Influence of Suneem Oil on Ecdysteroids Titer in the Haemolymph of the Cotton Leafworm, *Spodoptera littoralis* Larvae (Lepidoptera: Noctuidae)

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ABSTRACT

Radioimmunoassay was used to determine the titer of ecdysteroids during the penultimate and last larval instar of the cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) treated with suneem oil. The ecdysteroid titer was at very low levels during the early stage of the last larval instar. It reached its maximum peak at the prepupal phase. The major component of the haemolymph peak of ecdysteroid was 20-hydroxyecdysone in both penultimate and last larval instar. Feeding larvae with 1000 ppm suneem oil (containing about 0.1-10 ppm Azadirachtin) reduced and delayed the peak of haemolymph titers of molting hormone, ecdysone and 20-hydroxyecdysone more than the control larvae.

Key Words: Suneem Oil, Ecdysteroids Titer, Haemolymph, *Spodoptera littoralis*

INTRODUCTION

Botanical products are useful and desirable tools in pest management programs because they can be effective and often complement the actions of natural enemies (Ascher, 1993 and Schmutterer, 1990 & 1995).

One of the most efficient natural substances with molt-inhibiting activity is Azadirachtin, a tetranortriterpenoid plant (Neem tree, Meliaceae) limonoid with ecdysteroid like structure (Mordue (Luntz) and Blackwell 1993). It causes antifeedancy, growth reduction, molting inhibition, anatomical abnormalities as well as mortality, in a vast range of insects' species, many of them belonging to order Lepidoptera (Schmutterer, 1990; Isman, 1999 and Walter, 1999). In addition to controlling pests, some neem-based insecticides have negligible effects on beneficial and low environmental impacts (Schmutterer, 1995, Kelany *et al.*, 2003 and Haseeb *et al.*, 2004).

The growth regulatory effects of Azadirachtin are mostly concerned with its interference in the neuroendocrine system of the insects (Mordue and Nisbet 2000). The main hormones involved in growth regulation in insects are ecdysone and 20-hydroxyecdysone (molting hormones) and juvenile hormone (JH). They are respectively produced in the pro-thoracic glands and *corpora allata*, through stimulation of hormones secreted in the brain (Gilbert *et al.*, 2002).

The molting process is initiated by an increase of 20-hyroxyecdysone (20E) in the haemolymph and completed following a decline of 20E titer and the release of a peptide eclosion hormone. There are two major surges of ecdysone in the last larval instar of Lepidoptera. The first small surge leads to the cessation of feeding while the second large increase during the prepupal period induces pupation. Finally, pupal-adult transformation occurs at increased ecdysteroid titers but with very low levels of JH (Hoffmann and Lorenz, 1997 and Niimi Sakurai, 1997).

The cotton leafworm, *Spodoptera littoralis* (Boisduval) is an important pest mainly of cotton in Southern Europe, Africa and Middle East (Hosny *et al.*, 1986).

The present work was intended to evaluate the effect of the compound Suneem oil on the haemolymph ecdysteroids titer during penultimate and last larval instars of *S. littoralis*.

MATERIALS AND METHODS

Insect rearing

Larvae of *S. littoralis* were reared under long-day photoperiod (LD 16:8) at 25°C on semi-artificial diet prepared and described previously (Adel *et al.*, 1999). The experiments were set up in Petri dishes diameter 9cm. 30 larvae were used at 5th and 6th larval instars. The larvae were fed on diets containing suneem oil at the concentration of 1000 ppm.

Ecdysteroid extraction and quantification

Tested larvae were first anesthetized in water for 5 to 20 min. excess water was removed by sliding the larvae on an absorbent paper. Haemolymph samples were collected every 24 h (10 µl) from each larvae through a cut in the second thoracic leg of each larvae for analysis by the ecdysteroid

radioimmunoassay (RIA). Ecdysteroids were extracted from the haemolymph with methanol (100 µl) and the extracts were stored at 4°C until assayed. For RIA, extracts were centrifuged at 10,000 xg for 10 min to pellet precipitated protein. Ecdysteroid RIA was performed as described by (Warren and Gilbert, 1988).

