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COPPER SULPHATE INDUCED EXPRESSIONS OF DIGESTIVE ENZYMES AMYLASE, PROTEASE AND LIPASE IN INVERTEBRATE MODEL: *BELLAMYA BENGALENSIS* (L).

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ABSTRACT
Pollution is one of the undesirable side effects of increased industrialization, generally responsible for aquatic contamination. Heavy metals are major pollutants released into the environment. Biotoxicity of these metals against enzyme reactivity provides information about animal's health. Present investigation deals with intoxication of pre-determined mean LC_{50} concentration of heavy metal Copper sulphate ($CuSO_4$. $5H_2O$) as 0.56 ppm, for 24 hrs., 48 hrs., 72 hrs. and 96 hrs. Three different enzymes as amylase, lipases and protease activity were biochemically assessed from five different digestive organs including glands. Comparative data of enzyme expression against toxicity of heavy metal was interpreted. Enzymatic reactions were found significantly reduced as per exposure period and toxic dose. Results obtained were statistically documented and correlated with change in rate of biochemical reactions in invertebrate aquatic molluscan model as snail *Bellamya bengalensis* (L).

KEYWORDS: Enzyme expression, digestive organs, Copper sulphate, physiology of digestion, *Bellamya bengalensis.*

INTRODUCTION
Biological metabolisms are essential requirement for to maintain homeostasis of body. Enzymes as biocatalyst regulate the rate of physiological reactions to maintain health and also play critical role to overcome pathological conditions. Laoma and Rainbow, (2008) recorded, metal contamination in aquatic bodies and found responsible for change in the metabolic activities. Perić et. al., (2017) found variations of biomarkers response in mussels *Mytilus galloprovincialis* to low, moderate and high concentrations of organic chemicals and metals. Buschiazzoa et. al. (2004) studied capacity of bioaccumulation in two tropical oysters against dissolved metal concentration in the aquatic bodies. Dhivya et. al. (2011), noticed that. physiological and biochemical responses of brown mussel *Perna indica* were alternated due to interference of environmental factors. Researchers noted that, food and feeding mechanism can be associated for bioaccumulation of metallic components in the animal body along with habitat, seasonal changes or other affecting environmental parameters (Lawrence, 2007). Rajkumar and Milton, (2011) reported changes in biochemical components by induced effect of cadmium, copper, lead and zinc against exposure to *Perna viridis* under longterm toxicity test. Abdelkhalik et. al., (2013) studied heavy metal uptake and bioaccumulation in marine polychaete *Nereis succinea* and reported histopathology and biochemical alterations. Radlowska and Pempkowiak, (2002) noted stress-70 as indicator of heavy metals accumulation in blue mussel *Mytilus edulis.* Chandurvelan et. al., (2016) noticed Biomarker responses of mussels when exposed to disturbance to the aquatic media. Copper was found to be bioaccumulating in the freshwater snail *Lymnea eregra* and was used as biomarker of environmental pollution where, Bhavani et. al. (2003), reported bioabsorption and biomagnifications of cadmium and mercury by mussel-*Perna viridis* showing its toxic impact on the rate of biochemical reactions for the energetic. Energy metabolism of most of the gastropods known to be based on metabolic rate in which, Lawrence et. al., (2007) reported about digestive system of sea urchins which contains important enzymes including higher level of carboxylases and to a lesser extent, lipases and proteases. Copper is an essential element for all living organisms, particularly molluscs and crustaceans, which utilize copper-containing hemocyanin for oxygen transport (Viant et al., 2002).

Analysis of intracellular enzymatic activity provides valuable prognosis against health of experimental animal. Utilization of nutrients in aquatic animals found depends on concentration and activation of digestive enzymes (Rungruangsak et al., 2006; Areekijseree et al., 2006). Activities of digestive enzymes constitute a

physiological parameter affecting the digestive capacity where enzyme activities in body or cells (amylase, protease, and lipases) provide information about efficiency of species pertaining to feeding components (Ibarrola et al., 2000). Oliveira et. al.(2010), enlisted higher level of pollution of coastal lagoon using *Liza aurata* kidney oxidative stress and correlated it with alterations in biochemical changes. Number of researcher shown that, enzymes shows physical, chemical and catalytic features that could be of advantage in assessment of several biotechnological processes (Zhang and Kim, 2010) found useful to understand fate of digestive enzymes from crustacean in food biotechnology (Rossano et al., 2011).

