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# Hepato toxicity of lead in Indian major carp *Labeo rohita (Rohu)* - A Fourier transform infra red study

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## ABSTRACT

Acute toxicity test was performed on Indian major carp *Labeo rohita* exposed to different concentrations of lead for 96 h. The Median Lethal Concentration ($LC_{50}$) was found to be 34.30 ppm. The effects of lead accumulation and Bioconcentration factor (BCF) in different organs at sublethal concentration (33% 96 h $LC_{50}$) were estimated. The accumulation of lead and BCF was found to be in this order: Liver > Gill > Kidney > Muscle. Lead treatment showed significant reduction in protein content in liver, which was confirmed by Fourier Transform Infra Red spectral analysis. The liver damage was also confirmed by histology analysis. It was concluded that liver has the highest tendency to accumulate lead and affected by lead treatment. The present work could be used as a preliminary study to screen out the hepato protective agent (herbal drugs) against lead toxicity.

**Key Words:** Acute toxicity, Bioaccumulation, Bioconcentration factor, *Labeo rohita*, Fourier transform infra red spectroscopy

## INTRODUCTION

Heavy metals include a great variety of chemical elements that typically occur in low or trace amounts in the environment, all which have the potential to provoke toxic effects in organisms (Karthikeyan, 2007; Ambedkar and Muniyan, 2011). These are considered to be known toxicants and have received considerable attention due to their toxicity (Javed and Hayat, 1999). The accumulation of heavy metals in aquatic biota imposes acute disorder in aquatic organisms. Lead (Pb) is a non-essential and toxic heavy metal. It is cheap, useful, easy to mine, therefore ubiquitous- in air, food, water, soil, ceilings etc (Lead and Lead Compounds, 2011). Generally, Pb exists in both organic and inorganic forms in the environment. The divalent form of Pb (II) is the stable ionic form present in the environment and is the one most accumulated by aquatic organisms. Aquatic organisms are susceptible to both organic and inorganic lead from water and sediment, but uptake of inorganic lead is relatively a slow process (World Health Organization, 1989). Pb is entered into aquatic environment through erosion and leaching of soil, falling of lead-dust out, combustion of gasoline, industrial, municipal and agricultural waste discharge, run-off water deposits from streets and other surfaces as well as precipitation and loss of lead in fishing sinkers and scuba diving weights in inventories,etc., (Department of Water Affairs and Forestry, 1996). It is an immunotoxicant which through human exposure results in immune function changes and has the potential to adversely affect human health. Fish is a main part of food chain in aquatic environment and also a common source of protein. It contains a greater quantity of protein than any other living organism, contributing roughly about 75% of the weight of fish (Karthikeyan, 2012*). Labeo rohita* is a widely consumed telost fish in Tamil Nadu, India and its impact on human health condition cannot be negligible. In addition, it is also suitable for toxicity monitoring (Ramani, 2002). The accumulation of heavy metal results in the decrease in the total protein content which could possibly affect the enzyme mediated bio defense mechanisms of the fish, which in turn pose a serious threat to human beings by secondary poisoning through food chain (Vutukuru, 2005). So, it is essential to study the toxicity effects of lead in fish. Among the organs, Liver is a place where detoxification occurs in fish (Kaoud and El-Dahshan, 2010), Hence the present study is aimed to investigate the hepato toxicity of lead on biochemical composition especially proteins in *Labeo rohita* by using FTIR spectroscopy.

## MATERIALS AND METHODS

**Experimental Design:** The fresh water fish, *Labeo rohita* (Rohu) (8-10 cm length and 28 ± 0.6 g weight) was used for the toxicity tests. These were collected from ponds of northern districts of Tamil Nadu, India. The fish was acclimatized to laboratory conditions for a week. The fish was fed with standard powdered feed and were starved for 24 h prior to the experimentation. After acclimatization, the fish was transferred to aquaria/trough with the capacity of 15 l. The water quality parameters (temperature, dissolved oxygen, and pH) were maintained as recommended by USEPA, 1976. Analytical grade lead acetate (Janaki Scientific chemicals, India) was used as the metal toxicant. The acute toxicity test for lead was based on the standard method of USEPA (1995).Fish was divided into three groups of 6 each, with the first group serving as control and other groups as experimental groups.

