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Research Paper

Biochemical and Chromatographic profiles of freshwater fingerlings *Labeo rohita* (Hamilton) exposed to Lead and Copper Heavy Metal Toxicity with response to tissue-specific variations.

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Abstract: The fingerlings of freshwater carp, *Labeo rohita* were exposed to sub-lethal concentrations (2.5, 2.9, 3.5, and 3.9 ppm) of lead and copper (lead nitrate and copper sulfate respectively) for a duration of 24 and 48 hrs. Muscle, Gill, Liver, and Intestine tissues were dissected after each period of exposure and subjected to total protein and lipid profiling. Overall, protein levels depleted with increasing concentration of heavy metal exposure and duration. Decreases in the total lipid profile were also observed. The overall depletion of protein could correlate to the susceptibility of the fish tissues to biotic and abiotic stressors. The depletion of the lipid profile indicated the damage and growth suppression of the tissues by various enzymatic reactions DNA synthesis, transcriptional regulation, catalysis, nitric acid, and oxygen sensing. Further, we separated methionine, proline, threonine, arginine, tyrosine, and glycine from the pooled samples of protein using paper chromatography. One-dimensional thin-layer chromatogram determined the separation of major classes of lipids varying in polarity that includes cholesterol, esters, and phospholipids.

Keywords: Heavy metals, Paper chromatography, Thin-layer chromatography, amino acid, phospholipids, triglycerides.

Abbreviations: Cu- Copper, Pd- Lead, hrs.- Hours, RPM- Revolutions Per Minute, TLC- Thin Layer Chromatography, PPM- Parts Per Million, UV- Ultra Violet, RF- Retardation or Retention Factor, LC50- Lethal Concentration 50%

Introduction:

Fish is a vital source of food for humans as they grow in short periods using cheap food

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sources which are desirable for aquaculturists. Dietary intake of fish is sufficient to provide about 50-60% of an adult's daily protein requirement. Fishes are also good sources of all essential amino acids, fatty acids, vitamins, and minerals. Furthermore, the consumption of fish and fish protein helps in preventing cardiovascular diseases and other ailments (Singh et al.,2016). Considering the increasing demand for fish protein, the small-scale fish rearers, aquaculturists, and fish processing industries are making efforts to minimize crop loss potentially by curbing aquatic pollution. Despite the sustainable efforts, industrial discharge of heavy metals has been a menace affecting the dynamics of an aquatic environment. The accumulation of heavy metals in fish tissues (D'itriandD'itri,1977) is responsible for the alteration of the physiological and biochemical parameters in fishes. In another sense, fishes serve as indicators of aquatic pollution, although wide variations exist between fish species as far as metabolic pathways are concerned and no single fish species can act as a reliable indicator of pollution (Bradley and Morris, 1986; Dallinger et al., 1987; National technical information Service, 1987). Further, the survivability of fish depends not only on the health status of the ecosystem but also on the type and length of exposure and inherent toxicities of the metal toxicants (Brungs *et al*., 1977, Farombi et al.,2007, Vosyline et al.,2006 and Ashraj,2005).

Heavy metal pollution is dangerous to the aquatic environment because they persist, accumulates, and is harmful to aquatic organisms including fishes (Tamizhazhagan et al.,2016). Most heavy metals are natural constituents of the aquatic environment but some metals like cadmium, lead, and mercury are highly hazardous to aquatic biota (Mali, 2002). Higher concentrations of lead, cadmium, and mercury were found to be toxic to fish (Atta *et al*., 2012; Salah *et al*., 2013). Heavy metals like Copper (Cu), Lead (Pb), and Cadmium (Cd) are non-essential (Ahmed et al.,2015) even the sub-lethal concentrations of these metals cause severe effects on protein metabolism and animal behavior (Rex, Joseph, and Taiwo, 2017). It leads to significant structural changes in their protein metabolism by inactivating enzymatic pathways (Maheswaran and Devepaul,2008).

Lead (Pb) at elevated levels can cause severe damage to the tissue architecture of fish. Acute Pb toxicity results in respiratory asphyxiation and the disruption of ion-regulatory homeostasis mechanisms. Chronic effects are similar to those in humans, involving hematological and neurological dysfunction. Lead is taken up and elicits its effects by substituting calcium and potentially other essential divalent cations such as iron and zinc. Lead is having higher magnification in the food chain component in the ecosystem (Patnaik et al., 2011). Copper (Cu) is highly toxic in aquatic environments and has shown adverse effects in fish, invertebrates, and amphibians, with all three groups equally sensitive to chronic toxicity (U.S EPA, 1993; Horne and Dunson, 1995). Cushowbioconcentratation in many different organs of fish and mollusks is highly species-specific (Holan et al., 2017).

Mostly exposure to Cu has been manifested as histopathological alterations in fish such as necrosis, hepatic fibrosis, and congestion of blood vessels (Padrilah et al., 2017). Further, Cu toxicity is shown to cause negative impacts on chemoreception and the ability to respond appropriately to external stimuli as suggested in the Crayfish, *Orconectes rusticus* (Lahman et al., 2015). The aquatic organisms especially those habituating in the benthos region accumulate

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more in their tissues (Uwem et al., 2013). Continuous exposure to the habitat of an organism that will be consumed by humans may lead to Type II Diabetes, abnormal kidney function, and miscoordination of the nervous system. This heavy metal also acts as a carcinogenic agent (Mohamed et al.,2016). Even though Cu is considered a trace element when it goes above the internal concentration in tissues and exceeds over physiological detoxification mechanism. The toxic effect will be expelled in all physiological metabolism of organisms (Haoud Hussein A, 2015).

Labeo rohita is a promising carp species for aquaculture exploitation with its rapid growth and good market potential. In terms of value-added and processed fish products, this species showed great potential in Asian markets. The present study investigated and evaluated the acute toxicity of heavy metals Pb and Cu on the biochemical variation and behavioral responses of the Indian common carp, *Labeo rohita* in different tissues such as muscle, gills, liver, and intestine.

Materials and Methods

Collection of *L. rohita*

Healthy fingerlings of Indian major carp species *L.rohita* (Rohu) (n=24; 5 ± 0.2cm length and 7±2g weight) were collected from a local fishpond at Tailasahi village in Khordha district, Bhubaneswar, Odisha, India (Fig 1). Collection of Fingerlings from the pond of Tailasahi (Khurda Dist.). The fish were acclimatized under laboratory conditions [25 ± 2 ºC] in freshwater containers for one week at least before use. The tank containing fish was aerated with oxygen and hygiene was maintained by renewing the water every 24h. *L. rohita* fingerlings were fed daily with an artificial diet.

Heavy Metal Treatment and Biochemical estimations

Acute toxicity experiments were conducted for 24 and 48 h using a static bioassay technique and LC_{50} values were recorded. LC_{50} values for lead acetate were 2.5 and 2.9 ppm and those for copper were 3.5 and 3.9 ppm respectively. A total of 24 fishes were exposed to the heavy metals (12 fishes each for lead and copper exposure). A control group (n=12) was set with no heavy metal exposure. During the acute toxicity experiments, no food was provided to the fish. After acute (24 and 48 hrs) with lead and copper, the live fish (six from each group) were sacrificed and the tissues (gill, liver, intestine, and muscle) were excised, cleaned off the extraneous material, weighed, and used for biochemical estimations. These pooled samples were used for the estimation of the total protein by folin phenol method (Lowry *et al*., 1951), and total lipid by Thin-layer chromatography (Folch; 1956) methods. The biochemical analysis was repeated three times and the mean values of three readings were expressed in terms of mg/100 mg wet weight of tissue. The values recorded were compared with the control and percent changes were calculated for the presentation of the data.

The data obtained were subjected to statistical analysis to arrive at the arithmetic Mean, standard deviation, and standard error of the mean. To test the significance of differences observed between control and experimental groups, the levels of significance were determined by applying two-way ANOVA.] The data were analyzed using STATA (v.13.0) Program.

Preparation of Sample

The dissected gills, muscle, liver, and intestine samples were collected from each fish in an Eppendorf tube. Further, the process was performed by mixing phosphate buffer saline with gills, muscle, liver, and

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intestine samples in a hand homogenizer, and the filtrate was collected.