Digestive tract proved to be a principle site for synthesis and secretion of digestive enzymes essential for complete digestion and absorption of nutrients (Pauchet *et al;* 2008). Roberts et al. (2001) stated that, secretion of endopeptidases such trypsin and chymotrypsin (important enzymes in the initial stages of protein digestion) may require induction by substrate. Effects of classical invertebrate hormones on the expression profiles of a lipase gene from *H. armigera* have been studied by (Sui *et al*., 2008). Grillo *et al.,* (2007) critically described the role of TAG lipase in lipid digestion in the *Rhodnius prolixus* midgut and reported that, products of digestion were absorbed by the midgut epithelium and then used to synthesize complex lipids. Studies on these enzyme activity has helped in the development of more rapid and accurate *in vitro* nutritional digestibility (Areekijseree et al., 2006; Supannapong et al., 2008), with which, mollusca showed enormous amount of nutritional componants of digestive materials (Privitera et al., 2008; Wangensteen et al., 2011).

With the avalable literature we found that still more attention is required in the enzyme activity study specifically in toxicological point of view, so present investigation carried out to focus role of three different enzymes expressions against heavy metal copper sulphate toxicity from selected digestive organs and glands in freshwater snail *Bellamya bengalensis.* Results obtained were interpreted for the role of enzymes in the maintenance of rate of biochemical reactions to overcome the toxicity impact in the environment for the survivility.

MATERIAL AND METHODS

a) Animal under study

For the present investigation, freshwater prosobranch snail *Bellamya bengalensis* (Lamark) was selected. The experimental snails were acclimatized under laboratory conditions for a week. Animals having same size and weight (26-28 mm shell height and 2.8 to 3.5 gm weight) were selected for experiments. Two sets were prepared. In each set five troughs were used in which, one trough was used as control group and remaning four were used as experimental animals, Experimental animals were intoxicated with pre-determined mean LC_{50} concentration of water miscible heavy metal Copper sulphate ($CuSO_4$. $5H_2O$) at 0.56 ppm, for 24 hrs., 48 hrs., 72 hrs. and 96 hrs. respectively. After complition of intoxication periods, animals were carefuly dissected for digestive tract including oesophagus, intestine, stomach with associated salivary gland and hepatopancreas. All the tisseues were subjected for enzymological asssay by applying standerd protocal.

b) Enzyme studies

To study the enzymological alteration against toxic effect of heavy metal after exposure period 24 hrs, 48 hrs, 72 hrs and 96 hrs., tissues were processed for enzymological estimation as, amylase, lipases and protease by applying methods for Amylase (Ishaaya *et. al*, 1970); Lipase (Hayashi and Tappel, 1970); The protease activity from each of intoxicated group and tissue were determined by applying methods of Egauche and Iwamoto, (1982).

RESULTS AND DISCUSSSIONS

Biomechanisms of digestive enzymes constitute a number of physiological parameter affecting the digestive capacity of intoxicated animals, (Ibarrolai *et al*., 2000). In molluscan species the structure of α-amylase and its role in digestive gland were determined by electrophoretic seperations (Moal *et al;* 2000). It was reported that, amylase activity has promoted carbohydrate digestion in animals at initial stage (Areekijseree *et al.,* 2006; Supannapong *et al.,* 2008) and used as an indicator for assessment of altered carbohydrate metabolism. Carbohydrate digesting enzymes (carboxylase) lipid digesting enzymes (lipase) and protein digestive enzymes (protease), with their action provided biochemical information about toxicity induced stress on molluscan species (Abdel-Kader *et al*., 2005). Effect of heavy metals on living organisms can be multifold where most of results showed effect on histology, hematology and rate of enzyme activity in the organism. El-Khayat et. al., (2015) reported that, molluscs especially aquatic snails were reported as pollution biomarkers for metallic pollution. Accumulation of copper in tissue has reduced the activity of lysosomal hexoasaminidase in the digestive cells after three days of exposure whereas, metals aluminium, lead, mercury and cadmium has increased enzyme activity in the foot of marine mussels (Patel and Patel, 1985).

