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RESEARCH ARTICLE

Evaluation of the toxic effect of common pesticides cypermethrin, imidacloprid and Neem (*Azadirachta indica*) fruit extract, on earthworm (*Pheretima posthuma*)

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Abstract

The toxic effect of common pesticides i.e. cypermethrin and locally used Neem (Azadirachta indica) fruit extract has been investigated on adult earthworm (Pheretima posthuma) and compared with another commonly used pesticide imidacloprid. The earthworms were treated with the test compounds using feeding cum contact method to establish LD_{50} values. Major organs of the LD_{50} treated surviving earthworms were subjected to total protein extraction, estimation, SEC FPLC and SDS-PAGE analysis. The total protein contents of cypermethrin (which was found to be the most toxic, LD_{50} = 0.14 ppm) treated earthworms was 25.2, 37.2 and 38.5 mg/ml in the peristomium, clitellum and abdominal regions, respectively. Whereas Neem fruit extract treatment (which was found to be the less toxic LD_{50} = 0.48 ppm) demonstrated moderate effect on total protein concentrations (37.8, 54.8 and 66.4 mg/ml). Imidacloprid which is also a commonly used pesticide was found to be more toxic (LD_{50} = 0.24 ppm) to earthworms as compared to Neem fruit extract and showed protein concentrations of 27.7, 26.1 and 30.3 mg/ml in the peristomium, clitellum and abdominal regions, respectively. The untreated or control animals showed protein concentration of 63.9, 76.8 and 78.7 mg/ml in peristomium, clitellum and abdomen regions, respectively. These results were further confirmed by SEC FPLC profiles which demonstrate drastic differences in terms of the proteins and peptides compared to their respective controls. These results were further complemented by SDS PAGE analysis of extracted proteins of peristomium (head) region which revealed that cypermethrin is selectively more toxic to proteins of the head region as compared to imidacloprid and Neem fruit extract suggesting different mode of action of these pesticides on different organs of the earthworms. In conclusion, our results demonstrate for the first time not only the toxic effect of these commonly used pesticides but also the differential action on the secondary target organisms such as earthworms.

Introduction

Earthworms exert both beneficial and harmful effects. They are used: as bait for fishing; food in fish aquaria; in medicines; as enhancer of soil fertility, pH, soil texture, porosity and amendments of soil organic matter content as well as plant performance (Scheu, 2003). Earthworms also play a crucial role in nutrient cycling in soil ecosystem as well as the biological activity enhancement (Parmelee et al., 1998). In view of extensive use of pesticides in agricultural fields, earthworms may be used as indicator/biomarker for total soil metabolism, soil pollution or toxicity testing (Peakall, 1994; Van Straalen and Van Gestel, 1998; Paoletti, 1999; Spurgeon and Hopkin, 1999; Lock and Janssen, 2003; Brady and Weil, 2004).

Cypermethrin, a broad-spectrum synthetic pyrethroid insecticide, is widely used in agriculture and household for pest control (Mukhopadhyay et al., 2002; 2004). Following studies which indicate possible undesirable effect of cypermethrin on the endocrine, immune, and nervous systems of the animals it has been included in the list of environment disrupting chemicals “EDCs” (Wen and Liu 2005; Li and Li, 2007). On the other hand, imidacloprid (a chloronicotinyl nitroguanidine) is the major component of the many widely used insecticides used for the control of sucking insects, turf and soil insects, and relatively persistent in soils (Capowiez et al. 2005). Imidacloprid causes interference in transmission of stimuli in the neuronal pathway leading to paralysis and eventually death (Kidd and James, 1991).

Plants extract are supposed to be a good natural source of pesticides. In particular, Neem (Azadirachta indica) tree has attracted a global attention as a potential source of natural drugs as well as environment friendly pesticides (Raguraman and Singh, 1997; Joshi and Lockwood, 2000; Daniel, 2000; Kumar, 2002). All parts of the Neem tree have some biologically active components and different aspects of its uses have been explored (Khan and Ahmed, 2000; Ahmed et al., 2001; Koul, 2004; Athanassiou et al., 2005). In particular, Neem fruits extract have been found to possess pest repellent characteristics and thus utilized extensively in manufacturing natural biopesticides.

