J. Adv. Zool. 2017: 38(2): 164-177 **ISSN-0253-7214**

POTENTIAL OF NEEM SEED'S ACETONE EXTRACT ON THE HAEMOLYMPH AND FAT BODY BIOCHEMISTRY OF *CORCYRA CEPHALONICA* LARVAE (LEPIDOPTERA: PYRALIDAE)

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Received-19-09-2017 **Accepted-20-12-2017** **Published-31-12-2017**

ABSTRACT: Third instar larvae of Rice- moth, *Corcyra cephalonica* were exposed to sub-lethal doses (0.04, 0.06 and 0.08 %) of neem seed's acetone extract in order to evaluate its potential effects on the larval haemolymph and fat body biochemistry of this pest. It was observed that these sub- lethal doses caused a significantly dose-dependent perturbation in the metabolic flux that impairs the physiological fitness of the larva and contributes to the lethal action of this biopesticide.

KEYWORDS: *Corcyra cephalonica*, *Azadirachta indicta*, larval biochemistry.

INTRODUCTION

The post-harvest losses and quality deterioration due to storage pests are a major problem throughout the world[26]. The rice-moth, *Corcyra cephalonica* (Staint.) is a notorious pest of stored cereals and cereal products in Asia, Africa, North America, Europe and other tropical and subtropical regions of the world[15,43,25,55,2].

Persistent use of synthetic organic insecticides affect immune system of insects, develop resistance[13,87,88,86,83,61], toxicity to non-target organisms[72], cause residue in food grains[18], biomagnification, and of course pollute our own environment due to non-biodegradability, leading to biological imbalance due to the destruction of beneficial species such as parasites and predators of pests beside the destruction of pollinating insects such as honey bees. They affect human health posing problems also such as poisoning in man and other animals[54]. Thus, there is an urgent need to develop safe alternatives to synthetic insecticides for the protection of grain and grain products against insect infestations.

Botanical insecticides compared to synthetic ones may be safer for the environment, are generally, less expensive, easily processed and used by farmers and small scale industries[4]. Since, these insecticides are often active against a limited number of species, are often biodegradable to non-toxic products, and are potentially suitable for use in integrated pest management. They could lead to the development of new classes of safer insect control agents[34]. Neem derivative azadirachtin possesses insecticidal, ovicidal,

antifeedent and growth inhibiting effects against many insect and storage pests[1,66,80,46,30,27,28,40]. Although seed damage is not always reduced by neem materials at par with synthetic insecticides[68], the advantage of neem treatment is that it does not impair the germination of stored seed[24]. In India the time first demonstration of powdered neem kernel when mixed with wheat seed at a proportion of 1-2 to 100 (wt/wt) parts satisfactorily protected against *S. oryzae, R. dominica*, and *Trogoderma granarium* for 270, 320 and 380 days, respectively[31]. Similarly, ethanolic neem kernel extract, containing azadirachtin, at 75 mg/kg protected stored wheat against *R. dominica* for up to 48 weeks[59]. The insecticidal constituent azadirachtin, present in neem, *Azadirachta indica* A. Juss, and *Melia azedarach* L. (Meliaceae), is considered as promising alternative to synthetic insecticides[32,28,29].

Haemolymph is the only extra cellular fluid in the insect body. It freshly bathes the various internal organs and also enters the appendages and the tubular cavities of the wing veins. It consists of liquid plasma and numerous blood cells or haemocytes. The fat body is composed of irregular masses or lobes of rounded or polyhedral cells (trophocytes) which are usually unvacuolated and contain inclusions of various kinds[53,58]. It is a dynamic tissue discharging a variety of important functions like storage of various nutrients, detoxification of foreign chemicals and biosynthesis of circulating metabolites like a mammalian liver[33]. The fat body maintains a constant exchange relation with haemolymph[20].

