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Acute Toxicity of a Neem Seed Kernel based Biopesticide, Nimbecidine Plus on an Edible Fresh Water Crab, *Varuna litterata* (Fabricius, 1798)

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Abstract

Present study assesses toxic effects of a neem seed kernel based biopesticide, Nimbecidine Plus (a.i.- azadirachtin 1%) on the survival of the freshwater edible crab, *Varuna litterata* under laboratory conditions. The four-day acute static renewal bioassay test was performed to determine the LC50 values at different exposure period and the safe concentration using the probit analysis. Adult male crabs (mean length- 2.867 ± 0.4 cm; mean weight- 9.895 ± 4.179 g) were exposed to different concentrations (0, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 ppm) of Nimbecidine Plus. The LC50 values at various exposure periods were 14.940 ppm for 24hr; 10.602 ppm for 48hr; 7.673 ppm for 72hr and 6.284 ppm for 96hr. The upper confidence limits were 16.388, 12.033, 9.017 and 7.457 ppm for 24, 48, 72 and 96 hr and lower confidence limits were 13.437, 9.105, 6.188 and 4.924 ppm, respectively. Safe concentrations were found to range from 0.628 ppm to 2.514 ppm. Behavioural changes like erratic body movement, irregular locomotion and shivering of body were noticed in the treated crabs. Attention is warranted regarding use of the biopesticides in agricultural field to avoid drastic effects on non-target species, which are also used as food.

Keywords: Biopesticides, Nimbecidine Plus, LC50, Safe concentration, *Varuna litterata*.

Introduction

Extensive use of synthetic chemical pesticides has become an essential part of present day agricultural practices. As the chemical pesticides are not easily degraded in the environment, they contaminate water of the streams, lakes and ponds through the agricultural runoff and exert their harmful effects on the non-target aquatic flora and fauna (Patil et al., 2008). The alternate way to overcome these hazardous effects of the chemical pesticides is the use of natural biopesticides in the agricultural fields. Unlike synthetic chemical pesticides, biopesticides are naturally degradable, environment friendly and at certain concentrations destroy pest organisms (Unnithan, 1997).

Among the biopesticides of herbal origin, neem extract and various neem-based biopesticidies are being mostly used in the agricultural field to eradicate insect pests (Mondal et al., 2007). Neem (*Azadirachta indica* A. Juss), belonging to the family Meliaceae, is a traditional and highly esteemed medicinal tree in Indian sub-continent well known for its insecticidal, biomedical and pharmacological properties (Govindachari, 1992; ICAR, 1993; Biswas et al., 2002). Azadirahctin (a tetranotriterpenoid) is the principal active compound (Kraus *et al*., 1981; Broughton *et al*., 1986; Saxena, 1990) extracted from the neem, which have the pesticide property (Anjaneyulu and Misha, 1998). Field experiments have successfully demonstrated the potential of neem extract as a pest-control agent (Martinez, 2002; Kreutzweiser et al., 2004) and it is used in agricultural as well as aquaculture systems to control various predators, parasites and pathogenic bacteria (Dunkel and Ricilards, 1998; Das et al., 2002; Farah et al., 2006; Winkaler et al., 2007). Besides the pest control properties, the acute toxicity values of several neem preparations and pure azadirachtin for the laboratory animals and some non target species have been studied extensively (Gandhi et al., 1988; Osuala and Okwuosa, 1993; Wan et. al., 1996; Mahboob et al., 1998). The adverse effects of neem based biopesticides on the non-target organisms due to indiscriminate usage were also reported (Schmutterer and Holst 1987; Beckage et al. 1988; Price and Schuster 1991; Schöder, 1992; Omoregie and Okpanachi, 1992, 1997; Winkaler et al., 2007; Saravanan et al., 2010, 2011; Maitra et al., 2014).

The freshwater crab, *Varuna litterata* (Fabricius 1798), popularly known as 'Chiti Kankra' in West Bengal, is an edible and economically important Indian crab fauna (Devi et al., 2013) and widely distributed in different parts of India. It is a member of the crab family Varunidae and highly adapted in marine, estuarine and freshwater habitats and widely found in rivers, slow streams in monsoon drains, ponds, and pools of water and even in paddy fields (Pati et al., 2012). It has huge demand in the fish market for its delicious taste and its numbers compensate for its small size (Hora, 1933). It is also a very good supplementary diet of protein and lipid (Das et. al, 2015).

