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Comparative Toxicity of Spinetoram and Nitenpyram against Earthworm and their Effects on Protein Contents and Cholinesterase Activity

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Abstract. The earthworms' numbers and biodiversities are considered as an indicator of soil nature and fertility. Earthworms are very important soil organisms that aid in the decomposition of plant litter. In recent era earthworm obtain more attention in agriculture practices, so that in the present work to determined the comparative effects of nitenpyram and spinetoram on total amount of protein and cholinesterase activity of *Pheretima posthuma* Kinberg (Order: Haplotaxida and Family: Megaseolecide) earthworm. Total protein contents were found to be decreased significantly in the peristomium, clitellum, and abdomen regions under treatments of nitenpyram and spinetoram. Cholinesterase activities were inhibited in the peristomium, clitellum and abdomen under the exposures of nitenpyram and spinetoram, at the intervals of 30, 60 and 90 seconds in *P. posthuma.* Conclusively it is safe to say that spinetoram is better than neonicotinoids for the earthworms, therefore could be switched from neonicotiniods as an IPM component.

Keywords: enzyme, bio-pesticide, neonicotinoid, earthworm

Introduction

Nitenpyram is being used as a novel insecticide in the class of neonicotinoid pesticides (Tomlin, 1997). It has also been recognized as an effective systemic insecticide to the flea in feeding tests on cattle (Tinembart *et al*., 1999) and frequently used on foliar fields, especially, on horticultural fields, applied in attracted formulations for household uses against ants and cockroaches, also used in granular formulations to the management of amenity and meadow grasslands against ground dwelling insect pests. It could be applied in irrigation water to defend persistent of various crops and introduced into the timber to fighting the termites (Oliver *et al*., 2010). These insecticides are fetched 1/3 of the total pesticide traded (Simon-Delso *et al*., 2015). They could be applied through irrigating water to field crops and introduced into timber as termiticide (Oliver *et al*., 2010). Therefore, they could get way to the inner soil layers. Studies have proved that neonicotinoids could get way in the course of underground water, hence, damaging the agro soil atmosphere (Bacey, 2000). It not only effect on farming soil but also adversely affect the soil dwelling organisms thus, develop ultimate unfavorable effects on agro-ecosystems. On the other hand, earthworms have been observed plentiful in the most of the soil ecosystems and considered as reformer in the ground ecosystems, as they vigorously regulate the physical, chemical and natural of the polluted soils (Bottinelli *et al*., 2010; Binet *et al*., 1998; Jones *et al*., 1997). They have significant effect on richness and porosity of soil (Wang *et al*., 2004). Bustos-Oberg-n and Goicochea (2002) have pointed out that owing to extensive use of agro pesticides the danger of soil contamination has provoked a growing concern the world over.

Spinetoram is used as biopesticide. Biopesticides are used with an understanding that they are safer than other insecticides. Thus spinetoram is supposed to exerts low lethal impacts and least environmentally threaten product (DAS, 2008). The spinetoram showed highly successful results against variety of insects in various crop fields and pose reduced risk to natural enemies reported by Srivastava *et al*. (2008); El Kady *et al*. (2007); Mahmoud and Osman, (2007); Reita *et al*. (2003); Williams *et al*. (2003); Kirst *et al*. (1992). Spinetoram showed its impacts either by stomach and contact poison and degraded rapidly in the atmosphere as reported by Cisneros (2002) and directly exerted its impacts on receptors of γ-aminobutyric acid and nicotinic acetylecholine (Shimokawatoko *et al*., 2012). The pesticides are known to exert adverse effects on soil beneficial fauna e.g. the earthworms (Pfiffiner *et al*.,

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2014) as the earthworm has significant effect on richness and porosity of soil (Wang *et al*., 2004). Lawrence and London (1997) reported that the earthworms make the soil pH neutral as well. In view of importance of earthworms, growing trend of using neonicotinoids, presently effects of nitenpyram were evaluated against earthworm in comparison with spinetoram as an alternate pest controlling compound.

Materials and Methods

Identification of earthworm. The procured earthworms were already identified, however, for further confirmation; identification was made after purposed and designed key by Stephenson (1923) for the earthworm species identification. Body of *Pheretima posthuma* Kinberg is approximately 13-15 cm in length, having a brownish bright colour. The whole body consists of segments 100-120. The anterior of the body has peristomium, fewer segments are made a thick band called as clitellum and remaining of body after clitellum is called as abdomen. The genital pore of female found on ventral side of 14^{th} segment of the body, whereas genital pore of male on ventral side of 18^{th} segment of the body just behind to clitellum. Copulatory papillae present on the venereal side of segments no17^{th} and 19^{th} of the body. In the last segment, there is a vertical slit-like anus (Kotpal *et al*., 1987).