Centrifugation

The sample was then filtered using filter paper and centrifuged at 12000 RPM for 10 minutes. The supernatant was discarded into another Eppendorf tube and stored in the refrigerator at -20^{o} C until further experimental processes were executed.

Total Protein Estimation

Protein was estimated by the method of Lowry *et al*., (1951).

Solution A: 4 mg/mL NaOH and 20 mg/mL Na2CO3 in water Add 2 g of NaOH and 10 g of Na2CO3 to 400 mL water while stirring until completely dissolved, then adjust the volume to 500 ml.

Solution B: 10 mg/mL Potassium Sodium Tartrate and 5 mg/ mL CuSO4 in water Add 100 mg Potassium Sodium Tartrate and 50 mg CuSO4 (cupric sulfate) to 8 mL of water in a plastic falcon tube. Shake the mixture until the solids are completely dissolved, then adjust the volume to 10 ml. Mix solutions A and B and store them at 4˚C this is called Lowry's solution and should be usable for up to six months. (50:1 mix of solutions A and B)

200µl of each sample was taken in a test tube and 800µl of distilled water was added to it. Again 4.5ml of Lowry's solution was added and was incubated for 10 minutes

Then 0.5ml of Folin-Phenol reagent (Solution B) was added to the test tube and was again incubated for 30 minutes in dark.

The measurement process was carried out by spectrophotometer at 660nm and the OD value was recorded.

Paper Chromatography:

Apparatus: Glass beakers Whatman filter paper Petri dishes Measuring cylinder Developing chamber and capillary tubes etc.

Chemicals: n-butanol Glacial acetic acid Distilled water (4:1:5) Amino acids Ninhydrin reagents. Solvents system and its preparation methods 1. n-butanol and water are taken in 4:5 ratios in a conical flask and allowed to saturate for 24 hours. The saturated n-butanol and Glacial acetic acid are taken in the ratio of 4:1 which can be used as a solvent system (or) mobile phase.

Ascending paper chromatography paper is cut into15 cm × 20 cm and marks a line on the paper with the pencil at about 2 cm from the bottom and a 2 cm gap for each sample. With the help of a capillary tube, the samples are applied at different points on the starting line Now, roll the paper without touching the corner of all the sides and put a staple that is kept inside the beaker which contains the mobile phase. While placing the paper, the solvent level mustn't reach the starting line or the sample spots and paper shouldn't touch the walls of the beaker. After some time the solvent rises the paper or the stationary phase by capillary action and dissolves the sample. The components of the sample move along with the solvent in an upward direction. The paper is removed when it reaches the top and marked the level with the pencil. This level (or) height is called the "solvent front". By using UV light, ninhydrin spray (0.2gm ninhydrin powder mixed with 100ml butanol. 10ml butanol mixed with 90ml distilled water) examined the different spots. Each spot represents a specific component of the sample (D.A. Stiles and K. Ramaswamy, 1970).

The Results obtained from the separation of amino acids by paper chromatography were processed by marking the spots that appear on the chromatogram. The R_f value is calculated by the Distance traveled by the compound divided by the distance traveled by the solvent front.

Total Lipid Estimation

Preparation of sample:

The dissected Muscle, Gill, Liver, and Intestine samples were collected from each

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fish in an Eppendorf tube. Further, the process was performed by adding buffer solution and mixing it with the sample in the mortar, and was hand homogenized.

Centrifugation:

The sample was then filtered using filter paper and was centrifuged at 2000 RPM for 5 minutes. The supernatant was discarded into another Eppendorf tube and stored in the refrigerator at -20^{o} C until further experimental processes were executed.

Lipid extraction was carried out by Folch et al.,1957

Solution for Folch Method

Buffer: 100ml Chloroform was added with 50ml methanol in a 2:1 ratio and was kept at room temperature.

Solution A: 0.5g ferric Chloride Mixed with 10ml of distilled water. Then 3ml of Concentrated NH_3 and 25ml Glacial Acetic Acid were added to it.

Solution B: 25ml of H_2SO_4 mixed with 0.025gm Ferrous sulfate and was added in 25ml Glacial Acetic acid and 100ml of H_2SO_4.

This volume is made up to 200ml by adding concentrated H_2SO_4.

500 µl lipid sample mixed with 1.5ml of solution A and 1ml of solution B. After 20 minutes reading was taken at 560nm.

Thin Layer Chromatography (TLC):

Lipid samples extracted from *L.rohita* by modified Folch method. 5-10% of crude lipid in chloroform and methanol mixture (2:1) was put into a glass test tube with a glass stopper and stored frozen.

Solvent preparation:

Silica coated Plastic Plate used as Stationary Phase and Solvent for Mobile Phase Petroleum ether: diethyl ether: acetic acid at a ratio of 84:15:1 used.

Spraying agent: 0.6% potassium dichromate mixed with 55% H_2SO_4 and 45ml of Distilled water. Stored the reagent at 4^{0}c and all the precautionary measurements are taken care of while sprayed.

Applied the sample in an activated Silica pre-coated Plastic Plate as discrete spots 2.0cm from the bottom of the plate using a capillary tube without touching the corner of all sides. Placed the plate into a developing tank containing a developing solvent system. After sometimes the solvent rises the plate by capillary action. The components of the sample move along with the solvent in an upward direction. Removed the plate when the solvent reaches 10 cm from the spotted bottom of the plate. The plates were air-dried for about 30 minutes to remove the solvent. By using a detecting or spraying agent followed by heat treatment at 150-1800c for 15-20 minutes after spraying and exposure of the plates to UV light (Miwa et al.,1992). Identification of spots is done through comparison with the retention factor (R_f) of standard samples and analysis of qualitative reactions. The colored yellowish spots on a dark purple background could be identified. Each spot had a retention factor (R_f) which is equal to the distance migrated over the total distance covered by the solvent. The Rf value is calculated by the Distance traveled by the compound divided by the distance traveled by the solvent front.

Results:

Heavy metal toxicity proved in this study as one of the major threats to health issues in humans. The results showed the proximate composition of total lipids in the Muscle, Gill, Liver, and Intestine when the fingerlings were exposed to different concentrations of lead and copper heavy metals applied on 24hrs. and 48 hrs. duration. Physiological responses were determined and total protein and lipid content in *L.rohita* were compared with control and followed by Paper chromatography for amino acids separation

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and Thin layer chromatography for lipid separation have been carried out.

Our research results showed that total protein content decreased with the increase of Copper (Cu) heavy metal concentration in the Muscle and Intestine **(Fig.1A,1D**). In Gills and Liver, it reduced 3.5ppm and 3.9ppm levels of Copper (Cu) heavy metal toxicity **(Fig.1B,1C).** In the case of Muscles Fig.1A, it showed highly ******* = significant ($P \leq 0.001$). Also, in Intestine **Fig.1D** we observed increasing followed by decreasing with highly significant. In the case of Gills, **Fig.1B** showed significance at the level of ***** = significant ($P \leq 0.05$).

Heavy metal Lead (Pb) treated samples of Muscle, Liver and Intestine showed a decreasing trend of protein concentration compared with control samples **(Fig.2).** Except Gills which showed an increasing trend **Fig.1B**. The overall duration of 24 hrs. and 48hrs. did not show any difference at the increasing trend of concentration 2.5ppm, and 2.9ppm of Heavy metal Lead (Pb). In the case of the Liver and intestine **Fig.1C, 1D** showed highly significant at the level of ******* = significant ($P \leq 0.001$).

The separation technique of paper chromatography was performed in this study for the separation of amino acids in the pooled sample of the Heavy metal toxicity Lead (Pb) and Copper (Cu) treated and comprised of amino acid chains. The control variable accomplished the separation of each amino acid to be compared with the treated tissues of *L.rohita.* The measured distance traveled by the standard and the computed Rf value showed in **Table:1 and Table:2.**

Table1: Depicted the obtained Rf values of Amino acids treated by Heavy metal Lead (Pb) Toxicity by Paper Chromatography. The essential amino acid (EAAs) of Methionine, Phenylalanine in Muscle, and Tryptophan, Valine, and Leucine in Gill were present **(Fig.5A, 5B)**. The Non-Essential Fatty acid of Glycine, Proline, Alanine, Tyrosine, Serine, Glutamine, and Glutamic acid was present in the Liver and Intestine of heavy metal Lead (Pb) treated toxicity. The conditionally essential Amino acid of Proline is present in the gill **(Fig.5C, 5D)** of *L. rohita* . The amino acids were calculated based on the Retention value.

