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# Lead nitrate toxicity: its effects on hepatic extracellular matrix fibers, filamentous cytoskeleton and the mitigative potentials of *Morinda lucida* extract

Akinyemi K. Jolayemi [a], David O. Adeyemi [b,*], Paul O. Awoniran [a]

[a] Department of Human Anatomy, Faculty of Basic Medical Sciences, Redeemer's University, Ede, Osun State, Nigeria
[b] Department of Anatomy and Cell Biology, Faculty of Basic Medical Sciences, College of Health Science, Obafemi Awolowo University, Ile-Ife, Nigeria

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ABSTRACT

In this study, the effect of orally administered methanolic extract of *Morinda lucida* stem bark (MLSB) was tested for its efficacy to reverse lead nitrate-induced hepatotoxicity in Wistar rats. Thirty-six female rats were assigned into six groups (n = 6). Rats in group I received 2.2 mL/kg distilled water for 28 days, those in group II received 30 mg/kg lead nitrate for 14 days while those in groups III to VI received 30 mg/kg lead nitrate for 14 days followed by a treatment with 100, 250, 500 mg/kg BW MLSB extract and 0.2 mL/100 kg rats silymarin respectively for 14 days. They were sacrificed after 28 days after which biochemical, histological, and immunohistochemical parameters were examined. The results of this study showed a reduction of catalase and superoxide dismutase activities by lead nitrate. Deranged hepatic histomorphology was also observed intracellularly and extracellularly in lead nitrate-treated rats. Altered vimentin arrangement was also observed in lead nitrate-treated rats. However, 250 mg/kg BW dose of *Morinda lucida* significantly reversed some of these changes while the 500 mg had some toxic effect on liver tissue. We concluded that the extract at 250mg/kg BW dose may be a potential treatment for conditions associated with lead toxicity and other metallic particles.

## Introduction

Environmental threat to optimal health stems from many sources including lead compounds, lead-containing materials and other heavy metals (Wuana and Okieimen, 2011). Lead is a known toxic element contributed majorly by human activities and natural availability (Flora *et al.,* 2006). Its more recent applications include heat stabilizer in nylon and polyesters, as coating of photo thermographic paper, and rodenticides (Arrad, 2019). Lead nitrate exposure may also occur when blood is transfused from an exposed individual to a patient (Agyemang *et al.,* 2020). Once it gains access into the body it causes deadly damage to organ system of the body (Yu *et al.,* 2008). It affects the structure and function of various organs and also increases the rates of spontaneous abortion, fetal malformation, and low birth weight if exposed to during pregnancy (Litvinov et al., 1991; Agyemang *et al.,* 2020). Lead and its compounds accumulate in areas such as the blood, kidneys, liver, and brain causing serious damage to these vital structures (Sharma *et al.,* 2010; Agyemang *et al.,* 2020).

Since lead has such a detrimental effect on human health, the ideal situation would be to eliminate it from the workplace and replace it with materials that are similarly potent with little or no toxic effect. However, this is not always possible considering its widespread industrial applications (Karrari *et al.,* 2012). Various methods, products, and procedures, including the use of protective equipment, drugs, chelation therapy, and other alternative therapies have been tried to either prevent or ameliorate its toxic effects some of which were ineffective while the effective ones were compromised by side effects (Ezejiofor and Orisakwe, 2019; Mumtaz et al., 2020; Pecoraro et al., 2013).

Plants are the main source of various bioactive compounds with great therapeutic applications and minimal side effects which are used as raw materials in the pharmaceutical industry (Calixto, 2019). *Morinda lucida* Benth (Rubiaceae), also referred to as brimstone tree, is an ethnomedicinal plant which has been widely used in traditional medicine for several decades, particularly in the African continent. Various parts of the plant, including stem bark, leaves and root, have been applied in traditional medicine for the management of various pathological conditions such as malaria, diabetes, hypertension, inflammation, typhoid fever, cancer, cognitive disorders, sickle cell disease,

* Corresponding author.
*E-mail address:* david.adeyemi@oauife.edu.ng (D.O. Adeyemi).