In the present biochemical assessment part of digestive tract including oesophagus, intestine, stomach with associated salivary gland and hepatopancreas were subjected for comparative study of alteratons in the biochemical componants of enzyme expressions, which were recorded as folows:

a) Enzyme expressions in control group: Amylase activity of control group in snail *B. bengalensis* of different digestive organs, where salivary gland showed 44.47 mg maltose/mg protein/hrs, oesophagus contained 42.13 mg maltose/mg protein/hrs, intestine showed 24.65

mg maltose/mg protein/hrs, stomach has 16.07 mg maltose/mg protein/hrs and hepatopancreas was with 18.53 mg maltose/mg protein/hrs. Generally lipase activities of control group of snails were more in intestine and stomach. The salivary gland showed 7.65 mg Palmatic acid/ mg protein/hrs, in oesophagus 3.82 mg Palmatic acid/ mg protein/hrs, the intestine showed 14.23 mg Palmatic acid/ mg protein/hrs, stomach found 16.30 mg Palmatic acid/ mg protein/hrs and in hepatopancreas 8.16 mg Palmatic acid/ mg protein/hrs lipase activity observed. Protease activity in different digestive organs of control group were recorded where, salivary gland showed 2.26 mg Tyrosine/ mg protein/hrs, oesophagus 1.92 mg Tyrosine/ mg protein/hrs, intestine 2.29 mg Tyrosine/ mg protein/hrs, stomach showed 3.11 mg Tyrosine/ mg protein/hrs and hepatopancreas showed 3.84 mg Tyrosine/ mg protein/hrs. For the toxicity study values were compared with intoxicated groups of different exposure periods.

b) Effect of Copper sulphate on activity of amylase: Intoxication of copper sulphate has changed amylase activity after 24 hrs, it was 19.81 mg maltose/mg protein/hrs, at 48 hrs amylase 18.65 mg maltose/mg protein/hrs, after 72 hrs amylase activity found 11.17 mg maltose/mg protein/hrs and 9.83 mg maltose/mg protein/hrs was found after 96 hrs. The amylase activity in oesophagus after 24 hrs was 23.09 mg maltose/mg protein/hrs, after 48 hrs 10.53 mg maltose/mg protein/hrs, at 72 hrs 9.93 mg maltose/mg protein/hrs and amylase activity found 4.41 mg maltose/mg protein/hrs after 96 hrs exposure. Intestine has 14.82 mg maltose/mg protein/hrs after 24 hrs, at 48 hrs 9.40 mg maltose/mg protein/hrs, at 72 hrs 5.91 mg maltose/mg protein/hrs and after 96 hrs 5.15 mg maltose/mg protein/hrs was found. The stomach showed 10.23 mg maltose/mg protein/hrs amylase activity after 24 hrs, 4.02 mg maltose/mg protein/hrs after 48 hrs, 3.15 mg maltose/mg protein/hrs at 72 hrs, after 96 hrs 1.06 mg maltose/mg protein/hrs was noted. The hepatopancreas has amylase activity as 12.6 mg maltose/mg protein/hrs after 24 hrs, 7.65 mg maltose/mg protein/hrs at 48 hrs, 5.13 mg maltose/mg protein/hrs at 72 hrs, 5.11 mg maltose/mg protein/hrs at 96 hrs. The enzymatic data was reported in Table No. 01 and represented in Graph No. 01.

c) Effect of Copper sulphate on activity of lipase: After 24 hrs, salivary gland showed 1.82 mg Palmatic acid/ mg protein/hrs, after 48 hrs found 1.09 mg Palmatic acid/ mg protein/hrs, after 72 hrs it was 1.06 mg Palmatic acid/ mg protein/hrs and 0.70 mg Palmatic acid/ mg protein/hrs after 96 hrs of exposure. Oesophagus at 24 hrs was 2.30 mg Palmatic acid/ mg protein/hrs with lipase activity, after 48 hrs 2.11 mg Palmatic acid/ mg protein/hrs, after 72 hrs 2.09 mg Palmatic acid/ mg protein/hrs and after 96 hrs it was 1.93 mg Palmatic acid/ mg protein/hrs lipase activity. In intestine 3.42 mg Palmatic acid/ mg protein/hrs at 24 hrs, 1.29 mg Palmatic acid/ mg protein/hrs at 48 hrs, 0.76 mg Palmatic acid/ mg protein/hrs after 72 and at 96 hrs 0.67 mg Palmatic acid/ mg protein/hrs lipase activity was obtained. Stomach showed 1.42 mg Palmatic acid/ mg protein/hrs at 24 hrs, 0.77 mg Palmatic acid/ mg protein/hrs at 48 hrs, 0.62 mg Palmatic acid/ mg protein/hrs at 72 hrs and at 96 hrs it was 0.37 mg Palmatic acid/ mg protein/hrs. Hepatopancreas showed 1.70 mg Palmatic acid/ mg protein/hrs after 24 hrs, at 48 hrs 1.05 mg Palmatic acid/ mg protein/hrs, after 72 hrs 0.88 mg Palmatic acid/ mg protein/hrs and at 96 hrs 0.73 mg Palmatic acid/ mg protein/hrs lipase activity was recorded. Enzymatic data was reported in Table No. 02 and represented in Graph No. 02.