Rearing and culturing of earthworm. The procured earthworm was kept in the laboratory at around 20-25 °C with a humidity level above 80%, for the process of rearing. Rearing was carried out in wooden made rearing chambers on the similar soil from where they were initially collected. The soil was consisting of suitable organic materials for earthworm's development. A suitable amount of water as needed by earthworms was added in order to maintain moisture level. The earthworms were kept well protected in rearing chamber with muslin cloth from all predators, contamination and attrition. In the said experiment all the earthworms having age 20-40 days of maturity were used.

Spinetoram was purchased from an authorized pesticide dealer with the trade mark of Delegate 25% w/w and nitenpyram was procured with the trade mark of knockout 25% w/w. It is a product of Sygenta Corporation (both pesticides were procured from its authorized dealers in the Karachi city of Pakistan). Additionally, dilutions of these pesticides have been made and either pesticide for testing in sterile soil. LD_{50} of the spinetoram and nitenpyram were measured after based on Finney (1971). Standard deviation, Standard error and mortality rang at 95% confirmed limits have been measured as well.

Statistical analysis. ***Charle's Formula.*** To make various concentrations of under test compounds following formula was used for calculations.

$$C_1 V_1 = C_2 V_2$$

Where:

C_1 = original concentration of solute;
C_2 = final concentration of solvent;
V_1 = initial volume of solute;
V_2 = final volume of solvent;

Abbot's Formula. Obtained readings have been subjected to Abbot's formula that is as following:

Abbot's formula = % Kill– Kill in Control * 100*(100 – Control mortality)$^{-1}$

Standard deviation (S.D.). The S.D. in the mortality of all compounds taking place at different concentrations has been measured by following formula:

$$S.D. = [\Sigma(x - \bar{X})^2.(n-1)^{-1}]^{0.5}$$

Where:

$\bar{X}$ = Average of variable x;
X = Notation for variations of variable x;
S. D. = Standard deviation;
n = Total number of observations.

Standard error (S.E.).

$$S.E. = [SD^2. n^{-1}]^{0.5}$$

Where:

n = Total number of observations;
S. E. = Standard error;
S. D. = Standard deviation.

Range. Range at significance value of 95% confidence limit has been measured by using following formula:

$$Range = X \pm S.E. \times 1.96$$

Estimation of total contents of protein. To find out the effect of spinetoram and nitenpyram on the protein contents, ten mature earthworms were treated with

respective $LD_{50's}$ for 48 h. After 48 h the treated active worms were drawn out and relevant parts dissected out and homogenisation was prepared in a test tube by "Disperser" homogeniser for 30 min at a speed of 80% of the device in 3 mL de-ionized water. Subsequently, homogenised samples were centrifuged for 20 min at a speed of 15000 rpm.

A Sigma Italia test kit no. 10031 was used to estimate the total contents of protein (based on Biuret method). Five test tubes were used as control, blank, standard, spinetoram, nitenpyram and one milli litter of Biuret reagent was taken in all tubes. After that 20 micro liter of standard was taken in tubes and 20 liters of compounds (spinetoram and nitenpyram and control) were taken in particular tubes. After mixing almost half an hour, it was placed at 25 °C. The absorbance of sample and standard were calculated at 546 nm wavelength against the reagent blank.

Chemicals

Biuret Reagent; Standard protein 60 g/L; NaOH 0.75 mmol/L; Na-K-tartarate 20 mmol/L; KI 6 mmol/L; Cu_2SO_4 7 mmol/L

Determination of cholinesterase activity. For the determination of cholinesterase activity a kit from Boehringer Mannheim Gm Bh, No. 124125 was used. The method depends upon hydrolysis of acetylcholine by the action of cholinesterase (Knedel and Boettger, 1967).

Currently, the S-butyrylthiochloline iodide has been used as substrate. Whereas 2-nitrobenzoate (5, 5-dithiobis) has been used as indicator that is decreased by thiocholine released to form the yellow colored 5-mercapto-2-2 nitrobenzoate. The activity of cholinesterase is attained from the rate of colour pattern that is calculated photometrically.

