Effect of *Melia azedarach* Fruit Extract on Juvenile Hormone Titer and Protein Content in the Hemolymph of Two Species of Noctuid Lepidopteran Larvae [Insecta: Lepidoptera: Noctuidae]

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The effect of an enriched methanolic extract of *Melia azedarach* L. (Meliaceae) fruits on the size of the corpora allata (CA), the juvenile hormone (JH) titer and the protein content in the hemolymph of two lepidopteran pests in Egypt, *Spodoptera littoralis* (Boisd.) and *Agrotis ipsilon* (Hufn.), was studied. Different concentrations of the extract were incorporated into an artificial diet on which the larvae were allowed to feed. In *S. littoralis*, a significant reduction in the CA volume of larvae treated at the 1000 ppm extract level *vs* that of control larvae was observed. In *A. ipsilon*, a reduction was found in the right CA gland only. Larvae of both species that had fed on a diet with *Melia* extract had a higher mean JH-II titer in the hemolymph than did control larvae. In both species, the content of hemolymph protein was decreased significantly after feeding for 6 days on a diet treated with concentrations above 50 ppm extract, followed by 6 days on a normal diet. The results show that a *M. azedarach* fruit extract has an effect on the neuroendocrine control in the insects. The effect on the hemolymph protein levels is discussed in connection with changes in the morphology/physiology of the gut.
KEY WORDS: *Melia azedarach* methanol extract; *Agrotis ipsilon*; *Spodoptera littoralis*; Lepidoptera; juvenile hormone titer; corpora allata; larval development; hemolymph protein.

INTRODUCTION

The fruits of *Melia azedarach* L. (Persian lilac or chinaberry tree) (Meliaceae) contain compounds inducing insecticidal effects in various insects. The subject has been reviewed by Ascher *et al.* (1). The most potent compounds are tetranortriterpenoids, *e.g.* 1-cinnamoyl melianone and 1-cinnamoyl-3,11-dihydroxymeliacarpin (9). These compounds are insect growth regulators (IGRs), especially for lepidopteran pests. The treated larvae exhibited retarded growth and larval development was prolonged accordingly. High mortality was found in larval and pupal stages, generally due to molt disruption. Application of *M. azedarach* extract affected also the behavior and vitality of larvae and adults of *Spodoptera frugiperda* (J.E. Smith) (4,5,6). Extracts sprayed on twigs of *Pinus*

Received Feb. 2, 1998; received in final form Sept. 7, 1998; http://www.phytoparasitica.org posting Sept. 18, 1998.
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nigra strongly reduced growth of larval instars of *Thaumetopoea pityocampa* (Den. & Schiff.), which failed to cluster in a group and spin a silky nest; 1% extract was enough to cause 100% mortality (3).

Schmidt *et al.* (15) prepared a methanol extract of *M. azedarach* fruits from Greece enriched by washing with petrol ether and ethyl acetate and fed a diet containing 10–1000 ppm of this extract to *Spodoptera littoralis* (Boisd.) and *Agrotis ipsilon* (Hufn.) larvae. Starting the experiments from 3rd instars, the larvae of both species treated with 25 ppm and higher extract concentrations lost weight until pupation and the larval period was prolonged. Pupal weight was reduced significantly; with 100 ppm the larvae failed to pupate altogether. When larvae were treated with lower amounts of the extract, all reproduction parameters of the emerged adults studied were affected; *S. littoralis* was more sensitive than *A. ipsilon*.

These observations, especially the nature of the molting defects, suggest effects on the neuroendocrine system of the insects (5,6). This was pointed out by Rembold (12) for azadirachtin, the most active compound of *Azadirachta indica* Juss., but there are no data available yet for *M. azedarach* constituents. In insects, the most important hormones involved in the molting process are ecdysone and juvenile hormone (JH). This paper reports first results on the influence of *Melia* extract on the JH titer in larvae of *S. littoralis* and *A. ipsilon*. Because it is known that JH, produced by the corpora allata (CA), may also influence the proteins in the hemolymph and their uptake by the developing oocytes, also hemolymph protein was measured.