d) Effect of Copper sulphate on activity of protease: Intoxication of copper sulphate in experimental group has altered protease activity at different exposure periods in selected digestive organs, where salivary gland showed 1.77 mg Tyrosine/ mg protein/hrs after 24 hrs, at 48 hrs found 1.05 mg Tyrosine/ mg protein/hrs, After 72 hrs 0.50 mg Tyrosine/ mg protein/hrs and after 96 hrs 0.44 mg Tyrosine/ mg protein/hrs activity was found. In oesophagus, 1.52 mg Tyrosine/ mg protein/hrs at 24 hrs, at 48 hrs 0.97 mg Tyrosine/ mg protein/hrs activity was found. The 72 hrs and 96 hrs of exposure showed 0.90 mg Tyrosine/ mg protein/hrs and 0.81 mg Tyrosine/ mg protein/hrs of protease activity. Intestine has 1.32 mg Tyrosine/ mg protein/hrs at 24 hrs, 0.54 mg Tyrosine/ mg protein/hrs at 48 hrs, 0.46 mg Tyrosine/ mg protein/hrs at 72 hrs and 0.31 mg Tyrosine/ mg protein/hrs at 96 hrs. Stomach has protease activity after 24 hrs which showed 0.94 mg Tyrosine/ mg protein/hrs, at 48 hrs 0.89 mg Tyrosine/ mg protein/hrs, at 72 hrs 0.88 mg Tyrosine/ mg protein/hrs and at 96 hrs found 0.51 mg Tyrosine/ mg protein/hrs respectively. Hepatopancreas showed 1.15 mg Tyrosine/ mg protein/hrs at 24hrs, 48 hrs showed 0.84 mg Tyrosine/ mg protein/hrs, after 72hrs found 0.80 mg Tyrosine/ mg protein/hrs and at 96 hrs 0.18 mg Tyrosine/ mg protein/hrs activity was recorded. The enzymatic data was reported in Table No. 03 and represented in Graph No. 03.

Comparatively, it was noticed that copper induced amylase activity was decreased up to 78% in salivary gland, oesophagus has 90%, intestine with 79%, stomach 93% and 72% reduction was found in hepatopancreas. Lipase activity of copper sulphate after 96 hrs of salivary gland showed 91%, oesophagus 49%, intestine 95%, stomach 97% and 91% hepatopancreas depleted. Protease activity in copper sulphate activity in salivary gland showed 80%, oesophagus 58%, intestine 86%, stomach 83% and in hepatopancreas 95% of reduction was recorded, data was reported in table No. 04 and represented in graph No. 04.

Biotechnological research indicated that, some pesticides like organic phosphorus and carbomate directly affected on freshwater bivalve *Amblema plicata* (Van Erp *et al*., 2002; Doran *et al.*, 2001), and found significantly reduced enzyme activity in their tissues. Akinpelu *et al.*, (2012), supported inhibitory effect of saponin mixture

