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White Rot Fungus (*Pleurotus pulmonarius*) Cultivated on Lead Contaminated Rice Straw Induced Haematotoxicity and Lead Accumulation in Liver and Kidney of Wistar Rats

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Abstract

White rot fungi (*Pleurotus* species) are edible mushrooms known for their ability to bioaccumulate contaminants including metals from the environment. There is scarcity of information on the toxic effects of the bioaccumulated metals on organisms at higher trophic levels. In this study, we investigated alterations in haematological indices and erythrocyte morphology and lead bioaccumulation potentials in liver and kidney of Wistar rats fed aqueous extract of *Pleurotus pulmonarius* cultivated on lead (0, 10, 100, 200, 500 and 1000 mg/L) contaminated rice straw substrate. After 8 weeks of cultivation, mushroom did not germinate in the 1000 mg/L Pb contaminated rice straw. 1 g of mushroom harvested from each of the remaining concentrations showed significant ($p<0.001$) increase in Pb concentration. Rats exposed to 0.5 mL of the contaminated mushroom extracts for 30 days, showed decreased leucocyte, erythrocyte, haematocrit, platelet and haemoglobin concentrations. Abnormal erythrocyte morphologies like acanthocytes, schizocytes and tear drop were significantly higher in the Pb treated mushroom fed rats than the control. The kidney and liver of treated rats showed significant ($p<0.05$) increase in Pb concentration with higher Pb bioaccumulation factor in the kidney than the liver. Also insignificant decrease in body and organ weight was observed in treated rats than the control. Pb accumulation in *P. pulmonarius* increased liver and kidney Pb bioaccumulation and induce alterations in haematological indices and erythrocyte morphology in rats. This poses public health threat to humans and other tertiary consumers foraging white rot fungi from metal contaminated sites.

Keywords: Bioaccumulation potentials; Erythrocyte morphology; Hematological indices; Lead contaminated *Pleurotus pulmonarius*; Rats

Introduction

Human exposure to toxic chemicals may come from air, soil, water and food. Heavy metals are ubiquitous carcinogens with multiple sources of environmental contamination [1], and are transferred through aquatic and terrestrial food webs to humans and other animals posing health risk [2,3]. There is increasing utilization of mushrooms as important delicacies in most countries of the world due to their significant role in nutrition. They also serve as therapeutic food useful in preventing diseases such as hypertension, hypercholesterolemia and cancer [4,5], hence edible mushrooms are now widely cultivated throughout the world. There are increasing reports that these mushrooms are effective in bioremediation or biological treatment of polluted sites [6]. This is due to their ability to efficiently degrade, utilize and transform organic and inorganic chemicals in the substrates [7]. This process usually leads to increase in the accumulation of chemicals including toxic metals in their fruit bodies [8,9]. There is possibility that the accumulated chemicals can be transferred from one environmental matrix to another, which may lead to health problems and biodiversity loss [10-12]. It was recently reported that the consumption of mushrooms collected from the wild induced nausea, vomiting and increased Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) among human population, due to bioaccumulated chemicals in the consumed mushroom [13]. The altered liver enzymes and vomiting suggest abnormal functioning of the liver in the exposed human population.

Several studies focused on the evaluation of heavy metal accumulation potential of various species of mushrooms [14-16]. From these studies, *Pleurotus* species along with other species were found to be efficient in accumulating toxic metals such as Pb, Hg and Cd from polluted substrates into their fruit bodies [9,16]. However, information is scarce on the potential impact of the accumulated metal(s) on bio-accumulation potential in mammalian tissues and possible systemic toxicity. Pb is one of the toxic metals in the priority list of hazardous substances recommended for toxicological assessment [17]. Environmental and occupational exposure to this metal has been linked to increasing hematological disorder, neurological damage and cancer risk [18,19]. In 2010, 400 individuals died due to lead poisoning during unsafe mining and ore processing in Zamfara State, Nigeria [20]. This report suggests increasing exposure to Pb from various sources and its potential health impact in biota.

