Vol. 3, No. 4, pp. 357-370, (December2022)
DOI: 10.21608/aujes.2022.158168.1091
Aswan University Journal of Environmental Studies (AUJES)
Online ISSN: 2735-4237, Print ISSN: 2735-4229
Journal homepage: https://aujes.journals.ekb.eg/ E-mail: AUJES@aswu.edu.eg
Original research

Acute toxicity of some heavy metals on Melanoides tuberculata (Gastropoda: Thiaridae) as a bioindicator: a biochemical and molecular study

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Received: 23/8/2022 Accepted: 30/9/2022

Abstract:

This study evaluated the acute toxicity of four heavy metals (Pb, Zn, Mn, and Fe) to the freshwater gastropod *Melanoides*. For all metals, the LC50 increased as the mean exposure concentrations and periods decreased, respectively. The toxicity of these metals was arranged as follow Pb, then Zn, Fe, and Mn were, with Fe having the largest accumulation in the soft tissues. The survival rate of *M. tuberculata* (Stress on Stress) was decreased during the course of the exposure. The biochemical responses to heavy metal uptake in this snail showed that the Pb-treated group had the highest level of lipid peroxidation, but the actions of glutathione peroxidase (GPx), glutathione-S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD) revealed that the lowest activity of the enzyme was in the Pb-treated group, followed by the Zn-treated group, then Mn-treated group, while the Fe-treated group had the highest GPx, GST, CAT, and, SOD activity. The changes in the randomly amplified polymorphic DNA (RAPD) profiles could be induced by structural deformations produced by various kinds of DNA damage. The heavy metals accumulated in tissue of the gastropod *M tuberculata* caused oxidative stress and genetic changes.

Key words: Biochemical response; Genetic changes; RAPD; Oxidative stress; Water pollution.

1- Introduction

Water pollution is one of the most serious issues caused by urbanization, industrialization, and modern agricultural methods. It alters the physical, chemical, and biochemical properties of the environment and water bodies (Indra and Sivaji, 2006). Rivers that pass through farmland where pesticides and fungicides may have been used become contaminated, causing ecological harm. Furthermore, when metal trash is discharged into bodies of water from industrial sites where it may be deposited, a number of issues arise (Bashir et al., 2020). River discharge effluents may directly or indirectly harm aquatic life such as fish and snails (Ekpo et al., 2008; Abd El-Wakeil et al., 2014; Authman et al., 2013). Water pollution is a global issue because of mining, irrigation water drainage, and factory contributions (Soliman et al., 2014).

Heavy metals are pollutants that are harmful to aquatic creatures and, as a result, to individuals who rely on aquatic products for food because they cannot be eliminated biologically and have the potential to congregate in the habitats (Abo Elmagd et al., 2020).

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Heavy metals can accumulate in the tissues of aquatic animals to the point where tissue concentrations endanger both animal and human health (Ashraf, 2005; Abdel-Wahab et al., 2018). Thus, it is critical to conduct research using indigenous creatures to gather information on toxicants, ascertain the organism's susceptibility, and establish a legal cap for water that can safeguard water bodies.

Toxicology testing has been widely used as a technique for determining appropriate organisms for bioindicators and establishing chemical water quality criteria. Acute toxicity studies can provide quick, useful information, determine whether additional toxicity research is required, and help explain toxic impacts (Calow, 1993; Rand et al., 1995; Watts and Pascoe, 2000). Metals, unlike some organic insecticides, never biodegrade and cannot be broken down into less hazardous components (Jaishankar et al., 2014).

Understanding the concentration dependence of toxicity is required for metal contamination management. Dose-response correlations assist in evaluating the dangers and risks posed by environmental pollutants (Shuhaimi-Othman et al., 2012). Toxicology examination is an important method of determining the impact and long-term path of environmental toxins in water bodies. It is also frequently used to identify appropriate organisms for use as bioindicators and to establish chemical water quality standards. Toxicology can be tracked in a variety of ways, but mortality is the most commonly used measure (endpoint) (Luoma and Rainbow, 2008).