Table2: Depicted the obtained Rf values of Amino acids treated by Heavy metal copper (Cu) Toxicity. The Essential Amino acids of Leucine. Methionine, Threonine, Arginine, and Tryptophan, were presented **(Fig.6)** in Muscle, Gill, Liver, and Intestine of Heavy metal copper (Cu) treated *L.rohita.* The conditionally essential amino acid of Proline Glutamic acid and serine were present only in the Gill and Liver **(Fig.6B,6C)** of *L.rohita.*

The Total lipid level of *L.rohita* showed an overall decreasing level in Muscle, Gills, Liver, and Intestine when *L.rohita* was exposed to heavy metal copper (Cu) toxicity **(Fig.3).** Though there was fluctuation occurred based on heavy metal copper concentration (3.5ppm,3.9ppm), in Muscle, Gills, Liver and Intestine finally it showed decreasing level when compared with control. Except for gills all other samples **Fig.3A,3C, and 3D** were highly significant at the level of ******* = significant ($P \leq 0.001$).

Lead (Pb) heavy metal toxicity on the total lipid of *L.rohita* showed overall decreased concentration in Muscle, Gills, Liver, and Intestine (**Fig.4).** In the case of Gills, 48 hrs. Treated heavy metal Lead (Pb) concentration showed a high level of depletion **(Fig.4B**). Gill, Liver, and Intestine **(Fig.4B,4C,4D)** showed highly significant at the level of ******* = significant ($P \leq 0.001$) when the animal exposed to heavy metal Lead (Pb) toxicity.

Another separation technique of thin-layer chromatography was performed in this study for the separation of major lipid classes

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varying in polarity from cholesterol, and esters to phospholipids. The heavy metal copper (Cu) treated Muscle, Gill, Liver, and Intestine compared with the control. **Table:3** showed the Rf value on Thin layer chromatography and their respective lipid classes. The one-dimensional Thin layer chromatography was used for the less frequent phospholipids form and triglycerides form **(Fig.7).** The Phosphatidyl compounds were commonly present in Muscle, Gill, and Liver whereas in the Intestine it was absent (**Fig.7A,7B,7C**) instead Cardiolipin and Diglycerides were present (**7D**). Diglyceride and Triglyceride are found in Muscle, Liver, and Intestine.

Discussions:

Toxicity is a relative property of a chemical, which refers to its potency to induce harmful effects on an organism. It is a function of the concentration of the toxicant and the length of exposure of the animal (Wilkinson, 1976). It can also be regarded as the characteristic of an individual organism that responds to the heavy metal at a particular concentration or dose for a specific period.

The toxic effects may include both lethal and sub-lethal concentrations which may change the growth rate, development, reproduction, histopathology, biochemistry, physiology, and behavior (Rand and Petrocelli, 1985). Furthermore, metal pollution may lead to a shift in the community structure to metal-tolerant organisms, which are often capable of accumulating large amounts of metals (Rand and Petrocelli, 1985).

Metal ions and their complex exhibit widening toxicity to the organism that ranges from sub-lethal to lethal depending upon the time of exposure and the prevailing conditions in the ambient water (Goel, 1997). A low concentration of heavy metal salt does not trigger a quick and marked toxic pathological manifestation as otherwise observed in the case of acute toxicity (Rajan and Banerjee, 1993 a, b). In the evaluation of acute toxicity in the environment, the LC50 values (Median Tolerance Limit) are useful indices in measuring the acute toxicity of the test heavy metal, under certain environmental conditions.

The application of LC50 values has gained wide acceptance among toxicologists and is generally the most reliable test for assessing potential hazards to aquatic life (Brungs and Mount, 1978). LC_{50} values differ from species to species for the same toxicant due to the mode of action and responses of the animals (King, 1992). The toxicity tests were also influenced by the size, age, sex (Victoriamma and Radhakrishnaiah, 1982), nutrient supply (Arunachalam, 1980), and pH (Eisler, 1970).

Excessive intake of copper may lead to liver cirrhosis. Hepatic copper accumulation occurs in several cholestatic diseases and they play an important part in pathogenesis and can occasionally lead to neurological toxic effects. Copper overload in the newborn period when biliary excretion of copper is inefficient may establish a vicious cycle of copper accumulation and liver damage. In addition to copper, research indicates that iron deposition causes progressive liver cell damage and is involved in the pathogenesis of liver cirrhosis and fibrosis.

The present study was also initiated to find the susceptibility of the freshwater fish *Labeo rohita* to potentially hazardous heavy metals like lead and copper. An increase in the exposure period led to a decrease in LC_{50} values. From the fitted regression, an increase in the exposure period resulted in mortality and the values were directly proportional to the exposure period. Therefore, the acute toxicity test confirmed

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the potentially hazardous effects of lead on the experimental fish *Labeo rohita*.

The development of an in vitro fish ecotoxicological assessment tool would greatly enhance the ability to detect and screen the sub-lethal effects of aquatic pollutants (Kilemade and Mothersill, 2000). The induction and reliable assay methods for stress protein have a good potential as tools in monitoring toxic heavy metals. The most common sub-lethal effects observed due to altered environmental influence are behavioral changes. The behavior of the fish in heavy metal-polluted water varies according to test concentration (Sahai, 1990) The present study revealed that the behavior of fish remarkably changed due to the influence of lead and copper toxicity. The physiological responses of fish as evidenced by gulping and rapid opercula movement were indicative of respiratory inconveniences triggered by the absence of dissolved oxygen in the effluent. Similar findings with respiration were also recorded by Panigrahi and Mishra (1978).

Protein Biochemistry:

Pollution may induce certain biochemical changes in fish earlier to the manifestation of drastic cellular and systemic dysfunction, appropriate biochemical parameters could be used effectively as sensitive indicators (Aldrich, 1983).

Protein is an important constituent of animal tissue that plays an important role in cellular metabolism and regulates the process of interaction between intra and extra-cellular media, as a constituent of the cell membrane, but the protein metabolism was reported to alter due to the stress of various contaminants. Varieties of pollutants have proved to alter the protein metabolism in fish (Palanichamy et al., 1989).

Baskaran (1991) reported a depletion of protein content in the muscle and liver of Tilapia exposed to fenvalerate. Jeba Kumar et al. (1990) observed a decrease in the protein content of almost all tissues in *Ctenopharyngodon idellus* when exposed to sub-lethal and lethal concentrations of fenvalerate (Tilak et al.,2001).

Protein breakdown and synthesis proceed simultaneously in all the tissues. But during stress, the breakdown of protein occurs which acts as an alternative source of energy. The fish exposed to toxic stress stimulates Protein metabolism (Kabeer et al., 1981 b).

According to increasing concentration of heavy metal toxicity correlated well with the depletion of protein levels due to increased duration and test concentration**.** Radhakrishnaiah et al.(1991) also observed a decrease in the soluble, structural and total protein contents and an increase in free amino acid levels, and the activities of protease, alanine and aspartate aminotransferases were observed in all the organs at the exposure period.

The decrease in the protein content may be due to the necrosis of hepatocytes (Verma et al., 1984). Attributed the decrease in protein content of the liver of *Channa punctatus* exposed to factory effluent and ammonia respectively, to the availability of the decreased level of key enzyme and enhanced level of catabolism of amino acid as a consequence of the increased activity of glutamate dehydrogenase. Among all tissues, the liver showed higher protein content which might be due to a greater concentration of the enzyme. The liver is the site of metabolism. The liver plays an important role in the synthesis of proteins. The continuous reduction of Gill protein content was reported in *Anabas testudineus.* When exposed to sublethal concentration its quantity becomes variable, the same was reported by Osibona et al.,2006

Heavy metals are known to depress blood proteins in fish (Jana and Yopadhyaya,

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1987). The heavy metals may inhibit RNA synthesis resulting in lower content or they may somehow affect the uptake of amino acid in the polypeptide chains. Muscles rich in proteins, produce tissues for mechanical mobility, also Liver is rich in proteins and centers for metabolic activities. The protein depletion in all the tissues indicated that usage of the metabolic activity or due to utilization of keto acid to gluconeogenesis pathway for the synthesis of glucose or to maintain Osmo ionic regulation. The production of heat shock proteins or the destruction of free radicles could be a reason for cell apoptosis. (Shoba, K et al.,2007).