Pleurotus pulmonarius (White rot fungus), asides its nutritional and therapeutic values, is well known for its ability to accumulate metals and radioactive substances from contaminated substrates [21,22]. This feature also necessitated its use in the biological treatment of landfill leachate and effluent contaminated sites [23,24]. Consuming such *P. pulmonarius* (primary producer in a number of terrestrial ecosystems) by many higher consumers and humans may increase exposure to toxic metals. This study therefore aims at investigating the

haematotoxicity and Pb bioaccumulation potentials of the liver and kidney of Wistar rats fed *P. pulmonarius* cultivated on lead contaminated rice straw for 30 days.

Materials and Methods

Rice straw preparation and *P. pulmonarius* cultivation

Rice straw (substrate) used for the cultivation of the White rot mushroom was obtained from the International Institute of Tropical Agriculture (IITA), Ibadan. They were air dried for a period of 2 weeks and cut into 3-5 mm size using guillotine. Chopped rice straw was completely immersed in different concentrations (10, 100, 200, 500 and 1000 mg/L) of Lead chloride (Tianjin Yong Da Chemicals, China) solution and the control (distilled water) for 24 hr. The selected concentrations are the range of previously reported Pb levels in mushrooms [6,7]. 80 g of the rice straw from each of the selected concentrations were added into five (5) pre-cleaned bottles per concentration and covered with aluminum foil. The preparations were autoclaved at 121°C for 30 min for sterilization and allowed to cool to 25°C.

The different concentrations of the lead contaminated rice straw were inoculated with 10 g of inbred strain culture of *P. pulmonarius*, obtained from the Plant Physiology Laboratory, Department of Botany, University of Ibadan, Nigeria, and were kept in the incubator (28 ± 2°C) until they were completely ramified within 4 weeks (Figure 1a). The aluminum foil was carefully removed and the ramified mushroom wetted with distilled water to moisten it and transferred to the mushroom cultivation unit (Figure 1b). The fully developed fruit bodied mushrooms (cap and stipe) were harvested and dried at 50°C for 48 hr (Figure 1c).

Heavy metal analysis of the cultivated mushroom

Harvested mushroom fruiting bodies from each replicates of the different concentrations and control were dried and ground using Cutting Boll Mill. 1 g from each replicate was added into a graphite crucible and digested with 10 mL of 65% HNO_3 in a fume chamber digestion system for 15 min. The digested material was diluted in distilled water and analyzed using atomic absorption spectrometer (AAS) (Buck Scientific model 201 VGP, East Norwalk, USA) for Pb concentrations.

Animals and experimental design

Wistar rat (*Rattus novergicus*), 7-8 weeks old (mean ± SD body weight of 131.5 ± 3.04), obtained from the animal house unit of the Department of Physiology, College of Medicine, University of Ibadan, Nigeria, were acclimated to laboratory conditions of 26 ± 1°C and 12/12 h dark/light modes for 14 days prior to the experimental set up. The rats were fed with standard animal feed (Ladokun feed Nigeria®), with access to drinking water ad libitum. Five (5) rats per treatment group were randomly selected into the various concentrations of the metal treated mushroom and control.

The dried powdered grounded mushroom was measured and 10 g soaked in 100 ml of distilled water for 48 hr with regular stirring using clean glass rod to obtain aqueous extract [25]. The extracted solution was filtered using Whatmann® No. 42 filter paper to remove solid particles. 0.5 ml of the mushroom extract was orally administered to each rat for 30 consecutive days. Similar treatment was concurrently given to the control group, and the rats maintained in standard conditions according to guide for care and use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23) [26].

Hematological analysis and organ weight measurement

At the end of 30 day exposure, rats were fasted overnight, weighed and blood was collected from the orbital plexus using heparinized 70 ml micro-hematocrit capillary tubes into EDTA tubes. The recommended haemogram: Red blood cell (RBC) count, haemoglobin (Hb) content, platelet (PLT) count, haematocrit and total white blood cell (WBC) count [27] were measured to assess alterations in haematological indices using automated analyzer, (Abbott Hematology Analyzer Cell-Dyn 1700, 178 Abbott Laboratories, Abbott Park, Illinois, USA).