Biomonitoring techniques analyze not only the presence of these pollutants but, more crucially, their impact on the organisms by analyzing biomarkers, or measures that represent their effects at the molecular, cellular, organ, and organism level. Although these biomarkers are employed in monitoring, they do not replace population studies or chemical monitoring (Walsh et al., 1995). To effectively determine the amount of exposure of aquatic organisms to chemical pollutants, biomarkers for biomonitoring of natural aquatic systems employing bioindicator species should be employed (Sureda et al., 2011).

In environmental genotoxicity research, it is critical to assess the molecular effects of genotoxins on DNA damage and mutation. Randomly amplified polymorphic DNA (RAPD), a PCR-based technique, has demonstrated that several heavy metals not only cause morbidity in exposed species but also have the potential to cause genotoxicity (Cimino, 2006; Theodorakis et al., 2006; Azimi et al., 2013).

Generally, fresh water mollusk considered as a bio monitor for pollution. Few studies delt with the metal toxicity in the gastropod *Melanoisdes tuberculata* (family: **Thiaridae)** (Gardenfors et al., 1988; Lau et al., 1998; Bali et al., 1984; Mostafa et al., 2005).

The goals of this work are to determine the acute toxic effect of four heavy metals (Pb, Zn, Mn, and Fe) to the freshwater mollusk *M. tuberculata* and investigate their bioconcentration in the tissues after 96 hrs of exposure, as well as integrate molecular and biochemical signs to determine the mollusks' tolerance and capacity to absorb heavy metals. This is to foresee what might follow if water became polluted as a result of cruise ship activities and industrial drainage with such heavy metals for aquatic life and hence human life.

2- Materials and Methods

Toxicological test

Samples were collected from site next to the Cataract hotel in Aswan city.The snails were adapted for a week with dechlorinated tap water aerated via an air pump, before conducting

toxicological tests Shuhaimi-Othman et al. (2012). The stock standard solutions (100 mg\ L) of Pb, Zn, Mn, and Fe were prepared with analytical grade metallic salts of Pb (NO3)2, ZnSO4.7H2O, MnSO4.H2O, and FeCl3, respectively (Merck, Darmstadt, Germany). Adult snails from stocking containers (shell length roughly 1.5 2.0 cm, mean wet weight 22.5 ± 1.6 mg) were used. Using dechlorinated tap water to dilute a stock solution, metal solutions were prepared. A dechlorinated tap water was kept as a control, with a two-day solution replacement. The snails were not fed properly throughout the toxicity test. During the test, there were four separate treated groups, with five different concentrations of each toxic solution, ten samples, and a control group.

Every 24 hours, mortality was recorded. Dead animals were removed right away. The bioconcentration of metals in soft tissues based on the amounts utilized was calculated using the live snails at the end of day four. An atomic absorption spectrophotometer (AAS) (model iTE 3000 SERIES) was then used to measure the metals according to the method of (Federici et al., 2007).

Biochemical analysis

After being thoroughly cleaned with deionized water, tissue samples were crushed in an glacier crusher and pestle for 25 minutes with 0.01 M chilled phosphate buffer at pH 7, 4°C and 14,000 rpm (Bakshi et al., 2018; Banaee et al., 2019). The method for determining lipid peroxidation (LPO) was modified by Ohkawa et al. (1979). Using the Paglia and Valentine (1967) method, the activity of the enzyme glutathione peroxidase (GPx) was assessed in homogenates of tissues. The spectrophotometric technique established by Habig et al. (1974) was used to measure the glutathione-s-transferase (GST) activity in homogenates of tissues. The inhibition effect of superoxide dismutase (SOD) on epinephrine oxidation at 480 nm was utilized to determine the enzyme's performance (Misra and Fridovich, 1972). Catalase (CAT) activity was assessed using Beers and Sizer's method (1952). The assay was performed in accordance with the reagent kit's manufacturer guidelines (Biodiagnostic, Egypt).

DNA analyses

Genomic DNA (gDNA) was derived from frozen, alcohol-conserved tissues employing a conventional technique (Younis et al., 2022). Then, utilizing two ten-mer oligonucleotide primers (UBC 476 and UBC 477), RAPD-PCR was used to detect genetic variation (Yousif et al., 2009; Abdel-Halim et al., 2019). Minor modifications were made to Williams et al., (1990) instructions for RAPD PCR reaction. On ethidium bromide-stained agarose gels (1.5% w/v), the amplification products were examined, seen under UV light, and photographed (Jing et al. 2005).