Freshwater crabs are often exposed to biopesticide in their aquatic habitats through the agricultural runoff, but there is no such report of the acute toxicities of the widely used neem-based biopesticides, particularly on this freshwater crab, *V. litterata*. The objectives of the present study were to determine the acute toxicity of a popularly used neem biopesticide, Nimbecidine Plus on *V. litterata* at different exposure periods and also to determine the safe concentrations of this biopesticide into the aquatic habitats which in turn establish the levels of acceptability by the living organisms in the environment. The hypothesis of the present work is that Nimbecidine Plus has toxic effects on *V. litterata* and presence of a higher concentration in the aquatic habitats may lead to death of this fresh water crab species in the environment.

Materials and Methods

Test Animal

Live freshwater crabs, *Varuna litterata* (Fabricius 1798), were collected from the local fishermen of Birlapur, South 24 Parganas District, West Bengal, India. The supply of the crab was strictly from the freshwater streams, canals and ponds. The crab species was identified by Zoological Survey of India, Kolkata, India. Adult crabs weighing 9.895 ± 4.179 g with a mean carapace length of 2.867 ± 0.4 cm were brought to the laboratory and kept in huge plastic trough (16 L capacity) filled with tap water for 2 weeks for acclimatization. Water level was high enough to keep the crabs in submerged condition. The crabs were fed with rice, small pieces of prawns and fragmented mollusks and small twig of *Ipomoea* sp. The natural photoperiod was maintained.

Biopesticide

Nimbecidine Plus (manufactured by T. Stanes and Company) is a neem seed kernel based preparation containing min. 1% azadirachtin as active ingredient. It was procured from the Agriculture Office, Govt. of West Bengal, Howrah. It's a prepared liquid solution and mixed directly with water so it was directly used in different concentrations.

Experimental Procedure

To determine the LC50 value of Nimbecidine Plus, the four-day static renewal acute toxicity test (APHA, AWWA, WEF, 2012) was done in the laboratory using the probit analysis (Fenny, 1971). Adult male crabs (n=10) were kept in separate plastic trough (16 L capacity) containing 2 L tap water and exposed to each concentration of Nimbecidine Plus (0, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 ppm). Each experiment was carried out in triplicate. Behaviour of the test crabs was observed and the dead crabs were removed and recorded from time to time during 96 hr exposure

period. The water in the trough was renewed for every 24 hr and the required concentrations of Nimbecidine Plus were added daily in the treatment groups in order to maintain constant concentration during the experiment (Sprague, 1964). Feeding was withdrawn 24 hours prior to the experimentation to avoid the metabolic differences, if any due to differential feeding.

Safe Concentration Determination

To get a satisfactory safe permissible level of Nimbecidine Plus, the safe concentrations were determined after Hart et al. (1945), Edwards and Brown (1966) and Burdick (1967) and EIFAC (1983).

Statistical Analysis

At different exposure periods (24, 48, 72 and 96 hr), the mortality of the crabs was subjected to Probit analysis with the BIO-STAT (version 5.8.4.3, 2009) software to calculate the LC50 values and other associated statistical data.

Results

Crabs treated with Nimbecidine Plus exhibited some behavioural changes such as erratic body movement, irregular locomotion, heavy tremor, etc. Loss of balance was also noticed and ultimately they turn over ventrally. All the appendages were crinkled to the ventral side and in the higher concentrations (≥16 ppm) (Table 1) thoracic appendages (walking legs) were sometimes shed off from the body prior to death. The control group of crab showed no such signs.

Table 1: Cumulative mortality of the fresh water crab, *V. litterata* after 24, 48, 72 and 96 hr exposure to Nimbecidine Plus (n=10, for each concentration).

Dose Conc. (ppm)	No. of crab dead at			
	24 hr	48 hr	72 hr	96 hr
0	-	-	-	-
4	-	-	1	2
6	-	1	3	4
8	-	2	5	6
10	1	4	7	9
12	2	6	8	10
14	4	7	9	-
16	5	9	10	-
18	7	10	-	-
20	9	-	-	-
22	10	-	-	-