Aliquot of the blood samples were used to make thin blood films on pre-cleaned slides (three slides per rat), air-dried, fixed in absolute methanol for 30 min and stained in 5% Giemsa solution for 20 min to determine abnormal erythrocyte morphologies. 1000 erythrocytes were scored per rat for the presence of poikilocytosis (variations in red blood cell shapes) in accordance with Cheesebrough [28] procedures.

Body and organ weight measurement

Body weight of the treated and control rats was measured at the beginning of the experiment and once weekly for the duration of exposure. At the end of exposure, terminal body weight was also measured before blood collection, and rats were sacrificed by cervical dislocation. The liver and kidney were surgically removed, rinsed with ice-cold physiological saline, blotted dry and weighed (absolute organ weight) using Acculab® USA, Model-vic-303 electronic analytical weighing balance. The relative organ weight was determined using the formula: organ weight/ body weight × 100 g.

Lead accumulation measurement in liver and kidney tissues

The liver and kidney were placed in Pyrex dishes and oven dried to a constant weight at 60°C for a period of 48 hr. One gram of the dried liver and kidney tissues was accurately weighed into clean 100 mL Erlenmeyer flask and 5 mL Perchloric acid (70%) and 10 mL nitric acid (55%) (Merck Darmstadt, Germany) were added. Digestion was performed on a hotplate at 150-200°C for about 4 hr until a clear solution was obtained. The resultant residue was cooled to room temperature and made up to 50 mL using double distilled water [29]. Pb concentration in the tissues was determined using atomic absorption spectrophotometer (Buck Scientific model 201 VGP, USA). Sample analysis was performed in triplicate and Pb concentration in the liver and kidney expressed as mg/g dry weight.

Bioaccumulation factor (BAF, often used to compare the body burden of chemical in an organism with the degree of contamination in the substrate) of lead in the liver and kidney was calculated in accordance with Holwerda [30] with slight modification.

$$\text{Bioaccumulation factor (BAF)} = [M]_{treated} - [M]_{control}/[M]_{mushroom}$$

Where $[M]_{treated}$, $[M]_{control}$, and $[M]_{mushroom}$ are the Pb concentrations in the experimental group (liver and kidney of the rats exposed to various concentrations of mushroom), Pb concentrations in the control group (liver and kidney from the control rat), and Pb concentration in the mushroom cultivated in rice straw contaminated with various concentration of Pb, respectively.

Statistical analysis

All statistical analyses were conducted with Graphpad prism 5.0® computer programs. Data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine the differences among various groups. While Dunnett multiple posthoc-test was used to compare the level of significance ($p<0.05$) of each treated group with the control.

Results

Lead accumulation in mushroom, liver and kidney of rats and BAF in the liver and kidney

At 1000 mg/L of Pb contaminated rice straw, there was no *P. pulmonarius* fruit body germination even at 8 weeks after full ramification of the rice straw by the mycelium (Figure 1a).

Figure 1a: Completely ramified *P. pulmonarius* after 4 weeks of cultivation. 1b. Ramified mushroom germinated in the mushroom cultivation Unit. 1c. Harvested and dried fully developed fruit bodied mushrooms (cap and stipe).

