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BIOACCUMULATION OF HEAVY METALS MIXTURE AND ITS EFFECT ON DETOXIFICATION ENZYMES OF WOLF SPIDER, *PARDOSA OAKLEYI*

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ABSTRACT

The relationship between detoxifying enzymes and heavy metal body burden in the spider *Pardosa oakleyi* was assessed. Superficial layer of soil was sampled from three field sites which varied in their physico-chemical characteristics and was analyzed for heavy metals (Cr and Cu) contents. Spiders were also sampled from the same field sites and were analyzed for these metal contents. The activity of glutathione-S- transferase (GST), acetylcholiesterase (AChE), carboxylesterase (CarE) and Cytochrome P450 (Cyt P 450) was also assayed in the spiders. In the soil, the concentration of Cr ranged from 13 to 114 ug /g and Cu from 28 to 112 ug / g. Metal body burden in spiders from three sites also varied from 5 to 74 ug / g of Cr and 32 to 81 ug / g of Cu. A negative relationship was observed between concentration of Cr and Cu in the soil and in the body of spiders collected from the field site. A strong negative relationship was observed in the levels of GST, AChE and CarE and metal quantity in spider's body. Cytochrome P450 did not show any relationship with the metal body burden of spiders. The results indicate that high bioaccumulation of heavy metals of Cr and Cu leads to decrease activity of detoxifying enzymes.

Key words: - wolf spiders, bioaccumulation, chromium, copper, detoxifying enzymes inhibition.

INTRODUCTION

Metals are accumulating into the terrestrial and aquatic ecosystems globally due to many natural processes such as urbanization, industrial revolution and intensification of agriculture. Agricultural activities are adding rather large quantities of heavy metals in the soil through application of fertilizers and pesticides (Atafar *et al*., 2010; Malidareh *et al*., 2014). Accumulation and persistence of metals in the soil and their translocation via food webs or food chain is directly related with the sustainability of ecological systems (Wuana and Okieimen, 2011; Opaluwa *et al*., 2012). Accumulation of heavy metals in different layers of soil depends upon different factors such as its type, texture, quantity of organic matter and environmental conditions. All heavy metals present in the soil are not available to the soil organisms. The bioavilability of heavy metals depends upon their concentration present in the soil solution (Gambrell, 1994).

Accumulation of heavy metals varies between different groups of organisms due to the difference in their physiology, behavior, and trophic level (Hendrickx *et al*., 2003; Wilczek *et al*., 2008). Among invertebrates spider accumulate higher concentration of heavy metals (Hendrickx *et al*., 2004) and are regarded as macro-concentrator (Dallinger, 1993). They colonize metals contaminated areas where they successfully sustain their populations and respond at species, population and community level to any change in the environment (Jung *et al*., 2008; Babczyńska *et al*., 2012). Therefore, spiders are considered excellent biological indicators to explore the environmental health potentially contaminated with heavy metals.

Lycosids are ground dwelling spiders and are strong metal accumulator (Wilczek and Migula, 1996; Wilczek and Babczyńska, 2000), especially member of the genus *Pardosa* (Lagadic, 1999; Migula, 2000) due to their direct contact with polluted soil, their active hunting strategy and their types of prey organisms such as members of Diplopoda, Diptera, Collembola and Isopoda groups that are also known for storage of heavy metals (Nyffeler and Benz, 1981; Hendrickx *et al*., 2004; Wilczek *et al*., 2008; Jung *et al*., 2008).

Organism uses various mechanisms to deal with additional stress caused by heavy metals accumulation in their body. They either store metals in different organs of their body (Ludwig and Alberti, 1988) or eliminate them from their bodies via excretion or molting (Lee *et al*., 1978). They also exhibit a variety of enzymatic activities to metabolise or degrade different xenobiotics (Augustyniak *et al*., 2005; Wilczek *et al*., 2004, 2008). Spiders show resistance to bioavailable metals by increasing level of detoxifying enzymes above normal range. Zvereva *et al*. (2003) demonstrated that metal detoxification by enzymes favor the survival of organism in polluted environment. Enzymes protect cells from oxidative damage that inhibits antioxidant activity thus secure more important molecule like DNA, RNA and proteins (Augustyniak and Migula, 2000). There is an opinion that synthesis of detoxification enzymes is energetically more costly as compared to other detoxifying processes (Müller *et al*., 1994). Organisms

usually have energy constraints. For the production of high quantity of enzymes, they have to utilize energy resources that should be used in their maintenance and reproduction. This also decreases their fitness in natural conditions. In some cases metals directly interact with active site of enzymes and cause their inhibition in the organisms (Wilczek and Migula, 1996; Stone *et al*., 2002).