Separation of Amino Acid

Fish protein contains a well-balanced amino acid composition. Fish is composed of 16-18 amino acids based on the species type and seasonal variations (Kim et al., 2000). Fish contains well-balanced amino acid compositions consisting of eight essential amino acids and eight nonessential amino acids. Due to the rich amino acid content of fish, it is being utilized as fish meal, fish sauce, fertilizer, animal feed, and fish silage

Proteins extracted from the fish muscle contain several peptides that have many bioactivities such as antihypertensive, antithrombotic, immune-modulatory, and antioxidative properties (Kim et al., 2000). The bioactive peptides obtained from the fish muscle have anticoagulant and antiplatelet properties, which are the main reason behind the capability of peptides obtained from the fish to inhibit coagulation factors in the intrinsic pathway of coagulation (Je J. Y. et al., 2004). The protein obtained by the enzymatic hydrolysis of the fish muscle has several nutritional and functional properties from which many biologically active peptides can be obtained.

In this study presence of the essential amino acids, Methionine, leucine, tryptophan, and phenylalanine in heavy metal-treated samples emphasized the production of stress proteins that support the organism to withstand toxic conditions. Lead toxicity is higher effective than Copper heavy metal toxicity so, in Lead treated Liver and Intestine mostly absent. In absence of these amino acids suggested that reduction in the growth of animals, and they may present anorexia, besides anatomical signs of such deficiency. Amino acids are the building blocks of proteins (Walsh C. T et al., 2013). They have wide nutritional value, taste, medicinal action, and chemical properties. All amino acids are sold in different quantities each year. They are used as food additives, in pharmaceutical applications, feed, and food supplements. The amino acids such as arginine, glycine, glutamate, and histidine are used in protein pharmaceuticals as an excipient for drug development (Amraibure Odia and Oaikhena Zekeri Esezobor, 2017). The largest consumer of amino acids is the food flavoring industry which uses monosodium glutamate, alanine, aspartate, and arginine to improve the flavor of food (Francesco S.Dioguardi, 2011). The second largest consumer of amino acids is the animal feed industry which uses lysine, methionine, threonine, tryptophan, and others to improve the nutritional quality of animal feed. The amino acids can also use in various pharmaceutical applications such as protein purification and formulations and the production of antibiotics such as jadomycin.

Lipid Biochemistry:

Lipid was found more or less similar in all fishes under investigation. Lipid composition and distribution between and within the fish vary from species and are influenced by seasonal dietary variations. The range of lipid content in the edible part is approximately 0.5-18%. This depends on seasonal variations in feeding habits and

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regional differences in basic foods and nutrients. The lipid concentrations in the liver of *Clarias batracus* were seen to increase according to seasonal variations. The lower values of fat in the fish and the low-fat content of muscle are recognized due to their feeding nature (Klinger et al., 1996). In this study overall decrease in lipid concentration in Muscle, Gill, Liver, and Intestine due to heavy metal accumulation might affect cell membrane, and cell signal transduction through the interaction between lipids and biological systems reported by Martin-Perez Met al., 2021.

Lipid content is an essential organic constituent of the tissues of all animals and plays a key role in energy metabolism. Lipids are the best energy producer of the body next to carbohydrates. The decreased level of glycerol or inhibition of oxidative phosphorylation. George et al.,2013 reported that lipid is vital to embryogenesis, providing two third of energy by oxidation. Lipid act as reversed depots of energy from where the energy is supplied as and when required. Cholesterol and lipid levels were decreased in different organs of *Clarias batracuhus* exposed to lead nitrate (Katti et al.,1983).

Lipid Separation

Iverson et al. 2001, extracted lipids from fish samples using the original procedure described by Folch et al., 1957 and the results were compared to the Bligh and Dyer method of lipid extraction. The results suggested that both methods of extraction were highly correlated. In this study Triglycerides, Phosphatidyl compounds, and Cardiolipin were found. Cardiolipin is a Phospholipid present in Copper treated heavy metal in Gill and muscle mitochondrial inner membrane associated with mitochondrial functions. The accumulation of Cardiolipin in Gill and Muscle of *L.rohita* intake of human as a food meal may lead to dysfunction of Lipid metabolism (Kaviyarasi Reu et al.,2022) also Damage in the structure of myocardium, inclusive fibrosis in cardiac Tissue, Myocyte apoptosis and decreased contractility due to mitochondrial dysfunction.

Bell et al.2004, extracted lipids from the Atlantic salmon (*Salmo salar*) using the procedure described by using the Folch method and analyzed the free fatty acid in the fish. Saify et al.2004, extracted lipids from two species of the shark using chloroform/methanol (2:1) extraction method (Folch method) and evaluate the lipid composition of both species.

Fish oil contains two important polyunsaturated fatty acids called EPA and DHA or otherwise called omega-3 fatty acids. These omega-3 fatty acids have beneficial bioactivities including prevention of atherosclerosis, protection against maniac–depressive illness, and various other medicinal properties. A very recent study on Dietary supplementation of Diglyceride has antiviral and antibacterial properties and acts as an emulsifier to increase dietary lipid digestibility (Hanging Song et al., 2021). Fish oil can also be converted to non-toxic, biodegradable, environment-friendly biodiesel using chemical or enzymatic transesterification. The main lipid classes separated are hydrocarbons, cholesterol esters, methyl esters of fatty acids, triglycerides, fatty acids, 1,3-diglycerides, 1,2-diglycerides, cholesterol, monoglycerides, and phospholipids together with cerebrosides, which remain at the origin.

In extracts of naturally occurring lipids, caution should be used in attributing the dichromate reduction of material scraped from the origin entirely to phospholipids, since any very polar material in the extract will remain in this region. Marinetti 1964,

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subjected sialic acid-impregnated papers to a very short preliminary development with chloroform-methanol1:1 (containing 2% water) to cause the polar lipids to migrate away from other material at the origin. In those cases where only a total phospholipids value is required, it may be preferable to carry out phospholipids-phosphorus estimation. Skupski V.P. et al., 1964, on the material at the origin in place of the dichromate reaction. It is suggested that the lipid extract is first to run in a system suitable for phospholipids separation. Individual phospholipids fractions can then be scraped from the plate and quantified by the dichromate reduction method. Neutral lipids, which will be located near the solvent front in such systems, can then be eluted as a group and chromatographed and estimated by the procedure.

This study concluded that Lead acetate and Copper sulfate are toxic to aquatic organisms. Lead acetate has a profound influence on the biochemical analysis of major carp *L.rohita*. Fish absorbed metals through ingestion of water or contaminated food. Various types of pollution like industrial effluent, hazardous material mixing with the water bodies, and heavy metals have been shown to undergo bioaccumulation in the tissues of aquatic organisms on the consumption of fish, and these metals become transferred to man. The results simply a better understanding of the toxicological endpoint of these specific heavy metals and provides significant information on safe levels in the aquatic environment. Although the results do not explicitly indicate a manifestation of toxic effects, the possibility that deleterious effects could manifest after a long period of consumption of fish caught from the aquatic system, with trace metal contamination cannot be ruled out.

In the future, the Identification of sustainable biochemical markers for aquatic pollution especially relating to heavy metals toxicity. Correlation of elevated and down-regulated total protein concentration referred to as biochemical index with the specific metal contaminant of industrial sites. Understanding adaption of genes and proteins in the aquatic model system for an environmental impact assessment.

