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EFFECT OF BIOSAL (NEEM EXTRACT) ON THE COTTON STAINER *DYSDERCUS KOENIGII* (F.) (HEMIPTERA; PYRRHOCORIDAE), ITS IMPACT ON THE CONTENTS OF DNA, RNA, PROTEIN AND CHOLINESTERASE

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ABSTRACT

Application of Biosal (neem extract) to adult cotton stainer *Dysdercus koenigii* (F.) not only caused mortality, but also reduced nucleic acid and protein contents and activated cholinesterase. The LC_{50} value of Biosal was determined to be 11.47 mg/cm^2 against *D. koenigii* (F.) using the filter paper impregnation technique. Its effects on the content of nucleic acids (DNA, RNA), proteins and cholinesterase in the LC_{50} treated insects showed a decrease to 6.74%, 43.64% and 9.72%, respectively and activated cholinesterase by 49.95%.

Key-words: Biosal. *Dysdercus koenigii,* cotton pest, DNA, RNA, Protein, Cholinesterase.

INTRODUCTION

Neem phytopesticides have been shown to have different effects on pests and their natural enemies. Abudulai *et al.,*(2005) discovered that neem extracts had little effect on predatory insects, indicating their potential for integrated pest management. Raguraman and Rajasekaran (1996) found that neem oil and neem seed kernel extract were effective against brown locusts and leafhoppers in rice and promoted recolonization by predatory spiders. However, Khosravi and Sendi (2013) discovered that neem pesticides had a significant effect on the enzymatic activities in the midgut and the biochemical compounds in the hemolymph of the small mulberry flea Glyphodes pyloalis.

The cotton stainer *Dysdercus koenigii* (F.) belongs to a group of pyrrhocorids that have long been known as cotton pests. The bio-spray plays a very important role in the control of this pest. Although it is effective at a relatively high dose, it is the least toxic to the other beneficial insects and there has never been a reported case of resistance in this pest in the field. Many researchers have described the toxicity of neem compounds, such as Koul *et al.,* (1988), who studied the effect of Margosan-O against the red cotton bug. Nurulain *et al.,* (1989) reported the effect of ethanolic extracts (RB-a and RB-b) and (Margosan-O) against *Oxycarenus lugubris* Motschulsky. Bhathal *et al.,* (1991) reported that neem oil significantly reduced the hatching of *Dysdercus koenigii* (F.) eggs. Thomas and Hiradhar (1993) observed growth regulating effects in *Dysdercus cingulatus* (F.) by neem extracts. Ahmad *et al.,* (1995) tested two neem fractions (RB-a and RB-b) against *D. koenigii* using the injection method. Fakhri and Murad (2002) tested a neem formulation, Neemjeevan 0.3 E.C., against *Dysdercus koenigi* (F.) and observed the effect on growth and development of nymphs. Application to the adult cotton dyers caused not only mortality but also a reduction in the content of nucleic acids. Ahmad *et al.,* (1979) reported toxic effects of cypermethrin RB-a against the berry bug *Halys dentatus* (F.) at 0.08µg/cm2 and 200µg/cm2 respectively. The RNA content was reduced to 36.66% and 42.78% after treatment with RB-a and cypermethrin, respectively. Azmi *et al.,* (1979) determined the toxicity of Bakayan Berry (B.B.) extract against *H. dentatus* and its effect on nucleic acid content. RNA was reduced by 2.14% and DNA by 42.52%. It seems that Biosal could be used more effectively against *D.koengii* and would prove to be a human and environmentally friendly compound.

MATERIAL AND METHOD

Collection of insects:

Adults of *D. koenigii* were collected from the premises of Karachi University campus on *Thespesia populanea* (malvaceous) plant where they lived on leaves and inside the dry fruits of the host plant which resemble cotton. These insects were kept in glass jar, covered with muslin cloth. Leaves and fruits of host plant were provided as food.

Preparation of compounds:

Five selected concentrations i.e, 0.015, 0.031, 0.047, 0.062 and 0.078 μg/cm^2 of cypermethrin (10 E.C) were prepared from 0.05% stock solution.