Materials

Azadirachtin used was in a form called suneem oil which was obtained from Sunida Exports, India. The oil was vortexed in methanol and 500 µl of the emulsion containing 100 mg of the oil was added to 24.5 ml of the ascorbic acid solution just before it was stirred into the 75 gm blend of other food ingredients (Adel *et al.*, 1999). Samples of 100 g diet were prepared, containing 1000 ppm of the suneem oil (about 0.1-10 ppm Azadirachtin). For the control, pure solvent (500 µl methanol) was added to the diet.

Data obtained from experimental and the control insects were compared by paired Student's *t*-test.

RESULTS AND DISCUSSION

Haemolymph ecdysteroid titers throughout the penultimate and sixth larval instar

The ecdysteroid profile shows significant variations in the treated *S. littoralis* during the penultimate and last larval instar with the prepupal phase, inclusive. In the penultimate larval instar, 20-hydroxyecdysone (20E) titer was characterized by a significant increase to the level of about (314.33± 72.31ng/ml) at the day (L5/2). Only one significant peak of 20E titer was observed at the penultimate instar before ecdysis to the sixth (last) larval instar. There was a drastic reduction in the ecdysteriod titer prior to and after molting to the last larval instar (Table, 1 and Fig. 1).

In the last larval instar, there was an initial increase in the ecdysteroid titer between days (L6/1) and (L6/3) (22.38 ± 10.21 to 66.92 ± 18.41 ng/ml) and this increase was followed by a second rise between days of wandering larvae (WL) and early prepupae (ERPP) to a level of approximately 519 ± 157.3 ng/ml. Afterwards, the level increased rapidly to reach the maximum level of (2147.5± 658.4 ng/ml) on the prepupal phase (PP). Then, ecdysteroid titer dropped rapidly to a low of (530 ± 142.9 ng/ml), prior to the larval-pupal formation.

When the tested larvae were fed on diets, containing (1000 ppm) suneem oil, they showed significant reduction of the ecdysteriod titer (20E)

Table (1): Effects of Suneem oil at the concentration of 1000 ppm on the ecdysteroid titers during penultimate and (sixth) last larval instars of *S. littoralis*

Larval instar in days	20-Hydroxyecdysone equivalents (ng/ml haemolymph)	
	Control	Treated
L5/1	30.55± 7.40	93.00± 15.89*
L5/2	314.33± 72.31	60.52± 13.90*
L5/3		207.83± 56.93
L6/1	22.38± 10.23	14.81± 6.10*
L6/2	21.57± 9.63	60.88± 26.75*
L6/3	66.92± 18.41	48.52± 24.80*
WL	293.50± 54.11	176.13± 41.04*
ERPP	519.00± 157.30	468.00± 264.90*
PP	2147.± 658.4050	1959.17± 900.20*
P	530.33± 142.90[a]	658.50± 182.50[b]

Each value in the treatment and control represents the mean ± SE. The amount of haemolymph ecdysteroid was determined by RIA and expressed in ng of 20-E equiv./ml haemolymph. L5/1= day of the antepenultimate instar before ecdysis to the last instar; L6/1, L6/2, L6/3 = days 1, 2 and 3, respectively, of the last larval instar; WL = Wandering larvae; ERPP = Early Prepupae; PP = Prepupae, P = Pupae. A- Un treated pupae, b- Extended treated prepupae (EPP_T).

* Significant of differences from "un-treated" controls (taking into account the Student's *t*-test): $P < 0.05$.