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Table1: Obtained Rf values of Amino acids treated by Heavy metal Lead (Pb) Toxicity

Tissues	Compounds	Distance Travelled (cm)	Retention factor(Rf)
Muscle			
Control	-	-	-
24hrs (2.5ppm)	Tryptophan	5	0.66
24hrs (2.9ppm)	Valine	4.6	0.61
48hrs (2.5ppm)	Glycine, Valine	2, 4.6	0.26, 0.61
48hrs (2.9ppm)	Leucine	5.5	0.73
Gill			
Control	Phenylalanine	5.1	0.68
24hrs (2.5ppm)	Proline	4.3	0.413
24hrs (2.9ppm)	Proline	4.3	0.413
48hrs (2.5ppm)	Methionine	4.1	0.55
48hrs (2.9ppm)	Methionine	4.2	0.56
Liver			
Control	Alanine	2.9	0.38
24hrs (2.5ppm)	-	-	-
24hrs (2.9ppm)	Phenylalanine	5.1	0.68
48hrs (2.5ppm)	-	-	-
48hrs (2.9ppm)	-	-	-
Intestine			
Control	Tyrosine	3.4	0.45
24hrs (2.5ppm)	Serine	2.1	0.28
24hrs (2.9ppm)			
48hrs (2.5ppm)	Glycine	2	0.26
48hrs (2.9ppm)	Glutamine, Glutamic acid	1, 2.3	0.13, 0.30

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Table 2: Obtained Rf values of Amino acids treated by Heavy metal copper (Cu) Toxicity

Tissues	Compounds	Distance Travelled (cm)	Retardation factor
Muscle			
Control	Leucine	5.4	0.738
24hrs (3.5ppm)	Methionine	4.1	0.55
24hrs (3.9ppm)	Methionine	4.1	0.55
48hrs (3.5ppm)	Glutamic acid	2.3	0.30
48hrs (3.9ppm)	Tryptophan	5	0.66
Gill			
Control	Glutamic acid, Proline, Leucine ,	2.2, 4.3, 5.5	0.30, 0.55, 0.73
24hrs (3.5ppm)	-	-	-
24hrs (3.9ppm)	Tryptophan	5	0.66
48hrs (3.5ppm)	Tryptophan	5	0.66
48hrs (3.9ppm)	Glutamic acid, Valine	2.3, 4.6	0.30, 0.61
Liver			
Control	Alanine	2.9	0.38
24hrs (3.5ppm)	Serine	2.1	0.28
24hrs (3.9ppm)	Serine	2.1	0.28
48hrs (3.5ppm)	-	-	-
48hrs (3.9ppm)	Methionine	4.1	0.55
Intestine			
Control	Methionine	4.1	0.738
24hrs (3.5ppm)	-	-	-
24hrs (3.9ppm)	Arginine	1.7	0.20
48hrs (3.5ppm)	Threonine	2.6	0.35
48hrs (3.9ppm)	Arginine	1.7	0.20

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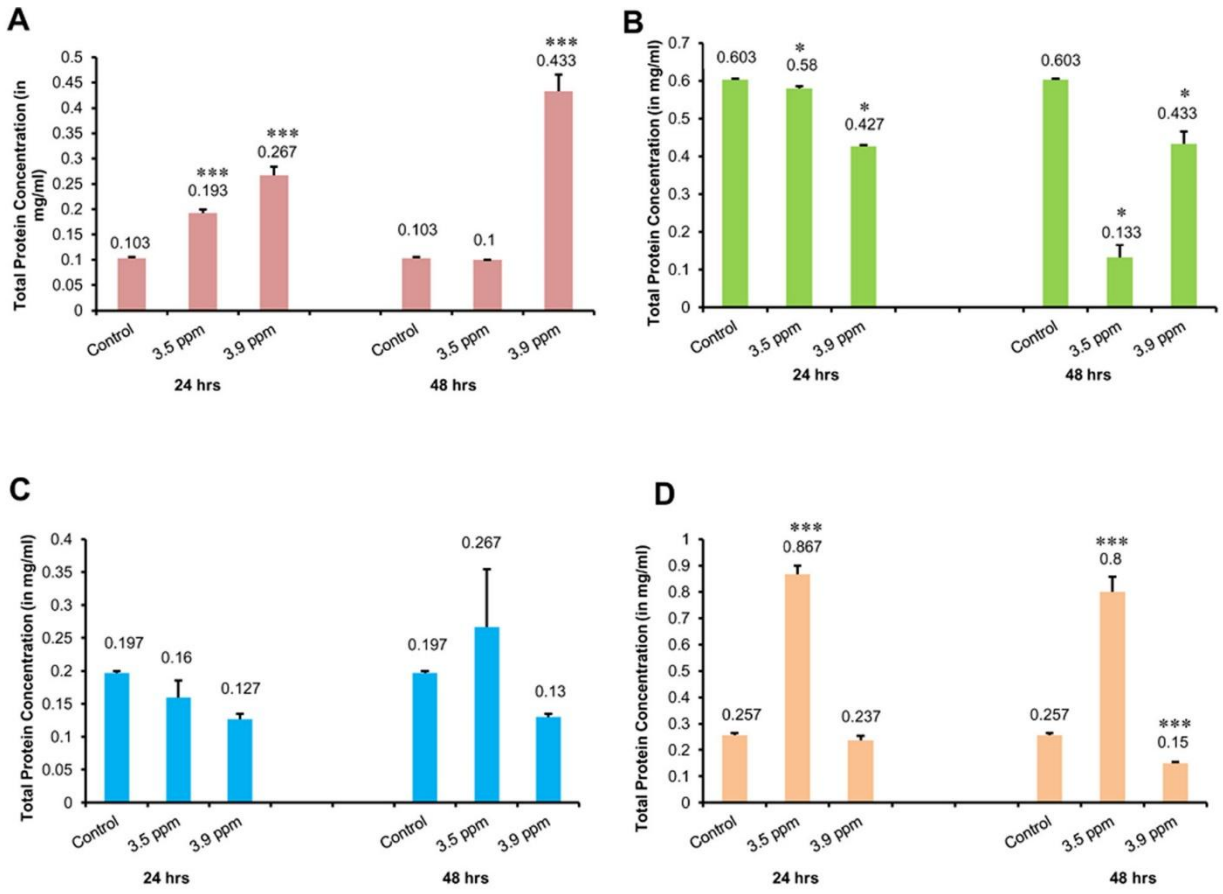

Figure1: Total Protein Concentration. Exposed under Copper Heavy Metal Toxicity. The data were analyzed using STATA (v.13.0) Program. ***** = significant ($P \leq 0.05$), ****** = significant ($P \leq 0.01$), ******* = significant ($P \leq 0.001$) for **A.** Muscle, **B.** Gill, **C.** Liver and **D.** Intestine.