Numerous Investigations have shown that botanicals/ plant extracts affect the biochemical constituents of various tissues in insects. Such plant extracts induced influence on total free amino acids have been investigated in various insects[5,64,81]. Protein contents influenced by different plant extracts have been explored in different tissues of the insects[23,78,85,5,64,62,81,57,41,10]. Similarly, changes in influenced by plant extracts in nucleic acids level[69,45,79,65] have resulted drastic perturbation in the metabolic flux of the larva leading to death. Plant products/biopesticides induced changes in the biochemical constituents of various tissues of insects may be regarded as one of the objective criteria permitting an assessment of effectiveness of botanical/biopesticides control measures against insect pests population in general and lepidopterous pests in particular.

Though, sufficient information exists on the reproductive and nutritional physiology of this lepidopterous pest[36,76,77,6,7]. In addition, the grubs and adults of lesser meal worm, *Alphitobius diaperinus* Panz. were found gregariously feeding on the eggs and larvae of *C. cephalonica* Staint.[17]. Influence of insecticidal agents of certain plant materials have also been reported against the ontogeny as well as the larval biochemistry of this lepidopterous pest[47,48,70,49,50,51,52], but the scientific contribution in relation to role of natural

plant products as safer insecticidal agents influencing life-cycle stages as well as biochemical constituents viz. total protein, total free amino acid and nucleic acids in haemolymph and fat body tissues of the larva of rice moth, *C. cephalonica*, pertaining to a specific age group, is completely wanting. Hence, as an objective of such programme the present research programme has been designed and carried out to examine into the impact of neem, *A. indica* A. Juss (Family-Meliaceae) seeds acetone extract at various doses, on the various biochemical constituents viz. total protein, total free amino acids and nucleic acids in the haemolymph and fat body tissues of the larva of rice-moth, *C. cephalonica*. Such knowledge, in turn, is likely to generate new insights into divising ways and means for controlling *C. cephalonica*, by disrupting its metabolic framework so that evolution of a new generation of this pest for the eventual establishment on stored cereals and cereal products can be considerably restricted.

MATERIALS AND METHODS

C. cephalonica adults were collected from Biological Control Station, Gorakhpur, U.P. A rich standard culture of this insect was maintained in the laboratory on normal dietary medium composed of coarsely ground jowar (*Sorghum vulgare*) mixed with 5% (w/w) powdered yeast inside large glass containers (150 mm diameter, 200 mm height) at 26 ± 1^0C and 93 ± 5% relative humidity (R.H.).

From the above culture whenever needed, newly emerged males and females were transferred to oviposition glass chambers (35 mm diameter, 200 mm height). Since *C. cephalonica* individuals do not feed during their adult stage, no food was provided to them during their confinement in these vessels. Eggs laid by the females were collected and then placed in glass chambers (consisting of 250 ml beakers) for hatching.

A. indica A. Juss (Family- Meliaceae) plant's seed was collected from local areas in Gorakhpur, Uttar Pradesh, India. Fresh neem seeds were properly washed with fresh tap water, air dried at room temperature for 10-15 days, pulverized in a mortar and pestle, crushed in an electric grinder and the powder so obtained was extracted in acetone in a Soxhlet assembly. The extract was utilized as insecticidal agent throughout the experiments.

For the preparation of different dose levels of neem seed acetone extract, in dietary media, a stock solution of known concentration of this extract was prepared in acetone and then adjusted via serial dilutions to achieve its different required concentrations. Now required volume of different concentrations of neem seed acetone was thoroughly mixed with the required quantity of normal food (roughly ground jowar mixed with 5% w/w yeast powder) to get different desired dose levels of acetone extracts of neem seed . This treated food was then air dried at room temperature to eliminate completely the organic solvents. For control purposes, the normal food was

thoroughly mixed with a required volume of acetone similar to that of treated food and then air dried in the same way.