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trypanosomiasis, onchocerciasis and various fevers (Adewole *et al.,* 2021). The many benefits derived from *Morinda lucida* is owed to the high contents of vitamins A, K and E, alkaloids flavonoids and other phytochemicals which are powerful antioxidant (Adeleye *et al.*, 2018). Other reported secondary metabolites include, tannins, anthraquinones, sterols, saponins, polyphenols, terpenoids, phenols and cardiac glycosides (Adewole *et al.,* 2021, Adeyemi *et al.,* 2022). The in vitro and in vivo experimental studies on various extracts, fractions and isolated compounds of M. lucida support the acclaimed pharmacological activities of the plant, such as antimalarial, antidiabetic, hypotensive, anti-inflammatory, immunostimulatory, antioxidant, antimicrobial, antiproliferative, cognitive-enhancement, anti-sickling, anti-try panosomal, anti-onchocercal, muscle relaxant, antifungal and anti-leishmanial activities. These evidence-based scientific reports lend credence to their traditional uses. (Adewole *et al.,* 2021). In the present study, its efficacy in the mitigation of lead nitrate induced hepatocellular injury will be examined.

## Materials and Methods

### *Materials and experimental rats*

Lead nitrate was purchased from Loba Chemie Laboratory Reagents and Fine Chemicals, (Mumbai, India). Silymarin tablet was procured from Micro Labs limited, (India., SYFH0048). Female Wistar rats (120 - 150 g) were bred in the laboratory animals' unit of the Department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife, Nigeria. They were given feed and water *ad libitum* and were allowed to acclimatize to the environment for one week before experimentation. The experimentation processes complied with the regulations outlined in the NIH Guide for the Care and Use of Laboratory Animals (NRC 2011) and the research protocol was approved by the Health Research and Ethics Committee of the Institute of Public Health, Obafemi Awolowo University, Ile - Ife, Nigeria.

### *Preparation of extract*

Fresh stem barks of *Morinda lucida* (MLSB) were obtained from the botanical garden, Obafemi Awolowo University, Ile-Ife, Nigeria. Authentication was done by a senior taxonomist in the Department of Botany of the same institution and a voucher specimen (reference number: IFE-17539) was deposited in the department's herbarium. The stem barks were chopped into small bits and air-dried at room temperature until a constant weight was attained. We pulverized a known mass in a milling machine (DIK – 2913) and extracted the powder three times with 70% methanol with a continuous stirring at room temperature for 24 h each. Whatman number 1 filter paper was used to filter the extract. The filtrate was concentrated in a vacuum rotary evaporator (Buchi), freeze dried in a lyophilizer and stored in a desiccator before the commencement of treatment.

### *Hepatotoxicity induction and treatment*

Thirty-six female rats were randomly assigned into six groups of six rats per group (n = 6). The duration of the experiment was 28 days. MLSB, silymarin were dissolved in distilled water (0.3 ml/ 100g rat). Group I (Normal control group) received distilled water only for 14 days. To induce liver toxicity, groups II to VI received 30 mg/kg Pb for 14 days. The extract (MLSB: 100, 250, 500 mg/kg BW respectively) was administered to groups III to V daily for 14 days, while Group VI was given Silymarin (standard drug; 2 mg/kg BW) 12 hourly for 14 days.

### *Sacrifice and surgical excision*

Twenty-four hours after the last administration of the extract and silymarin, all the experimental rats were sacrificed under diethyl ether anesthesia. An incision was made through the abdomen, the liver was resected and lobes were identified. The median lobe was cut into two portions, a portion was fixed in 10% neutral buffered formalin for microscopic examination while the other portion was frozen for the estimation of activity of antioxidant enzymes.

### *Histology, Histochemistry, and Immunohistochemistry*

Fixed tissues were processed for paraffin wax embedding as earlier described (Drury and Wallington, 1980; Adeyemi *et al.,* 2014). 5 μm thick sections were obtained on a rotary microtome (Leica RM 2125 RTS) and stained with hematoxylin and eosin, Gordon and Sweet's silver stain and Masson's trichrome stain for the demonstration of general histoarchitecture, reticulin and collagen fibers respectively. Avidin-biotin peroxidase complex method was used to perform immunohistochemistry for vimentin expression on liver specimens previously fixed in formalin and embedded in paraffin.