Chemicals

(i) 0.134M of phosphate buffer (pH 7.2) was made by mixing seven parts (v/v) of disodium hydrogen phosphate (47.8 g/L) and three parts of potassium dihydrogen phosphte (18.156 g/L).
(ii) 0.04M of acetylcholine was made by dissolving acetylcholine chloride (0.7266 g) in 100 ml of 0.001M of acetate buffer (pH 4.5).
(iii) Acetate buffer was made by mixing 28 mL of sodium acetate trihydrate (0.2M) with 22 mL of acetic acid (0.2M). 1 volume of reagent two has been diluted with nine volumes of reagent one used newly.
(iv) Freshly prepared solution of hydroxylamine hydrochloride (2M) was taken.
(v) In bidistilled water sodium hydroxide (3.5M) was prepared.
(vi) Newly prepared solution of alkaline hydroxy lamine through mixing the same volume of reagents'd' and 'e'.
(vii) Solution of HCL was made by mixing 1 volume of HCl (conc.) with two volumes of bidistilled water.
(viii) Ferric chloride (0.37M) solution was made in 10% HCl.
Each regent was stored at 3 °C.

Results and Discussion

In the carried out studies, the total amount of protein in different three regions of imago *Pheretima posthuma* earthworms i.e. peristomium, clitellum and abdomen have been observed by applying the treatments of nitenpyram and spinetoram after the period of 48 h.

Under the treatments of nitenpyram the total amount of protein were estimated as 38.8±0.46, 48.1±0.18 and 39.0±0.46 mg/mL to respective regions as peristomium, clitellum and abdomen in the earthworms *Pheretima posthuma* (Table 1). In the case of untreated earthworms the total protein amount was 70.2±0.46, 82.0±0.25 and 65.5±0.35 mg/mL in the regions i.e., peristomium, clitellum and abdomen (Table 1).

Under the exposures of spinetoram total protein content found to be 30.8±0.35, 34.0±0.22 and 44.5±0.28 mg/mL to respective regions as peristomium, clitellum and abdomen in the earthworms *Pheretima posthuma* (Table 2). While, in the case of untreated earthworms the total protein amount to be 72.0±0.25, 80.5±0.35 and 63.4±0.31 mg/mL in the respective regions i.e. peristomium, clitellum and abdomen (Table 2).

Faheem *et al*. (2012) evaluated the total amount of protein in the earthworm *Pheretima posthuma* under exposure for the period of 48 h of cypermethrin and neem fruit extract. Faheem *et al*. (2010) postulated that decrease in the total amount of protein owing to the harsh segregation take place in proteins of nucleic plasm as compared to the proteins content of cytoplasmic and also considered key role as bio-indicators of whole metabolic activities of all body cells, to play major role in the various function of all cells of organisms. Above results are in line with the present findings of decline

in protein contents under exposure of pesticides e.g., nitenpyram and spinetoram.

These proteins could be used for the energy production in the body of organism however, under the exposure to the pesticides resulted reduction in metabolic functions as reported by Granett and Leeling (1971). The toxicity of pesticides on protein contents was found in the hemolymph and proteins nature of stem borer of rice under the treatments of different insecticides by Saleem and Shakoori (1987). Chang *et al*. (1974) reported the toxic effect after the treatments of malathion and permethrin and on amount of protein in *Tribolium castaneum* (Order: Coleoptera and family: Cerambycidae). In current studies, the protein content in the earthworm some changes found in the region of abdomen under the treatment of spinetoram as compared to nitenpyram effects are in conformities (Faheem *et al*., 2012; 2010; Saleem and Shakoori, 1987; Chang *et al*., 1974).

The amount of protein in different organisms has been decreased after the treatment of insecticide as reported by Tabassum and Naqvi (2001); Mohamed and Hafez (2000). Javaid (1989) investigated the effects under the treatments of neem formulations against the bollworm larvae and the amount of protein was reduced as investigated by Gujar and Mehrota (1985). Saleem and Shakoori (1985) observed the reduction in protein content after the effect of permethrin and deltamethrin. The amount of protein has been reduced in the immature *Drosophila melanogaster* (Order: Diptera and family: Drosophilidae) after the Duter™ exposure. Other researchers also reported the impact of various insecticides on enzymes and proteins of animals (Naqvi *et al*., 1995; Yasmin *et al*., 1994; Rizvi *et al*., 1990;

Table 1. Impact of nitenpyram on total protein in peristomium, clitellum and abdomen regions of *Pheretima posthuma* earthworm.