To investigate the toxic effects of various doses of neem seed's acetone extract the larvae of *C. cephalonica* were allowed to feed on a normal dietary medium (kept inside 250 ml beakers) for exactly 15 days. On the 16th day, 25 third instar larvae were transferred to each similar rearing chambers containing 50 gms of dietary medium mixed and treated separately with different known dose levels of neem seed'sacetone extract .Experiments were conducted on 9 different concentrations of neem seed acetone extract (0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16%). Twenty five larvae were also kept on a normal dietary medium as control. On the completion of developmental cycle, percent adult emergence and percent pupal death was observed and on that basis percent pupation and percent larval death was calculated. Experiments were replicated five times and data so obtained were analysed statistically and expressed as the mean ± S.D.[49].

For biochemical estimations, out of various dose levels of this biopesticide mentioned above only such doses (0.04, 0.06 and 0.08%), were selected, which allowed the larvae to survive and develop but caused considerable effect in the internal biochemistry of the larva that could be easily detected and assessed to prove the effectiveness of different components of *A. indica* as biopesticidal control measures against this lepidopterous pest. For this purpose, freshly hatched larvae were allowed to feed on a normal dietary medium (kept inside 250 ml beakers) for 15 days. On the 16th day, 25 third instar larvae were transferred to each similar rearing chambers containing dietary medium mixed with 0.04, 0.06 and 0.08% dose levels of neem seed's acetone extract and were allowed to feed for 10 days. 25 larvae were kept as control with each set of experiment. On the completion of 25 days, 10-15 larvae from each set, experimental as well as control, were taken out and their haemolymph and fat body were separately collected and pooled in a manner outlined as thus:

Haemolymph was obtained from these larvae by making of a small puncture by means of a sharp needle at the dorsolateral side of the prothoracic segment and drawing the blood, easily oozing out through this puncture, into a fine glass capillary tube[35]. The haemolymph thus obtained from caterpillars was collected in a previously weighed small glass vial (12 mm diameter; 55 mm height). For each biochemical estimation, after ascertaining the weight of the haemolymph, a known volume of required solvent was added to prepare the homogenate.

Fat bodies were taken out from these larvae following careful dissections performed on a clean glass slide containing minute quantities of distilled water under a stereoscopic binocular microscope. The water and the flowed out haemolymph surrounding these tissues were then completely drained off with the help of absorbant paper. Later, this

fat body was weighed and swiftly mixed with known volume of required solvent to prepare the homogenate for each biochemical estimation.

The entire programme of biochemical estimation includes the quantitative measurement of total protein, total free amino acids and nucleic acids levels in haemolymph and fat body of the larva of rice-moth treated with sublethal doses of neem seed acetone extract as well as control.

The total protein was measured using bovine serum albumin as standard[39] while total free amino acid was estimated by using glycine solution as standard[75]. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) levels were determined using diphenylamine and orcinol reagent for DNA and RNA respectively[67]. Significant differences between treatment groups, in order to show dose-dependence, were determined by one way analysis of variance ($P < 0.05$ to $P < 0.001$)[74]. Student's t-test was applied to determine the significant differences between the corresponding treated groups and the controls ($P < 0.05$ to $P < 0.001$)[74].

RESULTS AND DISCUSSION

Sublethal doses i.e. 0.04%, 0.06% and 0.08% of *A. indica* seed's acetone extract caused a significantly dose-dependent ($P < 0.001$) reduction on the levels of total protein, DNA, RNA and RNA/DNA ratio and a significantly dose-dependent ($P < 0.001$) enhancement in the total free amino acids in haemolymph and fat body tissues of the larva of rice-moth, *C. cephalonica* (Table 1, 2 and 3). In control larval groups, the total protein content in the haemolymph and fat body was 69.126 and 12.661 µg/mg respectively. The maximum decrease in total protein level in haemolymph (30% of the control value) and fat body (43% of the control value) was observed in larvae treated with 0.08% of *A. indica* seed's acetone extract. Protein levels, in haemolymph, were reduced to 79% (54.609 µg/mg), 62% (42.858 µg/mg) and 30% (20.738 µg/mg) of the control while these levels, in fat body, were reduced to 88% (11.142 µg/mg), 59% (7.469 µg/mg) and 43% (5.444 µg/mg) of the control following treatment with 0.04%, 0.06% and 0.08% dose levels of *A. indica* seed's acetone extract respectively (Table 1).

