Pakistan J. Zool., vol. 43(6), pp. 1079-1084, 2011.

Laboratory Evaluation of Chitin Synthesis Inhibitors (Diflubenzuron and Buprofezin) Against *Aedes aegypti* Larvae From Lahore, Pakistan

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Abstract.- Dengue fever is a serious problem in Pakistan from the last few years. It is important to develop new control measures for the effective control of dengue vectors. Laboratory evaluation of two insect growth regulators, Diflubenzuron and Buprofezin 25% WP (wettable powder), chitin synthesis inhibitors, was carried out against *Aedes aegypti* (early 4th instars) larvae collected from natural containers (used tiers and plastic jars) at Lahore Pakistan. The primary objective was to determine the minimum effective dosage of each for larval mortality along with pupae reduction and adult emergence inhibition (EI). Laboratory bioassays with Buprofezin indicated LC_{50}-LC_{95} ranged 0.341-2.54ppm at 48h post exposure. Pupae formation was completely inhibited at 48h and 120h post exposure against 10 and 1 ppm respectively while 80% pupae reduction was observed against 0.0001ppm one week post exposure. No adults emerged against upto 1ppm, and 96% adult emergence was inhibited against 0.1ppm one week post exposure. In contrast LC_{50}-LC_{95} ranged 29-83ppm at 48h post exposure against Diflubenzuron indicated less effect at larval stages as compared to Buprofezin. Maximum pupae inhibition (100%) and adult emergence inhibition (EI) was observed against 1ppm and 0.1ppm diflubenzuron respectively at one week post exposure. Results indicated buprofezin cause high larval mortality while the effect of diflubenzuron was more toxic to pupae and adults emergence. Further studies in natural conditions are in progress to control dengue vectors in Pakistan.

Keywords: *Aedes aegypti*, Diflubenzuron, Buprofezin, Dengue fever, Pakistan

INTRODUCTION

Mosquitoes are important vector of human and animal diseases. The three genera of mosquitoes, *Anopheles*, *Aedes* and *Culex*, are the primary vectors for various pathogens. Human malarial parasites (*Plasmodium*), the filarial parasites (*Waucheria bancrofti* and *Brugia malayi*), and a number of arboviruses (yellow fever, dengue, encephalitis) are the major cause of human mortality and morbidity in the world.

Mosquitoes belonging to the above mentioned three genera are abundant in Pakistan. *Aedes aegypti* is an important vector of dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). *Ae. albopictus* commonly known as Asian tiger mosquito is also a competent vector of many viruses including dengue (CDC, 2001) and Eastern equine encephalitis (Mitchell *et al*., 1992). Both species commonly occur in the tropical and subtropical regions such as Eastern Mediterranean, Southeast Asia (India, Pakistan, Indonesia, Somalia, Malaysia, Philippines, Thailand and Japan etc), North and South America, Africa and Europe (O'Meara, 1997).

Almost half of the global population estimated to be at risk of dengue infection. An estimated 50 million dengue infections occur annually and approximately 2 – 5 billion people live in dengue endemic countries. It has been identified as a re-emerging disease in Southeast Asia. Revision of the International Health Regulations (WHA, 58.3:2006), stated that dengue may constitute a public health emergency of international concern.

Dengue is a flavivirus having four serotypes; DEN-1, DEN-2, DEN-3 and DEN-4 (Gubler and Kuno, 1997). All four dengue viruses are known to be present in Thailand, Philippines, Indonesia, Malaysia, Singapore, Vietnam and Burma (Russel *et al.*, 1969).

There is no vaccine or specific treatment for dengue to date. Therefore, control of DF/DHF depends on controlling the mosquito vectors. The use of larvicides is one of the vector control options, particularly in urban areas where indoor spraying of insecticides is not feasible and vector control operation mainly relies upon antilarval methods. During the past few years, considerable progress has been made in the development of natural and

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0030-9923/2011/0006-1079 $ 8.00/0

synthetic compounds, which affect the process of growth, development and metamorphosis of the target mosquito's species, known as insect growth regulators (IGRs) (Mulla, 1995; Graf, 1993). Diflubenzuron and Buprofezin interfere with chitin synthesis and are taken up more by ingestion rather than by contact. When the active ingredient is ingested by larvae, it disrupts the development of exoskeleton, resulting in death of the larvae (Ware, 2000; Ishaaya and Horowitz, 1998).