MATERIALS AND METHODS

Test insects

Spodoptera littoralis and *Agrotis ipsilon* were used as the test insects. They were reared on an artificial diet under constant laboratory conditions at 25±3°C, 40–50% r.h., and normal daylight; for further details, see Schmidt *et al.* (15).

Preparing the Melia *extract*

Unripe fruits of Greek origin were used for preparing the *M. azedarach* extract. The whole frozen fruits were ground with 80% methanol in a blender (50 g fruits/100 ml solvent), stirred for 1 h and filtered under reduced pressure. The residue was extracted once again with fresh methanol. The extracts were combined, filtered, concentrated *in vacuo* and lyophilized for 24 h. The dry crude extract was redissolved in 80% methanol and washed with the same volume of petroleum ether (b.p. 30–50°C) by stirring for half-an-hour.

After repetition of the procedure, the separated methanol extract was dried using a rotary evaporator and freeze dryer and then partitioned between the solvents water and ethyl acetate (1:1). The water layer was washed once again with fresh ethyl acetate. The ethyl acetate extract was concentrated in a rotary evaporator and redissolved in 80% methanol, giving a final concentration of 10% (w/v). By this procedure, a defatted extract with enriched insecticidal compounds was obtained (2). For the experiments, the extract was mixed with an artificial diet, prepared as described by Khalifa *et al.* (8). For the control, pure solvent (80% methanol) was added to the diet.

Quantification of juvenile hormone

The method of Rembold and Lackner (13) for extraction of JH from hemolymph and GC-MS analysis of the 10-dimethyl(nonafluorohexyl)silyloxy-11-methoxy-JH derivatives was employed. The limit of detectability of JH-II (no JH-III was present in the samples) according of this method is 0.05 pmol per injected sample. Each 1-ml methanol sample contained 5 pmol of ethyl JH-I-oate and 5 pmol of ethyl JH-III-oate as internal standards, and 5 μl of the hemolymph sample. Controls followed the same protocol, but without hemolymph. All the analyses were run at MPI for Biochemistry, Martinsried/Germany.

Fourth instar larvae of both species were kept on diets containing *Melia* extract at concentrations of 100 and 1000 ppm. The experiments were done in four replicates. Some larvae were fed on the control diet.

Three days after treatment, 5 μl of the hemolymph was taken from each larva of the treated and control groups using micropipets after cutting off the abdominal prolegs. The hemolymph was collected in a small vial containing 1 ml methanol and the internal standard.

Size of corpora allata

After the hemolymph had been taken from treated and control larvae, the head capsule was dissected in Ringer solution. The tissues around the CA were removed and the glands on both sides were contrasted with methylene blue. The size of both glands in each larva was measured using an ocular micrometer, and the volume was calculated as a rotation-ellipsoid by the formula ($r_1 > r_2$):

$$V(mm^3) = 4/3\pi \times \left[\frac{(r_1 + r_2)}{2}\right]^3$$

For measuring the size of CA, this formula is most suitable (16,17).

Protein content in the hemolymph

Quantitative protein determination was performed using the method of Lowry *et al.* (10). Third instar larvae of both insect species were fed on a diet containing 50 and 100 ppm *Melia* extract. These experiments and a control were carried out in five replicates. After a period of 3 and 6 days, respectively, 5 μl hemolymph was taken from each larva. Some individuals were reared for 6 days on treated diet and an additional 6 days on untreated diet, before hemolymph was collected. For protein determination, 2 ml solution A (0.2 ml 5% $CuSO_4$ + 0.8 ml 2.5% $C_4H_4KNaO_6$ (potassium sodium tartrate) dissolved in H_2O and made up to 50 ml by 4% Na_2CO_3 in 0.2 N NaOH) was added to the hemolymph, which was used as a replicate in a test tube and shaken for a few minutes. After incubation for 10 min, 0.2 ml Folin reagent was added to each tube and agitated for a further 20 min. The color density was measured in a photometer at 750 nm (Double Beam Spectrophotometer UV-200, Shimadzu). The amount of hemolymph protein was calculated using a calibration curve, prepared with bovine serum albumin (10–100 μg/ml).