## Biochemical Assays

### *Assay of catalase and SOD activities*

Activity of catalase in the liver homogenate was assayed based on the procedure described by Sinha (1972) as earlier reported (Adeyemi and Adewole 2019). The method was based on catalase decomposing hydrogen peroxide to form water and oxygen. Superoxide dismutase activity was determined in the liver using standard laboratory protocol as described by Misra and Fridovich (1972).

### *Photomicrography and image analysis*

The liver sections were examined under a LEICA research microscope (DM750) connected to a digital camera (LEICA ICC50). Permanent photomicrographs were taken at high power magnifications (400x, 1000x). To examine staining in liver tissue, a quantitative image analysis was done with Fiji image J software. Micrographs were opened on the software and the scale was set using the scale setting tool to convert measurements in pixels to microns and this was applied globally to all images following a previously reported method (Awoniran and Adeyemi, 2018). Mean gray-level values were obtained for reticulin, collagen, glycogen, and filamentous cytoskeleton.

### *Statistical analysis*

The results obtained from all quantitation were analyzed using graph pad prism statistical software 5.03. Analysis of variance (ANOVA) was performed to examine if there exists a significant difference between the groups mean and Students Newman-Keuls post hoc tests was performed for multiple comparison test in order to identify sample means that are different from each other The results obtained were presented as mean± S.E.M. Alpha level was set at 0.05.

## Results

### *Effect of lead nitrate and MLSB on the activities of catalase and SOD*

Lead nitrate significantly reduced the activity of catalase in group II (negative control) rats when compared with group I (normal control). However, in Group IV and V, MLSB has significantly increased its activity in a dose-dependent manner when compared with Group II (negative control) rats. Activity of SOD was also significantly inhibited by the lead nitrate treatment, which was significantly reversed by 250 and 500mg/kg BW MLSB treatment in groups IV and V rats when compared with untreated group II rats. The results of treatment with MLSB were in a similar to the effect exhibited by silymarin treatment in group VI rats (Table 1).

**Table 1**
Effects of Lead nitrate and MLSB on the Activities of Catalase and SOD.

| Groups | Antioxidant Enzymes Activities Catalase (μmol /min/mg Protein) | Superoxide Dismutase (Unit/min/mg Protein) |
|---|---|---|
| 1 | $4.5215 \pm 0.8637^{\alpha}$ | $0.7422 \pm 0.1418^{\alpha}$ |
| 2 | $2.0415 \pm 0.3065^{\beta}$ | $0.3351 \pm 0.0503^{\beta}$ |
| 3 | $2.8627 \pm 0.2536^{\beta}$ | $0.4699 \pm 0.0416^{\beta}$ |
| 4 | $3.6601 \pm 0.2150^{\alpha}$ | $0.6005 \pm 0.0339^{\alpha}$ |
| 5 | $3.7895 \pm 0.2698^{\alpha}$ | $0.6220 \pm 0.0443^{\alpha}$ |
| 6 | $3.9721 \pm 0.2265^{\alpha}$ | $0.6520 \pm 0.0371^{\alpha}$ |

Each value represents Mean ± SEM, n = 6 readings. Group with similar symbol are not significantly different while those with different symbols are significantly different.

*Effect of lead nitrate and MLSB treatment on liver histology*

The normal control group showed intact hepatic parenchymal cells, round nuclei with prominent nucleoli, apparent sinusoids, and normal simple squamous sinusoidal epithelium (endothelium). Lead nitrate inflicted extensive pathological damage such as karyopyknosis, karyolysis, cell and aberrant sinusoidal dilation with congestion. The treatment groups that received MLSB at 100, 250 mg/kg BW doses and silymarin at 2 mg/kg BW dose reversed some of these deranged features. However, focal areas of cell shrinkage and karyolysis remained irreversible in these groups. Group V that received MLSB at a dose of 500 mg/kg BW showed histopathological features similar to that of group II (lead nitrate only -treated rats) with aberrant dilation, congested sinusoids, and stretched sinusoidal endothelium (Fig. 1).