In Pakistan, DF/DHF outbreak occurred in 2006 reporting more than 5,522 cases by Nov. 2006, with more than 2,000 positive cases and 50 deaths, mostly (43) in Karachi. In 2008 seven deaths and more than 900 cases were reported from Lahore only. Recently in 2010 out of 36 districts in Punjab, 34 were reported with dengue cases along with many deaths. Therefore, dengue situation in Pakistan is alarming with tremendous risk of an epidemic.

There is no pre-existing information on mosquito vectors of dengue and their control in Pakistan. Current study was designed to evaluate two insect growth regulators, Buprofezin 25% WP, and Diflubenzuron, (chitin synthesis inhibitors) for the control of *Aedes aegypti* (early 4th instars) larvae collected from natural containers (used tiers and plastic jars) at Lahore, Pakistan. The primary objective was to determine the minimum effective dosage of each for larval mortality along with pupae reduction and adult emergence inhibition (EI).

MATERIALS AND METHODS

Mosquito samples

Field collected 4th instar larvae of *Ae. aegypti* were used for laboratory assays. The larvae were periodically collected from natural and artificial containers at Govt. College University, Lahore during July to November of 2007 and 2008. These larvae were reared in insectory using standard WHO procedure.

Diflubenzuron and Buprofezin

Diflubenzuron [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)] urea EC (Emulsifiable concentration) Pestanal Analytic® by standard Sigma Aldrich Corporation, and Buprofezin 25% WP (wettable powder), insect growth regulators (IGRs: chitin synthesis inhibitors) were used to evaluate efficacy of wild caught laboratory-reared early 4th instars of *Ae. aegypti* in laboratory.

Mosquito rearing and maintenance

Wild caught *Ae. aegypti* were reared in the laboratory under standardized conditions at 27±3ºC and 80±3% relative humidity (RH) and a photoperiod of 16:8 h (L:D). Larvae were maintained in batches of 200, each in 1000 ml of distilled water in steel trays (35x30x5 cm). Each batch of 1st instar larvae were fed on a yeast suspension in a steal pan to a final concentration of 0.02%. The second instart larvae were fed with finely ground powdered fish food available from local market whereas 3rd and 4th instar larvae were fed course fish food. Fish food was applied on the surface of water and allowed to spread evenly until pupae formation which is 9-12 days post hatching. Adults emerging within 24 h were maintained in cages of 30 cm^3 and fed on 10% glucose solution and water. Nulliparous females of the same age (4-6 days post emergence) were fed periodically on restricted albino mice to maintain the colony.

Laboratory bioassays

Bioassays were performed with 7 different concentrations of Buprofezin 25% WP and Diflubenzuron (100, 10, 1, 0.1, 0.01, 0.001, 0.0001 ppm). Stock solution (100 ppm) of technical grade of Diflubenzuron was prepared in acetone and distilled water by dissolving 50 mg of Diflubenzuron in 20 ml of acetone and then diluted with 480 ml of distilled water. Stock solution (100 ppm) of Buprofezin 25% WP was directly prepared in distilled water. From this stock solution appropriate serial dilutions *viz.* 10, 1, 0.1, 0.01, 0.001, 0.0001 ppm were made. Each concentration was replicated three times and three untreated cups were used as control in order to determine their active range and to find the minimum effective dose.

For bioassays, 25 fourth instar larvae of *Ae. aegypti* were placed in disposable cups (7.8 cm diameter) containing 200 ml of distilled water. Mortality in each concentration was assessed 24h post-treatment. The larvae were considered dead if

immobile and were unable to reach the water surface. Dead larvae were separated from the live larvae with the help of small brush. Pupae and adults emergence was recorded in each concentration. Larval mortality in treated cups was corrected for any mortality in corresponding controls.

Data analysis

Data from all replicates were pooled and analyzed using computer software SPSS for Probit-regression analysis to estimate the dosage response of exposed larvae (Finney, 1971). LC_{50} and LC_{95} were determined in each group. The efficacy of a formulation against tested mosquito was assessed as mean percent larval mortalities, pupae reduction and adults emergence inhibition and adjusted for any larval or pupal mortalities in corresponding controls with the formula of Mulla *et al.* (1974):

% inhibition of emergence = 100-100(T/C) where T is percent emergence in treated groups and C is percent emergence in control groups.