Nitenpyram	Mean of protein content (mg/mL)		S.D.	S.E.	Variance	Range at 95% confidence limit S.Ex1.96
Peristomium region	Treated	38.86	0.65	0.46	0.42	37.90-39.76
	Untreated (Control)	70.2	0.52	0.37	0.28	69.40-70.90
Clitellum region	Treated	48.1	0.26	0.18	0.07	47.70-48.40
	Untreated (Control)	82.0	0.80	0.25	0.65	80.80-83.00
Abdomen region	Treated	39.0	0.65	0.46	0.43	38.00-39.90
	Untreated (Control)	65.5	0.50	0.35	0.25	64.80-66.10

Table 2. Impact of spinetoram on total protein in peristomium, clitellum and abdomen regions of *Pheretima posthuma earthworm*.

Spinetoram	Mean of protein content (mg/mL)		S.D.	S.E.	Variance S.Ex1.96	Range at 95% confidence limit
Peristomium	Treated	30.6	0.26	0.18	0.07	30.00-31.10
Region	Untreated (Control)	72.0	0.36	0.25	0.15	71.50-72.40
Clitellum region	Treated	34.0	0.32	0.22	0.25	33.50-34.40
	Untreated (Control)	80.5	0.50	0.35	0.28	79.00-81.10
Abdomen region	Treated	44.5	0.40	0.28	0.16	43.90-45.00
	Untreated (Control)	63.4	0.45	0.31	0.20	62.80-45.00

Bradford, 1976). The reducing impact on protein revealed that the protein may be used for the production of energy in the organisms under the pesticide effect was identified to cause reduction of metabolic reserves as reported by Orr and Doner (1982). In the current investigation earthworm protein content was found less reduced in various regions under post-treatment of spinetoram as compared to nitenpyram treatments. During the current study, the protein amount has been found to be decreased in three parts of the adult earthworm under the exposure of spinetoram and nitenpyram. The decrease of total protein content agrees to the postulations of Tabassum and Naqvi (2001); Mohamed and Hafez (2000); Saleem and Shakoori (1985); Orr and Doner (1982).

Acetylcholinesterase (AChE) is responsible for of neurotransmitter and targeted by various pesticides (Ferrari *et al*., 2004; Smulders *et al*., 2003). Thus, AChE is used to detect insecticides toxicity (Sanchez-Hernandez and Moreno-Sanchez, 2002). The decreasing trend of cholinesterase in the earthworms provides the caution concerning injurious impacts of pesticides (Booth and O'Halloran, 2001). The earthworm has significant effect on richness and porosity of soil (Wang *et al*., 2004). Lawrence and London (1997) reported that the earthworms make the soil pH neutral as well. Bustos-Oberg-n and Goicochea (2002) have pointed out that owing to extensive use of agro pesticides, the danger of soil contamination has provoked a growing alarm the worldwide. In the present case of work, ChE inhibition in the *P. posthuma* under the treatments of nitenpyram to be 86.00%, 89.55% and 87.60% in the peristomium region, (Table 1), 57.11%, 19.42% and 0.41% in clitellum (Table 2) and 90.00%, 73.96% and 68.84% in abdomen at the respective period of time as 30, 60 and 90 seconds (Table 3). In the treatments of spinetoram ChE inhibition observed to be 81.33%, 87.22% and 95.11% in peristomium (Table 3), 51.62%,

Table 3. Effect of spinetoram and nitenpyram treatment on ChE activity in peristomium region of earthworm (Pheretima posthuma).

Pesticides	Parameters					
	Time (in second)	Mean (U/I), S.D(±)	Variance	Range at 95% confidence limit	Inhibition %	Enhancement %
Nitenpyram	30	210.7±0.36	0.13	210.2-211.1	86.00	-
	60	180.6±0.36	0.13	180.2-181.1	89.55	-
	90	231.1±0.79	0.63	230.0-232.2	87.60	-
Spinetoram	30	280.9±2.00	4.00	279.4-283.6	81.33	-
	60	220.8±1.04	1.08	219.3-222.2	87.22	-
	90	91.0±0.95	0.90	89.0-92.3	95.11	-
Control	30	1505.3±0.57	0.33	1504.2-506.0	00	00
	60	1728.8±0.80	0.65	1727.6-729.9	00	00
	90	1864.6±2.02	4.10	1861.7-867.4	00	00

Table 4. Effect of spinetoram and nitenpyram treatment on ChE activity in clitellum region of earthworm (*Pheretima posthuma*).