*Effects of lead nitrate and MLSB on hepatic vimentin*

Vimentin staining was expressed in hepatic parenchymal cells across the groups. However, there was a significant decrease in the mean gray value (chromogen intensity) in group IV and VI rats when compared with group II rats (negative control). The mean gray level in groups III and V rats were not significantly different from group II. Staining showed a unique expression (perinucleus, intracytoplasmic) in group II rather than the general expression pattern (enriched beneath the cell border / perisinusoidal) seen in other groups (Fig. 2).

*Effect of lead nitrate and MLSB treatment on reticulin distribution*

An intact reticulin support for the blood vessels and bile ducts in the portal tracts of the liver was observed in the micrographs of Group I and Group IV rats. Also, normal collagen type III fibrils arrangement of the perisinusoidal space (between the sinusoids and the hepatocytes) was observed in these groups. We observed a discontinuous highlight of reticulin fiber in group II rats and also noticed a discontinuation of reticulin support of terminal hepatic venule in groups III and V rats. There was no significant difference in the mean gray level across all groups (Fig. 3).

*Effect of lead nitrate and MLSB treatment on collagen distribution*

Normal collagen highlight of the portal tracts and vessel walls of liver tissue was observed across the groups. Collagen deposition was not noticed within the liver cells. There was no significant change in mean gray level across all groups (Fig. 4).

## Discussion

Cell-extracellular matrix interactions are important to the structure of tissue (Frantz *et al.*, 2010). The matrix contains collagen fibers (types I and III) among other components and it functions as essential support structure for cells and also controls communication between cells (Theocharis *et al.*, 2016). Any alteration to cell and the pericellular or interstitial extracellular matrix may irreversibly affect cell structure and function. This study examined the efficacy of MLSB in the mitigation of lead nitrate-induced cellular, cytoskeletal and extracellular alterations.

In the present study, we have shown that lead nitrate significantly reduced endogenous antioxidant activities that automatically exposes the body to the detrimental actions of free radicals. This is an indication that altered antioxidant enzymes activities have a significant contribution to lead nitrate-induced toxicity. Our result conforms with the previous report of Saxena and Flora, (2004) who also reported a significant decrease in superoxide dismutase activity in experimental rats exposed to lead nitrate. This affirms the role of free radical damage in lead nitrate toxicity. Sharma et al. (2010) reported that lead nitrate-induced liver damage is caused by altered pro-oxidant-antioxidant balance. The middle and high doses of the extract increased the antioxidant activities