In the present study, Neem fruit extract in comparison with two other known pesticides (i.e. cypermethrin and imidacloprid) has been studied for its deleterious effects on earthworms (Pheretima posthuma) with respect to total protein contents of various organs of this animal as a non-target organism. Our results clearly demonstrate that natural Neem fruit extract is less toxic to earthworms as compared to the other two synthetic pesticides. Results were further complemented by subjecting the total protein contents to size exclusion fast protein liquid chromatography and polyacrylamide gel electrophoresis.

Materials & Methods

Study Areas and Earthworm (Pheretima Posthuma) Collection

In the present work, earthworm (Pheretima Posthuma) was taken as a test organism as described earlier by Faheem and Khan (2010). In order to collect disease free and vigorous earthworms, field trips were conducted during February 2009 to November 2010 to the plant sales outlets, plantation plots and a variety of agro-farms situated in Sindh province of Pakistan.

Earthworm Culture and Rearing

After collection, earthworms were reared using soil of the respective collection field at 25ºC with 80% RH. The rearing media was a mixture of organic material, soil and compost etc. in appropriate amount of water. The soil was stirred periodically and kept moist. To protect the earthworms from crawling out and getting caught by omnivorous/carnivorous animals the containers were closed with muslin cloth. In the present study, one week old adult earthworms were used. The Neem (Azadirachta indica) fruit extract was prepared as ideally described by us (Khan and Ahmed, 2003). Cypermethrin (92%, technical grade) and imidacloprid were procured from the Pesticide Research Institute Karachi (PRIK). Serial dilutions of 0.04, 0.08, 0.16, 0.32 and 0.64 ppm from 1% stock solution were used for the treatment as recently described by us (Faheem and Khan, 2010).

Extraction and Estimation of Total Protein Contents

Ten adult earthworms were exposed with their respective LD_{50} for 48 hrs. The deleterious consequences of the exposure of cypermethrin, imidacloprid and Neem fruit extract on the total protein was analyzed after dissection of peristomium, clitellum and abdomen regions of the each treated surviving earthworm(s) and homogenization in 50 mM buffer of sodium phosphate and 0.01% NaN_3 (6.8 pH). The homogenized samples were centrifuged for 15 min at a speed of 14,000 rpm at 4ºC (Biofuge PrimoR, Heraeus Japan). The supernatant was collected and stored at -20ºC for further use. Total protein concentration of the samples was estimated by the Bradford (1976) method.

Chromatographic and Electrophoretic Analysis of Proteins

The chromatographic behavior of the total protein extracts (*ca.* 1 mg/ml) was analyzed by fast protein liquid chromatography (ÄKTA-design, GE Healthcare, UK) using a size exclusion column (TSK2000 SW, 7.5 x 300mm, Tosoh Bioscience, Japan). The column was equilibrated and eluted in 50 mM buffer of sodium phosphate and 0.01% NaN_3 (6.8 pH). The flow rate was maintained at 1 ml/min and the eluate was monitored at 280 nm. The data was analyzed by automated software UNICORN 5.0 (GE Healthcare, UK). In order to complement, the crude extracts (ca. 25-30 μg) were also subjected to polyacrylamide gel electrophoretic analysis (Mini-PROTEAN® 3

Cell, Bio-Rad Lab, UK) under dissociating and denaturing conditions (pH 8.8 in presence of SDS and ß-mercaptoethanol) as described by Laemmli (1970). The crude extracts were subjected to 10% gel and run at 140V for 50 minutes. Finally, the gels were stained using 0.2% Coomassie Brilliant Blue R-250 (Fluka, Germany) as described earlier (Ali et al., 1995; 2013).