**Determination of Median Lethal Concentration ($LC_{50}$):** To evaluate the fish viability and $LC_{50}$ of lead acetate, the second group fish was divided into six subgroups and exposed to different concentrations (14, 28, 42, 56, 70 and 84 ppm) of lead acetate. Stock solution of the test compound lead acetate and their dilutions was made according to the guidelines given in the standard methods (Organisation for Economic Co-operation and Development 1993). The mortality rate was determined from 24-96 h. Then the $LC_{50}$ was calculated by Probit analysis (Finney et al., 1948; Finney, 1955).

**Determination of Lead Accumulation:** The third group fish was exposed to 12 ppm which is 1/3 value of $LC_{50}$ for 4 days to determine lead accumulation in various organs like muscle, liver, kidney and gill. After 96 h of exposure to the above mentioned lead acetate concentration, *Labeo rohita* fingerlings (control and lead acetate exposed) were sacrificed and dissected. 0.2 g each of muscle, gill, kidney, and liver of fish was mixed with $HClO_3$, $HNO_3$ (Perchloric acid, Nitric acid) in 1:3 ratio and heated up to 60 ºC for 1 h to digest tissue samples. After the appearance of pale yellow color, the solution was cooled to room temperature and the final volume was made up to 50 ml by adding distilled water. Then the concentration of accumulated lead was determined by Inductively Coupled Plasma Atomic Emission Spectrophotometer (Perkin Elmer Optima 5300 DV Model).

**Histological Studies:** Histological analysis was performed on liver tissues of fish. Liver tissue was preserved in 10% formalin for 24 h and washed with 70% ethanol. It was processed to obtain five micron thick paraffin sections, then stained with Hematoxylin and Eosin (Kim Suvarna, 2013) and examined under Olympus BX51 light microscope

**Determination of Protein Content variation by FTIR:** Fourier Transform Infrared Spectroscopy (PE FT IR Model) was carried out before and after the lead acetate treatment to find the protein content variation in the tissues. For this analysis, muscle, gill, liver and kidney tissue samples were dried at 70 ºC to completely remove the moisture content and then the dried samples were ground into fine powder. The tissue powder samples were analyzed by KBr pellet method using FTIR.

## RESULTS

**Determination of $LC_{50}$ Concentration:** The fish in the control aquarium was observed to be healthy, normal and no mortality was recorded in it. In lead treated aquarium no mortality was observed at lead concentration of 14 ppm and 28 ppm after 96 h exposure. However, the fish exposed to the concentrations of 42, 56 and 70 ppm and 84 ppm of lead acetate showed 33.3, 66.6, 83.3 and 100% mortality after 96 h respectively. It was observed that the percentage and number of survivors decreased with increasing concentration of lead in fish.

**Table.1.Percentage of mortality of acute toxicity in lead acetate exposed *Labeo rohita* for 96 h**

| Type of Aquarium | Concentration of lead acetate (ppm) | No of alive fishes | Mortality (%) |
|---|---|---|---|
| Lead acetate treated aquarium | 14 | 6 | 0.0 |
| | 28 | 6 | 0.0 |
| | 42 | 4 | 33.3 |
| | 56 | 2 | 66.6 |
| | 70 | 1 | 83.3 |
| | 84 | 0 | 100 |
| Control aquarium | 0 | 6 | 0.0 |

**The accumulation of lead in various organs:** After the determination of $LC_{50}$ fish was exposed to 12 ppm which is 1/3 value of $LC_{50}$ for 4 days to determine lead accumulation in various organs. The accumulation of lead in various organs and whole body animal tissue is presented in table 2. The accumulation of lead in the organs was increased in the following order: Liver > Gill > Kidney > Muscle. The present study revealed that the highest degree of lead accumulation in liver and the lowest degree of accumulation in muscle.