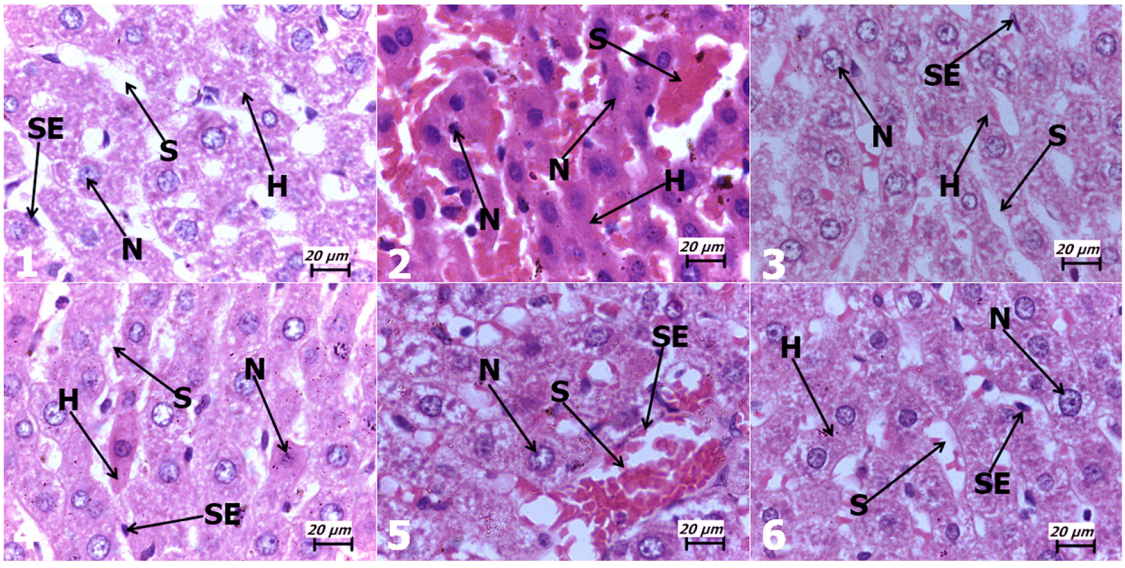


**Fig. 1.** Representative light micrographs of the liver sections subjected to Hematoxylin and Eosin stain. Observe in (1) intact hepatic parenchymal cells (H), round nuclei with prominent nucleoli (N), apparent sinusoids (S) and sinusoidal epithelium (SE) (2) karyopyknosis (dark and shrunken nucleus), karyolysis (N), dark, oval nuclei (mitotic figures) and aberrant sinusoidal dilation and congestion (S) (3 and 6) hepatic features are close to normal control's (4) focal areas of cell shrinkage (H) and karyolysis (N) (5) aberrant dilation and congestion of sinusoids, stretched sinusoidal endothelium (simple squamous epithelium) (SE). Scale bars - 20μm.

