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Effects of a Neem Sample on Protein Patterns of *Bactrocera cucurbitae*

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Abstract: *Bactrocera cucurbitae* (Coquillett) adults were reared with 5% honey solution, sugar, and protein-hydrolysate. Females were provided to lay eggs on larval rearing media in a bottle gourd. Twenty third-instar larvae of the same size and age were exposed to filter papers treated with varying doses of a neem sample in 90 mm diameter petri dishes for 24 h. A probit-mortality curve was drawn to determine the LC_{50} of the sample. LC_{50} dose of the sample was calculated as 5.6%. Thin layer chromatography was used to determine the effects of neem sample on the protein patterns of the treated insect. The Rf-values of separated proteins (peptides) were determined and compared to those of the untreated control.

Key Words: *Bactrocera cucurbitae*, insect rearing, protein patterns, *Azadirachta indica*

Introduction

With their ability for rapid distribution, high rate of reproduction, vast range of host plants, and good ecological adaptability, fruit flies are posing a large problem all over the globe. They are a big menace in vegetable and fruit production (Vargas and Carey, 1990; Khan et al., 1999). In Pakistan, fruit flies cause a loss of around 7 million Rupees to growers, annually (Khan et al., 1999).

The melon fruit fly, *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae), is distributed widely in temperate, tropical, and sub-tropical regions of the world. It has been reported to damage 81 host plants and is a major pest of cucurbitaceous vegetables. The extent of losses varies between 30% and 100%, depending on the cucurbit species and season. Its abundance increases when the temperature falls below 32 °C and the relative humidity ranges between 60% and 70% (Dhillon et al., 2005).

Fruit flies lay eggs inside the fruits, and upon hatching larvae start feeding on the pulp, thus rendering them unfit for human consumption. Once egg laying has taken place, chemical eradication becomes difficult. Therefore, flies can only be controlled either at the adult stage when they start hovering over the vegetation or just before pupation when the third-instar larvae come out of the infested fruit and are about to enter the soil for pupation (Agarwal et al., 1987).

Almost all the parts of the neem tree, *Azadirachta indica* (A.Juss), have some biologically deterrent activity against many insect pest species. Nevertheless, neem fruits have been proven to be the tree's main agent for combating pest insects (BOSTID, 1992; Schmutterer, 1995). Moreover, resistance does not develop in insects against neem (Vollinger, 1987, 1992, 1995; Naqvi and Tabassum, 1992). Many neem extracts were reported to have pesticidal activity (Naqvi, 1996). Naqvi et al. (1996a) reported LD_{50} as 0.6 and 0.64 µg/mg media against the melon fly *B. cucurbitae* for RB-b and RB-a neem extracts, respectively. The LC_{50} of RB-a (neem extract) by contact method on impregnated filter paper has been reported as 0.01% (Yasmin et al., 1995) and RB-b (neem extract) by feeding method has been reported as 17 µg/ml food (Khan et al., 2007) against fruit fly adults (*Drosophila* sp.).

Since most fruit fly infestations occur on commodities that are consumed without cooking, pesticide spray hazards remain an important concern. Therefore, use of a safe controlling strategy is of prime importance. Hence, it was aimed to test a neem sample against fruit flies as

an alternate to synthetic pesticides. It is well known that fruit fly larvae drop to the ground and pupate inside the earth; therefore, if the earth is treated underneath the plant canopy, the next generation could be checked by the reduction in the third-instar larvae pupating phase. In this study, third-instar *B. cucurbitae* larvae were treated with a neem sample and its effects on protein patterns were studied.

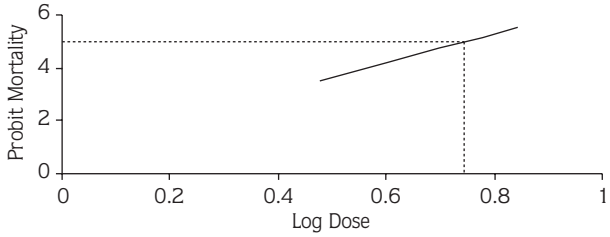

Figure. Mortality curve of *B. cucurbitae* under the effects of neem sample.