Results

Toxicity Profiles of the Analyzed Pesticides

Toxicity of some common and locally used pesticides such as cypermethrin, imidacloprid and Neem fruit extract were examined in terms of their LD_{50} and the effects on whole protein contents of different organs of adult earthworms (Pheretima posthuma) having age of 7 days were observed. As shown in figure 1, the LD_{50} of cypermethrin, imidacloprid and Neem fruit extract was found to be 0.14 ppm, 0.24 ppm and 0.48 ppm, respectively. It was observed that the order of efficacy by contact cum feeding method was found to be as cypermethrin > imidacloprid > Neem fruit extract (Figure 1).

Effect of Pesticides on Total Protein Contents

The total protein contents in the different body organs (i.e. peristomium, clitellum and abdominal regions) of the treated surviving adult earthworms (Pheretima posthuma) were also extracted and estimated. The total protein concentration in cypermethrin treated earthworms was found to be 25.2, 37.2 and 38.5 mg/ml in the peristomium, clitellum and abdominal regions, respectively. While, total protein contents in the imidacloprid treated earthworms was found to be 27.7, 26.1 and 30.3 mg/ml in the peristomium, clitellum and abdominal regions, respectively (Figure 2). On the other hand, in the Neem fruit extract treated earthworms, it was found to be 37.8, 54.8 and 66.4 mg/ml in the peristomium, clitellum and abdominal regions, respectively. In case of untreated control batches of the earthworms, the total protein contents were found to be 63.9, 76.8 and 78.7 mg/ml in the peristomium, clitellum and abdominal regions, respectively.

Group separation of proteins using SEC-FPLC

The effect of cypermethrin (CYP), imidacloprid (IMD) and Neem fruit extract (NE) on the total protein pattern of peristomium, clitellum and abdomen regions of earthworm Pheretima posthuma was also analyzed by SEC FPLC and the superimposed chromatograms of each region treated with pesticides and control are presented in figure 3a-c. In panel (a) the chromatogram (in green) shows the dominant effect of cypermethrin on peristomium region. The cypermethrin causes the suppression of peak at 4.8 min retention time, enhancement of peak at 12 min and appearance of two new peaks at 15.5 min and 19 min. The Neem fruit extract only affected the peak area between 10 to 12.5 min while chromatogram of imidacloprid treated peristomium region shown in black was not found to have significant effect on total protein separation as compared to control. The panel (b) in figure 3 shows the effect of three pesticides on clitellum region. The peak near retention time 5 min is suppressed by all used pesticide as compared to control while shoulder peaks at 12.5 min were observed after major peak 11.5 min. The panel (c) in figure 3 shows the effect three pesticides on abdominal regions. Two pesticides cypermethrin and imidacloprid significantly decrease the peak at 5 min while cypermethrin causes the increase in the intensity of peaks at 11.5 min and 14.5 min. The treatment with imidacloprid seems to affect the protein in the low molecular mass region as can be observed in the chromatograms (Figure 3a-c).

Evaluation of electrophoretic behavior of test and control samples

The SDS-PAGE gel (10%) in figure 4 shows the difference in the number and intensity of protein bands in the peristomium region of earthworm Pheretima posthuma treated with Neem fruit extract (Lane 1 from right), Control (Lane 2), imidacloprid (Lane 3) and cypermethrin (Lane 4). In control, approximately 10 protein bands can be observed easily. Lane 1 shows that Neem fruit extract causes the absence and decrease in the intensity of some protein bands, while Lane 3 revealed that pesticide imidacloprid over express (bands near 34kDa and 90kDa) and/or destroy some proteins. Cypermthrin treated sample in lane 4 also shows the over expression and disappearance of some proteins but very distinct than imidacloprid i.e. lane 3 (Figure 4).