Mean percent larval mortalities, percent pupae reduction and adults emergence inhibition caused by the two formulations was analyzed by one way ANOVA with Turkey's test for significance difference at 95% CL using computer software MINITAB (MTB12ST).

RESULTS

Laboratory bioassay with buprofezin 25% WP against early 4th instars of *Ae. aegypti* wild caught laboratory reared indicated LC_{50} 20.72 ppm and LC_{95} 76.1 ppm at 24 h post exposure. However, LC_{50}- LC_{95} drop dramatically (LC_{50} 0.34 ppm and LC_{95} 2.54 ppm) at 48 h post exposure indicating that the toxic effect was high with this exposure. In addition LC_{95} 1.67, 0.86, 0.58, 0.58 and 0.58 ppm was observed at 72, 96, 120, 144 and 168 h post exposure, respectively (Table I). These results indicated that action of buprofezin was highly effective by 96-144 h post exposure where LC_{95} dropped from 0.86 to 0.58 ppm at 24 h -120 h post exposure.

Mean percent larval mortality was 99±0.57, 98±1.7, 84±1.6, 67±1.0, 63±1.0, 59±1.0, 51±0.9 against 100, 10, 1, 0.1, 0.001 and 0.0001 ppm buprofezin, respectively (Table II).

Pupae formation was completely inhibited against 100, 10 and 1ppm, while 80% reduction was observed against as low as 0.0001 ppm. No adult emerged by 100-1ppm, while 96 and 92% adults emergence was inhibited against 0.1, 0.01 ppm, respectively (Table II). These results indicate that buprofezin is highly effective at low doses. No adult emerged in the presence of 1 ppm and 80% adult emergence was inhibited in the presence of 0.0001 ppm (Table II).

Table I.- Susceptibility of wild caught laboratory-reared *Aedes aegypti* larvae (early 4th instars) against diflubenzuron and buprofezin post one week exposure.

Time in hours	Diflubenzuron		Buprofezin	
	LC_{50}	LC_{95}	LC_{50}	LC_{95}
24	39	121.2	20.72	76.1
48	29	103.0	0.34	2.54
72	13	83.0	-	1.67
96	1.8	16.04	-	0.86
120	-	13.0	-	0.58
144	-	1.62	-	0.58
168	-	0.59	-	0.58

Probit-regression analysis with diflubenzuron indicated LC_{50} - LC_{95} 29-103 ppm after 48h exposure whereas LC_{95} was 1.62 and 0.59ppm after 144 and 168h, respectively (Table I). Mean percent larval mortality was 96±1.84, 78±2.39, 72±2.83, 54±1.83, 50±1.83, 41±1.3 and 32% ±0.97 against 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ppm, respectively (Table II). Hundred percent pupae reduction was observed in the presence of 1 ppm, while 68% pupae reduction was observed with 0.0001 ppm. Adult emergence was completely inhibited with 0.1 ppm while 84% adult emergence inhibition was observed in the presence of 0.0001 ppm (Table II). These results indicated diflubenzuron was more toxic at adult stage compared to burofezin which is more toxic to larvae (Table II). On comparison of the two IGRs there was no significant difference ($P > 0.05$) observed with regard to mean percent larval mortalities ($P = 0.235$), percent pupae reduction ($P = 0.498$) and percent adult emergence inhibition ($P = 0.679$) (Table II).

Table II.- Mean percent larval mortality, percent pupae reduction (% PR) and adults emergence Inhibition (%EI) of *Aedes aegypti* post one week exposure to diflubenzuron and buprofezin.

Concentrations in ppm	Diflubenzuron			Buprofezin		
	Mean % larval mortality	% PR	%EI	Mean % larval mortality	% PR	%EI
100	96 ±1.84	100	100	99 ±0.5	100	100
10	78 ±2.39	100	100	98 ±1.7	100	100
1.0	72 ±2.83	100	100	84 ±1.6	100	100
0.1	54 ±1.83	88	100	67 ±1.0	92	96
0.01	50 ±1.40	80	92	63 ±1.0	88	92
0.001	41 ±1.3	80	88	59 ±1.0	84	84
0.0001	32 ±0.97	68	84	51 ±0.9	80	80

DISCUSSION

There is no previous study related to the effect of IGRs against any species of mosquito in Pakistan. However, many studies against different IGRs such as diflubenzuron, pyriproxifen, and methoprene have been reported on the control of different species of mosquitoes in other areas of the world (Ali *et al.,* 1995; Nayar *et al.,* 2002; Arredondo-Jinenez and Valdez-Delgado, 2006; Jambulingam *et al.,* 2008).

