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EFFECT OF HEAVY METALS ON ANTIOXIDANT ENZYMES IN *OXYA HYLA HYLA* (ORTHOPTERA: ACRIDIDAE)

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ABSTRACT

Toxic effects of heavy metals on grass hopper species, *Oxya hyla hyla* (Serville) were studied by exposing *O.hyla hyla* to Cd^{+2}, Pb^{+2} and Hg^{+2} at different concentrations of $CdCl_2$, $PbCl_2$ and $HgCl_2$ for variable exposure time (24h, 50h and 75h) observing changes in the activities of antioxidant enzymes *[superoxide dismutase (SOD), catalase (CAT), peroxidase (POD)]* and biochemical composition of haemolymph. Significant accumulation of metals was noticed that alarmingly increases with increase in exposure time and dosage. At low metal concentrations, oxidative stress was expressed by SOD and this effect disappeared at high concentrations. Assayed activities of CAT, POD and LPO level were significantly accelerated and correlated positively as metal exposure time increased. A significant decrease in total soluble protein, sugar, lipids and glycogen contents was also observed due to metal exposure throughout the entire test period except after the first 24h of exposure at lower concentrations. The study proved *O.hyla hyla* (Orthoptera: Acrididae) and its antioxidant enzyme level as bio-indicators and bio-markers of biotic and abiotic stresses.

Keywords: Heavy metals, antioxidant enzymes, *Oxya hyla hyla,* Grasshopper.

INTRODUCTION

Organisms respond variably to environmental stresses by their physiological and biochemical mechanisms (Niu *et al.,* 2002). Scientific community many times has acknowledged the potential importance of dietary metal toxicity in natural ecosystems, yet insects are still under represented in such datasets (Davis *et al.,* 2001). Metal trace elements including Cd, Pb and Hg are non-biodegradable xenobiotics and no specific detoxifying enzymes are known for their metabolism. Increased presence of such metals in cells undergoes redox reactions that affect catalytic abilities of anti-oxidative enzymes (Cervera *et al.,* 2003).

These metal trace elements are often involved in oxidative stresses at cellular level and results in production of reactive oxygen species (ROS). ROS includes, superoxide radical (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) mainly affecting lipids, proteins, carbohydrates and nucleic acids in living organisms (Damien *et al.,* 2004). The importance of antioxidant enzymes is generally emphasized in the prevention of oxidative stresses by scavenging ROS. Antioxidant system, responsible for scavenging ROS comprises of several enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). SOD convert superoxide radicals to H_2O_2 whose accumulation is prevented in the cell by CAT and POD (Sharma e*t al.,* 2011). Super oxide dismutase, catalase and peroxidase are thus considered as primary enzymes as they are involved in direct elimination of ROS (Farima *et al.,* 2004). Lipid peroxidation, a most important cellular deteriorative change is one of the primary effects induced by oxidative stresses and occurs readily in tissues due to the presence of membrane rich in polyunsaturated highly oxidizable fatty acids (Arking *et al.,* 2002).

Carbohydrates, lipids and proteins acts as sources of energy production and greatly affect insect biology and morphogenetic activities as well (Niu *et al.,* 2002). According to Pracheta *et al.* (2009) oxidative damage to protein, lipids sugar and glycogen contents are also considered as a general measure of oxidative stress. So, this study was designed to study effect of Cd, Pb and Hg stress in relation to the activities of SOD, POD, CAT, LPO levels, essential metabolism and biochemical compositions of haemolymph in insects. *Oxya hyla hyla* (Orthoptera: Acridiidae) was selected as test insect as it is popularly known to possess many traits of a successful indicator of oxidative

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stresses (Azam *et al.,* 2015).

MATERIALS AND METHODS

Nymphs of *O. hyla hyla* (5th instar) were collected from nine vegetated sites in Gujrat district of Punjab province of Pakistan during the month of August (2014). Collected specimens of *O. hyla hyla* were acclimatized for seven days in laboratory. Water was replaced on daily basis and laboratory temperature was maintained between 20°C and 36.3°C with relative humidity of 60% and 98% respectively.