Method of treatment:

This insect was treated with filter paper impregnation method. Six petri dishes used in each experiment, five for different concentrations and one for control. The insecticide was applied by pipette to the filter paper, placed in petri dishes. Approximately 50 insects were released in each petri dish. A control batch of untreated insects were also kept for the determination of environmental effects and leaves of host plant also kept as a diet. Each petri dish was covered with another petri dish. Mortalities were noted after 24 h. Each of the experiment was repeated five times. The data was analyzed statistically; mortality curve was drawn on log-log graph paper to find out the LC_{50} of the tested compound.

Assay Procedure for DNA

Treated and untreated samples of 0.3mL and 0.6mL of supernatant of insects were taken for each test in separate test tubes but not in blank. The volume of the solutions was made up to 1.0 mL with 0.5 mL PCA and 3ml DPA reagent was added in each test tube as well as in blank. The solutions were mixed well and boiled in boiling water bath for about 30 minutes when blue colour appeared, absorbance was read at 595ηm against the blank. Total amount of DNA was calculated by the following formula.

$$conc\ of\ sample\ \mu\text{g/mg} = \frac{\text{O. D of sample}}{\text{O. D of std}} \times \frac{conc.of\ std}{amount\ of\ sample(in\ mgs)} \times 1$$

Assay Procedure for RNA

Treated and untreated samples of 0.2 mL and 0.4 mL of supernatant of insects were taken for each test in separate test tubes but not in blank. The volume of the solutions was made up to 1.0 ml with 0.5 mL PCA and 3 mL Orcinol reagent was added in each test tube as well as in blanks.
The solutions were mixed well and boiled in boiling water bath for about 30 minutes. When green colour appeared, then absorbance was read at 600ηm against the blanks. Total amount of RNA was calculated by the following formula:

$$conc\ of\ sample\ \mu\text{g/mg} = \frac{\text{O. D of sample}}{\text{O. D of std}} \times \frac{conc.of\ std}{amount\ of\ sample(in\ mgs)} \times 1$$

ESTIMATION OF TOTAL PROTEIN

Total protein was estimated by Lowry's method given in Lowry *et al.,* 1951.

a) Preparation of Reagents

i) Lowry's reagent A: (2% Na_2 CO_3 in 0.1 M NaOH)

Sodium Hydroxide (4gms) (Fluka, A.G.) was dissolved in distilled water. Then 20 gm of sodium carbonate was added to this solution and the volume was made up to one litre with distilled water.

ii) Lowry's Reagent B_1 (1% $CuSO_4$)

Copper Sulphate (10gms) (B.D.H) was dissolved in distilled water and the volume was made up to one litre.

iii) Lowry's Reagent B_2 (2% Na.K.tartarate)

Sodium Potassium Tartarate (20gms) (B.D.H) was dissolved in distilled water and the volume was made up to one litre.

iv) Lowry's Reagent - C

Two ml of Lowry's reagent B_2 was added to two ml of Lowry's B_1 while stirring. The solution was then mixed with 200ml of reagent A. and always freshly prepared.

v) Lowry's Reagent –E

Follin Phenol reagent (B.D.H) was diluted 1:1 (V/V) with distilled water before use.

b) Preparation of Proteins Standards

i) Stock Protein Standard (1mg / 1mL)

Bovine serum albumin (50mg. Sigma) was dissolved in distilled water and the volume was made up to 50mL.

ii) Working Protein Standard: (250µg / mL)

To 3mL of the stock protein standard solution (1mg/1mL), 9ml of distilled water was added and the contents were mixed.

c) Preparation of Standard Curve

For obtaining standard curve, 0.2, 0.4, 0.6, 0.8, and 1mL of working protein standard solution 250µg/mL were taken in separate test tubes and the volume was made up to 1mL with distilled water and 1mL distilled water was used as a blank. Then 5mL of reagent–C was added to each test tubes were incubated at room temperature for 45 minutes. Then 1ml of diluted reagent–E (1:1 V/V) was added to each tube and contents were mixed immediately the tubes by Vortex mixer model VM300 were kept at room temperature for 45 minutes again. A blue colour was developed. The absorbance was measured at 660ηm on thermospectronic spectrophotometer model Heλosε against the blank the standard was constructed by plotting optical density versus concentration.

d) Estimation of Total Protein in the Sample

0.1 and 0.2 mL of supernatant of treated and untreated samples were added to each test tube and then estimation of total protein was carried out as described for the standard.