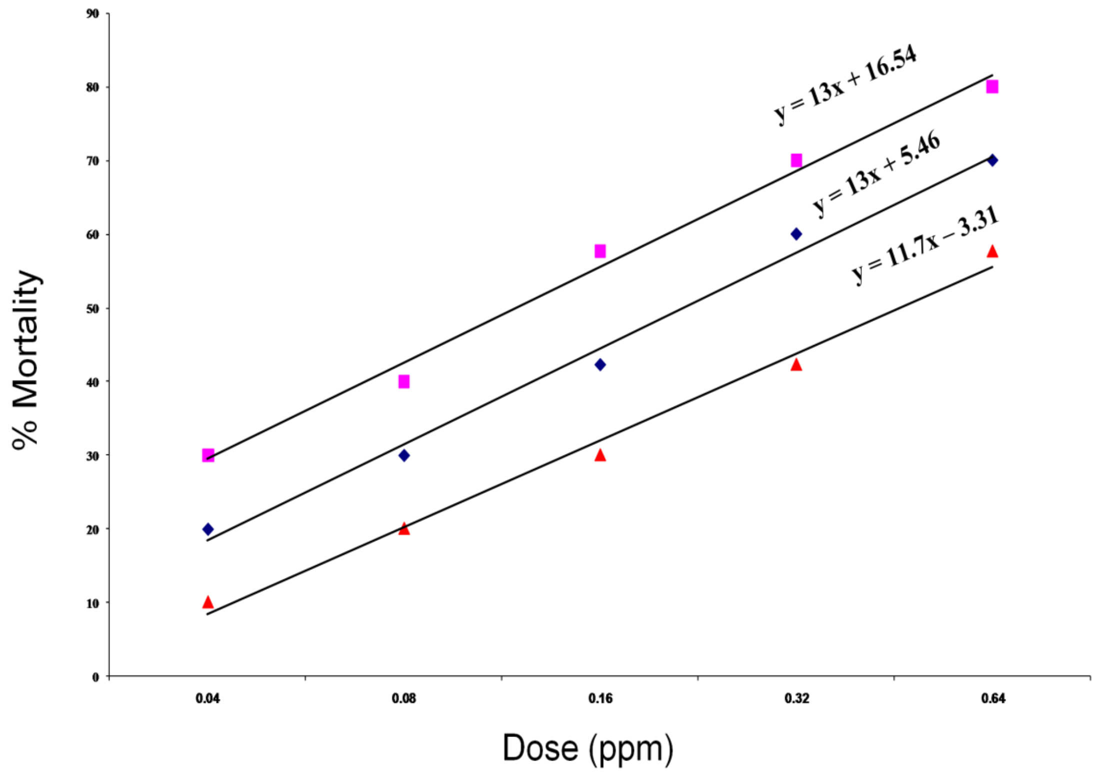

Figure 1. Mortality curves of earthworm Pheretima posthuma under the effect of cypermethrin (CYP, - ■ -), imidacloprid (IMP, -♦-) and Neem fruit extract (NE, -▲ -). Each point is the mean of duplicate (n=10). See “Materials & Methods” for details.