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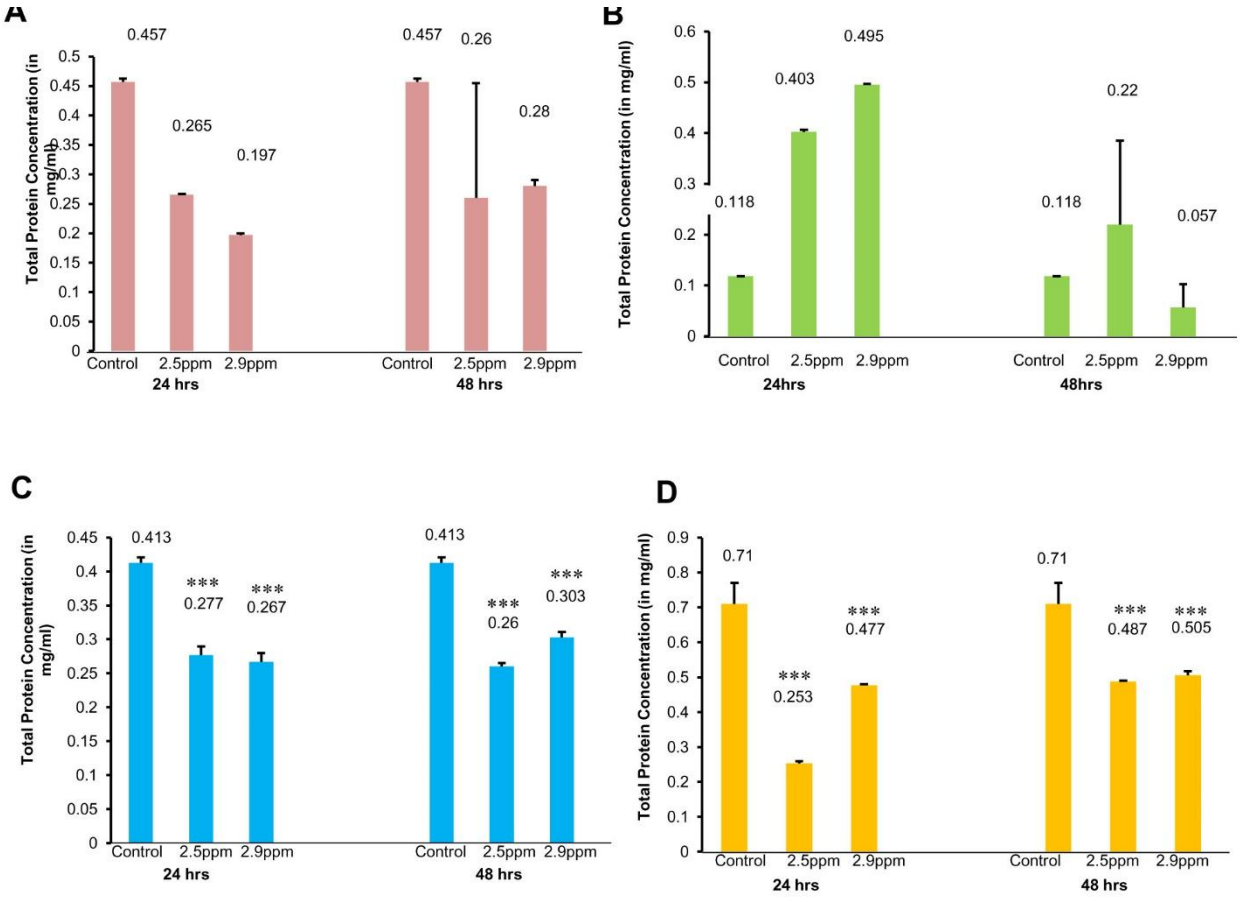

Figure 2: Total Protein Concentration. Exposed under Lead Heavy Metal Toxicity. The data were analyzed using STATA (v.13.0) Program. ***** = significant ($P \leq 0.05$), ****** = significant ($P \leq 0.01$), ******* = significant ($P \leq 0.001$) for **A.** Muscle, **B**. Gill, **C**. Liver and **D.** Intestine.

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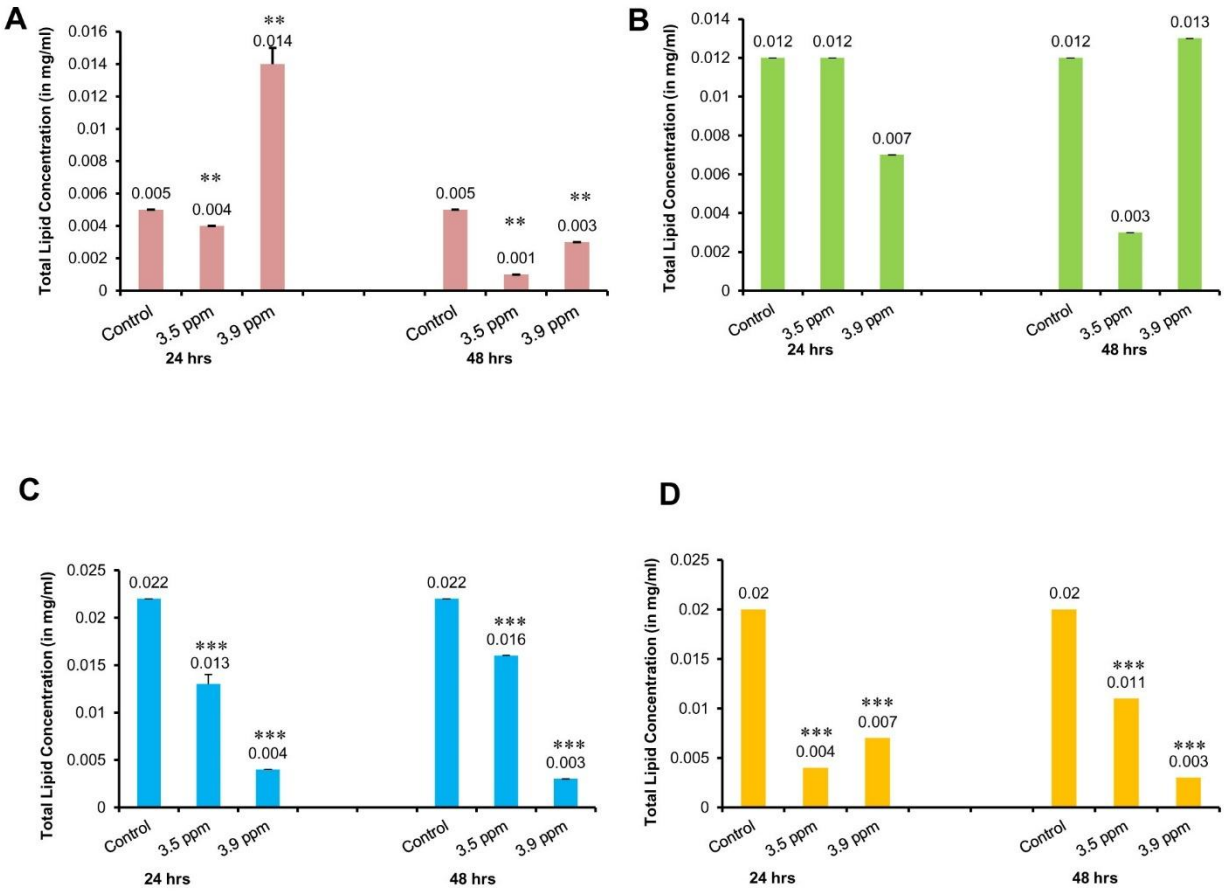

Figure 3: Total Lipid Concentration. Exposed under Copper Heavy Metal Toxicity. The data were analyzed using STATA (v.13.0) Program. ***** = significant ($P \leq 0.05$), ****** = significant ($P \leq 0.01$), ******* = significant ($P \leq 0.001$) for **A**. Muscle, **B.** Gill, **C.** Liver and **D.** Intestine.