Materials and Methods

Rearing of *Bactrocera cucurbitae* was carried out in insectary at temperature 25 ± 3 °C, 60 ± 5% relative humidity and 10:14 light:dark h photoperiods. Adult flies were provided with 5% honey solution, sugar, and protein-hydrolysate ad libitum. Water was provided in soaked cotton pads. The egg laying was achieved directly on the larval rearing medium, e.g., bottle gourd. Jumping out third-instar larvae of *B. cucurbitae* were collected for the exposure to the neem sample obtained by the courtesy of Professor Dr. S.N.H. Naqvi of Baqai Medical University, Karachi. The sample was a fraction of neem seed kernel oil containing around 0.3% azadirachtin. Dr. S.N.H. Naqvi communicated that the strength of the sample with azadirachtin was determined on LC MS at HEJ Research Institute of Chemistry, University of Karachi, Karachi. Effects of neem sample were tested by the contact method. Therefore, filter papers of the same size were placed in each 90 mm diameter petri dish and impregnated with the desired dose, i.e. 3%, 4%, 5%, 6%, and 7% of neem sample, and left overnight to dry. Then 20 insects of about the same size and age were released inside the petri dishes and kept there for 24 h. The insects were kept starving during the exposure period. Mortality count was performed after 24 h of treatment, according to Khan and Ahmed (2003) with some minor modifications. Each experiment was carried out in triplicate and the average values of the 10 experiments were analyzed. A probit-mortality curve was drawn and LC_{50} was calculated through probit analysis (Finney, 1964) and presented in the Figure and Table 1, respectively.

To determine the effects of the test compound on protein patterns in the treated insects, thin layer chromatography (TLC) was employed as described by Khan and Ahmed (2000). A day before the TLC assay, 30 insects were treated with LC_{50} of the respective test compound. The next day, 10 live insects were taken from each treated batch for TLC assay. Simultaneously, 10 untreated insects were taken as controls. All insect

Table 1. Toxicity of neem sample against *Bactrocera cucurbitae*.

S. No.	Dose	No. of Insects Exposed	No. of Insects Dead	Corrected % Mortality	Log Dose	Expected Probit
1	3%	50	07	11.34	0.477	3.48
2	4%	50	08	13.40	0.602	4.18
3	5%	50	17	31.96	0.699	4.72
4	6%	50	34	67.01	0.778	5.16
5	7%	50	35	69.07	0.845	5.54
6	Control	100	03	-	-	-

Regression Equation: Y = 4.77339+ 5.578758 {x – (0.7075042)}

χ^2 = Chi square (Heterogeneity factor) = 6.663903

LC_{50} = 5.599% {range at 95% limits 5.2-6.04}

LC_{90} = 9.497% {range at 95% limits 7.9-11.37}

LC_{99} =14.648% {range at 95% limits 10.97-19.57}

batches were crushed separately with the help of a mortar and pestle with 2.5 ml of methanol, and then homogenized in a Teflon Pyrex tissue grinder for 5 min at 1000 rpm. The homogenized samples were centrifuged at 3000 rpm for 30 min. The supernatants thus obtained were collected in test tubes. Ten microliters of each sample was spotted on the chromo-plate coated with a 500-micron layer of silica gel. The plate was kept for around 45 min in the chamber containing methanol as mobile phase solvent. Thereafter, Ninhydrin 1% solution was sprayed and the plate was kept in an oven for 5 min at 80 °C to develop bright protein spots. The Rf values of different metabolites (peptides) were calculated and are presented in Table 2.

Table 2. Rf values of different proteins of *B. cucurbitae* in neem treated and control batches.

Protein	Rf value	
	Control	Treated
I	10.230	-
II	-	7.823
III	-	3.800
IV	3.325	-
V	-	3.166
VI	2.557	Absent
VII	1.385	-
VIII	-	1.371
IX	1.291	-
X	-	1.266
XI	1.209	-
XII	-	1.187

