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Effects of NeemAzal on marker enzymes and hemocyte phagocytic activity of larvae and pupae of the vector mosquito *Aedes aegypti*

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ABSTRACT

Many of the neem based botanical biocides are currently studied to a greater extent because of the possibility of their use in eco-friendly control of pests and vectors. However, no report was available to assess the impact of neem based formulation, NeemAzal on marker enzymes and hemocyte mediated cellular immune responses of important vector mosquito *A. aegypti*. The NeemAzal found to exert larvicidal and pupicidal activities against *A. aegypti* developmental stages. The pupae appear to be more susceptible to the treatment. Further, a significant increase in the level of total protein (31%), α-carboxylesterase (121%), β-carboxylesterase (46%), acid phosphatase (62%) and alkaline phosphatase (37%) was observed in larvae upon exposure to NeemAzal. Moreover, treated pupae showed increased level of acetylcholinesterase (116%) and acid phosphatase (43%) while α-carboxylesterase (34%), β-carboxylesterase (12%) levels were simultaneously decreased, and no significant changes in alkaline phosphatase were noticed. Qualitative analysis also revealed that the exposure considerably modulated the larval β-carboxylesterase isoenzyme profile whereas little changes were noticed on phosphatases. On the other hand hemocyte viability of larvae (18%) and pupae (16%) as well as phagocytic ability of larval (48%) and pupal hemocytes (44%) against yeast target was significantly reduced upon NeemAzal exposure. We demonstrated for the first time that the NeemAzal differentially affected the marker enzymes and created immuno-suppressive state by reducing the phagocytic ability of hemocytes of larvae and pupae of *A. aegypti*.

Introduction

The occurrence of arthropod-borne diseases continues to pose significant health risk in many parts of the world. In recent years, mosquito-borne diseases have emerged as a serious public health problem in terms of morbidity, mortality and economic costs in countries of the South-East Asia region, including India (Vijayakumar et al., 2005; Garg et al., 2008). Among various mosquito-borne diseases, dengue fever, Japanese encephalitis and malaria occur world-wide in epidemic form almost on an annual basis (Tolle, 2009), and they globally threaten 2.5 billion people at any time point (WHO, 2013). In India, the recent outbreak of dengue fever transmitted by *Aedes aegypti* with an alarming report of 50,000 cases in 2012 stunned policy makers, thereby imposing urgent need for development of control measures (Garg et al., 2008; Raheel et al., 2011; Chaudhuri, 2013). At present, there is no specific treatment or vaccine available for dengue, and vector control is, therefore, the only available means of prevention of this deadly disease. However, the success of this approach appears to be limited, because of frequent reports of *A. aegypti* resistance to common class of insecticides including organochlorines, organophosphates, carbamates and pyrethroids (Hemingway and Ranson, 2000; Singh et al., 2011).

To overcome the known drawbacks of synthetic chemical insecticides, and the increasing cost of introduction of new chemical insecticides into the market, the focus is shifted towards alternative control methods using biopesticides (Casida and Quistad, 1998; Cantrell et al., 2012). The eco-friendly neem and *Bacillus thuringiensis* (*Bt*)-based products with potent antimosquito property currently favoured in the vector control programme all over the world, because of their effectiveness against broad range of insects, low environmental impact and less chance for development of resistance (Hall and Menn, 1998; Singh et al., 2011). However, certain disadvantages in applications of *Bt*-based products have been reported, which include limited field stability, ineffective against certain insects, and finally it also allows development of resistance in target insects (Chevillon et al., 2001; Ferré and Van Rie, 2002; Paris et al., 2011). Due to these undesirable features, usages of Bt-based products are not the most preferred commercial insecticides (Ferré and Van Rie, 2002). Alternatively, insecticidal botanicals are gaining more attention in vector control programme due to their eco-friendly nature and less chances for the development of resistance (Isman, 2006).

Azadirachtin is one of the most studied botanical insecticides from neem tree, *Azadirachta indica*, and it has been shown to be the main agent for combating mosquitoes (Su and Mulla, 1999; Suman et al., 2010). Azadirachtin is known to interfere in a wide range of physiological processes in mosquitoes and other insects. This botanical has been

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reported to bind to chemoreceptors thereby resulting in deterrent and antifeeding responses (Mordue and Blackwell, 1993). Besides, azadirachtin modulates ecdysteroid and juvenile hormone titers, which are well manifested by perceptible impairment of growth, moulting, ovarian development, oocyte ultrastructure, fecundity, and egg viability (Mordue and Blackwell, 1993; Su and Mulla, 1999; Lucantoni et al., 2006; Suman et al., 2010). Azadirachtin with such multitude or physiological effects has attracted greater attention worldwide to explore its insecticidal property against more than 200 insect species (Isman, 2008). In insects, esterases and phosphatases play important roles in various physiological processes (Krisch, 1971; Walter and Schütt, 1974; Urich, 1994). Esterases are involved in digestion, neuronal conduction, reproduction, metabolism of juvenile hormone, moulting and detoxification of insecticides to less toxic metabolites (Urich, 1994; Wheelock et al., 2005). Phosphatases are essential for tissue growth, cellular differentiation, metabolism of carbohydrate, nucleotide and ATP generation (Walter and Schütt, 1974; Urich, 1994). Therefore, these two classes of enzymes are considered as marker enzymes for assessment of impact of toxicants on biochemical or physiological processes of target insects (Smirle et al., 1996; Laranja et al., 2003; Nathan et al., 2008; Shekari et al., 2008; Smirle et al., 2010; Koodalingam et al., 2011, 2012; Pontual et al., 2012). Growing interest on application of neem-based products generated large amount of scientific information on the effects of especially azadirachtin on vector mosquitoes (Su and Mulla, 1998; Mulla and Su, 1999; Lucantoni et al., 2006; Ndione et al., 2006; Okumu et al., 2007; Lucantoni et al., 2010). However, systemic effects of neem-based products with respect to modulations in esterases and phosphatases in larvae and pupae of *A. aegypti* remain to be studied.