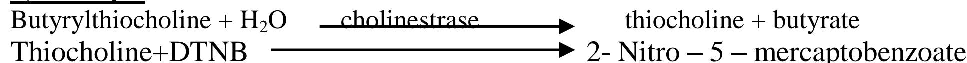
C) ESTIMATION OF ACETYLCHOLINESTERASE

Acetyl cholinesterase activity was measured through the same procedure given by Knedel and Boettagar (1967)

a) Colorimetric Method

Butrylcholinestrase hydrolyses butrylthiocholine to give thiocholine and butyrate. The reaction between thiocholine and DTNB (Dithiobisnitrobenzoate) gives 2- nitro –5- mercaptobenzoate, a yellow compound, which can be measured at 405ηm.

b) Principle



c) Reagent Composition

1Buffer / Chromogen
Phosphate buffer 50 mmol/L,PH, 7.7
DTNB
2 Substrate
Butrylthiocholine iodide 6 mmol/L.
Stability and Preparation of Reagents

i) Buffer / chromogen (solution 1)

Contents were dissolved in 30ml of redistilled water which remained, stable when stored for 6 weeks at + 2 to +8°C protected from light.

ii) Substrate (Solution 2)

The contents were dissolved in 1ml redistilled water which remained stable for 6 weeks at +2°C to +8 °C

d) Procedure

After preparation, reagents were kept for stability for 1 h at +15°C to +25°C. Room temperature for the experiment was maintained between 25°C to 30°C. A 1cm light path cuvette was taken. 1.50mL solution (1), 0.01mL sample and 0.05mL solution (2) were added and mixed. Initial absorbance of light at wavelength 405ηm against air was read immediately 0 sec, 30sec, 60 sec and 90 sec after the times started. The mean absorbance

changed per minute (A△ /min) was determined and used in the calculation. The activity of cholinesterase was calculated. The activity of cholinesterase was thus calculated by using the following formula

U/L (25°C/30°C= 11730X A △405ηm/min.

RESULTS

The toxicity of Biosal was found to be highly effective against *D. koenigii* and the LC_{50} of the tested substance was determined by plotting the average mortality values after 24 h of treatment on log-log paper as shown in Fig.1. The statistical analysis is shown in Table 1. According to Fig.1, an LC_{50} value of 11.47 µg/cm^2 was determined. Cypermethrin also had significant effects on the DNA and RNA content of *D. koenigii*. According to Tables 2, 3, 4 and 5, DNA content decreased to 6.74%, RNA content to 43.64%, protein content to 9.72% and activated cholinesterase to 49.95% in the samples treated with LC_{50}.

Table 1. Statistical analysis of toxicity of Biosal against adults of *D. koenigii* (Wild strain) after 24 hours of treatment.

Concentration (µg/cm^2)	% Average mortality $\bar{X}$	S.D. (±)	S.E. (±)	Range at 95% confidence limit Range = $\bar{X}$ ± S.E. x 2.571
Control	2	1.048	0.428	0.8991 – 3.1008
6.287	14	3.502	1.429	10.3260 – 17.6739
7.859	22	2.065	1.843	19.8326 – 24.1673
9.431	35	3.710	1.514	31.1065 – 38.8924
11.003	46	3.6696	1.498	42.1481 – 49.8518
12.575	66	2.875	1.173	62.9842 – 69.0157

$F_{0.001}$ (5,30) = 237.353 and $LSD_{0.05}$ = 3.06 (Duncan's Multiple Range Test)

Table 2. Estimation of DNA in adult *D. koenigii* after the LC_{50} treatment with Biosal.

Name of insecticides	$\bar{X}$ Mean µg/mg	S.D. ±	S.E. ±	Range = $\bar{X}$ ± S.E. x 4.303	% Decreased
Control	0.8736	0.005	0.0029	0.86105 – 0.8861	0.00
Biosal	0.8147	0.0067	0.0038	0.7979 – 0.83146	6.74

Table 3. Estimation of RNA in adult *D. koenigii* after the LC_{50} treatment with Biosal.