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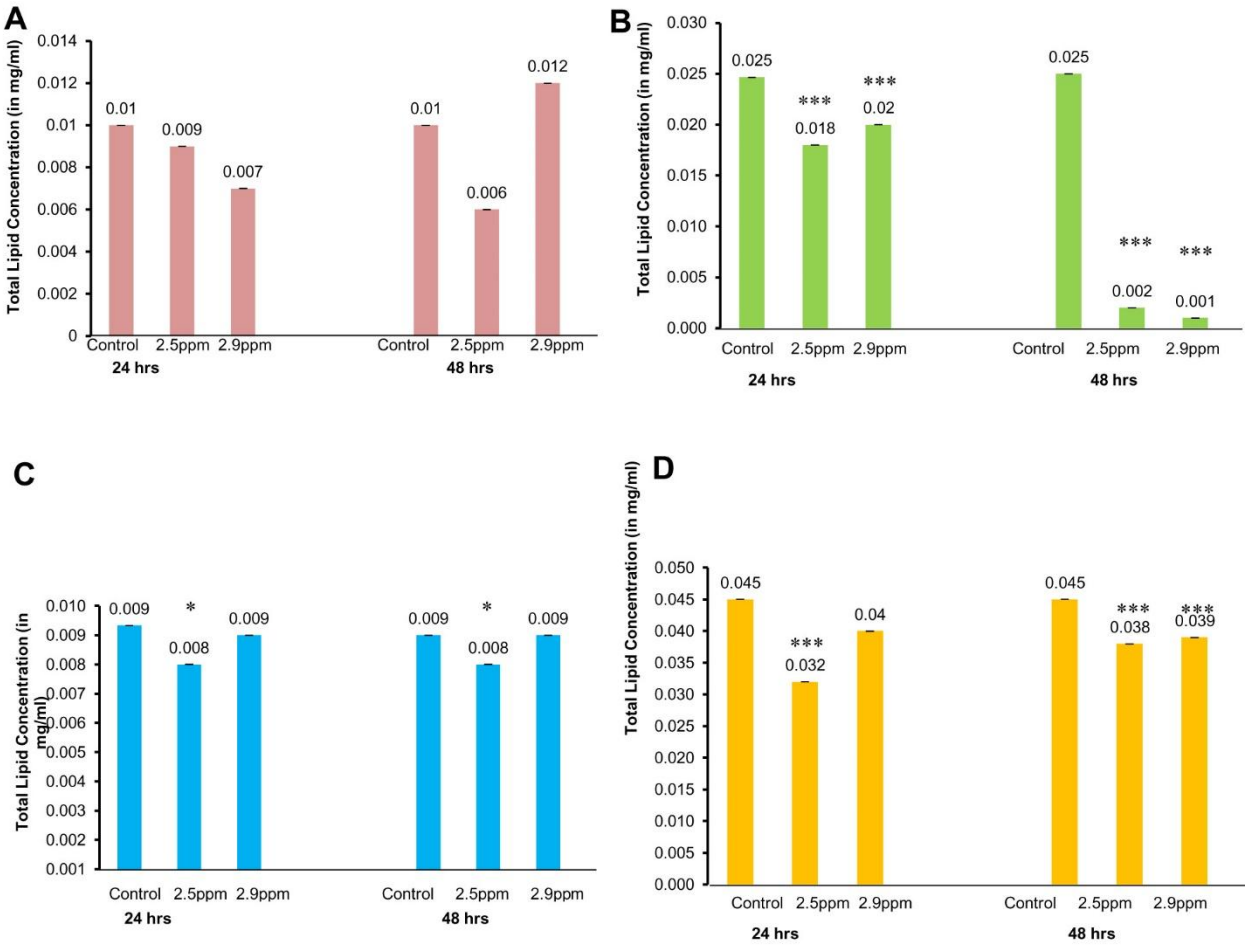

Figure 4: Total Lipid Concentration. Exposed under Lead Heavy Metal Toxicity. The data were analyzed using STATA (v.13.0) Program. ***** = significant ($P \leq 0.05$), ****** = significant ($P \leq 0.01$), ******* = significant ($P \leq 0.001$) for **A**. Muscle, **B.** Gill, **C**. Liver and **D.** Intestine.

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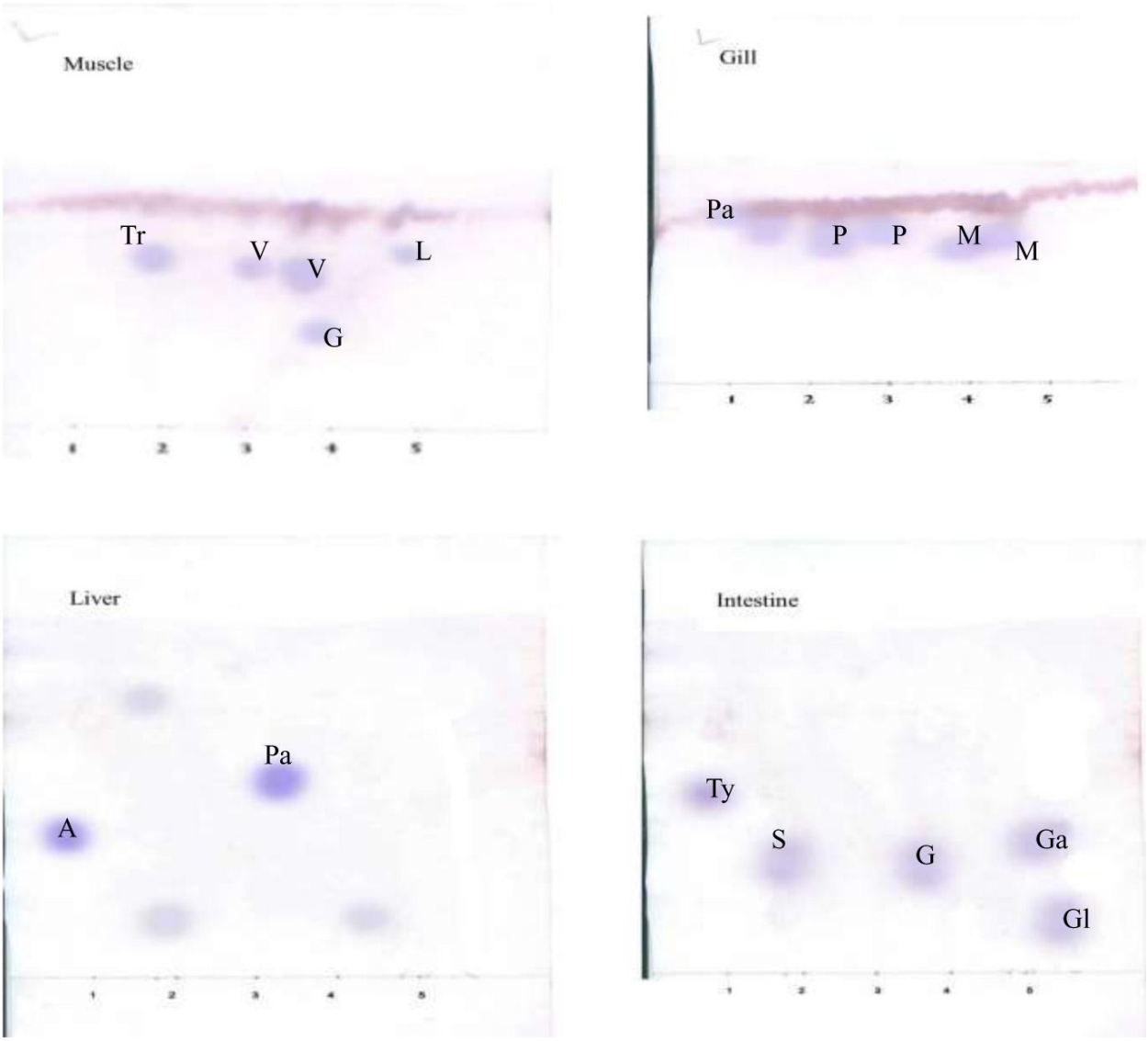

Figure 5: Paper Chromatography – Samples Exposed under Lead Heavy Metal Toxicity of **A**.Muscle, **B**.Gill, **C**.Liver, **D**. Intestine

1. Control , 2. 24hrs- 2.5ppm. , 3. 24hrs- 2.9ppm. , 4. 48hrs- 2.5ppm. ,5. 48hrs- 2.9ppm.

Abbreviations: Tr- Tryptophan, **V**- Valine, **G**- Glycine, **L**- Leucine, **A**- Alanine, **Pa**- Phenylalanine, **P**- Proline, **M**- Methionine, **Ty**- Tyrosine, **S**- Serine, **Ga**- Glutamic acid, **Gl**- Glycine.