Conc (mg/L)	*P. pulmonarius* cultivated in Pb contaminated rice straw (mg/g)	Liver (mg/g)	Kidney (mg/g)	BAF (liver)	BAF (kidney)
Control	BDL	BDL	BDL	-	-
10	0.017 ± 0.003	0.020 ± 0.002	0.006 ± 0.002	1.198 ± 0.020	0.359 ± 0.005
100	0.167 ± 0.033[a]	0.004 ± 0.002	0.276 ± 0.072[a]	0.024 ± 0.010	1.653 ± 0.200
200	0.270 ± 0.023[a]	0.006 ± 0.002	1.250 ± 0.210[b]	0.022 ± 0.003	4.630 ± 0.910
500	1.320 ± 0.023[a]	0.010 ± 0.001	4.582 ± 0.171[c]	0.008 ± 0.006	3.471 ± 0.072

Table 1: Mean Pb accumulation in *Pleurotus pulmonarius* cultivated in Pb contaminated rice straw and in liver and kidney of rat fed with the *P. pulmonarius* for 30 days, and bioaccumulated factors in liver and kidney of the rats. Values are in mean ± SD (means of five replicates). Superscripts differ significantly ([a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$) from the control using Dunnett's multiple post hoc test. BAF-Bioaccumulation factor. sBDL-Below Detectable Limits (assumed to be 0.00 ± 0.00).

There was concentration dependent significant ($p<0.001$) increase in Pb accumulation in the fruit bodies of *P. pulmonarius* cultivated in the Pb contaminated rice straw compared to the control (Table 1).

Similarly, Pb accumulation in the kidney of *P. pulmonarius* treated rats showed concentration dependent significant ($p<0.001$) increase compared to the control group. However lead accumulation in the liver of rats fed with the *P. pulmonarius* extract increased but was not significantly ($p=0.5985$) different from the control (Table 1). Bioaccumulated factor for Pb in the kidney of rats showed increased Pb transfer factor in accordance with Pb concentration in the mushroom, while the BAF of Pb in the liver did not increase in accordance with Pb concentration in the mushroom (Table 1).

Effects of Pb accumulated mushroom on body and organ weight change of rats

There was no significant change in the weekly body weight gain; 1st week ($p=0.5969$), 2nd week ($p=0.6215$), 3rd week ($p=0.8433$), 4th week ($p=0.6685$) and termination week ($p=0.1487$), of the treated rats compared to the control (Figure 2).

However percentage change of the terminal weight gain was lower in the treated rats compared to the control (Table 2). There was a concentration dependent insignificant ($p>0.05$) decrease in both absolute and relative liver and kidney weight gain in the treated rats compared to the control group (Table 2).

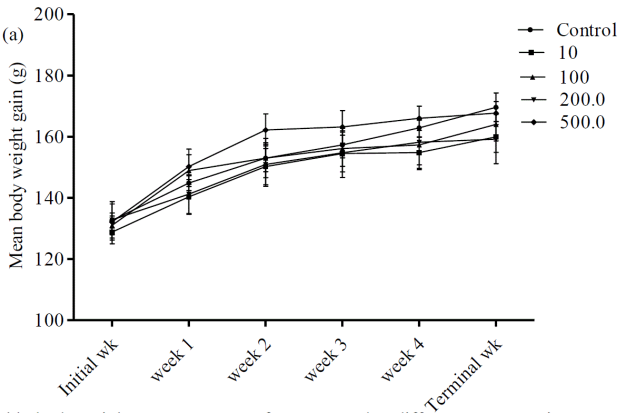

Figure 2: Weekly body weights (mean ± SD) of rats exposed to different concentrations of mushroom for 30 days and control.

Conc. (mg/L)	% Body weight	ALW (g)	RLW (g)	AKW (g)	RKW (g)
Control	27.9	5.40 ± 0.34	3.18 ± 0.11	0.98 ± 0.10	0.58 ± 0.03
10	24.15	5.28 ± 0.19	3.30 ± 0.06	0.80 ± 0.05	0.50 ± 0.10
100	25.19	5.18 ± 0.24	3.16 ± 0.13	0.80 ± 0.03	0.49 ± 0.02
200	19.88	5.00 ± 0.34	3.14 ± 0.76	0.80 ± 0.06	0.50 ± 0.09
500	26.95	4.98 ± 0.25	2.97 ± 0.38	0.78 ± 0.06	0.47 ± 0.07
-	-	p=0.7931	p=0.6813	p=0.2165	p=0.2017

Table 2: Percentage body weight gain, absolute and relative liver and kidney weight gain in rats fed with mushroom cultivated in Pb contaminated rice straw for 30 days. Values are in mean ± SD. ALW-Absolute liver weight (g), RLW-Relative liver weight (g). AKW-Absolute kidney weight (g), RKW-Relative kidney weight (g).