In present study, the quantity of Cr and Cu in the soil and bodies of adult *Pardosa oakleyi* collected from three different areas was measured. The accumulation level of these heavy metals in the body of *P. oakleyi* was assessed and its relationship with some detoxifying enzymes was calculated. For this purpose spider sampled from the same area were assayed for enzymes glutathione-S- transferase (GST), acetylcholiesterase (AChE), carboxylesterase (CarE) and Cytochrome P450 (Cyt P450). The suitability of these enzymes as biomarkers of heavy metals pollution was also assessed.

MATERIALS AND METHODS

Sampling Sites: Spiders were collected from agricultural fields located at three different sites of district Lahore, Pakistan. Site I was vegetable fields alongside Hudaira drain (HD) near Gajumata (31º23'N/74º21E). More than 100 industrial units and factories of different natures are situated all along HD and discharge their effluents into it. The studied field was irrigated with treated water of HD for many years. Site II was agricultural fields in the vicinity of River Ravi (31º36N/74º17E). The main sources of pollution in the River Ravi (RR) are municipal, agricultural and industrial wastes. Additionally, it also gets effluents from nine different polluted water drains of Lahore city. This field was irrigated with the water of RR. At each moon soon season the river is flooded and top soil of the studied field is replaced with new layer of soil Site III was agricultural fields at University of the Punjab (31º29N/74º21E), irrigated with ground water (PU). Moreover, in all fields, pesticides and fertilizers are used according to the need.

Sampling: Adult specimens of *P. oakleyi* (Family lycosidae) were hand collected from July to September, 2013 and 2014 from all the selected sites. Maturity of the spider was determined according to Foelix (1996). Collected specimens were transported to the laboratory in separate glass vials. Of the thirty spiders collected from each sampling sites, ten were killed and stored in the freezer to estimate concentration of heavy metals in their bodies. The remaining individuals were maintained in the laboratory and used for the estimation of enzymes.

From each site, three top soil samples (approximately 20 cm deep and weighing 500 g each) were taken from different locations and after drying stored in glass jars for quantitative estimation of heavy metals.

Metal Analysis: For heavy metal analysis, frozen spiders were washed with 1% HNO_3 solution and dried for 48 hours at 70°C before digestion and weighed. Then 25 mg of dried spiders were digested in 5 ml of ultra pure 65% HNO_3 and 2ml of 20% H_2O_2 (two times) at 130°C. The solution was reduced to 2 ml and then diluted upto 20 ml using 1% HNO_3 (Tack *et al*., 2000). Concentrations of heavy metals (Cr and Cu) were determined using Atomic absorption spectrophotometer (z-5000).

Soil samples were completely dried at 70 °C for 72 hours. The dried soil (1g) samples were subjected to acid digestion in 15 ml of 0.1 N HCl and HNO_3 (3:1v/v) solution at 100 ºC and reduced until it became 5 ml (Jung and Lee, 2012). It was then filtered with Whatman filter paper number 42. The filtrate was then diluted with distilled water up to 50 ml. Concentrations of heavy metals were determined using Atomic absorption spectrophotometer.

Preparation of sample and Enzyme assays: Single adult specimen of *P. oakleyi* was anaesthetized on the ice and homogenized in 1 ml of ice-cold 0.02 M sodium phosphate buffer, pH 7 using Teflon-glass homogenizer. Homogenate was centrifuged at 10,000 g for 10 minutes at 4 ºC. Supernatant obtained was used immediately to estimate total protein and in enzyme assays**.**

Total protein concentration in the supernatant was determined by Bradford method using Bovine Serum Albumen (BSA) as a standard (Bradford, 1976). Glutathione-S-transferase activity was determined by rate of conjugation of CDNB (1-chloro-2, 4 dinitrobenzene) towards glutathione as a substrate. The total volume of the mixture was 3ml, containing 100 µl of supernatant, 1.0 mM reduced Glutathione, 1.0 mM CDNB and 1.0mM phosphate buffer (pH 6.5). Change in absorbance was recorded at 340 nm after 5 minutes (Fan *et al*., 2007). Absorbance value was expressed as nmol of CDNB-GSH conjugate produced per minute per mg of protein.