The present study documents excellent larvicidal effect and high toxicity for adult emergence against two chitin synthesis inhibitors (diflubenzuron and buprofezin), at various concentrations against wild caught early 4th instars larvae of *Ae. aegypti* in the laboratory. Overall effect was dose dependent for both IGRs used in this study. Higher doses (100, 10, 1 ppm) caused high larval mortalities and completely inhibited adult emergence, while the minimum dose (0.0001 ppm) also caused acute toxicity (> 80% adult emergence inhibition). However, diflubenzuron was more toxic at adult stage where complete emergence inhibition (EI) was observed against 0.1 ppm as compared to 1ppm buprofezin. In contrast mean percent larval mortality was high (> 50% against 0.0001 ppm buprofezin as compared to 32% against the same dose of diflubenzuron). These results indicated buprofezin caused high larval mortality, while diflubenzuron was more toxic to pupae and adults emergence.

There is no previous report against buprofezin in controlling any species of mosquitoes. It is mainly larvicide and acts through the inhibition of chitin formation, resulting in abnormal endocuticular deposition which causes abortive molting and finally death of the insect pest (Izawa *et al*., 1985). In China, buprofezin has been reported highly effective against homopteran pests such as the plant hopper, with very low risks to the environment and humans (Asia *et al.,* 1985; Nagata, 1986).

Effects of buprofezin was studied in laboratory on survival and development of immature *Chrysoperla rufilabris* (Neuroptera: Chrysopidae) where higher concentrations of buprofezin (500 ppm, 1000ppm) reduced survival rates (17-47%) and prolonged development from first instar (treated at this level) to adult emergence by 2-3 days. The present study documents excellent larvicidal efficacy with 100% pupae reduction and adults emergence inhibition as low as against 1 ppm dose of buprofezin while 80% adults emergence inhibition was observed against minimum concentration of 0.0001 ppm and the remaining 20% adults emergence was delayed 2-3 days than control.

In a comparative study pyriproxifen induced 52.7-100% at 0.02 ppm and 93-100% at 0.05 ppm emergence inhibition in the laboratory against late 3rd – early 4th instars larvae of *Ae. albopictus* after one week of treatment (Nayar *et al*., 2002). In addition authors also reported that pyriproxifen was more active in terms of magnitude and duration of activity at equal concentrations of active ingredients of the two IGRs: pyriproxifen and s-methoprene. In our study 88-80 % pupae reduction and 92% adults EI was observed in the presence of 0.01 ppm buprofezin and diflubenzuron one week after exposure which is slightly lower than the above

mentioned study in laboratory. It could be due the difference in the species of mosquitoes and different class of IGRs.

According to Fournet *et al.* (1993) diflubenzuron cause higher larval mortality than OMS 2017. They observed 17% larval mortality against 0.001mg/liter of diflubenzuron in laboratory bioassays while in this study 41% larval mortality was observed against the same treatment. According to their study 30% emergence inhibition was observed in 0.00026mg/liter, whereas in current study >80% adults emergence inhibition occurred against 0.0001mg/liter of both IGRs one week after exposure

Compared to other IGRs like methoprene cause higher mortality in early 4th instars larvae and newly formed pupae of *Ae. aegypti*, Diflubenzuron promoted mortality mainly during larval-pupal intermediate form (Silva and Mendes, 2002). In current study diflubenzuron was found highly effective for inhibition of adults emergence, where complete (100%) adult emergence inhibition was observed in the presence of 0.1 ppm

In conclusion, *Ae. aegypti* was highly susceptible to buprofezin and diflubenzuron (chitin synthesis inhibitors) in the laboratory conditions. Furthermore, the effective dose to kill 100% of the larval population and adults emergence inhibition in the laboratory has been shown to be extremely low. These IGRs may have great potential for inclusion in integrated vector management operations. Further studies on integrated control in natural population are recommended for *Aedes* mosquitoes to control dengue disease in Pakistan.

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(Received 10 December 2010, revised 11 January 2011)