Specimens of *O. hyla hyla* were injected with metal salt solutions (4ul, i.p.) at different doses of Cd^{+2}, Pb^{+2} and Hg^{+2} (prepared by dissolving $CdCl_2$, $PbCl_2$ and $HgCl_2$ in triple distilled water) in variable concentrations (i.e. 0, 0.50×10^{-4}, 1.10×10^{-4}, and $1.55\times10^{-4}gg^{-1}$) of body weight at two to three of abdominal segments. The number of dead insects was determined and used for the calculation of LD50 with probit analysis. The LC_{50} for *O. hyla hyla* was kept $110.21\times10^{-4}gg^{-1}$. Equal volume of triple distilled water was injected to control insect specimens. Each dose was repeated three times to every of 20 – 22 individuals. Every metal was administered in three different doses. Specimens of *O. hyla hyla* were divided into two groups, with one used for SOD, CAT, POD, LPO analysis while the other group was used for biochemical compositions of haemolymph.

For determination of enzyme activity insect samples were anaesthetized by ice method (Lee *et al*. 2005) with their wings and legs excised. Samples were homogenized at 0–4°C in 2.5 ml 0.05 M Sorensen buffer, pH 7.4 containing 0.01 $molL^{-1}$ Tris–HCl. The homogenates were filtered and centrifuged for 10 min at 15,000g. For haemolymph extraction insects were dissected and haemolymph was collected using a glass capillary in a sterilized eppendorf tube and was stored at -70°c until analysis.

To determine activity of SOD, Kakkar *et al*. (1984) with some modification of Das et al. (2000) was followed. Catalase activity was assayed following Luck (1974). CAT activity was determined through spectrophotometer by measuring the decrease of absorbance at 240 nm due to H_2O_2 decomposition. Lipid peroxidation extent was measured according to Ohkawa *et al*. (1979). The content of total sugar in each haemolymph sample was determined following Kaufmann and Brown (2008).

RESULTS

Enzyme Activities

Apparent heavy metal exposure time-dependent differences in enzyme activity patterns were observed in the specimens of *O. hyla hyla*. The mean activity of Superoxide dismutase (SOD) was highest but decreased about 2–3 times with increase in heavy metal exposure time and dose (Figure 1). Catalase (CAT) activity was lowest at 0.50×10-4 gg^{-1}, and it increased with the exposure time after every next dose. Whereas mean values for Peroxidase (POD) increased after 50h of dosage (1.10×10^{-4} gg^{-1}) and then decreased again at $1.55\times10^{-4}gg^{-1}$. SOD, CAT and POD activities were determined to be significantly changed and there was statistically significant increase in the CAT, POD activity but significant decrease in SOD activity (Figure 1).

Heavy metals and their exposure time (Pb^{+2}, Cd^{+2}, Hg^{+2}) showed insignificant results at $p<0.05$ (Table 1). Enzymes (SOD, POD, CAT), BPA, LPO LEVELS and biochemical parameters showed significance at $p<0.05$.

Effect of Metal Exposure on protein, total sugar, lipids and glycogen concentration

A significant decrease in the contents of total sugar, lipids and glycogen values in the hemolymph occurred due to Cd^{+2}, Pb^{+2}, Hg^{+2} exposure throughout the entire tested period of 75 hours. Activity of Lipid Peroxidation significantly increased after every dose of Cd^{+2}, Pb^{+2} and Hg^{+2} exposures. In contrast, when exposed to Pb^{+2}, activity of Bradford Protein assay was not apparently altered. But total sugar contents in the haemolymph decreased strikingly over the whole tested time, except during earlier 24h of Cd^{+2} exposure. A significant decrease was however noticed when they were not apparently altered except after 24h of Cd^{+2} exposures. Glycogen content also displayed a slight decrease in total contents after exposure to Pb^{+2}, Cd^{+2} and Hg^{+2}. Thus, it is suggested that Cd^{+2}, Pb^{+2}, Hg^{+2} exposure shows significant adverse impact on the antioxidant enzyme activities as well as metabolism in grasshopper, depending on its exposure time and dosage (Figure 2).