Haematotoxic effects of Pb accumulated mushroom in rats

Table 3 shows the results of alterations in haematological indices of rats fed with aqueous extract of Pb contaminated *P. pulmonarius* for 30 days. There was insignificant decrease in RBC (p=0.3411), HCT (p=0.5045) and HGB (p=0.4066), but WBC (p=0.0520) and PLT (p=0.0513) of the treated rats marginally decreased compared to the control.

There was significant increase in acanthocytes (p<0.001; crenated red blood cells), schizocytes (p<0.001; fragmented red blood cells), target cells or codocytes (p<0.001; Heinz bodies resembling bull eyes), but insignificant increase in tear drops (p=0.1357; red blood cells with spindle or sickle shape) in rats fed with aqueous solution of Pb contaminated *P. pulmonarius* compared to the control (Figure 3). The various erythrocytes morphologies scored in the treated and control rats are presented in Figure 4.

Conc. (mg/L)	RBC (× 106 µL)	HCT (%)	HGB (g/dL)	PLT (× 106 µL)	WBC (× 103 µL)
Control	6.88 ± 0.12	40.40 ± 0.75	13.86 ± 0.29	129.0 ± 132.7	68.00 ± 8.09
1	6.70 ± 0.35	39.80 ± 1.83	13.70 ± 0.59	96.8 ± 106.7*	54.30 ± 4.72
10	6.82 ± 0.02	39.60 ± 1.50	13.52 ± 0.39	124.6 ± 170.8	54.00 ± 2.65
100	6.30 ± 0.19	37.37 ± 1.02	12.76 ± 0.41	110.6 ± 142.6	64.00 ± 7.45
200	6.59 ± 0.04	37.80 ± 1.11	13.04 ± 0.26	119.2 ± 240.3	6.50 ± 3.84*
500	(p=0.3411)	(p=0.5045)	(p=0.4066)	(p=0.0513)	(p=0.0520)

Table 3: Hematological profile of rats fed with mushroom cultivated in Pb contaminated rice straw for 30 days. End points represent mean ± SD for 5 rats. RBC (Red blood cell count); HGB (Hemoglobin concentration); HCT (Percentage Hematocrit); PLT (platelets); WBC (White blood cell count); Superscripts differ significantly (*p<0.05) from the control using Dunnett's multiple post hoc test.

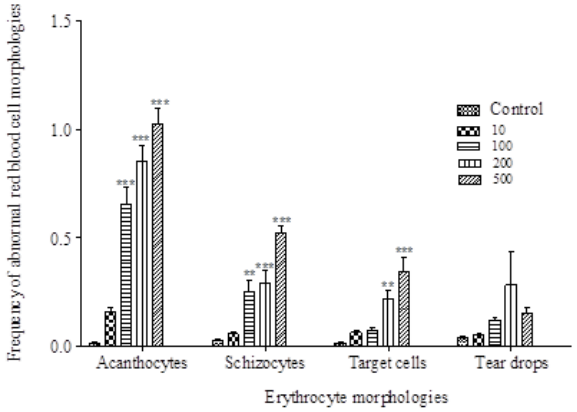

Figure 3: Frequencies of abnormal erythrocyte morphologies in rats fed aqueous extracts of *P. pulmonarius* cultivated in Pb contaminated rice straw.