Statistical analysis

Analysis of probit line regression on MedCalc application software (version 19.5) was used to test LC50.

The software Statistical Package for the Social Sciences (SPSS: version 23) was used to calculate Multiple Range Comparison (LSD) to detect the distinct variance between the means (Sparks, 2000). Where, Probability value ≤ 0.05 was defined as significant, however, the value >0.05 was defined as non-significant, whereas those less than 0.01 were defined as highly significant.

2- Results and Discussion

The aquatic snail *M. tuberculata* was employed as a bioindicator in this study, using integrating molecular and biochemical approaches, to assess the influence of heavy metals on the

aquatic environment as potential contaminants. The snails were exposed to varying levels of Zn, Pb, Fe, and Mn in a laboratory for four days.

Throughout the experiment, all of the control animals survived, and were kept in dechlorinated tap water. Mortality increased with an increase in concentration and time exposure. The most hazardous metal for *M. tuberculata* was Pb, then Zn, Fe, and Mn. As shown in Fig. 1, the present study showed that the mean lethality concentrations (LC50) for four days of Zn, Fe, Pb, and Mn to *M. tuberculata* were 3.3 mg/l, 17.1 mg/l, 5.6 mg/l, and 36.2 mg/l. Other research with various snails demonstrated toxicity in varying ways. According to Luoma and Rainbow (2008), different organisms will respond to metals to different degrees of toxicity. Khangarot and Ray (1987), (1988) demonstrated that the toxicity ranking with *Lymnaea luteola* was Cd higher than Ni higher than Zn; with *Viviparus bengalensis*, Gupta et al. (1981), and Gadkari and Marathe (1983) determined the toxicity ranking with *V. bengalensis* , it was Zn higher than Cd higher than Pb higher than Ni.