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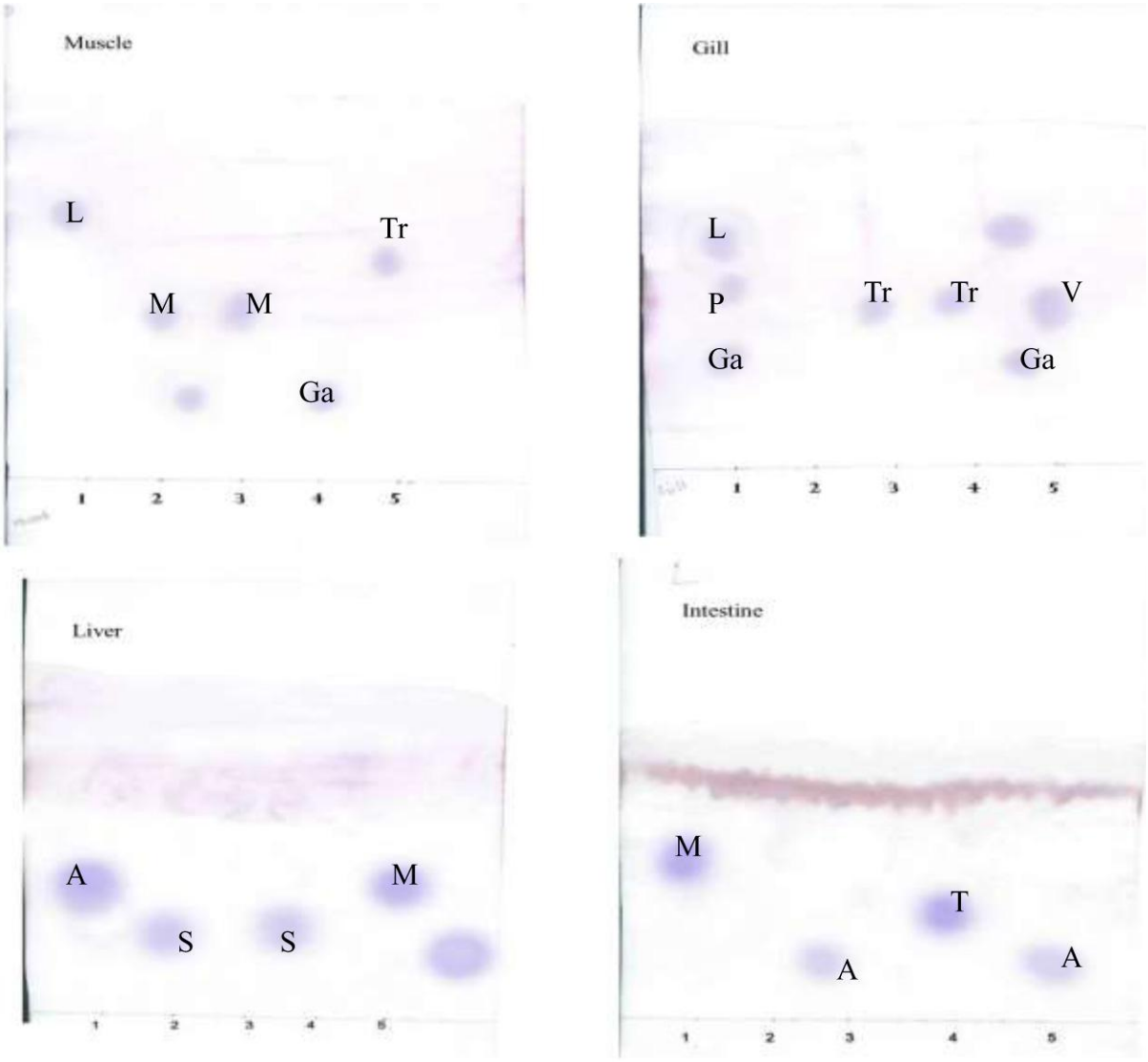

Figure 6: Paper Chromatography – Samples Exposed under Copper Heavy Metal Toxicity of **A**.Muscle, **B**.Gill, **C**.Liver, **D**. Intestine

1. Control , 2. 24hrs- 3.5ppm. , 3. 24hrs- 3.9ppm. , 4. 48hrs- 3.5ppm. , 5. 48hrs- 3.9ppm

Abbreviations: L- Leucine, **M**- Methionine, **Ga**- Glutamic acid, **Tr**- Tryptophan, **P**- Proline, **V**- Valine, **A**- Alanine, **Ty**- Tyrosine, **S**- Serine.

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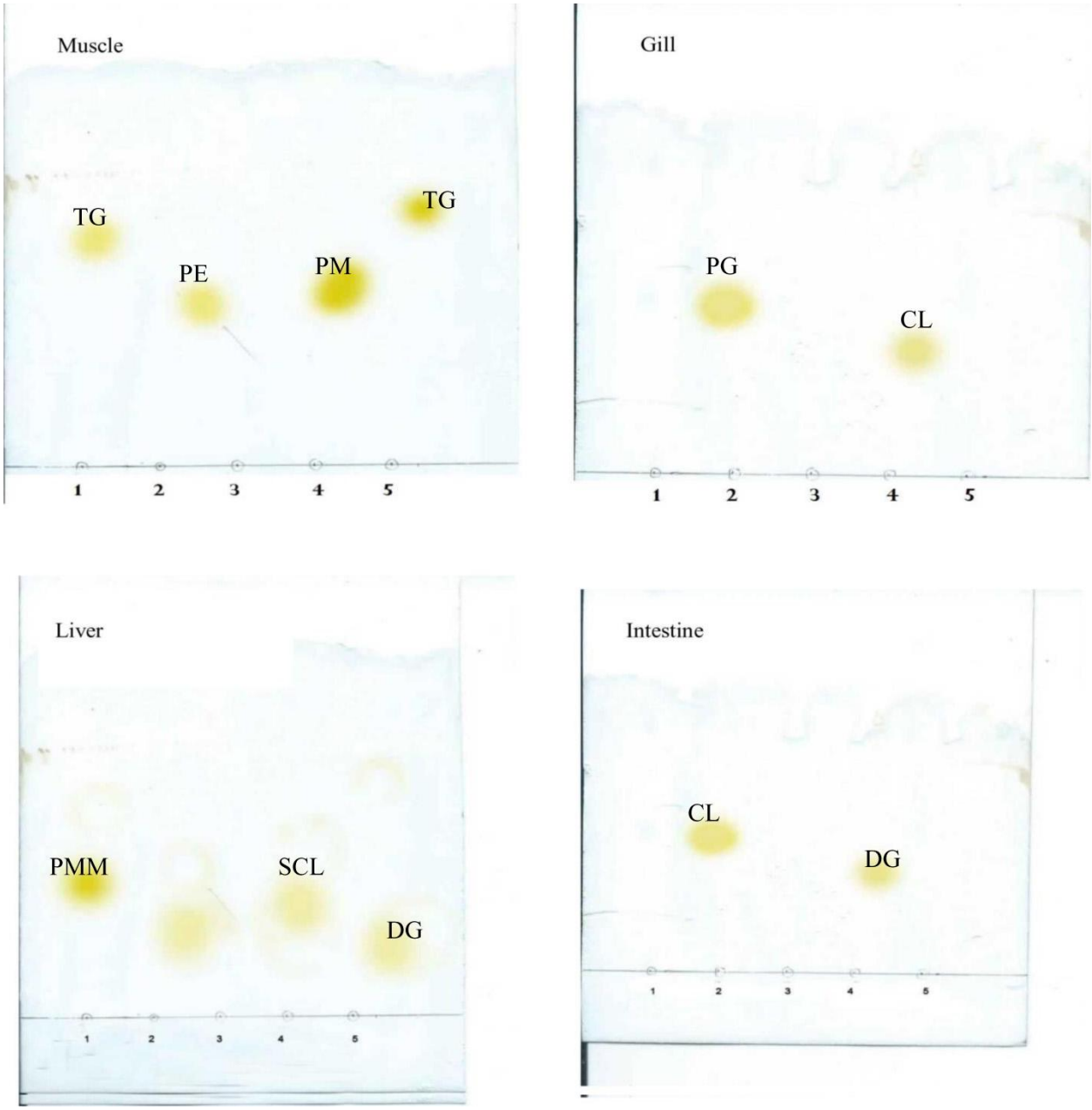

Figure 7: Thin layer Chromatography – Samples Exposed under Copper Heavy Metal Toxicity of **A.**Muscle, **B**.Gill, **C**.Liver, **D**. Intestine
1. Control , 2. 24hrs- 3.5ppm. , 3. 24hrs- 3.9ppm. , 4. 48hrs- 3.5ppm. , 5. 48hrs- 3.9ppm.
Abbreviations: **TG**- Triglyceride, **PE**- Phosphatidylethanolamine, **PM**-Phosphatidyl monoamine, **PG**- Phosphatidylglycerol, **CL**- Cardiolipin, **PMM**- Phosphatidyl monomethyl ethanolamine, **SCL**- Synthetic cardiolipin, **DG-** Diglyceride.