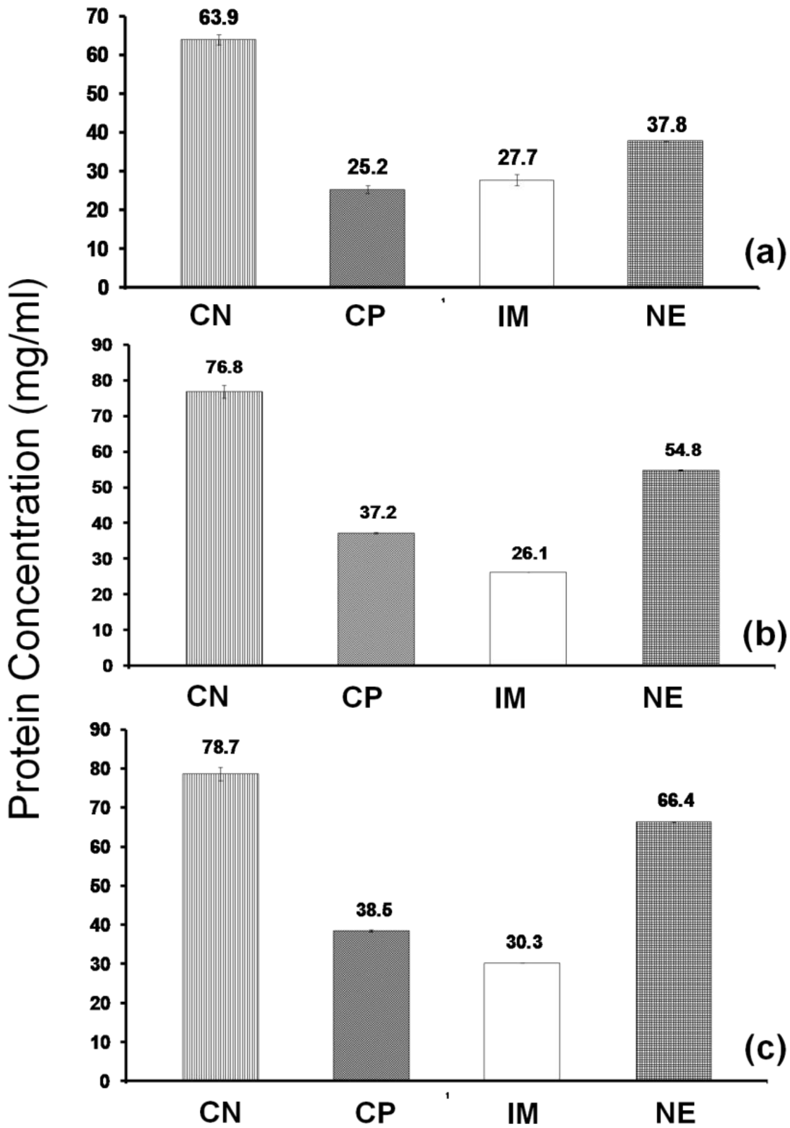

Figure 2. Comparative effect of cypermethrin (CP), imidacloprid (IM) and Neem fruit extract (NM) on the extracted total protein concentrations measure in the peristomium (a), clitellum (b) and abdomen (c) regions of LD_{50} treated earthworm (Pheretima posthuma). In each panel "CN" represents the untreated controls. Values plotted are the mean of two experiments (n= 10) ±SD.