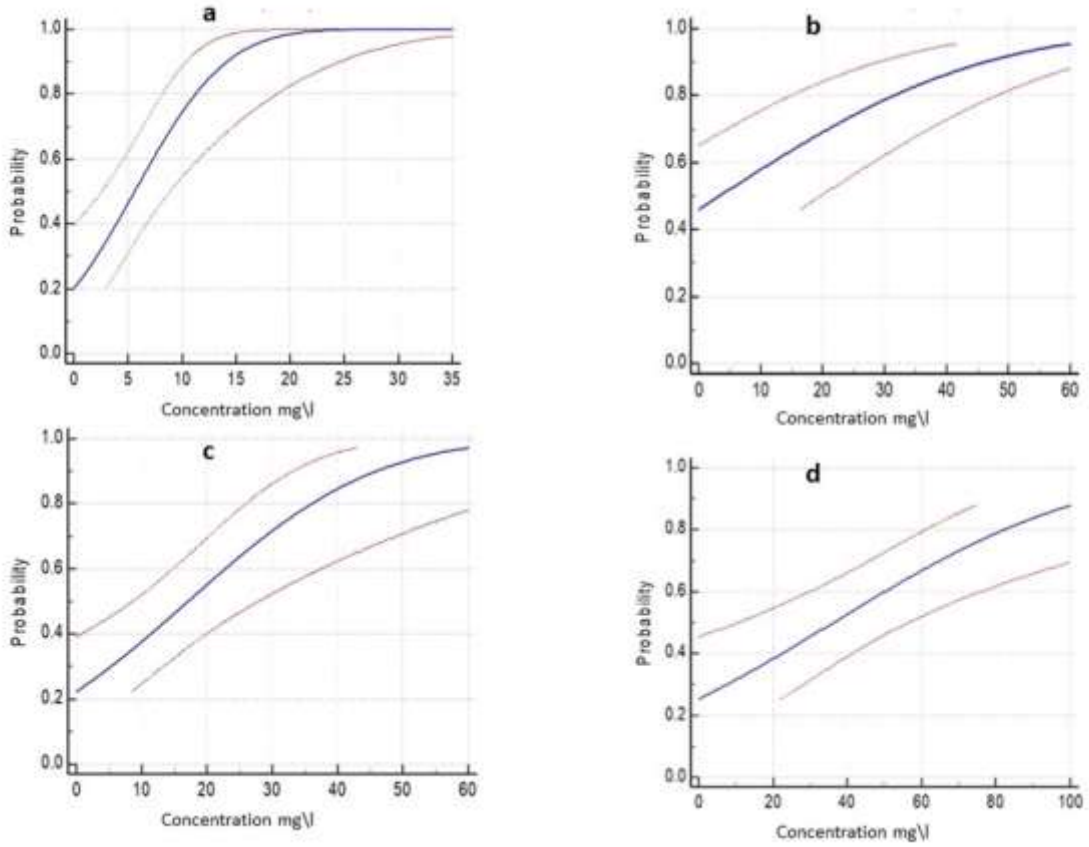

Fig.1 Probit line regression graph of mortality after 96 hrs in toxicity test for *Melanoides tuberculata*. (a) Pb toxicity test, (b) Zn toxicity test, (c) Fe toxicity test and (d) Mn toxicity test.

Metal acute toxicity studies on freshwater mollusks revealed that the 96-hour LC50 values for Pb were 6.82 mg/l (Shuhaimi-Othman et al., 2012), 14.0 mg/l (Cairns et al., 1976), and 2.54 mg/l (Gadkari and Marathe, 1983). And 96 h-LC50 of Zn was 3.90 mg/l (Shuhaimi-Othman, et al., 2012), 10.49 mg/l (Khangarot, et al., 1982), and 0.64 mg/l (Gupta, et al.,1981). Furthermore, the 96 h-LC50 of Fe was found to be 8.49 mg/l (Shuhaimi-Othman et al., 2012),

12.09 mg/l (Birge et al., 1985), and 76.0 mg/l (Nishiuchi and Yoshida, 1972). In addition, the 96 h-LC50 of Mn was 45.59 mg/l (Shuhaimi-Othman, et al., 2012) and 100.0 mg/l (Tomasik, et al., 1995). Interestingly, *M. tuberculata was* found to have less metal sensitivity as compared to other taxa.

Because the parameters of the test waters (namely their water hardness, pH, and temperature) differed from those reported in the literature, it was difficult to directly compare the toxicity levels found in this study with those found in the literature. Despite employing adult snails and similar water hardness (soft water), as well as diverse organism types, maturities, and dimensions, as well as different testing procedures (water hardness and quality), the toxicity reported in previous studies varies from the findings of this work (McCahon and Pascoe 1988; Ebrahimpour et al. 2010).

By detecting the survival (Stress on Stress (SoS)) rate during the duration of exposure (96 hrs) in the five different concentrations and control, it was decreased, as shown in Table (1), our study indicated that contaminant exposure is likely to reduce survival rate with time exposure, which agrees with previous findings (Smaal et al., 1991; Veldhuizen-Tsoerkan et al., 199l; Viarengo, et al. 1995). These findings demonstrate that SoS response can significantly indicate the effects of brief exposure to low levels of contaminants while also validating the effects of various types of pollutants on organisms. In a circumstance comparable to that of a field setting, the SoS reaction can also demonstrate how exposed an organism is to a range of pollutants at extremely low concentrations. The findings are consistent with those of Viarengo et al. (1995). When other metabolic metrics commonly utilized in monitoring programs, such as Adenylate Energy Charge, glycogen, or succinate levels, fail to detect a stress syndrome (Veldhuizen-Tsoerkan et al., 1991). Furthermore, the methodology employed to test the stress-on-stress reaction is simple, rapid, inexpensive, and requires no special equipment. As a result, SoS response may be utilized as an indicator of overall stress at the organismal level and as a monitoring tool to analyze contaminated coastal areas.

Table (1). Survival rate (Stress on Stress (SoS)).

Toxic solution	Stress on Stress (SoS) %
Zn	100-10 %
Pb	100-0 %
Fe	100-10 %
Mn	100-10 %

Bioconcentration of Pb, Fe, Zn, and Mn in living *M. tuberculata* is revealed in Figure 2.