**Table.2.Bioaccumulation of lead in the organs of *Labeo rohita***

| Organ | Control in (mg/g) | Pb treated in (mg/g) | Bioconcentration factor(BCF) |
|---|---|---|---|
| **Gill** | 0.038±0.007 | 13.83±0.270 | 4.61±0.090 |
| **Liver** | 0.094±0.010 | 20.10±0.793 | 6.70±0.264 |
| **Muscle** | 0.016±0.004 | 11.28±0.685 | 3.76±0.228 |
| **Kidney** | 0.014±0.005 | 12.46±0.210 | 4.15±0.070 |

Table 2 shows the Bioconcentration factor (BCF) of different organs (gill, liver, kidney and muscle) of *Labeo rohita*. It was observed that liver has the highest BCF value followed by gill, kidney and muscle.

**Determination of Protein content variation by FTIR:** The effect of lead on protein content of *Labeo rohita* on liver, kidney, gill and muscle tissues was studied by using FTIR spectra. A FTIR spectrum was recorded through scanning in the range 4000-400cm$^{-1}$ for control and lead treated samples. The scanning results were given in percentage of absorbance on specific wave number for every amide functional group in each sample. Each amide has specific marker group. The vibrational assignments of FTIR spectra for the control and lead treated fish tissue samples were presented in table 3.

**Table.3.The frequency assignment of FTIR absorption spectra for control and lead treated tissues of *Labeo rohita***

| Wave Number (cm$^{-1}$) | | | | Vibrational Assignments |
|---|---|---|---|---|
| **Control Gill** | **Pb treated Gill** | **Control Muscle** | **Pb treated Muscle** | |
| 3295 (s) | 3390 (m) | 3286 (s) | 3276 (s) | O–H/N–H stretching |
| 2936 (m) | 2951 (m) | 2928 (m) | 2927 (m) | $CH_3$ asym. stret.: lipid, protein |
| 1646 (s) | 1657 (s) | 1656 (s) | 1655 (s) | C = 0 stretching (amide I) |
| 1536 (s) | 1552 (m) | 1533 (m) | 1551 (m) | N–H bending (amide II) |
| 1246 (m) | 1241 (w) | 1232 (w) | 1240 (w) | C–N stretching (amide III) |
| **Wave Number (cm$^{-1}$)** | | | | **Vibrational Assignments** |
| **Control Liver** | **Pb treated Liver** | **Control Kidney** | **Pb treated Kidney** | |
| 3295 (s) | 3298 (m) | 3286 (s) | 3286 (s) | O–H/N–H stretching |
| 2953 (m) | 2951 (m) | 2928 (m) | 2927 (m) | $CH_3$ asym. stret.: lipid, protein |
| 1664 (s) | 1675(m) | 1656 (s) | 1684 (m) | C = 0 stretching (amide I) |
| 1532 (s) | 1550(m) | 1543 (m) | 1552 (w) | N–H bending (amide II) |
| 1240 (m) | 1245 (w) | 1232 (w) | 1232 (w) | C–N stretching (amide III) |

s – Strong; m – medium; w – weak;

Fig.1 represents the FTIR spectra of proteins in selected tissues (liver, kidney and muscle) before and after the lead treatment. In all the spectra, the amide I bands were occurred within 1646-1684 cm$^{-1}$ region and amide II bands in the region 1536-1551 cm$^{-1}$for control and lead treated tissues respectively.