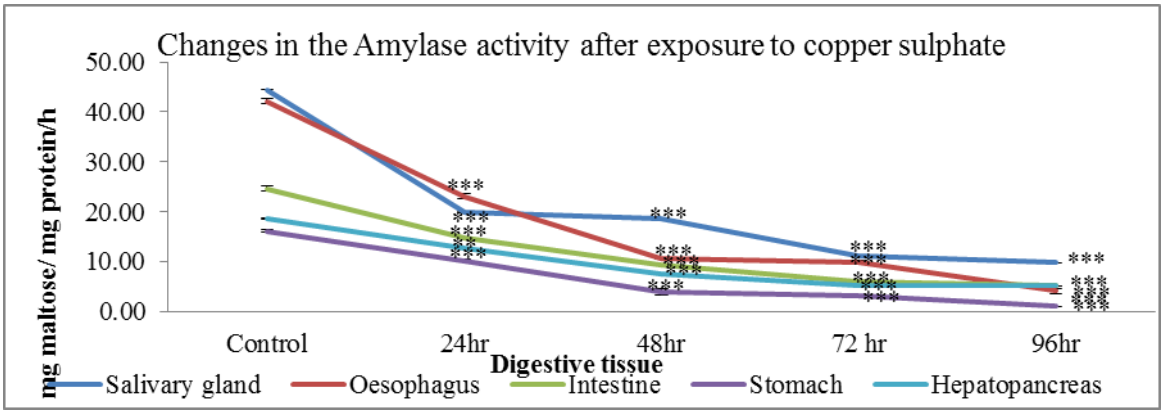
against biochemical reactions of freshwater snail of *Lybicus lanistes*. Carbohydrate digesting enzymes (e.g. carboxylase) lipid degrading enzymes (e.g. lipase) and protein digestive protease, were recorded among targeted enzymes which provided accurate information on the molluscicide induced stress on molluscan species (Abdel-Kader *et al*., 2005). Al Daihan (2008), noted stepwise reduced activity of α-amylase and lipase with delayed development of *Schistosome parasite*.

Exposing *Cnaphalocrocis medinalis* (Guenee) invertebrate to sub-lethal doses of *Bacillus thuringiensis* (Kurstaki) in the laboratory documented minimised digestive enzyme activities (Senthil Nathan *et al*., 2006). Fenvalerate treatment reduces a reduction activity of amylase, protease and sucrose (Vyijayanthi and Subramanyam, 2002). Imbalance in enzyme-substrate complex and inhibition of peristaltic movement of the gut might have inhibited the enzyme activities (Senthil Nathan *et al;* 2006). Carbaryl and γ-BHC caused damage to the epithelial cells of midgut of *Chaphalocrcocis madinalis* depleting activity of digestive enzymes. Mohamed *et al.*, (2000) reported that, haemolymph glucose and tissue glycogen of *Melanoid tuberculata* subjected to different kinds of stress. Similarly, El-Ansary *et al.*, (2000) documented an increased glycolytic enzyme activity in *B. alexandrina* snails infected with *S. Mansoni*. Under toxicity decreased level of glycogen with increased lactate content has indicated the diversion of pyruvate, the end product of glycolysis, for anaerobic metabolism instead of incorporating it into aerobic reaction of Krebs cycle (Satyparameshwar *et al.,* 2006).

Similarly Sultana and Lomte, (2000) reported decreased amylase activity in *Lamilidan marginalis* and concluded that mercury chloride and copper chloride were potent inhibitors of amylase activity. Shobha *et al.,* (2000) studied alterations in activity of dehydrogenase in a freshwater fish, *Tilapia mossambica* when exposed to arsenic toxicity. Al-Daihan, (2008) showed the enzymatic activities of ALP, ACP, amylase, lipase and glucose in control and *S. nigrum* treated *B. arabica* snails and reported higher depletion in the biochemical activity and stressed conditions. The enhanced activity of alkaline phosphotase in the hepatopancreas might be due to activation of intracellular energy consuming because, the alkaline phosphotase facilitate the breakdown of ATP to ADP and organic phosphate, thereby making free energy available for metabolic processes (Botham and Mayes, 2003).

Table No. 1: Alteration in the Amylase activity (mg maltose/mg protein/hrs) of different digestive tissues of freshwater Snail *Bellamya bengalensis* after exposed to Copper sulphate. All the values are mean of three observations ± Standard deviation, P<0.001=*, P<0.01=**.**