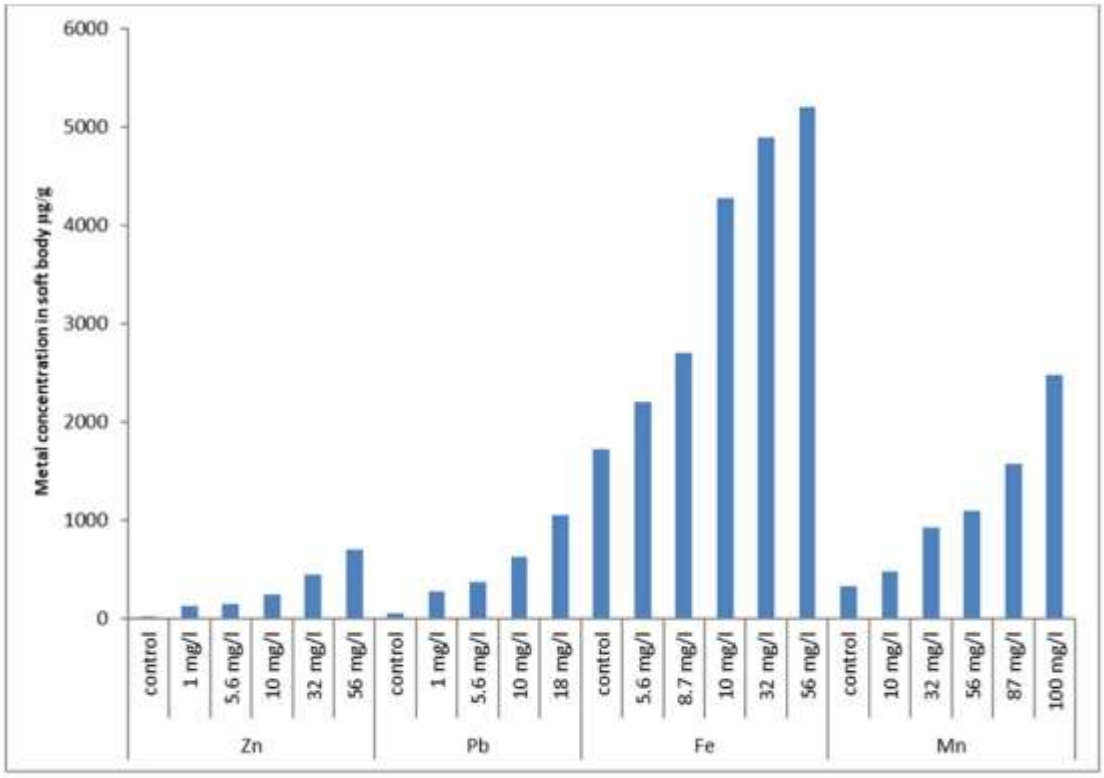

Fig. 2 Bioconcentration of Zn, Pb, Fe and Mn (mean) in *Melanoides tuberculata* soft tissues (µg/ g) after 4-day exposure to different concentrations of Zn, Pb, Fe and Mn.

Live snail bioconcentration information was acquired of five Zn concentrations (1 mg/l, 5.6 mg/l, 10 mg/l, 32 mg/l, and 56 mg/l), Fe concentrations (5.6 mg/l, 8.7 mg/l, 10 mg/l, 32 mg/l, and 56 mg/l), Mn concentrations (10 mg/l, 32 mg/l, 56 mg/l, 86 mg/l, and 100 mg/l), and four Pb (1 mg/l, 5.6 mg/l, 10 mg/l, and 18 mg/l) concentration exposures. Zn bioconcentration ranged from 125 to 700 µg/g, Pb bioconcentration from 50 to 1050 µg/g, Fe bioconcentration from 2206 to 5200 µg/g, and Mn concentration from 475 to 2475 µg/g. In the *M. tuberculata*, as concentration exposure rises, so does bioconcentration. Moolman et al. (2007) stated comparable results in the accumulation of Zn and Cd in two freshwater gastropods (*M. tuberculata* and *Helisoma duryi*). These findings support Luoma and Rainbow's (2008) claim that aquatic species' absorption of toxic elements from solution principally relies on concentration. This variation is most likely due to the study's modest (four-day) metal exposure. Aquatic organisms' feeding habits (Mance, 1990), growth rate and age (Lau et al., 1998; Pentreath, 1976), and metal bioavailability—which is heavily influenced by the hardness, pH, and acid-volatile sulfide content of the water—are all factors that may influence heavy metal bioaccumulation in aquatic organisms (Besser, 1996). Because the rate of gain in bodyweight of the organism's tissue and shell is greater than the concentration of collected metals, creatures that grow at a faster rate have relatively low levels of metal in their systems (Lau et al. 1998).