Name of insecticides	$\bar{X}$ Mean µg/mg	S.D. ±	S.E. ±	Range = $\bar{X}$ ± S.E. x 4.303	% Decreased
Control	18.5833	0.1301	0.0751	18.260 – 18.906	0.00
Biosal	10.4720	0.0555	0.0320	10.3341 – 10.6098	43.64

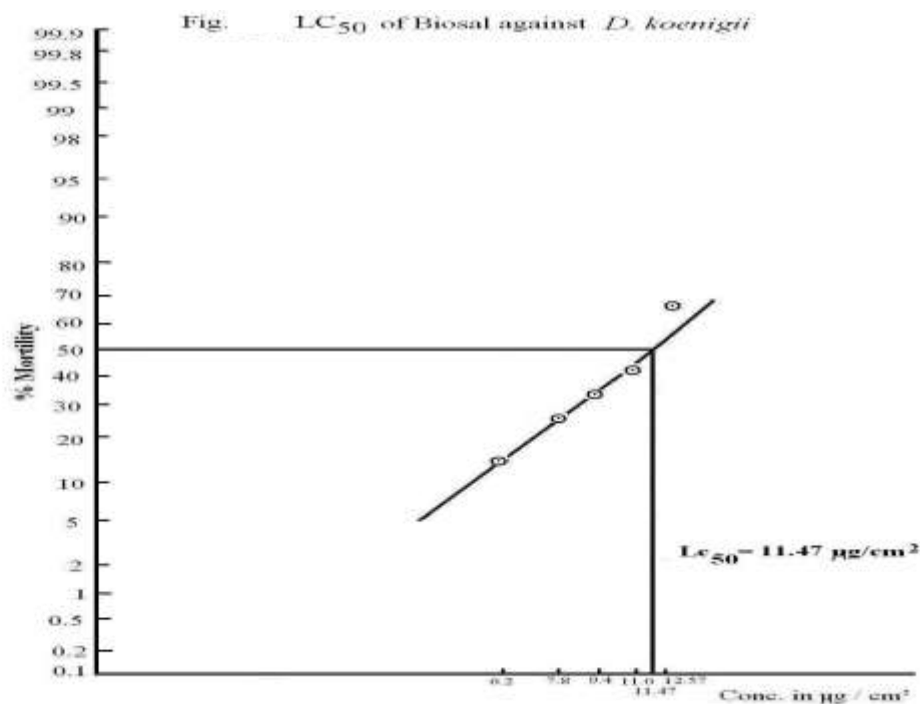
Table 4. Estimation of protein in adult *D. koenigii* after the LC_{50} treatment with Biosal.

Name of insecticides	$\bar{X}$ Mean µg/mg	S.D. ±	S.E. ±	Range = $\bar{X}$± S.E. x 4.303	% Decreased
Control	154.2857	0.7143	0.4124	152.511 – 156.060	0.00
Biosal	139.28001	1.1284	0.651	138.23731 – 142.0837	9.72

Table 5. Estimation of cholinesterase in adult *D. koenigii* after the LC_{50} treatment with Biosal.

Name of insecticides	$\bar{X}$ Mean µg/mg	S.D. ±	S.E. ±	Range = $\bar{X}$ ± S.E. x 4.303	% Inhibition
Control	62.58	6.754	3.900	45.7978 – 79.362	0.00
Biosal	93.84	-	-	-	+49.95

+ = Activation



DISCUSSION

The biosal is a neem product and active ingredient of biosal is azadirachtin. Many scientists investigated effect of neem product i.e., antifeedant, insecticidal, metamorphosis disruption, growth disruption, ovipositional deteterent/ovicidal, influencing fertility/ reproduction, insecticide synergisation and performance in practical insect control and crop response, against insects. The present investigation revealed that the toxicity of biosal against *D. koenigii* was 11.47 mg/cm^2 . This was higher concentrations to show the effectiveness of this compound as toxic agent against this bug. Because insecticidal performance of neem products against most insects is not as dramatic as that of synthetic insecticides and for equivalent, effectiveness, considerably higher doses are required.