The total free amino acid content, in control larvae, was 87.512 and 10.996 µg/mg in haemolymph and fat body respectively. Larvae treated with 0.08% dose level of *A. indica* seed's acetone extract showed a maximum enhancement in the total free amino acid level in haemolymph (165% of the control) and fat body (171% of the control). Total free amino acid levels, in haemolymph, were increased to 107% (93.638 µg/mg), 125% (109.390 µg/mg) and 165 % (144.395 µg/mg) of the control while these levels, in fat body, were increased to 126% (13.855 µg/mg), 150% (16.494 µg/mg) and 171% (18.803 µg/mg) of the control following treatment with 0.04%, 0.06% and 0.08% dose levels of

A. indica seed's acetone extract respectively (Table 1).

Table 1. Changes in the total protein and total free amino acid levels in the haemolymph and fat body of the larva of rice-moth, *C. cephalonica* treated with neem seed acetone extract

Percent neem seed acetone extract concentration	Total protein # (µg/ mg, wet wt.)		Total free amino acid # (µg/ mg, wet wt)	
	Haemolymph	Fat body	Haemolymph	Fat body
Control (untreated)	69.126 ± 2.121 (100)	12.661 ± 0.661 (100)	87.512 ± 3.822 (100)	10.996 ± 0.848 (100)
0.04	54.609 ± 2.167 (79)	11.142 ± 0.589 (88)	93.638 ± 4.016 (107)	13.855 ± 1.201 (126)
0.06	42.858 ± 2.044 (62)	7.469 ± 0.532 (59)	109.390 ± 4.266 (125)	16.494 ± 1.062 (150)
0.08	20.738 ±1.536 (30)	5.444 ± 0.442 (43)	144.395 ± 4.607 (165)	18.803 ±1.452 (171)

Values are expressed as the mean ± s.e. of six replicates.
Values in the parentheses indicate the percentage change, with control values taken as 100%.
Student's t-test showed significant differences ($P < 0.05$ to $P < 0.001$) between the corresponding treated groups and the controls.
Analysis of variance showed that the response to the neem seed acetone extract was dose dependent $P < 0.001$.

Table 2. Changes in the DNA and RNA levels in the haemolymph and fat body of the larva of rice-moth, *C. cephalonica* treated with neem seed acetone extract

Percent neem seed acetone extract concentration	DNA# (µg/ mg, wet wt.)		RNA # (µg/ mg, wet wt)	
	Haemolymph	Fat body	Haemolymph	Fat body
Control (untreated)	10.610 ± 0.648 (100)	6.413 ± 0.528 (100)	15.942 ± 0.884 (100)	10.986 ± 0.664 (100)
0.04	9.230 ± 0.662 (87)	5.674 ± 0.468 (88)	12.913 ± 0.732 (81)	9.008 ± 0.512 (82)
0.06	8.276 ± 0.582 (78)	4.905 ± 0.332 (76)	9.406 ± 0.552 (59)	7.251 ± 0.502 (66)
0.08	7.321 ± 0.568 (69)	4.037 ± 0.398 (63)	6.536 ± 0.531 (41)	5.383± 0.486 (49)

Values are expressed as the mean ± s.e. of six replicates.
Values in the parentheses indicate the percentage change, with control values taken as 100%.
Student's t-test showed significant differences ($P< 0.05$ to $P< 0.001$) between the corresponding treated groups and the controls.
Analysis of variance showed that the response to the neem seed acetone extract was dose dependent $P< 0.01$.