Statistical treatment

For statistical analysis, Student's t-test was applied.

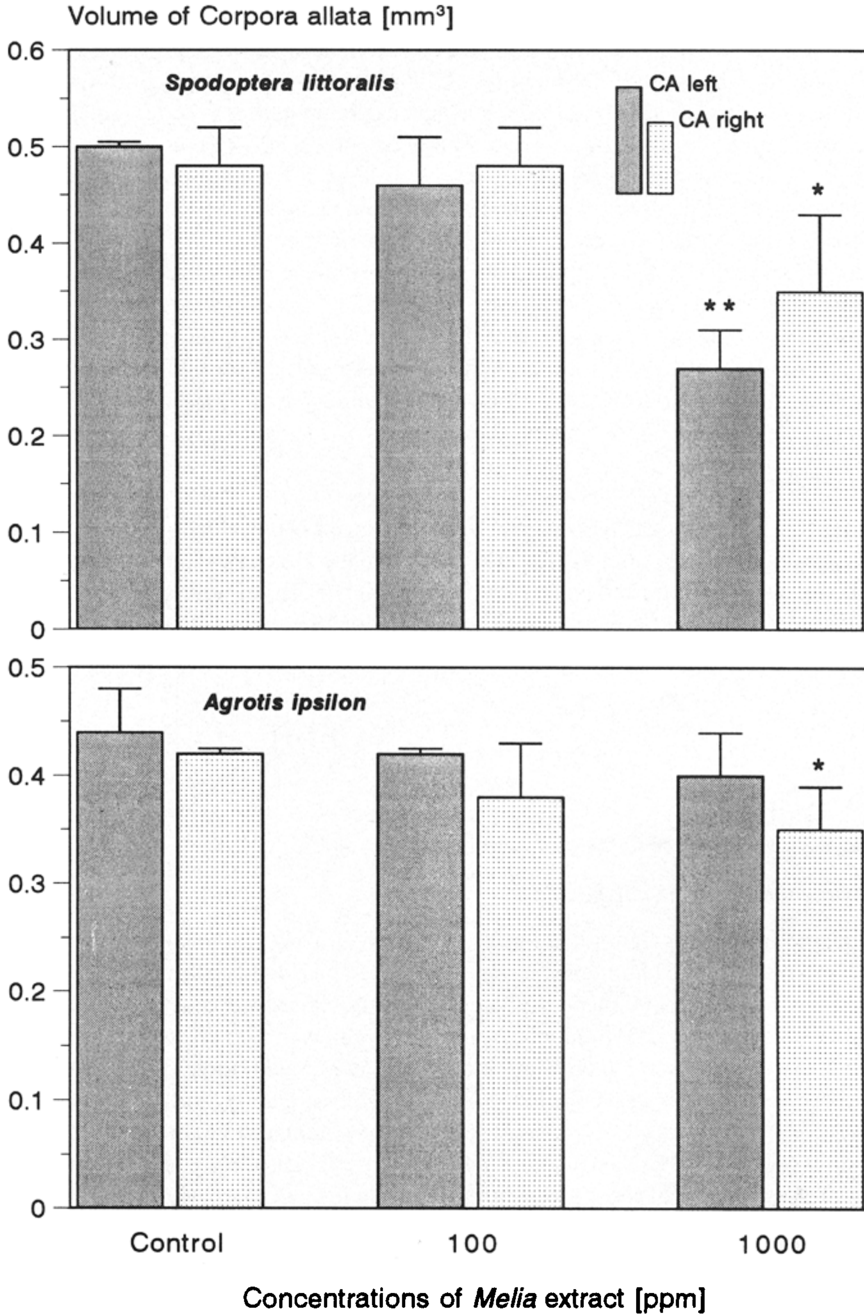

Fig. 1. Effect of *Melia* extract on the volume of corpora allata (±SD) of 4th instar larvae of *Spodoptera littoralis* and *Agrotis ipsilon*. Significantly different from the control * $P < 0.05$, ** $P < 0.01$; four replicates.