Among them Ahmad *et al*. (1995) observed the efficacy of RB-a and RB-b, the two neem fractions on comparison with cypermethrin against *Dysdercus koenigii.* The LC_{50} for RB-a was 2.8%, for RB-b was 3.2% and of cypermethrin was found to be 0.00052%. The same author Ahmad *et al*. (1997) also reported the effect of cypermethrin and RB-a (neem extract) on toxicity and nucleic acid content of *Halys dentatus* and observed the mortality after 24h of treatment. The LC_{50} value of cypermethrin was found to be 0.08µg/cm^2. The RNA and DNA contents were decreased after insecticide treatment. This decreased was higher in cypermethrin than in RB-a (neem extract), On the same insect Azmi *et al*., (1997) studied the effect of cyflurin (pyrethroid) and Bakayan Berry *Melia azedrach* extract on nucleic acid of *Halys dentatus*. They found the LC_{50} value of cyfluthrin b as 0.1 µg/cm^2, and RB-a extract as 150 µg/cm^2.The DNA and RNA decreased after the treatment of these compounds. Ahmad *et al*., (1998) estimated quantitatively the DNA content in the ovaries and testes of legume bug *Piezodrous hybneri* in untreated and cypermethrin treated insects. The LC_{50} concentrations(0.004%) of cypermethrin caused significant effect on DNA of ovaries and testes. The DNA contents decreased up to 40.57% and 54.12% in ovaries and testes of *Piezodrous hybneri* respectively. Ahmad and Perveen (1992) estimated DNA content in testes of cotton stainer *Dysdercus koenigii.*

Koul (1988) studied the effect of neem formulation, Margosan-O against red cotton bugs. He observed that continuous treatment with Margosan-O produced significant effect in red cotton bug, while at 100 ppm complete mortality of larvae occurred. This showed that these insects also appeared to be susceptible to Margosan-O which could be used in its control. Nurulain *et al.* (1989) reported LD_{50} of two ethanolic crude extracts of neem (RB-a and RBA) and neem origin insecticide (Margosan-O) against *Oxycarenus lugubris*, in laboratory. The LD_{50} of these compounds were found to be 5.8%, 9.2% and 0.017% respectively. Ahmad *et al*. (1995) tested the two neem fractions (RB-a and RB-b) against *D. koenigii* by using injection method. The LC_{50} of RB-a and RB-b were found to be 2.8% and 3.2% respectively.Heyde *et al*. (1984); and Siddiqui *et al*. (2003) worked on the neem oil and neem extract as potential insecticides for control of many insects. Rembold *et al*. (1987) described structure and biological

activity of azadirachtin. Koch (1982) reported that crude methanolic neem seed extracts caused morphogenetic, effects in *D. fasciatus,* after topical applications. Osche (1982) studied the result of topical applicantion of crude extracts of neem on the metamorphosis of *D.fasciatus*. All stages from 2nd to 5th nymphal instars proved susceptible and effects were noted either on the stage that was treated or on the subsequent stage. The results were often fatal, asymmetry of the body was especially conspicuous after moulting. When 5th instar nymphs were treated topically, the fecundity to the resulting adults and hatching of the larvae from newly laid eggs of treated insects was lowered. Koul (1984a, 1984b) tested the azadirachtin-I interaction with the development of *D. koenigii* and azadirachtin-ll interaction with reproductive behaviour which impaired embryogenesis in *D. koenigii*. Dorn *et al*., (1986) worked on the effect of azadirachtin on the moulting cycle of endocrine system and ovaries in the last instar larvae of *Oncopeltus fasciatus* and found that low doses of azadirachtin injected newly moulted last instar larvae of the *O. fasciatus* prolonged the intermittent intermoult stage. Medium and high dose suppressed adult ecdysis and the larvae became permanent larvae, the longevity of which increased after using the dose. Although medium dose prevented ecdysis apolysis and secretion of adult cuticle took place. Bhathal *et al*., (1991) reported that neem oil considerably reduced hatching of eggs of different age groups of *D. koenigii*. Neem oil both at 5000 ppm and 2500 ppm proved to be effective ovicidal for the eggs of young age groups. Thomas and Hiradhr (1993) observed growth regulatory effects in *D. cingulatus* by *Azadirachta indica.* Neem extracts interfered with the reproductive performance in dose dependent time specific manner. Fifth instar exhibited a pronounced influence compared to treatment of adult females. The inhibitory effect of these compounds on oocytes differentiation, fecundity hatchability and sterility suggested a general imbalance in the physiological pathways co-relating them. As these were hormone regulatory processes and an alteration in hormone titer could be implicated. Fakhri and Murad (2002) tested a neem formulation Neemjeevan 0.3 E.C. against *D. koenigii* by ingestion method. There different concentrations i.e., 0.02%, 0.03% and 0.04% affected the growth and development of nymphs. Shannag *et al.*, (2014) tested Neem-based formulations were highly effective in aphid population suppression.

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