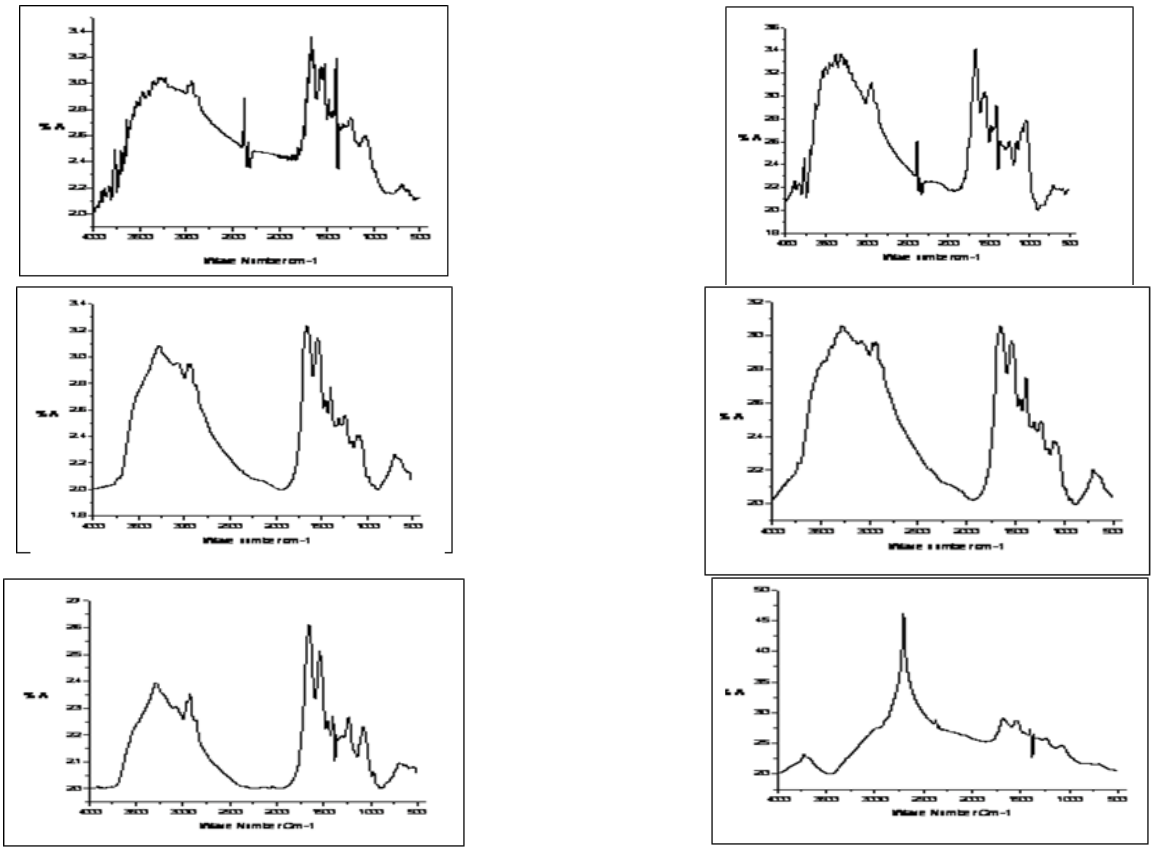

**Fig.1.FTIR spectra of proteins in liver tissue (A &B), muscle tissue (C &D) and kidney tissue (E&F) of fish before and after the lead treatment**

The ratio of amide II & amide I was decreased for the entire tissue sample after lead treatment from the control (table 4). It was observed that C-H stretching region which includes the $CH_3$ asymmetric stretching (2937-2953 $cm^{-1}$) and O–H/N–H stretching in the region 3276-3390 $cm^{-1}$ respectively. The broad band centered at ~3295 $cm^{-1}$ was assigned as the O–H stretching with small contribution from the amide bands of protein. These results were consistent with the observed FTIR spectra for proteins (Table 3). (Severcan and Parvez, 2012; Chiriboga et al., 2006).It was observed that amide I & amide II peak intensities were increased. There was also a shift in amide I absorption frequency towards high wave number and changes in band intensity. Fig. 1A and Fig. 1B show the significant difference between the control and lead treated liver tissue and no significant differences were observed in muscle and kidney tissue (Fig. 1C, 1D and Fig. 1E, 1F).

