedarach* combination can provide control of *S. littoralis* larval populations, which is comparable with, superior to, that provided by a botanical insecticide or virus alone. In addition, there were greater mean larval weight loses in larvae fed on combined treatment of extract and NPV as compared to control larvae. The present finding also showed reduced development rate in treated larvae and this similar to Nathan and Kalaivani, (2005) using azadirachtin (AZA) and *Spodoptera* nucleopolyhedrovirus (SplNPV). It may be concluded from this study that extended larval period is coupled with lower developmental rate with increased mortality. A similar result was reported by Shapiro *et al.*, (1994) using aqueous neem extract in combination with (*Lymantria dispar* L.) gypsy moth NPV.

CONCLUSION

Melia extract and *SINPV* combination can provide control of *S. littoralis* larval populations, which is comparable with, superior to, that provided by a botanical insecticide or virus alone. The greatest advantage of *SINPV* and methanol extract of *M. azedarach* combination is the potential decrease in the pathogen dosage required to kill larvae, the faster kill and negative effects on growth and development which could result lesser feeding damage to the host plant.

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تأثير الخلط بين مستخلص الميثانول للزئذخت والفيروس النووي لدودة ورق القطن علي دودة ورق القطن

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أجريت الدراسة في هذا البحث بغرض اختبار سمية مستخلص الميثانول لنبات الزئذخت والفيروس النووي لدودة ورق القطن ضد حشرة دودة ورق القطن العمر اليرقي الثاني والرابع في المعمل. أظهر الفيروس المختبر تأثير معنوي عالي السمية بالنسبة للعمرين الثاني والرابع، بينما كان للمستخلص تأثير عالي السمية بالنسبة للعمر اليرقي الثاني وأقل سمية ضد العمر الرابع. وقد وجد ان المستخلص يؤدي الى خفض وزن اليرقات بشكل معنوي مقارنة باليرقات غير المعاملة. وقد أدى الخلط بين مستخلص الزئذخت والفيروس الى تحسين خواص الفيروس في المكافحة من حيث قدرته على قتل اليرقة اسرع و ايضا تقليل وزن و نمو اليرقات مما يقلل من التلف الممكن حدوثه نتيجة التغذية.