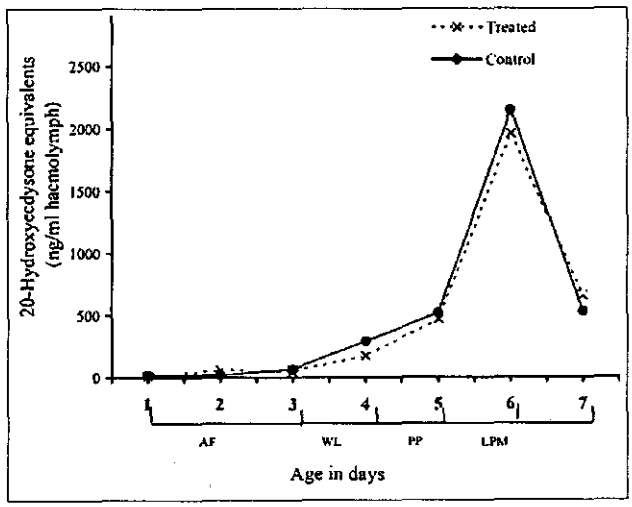

Fig. (1): Haemolymph 20-hydroxyecdysone titer of *S. littoralis* during the fifth and sixth (last) larval instar. Results are expressed in ng of 20-E equiv./ml haemolymph.

AF- active feeding; WL- wandering larva; PP- prepupal; LPM-larval pupal moult. $L5/2_{co}$–end of the penultimate control instar, $L5/3_T$–extended treated larvae at the end of the penultimate instar. PP_{co}= untreated prepupae, PP_T=treated prepupae, EPP_T= extended treated prepurae.

than the control larvae (Table 1). In the penultimate instar, the peak of 20E titer was delayed and had significant reduction in the hormone level (207.83 ± 56.93 ng/ml) at the day (L5/3T) in the extended larval stage. Then, the ecdysteroid titer reduced to the level (14.81± 6.10 ng/ml) at the beginning of the last larval instar L6/1 (Fig. 1). The titer remained at levels ranged between 60.88± 26.75 and 176.13 ± 41.04 ng/ml until the wandering larval stage (WLT). Then, it was followed by an increased peak of (1959.2± 900.2 ng/ml) at the prepupal phase (pp_T). It was significantly reduced and occurred one day later than the control. After that ecdysteroid level declined to express a value of (658.5 ± 182.5 ng/ml) on the extended prepupae (EPP_T) (Fig. 1).

Table (2) presents means and standard errors of the haemolymph ecdysone titers in treated and untreated 5th and 6th larval instars of *S. littoralis*. The titers are expressed as ecdysone equivalents ng/ml haemolymph. In the control larvae, the titers of ecdysone rose to a peak of (7.28 ng of E equiv. /ml) in the second third of the penultimate larval instar around day (L5/2), and then declined to the level 6.08 ng/ml at the beginning of the last larval instar L6/1 (Fig. 2). When the larvae ceased feeding and initiated wandering, the titer increased to the maximum peak of 8.32 ng/ml haemolymph at prepupal phase, then, the titer declined to metamorphic pupae.

In suneem oil–treated larvae, the peak of ecdysone was delayed and increased in titer to reach 9.34 ng/ml haemolymph. It was more than the control in the extended larvae (L5/3), whereas the controls were ecdysed to the last larval instar at day (L6/1). Then, the hormone titer of the treated larvae were reduced to 1.85 ng/ml ($P < 0.05$ Table 2) at the first day of the 6th larval instar and then slightly rose at different rates (2.03 – 2.98 ng/ml) on the prepupal phase (Fig. 2).

As shown in Fig. (3), there is a temporal relationship between 20-hydroxyecdysone and ecdysone during the 5th and 6th larval instars. Throughout the 5th instar, ecdysone was detected at a steady concentration of about (5.30± 0.42 to 7.28± 1.72 ng/ml) Table 2. During the last day of the 6th larval instar development, ecdysone titers rose to (8.32 ± 1.99 ng/ml) in the prepupae (Table 2). While 20-hdroxyecdysone was found at the end of the 5th instar (314.33 ± 72.31 ng/ml) Table (1). The haemolymph 20E was very low at the beginning of the sixth (last) instar, but it reached the maximum at the onset of the prepupal phase (2147.50± 658.40 ng/ml) Table (1). The level of ecdysone was always low during the penultimate and 6th larval instar of *S. littoralis*.