Results and Discussion

The LC_{50} was calculated as 5.6% while the log probit regression line was calculated as Y = 4.773 + 5.579 (x-0.7075) with heterogeneity factor (chi square) 6.66 (Table 1 and Figure). Nurulain et al. (1994) studied the toxic effect of crude neem seed kernel extract (RB-a) against *Musca domestica* (PCSIR strain) and found its LD_{50} to be 5.5 µg/fly. Naqvi et al. (1989) worked on RBU-9, RB-b (neem extracts) and Margosan-O™ against white flies *Aleurobus barodensis* in field conditions and found Margosan-O™ to be more effective than the 2 crude extracts of neem. Naqvi et al. (1994) studied neem fractions against fourth-instar larvae of *Aedes aegypti*. They reported LC_{50} values of 350, 490, and 340 ppm for RBU-9, RB-b, and Margosan-O™, respectively. Naqvi et al. (1995) determined the quantity 154.5 ppm as the LC_{50} of Margosan-O™ against fourth-instar *Culex fatigans*. Presently, the LC_{50} value of neem sample was found to be 5.6%, which is much lower than that reported by Naqvi et al. (1994); this difference could be due to the difference in insect species and life stage of insects. Nurulain et al. (1989) reported the same pattern against *Oxycarenus lugubris* in laboratory trials with LD_{50} of Margosan-O™ as 0.0171% in comparison with malathion (0.0039%). Jahan et al. (1990) reported the LC_{50} of Margosan-O™ against *M. domestica* to be 0.0018%. Naqvi et al. (1995) reported that the LC_{50} of Margosan-O™ was 0.25 µg/insect against the adult housefly in comparison with 2 neem fractions (NC = 0.85 µg/insect and H-34 = 1.8 µg/insect). Naqvi et al. (1996a) reported LD_{50} values of 0.6, 0.64, and 0.96 µg/mg media for RB-b, RB-a (neem extracts), and Azodrin, respectively, against fruit flies. In our study, the LC_{50} value of neem was 5.6%, which is higher than that reported by Naqvi et al. (1996a, 1996b) and other authors. These differences could be due to differences in insect populations, life stage of insects, and the neem samples tested as Nurulain et al. (1997) reported 1.8, 0.56, and 0.25 µg/fly LD_{50} values of H-34, N6-b (neem extracts), and Margosan-O™ against houseflies. This report confirms toxicity variations between different neem samples. Sharma et al. (1984) reported that 0.1% (1000 ppm) of methanol soluble fraction of fresh kernel caused 78% larval mortality of *Mythimna separate* (Walker). Sombatsiri and Tigvattanont (1984) reported that 0.1% methanolic neem seed kernel extract produced 91.4% mortality of *Schistocerca* sp. in the third-instar larvae as compared to 30% mortality of *Plutella xylostella* L. in the fourth-instar larvae. Sombatsiri and Temboonkeat (1987) reported LC_{50} values of second and fourth-instar larvae of diamond back moth *P. xylostella* treated with aqueous extract as 0.84% (8400 ppm) and 0.86% (8600 ppm), respectively. These findings are in accordance with the present findings; some variations in doses may be due to variations in the type of extract or the insect used. Yasmin et al. (1995) determined the toxic dose of RB-a (neem extract) against adults of *Drosophila melanogaster* by contact method and calculated LC_{50} as 0.01% at 24 h of post treatment from the mean values on log probit graph paper. Similar reports have been published by Akhtar et al. (1987), Jahan et al. (1990), Tabassum et al. (1994), Munir et al. (1997), Naqvi et al. (1995, 1996a, 1996b, 1999), and Khan et al. (2007).

The above reports are generally in accordance with the present report, with some variations that could be due to differences in insect species, life stage of the subject, and nature of the sample tested.

Ahmed and Naqvi (1985), Rizvi et al. (1986), Yasmin et al. (1994), and Ganesalingan (1993) studied the effects of various pesticides on protein patterns using TLC. The same technique was employed in the present study. With respect to the Rf values, control and treated insect batches, all proteins were designated in Roman numerals (Table 2). Protein I (Rf-value 10.230) was detected in the control, but was absent in the treated insect batch. However, proteins II and III appeared with Rf values 7.823 and 3.8 in the treated insect batch only, which were probably the metabolites of protein I under the effects of the neem sample. Similarly, protein IV (Rf value 3.325) appeared in the control and was absent in the treated insect batch, which probably degraded under the effects of treatment and appeared as protein V (Rf value 3.166). Protein VI (Rf value 2.557) and protein VII (Rf value 1.385) appeared only in the control and were absent in the treated insect batch. However, protein VI was either completely degraded or stunted under the effects of the treatment. Proteins VII (Rf value 1.385), IX (Rf value 1.29), and XI (Rf value 1.209) appeared only in the control and proteins VIII (Rf value 1.371), X (Rf value 1.266), and XII (Rf value 1.187) were detected only in the treated insect batch; later proteins might be metabolites of the former proteins, under the effects of the neem sample. Naqvi et al. (1992), Naqvi and Schmidt (1993), Naqvi et al. (1995), Naqvi and Aslam (1996), and Khan and Ahmed (2000) reported that neem exerts some phagodeterrent and growth regulator effects, which disrupt insect growth. Those reports indicate probable effects of neem compounds on proteins. The present study, based on TLC, therefore confirms the effects of neem compounds on various proteins.

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