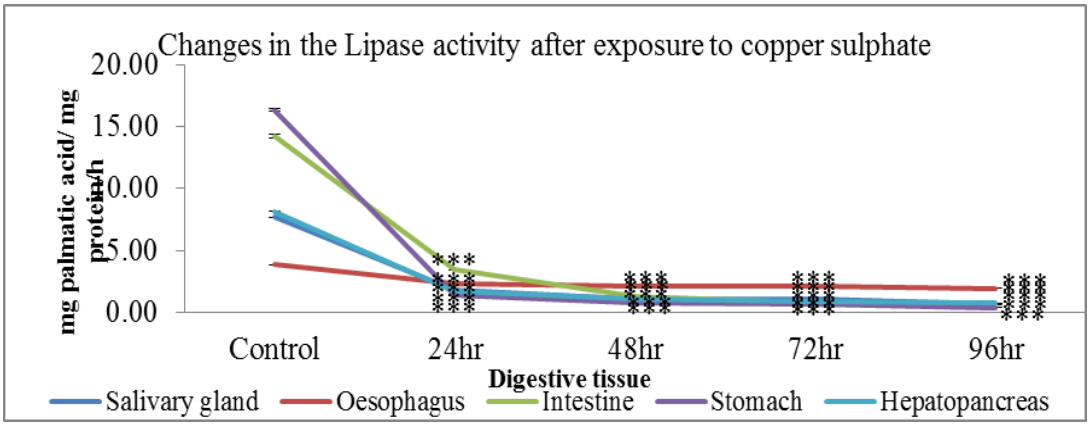
Tissue	Control	Exposure periods			
		24hrs.	48hrs.	72hrs.	96hrs.
Salivary gland	44.47±0.03	19.81±0.01 ***	18.65±0.01 ***	11.17±0.06 ***	9.83±0.02 ***
Oesophagus	42.13±0.46	23.09±0.44 ***	10.53±0.06 ***	9.93±0.42 ***	4.14±0.51 ***
Intestine	24.65±0.52	14.82±0.09 ***	9.40±0.01 ***	5.91±0.01 ***	5.15±0.03 ***
Stomach	16.07±0.13	10.23±0.02 ***	4.02±0.02 ***	3.15±0.00 ***	1.06±0.01 ***
Hepatopancreas	18.53±0.23	12.6±0.2 **	7.65±0.64 ***	5.13±0.06 ***	5.11±0.01 ***



Graph No. 1: Alteration in the Amylase activity (mg maltose/mg protein/hrs) of different digestive tissues of freshwater Snail *Bellamya bengalensis* after exposed to Copper sulphate.

Table No. 2: Alteration in the Lipase activity (mg palmatic acid/ mg protein/hrs) of different digestive tissues of freshwater snail *Bellamya bengalensis* after exposed to Copper sulphate. All the values are mean of three observations ± Standard deviation, P<0.001=*.**

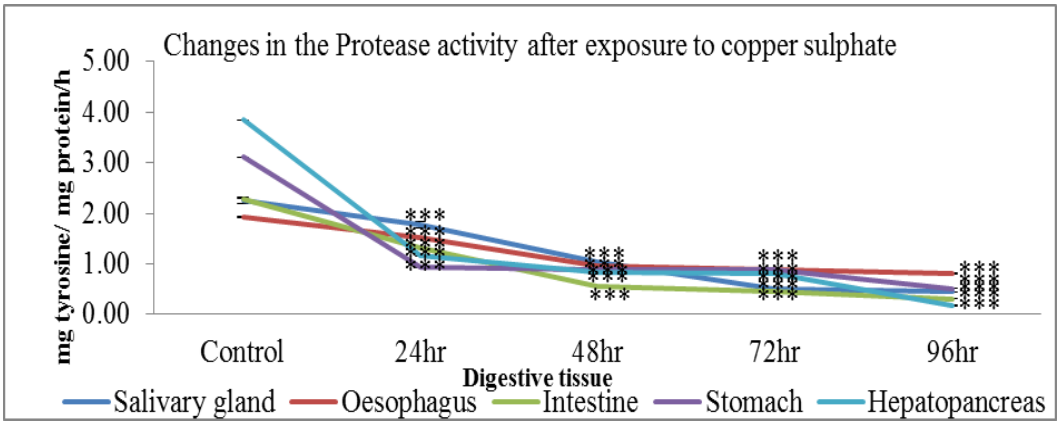
Tissue	Control	Exposure periods			
		24hrs.	48hrs.	72hrs.	96hrs.
Salivary gland	7.65±0.02	1.82±0.02 ***	1.09±0.01 ***	1.06±0.01 ***	0.70±0.01 ***
Oesophagus	3.82±0.02	2.30±0.02 ***	2.11±0.02 ***	2.09±0.010 ***	1.93±0.02 ***
Intestine	14.23±0.15	3.42±0.03 ***	1.29±0.02 ***	0.76±0.02 ***	0.67±0.01 ***
Stomach	16.30±0.10	1.42±0.02 ***	0.77±0.03 ***	0.62±0.03 ***	0.37±0.02 ***
Hepatopancreas	8.16±0.02	1.70±0.02 ***	1.05±0.02 ***	0.88±0.03 ***	0.73±0.01 ***



Graph No. 2: Alteration in the Lipase activity (mg palmatic acid/ mg protein/hrs) of different digestive tissues of freshwater snail *Bellamya bengalensis* after exposed to Copper sulphate.