Pesticides	Parameters					
	Time (in second)	Mean (U/I), S.D(±)	Variance	Range at 95% confidence limit	Inhibition %	Enhancement %
Nitenpyram	30	182.5±2.83	8.05	178.5-86.4	57.11	-
	60	307.3±1.52	2.33	305.1-309.4	19.42	-
	90	290.5±0.5	0.25	289.8-291.1	00.41	-
Spinetoram	30	205.9±1.00	1.00	204.5-207.2	51.62	-
	60	271.5±1.45	2.11	269.5-273.4	28.81	-
	90	224.9±0.85	0.73	223.7-226.0	22.90	-
Control	30	425.6±0.55	0.30	424.8-426.3	00	00
	60	381.4±1.36	1.85	379.5-383.2	00	00
	90	291.7±1.62	2.65	287.4-293.9	00	00

Table 5. Effect of spinetoram and nitenpyram treatment on ChE activity in abdomen region of earthworm (*Pheretima posthuma*).

Pesticides	Parameters					
	Time (in second)	Mean (U/I),S. D (±)	Vari-ance	Range at 95% confidence limit	Inhibition %	Enhancement %
Nitenpyram	30	160.9±1.01	1.03	159.5-162.2	90.00	-
	60	361.8±1.60	2.85	359.5-364.0	73.96	-
	90	275.3±1.52	2.33	273.1-277.4	68.84	-
Spinetoram	30	566.3±1.52	2.33	564.1-568.2	64.89	-
	60	357.2±1.05	1.12	355.7-358.6	74.29	-
	90	196.5±0.5	0.25	195.8-197.1	77.76	-
Control	30	1613.0±2.00	4.0	1610.2-1615.7	00	00
	60	1389.8±0.76	0.58	1388.7-1390.8	00	00
	90	883.7±0.62	0.39	882.8-884.50	00	00

28.81% and 22.90% in clitellum (Table 4) and 64.89%, 74.29% and 77.76% abdomen region at the respective period of time as 30, 60 and 90 seconds (Table 5). Present results are in accordance to Shimokawatoko, *et al*. (2012). They also found that spinetoram directly influences the effects on receptors of nicotinic acetylecholine. Mineau (1993) studied that activity of cholinesterase in wild birds has been decreased after the treatment of carbamate and organophosphate. Shakoori *et al*. (1994) reported that the cholinesterase inhibition in larvae of *Tribolium castaneum* (Herbst.) after the treatment of Sumicidin super. Gard & Hooper (1995) reported the carbamate and organophosphorus as potential acetylcholinesterase inhibitors at nerve synapses. The cholinesterase activities in control of 22 days old adult of daphnids stated expressed per protein content were found as 84.28±4.84 l/mol/min/mg proteins, 87.26±6.67 nmol/min/mg protein and 0.61±0.043 nmol/min/mg protein (Barata *et al*., 2005; Diamantino *et al*., 2000; Guilhermino *et al*.1996). Kristoff *et al*. (2006) reported the assessment of sample recoveries in the earthworm *Lumbricus variegatus*, under exposure of azinphos-methyl, inhibition of ChE observed to be 90%. Under treatment of chlorpyrifos the ChE activities found up to 40% inhibition (Aamodt *et al*., 2007). In the carried out work, cholinesterase activity was mostly found inhibited under exposure of nitenpyram and spinetoram. In the present study, cholinesterase inhibition activities were observed in the three regions of earthworm as in the peristomium region approximately equal, but in the region of clitellum found a little variation at intervals of 30, 60 sec of time and at the interval of 90 sec least inhibition found at post treatments of nitenpyram as compared to under treatments of spinetoram and in the region of abdomen low inhibition found under the treatment of spinetoram as compared under the treatments of nitenpyram. The previous reports showed that the nitenpyram being as (neonicotinoid) is toxic as compared to spinetoram (bio-pesticide). The difference may be due to immediate neurotoxic effect of pesticides that kill the organism by directly inhibiting neuromuscular enzyme that is accordance with above cited researchers in same paragraph.

Conclusion

Conclusively it is safe to say that spinetoram is better than neonicotinoids for the earthworms, therefore could be switched from neonicotiniods as an IPM component.

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