Table 3. Alterations in the RNA/DNA ratio in haemolymph and fat body of the larva of rice-moth, *C. cephalonica* treated with neem seed acetone extract

Percent neem seed acetone extract concentration	RNA/DNA Ratio	
	Haemolymph	Fat body
Control (untreated)	1.502 (100)	1.713 (100)
0.04	1.399 (93)	1.587 (93)
0.06	1.136 (76)	1.478 (86)
0.08	0.893 (59)	1.333 (78)

The values in the parentheses indicate the percentage change, with control value taken as 100%.

The RNA/DNA ratio, in control larvae, was 1.502 in haemolymph and 1.713 in fat body. The maximum decrease in this ratio, in haemolymph (59%of the control value) and fat body (78% of the control value) was observed in larvae treated with 0.08% of *A. indica* seed's acetone extract. The RNA/DNA ratios, in haemolymph, were reduced to 93% (1.399 µg/mg), 76% (1.136) and 59% (0.893) of the control while these ratios in fat body, were reduced to 93% (1.587), 86% (1.478) and 78% (1.333) of the control following treatment with 0.04%, 0.06% and 0.08% dose levels of *A. indica* seed's acetone extract respectively (Table 3).

The present investigation reveals several essential and interesting informations concerning some of the so far unexplored natural plant product (biopesticide) induced changes on in the levels of total protein, total free amino acid, DNA, RNA and RNA/DNA in the haemolymph and fat body tissues of the larva, pertaining to a specific age group, of the rice-moth, *C. cephalonica*. The findings are discussed here in the light of the influence of insecticidal agents on the basic extrinsic as well as intrinsic cellular mechanisms such as transport, synthesis, degradation and storage in relation to the aforesaid biochemical constituents to come to some such conclusions which may in future help in divising ways and means for the effective control of this lepidopterous pest.

The toxicity of *A. indica* seed's acetone extract increased with increase in its concentration on the developmental stages i.e. larvae, pupae and adults[49]. The azadirachtin (a tetranortriterpenoid) present in the neem seed is a strong antifeedant[12], acts as a potent growth inhibitor at microgram levels[63,71,42] and has insecticidal properties[56]. The most

defined effects are: (a) delay and/or inhibition of molt into the successive instar, (b) disturbance of the molting process, and (c) delay, disturbance or inhibition of ovarian development.

Proteins are among the most complex of all known chemical compounds and also the most characteristic of living organism. They serve as an important internal environmental factor for the metabolism, especially having a close relation with fat body, metamorphic hormone, trehalose and sex hormone during development and metamorphosis[38]. Protein synthesized in the early instars of the larval fat body (the main site of protein synthesis of blood protein) are subsequently released into the surrounding blood which, in later instars are sequestered from the blood into the fat body. Regarding their synthesis have suggested that in *Drosophila* amino acids are first incorporated into peptides[73] and later enter into proteins[82].

In the present investigation, all the three sublethal doses of *A. indica* seed's acetone extract caused a significantly dose-dependent ($P < 0.001$) reduction in the level of total protein in both the tissues of the larva (Table 1). Earlier investigations have revealed that botanical insecticides (natural plant products/biopesticides) and synthetic pyrethroids influenced the biochemistry of insect pests. Application of *Annona squamosa* seed extracts caused a significant reduction in protein content in the nymphs of *Dysdercus koenigii*[5]. In a similar way, *Polyscias quilfolei*[60] extracts and azadirachtin[78] have also been reported to cause significant alteration in protein contents in certain other insects. The present results are also in agreement with findings of the above workers[5,60,78].

One of the most characteristic features of insect haemolymph is the high level of free amino acids[11,19,21,22,84,16,14,20] whereas insect fat body is an active site for the intermediary metabolism of these amino acids[33,14]. The high concentration of free amino acid is believed to play an important role in osmoregulation[8,3] ; buffering of the blood to some extent, energy production for flight and cocoon construction[84] with the predominant function of serving as units for protein synthesis[11] and taking part in other metabolic activities.