Acetylcholine esterase (AChE) activity was estimated according the method of Ellman *et al*. (1961) using acetylthiocholine-iodide (ATChI) as a substrate. The reaction mixture consisting of 1.5 mM ATChI, 1.0 mM DTNB (5.5-dithio-bis 2-nitrobenzoic acid), and 100 µl of supernatant was prepared in a final volume of 3ml with 0.1 M phosphate buffer (pH 8.0). Absorbance was recorded at 412 nm after 30 minutes incubation at 30°C and represented as nmol AChI per minute per mg of protein.

Carboxylesterase (CarE) activity of supernatant was measured using Van Asperen method (1962) with some modifications. 100 µl of supernatant and 0.3mM of α-Naphthyl acetate (1-NA) was mixed and prepared with 0.1 M phosphate buffer, pH 7.0 in a final volume of 3 ml. The mixture was incubated at 30˚C for 30 minutes. Then

reaction was stopped by adding 1 ml of fast Blue B salt-sodium dodecylsulphate solution (2 parts of 1% Fast Blue B salt and 5 parts of 5% SDS). After 15 minutes the optical density was measured at 600 nm and expressed as nmol NPA per minute per mg of protein.

General Oxidase activity was determined following method of Martin *et al*. (2002). For estimation, 160 µl aliquot of enzyme source mixed with 640 µl of 62.5 mM potassium phosphate buffer (pH 7.2), 1600 µl of TMBZ solution (3,3́,5,5́- tetramethylbenzidine) and 25 µl of 3% hydrogen peroxide. The mixture was incubated at 25 °C for 30 minutes and absorbance was recorded at 630 nm. A standard curve was prepared using different concentration of cytochrome C. Total oxidase activity was expressed as nmol equivalent Cyt P450/mg protein by using standard curve.

Data Analysis: The metal bioaccumulation factor (BAF) in spiders was calculated following Jung and Lee (2012). Metal body burden (MBB) of spider was calculated by adding quantity of Cu and Cr in the body of the spider. All the relationships were determined using Pearson's correlation. ANOVA was employed to assess the differences in the concentrations of detoxifying enzymes of spiders collected from the three studied sites. Where significant difference was recorded, means were separated using Tukey´s posthoc test. All statistical analyses were performed using Minitab 17.

RESULTS

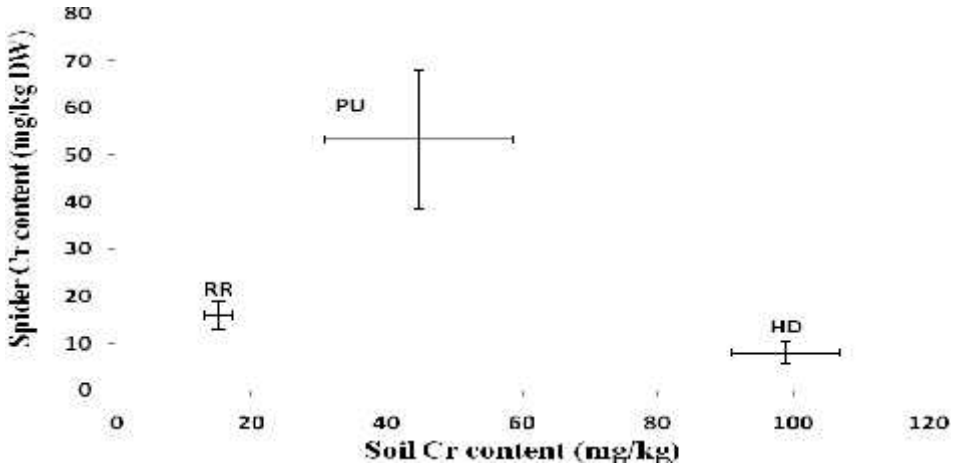
The highest concentrations of Cu and Cr in soil were recorded from HD, followed by PU and RR (Fig. 1). The quantity of heavy metals was significantly different between the sites (Cu: df = 2,22, F= 181.19, $p < 0.001$; Cr: df = 2,22, F= 173.92, $p < 0.001$). The concentration of Cu and Cr in the bodies of spiders collected from the three studied sites also differed from each other (Cu: df = 2,22, F= 6.61, p = 0.006; Cr: df = 2,22, F= 76.23, $p < 0.001$). Average concentration of Cu and Cr was high in spiders collected from RR and was low in spiders collected from HD sites (Fig. 1). Negative correlation was observed between metal content in soil and in the body of spider (Cu: r = - 0.475; p = 0.003 and Cr: r = - 0.259; p = 0.127). Metal bioaccumulation factor (BAF) of spiders collected from three field sites, also differ from each other. Highest quantity of metals was accumulated by spiders collected from RR and least from HD (Fig 2).