In mosquitoes, hemocytes are important components in cellular as well as humoral immune responses by performing phagocytosis, encapsulation, nodule formation and cytotoxicity (Hillyer, 2010). Hemocytes mount an effective immune challenge against invading microorganisms and parasites through phagocytosis, thereby maintaining immune homeostasis in mosquitoes (Hernández-Martinez et al., 2002; Blandin and Levashina, 2007). Among various hemocyte morphotypes detected in systemic circulation of mosquitoes, mainly granulocytes rarely plasmatocytes or oenocytoids have been reported to phagocytose various abiotic and biotic targets (Drif and Brehélin, 1983; Hernández-Martinez et al., 2002; Hillyer et al., 2003, 2005; Blandin and Levashina, 2007). A few investigators have attempted to examine changes in hemocytic responses of the insects as a novel approach to elucidate the impact of plant secondary metabolites on immuno-defence process. In most of such studies, alterations in hemogram, viability and ultrastructure of hemocytes were assessed (Ayyangar and Rao, 1990; Azambuja and Garcia, 1992; Tikku et al., 1992; Peter and Anandakrishnan, 1995; Ayaad et al., 2001; Sharma et al., 2003, 2008). On the other hand, only three recent studies have examined the effect of botanicals on the functional capability of insect hemocytes by directly assessing the hemocytic ability to bind and phagocytose foreign cell in vitro (Figueiredo et al., 2006; Zibaee and Bandani, 2010; Koodalingam et al., 2013). It is pertinent to note that infectious fungal spores (microsporida), bacteria (Wolbachia) and virus (densovirus) possess inherent potency to knock down mosquito population (Kurtz et al., 2000; Gu et al., 2011; Hurk et al., 2012). The deleterious effect of these pathogens on mosquitoes has led investigators to question whether they impair the immune system of mosquitoes, thereby making them more susceptible to infection by opportunistic pathogens (James and Xu, 2012). Earlier studies have demonstrated that the NeemAzal (a commercial azadirachtin formulation) exhibits anti-mosquito activity by reducing the survival, fecundity and adult emergence of three important vector mosquito species (Boschitz and Grunewald, 1994; Gunasekaran et al., 2009). However, its impact on biochemical marker enzymes and hemocyte-mediated cellular immune responses of the vector mosquitoes remains to be investigated. Therefore, the objective of the current study was focused on a comparative analysis of the effect of NeemAzal on two marker enzymes (esterases and phosphatases) as well as viability and phagocytic activity of hemocytes of larvae and pupae of dengue vector mosquito *Aedes aegypti*.

Materials and methods

Fine chemicals and reagents

Acetylthiocholine iodide, DTNB reagent (5-5-dithiobis 2-nitro benzoic acid), Fast blue salt, α- or β-naphthyl acetate were obtained from Himedia (Mumbai, India), *p*-nitrophenyl phosphate, *p*-nitrophenol and α- or β-naphthol were purchased from Ranbaxy (Mohali, India). All other chemicals and reagents used were of the highest analytical grade.

Mosquito rearing

The eggs of *A. aegypti* obtained from the laboratory reared mosquitoes were allowed to hatch out under the controlled laboratory conditions (Koodalingam et al., 2009). The newly hatched fourth instar larvae and pupae were used in all the experimental studies. Mosquito larvae were reared in plastic bowls filled with unchlorinated tap water. Larval food was supplied daily containing a mixture of powdered dog biscuit and brewer's yeast at 3:1 ratio.

Preparation of NeemAzal stock solution

The botanical biocidal formulation NeemAzal T/S 1.5 EC was purchased from E.I.D. Parry, Chennai, India. This neem formulation contains 10,000 ppm of azadirachtin. Based on initial stock solution (10,000 ppm) various test concentrations were freshly prepared by suitably diluting the NeemAzal with unchlorinated tap water.

Toxicity assay

The test media were prepared with different concentrations of NeemAzal: 1, 2, 5, 10, 15 and 20 ppm, for the exposure of the larvae and pupae. Batches of 10 newly hatched fourth instar larvae or pupae were introduced into 50 ml of test medium. This experiment was performed with five replicates. Control containers received equal volume of tap water instead of NeemAzal. The percentage of mortality of larvae and pupae was recorded after 24 hour exposure period.

Preparation of whole body homogenates

The live control fourth instar larvae and pupae as well as the NeemAzal exposed larvae and pupae were washed with double distilled water, and the adhering water was completely removed from the body surface by blotting with tissue paper. The larvae and pupae (each 10 individual) were separately homogenized in eppendorf tubes using a Teflon hand homogenizer in 500 µl of ice-cold 0.9% saline for eventual estimation of total proteins, esterases and phosphatases activity. For qualitative analysis of esterase and phosphatases by native PAGE, the whole body homogenates of control and exposed larvae as well as pupae were prepared as described earlier, except that 100 µl of Tris-glycine (pH 8.3) was used as homogenization buffer.

Quantitative analysis of biochemical constituents

Determination of protein concentration. The proteins present in the homogenates of larvae and pupae were first precipitated with 80% ethanol and the proteins present in the precipitate were estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Acetylcholinesterase assay. The acetylcholinesterase activity was determined by the procedure of Ellman et al. (1961) as described in Koodalingam et al. (2012) using acetylthiocholine iodide as a substrate.