**Table.4.Amide II to Amide I ratio**

| Organs | Control | Pb treated | % Variation |
|---|---|---|---|
| **Gill** | 0.790±0.020 | 0.780±0.040 | 1.29±1.299 |
| **Muscle** | 0.943±0.017 | 0.923±0.046 | 2.12±3.101 |
| **Liver*** | 0.906±0.014 | 0.769±0.014 | 15.19±1.250 |
| **Kidney** | 0.952±0.003 | 0.843±0.040 | 11.45±4.173 |

***Significant reduction in protein content (p< 0.01)**

It was observed from the spectra that the ratio of absorption of intensity of the band amide II/amide I for the liver decreases from 0.906 to 0.769 which corresponds to 15.19±1.250 % by value. Among the four different tissues, liver has the highest variation in the amide II to amide I ratio for lead treated sample compared to control. It shows significant reduction in protein content ($p < 0.01$) in liver due to lead treatment. Kidney has the next highest variation in that ratio with 11.45±4.173 % followed by muscle and gill (Liver > Kidney > Muscle > Gill).

**Histology:** The control group liver tissue (Fig. 2A) generally exhibited a normal architecture with polygonal-shaped hepatocytes. These hepatocytes are located among blood capillaries called sinusoids, forming cord like structures known as hepatic cell cords. The liver of lead acetate exposed fish (Fig. 2B) showed clear large vacuoles found between hepatocytes, congestion of blood vessel, leukocytic infiltration, necrosis and degeneration of blood vessel hypertrophy.

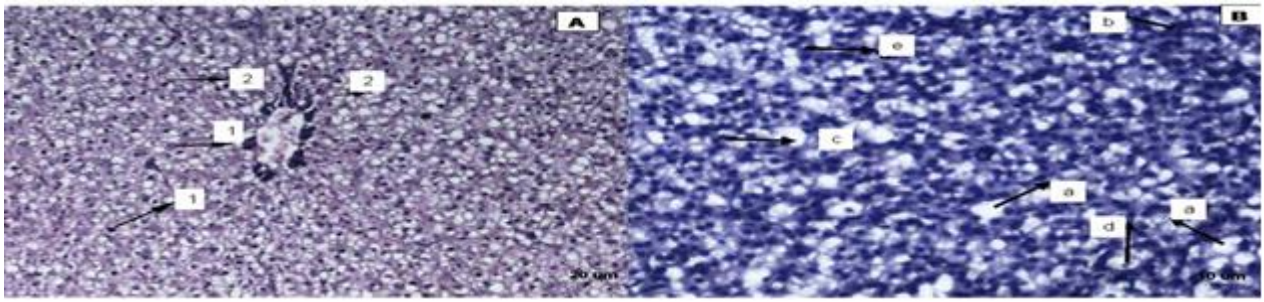


**Fig.2.A Photomicrograph of the liver section of Control fish (A) showing 1- Hepatocytes with a nucleus, 2-Sinusoid (Scale Bar = 20 µm) and lead acetate exposed fish (B)showing a- Vacuolization of nucleus, b-Cytoplasmic degeneration in hepatocytes, c- Congestion of blood vessels, d- Leukocyte infiltration, e-Hypertrophy of hepatocytes (Scale Bar = 10 µm)**

## DISCUSSION

Fish is a possible mediator which can transfer nutrients (proteins and omega fatty acids) to human. Unfortunately, from the surrounding water, it may absorb dissolved heavy metals that may accumulate in various tissues, organs and even be magnified in the food chain/web (Javed, 2003). The accumulated heavy metal poses serious threats to man. Hence, the Studies on the accumulation of heavy metals in various organs of the fresh water fish are very essential due to its toxic effects. Lead accumulation was different for different organs, suggesting different functional capability to regulate the metal adsorption. However, Liver had highly accumulated lead than muscle, gill and kidney (Liver > Gill > Kidney >Muscle). The same was reported for arsenic in *Labeo rohita* (K. Pazhanisamy et al., 2007; Swati et al., 2012). This will lead to liver damage. The reason behind the liver damage was due to decrease in the level of protein, lipids, glycogen and metabolic enzymes (Zodape, 2010).The liver of fish was important organ for ecotoxiocological study and it was the prime site for accumulation of lead (Ahmed and Bibi, 2010; Vinodhini and Narayanan, 2008; Lal Shah and Ahmet Altindau, 2005). Accumulation of lead in Gill which comes next in the order due to its large surface area's contact with the water. Since, Kidney is the doorway for heavy metal detoxification in the body, accountable amount of lead accumulation was observed. Heavy metals were uniformly spread over the body muscles. Hence, the muscles are known to have less accumulation of lead than other organs.