AChE activity (in nmole/min/mg protein) differed significantly in spiders collected from the three study sites (df = 2,102; F = 37.49, $p < 0.001$; Fig. 3a). Highest activity was recorded in spiders collected from HD (95.3 ± 11.6) site. Quantity of AChE was high in spider collected from PU (39.3 ± 1.7) compared to RR (13.7 ± 1.46) sites. AChE concentration was negatively related with MBB of spider (r = - 0.693, p = 0.039) .

Spiders showed variable CarE activity (in nmole/min/mg protein) from the three sites (df = 2,91; F = 5.28, p = 0.007; Fig. 3b). Highest activity was recorded from HD (340.5 ± 49.0) site. The spiders at PU (191.9 ± 24.0) and RR (190.5 ± 37.4) produced similar concentrations of CarE enzyme. CarE quantity showed no significant correlation with MBB (r = - 0.150, p = 0.70).

Activity of GST in spiders collected from all three sites differ significantly (df = 2,102; F= 81.07, $p < 0.001$). The highest GST activity was recorded in spider (in nmole/min/mg protein) from HD site (506.5 ± 42.3), followed by PU site (371.4 ± 12.2) and lowest from RR site (24.75 ± 2.56) (Fig. 3c). GST activity in spider showed a significant negative relationship with MBB (r = - 0.627, p = 0.049).

The highest Cyt P450 values (mg of protein) were recorded in spiders collected from PU (1.67 ±0.01) and it significantly differed in spiders collected from the other two sites (df = 2,57; F= 26.22, $p < 0.001$; Fig. 3d). Least value was recorded from RR site (0.33 ± 0.03). Cyt P450 level did not show any significant relationship with MBBI (r = - 0.264, p = 0.493).



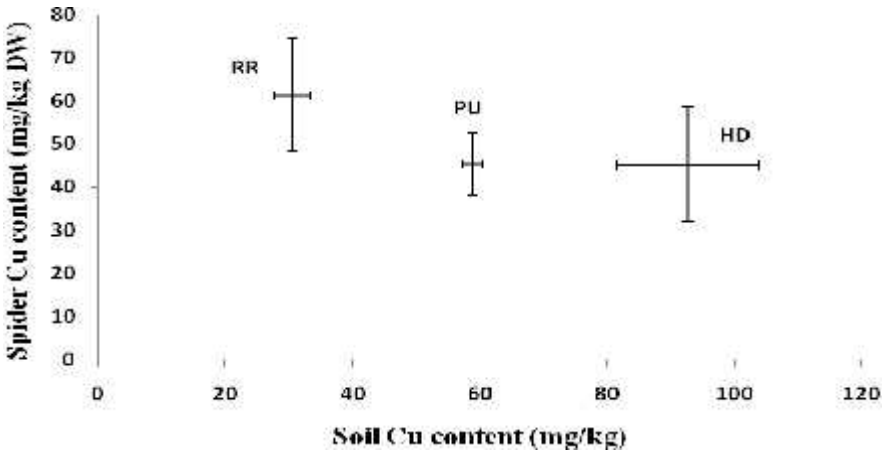


Fig.1. Quantity of Cr and Cu metals in the soil and body of the spiders, *Pardosa oakleyi* (mean ± standard deviation). HD= Hudiara Drain; PU= University of the Punjab; RR = River Ravi.