The cumulative mortality of the fresh water crab, *V. litterata* after exposure to various concentrations of Nimbecidine Plus for 24, 48, 72 and 96 hr have been shown in Table 1. The LC50 values at various exposure periods were 14.940 ppm for 24hr; 10.602 ppm for 48hr; 7.673 ppm for 72hr and 6.284 ppm for 96hr. The LC50 values and their 95% upper and lower Fiducial limits, Regression equations, Chi-square values and Correlation coefficients were shown in Table 2. The Chi-square values indicate no significant differences ($P > 0.05$) between observed and expected mortality responses i.e. no large random deviations of the observed data from the Log-probit model. It also indicates that the crab populations used in the experiments were homogenous. A strong positive correlation exists between percentage mortality and the dose concentrations in each exposure period. The positive correlation coefficients (r) indicate that percentage mortality increases significantly ($P < 0.01$) with increasing dose concentrations. The plot of Finney's probits against Log10 concentration for calculating LC50 value of Nimbecidine Plus for 24, 48, 72 and 96 hr has been depicted in Figures 1, 2, 3 and 4.

Table 2: LC50 values, 95% Fiducial limits, Regression equations, Chi-square values and Correlation Coefficients for the Nimbecidine Plus at different exposure periods for the fresh water crab, *V. litterata*.

Exposure period (hr)	LC50 value (ppm) ± SE	Regression Equation Y = bX + c	95% Fiducial limits		Chi-square (χ^2)	Correlation Coefficient (r)
			Lower	Upper		
24	14.940 ± 0.757	Y= 8.9617x - 5.5242	13.437	16.388	0.550	0.98586*
48	10.602 ± 0.755	Y= 6.4826x - 1.6471	9.105	12.033	0.552	0.98598*
72	7.673 ± 0.738	Y= 5.0642x + 0.5183	6.188	9.017	0.133	0.99699*
96	6.284 ± 0.667	Y= 5.6491x + 0.4909	4.924	7.457	0.621	0.98331*

*Values indicate significance at 0.01 levels ($P < 0.01$)

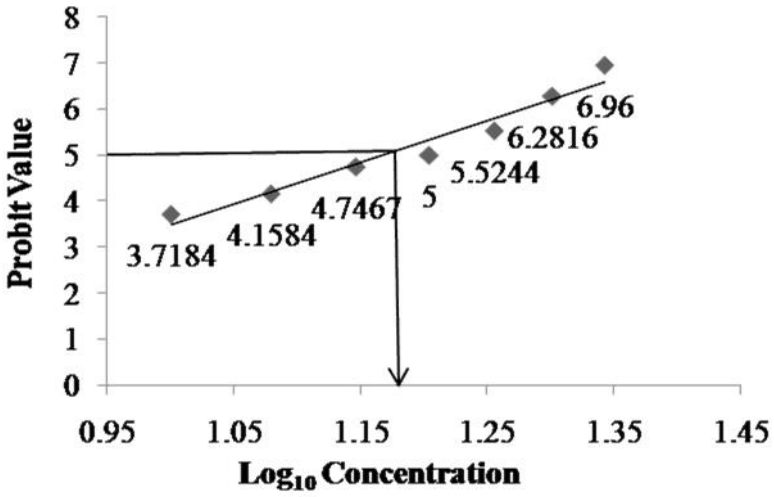

Figure 1: Plot of probits and predicted regression (linear) line for Nimbecidine Plus to the freshwater crab, *V. litterata* after 24 hr exposure.

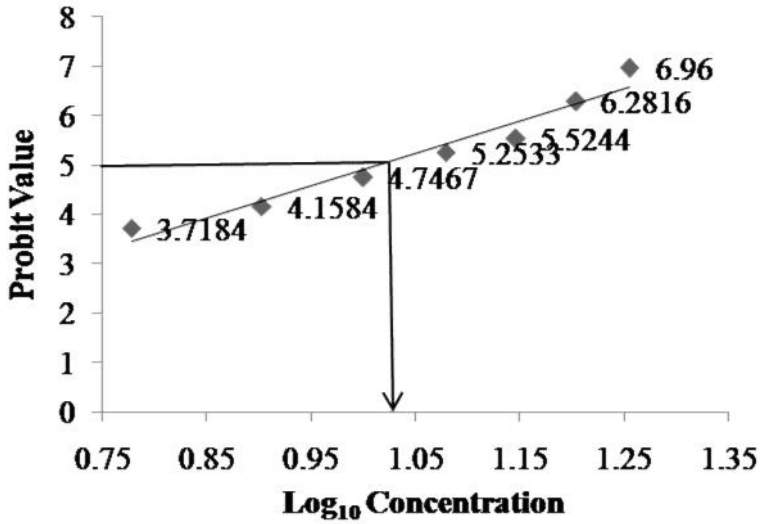

Figure 2: Plot of probits and predicted regression (linear) line for Nimbecidine Plus to the freshwater crab, *V. litterata* after 48 hr exposure.