Bradford Protein Assays and Lipid Peroxidation Assays showed significant effect due to heavy metal exposure at 5% p level. Similarly, Sugar, Glycogen, Lipids Fractions are also significantly affected by heavy metal exposure time.

Correlation Analysis

Assayed activity of SOD (Superoxide dismutase), POD (Peroxidase), CAT (Catalase) was correlated with body concentration of metals and exposure time. The (SOD) Superoxide dismutase activity of *O. hyla hyla* from control insects was highest and it decreased with metal exposure time. SOD activity was two times higher by Cd^{+2} than in the case of Pb^{+2} and Hg^{+2}. Positive correlations were documented between SOD activity at 24h and 75h, while it correlated negatively at 50h. Catalase had the highest activity of assayed antioxidant enzymes and its activity correlated positively with each exposure time interval. There were no significant time dependent differences in POD activity except at 24h. CAT, POD, SOD was significantly affected by treatment with heavy metals (Pb^{+2}, Cd^{+2}, Hg^{+2}) and durations (24h, 50h and 75h) as shown in Table (3&4).

Bivariate correlation analysis showed that soluble protein concentration, total sugar, lipids, glycogen contents in haemolymph of metal (Cd^{+2}, Pb^{+2}, Hg^{+2}) exposed insects after each exposure were obviously reduced. Cd^{+2} exposures (with progressive increase in time) distinctly caused a more striking drop in soluble protein and sugar levels. Sugar content was 3 – 5 times lowered at 50h and 5 – 6 times lowered at 75h of heavy metal exposure. Very strong correlation was observed between control and 24h exposure time for BPA, Sugar, Glycogen and lipids. But moderate positive correlation was observed at 50h for total soluble proteins, sugar, glycogen and lipids contents. Glycogen and lipids showed significant results and a positive correlation at each exposure time (Table 5& 6).

Regression Analysis

Linear regression analysis for enzymes was also applied for the data analysis. Assumptions of normality for dependent variables and assumptions of linear regression were tested and concluded satisfactory. Result of linear regression (Table 7) indicates Superoxide dismutase (SOD) as dependent and LPO, BPA, Sugar, Glycogen, Lipids as independent variables. Simple regression (pair wise) was also run over the whole data that shows model containing LPO as dependent variable is significant at 5% and LPO explain 80% variation of superoxide dismutase (SOD) as indicated in Table (7). While the model that contain sugar as independent variable explains moderate level of variation of SOD and overall model is significant at 5%. However, all the other pair wise models are insignificant and explained poor variation of dependent variables.

A multiple regression model was performed (Table 8) using Superoxide dismutase (SOD) as dependent variable and LPO, Sugar, Glycogen as dependent variable (which were significant in table-7). It indicates that all the coefficients of regression model are significant and Superoxide dismutase (SOD) is 81% due to independent LPO, Sugar and Glycogen. Also, overall model fitness is tested and concluded that model is significant at 5% level of significance.

Another multiple linear regression model using BPA and Lipids as independent variables (which were insignificant in Table-7) and SOD as dependent variable was performed (Table 9). Results of this multiple regression show that the model is insignificant and there is no variation in SOD due to BPA and Lipids. Hence from the Tables (7, 8 & 9) it can be concluded that LPO, Sugar and Glycogen are separately and together significantly affect variation of SOD. On the other hand, BPA and Lipids does not affect variation of SOD in both cases. So, it can be easily said that LPO, Sugar and glycogen are the only three factors that affect enzyme activity of Superoxide dismutase (SOD).