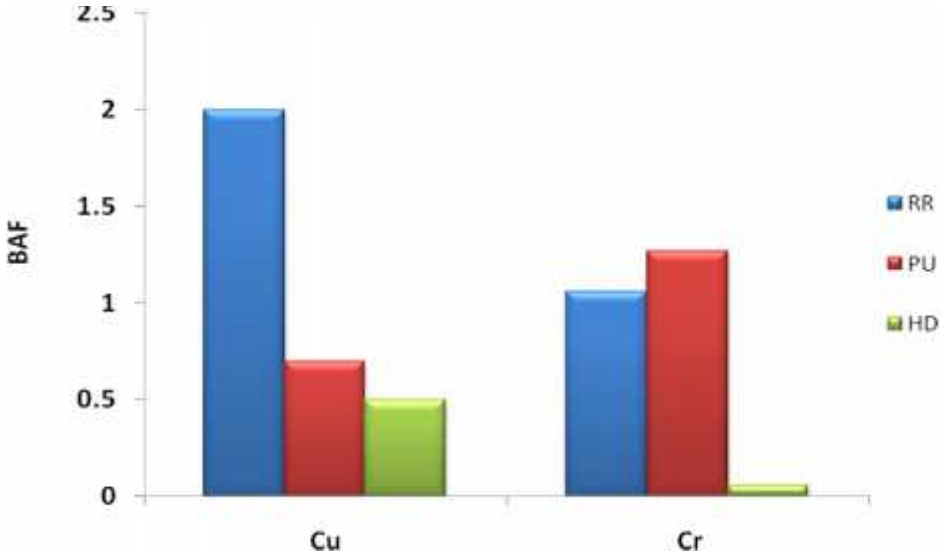
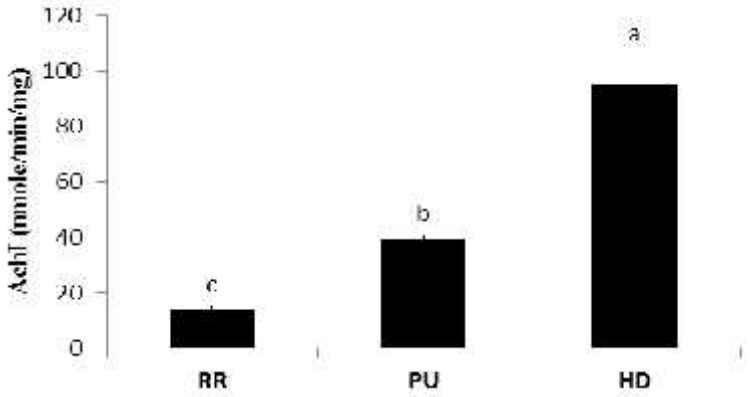


Fig.2. Bioaccumulation of Cu and Cr in the body of *Pardosa oakleyi* collected from agriculture fields located near Hudiara drain (HD), University of the Punjab (PU) and River Ravi (RR).

(a)