The histopathological lesions in the liver may be attributed to enzymatic changes or metabolic changes due to contaminated water. The liver of lead acetate exposed fish showed clear large vacuoles found between hepatocytes, congestion of blood vessel, leukocytic infiltration, necrosis and degeneration of blood vessel hypertrophy. Congestion is a blood circulation disturbance due to increased volume of the blood in the blood capillary. Vacuolar degeneration is known as an acute swelling of the organ. In addition, loose arrangement of hepatic cells and hepatocytes with large intracellular and intercellular vacuoles were observed (Rejeki, 2008).

Heavy metal treatment in general, decreases protein content which was confirmed by FTIR spectral analysis. Amide is the easiest protein group marker to be identified in protein samples. Therefore, to determine protein content of a sample, the easiest way is to detect the amide in it. Various types of amide show specific functional groups in different protein samples. (Sjahfirdi et al., 2012).The absorption signals from protein secondary structural elements arising from specific vibrations superimpose under the amide regions, which are amides I to V and amide A & B. Amide I & II regions are generally preferred in protein structure analysis. Amide III & V inherit structural information as well, but are quiet complex bands. These bands are affected by several factors such as environmental conditions and from the alterations like heavy metal toxicity (Severcan and Parvez, 2012*;* Kong and Shaoning, 2007). The wave number for amide I and amide II was obtained from FTIR spectra for control and lead treated tissues. The shift in the amide I band wave number in lead intoxicated tissues indicates conformational changes of the protein backbone. The changes in peak position of amide bands may quantitatively reflect alterations in amino acid side-chain of proteins (Andreas, 2000).Since, the amide I band intensities play a predominant role in structure of protein, this changes indicate either a structural rearrangement of the existing protein or the expression of new set of proteins (Karthikeyan, 2012*).* In addition, the hydrogen bonding plays an important role in the broadening of the spectral bands (Besley, et al., 2004). Further, the changes in the amide band indicate the altered protein secondary structure due to effect of heavy metal intoxication (Senthamilselvan et al., 2012; Van de Weert et al., 2001). The band observed around 2927 $cm^{-1}$ was assigned to $CH_2$ asymmetric stretching due to lipids. The decreased band areas of asymmetric $CH_2$ stretching modes observed in the lead intoxicated tissues suggests the decreased composition of the acyl chains in the lead intoxicated tissues of *Labeo rohita*. Similar results were reported by Palaniappan and Vijayasundaram (2008).

The ratio of the intensities of the amide II and amide I vibrational bands of protein components was used to analyze pure proteins in order to evaluate their denaturation state (Bramanti et al., 1992). Since the amide absorptions are sensitive to protein conformations, an increase or a decrease in this ratio could be attributed to changes in the composition of the whole protein pattern. The decrease in the ratio of intensities of amide II and amide I between control and lead treated tissue was observed. These decreased ratio reflect an alteration in the level of total proteins due to heavy metal treatment (Karthikeyan, 2012*).*Reduction in protein content showed the following order: liver > kidney > muscle > gill. These results suggested that the relative concentration of protein to water in the tissue membrane is considerably lower in heavy metal treated tissues with that of control. The decrease in the protein content in liver was high when compared to other organs, it may be due to diversification of energy through gluconeogenis to meet the impending energy demands when the animals are under acute toxicity induced stress. The protein was utilized for cell repair and tissue organization. The activity of proteolytic enzymes was increased resulting in decreased protein levels. This is in line with the results of nickel and mercury toxicity induced biochemical changes in the muscle tissues of *Lates Calcarifer* (Senthamilselvan et al., 2012).Finally, it may be concluded that the protein content varies significantly in the lead exposed fishes and FTIR results will have a promising application in heavy metal toxicity studies for *Labeo rohita.* In view of future work, efforts designed to find the structural level changes in the proteins and marker enzymes due to lead toxicity.

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