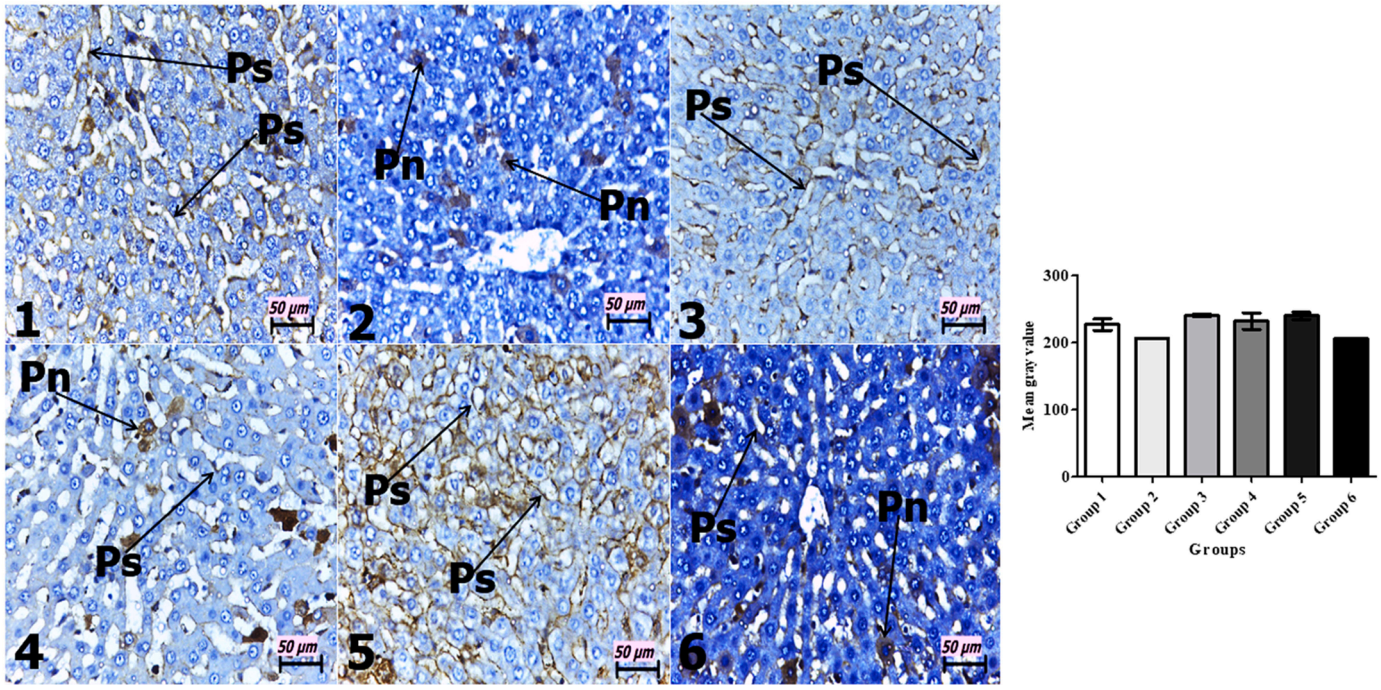


**Fig. 2.** Representative light micrographs of the liver sections subjected to immunostaining for vimentin. Positive reactivity was seen across the groups evidenced by the brown DAB color. Strictly perisinusoidal (Ps) distribution of vimentin was observed in groups 1, 3, and 5. However, both perisinusoidal (Ps) and perinuclear (Pn) distributions were observed in groups 2, 4, 6. The bar chart represents vimentin staining intensity across the groups. Values are the mean ± SEM of six animals per treatment group. $^{\alpha}$ Significant differences ($p < 0.05$) from negative control. Scale bars - 50µm.

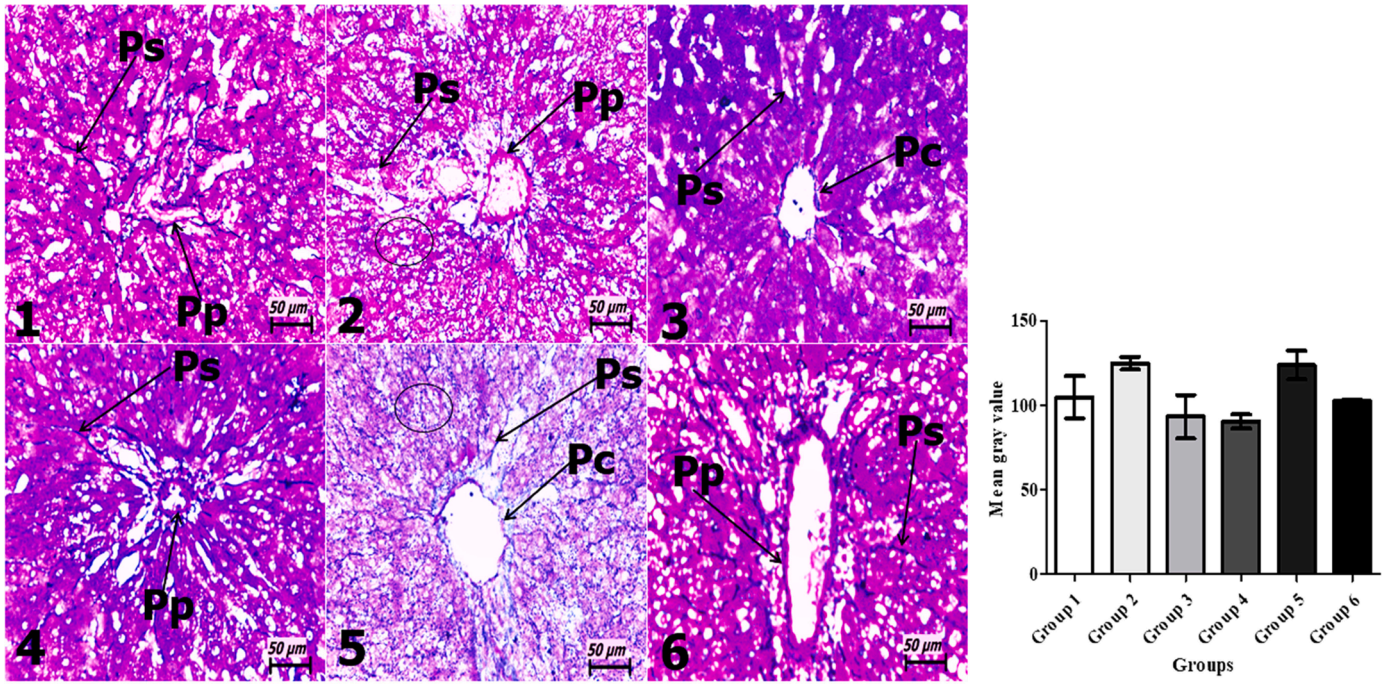


**Fig. 3.** Representative light micrographs of the liver sections subjected to silver stain for reticular fiber. Observe the continuous black highlight of reticular fiber around the hepatic portal area (Pp) and perisinusoidal space (Ps) in groups 1, 4, and 6. Group 2 shows areas of discontinuous reticular fiber distribution around the portal area (Pp) and in the perisinusoidal space (Pp) while 3 and 6 show faint to no reticulin distribution around these spaces. The bar chart represents the mean gray level of reticulin highlighted in each group. No statistically significant difference in reticulin distribution was seen. Values are mean ± SEM. Scale bars- 50µm.

which is a pointer to its ability at those doses to augment endogenous antioxidant levels and scavenge free radicals. The effect observed is attributable to the presence of flavonoids and phenolic compounds in the extract, which are reputed for high antioxidant activities as previously reported by Osuntokun *et al.* (2016), and Xu *et al.* (2018).