DISCUSSION

Heavy metals are one of the most important environmental variables that affect invertebrates (Bale et al. 2002). To understand oxidative stress induced in grasshoppers by environmental pollution, an index of oxidative stress (LPO), as well as the activity of antioxidant enzymes (CAT, POD and SOD) was measured. Result clearly demonstrated that in *O. hyla hyla* heavy metals accumulation was accompanied by significant increase in the activities of antioxidant enzymes. These observations are in accordance with the findings of An and Choi (2010) and Yang *et al.* (2010).

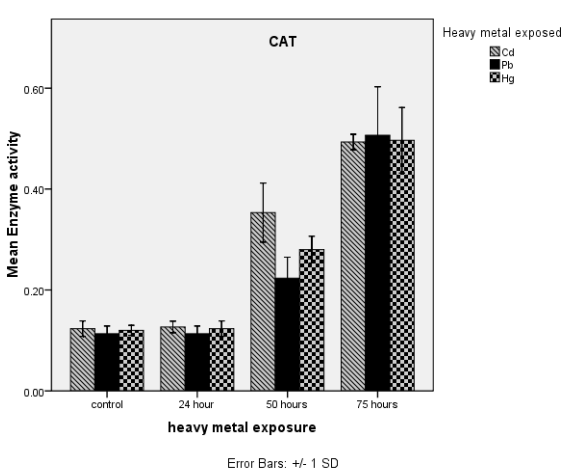
Among these antioxidant enzymes in insects, CAT has been solely responsible for scavenging H_2O_2, because insects are deficient in Selenium-dependent glutathione peroxidase, a natural scavenger present in many other organisms (Ahmad and Pardini 1990; Sohal *et al.,* 1990). However, CAT removes H_2O_2 only at high cellular concentrations and is inefficient at low concentration of H_2O_2 (Ahmad *et al.,* 1990). In this study, CAT activity of *O. hyla hyla* increased significantly as a result of metal-exposure. The overall expression of CAT under metal exposure resulted in the enzyme enhanced removal of H_2O_2 and hence, its prevention of damage by oxidative stress.

Mathews *et al*. (1997) and Lee *et al.* (2005) stated that in addition to CAT, insects show POD activities that break down H_2O_2. In present study POD activities also increased significantly under metal stress compared to the control and these findings are in accordance to those of Corona and Robinson (2006). Superoxide dismutase plays an important role in defense against oxidative stresses induced by accumulation of heavy metals. In the current study, SOD activities significantly increased due to heavy metal exposure at 24h and progressively decrease up to 75h. The high site-dependent variation of enzymatic responses in *O. hyla hyla* from metal exposure time gradient seems to be a very important indicator of metal stress.

In the present study total haemolymph, sugar, glycogen and lipids contents in metal exposed *O. hyla hyla* were observed to be decreased significantly except at 24h. Total soluble protein content in metal-exposed insect species however showed no significant increase except at 50h interval.

It is thus concluded that there is a very strong correlation and regulation between metal exposure and metabolic rate and heavy metals causes oxidative stresses which leads to significantly enhanced CAT, POD, LPO and SOD activities as a defense mechanism in response to metal-exposure in grasshopper (*O. hyla hyla)*. However prolonged exposure to metals results in decreased activities of SOD, accompanied by impaired antioxidant capacity and high level of oxidative stresses.