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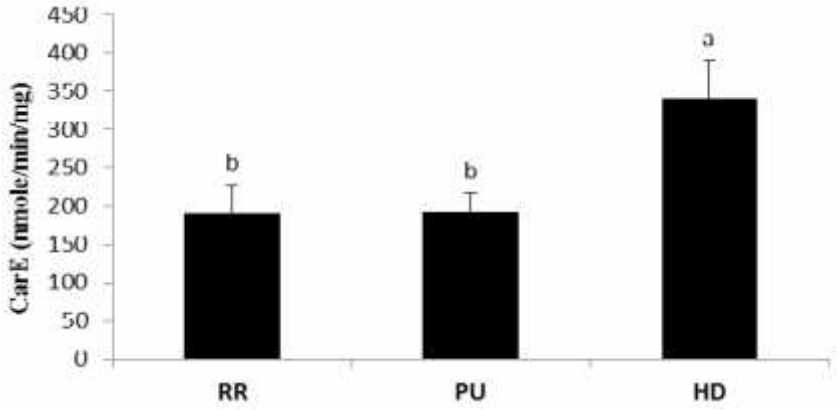
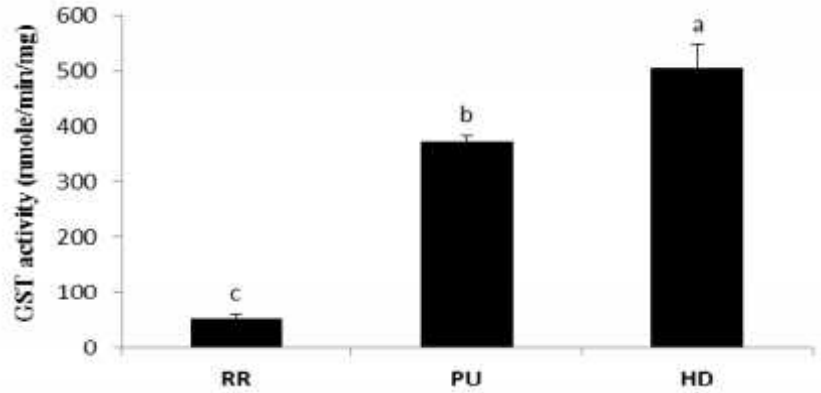


(c)



(d)

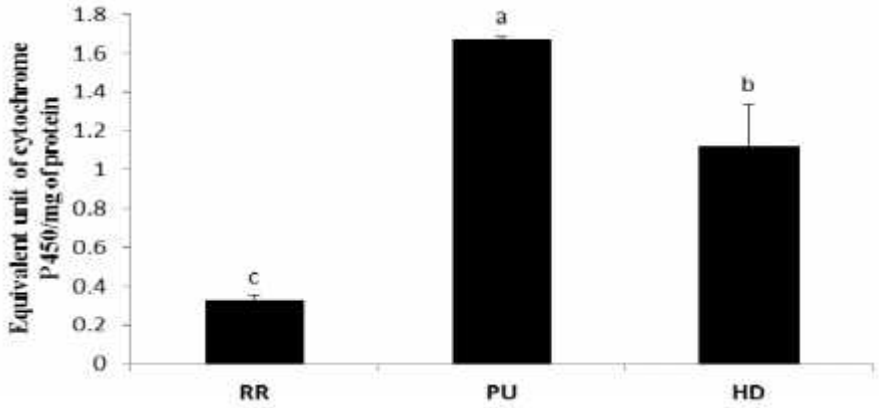


Fig. 3. Activity of (a) acetylcholinesterase (AChE); (b) carboxylesterase (CarE): (c) glutathione S-transferase (GST); and (d) oxidases (Cyt P450) in the tissues of *Pardosa oakleyi* collected from polluted sites. Values with same letter did not differ significantly (ANOVA, Tukey's test, $p < 0.05$).

DISCUSSION

In this study, the highest concentration of both metals, Cu and Cr, was recorded in the soil at HD and lowest in the body of *P. oakleyi* collected from HD. The accumulated amounts of Cu and Cr in the body of spiders were less than the highest concentration of these metals in the soil. However, in spider populations of HD and RR, bioaccumulation level of Cu was higher than Cr. In spider population of PU quantity of Cr was more than Cu. It has been previously reported that bioavailability of metals in different areas varies due to difference in the texture of soil, organic content, salinity, pH and cation exchange capacity (Gambrell, 1994; Tam and Wong, 1999). In the present study, metal body burden of spiders may be related to the available metal contents in the soil. Gambrell *et al*. (1991) reported increased soluble concentration of Cr, Cu and Cd with the increase of salinity in brackish marsh soil. These results correspond with our observation of high Cu and Cr contents in the body of spiders residing near the River Ravi (RR).