Lead nitrate altered liver cell structure, dilated and congested the microcapillary system of the liver, induced karyopyknosis and caused significant cell proliferation evident by numerous mitotic figures. Karyopykosis observed in this study is an irreversible condensation of chromatin in the nucleus of a cell undergoing necrosis or apoptosis

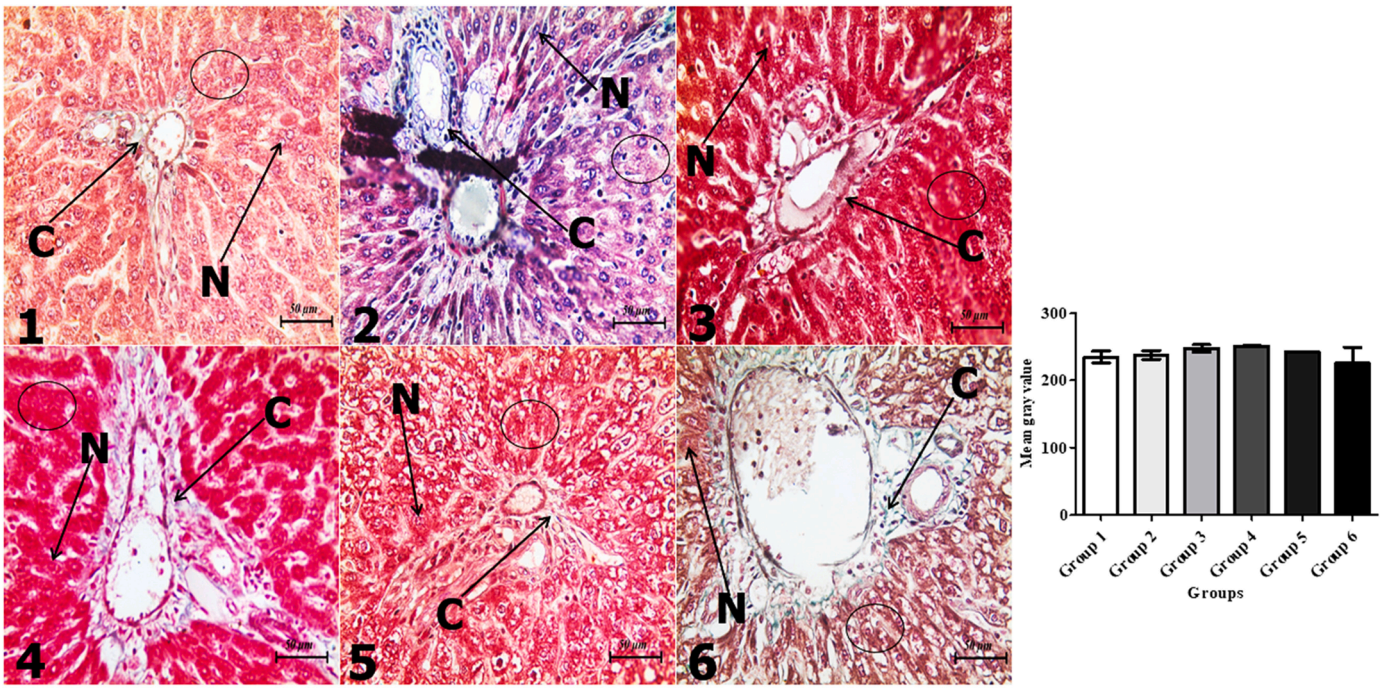


**Fig. 4.** Representative light micrograph of the liver sections subjected to Masson's trichrome stain for collagen type 1. Observe the green color highlight of the portal tract (C), different shades of red highlight of the hepatocytes (circle), and the blue highlight of the nuclei (N) of the cells across the groups. The bar chart represents the mean gray level of collagen highlighted across the six groups. No statistically significant difference in collagen highlight was seen. Values are mean ± SEM. Scale bars - 50µm.