Table No. 3: Alteration in the Protease activity (mg tyrosine/ mg protein/hrs) of different digestive tissues of freshwater snail *Bellamya bengalensis* after exposed to Copper sulphate. All the values are mean of three observations ± Standard deviation, P<0.001=*.**

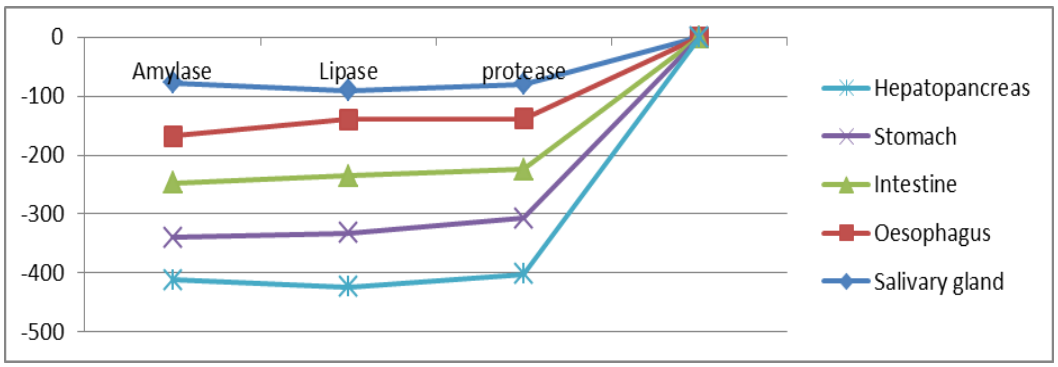
Tissue	Control	Exposure periods			
		24 hrs.	48hrs.	72hrs.	96hrs.
Salivary gland	2.26±0.06	1.77±0.01 ***	1.05±0.00 ***	0.50±0.01 ***	0.44±0.01 ***
Oesophagus	1.92±0.81	1.52±0.01 ***	0.97±0.00 NS	0.90±0.01 *	0.81±0.02 *
Intestine	2.29±0.01	1.32±0.01 ***	0.54±0.01 ***	0.46±0.01 ***	0.31±0.0 ***
Stomach	3.11±0.01	0.94±0.01 ***	0.89±0.01 ***	0.88±0.01 ***	0.51±0.0 ***
Hepatopancreas	3.84±0.01	1.15±0.01 ***	0.84±0.01 ***	0.80±0.01 ***	0.18±0.0 ***



Graph No. 3: Alteration in the Protease activity (mg tyrosine/ mg protein/hrs) of different digestive tissues of freshwater snail *Bellamya bengalensis* after exposed to Copper sulphate.

Table No. 4: Percent declined enzymes activity of digestive organs of freshwater snail *B. bengalensis* against Copper sulphate after 96 hrs.

Enzymes	Organs under study (% values)				
	Salivary gland	Oesophagus	Intestine	Stomach	Hepatopancreas
Amylase	-78	-90	-79	-93	-72
Lipase	-91	-49	-95	-97	-91
protease	-80	-58	-86	-83	-95



Graph No. 4: Percent declined in enzymes activity of digestive organs of freshwater snail *B. bengalensis* against Copper sulphate after 96 hrs.

CONCLUSION

Present study conclude that, catalytic activity of important digestive enzymes as amylase, lipase and protease were decreased after induction at 24 to 96 hrs of exposure. Results showed that, Copper sulphate induction to the snail became toxic as per the exposure periods and has inhibited synthesis and secretion of digestive enzymes, which was reflected by a disturbed digestion process. Comparaively all enzymes were unable to maintain the rate of reaction as per selected organs. Maximum deplition in the rate of reaction was found in hepatopancreatic cells as is the major targeted gland for to run biochemical process and maintenance of energetic. Snails of experimental groups were more slugish and unable to move in the trough showing the toxic impact of the copper sulphate. Results about depleted rate of enzyme reaction conforms the toxic potential of copper sulphate with its bioaccumulation and magnification capacity. So it is challenge before us to control the severe contamination of heavy metals including copper sulphate in the aquatic and terrestrial media for development and survivality of floral and faunal components of food chain.

Conflicts of Interest: The author declares that no conflicts of interest.

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