Rees (1985) stated that in Lepidoptera, ecdysone biosynthesis is regulated by the prothoracicotropic hormone (PTTH), which is released from the brain-corpora cardiaca complex. Ecdysone is converted by a 20-monooxygenase in the peripheral tissues to the major molting hormone; 20-hydroxyecdysone. Then, it acts on target tissues such as the epidermis to elicit hormonal effects (Warren *et al.*, 1988 and Gilbert *et al.*, 2002). Transportation of ecdysteroids in haemolymph by a highly specific protein had been

Table (2): Effects of Suneem oil at the concentration of 1000 ppm on the ecdysteroid titers during (penultimate) and last larval instars of *S. littoralis*

Larval Instar in days	Ecdysone equivalents (ng/ml haemolymph)	
	Control	Treated
L5/1	5.30 ± 0.42	3.63 ± 0.52*
L5/2	7.28 ± 1.72	6.00 ± 0.30*
L5/3		9.34 ± 3.97
L6/1	6.08 ± 1.54	1.85 ± 0.00*
L6/2	1.10 ± 0.00	2.03 ± 0.17*
L6/3	2.35 ± 0.55	1.93 ± 0.12*
WL	1.90 ± 0.29	2.33 ± 0.44*
PP	8.32 ± 1.99	2.98 ± 0.34* [b]
P	2.38 ± 0.32[a]	

§ Explanation as for Table 1.
a- Un-treated pupae, b- Treated prepupae (PP_T).
* Significant of differences from "un-treated" controls (taking into account the Student's *t*-test):$P < 0.05$.

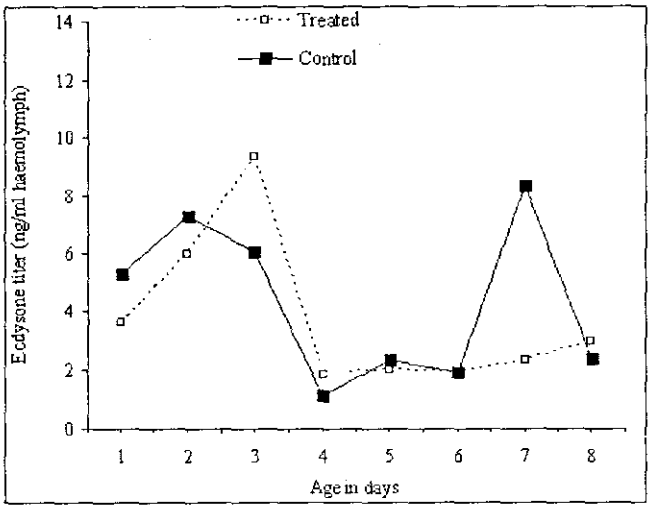

Fig. (2): Haemolymph Ecdysone titer of *S. littoralis* during the penultimate and last larval instar. Results are expressed in ng of E equiv./ml haemolymph.
L5/2– end of the penultimate control instar.
L5/3– extended treated larvae at the end of the penultimate instar.

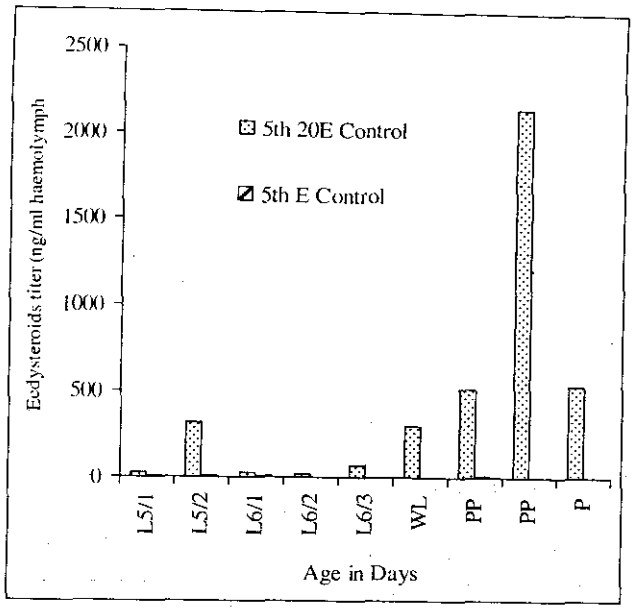

Fig. (3): 20-Hydroxyecdysone and ecdysone titers in the haemolymph of *S. littoralis* during the penultimate, last larval instar and the prepupae.

only reported so far by (Feyereisen, 1980), who was able to confirm the existence of an ecdysteroid binding protein.