Biomarkers do have the potential to determine the frequency of polluted contact (Berra et al., 2002). As shown in Fig. 3, comparing LPO activity revealed that the Pb-treated group had the highest level of the enzyme's activity with values ranging between (20.6 nmol/mg tissue and 16.1 nmol/mg tissue) with highly significant differences ($p \leq 0.01$). Cellular LPO is an important

indicator of oxidative stress and is linked to accelerated cell aging (Cipak Gasparovic et al., 2017). This finding is consistent with earlier findings on macro-invertebrates, which showed that the peroxidase pool increased LPO and antioxidant enzyme activity decreased (Doyotte et al. 1997; Choi et al. 2001). GPx activity displayed that the least action of the enzyme was in the Pb-treated group. It ranged between (35 µM/min./mg tissue and 41.6µM/min./mg tissue). The decreased GPx action in the Pb-treated group is consistent with the findings of Farid et al. (2009). The increase in ROS production may be the cause of the GPx activity's decline. By matching GST activity, it was discovered that the enzyme activity was lower in the Pb-treated group. It was in the range of (7.3µM/min./mg tissue and 9.9µM/min./mg tissue). While matching SOD activity, it showed that the least action of the enzyme was in the Pb-treated group. It was in the range of (13.2U/min./mg tissue and 19.2U/min./mg tissue) with highly significant differences ($p \leq 0.01$). Nevertheless, contrasting CAT assay demonstrated that the Pb-treated group had diminished enzyme activity. It was in the range of (3.9U/min./mg tissue and 4.6U/min./mg tissue). The decrease of GST, SOD, and CAT activity in the study could be related to changes in antioxidant enzyme activity and other oxidative stress biomarkers may contribute to metabolic malfunction, which agrees with Farombi et al. (2007) and Joseph and Kafilat (2012). According to LSD multiple correlations between GST, LPO, GPx, SOD, CAT, and (control and treated groups), there were multiple differences as shown in table (2). The results of LSD of enzyme activity showed that there were significant differences between the treatment groups and the control group. This reveals that LPO, GPx, GST, SOD, and CAT activities are impacted by the duration and stress level.