All the sub-lethal doses of *A. indica* seed'sacetone extract caused a significantly dose- dependent ($P < 0.001$) enhancement in the level of total free amino acid in both the tissues of this larva (Table 1). Active compounds extracted from seed of *A. squamosa* has enhanced the amino acid content in *Dysdercus koenigii* possibly due to this biopesticide induced depletion of protein and/or inhibition of amino acid incorporation into protein[64]. Similarly, botanical insecticides induced alterations in the free amino acid contents of *Spodoptera litura*[81]. Since, *A. indica* seed's acetone extract, in the present study, decreased the protein level in the haemolymph and the fat body of the larva

of this moth as stated earlier, it may be concluded that a rise in the total free amino acid level in both the tissues is plausibly on account of protein depletion and/or inhibition of amino acid incorporations into proteins as suggested in case of *D. koenigii* expose to *A. squamosa*[64] and *Spodoptera litura*[81] treated with botanical insecticides.

RNA content can be considered as an index of the capacity of organism for protein synthesis where as DNA content provides an estimate of cell number. The RNA/DNA ratio is, therefore, a measure of protein synthetic capacity per cell[9,37]. Literatures concerning botanical insecticides and pyrethroid induced changes in the nucleic acid levels with special reference to insects are far from adequate[69,45,79].

In the present investigation, all the sublethal doses of *A. indica* seed's acetone extract caused a significantly dose- dependent ($P < 0.01$) reduction in the levels of DNA and RNA (Table 2) and a significant reduction in RNA/DNA ratio (Table 3) in both the tissues of the larva of this pest. Botanicals (neem compounds) and pyrethroid have been shown to inhibit the nucleic acid level in *M. domestica* L.[44]. Similar findings have also been observed in case of pulse beetle, *Callosobruchus analis* L. following treatment with neem compounds NfC (Neutral fraction C- which is a crude extract of whole neem seed) and NC (Nimolicine, Azadirachtin)[79]. But, sublethal and lethal doses of fenpropathrin (a pyrethroid) did not change much of the RNA and DNA levels in the larvae of *T. castaneum* (Herbst.)[69]. The reduction in the DNA and RNA levels, in the present study, may be due to interference of active ingredient of *A. indica* seed's acetone extracts with the synthesis site of nucleic acids. Our findings are in accordance with the results of *Musca domestica* L.[44] treated with neem compounds and pulse beetle, *Callosobruchus analis* L.[79] exposed to NfC. who also reported similar possibility of the decreased DNA and RNA due to the action of neem compounds NfC and NC (Nimolicine, Azadirachtin) on nucleic acids synthesis. As stated earlier, the two parameters- RNA content and RNA/DNA ratio, show a significant correlation with protein content. Thus, the protein content depends on its synthesis in which RNA plays a vital role. Data in the present study also demonstrate the reduction in the total protein level in both the tissues of the larva following treatment with *A. indica* seed's acetone extract. Therefore, it may be presumed that the synthesis of protein is inhibited due to inhibition of RNA. It may also be presumed that reduction in protein level may be due to the involvement of *A. indica* seed's acetone extracts in influencing the transport of amino acids as well as their incorporation into the polypeptide chain. The enhancement in total free amino acid level further supports the above presumption.

The findings reported in the present work reveal that sublethal doses of *A. indica* seed's acetone extract disrupts the metabolic

balance of the larva resulting into reduced levels of total protein, DNA, RNA and RNA/DNA ratio and enhanced levels of total free amino acids. These biochemical perturbations in the metabolic framework may impair the physiological fitness of the larva and thereby contribute to the lethal action of ingradients present in *A. indica* seeds acetone extract.

ACKNOWLEDGEMENT

S.K. Tiwari is thankful to U.G.C. New Delhi for providing financial support (F.No. 34-478/ 2008, SR).

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