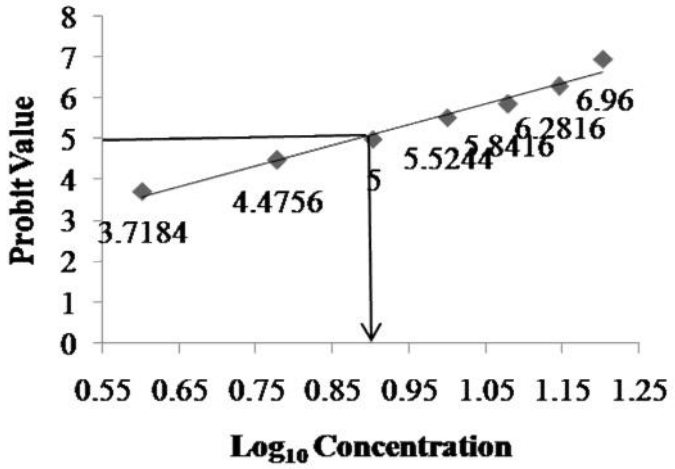

Figure 3: Plot of probits and predicted regression (linear) line for Nimbecidine Plus to the freshwater crab, *V. litterata* after 72 hr exposure.

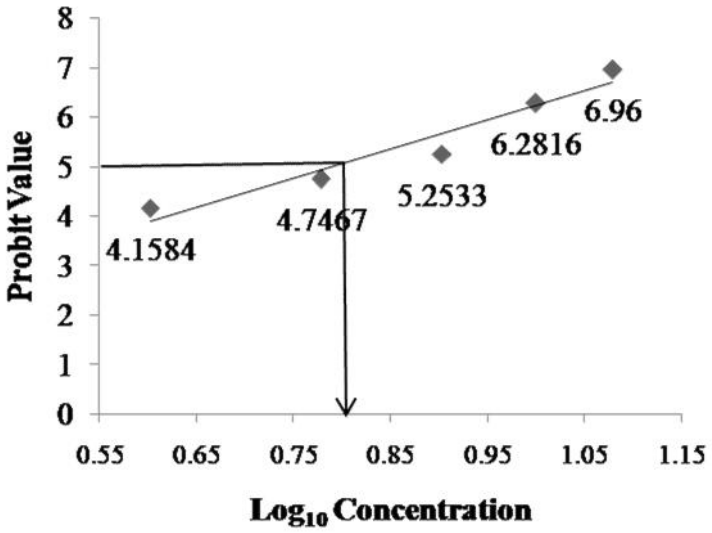

Figure 4: Plot of probits and predicted regression (linear) line for Nimbecidine Plus to the freshwater crab, *V. litterata* after 96 hr exposure.

The safe concentrations calculated for the fresh water crab, *V. litterata* exposed to the Nimbecidine Plus are shown in Table 3. The minimum safe permissible concentration was found to be 0.628 ppm according to the method of Burdick (1967) and EIFAC (1983) while the maximum value was observed as 2.514 ppm according to the method of Edwards and Brown, 1966 and according to the method of Hart et al. (1945) the middle most value was 1.602 ppm. As all the aforementioned methods were reported in published scientific works, the present effort of applying these three methods was committed to get a range of safe permissible concentrations of the Nimbecidine Plus to the fresh water crab, *V. litterata* and it was found to be 0.628 ppm to 2.514 ppm (Table 3).

Table 3: Safe concentrations of Nimbecidine Plus to the fresh water crab, *V. litterata*

Safe concentration (ppm) according to		
Hart et al. (1945)	**Edwards and Brown (1966)**	**Burdick (1967) and EIFAC (1983)**
1.602	2.514	0.628