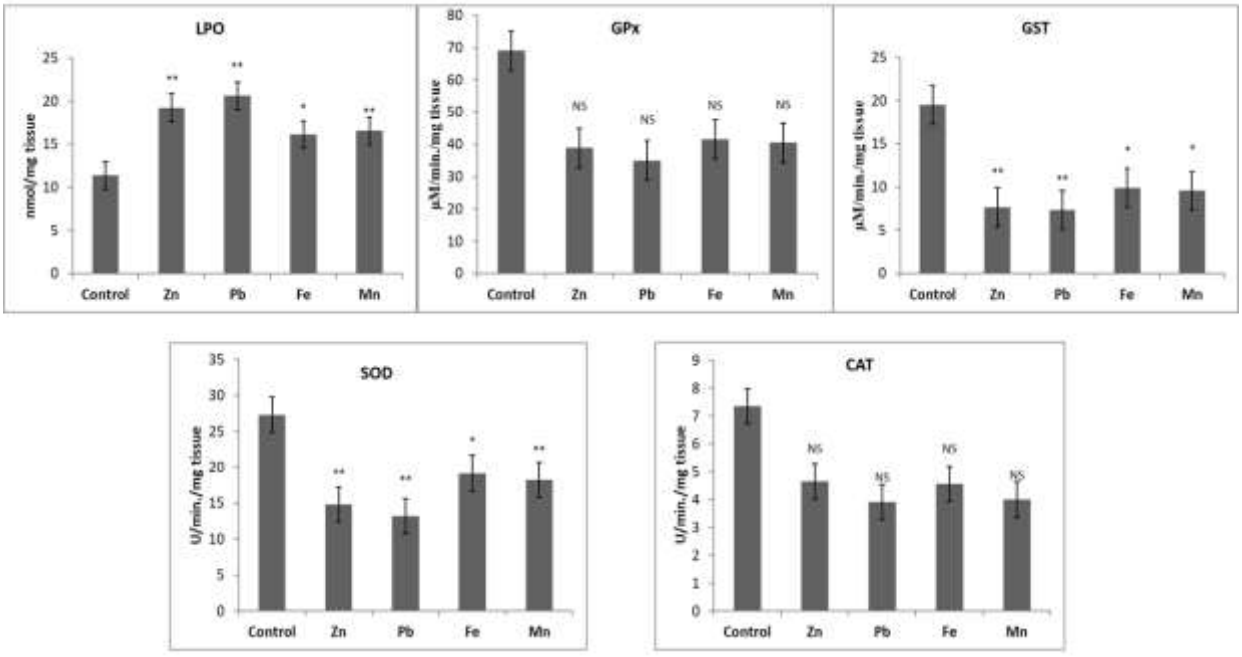

Fig. 3 Enzymatic activities activity. Lipid peroxidation (LPO), glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT).

The RAPD-PCR technique was employed in this investigation to assess the possible acute (4-day) genotoxicity of Pb, Zn, Mn, and Fe in *M. tuberculata*, which resulted in different and distinctive finger patterns derived from the DNA. As appeared in Fig. 4, only an amplified band with a high intensity of 400 bp could've been produced by the UBC 476 primer using DNA

derived from the control and Fe = 10 mg/l groups. The Zn = 10mg/l group has two low-intensity bands ranging from 600bp to 800bp. As shown in Fig. 5, there were five amplified fragments with a low intensity ranging between 200 bp and 600 bp that could be generated by UBC 477primer using DNA extracted from the control group. The appearance of the band ranged between 600bp and 800bp with low intensity in Mn = 8.7mg/l and another band with low intensity ranged between 400bp and 600bp. The appearance of bands with high intensity ranged around 600bp in the Fe=3.2mg/l group, Zn=3.2mg/l group, and Zn=10mg/l group, and with low intensity in the Fe=10mg/l group, Pb=0.75mg/l, and Pb=10mg/l groups, with the appearance of new bands with low intensity ranged around 400bp and 800bp in the Fe=10mg/l group. Primers utilized in *M. tuberculata* DNA subjected to Pb, Zn, Mn, and Fe resulted in RAPD designs different from the samples of control. This demonstrated that heavy metal exposure to DNA samples resulted in polymorphism areas in the *M. tuberculata* genome. This supports Abumourad et al., 2012. The key modifications in the current study's RAPD profiles were the addition or removal of distinct bands, as well as alterations in their strength. These changes could be attributed to possible structural changes in DNA caused by possible various types of DNA breakage (Arillo et al. 1981).

Table (2). LSD multiple comparisons for *Melanoides tuberculata* between superoxide dismutase (SOD), Lipid peroxidation (LPO), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and catalase (CAT).

Sites & treated groups	SOD	LPO	GST	GPX	CAT
Control & ZN	**	**	**	NS	NS
Control & Pb	**	**	**	NS	NS
Control & Fe	*	*	*	NS	NS
Control & Mn	**	**	*	NS	NS
Pb & Zn	NS	NS	NS	NS	NS
Fe & Mn	NS	NS	NS	NS	NS
Fe & Zn	NS	**	NS	NS	NS
Fe & Pb	**	**	NS	NS	NS
Mn & Zn	NS	**	NS	NS	NS
Mn & Pb	NS	**	NS	NS	NS

(*) the mean difference is significant at the 0.05 level, (**) the mean difference is significant at the 0.01 level and (NS) the mean difference is not significant.