Metals concentration in the body of spiders also varies in populations of a species and within individuals of a population. Previous studies have shown that metal body burden of a species depend on its habitat, food, hunting strategy, soil properties, metal accumulation and excretion rate (Foelix, 1996; Wilczek *et al*., 2004). In lycosid spiders, considerable heavy metal concentrations are due to their high predation rate and high metal contents in bodies of their prey such as Collembola, dipteran larvae and other spiders (Wilczek and Babczyńska, 2000; Jung and Lee, 2012). Heavy metal accumulation in spiders is also related with metal excretion rate in the feces and production rate of metal containing granules. Cu is stored in the granules that originate from lysosomal system and contain acid phosphate. It is also probable that concentrations of Cu and Cr are regulated to a certain level in the body of spiders that is why their concentration was less than background concentration in many studied samples (Hussein *et al*., 2006; Del Toro *et al*., 2010). It is reported that elimination of heavy metals like Cd, Zn, Pb and Cu was generally low in spiders compared to other invertebrates. In *Pardosa* sp. half of the total accumulated Cd was released from the body in more than three months (Karamarz, 1999).

Different detoxifying enzymes like GST, AChE, CarE and Cyt P450 are present in all biota and can be induced in the organisms by endogenous and exogenous xenobiotics (Wilczek *et al*., 2004, 2008; Augustyniak *et al*., 2005). They are easily measureable and used as biomarker in ecotoxicological studies. In the present study, the relationship between metal accumulation and level of some selected detoxification enzymes in the spider was assessed.

Activity of GST and AChE showed negative relationship with metal body burden of spider in the present study. There is a general trend of increase in GST and AChE activity with the increase of xenobiotics (Sun *et al*., 2008). But in ground beetles no difference was found in GST level in male collected from metal polluted areas (Stone *et al*., 2002). Similarly in *Pardosa lugubris* a strong negative correlation was found between heavy metals and GST activity in male spider, collected from less polluted site (Wilczek *et al*., 2004). Schmidt *et al*. (1992) reported reduced AChE in grasshopper *Aiolopus thalassinus* when exposed for 8 weeks to 8 or 80 ppm of Cd. Wilczek *et al*. (2003) also found lower AChE activity in spiders collected from medium polluted site compared to low or high polluted sites. It might be due to the blocking of active centers of enzyme by the metal in the spider inhabiting medium polluted site. Spiders of highly polluted areas may have developed some compensatory mechanisms which cause increase synthesis of enzyme molecule and high AChE activity (Qin *et al*., 2012).

The level of CarE showed no clear relationship with the quantity of xenobiotics in many studies (Wilczek and Migula, 1996; Stone *et al*., 2002). Spiders *Pardosa lugubris* and *Agelena labyrinthica* collected from the polluted sites of forest and grass land have high activity of CarE compared to those collected from unpolluted sites (Sun *et al*., 2008). In ground beetle (Coleoptera, Carabidae) the activity of CarE was also high in specimens collected from polluted areas than reference site (Stone *et al*., 2002).

Monoxygenases (cytochrome P450) are involved in metabolism of a variety of toxicants and several endogenous compounds (Scott, 1999; Kasai and Scott, 2000). However, no relationship between Cyt P450 and metal quantity in the body of *P. oakleyi* was discovered in present study. In earthworms the level of cytochrome decrease with increasing distance from metal source (Lukkari *et al*., 2004). Henczová *et al*.(2006) reported that fresh water fishes exposed to Cd showed inhibition of monoxygenases. In another study, Henczová *et al*.(2008) reported higher activity of monoxygenases in silver carp fish than Wels after exposure to dose of 10 mg/ l of Cu doses.

The results of the present study suggested that heavy metal contents strongly affect activity of the spider *P. oakleyi* triggering inhibition of enzymes at high metal concentration. Activity of these enzymes was related with the contents of heavy metals or xenobiotics in the body of the spider. During the study only two metals were recorded in the soil and body of the spiders. Many other pollutants would also be bioavailable and accumulate in the spider body increasing stress to the detoxifying enzymes. Along with that maintaining high quantity of detoxifying enzymes requires high energy allocation. For their survival *P. oakleyi* has to utilize a big portion of its body energy in the repair of the organ

damaged by bioaccumulation of heavy metals. So their will be trade off between maintenance of the organs and production of detoxifying enzymes. That is why *P. oakleyi* specimens collected from polluted sites did not exhibit any apparent intoxicated symptoms. On the basis of this study, it is suggested that *P. oakleyi* can be used as bioindicator of heavy metals in the soil.

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