Discussion

Results of the acute toxicity of the neem extracts and the neem based biopesticides have been reported by different workers. Winkaler et al. (2007) have reported the 24 hr LC50 of neem leaf extract for juveniles of the neotropical freshwater fish, *Prochilodus lineatus* as 4.8 g L^{-1}. The 96 hr LC50 of water-extract of mesocarp of neem fruit for hybrid fish, *Heteroclarias* has been found to be 81.28 mg L^{-1} (Akinwande et al., 2007). Acute toxicity of neem based biopesticides, Nimbecidine (EC-Azadirachrtin 0.03%) and Neem Gold (EC-Azadirachtin A 0.15%) for the fingerlings of a freshwater loach, *Lepidocephalichthys guntea* were determined by Mondal et al. (2007) and the 96hr LC50 values were found to be 0.135 mg $L^{-1}$and 0.525 mg L^{-1} respectively. Stalin et al. (2008) have observed the 96hr LC50 value of azadirachtin to the fish, *Poecilia reticulata* as 0.011 mg L^{-1} while Ansari and Ahmad (2010) have reported the 96 hr LC50 value of Neemgold to the Zebrafish, *Danio rerio* as 2.980 µg L^{-1}. Acute toxicity of Nimbecidine (EC Azadirachtin 0.03%) have also been studied on the adult heteropteran male insect, *Sphaerodema rusticum* (Shoba et al., 2010) and the 96hr LC50 value was observed to be 0.0028 ppm. In another toxicity studies, the 24hr LC50 values of neem leaves extract to the Indian major carps, *Cirrhinus mrigala* and *Labeo rohita* were found to be 1.035 g L^{-1} (Saravanan et al., 2010, 2011). Similarly, Kumar et al. (2012) have studied the acute toxicity of azadirachtin to a teleost, *Heteropneustes fossilis.* They have also determined the LC50 values at different exposure period as 173.06 mg L^{-1} for 24 hr; 80.69 mg L^{-1} for 48 hr; 58.57 mg L^{-1} for 72 hr and 52.35 mg L^{-1} for 96 hr. The acute toxicity of azadirachtin on the freshwater cat fish, *Pangasius hypophthalmus* have been reported by Suresh Babu et al. (2013) and found the LC50 values at various exposure periods as 165.72 mg L^{-1} for 24 hr; 95.17mg L^{-1} for 48 hr; 62.48 mg L^{-1} for 72 hr and 55.76 mg L^{-1} for 96 hr. Maitra et al. (2014) have determined the 96 hr LC50 value of an azadirachtin-based bioagrocontaminant, Neemsheild on the major carp, *Labeo rohita* as 44.61 ppm. From the above discussion it is shown that the LC50 values are differing in species to species for the same toxicants due to the mode of action and responses of the animals (Nisha et al., 2016) to this particular toxicant. The toxicity levels were also influenced by the size, age (Saunders et al., 1983) and sex (Victoriamma and Radhakrishnaiah, 1982) of the animal and also by the nutrient supply (Arunachalam et al., 1980).

Most of the published reports of the acute toxicity of the neem extract and the neem based pesticides are limited on the vertebrates specially the different fish species. The present experiment has enlightened the crab as an aquatic invertebrate species model for the acute toxicity test of the neem based biopesticide. The results of the present study indicate that the Nimbecidine Plus is toxic to the freshwater crab, *V. litterata* and may cause high mortality when large amounts reach the water reservoirs. Farmers often use a dose of 20 ppm or sometimes it may reach up to 30 ppm as it is assumed that a surplus dose of the biopesticide is much more effective to eradicate the pest. But, such excessive amount of the biopesticide increases the risk of contaminating the aquatic systems as agriculture run-off. It is observed from the present experiment that the LC50 values (Table 2) of the Nimbecidine Plus to the fresh water crab, *V. litterata* at each exposure period were lower than the actual dose used in the agricultural field. The safe permissible concentration (maximum value 2.514 ppm) (Table 3) for the crab was also found to be lower compared to the dose applied by the farmers. Safe permissible concentrations of any pesticide in the aquatic environment are highly useful in establishing the limits of acceptability of the pesticide by the aquatic animals (Roopadevi and Somashekar, 2012). If the amount of the biopesticide reaching the aquatic systems exceeds the observed lethal concentrations, it might be deleterious for the survival of this economically important crab species in the environment.

Conclusion

Generally most of the pest organisms belong to the lower trophic level of the food chain in an ecosystem. Neem extracts and the neem based biopesticides are shown to have lethal actions not only on the lower trophic level pest organisms, but also on animals of the higher trophic level of the food chain. However, no attention has been paid to small invertebrates such as crabs, prawns, gastropods, bivalves, etc, which are also used as food. Hence, further study is warranted to understand the extent of such undesirable effects of the biopesticides on various economically and ecologically important fauna of the aquatic ecosystem.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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