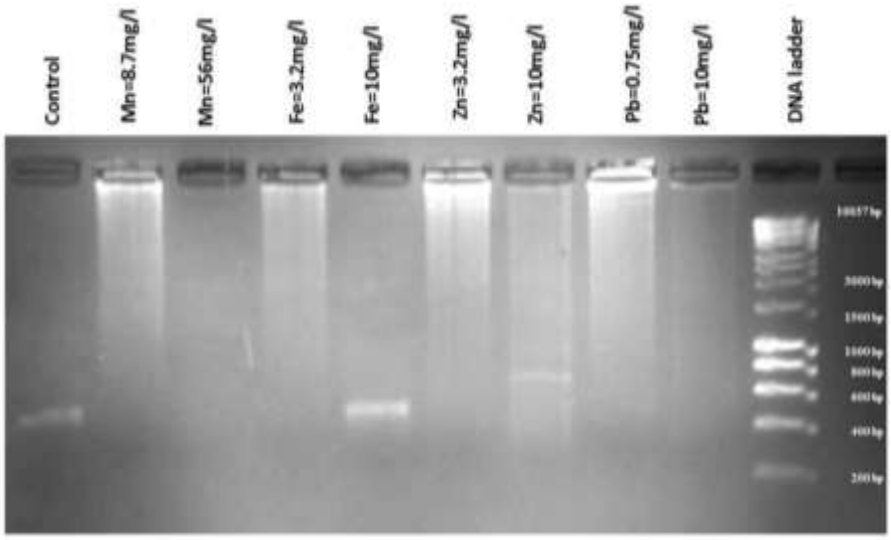

Fig. 4 RAPD profiles of different groups of genomic DNA represents PCR products with primer (UBC 476).

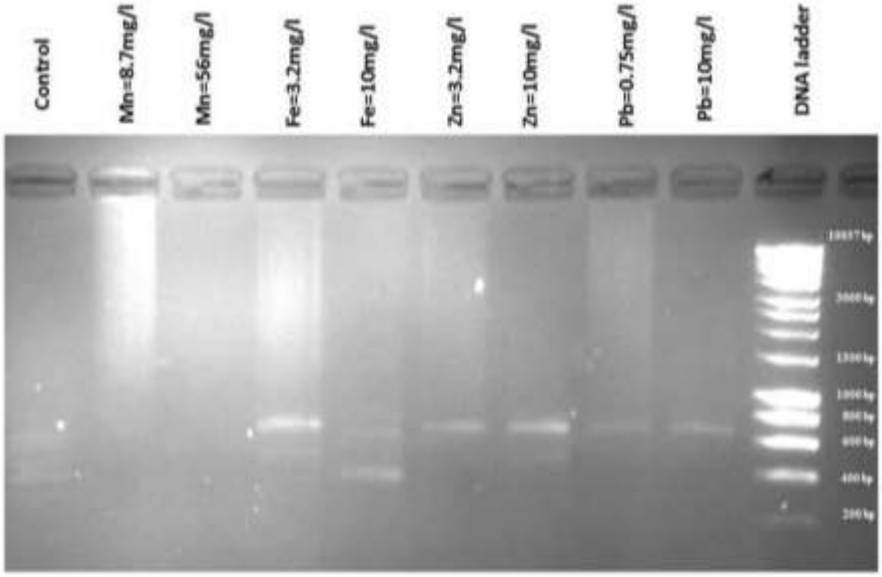

Fig. 5 RAPD profiles of different groups of genomic DNA represents PCR products with primer (UBC 477).

Conclusion

These findings suggest that *M. tuberculata* may possibly be used as a bioindicator for metal contamination and toxicological assessment. Laboratory studies using integrated biochemical markers (GPx, SOD, LPO, GST, and CAT) and RAPD-PCR revealed the existence of toxicity due to the examined heavy metals (Pb, Mn, Zn, and Fe), which accumulate in living tissues and induce oxidative stress and genetic alterations. Pb was the most hazardous to be the most hazardous metal, followed by Zn, Fe, and Mn. For the level of biococentration in *M. tuberculata*'s soft tissues Fe and Mn had the highest level howeverle Zn had the lowest one. SoS diminishes as concentration and exposure time increase. To sum up, this study shade light in the potential human

and industrial practices that may contribute in the accumulation of heavy metals in rivers and other bodies of freshwater threaten aquatic life and, hence, human life

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