(Abdelhalim and Jarrar, 2011). Congestion has been previously reported to result from the stagnation of blood in the capillaries due to poor venous outflow (Damjanov, 2009, Simonetto *et al.,* 2015). It is more often than not due to some circulatory related pathology in veins towards the congested area (Simonetto *et al.,* 2015). Hepatic veins also dilate in response to increased pressure. Chronic vascular congestion can result in a serious morphological derangement in affected organs as a result of a reduced blood flow to the affected tissues and if occurring over long time scales results in ischemia to the affected capillaries and surrounding parenchyma which in this case are the hepatocytes (Damjanov, 2009). The necrosis and the associated pyknosis observed in this study could then be attributed to ischemia, a reduction in blood flow resulting in decreased oxygen and nutrient supplies to liver tissue. The increased mitotic figures observed in this study is an indication that lead nitrate can stimulate cell proliferation in the liver of female Wistar rats. This result conforms with the report of Columbano *et al.* (1984) who also reported necrosis and high mitotic figures when lead nitrate was administered to experimental rats. Methanolic extract of MLSB at 100 and 400mg/kg BW reversed some of the morphological alterations reported in this study while the 500 mg/kg BW dose induced several adverse side effects. It could be that this dose was too high for the targeted blood level for an appreciable morphological effect. Our results are in agreement with data from the literature (Kumar *et al.,* 2011, Haouas *et al.,* 2014).

Histological examination of extracellular matrix fibers showed an insignificant increase in the quantitative (deposition) but not qualitative and arrangement of extracellular matrices containing collagen types III in lead nitrate only treated rats while type I deposition decreased insignificantly. Extracellular matrix provides tissue with tensile strength, elasticity, and resistance to compressive forces (Muiznieks and Keeley, 2013). The quality of its arrangement is very significant to its function while the quantitative presence of its components like fibers is associated with certain pathologies such as hepatic fibrosis or cirrhosis and even cancer (Young *et al.,* 2006). A significant increase in deposition of fibers wasn't noticed in this study. However, 100 and 400mg/kg BW doses of extract reduced the deposition insignificantly in a similar fashion to what was observed in the normal control rats. This effect was probably conferred by flavonoids which are known as effective anti-fibrotic compounds (Wu *et al.,* 2018).

We observed positive vimentin expression across the groups. In quantity, the expression was enhanced by lead nitrate while it was declined significantly by silymarin and 500mg/kg BW extract when compared with negative control. However, in arrangement, it appeared abnormally localized in negative control rats and silymarin treated rats when placed side by side with the normal control and extract treated groups. Strnad *et al.,* (2008) reported that liver perisinusoidal cells express vimentin as their major intermediate filament protein network. Rather than perisinusoidal, it appeared condensed in the cytoplasm, directly around the nucleus in those groups. There are several reports on cytoskeletal changes in various liver diseases including alcoholic liver disease, intrahepatic cholestasis and hepatocellular carcinoma (Okanoue et al., 1985; Strnad *et al.,* 2008). Omary *et al.,* (2004); Ku *et al.,* (2007); Herrmann *et al.,* (2007) all preciously reported that intermediate filaments disruption results in increased mechanical fragility in tissues. This suggests that vimentin has a role in some liver injury in which lead nitrate hepatotoxicity may be inclusive.

## Conclusion

Findings in this study have shown that the middle dose of MLSB administered was a potent treatment for lead nitrate-induced extra- and intrahepatocellular injury in Wistar rats. We suggest that the effects of MLSB in reversal of lead nitrate-induced extra- and intrahepatocellular injury could be due to its ability to scavenge free radicals and augment endogenous antioxidants.

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## Ethical statement

The processes of the animal experiment complied with the regulations outlined in the NIH Guide for the Care and Use of Laboratory Animals and the research protocol was approved by Health Research and Ethics Committee of the Institute of Public Health, Obafemi Awolowo University, Ile - Ife, Nigeria.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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