RESULTS

Effect on the volume of CA

The data obtained for the size of CA are presented in Figure 1 for both *S. littoralis* and *A. ipsilon*. The volume of the right and left glands of *S. littoralis* decreased significantly at 1000 ppm *Melia* extract in comparison with the control. In *A. ipsilon*, a significant reduction in volume was found only in the right CA; right and left glands can thus have different volumes.

Effect on the JH-II titer in the hemolymph

In both species of larvae, JH-II was the main juvenile hormone; only traces of JH-I were found. The amount of JH-II increased in the treatments with 100 and 1000 ppm *Melia* extract compared with larvae fed on the control diet (Fig. 2). The JH titer in *S. littoralis* was affected more than in *A. ipsilon*. In the 1000 ppm extract treatment, it increased to 0.273 pmol in the former and 0.088 pmol in the latter in comparison with the controls, where very low amounts were found (0.03 and 0.05 pmol, respectively).

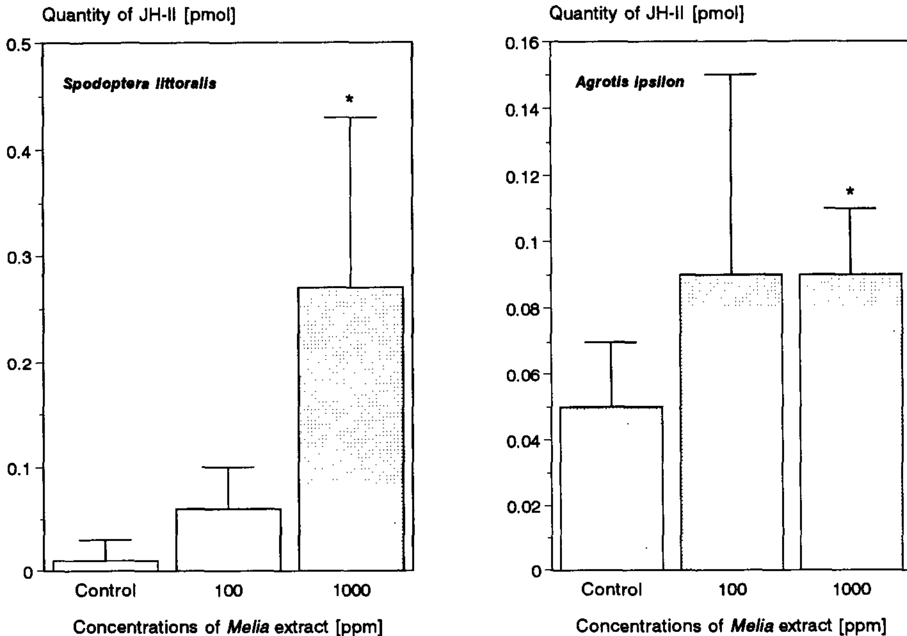

Fig. 2. Effect of *Melia* extract on the JH-II titer in the hemolyph (amount in 5 μl, ±SD) of 4th instar larvae of *Spodoptera littoralis* and *Agrotis ipsilon*. Significantly different from the control * $P < 0.05$; four replicates.