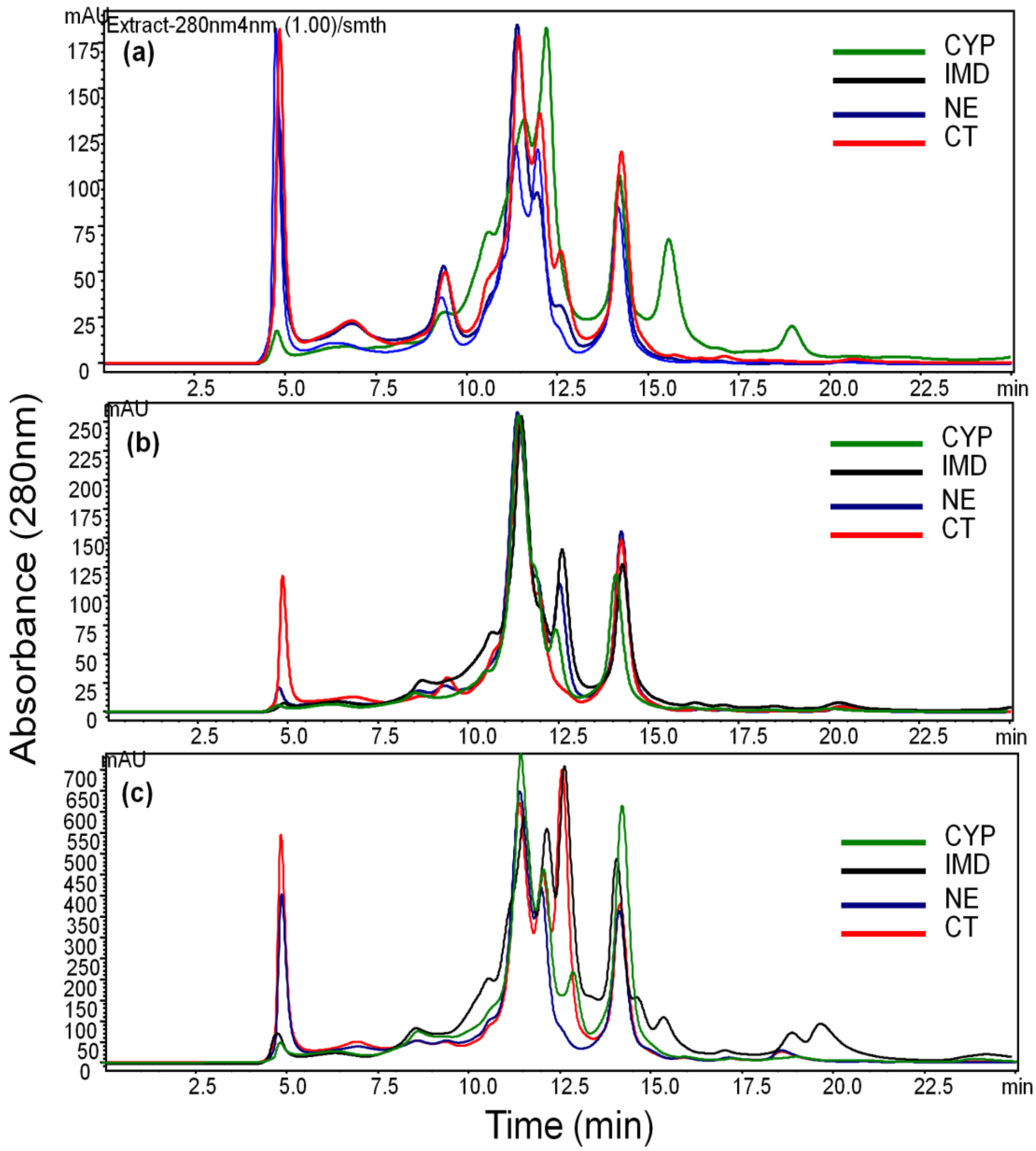

Figure 3. Comparative effect of cypermethrin (CYP), imidacloprid (IMD) and Neem fruit extract (NE) on the total protein pattern of earthworm Pheretima posthuma, on peristomium (a), clitellum (b) and abdomen (c) regions, as analyzed by SEC FPLC. In each chromatographic panel, profile "CT" represents the control. See "Materials & Methods" for details.