A



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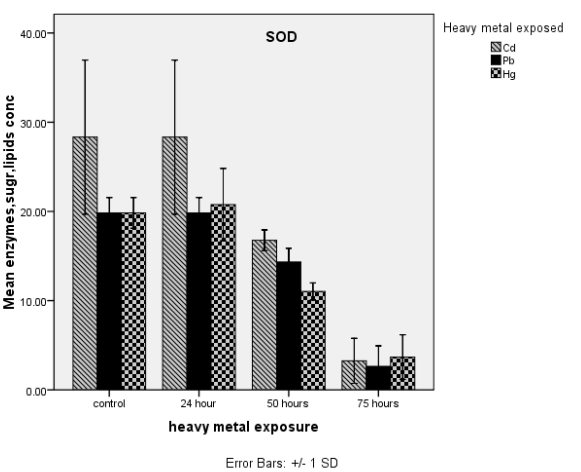

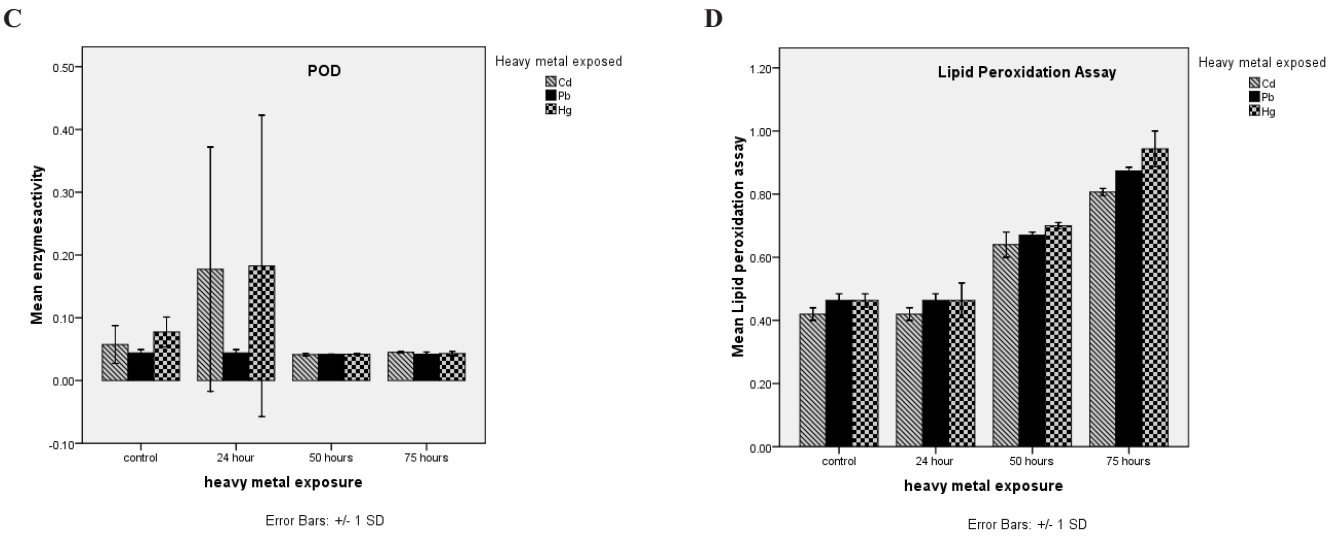

Fig. 1
Activity of CAT, SOD, POD and LPO Level in control group and after each exposure time to three different concentrations of heavy metals used for dosage in *Oxya hyla hyla (Serville)*(Order Orthoptera).