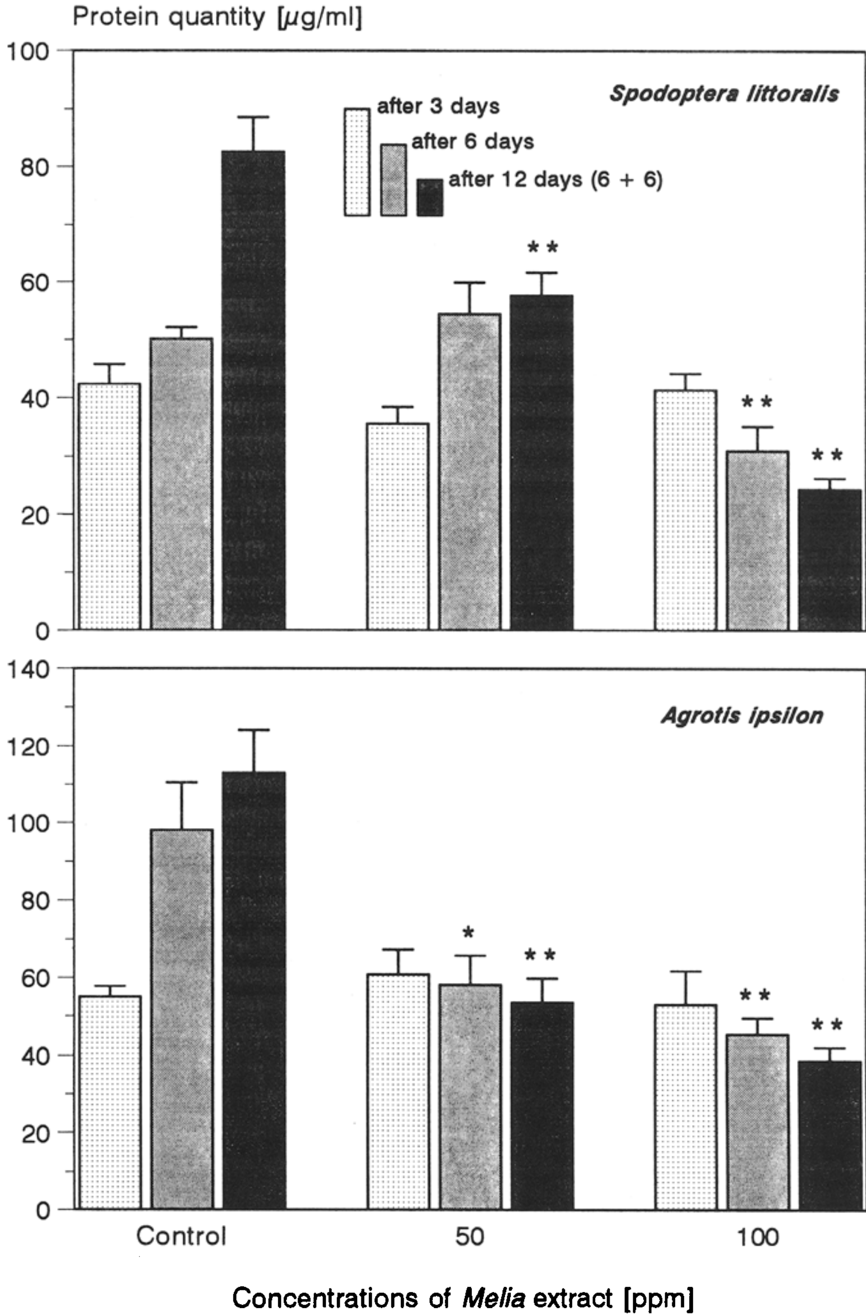

Fig. 3. Effect of *Melia* extract on the hemolymph protein content (±SD) of 3rd to 4th instar larvae of *Spodoptera littoralis* and *Agrotis ipsilon*. Significantly different from the control * $P < 0.05$, ** $P < 0.01$; five replicates.

Effect on the amount of hemolymph protein

The protein content was determined quantitatively in the larvae of both species fed on diet concentrations of 50 and 100 ppm *Melia* extract for 3 and 6 days, but also for 6 days followed by 6 days on untreated diet. The data obtained for *S. littoralis* showed no significant difference between treatment with 50 ppm *Melia* extract and the control after 3- and 6-day feeding periods (Fig. 3). When *S. littoralis* larvae were allowed to feed for 12 days (6 on treated diet + 6 on normal diet), the amount of hemolymph protein decreased significantly compared with the control. In *A. ipsilon*, the reduction on 50 ppm was obvious already after 6 days (Fig. 3).