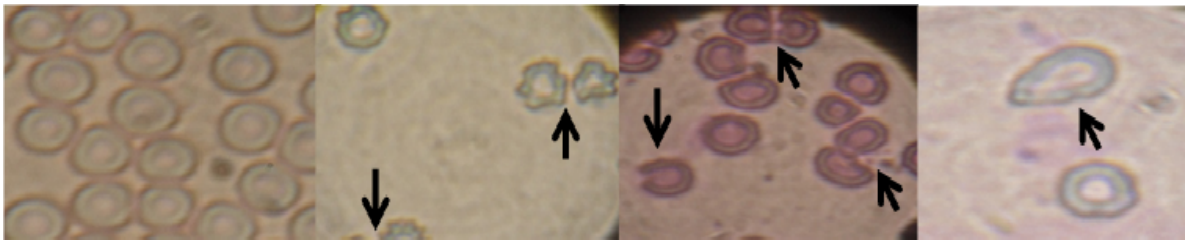

Figure 4: Abnormal erythrocyte morphologies (arrowed) observed in rats fed aqueous extracts of *P. pulmonarius* cultivated in Pb contaminated rice straw (a) normal erythrocytes from the control rats (b) acanthocytes (crenated): cells with shrink cell membranes (c) schizocytes (fragmented red blood cells) (d) tear drops (RBCs with spindle shape).

Discussion

There are increasing reports that macromycetes (both wild and cultured) species are hyperaccumulators of heavy metals. Researchers have concentrated efforts in harnessing this unique property of the mushrooms in cleaning up contaminated environment without considering health risk associated with the possibility of consuming such mushrooms [9,31,32]. Concentration dependent significant Pb accumulation in *P. pulmonarius* cultivated in Pb contaminated rice straw suggests that chitin, chitosan, glucan and amino polysaccharide present in the cell wall readily absorb Pb and passed it to the fruit bodies. Pb primarily binds to the amine-N of chitin which acts as the nucleation site for the absorption of this metal into the mushrooms [33,34]. This assertion is supported by the report that Cd accumulation in *Neurospora crassa* was via N-acetylglucosamine polymer and chitin present in the fungal cell wall [35]. Furthermore, these biological molecules in the cell wall were isolated from a white rot fungal species (*Coriolopsis polyzona*) in an attempt to understand their role in bioaccumulation. They were used as biocatalyst and succeeded in removing endocrine disrupting chemicals, nonylphenol, bisphenol A and personal care product ingredient, triclosan, from contaminated substrates [24]. The finding that *P. pulmonarius* cultivated on 1000 mg/L of the Pb contaminated rice straw did not germinate suggests that the Pb concentration was toxic to the biological molecules which possibly led to denaturation and cell death [36]. Pb accumulation in *P. pulmonarius* herein is consistent with Kumhomkul and Panich-pat [37] report that straw mushroom (*Volvariella volvacea*) cultivated on lead contaminated substrate similarly bioaccumulated Pb into its fruit bodies.

Collection and consumption of wild mushrooms which are traditionally social outing in most countries in Europe, Asia, and the United States [38], constitute major source of food and ingredients for traditional medicine in African [39]. Pb accumulation in *P. pulmonarius* cultivated at higher concentrations of the Pb contaminated straw exceeded the maximum standards (0.3 mg/kg wet weight for Pb in cultivated fungi) established for food contaminants by FAO/WHO [40]. Rats fed aqueous extract of *P. pulmonarius* accumulated Pb had increased Pb bioaccumulation in the kidney and liver. This was higher and exhibited concentration dependent increase in the kidney. Higher concentration of the Pb in the kidney may be attributed to the presence of divalent metal transporter 1 (DMT-1) which transported the Pb from the gastrointestinal tract to renal tissue of the treated rats [41,42]. It is also possible that Pb when in the blood formed conjugation with cysteine or histidine and was transported to the proximal tubule via sodium amino acid cotransporter [43]. Toxic metals (including Pb^{2+}) in mammalian systems are potent inducers of metallothioneins and glutathione in the renal and liver tissues. These peptides protect against heavy metal toxicity by trapping the metal inside the cells; this may account for the bioaccumulation of Pb in the organs [44,45]. However, due to regular renewal of the hepatocytes during intoxication in the liver, the Pb^{2+} were released from the metallothioneins and glutathione conjugates back into the systemic circulation and are subsequently sent to the kidney [46]. This possibly resulted in the higher bioaccumulation of Pb in the kidney than the liver of the treated rats. Increase BAF of Pb^{2+} in the kidney of treated rats corroborated the higher Pb bioaccumulation in the kidney. Since the BAF value of Pb in the liver was less than 1 for virtually all the concentrations of the Pb contaminated *P. pulmonarius* treated rats except 10 mg/L, it is described as transferred factor (TF) [47]. The body burden of bioaccumulated Pb in the mushroom induced decrease terminal body weight and decrease absolute and relative kidney and liver weight gain in the treated rats. This suggests that the accumulated Pb affected the organ weights in the course of Pb sequestration by the organs [48]. This is in agreement with the report that heavy metal bioaccumulation in the mammalian liver and kidney altered the absolute and relative liver and kidney weight [49].