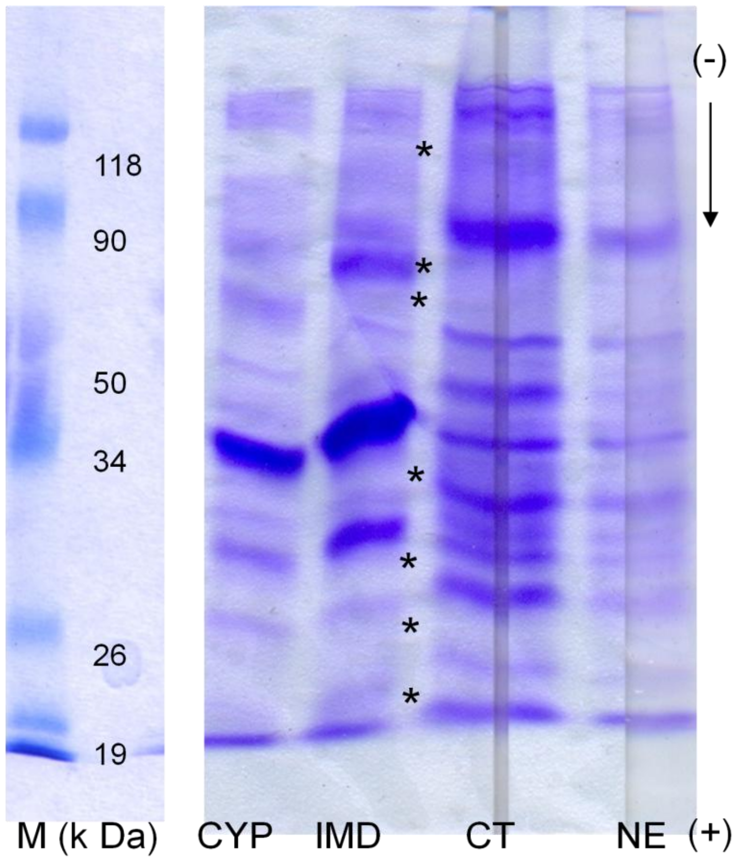

Figure 4. Effect of cypermethrin (CYP), imidacloprid (IMD) and Neem fruit extract (NE) on the total protein expression pattern analyze by 10% SDS PAGE in the peristomium (head) regions of earthworm Pheretima posthuma. Lane “CT“ represents the control. Asterisks represents the differential band patterns and arrow indicates the electrophoretic mobility. Lane M, known protein marker with annotated molecular weight in kilodalton (kDa).

Discussion

Toxicity profiles of at least cypermethrin in different non-targeted organisms have been extensively evaluated and reported in the past. For example, the effect of alpha-cyprmethrin on the behavior of male guppies revealed the LC_{50} value of 17.94 μg/L (Yılmaz et al., 2004; Sarıkaya, 2009). Likewise, studies by Wendt-Rasch and coworkers (2003) on the crustacean, rotifer, periphyton and phytoplankton showed minimum inhibitory concentration of toxicant as 0.13 μg/L.

In the present work, toxicity of some common and locally used pesticides such as cypermethrin, imidacloprid and Neem fruit extract were examined in terms of their LD_{50} and the effects on whole protein contents of different organs of adult earthworms (Pheretima posthuma) having age of 7 days were observed. As shown in figure 1, the LD_{50} of cypermethrin, imidacloprid and Neem fruit extract was found to be 0.14 ppm, 0.24 ppm and 0.48 ppm, respectively. It was observed that the order of efficacy by contact cum feeding method was found to be as cypermethrin > imidacloprid > Neem fruit extract (Figure 1).

Effect of various pesticides on total protein contents and different enzymatic activities in different organisms have also been extensively studied by a number of workers. Earlier Sak and coworkers (2006) reported that the level of protein in all stages and sexes of the wasp tend to be reduced after treatment with cypermethrin as compared to controls. Likewise, Mosleh and coworkers (2003) reported the effects pertaining to aldicarb, cypermethrin, profenofos, chlorfluazuron, atrazine, and metalaxyl on the soluble protein of the LC_{25} treated earthworm. As compared to other tested pesticides, the aldicarb appeared to be the most toxic. Vijayaraghavan and coworkers (2002) also reported that the total protein increases from larval to pupal transition whereas a decreases has been observed in larvae of Spodoptera litura Fabr. (Lepidoptera: Noctuidae) exposed to various insecticides. Ribeiro and coworkers (2001) also observed a reduction in protein content and associated it with physiological adaptability for compensation of insecticidal stress. In contrast, Mujeeb (2000) consider the elevated protein content in beetles of the stored grain pests Tribolium castaneum (Herbst.) to be due to treatment effects of malathion and primiphosmethyl. Gill and coworkers (1990) reported the impact of an organophosphate in Punctius conchonius and demonstrated decline in the contents of protein. Similarly, Tripathi and Shukla (1991) observed changes in protein pattern in Clarius batrachus after methyl parathion treatment. Saleem and coworkers (1998) also reported reduction in total protein contents of T. castaneum larvae after 4 days treatment of Ripcord 20EC (cypermethrin). Likewise, a decline in protein contents following pesticide treatment has been observed in different organisms (Ahmad et al., 2000; Tabassum and Naqvi, 2001). In conclusion not restricted to different pesticides, even different plant extract based agro-treatments also effect proteins as well as nucleic acid contents of many secondary target organisms (Naqvi et al., 1994; Azmi et al., 1997; 1998; Nurulain et al., 1997; 2000; Ahmad et al., 2003).