Remarkable changes in ecdysteroid titer are found throughout the life of an insect. Preceding molting or pupation, larvae cease feeding due to high levels of ecdysteroids (Langelan *et al.*, 2000 and Westerlund, 2004). Obtained results showed that during the penultimate larval instar of *S. littoralis*, also appeared a high level of the ecdysteroid titers on day (L5/2) before ecdysis to the last larval instar (Figs. 1 & 2). During the last larval instar there were two critical peaks, the commitment and the pre-pupal peaks prior to a metamorphic molt. This phenomenon was studied mainly in Lepidoptera and reported by (Dean *et al.*, 1980; Sehnal, 1989 and Lafont *et al.*, 2005). Ecdystroid titers (20E) in normal larvae have two peaks the first at ceased feeding (293.5 ng/ml haemolymph) and reached the highest peak (2147.5 ng/ml haemolymph) at the prepupal phase. On the other hand, haemolymph ecdysone titer reached the peak of (8.32 ng/ml) in the late of the instar. A similar high ecdysteroid titer was observed during the last larval stage of some lepidopteran insects, e.g. *Heliothis virescens* (Barnby and Klock, 1990) and *Manduca sexta* (Keshan *et al.*, 2006). Warren *et al.*, (2006) recorded that also during the last larval instar of *Drosophila melanogaster*. In lepidopteran insects, this increase in ecdystroid titer is responsible for reprogramming of epidermal commitment from larval-pupal cuticle, and therefore for the well documented behavior and other accompanying prodromes of the larval-pupal metamorphic molt (Riddiford, 1995).

Azadirachtin based IGRs are very selective ecdysone antagonists (Tunaz, 2004). This was attributed to a disruption of endocrine events as the down-regulation of haemolymph ecdysteroid level through the blockage of release of prothoracicotropic hormone, from the brain-corpus cardiacum complex, or to a delay in the appearance of the last ecdysteroid peak showing a complete molt inhibition (Anibal 2007). It seems likely that ecdysis in treated larvae was inhibited by disturbed ecdysteroid regulation shortly before larval ecdysis (Schluter *et al.*, 1985).

Numerous studies have shown that Azadirachtin treatment modifies ecdysteroid titer in the migratory locust, *Locusta migratoria*, in the (milk weed bug), *Oncopeltus fasciatus* (Mordue (Luntz) and Nisbet, 2000) and the tobacco hornworm, *Manduca sexta* (Schluter *et al.*, 1985), mainly by delaying the occurrence of the haemolymph ecdysteroid peak.

Therefore, obtained results suggest that suneem oil treatment affects the pre-pupal surge of ecdystroid. The formation of extended larvae is possibly due to the absence of the required titer of ecdystroid needed for normal pupation. A similar result was obtained by Josephrajkumar *et al.*, (1999), who demonstrated also that Azadirachtin-treated larvae of *H. armigera* had a reduced ecdysteroid titer in the extended larval stage. *In vivo*, suneem oil (containing about 0.1 – 10 ppm Azadirachtin) might influence the release and turnover of prothoracicotropic hormone (PTTH) activity and consequently affect the release of ecdystroids from the pro-thoracic glands. Garcia *et al.*, (1990), suggested that the synthesis and release of PTTH was deficient in the Azadirachtin-treated *Rhondius prolixus* larvae.

The overall profile of obtained results showed that 20-hydroxyecdysone was the dominant ecdysteroid in the penultimate and last instar larvae of *S. littoralis*. The level of ecdysone was always low which was indicating its rapid conversion to 20E. Similar results were found with the same species *S. littoralis* by Jarvis *et al.*, (1994) and other insect species e.g. *Manduca sexta* (Langelan *et al.*, 2000), desert locust, *Schistocerca gregarira* (Tawfik and Sehnal 2003) and *S. frugiperda* (Westerlund 2004).

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