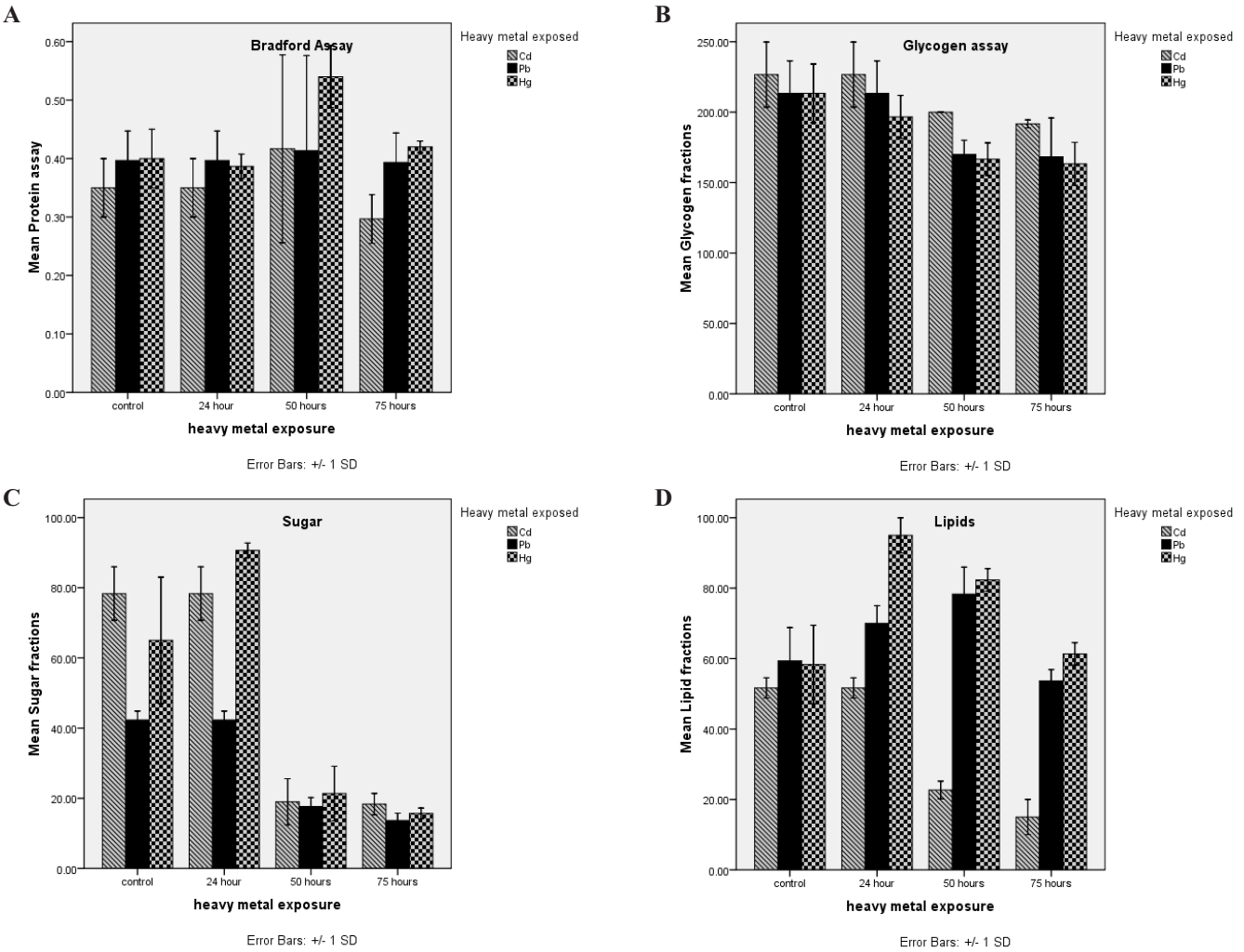

Fig. 2
Contents of total soluble proteins, glycogen, sugar and lipids mg/mL in the hemolymph of *Oxya hyla hyla (Serville)* due to cadmium (Cd^{+2}), Lead (Pb^{+2}), Mercury (Hg^{+2}) exposure.

Table 1
Test statistics for heavy metal exposure time.

Group	Test	Statistics	Significance
Exposure Time	Kruskal-Wallis Test	.353	.950
	Jonckheere-Terpstra Test	2152	.848
Anti-Oxidant Enzyme (CAT, SOD,POD)	Kruskal-Wallis Test	90.066	.000*
	Jonckheere-Terpstra Test	149.500	.001*
Type of Heavy Metals (Pb, Cd, Hg) exposed	Kruskal-Wallis Test	.962	.618
	Jonckheere-Terpstra Test	5779.500	.319

*significant at $p<0.05$

Table 2
Test statistics for BPA, LPO levels, sugar, glycogen, lipids fractions at different exposure time.