Haematological testing in rodents during toxicity evaluations is generally acknowledged as part of safety assessment of chemicals [27]. It serves as sensitive predictors for effects in humans; since both are mammals with similarities in their kinetics, metabolic profile and dynamics [50]. Also changes in haematological indices have been associated with haematopathology, physiological and immunological changes [51]. Decrease erythrocytes (RBC), haemoglobin (Hb), haematocrits (Hct), platelet and total white blood cell counts (although statistically insignificant) compared to the control presents haematotoxic effects of the absorbed Pb on the blood indices possibly due to interference with the haemopoietic system and or heme synthetic pathway. This assertion follows the reports that Pb targets heme synthetic pathway which leads to the inhibition of heme and hemoglobin synthesis [52] and may cause damage to the hemopoietic system [53]. Decrease RBC, platelet counts, percentage hematocrit and hemoglobin concentration has been reported as indicator of anemia, stress disorder, failure in oxygen carrying capacities of red blood cells and poor health status due to exposure to toxic metals [54-56]. Quantitative investigation of total white blood cells (nonspecific immune cells) forms basic screening usually included in immunotoxic studies [57]. WBCs are useful in signaling clinically relevant haematologic changes that may result in clinical identifiable autoimmune disorders and various forms of leukemia [58]. The significant decrease in total leucocyte count observed in the treated rats may be related to suppression or inhibitory mechanisms in the

production process during haemopoiesis and may lead to immunosuppression in treated rats.

Altered erythrocyte morphologies in the treated rats corroborated the observed decreased haematological indices. When absorbed from the gastrointestinal tract, plasma Pb^{2+} is formed as non-diffusible (protein-bound) and diffusible (complexed and ionized) forms. Either of these forms is capable of inducing disturbances in the osmoregulatory system of the blood cells [59] and/or oxidative injury to the cell membrane [60]. This may enhance leakage or denaturation of hemoglobin [61], which produced the abnormal erythrocyte morphologies in the treated rats. It is possible that the Pb^{2+} generated free radicals and lipid peroxidation (LPO) which induced different shapes of the vulnerable erythrocytes [62,63], via decreased membrane fluidity, increased membrane permeability, inactivation of membrane-bound enzymes, and loss of essential fatty acids alterations in the structure and functions of the cell membrane [64]. Pb is also capable of altering the properties of erythrocyte membranes directly by rendering them more fragile and permeable, hence may result in cell swelling, deformation and damage [53]. This may account for the different abnormal erythrocytes observed in the treated rats compared to the control.

Plurotus pulmonarius cultivated in Pb contaminated rice straw accumulated Pb into its fruit bodies in a concentration dependent manner. Rats fed aqueous extracts of this mushroom significantly bioaccumulated Pb in its kidney and liver with high bioaccumulation factor in the kidney. The accumulated Pb possibly induced decrease body and organ weights, alterations in hematological indices and abnormal red blood cell morphology. Lead poisoning recognized as a major public health issue, mostly in developing countries, can be increased through the consumption of Pb contaminated plants most importantly mushrooms.

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