In the present work, the total protein contents in the different organs (i.e. peristomium, clitellum and abdominal regions) of the treated surviving adult earthworms (Pheretima posthuma) were also extracted and estimated. The total protein concentration in cypermethrin treated earthworms was found to be 25.2, 37.2 and 38.5 mg/ml in the peristomium, clitellum and abdominal regions, respectively. While, total protein contents in the imidacloprid treated earthworms was found to be 27.7, 26.1 and 30.3 mg/ml in the peristomium, clitellum and abdominal regions, respectively. On the other hand, in the Neem fruit extract treated earthworms, it was found to be 37.8, 54.8 and 66.4 mg/ml in the peristomium, clitellum and abdominal regions, respectively. In untreated control batches of the earthworms on the other hand, the total protein contents were found to be 63.9, 76.8 and 78.7 mg/ml in the peristomium, clitellum and abdominal regions, respectively (Figure 2). This study clearly revealed a drastic decrease in total protein contents of the pesticides treated earthworms as compared to untreated controls. Moreover, the total protein depletion effect is more significant in cypermethrin and imidacloprid treated earthworms as compared to the Neem fruit extract which nicely correlated with their respective toxicities in terms of LD_{50} values.

In order to complement overall protein depletion in different organs of the earthworms observed as a result of different pesticide treatments, the extracted proteins were subjected to size exclusion fast protein liquid chromatography. SEC FPLC profiles not only demonstrate major differences in terms of the proteins and peptides depleted as compared to control, but also provide clear differentiation between the different pesticides as well as their distinct effect in different organs of the earthworms. For example, cypermethrin which is found to be more toxic to earthworms, both in terms of LD_{50} value (Figure 1) and the maximum depletion of total protein concentration (Figure 2), demonstrate toxic effect on high molecular weight proteins in all three organs (i.e. peristomium, clitellum and abdomen) of the earthworms as apparent from the major protein peaks at retention time of 4.8 min (Figure 3a-c). This depletion of high molecular weight proteins are also complemented by the increase in low molecular weight proteins and peptides peaks which appears at the end of each chromatograms compared to their respective controls. Moreover, the depletion effect is more than 90% in each organ as compared to control samples. Similarly, imidacloprid, the second most toxic tested pesticide, revealed the same effects as cypermethrin in the clitellum and abdomen region but interestingly shows almost no effect on peristomium or head region as compared to controls suggesting the different mode of action of the two pesticides in different organs of the earthworms. Results of the above mentioned SEC FPLC also demonstrate that despite identical protein contents (Figure 2) in the two pesticides treated earthworms, the type of proteins depleted or expressed are quite different (Fig. 3a-c). On the other hand, locally used plant natural pesticide i.e. Neem fruit extract was found to be the least toxic as compared to their respective controls and other two organic pesticides despite revealed more selective effect on abdominal region proteins as compared to peristomium and clitellum regions (Figure 3b).

A representative example of the under study pesticides effect on peristomium (head) region of the earthworms was also demonstrated by a 10% SDS-PAGE analysis (Figure 4). Results revealed overall protein depletion as compare to untreated control and demonstrate that the cypermethrin is more and selectively toxic to head region than imidacloprid and Neem fruit extract which not only complement the SEC FPLC results (Figure 3a) but also suggest that the combination of these two techniques can be used for the analysis of mode of action of these pesticides on different organs of the earthworms and/or other non-target organisms. As very little is known so far about the different proteins and/or proteome of earthworms, characterization of the different proteins depleted or over-expressed, for example a 34 kDa major band in cypermethrin and imidacloprid treated earthworms would be an interesting target to analyze in the future studies (Figure 4).

In conclusion, our results for the first time not only demonstrate the toxic effect of these under used pesticides but also the differential and/or different mode of action on the secondary target organisms such as earthworms (Pheretima Posthuma).

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