Group	Test	Statistics	Significance
Exposure Time	Kruskal-Wallis Test	.789	0.852
	Jonckheere-Terpstra Test	5842	.551
LPO LEVELS, BPA, Sugar, Glycogen, Lipids Cd^{+2}, Pb^{+2}, Hg^{+2}	Kruskal-Wallis Test	31.607	.000*
	Jonckheere-Terpstra Test	149.500	.000*
	Kruskal-Wallis Test	.962	.618
	Jonckheere-Terpstra Test	5779.5	.319

* significant at $p<0.05$

Table 3
Correlation between CAT, SOD Enzyme Activities in Grasshopper.

Heavy Metal Exposure	CAT				SOD			
	Control	24 hours	50 hours	75 hours	Control	24 hours	50 hours	75 hours
Control	1				1	.954**	.336	.312
24 hours	.946**	1				1	.218	.418
50 hours	.138	.140	1				1	-.124
75 hours	-.437	-.294	-.079	1				1

Table 4
Correlation between POD, LPO Level Activities in Grasshopper.

Heavy Metal Exposure	POD				LPO Level			
	Control	24 hours	50 hours	75 hours	Control	24 hours	50 hours	75 hours
Control	1				1	.469	.505	.542
24 hours	.253	1				1	.354	.356
50 hours	-.631	-.181	1				1	.597
75 hours	.434	.274	-.325	1				1

Table 5
Correlation between Content of Total Soluble Protein, Sugar, in Hemolymph.

Heavy Metal Exposure	BPA				SUGAR			
	Control	24 hours	50 hours	75 hours	Control	24 hours	50 hours	75 hours
Control	1				1	.735*	.439	.611
24 hours	.946**	1				1	.407	.395
50 hours	-.526	-.616	1				1	-.206
75 hours	.417	.414	.138	1				1

Table 6
Correlation between Content of Glycogen and Lipids in Hemolymph

Heavy Metal Exposure	GLYCOGEN				LIPID			
	Control	24 hours	50 hours	75 hours	Control	24 hours	50 hours	75 hours
Control	1				1	.264	.342	.400
24 hours	.753*	1				1	.837**	.887**
50 hours	.338	.358	1				1	.981**.
75 hours	.175	.222	.723*	1				1

Table 7
Test of Regression Analysis by SOD as dependent.

Variables		Correlation	Regression coefficients				Significance	
Dependent	Independent	r	β??	β?	β?	β?	R^2	P
SOD	LPO,Sugar, Glycogen	0.80	48.2	-45.2*	0.03	-0.03	0.81	45.3*

Table 8
SOD as dependent and LPO, Sugar, Glycogen as independent.

Variables		Correlation	Regression coefficients		R square	F value
Dependent	Independent	r	β??	β?	R^2	F
SOD	LPO	0.896	43.49*	-45.49	0.80	138.9*
	BPA	0.13	21.7	-15.07	0.02	0.652
	Sugar	0.74	5.5*	0.24*	0.55	42.18*
	Glycogen	0.61	-25.23*	0.209*	0.38	20.79
	Lipids	0.17	11.56	0.071	0.03	1.04

Table 9
SOD as dependent and BPA, Lipids as independent.

Variables		Correlation	Regression coefficients			R square	F value
Dependent	Independent	R	β??	β?	β?	R^2	F
SOD	BPA, Lipids	0.27	19.74	-26.1	0.11	0.08	1.39

AUTHORS' CONTRIBUTION

Iqra Azam conceived the idea and designed the experiments. Sumera Afsheen supervised the whole research work. Muhammad Kaleem Sarwar conducted the laboratory experiments and heavy metal analysis. Ahmad Zia carried out the specimen identification. Abdul Rauf Bhatti helped in statistical analysis and paper write up.

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