By feeding on a diet containing 100 ppm extract, significant differences were found between treated and untreated larvae already after 6 days; the hemolymph protein was significantly reduced in both species. When the larvae were fed on the same concentration for 6 days, followed by a further 6 days on a normal diet, the amount of hemolymph protein did not recover. It remained very low: levels of 24.4 μg/ml in comparison with 82.6 μg/ml in the control larvae in *S. littoralis*, and of 38.6 μg/ml in comparison with 113.0 μg/ml in the control larvae in *A. ipsilon*.

DISCUSSION

In previous investigations with *Melia* extract mortality was caused mainly by molt disruption and morphogenetic defects. The fact that larval–pupal intermediates are found after treatment with *Melia* extract, specifically suggests an imbalance of the JH regulation in the insects (5,6,15). Ecdysone triggers the molting, but JH controls the degree and direction of differentiation at each molt.

In the test insects *S. littoralis* and *A. ipsilon*, the JH concentration of the hemolymph was indeed altered after treatment with *Melia* extract. In the midst of the 4th instar it can be expected that the JH titer would reach again a low level, as found in the control larvae. Especially in *S. littoralis*, the JH titer was significantly higher in larvae fed on a diet with 1000 ppm *Melia* extract than on untreated diet. A too high JH level is probably responsible also for the insufficient expansion of pupal features, because pupal molting after treatment with *Melia* extract causes a high percentage of intermediates with many larval characters. Gujar and Mehrotra (7) suggest that also azadirachtin has juvenilizing effects on *Spodoptera litura* Fabr., leading to larval–pupal intermediates. However, further experiments are necessary to verify this hypothesis for *M. azedarach* components.

Spodoptera littoralis larvae treated with 1000 ppm *Melia* extract had a significantly smaller volume of right and left CA than did control larvae. In treated larvae of *A. ipsilon*, a significant decrease in the CA volume was found only in the right gland. This may be due, above all, to the fact that treated larvae grow slower and attain a smaller size in the same stage than do untreated larvae. Schulz (18) stated that the cells of CA and corpora cardiaca of female *Epilachna varivestis* Muls. (Col. Coccinellidae) were destroyed as a result of neem extract treatment. Schlüter (14) studied the molting inhibition by azadirachtin on the same insect and found a complete destruction of the epidermis.

In both insect species we studied, the protein content in the hemolymph was significantly lowered after treatment with various extract concentrations. Also larvae fed for 6 days on a treated diet followed by 6 days on a normal diet still showed a reduced amount of hemolymph protein.

Rao and Subrahmanyam (11) found a considerable decrease of protein in females of *Schistocerca gregaria* between the 5th and 8th day after treatment when 1–8 $\mu g/g$ azadirachtin had been injected, which coincided with a decrease of the level of amino acids in the hemolymph during this period. Histological studies showed that the cells of different layers of the midgut of the lepidopteran larvae treated with 100 ppm *Melia* extract were affected, the peritrophic membrane disappeared and the epithelial cells degenerated (15). This indicates that proper digestion and absorption of food components is possible only to a reduced degree. In this way, insects may lose their appetite and feeding inhibition may occur. The relation between the consumed quantity of food and the weight gain of the larvae showed that with increasing *Melia* extract concentration, the larvae not only reduced their food intake but also digested and/or metabolized smaller amounts in a less effective way (6).

It is not surprising, therefore, that after treatment with *Melia* extract also the protein content is lowered in the hemolymph. This again might be a reason that less reserve material – which is necessary for egg development in the adult stage – could be stored in the larval body. Treatment with *M. azedarach* extract at the larval stage in fact reduced the number of eggs laid by the developed adults of *S. frugiperda* (6) and our test insects (15). *Melia* extract seems to have an insecticidal effect like a stomach poison, resulting in reduction of larval weight in comparison with the control larvae (4,6,15).

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